FEBS 29215 FEBS Letters 579 (2005) 797–802

Structural organization of the twin-arginine translocation system in *Streptomyces lividans*

Sophie De Keersmaeker, Lieve Van Mellaert, Kristien Schaerlaekens, Wesley Van Dessel, Kristof Vrancken, Elke Lammertyn, Jozef Anné*, Nick Geukens

Laboratory of Bacteriology, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

Received 7 December 2004; revised 17 December 2004; accepted 17 December 2004

Available online 11 January 2005

Edited by Gerrit van Meer

Abstract **The twin-arginine translocation (Tat) system exports folded proteins across bacterial cytoplasmic membranes. Recently, genes encoding TatA, TatB and TatC homologues were identified in *Streptomyces lividans* and the functionality of the Tat pathway was demonstrated. Here, we have examined the localization and structural organization of the Tat components in *S. lividans*. Interestingly, besides being membrane-associated proteins, *S. lividans* TatA and TatB were also detected in the cytoplasm. TatC could only be detected in isolated membrane fractions. Whereas all TatC was found to be stably inserted in the membrane, part of membrane-associated TatA and TatB could be extracted following high salt, sodium carbonate or urea treatment suggesting a more loose association with the membrane. Finally, we have analyzed Tat complexes that could be purified from an *S. lividans* TatABC overproducing strain. From the cytoplasmic membrane, two types of high molecular mass Tat complexes could be isolated having a similar composition as those isolated from *Escherichia coli*. In the cytoplasm, TatA and TatB were detected as monomer or as homo-oligomeric complexes.**

Keywords: Twin-arginine translocase; Membrane protein complex; *Streptomyces lividans*

1. Introduction

All cells need to transport proteins across their membranes. In bacteria, the Sec pathway is the most widely used mechanism to transport proteins either co- or post-translationally across the cytoplasmic membrane [1]. Translocation by the Sec system occurs by a threading mechanism in which the precursor must adopt an unfolded conformation. Besides this well-characterized pathway, another route for protein transport across the cytoplasmic membrane exists in bacteria, notably the twin-arginine translocation (Tat) pathway (recently reviewed in [2]). This pathway has the remarkable feature to export proteins which have already obtained some degree of tertiary structure across the cytoplasmic membrane. Proteins destined for export by the Tat system are synthesized as precursors with a cleavable N-terminal signal peptide containing a conserved twin-arginine sequence S/T-R-R-x-φ-φ that is essential for transport. This translocation process is energized solely by the transmembrane proton electrochemical gradient [3] and not by nucleoside triphosphates, which are essential energy sources in the Sec-dependent transport. In *E. coli*, the integral membrane proteins TatA, TatB, TatC and TatE have been shown to be components of the Tat pathway, although little is known about their exact function. From detergent-solubilized *E. coli* membranes, a large complex of approximately 650 kDa containing TatA, TatB and TatC and a second complex, TatAB with a molecular mass between 350 and 650 kDa have been isolated. Both complexes are assumed to come together in the cytoplasmic membrane to form the functional Tat translocase [2]. TatABC complexes with a similar molecular mass and composition could also be purified from two other proteobacteria, notably *Salmonella* Typhimurium and *Agrobacterium tumefaciens* [4].

Recently, *tat* genes which encode homologues of *E. coli* TatA, TatB and TatC were identified in *Streptomyces lividans* [5]. *S. lividans* is a Gram-positive soil bacterium with a naturally high secretion capacity and therefore of industrial interest as a host for heterologous protein production [6,7]. Functionality of the Tat pathway in *S. lividans* has been shown by evaluating the secretion efficiency of the chimeric preTorA23K, *S. lividans* xylanase C and *Streptomyces antibioticus* tyrosinase in wild-type *S. lividans* and δ*tatB* and δ*tatC* single mutants [5,8]. The *S. lividans* δ*tatB* and δ*tatC* single mutants showed an aberrant phenotype, notably growth retardation, a dispersed growth in liquid medium and an impaired morphological differentiation on solid medium [8]. These findings together with the high number of predicted Tat substrates (230 precursors), suggest that the Tat system in *Streptomyces* may be more important for protein export than in other bacteria [8].

A detailed knowledge of the structural organization and function of all Tat components in *S. lividans* is of great impor-

* Corresponding author. Fax: +32 16 337340.
E-mail addresses: Sophie.Dekeersmaeker@rega.kuleuven.ac.be (S.D. Keersmaeker), Lieve.Vanmellaert@rega.kuleuven.ac.be (L.V. Mellaert), Kristien.Schaerlaekens@bio.kuleuven.ac.be (K. Schaerlaekens), Wesley.Vandessel@gmail.com (W.V. Dessel), Kristof.Vrancken@rega.kuleuven.ac.be (K. Vrancken), Elke.Lammertyn@rega.kuleuven.ac.be (E. Lammertyn), Jozef.Anne@rega.kuleuven.ac.be (J. Anné), Nick.Geukens@rega.kuleuven.ac.be (N. Geukens).

