Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1758 (2006) 443–451
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

The $TatA_d$ component of the *Bacillus subtilis* twin-arginine protein transport system forms homo-multimeric complexes in its cytosolic and membrane embedded localisation

Martin Westermann [b,1], Ovidiu I. Pop [a,1], Roman Gerlach [a,2], Thomas R. Appel [c], Wiebke Schlörmann [b], Sandra Schreiber [a], Jörg P. Müller [a,*]

[a] *Institut für Molekulare Zellbiologie, Klinikum der Friedrich-Schiller-Universität Jena, Drackendorfer Str. 1, D-07747 Jena, Germany*
[b] *Elektronenmikroskopisches Zentrum, Klinikum der Friedrich-Schiller-Universität Jena, Ziegelmühlenweg 1, D-07743 Jena, Germany*
[c] *Institut für Molekulare Biotechnologie, Beutenbergstr. 11, 07745 Jena, Germany*

Received 4 August 2005; received in revised form 27 February 2006; accepted 9 March 2006
Available online 7 April 2006

Abstract

The twin arginine translocation (Tat) system has the capacity to transfer completely folded proteins across the bacterial cytoplasmic membrane and the thylakoid membrane of plant chloroplasts. The most abundant TatA protein of this system has been suggested to form the protein conducting channel. Here, the molecular organisation of soluble and membrane embedded *Bacillus subtilis* $TatA_d$ was analysed using negative contrast and freeze-fractured electron microscopy. In both compartments, the protein showed homo-oligomerisation. In aqueous solution, $TatA_d$ formed homo-multimeric micelle-like complexes. Freeze-fracture analysis of proteoliposomes revealed self association of membrane-integrated $TatA_d$ independent from $TatC_d$, the second component of this transport system. Immunogold labelling demonstrated that the substrate prePhoD was co-localised with membrane-integrated $TatA_d$ complexes.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Protein export; Twin-arginine translocation; *Bacillus subtilis*; Complex formation; TatA

1. Introduction

Besides the Sec-dependent protein translocation system transporting unfolded proteins through the cytosolic membrane of the bacterial cell, the Tat-dependent system transports proteins frequently associated with cofactors prior translocation [1]. Therefore, it is believed that it has the capacity to export folded proteins [2]. Proteins destined for export by the Tat system are synthesised as precursors with a signal peptide containing an almost invariant twin-arginine sequence motif [3]. While the Sec-dependent transport system needs activity of translocation ATPase SecA, the Tat-dependent system requires neither stromal or cytosolic factors nor nucleoside triphosphates for translocation across the membrane [1,4,5]. As demonstrated first for the Tat-homologous system in the thylakoid membrane of plant chloroplasts, it is totally reliant on the membrane pH gradient [6,7]. In vitro translocation systems using *E. coli* components demonstrated that transport is energised exclusively by the transmembrane proton electrochemical gradient [8,9].

Three proteins have been shown to be essential for Tat function in both bacteria and chloroplasts. In *E. coli* TatA, TatB and TatC proteins are encoded in one operon [10–13]. A fourth unit, TatE, appears to be functionally redundant [14]. The sequence-related proteins TatA and TatB are anchored in the cytoplasmic membrane via the amino-proximal alpha-helical domain [10]. TatC contains six calculated transmembrane spanning domains [10,15]. Gouffi and co-workers proposed function-linked changes of TatA and TatC topologies for the mechanism of folded protein translocation [16].

* Corresponding author. Tel.: +49 3641 932 5671; fax: +49 3641 932 5652.
E-mail address: joerg.mueller2@med.uni-jena.de (J.P. Müller).
[1] Both authors contributed equally to this work.
[2] Current address: Institut für klinische Mikrobiologie, Immunologie und Hygiene der Friedrich-Alexander-Universität Erlangen-Nürnberg, Wasserturmstrasse 3-5, D-91054 Erlangen, Germany.

0005-2736/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2006.03.018

Currently the targeting of the twin-arginine precursors to the translocase unit and the structure of the Tat transport system is not completely understood. The molecular ratio of TatA: TatB: TatC in the cytosolic membrane of *E. coli* has been estimated to be about 40:2:1 [14,17]. The *E. coli* system showed various composition of proteins present in purified Tat complexes. A large complex of approximately 600 kDa containing TatABC proteins has been purified from detergent-solubilised *E. coli* membranes [2,18]. Bolhuis and co-workers have suggested that TatB and TatC proteins form a functional and structural unit of the twin-arginine translocase [2], while the membrane spans of TatC are the only determinants for the complex assembly [19]. A purified Tat complex with a molecular weight of 600 kDa contained varying levels of TatA indicating TatA may participate in a separate complex lacking TatB [2,20]. Sargent and co-workers purified a similar-sized TatA/B complex. An electron microscopically detected double layered ring structure of the TatA/B complex indicated that these proteins could form the protein-conducting channel [17]. Independently, overexpressed TatA protein formed a self-associated complex of 460 kDa indicating that no additional components are required for homo-oligomerisation of membrane-localised *E. coli* TatA [21]. The analysis of functional TatABC systems purified from *E. coli*, *Salmonella thyphimurium* and *Agrobacterium tumefaciens* showed that the complexes share a common structural feature [22]. A recent report of Gohlke and co-workers shows that *E. coli* TatA forms ring-shaped structures of variable diameter supporting the proposal that TatA forms the protein-conducting channel [23]. In the plant thylakoid membrane two types of Tat complexes have been identified: one consisting of cpTatC and Hcf106 (orthologues of bacterial TatC and TatB) and one consisting of Tha4 (the TatA orthologue) [24]. It has been suggested that Tha4 is required for the transport steps following precursor recognition [24,25].

While most bacterial and plant Tat systems contain three TatABC proteins, several bacterial and archaeal species miss a TatB-like protein [26,27]. Thus, at least one copy of a TatA homologue and one copy of a TatC homologue are required for a functional Tat pathway [10,13,15]. Blaudeck and co-workers suggest that in TatAC systems, the TatA protein represents a bifunctional component fulfilling both the TatA and TatB functions [28]. The *B. subtilis* genome encodes three TatA-like and two TatC-like proteins [26,29]. Despite the frequent presence of twin-arginines in the N-domain of signal peptides so far only two substrates were shown to be strictly transported Tat dependent. The secretion of prePhoD, a *B. subtilis* phosphodiesterase was dependent on the expression of the $tatA_d/tatC_d$ genes co-localised with *phoD* in one operon [30,31]. The second *tatA/tatC* pair ($tatA_y/tatC_y$) was required for the export of YwnB, obviously forming a second minimal Tat translocase unit [32]. In spite of its calculated membrane-spanning domain and beside its expected membrane-integrated localisation, $TatA_d$ was found also soluble in the cytosol [33]. Cytosolic $TatA_d$ showed sequence specific affinity for the twin-arginine motif of the prePhoD signal peptide elucidating its role as target site for pre-protein $TatA_d$ interaction. These features suggest that $TatA_d$ interacts prior to membrane integration with its pre-protein substrate and could therefore assist targeting of twin-arginine pre-proteins. Interestingly, besides being membrane-associated, *Streptomyces lividans* TatA and TatB proteins were also detected in a cytosolic localisation [34].

