Characterisation of the twin-arginine protein translocation pathway of *Bacillus subtilis*

Dissertation

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1 Introduction

1.1 The genus *Bacillus*

The genus *Bacillus* is one of the most ubiquitous and diverse group of bacteria, with species being spread in the soil and associated water sources such as rivers, coastal waters (seaside), and estuaries (Harwood, 1992). The representatives of the genus are rod-shaped, Gram positive bacteria, characterised by their ability to produce robust endospores in response to adverse environmental conditions. Although most bacilli are harmless saprophytes, some species are known pathogens of humans (*B. anthracis* and *B. cereus*) or animals (*B. anthracis, B. thuringiensis, B. popilliae*). As soil microorganisms, bacilli species secrete numerous enzymes, enabling them to degrade a variety of substrates and thus to succeed in a complex and continuously changing environment. The ability of bacilli to secrete large quantities of proteins directly into the growth medium, coupled with the possibility to grow in large scale fermentors and their established safety record, make them prime candidates as "Cell Factories" (Simonen & Palva, 1993; Braun *et al.*, 1999). Some species, including *Bacillus licheniformis, B. amyloliquefaciens* and *B. subtilis*, have already a long commercial history as GRAS (Generally Regarded as Safe) organisms. Secretory protein production in bacilli is a major biotechnological tool with a market of over $1 billion per year (see table 1) (van Dijl *et al.*, 2002).

The best studied *Bacillus* species is *Bacillus subtilis*, a soil bacterium used as model system for a large variety of industrial applications. Despite its apparent advantages, the use of *B. subtilis* for the production of heterologous proteins has been limited. One important limitation is the synthesis of several extracellular proteases which cause substantial degradation of secreted foreign proteins (Wu *et al.*, 1991; Harwood, 1992). Strains lacking all but one of these proteases showed a reduced protease activity with 99,5% and an improvement in the heterologous product stability (Wu *et al.*, 1991). A second important restriction in the use of *B. subtilis* for secretory protein production has been the limited knowledge about its secretion machinery (Harwood, 1992; van Dijl *et al.*, 2002). These problems can be resolved by a better understanding of its general physiology, particularly of the secretion apparatus.

In a medical context, this inoffensive microbe, which produces proteins involved in the biosynthesis of antibiotics, is a valuable model for the study of pathogenic bacilli, such as *B. anthracis* and *B. cereus*.

Products from *Bacillus* species		
Enzymes	Alkaline phosphatases	• Laundry detergents • Leather industry
	Amylases	• Starch conversion to high fructose corn syrup (HFCS) • Desizing of textiles • Paper industry • Detergents
	Cellulases	• Clearing solutions
	Glucanase	• Brewing industry
	Glucose isomerase	• Starch industry
	Neutral proteases	• Leather industry • Baking • Aspartame synthesis
	Xylanase	• Paper industry
Vitamins	Riboflavins; Folic acid	• Food industry • Pharmaceutical industry
Insecticides	*B. thuringiensis* crystal proteins	• Agriculture

Table 1. **Biotechnological products obtained using *Bacillus* species.**

1.2 Protein transport across the bacterial cytosolic membrane

1.2.1 Cellular compartmentation in bacteria.

Unlike the eukaryotic cells, prokaryots lack subcellular membrane mediated compartmentation. Still, a few compartments can be distinguished also in bacterial cells.

- The cytosol, is the organic matrix, which is the site for the most important biological processes in all living cells.

- The cytosolic membrane (or plasma membrane) surrounds the cytosol and is generally made of a phospholipid bilayer. The membrane usually embeds a large subset of structural proteins and enzymes. The cytosolic membrane has many important cellular functions: it ensures the cell integrity by maintaining the gradient between the concentrated cytosol and the outer medium, provides a stable site for different enzymatic reactions and contains different types of translocases (Madigan M.T. *et al.*, 2003).

- The cell wall is present in all bacteria, except *Mycoplasma* species. It has a different organization in Gram negative and Gram positive bacteria. In *E. coli*, as an example of Gram negative

bacteria, the cell wall consists of a single, thin layer of peptidoglycan (carbohydrate polymers cross-linked by peptides). In Gram positive bacteria like *B. subtilis*, the cell wall is much thicker, being made of multiple layers of peptidoglycan embedded with teichoic and teichuronic acids. The role of the cell wall is very important. Particularly, it maintains the shape and the stability of the cell (Madigan M.T. *et al.*, 2003).

- The outer membrane is present only in Gram negative bacteria. It consists of a lipid bilayer, but unlike the cytosolic membrane, it is not solely made of phospholipids. The external layer contains in addition polysaccharides and proteins. The outer membrane is also called lipopolysaccharide layer (LPS). Although the major function of the outer membrane is structural, one of its biological roles is to keep certain enzymes, which are present outside of cytosolic membrane, from diffusing away from the cell. Therefore, it forms a second membrane-bordered compartment called periplasm. Another property of the outer membrane is that it is frequently toxic to animals. The lipid portion of the LPS contains a toxin named Lipid A, which is responsible for most of the pathogenic effects associated with harmful Gram negative bacteria (Madigan M.T. *et al.*, 2003).

- The periplasm. In Gram negative bacteria, the periplasm is the space between the cytosolic membrane and the outer membrane. It has a gel-like consistency and is the action place of different hydrolytic enzymes, binding proteins involved in translocation events, and chemoreceptors (Madigan M.T. *et al.*, 2003). The estimated protein content of the *E. coli* periplasm, for example, ranges between 4% and 16% of the total cellular protein (Nossal & Heppel, 1966; Ames *et al.*, 1984).

In Gram positive bacteria, due to the lack of an outer membrane, a real, membrane-enclosed periplasmic space cannot be accounted. Though, an important number of proteins were constantly detected in the protoplast supernatant fraction (PSF) of different bacilli. For example, approximately 9% of the total cellular protein content of *B. subtilis*, has been found in the PSF (Pooley *et al.*, 1996). In *B. subtilis*, proteins retained in this cell compartment include DNases, RNases, proteases (Babe & Schmidt, 1998), enzymes involved in the synthesis of peptidoglycan, and cell wall hydrolases (Blackman *et al.*, 1998) that are involved in cell wall turnover during cell growth, cell division, sporulation, and germination. Some cell wall bound proteins (CWBP) such as WapA and WprA possess cell wall retention signals consisting of a variable number of repeated domains in the mature part of the protein with affinity for components of the cell wall (Tjalsma *et al.*, 2000). Thus, Gram positive bacteria do have a functional periplasm, even though it is not confined to a periplasmic space (Fig. 1) (Merchante *et al.*, 1995).

Eubacteria export numerous proteins across the plasma membrane into either the periplasmic space (Gram negative species), or into the periplasm and growth medium (Gram positive species) (van Dijl *et al.*, 2002). Most proteins that are predetermined for transmembrane transport are generally synthesised as precursor proteins (pre-proteins) containing a signal sequence localised at the amino-terminal end. This sequence plays the function of a "ZIP code", leading the protein to a specific transport system in the cytosolic membrane (Blobel & Dobberstein, 1975). During, or shortly after the export event, the signal sequence is cleaved off by specialised enzymes called signal peptidases

(Dalbey *et al.*, 1997).

Various transport mechanisms have evolved to allow proteins to cross the membrane without disturbing its barrier function (de Keyzer *et al.*, 2003). All these mechanisms are active processes, requiring the hydrolysis of energy-rich molecules (e.g. ATP, GTP) or the proton motive force at the membrane (PMF). To date, there are a few fundamentally different export pathways described.

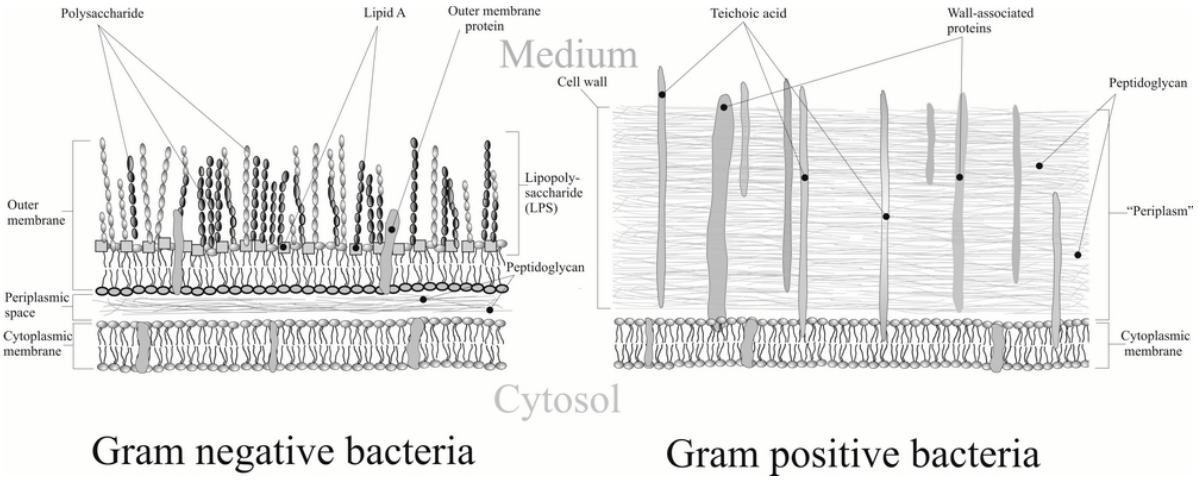

Figure 1. **Comparative view of the bacterial cell envelope of Gram negative and Gram positive bacteria.** (see text for details)

1.2.2 The ABC transport system.

The ABC (***A***TP-***B***inding ***C***assette) transport systems is one of the active transporters of the cell. It is widespread in archaea, eubacteria, and eukaryotes (Higgins, 1992). It is also known as the periplasmic binding protein-dependent transport system in Gram-negative bacteria and as binding lipoprotein-dependent transport system in Gram-positive bacteria. The chemical nature of the substrates handled by ABC transporters is extremely diverse, from inorganic ions to sugars and large polypeptides (Higgins & Linton, 2001). The protein substrates of this pathway lack a cleavable signal sequence but possess instead a carboxi-terminal peptidic secretion signal (Binet *et al.*, 1997; Thanassi & Hultgren, 2000).

The majority of bacterial ABC transporters import essential nutrients that are delivered to them by specific binding proteins (Nikaido & Hall, 1998). These proteins bind their substrates selectively and with high affinity, which is thought to ensure the specificity of the transport reaction. There are bacterial ABC transporters also serving as exporters. Particularly, they mediate the extrusion of toxic and hydrophobic molecules having a role to drug and antibiotic resistance of infectious pathogens (Young & Holland, 1999). Those molecules are transported from the cytoplasm across the inner and outer membrane without a periplasmic intermediate (Higgins, 1992).

A typical ABC transport system consists of two integral membrane proteins (permeases), two peripheral membrane proteins that bind and hydrolyze ATP, and a periplasmic substrate-binding

protein. In Gram positive bacteria, the periplasmic substrate-binding protein is replaced by a binding lipoprotein. The ATP-binding protein is the most conserved component, the membrane protein is less conserved, and the substrate-binding protein is the most divergent in terms of sequence similarity (Tam & Saier, Jr., 1993; Saurin & Dassa, 1994).

1.2.3 The general secretory pathway

The vast majority of secretory proteins are translocated via the general ***sec***retory (Sec) pathway. These proteins are synthesised in the cytosol as pre-proteins containing an N-terminal signal sequence that generally comprises of 16 to 30 amino acids (von Heijne, 1998). These signal sequences contain a positively charged N-terminal domain, a nonpolar α-helical hydrophobic H-domain, and a polar C-domain. The C-domain contains the cleavage sequence recognised by the enzyme signal peptidase. This cleavage sequence follows the general motif A-X-A in which A represents the amino acid alanine and X is another amino acid. Proteins designed to integrate into the cytosolic membrane usually do not have a cleavable signal sequence, and instead, their hydrophobic core functions as an anchor signal (de Keyzer *et al.*, 2003).

1.2.3.1 The general secretory pathway of *Escherichia coli*

In *Escherichia coli*, some proteins destined for transmembrane transport are targeted to the cytosolic membrane post-translationally via the molecular chaperone SecB. Many membrane proteins and some pre-proteins are targeted co-translationally to the membrane via the signal recognition particle (SRP) (Fekkes & Driessen, 1999; Herskovits *et al.*, 2000). Both pathways are targeting their substrates to the membrane at the same translocase unit (Valent *et al.*, 1998).

The Sec translocase is a large protein complex that mediates protein transport into or across the cytosolic membrane. It consists of the SecYEG protein complex, the cytosolic SecA, YidC and the SecDFYajC protein complex (Duong & Wickner, 1997; Prinz *et al.*, 2000; Scotti *et al.*, 2000). The SecYEG is a membrane embedded protein complex, which is forming the protein-conducting pore of the translocase. SecA exists as a dimer and functions as a molecular motor that couples the energy of ATP binding and hydrolysis to the stepwise translocation of pre-proteins (van der Wolk J. *et al.*, 1993). YidC is a membrane protein which works independently or together with SecYEG complex to insert proteins into the cytosolic membrane (Scotti *et al.*, 2000; Samuelson *et al.*, 2000; Driessen *et al.*, 2001). SecDFYajC is another membrane integrated protein complex with an accessory function. SecD and SecF are involved in the modulation of the catalytic cycle of SecA (Economou *et al.*, 1995) and in the maintenance of the proton motive force. YajC, the third component of this complex, is a membrane protein without known function (Arkowitz & Wickner, 1994; Duong & Wickner, 1997).

SecB mediated targeting. SecB is a cytosolic chaperone of 17 kDa (Kumamoto *et al.*, 1989) organised as a homotetramer (Muren *et al.*, 1999; Xu *et al.*, 2000). It has the function to mediate targeting of pre-proteins to the Sec translocase. After binding the substrate, SecB interacts with the SecYEG-bound SecA (Hartl *et al.*, 1990; de Keyzer *et al.*, 2003). While the mature part of the pre-

protein is bound by SecB, the signal peptide is interacting with SecA, increasing the overall SecB-SecA affinity (Fekkes *et al.*, 1997). Subsequently, the affinity between SecB and the pre-protein is weakened. Finally, ATP binds to SecA and the pre-protein is completely released from SecB. The latter is also set free by SecA, being ready to be recycled. This is the end point of SecB mediated targeting and the beginning of the translocation process itself (de Keyzer *et al.*, 2003).

SRP mediated targeting. This is a co-translational process, which is best studied for the endoplasmic reticulum of mammals (for a review see Keenan *et al.*, 2001). SRP-dependent targeting in prokaryotes follows the same mechanism as in eukaryotes, but the components involved have less complex subunit composition (Herskovits *et al.*, 2000). *E. coli* SRP is a nucleo-protein complex, consisting of a 4,5 S RNA and a 48 kDa GTP-ase named Ffh (Poritz *et al.*, 1990; de Keyzer *et al.*, 2003). It interacts specifically with the signal sequence of nascent pre-proteins and hydrophobic regions of nascent membrane proteins (Luirink *et al.*, 1992; Valent *et al.*, 1997). The SRP-ribosomal-nascent chain (RNC) complex interacts with the bacterial SRP receptor FtsY (Bernstein *et al.*, 1989), which can bind to the membrane via phospholipids (de Leeuw *et al.*, 2000). The FtsY-Ffh interaction facilitates GTP binding and subsequent GTP hydrolysis, which dissociates the RNC-SRP-FtsY complex. As a result, the RNC is released in the proximity of the translocase (de Keyzer *et al.*, 2003) being ready to be inserted into the membrane. However, the exact mechanism of this last step is not clearly understood. From this point, both SecB and SRP targeting pathways, converge and are continued with the translocation process through the SecYEG channel (Valent *et al.*, 1998).

The Sec translocation mechanism. The SecYEG complex together with SecA, forms the core unit of the Sec translocase. The driving forces of the translocation process are mediated by ATP hydrolysis (Chen & Tai, 1986) and the proton motive force (Daniels *et al.*, 1981; Enequist *et al.*, 1981; Zimmermann & Wickner, 1983; Bakker & Randall, 1984; Bayan *et al.*, 1993). These energy sources act at different stages (Tani *et al.*, 1989; Geller & Green, 1989; Tani *et al.*, 1990). However, the initiation of the translocation process is accomplished via ATP hydrolysis (Bakker & Randall, 1984). The SecYEG-bound SecA exist in an ADP-bound form and the binding of the pre-protein stimulates the ADP/ATP exchange (Lill *et al.*, 1989; Matsuyama *et al.*, 1990). The subsequent ATP hydrolysis results in the translocation of the signal peptide to the periplasmic face of the membrane (Schiebel *et al.*, 1991). Once the pre-protein enters the translocation channel, it is released from SecA. The latter can either dissociate from the membrane or rebind to the partially translocated protein. If rebinding of SecA occurs, another 2-2,5 kDa portion of the polypeptide is translocated, mediated by another ATP binding/hydrolysis cycle (van der Wolk *et al.*, 1997). Following this scenario, SecA can translocate the protein in a stepwise manner (van der Wolk *et al.*, 1997). The initiation of the translocation can be stimulated by the PMF, which affects binding and insertion of signal peptides into the cytosolic membrane (van Dalen *et al.*, 1999). After this step, the translocation is further driven by the PMF. It is even possible that PMF completes a translocation process in the absence of ATP (Tani *et al.*, 1990; Schiebel *et al.*, 1991; Driessen & Wickner, 1991). However, the PMF can promote the translocation efficiently only when SecA is no longer connected with the translocating protein (Schiebel *et al.*,

1991; van der Wolk *et al.*, 1997). Thus, if SecA concentration is high, the reaction is shifted to the ATP-dependent cycle and the necessity for PMF decreased (Yamada *et al.*, 1989; Shiozuka *et al.*, 1990). Despite of these observations, the ATP or PMF translocation modes are often complemental (de Keyzer *et al.*, 2003). The mechanism by which the energy from ATP hydrolysis is translated in pre-protein movement through the translocation pore is not yet understood.

After translocation, the pre-protein is processed. This means its signal peptide is cleaved off by specialised membrane embedded serine-proteases called signal peptidases (SPase) (Dalbey *et al.*, 1997). In *E. coli*, a type I SPase responsible for most of the processing activities is LepB. After being cleaved off from the mature protein, the signal peptide is degraded by a signal peptide peptidase (SPPase) such as SppA (Hussain *et al.*, 1982). If the SPPase is missing, the activity of the signal peptidase itself is decreasing, perhaps due to the accumulation of certain signal peptides (Bolhuis *et al.*, 1999).

1.2.3.2 The general secretory pathway in *Bacillus subtilis*

In *B. subtilis*, the Sec pathway is generally similar organised as in *E. coli*. However, a few differences have been observed. The signal peptides of *B. subtilis* pre-proteins have the same structure but they are usually 5 to 7 amino acids longer and more rich in hydrophobic residues than those of *E. coli* (von Heijne, 1985). The targeting of pre-proteins in *B. subtilis* is mediated by SRP, which consist of Ffh GTP-ase and a small cytoplasmic RNA (scRNA) (Tjalsma *et al.*, 2000). Since no SecB homologue is found in *B. subtilis*, it is supposed that SRP is involved in targeting of almost all pre-proteins and membrane proteins in this organism. Unlike *E. coli* SRP, which binds mainly nascent chains of membrane proteins, the SRP complex from *B. subtilis* binds also to the signal peptides of completely synthesised pre-proteins (Bunai *et al.*, 1996). This affinity is likely to be favored by the relative long and hydrophobic signal sequence of the pre-protein. The SRP-pre-protein complex binds the SRP-receptor FtsY, and is then targeted to the membrane (Tjalsma *et al.*, 2000). A remarkable characteristic of *B. subtilis* Ffh is that it binds to SecA with high affinity. The Ffh-pre-protein complex is up to 30-fold more efficiently recognised by SecA than the pre-protein alone (Bunai *et al.*, 1999). Deletion of *ffh* gene in *B. subtilis* reduced the protein export approximately 80% (Hirose *et al.*, 2000). Moreover, the depletion of SRP receptor FtsY resulted in accumulation of β-lactamase precursor in the cytosol of *B. subtilis* (Oguro *et al.*, 1996). These data suggest that both Ffh and FtsY are key components for targeting the general secretory pathway, and that SRP driven targeting is predominant in *B. subtilis.*

Another possible targeting factor for the secretory proteins in *B. subtilis* is CsaA. This is a cytoplasmic chaperone capable to complement the growth defect of *E. coli secA51*(ts), *dnaK756, dnaJ259 and grpE280* mutants (Müller *et al.*, 1992; Müller *et al.*, 2000). CsaA interacts with unfolded precursor and has affinity to SecA (Müller *et al.*, 2000). In response to *csaA* suppression, only few secretory proteins are reported to be lost. Therefore, the importance of CsaA for the general secretory pathway in *B. subtilis* has to be further investigated.

The translocase of *B. subtilis* is similar to that of *E. coli*. Homologues of all *E. coli* translocase components have been found in *B. subtilis*. ATPase SecA energize the translocation while SecYEG protein complex is forming the translocation channel. Notably, SecA shows a much weaker affinity to the SecYEG complex when compared with *E. coli* (Swaving *et al.*, 1999). Therefore, it has been suggested that it could fulfill an additional function as an export-specific chaperone (Herbort *et al.*, 1999). *SecD and secF* genes are naturally unified, their expression resulting in the production of the siamese twin SecDF, which is only necessary for maintaining a high secretion capacity (Bolhuis *et al.*, 1998).

The translocation mechanism follows the same hypothetical model described for *E. coli*. After emerging at the outer side of the cytosolic membrane, the signal peptides are cleaved off by signal peptidases. *B. subtilis* genome encodes multiple copies of type I signal peptidases (van Dijl *et al.*, 1992; Meijer *et al.*, 1995; Bolhuis *et al.*, 1996; Tjalsma *et al.*, 1997; Tjalsma *et al.*, 1998; Tjalsma *et al.*, 1999). Although they seem to have, at least partly, an overlapping function, the particular role of each of these signal peptidases is not completely elucidated. After processing, the signal peptide peptidase (SppA) degrade the cleaved-off signal peptides.

After leaving the translocase at the outer side of the membrane, the mature proteins have to fold into their native conformation. Since the *B. subtilis* periplasm is the physiological site of several proteases, rapid and correct folding is essential because even unfolded or partly unfolded proteins are very sensitive to proteases (Tjalsma *et al.*, 2000). The folding process is mediated by several extracellular folding catalysts, also called foldases. One of the *B. subtilis* active foldases is PrsA, a lipoprotein that is anchored to the outer side of the cytosolic membrane (Kontinen *et al.*, 1991; Kontinen & Sarvas, 1993). PrsA is essential for cell viability. A *prsA* strain is defective in secreting normal amounts of degradative enzymes, most probably due to the increased sensitivity of the secreted proteins to proteolysis (Kontinen *et al.*, 1991; Kontinen & Sarvas, 1993; Jacobs *et al.*, 1993). Although the folding catalysts are not part of the translocation machinery per se, they do have a role for the stability of the secreted products.

1.2.4 The Tat translocation pathway

The Tat pathway is another signal peptide-dependent translocation route. It is entirely different from the Sec pathway by means of energy requirements and substrate conformation during the translocation event. It mediates transport of folded proteins without the consumption of ATP. It is instead totally reliant on the membrane pH gradient (ΔpH). The signal peptides of Tat transported pre-proteins contain a characteristic, almost invariant twin-arginine motif (RR), hence the name of ***t***win-***a***rginine ***t***ranslocation (Tat) pathway. This pathway exists in most bacteria, archaea, plant thylakoids, and in mitochondria of some higher plants (Bogsch *et al.*, 1998).

1.2.4.1 D**pH translocation system in plant thylakoids.**

This transport pathway was first identified in plant chloroplasts, in which is involved in the import of stromal proteins into the thylakoid lumen. Initially, Cline and co-workers have discovered that the LHCP (***l***ight-***h***arvesting ***c***hlorophyll a/b ***p***rotein) insertion into thylakoid membrane, and the translocation of OE33, OE23 and OE17 (the 33-, 23-, and 17-kDa subunits of the oxygen-evolving complex) are transport processes mediated by different energy sources (Cline *et al.*, 1992). More precisely, the insertion of LHCP and translocation of OE33 is mediated by the thylakoidal Sec-like pathway with ATP hydrolysis, whereas the translocation of both OE23 and OE17 is Sec-independent but reliant on the pH gradient at the membrane. This Sec-independent transport route was thus called ΔpH-dependent transport pathway. In addition, the translocation of the latter two substrates occurred in absence of any soluble cofactors (Cline *et al.*, 1992). Comparative studies using chimera proteins demonstrated that the signal sequence of the pre-protein is important for directing the transport to either Sec- or ΔpH-dependent pathway (Henry *et al.*, 1994). A hybrid consisting of the signal sequence of the ΔpH-dependent OE23 and mature sequence of the Sec-dependent plastocyanin, was transported in a ΔpH-dependent manner (Henry *et al.*, 1994). Opposite, when the OE33 Sec signal sequence was linked with the mature sequence of ΔpH-dependent OE17, the hybrid protein was translocated in a Sec-dependent manner (Henry *et al.*, 1994). Thus, the signal peptides of those substrates were demonstrated to determine the transport path. A direct sequence comparison of the two types of signal peptides has shown that all known ΔpH-dependent chloroplast pre-proteins contain two consecutive arginine residues in the N-domain. The substitution of any of the two residues with lysine, blocked the ΔpH-dependent translocation route. None of the tested Sec-dependent sequences have this motif (Chaddock *et al.*, 1995; Henry *et al.*, 1997).

The first identified component of the ΔpH-dependent pathway was Hcf106 (for high-chlorophyll fluorescence) found in maize chloroplasts (Voelker & Barkan, 1995; Settles *et al.*, 1997). This is a membrane protein possessing a single transmembrane domain near the N-terminus, an amphiphatic helix, and an acidic hydrophilic domain. The amphiphatic and hydrophilic domains are oriented in the stroma, suggesting a receptor role (Settles *et al.*, 1997). A second protein involved in the ΔpH transport mechanism is the Tha4 (for ***th***ylakoid ***a***ssembly ***4***) whose gene sequence and

protein topology is related with *hcf106* gene and Hcf106 protein respectively (Walker *et al.*, 1999). The third component of this pathway was deduced from information regarding the bacterial transport machinery. It is called cpTatC (chloroplast TatC) and is predicted to be a membrane protein with six spanning domains, with its N- and C-terminus oriented in the chloroplast stroma (Bogsch *et al.*, 1998; Mori *et al.*, 2001). Quantitative immunoblotting measurements of pea thylakoids suggest a relative Tha4/Hcf106/cpTatC stoichiometry of 8:5:1 (Mori *et al.*, 2001). Although this molecular ratio does not provide any information about the structure of the translocase, it gives some insight about the importance of ΔpH transport components.

According to the endosymbiotic hypothesis, chloroplasts evolved from cyanobacteria. Subsequently, it was hypothesized that the transport of proteins into the thylakoid would employ a prokaryote-like mechanism (Smeekens *et al.*, 1990). The newly described ΔpH-dependent transport appeared to have eukaryotic origins because of its unique features (at the time, not known in prokaryotes) and because of the lack of similar substrates in cyanobacteria. This statement was in contrast with the Sec system, which transports proteins also found in cyanobacteria (Robinson & Klosgen, 1994; Cline & Henry, 1996). However, the discovery of bacterial homologues for all identified components of the ΔpH-dependent pathway, as well as the observed similarities between the signal peptides of some bacterial pre-proteins and plastidial ΔpH-dependent pre-proteins (von Heijne *et al.*, 1989), demonstrated that this system is a genuine prokaryotic innovation (Keegstra & Cline, 1999).

The mechanism of the ΔpH-dependent transport system is not yet understood. The ΔpH-dependent thylakoidal pre-proteins are synthesised in the cytosol, usually with a bipartite signal sequence necessary for the transport across both the chloroplast envelope and the thylakoidal membrane (Henry *et al.*, 1994). The twin-arginine containing signal peptide directs the pre-protein to the ΔpH-dependent translocase from the thylakoidal membrane. The Hcf106 and cpTatC components form a membrane complex with the role of receptor for the pre-proteins. In fact, cpTatC alone is likely to contain a Tat signal peptide binding site because complexes containing only Hcf106 do not bind the pre-protein (Cline & Mori, 2001). Interestingly, *in vitro* studies showed that pre-proteins are able to bind to the receptor complex also in the absence of the pH gradient. Thus, the pH gradient is not a prerequisite for the membrane recognition of the substrate (Ma & Cline, 2000).

After substrate recognition, Tha4 binds to the cpTatC-Hcf106 complex (Mori & Cline, 2002). In order to bind to this complex, Tha4 requires, beside a functional RR-pre-protein, also the pH gradient at the membrane. Tha4 has a transient affinity to the (Hcf106-cpTatC) complex. This affinity dissipates upon the completion of the translocation process. Such an assembly-disassembly system could explain, how the ΔpH system can form translocases to accommodate folded protein of varied sizes (Mori & Cline, 2002).

The existence of a ΔpH-dependent transport system, in addition to the known ATP-dependent pathway was surprising. Although it is possible that such redundancy provides a backup system for

the cellular transport systems, it is more likely that the different pathways exist to accommodate the specific assembly characteristics of the different groups of proteins that follow each pathway. For example, the chloroplast ΔpH system is able to transport Sec substrates if they are featured with twin-arginine signal peptides. In contrary, the Sec pathway can only hardly, if at all, transport an ΔpH-dependent substrate linked to a Sec signal peptide. This suggests that the ΔpH-dependent transport has evolved especially to transport proteins that, by their nature, have the tendency to fold tightly and are impossible to be threaded through a Sec pore (Creighton *et al.*, 1995; Keegstra & Cline, 1999).

1.2.4.2 The Tat system in *E. coli*

The *E. coli* Tat pathway is likely to be similar with the ΔpH-dependent pathway recently identified in chloroplasts (Wu *et al.*, 2000). In *E. coli*, as well as in many other bacteria, the homologues of chloroplast *hcf106* and *tha4* have been discovered (Bogsch *et al.*, 1998). Almost all efforts to describe the prokaryotic ΔpH protein transport system have been focused on *E. coli.*

1.2.4.2.1 Tat targeting motifs

Tat signal peptides are structurally similar with Sec signal peptides. They have the same tripartite organisation in which a positively charged amino-terminal region is followed by a hydrophobic H-region and then a hydrophilic C-region containing the cleavage site for the enzyme signal peptidase. However, in a few aspects the signal peptides of Tat substrates differ from their Sec analogues: i) Contain targeting information necessary for the recognition by the Tat translocase; ii) Possess a possible "Sec avoidance" motif localised in C- and H-domain; iii) Are frequently longer than their Sec counterparts (Fig. 2);

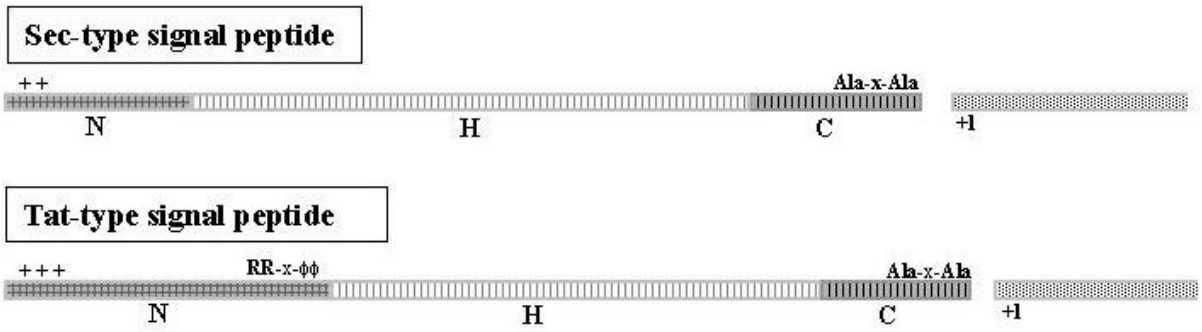

Figure 2. **The general structure of Tat- and Sec-type signal peptides** (see text for details).

Tat signal peptides contain a conserved amino acid sequence motif at the N-region/H-region boundary. For Gram-negative bacteria this motif can be defined as (S/T)-R-R-x-F-L-K. The two consecutive arginine residues are almost invariant. The frequency of the other motif residues is greater than 50% and x is normally a polar amino acid (Berks, 1996; Berks *et al.*, 2000; Stanley *et al.*, 2000). When in addition, other bacterial, archaeal or thylakoidal RR-signal peptides are taken in

consideration, the general motif can be defined as R-R-x-ϕ-ϕ, where ϕ is a hydrophobic residue (Tjalsma *et al.*, 2000).

Tat signal peptides frequently contain basic residues in the C-region. Such amino acids are almost always absent from the equivalent position of Sec signal peptides (von Heijne, 1986). This has led to the hypothesis that such residues may form a "Sec-avoidance" motif (Bogsch *et al.*, 1997), canalising the Tat substrates only to the Tat translocase. In addition, the H-regions of Tat signal peptides are significantly less hydrophobic than those of Sec targeting signals (Cristobal *et al.*, 1999). This feature could also act to avoid misrouting of the Tat-dependent pre-proteins to other targeting pathways such as SecA or SRP, whose substrates possess signal peptides with a more hydrophobic residue composition (Cristobal *et al.*, 1999).

Because the basic residues in the C-domain are not present in all Tat signal peptides (Berks, 1996) and because comparable levels of H-region hydrophobicity can re-route Sec substrates to SRP targeting pathway (Valent *et al.*, 1997), none of the above-mentioned "Sec-avoidance" theories can fully explain this hypothetical view of how particular substrates are canalised to the specific translocation units.

Tat signal peptides are with an average of 14 amino acids longer than their Sec counterparts (Berks, 1996). Most of this extension is attributed to the averaged 11 amino acids longer N-region (Cristobal *et al.*, 1999).

The high sequence conservation throughout the entire Tat consensus sequence suggests a broader Tat recognition signal, beyond the strict twin-arginine residues. Nevertheless, only the role of twin-arginine motif was subject to detailed investigation. Site-directed mutagenesis experiments have demonstrated the important role of this sequence in the translocation process (Halbig *et al.*, 1999; Gross *et al.*, 1999). However, some proteins are still exported Tat-dependently despite missing the twin-arginine motif. *E. coli* preSufI protein was transported Tat-dependent, if either the first or the second arginine was replaced by lysine. Interestingly, the export was abolished if the arginine residue was replaced with an alanine residue (Stanley *et al.*, 2000). For *E. coli* TMAO reductase TorA, the transport was not inhibited when the first, but not the second, arginine residue was substituted with a lysine residue (Buchanan *et al.*, 2001). Although very rare, unusual Tat motifs occur also naturally. The signal peptide of the TtrB subunit of *Salmonella enterica* tetrathionate reductase, contains a lysine-arginine motif but is nevertheless transported Tat-dependent (Hinsley *et al.*, 2001). More intriguing, the signal sequence of *E. coli* pre-pro-penicillin amidase, which comprises of two arginine residues separated by one asparagine (S-R-N-R-x-F-L-K) is translocated Tat-dependent (Ignatova *et al.*, 2002). These and other exceptions have switched the initial statement of "invariant RR motif" to "almost invariant RR motif". These examples of Tat signal peptides lacking a consensus arginine pair expand the range of sequences that can target proteins Tat-dependently. Moreover, some proteins synthesised with a twin-arginine signal peptide are not transported via the Tat pathway. All these exceptions, suggest that more profound studies of the Tat targeting motifs are necessary.

1.2.4.2.2 The substrates for twin-arginine translocation of *E. coli*

The vast majority of bacterial Tat substrates are proteins containing different types of redox-active cofactors. During cofactor acquisition in the cytosol, these substrates attain a folded conformation (Berks, 1996) and are subsequently translocated across the membrane. If the Tat pathway is blocked, the substrates fail to be exported and accumulate in the cytosol. The accumulated substrates already acquired their cofactors (Sargent *et al.*, 1998) suggesting that cofactor association is a prerequisite for export. This thesis has been demonstrated by Santini & co-workers for an *E. coli* strain that cannot export molybdoenzymes because it fails to produce its molybdopterin cofactor (Santini *et al.*, 1998). The lack of soluble cofactor results in cytosolic accumulation of unfolded and therefore, Tat-incompatible substrates.

Some proteins that do not associate with cofactors are transported via the Tat-dependent pathway. For example, the *E. coli* Tat pathway transports two amidase isoenzymes involved in the cleavage of the division septum (Stanley *et al.*, 2001). Some cofactorless proteins involved in pathogenicity are also transported Tat-dependent. For example, in *Pseudomonas aeruginosa* the Tat system proved essential for the export of phospholipases, proteins involved in pyoverdine-mediated iron-uptake, motility, and biofilm formation. In a rat lung model, a *tatC* mutant strain did not cause a heavy inflammatory host response compared with wild type, indicating that *tatC* mutant cells are attenuated for virulence (Ochsner *et al.*, 2002).

Finally, some heterologous substrates can be transported Tat-dependent, if fused with Tat signal peptides. For example, the Sec-dependent protein *b*-lactamase and the periplasmic P2 domain of signal peptidase (Niviere *et al.*, 1992; Stanley *et al.*, 2002), or the reporter protein GFP (Thomas *et al.*, 2001). All these heterologous proteins do not seem to require cofactors in order to be transported Tat-dependent. Therefore, the Tat system could be important for the industrial production of heterologous proteins which have proven incompatible with the Sec transport system (Palmer & Berks, 2003).

1.2.4.2.3 The components of *E. coli* twin-arginine translocation system

E. coli Tat system involves four proteins with calculated membrane-spanning domains (Settles *et al.*, 1997; Bogsch *et al.*, 1998; Weiner *et al.*, 1998). TatA/TatE and TatB are sequence-related proteins that are homologous to Tha4 and Hcf106 of the ΔpH-dependent thylakoid import pathway (Settles *et al.*, 1997; Sargent *et al.*, 1998; Weiner *et al.*, 1998; Chanal *et al.*, 1998; Walker *et al.*, 1999). They have one predicted amino terminal transmembrane helix, followed by an amphiphatic helix and an unstructured C-terminal tail (Settles *et al.*, 1997). The amphiphatic helices are localised at the cytoplasmic side of the membrane because they are accessible to proteases from cytosolic but not from periplasmic side of the cytosolic membrane (Porcelli *et al.*, 2002). For TatA and TatB proteins, both helices have been proved to be critical for their function, while the C-terminal domain is not important (Lee *et al.*, 2002). In addition, the "hinge" region between the two helices appears to be important for both proteins (Barrett *et al.*, 2003).

Gouffi and co-workers proposed a dual topology for TatA protein. During the translocation

process, the amphiphatic helix of this protein is supposed to flip in and out of the membrane. This molecular rearrangement is discussed to be the promoter of the translocation assembly (Gouffi *et al.*, 2003).

TatA has approx. 60% sequence similarity with TatE protein. The role of these two proteins is not elucidated but it is observed that they show a partial overlapping function. Only a *tatA/tatE* double mutant blocks the Tat-dependent export, whereas single mutants of either *tatA* or *tatE* slightly decrease the export efficiency (Sargent *et al.*, 1998). The *tatA* mutant shows, however, a stronger Tat inhibitory effect than the *tatE* mutant. Genetic studies confirmed this difference, indicating that *tatA* is transcribed and translated at approx. 100-fold higher level than *tatE* (Jack *et al.*, 2001).

TatB protein shares 25% amino acid identity with TatA and TatE (Palmer & Berks, 2003). An in-frame deletion of *tatB* is sufficient to abolish the export of endogenous Tat substrates (Ize *et al.*, 2002). Moreover, a *tatB* mutant cannot be complemented by additional gene copies of *tatA/tatE*. It is therefore believed that TatB fulfils a distinct function in the translocation process (Sargent *et al.*, 1998).

TatC, the fourth protein known to be involved in the Tat system of *E. coli*, is predicted to be an integral membrane protein with six transmembrane helices, with both N- and C-terminal ends localised in the cytosol (Sargent *et al.*, 1998; Yen *et al.*, 2002; Behrendt *et al.*, 2004). A recent topology study used PhoA and LacZ as markers for periplasmic and cytosolic localisation. The measured activity of those markers confirmed the predicted TatC topology with six membrane helices (Behrendt *et al.*, 2004). A previous topology study revealed only four transmembrane helices (Gouffi *et al.*, 2003). The TatC protein seems to be a key component of the Tat pathway (Bogsch *et al.*, 1998). It shows the highest degree of amino acid conservation. 21 amino acids are strictly conserved in eubacteria, 7 of these being also conserved in eukaryotic homologues. Most of these conserved residues are part of the cytosolic loops and have an essential role for protein export via the Tat pathway (Buchanan *et al.*, 2002; Allen *et al.*, 2002).

The *tatA*, *tatB* and *tatC* are the first three genes of the *tatABCD* operon (Weiner *et al.*, 1998), while *tatE* forms an independent transcriptional unit (Fig.3)(Berks *et al.*, 2000; Palmer & Berks, 2003). Both transcription units are expressed constitutively in *E. coli*, indicating a requirement for the Tat pathway under all growth conditions (Jack *et al.*, 2001). The *tatABC* genes in *E.coli* and other related bacteria are co-transcribed with *tatD*. This fourth gene of the *tat* operon is the most widespread Tat component, homologue sequences being found in all complete genomes except of *Archaeoglobus fulgidus* (Wu *et al.*, 2000). Ironically, TatD encodes a cytoplasmic protein with nuclease activity. This protein is shown to be not essential for the Tat machinery (Settles & Martienssen, 1998; Wexler *et al.*, 2000).

The Tat system is not universally conserved, half of the complete sequenced bacterial genomes lack the *tat*-like genes (Wu *et al.*, 2000). However, in the organisms with a functional Tat system, the distribution of known *tat* genes is heterogeneous. For example, the *tatC* gene is present in all complete bacterial genomes bigger that 2 Mb, but is absent from the genomes smaller than 1 Mb, and also

absent from half of those between 1 and 2 Mb. A similar distribution is valid for *tatA*-like genes. At least one copy of *tatA* is present in genomes containing *tatC*, but is lacking from the genomes where *tatC* is missing (Wu *et al.*, 2000). At least one *tatA/tatC* pair seems to be required for a functional Tat system, though in many organisms a *tatB* gene is also present.