Abbreviations: DMSO, dimethyl sulfoxide; GST, gluthatione *S*-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; NHS, *N*-hydroxysuccinimide; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid

0014-5793/$30.00
doi:10.1016/j.febslet.2004.12.059

tance for improving the yield of secreted recombinant proteins in this bacterium via the Tat pathway. In this study, we investigated the localization and structural organization of the Tat components in *S. lividans*. To this purpose, fractionation experiments were performed. Next, membrane protein extraction experiments were used to examine the strength by which the Tat components are associated with the cytoplasmic membrane. Finally, we determined in which membrane-associated and cytoplasmic complexes the *S. lividans* Tat components participate. Therefore, TatA, TatB and TatC were simultaneously overproduced in *S. lividans* and the resulting Tat complexes were purified using ion-exchange and gel filtration chromatography. The type and composition of the obtained Tat complexes were compared with those of *E. coli* and *Bacillus subtilis*.

2. Materials and methods

2.1. Strains, media and growth conditions

Escherichia coli strain TG1 was used as host for cloning purposes [9]. Cultures were grown at 37 °C (300 rpm) in Luria–Bertani medium, supplemented with the appropriate antibiotics. *S. lividans* TK24 and its derivatives were precultured in 5 ml phage medium [10] supplemented with thiostrepton (10 μg/ml) or kanamycin (50 μg/ml), if necessary, and grown at 27 °C with continuous shaking at 300 rpm for 48 h. After homogenizing the mycelium, the strains were inoculated in liquid NM medium [11]. For solid medium, MRYE [8] was used supplemented with thiostrepton (50 μg/ml) or kanamycin (50 μg/ml), if applicable. Protoplast formation and subsequent transformation of *S. lividans* were carried out as described by Kieser et al. [12].

2.2. DNA manipulations and vector constructions

For all DNA manipulations standard techniques were followed [9,12]. Restriction endonucleases and DNA modifying enzymes were from Invitrogen and Roche Diagnostics. DNA sequence analysis was carried out according to the dideoxy chain termination method with the Thermo Sequenase Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Biosciences) on an ALFexpress apparatus (Amersham Biosciences). Oligonucleotides used in this work are listed in Table 1.

In order to raise antibodies against *S. lividans* TatA, TatB and TatC, these proteins were overexpressed and purified. Therefore, corresponding genes were fused to the gluthatione *S*-transferase (GST) gene of *Schistosoma japonicum* and expressed in *E. coli.* To this purpose, the respective *S. lividans tat* genes were amplified by PCR in the presence of 10% dimethyl sulfoxide using *S. lividans* chromosomal DNA as template with the oligonucleotides TatA5-TatA3, TatB5-TatB3 or TatC5-TatC3. The resulting DNA fragments were cloned into pGEM-T Easy (Promega) and the DNA sequences were verified. Subsequently, *tatA* and *tatB* were cloned as *Bam*HI–*Eco*RI fragments in the *Bam*HI–*Eco*RI treated GST gene fusion vector pGEX-4T-1 (Amersham Biosciences), whereas the *tatC* gene was inserted as an *Eco*RI–*Not*I fragment in this vector. The resulting expression plasmids were designated pGEXTatA, pGEXTatB and pGEXTatC.

In order to overproduce TatABC in *S. lividans*, the *tatAC* operon and *tatB* gene were cloned in the *E. coli*/*Streptomyces* shuttle vector pIJ2587 [13] under control of their own promoter. Therefore, the *tatAC* operon and the *tatB* gene were amplified by PCR with the oligonucleotides Tat14-Tat11 and TatB1-TatBNNID, respectively. After cloning of these PCR fragments in pGEM-T Easy, DNA sequences were verified and the resulting pGEMTatAC and pGEMTatB plasmids were digested with *Eco*RI and *Bam*HI, respectively. The two generated DNA fragments were successively ligated into the *Bam*HI and *Eco*RI site of pIJ2587 resulting in pIJ2587TatABC.