To get further insight in the function of TatA, we studied the structural features of the *B. subtilis* $TatA_d$ both soluble as well in its membrane-integrated localisation. Purified soluble $TatA_d$ formed multimeric assemblies of round micelle-like complexes. Absence of lipids argue for TatAd assembly prior to membrane insertion. $TatA_d$ reconstituted in liposomes formed distinct homo-multimeric structures. The substrate prePhoD was co-localised with reconstituted $TatA_d$. TatCd-containing proteoliposomes revealed that the protein neither contributed to $TatA_d$ complex formation nor showed multimerisation. The data presented in this work suggest that $TatA_d$ has a targeting function for its substrate prePhoD and forms the export channel for PhoD.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

E. coli strains TG1(pREP4) containing plasmids pQE9$tatA_d$, pQE60$tatC_d$ or pQE9phoDp were used to overexpress proteins [33,35]. *E. coli* strains were grown aerobically at 37 °C in TY medium [36]. As required, media were supplemented with ampicillin (100 μg/ml), kanamycin (40 μg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM) [37]. To induce the phoD operon *B. subtilis* 168 was grown in low phosphate defined medium (LPDM), as described [38]. *B. subtilis* strain 168(*pREP9*$tatA_d/C_d$) allowing IPTG-inducible overexpression of $tatA_d$ and $tatC_d$ [33] was grown in TY medium to mid-exponential phase and expression of tat genes was induced with 1 mM IPTG for one h. Membrane-free cell extract of *B. subtilis* was prepared as described [33].

2.2. Purification of His6-tagged proteins

His_6-$TatA_d$, $TatC_d$-His_6 and His_6-prePhoD were purified using *E. coli* strains TG1(pREP4, pQE9$tatA_d$), TG1(pREP4, pQE60$tatC_d$), TG1(pREP4, pQE9$phoD_p$) as described [33]. Purity and identity of the proteins was examined by SDS-PAGE and subsequent immunodetection as demonstrated previously [33].

2.3. Thin layer chromatography (TLC)

Lipids were extracted from 100 μl samples by adding 250 μl chloroform, 250 μl methanol and 125 μl 1 M KCl-solution. After vortex-mixing the phases were separated by centrifugation and the lower phase was collected. TLC was performed on 10×10 cm silica gel precoated glass plates with 2.5 cm preconcentration zone (Macherey and Nagel, Düren, Germany). The plates were predeveloped in solvent 1 (25:25:25:10:9 – by volume – methyl acetate:1-propanol:chloroform:methanol:0.25% KCl in water) and activated by heating at 110 °C for 10 min [34]. Lipid extracts were applied on the TLC plates as 1–8 μl aliquots using a hamilton syringe. After evaporation of the solvents by a stream of cold air the plate was developed in solvent 1 until the solvent front had run 4.7 cm out of the preconcentration zone. Plates were dried thoroughly by hot air, and developed to full length in solvent 2 (n-hexane: diethyl ether: acetic acid 75:23:2). After drying by hot air, the third development was carried out to full length using n-hexane.

The dried plate was stained by dipping (1 to 4 s) into an aqueous solution containing 10% copper sulphate and 8% phosphoric acid. Excessive staining solution was removed by placing the plate upright on paper towels for 30 s. Finally the plates were heated in an oven at 170 °C for 5–8 min in order to

develop dark-coloured lipid bands on white background. As controls, lipid standards had been prepared by dissolving commercially available lipids (Sigma-Aldrich) in 1,2-dichloropropane/1-propanol 2:1.

2.4. Freeze-fracture electron microscopy

Liposomes were concentrated by centrifugation and resuspended in PBS containing 15% (v/v) glycerol. Aliquots were enclosed between two 0.1 mm copper profiles as used for the sandwich double-replica technique. The sandwiches were rapidly frozen by plunging them into liquid propane, cooled by liquid nitrogen. Freeze-fracturing was performed in a BAF400T (BAL-TEC, Liechtenstein) freeze-fracture unit at −150 °C using a double-replica stage. The fractured samples were shadowed without etching with 2.0–2.5 nm platinum/carbon at an angle of 35°. The electron gun evaporation of platinum/carbon was controlled by a thin-layer quartz crystal monitor.

2.5. Negative staining electron microscopy

Soluble His_6–$TatA_d$ complexes purified via sucrose density gradient centrifugation were adsorbed to carbon coated 400 mesh copper grids, washed in distilled water and negatively stained with 2% uranyl acetate for 1 min.

2.6. Fracture labelling of prePhoD

For freeze-fracture immunogold labelling and subsequent electron microscopy the freeze-fracture replica were transferred to a digesting solution (2.5% SDS in 10 mM Tris buffer pH 8.3 and 30 mM sucrose) and incubated over night according to Fujimoto [39]. The replica were washed four times in PBS buffer and treated with PBS + 1% bovine serum albumine (BSA) for 30 min. Next, they were placed in PBS containing 0.5% BSA and monospecific antibodies against PhoD [33] (dilution 1:20) for 1 h. Subsequently, the replica were washed four times with PBS and placed on a 1:50 diluted solution of the second gold-conjugated antibody (goat anti-rabbit IgG with 10 nm gold, British Biocell International, Cardiff, UK) in PBS containing 0.5% BSA for 1 h. After immunogold labelling, the replica were immediately rinsed several times in PBS, fixed with 0.5% glutaraldehyde in PBS for 10 min at room temperature, washed 4 times in distilled water and finally picked onto Formvar-filmed copper grids for viewing in an EM 902 electron microscope (Zeiss, Oberkochen, Germany). Freeze-fracture micrographs were preferentially mounted with direction of platinium shadowing from bottom to top.

2.7. SDS-PAGE and Western blot analysis

Protein SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared as described by Laemmli [40]. After separation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Schleicher and Schüll) as described by Towbin [41]. Proteins were visualised using silver staining or monospecific antibodies against $TatA_d$, $TatC_d$ and alkaline phosphatase-conjugated goat anti-rabbit antibodies (SIGMA) according to the manufacturer's instructions.

2.8. Sucrose gradient density centrifugation

Linear sucrose gradients ranging from 5 to 25% in PBS with a total volume of 11 ml were generated in a SW40 polyallomer tube using a gradient maker. Proteins resuspended in 0.2 ml PBS to a concentration of 1 mg/ml were loaded onto the gradient and subsequently centrifuged at 200,000×*g* for 16 h. 1 ml samples were collected and processed further for immunoblot analysis. Molecular weight standards were prepared in the same way as the samples to 1 mg/ml in PBS, and loading 0.2 ml of the mixture was loaded on identically prepared gradients. Molecular weight standards were resolved on standard SDS-PAGE following staining with Coomassie Brilliant Blue.