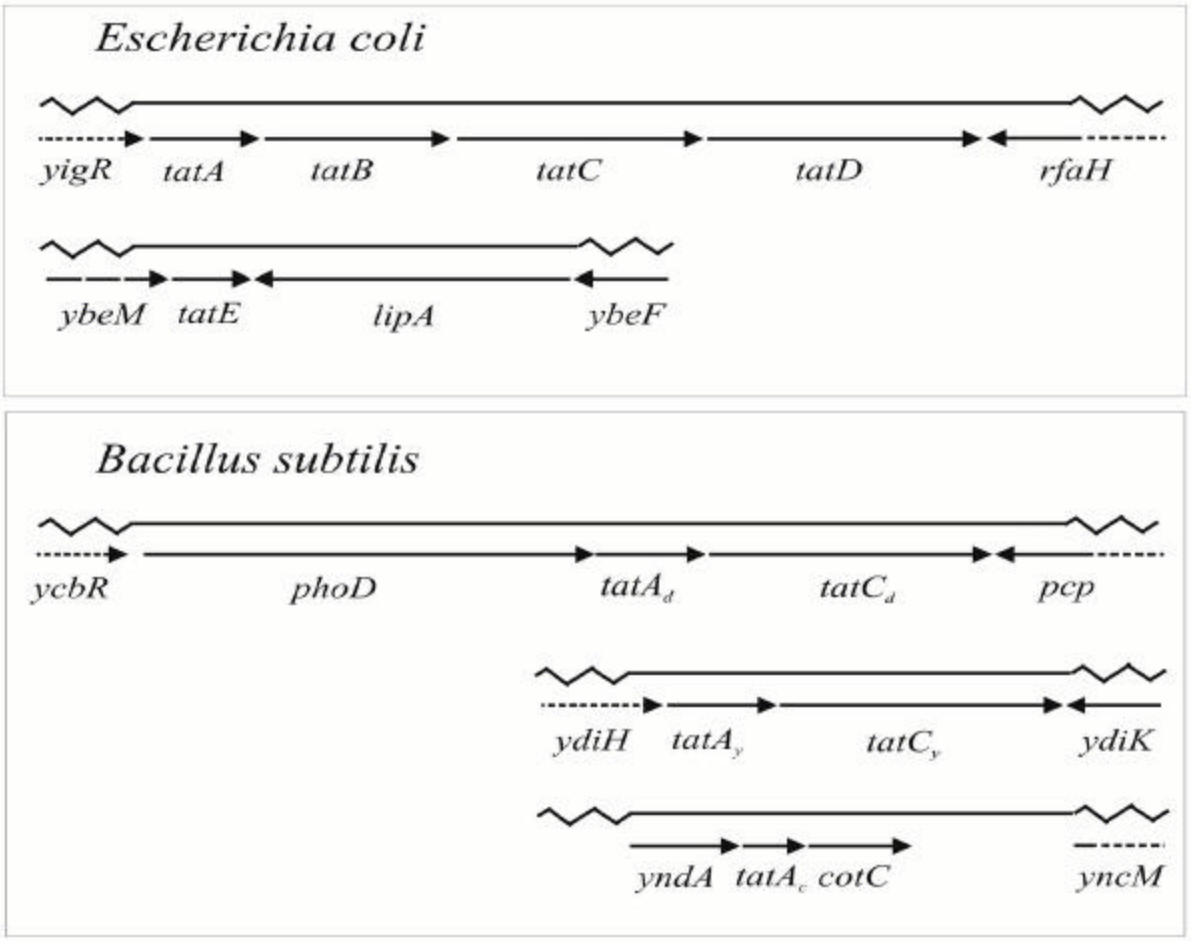

Figure 3. **The *tat* regions of *Escherichia coli* and *Bacillus subtilis*** (from Jongbloed *et al.*, 2000; see text for details)

1.2.4.2.4 The mechanism of twin-arginine translocation of *E. coli*

The Tat system has the special feature to transport folded proteins of variable size across the cytoplasmic membrane without destabilising the membrane equilibrium (Teter & Theg, 1998). Taking in account the great challenge of keeping membrane integrity, a few transport models were suggested (Berks *et al.*, 2000). 1) Vesicular transport, in which cytosolic born vesicles could encapsulate the transported substrate, subsequently fusing with cytosolic membrane. 2) Gated hydrophilic pore formed by the Tat apparatus. 3) The Tat translocon could permanently seal the substrate throughout the bilayer.

Since cytosolic membrane vesicles are not reported in bacteria, the vesicular model can be excluded. For the other two possibilities, the translocation pore/channel should be able to accommodate the folded proteins. The largest known Tat substrate is the FdnGH subcomplex of *E. coli* formate dehydrogenase-N complex with a size of 142 kDa (Berg *et al.*, 1991). Therefore, the pore should have a diameter ranging between 60Å and 70Å (Berks *et al.*, 2000; Robinson & Bolhuis, 2001).

Despite the fact that the essential Tat components are identified, only little is currently known about the Tat translocase organisation and mechanism. The folded substrate must be translocated across the membrane through a proteinaceous channel with aqueous properties. A prerequisite for understanding the Tat mechanism would be the isolation of such functionally active Tat complex. Some information could be derived from the calculated molecular ratio of the Tat components. Studies of chromosomal translational fusions revealed that in *E. coli* the TatA/TatB/TatC proteins are produced at a molecular ratio of 25 : 1 : 0,5 (Jack *et al.*, 2001). This is consistent with the TatA/TatB ratio of 20 : 1 determined by quantitative immunoblotting in *E. coli* cells (Sargent *et al.*, 2001). Research in *E. coli* led to the isolation of membrane localised TatA/TatB/TatC (Bolhuis *et al.*, 2001; de Leeuw *et al.*, 2002) or TatA/TatB (Sargent *et al.*, 2001) complexes with a size of ~ 600 kDa. Because those complexes are not yet proven to be functionally active, the information about the structure of the Tat translocase remains pure speculative and contradictory. Bolhuis and co-workers suggested that TatB and TatC proteins form a stable complex, while TatA has a weaker affinity. Therefore, TatB and TatC are suggested to form the structural and functional unit of the *E. coli* Tat translocase, whereas TatA could have a different role in the translocation process (Bolhuis *et al.*, 2001). Opposite, a report from Sargent and co-workers demonstrated that the isolated TatA/TatB complex forms a double-layered ring structure with a central cavity of ~ 65Å in diameter, as seen in negative stain electron micrographs (Sargent *et al.*, 2001).

It is, however, generally believed that *E. coli* Tat translocase consists of a TatB/TatC pair, with the possibility that TatA plays a role in the translocation channel assembly. This is in accordance with the *in vitro* studies, which demonstrated that TatA, TatB, and TatC are necessary and sufficient for a functional translocation system (Yahr & Wickner, 2001; Alami *et al.*, 2002; Alami *et al.*, 2003).

The translocation mechanism itself is poorly described. The folded cofactor-containing pre-proteins are targeted to the membrane by a yet unknown mechanism. Likewise in the thylakoid

system, a permanent TatBC protein complex fulfils the substrate-receptor role, especially due to the high sequence conservation of TatC (Berks *et al.*, 2000; de Leeuw *et al.*, 2002). Binding of the substrate to this complex does not require the ΔpH (Alami *et al.*, 2002). This first step is supposed to induce a conformational change in the Tat membrane complex, initiating the formation of a translocation channel. It is suggested that TatA might play the major structural role in forming this aqueous conducting channel, via function-linked changes in its topology (Gouffi *et al.*, 2003). In order to form a pore of a sufficient size, more than 20 α-helices of this small protein should be involved (Berks *et al.*, 2000). This would be consistent with the calculated molar excess of TatA/Tha4 from bacteria and thylakoids. After the conducting channel is formed, the folded substrate is passed through in the presence of the pH gradient at the membrane. When the substrate is approaching the outer side of the cytosolic membrane, its signal peptide becomes accessible to SPases. The processing event is marking the end of the tranlocation process.

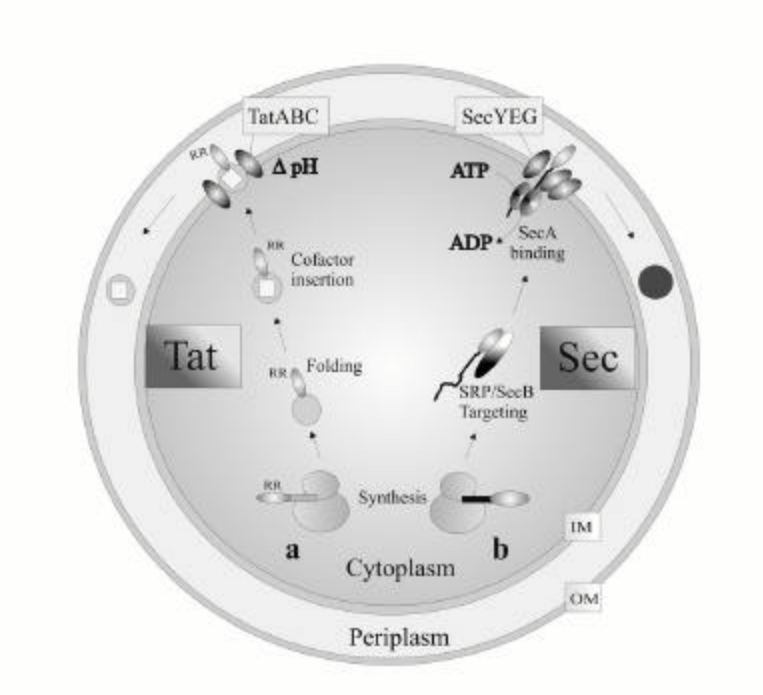

Figure 4. **Protein export pathways in *E. coli.***

a) *The twin-arginine translocatio*n pathway *(Tat)*. Most, (but not all) of the substrates for this pathway bind redox cofactors and function in the periplasm. After synthesis in the cytoplasm, the substrates are predicted to fold and then bind the cofactor (represented by white square) after which the pre-protein is believed to be transferred through the Tat apparatus in a folded form and finally undergo processing to the mature size.

b) *The general secretory pathway (Sec)*. Soon after emerging from the ribosome, the pre-protein is usually bound by the SRP complex or SecB protein, which prevents folding of the mature domain. The substrate is then transferred to SecA, which drives ATP-dependent translocation through the SecYEG complex, after which the precursor is processed to the mature size and allowed to refold (from Robinson & Bolhuis, 2001).

1.2.4.3 The Tat system in *Archaea*

Archaea represents a group of prokaryotes that resemble in part the bacteria and in part the eukaryotes. Since many archaeal species are found in very extreme life conditions, it is likely that they have specific adaptation in their protein translocation pathways. However, what is known about the protein transport systems in archaea is mostly based on genomic sequencing (Hutcheon & Bolhuis, 2003). In several archaea species, homologues of known Tat components have been identified (Yen *et al.*, 2002; Hutcheon & Bolhuis, 2003). These Tat components are TatA/E- and TatC-like proteins. No archaeal species analysed to date, are encoding a TatB-like protein (Pohlschröder *et al.*, 2004). TatA/E-like proteins are present in one to three copies and have similar predicted topology. Archaeal TatC is very similar with the bacterial TatC and exists in one ore two copies. Generally, TatC proteins have the same topology like in bacteria, except for *Halobacterium* sp. NRC-1, in which TatC2 protein has 14 spanning domains. This exist as a natural fusion between two TatC molecules (Bolhuis, 2002) and might represent an adaptation for a very high salt environment. Another interesting observation is that the methanophilic *Methanopyrus kandlery* contains only one TatA/E-like protein but no TatC protein. If at least one of the three putative Tat substrates identified in this organism is really transported Tat-dependent, then the only TatA/E-like protein must be a multifunctional component or, some completely different partners must be involved (Hutcheon & Bolhuis, 2003). However, it cannot be excluded that the lack of a TatC homologue has been overlooked due to a sequencing error.

All putative archaeal Tat substrates are synthesised with a twin-arginine containing signal peptide, as calculated with the SignalP 2.0 signal peptide prediction server (Bolhuis, 2002). Interesting, genomic data suggested that the halophilic archaea group, have routed almost all of their exported proteins to the Tat pathway (Rose *et al.*, 2002). For example, in *Halobacterium* sp. NRC-1, 18 putative Tat substrates were identified. This represent 40% of the exported proteins synthesised with a cleavable signal peptide (Bolhuis, 2002). The reason for having so many proteins destined to be exported via the Tat pathway may be the very high intracellular and extracellular salt concentration (Hutcheon & Bolhuis, 2003). In such conditions, the protein must quickly fold in order to prevent aggregation, such folded proteins being no longer compatible with the Sec system but rather adapted for the Tat export system (Bolhuis, 2002; Hutcheon & Bolhuis, 2003). The substantial use of the Tat pathway seems a common adaptation for all Archaea living in high salt environments.

For other Archaea, the Tat pathway is, as in bacteria, used to transport substrates that bind cofactors in the cytosol. For example, in *Thermoplasma acidophilum* and *T. volcanicum*, all predicted Tat substrates are Rieske iron-sulphur proteins (Berks, 1996). Some putative Tat substrates were found in Archaea species that completely lack Tat components (for example, *Pyrococcus sp.*). However, none of these substrates were predicted to bind cofactors, implying a non-necessity for the Tat pathway (Berks, 1996). Although a big number of putative Tat substrates are predicted, there is almost no experimental evidence that these substrates are transported in a Tat-dependent manner.

The organisation, energetics and mechanism of the archaeal Tat system is unknown. The pH gradient at the membrane, which is supposed to drive the Tat export, can be sometimes perturbed due

to the variety of extreme environments these organisms are faced to. For example, acidophiles such as *Thermoplasma acidophilum* have a reversed membrane potential, and the PMF depends mostly on the ΔpH, whereas in alkaliphiles such as *Natronobacterium pharaonis*, the PMF depends completely on the membrane potential. Therefore, the use of a different energy source for the Tat transport cannot be excluded (Hutcheon & Bolhuis, 2003).

1.2.4.4 The Tat system of *B. subtilis*

1.2.4.4.1 The signal peptides and the substrates of Tat-dependent export system of *B. subtilis*

The complete sequencing of the *B. subtilis* genome (strain 168) has revealed that this organism encodes approximately 4100 proteins (Kunst *et al.*, 1997; Kobayashi *et al.*, 2003). Computer-assisted predictions were made to identify putative Tat-dependent proteins. The research was based on the presence of the twin-arginine as well as the lysine-arginine motifs, immediately in front of the hydrophobic core of the signal peptides. In addition, the presence of a SPase cleavage site was also taken in consideration (Jongbloed *et al.*, 2000). The search revealed that 69 proteins are synthesised with a potential RR or KR-signal peptide, and are therefore, potential substrates for the Tat pathway.

Like in *E. coli*, the presence of a RR or KR motif in the N-terminal part of the signal peptide does not necessarily lead the protein through the Tat route, but is a prerequisite for a possible Tat export. A detection of the secretory proteins in 2D gel electrophoresis experiments showed that only 14 out of 69 proteins were exported in the growth medium. A comparison of the extracellular proteomes of wild type *B. subtilis* and a *tat* mutant strain depleted for all Tat proteins showed that only PhoD is transported in a strictly Tat-dependent manner (Jongbloed *et al.*, 2000). PhoD is an enzyme with alkaline phosphatase and phosphodiesterase activities, with a mass of 62.7 kDa. N-terminal sequencing of the released protein (molecular mass 56.6 kDa) indicated that a signal peptide of 56 amino acids is cleaved before secretion (Eder *et al.*, 1996). Despite the fact that was not detected by proteomic analysis, the YwbN protein is also transported Tat-dependent (Jongbloed, 2002). Interesting, this protein with unknown function is synthesised in all living conditions. PhoD and YwbN have signal peptides that fulfill the most stringent criteria for a Tat-dependent substrate.

1.2.4.4.2 The components of the Tat pathway

While most bacteria contain one copy of *tatA* and *tatC* (Wu *et al.*, 2000), sequencing of bacilli genomes indicates that the chromosome encodes multiple copies of TatA and TatC proteins. A TatB-like protein appear to be absent in bacilli. In particular, *B. subtilis* contains two copies of *tatC*-(denoted $tatC_d$ and $tatC_y$) and three copies of *tatA*-like genes (denoted $tatA_d$, $tatA_c$ and $tatA_y$) (Kunst *et al.*, 1997). Both *tatC* genes are localised directly downstream of a *tatA* gene (Jongbloed *et al.*, 2000). The $tatA_d/tatC_d$ gene pair is localised downstream of *phoD* gene and is co-regulated with the expression of *phoD* (Fig.3). Moreover, the genes from this operon (denoted *phoD* operon) are expressed only when the cells face a growth stress regarding the lack of phosphorus in the environment. This suggests, opposite to *E. coli*, that at least partly, the *B. subtilis* Tat

system is not required under normal growth conditions. A *B. subtilis* strain deleted for all *tat* genes did not show impaired viability in normal growing conditions (Jongbloed *et al.*, 2000).

1.2.4.4.3 The mechanism of the Tat export

It is demonstrated that for the translocation of PhoD, the presence of $tatC_d$ is crucial. The inactivation of the second copy of *tatC* ($tatC_y$) is not affecting the export of PhoD. A $tatC_d/tatC_y$ mutant completely blocked the secretion of PhoD while the transport of the Sec-dependent PhoB remained unaffected. Thus, $TatC_d$ is considered a specificity determinant for the secretion of PhoD via the Tat pathway (Jongbloed *et al.*, 2000). Interesting, also for the second Tat substrate YwbN, a *tatC* gene seems to be essential. In this case only $tatC_y$ is required for the export process. This clearly indicates the existence of at least two parallel Tat pathways in *B. subtilis* (Jongbloed, 2002). The organisation of the translocase and the translocation mechanism are not yet described but will make the subject of the present work.

1.3 The scope of the thesis.

The detailed understanding of bacterial protein transport was and still is one of the most important topics of the microbiological research of the last decades. The general Sec-dependent transport pathway of bacteria is ubiquitously distributed among the three kingdoms of life and has been studied extensively in bacteria and eukaryotic organisms. This resulted in a detailed understanding of the particular steps involving pre-protein recognition, targeting, translocation and structure of the Sec translocase. Still, a long list of open questions remains to be answered. In contrary, the newly described Tat export system distributed among bacteria, archaea and plant chloroplasts has been discovered very recently and it is, therefore, only poorly understood. Particularly, it is completely unknown, how three-dimensional folded protein can pass biological membranes without leakage of the cell. In addition, this transport system is independent of ATP hydrolysis and does nor require soluble co-factors. Most of the studies published so far focused on the Tat systems of *E. coli* and plant thylakoids. The Tat export mechanism is described neither in the bacterial nor in the thylakoidal system.

The work presented here has the aim to study a Tat-dependent protein export system of *B. subtilis*. The *tat* genes and a number of putative Tat substrates have been identified in this organism. The phosphodiesterase PhoD, is the only protein demonstrated to be transported in a strictly Tat-dependent manner. These first pieces of information suggested that *B. subtilis* has a functional Tat system that might differ from the one of *E. coli* or plant thylakoids.

B. subtilis already has a long history in the industrial production of various proteinsl. Therefore, the potential ability of this bacterium to transport folded heterologous proteins via the twin-arginine translocation system is of highly biotechnological interest.

The major questions addressed in this work are:

1) It is known that the Tat components have a certain degree of sequence conservation among different species. Can this similarity be generalised for different Tat systems? Are *B. subtilis* Tat substrates recognised by the *E. coli* Tat system?

2) One of the features of a Tat substrate is its specific (almost invariant) twin-arginine motif from the signal peptide. While not all substrates with a twin-arginine containing signal peptide are directed through the Tat translocase, almost all proteins exported Tat-dependent must have such a motif in their signal peptides. Is this twin-arginine sequence from the signal peptides the only informational motif that leads the pre-proteins through the Tat system?

3) *B. subtilis* is proved to have multiple copies of *tatA* and *tatC* genes. It is known that $TatC_d$ (but not $TatC_y$) is involved in the export of PhoD. What determines the selectivity of PhoD export of *B. subtilis*?

4) Which is the molecular and structural organisation of the Tat translocase of *B. subtilis*?

5) What are the physiological affinities between the substrate PhoD and the Tat components?

6) How is the protein transported through the cytosolic membrane via the Tat system?

7) Which signal peptidases are responsible for the processing of the pre-protein?

8) How is the general Tat mechanism organised?

9) What are the diferences of the *B. subtilis* Tat export system when compared to the TatABC systems of *E. coli* or plant thylakoids?

10) Could *B. subtilis* Tat export system have potential for biotechnological applications?

1.4 Publications included in the cumulative dissertation

Published articles:

1. The twin-arginine signal peptide of PhoD and the $TatA_d/C_d$ proteins of *Bacillus subtilis* form an autonomous Tat translocation system.

Ovidiu Pop, Ulrike Martin, Christian Abel, and Jörg P. Müller.

The Journal of Biological Biochemistry, Vol.277, No.5, Issue of February 1, pp.3268-3273, 2002.

This article describes the minimal necessity for a functional Tat translocation system from *B. subtilis*. A single pair of *tat* genes (*$tatA_d/tatC_d$*) from *B. subtilis* is sufficient to transport the twin-arginine substrate PhoD in the presence of the pH gradient at the membrane. In addition, it was demonstrated that a *B. subtilis* substrate (PhoD) is not recognised by the *E. coli* Tat machinery.
Some plasmid constructions were created by Ulrike Martin and Christian Abel previously. I carried out about 50% of the cloning work and almost all of the microbial and protein biochemical studies. Jörg Müller supervised the work and helped in writing the manuscript.

2. Sequence specific binding of prePhoD to soluble $TatA_d$ complexes indicates protein mediated targeting of the Tat export in *Bacillus subtilis*.

Ovidiu Pop, Martin Westermann, Rudolf Volkmer-Engert, Daniela Schulz, Cornelius Lemke, Sandra Schreiber, Roman Gerlach, Reinhard Wetzker, and Jörg P. Müller.

The Journal of Biological Biochemistry, Vol.278, No.40, Issue of October 3, pp.38428-38436, 2003.

This article describes for the first time the presence of soluble TatAd in the cytosol of the *B. subtilis* cell. We demonstrated *in vivo* and *in vitro* the affinity of $TatA_d$ to prePhoD, particularly to the twin-arginine motif. We concluded that $TatA_d$ acts as a targeting factor for PhoD. These results demonstrated a novel characteristic of the *B. subtilis* Tat pathway, which differs substantially of *E. coli* and plant thylakoid Tat system.

Martin Westermann carried out the freeze fracture studies and the electron microscopy studies. Rudolf Volkmer-Engert und Daniela Schulz delivered the peptide libraries. Cornelius Lemke carried out the ultrathin sections of the cells. Sandra Schreiber cloned hybrid genes encoding TAP-tagged $TatA_d$ proteins and carried out TAP purification experiments. Roman Gerlach was involved in the detection of the $TatA_d$ binding motif. Reinhard Wetzker contributed and supervised the work with the TAP-tagged proteins. I carried out $1/3^{rd}$ of the cloning work, most of the microbial, protein biochemical and immunological work, the immunogold labelling of the ultrathin sections. I used the peptide libraries to characterise the binding motif of $TatA_d$. Jörg Müller supervised the work and helped in writing the manuscript.

3. Tat-dependent export of *E. coli* phytase AppA by using the PhoD-specific transport system of *Bacillus subtilis.*

Roman Gerlach, Ovidiu Pop and Jörg P. Müller.Journal of Basic Microbiology, 44 (2004) 5, 351–359.

This article demonstrates the potential of the *B. subtilis* Tat system for the industrial production of heterologous proteins. The *E. coli* phytase was expressed and secreted in *B. subtilis* by the PhoD specific Tat export system. In addition, it demonstrated that the cell wall protease WprA is involved in the degradation of heterologous Tat-dependent exported proteins.

Roman Gerlach did most of the cloning work, strain constructions and immunological protein detection of AppA. I carried out about 1/3rd of the cloning work and together with Roman Gerlach the immunological detection of AppA via pulse chase and immunoprecipitation experiments. Jörg Müller supervised the experimental work and was involved in writing the manuscript.

Submitted articles:

4. Dual localisation of homo-multimeric TatA elucidates its function in Tat-dependent protein targeting.

Ovidiu Pop, Martin Westermann, Roman Gerlach and Jörg P. Müller.

submitted for publication in Journal of Molecular Biology, ref. code: JMB-D-04-00516 from July 2004.

This article elucidates the $TatA_d$ mediated targeting in the PhoD translocation process. The $TatA_d$ protein proved to be key component for the Tat system. $TatA_d$ forms homo-multimers in the cytosol (where it interacts with the twin-arginine substrate PhoD) as well as in the cytosolic membrane (where it most likely forms the protein-conducting channel). In addition, $TatC_d$ seems to be not involved in the structure of the translocation pore, but plays a role as receptor for $TatA_d$- prePhoD-complex.

Martin Westermann carried out the freeze fracture studies and the electron microscopical studies. Roman Gerlach amplified and purified $TatC_d$ and was involved in the sucrose density sedimentation experiments, as well as in the electron microscopy studies of soluble $TatA_d$. I radiolabelled and purified all other proteins used in the study, did most of the microbial work, carried out the reconstitution of proteoliposomes and carried out the extraction experiments. Jörg Müller supervised the work and helped in writing the manuscript.

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The Twin-arginine Signal Peptide of PhoD and the $TatA_d/C_d$ Proteins of *Bacillus subtilis* Form an Autonomous Tat Translocation System*

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The bacterial twin-arginine translocation (Tat) pathway has been recently described for PhoD of *Bacillus subtilis*, a phosphodiesterase containing a twin-arginine signal peptide. The expression of *phoD* is co-regulated with the expression of $tatA_d$ and $tatC_d$ genes localized downstream of *phoD*. To characterize the specificity of PhoD transport further, translocation of PhoD was investigated in *Escherichia coli*. By using gene fusions, we analyzed the particular role of the signal peptide and the mature region of PhoD in canalizing the transport route. A hybrid protein consisting of the signal peptide of β-lactamase and mature PhoD was transported in a Sec-dependent manner indicating that the mature part of PhoD does not contain information canalizing the selected translocation route. Pre-PhoD, as well as a fusion protein consisting of the signal peptide of PhoD (SP_{PhoD}) and β-galactosidase (LacZ), remained cytosolic in the *E. coli*. Thus, SP_{PhoD} is not recognized by *E. coli* transport systems. Co-expression of *B. subtilis* $tatA_d/C_d$ genes resulted in the processing of SP_{PhoD}-LacZ and periplasmic localization of LacZ illustrating a close substrate specificity of the $TatA_d/C_d$ transport system. While blockage of the Sec-dependent transport did not affect the localization of SP_{PhoD}-LacZ, translocation and processing was dependent on the pH gradient of the cytosolic membrane. Thus, the minimal requirement of a functional Tat-dependent protein translocation system consists of a twin-arginine signal peptide-containing Tat substrate, its specific TatA/C proteins, and the pH gradient across the cytosolic membrane.

The existence of a protein export pathway structurally and mechanistically similar to the ΔpH-dependent pathway used for importing chloroplast proteins into the thylakoid has been shown for a variety of bacteria (1–3). Despite the fact that the mechanism of targeting and the transport of folded proteins via the ΔpH/Tat[1] route is not yet understood, some common features characterize these translocation systems (for reviews, see Refs. 4 and 5). It has been shown that the *Escherichia coli* Tat system involves four proteins with calculated membrane-spanning domains (6–8). TatA/TatE and TatB are sequence-related proteins that are homologous to Tha4 and Hcf106 of the ΔpH-dependent thylakoid import pathway (7–11). Chloroplast cpTatC has been described recently as the ortholog of *E. coli* TatC (12). While TatB and TatC appear to play a pivotal role in the Tat-dependent protein translocation in *E. coli*, TatA and TatE seem to fulfill complementary functions as the deletion of TatA or TatE does not block export, while the TatA/TatE double deletion drastically inhibits export (7). Expression studies suggested that *tatE* may be a cryptic gene duplication of *tatA* (13). An *in vitro* reconstituted translocation system demonstrated the necessity of TatA, TatB, and TatC for a functional *E. coli* Tat-dependent translocation system (14). The information about the structure of the Tat translocase is contradictory. While Bolhuis *et al.* (15) suggested that TatB and TatC proteins form a functional and structural unit of the *E. coli* Tat translocase, a recent report from Sargent *et al.* (16) demonstrated a double-layered ring structure with a central cavity of a complex consisting of TatA and TatB.

The presence of genes encoding TatA- and TatC-like proteins as well as the synthesis of exported proteins containing twin-arginine signal peptides are strong indications for the existence of the Tat pathway in eubacteria (17). While most of the bacteria contain one copy of *tatA* and one copy of *tatC* (5) sequencing of bacilli genomes (*i.e. Bacillus subtilis*, *Bacillus halodurans*, and *Bacillus stearothermophilus*) indicated the presence of multiple TatA and TatC proteins. In particular, *B. subtilis* contains two *tatC*- and three *tatA*-like genes (18). Both *tatC* genes are localized directly downstream from a *tatA* gene (19). A TatB-like protein appears to be absent from bacilli.

The recently described transport of PhoD of *B. subtilis* revealed that TatC could act as a specificity determinant for this process. While the inactivation of the $tatC_d$ completely inhibited the secretion of PhoD, the inactivation of the second *tatC* gene ($tatC_y$) had no effect on the secretion of PhoD (19). This observation was the first indication for the existence of multiple Tat pathways in a single bacterial cell with separate substrate specificity. PhoD is a secretory protein with a twin-arginine signal peptide. We have shown previously that it is efficiently transported across the cytosolic membrane but only inefficiently processed. Slow processing of the enzymatically active precursor was shown to keep the protein at the outer surface of the cell envelope (20). The *tatA*/*tatC* gene pair (designated $tatA_d/C_d$), localized downstream from *phoD*, is co-regulated with the expression of *phoD* (19).

To investigate the specificity of the PhoD transport further, we analyzed its transport in *E. coli*. By using gene fusion technology the particular role of the signal peptide and the mature region of PhoD in canalizing the transport was investigated. A fusion protein consisting of the signal peptide of β-lactamase (Bla) and mature PhoD was transported in a Sec-dependent manner. PhoD, as well as the fusion protein consisting of the signal peptide of PhoD (SP_{PhoD}) and LacZ, was shown

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[1] The abbreviations used are: Tat, twin-arginine translocation; SP, signal peptide; Bla, β-lactamase; IPTG, isopropyl-β-D-thiogalactopyranoside.

TABLE I
Plasmids and strains

Cm^r, chloramphenicol resistance marker; Ap^r, ampicillin resistance marker; Km^r, kanamycin resistance marker; Tc^r, tetracycline resistance marker; Em^r, erythromycin resistance marker; RBS, ribosome binding site.

Plasmids	Relevant properties	Ref.
pAR3	pACYC184-derived plasmid carrying the *araB* promoter operator and the *araC* repressor gene from *S. typhimurium*; Cm^r	27
pAR3*phoD*	pAR3 derivative; carrying the *phoD* gene; Cm^r	This work
pAR3*phoD′-lacZ*	pAR3 derivative; carrying a fusion gene consisting of the signal sequence region of *phoD* and *lacZ*; Cm^r	This work
pQE9	pBR322-based vector for IPTG-inducible synthesis of His_6-tagged proteins; Ap^r	Qiagen
pREP4	plasmid; containing $lacI^q$ repressor gene; Km^r	Qiagen
pORI24	plasmid; replicates only in *E. coli* rep^+ strains; Tc^r	37
pMUTIN2	pBR322-based integration vector for *B. subtilis*; containing a multiple cloning site downstream of the P_{SPAC} promoter, and a promoterless *lacZ* gene preceded by the RBS of the *spoVG* gene; Ap^r; Em^r	38
pMUTIN2*bla-phoD*	pMUTIN2 derivative; carrying a fusion gene consisting of signal sequence region of *bla* and *phoD*	This work
pQE9$tatA_d/C_d$	pQE9 derivative; carrying the *B. subtilis* $tatA_d/C_d$ genes	This work
pFAT44	pMAK705 (39)-derived plasmid containing in-frame deletion of *E. coli tatE*	7
pFAT126	pMAK705-derived plasmid containing in-frame deletion of *E. coli tatABCD*	40

Strains	Relevant properties	Ref.
E. coli		
TG1	F^- *araD139* Δ(*ara-leu*)*7696* Δ(*lac*)*X74 galU galK hsdR2 mcrA mcrB1 rspL*	41
TG1 Δ*tatABCE*	TG1 Δ*tatABCE*	This work
B. subtilis		
168	*trpC2*	18

to be export-incompetent in *E. coli*. The co-expression of the *phoD*-associated *B. subtilis* gene pair $tatA_d/C_d$ resulted in the processing and the translocation of SP_{PhoD}-LacZ. This transport was shown to be ΔpH-dependent. Since the transport of SP_{PhoD}-LacZ was independent of *E. coli tat* genes, the minimal requirement of a Tat transport system consists of a twin-arginine signal peptide-containing substrate, an adopted TatA/C pair, and the pH gradient across the bacterial cytosolic membrane.

EXPERIMENTAL PROCEDURES

Plasmids, Bacterial Strains, and Media—Table I lists the plasmids and bacterial strains used. TY medium (tryptone/yeast extract) contained Bacto tryptone (1%), Bacto yeast extract (0.5%), and NaCl (1%). For pulse-chase labeling experiment M9 minimal medium was prepared as described previously (21). When required, media were supplemented with ampicillin (100 μg/ml), kanamycin (40 μg/ml), chloramphenicol (20 μg/ml), tetracycline (12.5 μg/ml), arabinose (0.2%), isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM), nigericin (1 μM), and/or sodium azide (3 mM). [^{35}S]Methionine was provided by Hartman Analytic (Braunschweig, Germany), and the ^{14}C-Labeled molecular weight marker was from Amersham Biosciences, Inc..

DNA Techniques—Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of *E. coli* were carried out as described in Sambrook *et al*. (22). Restriction enzymes were from MBI Fermentas. PCR was carried out with the VENT DNA polymerase (New England Biolabs).

To construct pAR3*phoD*, the *phoD* gene including its ribosome binding site was amplified from the chromosome of *B. subtilis* strain 168 by PCR using the primers P1 (5′-GAG GAT CCA TGA GGA GAG AGG GGA TCT TGA ATG GCA TAC GAC-3′) containing a *Bam*HI site and P2 (5′-CGA TCC TGC AGG ACC TCA TCG GAT TGC-3′) containing a *Pst*I site. The amplified fragment was cleaved with *Bam*HI and *Pst*I and cloned in the corresponding sites of pAR3. The resulting plasmid pAR3*phoD* allowed the arabinose-inducible expression of wild type *phoD* in *E. coli.*

To construct a gene fusion between *bla* and *phoD* genes, *phoD* deleted for its signal sequence was amplified using primer P3 (5′-GTA GGA TCC GCG CCT AAC TTC TCA AGC-3′) containing a *Bam*HI site and primer P2 containing a *Pst*I site. The amplified fragment was cleaved with *Bam*HI and *Pst*I and cloned in the corresponding sites of pUC19, resulting in plasmid pUC19′*phoD*. Next the 5′-region of β-lactamase encoding its signal sequence was amplified from plasmid pBR322 by PCR with primer B1 (5′-ATA GAA TTC AAA AAG GAA GAG TAT G-3′) containing an *Eco*RI site and primer B2 (5′-CTG GGG ATC CAA AAA CAG GAA GGC-3′) containing a *Bam*HI site. The amplified PCR fragment was cleaved with *Bam*HI and *Eco*RI and inserted into pUC19′*phoD*, cleaved with the same restriction enzymes, which resulted in plasmid pUC19*bla-phoD*. For easy selection of recombinant clones, plasmid pORI24, containing a tetracycline resistance gene, was inserted 3′ of the *bla-phoD* gene fusion using an unique *Pst*I site. From the resulting plasmid pUC19*bla-phoD*-Tc an *Eco*RI-*Bgl*II fragment containing *bla-phoD* and the tetracycline resistance gene of pORI24 was isolated and inserted into pMUTIN2 cleaved with *Eco*RI and *Bam*HI. In the plasmid pMutin2*bla-phoD* the *bla-phoD* gene fusion is under control of the IPTG-inducible P_{SPAC} promoter.

To construct a gene fusion consisting of the signal sequence of *phoD* and *lacZ*, a DNA fragment encoding the signal peptide of PhoD and the translational start site of *phoD* was amplified by PCR with primer P1 containing a *Bam*HI site and primer P4 (5′-GAG AAG GTC GAC GCA GCA TTT ACT TCA AAG GCC CC-3′) containing a *Sal*I site and inserted into the corresponding sites of pORI24 resulting in plasmid pORI24*phoD′*. Next, the *lacZ* gene lacking nine 5′-terminal codons was amplified using primer L1 (5′-ACC GGG TCG ACC GTC GTT TTA CAA CG-3′) containing a *Sal*I site and primer L2 (5′-GGG AAT TCA TGG CCT GCC CGG TT-3′) containing an *Eco*RI site and subsequently inserted into the corresponding sites of pORI24*phoD′*. The resulting plasmid pORI24*phoD′-lacZ* was linearized with *Bam*HI and inserted into pAR3 cleaved with *Bgl*II. The resulting plasmid pAR3*phoD′-lacZ* allows the arabinose-inducible expression of the *phoD′-lacZ* gene fusion.

To obtain a plasmid that mediates an inducible overexpression of $tatA_d/tatC_d$ of *B. subtilis*, the DNA region containing these genes including their ribosome binding sites was amplified by PCR with the primer T1 (5′-CAA GGA TCC CGA ATT AAG GAG TGG-3′) containing a *Bam*HI site and primer T2 (5′-GGT CTG CAG CTG CAC TAA GCG GCC GCC-3′) containing a *Pst*I site. The amplified fragment was cleaved with *Bam*HI and *Pst*I and cloned into the corresponding sites of pQE9 (Qiagen) resulting in pQE9$tatA_d/C_d$.

To obtain TG1 Δ*tatABCDE*, plasmids pFAT44 and subsequently pFAT126 covering in-frame deletions of *E. coli tatE* and *tatABCD* genes, respectively, were transferred to the chromosome of TG1 as described (7). Mutant strain TG1 Δ*tatABCDE* was verified phenotypically by mutant cell septation phenotype, hypersensitivity to SDS, and resistance to P1 phages as described previously (23).

SDS-PAGE and Western Blot Analysis—SDS-PAGE was carried out as described by Laemmli (24). After separation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Schleicher and Schüll) as described by Towbin *et al.* (25). Proteins were detected using specific antibodies against PhoD (20), β-galactosidase (5 Prime → 3 Prime, Inc., Boulder, CO), pro-OmpA (provided by R. Freudl), SecB (laboratory collection), and alkaline phosphatase-conjugated goat anti-rabbit antibodies (Sigma) according to the instructions of the manufacturer.

Protein Chase Experiments, Immunoprecipitation, and Quantification of Protein—Pulse-labeling experiments of *E. coli* strains were performed as described earlier (21). Cultures were pulse-labeled with 100 μCi of [^{35}S]methionine and chased with unlabeled methionine, and then samples were taken at the times indicated immediately followed by precipitation with trichloroacetic acid (0 °C). After cell lysis proteins

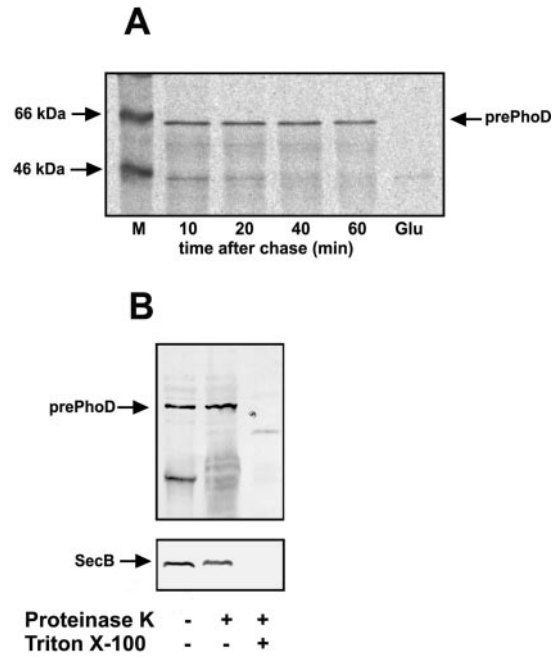

FIG. 1. **Processing and localization of pre-PhoD in *E. coli* TG1.** *A*, *E. coli* TG1 carrying plasmid pAR*phoD* encoding wild type PhoD was grown in M9 minimal medium to early logarithmic phase. 1 h prior to labeling expression of *phoD* was induced with IPTG (1 mM). Cells were labeled for 1 min with [^{35}S]methionine after which nonradioactive methionine was added. Samples were withdrawn at chase times 10, 20, 40, and 60 min and subjected to immunoprecipitation with monospecific antibodies against PhoD followed by SDS-PAGE using a 10% polyacrylamide gel and fluorography. *M*, molecular mass marker; *Glu*, uninduced control. *B*, *in vivo* protease mapping of PhoD in *E. coli* TG1(pAR3*phoD*). Cells were converted to spheroplasts and treated with proteinase K or with proteinase K and Triton X-100 or remained untreated as indicated. Localization of pre-PhoD is indicated. Accessibility of proteinase K to the cytosol was analyzed by monitoring SecB in a 15% polyacrylamide gel. PhoD and SecB were detected by monospecific antibodies.

were precipitated with specific antibodies against PhoD (20), OmpA, β-galactosidase, or β-lactamase (5 Prime → 3 Prime, Inc.). Relative amounts of radioactivity were estimated by using a phosphorimaging system (Fuji) and the associated image analytical software PC-BAS.

In Vivo Protease Mapping—In vivo protease mapping was carried out according to Kiefer *et al.* (26). For spheroplast formation, cells were grown in TY medium to exponential growth. To induce the plasmid-encoded genes the medium was supplemented with arabinose (0.2%) and/or IPTG (1 mM) for 60 min. After spheroplast formation cells were treated with proteinase K (Sigma) or with proteinase K and Triton X-100 or remained untreated. PhoD or SP_{PhoD}-LacZ were detected by Western blotting. Detection of cytosolic SecB revealed the proteinase K resistance of Triton X-100-untreated spheroplasts.