Table 1
Oligonucleotides used in this work

Name sites	Sequence (5′–3′ direction)	Restriction sites
TatA5	TAGGATCCTTCGGAAGGCTCGGCGC	*Bam*HI
TatA3	TAGAATTCTCAGCGCTTGGTCGTGTCC	*Eco*RI
TatB5	TAGGATCCTTCAATGACATAGGCGC	*Bam*HI
TatB3	TAGAATTCTCAGGTGGCGTCCATGTCG	*Eco*RI
TatC5	TAGAATTCCCGCTCGCGAACACCTTCG	*Eco*RI
TatC3	TAGCGGCCGCTCAGGTCACGTCGTCGTAGC	*Not*I
TatB1	TAGGATCCTGCCAAGGGCGGCGACGGCG	*Bam*HI
TatBNNID	TAGGATCCCCGCGCGGGCGTCGCCTTC	*Bam*HI
Tat14	AGGAGCAAGGACTGTGAG	
Tat11	CGGAGTTCACGCTGTTGT	

Restriction endonuclease cleavage sites are underlined.

2.3. Expression and purification of GST tagged S. lividans Tat proteins

For the expression of TatA, TatB and TatC as GST fusion proteins, *E. coli* TG1 was transformed with the expression plasmids pGEX-TatA, pGEXTatB and pGEXTatC, respectively. Transformants were grown at 37 °C to an OD_{600} of 0.6 and expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.1 mM as described [14]. Subsequently, the cultures were grown at 27 °C for an additional 4 h.

Escherichia coli TG1 cells producing GST-TatA, GST-TatB or GST-TatC were harvested by centrifugation (10 min, 5000 × *g*, 4 °C) and resuspended in 25 ml lysis buffer (50 mM Tris–HCl, pH 8.0, 20% sucrose and 10% glycerol). Cells were lysed by passing them three times through a French Pressure cell at 69 MPa. To the lysates obtained from *E. coli* TG1 cells containing pGEXTatA or pGEXTatB, Triton X-100 was added to a final concentration of 0.5% to aid in solubilization of the fusion proteins. After 30 min of mixing at 4 °C, samples were cleared by centrifugation (20 min, 12 000 × *g*, 4 °C). The lysate obtained from *E. coli* TG1 [pGEXTatC] cells was subjected to ultracentrifugation (2 h, 100 000 × *g*, 4 °C). The sedimented membranes were resuspended in 5 ml solubilization buffer (50 mM Tris–HCl, pH 8.0, 0.5% Triton X-100). Upon incubation for 1 h at 4 °C, the sample was recentrifuged (1 h, 100 000 × *g*, 4 °C) to remove membranes.

Cleared samples were incubated for 1 h with 2 ml gluthatione–Sepharose 4 Fast Flow matrix (Amersham Biosciences), equilibrated with 50 mM Tris–HCl, pH 8.0, 0.5% Triton X-100. Next, the resulting samples were loaded on an empty polypropylene column (Qiagen). After extensive washing with phosphate-buffered saline, GST-Tat fusion proteins were eluted with 10 mM reduced gluthatione in 50 mM Tris–HCl pH 8.0. Fractions of 1.5 ml were collected and analyzed for purity by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Coomassie brilliant blue staining.

2.4. Antibody preparation

Tat antisera were raised in pfd:Hollander rabbits against the GST-Tat proteins electroeluted from the obtained affinity-purified GST-TatA, GST-TatB and GST-TatC preparations. The fusion proteins were injected twice with an interval of two weeks. After a further two weeks, blood was sampled. After an overnight incubation at 4 °C, the obtained sera were cleared by centrifugation (5 min, 150 × *g*). Next, the Tat antisera were affinity purified using GST-TatA, GST-TatB and GST-TatC proteins immobilized on *N*-hydroxy-succinimide-activated Sepharose HiTrap column (Amersham Biosciences) following the manufacturer's recommendations.

2.5. SDS–PAGE and Western blot analysis

Proteins were separated by SDS–PAGE and visualized by Coomassie Brilliant Blue or by Western blotting and immunodetection with specific antibodies against the proteins in combination with a suited alkaline phosphatase-conjugated goat anti-rabbit antibody (Sigma) and the chemiluminescent substrate CDP-Star™ (Western Star Kit, Tropix).

2.6. Membrane protein extraction

Wild-type *S. lividans* TK24 was precultured in 5 ml phage medium at 27 °C with continuous shaking at 300 rpm for 48 h. The mycelium was subsequently homogenized and inoculated (2%) in NM medium. After 24 h of growth, the mycelium was harvested by centrifugation and the cells were lysed in a French Pressure cell. After removal of the cell debris by centrifugation (20 min, 12 000 × *g*), four times 5 ml cell lysate

was centrifuged for 2 h at 100 000 × g. The sedimented membranes were each resuspended in 2.5 ml buffer, either in 10 mM Tris–HCl, pH 8.0, containing 500 mM KCl, in 100 mM Na_2CO_3, pH 11.0, in 4 M urea or 10 mM Tris–HCl, pH 8.0, containing 1% Triton X-100. Upon incubation for 15 min at 4 °C, the samples were recentrifuged for 2 h at 100 000 × g. The supernatant was collected and the pellet of each sample was resuspended in 2.5 ml buffer containing 10 mM Tris–HCl, pH 8.0, and 1% Triton X-100, followed by an additional incubation of 15 min at 4 °C. Equal amounts were analyzed by SDS–PAGE, Western blotting and immunodetection with Tat-specific antibodies.