2.9. Preparation of proteoliposomes

Purified His6-TatAd, TatCd-His6 and His6-prePhoD proteins were incubated with total membrane polar lipids at a protein–lipid ratio of 1:100 in 2 ml PBS (20 mM NaCl, 2.7 mM KCl, 1.3 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.3) containing 1% octyl-glucoside for 30 min at room temperature. Bio-Beads SM-2 (Bio-Rad) were then added at a concentration of 160 mg per ml. To release the non-incorporated proteins, liposomes were subjected to ultra-sonication and subsequently washed twice with PBS. Protein content of the proteoliposomes was detected by silver staining or western blotting.

3. Results

3.1. Soluble $TatA_d$ forms high molecular weight complexes

We previously demonstrated that $TatA_d$ specifically interacts with the signal peptide of prePhoD [33]. Sucrose gradient density centrifugation was used to get insight in the molecular organisation of soluble $TatA_d$ interacting with prePhoD. His_6-tagged $TatA_d$ was purified from *E. coli* TG1(pREP4, pQE9-*tatA*$_d$) by Ni^{2+}-NTA affinity chromatography. Despite its calculated membrane localisation, purification of $TatA_d$ yielded in highly pure hexahistidine-tagged protein both under denatured and native conditions following standard protocols. In order to study lipid association of the protein, lipid content of preparations were analysed using high resolution thin layer chromatography. Soluble His_6-$TatA_d$ which was purified under native conditions did not contain visible lipid contamination (Fig. 1, lane 1). In parallel, His_6-$TatA_d$ was purified from *E. coli* in presence of 8 M urea and 1% octylglucoside. This yielded in a protein preparation associated in bacterial lipids (Fig. 1, lane 2). The co-purified lipids reflected the composition of the bacterial membrane.

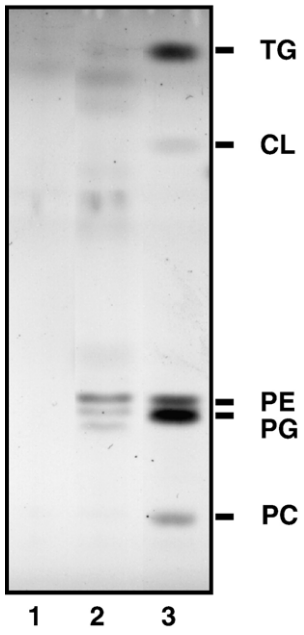

Fig. 1. Soluble $TatA_d$ does not contain lipids of the bacterial membrane. Thin layer chromatography was carried out using His_6-$TatA_d$ purified under native conditions (lane 1) or purified in presence of 8 M urea (lane 2). Similar amounts of protein (10 μg each) were loaded. For comparison lipid standards TG (triglyceride), CL (cationic lipids), PE (phosphatidylethanolamine), PG, (phosphatidylglycerol) and PC (phosphatidylcholine) (1 μg each) were loaded (lane 3).

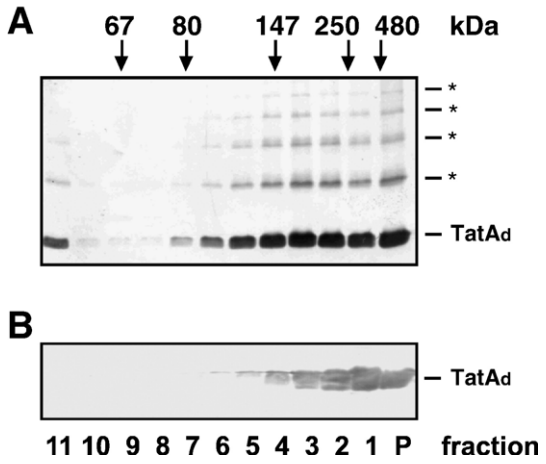

Fig. 2. Soluble $TatA_d$ forms homo-multimeric complexes. Distribution of His_6–$TatA_d$ across 5–25% sucrose density gradient. Sedimentation was carried out with purified $TatA_d$ (A). For comparison, a membrane-free cell lysate of *B. subtilis* strain 168(pREP9$tatA_d/C_d$) was sedimented under identical conditions (B). Gradients were fractionated to 11 fractions and the pellet and subsequently characterised via SDS-PAGE and Western blotting using specific $TatA_d$ antiserum. Sedimentation of marker proteins is indicated at the top.

Purified protein prepared under native conditions was separated on a linear 5–25% sucrose gradient. Individual fractions were assayed immunologically for the amount of $TatA_d$ (Fig. 2A). In order to calculate the molecular size of the molecules in the particular fractions, localisation of molecular weight standards were determined in sucrose gradients prepared under the same conditions. A significant fraction of $TatA_d$ was found in the pellet of the gradients. One peak of soluble $TatA_d$ complexes sedimented with an apparent size between 150 to 250 kDa, demonstrating the formation of large homo-oligomeric $TatA_d$ complexes. Only a minor fraction floated on top of the gradient indicating the presence of single molecules. Interestingly, immunodetection of $TatA_d$ after SDS-PAGE showed a ladder of oligomeric complexes which were not completely dissolved during electrophoresis (Fig. 2A).

To study whether $TatA_d$ shows a similar complex formation in the natural host, membrane-free cytosolic proteins of *B. subtilis* 168(pREP9$tatA_d/C_d$) were fractionated via gradient sedimentation under essentially identical conditions. Most $TatA_d$ protein was identified in the bottom fractions of the gradient as well as in the pellet (Fig. 2B). This demonstrated that homo-oligomerisation of soluble $TatA_d$ occurs also in the natural host and is not an experimental artefact.

In parallel, oligomerisation of purified $TatA_d$ was characterised via gel chromatography using a Superdex 200 column. Most of the protein was eluted as a peak at a position in the chromatograph corresponding to an apparent molecular mass between 100 and 150 kDa ([33]; data not shown). Since the molecular mass of recombinant His_6-$TatA_d$ is 9.6 kDa, it can be concluded that soluble $TatA_d$ formed homo-multimeric complexes.