RESULTS

PhoD Is Not Transported in E. coli—The initial aim was to test whether PhoD could be exported by the Tat pathway in *E. coli*. For this purpose we placed the gene encoding this peptide under the control of the P_{BAD} promoter of *Salmonella typhimurium* localized at plasmid pAR3 (27). The resulting plasmid allowed the arabinose-inducible enzymatically active production of PhoD in *E. coli* TG1 (data not shown). Since phosphodiesterase is highly toxic to *E. coli* after induction of PhoD synthesis cell growth immediately ceased. To assay transport of PhoD in *E. coli* TG1(pAR*phoD*) pulse-chase experiments were performed. As shown in Fig. 1*A* no processing of the wild type pre-PhoD was observed even 60 min after chase, indicating that pre-PhoD was not translocated by the *E. coli* Tat machinery. Localization of PhoD was further analyzed by *in vivo* protease mapping. As shown in Fig. 1*B* pre-PhoD was not accessible to proteinase K at the outer side of the cytosolic membrane, demonstrating that PhoD remained in a cytosolic localization.

PhoD Can Be Transported via the Sec-dependent Protein Translocation Pathway—Absence of pre-PhoD processing in *E. coli* could be due to inefficient recognition of the signal peptide of PhoD by the *E. coli* Tat machinery or due to the nature of the mature part of the PhoD peptide. This *B. subtilis* protein could have unexpected folding characteristics or the necessity of cofactors not present in *E. coli*. To address this question, the DNA encoding the mature peptide of PhoD was fused to the region encoding the signal peptide of β-lactamase (SP_{Bla}). The resulting gene fusion was cloned into the pMUTIN2 vector containing an IPTG-inducible P_{SPAC} promoter allowing the synthesis of the SP_{Bla}-PhoD peptide. The transport and processing of this fusion protein was analyzed by immunoblotting of whole cell extracts of *E. coli* strain TG1(pMUTIN2*bla-phoD*). As shown in Fig. 2*A*, *lane 2*, SP_{Bla}-PhoD was completely converted to a protein with a molecular weight of mature PhoD indicating the efficient transport of the protein. To elucidate the export path used for SP_{Bla}-PhoD translocation, Sec-dependent transport was selectively inhibited by addition of sodium azide. While the presence of sodium azide abolished conversion of SP_{Bla}-PhoD to PhoD, addition of nigericin did not retard processing of SP_{Bla}-PhoD (Fig. 2*A*, *lanes 3* and *4*). To analyze the Sec dependence of SP_{Bla}-PhoD transport in a more detailed manner, expression of *bla-phoD* in *E. coli* TG1(pMUTIN2*bla-phoD*) was induced in the presence or absence of sodium azide, pulse-labeling with [^{35}S]-methionine was carried out, and PhoD was subsequently immunoprecipitated. Fig. 2*B* demonstrates the kinetics of conversion of SP_{Bla}-PhoD to mature PhoD. The presence of sodium azide significantly retarded maturation of SP_{Bla}-PhoD (Fig. 2*C*). To demonstrate that azide was effective in inhibiting Sec-dependent translocation, the processing of pro-OmpA was monitored from the same cultures. While in untreated culture pro-OmpA was quickly converted into its mature form, in the azide-treated culture processing of pro-OmpA was efficiently retarded (Fig. 2, *D* and *E*). These data indicate that PhoD can be transported in *E. coli* in a Sec-dependent manner. Thus, it can be concluded that the mature PhoD peptide is not canalizing the export route and does not prevent efficient transport or processing.

The Signal Peptide of PhoD Cannot Mediate Transport of LacZ in E. coli Wild Type Cells—It has been shown that signal peptides containing a twin-arginine motif can canalize transport of heterologous proteins via the Tat-dependent translocation route (for a review, see Ref. 5). The signal peptide of the *E. coli* trimethylamine-*N*-oxide reductase (TorA) has been successfully used to mediate Tat-dependent transport of the thylakoidal protein 23K, the glucose-fructose oxidoreductase of *Zymomonas mobilis* and green fluorescent protein (3, 7, 28, 29). To test whether the signal peptide of PhoD is recognized by the *E. coli* Tat machinery and could canalize the transport of a protein in *E. coli*, we constructed a gene fusion consisting of the DNA region encoding the signal peptide of PhoD (SP_{PhoD}) and the *lacZ* gene encoding β-galactosidase as a reporter protein. The gene hybrid was inserted into plasmid pAR3 resulting in plasmid pAR3*phoD′-lacZ*. Induction of production of the SP_{PhoD}-LacZ fusion protein in *E. coli* TG1 resulted in $LacZ^+$ colonies (data not shown). Hence, correct folding and tetramerization of the peptide as a prerequisite for its activity does occur in *E. coli*.

To analyze whether the signal peptide of PhoD could mediate translocation of LacZ into a extracytosolic localization, we studied localization of LacZ by using *in vivo* protease mapping. As shown in Fig. 3*A* no processing of SP_{PhoD}-LacZ could be observed. The SP_{PhoD}-LacZ fusion protein was not susceptible to

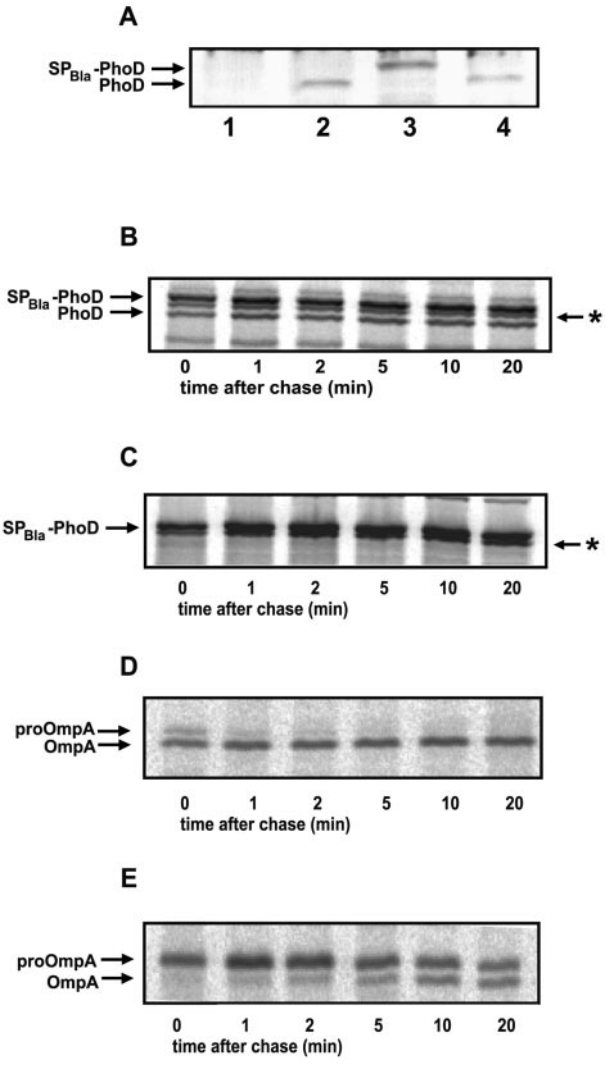

FIG. 2. **Induction and processing of SP_{Bla}-PhoD in *E. coli* TG1.** *A*, *E. coli* TG1(pMUTIN2*bla-phoD*) was grown in TY medium to logarithmic growth phase. Expression of *bla-phoD* was induced with IPTG (1 mM, *lanes 2–4*) or remained uninduced (*lane 1*). At the time of induction cultures were treated with sodium azide (3 mM, *lane 3*) or with nigericin (1 μM, *lane 4*) or remained untreated (*lane 2*). Samples were taken 20 min after induction of SP_{Bla}-PhoD and lysed, and cell extracts were analyzed by SDS-PAGE using a 10% polyacrylamide gel. *B–E*, TG1(pMUTIN2*bla-phoD*) was grown in M9 minimal medium to early logarithmic phase. 1 h prior to labeling, expression of *phoD* was induced with IPTG (1 mM). While one culture remained untreated (*B* and *D*), the other was treated with sodium azide (3 mM) upon induction (*C* and *E*). Cells were labeled for 1 min with [^{35}S]methionine after which nonradioactive methionine was added. Samples were withdrawn at times after chase as indicated in the figures and subjected to immunoprecipitation with antibodies against PhoD (*B* and *C*) or against OmpA (*D* and *E*) followed by SDS-PAGE using a 12.5% polyacrylamide gel and fluorography. Localization of SP_{Bla}-PhoD and mature PhoD is indicated. *, unspecific cross-reacting band.

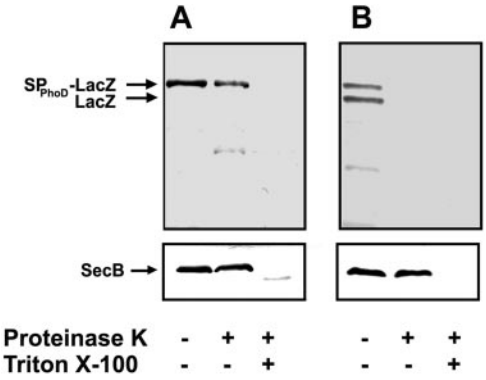

FIG. 3. **Localization of SP_{PhoD}-LacZ in *E. coli* TG1 in the absence or presence of *B. subtilis* $tatA_d/C_d$.** *E. coli* TG1 strains carrying either plasmid pAR3*phoD′-lacZ* (*A*) or plasmids pAR3*phoD′-lacZ*, pREP4, and pQE9$tatA_d/C_d$ (*B*) were grown in TY medium to exponential growth, and expression of *phoD′-lacZ* and $tatA_d/C_d$ was induced for 1 h with arabinose (0.2%) and IPTG (1 mM), respectively. Subcellular localization of SP_{PhoD}-LacZ was detected by *in vivo* protease mapping according to Fig. 1*B*. SP_{PhoD}-LacZ and SecB were monitored by antisera against LacZ and SecB. Bands representing SP_{PhoD}-LacZ, LacZ, and SecB are indicated.

protease digestion in spheroplasts. When spheroplasts were destroyed by addition of Triton X-100, the unprocessed SP_{PhoD}-LacZ protein became protease-sensitive. The reliability of the method was verified by using the cytosolic protein SecB as internal control. In spheroplasts SecB was resistant to proteinase K but was digested after solubilizing the spheroplasts with Triton X-100.

Export of SP_{PhoD}-LacZ Fusion Protein in E. coli Needs the Presence of the B. subtilis $TatA_d$ and $TatC_d$ Transport Components—The data demonstrated above indicate that the Tat system of *E. coli* does not mediate transport of pre-PhoD or of the SP_{PhoD}-LacZ fusion protein. Absence of translocation could be due to the necessity of additional components for the translocation of PhoD present only in *B. subtilis* or due to the specificity of recognition of pre-PhoD as a Tat-dependent substrate. To test the latter hypothesis, the *B. subtilis* $tatA_d/C_d$ gene pair was coexpressed in *E. coli* strains TG1(pAR*phoD*) and TG1(pAR*phoD′-lacZ*).

To study the effect of $TatA_d/C_d$ proteins on localization of PhoD, strain TG1(pAR*phoD*, pREP4, pQE9$tatA_d/C_d$) expression of *phoD* as well as $tatA_d/C_d$ was induced with arabinose and IPTG. Unexpectedly, no PhoD could be detected in strain TG1(pAR*phoD*, pREP4, pQE9$tatA_d/C_d$) using Western blotting (data not shown). Induction of $TatA_d/C_d$ proteins in strain TG1(pAR*phoD′-lacZ*, pREP4, pQE9$tatA_d/C_d$) resulted in stable co-production of $TatA_d/C_d$ and SP_{PhoD}-LacZ (data not shown). SP_{PhoD}-LacZ processing was analyzed in the presence and absence of $TatA_d/C_d$ using pulse-chase labeling and subsequent immunoprecipitation with specific antibodies against LacZ. While in TG1(pAR*phoD′-lacZ*) no processing of SP_{PhoD}-LacZ could be observed (Fig. 4*A*), in strain TG1(pAR*phoD′-lacZ*, pREP4, pQE9$tatA_d/C_d$) the peptide was at least partially processed (Fig. 4*B*).

Since processing of the translocation product is an indication of membrane translocation but does not necessarily prove that export of the protein has occurred, we examined whether LacZ was localized in the periplasmic space in TG1(pAR*phoD′-lacZ*, pREP4, pQE9$tatA_d/C_d$). To monitor localization of the LacZ peptide, cells of strain TG1(pAR*phoD′-lacZ*, pREP4, pQE9$tatA_d/C_d$) were converted to spheroplasts and treated with proteinase K. As shown in Fig. 3*B* after co-expression of $tatA_d/C_d$, SP_{PhoD}-LacZ was completely susceptible to protease digestion in spheroplasts. Unexpectedly, both the processed form and the precursor of the fusion protein were accessible to the protease treatment. The presence of SecB after proteinase K treatment demonstrated the stability of spheroplasts. These results clearly show that the SP_{PhoD}-LacZ fusion protein is exported into the periplasmic space of *E. coli* when the *B. subtilis* $tatA_d/C_d$ genes are co-expressed.

$TatA_d/C_d$-mediated Transport of SP_{PhoD}-LacZ Needs ΔpH-dependent Gradient at the Cytosolic Membrane and Is Sec-independent—To directly prove that the membrane translocation of the system is dependent on the pH gradient across the cytosolic membrane, Sec- or Tat-dependent protein translocation pathways were selectively blocked. Localization of SP_{PhoD}-LacZ in TG1(pAR*phoD′-lacZ*, pREP4, pQE9$tatA_d/C_d$) was de-

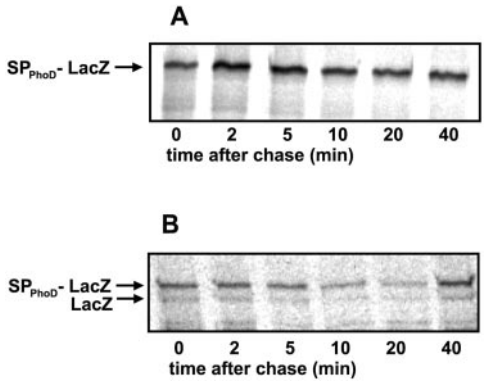

FIG. 4. **Processing of SP$_{PhoD}$-LacZ in *E. coli* TG1 co-expressing *B. subtilis tatA$_d$/C$_d$*.** *E. coli* strains TG1(pAR3*phoD'-lacZ*) (*A*) and TG1(pAR3*phoD'-lacZ*, pREP4, pQE9*tatA$_d$/C$_d$*) (*B*) were grown in M9 minimal medium to early logarithmic phase, labeled for 1 min with [^{35}S]methionine, and subsequently chased with nonradioactive methionine. Samples were taken at the indicated chase times and further processed by immunoprecipitation with antiserum against LacZ followed by SDS-PAGE using a 7.5% polyacrylamide gel and fluorography. Bands representing SP$_{PhoD}$-LacZ and LacZ are indicated.

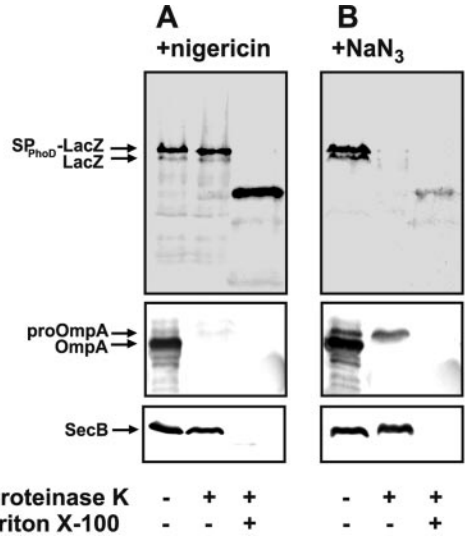

FIG. 5. **TatA$_d$/C$_d$-mediated transport of SP$_{PhoD}$-LacZ in *E. coli* is ΔpH-dependent.** *E. coli* TG1(pAR3*phoD'-lacZ*, pREP4, pQE9*tatA$_d$/C$_d$*) was grown in TY medium to exponential growth, and nigericin (1 μM) (*A*) or sodium azide (3 mM) (*B*) was added to the cultures prior to induction of gene expression. Localization of LacZ and OmpA was analyzed by *in vivo* protease mapping as described in Fig. 3. Western blotting was performed for immunological detection of LacZ, OmpA, and SecB with specific antibodies. Bands representing SP$_{PhoD}$-LacZ, LacZ, pro-OmpA, OmpA, and SecB are indicated.

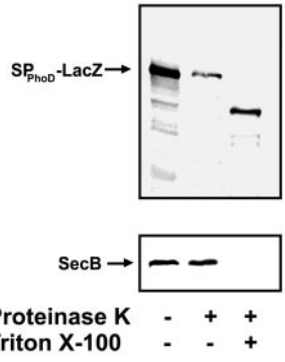

FIG. 6. **Localization of SP$_{PhoD}$-LacZ in *E. coli* strain deleted for *tatABCDE* but containing *B. subtilis tatA$_d$/tatC$_d$*.** *E. coli* strain TG1 Δ*tatABCDE*(pAR3*phoD'-lacZ*, pREP4, pQE9*tatA$_d$/C$_d$*) was grown in TY medium, and synthesis of SP$_{PhoD}$-LacZ and TatA$_d$/C$_d$ was induced and analyzed by *in vivo* protease mapping as described in Fig. 3. LacZ and SecB were visualized by SDS-PAGE and Western blotting using specific antibodies.

tected by Western blotting after *in vivo* protease mapping. Nigericin, an ionophore inhibiting the Tat-dependent protein translocation as a result of dissipating the ΔpH (30), did efficiently block both processing and translocation of SP$_{PhoD}$-LacZ (Fig. 5*A*). The addition of an equal volume of methanol used to disperse nigericin had no effect on localization of SP$_{PhoD}$-LacZ (data not shown). Treatment of the culture with sodium azide, which inhibits Sec-dependent protein export by interfering with the translocation-ATPase activity of the SecA protein (31), did result in accumulation of pro-OmpA but did not affect the localization and the processing of the SP$_{PhoD}$-LacZ fusion protein (Fig. 5*B*).

Tat$_d$/C$_d$-mediated Transport of SP$_{PhoD}$-LacZ Is Not Assisted by E. coli Tat Components—To exclude co-operative action of *B. subtilis* and *E. coli* Tat proteins, *E. coli* strain TG1 was deleted for *tatABCDE* genes and subsequently transformed with plasmids pAR*phoD'-lacZ*, pREP4, and pQE9*tatA/C$_d$*. Processing and localization of the SP$_{PhoD}$-LacZ fusion protein was analyzed under identical conditions as described for the *E. coli tat*$^+$ strain. As shown in Fig. 6 in the absence of the *E. coli tatABCDE* genes most of SP$_{PhoD}$-LacZ was protease-accessible demonstrating the extracytosolic localization of the fusion protein. The resistance of SecB to the proteolytic digestion demonstrated the stability of the spheroplasts (Fig. 6). Surprisingly, no processing of the SP$_{PhoD}$-LacZ fusion protein could be observed in the absence of *tatABCDE*.

DISCUSSION

In the present report we have shown that the export signals of PhoD, a Tat-dependent transported phosphodiesterase of *B. subtilis*, was incompatible with the Tat machinery of *E. coli*. While the mature part of PhoD could be exported efficiently with the help of the export signals of Sec-dependent β-lactamase, wild type PhoD or a fusion protein consisting of the signal peptide of PhoD and LacZ remained cytosolic. The co-expression of the *phoD*-associated genes *tatA$_d$* and *tatC$_d$* mediated the Tat-dependent translocation of the SP$_{PhoD}$-LacZ fusion protein. Since transport of SP$_{PhoD}$-LacZ was blocked in the presence of nigericin but not in the presence of sodium azide, it can be concluded that SP$_{PhoD}$-LacZ is transported in a Sec-independent manner. Transport of SP$_{PhoD}$-LacZ in an *E. coli tatABCDE* strain revealed that transport was independent of the *E. coli* Tat components. These data show that the minimal requirement of a specific Tat-dependent protein translocation system is consisting of a pair of TatA and TatC proteins, a signal peptide specifically recognized by these Tat components, and the existence of the ΔpH gradient across the cytosolic membrane.

PhoD as well as SP$_{PhoD}$-LacZ is not recognized by the *E. coli* Tat system. Our previous results obtained in *B. subtilis* revealed that transport of PhoD is mediated by TatC$_d$ but is independent of the TatC$_y$ protein (19). These observations implied that *B. subtilis* contains at least two specific routes for Tat translocation. Further, *E. coli tat* strains could not be complemented by its *B. subtilis* Tat proteins (19). Finally, absence of *E. coli tat* genes did not prevent TatA$_d$/C$_d$-mediated transport of SP$_{PhoD}$-LacZ in *E. coli*. These data strongly implicated that transport of hybrid peptides consisting of the signal peptide of PhoD, the reporter protein, and the TatA$_d$/C$_d$ protein pair form an autonomous Tat translocation system, and the recognition of Tat substrates is a selective process determined by multiple special protein-protein interactions between a given Tat substrate and its specific Tat proteins.

Most of the twin-arginine signal peptide-containing *E. coli*

precursors associate in the cytosol with cofactors (17). As shown for a mutant glucose-fructose oxidoreductase, association with its cofactor NADP was a prerequisite for the efficient export of the protein (30). We currently cannot exclude that PhoD needs the association of cofactors prior to translocation. Since the protein could be exported efficiently by using the Sec-dependent signal peptide of β-lactamase, necessity of a *B. subtilis*-specific cofactor association prior to transport appears to be unlikely.

The efficiency of SP_{Bla}-PhoD processing in *E. coli* far exceeded transport kinetics observed for wild type PhoD observed in the gene donor strain. While the half-life of pre-PhoD in *B. subtilis* far exceeded 40 min (20), the half-life of the SP_{Bla}-PhoD precursor in *E. coli* was about 10 min. Slow processing kinetics of pre-PhoD could be due to slower transport kinetics of the Tat pathway compared with the efficient Sec-dependent pathway. Overexpression of *phoD* in *E. coli* was highly toxic for the cell. Immediately after induction of PhoD synthesis cell growth was blocked. Most likely cytosolic phosphodiesterase activity was highly detrimental for *E. coli*. After co-expression of $tatA_d/C_d$ no PhoD could be detected by using Western blotting. Co-expression of $TatA_d/C_d$ proteins obviously resulted in additional impairment of cell viability preventing further protein synthesis of PhoD or raising protease degradation of the heterologous peptide. The induction of the SP_{PhoD}-LacZ fusion protein was not lethal for the *E. coli* cell. Several signal peptide-LacZ fusion proteins were previously used for studies of the Sec-dependent protein transport in *E. coli*. These fusion proteins were usually not transported through the cytosolic membrane. High level induction of these proteins was frequently detrimental for *E. coli* due to jamming of the Sec machinery (32–36). Induction of production of SP_{PhoD}-LacZ was not toxic for *E. coli* either in the absence or in the presence of *B. subtilis* $TatA_d/C_d$ proteins. Since SP_{PhoD} is not recognized by the *E. coli* Sec or Tat machinery the fusion protein remained cytosolic. Co-induction of *B. subtilis* $TatA_d/C_d$ proteins transported SP_{PhoD}-LacZ independent of *E. coli*-specific transport paths. Therefore, its translocation did not interfere with essential export functions of the cell. Tat-mediated export of LacZ is consistent with the capacity of the Tat translocation system to transport proteins that are probably folded prior to translocation.

Despite the fact that SP_{PhoD}-LacZ was partially processed only in *E. coli* TG1(pAR*phoD′-lacZ*, pREP4, pQE9$tatA_d/C_d$), the protein was entirely proteinase K-accessible. This observation indicates that the $TatA_d/C_d$ components transport the protein efficiently through the cytosolic membrane, but cleavage of the signal peptide by *E. coli* LepB was inefficient. In *E. coli* TG1 Δ*tatABCE*(pAR*phoD′-lacZ*, pREP4, pQE9$tatA_d/C_d$) no processing of SP_{PhoD}-LacZ could be observed. At the moment there is no experimental knowledge about whether, when, and how the *E. coli* leader peptidase LepB cleaves signal peptides of Tat substrates. The presence of *E. coli* Tat components could be a prerequisite for cleavage of twin-arginine leader peptides by LepB.

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REFERENCES

1. Dalbey, R. E., and Robinson, C. (1999) *Trends Biochem. Sci.* **24,** 17–22
2. Settles, A. M., and Martienssen, R. (1998) *Trends Cell Biol.* **8,** 494–501
3. Wexler, M., Bogsch, E. G., Klösgen, R. B., Palmer, T., Robinson, C., and Berks, B. C. (1998) *FEBS Lett.* **431,** 339–342
4. Robinson, C., and Bolhuis, A. (2001) *Nat. Rev. Mol. Cell. Biol.* **2,** 350–356
5. Wu, L. F., Ize, B., Chanal, A., Quentin, Y., and Fichant, G. (2000) *J. Mol. Microbiol. Biotechnol.* **2,** 179–189
6. Bogsch, E. G., Sargent, F., Stanley, N. R., Berks, B. C., Robinson, C., and Palmer, T. (1998) *J. Biol. Chem.* **273,** 18003–18006
7. Sargent, F., Bogsch, E. G., Stanley, N. R., Wexler, M., Robinson, C., Berks, B. C., and Palmer, T. (1998) *EMBO J.* **17,** 3640–3650
8. Weiner, J. H., Bilous, P. T., Shaw, G. M., Lubitz, S. P., Frost, L., Thomas, G. H., Cole, J. A., and Turner, R. J. (1998) *Cell* **93,** 93–101
9. Settles, A. M., Yonetani, A., Baron, A., Bush, D. R., Cline, K., and Martienssen, R. (1997) *Science* **278,** 1467–1470
10. Chanal, A., Santini, C., and Wu, L. (1998) *Mol. Microbiol.* **30,** 674–676
11. Walker, M. B., Roy, L. M., Coleman, E., Voelker, R., and Barkan, A. (1999) *J. Cell Biol.* **147,** 267–276
12. Mori, H., Summer, E. J., and Cline, K. (2001) *FEBS Lett.* **501,** 65–68
13. Jack, R. L., Sargent, F., Berks, B. C., Sawers, G., and Palmer, T. (2001) *J. Bacteriol.* **183,** 1801–1804
14. Yahr, T. L., and Wickner, W. T. (2001) *EMBO J.* **20,** 2472–2479
15. Bolhuis, A., Mathers, J. E., Thomas, J. D., Barrett, C. M., and Robinson, C. (2001) *J. Biol. Chem.* **276,** 20213–20219
16. Sargent, F., Gohlke, U., De Leeuw, E., Stanley, N. R., Palmer, T., Saibil, H. R., and Berks, B. C. (2001) *Eur. J. Biochem.* **268,** 3361–3367
17. Berks, B. C., Sargent, F., and Palmer, T. (2000) *Mol. Microbiol.* **5,** 260–274
18. Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Brouillet, S., Bruschi, C. V., Caldwell, B., Capuano, V., Carter, N. M., Choi, S. K., Codani, J. J., Connerton, I. F., Danchin, A., *et al.* (1997) *Nature* **390,** 249–256
19. Jongbloed, J. D. H., Martin, U., Antelmann, H., Hecker, M., Tjalsma, H., Venema, G., Bron, S., van Dijl, J. M., and Müller, J. (2000) *J. Biol. Chem.* **275,** 41350–41357
20. Müller, J. P., and Wagner, M. (1999) *FEMS Microbiol. Lett.* **180,** 287–296
21. Müller, J., Walter, F., van Dijl, J. M., and Behnke, D. (1992) *Mol. Gen. Genet.* **235,** 89–96
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
23. Stanley, N. R., Findlay, K., Berks, B. C., and Palmer, T. (2001) *J. Bacteriol.* **183,** 139–144
24. Laemmli, U. K. (1970) *Nature* **227,** 680–685
25. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
26. Kiefer, D., Hu, X., Dalbey, R., and Kuhn, A. (1997) *EMBO J.* **16,** 2197–2204
27. Perez-Perez, J., and Gutierrez, J. (1995) *Gene* (*Amst.*) **158,** 141–142
28. Blaudeck, N., Sprenger, G. A., Freudl, R., and Wiegert, T. (2001) *J. Bacteriol.* **183,** 604–610
29. Thomas, J. D., Daniel, R. A., Errington, J., and Robinson, C. (2001) *Mol. Microbiol.* **39,** 47–53
30. Santini, C. L., Ize, B., Chanal, A., Müller, M., Giordano, G., and Wu, L. F. (1998) *EMBO J.* **17,** 101–112.
31. Oliver, D. B., Cabelli, R. J., Dolan, K. M., and Jarosik, G. P. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 8227–8231
32. Bassford, P. J., Jr., Silhavy, T. J., and Beckwith, J. (1979) *J. Bacteriol.* **139,** 19–31
33. Rasmussen, B. A., Bankaitis, V. A., and Bassford, P. J., Jr. (1984) *J. Bacteriol.* **160,** 612–617
34. Tommassen, J., Leunissen, J., van Damme-Jongsten, M., and Overduin, P. (1985) *EMBO J.* **4,** 1041–1047
35. Ito, K., Akiyama, Y., Yura, T., and Shiba, K. (1986) *J. Bacteriol.* **167,** 201–204
36. Voorhout, W., De Kroon, T., Leunissen-Bijvelt, J., Verkleij, A., and Tommassen, J. (1988) *J. Gen. Microbiol.* **134,** 599–604
37. Leenhouts, K., Buist, G., Bolhuis, A., ten Berge, A., Kiel, J., Mierau, I., Dabrowska, M., Venema, G., and Kok, J. (1996) *Mol. Gen. Genet.* **253,** 217–224
38. Vagner, V., Dervyn, E., and Ehrlich, S. D. (1998) *Microbiology* **144,** 3097–3104
39. Hamilton, C. M., Aldea, M., Washburn, B. K., Babitzke, P., and Kushner, S. R. (1989) *J. Bacteriol.* **171,** 4617–4622
40. Wexler, M., Sargent, F., Jack, R. L., Stanley, N. R., Bogsch, E. G., Robinson, C., Berks, B. C., and Palmer, T. (2000) *J. Biol. Chem.* **275,** 16717–16722
41. Carter, P., Bedouelle, H., and Winter, G. (1985) *Nucleic Acids Res.* **13,** 4431–4443

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Sequence-specific Binding of prePhoD to Soluble $TatA_d$ Indicates Protein-mediated Targeting of the Tat Export in *Bacillus subtilis*

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The Tat (twin-arginine protein translocation) system initially discovered in the thylakoid membrane of chloroplasts has been described recently for a variety of eubacterial organisms. Although in *Escherichia coli* four Tat proteins with calculated membrane spanning domains have been demonstrated to mediate Tat-dependent transport, a specific transport system for twin-arginine signal peptide containing phosphodiesterase PhoD of *Bacillus subtilis* consists of one TatA/TatC ($TatA_d$/$TatC_d$) pair of proteins. Here, we show that $TatA_d$ was found beside its membrane-integrated localization in the cytosol were it interacted with prePhoD. prePhoD was efficiently co-immunoprecipitated by $TatA_d$. Inefficient co-immunoprecipitation of mature PhoD and missing interaction to Sec-dependent and cytosolic peptides by $TatA_d$ demonstrated a particular role of the twin-arginine signal peptide for this interaction. Affinity of prePhoD to $TatA_d$ was interfered by peptides containing the twin-arginine motif but remained active when the arginine residues were substituted. The selective binding of $TatA_d$ to peptides derived from the signal peptide of PhoD elucidated the function of the twin-arginine motif as a target site for pre-protein $TatA_d$ interaction. Substitution of the binding motif demonstrated the pivotal role of basic amino acid residues for TatA binding. These features suggest that TatA interacts prior to membrane integration with its pre-protein substrate and could therefore assist targeting of twin-arginine pre-proteins.

Bacteria have two distinct pathways for the export of proteins across the cytoplasmic membrane. The majority of the periplasmic proteins are exported in an unfolded conformation via the Sec pathway, which is promoted by ATP hydrolysis (1–4). Proteins translocated by this pathway are targeted to the membrane-embedded proteinaceous Sec pore by soluble targeting factors (1). The transport is mediated by N-terminal signal peptides that are similar structurally but do not show sequence conservation (5).

Several proteins use an alternate translocation path. Because of its highly conserved twin-arginine sequence motif present in the signal peptides of proteins using this way, it is called Tat[1] (twin-arginine translocation) pathway (6–8). It was originally identified in chloroplasts (9, 10) and has been described recently (9, 11) for *Escherichia coli*. The currently only known driving force of the translocation is the pH gradient at the membrane (9, 12). Because most of the Tat substrates require the incorporation of cofactors or subunit association (13–15), the Tat pathway appears to be responsible for proteins incompatible with the Sec pathway (16). By a not yet identified control mechanism the Tat system exports only Tat substrates that attained a native conformation (15–17).

The currently best characterized Tat system of *E. coli* consists of four proteins with calculated membrane spanning domains (10, 18, 19). Sequence analysis predicts that TatA, TatB, and TatE are proteins that comprise a transmembrane N-terminal α-helix followed by an amphiphatic α-helix at the cytoplasmic side of the membrane (20, 21). TatC, the fourth protein known to be involved in the Tat system of *E. coli*, has six predicted transmembrane helices (20). Topology determination revealed that TatC contains four transmembrane helices (18). Although *tatB* and *tatC* appear to play a pivotal role in the Tat-dependent protein translocation in *E. coli* (11, 19, 20), TatA and TatE can, at least partially, functionally substitute each other (20). By analyzing the presence of Tat substrates and components of the Tat machinery surveys of prokaryotic genomes indicate that the Tat pathway is wide spread among bacteria and archaea (8, 22). The standard Tat systems (such as in *E. coli*) consist of one TatC, at least one copy of TatA/E, and TatB (23). In certain prokaryotes a TatB homolog appears to be absent.

Currently there exist only weak ideas about the targeting of the twin-arginine precursors to the translocase unit and the structure of the Tat transport system. *In vitro* work with the plant thylakoid Tat system demonstrated that no soluble factors are required of Tat-dependent export (24) and that TatA is required for the transport steps following precursor recognition (25, 26). The TatA/B/E proteins have been predicted to act as membrane receptors for Tat substrates (22) or to form the export channel itself (27–31). The targeting of Tat-dependent

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[1] The abbreviations used are: Tat, twin arginine translocation; IPTG, isopropyl-β-D-thiogalactopyranoside; PBS, phosphate-buffered saline.

TABLE I
Plasmids and strains

Stains/plasmids	Relevant properties	Sources
Plasmids		
pAR3*phoD*	pAR3 derivative; carrying the *phoD* gene; Cm^r	Ref. 33
pAR3*phoD-lacZ*	pAR3 derivative; carrying a fusion gene consisting of the signal sequence region of *phoD* and *lacZ;* Cm^r	Ref. 33
pREP4	Plasmid, containing *lacI* repressor gene; Km^r	Qiagen
pQE9$tatA_d$	pQE9 derivative; allows IPTG-inducible synthesis of His-$TatA_d$	This work
pQE60$tatC_d$	pQE9 derivative; allows IPTG-inducible synthesis of $TatC_d$-His	This work
pQE9$tatA_d/C_d$	pQE9 derivative; allows the IPTG-inducible expression of the *B. subtilis* $tatA_d/C_d$ wild type genes	Ref. 33
pQE9$phoD_p$	pQE9 derivative; allows IPTG-inducible synthesis of His-prePhoD	Ref. 33
pQE9$phoD_m$	pQE9 derivative; allows IPTG-inducible synthesis of signal peptide less His-PhoD	This work
pQEC	pQE9 derivative; allows IPTG-inducible synthesis of CopR	Ref. 57
pQE9$yvaY_p$	pQE9 derivative; allows IPTG-inducible synthesis of His-preYvaY	This work
pQE9$yvaY_m$	pQE9 derivative; allows IPTG-inducible synthesis of mature His-YvaY	This work
pREP9	*E. coli- B. subtilis* shuttle plasmid containing IPTG-inducible P_{pac} promotor; Km^r, Cm^r	Ref. 72
pREP9$tatA_d/C_d$	pREP9 derivative; allows IPTG-inducible expression of the *B. subtilis* $tatA_d/C_d$ wild type genes	This work
Strains		
E. coli		
TG1	F^-; *araD*139; Δ(*ara-leu*)7696; Δ(*lac*)X74; *galU; galK; hsdR*2; *mcrA; mcrB*1; *rspL*	Ref. 35
B. subtilis		
168	*trpC2*	Ref. 32
GCH871	168 *phoR12 trpC2;* allows IPTG-inducible induction of *phoD* operon; Cm^r	Ref. 40
168::pORI22-$tatA_d$-TAP	168 derivative strain; allows pho regulon-inducible synthesis of $TatA_d$-TAP; Em^r	This work
168::pORI22-$tatA_d$-TAP	168 derivative strain; allows pho regulon-inducible synthesis of $TatA_d$-TAP; Em^r	This work
MH5444	*trpC2 pheA1 phoD*♌pSE4; deleted for *phoD;* containing promotorless $tatA_d/tatC_d$, Cm^r	Ref. 38

iron-sulfur protein HiPIP to the membrane appears to be independent of the Tat components (15). The use of the Tat system by redox proteins in many bacteria or preferably non-redox proteins in other species, as well as the different composition of the Tat proteins, indicates that the Tat system is of heterogeneous nature (8, 22, 23).

Analysis of Tat-like proteins in *Bacillus subtilis* revealed that the genome encodes three TatA and two TatC-like proteins (31). TatB-like proteins appear to be absent of *B. subtilis* and of other sequenced bacilli (22, 32). Translocation of the *B. subtilis* protein PhoD containing a twin-arginine signal peptide was shown to be dependent on the expression of a *tatA tatC* pair (33). These genes (designated $tatA_d$ and $tatC_d$) are co-localized with *phoD* in one operon resulting in co-regulated expression of *phoD* and $tatA_d/C_d$. A second copy of *tatC* ($tatC_y$) was not required for PhoD export (31). The PhoD-specific transport system was functionally active in *E. coli*. Although PhoD or a fusion consisting of the signal peptide of PhoD and LacZ was not recognized by the *E. coli* Tat components, co-expression of *B. subtilis* $tatA_d/tatC_d$ resulted in translocation of SP_{PhoD}-LacZ. This transport was shown to be Δ pH-dependent. These studies revealed that the minimal requirement of a Tat transport system consists of a TatA/TatC pair, a twin-arginine signal peptide, and the pH gradient at the bacterial cytoplasmic membrane (33). PhoD is the only known substrate of the $TatA_d$/$TatC_d$ system (31, 34).

The *B. subtilis phoD* gene encodes an secretory enzyme with alkaline phosphatase and phosphodiesterase activities (36). Slow processing maintains the protein in a cell wall-associated localization before release (37). It has been demonstrated that PhoD is a member of the so-called Pho regulon of *B. subtilis* (38). The Pho regulon comprises a group of genes that are induced in response to the depletion of inorganic phosphate in the growth medium and is regulated by the two component signal transduction system PhoR/PhoP (39). A *phoR12* mutation in *B. subtilis* strain GCH871 was shown to be functionally active under phosphate replete conditions resulting in the induction of Pho regulon genes (40).

To investigate the selectivity and specificity of the PhoD transport system further, we analyzed the localization and affinity of $TatA_d$ by combining genetic and *in vitro* approaches in *B. subtilis* and *E. coli*. Unexpectedly, we found $TatA_d$ in the cytoplasmic membrane, as well as in the cytosol. By using purified $TatA_d$ and prePhoD we demonstrated the interaction of both peptides. Inefficient binding of $TatA_d$ to mature PhoD or Sec-dependent or cytosolic proteins demonstrated the particular role of the twin-arginine-containing signal peptide of PhoD in the recognition process. The selective affinity of $TatA_d$ to peptides derived from the signal peptide of PhoD showed that the twin-arginine motif acts as a binding site of $TatA_d$. Substitution of amino acid residues of the binding motif elucidated the role of particular amino acids of this motif for $TatA_d$ recognition.

EXPERIMENTAL PROCEDURES

Plasmids, Bacterial Strains, and Media—Table I lists the plasmids and bacterial strains used. TY medium (tryptone/yeast extract) contained Bacto tryptone (1%), Bacto yeast extract (0.5%), and NaCl (1%). For pulse labeling of proteins M9 minimal medium was used (41). To induce or repress the *phoD* operon in *B. subtilis* 168 cultures were grown in low or high phosphate defined medium, as described (40). *B. subtilis phoR12* was cultivated in absence of isopropyl-β-D-thiogalactopyranoside (IPTG). Induction of phosphate starvation response was monitored by determining alkaline phosphatase activity as described previously (39). When required, media were supplemented with ampicillin (80 μg/ml), kanamycin (20 μg/ml), chloramphenicol (20 μg/ml or 5 μg/ml), tetracycline (12.5 μg/ml), erythromycin (5 μg/ml), arabinose (0.2%), or IPTG (1 mM).

DNA Techniques—Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of *E. coli* were carried out as described by Sambrook *et al.* (42). Restriction enzymes were from MBI Fermentas. PCR was carried out with the VENT DNA polymerase (New England Biolabs) using chromosomal DNA of *B. subtilis* 168 as template.

To construct pQE9$phoD_m$, the *phoD* gene lagging the 5′-terminal region encoding the signal sequence was amplified from the chromosome of *B. subtilis* strain 168 by PCR using the primers P1 (5′-GTA GGA TCC GCG CCT AAC TTC TCA AGC-3′) containing a *Bam*HI site and P2 (5′-CGA TCC TGC AGG ACC TCA TCG GAT TGC-3′) containing a *Pst*I site. The amplified fragment was cleaved with *Bam*HI and *Pst*I and cloned in the corresponding sites of pQE9. The resulting plasmid pQE9*phoD* allowed the IPTG-inducible synthesis of N-terminally His_6-tagged mature PhoD in *E. coli*.