2.7. Purification of S. lividans Tat complexes

S. lividans TK24 [pIJ2587TatABC] was precultured in 5 ml phage medium for 48 h. After homogenization of the mycelium, 2 ml was used to inoculate 100 ml NM medium. After 24 h of growth, the mycelium was harvested by centrifugation. Isolation of the cytoplasmic and membrane fractions from *S. lividans* and subsequent extraction of membrane proteins by the addition of detergent solution to the latter fraction was performed as described previously [15].

Prior to applying the cytoplasmic proteins or the solubilized membrane proteins on an ion-exchange column, the buffer of the samples was changed to 50 mM NaH_2PO_4, pH 5.0, 0.2% Triton X-100 (anion-exchange column) or 50 mM Tris–HCl, pH 7.0, 0.2% Triton X-100 (cation-exchange column) using gel filtration on a PD-10 column (Amersham Biosciences). The resulting sample was then applied either to a 3 ml pre-equilibrated HiTrap™ SP-FF column (Amersham Biosciences) or to a HiTrap™ Q-HP column. Bound proteins were eluted with 50 mM NaH_2PO_4, pH 5.0, 0.2% Triton X-100 or 50 mM Tris–HCl, pH 7.0, 0.2% Triton X-100 using a linear salt gradient from 0 to 3 M NaCl in 10 column volumes. From both columns, fractions enriched in TatA, TatB or TatC, as shown by SDS–PAGE and Western blotting followed by immunodetection with Tat antibodies, were pooled, concentrated and transferred to the gel filtration buffer (50 mM NaH_2PO_4, pH 7.0, 150 mM NaCl, 0.2% Triton X-100) similarly as described above. The concentrated sample was finally applied to a pre-equilibrated HiPrep™ Sephacryl™ S-300 HR gel filtration column (Amersham Biosciences). Protein elution was followed by monitoring the OD_{280}. Eluted fractions were screened for the presence of Tat components by SDS–PAGE of the TCA-precipitated (10% final concentration) proteins and Western blotting using Tat antibodies. Elution positions of marker proteins (HMW Gel Filtration Calibration Kit, Amersham Biosciences) were used to estimate the molecular mass of eluted Tat complexes.

3. Results

3.1. Localization of the different Tat subunits in S. lividans

For the localization of the Tat components in *S. lividans*, we generated polyclonal antisera against *S. lividans* TatA, TatB and TatC by immunizing rabbits with purified GST-Tat proteins. The obtained antisera were then affinity-purified and tested for their specificity by Western blotting analysis. The TatA antiserum recognized a single protein of 16 kDa in total cell lysates from wild-type *S. lividans* TK24 cells, while the TatB and TatC antisera recognized proteins of 19 and 33 kDa, respectively. No cross-reactivity of the affinity-purified Tat antisera was thus observed.

Fractionation of the *S. lividans* cell lysate into a cytoplasmic and membrane fraction [15] was performed for localization of the TatA, TatB and TatC components. Proteins present in the total cell lysate, the cytoplasmic and membrane fraction of wild-type *S. lividans* were separated by SDS–PAGE followed by Western blotting and immunodetection with the Tat antisera (Fig. 1). As expected, the TatC component, having 6 putative transmembrane anchors, was only detected in the membrane fraction of *S. lividans*. Interestingly, *S. lividans* TatA and TatB, having one putative N-terminal transmembrane anchor, were identified both in the membrane as well as in the cytoplasmic fraction. Scanning of the films was used to estimate the percentage of cytoplasmically localized TatA and TatB. Approximately 49% and 36% of TatA and TatB, respectively, were found to reside in the cytoplasm. These experiments suggest that TatA and TatB can be present in *S. lividans* in two different states, as a soluble cytoplasmic protein and as a membrane-associated protein. As seen in Fig. 1, both forms have an identical molecular mass demonstrating that the cytoplasmic localization of TatA and TatB is not resulting from proteolytical cleavage of several amino acids from the N-terminus, containing the transmembrane anchor (amino acids 5–22 for TatA and TatB; predicted by the TMHMM v2.0 software).