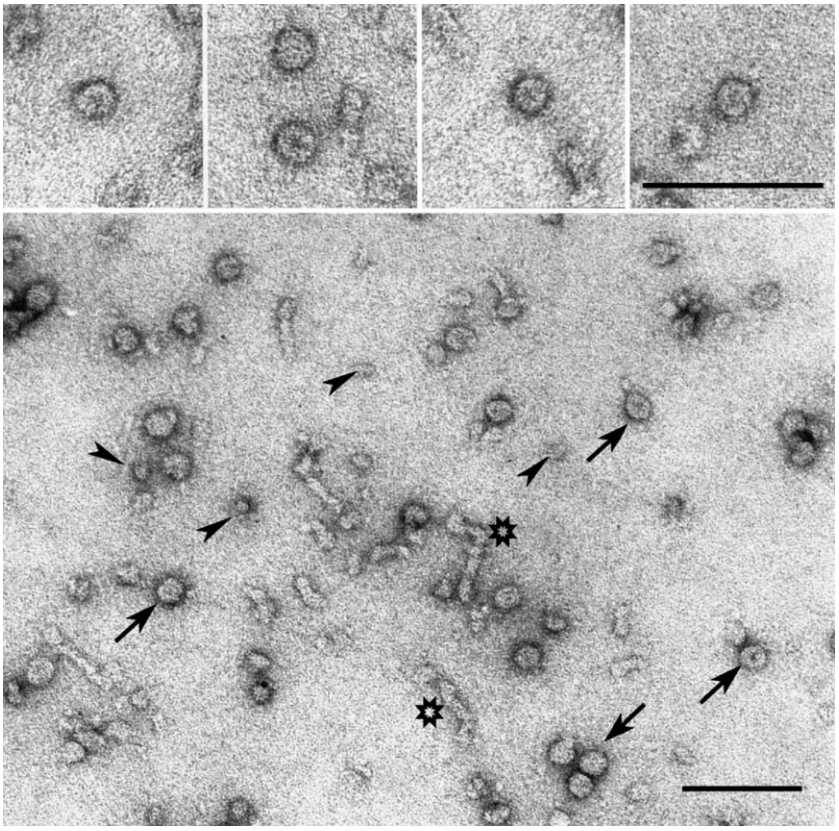

Fig. 3. Negative staining electron microscopy of soluble $TatA_d$ complexes. His_6–$TatA_d$ complexes with a molecular weight of 150–250 kDa were prepurified via sucrose density gradient centrifugation. They were adsorbed on carbon coated Formvar filmed copper grids and stained with 2% uranyl acetate. Small particles with diameter of about 12 nm (arrowheads), elongated particles of maximal 100 nm in length and 12 nm in diameter (asterisks) and large round particles with a diameter of 25 nm (arrows and detail views) were found. Scale bar represents 100 nm.

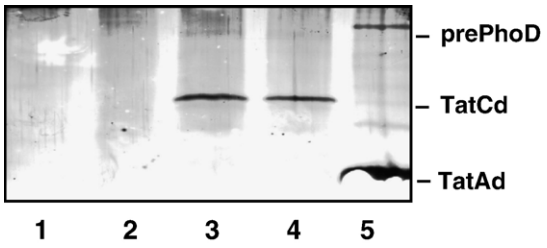

Fig. 4. Protein content of proteoliposomes. Purified proteins were reconstituted with bacterial lipids as indicated. The proteoliposomes were sonicated, washed and analysed for their protein content via SDS-PAGE and subsequent silver staining. Liposomes were prepared in presence of His_6–$TatA_d$ (lane 5), $TatC_d$–His_6 (lanes 3 and 4) or His_6–prePhoD (lanes 2, 4 and 5) or in absence of any protein (lane 1). Protein contents of proteoliposomes was analysed.

3.2. Structure of soluble homo-multimeric $TatA_d$ complexes

To obtain information about the structure of the protein, $TatA_d$ complexes with an apparent molecular weight between 150 and 250 kDa fractionated from sucrose density gradients were dialysed and subjected to negative contrast electron microscopy. His_6-$TatA_d$ was adsorbed on carbon-coated Formvar filmed copper grids and stained with 2% uranyl acetate. Two distinct classes of round particles could be observed. While the small structures had a diameter of about 12 nm (Fig. 3, arrowheads), the large class showed round particles with a diameter of 25 nm (Fig. 3, arrows). The latter had a pronounced staining in the centre indicating that particles had an aqueous centre. According to their size, $TatA_d$ complexes have an estimated oligomeric organisation unifying 15–25 molecules to the 12 nm particles and 50–75 molecules to the 25 nm particles. Essentially similar $TatA_d$ structures were observed when $TatA_d$ complexes were sedimented in presence of prePhoD (data not shown).

Besides round structures, vermicular structures with a diameter of 12 nm (Fig. 3, asterisks) and 25 nm (data not shown) were observed. Their presence indicated that both classes of particles had the tendency to pile up. Extended vermicular structures were found in the TatA fractions obtained from the pellet of the sucrose gradients (data not shown).

3.3. $TatA_d$ forms a homo-multimeric complex in reconstituted membranes

Due to the formation of homo-multimeric complexes and its abundance in the Tat translocase unit *E. coli* TatA was postulated to be involved in the formation of the protein transporting channel [21]. Its three-dimensional structure confirmed this thesis [23]. In order to analyse this feature for *B. subtilis* $TatA_d$, we next studied the possibility to form self-associated multimeric structures in the hydrophobic bilayer of the bacterial membrane. Proteoliposomes were formed by co-reconstitution of purified His_6-$TatA_d$ with total *E. coli* polar lipids. Membrane integration of $TatA_d$ was

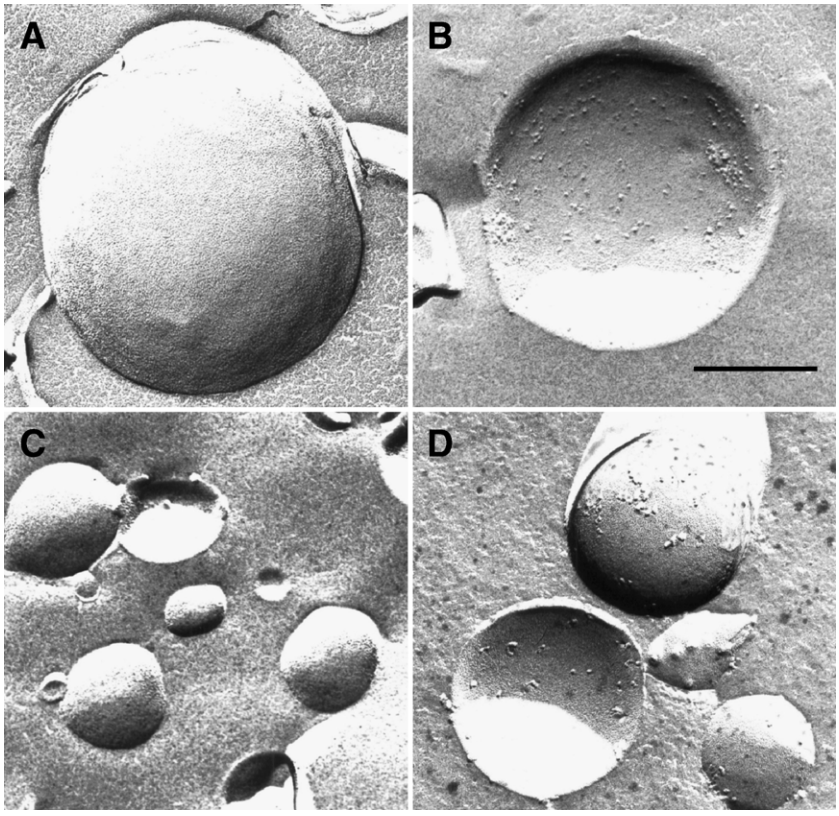

Fig. 5. Membrane-integrated $TatA_d$ forms oligomeric complexes. Electron micrographs of freeze-fractured proteoliposomes containing His_6–$TatA_d$ (B, D), $TatC_d$–His_6 (C). For comparison, liposomes were prepared in the absence of proteins (A). Purified proteins were reconstituted in bacterial membrane lipids and subsequently freeze-fractured. Scale bar represents 250 nm.

