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Biochemical and Biophysical Research Communications 335 (2005) 973–982
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Functional analysis of TatA and TatB in *Streptomyces lividans* ☆

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Received 27 July 2005
Available online 9 August 2005

Abstract

Recently, genes encoding TatA, TatB, and TatC homologues were identified in *Streptomyces lividans* and the functionality of the twin-arginine translocation (Tat) pathway was demonstrated. Previously, we have shown that TatC is indispensable for Tat-dependent secretion in *S. lividans*. In the present work, we demonstrate that as TatB, *S. lividans* TatA is important but not essential for efficient secretion of xylanase C and tyrosinase. The results presented here indicate that in the presence of TatC, still partially functional translocation systems composed of TatAC or TatBC can be formed, suggesting that TatA and TatB have at least partially overlapping activities. However, the dissimilar effect caused by a *tatA* deletion or a *tatB* deletion on Tat-dependent secretion together with the fact that TatA cannot fully functionally substitute TatB and vice versa indicates that in *S. lividans* TatA and TatB are not functionally equivalent. Interestingly, soluble GST-tagged TatA and TatB were able to specifically bind Tat-dependent preproteins. The ability to bind Tat-dependent preproteins together with their cytoplasmic localization in *S. lividans* strongly suggests that both TatA and TatB, independently or associated, serve to recruit Tat-dependent preproteins to the translocase.

Keywords: *Streptomyces lividans*; Protein secretion; Twin-arginine translocation

The majority of bacterial proteins with an extracytoplasmic location are exported across the cytoplasmic membrane via the Sec pathway. These proteins, made as precursors with an amino-terminal signal peptide, are directed to the Sec apparatus in an unfolded or loosely folded conformation [1]. More recently, a fundamentally different pathway for protein transport across the cytoplasmic membrane was discovered in bacteria, archaea, and plant chloroplasts, called the twin-arginine translocation (Tat) pathway. This secretion pathway differs from the Sec pathway in its ability to transport proteins of varying dimensions that have already obtained some degree of tertiary structure in the cytoplasm prior to export (reviewed by [2,3]). Besides differences in the conformation state of the preproteins, additional features distinguish the Tat pathway from the Sec pathway. Tat-dependent substrates possess a cleavable N-terminal signal peptide containing an S/T-R-R-x-φ-φ consensus sequence that is essential for transport [4,5]. Furthermore, this translocation process is energized by the transmembrane proton electrochemical gradient [6] and not by nucleoside triphosphates, which are essential energy sources in the Sec-dependent transport.

The currently best-characterized Tat system is that of *Escherichia coli*. The minimal components of the *E. coli* Tat translocase are TatA, TatB, and TatC, which are exclusively inserted in the membrane. TatA and TatB

☆ *Abbreviations:* EDC, *N*-ethyl-*N*′-(3-dimethyl-amino-propyl)-carbodiimide hydrochloride; GST, gluthatione *S*-transferase; NHS, *N*-hydroxysuccinimide; PMSF, phenylmethylsulfonyl fluoride; SAM, self-assembled monolayer.

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.07.165

are sequence-related proteins that perform distinct functions in the Tat system. Whereas *E. coli* TatA is likely involved in pore formation, TatB seems to function as a mediator between substrate recognition by TatC and subsequent translocation [3]. Although the mechanism of Tat-dependent secretion is not fully understood, current evidence from in vitro experiments using isolated *E. coli* membranes suggests that substrates bind initially to the TatBC complex which acts as a receptor element of the Tat pathway [7,8]. In the presence of substrate and a proton-motive force, the TatA complex will be recruited to form an active translocation unit [8,9].

Recently, *tat* genes which encode homologues of the known Tat system components TatA, TatB, and TatC were identified in *Streptomyces lividans* and the functionality of this secretion pathway has been demonstrated [10,11]. Although the *E. coli* Tat components are all exclusively membrane-integrated proteins, *S. lividans* TatA and TatB could also be present as soluble cytoplasmic proteins [12]. Details about the function of these Tat components and the operating mechanism of this secretion system in *S. lividans* have yet to be resolved. In this work, we examined the role of TatA and TatB in *S. lividans*. A functional analysis of these proteins was carried out in order to evaluate if they are equivalent to their well-documented *E. coli* counterparts or exhibit a different role in Tat secretion in *S. lividans*, whether they are functionally exchangeable, and if the soluble forms of both proteins may be involved in preprotein binding.

Materials and methods

Strains, media, and growth conditions. Escherichia coli strain TG1 was used as host for cloning purposes [13]. 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 [14] supplemented with thiostrepton (50 μg/ml), apramycin (50 μg/ml) or kanamycin (50 μg/ml), if necessary, and grown at 27 °C with continuous shaking at 300 rpm for 48 h. After homogenization of the mycelium, the strains were inoculated in liquid NM medium [15]. For solid medium, MRYE was used [16] supplemented with thiostrepton (50 μg/ml), apramycin (50 μg/ml) or kanamycin (50 μg/ml), if applicable. Protoplast formation and subsequent transformation of *S. lividans* were carried out as described in [17].