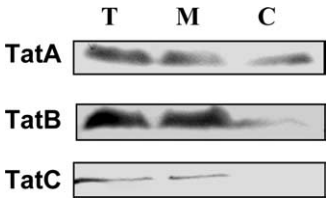

Fig. 1. Localization of *S. lividans* TatA, TatB and TatC. Equal amounts of total cell lysate (T), solubilized membrane proteins (M) and cytoplasmic proteins (C) prepared from *S. lividans* wild-type were separated on a 15% SDS–PAA gel. The Tat proteins were visualized after Western blotting using Tat-specific antisera and a chemiluminescent detection procedure.

3.2. Extraction of the Tat proteins from the cytoplasmic membrane

Once it was clear that TatA and TatB could be present as soluble cytoplasmic proteins in *S. lividans*, we wanted to investigate how stably their membrane-associated forms were integrated in the membrane. To this purpose, membrane fractions [15] isolated from wild-type *S. lividans* were treated with high salt, sodium carbonate, urea and a detergent (Triton X-100). High salt, sodium carbonate and urea are assumed to extract peripheric membrane proteins, whereas Triton X-100 is generally used to extract integral membrane proteins. Proteins extracted by the respective solutions and proteins remaining in the membrane after treatment were separated by SDS–PAGE followed by Western blotting and immunodetection with the TatA, TatB and TatC antisera. Fig. 2 shows that about 36%

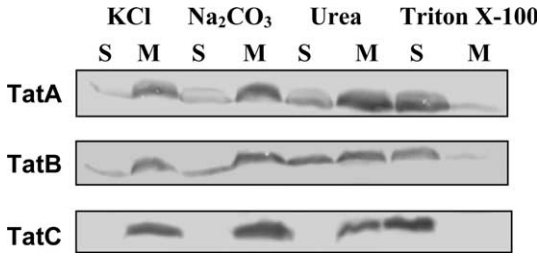

Fig. 2. Membrane extraction experiments. Membrane fractions from *S. lividans* wild-type were isolated and treated with 10 mM Tris–HCl, pH 8, 500 mM KCl, with 100 mM Na_2CO_3, with 4 M urea or with 10 mM Tris–HCl, pH 8, 1% Triton X-100. The solubilized proteins (S) and the membrane pellet (M) were separated on a 15% SDS–PAA gel. The Tat proteins were visualized after Western blotting using Tat-specific antisera and a chemiluminescent detection procedure.

of TatA and TatB could be extracted using high salt and sodium carbonate. When membranes were treated with urea, about 54% of membrane-associated TatA and TatB could be extracted, whereas the detergent Triton X-100 extracted almost all TatA and TatB from the membrane. These experiments suggest that TatA and TatB can be membrane-associated in two manners, loosely associated as a kind of peripherical membrane protein and stably inserted as an integral protein. As expected, TatC could only be extracted from the membrane after treatment with detergents.

3.3. Structural organization of the Tat proteins in S. lividans

3.3.1. Overproduction of TatA, TatB and TatC in S. lividans. In order to investigate in which complexes TatA, TatB and TatC participate, the respective *tat* genes were simultaneously overexpressed in *S. lividans*. To this purpose, the *tatAC* operon and the *tatB* gene under the control of the native regulatory sequences were cloned into pIJ2587 resulting in pIJ2587TatABC.

To test the plasmid-born overproduction of TatA, TatB and TatC, *S. lividans* wild-type and *S. lividans* [pIJ2587TatABC] were cultured in liquid medium. Equal amounts of cytoplasmic proteins and 0.2% Triton X-100 solubilized membrane proteins prepared from *S. lividans* wild-type and *S. lividans* [pIJ2587TatABC] were separated by SDS–PAGE and analyzed by Western blotting and immunodetection with the Tat antisera (data not shown). Obtained results indicate that in *S. lividans* [pIJ2587TatABC] all Tat components accumulated to levels that were a factor two higher than to those in wild-type *S. lividans* cells. Also the level of cytoplasmically localized TatA and TatB in *S. lividans* [pIJ2587TatABC] was higher than those in wild-type *S. lividans* cells.

3.3.2. Isolation of Tat complexes from the cytoplasmic membrane. Having established that in *S. lividans* [pIJ2587-TatABC] the amount of the Tat components in the cytoplasmic membrane as well as in the cytosol was significantly increased, membrane fractions of this strain were used as a starting point for the isolation of complexes in which these proteins participate.

Therefore, proteins present in the membrane fraction were first solubilized by treating the membranes with 0.2% Triton X-100 and then subjected to ion exchange and gel filtration chromatography column.

Since the p*I* value of the Tat components is significantly different (p*I*[TatA] = 9.52, p*I*[TatB] = 4.95, p*I*[TatC] = 4.64), two different purification strategies were selected. This allowed to investigate if the nature and composition of the purified Tat complexes were different when either TatA or TatB/TatC were specifically bound on an ion-exchange column.