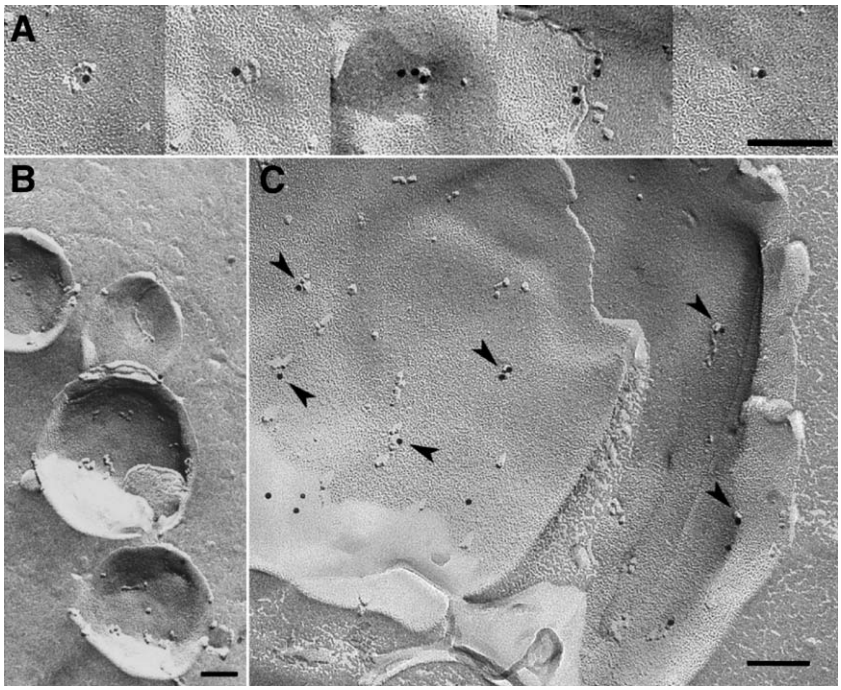

Fig. 6. Co-localisation of prePhoD with $TatA_d$ by freeze-fracture cytochemistry. Proteoliposomes formed in presence of His_6–$TatA_d$ (B) or His_6–$TatA_d$ and His_6–prePhoD (A and C) were freeze-fractured and subsequently labelled with PhoD-specific antibodies and 10 nm gold-conjugated secondary antibodies. Scale bar represents 100 nm.

monitored by analysing the protein content of the membrane vesicles using SDS-PAGE and subsequent silverstaining of sonicated and washed proteoliposomes (Fig. 4). Proteoliposomes containing $TatA_d$ were identified by freeze-fracture analysis. While in the absence of $TatA_d$, phospholipid vesicles formed smooth membranes without intramembrane particles (Fig. 5A), $TatA_d$-containing freeze-fractured proteoliposomes showed visible intramembrane particulate structures (Fig. 5B). Thus, it can be concluded that these structures were formed by self-association of $TatA_d$. The approximate size of these membrane particles was about 10 nm. Infrequently, beside round 10 nm particles, some $TatA_d$ complexes were extended to long shaped vermicular and dumbbell-like structures (Figs. 5D and 6A).

Next, proteoliposomes were reconstituted with purified $TatC_d$. As monitored (Fig. 4, lanes 3 and 4), these vesicles contained a substantial amount of $TatC_d$, indicating the efficient integration of $TatC_d$ in the membrane. Freeze-fracturing of these membrane vesicles did not result in visible intramembrane particle structures, demonstrating absence or a low degree of oligomerisation of $TatC_d$ (Fig. 5C). Reconstitution of the Tat complex using a $TatA_d$: $TatC_d$ ratio of 10:1 did result in structures identical to the $TatA_d$-containing proteoliposomes (data not shown).

3.4. Membrane-integrated $TatA_d$ has affinity for prePhoD

The prerequisite of $TatA_d$ to fulfil a function as translocator would be the interaction of membrane-integrated $TatA_d$ for its substrate prePhoD. In order to prove this hypothesis we next studied the localisation of prePhoD reconstituted in $TatA_d$ proteoliposomes. Membrane vesicles were formed in presence of $TatA_d$ and prePhoD, washed and further processed for freeze-fracture immuno-cytochemistry. In presence of $TatA_d$ an efficient integration of prePhoD could be observed (Fig. 4, lane 5). Essentially similar retention of prePhoD was observed when proteoliposomes were reconstituted with $TatA_d$ and $TatC_d$ (data not shown). In presence of $TatC_d$ or absence of any Tat protein, almost no prePhoD was retained in the liposomes (Fig. 4, lanes 4 and 2). Freeze fracture immunogold labelling of prePhoD in $TatA_d$ containing proteoliposomes revealed that membrane-associated prePhoD was co-localised with the particulate $TatA_d$ structures (Fig. 6C). Interestingly, most immunogold particles were detected in close proximity to symmetric 10 nm particles as well as elongated filamentous $TatA_d$ particles (Fig. 6A). $TatA_d$ vesicles untreated with prePhoD were hardly labelled with immunogold particles, elucidating the specificity of prePhoD antibodies (Fig. 6B and Table 1). No immunogold labelling of prePhoD was observed in protein-free phospholipid vesicles incubated with prePhoD under identical conditions (Table 1).

Table 1
Efficiency of immunogold labelling of prePhoD in liposomes reconstituted with $TatA_d$, $TatA_d$ and prePhoD or no protein

Liposomes containing	prePhoD labelling (labels/μm^2)
No protein	1.01±0.2
$TatA_d$	1.22±0.2
$TatA_d$ and prePhoD	16.0±4.0

Immunogold labels were counted from randomly selected areas of freeze fractured membranes.