To overexpress $tatA_d$ and $tatC_d$ genes in *E. coli*, genes were amplified

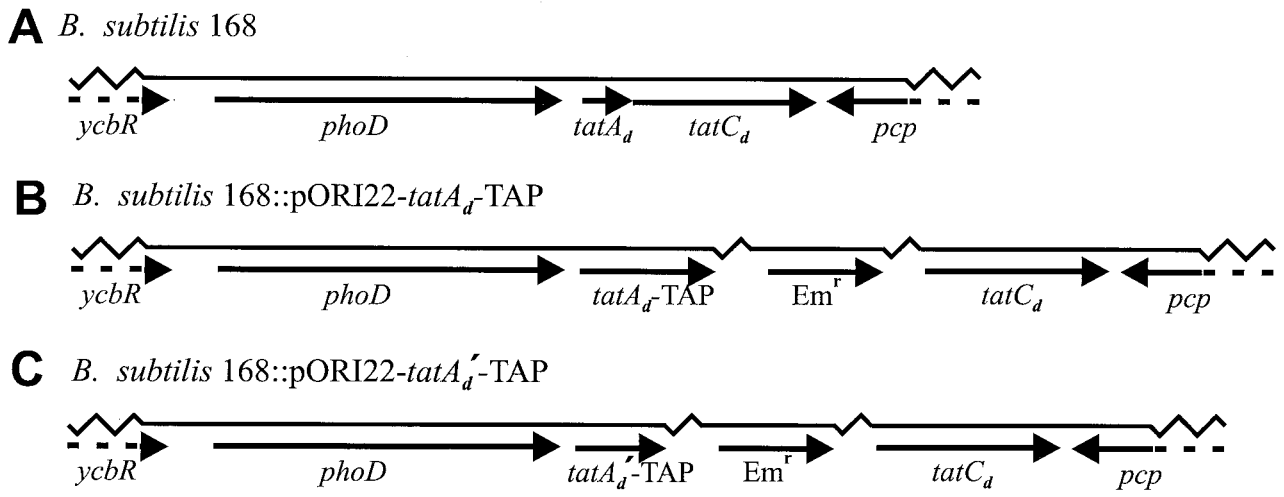

FIG. 1. **Construction of mutant strains of *B. subtilis* $tatA_d$-TAP.** Schematic presentation of the *phoD* operon of *B. subtilis* 168 (*A*), 168::pORI22-$tatA_d$-TAP (*B*), and 168::pORI22-$tatA_d'$-TAP (*C*). By a Campbell-type integration of the pORI22-derivative plasmids were integrated into the chromosome of *B. subtilis* 168. *B. subtilis* 168::pORI22-$tatA_d$-TAP or 168::pORI22-$tatA_d'$-TAP allowed the phosphate starvation-inducible synthesis of fusion proteins consisting of $TatA_d$ and TAP epitopes or the ATG start codon of $TatA_d$ and TAP, respectively. The $tatC_d$ gene in the latter strains was placed under the control of the in *B. subtilis* constitutive *repC* promoter.

using primers A_d1 (5′-GTG GGA TCC ATG TTT TCA AAC ATT GG-3′) containing a *Bam*HI site and primer A_d2 (5′-CCT CTG CAG CAT TCA GCC CGC G-3′) containing a *Pst*I site and C_d1 (5′-TTT CCA TGG ATA AAA AAG AAA CCC-3′) containing a *Nco*I restriction site and C_d2 (5′-GCG GGA TCC GGC CGC CGC TGT TTC TTC-3′) containing a *Bam*HI restriction site, respectively. The $tatA_d$ fragment was cleaved with *Bam*HI and *Pst*I and cloned in the corresponding sites of pQE9. The resulting plasmid pQE9$tatA_d$ allowed the IPTG-inducible synthesis of His_6-$TatA_d$. The $tatC_d$ fragment was cleaved with *Nco*I and *Bam*HI and subsequently cloned into pQE60, cleaved with *Nco*I and *Bgl*II. The resulting plasmid pQE60$tatC_d$ allowed the synthesis of TatC-His_6.

To amplify the DNA region encoding preYvaY primer Y1 (5′-ATG GAT CCA TGA AAA GTA AAT TAC TTA GGC T-3′), incorporating a *Bam*HI restriction site, and primer Y2 (5′-TAA AAG CTT ATT GAT GAA TCA ATT TT-3′), incorporating a *Hin*dIII restriction site, were used. For amplification of the DNA fragment encoding the mature part of YvaY primer Y3 (5′-CAG GAT CCA AAG AAA ACC ATA CAT TT-3′), incorporating a *Bam*HI restriction site, and primer Y2 were used. The PCR fragments were digested with *Bam*HI and *Hin*dIII and inserted 3′-terminal of the His coding region of pQE9 digested with the same enzymes. The resulting plasmids pQE9$yvaY_p$ and pQE9$yvaY_m$ were transformed into *E. coli* TG1(pREP4).

To synthesize epitope-tagged $TatA_d$ the DNA region encoding *phoD* and $tatA_d$ was amplified from the chromosome of *B. subtilis* 168 by using primer P1 (33) and primer T1 (5′-CG GAA TTC CAT AAT TTC CAC TCC TTA ATT CGT GA-3′) containing an *Eco*RI site. A *Hin*dIII-*Eco*RI DNA fragment encoding the 359 3′-terminal amino acids of *phoD* and $tatA_d$ was fused to the DNA region encoding the TAP (calmodulin-binding-protein and protein A) epitopes (43) resulting in an in-frame fusion of $tatA_d$ and TAP. The '*phoD*-$tatA_d$-TAP DNA fragment was placed on the *B. subtilis* nonreplicative vector pORI22 (44) and was transformed into the *B. subtilis* 168. Campbell-like integration into the chromosome resulted in *B. subtilis* strain 168::pORI22-$tatA_d$-TAP (Fig. 1). As a control a DNA fragment encoding *phoD* and the start codon of $tatA_d$ was amplified using primer P1 and A2 (5′-CG GAA TTC GCC CGC GTT TTT GTC CTG CTT TAC CGC-3′) containing an *Eco*RI site and fused to the TAP region resulting in the fusion of the start codon of $tatA_d$ and TAP. After subcloning of the DNA fragment into pORI22 and transformation into *B. subtilis*, strain 168::pORI22-$tatA_d'$-TAP was obtained (Fig. 1). Appropriate mode of integration of pORI22-derived plasmids into the chromosome of *B. subtilis 168* was validated by PCR amplification of junction fragments. For inducible expression of $tatA_d/tatC_d$ genes in *B. subtilis* a 1046-bp *Bam*HI-*Pst*I DNA fragment from pQE9$tatA_d/C_d$ (33) was inserted into pREP9 (45) resulting in pREP9$tatA_d/C_d$.

Freeze-fracture Electron Microscopy—Cells were concentrated by centrifugation, washed two times with growth medium, and resuspended in 10% (v/v) of the initial volume of growth medium containing 15% (w/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 evaporation of platinum/carbon with electron guns was controlled by a thin-layer quartz crystal monitor.

Freeze-fracture Labeling—For freeze-fracture immunogold labeling 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 overnight (46). The replica were washed four times in PBS buffer and treated with PBS + 1% bovine serum albumin for 30 min. Next they were placed in PBS containing bovine serum albumin (0.5%) and monospecific antibodies against $TatA_d$, SecY, or DnaK (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 of gold; British Biocell International, Cardiff, UK) in PBS containing 0.5% bovine serum albumin for 1 h. After immunogold labeling, the replica were immediately rinsed several times in PBS, fixed with 0.5% glutaraldehyde in PBS for 10 min at room temperature, washed four times in distilled water, and finally picked onto Formvar-coated grids for viewing in an EM 902 electron microscope (Zeiss, Oberkochen, Germany). Freeze-fracture micrographs were mounted with direction of shadowing from bottom to top at a magnification as indicated in the figures.

Post-embedding Labeling of B. subtilis—Phosphate-starved *B. subtilis* cells were embedded in Lowicryl K4M as described (37). Ultrathin sections of Lowicryl-embedded cells were mounted on nickel grids with carbon-coated Formvar films and subsequently labeled with specific rabbit antibodies and goat anti-rabbit IgG conjugated to 10 nm of gold (British Biocell International, Cardiff, UK) as a secondary antibody (37). Control experiments were performed by staining sections under similar conditions by omitting the specific antibodies.

Cell Fractionation of B. subtilis and E. coli—B. subtilis cells were harvested and resuspended in PBS buffer (140 mM NaCl, 2.7 mM KCl, 1.3 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.3) containing 5 mM phenylmethylsulfonyl fluoride. Cell suspension was passed three times through a French press at 16,000 lb/in^2. Unbroken cells were removed by centrifugation at 10,000 × *g* for 10 min. To obtain membrane-free cytosolic protein cell lysate was centrifuged at 150,000 × *g* for 2 h at 4 °C, and pellet was used as membrane fraction. Cytosolic and membrane fractions of *E. coli* were obtained from spheroblasted cells. Spheroblasts were lysed in 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$ and subsequently centrifuged at 100,000 × *g* for 1 h. Pellets contained membrane fraction and supernatant cytosolic protein.

SDS-PAGE and Western Blot Analysis—SDS-PAGE were prepared as described (47). After separation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Schleicher & Schüll) (48). Proteins were visualized using monospecific antibodies against PhoD (37), $TatA_d$, or $TatC_d$ and alkaline phosphatase-conjugated goat anti-rabbit antibodies (Sigma) according to the manufacturer's instructions.

Purification of His-tagged Proteins—His-tagged proteins were prepared from IPTG-induced *E. coli* TG1(pREP4, pQE9*tatA$_d$*), TG1(pREP4, pQE60*tatC$_d$*), TG1(pREP4, pQE9*phoD$_p$*), TG1(pREP4, pQE9*phoD$_m$*), TG1(pREP4, pQEC), TG1(pREP4, pQE9*yvaY$_p$*), and TG1(pREP4, pQE9*yvaY$_m$*) cultures as abundant proteins and purified by nickel-nitrilotriacetic acid affinity chromatography. The purification of TatC$_d$, CopR, PhoD, and YvaY proteins were carried out under denaturating conditions. Soluble His$_6$-TatA$_d$ was purified under native conditions with sodium phosphate buffer under standard conditions (Qiagen).

Preparation and Purification of Antibodies against TatA$_d$—Purified His$_6$-TatA$_d$ or TatC$_d$-His$_6$ emulsified in MPL + TDM + CWS adjuvant (catalog number M6661; Sigma) at 100 μg/ml were used to immunize New Zealand White rabbits (Charles River, Hilden, Germany). Specific antibodies were affinity-purified from the sera by adsorption to and elution (with 0.1 M glycine at pH 2.5) from nitrocellulose (37). As proved by Western blotting, monospecific antibodies specifically cross-reacted with TatA$_d$ or TatC$_d$.

In Vivo Labeling of His-tagged Proteins—[^{35}S]-Labeled His$_6$-tagged proteins were obtained by pulse labeling of *E. coli* cultures. Strains were grown in M9 minimal medium, expression of genes of interest was induced for 15 min, and cultures were labeled in 50 μCi of [^{35}S]methionine for 5 min. Subsequent purification using nickel-nitrilotriacetic acid affinity chromatography was carried out essentially as described above.

Co-immunopurification—Purification of TAP proteins was carried out following standard procedures (43). Cytosolic fractions of *B. subtilis* cell lysates were prepared in purification buffer IPP150 (43) as described above.

Co-immunoprecipitation—Binding of His$_6$-tagged [^{35}S]-labeled proteins to unlabeled TatA$_d$ was measured as follows: [^{35}S]-labeled proteins were incubated with 10-M excess of His$_6$-TatA$_d$ (total 0.4 μg) at room temperature in 50 μl of PBS buffer supplemented with 0.5% *n*-octyl-β-D-glucothiopyranoside if indicated. For competition experiments synthetic peptides QNNTFDRRKFIQGAGKIAG or QNNTFDAAAFIQGAGKIAG (0.4 M) were added to the reaction mixture. After 60 min, 50 μl of PBS buffer containing 1 μl of monospecific antibodies against the unlabeled protein, pre-complexed with 10 μl of Dynabeads (Dynal, Oslo, Norway), were added, and the mixture was further incubated for 60 min while shaking. Subsequently, the protein A Dynabeads were washed five times with 500 μl of PBS buffer. [^{35}S]-Labeled proteins bound to protein A beads were counted in scintillation liquid. Immunoprecipitation experiments were carried out at least three times.

Synthesis and Screening of the Cellulose-bound Peptide Arrays—Peptide arrays were prepared by automated spot synthesis using the AMS SPOT-robot (Abimed, Langenfeld, Germany) (49, 50). Before screening, the membranes were washed in methanol for 10 min, three times in TBS buffer (50 mM Tris, 137 mM NaCl, 27 mM KCl, pH 8.0), and subsequently incubated in blocking buffer (10% GENOSYS SU-07–250; 5% sucrose, TBST buffer (TBS with 0.05% Tween)) for 3 h. After washing with TBST buffer peptide arrays were incubated with [^{35}S]-labeled His$_6$-TatA$_d$ (50 ng/ml; 10,000 cpm/ml) in blocking buffer for 16 h at room temperature with gentle shaking. Unbound protein was washed out with TBST buffer. Amount of retained [^{35}S]-labeled protein was quantified using phosphorimage analysis. Relative amounts of radioactivity were estimated by using a phosphorimager (Fuji) and associated image analytical software PC-BAS.

RESULTS

Immunogold Labeling of TatA$_d$—The *E. coli* Tat system has the ability to transport folded proteins and enzyme complexes across the cytoplasmic membrane (22, 27). To transport these folded peptides would result in a translocation pore with a minimum diameter of 5 nm (51). We have shown that the TatA$_d$/C$_d$ transport system of *B. subtilis* is able to transport PhoD with a molecular mass of 62.7 kDa, as well as a hybrid protein consisting of the signal peptide of PhoD and LacZ, resulting in a molecular mass of 120 kDa (31, 33), resulting in a necessarily similar sized translocation pore. Because freeze-fracture cytochemistry is known to be a powerful technique to study macromolecular architecture of biomembranes (46, 52) we used this method to investigate the cytoplasmic membrane of *B. subtilis* and *E. coli* containing TatA$_d$/C$_d$ proteins that could form detectable structures. Immunogold labeling of TatA$_d$ with monospecific antibodies was carried out to localize TatA$_d$ and to identify visible ultrastructures and TatA$_d$-containing translocation particles. Immunogold labeling of TatA$_d$ in freeze-fractured membranes of *B. subtilis* 168 cells grown under phosphate starvation revealed an even distribution of TatA$_d$ in the cell envelope. Interesting, most immunogold labels appeared to be clustered both at the protoplasmic (PF) and the exoplasmic side (EF) of the cytoplasmic membrane (Fig. 2, *A* and *B*). Absence of immunogold labeling of freeze fractures of *B. subtilis* 168 wild type cells grown under phosphate replete conditions demonstrated specificity of labeling (Supplemental Fig. 1*C*). Because no labeling of gold-conjugated anti rabbit antibody could be observed in absence of primary antibodies, unspecific binding of the secondary antibody could be excluded (data not shown). To rule out that the monospecific TatA$_d$ antibodies cross-reacted with another phosphate starvation-induced cytosolic protein, we compared the immunodetection of soluble *B. subtilis* proteins between cell extracts obtained from cells grown under phosphate replete and phosphate depleted conditions. No other proteins, except for TatA$_d$, could be detected in the Western blot (data not shown). Unexpectedly, a substantial amount of gold particles was localized at the cytosol indicating that *B. subtilis* TatA$_d$ is not an exclusively membrane integrated protein (Fig. 2*A*). Artificial induction of the phosphate starvation response by using *B. subtilis* 168 *phoR12* resulted in similar distribution of TatA$_d$ except for a higher expression level of TatA$_d$ (Supplemental Fig. 1, *A*, *B*, and *D*). Again clusters of TatA$_d$-labeled protein could be observed but were not linked to vesicle-like structures in the membrane (Supplemental Fig. 1*A*). To validate immunogold labeling for protein localization, freeze-fractured cells were immunogold-labeled with antibodies against the chaperone DnaK and integral membrane protein SecY, a part of the Sec-translocase unit (3). As expected, the chaperone DnaK could be detected in the cytosol only (Supplemental Fig. 2*B*), and immunogold labeling of SecY indicated that SecY was predominantly localized in the cytoplasmic membrane (Supplemental Fig. 2*A*).

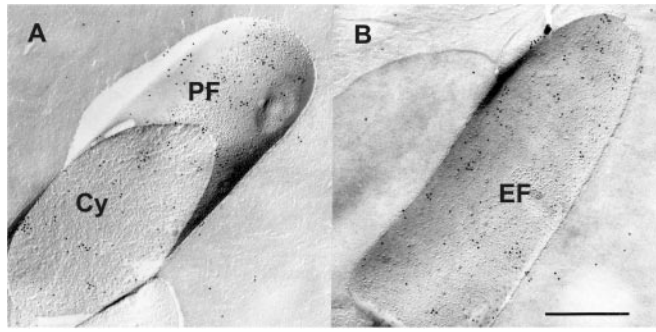

FIG. 2. **Localization of TatA$_d$ in *B. subtilis*.** Cells of *B. subtilis* 168 was grown in low phosphate defined medium to phosphate starvation, freeze-fractured, and subsequently labeled with TatA$_d$-specific antibodies and 10 nm of gold-conjugated secondary antibody. Electron micrographs demonstrate the protoplasmic face (*PF*; *A*) and the fracture through the cytosol (*Cy*; *A*) and the exoplasmic face (*EF*; *B*) of the cytoplasmic membrane. *Scale bar* represents 0.2 μm.

Localization of TatA$_d$ was further elucidated by immunogold labeling of ultrathin sections of *B. subtilis* 168 cells grown under phosphate starvation. Again beside the expected membrane associated localization of the gold particles, about 50% could be detected in the cytosol of the cell (Fig. 3). As a control DnaK and SecY protein were immunogold-labeled in *B. subtilis* 168. Although DnaK was found in the cytosol, SecY was membrane-associated (Supplemental Fig. 3).

To elucidate whether the localization of TatA$_d$ was depending on the presence of prePhoD, we analyzed its localization in a *B. subtilis phoD* strain. Strain MH5444 deleted for *phoD* was transformed with plasmid pREP9*tatA$_d$/C$_d$* allowing the IPTG-

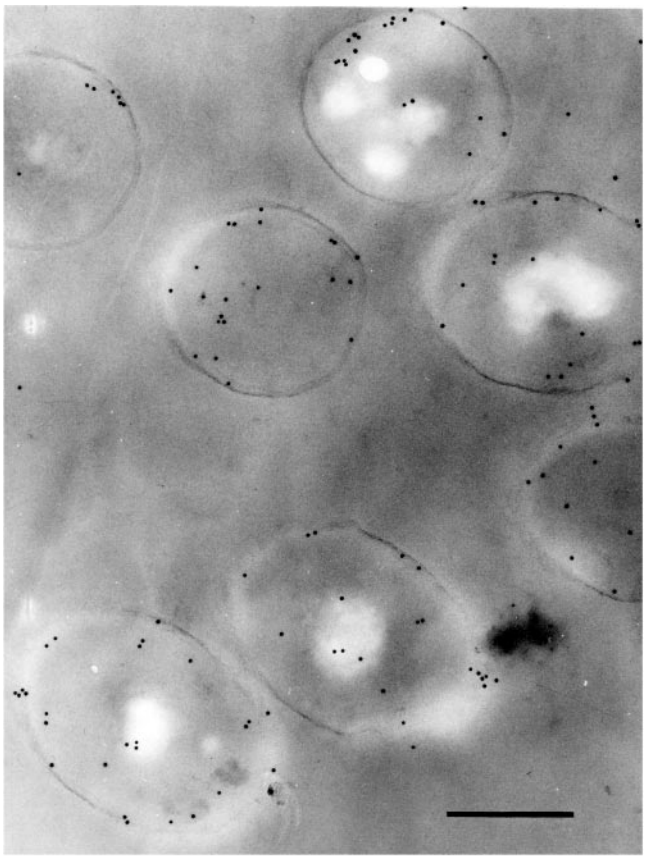

FIG. 3. **Localization of $TatA_d$ in *B. subtilis* 168.** Ultrathin sections of phosphate-starved cells were incubated with $TatA_d$-specific antiserum and subsequently incubated with anti-rabbit IgG-gold. *Scale bar* represents 0.2 μm.

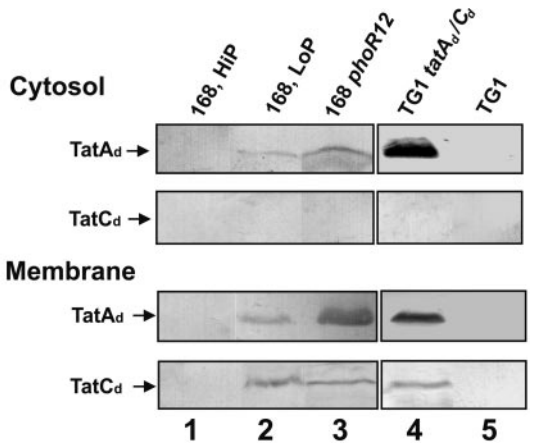

FIG. 4. **Subcellular localization of $TatA_d$ and $TatC_d$ in *B. subtilis* and *E. coli*.** The presence of $TatA_d$ in cytoplasmic and membrane fractions of *B. subtilis* (*lanes 1–3*) and *E. coli* strains (*lanes 4* and *5*) was detected by using Western blotting with monospecific antibodies against $TatA_d$ and $TatC_d$. *B. subtilis* strain 168 was grown in low phosphate defined medium to phosphate starvation (*lane 2*); *B. subtilis* strains 168 (*lane 1*) and GCH871 (*lane 3*) were grown in high phosphate defined medium, *E. coli* strains TG1(pREP4, pQE9*tatA*$_d$/*C*$_d$) (*lane 4*) and TG1(pREP4) (*lane 5*) were grown in TY medium to exponential growth, and expression of *tat* genes was induced for 1 h with IPTG (1 mM). *Bands* representing $TatA_d$ and $TatC_d$ are indicated.

inducible synthesis of the $TatA_d$/$TatC_d$ proteins. MH5444-(pREP9*tatA*$_d$/*C*$_d$) was grown to phosphate starvation, expression of Tat proteins was induced, and localization of $TatA_d$ was detected using immunogold labeling of freeze-fracture cells essentially as described above. No $TatA_d$ could be detected in the cytosol of the cell (data not shown).

Because the PhoD-specific $TatA_d$/$TatC_d$ translocation system has been demonstrated to be functionally active in *E. coli* (33), we analyzed the localization of $TatA_d$ in *E. coli*. Cells of *E. coli* TG1(pREP4, pQE9*tatA*$_d$/*C*$_d$)-expressing *B. subtilis* $TatA_d$/$TatC_d$ proteins were analyzed by freeze-fracture technique with subsequent immunogold labeling of $TatA_d$. Like in *B. subtilis*, immunologically detected $TatA_d$ was localized at the cytoplasmic membrane, as well as in the cytosol (Supplemental Fig. 4).

TatA$_d$ *Is Localized in the Cytoplasmic Membrane and the Cytosol*—The above results indicated that $TatA_d$ is not exclusively localized in the membrane. Immunological detection of $TatA_d$ in cytosolic cell fractions of *B. subtilis* strains 168, GCH871, and TG1(pREP4, pQE9*tatA*$_d$/*C*$_d$) confirmed that a substantial amount of the protein was localized in the cytosol (Fig. 4). Absence of membrane proteins in the cytosolic fraction was monitored by detecting $TatC_d$. No $TatC_d$ could be detected in the cytosolic fractions either of *B. subtilis* strains or of *E. coli* TG1(pREP4, pQE9*tatA*$_d$/*C*$_d$) (Fig. 4).

A kinetic study revealed that cytosolic localization of $TatA_d$ in phosphate-starved *B. subtilis* 168 was variable. At the onset of phosphate starvation substantial amounts of $TatA_d$ was detected in the cytosol. Upon ongoing starvation cytosolic $TatA_d$ decreased (data not shown). The fraction of $TatA_d$ that was co-purified with membranes, resisted carbonate extraction, indicating that this protein is membrane-integrated (data not shown).

Co-purification of TatA$_d$ *with prePhoD—In vivo* synthesized TAP epitope-tagged proteins have been successfully used to demonstrate the interaction between proteins in yeast, plants, and mammalian cells (53–55). To elucidate the affinity of $TatA_d$ for its substrate prePhoD, TAP-tagged $TatA_d$ was synthesized in *B. subtilis*. Strains 168::pORI22-*tatA*$_d$-TAP and 168::pORI22-*tatA*$_d$′-TAP were grown to phosphate starvation, and TAP peptides were subsequently purified from the cytosolic fraction using IgG-Sepharose beads (43). Both $TatA_d$′-TAP and $TatA_d$-TAP peptides could be detected in the cytosolic fraction. Strain 168::pORI22-*tatA*$_d$′-TAP mediating the synthesis of the N-terminal methionine of $TatA_d$ with the TAP epitope ($TatA_d$′-TAP) showed inducible synthesis of a protein with the molecular weight of the TAP epitope (Fig. 5, *lane 2*). Strain 168::pORI22-*tatA*$_d$-TAP produced a protein according to the molecular weight of $TatA_d$-TAP. $TatA_d$-TAP was detected by protein A (Fig. 5, *lane 1*), as well as $TatA_d$ antibodies (data not shown). IgG-purified TAP peptides were assayed for presence of PhoD. Although prePhoD was co-purified with $TatA_d$-TAP, no PhoD could be co-purified with $TatA_d$′-TAP (Fig. 5). This result demonstrated the *in situ* interaction of $TatA_d$ and prePhoD in the cytosol of the cell.

TatA$_d$ *Has Affinity for prePhoD*—After demonstrating interaction of $TatA_d$ with prePhoD in the cytosol, co-immunoprecipitation experiments were carried out to further investigate specificity of this interaction *in vitro*. Complex formation of purified [^{35}S]-labeled His_6-prePhoD (Table II) with purified His_6-$TatA_d$ was assessed by immunoprecipitation with $TatA_d$-specific antibodies. Taking into account that soluble $TatA_d$ forms high molecular weight homomultimers,[2] 10-M excess of $TatA_d$ was used to co-immunoprecipitate peptides. His_6-prePhoD could be co-immunoprecipitated with His_6-$TatA_d$, whereas only low levels of His_6-prePhoD were immunoprecipitated when either the $TatA_d$ or the $TatA_d$ antibodies were omitted from the mixture (Table II).

To analyze the specificity of $TatA_d$ we investigated affinity to

[2] O. Pop and J. P. Müller, unpublished data.

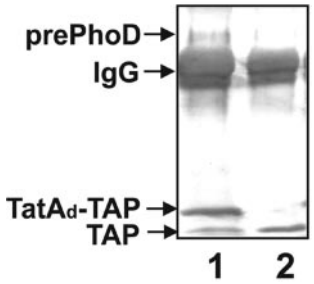

FIG. 5. **Co-purification of $TatA_d$ and prePhoD.** *B. subtilis* strains 168::pORI22-*tatA$_d$*-TAP (*lane 1*) and 168::pORI22-*tatA$_d$*′-TAP (*lane 2*) were grown to phosphate starvation for 2 h. Cells were lysed, and TAP-containing peptides were purified from the cytosolic fraction by using IgG beads. Purified TAP proteins were separated via SDS-PAGE and subsequently detected by using Western blotting with monospecific antibodies against protein A and PhoD. Bands representing $TatA_d$-TAP, $TatA_d$′-TAP (*TAP*), IgG, and prePhoD are indicated.

TABLE II

Co-immunoprecipitation of $TatA_d$ with purified [^{35}S]-His_6 proteins

Purified [^{35}S]-labeled proteins were immunoprecipitated with TatA-specific antibodies pre-complexed with protein A Dynabeads. In parallel, $TatA_d$ or $TatA_d$ antibodies were omitted from the reaction as indicated. Immunoprecipitates were collected, and the amount of bound [^{35}S]-labeled proteins was quantified by counting in scintillation liquid. Amount of bound prePhoD was quantified by counting in scintillation liquid. Bound prePhoD is indicated in percent of input. Data were obtained from a representative experiment.

His_6 protein	$TatA_d$	pAb[a] $TatA_d$	Bound [^{35}S]protein
prePhoD	+	+	58.6
	+	−	13.0
	−	+	21.0
PhoD	+	+	25.0
	+	−	3.3
	−	+	12.0
preYvaY	+	+	8.5
	+	−	2.9
	−	+	17.7
YvaY	+	+	4.4
	+	−	6.0
	−	+	12.3
CopR	+	+	12.0
	+	−	8.0
	−	+	23.4

[a] pAb, polyclonal antibodies.

radiolabeled mature PhoD, to the precursor and mature Sec-dependent *B. subtilis* protein YvaY (56), as well as CopR, a cytosolic protein involved in replication control of plasmids (57). His_6-tagged proteins were purified as abundant proteins (Supplemental Fig. 5). As shown in Table II only 25% of mature His_6-PhoD could be co-immunoprecipitated by His_6-$TatA_d$ compared with 58% of His_6-prePhoD (Table II). Because the amount of bound mature [^{35}S]-His_6-PhoD was hardly higher when antibodies or $TatA_d$ were omitted, only weak interaction can be concluded. Amounts of co-immunoprecipitated [^{35}S]-labeled His_6-preYvaY, His_6-YvaY, and His_6-CopR fairly protruded the level of bound protein when $TatA_d$ or $TatA_d$ antibody was omitted, demonstrating no interaction with Sec-dependent or cytosolic proteins. These data indicate that the signal peptide of PhoD contains specific information mediating the affinity to $TatA_d$. Essentially similar results were obtained when octylglucoside was omitted from the reactions.

TatA$_d$ Specifically Binds to Twin-arginine Peptides of the Signal Peptide of PhoD—Cellulose-bound peptide arrays have been used successfully to characterize substrate binding motifs of proteins (58). To determine the sequence-specific information necessary for binding of $TatA_d$ to prePhoD, we screened a cellulose-bound peptide scan of the N-terminal region of prePhoD for $TatA_d$ binding. The peptide scan was composed of 20-mer peptides that overlap by 19 residues over the sequence of 60 N-terminal localized amino acids and covering the 56-amino acid residue-long signal peptide of PhoD. The cellulose-bound peptides were incubated with [^{35}S]-labeled His_6-$TatA_d$. $TatA_d$ showed selective affinity to peptides containing the twin-arginine motif (Fig. 6*A*). Binding was most pronounced when these residues were localized at the N-terminal flexible end of the peptide and gradually decreased when they moved to the C-terminal end of the peptides. Optimal affinity was observed for the peptide 26 containing $R_{26}R_{27}$ at its N-terminal end. Interesting, peptide starting with amino acids $R_{27}K_{28}$ also showed pronounced binding (Fig. 6*B*). In addition, affinity could be observed to peptides containing amino acid residues $K_{13}L_{14}K_{15}$ (numbers indicate amino acid position in prePhoD). Binding of [^{35}S]-labeled SecB, the targeting factor of the Sec translocation system of *E. coli*, to the PhoD signal peptide library was different from $TatA_d$ peptide recognition (data not shown) and followed rules identified by Knoblauch *et al.* (59).

To study the role of particular amino acids of the proposed binding motif, the 10 N-terminal amino acid residues of the twin-arginine containing peptide D**RR**KFIQGAGKIAGLS-$LGLT_{25-44}$ were substituted each in turn by all gene-encoded amino acids (Fig. 7). Substitution of the basic amino acid RRK cluster decreased binding of $TatA_d$ most seriously. In this region amino acid substitution except of arginine, histidine, or lysine interfered with the $TatA_d$ peptide interaction. Replacement by acidic amino acids almost abolished affinity of $TatA_d$. Substitution of N-terminal aspartic acid by none acidic residues stimulated binding than compared with the wild type peptide. Replacements of amino acids localized C-terminal of the RRK motif hardly altered binding. Substitutions by arginine, lysine, or histidine stimulated $TatA_d$ binding in the N-terminal half of the peptide. Despite its low affinity, peptide SFQNNTFD**RR**KFIQGAGK$_{18-35}$ was selected to study the role of amino acid residues localized N-terminal of the twin-arginine motif for $TatA_d$ recognition. Substitution of residues Ser-18 to Phe-24 each in turn by all gene-encoded amino acids hardly changed binding to $TatA_d$ (data not shown). Again, charge alteration had most pronounced effects. Although substitution by aspartic acid or glutamic acid reduced binding, introduction of basic amino acids stimulated binding above the $TatA_d$ affinity observed for the wild type-derived peptide (data not shown).

To elucidate the function of the RRK sequence motif for the interaction of $TatA_d$ with prePhoD further, co-immunoprecipitation experiments were carried out in the presence of synthetic peptide containing the twin arginine. Addition of QNNTFD**RRK**FIQGAGKIAG peptide significantly reduced co-immunoprecipitation of prePhoD by $TatA_d$ (Table III). In contrast, peptide QNNTFD**AAA**FIQGAGKIAG containing a substitution of the RRK sequence motif showed less interference of the prePhoD-$TatA_d$ interaction (Table III).

DISCUSSION

Several components with one or six calculated membrane spanning domains have been described to mediate the transport of twin-arginine signal peptide-containing proteins (22). Therefore, Tat-dependent protein export is believed to be independent of soluble cytosolic factors (60). In the present study we demonstrate that a substantial fraction of $TatA_d$ protein of the PhoD-specific *B. subtilis* translocation system can be found beside its expected membrane-integrated localization in the cytosol. Soluble $TatA_d$ was functional active as demonstrated by its affinity to its substrate prePhoD and sequence-specific interaction with twin-arginine containing peptides. Therefore, we currently favor the thesis that $TatA_d$ on its way to the cytoplasmic membrane could fulfill a function as targeting factor for Tat substrate prePhoD.

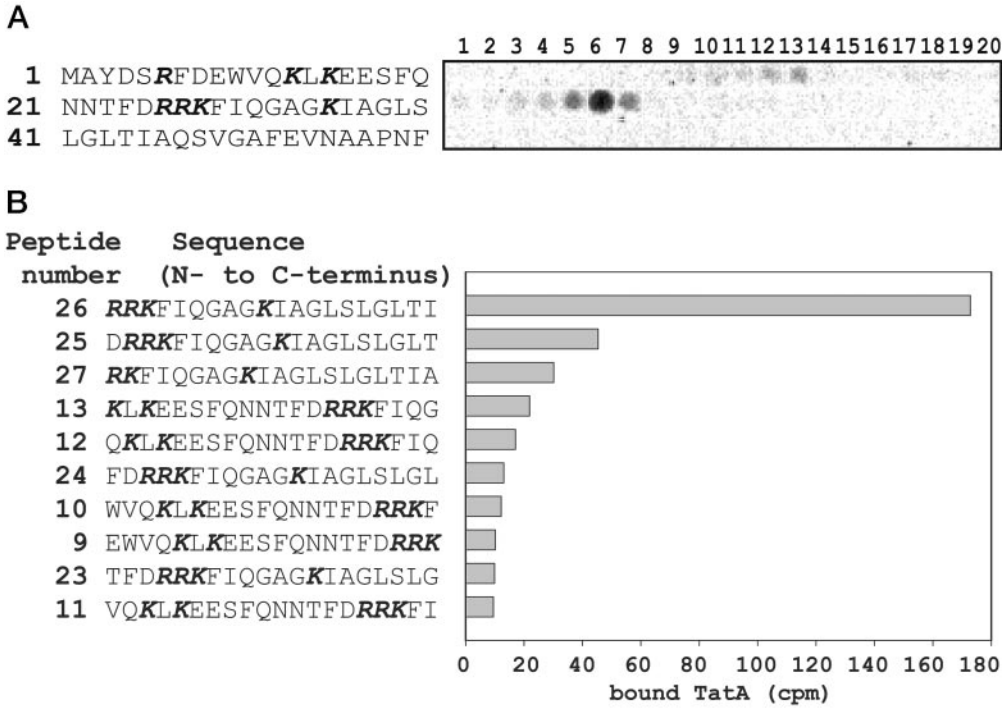

FIG. 6. **$TatA_d$ binding to cellulose-bound peptide scan of prePhoD.** A scan composed of 20-mer peptides derived from the prePhoD sequence that overlap by 19 residues was screened for $TatA_d$ binding. The position of arginine and lysine residues are *bold* and in *italics*. The peptide scan (*A*) was incubated with [^{35}S]-His_6-$TatA_d$, and the amount of $TatA_d$ bound to peptides was visualized by phosphorimaging. The *numbers* on the *left* indicate the first residue of the first *spot* of each *row*. In *B*, amount of bound $TatA_d$ of the 10 best binding peptides of the scan is shown. The *numbers* on the *left* indicate the position in the scan, and the *horizontal bars* demonstrate amount of activity detected.

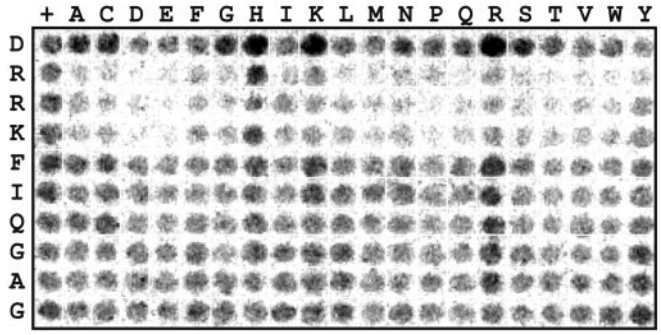

FIG. 7. **Substitution analysis of the twin-arginine binding motif of $TatA_d$.** N-terminal 10 amino acids of peptide DRRKFIQGAGKIAGLSLGLT (indicated on the *left*) derived from the signal peptide of PhoD were substituted by amino acids indicated on the *top*. *Lane* + indicates wild type sequence. Peptide matrix was treated with [^{35}S]-labeled His_6-$TatA_d$ as described.

TABLE III
Co-immunoprecipitation of $TatA_d$ with [^{35}S]-prePhoD

Purified $TatA_d$ and [^{35}S]-prePhoD proteins were mixed in PBS buffer containing 0.5 % *n*-octyl-β-D-glucothiopyranoside. After 60 min of incubation at room temperature, $TatA_d$-specific antibodies pre-complexed with protein A Dynabeads were added, and the incubation was continued for another 60 min. Amount of bound prePhoD was quantified by counting in scintillation liquid. Bound prePhoD is indicated in percent of input with standard deviation.

$TatA_d$	pAb[a] $TatA_d$	Peptide	Bound prePhoD
+	+	–	56.0 ± 14.0
+	+	QNNTFDRRKFIQGAGKIAG	13.0 ± 1.2
+	+	QNNTFDAAAFIQGAGKIAG	38.2 ± 4.5

[a] pAb, polyclonal antibodies.

We have shown previously (33) that the transport of PhoD can be mediated by only two Tat proteins, one similar to TatA and one similar to TatC of *E. coli*. To investigate the function of TatA proteins further, we studied the distribution of the $TatA_d$ protein in *B. subtilis* and in *E. coli* by using freeze-fracture technique and subsequent immunogold labeling. In *B. subtilis*, membrane-bound $TatA_d$ was equally distributed at both sides of the freeze-fractured cytoplasmic membrane. Immunogold-labeled protein was found to be aggregated with proteinaceous structures with a size of less than 5 nm. Absence of larger structures stimulates the hypothesis that the transport of proteins is independent of vesicle formation, which would be visible in the freeze-fractured membranes (22). Freeze-fractured membranes of *E. coli* cells overexpressing $TatA_d$/$TatC_d$ demonstrated the uniform distribution of $TatA_d$ over the surface of the cell envelope.

In addition to its expected membrane-associated localization, freeze fractures through the cytosol indicated that a substantial amount of $TatA_d$ was localized in the cytosol in both bacterial systems. Ultrathin sections of *B. subtilis* cells confirmed an abundant localization of $TatA_d$ in the cytosol. Immunogold labeling of reference proteins demonstrated the reliability of both freeze-fracture analysis and labeling of ultrathin sections. The cytosolic chaperone DnaK could be detected in the cytosol, and SecY was detected predominately in the cytoplasmic membrane. Cytosolic localization of $TatA_d$ was first observed in *E. coli* TG1(pREP4, pQE9*$tatA_d$*) cells overexpressing His_6-$TatA_d$. An abundant amount of His_6-$TatA_d$ was soluble after sonication of the cells under native buffer conditions (data not shown). This unexpected observation was of crucial interest, because soluble $TatA_d$ is functional as it could be demonstrated to bind prePhoD- and prePhoD-derived peptides specifically. Identification of $TatA_d$ in membrane-free cell extracts of *B. subtilis* 168 confirmed the cytosolic localization of $TatA_d$. Absence of $TatC_d$ in the cytosolic fractions of *B. subtilis*, as well as *E. coli*, demonstrated absence of membrane proteins in the cytosolic fraction. Interestingly, the amount of soluble $TatA_d$ varied in dependence of the induction time of the *phoD* operon. Because the amount of cytosolic $TatA_d$ decreased during prolonged phosphate starvation, presence of soluble $TatA_d$ might

depend on the availability of newly synthesized prePhoD substrate to be targeted to the membrane. Absence of cytosolic $TatA_d$ in a strain not producing prePhoD confirmed this thesis. Thus, transient presence of cytosolic homomultimeric $TatA_d$ is linked to its substrate prePhoD. In addition, this observation points out that $TatA_d$ interacts specifically with prePhoD.

Co-immunoprecipitation demonstrated preferred binding of $TatA_d$ to prePhoD. The weak co-purification of mature PhoD indicated that secondary, but obviously less important binding sites, might be present in the mature part of the protein. The Sec-dependent transported *B. subtilis* protein preYvaY, mature YvaY, or cytosolic CopR were not recognized by $TatA_d$. It can therefore be concluded that the twin-arginine signal peptide is the preferred binding site of $TatA_d$. At the moment we can not quantify the stoichiometric ratio of the $TatA_d$-prePhoD complexes. Gel filtration of purified $TatA_d$ indicated that the soluble protein forms complexes bigger than 100 kDa indicating that functional $TatA_d$ acts as a homomultimeric protein.[2]

Affinity of $TatA_d$ to a 20-mer peptide library of the N-terminal region of PhoD elucidated sequence specificity of $TatA_d$ recognition. Selective binding to peptides containing the twin-arginine motif could be observed. The different binding pattern of SecB to the peptide library demonstrated that binding was motif-specific. Recognition of the sequence motif was position-dependent. Localization of the motif at the flexible N-terminal end resulted in stronger $TatA_d$ peptide interaction and gradually decreased when the motif moved to the C-terminal attachment site of the peptide. Accessibility of the recognition motif for $TatA_d$, especially because we observed that $TatA_d$ forms large homomultimeric complexes, might be sterically hampered if it is localized close to the inflexible C terminus of the peptide. Therefore, preferred binding of peptides with N-terminal localized RRK can be explained. The use of 20-mer peptides might underestimate the role of the secondary and tertiary structures of the signal peptide of PhoD for $TatA_d$ recognition. Still, selective binding to twin-arginine motif-containing peptides indicated that the twin-arginine motif is the target site recognized by $TatA_d$ and could play, in general, an essential role for the interaction of the Tat-translocase component TatA with its substrates. This result is consistent with the observation that $TatA_d$ binds preferable prePhoD, but less efficiently the mature PhoD as shown by co-immunoprecipitation.