TatA-mediated purification of Tat complexes. At pH 7, TatA is positively charged and can be captured by a cation-exchange column. In these conditions, TatB and TatC will have a net negative charge and can therefore only be purified when bound to TatA. Under these conditions, almost all TatB and TatC were retained on the column supposed to be bound to TatA (data not shown). Elution fractions containing TatA were further analyzed by gel filtration chromatography. The resulting chromatogram is shown in Fig. 3. Western blotting analysis showed that TatA and TatB together with TatC were found in a complex with an apparent molecular mass of 585 kDa. In addition, a smaller complex containing TatA and TatB with an apparent molecular mass of 235 kDa was identified. In

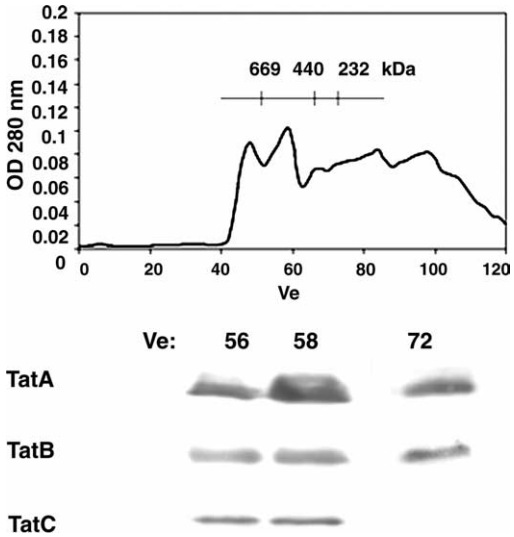

Fig. 3. TatA-mediated purification of Tat complexes. Solubilized membrane proteins from *S. lividans* [pIJ2587TatABC] were applied on a HiTrap SP column. TatA-containing fractions from the HiTrap SP column were pooled, concentrated and subjected to gel filtration chromatography on a Sephacryl S-300 column. The 280 nm absorbance of the column eluant was plotted and eluted fractions, precipitated with 10% TCA, were screened for the presence of TatA, TatB and TatC by SDS–PAGE, Western blotting and chemiluminescent detection using Tat-specific antisera. The elution volume (V_e) of standard proteins is indicated above the column profile. Standard proteins used are thyroglobulin (669 kDa), ferritin (440 kDa) and catalase (232 kDa).

independent purification procedures, the molecular mass of the TatABC complex was very similar, whereas that of the TatAB complex varied between 200 and 400 kDa.

TatB/TatC-mediated purification of Tat complexes. When solubilized membrane proteins are in a buffer at pH 7, TatB and TatC are negatively charged and can be captured by an anion-exchange column. In these conditions, TatA will have a net positive charge and can only be purified when bound to TatB or TatC. Under these conditions, a significant amount of TatA was found to be retained on the column (data not shown). The TatB/TatC-containing fractions were then pooled and subjected to gel filtration chromatography. Using this approach, obtained results were quite similar as for the TatA-mediated purification strategy. TatB and TatC were found to coelute with TatA to form a complex with an apparant molecular mass of 585 kDa. A second complex containing only TatA and TatB with a molecular mass varying between 200 and 400 kDa, was found.

3.3.3. Isolation of Tat complexes from the cytoplasm. After we had seen that the Tat proteins were able to assemble in hetero-oligomeric complexes in the cytoplasmic membrane, we investigated whether the soluble TatA and TatB proteins were also present in the cytoplasm as a part of a complex. To this purpose, cytoplasmic proteins were isolated from *S. lividans* [pIJ2587TatABC] and subjected to ion exchange and gel filtration chromatography as described above. The TatA-mediated purification strategy demonstrated that TatA could be present in the cytoplasm as a monomer. In addition homo-oligomeric TatA complexes could be detected (data not shown). Also via the TatB-mediated purification strategy, no evidence for a cytoplasmic TatAB complex could be obtained. The majority

of cytoplasmic TatB seems to be present as monomer, although sometimes the presence of homo-oligomeric TatB complexes could be detected.

In summary, whereas two different high molecular mass Tat complexes, a TatABC and a TatAB complex, were found in the membrane of *S. lividans* [pIJ2587TatABC], the cytoplasmically localized TatA and TatB proteins were shown to be monomeric or to participate into homo-oligomeric complexes.