4. Discussion

Due to its selective affinity to prePhoD, cytosolic $TatA_d$ was suggested to be involved in the targeting of its substrate [33]. In order to elucidate the nature of $TatA_d$ mediating this interaction, we analysed the molecular organisation of $TatA_d$ in aqueous solution and in reconstituted membranes. In solution, the protein formed homo-oligomeric micelle-like assemblies. Besides the complex formation in aqueous solutions, $TatA_d$ formed multimeric complexes also in proteoliposomes. Membrane-embedded $TatA_d$ showed affinity for its substrate prePhoD. Since the presence of $TatC_d$ was not a prerequisite for membrane complex formation, we suggest that $TatA_d$ is actively involved in substrate recognition as well as in the formation of the protein translocating channel of this Tat transport system. $TatC_d$ did not show multimeric structures nor affected $TatA_d$ complex formation. The function of $TatC_d$ in recognition of the prePhoD–$TatA_d$ complex will be addressed elsewhere.

A recently described special feature of the Tat system of *B. subtilis* was the partial cytosolic localisation of $TatA_d$ [33]. As demonstrated above, soluble $TatA_d$ associates in homo-oligomeric round particles with a size of about 12 or 25 nm in diameter as well as in elongated particles of 12 nm in diameter. How can its cytosolic localisation be explained? TatA proteins are amphiphilic molecules consisting of a calculated transmembrane segment followed by an amphiphatic helix and a hydrophilic C-terminus. To maintain its cytosolic localisation soluble $TatA_d$ assembles in micelle-like structures: The round 12 nm particles are likely to be protein micelles consisting of a single $TatA_d$ layer exposing the hydrophilic C-terminus to the aqueous cytosol. The hydrophobic N-terminus of the molecules is forming a nonpolar interior of the complex. The additionally observed filamentous elongated particles with 12 nm in diameter could be interpreted as $TatA_d$ molecules aggregated as cylinder-micelle-like structures. The large round 25 nm particles with a pronounced staining in the middle indicating an aqueous centre are likely rings of cyclic cylinder-micelle structures. The shape of the negative stained particles does not necessarily reflect the complex formation under physiological conditions, but it explains the soluble state of this amphiphatic protein. We recently demonstrated that the double-arginine containing signal peptide of prePhoD is specifically interacting with $TatA_d$ [33]. The hydrophobic centre of the observed 12 nm protein micelles could accommodate the calculated hydrophobic α-helical domain of the PhoD signal peptide. The C-terminal region of $TatA_d$, rich in negative charged amino acid residues could undergo polar interaction with the double-arginine motif. We are currently pursuing this line of research further.

Urea-mediated purification of $TatA_d$ resulted in a protein preparation with a high content of membrane lipids obviously removing the membrane-associated protein fraction as well. Since purification of soluble $TatA_d$ complexes carried out under native conditions in absence of detergents did not contain a substantial amount of lipids it can be suggested that oligomeric $TatA_d$ complexes are formed prior to membrane association.

In order to calculate the number of molecules forming soluble $TatA_d$ complexes, purified protein was separated via gel filtration and sucrose density centrifugation. Both experimental approaches demonstrated homo-multimeric assemblies of the protein. Soluble complexes showed particles with an apparent molecular weight of 150 to 250 kDa, reflecting a number of at least 15 $TatA_d$ molecules per unit. Soluble wild type $TatA_d$ expressed in the gene donor *B. subtilis* strain showed also self-association. The molecular weight of these assemblies was even higher than using purified His_6-tagged $TatA_d$. Differences in molecular size of the assemblies between native $TatA_d$ to the purified His_6-tagged protein might be due to the preparation of membrane-free cell extracts from *B. subtilis* and the His_6-modification of the other. Currently it cannot be excluded that $TatA_d$ assembles with additional cytosolic factors. As shown previously by freeze-fracture cytochemistry, most immunogold-labelled $TatA_d$ was localised in oligomeric clusters in the cytosol [33]. This observation further confirms the in situ self-assembly of the protein in the cytosol in its natural host.

Besides the complex formation in an aqueous environment, $TatA_d$ formed homo-multimeric assemblies in the lipid bilayer of reconstituted bacterial membranes. Round, currently not further classified intramembrane particles with an average size of 10 nm could be observed in parallel with vermicular and dumbbell-like structures in freeze-fractured membranes. As demonstrated for *E. coli* TatA, the membrane accommodates the hydrophobic domain of the protein [21]. The 10 nm assemblies are expected to form a structure where the hydrophobic α-helix of the molecules mediates the contact to the phospholipid bilayer, while their amphiphatic domain forms a hydrophilic centre. A similar structural feature was recently suggested by Gohlke and co-workers [23]. Reconstituted $TatA_d$ complexes do not necessarily result in physiologically active translocation units. The use of $TatA_d/C_d$ containing energised proteoliposomes for in vitro translocation of prePhoD was unsuccessful so far (our unpublished data), but oligomerisation of membrane-integrated $TatA_d$ provides further evidence that TatA actively contributes to the formation of a protein translocation channel, which would mediate transmembrane transport of Tat substrates.

Affinity of soluble $TatA_d$ to prePhoD was studied previously using co-immunoprecipitation. Neither a cytosolic nor a Sec-dependent transported protein could be co-purified via $TatA_d$ [33]. Co-localisation of $TatA_d$ particles with prePhoD in reconstituted proteoliposomes demonstrates affinity for its substrate in its membrane-embedded localisation, the prerequisite for mediation of the translocation. Since proteoliposomes which were reconstituted with $TatC_d$ alone did not show visible particle formation, it can be concluded that $TatC_d$ does not self-assemble to large oligomeric complexes. This observation is in agreement with data obtained from *E. coli*, where it has been shown that $TatC_d$ is not the abundant component of the TatABC complex [21] and does not contribute to the formation of the protein transport channel. Furthermore, neither prePhoD nor $TatC_d$ affected multimerisation or particulate shape of $TatA_d$ assemblies. Taken together, it can be concluded that $TatC_d$ is (i) not forming homo-multimeric membrane-integrated complexes,

(ii) not affecting the mode of $TatA_d$ oligomerisation and (iii) probably not significantly contributing to the formation of a protein-conducting channel. $TatC_d$ might be involved in the recognition of the $TatA_d$–prePhoD-complex.

In the proposed model for Tat-dependent protein translocation in *E. coli* and in the plant thylakoid system, TatA assembles with TatC–TatB complexes transiently after interaction with the Tat substrate [24,25,42] or different sized TatA complexes are present in the membrane [23]. The different stoichiometry of Tat proteins in purified complexes suggested that the composition of the Tat translocase might vary depending on the size of the substrate to be exported [17,18,21]. The Tat substrates are translocated in a folded conformation while maintaining the pH gradient at the membrane [43]. Therefore, a flexible Tat translocation unit allowing efficient gating is a prerequisite to maintain the pH gradient at the membrane upon ongoing protein translocation. The study of the three-dimensional structure of *E. coli* TatA showed classes of ring-shaped structures ranging from 85 to 130 Å in which the internal channels are large enough to accommodate known Tat substrate proteins [23]. Reconstituted $TatA_d$ complexes with a diameter of 10 nm could mediate the transport of their substrate prePhoD.