DNA manipulations, and vector constructions. For all DNA manipulations standard techniques were followed [13,17]. 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 and plasmids used in this work are listed in Table 1.

For the construction of an *S. lividans* Δ*tatA* mutant, the *neo* gene of plasmid pBSKAN [10] was removed by *Pst*I/*Eco*RI digestion, replaced by the apramycin resistance gene *aac(3)IV*, and amplified with the primers apr1 and apr2 using the same restriction sites resulting in pBSAPR. Then, the surrounding regions of the *tatA* gene were cloned at both sites of *aac(3)IV*. Therefore, a 1034-bp upstream fragment was amplified by PCR with the primers del1 and del2 containing a *Bam*HI and a *Pst*I restriction site, respectively. A downstream fragment of 962 bp, encoding the *tatC* gene, was amplified using the primers del3 and del4, containing an *Eco*RI and an *Eco*RV restriction site, respectively. Both fragments were cloned in pGEM-T Easy and subsequently, isolated with the appropriate restriction enzymes. The *tatA* downstream fragment was inserted in the *Eco*RI/*Eco*RV-treated pBSAPR, followed by the insertion of the *tatA* upstream fragment in the *Bam*HI/*Pst*I-treated vector. Following this, the resulting plasmid was digested with *Eco*RI to allow the insertion of the 274-bp *tatA* promoter region, amplified by PCR using the primers tat1′ and del2′, between the *aac(3)IV* and the *tatC* gene. As such, *tatC* is still expressed from its native *tatA* promoter in the *S. lividans* Δ*tatA* mutant. From the resulting plasmid, an *Xba*I/*Hin*dIII fragment consisting of the *aac(3)IV* gene flanked by the upstream and downstream region of *tatA* was isolated and subsequently cloned in the corresponding sites of the *E. coli*/*Streptomyces* shuttle vector pGM160 [18], containing a temperature-sensitive *ori*, resulting in pGMΔtatA. *S. lividans* protoplasts were transformed with pGMΔtatA and after 48 h of growth of the selected transformants in phage medium, a temperature shift to 40 °C was carried out to promote homologous recombination of the plasmid-located DNA fragment with their chromosomal counterparts. The knockout of the *tatA* gene was confirmed by PCR and Southern blot analysis.

For complementation studies of the *S. lividans tatA* and *tatB* deletion mutants, we used pSETtatB [11] and pSETtatA. For construction of the latter plasmid, the integrative vector pSET152 [19] was digested with *Sac*I to remove the *aac(3)IV* gene and to replace it by the *neo* gene of pBSKAN [10]. Next, an *Xba*I/*Eco*RV fragment containing *tatA* and its promoter region obtained by PCR amplification using the primers tatA3′ and tatA5′ was ligated into the corresponding sites of the modified pSET152, resulting in the vector pSETtatA.

Activity assays. To measure xylanase activity, the dinitrosalicylic acid assay [20] was used. *S. lividans* derivatives were precultured in phage medium for 48 h. After homogenization, 1 ml of preculture was used to inoculate 50 ml NM medium. After 24 h of growth, the culture was centrifuged (10 min, 4000*g*, 4 °C) and the supernatant was diluted in the assay buffer. Next, the amount of reducing sugar was quantified as described. One unit of xylanase was defined as the amount of enzyme that produces 1 mg of reducing sugar in 10 min at 60 °C from a saturated xylan solution.

Tyrosinase activity was measured using the dopachrome assay procedure with L-dihydroxyphenylalanine (L-DOPA) as a substrate [21]. *S. lividans* derivatives were precultured in phage medium for 48 h. One milliliter of homogenate was used to inoculate 50 ml of tyrosinase production medium [21]. After 6 h of growth, extracellular culture fractions were obtained by centrifugation of the culture (10 min, 4000*g*, 4 °C) and diluted in the appropriate assay buffer. Following this, the amount of dopachrome was quantified in the supernatant as described [21]. One unit of tyrosinase is defined as the amount of enzyme that converts 1 μmol L-DOPA/min into dopachrome.

Protein purification. TatA, TatB, and TatC were expressed as GST fusion proteins as described [12]. To purify soluble GST-TatA and GST-TatB, *E. coli* TG1 cells producing GST-TatA or GST-TatB, respectively, 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 (SLM-aminco) at 69 mPa. The obtained cell lysates were subjected to ultracentrifugation (2 h, 100,000*g*, 4 °C). The supernatants were collected, incubated with 2 ml glutathione–Sepharose 4 Fast Flow matrix (Amersham Biosciences), and equilibrated with 50 mM Tris–HCl, pH 8.0, 0.5% Triton X-100. Next, the resulting samples were purified under standard conditions following the manufacturer's recommendations and analyzed for purity by sodium dodecyl sulfate–