Additional evidence about the role of particular amino acids for $TatA_d$ binding was obtained by substitution of 10 N-terminal amino acid residues of peptide D**RR**KFIQGAGKI-AGLSLGLT_{25-44} localized in the signal peptide of PhoD. Substitution of the twin-arginine motif unambiguously demonstrated the essence of these residues for $TatA_d$ recognition. Beside substitution of the arginine residues, replacement of the lysine residue had similar consequences for $TatA_d$ binding. Therefore, it can be speculated that this third basic residue belongs to the recognition motif of $TatA_d$. Surprisingly, binding of $TatA_d$ to peptides with altered RRK cluster was mainly charge-dependent. Although conservative substitution hardly reduced binding, uncharged amino acids reduced and acidic amino acid residues abolished $TatA_d$ binding. Remarkably, variation of amino acids localized C-terminal or N-terminal of the RRK cluster had only inferior effects on $TatA_d$ binding indicating that these amino acid residues are not essentially involved in the $TatA_d$ recognition. Based on these data we tend to speculate that RRK_{26-28} is involved in the $TatA_d$ recognition motif. This indicates that the proposed conserved (S/T)RR*X*-FLK sequence motif of the Tat signal peptides (27) might be involved in recognition of other Tat components. Moreover, it cannot be excluded that $TatA_d$, functionally active in the cytosol of the *B. subtilis* cell, is acting different from TatA proteins of other organisms and therefore shares different recognition specificities. Absence of a TatB-like protein indicates that the Tat export in *B. subtilis* might be functionally different from *E. coli*. Most of the bacterial and plant Tat signal peptides studied demonstrated that both arginine residues of the consensus motif were critically important for the Tat transport (7, 13, 61–64). However, it has been demonstrated recently that a single lysine substitutions for arginine either naturally occurring (65) or replaced experimentally (61) were still transported in a Tat-dependent manner. Our data confirm that one of the arginine residues and, in addition, the lysine residue can be substituted by another positively charged amino acid residue and will still be recognized by $TatA_d$. The physiological relevance of this observation is currently being studied in more detail.

Interference of twin-arginine peptides with Tat substrates for interaction with Tat components has been first demonstrated in an *in vitro* translocation system by Alami and coworkers (66). We demonstrated that co-immunoprecipitation of prePhoD by $TatA_d$ was interfered by addition of a peptide containing the RRK motif. Substitution of this motif by alanine residues resulted in a far lesser extent of this interference. These data confirmed that interaction of soluble peptides is sequence-specific, and co-immunoprecipitation experiments reflect *in vivo* function of $TatA_d$. The discrepancy between the efficient interference of peptide QNNTFDRRKFIQGAGKIAG in the co-purification of prePhoD by $TatA_d$ and the inefficient recognition in the cellulose-bound peptide library can be explained by a higher flexibility of soluble peptide.

Despite the fact that TatA has an unusual and not very hydrophobic predicted structure in which only the extreme N terminus has the potential to form a classic hydrophobic transmembrane helix, TatA of *E. coli* has been found membrane-associated only (67, 68). Tha4, the plant TatA orthologue, was localized entirely in the membrane of thylacoids (51, 69). Other proteinaceous factors having affinity to TatA proteins could possibly mediate the targeting of selected substrates (70). *In vitro* translocation systems established for the *E. coli* Tat system demonstrated that no soluble factors are necessary to obtain protein translocation into inverted membrane vesicles (66, 71). Vesicle-based transport systems might underestimate the role of peptide-mediated targeting. In addition, because Tat substrates fold prior to translocation, the necessity for a fast targeting process to maintain export competence might be reduced compared with the Sec translocation system. Cytosolic localization of $TatA_d$ could reflect that Tat translocation in *B. subtilis* acts functionally different from other systems. Although soluble $TatA_d$ could mediate targeting of newly synthesized prePhoD to the translocation site, membrane-integrated $TatA_d$ could be involved in the translocation process. Structural and functional data of homomultimeric $TatA_d$ complexes present in the cytosol, as well as in the membrane,[3] will help to uncover the relevance of the dual localization of $TatA_d$.

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REFERENCES

1. Driessen, A. J., Manting, E. H., and van der Does, C. (2001) *Nat. Struct. Biol.* **8,** 492–498
2. Economou, A. (1999) *Trends Microbiol.* **7,** 315–320
3. Driessen, A. J., Fekkes, P., and van der Does, J. P. (1998) *Curr. Opin. Microbiol.* **1,** 216–222
4. Mori, H., and Ito, K. (2001) *Trends Microbiol.* **9,** 494–500
5. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) *Int. J. Neural Syst.* **8,** 581–599
6. Berks, B. C. (1996) *Mol. Microbiol.* **22,** 393–404

[3] M. Westermann, unpublished data.

7. Chaddock, A. M., Mant, A., Karnauchov, I., Brink, S., Herrmann, R. G., Klosgen, R. B., and Robinson, C. (1995) *EMBO J.* **14,** 2715–2722
8. Dilks, K., Rose, R. W., Hartmann, E., and Pohlschroder, M. (2003) *J. Bacteriol.* **185,** 1478–1483
9. Santini, C. L., Ize, B., Chanal, A., Muller, M., Giordano, G., and Wu, L. F. (1998) *EMBO J.* **17,** 101–112
10. Settles, A. M., Yonetani, A., Baron, A., Bush, D. R., Cline, K., and Martienssen, R. (1997) *Science* **278,** 1467–1470
11. Weiner, J. H., Bilous, P. T., Shaw, G. M., Lubitz, S. P., Frost, L., Thomas, G. H., Cole, J. A., and Turner, R. J. (1998) *Cell* **93,** 93–101
12. Cline, K., Ettinger, W. F., and Theg, S. M. (1992) *J. Biol. Chem.* **267,** 2688–2696
13. Halbig, D., Wiegert, T., Blaudeck, N., Freudl, R., and Sprenger, G. A. (1999) *Eur. J. Biochem.* **263,** 543–551
14. Rodrigue, A., Chanal, A., Beck, K., Muller, M., and Wu, L. F. (1999) *J. Biol. Chem.* **274,** 13223–13228
15. Bruser, T., Yano, T., Brune, D. C., and Daldal, F. (2003) *Eur. J. Biochem.* **270,** 1211–1221
16. DeLisa, M. P., Samuelson, P., Palmer, T., and Georgiou, G. (2002) *J. Biol. Chem.*
17. Thomas, J. D., Daniel, R. A., Errington, J., and Robinson, C. (2001) *Mol. Microbiol.* **39,** 47–53
18. Gouffi, K., Santini, C. L., and Wu, L. F. (2002) *FEBS Lett.* **525,** 65–70
19. Bogsch, E. G., Sargent, F., Stanley, N. R., Berks, B. C., Robinson, C., and Palmer, T. (1998) *J. Biol. Chem.* **273,** 18003–18006
20. Sargent, F., Bogsch, E. G., Stanley, N. R., Wexler, M., Robinson, C., Berks, B. C., and Palmer, T. (1998) *EMBO J.* **17,** 3640–3650
21. Settles, A. M., and Martienssen, R. (1998) *Trends Cell Biol.* **8,** 494–501
22. Wu, L. F., Ize, B., Chanal, A., Quentin, Y., and Fichant, G. (2000) *J. Mol. Microbiol. Biotechnol.* **2,** 179–189
23. Yen, M. R., Tseng, Y. H., Nguyen, E. H., Wu, L. F., and Saier, M. H., Jr., (2002) *Arch. Microbiol.* **177,** 441–450
24. Robinson, C., and Mant, A. (1997) *Trends Plant Sci.* **2,** 431–437
25. Cline, K., and Mori, H. (2001) *J. Cell Biol.* **154,** 719–729
26. Mori, H., and Cline, K. (2002) *J. Cell Biol.* **157,** 205–210
27. Berks, B. C., Sargent, F., and Palmer, T. (2000) *Mol. Microbiol.* **35,** 260–274
28. Bolhuis, A., Bogsch, E. G., and Robinson, C. (2000) *FEBS Lett.* **472,** 88–92
29. Bolhuis, A., Mathers, J. E., Thomas, J. D., Barrett, C. M., and Robinson, C. (2001) *J. Biol. Chem.* **276,** 20213–20219
30. Sargent, F., Gohlke, U., De Leeuw, E., Stanley, N. R., Palmer, T., Saibil, H. R., and Berks, B. C. (2001) *Eur. J. Biochem.* **268,** 3361–3367
31. Jongbloed, J. D., Martin, U., Antelmann, H., Hecker, M., Tjalsma, H., Venema, G., Bron, S., van Dijl, J. M., and Muller, J. (2000) *J. Biol. Chem.* **275,** 41350–41357
32. Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Brouillet, S., Bruschi, C. V., Caldwell, B., Capuano, V., Carter, N. M., Choi, S. K., Codani, J. J., Connerton, I. F., and Danchin, A. (1997) *Nature* **390,** 249–256
33. Pop, O., Martin, U., Abel, C., and Muller, J. P. (2002) *J. Biol. Chem.* **277,** 3268–3273
34. Jongbloed, J. D., Antelmann, H., Hecker, M., Nijland, R., Bron, S., Airaksinen, U., Pries, F., Quax, W. J., van Dijl, J. M., and Braun, P. G. (2002) *J. Biol. Chem.* **277,** 44068–44078
35. Carter, P., Bedouelle, H., and Winter, G. (1985) *Nucleic Acids Res.* **13,** 4431–4443
36. Yamane, K., and Maruo, B. (1978) *J. Bacteriol.* **134,** 108–114
37. Müller, J. P., and Wagner, M. (1999) *FEMS Microbiol. Lett.* **180,** 287–296
38. Eder, S., Shi, L., Jensen, K., Yamane, K., and Hulett, F. M. (1996) *Microbiology* **142,** 2041–2047
39. Hulett, F. M., Bookstein, C., and Jensen, K. (1990) *J. Bacteriol.* **172,** 735–740
40. Müller, J. P., An, Z., Merad, T., Hancock, I. C., and Harwood, C. R. (1997) *Microbiology* **143,** 947–956
41. Muller, J., Walter, F., van Dijl, J. M., and Behnke, D. (1992) *Mol. Gen. Genet.* **235,** 89–96
42. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
43. Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B. (1999) *Nat. Biotechnol.* **17,** 1030–1032
44. Leenhouts, K., Bolhuis, A., Venema, G., and Kok, J. (1998) *Appl. Microbiol. Biotechnol.* **49,** 417–423
45. Le Grice, S. F. (1990) *Methods Enzymol.* **185,** 201–214
46. Fujimoto, K. (1997) *Histochem. Cell Biol.* **107,** 87–96
47. Laemmli, U. K. (1970) *Nature* **227,** 680–685
48. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
49. Frank, R. (1992) *Tetrahedron* **48,** 9217–9232
50. Wenschuh, H., Volkmer-Engert, R., Schmidt, M., Schulz, M., Schneider-Mergener, J., and Reineke, U. (2000) *Biopolymers* **55,** 188–206
51. Robinson, C., and Bolhuis, A. (2001) *Nat. Rev. Mol. Cell. Biol.* **2,** 350–356
52. Takizawa, T., and Robinson, J. M. (2000) *Histol. Histopathol.* **15,** 515–522
53. Gavin, A. C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J. M., Michon, A. M., Cruciat, C. M., Remor, M., Hofert, C., Schelder, M., Brajenovic, M., Ruffner, H., Merino, A., Klein, K., Hudak, M., Dickson, D., Rudi, T., Gnau, V., Bauch, A., Bastuck, S., Huhse, B., Leutwein, C., Heurtier, M. A., Copley, R. R., Edelmann, A., Querfurth, E., Rybin, V., Drewes, G., Raida, M., Bouwmeester, T., Bork, P., Seraphin, B., Kuster, B., Neubauer, G., and Superti-Furga, G. (2002) *Nature* **415,** 141–147
54. Rivas, S., Romeis, T., and Jones, J. D. (2002) *Plant Cell* **14,** 689–702
55. Westermarck, J., Weiss, C., Saffrich, R., Kast, J., Musti, A. M., Wessely, M., Ansorge, W., Seraphin, B., Wilm, M., Valdez, B. C., and Bohmann, D. (2002) *EMBO J.* **21,** 451–460
56. Müller, J. P., Ozegowski, J., Vettermann, S., Swaving, J., Van Wely, K. H., and Driessen, A. J. (2000) *Biochem. J.* **348,** 367–373
57. Steinmetzer, K., and Brantl, S. (1997) *J. Mol. Biol.* **269,** 684–693
58. Gerharz, C. D., Reinecke, P., Schneider, E. M., Schmitz, M., and Gabbert, H. E. (2001) *Urology* **58,** 821–827
59. Knoblauch, N. T., Rudiger, S., Schonfeld, H. J., Driessen, A. J., Schneider-Mergener, J., and Bukau, B. (1999) *J. Biol. Chem.* **274,** 34219–34225
60. Robinson, C. (2000) *Biol. Chem.* **381,** 89–93
61. Stanley, N. R., Palmer, T., and Berks, B. C. (2000) *J. Biol. Chem.* **275,** 11591–11596
62. Dreusch, A., Burgisser, D. M., Heizmann, C. W., and Zumft, W. G. (1997) *Biochim. Biophys. Acta* **1319,** 311–318
63. Henry, R., Carrigan, M., McCaffrey, M., Ma, X., and Cline, K. (1997) *J. Cell Biol.* **136,** 823–832
64. Gross, R., Simon, J., and Kroger, A. (1999) *Arch. Microbiol.* **172,** 227–232
65. Hinsley, A. P., Stanley, N. R., Palmer, T., and Berks, B. C. (2001) *FEBS Lett.* **497,** 45–49
66. Alami, M., Trescher, D., Wu, L. F., and Muller, M. (2002) *J. Biol. Chem.* **277,** 20499–20503
67. Sargent, F. (2001) *Trends Microbiol.* **9,** 196–198
68. De Leeuw, E., Porcelli, I., Sargent, F., Palmer, T., and Berks, B. C. (2001) *FEBS Lett.* **506,** 143–148
69. Mori, H., Summer, E. J., Ma, X., and Cline, K. (1999) *J. Cell Biol.* **146,** 45–56
70. Oresnik, I. J., Ladner, C. L., and Turner, R. J. (2001) *Mol. Microbiol.* **40,** 323–331
71. Yahr, T. L., and Wickner, W. T. (2001) *EMBO J.* **20,** 2472–2479
72. Le Grice, S. F. (1990) *Methods Enzymol.* **185,** 201–214

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Tat dependent export of *E. coli* phytase AppA by using the PhoD-specific transport system of *Bacillus subtilis*

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It has been shown recently that the twin-arginine signal peptide of *Bacillus subtilis* phosphodiesterase PhoD (SP_{PhoD}) can mediate Tat dependent transport of proteins via its specific Tat-transport components. In order to test the use of Tat dependent transport signals for heterologous product synthesis, *Escherichia coli* phytase AppA was expressed under control of PhoD-specific export signals in *B. subtilis*. Induction of Tat components $TatA_d$/$TatC_d$ was mediated by using a functionally altered PhoR/PhoP signal transduction system which regulates the expression of these components. AppA was highly susceptible to host specific extracellular proteases. Expression of *appA* in *B. subtilis wprA* strain resulted in the stable production of AppA. A fusion protein consisting of SP_{PhoD} and mature AppA remained unprocessed, while introduction of the AppA signal peptidase cleavage site resulted in efficient processing of the fusion protein.

Phytases are hydrolytic enzymes that improve the bio-availability of phosphate from phytate, the major phosphorus form in plant based animal feed. Since some animals lack this enzyme, they excrete large amounts of phosphate. For that reason environmental pollution due to high-phosphate manure results in the accumulation of phosphorus at various locations. This has raised to serious ecological problems. Therefore, phytases appear of significant value in effectively controlling phosphate pollution. Different microbial phytase expression and secretion systems including those of bacteria, yeast and fungi have been developed (reviewed in PANDEY *et al*. 2001).

Sec dependent secretion of recombinant proteins from Gram-negative eubacterial or eukaryotic origin is often inefficient because of a variety of bottlenecks in the secretion pathway. Inefficient translocation due to fast or export incompetent folding prior to translocation is one of the potential hindrances of expressing particular heterologous proteins. Therefore, the recently described Tat dependent transport system which is able to export folded proteins has high potential to circumvent this problem. Several signal peptide-LacZ fusion proteins previously used for studies of the Sec dependent protein transport in *E. coli* were not transported through the cytosolic membrane. High level induction of these proteins is frequently detrimental for *E. coli* due to jamming of the Sec-machinery (ITO 1982, RASMUSSEN *et al*. 1984, TOMMASSEN *et al*. 1985, VOORHOUT *et al*. 1988). The efficient translocation of LacZ through the cytosolic membrane of *E. coli* by using the PhoD-specific Tat dependent export system of *B. subtilis* has been recently described (POP *et al*. 2002).

The bacterial Tat dependent protein transport system has been initially described for *E. coli* (reviewed in WU *et al*. 2000, ROBINSON and BOLHUIS 2001, DILKS *et al*. 2003). It is used by proteins that either fold too quickly or have to fold in the cytosol due to co-factor association. A highly conserved twin-arginine sequence motif in the signal peptide targets these proteins via the Tat transport path. The pH gradient at the cytosolic membrane is the driving force of the Tat transport (ROBINSON 2000). Four proteins with calculated membrane spanning domains are involved in this transport process (WEINER *et al*. 1998, SAR-

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GENT *et al*. 1998, BOGSCH *et al*. 1998). TatA, TatB and TatE are sequence related proteins with one predicted transmembrane helix. TatC, the fourth protein known to be involved in the Tat system of *E. coli*, has six predicted transmembrane helices (SARGENT *et al*. 1999). While TatB and TatC appear to play a pivotal role in the Tat dependent protein translocation in *E. coli* (SARGENT *et al*. 1998, WEINER *et al*. 1998, BOGSCH *et al*. 1998), TatA and TatE have an overlapping function (SARGENT *et al*. 1999). During or shortly after translocation, signal peptides are cleaved off by signal peptidases recognising an Ala-X-Ala cleavage site.

The *B. subtilis* genome encodes three TatA-like and two TatC-like proteins (JONGBLOED *et al*. 2000). TatB-like proteins appear to be absent in *B. subtilis* and in other sequenced bacilli (KUNST *et al*. 1997, WU *et al*. 2000, POP *et al*. 2002). Translocation of the *Bacillus subtilis* phosphodiesterase PhoD which contains a twin-arginine signal peptide, was dependent on the expression of $tatA_d$ and $tatC_d$, co-localised with *phoD* in one operon (JONGBLOED *et al*. 2000, POP *et al*. 2002). The PhoD-specific transport system was functionally active in *E. coli* (POP *et al*. 2002). A protein fusion consisting of the signal peptide of PhoD and LacZ (SP_{PhoD}-LacZ) was translocated after co-expression of *B. subtilis* $tatA_d/tatC_d$. These studies revealed that the minimal requirement of a Tat transport system consists of a TatA/TatC pair, a twin-arginine signal peptide and the pH gradient at the bacterial cytosolic membrane (POP *et al*. 2002). $TatA_d$ was found beside of its expected membrane integrated localisation soluble in the cytosol. Soluble homoo-ligomeric $TatA_d$ complexes had affinity for prePhoD (POP *et al*. 2003).

The *phoD* operon is a member of the so-called Pho regulon of *B. subtilis* (EDER *et al*. 1996). The Pho regulon consists of a group of genes which are induced in response to the depletion of inorganic phosphate in the growth medium and is regulated by the two component signal transduction system PhoR/PhoP (HULETT *et al*. 1994). A *phoR12* mutation in *B. subtilis* strain GCH872 was functionally active under phosphate replete conditions resulting in the induction of Pho regulon genes (MÜLLER *et al*. 1997). This strain allows the production and secretion of PhoD upon the induction of the *phoR12* expression. In order to prove the functionality of the PhoD-specific transport system further, SP_{PhoD} mediated export of *E. coli* phytase AppA was characterised in *B. subtilis*. Our data show the functionality of $TatA_d/TatC_d$ translocase for heterologous product synthesis.

Materials and methods

Plasmids, bacterial strains and media: Table 1 lists the plasmids and bacterial strains used in this study. TY medium contained Bacto tryptone (1%), BACTO yeast extract (0.5%) and NaCl (1%). Pulse-labelling of B. subtilis strains was carried out in high phosphate defined medium (HPDM) were methionine was omitted from the medium (MÜLLER et al. 1997). To induce the phoD promoter *B. subtilis phoR12* strains were grown in HPDM with the presence of isopropyl-β-D-thiogalactopyranoside (IPTG). Induction of the phosphate starvation response was monitored by determining alkaline phosphatase activity as described previously (HULETT et al. 1990). When required, media was supplemented with ampicillin (Ap, 100 μg/ml), kanamycin (Km, 20 μg/ml or 5 μg/ml), chloramphenicol (Cm, 20 μg/ml or 5 μg/ml), tetracycline (Tc, 12.5 μg/ml or 5 μg/ml), IPTG (1 mM), for both *E. coli* or *B. subtilis*, respectively.

DNA techniques: Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of *E. coli* were carried out as described in SAMBROOK *et al.* (1989). Restriction enzymes were obtained from MBI FERMENTAS. PCR (polymerase chain reaction) was carried out with the VENT DNA polymerase (New England BIOLABS) using chromosomal DNA of *E. coli* TG1 as the template.

To construct a gene fusion between *phoD* and *E. coli appA* the *appA* gene lacking the 5′-terminal region encoding the signal sequence was amplified from the chromosome of *E. coli* strain TG1 by PCR using the primers X1 (5′-CCC CTC GAG TGC ATT CGC TCA GCG TGA GCC GG-3′) containing a *Xho*I site, and E1 (5′-GGC GAA TTC ATT ACA AAC TGC ACG CCG GT-3′)

Table 1
Bacterial strains and plasmids

B. subtilis strains		
168	*trp*C2	(ANAGNOSTOPOULOS 1961)
GCH872	168 *phoR*::pDKphoR::pDIAphoR12 (P_{phoPR}*phoR* P_{spac}*phoR12*), Cm^r, Km^r	(MÜLLER *et al.* 1997)
DN 1885	168 derivative strain, *amyE*, *amyR2*, Em^r	(DIETRICHSEN *et al.* 1990)
DN 1885 *wprA*	DB1885::pMUTIN′*wprA*′, Em^r	K. STEPHENSON, unpublished
DN 1885 *wprA phoR12*	DB1885::pMUTIN′*wprA*′ *phoR*::pDKphoR::pDIAphoR12 (P_{phoPR}*phoR* P_{spac}*phoR12*), Cm^r, Km^r, Em^r	this work
plasmids		
pBluescript	*lacZ*, fl (+), ColE1 ori, Ap^r	Stratagene
pREP9	*E. coli* – *B. subtilis* – shuttle plasmid, P_{Spac}, *lacI*, Km^r, Cm^r	(LE GRICE 1990)
pORI24	plasmid replicates only in *E. coli rep*$^+$ strains, Tc^r	(LEENHOUTS *et al.* 1998)
pREP9-SPPD-*appA1*	pREP9 derivative plasmid, carrying a fusion gene consisting of the signal sequence region of *phoD* and *appA*, Km^r, Cm^r, Tc^r	this work
pREP9-SPPD-*appA2*	pREP9 derivative plasmid, carrying a fusion gene consisting of the signal sequence region of *phoD* and *appA* including its signal peptide cleavage site, Km^r, Cm^r, Tc^r	this work

containing a *Eco*RI site. The amplified fragment was cleaved with *Xho*I and *Eco*RI. Subsequently, the fragment was cloned in the corresponding sites of pBluescript SK+ (Stratagene). To produce AppA including its own signal peptide cleavage site, the *appA* gene lacking 19 5′-terminal codons of the signal sequence was amplified from the chromosome of TG1 by PCR using the primers E1 and X2 (5′-GGC GAA TTC ATT ACA AAC TGC ACG CCG GT-3′) containing a *Xho*I site. The amplified fragments were cleaved with *Xho*I and *Eco*RI, and subsequently cloned in the corresponding sites of pBluescript SK+ (Stratagene). In order to obtain gene fusions consisting of the signal sequence of *phoD* and '*appA* fragments, the plasmid pORI24*phoD*' was first linearized with *Sal*I and then integrated into the *Xho*I site of the pBluescript*appA* plasmids. Plasmids containing the *phoD-appA* gene fusion were digested with *Eco*RI and recircularised to release the pBluescript sequences. The resulting pORI24*phoD-appA* plasmids were linearized with *Bam*HI and inserted into the *Bam*HI site of pREP9. Plasmids pREP9*phoD-appA1* and pREP9*phoD-appA2* allowed the IPTG inducible synthesis of fusion proteins consisting of SP_{PhoD} and AppA.

SDS-PAGE and Western blot analysis: SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by LAEMMLI (1970). After separation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane (SCHLEICHER and SCHÜLL) as described by TOWBIN *et al.* (1992). Proteins were visualised using monospecific antibodies against $TatA_d$ (laboratory collection) and alkaline phosphatase-conjugated goat anti-rabbit antibodies (SIGMA) according to the manufacturer's instructions.

Pulse-chase experiments, immunoprecipitation and quantification of protein: Pulse-labelling experiments of *B. subtilis* strains was performed as described earlier (MÜLLER and WAGNER 1999). Cultures were pulse labelled with 100 µCi [^{35}S]methionine, chased with an excess of unlabelled methionine and samples were taken at the times indicated immediately followed by precipitation with trichloracetic acid (0 °C). After cell lysis proteins were precipitated with specific antibodies against AppA. Samples were assayed via SDS-PAGE. Relative amounts of radioactivity were estimated by using a PhosphoImager (Fuji) and associated image analytical software PC-BAS.

Results

Inducible expression of appA in B. subtilis

The 56 amino acid residues long signal peptide of PhoD containing the twin-arginine motif (SP_{PhoD}, Fig. 1) has been successfully used to export β-galactosidase in a Tat dependent manner in *E. coli* (POP *et al*. 2002). In order to study its functionality in *B. subtilis*, gene fusions encoding SP_{PhoD} and AppA of *E. coli* were constructed (Fig. 1). Plasmid pREP9*phoD-appA2* encodes a hybrid protein of SP_{PhoD} and 'AppA deleted for its signal peptide. Since the signal peptide cleavage site of prePhoD is inefficiently processed in *B. subtilis* (MÜLLER and WAGNER 1999), plasmid pREP9*phoD-appA1* encoding a hybrid protein containing SP_{PhoD} and AppA including its signal peptidase cleavage site was constructed. AppA contains a standard signal peptide with a typical cleavage site for signal peptidases (Fig. 1). IPTG-mediated induction of expression of SP_{PhoD}-AppA peptides in *B. subtilis* 168 (pREP9*phoD-appA1*) and 168 (pREP9*phoD-appA2*) was monitored by immunological detection of AppA in total cell extracts. No AppA was detected in either strain. Activity of host specific cytosolic proteases might degrade the synthesis of the AppA peptides (data not shown).

In order to export SP_{PhoD}-AppA fusion peptides PhoD-specific translocase components $TatA_d/TatC_d$ were induced. *B. subtilis* strain GCH872 allows the IPTG-inducible induction of phoR12. This results in the induction of the Pho regulon independent of the concentration of inorganic phosphate in the growth medium (MÜLLER *et al.* 1997). Induction of the Pho regulon was monitored by analyzing the induction of alkaline phosphatase activity (data not shown). As demonstrated in Fig. 2, expression of $tatA_d$ was induced in response to the induction of phoR12. In the uninduced strains no $TatA_d$ could be detected. This indicates that the *phoD* operon is regulated by the PhoR/PhoP signal transduction system.

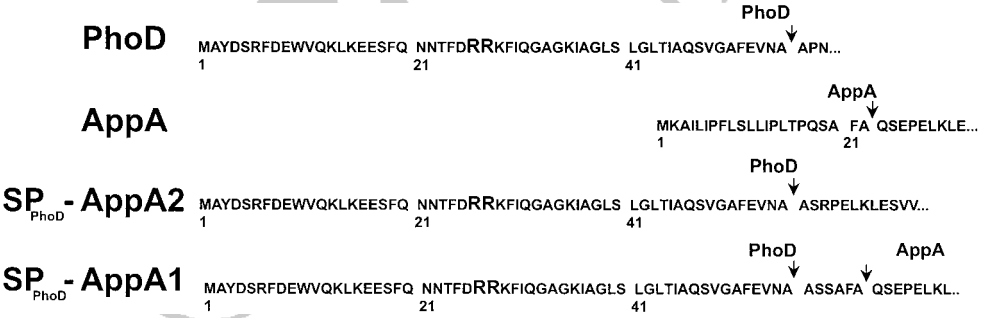

Fig. 1
Signal peptides of PhoD, AppA and PhoD-AppA fusion proteins.
Signal peptide sequences of PhoD, AppA, SP_{PhoD}-AppA fusion proteins. Their signal peptide cleavage sites (arrows) are indicated

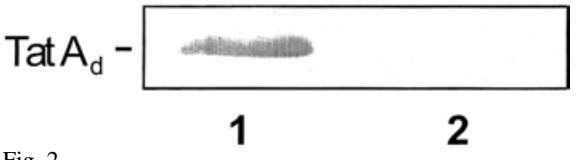

Fig. 2
Induction of PhoD transport components $TatA_d$ in *B. subtilis phoR12*.
B. subtilis GCH872 was grown in TY medium, induction of the *phoR12* was mediated by addition of 1 mM IPTG (lane 1) or remained uninduced (lane 2). 1 hour after induction cells were harvested and lysed and cleared lysates were separated via SDS-PAGE. $TatA_d$ was detected via Western blotting using monospecific antibodies

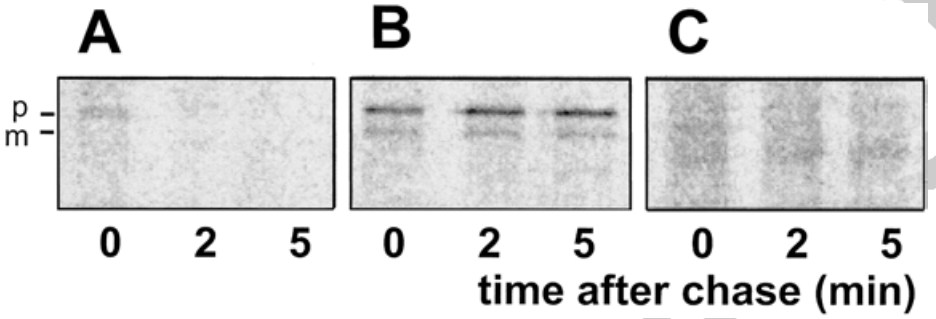

Fig. 3
Induction and processing of SP_{PhoD}-AppA fusion protein in *B. subtilis*.
Strains GCH872(pREP9*phoD-appA2*) (A, B) and GCH872(pREP9*phoD-appA1*) (C) were grown in synthetic medium to early logarithmic growth phase. 1 hour prior labelling expression of the *phoD* operon and the *phoD-appA* hybrid genes was induced with 1 mM IPTG (B, C) or remained uninduced (A). Cells were labelled for 1 min with [^{35}S]methionine, after which non-radioactive methionine was added. Samples were withdrawn at times after chase as indicated in the figures and subjected to immunoprecipitation with antibodies against AppA, followed by SDS-PAGE using a 12.5% polyacrylamide gel and fluorography. Localisation of SP_{PhoD}-AppA and mature AppA is indicated

In strain GCH872 (pREP9*phoD-appA2*) a protein band cross reacting with antibodies against AppA was detected in response to IPTG-mediated gene induction. On the other hand, in strain GCH872 (pREP9*phoD-appA1*) no inducible synthesis of AppA was detectable (data not shown). In order to monitor processing of SP_{PhoD}-AppA2, strain GCH872 (pREP9*phoD-appA2*) was pulse-labelled with [^{35}S]methionine, chased with excess of unlabelled methionine and subsequently AppA was immunoprecipitated. In absence of IPTG no AppA could be immunoprecipitated (Fig. 3A). IPTG mediated induction of *phoR12* and *phoD-appA2* resulted in the synthesis of SP_{PhoD}-AppA2. Most of the immunoprecipitated AppA had a molecular weight equal to that of the expected unprocessed protein. Therefore, it can be concluded that the hybrid protein was only inefficiently processed (Fig. 3B). Immunoprecipitation of AppA of pulse labelled cells of GCH872 (pREP9*phoD-appA1*) showed only traces of protein vastly disappearing upon the chase period (Fig. 3C).

Deletion of wprA stabilizes AppA

Despite the use of the same expression signals to produce SP_{PhoD}-AppA2 or SP_{PhoD}-AppA1, only the AppA variant with the inefficient PhoD processing site could be detected in significant amounts. Reduced stability of AppA in GCH872 (pREP9*phoD-appA1*) can be attributed due to fast degradation after transport of the heterologous protein. The depletion of WprA, a cell wall associated subtilisin protease resulted in enhanced stability of heterologous proteins (STEPHENSON and HARWOOD 1998). In order to analyse if WprA is involved in degradation of AppA, *B. subtilis wprA* knockout strain DN1885 *wprA* was transformed with *appA* plasmids. Transformation of chromosomal DNA derived from strain GCH872 resulted in DN1885 *wprA phoR12*, a strain allowing the IPTG-mediated induction of the *phoD* operon.

Both, in DN1885 *wprA phoR12* (pREP9*phoD-appA2*) as well as DN1885 *wprA phoR12* (pREP9*phoD-appA1*) stable inducible synthesis of AppA was obtained. Processing of SP_{PhoD}-AppA proteins was analysed via pulse-chase labelling with subsequent immunoprecipitation of AppA. Only 25% of the SP_{PhoD}-AppA2 protein synthesised was converted into mature AppA up to 20 minutes post-chase (Fig. 4A). In contrast, SP_{PhoD}-AppA1 containing

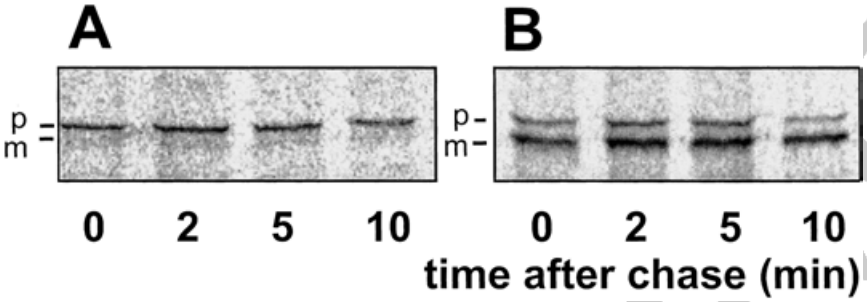

Fig. 4
Kinetics of processing and release of SP_{PhoD}-AppA fusion proteins in *B. subtilis wprA* strains.
B. subtilis strains DN1885 *wprA phoR12* (pREP9*phoD-appA1*) (A) and DN1885 *wprAphoR12* (pREP9*phoD-appA2*) (B) were grown and induced under essential identical conditions like *B. subtilis* $wprA^+$ strains described in Fig. 2. Samples were withdrawn at times after chase as indicated in the figures. Processing of AppA was assayed by immunoprecipitation with antibodies, followed by SDS-PAGE using a 12.5% polyacrylamide gel and fluorography. Localisation of SP_{PhoD}-AppA and mature AppA is indicated

signal peptidase cleavage site of AppA was efficiently converted in its mature form (Fig. 4B). Similar amounts of immunoprecipitated protein during the chase period indicated that the protein remained stable during the post-chase period.

Discussion

In the present report we have shown that the export signals of PhoD, a Tat dependent transported phosphodiesterase, was able to mediate export of *E. coli* phytase AppA in *B. subtilis*. The *phoR12* genetical background, artificially inducing the Pho regulon of *B. subtilis* was used for induction of the *phoD* operon encoding the $tatA_d/tatC_d$ genes. *B. subtilis wprA* strains were used to stabilize AppA in its heterologous host. Upon co-induction of the PhoD specific transport components $TatA_d/TatC_d$ via the PhoR/PhoP two components signal transduction system, AppA could be stably overexpressed and processed in *B. subtilis.* These data provide further evidence that the autonomous PhoD specific transport system is able to transport heterologous Sec dependent proteins.

We have shown previously that the twin-arginine signal peptide of PhoD targets PhoD or LacZ to a noncytosolic localisation. The Tat transport system consisting of $TatA_d/TatC_d$ exports SP_{PhoD} fusion peptides in a Tat dependent manner in *B. subtilis* as well as in *E. coli* (JONGBLOED *et al*. 2000, POP *et al*. 2002). SP_{PhoD} contains characteristics different from other bacterial Sec- or Tat signal peptides (NIELSEN *et al*. 1997). Due to the N-terminal extension, the signal peptide comprising of 56 amino acid residues and is therefore, the longest known *B. subtilis* signal peptide (TJALSMA *et al*. 2000). In addition, its cleavage site does not contain the Ala-x-Ala standard cleavage site. Therefore, transport of prePhoD far exceeds processing. Due to inefficient processing prePhoD remains localised at the outer site of the membrane in *B. subtilis* (MÜLLER and WAGNER 1999). SP_{PhoD} targets PhoD to the $TatA_d/TatC_d$ translocase complex (POP *et al*. 2002). SP_{PhoD}-AppA1 containing a standard Ala-x-Ala processing site was efficiently processed by $TatA_d/TatC_d$ in *B. subtilis.* It can be concluded that inefficient processing is caused by inefficient recognition of the signal peptide cleavage site rather than the N-terminal extension of $SP_{PhoD.}$ About 80% of SP_{PhoD}-AppA1 was processed at the end of the 1 min labelling period. Since no processing of SP_{PhoD}-AppA could be observed in absence of $TatA_d/TatC_d$ proteins, it can be concluded

that the Tat dependent transport system can effectively transport peptides in parallel to Sec dependent transport.

Heterologous proteins are often targets for host specific intracellular as well extracellular proteases. Several attempts to stabilize heterologous proteins have been made. Beside the inactivation of extracellular proteases (WU *et al*. 1991, YE *et al*. 1996, MURASHIMA *et al*. 2002) inactivation of cell wall associated protease WprA (STEPHENSON and HARWOOD 1998, LEE *et al*. 2000) has been demonstrated to stabilise production of recombinant proteins. WprA is a protein involved in cell wall associated quality control, which efficiently removes misfolded or incompletely synthesized proteins (TJALSMA *et al*. 2000). Increased stability of AppA after translocation in *B. subtilis wprA* indicates that WprA is also involved in proof reading of Tat dependent proteins. Since AppA degradation was more pronounced for a hybrid protein efficiently processed than compared to the SP_{PhoD}-AppA2 remained unprocessed after translocation indicates WprA does not act at the surface of the cytosolic membrane.

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References

ANAGNOSTOPOULOS, C. J., 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol., **81**, 741–746.

BOGSCH, E. G., SARGENT, F., STANLEY, N. R., BERKS, B. C., ROBINSON, C. and PALMER, T., 1998. An essential component of a novel bacterial protein export system with homologues in plastids and mitochondria. J. Biol. Chem., **273**, 18003–18006.

DIETRICHSEN, B., WEDSTED, U., HEDEGAARD, L., JENASEN, B. R. and SJONHOLM, C., 1990. Cloning of aldB, which encodes alpha-acetolactate decarboxylase, an exoenzyme from *Bacillus subtilis*. J. Bacteriol., **172**, 4315–4321.

DILKS, K., ROSE, R. W., HARTMANN, E. and POHLSCHRODER, M., 2003. Prokaryotic utilization of the twin-arginine translocation pathway: a genomic survey. J. Bacteriol., **185**, 1478–1483.

EDER, S., SHI, L., JENSEN, K., YAMANE, K. and HULETT, F. M., 1996. A *Bacillus subtilis* secreted phosphodiesterase/alkaline phosphatase is the product of a Pho regulon gene, *phoD*. Microbiology, **142 (Pt 8)**, 2041–2047.

HULETT, F. M., BOOKSTEIN, C. and JENSEN, K., 1990. Evidence for two structural genes for alkaline phosphatase in *Bacillus subtilis*. J. Bacteriol., **172**, 735–740.

HULETT, F. M., LEE, J., SHI, L., SUN, G., CHESNUT, R., SHARKOVA, E., DUGGAN, M. F. and KAPP, N., 1994. Sequential action of two-component genetic switches regulates the PHO regulon in *Bacillus subtilis*. J. Bacteriol., **176**, 1348–1358.

ITO, K., 1982. Purification of the precursor form of maltose-binding protein, a periplasmic protein of *Escherichia coli.* J. Biol. Chem., **257**, 9895–9897.

JONGBLOED, J. D., MARTIN, U., ANTELMANN, H., HECKER, M., TJALSMA, H., VENEMA, G., BRON, S., VAN DIJL, J. M. and MUELLER, J. P., 2000. TatC is a specificity determinant for protein secretion via the twin-arginine translocation pathway. J. Biol. Chem., **275**, 41350–41357.

KUNST, F., OGASAWARA, N., MOSZER, I., ALBERTINI, A. M., ALLONI, G., AZEVEDO, V., BERTERO, M. G., BESSIERES, P., BOLOTIN, A., BORCHERT, S., BORRISS, R., BOURSIER, L., BRANS, A., BRAUN, M., BRIGNELL, C., BRON, S., BROUILLET, S., BRUSCHI, C. V., CALDWELL, B., CAPUANO, V., CARTER, N. M., CHOI, S. K., CODANI, J. J., CONNERTON, I. F., DANCHIN, A. *et al*., 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature, **390**, 249–256.

LAEMMLI, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, **227**, 680–685.

LE GRICE, S. F., 1990. Regulated promoter for high-level expression of heterologous genes in *Bacillus subtilis*. Methods Enzymol., **185**, 201–214.