4. Discussion

The most interesting finding of this work is certainly the dual localization of TatA and TatB in *S. lividans*, notably as membrane-associated protein and soluble cytoplasmic protein. For both Tat components, the molecular mass of the cytoplasmic and membrane-associated protein is the same, indicating that the cytoplasmic form is not resulting from proteolytic cleavage of the membrane-associated form and consequently is a distinct state of the same protein. In *B. subtilis*, the TatAd protein was also shown to be present in the cytoplasm and was suggested to assist in targeting of twin-arginine preproteins to the translocase [16]. The physiological role of cytoplasmically localized TatA and TatB in *S. lividans* is, however, still unclear. A similar role as *B. subtilis* TatAd in preprotein targeting is the most probable hypothesis, but remains to be experimentally confirmed. It should however be noted that, as *B. subtilis* TatAd, *S. lividans* TatA and TatB are present as soluble cytoplasmic proteins which can form homomultimeric complexes [16]. In addition, when overproduced in *E. coli*, an abundant amount of *S. lividans* TatA and TatB was also present in the cytoplasm (data not shown).

Membrane protein extraction experiments showed that only a part of TatA and TatB is stably integrated in the cytoplasmic membrane. The other part could be extracted using high salt, sodium carbonate or urea, and is therefore only loosely associated with the cytoplasmic membrane as a kind of peripheric protein. Although these results were initially surprising, they support the idea of TatA and TatB being involved in protein targeting to the translocase. Peripheric TatA and TatB could then result from the association of both targeting factors with the cytoplasmic membrane.

The cytoplasmic localization of *S. lividans* TatB makes it an intriguing protein. In Gram-positive bacteria, a TatB-like protein has only been identified so far in *Streptomyces* and other *Actimomycetales*. In *S. lividans*, the protein was annotated previously as TatB because it was most similar to TatB-family proteins and because of its length. However, its cytoplasmic localization seems to be typical of TatA. Because of these findings, *S. lividans* TatB can be seen as a hybrid between TatA and TatB family proteins. A further support for this hypothesis is found in the presence of an FGP sequence at the border of the transmembrane and amphipathic helices, which is a combination of the FG and GP characteristic residues present in TatA and TatB proteins, respectively [17].

To date, the structural organization of purified Tat complexes from *E. coli*, *S.* Typhimurium and *A. tumefaciens* have been described [4]. So far, no data of purified Tat complexes of Gram-positive bacteria were reported. In the present study, we have purified a TatABC complex from an *S. lividans* strain coordinately overexpressing the known *tat* genes. The estimated molecular mass of this TatABC complex (585 kDa) was found to be slightly smaller than the apparent molecular mass of the *E. coli* TatABC complex (650 kDa) [2]. The TatA protein was also identified in a second complex together with the TatB protein. The estimated molecular mass of this TatAB complex varies somewhat between 200 and 400 kDa, which could refer to a looser association of the TatA component in this complex. Similarly, the apparent molecular mass of the *E. coli* TatAB complex was known to vary depending on the purification protocols (350–650 kDa). The purification of an *S. lividans* TatAB complex indicates direct protein–protein interactions between the TatA and TatB components. Whether direct protein–protein interactions are also present between TatA–TatC and TatB–TatC is subject of ongoing research. Tat complexes of similar composition and molecular size could also be purified from membrane fractions isolated from wild-type *S. lividans*, however the yield was significantly lower (data not shown). Though, this strongly suggests that the isolated Tat complexes are no artefacts resulting from overproduction of TatA, TatB and TatC. Notwithstanding the sequence diversity amongst the Tat proteins of different bacteria, similar Tat complexes were purified from *S. lividans* and Gram-negative proteobacteria. This suggests that, despite the observed differences of the Tat subunits, the final structural assembly of Tat components is widely conserved.

Nevertheless, the presence of cytoplasmically localized TatA and TatB in *S. lividans* was not shown in *E. coli* [18] and may suggest that the mechanism is more like in *B. subtilis*, where Tat proteins are also involved in preprotein targeting [16]. Our data agree with the idea of the *S. lividans* Tat system being a dynamic system in which the cytoplasmic TatA and TatB function to target preproteins to the translocase and the integral TatA and TatB proteins constitute together with TatC the actual translocase.

This report was a first step in the analysis of the *S. lividans* Tat pathway at a molecular level. Revealing the role of each Tat component in *S. lividans* in substrate binding and subsequent export and unravelling the working mechanism is subject of further research. This could eventually lead to modulation of this pathway to improve the secretion yield of recombinant proteins in *S. lividans* via the Tat pathway.

Acknowledgments: This work was supported by grants QLK-2000-00122 from the European Union, G.0271.98 from FWO and OT/00/37 from K.U.Leuven Onderzoeksfonds. N.G. is a postdoctoral fellow of K.U.Leuven Onderzoeksfonds (PDM/03/270). S.D.K. and W.V.D. are research fellows of IWT (Vlaams Instituut voor de bevordering van het Wetenschappelijk-Technologisch onderzoek in de industrie).