The prerequisite for the translocation of prePhoD is the targeting to and integration into the lipid bilayer of the membrane. Demonstration of prePhoD-$TatA_d$ complexes in the cytosol and the membrane indicates that $TatA_d$ could trigger this process. Topology changes shown for *E. coli* TatA by Gouffi and co-workers might assist this necessary transfer event [16]. It is currently not understood, if $TatA_d$, after mediating the targeting and the transport of prePhoD, is recycled to the cytosol or stays in the membrane. Short half-life of $TatA_d$ in *B. subtilis* indicates that it is, once integrated into the membrane, rapidly degraded by host-specific proteases (our unpublished data). Thus, it can be concluded that $TatA_d$ is faced to a one way use and subsequently degraded from the membrane. By using an in vitro approach has been recently demonstrated by Shanmugham and co-workers that Tat-preproteins interact tightly with a model membrane consisting of only phospholipids. Based on our we demonstrate that presence of $TatA_d$ is the prerequisite for membrane interaction of prePhoD [44].

Taken together, the data presented here and before [33] demonstrate that $TatA_d$ forms homo-multimeric assemblies with affinity for its substrate prePhoD both in cytosolic and membrane localisation. Mechanistically, we favour a model where soluble $TatA_d$ complexes recognise prePhoD and mediate its transport to the membrane. Here the prePhoD–$TatA_d$ complex is recognised by $TatC_d$ and mediates the integration of the heteromeric complex into the membrane (our unpublished data). Upon reorganisation of the complexed $TatA_d$ it could form the protein export channel for its substrate.

According to our recent finding and data demonstrated above, the *B. subtilis* TatAC transport system acts fundamentally different compared to the TatABC systems described in *E. coli* and plant thylakoids [45]. While in the bacterial TatABC systems specialised cytosolic chaperones are involved in recognition and targeting of substrates [46–49], $TatA_d$ might be engaged for this function in the *B. subtilis* $TatA_d$/$TatC_d$ system. Absence of substrate co-factor association of the substrate could be the explanation for this difference.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft. We thank Anne Ulrich, Karl-Jürgen Halbhuber and Walter Richter for many helpful discussions.

References

[1] B.C. Berks, F. Sargent, T. Palmer, The Tat protein export pathway, Mol. Microbiol. 35 (2000) 260–274.
[2] A. Bolhuis, J.E. Mathers, J.D. Thomas, C.M. Barrett, C. Robinson, TatB and TatC form a functional and structural unit of the twin-arginine translocase from *Escherichia coli*, J. Biol. Chem. 276 (2001) 20213–20219.
[3] B.C. Berks, A common export pathway for proteins binding complex redox cofactors? Mol. Microbiol. 22 (1996) 393–404.
[4] E.H. Manting, A.J. Driessen, *Escherichia coli* translocase: the unravelling of a molecular machine, Mol. Microbiol. 37 (2000) 226–238.
[5] M. Müller, Twin-arginine-specific protein export in *Escherichia coli*, Res. Microbiol. 156 (2005) 131–136.
[6] R.M. Mould, C. Robinson, A proton gradient is required for the transport of two lumenal oxygen-evolving proteins across the thylakoid membrane, J. Biol. Chem. 266 (1991) 12189–12193.
[7] K. Cline, W.F. Ettinger, S.M. Theg, Protein-specific energy requirements for protein transport across or into thylakoid membranes. Two lumenal proteins are transported in the absence of ATP, J. Biol. Chem. 267 (1992) 2688–2696.
[8] T.L. Yahr, W.T. Wickner, Functional reconstitution of bacterial Tat translocation in vitro, EMBO J. 20 (2001) 2472–2479.
[9] M. Alami, D. Trescher, L.F. Wu, M. Müller, Separate analysis of twin-arginine translocation (Tat)-specific membrane binding and translocation in *Escherichia coli*, J. Biol. Chem. 277 (2002) 20499–20503.
[10] F. Sargent, S.P. Ballantine, P.A. Rugman, T. Palme, D.H. Boxer, Reassignment of the gene encoding the *Escherichia coli* hydrogenase 2 small subunit — identification of a soluble precursor of the small subunit in a hypB mutant, Eur. J. Biochem. 255 (1998) 746–754.
[11] F. Sargent, N.R. Stanley, B.C. Berks, T. Palmer, Sec-independent protein translocation in *Escherichia coli*. A distinct and pivotal role for the TatB protein, J. Biol. Chem. 274 (1999) 36073–36082.
[12] J.H. Weiner, P.T. Bilous, G.M. Shaw, S.P. Lubitz, L. Frost, G.H. Thomas, J.A. Cole, R.J. Turner, A novel and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins, Cell 93 (1998) 93–101.
[13] E.G. Bogsch, F. Sargent, N.R. Stanley, B.C. Berks, C. Robinson, T. Palmer, An essential component of a novel bacterial protein export system with homologues in plastids and mitochondria, J. Biol. Chem. 273 (1998) 18003–18006.
[14] R.L. Jack, F. Sargent, B.C. Berks, G. Sawers, T. Palmer, Constitutive expression of *Escherichia coli* tat genes indicates an important role for the twin-arginine translocase during aerobic and anaerobic growth, J. Bacteriol. 183 (2001) 1801–1804.
[15] M.R. Yen, Y.H. Tseng, E.H. Nguyen, L.F. Wu, Sequence and phylogenetic analyses of the twin-arginine targeting (Tat) protein export system, Arch. Microbiol. 177 (2002) 441–450.
[16] K. Gouffi, F. Gerard, C.L. Santini, L.F. Wu, Dual topology of the *Escherichia coli* TatA protein, J. Biol. Chem. 279 (2004) 11608–11615.
[17] F. Sargent, U. Gohlke, E. DeLeeuw, N.R. Stanley, T. Palmer, H.R. Saibil, B.C. Berks, Purified components of the *Escherichia coli* Tat protein transport system form a double-layered ring structure, Eur. J. Biochem. 268 (2001) 3361–3367.
[18] E. De Leeuw, T. Granjon, I. Porcelli, M. Alami, S.B. Carr, M. Muller, F. Sargent, T. Palmer, B.C. Berks, Oligomeric properties and signal peptide binding by *Escherichia coli* Tat protein transport complexes, J. Mol. Biol. 322 (2002) 1135–1146.