Lee, S. J., KIM, D. M., BAE, K. H., BYUN, S. M. and CHUNG, J. H., 2000. Enhancement of secretion and extracellular stability of staphylokinase in *Bacillus subtilis* by *wprA* gene disruption. Appl. Environ. Microbiol., **66**, 476–480.

LEENHOUTS, K., BOLHUIS, A., VENEMA, G. and KOK, J., 1998. Construction of a food-grade multiple-copy integration system for *Lactococcus lactis*. Appl. Microbiol. Biotechnol., **49**, 417–423.

MÜLLER, J. P., AN, Z., MERAD, T., HANCOCK, I. C. and HARWOOD, C. R., 1997. Influence of *Bacillus subtilis phoR* on cell wall anionic polymers. Microbiology, **143 (Pt 3)**, 947–956.

MÜLLER, J. P. and WAGNER, M., 1999. Localisation of the cell wall-associated phosphodiesterase PhoD of *Bacillus subtilis.* FEMS Microbiol. Lett., **180**, 287–296.

MURASHIMA, K., CHEN, C. L., KOSUGI, A., TAMARU, Y., DOI, R. H. and WONG, S. L., 2002. Heterologous production of *Clostridium cellulovorans engB*, using protease-deficient *Bacillus subtilis*, and preparation of active recombinant cellulosomes. J. Bacteriol., **184**, 76–81.

NIELSEN, H., ENGELBRECHT, J., BRUNAK, S. and VON HEIJNE, G., 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng., **10**, 1–6.

PANDEY, A., SZAKACS, G., SOCCOL, C. R., RODRIGUEZ-LEON, J. A. and SOCCOL, V. T., 2001. Production, purification and properties of microbial phytases. Bioresour. Technol., **77**, 203–214.

POP, O., MARTIN, U., ABEL, C. and MUELLER, J. P., 2002. 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**, 3268–3273.

POP, O., WESTERMANN, M., VOLKMER-ENGERT, R., SCHULZ, D., LEMKE, C., SCHREIBER, S., GERLACH, R., WETZKER, R. and MUELLER, 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.

RASMUSSEN, B. A., BANKAITIS, V. A. and BASSFORD, P. J., JR., 1984. Export and processing of MalE-LacZ hybrid proteins in *Escherichia coli*. J. Bacteriol., **160**, 612–617.

ROBINSON, C., 2000. The twin-arginine translocation system: a novel means of transporting folded proteins in chloroplasts and bacteria. Biol. Chem., **381**, 89–93.

ROBINSON, C. and BOLHUIS, A., 2001. Protein targeting by the twin-arginine translocation pathway. Nat. Rev. Mol. Cell Biol., **2**, 350–356.

SAMBROOK, J., FRITSCH, E. F. and MANIATIS, T., 1989. Moleclar Cloning: a Laboratory Manual. Cold Spring Harbor NY: Cold Spring Harbor Laboratory.

SARGENT, F., BOGSCH, E. G., STANLEY, N. R., WEXLER, M., ROBINSON, C., BERKS, B. C. and PALMER, T., 1998. Overlapping functions of components of a bacterial Sec-independent protein export pathway. EMBO J., **17**, 3640–3650.

SARGENT, F., STANLEY, N. R., BERKS, B. C. and PALMER, T., 1999. Sec-independent protein translocation in *Escherichia coli*. A distinct and pivotal role for the TatB protein. J. Biol. Chem., **274**, 36073–36082.

STEPHENSON, K. and HARWOOD, C. R., 1998. Influence of a cell-wall-associated protease on production of alpha-amylase by *Bacillus subtilis*. Appl. Environ. Microbiol., **64**, 2875–2881.

TJALSMA, H., BOLHUIS, A., JONGBLOED, J. D., BRON, S. and VAN DIJL, J. M., 2000a. Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome. Microbiol. Mol. Biol. Rev., **64**, 515–547.

TOMMASSEN, J., LEUNISSEN, J., DAMME-JONGSTEN, M. and OVERDUIN, P., 1985. Failure of *E. coli* K-12 to transport PhoE-LacZ hybrid proteins out of the cytoplasm. EMBO J., **4**, 1041–1047.

TOWBIN, H., STAEHELIN, T. and GORDON, J., 1992. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. 1979. Biotechnology, **24**, 145–149.

VOORHOUT, W., DE KROON, T., LEUNISSEN-BIJVELT, J., VERKLEIJ, A. and TOMMASSEN, J., 1988. Accumulation of LamB-LacZ hybrid proteins in intracytoplasmic membrane-like structures in *Escherichia coli* K12. J. Gen. Microbiol., **134 (Pt 3)**, 599–604.

WEINER, J. H., BILOUS, P. T., SHAW, G. M., LUBITZ, S. P., FROST, L., THOMAS, G. H., COLE, J. A. and TURNER, R. J., 1998. A novel and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins. Cell, **93**, 93–101.

WU, L. F., IZE, B., CHANAL, A., QUENTIN, Y. and FICHANT, G., 2000. Bacterial twin-arginine signal peptide-dependent protein translocation pathway: evolution and mechanism. J. Mol. Microbiol. Biotechnol., **2**, 179–189.

WU, X. C., LEE, W., TRAN, L. and WONG, S. L., 1991. Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. J. Bacteriol., **173**, 4952–4958.
YE, R., YANG, L. P. and WONG, S.-L., 1996. Construction of protease deficient *Bacillus subtilis* strains for expression studies: inactivation of seven extracellular protease and the intracellular LonA protease. In: Proceedings of the International Symposium on Recent Advances in Bioindustry (S. K. HONG und W. J. LIM, eds.), pp. 160–169. **Erscheinungsort + Verlag??**

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*** Manuscript**

Dual localisation of homo-multimeric TatA elucidates its function in Tat-dependent protein targeting

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Summary

We recently demonstrated that a $TatA_d/TatC_d$ pair of proteins forms a functional Tat-dependent protein export system for the secretory phosphodiesterase PhoD of *Bacillus subtilis*. Soluble $TatA_d$ was specifically interacting with its twin-arginine signal peptide. Here we show that $TatA_d$ forms homo-multimeric protein complexes both in aqueous solutions and in membranes. In aqueous solution $TatA_d$ formed distinct micelle like complexes co-sedimenting with prePhoD. Freeze fracture analysis of proteoliposomes revealed homo-multimerisation of membrane integrated $TatA_d$. Immunogold labelling demonstrated that prePhoD was co-localised with membrane integrated $TatA_d$ complexes. Our data indicate that $TatA_d$ complexes are involved in targeting of prePhoD and form the protein conducting channel for Tat-dependent protein transport.

Introduction

Beside the Sec-dependent protein translocation system transporting unfolded proteins through the cytosolic membrane of the bacterial cell, the recently discovered Tat-dependent system transports proteins frequently associated with cofactors prior translocation [1]. It is therefore believed that it has the capacity to export folded proteins [2]. While ongoing research resulted in a detailed knowledge of the energetic, kinetic and structural prerequisites required for Sec-dependent protein translocation [3-5], only limited knowledge of the mechanism, how folded proteins get transported through the membrane via the Tat system does currently exist.

Proteins destined for export by the Tat-system are synthesised as precursors with a signal peptide containing an almost invariant twin-arginine sequence motif [6]. 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 thylakoidal or cytoplasmic membrane [3]. As demonstrated first for the Tat-homologous system in the thylakoid membrane of plant chloroplasts, it is totally reliant on the membrane pH gradient [7;8]. *In vitro* translocation systems using *E. coli* components, demonstrated that transport is energized exclusively by the transmembrane proton electrochemical gradient [9;10].

In *E. coli* TatA, TatB and TatC proteins have been demonstrated to be essential for Tat-dependent protein transport [11-14]. The TatA homologous protein TatE is functionally redundant [15]. The sequence related proteins TatA and TatB are anchored in the cytoplasmic membrane via the amino-proximal alpha-helical domain [11]. Gouffi and co-workers recently proposed function-linked changes of TatA and TatC topologies for the mechanism of folded protein translocation [16].

Currently there exist only weak ideas about the targeting of the twin-arginine precursors to the translocase unit and the structure of the Tat transport system. A large complex of approximately 600 kDa containing TatABC proteins have been purified from detergent-solubilised *E. coli* membranes [2;17]. Purified Tat protein complexes had a very different subunit composition. The molecular ratio of TatA: TatB: TatC in the cytosolic membrane of *E. coli* has been estimated to be about 40: 2: 1 [15;18]. Bolhuis and co-workers have suggested that TatB and TatC proteins form a functional and structural unit of the twin-arginine translocase [2]. The purified Tat complexes with a molecular weight of 600 kDa contain varying levels of TatA indicating TatA may participate in a separate complex lacking TatB [2;19]. Sargent and co-workers purified a similar sized TatA/B complex. An electron microscopically detected double layered ring structure of the TatAB complex indicated that these proteins could form the protein conducting channel [18]. 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 [20]. 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) [21]. It has been suggested that Tha4 is required for the transport steps following precursor recognition [21;22]. A recent study

demonstrated that functional TatABC systems purified from *E. coli*, *Salmonella thyphimurium* and plant thylacoides of *Agrobacterium tumefaciens* had common structural features [23].

While most bacterial and plant Tat systems contain three TatABC proteins, several bacterial and archaeal species miss a TatB-like protein [24;25]. Thus, at least one copy of a TatA homolog and one copy of a TatC homolog are required for a functional Tat pathway [11;14;26]. The *B. subtilis* genome encodes three TatA and two TatC-like proteins [24;27]. Despite the frequent presence of twin-arginines in the N-domain of signal peptides the only substrate strictly transported Tat dependent identified so far is PhoD, a secretory *B. subtilis* phosphodiesterase [28;29]. We have demonstrated that the expression of *tatA$_d$/tatC$_d$* genes co-localised with *phoD* in one operon was essential and sufficient to export the PhoD [30]. The second copy of a *tatC* (*tatC$_y$*) was not required for PhoD export [27]. Surprisingly, TatA$_d$ was found beside its expected membrane integrated localisation, also soluble in the cytosol there it showed affinity for prePhoD [31]. The selective binding of TatA$_d$ to peptides derived from the signal peptide of prePhoD elucidated the function of the twin-arginine motif as target site for pre-protein TatA$_d$ interaction. These features suggest that TatA$_d$ interacts prior membrane integration with its pre-protein substrate and could therefore assist targeting of twin-arginine pre-proteins.

To get further insight in the function of TatA$_d$, we studied the structural features of the protein both soluble as well in its membrane integrated localisation. Purified soluble TatA$_d$ formed homo-multimeric round micelle-like complexes. Sedimentation of TatA$_d$ with its substrate prePhoD demonstrated affinity of both proteins. TatA$_d$ reconstituted in liposomes formed distinct homo-multimeric structures. PrePhoD was co-localised with reconstituted TatA$_d$. Since TatC$_d$ neither contributed to TatA$_d$ complex formation nor showed itself oligomerisation, our data indicate that homo-multimeric TatA$_d$ could form the protein conducting channel of the Tat system in the membrane.

RESULTS

Soluble $TatA_d$ forms high molecular weight complexes with affinity for prePhoD

We previously demonstrated that $TatA_d$ selectively interacts with prePhoD. Sucrose gradient density centrifugation was used to get insight in the molecular organisation of soluble $TatA_d$ interacting with prePhoD. His_6-tagged $TatA_d$ as well as prePhoD were purified from *E. coli* producing strains by Ni^{2+}-NTA affinity chromatography. The purity and identity was elucidated by SDS-PAGE and subsequent immunodetection as demonstrated previously [31]. 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. Purified protein was separated on a linear 5 - 25% sucrose gradient. Individual fractions were assayed for the amount of $TatA_d$ immunologically (Fig. 1A). 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. Interesting, immunodetection of $TatA_d$ after SDS-PAGE showed a ladder of oligomeric complexes which were not completely dissolved during electrophoresis. To study, if $TatA_d$ shows a similar complex formation in the natural host, a membrane free cell extract of *B. subtilis* 168(pREP9*tatA$_d$/C$_d$*) was 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. 1D). This demonstrated that homo-oligomerisation occurs *in vivo* and is not an experimental artefact.

In parallel gradient centrifugation was carried out with purified prePhoD. The vast majority prePhoD was found in the pellet of the gradient indicating that the protein aggregated during centrifugation (Fig. 1B). Further, sedimentation was carried out with $TatA_d$ and prePhoD in a molecular ratio of 10:1 (Fig. 1C). Beside the localisation of the proteins in the pellet fraction some of the prePhoD was found in the same density horizon like $TatA_d$ with a molecular weight of about 150 to 250 kDa. This observation indicates that soluble homo-oligomeric $TatA_d$ complexes show affinity for prePhoD. In addition, the presence of $TatA_d$ obviously partially suppressed aggregation of prePhoD.

Oligomerisation of purified $TatA_d$ was further characterised via gel chromatography using a Superdex200 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 (data not shown). Since the molecular mass of recombinant His_6-$TatA_d$ is 9633 Da, it can be concluded that soluble $TatA_d$ formed large homo-multimeric complexes.

Structure of soluble homo-multimeric $TatA_d$ complexes

To obtain further insight into the structural properties and oligomeric state, we collected $TatA_d$ complexes with an apparent molecular weight between 150 and 250 kDa from sucrose density

gradients. The protein was dialysed and subjected to negative contrast electron microscopy. His_6-$TatA_d$ was adsorbed on carbon coated Formvar filmed copper grids and stained the 2% uranyl acetate. Two distinct classes of round round particles could be observed. While the smaller ones had a diameter of about 12 nm, the large class showed round particles with a diameter of 25 nm (Fig. 2, arrowheads). The latter had a pronounced staining in the centre indicating that particles had a aqueous centre. According to their size, $TatA_d$ complexes could have a estimated oligomeric organisation unifying 15-25 molecules to the 12 nm particles and 50-75 molecules to the 25 nm particles. Beside round structures vermicular structures with a diameter of 12 nm (Fig. 2) and 25 nm (data not shown) were observed. Their presence indicated that both classes of particles had the tendency to pile up.

$TatA_d$ is soluble in aqueous and hydrophobic solutions

TatA proteins are an essential component of the Tat translocation system. If *B. subtilis* $TatA_d$ is acting as a targeting factor for its substrate prePhoD the soluble protein complex should alter its structure to integrate into the cytosolic membrane. To mimic the hydrophobic environment of biological membranes, purified $TatA_d$ complexes dissolved in PBS were treated with the apolar organic solvent chloroform. TatAd dissolved in 200 µl PBS were incubated with an equal volume of chloroform and thoroughly mixed. At time points indicated, samples were separated by centrifugation (30 minutes, 15 000g) and the two fractions were concentrated and separated via SDS-PAGE (Fig 3A). The immunological analysis of these fractions demonstrated that $TatA_d$ was extracted from the hydrophilic PBS environment into the hydrophobic chloroform solution in a time dependent manner. For comparison lysozyme were treated under identical conditions. No protein was extracted into the hydrophobic fraction, as seen in the Commassie stained gels (Fig 3B). Chloroform extracted material was also processed by negative staining with subsequent electron microscopy. No oligomeric $TatA_d$ complexes were found in the chloroform fraction (data not shown).

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

A prerequisite for transport of proteins through a cytosolic membrane is the formation of a protein conducting channel. The demonstration of the formation of a self-associated homo-multimeric complex of *E. coli* TatA indicated that this protein has the potential to form visible structures in membranes [20]. According to the data obtained in *E. coli* by Porcelli and co-workers, TatA probably fulfils the role of a protein-conducting channel in the cytosolic membrane [20]. Therefore, we next studied if $TatA_d$ forms multimeric structures in the hydrophobic bilayer of bacterial membranes. Proteoliposomes were formed by co-reconstitution of purified $TatA_d$ with total *E. coli* polar lipids. Membrane localisation of $TatA_d$ was monitored by immunological detection of proteins in the membrane vesicles (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 multimerisation of membrane integrated $TatA_d$. The approximate size of these intramembrane particles was about 10 nm. Infrequently, beside round 10 nm particles, some $TatA_d$ complexes were extended to long shaped filamentous and dumbbell-like structures (Fig. 5D).

Next, proteoliposomes were reconstituted with purified $TatC_d$. As monitored immunologically, these vesicles contained a substantial amount of $TatC_d$, indicating the efficient integration of $TatC_d$ in the membrane (Fig. 4). Freeze fracturing of these membrane vesicles did not result in visible intramembrane particulate 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).

Membrane integrated $TatA_d$ has affinity for prePhoD

The prerequisite of $TatA_d$ to fulfil a function as translocator would be the affinity of membrane integrated $TatA_d$ for its substrate prePhoD. In order to prove this thesis 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. Subsequent immunogold labelling of prePhoD revealed that membrane associated prePhoD was co-localised with the particulate $TatA_d$ structures (Fig. 6). Interesting, immunogold particles were detected in close proximity to symmetric 10 nm particles as well as elongated filamentous $TatA_d$ particles. $TatA_d$ vesicles untreated with prePhoD were not labelled with immunogold particles, elucidating the specificity of prePhoD antibodies (data not shown). No immunogold labelling of prePhoD was observed in protein free phospholipid vesicles incubated with prePhoD under identical conditions (data not shown).

DISCUSSION

Due to its affinity for its substrate prePhoD, cytosolic $TatA_d$ was suggested to act as a targeting factor upon selective binding of its substrate [31]. In order to get further insight in the nature of this interaction we analysed the molecular organisation of $TatA_d$ in aqueous solution and in hydrophobic environment. In solution, the protein formed homo-oligomeric complexes with affinity for prePhoD. Beside the complex formation in aqueous solutions, $TatA_d$ formed multimeric complexes also in proteoliposomes. Their affinity for prePhoD demonstrated the potential function of these $TatA_d$ particles in protein translocation. 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 conducting channel of the Tat transport system.

A recently described special feature of the Tat system of *B. subtilis* was the partial cytosolic localisation of $TatA_d$ [31]. As demonstrated above, soluble $TatA_d$ forms homo-oligomeric round particles with a size of about 12 or 25 nm in diameter. TatA proteins are amphiphilic molecules. They consist of a calculated transmembrane segment followed by an amphiphatic helix and a hydrophilic C terminus. Therefore, circular structures of soluble $TatA_d$ complexes can be explained as micelle-like structures. The small 12 nm particles could be micelles of a single $TatA_d$ layer exposing the hydrophilic C terminus to the aqueous cytosol while forming a nonpolar interior. Pronounced negative contrast staining of the middle of the 25 nm particles indicated an aqueous centre. Therefore they can be explained as a double ring structure forming membrane-like spherical bilayers. The hydrophobic N-terminal helix of the molecules would form the middle of the bilayer. The hydrophilic C-terminus of the $TatA_d$ molecules in the outer layer is exposed to the environment and the one of the inner layer is forming the hydrophilic centre. The shape of these particles does not necessarily reflect the complex formation under physiological conditions but it provides additional information explaining the soluble state of this amphiphatic protein. We recently demonstrated that the double-arginine containing signal peptide of prePhoD is interacting with $TatA_d$ [31]. The hydrophobic centre of the observed 12 nm micelles could accommodate the calculated hydrophobic α-helical domain of the PhoD signal peptide. The $TatA_d$ C-terminal region 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.

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 a homo-multimerisation 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 multimerisation. Their molecular weight was even higher than purified His_6-tagged $TatA_d$.

Beside the complex formation in aqueous environment, $TatA_d$ formed homo-multimeric complexes in the lipid bilayer of a reconstituted bacterial membrane. Round, no further structured intramembrane particles with an average size of 10 nm could be observed in parallel with filamentous

and dumbbell-like structures. Here, the hydrophobic part of the molecules should mediate the contact to the membrane and the hydrophilic part of the molecules should form a hydrophilic centre. Reconstituted $TatA_d$ complexes do not necessarily result in physiological active translocation units. The use of $TatA_d/C_d$ containing 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 proteins actively contribute to the formation of a protein translocation unit, which would mediate transmembrane transport of Tat substrates. Despite the different shape of the membrane integrated $TatA_d$ complexes, the observed co-localisation with prePhoD indicates affinity for their substrate.

Proteoliposomes containing only $TatC_d$ did not show particle formation. Furthermore, neither prePhoD nor $TatC_d$ affected multimerisation or particulate shape of $TatA_d$ complexes. Thus, 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) not likely to significantly contribute to the formation of a protein conducting channel.

In the proposed model for Tat-dependent protein translocation in *E. coli* and in the plant thylakoid system, TatA proteins assemble with the TatC-TatB complexes transiently after interaction with the Tat substrate [21 ;22 ;32]. 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 ;20]. The Tat substrates are translocated in a folded conformation while maintaining the pH gradient at the membrane [33]. 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 absence of a detectable cavity in the middle of $TatA_d$ particles indicates that multimerisation does not result in the formation of a pore like structure.

What is the expected number of $TatA_d$ molecules forming a Tat translocase unit? The calculated molecular ratio of the TatABC complex in *E. coli* is 40:2:1. This results in a membrane integrated particle containing at least 40 TatA molecules [15]. A self associated 460 kDa TatA complex purified by Porcelli and co-workers consists of about 50 molecules [20]. The size of observed $TatA_d$ complexes, indicates that at least a similar number of molecules would contribute to the formation of a transport system mediating the prePhoD translocation.

The prerequisite for the translocation of prePhoD bound to cytosolic $TatA_d$ complexes is the targeting to and integration into the lipid bilayer of the membrane. Dual localisation of prePhoD-$TatA_d$ complexes indicate that $TatA_d$ triggers this process. Extraction of $TatA_d$ into the hydrophobic solvent chloroform indicates that the chemical properties of this protein allow the transfer from the aqueous to a hydrophobic environment. Topology changes of TatA recently reported by Gouffi and co-workers, might assist this necessary transfer event [16]. Possible receptor sites at the membrane, the driving force of this integration and the kinetics of this event will be addressed elsewhere. Taken together, data presented here demonstrate that $TatA_d$ forms homo-multimeric complexes with affinity for its

substrate prePhoD both in aqueous and membrane localisation. Mechanistically, we favour a model, where soluble $TatA_d$ complexes recognise prePhoD and mediate its transport to the membrane. Here, via a not yet understood mechanism, the $TatA_d$ complex integrates into the membrane and forms the protein conducting channel for its substrate prePhoD.

MATERIALS and METHODS

Bacterial strains, plasmids and media

E. coli strains TG1(pREP4) (QIAGEN, Hilden, Germany) was used to overexpress proteins. Plasmids pQE9tatA_d, pQE60tatC_d and pQE9phoD_p have been described [30 ;31]. *E. coli* was grown aerobically at 37°C in TY Medium [34]. As required, media were supplemented with ampicillin (100 µg/ml), kanamycin (40 µg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM). *B. subtilis* strain 168(pREP9*tatA$_d$/C$_d$*) allowing IPTG-inducible overexpression of *tatA$_d$* and *tatC$_d$* [31] was grown in TY medium to mid-exponential phase and expression of *tat* genes was induced with 1mM IPTG for one hour. Membrane-free cell extract of *B. subtilis* was prepared as described [31].

Purification of His_6-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 [31].

Freeze-fracture electron microscopy

Liposomes were concentrated by centrifugation resuspended in PBS containing 15 % (w/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 evaporation of platinum/carbon with electron guns was controlled by a thin-layer quartz crystal monitor.

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 [35]. The replica were washed four times in PBS buffer and treated with PBS +1 % bovine serum albumine (BSA) for 30 minutes. Next they were placed in PBS containing BSA (0.5 %) and monospecific antibodies against PhoD (dilution 1:20) for 1 hour. 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 minutes 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 mounted with direction of shadowing from bottom to top.

SDS-PAGE and Western blot analysis

Protein SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared as described by Laemmli [36]. After separation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Schleicher and Schüll) as described by Towbin *et al.* [37]. Proteins were visualised using monospecific antibodies against $TatA_d$, $TatC_d$ and alkaline phosphatase-conjugated goat anti-rabbit antibodies (SIGMA) according to the manufacturer's instructions.

Sucrose gradient density centrifugation

A linear sucrose gradients ranging from 5 to 25 % in PBS with a total volume of 11 ml were generated in a SW40 polyallomer tube with 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 000g for 16 hours. 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.

Preparation in proteoliposomes

Purified His_6-$TatA_d$, $TatC_d$-His_6 and His_6-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 ultrasonication and subsequently washed twice with PBS. Protein content of the proteoliposomes was detected by western blotting.

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FIGURES

Figure 1: **Homo-multimeric $TatA_d$ complexes co-sediment with prePhoD**. Distribution of His_6-$TatA_d$ and His_6-prePhoD across 5 - 25% sucrose density gradient. Sedimentation was carried out with purified $TatA_d$ (A), prePhoD (B) and $TatA_d$ and prePhoD proteins (C) in a molecular ratio of 10:1. for comparison, membrane free cell lysate of *B. subtilis* strain 168(pREP9tatA_d/C_d) was sedimented under identical conditions (D). Gradients were fractionated to 11 fractions and the pellet and subsequently characterised via SDS-PAGE and Western blotting using specific antisera. Sedimentation of marker proteins is indicated at the top.

Figure 2: **Negative contrast straining of soluble $TatA_d$.** His_6-$TatA_d$ complexes with a molecular weight of 150- 250 kDa were prepurified via sucrose density gradient centrifugation. They were subjected to were adsorbed on carbon coated Formvar filmed copper grids and stained with 2% uranyl acetate. Small particles with diameter of about 12 nm (arrowheads) and large round particles with a diameter of 25 nm (arrows and detail views) were found. Scale bar represents 100 nm.

Figure 3: **$TatA_d$ is soluble in aqueous and hydrophobic solutions.** 32 µg His_6-$TatA_d$ or 160 µg lysozyme dissolved in 200 µl PBS, were thoroughly mixed with an equal volume of chloroform. At time points indicated samples were centrifuged and aqueous and chloroform fraction were applied for SDS-PAGE. B – protein dissolved in buffer, C – protein extracted to chloroform.

Figure 4: **Protein content of proteoliposomes**. Purified proteins were reconstituted with bacterial lipids, sonicated washed and analysed for their content of proteins. Proteoliposomes reconstituted with $TatA_d$ (lanes 1 and 4), $TatA_d$ and $TatC_d$ (lanes 2 and 5) and prePhoD, $TatA_d$ and $TatC_d$ (lanes 3 and 6) were analysed via SDS-PAGE and subsequent Western blotting using antibodies against $TatC_d$ (lanes 1 - 3) or $TatA_d$ (lanes 4 - 6).

Figure 5: **Membrane integrated $TatA_d$ forms oligomeric complexes**. Electron micrographs of freeze fractured proteoliposomes containing His_6-$TatA_d$ (B, D), His_6-$TatC_d$ (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.

Figure 6: **Co-localisation of prePhoD with $TatA_d$. P**roteoliposomes formed in presence of His_6-$TatA_d$ and prePhoD were freeze fractured and subsequently labelled with PhoD-specific antibodies and 10 nm gold-conjugated secondary antibody. Scale bar represents 100 nm.

Reference List

1. Berks, B. C., Sargent, F., & Palmer, T. (2000). The Tat protein export pathway. *Mol. Microbiol.* **35**, 260-274.

2. Bolhuis, A., Mathers, J. E., Thomas, J. D., Barrett, C. M., & Robinson, C. (2001). TatB and TatC form a functional and structural unit of the twin-arginine translocase from Escherichia coli. *J. Biol. Chem.* **276**, 20213-20219.

3. Manting, E. H. & Driessen, A. J. (2000). Escherichia coli translocase: the unravelling of a molecular machine. *Mol. Microbiol.* **37**, 226-238.

4. Economou, A. (2002). Bacterial secretome: the assembly manual and operating instructions (Review). *Mol. Membr. Biol.* **19**, 159-169.

5. de Keyzer, J., van der, D. C., & Driessen, A. J. (2003). The bacterial translocase: a dynamic protein channel complex. *Cell Mol. Life Sci.* **60**, 2034-2052.

6. Berks, B. C. (1996). A common export pathway for proteins binding complex redox cofactors? *Mol. Microbiol.* **22**, 393-404.

7. Mould, R. M. & Robinson, C. (1991). A proton gradient is required for the transport of two lumenal oxygen-evolving proteins across the thylakoid membrane. *J. Biol. Chem.* **266**, 12189-12193.

8. Cline, K., Ettinger, W. F., & Theg, S. M. (1992). 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**, 2688-2696.

9. Yahr, T. L. & Wickner, W. T. (2001). Functional reconstitution of bacterial Tat translocation in vitro. *EMBO J.* **20**, 2472-2479.

10. Alami, M., Trescher, D., Wu, L. F., & Muller, M. (2002). Separate analysis of twin-arginine translocation (Tat)-specific membrane binding and translocation in Escherichia coli. *J. Biol. Chem.* **277**, 20499-20503.

11. Sargent, F., Ballantine, S. P., Rugman, P. A., Palmer, T., & Boxer, D. H. (1998). 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**, 746-754.

12. Sargent, F., Stanley, N. R., Berks, B. C., & Palmer, T. (1999). Sec-independent protein translocation in Escherichia coli. A distinct and pivotal role for the TatB protein. *J. Biol. Chem.* **274**, 36073-36082.

13. Weiner, J. H., Bilous, P. T., Shaw, G. M., Lubitz, S. P., Frost, L., Thomas, G. H., Cole, J. A., & Turner, R. J. (1998). A novel and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins. *Cell* **93**, 93-101.

14. Bogsch, E. G., Sargent, F., Stanley, N. R., Berks, B. C., Robinson, C., & Palmer, T. (1998). An essential component of a novel bacterial protein export system with homologues in plastids and mitochondria. *J. Biol. Chem.* **273**, 18003-18006.

15. Jack, R. L., Sargent, F., Berks, B. C., Sawers, G., & Palmer, T. (2001). Constitutive expression of Escherichia coli tat genes indicates an important role for the twin-arginine translocase during aerobic and anaerobic growth. *J. Bacteriol.* **183**, 1801-1804.

16. Gouffi, K., Gerard, F., Santini, C. L., & Wu, L. F. (2004). Dual topology of the Escherichia coli TatA protein. *J. Biol. Chem.* **279**, 11608-11615.

17. De Leeuw, E., Granjon, T., Porcelli, I., Alami, M., Carr, S. B., Muller, M., Sargent, F., Palmer, T., & Berks, B. C. (2002). Oligomeric properties and signal peptide binding by Escherichia coli Tat protein transport complexes. *J. Mol. Biol.* **322**, 1135-1146.

18. Sargent, F., Gohlke, U., De Leeuw, E., Stanley, N. R., Palmer, T., Saibil, H. R., & Berks, B. C. (2001). Purified components of the Escherichia coli Tat protein transport system form a double-layered ring structure. *Eur. J. Biochem.* **268**, 3361-3367.

19. Bolhuis, A., Bogsch, E. G., & Robinson, C. (2000). Subunit interactions in the twin-arginine translocase complex of Escherichia coli. *FEBS Lett.* **472**, 88-92.

20. Porcelli, I., De Leeuw, E., Wallis, R., van den Brink-van der Laan, de Kruijff, B., Wallace, B. A., Palmer, T., & Berks, B. C. (2002). Characterization and membrane assembly of the TatA component of the Escherichia coli twin-arginine protein transport system. *Biochemistry* **41**, 13690-13697.

21. Cline, K. & Mori, H. (2001). Thylakoid DeltapH-dependent precursor proteins bind to a cpTatC-Hcf106 complex before Tha4-dependent transport. *J. Cell Biol.* **154**, 719-729.

22. Mori, H. & Cline, K. (2002). A twin arginine signal peptide and the pH gradient trigger reversible assembly of the thylakoid [Delta]pH/Tat translocase. *J. Cell Biol.* **157**, 205-210.

23. Oates, J., Mathers, J., Mangels, D., Kuhlbrandt, W., Robinson, C., & Model, K. (2003). Consensus structural features of purified bacterial TatABC complexes. *J. Mol. Biol.* **330**, 277-286.

24. Wu, L. F., Ize, B., Chanal, A., Quentin, Y., & Fichant, G. (2000). Bacterial twin-arginine signal peptide-dependent protein translocation pathway: evolution and mechanism. *J. Mol. Microbiol. Biotechnol.* **2**, 179-189.

25. Dilks, K., Rose, R. W., Hartmann, E., & Pohlschroder, M. (2003). Prokaryotic utilization of the twin-arginine translocation pathway: a genomic survey. *J. Bacteriol.* **185**, 1478-1483.

26. Yen, M. R., Tseng, Y. H., Nguyen, E. H., Wu, L. F., & Jr, S. M. (2002). Sequence and phylogenetic analyses of the twin-arginine targeting (Tat) protein export system. *Arch. Microbiol.* **177**, 441-450.

27. Jongbloed, J. D., Martin, U., Antelmann, H., Hecker, M., Tjalsma, H., Venema, G., Bron, S., van Dijl, J. M., & Muller, J. (2000). TatC is a specificity determinant for protein secretion via the twin-arginine translocation pathway. *J. Biol. Chem.* **275**, 41350-41357.

28. Jongbloed, J. D., Antelmann, H., Hecker, M., Nijland, R., Bron, S., Airaksinen, U., Pries, F., Quax, W. J., van Dijl, J. M., & Braun, P. G. (2002). Selective contribution of the twin-arginine translocation pathway to protein secretion in Bacillus subtilis. *J. Biol. Chem.* **277**, 44068-44078.

29. van Dijl, J. M., Braun, P. G., Robinson, C., Quax, W. J., Antelmann, H., Hecker, M., Muller, J., Tjalsma, H., Bron, S., & Jongbloed, J. D. (2002). Functional genomic analysis of the Bacillus subtilis Tat pathway for protein secretion. *J. Biotechnol.* **98**, 243-254.

30. Pop, O., Martin, U., Abel, C., & Muller, J. P. (2002). 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**, 3268-3273.

31. Pop, O. I., Westermann, M., Volkmer-Engert, R., Schulz, D., Lemke, C., Schreiber, S., Gerlach, R., Wetzker, R., & Muller, 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.

32. Mori, H., Summer, E. J., & Cline, K. (2001). Chloroplast TatC plays a direct role in thylakoid (Delta)pH-dependent protein transport. *FEBS Lett.* **501**, 65-68.

33. Alder, N. N. & Theg, S. M. (2003). Energetics of protein transport across biological membranes. a study of the thylakoid DeltapH-dependent/cpTat pathway. *Cell* **112**, 231-242.

34. Müller, J. P. (1996). Influence of impaired chaperone or secretion function on SecB production in Escherichia coli. *J. Bacteriol.* **178**, 6097-6104.

35. Fujimoto, K. (1997). 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**, 87-96.

36. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

37. Towbin, H., Staehelin, T., & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U. S. A* **76**, 4350-4354.

Figure1

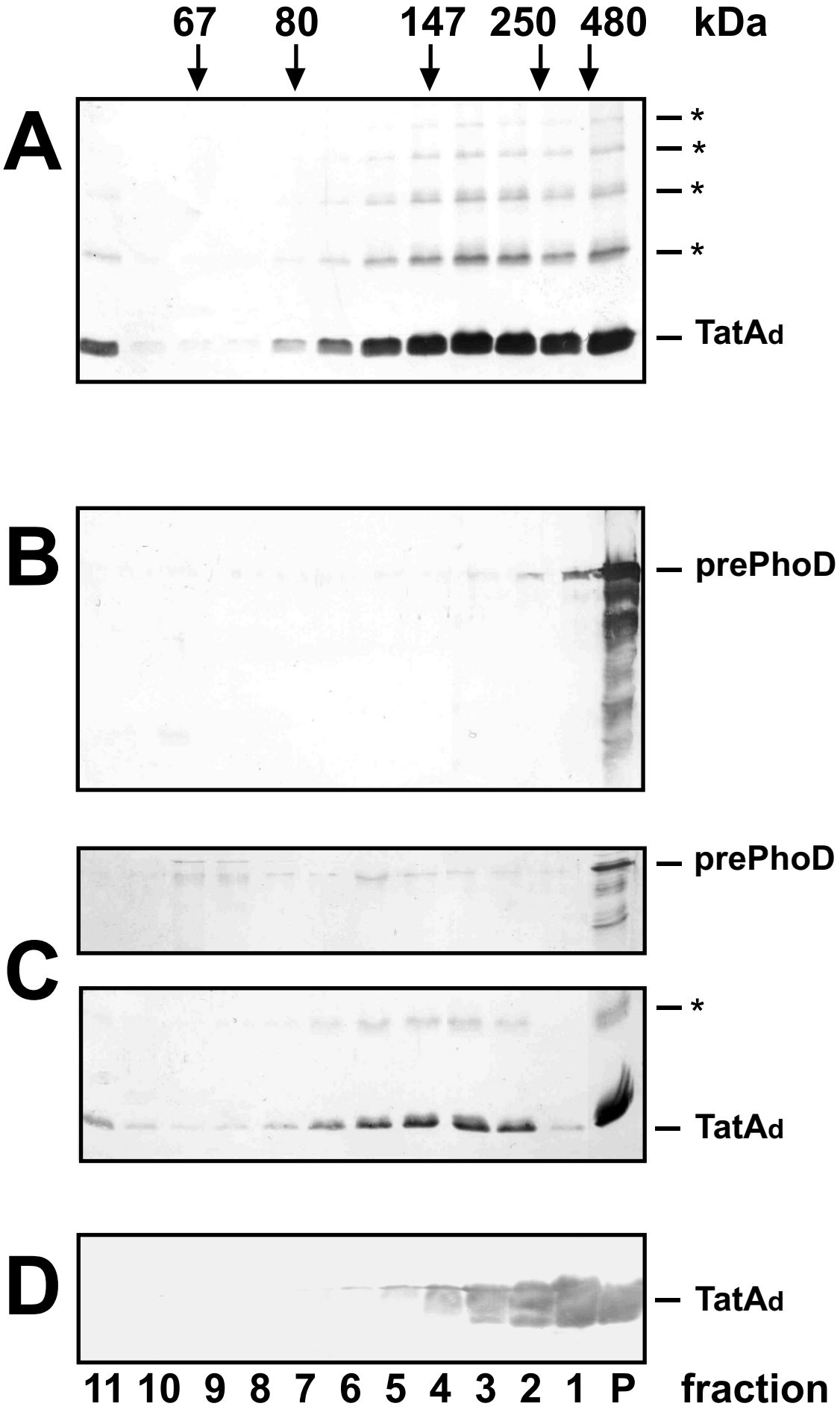
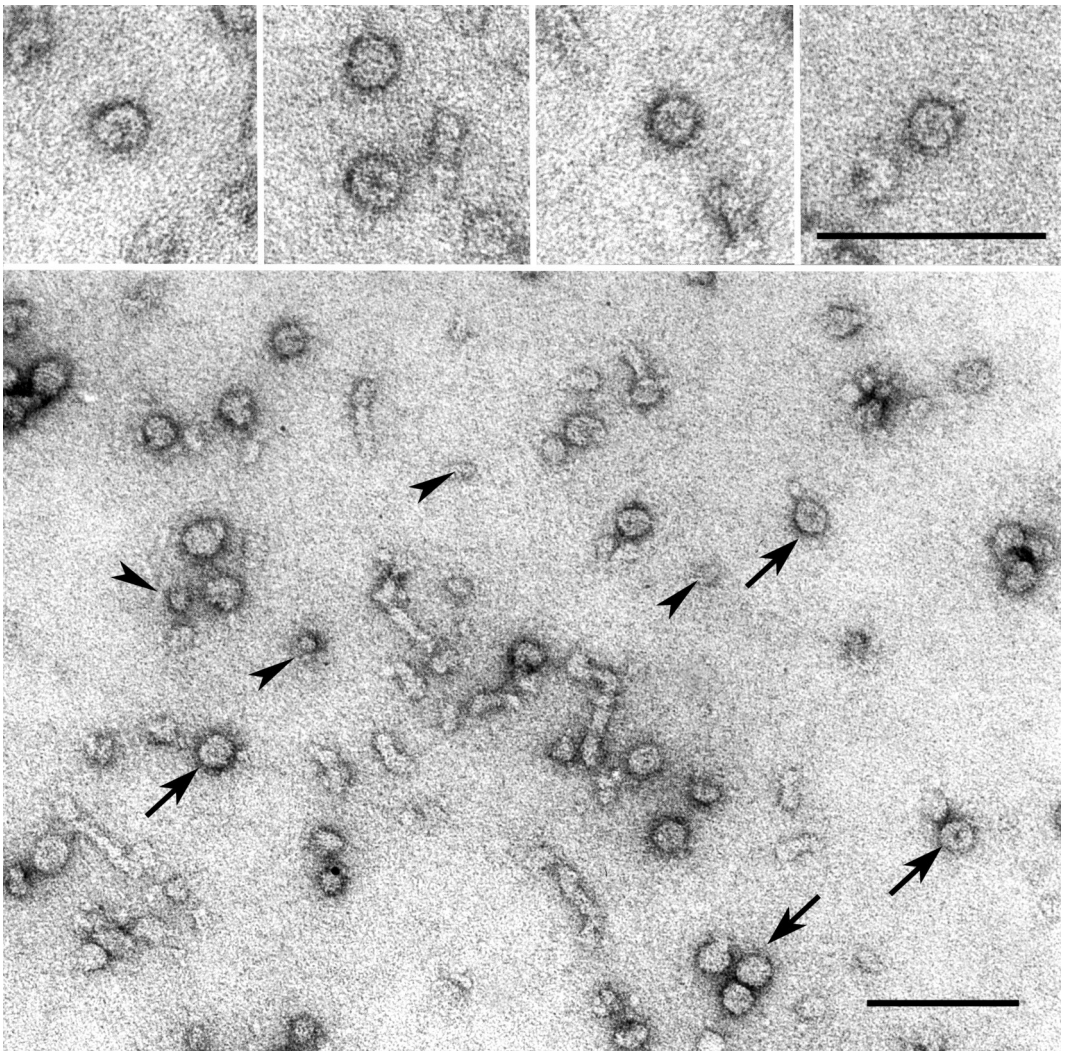