References

[1] Mori, H. and Ito, K. (2001) The Sec protein-translocation pathway. Trends Microbiol. 9, 494–500.
[2] Berks, B.C., Palmer, T. and Sargent, F. (2003) The Tat protein translocation pathway and its role in microbial physiology. Adv. Microb. Physiol. 47, 187–254.
[3] Yahr, T.L. and Wickner, W.T. (2001) Functional reconstitution of bacterial Tat translocation in vitro. EMBO J. 20, 2472–2479.
[4] Oates, J., Mathers, J., Mangels, D., Kuhlbrandt, W., Robinson, C. and Model, K. (2003) Consensus structural features of purified bacterial TatABC complexes. J. Mol. Biol. 330, 277–286.
[5] Schaerlaekens, K., Schierová, M., Lammertyn, E., Geukens, N., Anné, J. and Van Mellaert, L. (2001) Twin-arginine translocation pathway in *Streptomyces lividans*. J. Bacteriol. 183, 6727–6732.

[6] Van Mellaert, L. and Anné, J. (2001) in: Focus on Biotechnology. Volume I: Novel Frontiers in the Production of Compounds for Biomedical Use Gram-positive Bacteria for the Heterologous Production of Biopharmaceutical Compounds (Van Broekhoven, F., Shapiro, F. and Anné, J., Eds.), pp. 277–300, Kluwer Academic publishers, Dordrecht.
[7] Pierce, J.J., Robinson, S.C., Ward, J.M., Keshavarz-Moore, E. and Dunnill, P. (2002) A comparison of the process issues in expressing the same recombinant enzyme periplasmically in *Escherichia coli* and extracellularly in *Streptomyces lividans*. J. Biotechnol. 92, 205–215.
[8] Schaerlaekens, K., Van Mellaert, L., Lammertyn, E., Geukens, N. and Anné, J. (2004) The importance of the Tat-dependent protein secretion pathway in *Streptomyces* as revealed by phenotypic changes in *tat* deletion mutants and genome analysis. Microbiology 150, 21–31.
[9] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York.
[10] Korn, F., Weingärtner, B. and Kutzner, H.J. (1978) in: Genetics of the Actinomycetales. A Study of Twenty Actinophages: Morphology, Serological Relationship and Host Range (Freechsen, E., Tarnak, I. and Thumin, J.H., Eds.), pp. 251–270, Fisher G., Stuttgart.
[11] Van Mellaert, L., Dillen, C., Proost, P., Sablon, E., Deleys, R., Van Broekhoven, A., Heremans, H., Van Damme, J., Eyssen and H and Anné, J. (1994) Efficient secretion of biologically active mouse tumor necrosis factor alpha by *Streptomyces lividans*. Gene 150, 153–158.
[12] Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F. and Hopwood, D.A. (2000) Practical *Streptomyces* Genetics, John Innes Foundation, Norwich.
[13] van Wezel, G.P., White, J., Hoogvliet, G. and Bibb, M.J. (2000) Application of redD, the transcriptional activator gene of the undecylprodigiosin biosynthetic pathway, as a reporter for transcriptional activity in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*. J. Mol. Microbiol. Biotechnol. 2, 551–556.
[14] Studier, F., Rosenberg, A., Dunn, J. and Dubendorff, J. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. 185, 60–99.
[15] Geukens, N., Lammertyn, E., Van Mellaert, L., Schacht, S., Schaerlaekens, K., Parro, V., Bron, S., Engelborghs, Y., Mellado, R. and Anné, J. (2001) Membrane topology of the *Streptomyces lividans* type I signal peptidases. J. Bacteriol. 183, 4752–4760.
[16] Pop, O.I., Westermann, M., Volkmer-Engert, R., Schulz, D., Lemke, C., Schreiber, S., Gerlach, R., Wetzker, R. and Müller, J.P. (2003) Sequence-specific binding of prePhoD to soluble TatAd indicates protein-mediated targeting of the Tat export in *Bacillus subtilis*. J. Biol. Chem. 278, 38428–38436.
[17] Hicks, M.G., de Leeuw, E., Porcelli, I., Buchanan, G., Berks, B.C. and Palmer, T. (2003) The *Escherichia coli* twin-arginine translocase: conserved residues of TatA and TatB family components involved in protein transport. FEBS Lett. 539, 61–67.
[18] de Leeuw, E., Granjon, T., Porcelli, I., Alami, M., Carr, S.B., Mûller, M., Sargent, F., Palmer, T. and Berks, B.C. (2002) Oligomeric properties and signal peptide binding by *Escherichia coli* Tat protein transport complexes. J. Mol. Biol. 322, 1136–1146.