[19] D. Mangels, J. Mathers, A. Bolhuis, C. Robinson, The core TatABC complex of the twin-arginine translocase in *Escherichia coli*: TatC drives assembly whereas TatA is essential for stability, J. Mol. Biol. 345 (2005) 415–423.
[20] A. Bolhuis, E.G. Bogsch, C. Robinson, Subunit interactions in the twin-arginine translocase complex of *Escherichia coli*, FEBS Lett. 472 (2000) 88–92.
[21] I. Porcelli, E. De Leeuw, R. Wallis, B. van den Brink-van der Laan, B. de Kruijff, B.A. Wallace, T. Palmer, B.C. Berks, Characterization and membrane assembly of the TatA component of the *Escherichia coli* twin-arginine protein transport system, Biochemistry 41 (2002) 13690–13697.
[22] J. Oates, J. Mathers, D. Mangels, W. Kuhlbrandt, C. Robinson, K. Model, Consensus structural features of purified bacterial TatABC complexes, J. Mol. Biol. 330 (2003) 277–286.
[23] U. Gohlke, L. Pullan, C.A. McDevitt, I. Porcelli, E. deLeeuw, T. Palmer, H.R. Saibil, B.C. Berks, The TatA component of the twin-arginine protein transport system forms channel complexes of variable diameter, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 10482–10486.
[24] K. Cline, H. Mori, Thylakoid DeltapH-dependent precursor proteins bind to a cpTatC–Hcf106 complex before Tha4-dependent transport, J. Cell Biol. 154 (2001) 719–729.
[25] H. Mori, K. Cline, A twin arginine signal peptide and the pH gradient trigger reversible assembly of the thylakoid [Delta]pH/Tat translocase, J. Cell Biol. 157 (2002) 205–210.
[26] L.F. Wu, B. Ize, A. Chanal, Y. Quentin, G. Fichant, Bacterial twin-arginine signal peptide-dependent protein translocation pathway: evolution and mechanism, J. Mol. Microbiol. Biotechnol. 2 (2000) 179–189.
[27] K. Dilks, R.W. Rose, E. Hartmann, M. Pohlschröder, Prokaryotic utilization of the twin-arginine translocation pathway: a genomic survey, J. Bacteriol. 185 (2003) 1478–1483.
[28] N. Blaudeck, P. Kreutzenbeck, M. Müller, G.A. Sprenger, R. Freudl, Isolation and characterization of bifunctional *Escherichia coli* TatA mutant proteins that allow efficient tat-dependent protein translocation in the absence of TatB, J. Biol. Chem. 280 (2005) 3426–3432.
[29] J.D. Jongbloed, U. Martin, H. Antelmann, M. Hecker, H. Tjalsma, G. Venema, S. Bron, J.M. van Dijl, J. Müller, TatC is a specificity determinant for protein secretion via the twin-arginine translocation pathway, J. Biol. Chem. 275 (2000) 41350–41357.
[30] J.D. Jongbloed, H. Antelmann, M. Hecker, R. Nijland, S. Bron, U. Airaksinen, F. Pries, W.J. Quax, J.M. van Dijl, P.G. Braun, Selective contribution of the twin-arginine translocation pathway to protein secretion in *Bacillus subtilis*, J. Biol. Chem. 277 (2002) 44068–44078.
[31] J.M. van Dijl, P.G. Braun, C. Robinson, W.J. Quax, H. Antelmann, M. Hecker, J. Müller, H. Tjalsma, S. Bron, J.D. Jongbloed, Functional genomic analysis of the *Bacillus subtilis* Tat pathway for protein secretion, J. Biotechnol. 98 (2002) 243–254.
[32] J.D. Jongbloed, U.A.H. Grieger, M. Hecker, R. Nijland, S. Bron, J.M. van Dijl, Two minimal Tat translocases in *Bacillus*, Mol. Microbiol. 54 (2004) 1319–1325.
[33] O.I. Pop, M. Westermann, R. Volkmer-Engert, D. Schulz, C. Lemke, S. Schreiber, R. Gerlach, R. Wetzker, J.P. Müller, Sequence-specific binding of prePhoD to soluble TatAd indicates protein-mediated targeting of the Tat export in *Bacillus subtilis*, J. Biol. Chem. 278 (2003) 38428–38436.
[34] S. De Keersmaeker, L. Van Mellaert, K. Schaerlaekens, W. Van Dessel, K. Vrancken, E. Lammertyn, J. Anne, N. Geukens, Structural organization of the twin-arginine translocation system in *Streptomyces lividans*, FEBS Lett. 579 (2005) 797–802.
[35] O. Pop, U. Martin, C. Abel, J.P. Müller, The twin-arginine signal peptide of PhoD and the TatAd/Cd proteins of *Bacillus subtilis* form an autonomous Tat translocation system, J. Biol. Chem. 277 (2002) 3268–3273.
[36] J.P. Müller, Influence of impaired chaperone or secretion function on SecB production in *Escherichia coli*, J. Bacteriol. 178 (1996) 6097–6104.
[37] J. Müller, F. Walter, J.M. van Dijl, D. Behnke, Suppression of the growth and export defects of an *Escherichia coli* secA(Ts) mutant by a gene cloned from *Bacillus subtilis*, Mol. Gen. Genet. 235 (1992) 89–96.
[38] J.P. Müller, Z. An, T. Merad, I.C. Hancock, C.R. Harwood, Influence of *Bacillus subtilis* phoR on cell wall anionic polymers, Microbiology 143 (Pt. 3) (1997) 947–956.
[39] K. Fujimoto, SDS-digested freeze-fracture replica labeling electron microscopy to study the two-dimensional distribution of integral membrane proteins and phospholipids in biomembranes: practical procedure, interpretation and application, Histochem. Cell Biol. 107 (1997) 87–96.
[40] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[41] H. Towbin, T. Staehelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, Proc. Natl. Acad. Sci. U. S. A. 76 (1979) 4350–4354.
[42] H. Mori, E.J. Summer, K. Cline, Chloroplast TatC plays a direct role in thylakoid (Delta)pH-dependent protein transport, FEBS Lett. 501 (2001) 65–68.
[43] N.N. Alder, S.M. Theg, Energetics of protein transport across biological membranes. A study of the thylakoid DeltapH-dependent/cpTat pathway, Cell 112 (2003) 231–242.
[44] A. Shanmugham, H.W. Wong Fong Sang, Y.J.M. Bollen, H. Lill, Membrane binding of twin arginine preproteins as an early step in translocation, Biochemistry 45 (2006) 2243–2249.
[45] B.C. Berks, T. Palmer, F. Sargent, The Tat protein translocation pathway and its role in microbial physiology, Adv. Microb. Physiol. 47 (2003) 187–254.
[46] R.L. Jack, G. Buchanan, A. Dubini, K. Hatzixanthis, T. Palmer, F. Sargent, Coordinating assembly and export of complex bacterial proteins, EMBO J. 23 (2004) 3962–3972.
[47] J. Behrendt, K. Standar, U. Lindenstrauss, T. Bruser, Topological studies on the twin-arginine translocase component TatC, FEMS Microbiol. Lett. 234 (2004) 303–308.
[48] T. Brüser, C. Sander, An alternative model of the twin arginine translocation system, Microbiol. Res. 158 (2003) 7–17.
[49] T. Brüser, T. Yano, D.C. Brune, F. Daldal, Membrane targeting of a folded and cofactor-containing protein, Eur. J. Biochem. 270 (2003) 1211–1221.