Figure 1) Pop *et al.*

Figure2



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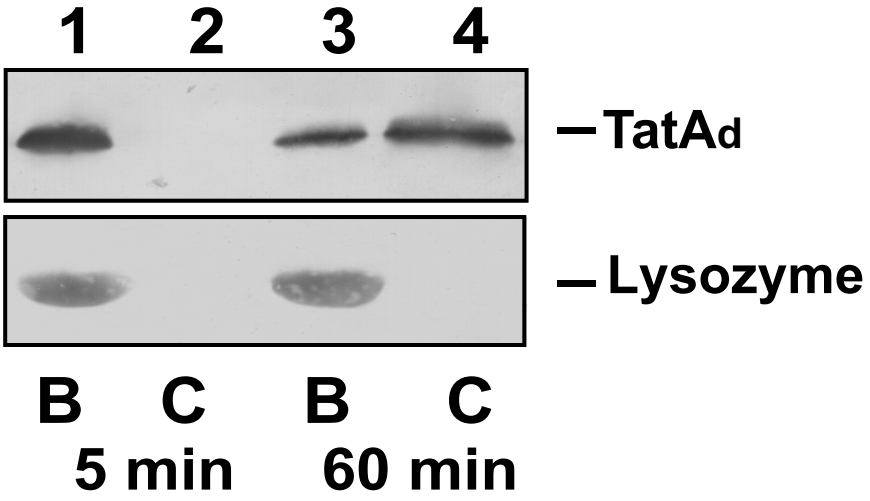

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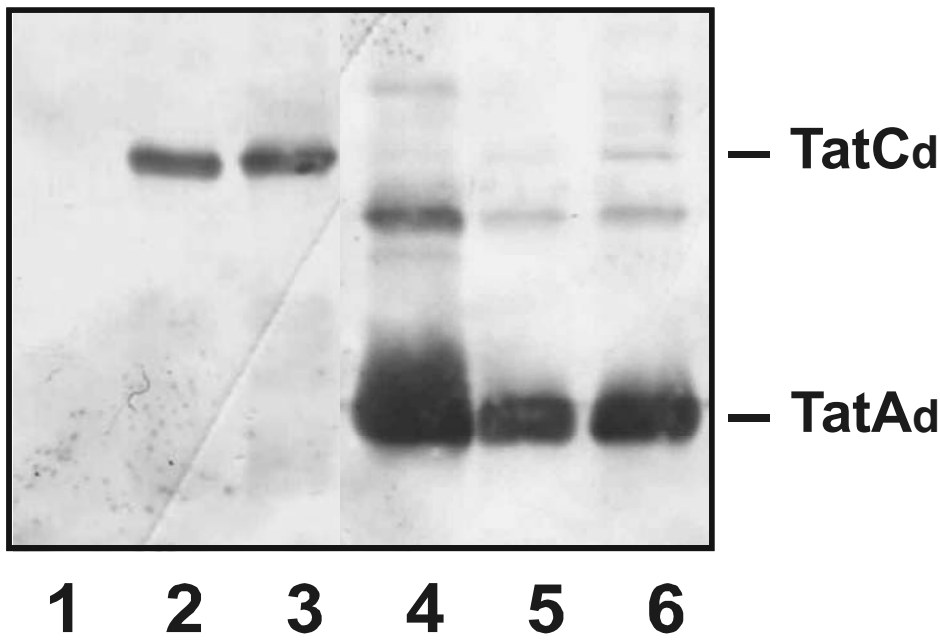

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Figure5

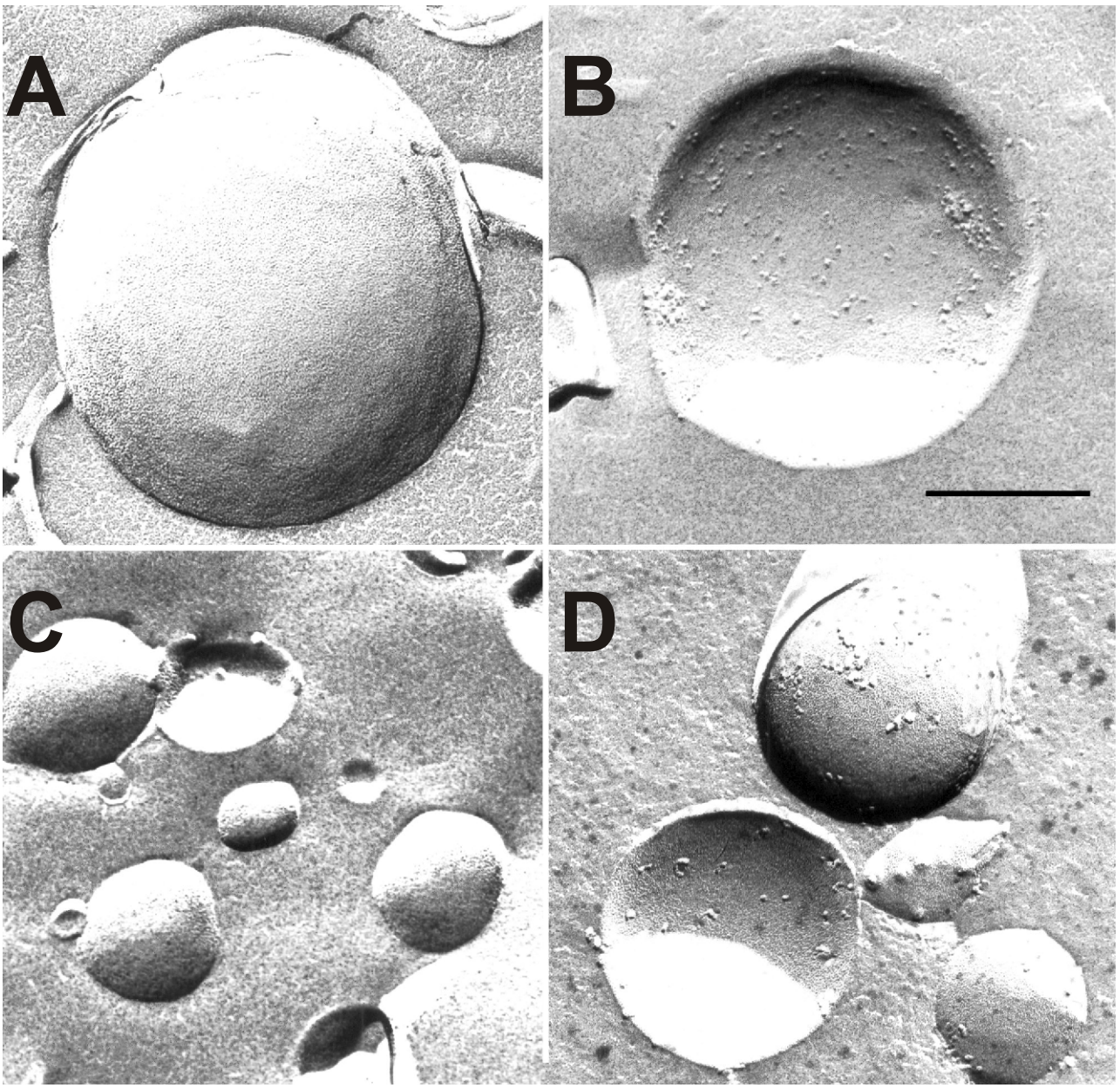

Figure 5) Pop *et al.*

Figure6

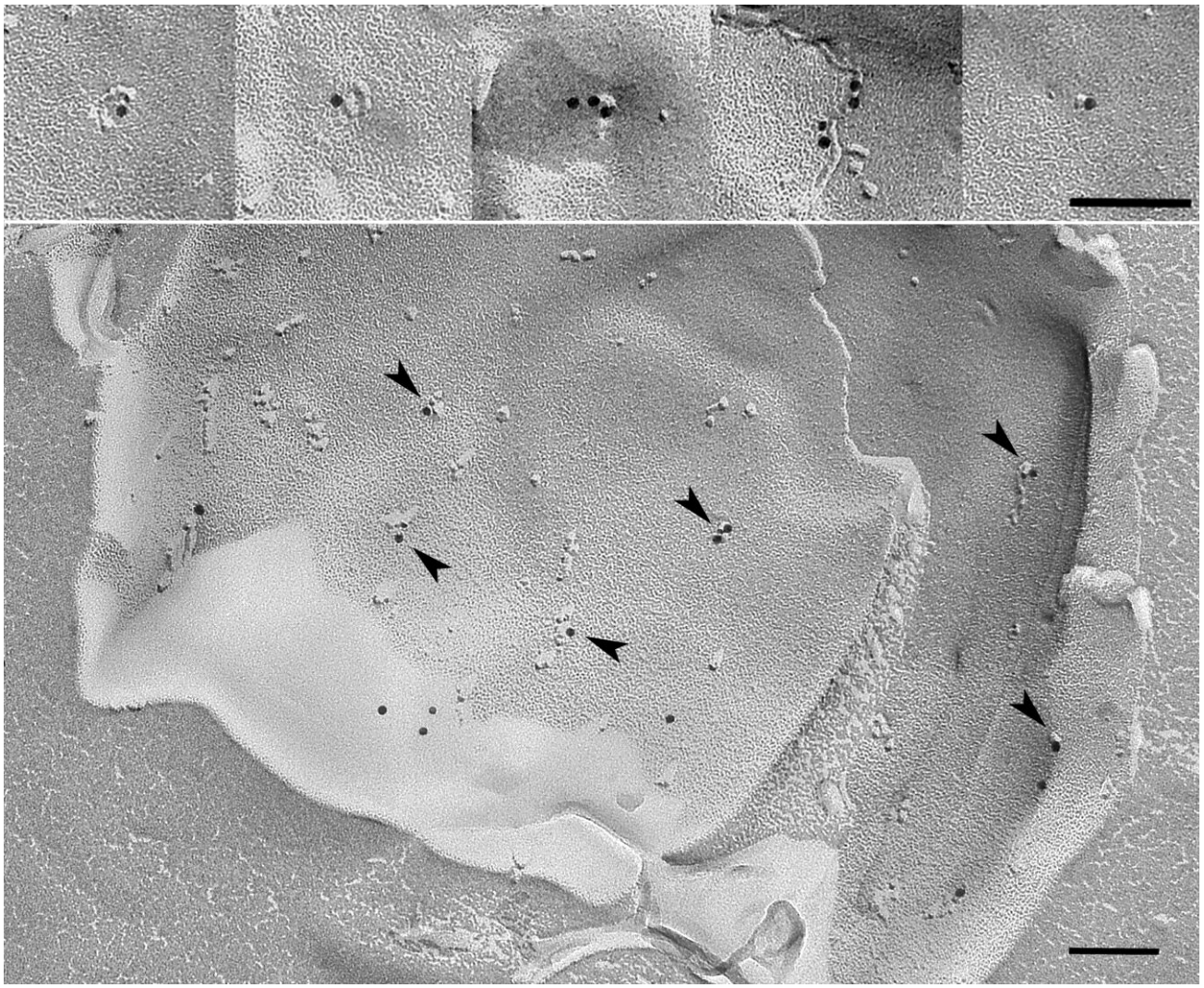

Figure 6) Pop *et al.*

2. Results and discussion

The recently described Tat system has the unusual capacity to transport completely folded proteins while maintaining the membrane barrier function. In addition, the transport process works without the need of ATP hydrolysis. These interesting features have stirred the scientific world in the last years. The current knowledge regarding the Tat system is mainly based on research in *E. coli* and plant thylakoids. However, Tat homologues have been identified in most bacteria, archaea, plant thylakoids and mitochondria of some higher plants (Bogsch *et al.*, 1998).

B. subtilis is an organism widely used in the industrial production of various proteins. Its Tat system could reveal new perspectives for the secretory production of heterologous proteins, which are not compatible with the general secretory pathway. In the present work I assessed a significant contribution for characterisation of the twin-arginine translocation system in this organism.

2.1 The phosphodiesterase PhoD is a substrate of the *B. subtilis* Tat export system

PhoD is a member of the so-called Pho regulon of *B. subtilis* which is regulated by the two component signal transduction system PhoR/PhoP (Hulett *et al.*, 1990). When growth of *B. subtilis* becomes limited by the availability of inorganic phosphate, the Pho regulon is induced by the response regulator PhoP, which is activated by PhoR, a histidine protein kinase activated upon phosphate starvation response. As a result, a group of genes/operons is induced. The products of these genes are represented by enzymes involved in phosphate uptake, phosphate conservation and the utilisation of alternative phosphate containing substrates (Takemura *et al.*, 1996; Müller *et al.*, 1997). PhoD is such an enzyme with alkaline phosphatase and phosphodiesterase activities. PhoD is efficiently transported across the cytosolic membrane but remains localised at the surface of the cell wall as a result of an inefficient processing (Müller *et al.*, 1997). The slowly cleaved signal peptide might function as a membrane anchor, maintaining the enzyme cell associated.

To facilitate studies of the phosphate starvation response, a strain with an altered PhoR protein was isolated by *in vitro* mutagenesis (Müller *et al.*, 1997). This *phoR12* mutation leads to a constitutive expression of activated PhoR. This results in the induction of the phosphate starvation response under phosphate replete conditions.

2.1.1 The wild type PhoD is not transported by the *E. coli* Tat system

Since the twin-arginine motif is conserved throughout in bacteria, a first question to be addressed was whether *B. subtilis* PhoD is compatible with the Tat system of another bacterial species. To check the transport specificity of this substrate, wild type PhoD and mature PhoD were produced in *E. coli*, and their localisation subsequently monitored (Pop *et al.*, 2002). While in *B. subtilis*, the

toxic effects of this enzyme are obviously neutralised by a very efficient translocation system, induction of *phoD* gene in *E. coli* decreased the cell growth drastically, most likely due to the enzymatic activity of this protein. Nevertheless, the transport of PhoD in *E. coli* was analysed via pulse-chase experiments. The PhoD precursor was not processed even at 60 minutes after chase, indicating that it was not translocated by the *E. coli* transport systems (Pop *et al.*, 2002). Similar results were obtained from *in vivo* protease mapping experiments, in which PhoD was shown to remain in a cytosolic localisation (Pop *et al.*, 2002).

In order to elucidate possible barriers located in the mature part of PhoD, we constructed a hybrid protein consisting of SP_{PhoD} and β-galactosidase. This SP_{PhoD}-LacZ fusion was then produced in a wild type *E. coli* strain. To analyse whether the signal peptide of PhoD could mediate the translocation of LacZ into the periplasm of *E. coli*, we studied the localisation of LacZ by using *in vivo* protease mapping experiments. *E. coli* Tat machinery did not recognise the signal peptide of PhoD and the hybrid protein remained cytosolic (Pop *et al.*, 2002). This demonstrated that *E. coli* Tat machinery cannot use the Tat signal peptide of PhoD as a translocation signal. The reason why the signal peptide of PhoD is not compatible with the *E. coli* transport systems, is a question that remains to be answered.

2.1.2 Replacement of Tat signal peptide by a Sec signal peptide results in Sec-dependent export of PhoD

To analyse whether an *E. coli* Sec signal peptide could assist the export process of PhoD, we constructed a fusion protein consisting of the signal peptide of the Sec-dependent β-lactamase (SP_{Bla}) and the mature PhoD. Interesting, as shown by immunoblotting of whole cell extract of *E. coli* TG1 cells producing SP_{Bla}-PhoD, the hybrid protein was processed to the mature form, indication that it was translocated. Since β-lactamase is a typical *E. coli* Sec substrate, it was likely that its signal peptide routed the hybrid to the Sec-pathway. Because Tat and Sec systems are driven by different types of energy (ΔpH and ATP), selective inhibition of those translocation routes can gain crucial information about the identity of the involved transport system. Nigericin is an ionophore that disrupts the pH gradient at the membrane and suppresses therefore the Tat specific energy source (Santini *et al.*, 1998). Sodium azide is an ATPase inhibitor, which is commonly used to block the Sec transport pathway (Oliver *et al.*, 1990). The treatment with sodium azide fully blocked the conversion of the hybrid precursor to mature form, whereas nigericin did not interfere with the export process of SP_{Bla}-PhoD (Pop *et al.*, 2002). This result demonstrated that the fusion SP_{Bla}-PhoD expressed in *E. coli* was transported in a Sec-dependent manner.

Based on these data, we can conclude that prePhoD was not exported by the *E. coli* transport systems because of the incompatibility of its signal peptide with either Tat or Sec export systems. Besides, the fact that prePhoD or SP_{PhoD}-LacZ are not transported Sec-dependently, supports the thesis of a "Sec-avoidance motif" present in the Tat signal peptides (Bogsch *et al.*, 1997). Moreover, mature

PhoD itself is compatible with a heterologous Sec transport system, if fitted with a proper Sec signal peptide. This demonstrated that the mature PhoD itself does not contain a motif that could suppress the effect of a Sec signal peptide. In adition, its export seems not assisted by special cofactors.

2.1.3 The signal peptide of PhoD canalises the export to the Tat system.

The inability of *E. coli* Tat system to recognise and translocate prePhoD and SP_{PhoD}-LacZ fusion suggests that the twin-arginine signal peptide of PhoD is likely to be recognised only by the mechanism evolved to translocate this phosphodiesterase in the original host. In *B. subtilis*, the *phoD* gene is localised in the same operon with two *tat* genes (*tatA*$_d$/*tatC*$_d$). This operon is induced as a response to phosphate starvation. Therefore, the involvement of the co-regulated *tatA*$_d$/*tatC*$_d$ genes in the translocation process of PhoD seemed a reasonable thesis. To investigate this assumption, the *B. subtilis tatA*$_d$/*C*$_d$ gene pair was co-expressed in *E. coli* strains producing wild type PhoD or the SP_{PhoD}-LacZ fusion. Export was analysed using pulse-chase and subsequent immunodetection experiments. In the absence of $TatA_d/C_d$, the hybrid protein remained unprocessed even at 40 minutes after chase, while in the strain co-expressing *B. subtilis tat* genes *tatA*$_d$/*C*$_d$ the SP_{PhoD}-LacZ fusion was partly processed, already at the beginning of the chase period (Pop *et al.*, 2002). Since processing of a pre-protein occurs only during or after the translocation event, it can be concluded that the SP_{PhoD}-LacZ fusion was transported across the cytosolic membrane.

To monitor the localisation of the LacZ peptide in an *E. coli* strain expressing *B. subtilis* Tat components, *in vivo* protease mapping experiments were performed. Both, the processed and the precursor form of the fusion protein, were accessible to proteinase K, suggesting that the hybrid protein was successfully translocated across the membrane but only inefficiently processed. The low processing efficiency suggested an insufficient recognition of the signal peptidase cleavage site by the *E. coli* LepB (Pop *et al.*, 2002). This result resembles the inefficient processing occurred in the wild type *B. subtilis*, where the translocated PhoD remained attached to the membrane via its signal peptide (Müller & Wagner, 1999).

In addition, it was demonstrated that the translocation of the SP_{PhoD}-LacZ fusion in *E. coli* does not require *B. subtilis* specific cofactors.

The selective energy inhibition assays were carried out to differentiate whether SP_{PhoD}-LacZ was transported Sec- or Tat-dependent. The addition of nigericin abolished both the processing and the translocation of SP_{PhoD}-LacZ. The treatment with sodium azide had no effect on SP_{PhoD}-LacZ translocation but severely inhibited the export of the Sec-dependent control substrate proOmpA (Pop *et al.*, 2002). Thus, it can be concluded that the heterologous fusion SP_{PhoD}-LacZ was transported in a Tat-dependent manner, independent of ATP hydrolysis.

Since the transport of SP_{PhoD}-LacZ fusion required only two components from the *B. subtilis* Tat system, a possible involvement of *E. coli* Tat components was investigated. The $TatA_d/TatC_d$ proteins and the SP_{PhoD}-LacZ hybrid were produced in an *E. coli* strain deleted for *tatABCDE* genes and an *in*

vivo protease mapping experiment was performed. Most of SP_{PhoD}-LacZ was protease accessible indicating a non-cytosolic localisation. Intriguingly, no processing of the substrate was observed. Although the relation between the presence of *E. coli* Tat components and the activity of LepB is not yet investigated, it seems that the deletion of *tat* genes strongly affected the LepB ability to cleave Tat signal peptides. This result demonstrated unambiguously that in a heterologous system, a particular twin-arginine signal peptide is recognised as a Tat motif only by the Tat components present in the host of origins (Pop *et al.*, 2002).

Taken together, the data presented underlines the minimal necessity for a functional Tat export system of *B. subtilis*. The twin-arginine signal peptide of PhoD, two of the five Tat components encoded by the *B. subtilis* chromosome ($TatA_d$ and $TatC_d$), and the membrane pH gradient as energy source, form an autonomous Tat export unit (Pop *et al.*, 2002).

2.1.4 The signal peptide of PhoD canalises the export of *E. coli* phytase in *B. subtilis.*

Phytase is a natural enzyme used as feed additive to improve the nutritional quality of phytic acid found in rich feed components (mainly wheat bran for pigs). Phytase can be purified from transgenic microorganisms such as fungi (*Aspergillus niger*), yeasts (*Hansenula polymorpha*) or bacteria (*Escherichia coli*) (Pandey *et al.*, 2001). Phytase has also been produced recently in transgenic canola (*Brassica napus*), alfalfa (*Medicago sativa*) and rice (*Oryza sativa*) (Ullah *et al.*, 2002; Hong *et al.*, 2004). The direct use of these plants as feeds provide the same benefits as adding the purified enzyme to conventional crop diets. However, the most efficient way to produce phytase remains the use of genetically modified microorganisms. A potential successful candidate could be the Gram positive bacterium *B. subtilis*, which is already used to produce a large number of industrial enzymes. It was already demonstrated that *B. subtilis* Tat system is able to export proteins which were not compatible with *E. coli* Sec system. It is also demonstrated that twin-arginine signal peptides can canalise heterologous substrates via the Tat pathway (Wu *et al.*, 2000). In addition, we showed that the signal peptide of PhoD (assisted by $TatA_d$ and $TatC_d$) can canalise heterologous substrates through the Tat system (Pop *et al.*, 2002). Therefore, the use of the Tat A_d/C_d system of *B. subtilis* for production of heterologous proteins was analysed.

In order to check whether *E. coli appA* gene (phytase) could be expressed and secreted in *B. subtilis*, a fusion protein consisting of the signal peptide of PhoD and the *E. coli* AppA (SP_{PhoD}-AppA) was constructed. *B. subtilis* GCH872 *phoR12* allows the artificial induction of the *phoD* operon. This strain was used as host to express SP_{PhoD}-AppA. Without the induction of *$tatA_d/C_d$* genes, the hybrid accumulated in the cytosol. By using the *phoR12* background, the Pho regulon was artificially induced and the SP_{PhoD}-AppA fusion exported. Though, the secreted heterologous enzyme was susceptible to be degraded by the exoprotease machinery of *B. subtilis.* When a strain depleted for the cell wall associated subtilisin WprA (strain *B. subtilis* DN1885 *wprA phoR12*) was used, the overall stability of the exported heterologous product was increased (Gerlach *et al.*, 2004).

Interesting, the AppA signal peptidase cleavage site (AFA-QS) was far better processed by the *B. subtilis* signal peptidases compared with the original cleavage sequence of PhoD signal peptide (VNA-AP). This was an additional confirmation of why in wild type *B. subtilis*, the PhoD is processed only partially (Müller & Wagner, 1999).

Taken together, it can be concluded that the *E. coli* phytase can be expressed and exported by the heterologous host *B. subtilis*. The export of this heterologous enzyme is Tat-dependent and is mediated by the PhoD-specific transport system of *Bacillus subtilis*. For a productive export, a more efficient signal peptidase cleavage site and an exoprotease defective *B. subtilis* strain should be considered.

This result demonstrates that the combined advantages of *B. subtilis* and the Tat system can lead toward a useful biotechnological tool. However, before a production process could be suggested, the proper folding, the adequate activity, and the purity of the final product must be carefully investigated.

2.2 Components of *B. subtilis* Tat pathway

In most bacteria and in plant thylakoids the Tat system consists of TatA, TatB, and TatC proteins (Wu *et al.*, 2000). Despite the fact that some substrates need to incorporate cofactors in order to become export-competent, no other soluble components are known to be required for the targeting process itself. Bacilli and archaea genomes encode only two types of Tat proteins, TatA and TatC, though some times they contain multiple copies of these *tat* genes. In *B. subtilis*, three TatA and two TatC proteins are present (Jongbloed *et al.*, 2002). As demonstrated above, the PhoD-specific transport system of this bacterium consists of only one pair of TatA/TatC proteins. The $tatA_d/tatC_d$ gene pair was shown to translocate the SP_{PhoD}-LacZ fusion protein in *E. coli* without the need of any specific *B. subtilis* cofactor (Pop *et al.*, 2002). The apparent simplicity of *B. subtilis* Tat systems and the extremely small number of translocated substrates, suggest that this export system has evolved as a high adaptation for exporting a very particular substrate in very particular circumstances.

2.2.1 $TatA_d$ is a protein with dual topology

As mentioned above, *B. subtilis* genome encodes three different TatA proteins: $TatA_d/TatA_c/TatA_y$. The $tatA_d$ gene is localised in the same operon with *phoD*, hence the name "$tatA_d$".$TatA_d$ is a typical TatA protein with an apparent size of 7,36 kDa. It is predicted to have one amino terminal transmembrane helix, followed by an amphiphatic helix and then an unstructured C-terminal end. For the *E. coli* TatA, both helices are proved to be essential for their function, while the C-terminal domain is not important (Lee *et al.*, 2002)

Figure 5. Computer prediction of TatA protein structure. The grey cylinders represent the membrane and amphiphatic helices; the arrow a beta-strand and the black line an unstructured domain.

Information about topology of *B. subtilis* Tat components are acquired only from computer prediction studies. In order to get further insight into the structure of *B. subtilis* translocase, the cytosolic membrane of *B. subtilis* expressing the *phoD* operon was investigated by freeze-fracture cytochemistry. This is a powerful technique to study the macromolecular architecture of biomembranes (Fujimoto, 1997). Subsequent immunogold labelling of freeze fractured *B. subtilis* membranes, using monospecific antibodies against $TatA_d$ was carried out to localise this protein and to identify possible $TatA_d$-containing membrane structures. $TatA_d$ was found evenly distributed at both sides of the cytosolic membrane, confirming its predicted topology. Strikingly, a substantial amount of gold particles was localised in the cytosol (Pop *et al.*, 2003). Similar results were obtained using *B. subtilis* GCH872 *phoR12* or *E. coli* strain expressing $tatA_d/C_d$. Given the attraction of this observation, different control experiments were performed. *B. subtilis* cells grown in phosphate rich media cannot express the Tat components. These cells were freeze-fractured and subsequent immunogold detection using antibodies against $TatA_d$ was carried out. The absence of gold particles demonstrated the specificity of the antibody. Under similar conditions, freeze-fractured cells were immunogold labelled with antibodies against the chaperone DnaK and the integral membrane protein SecY. As expected, the chaperone DnaK was detected only in the cytosol and SecY was predominantly localised in the cytosolic membrane (Pop *et al.*, 2003). In order to eliminate any uncertainty regarding the results obtained via freeze fracture experiments, localisation of $TatA_d$ was further elucidated by classical cell fractionation experiments as well as by immunogold labelling of ultrathin sections of phosphate-starved *B. subtilis* 168 cells. Again, beside the expected membrane associated localisation, a substantial amount of $TatA_d$ was detected in the cytosol of the cell. As control in the ultrathin sectioning experiments, DnaK and SecY were immunogold-labelled in parallel, showing identical distribution like in freeze fractured cells: DnaK was localised in the cytosol and SecY was found in the membrane (Pop *et al.*, 2003). In conclusion, different detection methods demonstrated unambiguously that *B. subtilis* $TatA_d$ is not, as predicted, an exclusive membrane protein but is also localised in the cytosol of the cell.

2.2.2 $TatA_d$ operates as targeting factor for PhoD

The dual topology of $TatA_d$ could be essential in understanding of the Tat export system of *B. subtilis*. This observation is even more interesting, since it is the first report that shows the existence of a soluble Tat component, while it is claimed that targeting and translocation via the Tat/ΔpH pathway function without the involvement of soluble proteins cofactors (Cline *et al.*, 1992). What could be the function of a soluble $TatA_d$ fraction? In order to address this question we searched for possible interrelations of soluble $TatA_d$ with the other participants in the translocation process, particularly with the translocated substrate PhoD. In order to study a possible correlation with the substrate PhoD, the localisation of $TatA_d$ was investigated in a strain deleted for the *phoD* gene. Interestingly, in the absence of PhoD, $TatA_d$ was entirely localised in the membrane (our unpublished data). This observation indicates indirectly that soluble $TatA_d$ might be necessary for targeting of prePhoD to the membrane.

In addition, kinetic studies revealed that cytosolic localisation of $TatA_d$ in phosphate-starved *B. subtilis* 168 was time variable. At the onset of phosphate starvation substantial amounts of TatAd were detected in the cytosol, while upon ongoing starvation cytosolic $TatA_d$ decreased concomitant with increasing the amount of membrane integrated $TatA_d$ (Pop *et al.*, 2003). These results excluded the possibility that the "soluble $TatA_d$" fraction could be only a natural transient protein fraction, newly synthesised in the cytosol and on the way to the membrane.

Since $TatA_d$ exists also soluble in the cytosol playing a potential role in the targeting process of prePhoD, its affinity to this substrate was further investigated. Using the tandem affinity purification technology (TAP), a new method for purifying protein complexes under native conditions, it is possible to demonstrate the interaction between partner proteins *in situ* (Gavin *et al.*, 2002). The TAP tag is a tandem epitope consisting of two binding modules: the ProteinA and the calmodulin binding protein. To elucidate the possible interaction of $TatA_d$ with prePhoD, a DNA region encoding the TAP tag was fused at the C-terminal end of *$tatA_d$* gene. In parallel, the ATG start codon of *$tatA_d$* (*$tatA_d$'*) was also linked to the TAP encoding region. The resulting fusions *$tatA_d$-TAP* and *$TatA_d$'-TAP* were first integrated into the *phoD* operon of the *B. subtilis* 168 chromosome and then synthesised under phosphate depleted conditions. The proteins were subsequently purified from the cytosol using the standard TAP protocols. After purification, the TAP peptides were assayed for the presence of PhoD. Although no PhoD could be co-purified with the TAP tag alone ($TatA_d$'-TAP), some prePhoD was co-purified with $TatA_d$-TAP. This result demonstrated the *in situ* interaction between $TatA_d$ and prePhoD and confirmed once more, the presence of soluble $TatA_d$ in the cytosol of the cell. This was the first direct indication that $TatA_d$ may act as a targeting factor for its substrate PhoD (Pop *et al.*, 2003).

In parallel, the affinity between $TatA_d$ and prePhoD was further analysed via co-immunoprecipitation experiments, using purified His-tagged [^{35}S]-prePhoD and purified His-tagged $TatA_d$, and $TatA_d$ monospecific antibodies (Pop *et al.*, 2003). PrePhoD was efficiently co-immunoprecipitated (60% from the input), whereas only low levels of precursor were immunoprecipitated when either $TatA_d$ or $TatA_d$-antibodies were omitted from the mixture.

To analyse the interaction specificity, we investigated affinity of $TatA_d$ to the radiolabelled mature PhoD (m-PhoD), the Sec-dependent *B. subtilis* preYvaY and m-YvaY proteins, and CopR, a cytosolic protein involved in replication control of plasmids. Except of m-PhoD, which was precipitated in proportion of 25%, all the other proteins showed affinities around the background level. These data indicate that particularly the signal peptide of PhoD contains the information that mediates the affinity to $TatA_d$, playing a crucial role in its recognition process. However, the weak affinity showed by m-PhoD could attest as well, a role of this part of the protein in stabilising the complex made with $TatA_d$.

2.2.3 $TatA_d$ has a sequence-specific affinity with the signal peptide of PhoD

Because the signal peptide of PhoD was shown to be important for the affinity to $TatA_d$, we suggested that the twin-arginine motif could be a specific target for the soluble $TatA_d$. Cellulose-bound peptide arrays have been successfully used to characterise substrate binding motifs of different proteins (Gerharz *et al.*, 2001). Therefore, we used a cellulose-bound peptide scan consisting of 20-mer peptides that overlap by 19 residues over the sequence of the 60 N-terminal localised amino acids and covering the 56-amino acid residue-long signal peptide of PhoD (Pop *et al.*, 2003). Interesting, purified radiolabelled $TatA_d$ showed highest affinity to peptides containing the twin-arginine motif ($R_{26}R_{27}$), especially when these residues were localised at the N-flexible end of the peptides. The affinity was observed, though to a lesser extent, also for peptides starting with RK residues ($R_{27}K_{28}$), indicating that lysine could be actively involved in the recognition motif. Purified radiolabelled SecB showed a different binding pattern, confirming the specificity of the method (Pop *et al.*, 2003).

Supplementary evidence about the role of particular amino acids for $TatA_d$ binding was obtained by substitution each in turn by all of 10 N-terminal amino acid residues of the twin-arginine containing peptide D**RR**KFIQGAGKIAGLSLGLT$_{25\text{-}44}$. Binding pattern showed that substitution of the twin-arginine motif decreased binding of $TatA_d$ most seriously. In addition, substitution of lysine residue following the RR motif also decreased the affinity for $TatA_d$. This demonstrated the particular importance of the twin-arginine motif for $TatA_d$ affinity (Pop *et al.*, 2003).

The co-immunoprecipitation experiments allowed to study the role of the signal peptide of PhoD as a whole. Therefore, we repeated this experiment in the presence of synthetic peptides containing the twin-arginine motif. The addition of QNNTFD**RRK**FIQGAGKIAG peptide, significantly decreased the amount of co-immunoprecipitated prePhoD by $TatA_d$. In contrast, the peptide QNNTFD**AAA**FIQGAGKIAG in which the **RRK** sequence motif was replaced by an **AAA** sequence affected the precipitation of prePhoD to a much lesser extent. The competition between synthetic RR-peptides and the signal peptide of PhoD in binding of $TatA_d$, confirmed the specific affinity of $TatA_d$ to PhoD, particularly to the twin-arginine containing signal peptide (Pop *et al.*, 2003).

Although the obtained data might bear an inevitable *in vitro* limitation factor, these studies clearly revealed that the RRK$_{26\text{-}28}$ sequence from PhoD signal peptide represents the recognition motif for soluble $TatA_d$.

2.2.4 Cytosolic $TatA_d$ is organised in soluble homo-oligomers

$TatA_d$ is an amphiphilic molecule, which consist of a calculated transmembrane segment followed by an amphiphatic helix and a hydrophilic C-terminus. The high molecular ratio of this protein over the other Tat components suggests that its physiological functions may be associated with the organisation in molecular complexes rather than single molecules. As it can be easily purified in a soluble form, its molecular organisation was investigated, both in hydrophilic and hydrophobic environments.

Sucrose density gradient centrifugation experiments are successfully used to separate protein complexes of different sizes. We employed this method in order to find out the molecular organisation of soluble $TatA_d$. Purified $TatA_d$ protein was loaded onto such sucrose gradient and subsequently ultra-centrifuged. The resulting gradient fractions were assayed by western blotting using specific $TatA_d$ antibodies (Pop *et al.* submitted for publication). A significant fraction of $TatA_d$ was found in the pellet of the gradients indicating that the protein formed large aggregates. Another big fraction of soluble $TatA_d$ was sedimented at the position in the gradient corresponding to the 150 - 250 kDa window, demonstrating the formation of large homo-oligomeric complexes. Essentially similar results were obtained using, instead of purified $TatA_d$, membrane free cell extract of *B. subtilis* strain over-expressing $tatA_d/C_d$ (Pop *et al.* submitted for publication). This demonstrated that homo-oligomerisation occurs also *in vivo* and it is not an experimental artefact.

To obtain further insight into the structural properties of $TatA_d$, the 150-250 kDa complexes from sucrose density gradients were subjected to negative contrast electron microscopy (Pop *et al.* submitted for publication). The micrographs revealed two different classes of ring-shaped particles with diameters of 12 and 25 nm respectively. They can be explained as micelle-like structures consisting of one or two protein layers. The small ones can be formed by one layer of 15 to 25 $TatA_d$ molecules oriented with the hydrophobic transmembrane helix to the middle and thus, exposing the amphiphatic segment to the hydrophilic environment. The big particles can consist of two layers of 50 to 75 $TatA_d$ molecules oriented with the hydrophobic transmembrane helices to one another forming the median region of the "protein bilayer", whereas the amphiphatic segments are facing the hydrophilic exterior or are enclosing a hydrophilic cavity. In addition to these particles, filamentous structures were also detected. Since they have about the same diameters like the described particles, it can be suggested that both 12 and 25 nm particles have the tendency to pile up and form filaments.

2.2.5 PrePhoD has affinity to $TatA_d$ homo-oligomeric complexes

In parallel, gradient centrifugation was carried out with purified prePhoD (Pop *et al.* submitted for publication). The vast majority of prePhoD was found in the pellet of the gradient indicating that the protein aggregated during centrifugation. Next, sedimentation was carried out with a mixture of $TatA_d$ and prePhoD in a molecular ratio of 10 : 1. Surprisingly, not all prePhoD was localised in the pellet. The protein sedimented partly in the same density horizon as $TatA_d$, corresponding to a molecular

weight of about 150 to 250 kDa. This observation indicates that soluble homo-oligomeric $TatA_d$ complexes show affinity for prePhoD. With other words, the presence of $TatA_d$ abolished the aggregation for a part of prePhoD molecules, suggesting a tight physiological interaction between both molecules.

2.2.6 Soluble $TatA_d$ integrates in hydrophobic membranes

In order to fulfil the proposed targeting function, soluble $TatA_d$ complexes should have the capacity to cope similarly with both hydrophilic and hydrophobic environments. *In vivo*, after interacting with prePhoD, the soluble $TatA_d$ complexes could integrate into the membrane forming the translocon. The same concept was applied to investigate the putative dual behaviour of $TatA_d$ complexes *in vitro*. By combining an aqueous $TatA_d$ solution (mimicking the hydrophilic cytosol) and the apolar solvent chloroform (mimicking the hydrophobic cytosolic membrane), it was possible to extract, to some extent, the soluble protein into the hydrophobic chloroform fraction. As a control, the hydrophilic protein lysozyme remained 100% in the aqueous solution. This result demonstrated that the amphiphatic protein $TatA_d$ can shift from a soluble to a hydrophobic environment (Pop *et al.* submitted for publication).

However, chloroform is just a hydrophobic medium that can affect protein-protein and lipid-protein interactions, which normally take place in a real membrane environment. To check the organisation of $TatA_d$ in a more appropriate surrounding, we investigated its molecular organisation in the hydrophobic bilayer of a biological membrane. Therefore, proteoliposomes were reconstituted with total *E. coli* polar lipids in the presence of purified $TatA_d$. The purified protein was successfully embedded into the lipid bilayer as monitored by immunological detection. Freeze fracture experiments of the reconstituted proteoliposomes revealed visible intramembrane particulate structures. Since those particles were missing from protein-free liposomes, it can be concluded that they were made by multimerisation of membrane integrated $TatA_d$. The approximate size of these intramembrane particles was about 10 nm. Here, the hydrophobic part of the molecules should mediate the contact to the membrane, and the hydrophilic part of the molecules should form an aqueous core. Infrequently, beside round 10 nm particles, some $TatA_d$ complexes were extended to long shaped filamentous and dumbbell-like structures. The fact that $TatA_d$ can integrate in the membrane as multimeric units reflects a very flexible molecular organisation of those complexes (Pop *et al.* submitted for publication).

As demonstrated above, the soluble $TatA_d$ complexes interact with the substrate prePhoD *in vivo.* Because during the integration into the hydrophobic bilayer $TatA_d$ is likely to undergo a structure alteration, this may affect the affinity with already bound prePhoD. To analyse if membrane integrated $TatA_d$ complexes have affinity to prePhoD, liposomes were formed in the presence of both proteins and localisation of prePhoD was assayed via freeze fracture cytochemistry and subsequent immunogold labelling. The gold particles were co-localised with the above-described $TatA_d$ 10 nm particles. This observation suggested that membrane integrated $TatA_d$ has affinity for prePhoD.

Because PhoD is a hydrophilic protein which cannot , by itself, integrate in a hydrophobic environment, the only possibility is to be localised in a sort of aqueous cavity enclosed/formed in the middle of $TatA_d$ complex (Pop *et al.* submitted for publication).

The molar excess of $TatA_d$, the observed $TatA_d$ membrane complexes, the ability of soluble $TatA_d$ to integrate into membranes, are indications pointing toward $TatA_d$ as forming the protein-conducting channel used to export folded proteins by the Tat system of *B. subtilis*. Still, relevant *in vivo* data is not yet demonstrated. Whether or not $TatA_d$ is really forming the translocation pore assembly in a living cell remains a question to be answered.

2.2.7 $TatC_d$ is a genuine membrane protein with potential receptor function

$TatC_d$, the second protein shown to be involved in the *B. subtilis* PhoD export system, is an integral membrane protein predicted to have six spanning domains. For *E. coli* TatC, the N- and C- terminal helices are shown to be localised in the cytosol (Sargent *et al.*, 1998; Yen *et al.*, 2002; Behrendt *et al.*, 2004).

In order to get further insight in the molecular organisation of this membrane protein, proteoliposomes were reconstituted with purified $TatC_d$ and total *E. coli* polar lipids. As monitored immunologically, these vesicles contained a substantial amount of $TatC_d$, indicating the efficient integration of $TatC_d$ in the hydrophobic membrane. Freeze fracturing of these membrane vesicles demonstrated the absence of any visible membrane structure suggesting a low degree of $TatC_d$ oligomerisation. Co-reconstitution of $TatA_d$ and $TatC_d$ at a ratio of 10 : 1 did result in similar structures observed in the $TatA_d$ containing proteoliposomes (data not shown). It can be therefore assumed that $TatC_d$ does not structurally contribute to the formation of the translocation pore (Pop *et al.* submitted for publication).

What could be then the functional role of $TatC_d$ in *B. subtilis*? In order to preserve the membrane barrier function, the Tat translocase should be a dynamic machinery. The six spanning helices of TatC indicate a quite inflexible protein topology. In *E. coli*, TatC has been predicted to be the receptor for the Tat substrates (Bogsch *et al.*, 1998). To fulfil this function in *B. subtilis*, membrane integrated $TatC_d$ should show affinity to the substrate prePhoD and/or to the prePhoD containing $TatA_d$ complexes. To analyse this hypothesis, first, the affinity between $TatA_d$ and $TatC_d$ was analysed by using column bound $TatC_d$ and subsequent treatment with purified His_6-$TatA_d$. A substantial amount of purified His_6-tagged protein remained bound to the column (Schreiber *et al.* in preparation). This was a first indication that both proteins show affinity to each other.

Similarly, soluble $TatA_d$ showed affinity to inverted membrane vesicles containing $TatC_d$. As a control, no $TatA_d$ was found bound to $TatC_d$-free membrane vesicles. To elucidate this relationship further, we studied the affinity of purified [^{35}S]-labelled His_6-$TatA_d$ to a cellulose bound $TatC_d$ peptide library. Interesting, $TatA_d$ showed a selective affinity, particularly to the sequences corresponding to the predicted cytosolic loops of $TatC_d$ (Schreiber *et al.* in preparation). These data strongly suggest that *B. subtilis* $TatC_d$ could act as a receptor for $TatA_d$.

In order to elucidate the receptor function of $TatC_d$ further, we studied the kinetic integration of

$TatA_d$ into the cytosolic membrane. Pulse chase experiments demonstrated that $TatA_d$ shifts its localisation from the cytosol to the membrane with a variable rate, depending if the cells express or not *$tatC_d$* gene. When $TatC_d$ was absent, the $TatA_d$ integration in the membrane was a slow process compared with the case in which $TatC_d$ was produced (Schreiber *et al*. in preparation). This kinetic study elucidated the special affinity between the two proteins, supporting the $TatC_d$ receptor thesis.

2.3 The role of type I signal peptidases of *B. subtilis* in the processing of PhoD

The processing of the bacterial secretory pre-proteins by the signal peptidases is essential for their release into the environment. After emerging at the outer side of the cytosolic membrane, the signal peptides of the secretory pre-proteins are cleaved off by signal peptidases. *B. subtilis*, chromosomally encodes multiple paralogues type I signal peptidases (SipS, SipT, SipU, SipV, and SipW) (van Dijl *et al.*, 1992; Bolhuis *et al.*, 1996; Tjalsma *et al.*, 1997; Tjalsma *et al.*, 1998). Given this large number of signal peptidases, a first question to be addressed was whether certain signal peptidases of *B. subtilis* have a dedicated function for the processing of PhoD. In order to elucidate this aspect, the effects of single and multiple *sip* mutations on the release of mature PhoD into the growth medium were investigated. First, single *sip* mutants of all five chromosomally encoded signal peptidases (Δ*sipS*, Δ*sipT*, Δ*sipU*, Δ*sipV*, *and* Δ*sipW*) were grown in phosphate depleted conditions and their extracellular proteome analysed by 2D gel electrophoresis. The secretion of PhoD in all five single *sip* mutants tested was not affected (Jongbloed, 2002). As a control, no PhoD was detected in a double $tatC_d$-$tatC_y$ mutant (Jongbloed *et al.*, 2000). These data demonstrate that none of the type I signal peptidases of *B. subtilis* have a particular role for the processing of PhoD.

Since the function of type I signal peptidases of *B. subtilis* appears to be redundant, at least for the processing of Sec-dependent substrates (Tjalsma *et al.*, 1997; Antelmann *et al.*, 2001), the secretion of the Tat dependent PhoD was analysed for multiple *sip* mutants strains. Thus, quadruple *sip* mutants Δ*sipSUVW* and Δ*sipTUVW* were investigated in the genetic background of strain 8G5, a non-sporulating derivative of *B. subtilis* 168. As observed for the single *sip* mutant strains, multiple *sip* mutations had no effect on the secretion of PhoD. It can be therefore concluded that none of the minor type I signal peptidases are important for the PhoD secretion and in addition, the presence of either SipS or SipT is sufficient for this process (Jongbloed, 2002). The next question to be addressed was whether there is any difference between the activity of these two major type I signal peptidases SipS and SipT, regarding the processing of the Tat substrate PhoD. In order to answer this question, *in vivo* processing of PhoD was analysed in the quadruple *sip* mutant strains Δ*sipSUVW* and Δ*sipTUVW*. Despite the low level of secreted PhoD, the detection of both, the precursor and the mature form of PhoD in the cell bound fraction revealed a similar processing efficiency for the SipS and SipT (Pop *et al.* in preparation). To get further insight in the activity of SipS and SipT, the processing of purified His-tagged prePhoD was investigated *in vitro*, using purified His-tagged SipS and SipT. Interesting, no processed PhoD was detected even after 60 minutes of incubation with the Sip proteins. As controls, the purified His-tagged precursor of *Bacillus licheniformis* α-Amylase (preAmyL) and a fusion protein consisting of the signal peptide of PhoD and the mature domain of *E. coli* maltose binding protein (SP_{PhoD}-MalE), were treated under similar conditions. Both substrates were processed after only one minute of incubation, by either SipS or SipT, with a similar efficiency (Pop *et al.* in preparation). This

demonstrated that the purified Sip proteins were enzymaticaly active and that the original cleavage sequence of PhoD signal peptide (VNA-AP) is recognised by both major type I signal peptidases. The lack of processing observed when purified His-tagged prePhoD was used can be explained as result of fast aggregation of the purified substrate. Subsequently, the signal peptidase cleavage site was not accessible to the Sip proteins. In conclusion, the present data indicate that for the processing of PhoD, any of the two major type I signal peptidases of *B. subtilis* is sufficient. However, further experiments are currently carried out in order to achieve a fine tuning regarding the possible differences in the activity of the two major type I signal peptidases SipS and SipT in the processing of Tat substrate PhoD.

2.4 The mechanism of the Tat export of *B. subtilis*

While all studies published so far describe Tat systems involving TatABC proteins, *B. subtilis* lacks a TatB-like protein. The involvement of only two Tat components together with the demonstrated dual localisation of $TatA_d$ clearly shows, that the *B. subtilis* Tat system represents a different export mechanism. The data obtained during my Ph.D. period, together with result obtained before, provide some first indications of how the transport of the Tat substrate PhoD could be organised. Further studies are still necessary to get more insight into the particular steps of this translocation system. Based on our current data, the following model for the transport of PhoD in *B. subtilis* can be suggested (Fig.6):

1) *Induction of the substrate and transport system components.* When *B. subtilis* is growing in an environment limited for inorganic phosphate, the cellular phosphate starvation response is induced. This response includes the expression of the *phoD* operon encoding the secretory phosphodiesterase PhoD and the Tat components $TatA_d$ and $TatC_d$. So far, PhoD is the only protein transported via this transport system.

2) *Folding of prePhoD and multimerisation of $TatA_d$.* The newly synthesised prePhoD is most likely folded in the cytosol. In parallel, produced $TatA_d$ forms soluble homo-multimeric complexes. These complexes may be micelle-like particles, which could have a hydrophobic centre and a hydrophilic envelope.

3) *Complex formation of $TatA_d$ with its substrate prePhoD.* In the cytosol soluble $TatA_d$ complexes bind their substrate prePhoD, which is recognised preferably via the twin-arginine motif from its signal peptide.

4) *Targeting.* The $TatA_d$-prePhoD complex moves to the membrane where it interacts with $TatC_d$. $TatC_d$ fulfils the function of a receptor at the surface of the cytosolic membrane.

5) *Membrane integration.* After the contact with the $TatC_d$ receptor the $TatA_d$-prePhoD complex integrates into the cytosolic membrane driven by the pH gradient. During this intergration process the $TatA_d$ complex inverts its conformation. The hydrophobic helices mediate the contact to the lipid bilayer and the hydrophilic part of the protein forms the protein conducting channel.

6) *Translocation.* PrePhoD is exported through this protein-conducting channel. The translocation process is mediated by the pH gradient at the membrane. After the export is completed, the conducting channel is closed and the substrate PhoD moves laterally from the translocase.

7) *Processing.* During or shortly after translocation, the signal peptide of PhoD is recognised and processed by specialised signal peptidases (✂). Depending of the efficiency of processing, the mature PhoD remains membrane-anchored via its signal peptide or is released into the cell wall and the growth medium. The cleaved signal peptide is degraded in the membrane via specific signal peptide peptidases.

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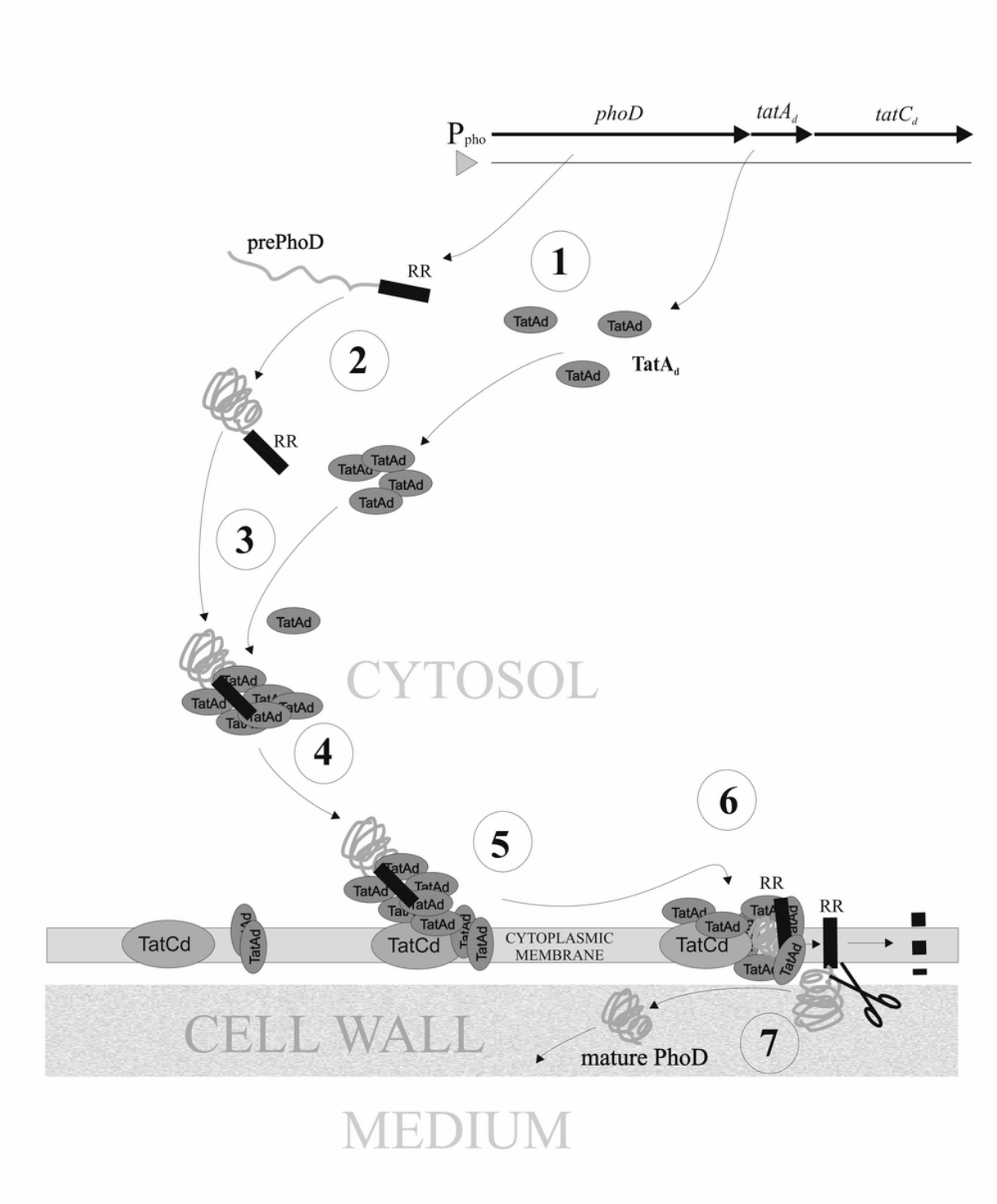

Figure 6. The translocation model of the PhoD specific Tat transport of *B. subtilis* (see text for details).

Summary

The newly discovered twin-arginine translocation (Tat) system has the capability to transport folded proteins through the cytosolic membrane. Proteins transported via this transport system are synthesised as pre-proteins with an N-terminal localised signal peptide containing an almost invariant twin-arginine sequence motif. The pH gradient at the membrane is currently the only known driving force mediating the transport of the pre-proteins through the Tat pathway. This translocation system was initially described for the transport of proteins into plant thylakoids. In the meanwhile, Tat pathway has been described for a variety of eubacterial organisms. Because of its ability to transport fully folded proteins, the possible biotechnological implications of the Tat system have raised the scientific interest. Due to its capacity to secrete a large amount of proteins into the growth medium, the Gram positive bacterium *Bacillus subtilis* has a long history for the large scale production of proteins. The special capabilities of the Tat system could convey to a even more powerful tool for the secretory production of heterologous proteins.

The presence of TatA- and TatC-like proteins as well as pre-proteins carrying twin-arginine signal peptides, indicated that the Tat export probably exists also in *B. subtilis*. It has been demonstrated previously that the transport of PhoD, a phosphodiesterase synthesised with a twin-arginine signal peptide, was dependent on the $TatC_d$ protein. *PhoD* and the genes encoding the components $TatA_d$ and $TatC_d$ are localised in one operon. As a response to phosphate starvation, all three genes are simultaneously induced. We demonstrated that $TatA_d$ and $TatC_d$ form an autonomous translocation system in the donor strain and in heterologous organisms. Particularly, they mediate the transport of PhoD, as well as hybrid proteins containing the signal peptide of PhoD as translocation motif. Precisely, we showed that the twin-arginine signal peptide of PhoD canalised the Tat-dependent transport of ß-galactosidase LacZ in *E. coli* and the *E. coli* phytase AppA in *B. subtilis.*

In order to get insight in the mechanism, how PhoD is transported in a Tat-dependent manner, we studied the biochemical characteristics of the translocase components. $TatA_d$ is a protein with one calculated membrane spanning domain. Nevertheless, besides its predicted membrane integrated localisation, was found also soluble in the cytosol. The soluble $TatA_d$ forms homo-multimeric particles, which have affinity to prePhoD, particularly to its twin-arginine motif from the signal peptide. According to our data, we suggest that $TatA_d$ play the role as targeting factor for prePhoD. Besides, $TatA_d$ was found to form detectable structures also in the membranes of reconstituted liposomes. These homo-multimeric complexes had affinity to prePhoD. Since $TatC_d$ does not have the tendency to form complexes in the hydrophobic bilayer, it can be concluded that $TatA_d$ may significantly contribute to the formation of the translocation channel, which conducts prePhoD through the cytosolic membrane.

Despite that fact that the detailed mechanism of the PhoD specific Tat-dependent transport is not known yet, data presented here brought a significant contribution in describing the particular steps for the Tat-dependent transport of folded proteins through biological membranes.

Zusammenfassung

Das erst vor kurzem beschriebene Doppelarginin-Proteintransport- (Tat-) System hat die Fähigkeit, bereits gefaltete Proteine durch die cytosolische Membran zu transportieren. Proteine, welche diese Exportroute nutzen, werden als Vorläuferproteine mit einem N-terminal lokalisierten Doppelarginin-Signalpeptid synthetisiert. Dieses invariante Sequenzmotiv kanalisiert den Tat-abhängigen Proteintransport. Der pH-Gradient der Membran ist die gegenwärtig einzig bekannte Engeriequelle, die den Translokation dieser Vorläuferproteine vermittelt. Das Tat-System wurde zuerst für den Import von Proteinen in pflanzliche Thylakoide beschrieben. Inzwischen wurde die Existenz dieser Exportroute auch in einer Reihe eubakterieller Organismen nachgewiesen. Aufgrund der spezifischen Fähigkeit gefaltete Proteine zu transportieren, besteht ein großes Interesse, dieses System für biotechnologisches Anwendungen zu nutzen. Durch die Möglichkeit, große Proteinmengen in das Wachstumsmedium zu sekretieren, wird das Bakterium *Bacillus subtilis* seit langem für die industrielle Herstellung von Proteinen. Durch den Einsatz des Tat-Systems besteht nun die potentielle Möglichkeit, in diesem Organismus weitere heterologe Proteine zu erzeugen, die sich aufgrund zu schneller Faltung bisher nur ineffizient sekretorisch produzieren ließen.

Die Anwesenheit von TatA- und TatC-ähnlichen Proteinen sowie von Vorläuferproteinen, die ein Doppelarginin-Signalpeptid tragen, deutete darauf hin, dass der Tat-Transportweg auch in *B. subtilis* existiert. So wurde bereits nachgewiesen, dass der Transport von PhoD, einer Phosphodiesterase, die mit einem Doppelarginin-Signalpeptid synthetisiert wird, von TatC abhängig war. *PhoD* und die Gene, welche die Komponenten $TatA_d$ und $TatC_d$ kodieren, sind in einem Operon lokalisiert. In Antwort auf Phosphatverarmung werden alle drei Gene simultan exprimiert. Wir haben gezeigt, dass $TatA_d$ und $TatC_d$ ein autonomes Translokationssystem sowohl in *B. subtilis* als auch in heterologen Systemen bildet. Es vermittelt den Transport von PhoD sowie von Hybridproteinen, die das Signalpeptid von PhoD als Translokationssignal tragen. Wir haben nachgewiesen, dass sowohl das Doppelarginin-Signalpeptid von PhoD den Tat-abhängigen Transport der ß-Galaktosidase LacZ in *E. coli* als auch der *E. coli* Phytase AppA in *B. subtilis* vermittelt.

Um weiter Einblicke in den Mechanismus des Tat-abhängigen Transports von PhoD zu erhalten, untersuchten wir die biochemischen Eigenschaften der Translokasekomponenten. $TatA_d$ ist ein Protein mit einer kalkulierten transmembranspannenden Domäne. Trotzdem konnten wir es sowohl in der Membran als auch im Cytosol nachweisen. Lösliches $TatA_d$ bildet homomultimere Komplexe, welche Affinität zu prePhoD, speziell zum Doppelargininmotiv des Signalpeptides, haben. Aus diesen Ergebnissen kann man schlussfolgern, dass $TatA_d$ eine Funktion beim Targeting von prePhoD besitzt. Weiterhin wurde demonstriert, dass $TatA_d$ detektierbare Strukturen in Membranen rekonstituierter Liposomen bildet. Diese homomultimeren Komplexe hatten Affinität zu prePhoD. Daraus lässt sich

schließen, dass $TatA_d$ maßgebend an der Bildung des transmembranalen Exportkanals beteiligt ist, der die Translokation von PhoD vermittelt.

Obwohl der vollständige Mechanismus des Tat-abhängigen Transportsystems von PhoD noch nicht bekannt ist, liefern die hier dargestellten Ergebnisse einen wichtigen Beitrag zum Verständnis des Transportes gefalteter Proteine durch die cytosolische Membran.

References

Alami, M., Luke, I., Deitermann, S., Eisner, G., Koch, H.G., Brunner, J. & Müller, M. (2003). Differential interactions between a twin-arginine signal peptide and its translocase in *Escherichia coli. Mol. Cell,* **12,** 937-946.

Alami, M., Trescher, D., Wu, L.F. & Müller, M. (2002). Separate analysis of twin-arginine translocation (Tat)-specific membrane binding and translocation in *Escherichia coli*. *J. Biol. Chem.,* **277,** 20499-20503.

Allen, S.C., Barrett, C.M., Ray, N. & Robinson, C. (2002). Essential cytoplasmic domains in the *Escherichia coli* TatC protein. *J. Biol. Chem.,* **277,** 10362-10366.

Ames, G.F., Prody, C. & Kustu, S. (1984). Simple, rapid, and quantitative release of periplasmic proteins by chloroform. *J. Bacteriol.,* **160,** 1181-1183.

Antelmann, H., Tjalsma, H., Voigt, B., Ohlmeier, S., Bron, S., van Dijl, J.M. & Hecker, M. (2001). A proteomic view on genome-based signal peptide predictions. *Genome Res.,* **11,** 1484-1502.

Arkowitz, R.A. & Wickner, W. (1994). SecD and SecF are required for the proton electrochemical gradient stimulation of preprotein translocation. *EMBO J.,* **13,** 954-963.

Babe, L.M. & Schmidt, B. (1998). Purification and biochemical analysis of WprA, a 52-kDa serine protease secreted by *B. subtilis* as an active complex with its 23-kDa propeptide. *Biochim. Biophys. Acta,* **1386,** 211-219.

Bakker, E.P. & Randall, L.L. (1984). The requirement for energy during export of beta-lactamase in *Escherichia coli* is fulfilled by the total protonmotive force. *EMBO J.,* **3,** 895-900.

Barrett, C.M., Mathers, J.E. & Robinson, C. (2003). Identification of key regions within the *Escherichia coli* TatAB subunits. *FEBS Lett.,* **537,** 42-46.

Bayan, N., Schrempp, S., Joliff, G., Leblon, G. & Shechter, E. (1993). Role of the protonmotive force and of the state of the lipids in the in vivo protein secretion in *Corynebacterium glutamicum*, a gram-positive bacterium. *Biochim. Biophys. Acta,* **1146,** 97-105.

Behrendt, J., Standar, K., Lindenstrauss, U. & Brüser, T. (2004). Topological studies on the twin-arginine translocase component TatC. *FEMS Microbiol. Lett.,* **234,** 303-308.

Berg, B.L., Baron, C. & Stewart, V. (1991). Nitrate-inducible formate dehydrogenase in *Escherichia coli* K-12. II. Evidence that a mRNA stem-loop structure is essential for decoding opal (UGA) as selenocysteine. *J. Biol. Chem.,* **266,** 22386-22391.

Berks, B.C. (1996). A common export pathway for proteins binding complex redox cofactors? *Mol. Microbiol.,* **22,** 393-404.

Berks, B.C., Sargent, F. & Palmer, T. (2000). The Tat protein export pathway. *Mol. Microbiol.,* **35,** 260-274.

Bernstein, H.D., Poritz, M.A., Strub, K., Hoben, P.J., Brenner, S. & Walter, P. (1989). Model for signal sequence recognition from amino-acid sequence of 54K subunit of signal recognition particle. *Nature,* **340,** 482-486.

Binet, R., Letoffe, S., Ghigo, J.M., Delepelaire, P. & Wandersman, C. (1997). Protein secretion by Gram-negative bacterial ABC exporters-a review. *Gene,* **192,** 7-11.

Blackman, S.A., Smith, T.J. & Foster, S.J. (1998). The role of autolysins during vegetative growth of *Bacillus subtilis* 168. *Microbiology,* **144 (Pt 1),** 73-82.

Blobel, G. & Dobberstein, B. (1975). Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. *J. Cell Biol.,* **67,** 835-851.

Bogsch, E., Brink, S. & Robinson, C. (1997). Pathway specificity for a delta pH-dependent precursor thylakoid lumen protein is governed by a 'Sec-avoidance' motif in the transfer peptide and a 'Sec-incompatible' mature protein. *EMBO J.,* **16,** 3851-3859.

Bogsch, E.G., Sargent, F., Stanley, N.R., Berks, B.C., Robinson, C. & Palmer, T. (1998). An essential component of a novel bacterial protein export system with homologues in plastids and mitochondria. *J. Biol. Chem.,* **273,**

18003-18006.

Bolhuis, A. (2002). Protein transport in the halophilic archaeon *Halobacterium* sp. NRC-1: a major role for the twin-arginine translocation pathway? *Microbiology,* **148,** 3335-3346.

Bolhuis, A., Broekhuizen, C.P., Sorokin, A., van Roosmalen, M.L., Venema, G., Bron, S., Quax, W.J. & van Dijl, J.M. (1998). SecDF of *Bacillus subtilis*, a molecular Siamese twin required for the efficient secretion of proteins. *J. Biol. Chem.,* **273,** 21217-21224.

Bolhuis, A., Mathers, J.E., Thomas, J.D., Barrett, C.M. & Robinson, C. (2001). TatB and TatC form a functional and structural unit of the twin-arginine translocase from *Escherichia coli*. *J. Biol. Chem.,* **276,** 20213-20219.

Bolhuis, A., Matzen, A., Hyyrylainen, H.L., Kontinen, V.P., Meima, R., Chapuis, J., Venema, G., Bron, S., Freudl, R. & van Dijl, J.M. (1999). Signal peptide peptidase- and ClpP-like proteins of *Bacillus subtilis* required for efficient translocation and processing of secretory proteins. *J. Biol. Chem.,* **274,** 24585-24592.

Bolhuis, A., Sorokin, A., Azevedo, V., Ehrlich, S.D., Braun, P.G., de Jong, A., Venema, G., Bron, S. & van Dijl, J.M. (1996). *Bacillus subtilis* can modulate its capacity and specificity for protein secretion through temporally controlled expression of the *sipS* gene for signal peptidase I. *Mol. Microbiol.,* **22,** 605-618.

Braun, P., Gerritse, G., van Dijl, J.M. & Quax, W.J. (1999). Improving protein secretion by engineering components of the bacterial translocation machinery. *Curr. Opin. Biotechnol.,* **10,** 376-381.

Buchanan, G., Leeuw, E., Stanley, N.R., Wexler, M., Berks, B.C., Sargent, F. & Palmer, T. (2002). Functional complexity of the twin-arginine translocase TatC component revealed by site-directed mutagenesis. *Mol. Microbiol.,* **43,** 1457-1470.

Buchanan, G., Sargent, F., Berks, B.C. & Palmer, T. (2001). A genetic screen for suppressors of *Escherichia coli* Tat signal peptide mutations establishes a critical role for the second arginine within the twin-arginine motif. *Arch. Microbiol.,* **177,** 107-112.

Bunai, K., Takamatsu, H., Horinaka, T., Oguro, A., Nakamura, K. & Yamane, K. (1996). *Bacillus subtilis* Ffh, a homologue of mammalian SRP54, can intrinsically bind to the precursors of secretory proteins. *Biochem. Biophys. Res. Commun.,* **227,** 762-767.

Bunai, K., Yamada, K., Hayashi, K., Nakamura, K. & Yamane, K. (1999). Enhancing effect of *Bacillus subtilis* Ffh, a homologue of the SRP54 subunit of the mammalian signal recognition particle, on the binding of SecA to precursors of secretory proteins *in vitro*. *J. Biochem. (Tokyo),* **125,** 151-159.

Chaddock, A.M., Mant, A., Karnauchov, I., Brink, S., Herrmann, R.G., Klosgen, R.B. & Robinson, C. (1995). A new type of signal peptide: central role of a twin-arginine motif in transfer signals for the delta pH-dependent thylakoidal protein translocase. *EMBO J.,* **14,** 2715-2722.

Chanal, A., Santini, C. & Wu, L. (1998). Potential receptor function of three homologous components, TatA, TatB and TatE, of the twin-arginine signal sequence-dependent metalloenzyme translocation pathway in *Escherichia coli. Mol. Microbiol.,* **30,** 674-676.

Chen, L. & Tai, P.C. (1986). Effects of nucleotides on ATP-dependent protein translocation into *Escherichia coli* membrane vesicles. *J. Bacteriol.,* **168,** 828-832.

Cline, K., Ettinger, W.F. & Theg, S.M. (1992). 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,** 2688-2696.

Cline, K. & Henry, R. (1996). Import and routing of nucleus-encoded chloroplast proteins. *Annu. Rev. Cell Dev. Biol.,* **12,** 1-26.

Cline, K. & Mori, H. (2001). Thylakoid DeltapH-dependent precursor proteins bind to a cpTatC-Hcf106 complex before Tha4-dependent transport. *J. Cell Biol.,* **154,** 719-729.

Creighton, A.M., Hulford, A., Mant, A., Robinson, D. & Robinson, C. (1995). A monomeric, tightly folded stromal intermediate on the delta pH-dependent thylakoidal protein transport pathway. *J. Biol. Chem.,* **270,** 1663-1669.

Cristobal, S., de Gier, J.W., Nielsen, H. & von Heijne, G. (1999). Competition between Sec- and TAT-dependent protein translocation in *Escherichia coli. EMBO J.,* **18,** 2982-2990.

Dalbey, R.E., Lively, M.O., Bron, S. & van Dijl, J.M. (1997). The chemistry and enzymology of the type I signal

peptidases. *Protein Sci.,* **6,** 1129-1138.

Daniels, C.J., Bole, D.G., Quay, S.C. & Oxender, D.L. (1981). Role for membrane potential in the secretion of protein into the periplasm of *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A,* **78,** 5396-5400.

de Keyzer, J., van Der, D.C. & Driessen, A.J. (2003). The bacterial translocase: a dynamic protein channel complex. *Cell Mol. Life Sci.,* **60,** 2034-2052.

de Leeuw, E., Granjon, T., Porcelli, I., Alami, M., Carr, S.B., Müller, M., Sargent, F., Palmer, T. & Berks, B.C. (2002). Oligomeric properties and signal peptide binding by *Escherichia coli* Tat protein transport complexes. *J. Mol. Biol.,* **322,** 1135-1146.

de Leeuw, E., te, K.K., Moser, C., Menestrina, G., Demel, R., de Kruijff, B., Oudega, B., Luirink, J. & Sinning, I. (2000). Anionic phospholipids are involved in membrane association of FtsY and stimulate its GTPase activity. *EMBO J.,* **19,** 531-541.

Driessen, A.J., Manting, E.H. & van Der, D.C. (2001). The structural basis of protein targeting and translocation in bacteria. *Nat. Struct. Biol.,* **8,** 492-498.

Driessen, A.J. & Wickner, W. (1991). Proton transfer is rate-limiting for translocation of precursor proteins by the *Escherichia coli* translocase. *Proc. Natl. Acad. Sci. U. S. A,* **88,** 2471-2475.

Duong, F. & Wickner, W. (1997). The SecDFyajC domain of preprotein translocase controls preprotein movement by regulating SecA membrane cycling. *EMBO J.,* **16,** 4871-4879.

Economou, A., Pogliano, J.A., Beckwith, J., Oliver, D.B. & Wickner, W. (1995). SecA membrane cycling at SecYEG is driven by distinct ATP binding and hydrolysis events and is regulated by SecD and SecF. *Cell,* **83,** 1171-1181.

Eder, S., Shi, L., Jensen, K., Yamane, K. & Hulett, F.M. (1996). A *Bacillus subtilis* secreted phosphodiesterase/alkaline phosphatase is the product of a Pho regulon gene, *phoD*. *Microbiology,* **142 (Pt 8),** 2041-2047.

Enequist, H.G., Hirst, T.R., Harayama, S., Hardy, S.J. & Randall, L.L. (1981). Energy is required for maturation of exported proteins in *Escherichia coli*. *Eur. J. Biochem.,* **116,** 227-233.

Fekkes, P. & Driessen, A.J. (1999). Protein targeting to the bacterial cytoplasmic membrane. *Microbiol. Mol. Biol. Rev.,* **63,** 161-173.

Fekkes, P., van Der, D.C. & Driessen, A.J. (1997). The molecular chaperone SecB is released from the carboxy-terminus of SecA during initiation of precursor protein translocation. *EMBO J.,* **16,** 6105-6113.

Fujimoto, K. (1997). 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,** 87-96.

Gavin, A.C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J.M., Michon, A.M., Cruciat, C.M., Remor, M., Hofert, C., Schelder, M., Brajenovic, M., Ruffner, H., Merino, A., Klein, K., Hudak, M., Dickson, D., Rudi, T., Gnau, V., Bauch, A., Bastuck, S., Huhse, B., Leutwein, C., Heurtier, M.A., Copley, R.R., Edelmann, A., Querfurth, E., Rybin, V., Drewes, G., Raida, M., Bouwmeester, T., Bork, P., Seraphin, B., Kuster, B., Neubauer, G. & Superti-Furga, G. (2002). Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature,* **415,** 141-147.

Geller, B.L. & Green, H.M. (1989). Translocation of pro-OmpA across inner membrane vesicles of *Escherichia coli* occurs in two consecutive energetically distinct steps. *J. Biol. Chem.,* **264,** 16465-16469.

Gerharz, C.D., Reinecke, P., Schneider, E.M., Schmitz, M. & Gabbert, H.E. (2001). Secretion of GM-CSF and M-CSF by human renal cell carcinomas of different histologic types. *Urology,* **58,** 821-827.

Gerlach, R., Pop, O. & Müller, J. (2004). Tat dependent export of *E.coli* phytase AppA by using the PhoD-specific transport system of *Bacillus subtilis*. *Journal of basic Microbiology.***44** (5) 351-359

Gouffi, K., Gerard, F., Santini, C.L. & Wu, L.F. (2003). Dual topology of the *Escherichia coli* TatA protein. *J. Biol. Chem.* **279**(12):11608-15

Gross, R., Simon, J. & Kroger, A. (1999). The role of the twin-arginine motif in the signal peptide encoded by the hydA gene of the hydrogenase from *Wolinella succinogenes*. *Arch. Microbiol.,* **172,** 227-232.

Halbig, D., Wiegert, T., Blaudeck, N., Freudl, R. & Sprenger, G.A. (1999). The efficient export of NADP-

containing glucose-fructose oxidoreductase to the periplasm of *Zymomonas mobilis* depends both on an intact twin-arginine motif in the signal peptide and on the generation of a structural export signal induced by cofactor binding. *Eur. J. Biochem.,* **263,** 543-551.

Hartl, F.U., Lecker, S., Schiebel, E., Hendrick, J.P. & Wickner, W. (1990). The binding cascade of SecB to SecA to SecY/E mediates preprotein targeting to the *E. coli* plasma membrane. *Cell,* **63,** 269-279.

Harwood, C.R. (1992). *Bacillus subtilis* and its relatives: molecular biological and industrial workhorses. *Trends Biotechnol.,* **10,** 247-256.

Henry, R., Carrigan, M., McCaffrey, M., Ma, X. & Cline, K. (1997). Targeting determinants and proposed evolutionary basis for the Sec and the Delta pH protein transport systems in chloroplast thylakoid membranes. *J. Cell Biol.,* **136,** 823-832.

Henry, R., Kapazoglou, A., McCaffery, M. & Cline, K. (1994). Differences between lumen targeting domains of chloroplast transit peptides determine pathway specificity for thylakoid transport. *J. Biol. Chem.,* **269,** 10189-10192.

Herbort, M., Klein, M., Manting, E.H., Driessen, A.J. & Freudl, R. (1999). Temporal expression of the *Bacillus subtilis* secA gene, encoding a central component of the preprotein translocase. *J. Bacteriol.,* **181,** 493-500.

Herskovits, A.A., Bochkareva, E.S. & Bibi, E. (2000). New prospects in studying the bacterial signal recognition particle pathway. *Mol. Microbiol.,* **38,** 927-939.

Higgins, C.F. (1992). ABC transporters: from microorganisms to man. *Annu. Rev. Cell Biol.,* **8,** 67-113.

Higgins, C.F. & Linton, K.J. (2001). Structural biology. The xyz of ABC transporters. *Science,* **293,** 1782-1784.

Hinsley, A.P., Stanley, N.R., Palmer, T. & Berks, B.C. (2001). A naturally occurring bacterial Tat signal peptide lacking one of the 'invariant' arginine residues of the consensus targeting motif. *FEBS Lett.,* **497,** 45-49.

Hirose, I., Sano, K., Shioda, I., Kumano, M., Nakamura, K. & Yamane, K. (2000). Proteome analysis of *Bacillus subtilis* extracellular proteins: a two-dimensional protein electrophoretic study. *Microbiology,* **146 (Pt 1),** 65-75.

Hong, C.Y., Cheng, K.J., Tseng, T.H., Wang, C.S., Liu, L.F. & Yu, S.M. (2004). Production of two highly active bacterial phytases with broad pH optima in germinated transgenic rice seeds. *Transgenic Res.,* **13,** 29-39.

Hulett, F.M., Bookstein, C. & Jensen, K. (1990). Evidence for two structural genes for alkaline phosphatase in *Bacillus subtilis*. *J. Bacteriol.,* **172,** 735-740.

Hussain, M., Ozawa, Y., Ichihara, S. & Mizushima, S. (1982). Signal peptide digestion in *Escherichia coli*. Effect of protease inhibitors on hydrolysis of the cleaved signal peptide of the major outer-membrane lipoprotein. *Eur. J. Biochem.,* **129,** 233-239.

Hutcheon, G.W. & Bolhuis, A. (2003). The archaeal twin-arginine translocation pathway. *Biochem. Soc. Trans.,* **31,** 686-689.

Ignatova, Z., Hornle, C., Nurk, A. & Kasche, V. (2002). Unusual signal peptide directs penicillin amidase from *Escherichia coli* to the Tat translocation machinery. *Biochem. Biophys. Res. Commun.,* **291,** 146-149.

Ize, B., Gerard, F., Zhang, M., Chanal, A., Voulhoux, R., Palmer, T., Filloux, A. & Wu, L.F. (2002). In vivo dissection of the Tat translocation pathway in *Escherichia coli*. *J. Mol. Biol.,* **317,** 327-335.

Jack, R.L., Sargent, F., Berks, B.C., Sawers, G. & Palmer, T. (2001). Constitutive expression of *Escherichia coli tat* genes indicates an important role for the twin-arginine translocase during aerobic and anaerobic growth. *J. Bacteriol.,* **183,** 1801-1804.

Jacobs, M., Andersen, J.B., Kontinen, V. & Sarvas, M. (1993). *Bacillus subtilis* PrsA is required in vivo as an extracytoplasmic chaperone for secretion of active enzymes synthesized either with or without pro-sequences. *Mol. Microbiol.,* **8,** 957-966.

Jongbloed, J.D. (2002) Specificity determinants for protein secretion in *Bacillus subtilis*. Ph.D. Thesis, Rijksuniversiteit Groningen.

Jongbloed, J.D., Antelmann, H., Hecker, M., Nijland, R., Bron, S., Airaksinen, U., Pries, F., Quax, W.J., van Dijl, J.M. & Braun, P.G. (2002). Selective contribution of the twin-arginine translocation pathway to protein secretion in *Bacillus subtilis*. *J. Biol. Chem.,* **277,** 44068-44078.

Jongbloed, J.D., Martin, U., Antelmann, H., Hecker, M., Tjalsma, H., Venema, G., Bron, S., van Dijl, J.M. & Müller, J. (2000). TatC is a specificity determinant for protein secretion via the twin-arginine translocation pathway. *J.*

Biol. Chem., **275,** 41350-41357.

Keegstra, K. & Cline, K. (1999). Protein import and routing systems of chloroplasts. *Plant Cell,* **11,** 557-570.

Keenan, R.J., Freymann, D.M., Stroud, R.M. & Walter, P. (2001). The signal recognition particle. *Annu. Rev. Biochem.,* **70,** 755-775.

Kobayashi, K., Ehrlich, S.D., Albertini, A., Amati, G., Andersen, K.K., Arnaud, M., Asai, K., Ashikaga, S., Aymerich, S., Bessieres, P., Boland, F., Brignell, S.C., Bron, S., Bunai, K., Chapuis, J., Christiansen, L.C., Danchin, A., Debarbouille, M., Dervyn, E., Deuerling, E., Devine, K., Devine, S.K., Dreesen, O., Errington, J., Fillinger, S., Foster, S.J., Fujita, Y., Galizzi, A., Gardan, R., Eschevins, C., Fukushima, T., Haga, K., Harwood, C.R., Hecker, M., Hosoya, D., Hullo, M.F., Kakeshita, H., Karamata, D., Kasahara, Y., Kawamura, F., Koga, K., Koski, P., Kuwana, R., Imamura, D., Ishimaru, M., Ishikawa, S., Ishio, I., Le Coq, D., Masson, A., Mauel, C., Meima, R., Mellado, R.P., Moir, A., Moriya, S., Nagakawa, E., Nanamiya, H., Nakai, S., Nygaard, P., Ogura, M., Ohanan, T., O'Reilly, M., O'Rourke, M., Pragai, Z., Pooley, H.M., Rapoport, G., Rawlins, J.P., Rivas, L.A., Rivolta, C., Sadaie, A., Sadaie, Y., Sarvas, M., Sato, T., Saxild, H.H., Scanlan, E., Schumann, W., Seegers, J.F., Sekiguchi, J., Sekowska, A., Seror, S.J., Simon, M., Stragier, P., Studer, R., Takamatsu, H., Tanaka, T., Takeuchi, M., Thomaides, H.B., Vagner, V., van Dijl, J.M., Watabe, K., Wipat, A., Yamamoto, H., Yamamoto, M., Yamamoto, Y., Yamane, K., Yata, K., Yoshida, K., Yoshikawa, H., Zuber, U. & Ogasawara, N. (2003). Essential *Bacillus subtilis* genes. *Proc. Natl. Acad. Sci. U. S. A,* **100,** 4678-4683.

Kontinen, V.P., Saris, P. & Sarvas, M. (1991). A gene (prsA) of *Bacillus subtilis* involved in a novel, late stage of protein export. *Mol. Microbiol.,* **5,** 1273-1283.

Kontinen, V.P. & Sarvas, M. (1993). The PrsA lipoprotein is essential for protein secretion in *Bacillus subtilis* and sets a limit for high-level secretion. *Mol. Microbiol.,* **8,** 727-737.

Kumamoto, C.A., Chen, L., Fandl, J. & Tai, P.C. (1989). Purification of the *Escherichia coli secB* gene product and demonstration of its activity in an in vitro protein translocation system. *J. Biol. Chem.,* **264,** 2242-2249.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A.M., Alloni, G., Azevedo, V., Bertero, M.G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S.C., Bron, S., Brouillet, S., Bruschi, C.V., Caldwell, B., Capuano, V., Carter, N.M., Choi, S.K., Codani, J.J., Connerton, I.F., Danchin, A. & . (1997). The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature,* **390,** 249-256.

Lee, P.A., Buchanan, G., Stanley, N.R., Berks, B.C. & Palmer, T. (2002). Truncation analysis of TatA and TatB defines the minimal functional units required for protein translocation. *J. Bacteriol.,* **184,** 5871-5879.

Lill, R., Cunningham, K., Brundage, L.A., Ito, K., Oliver, D. & Wickner, W. (1989). SecA protein hydrolyzes ATP and is an essential component of the protein translocation ATPase of *Escherichia coli*. *EMBO J.,* **8,** 961-966.

Luirink, J., High, S., Wood, H., Giner, A., Tollervey, D. & Dobberstein, B. (1992). Signal-sequence recognition by an *Escherichia coli* ribonucleoprotein complex. *Nature,* **359,** 741-743.

Ma, X. & Cline, K. (2000). Precursors bind to specific sites on thylakoid membranes prior to transport on the delta pH protein translocation system. *J. Biol. Chem.,* **275,** 10016-10022.

Madigan M.T., Martinko J.M., & Parker J. (2003). *Brock Biology of Microorganisms* -Pearson Education, Inc, 55-101

Matsuyama, S., Kimura, E. & Mizushima, S. (1990). Complementation of two overlapping fragments of SecA, a protein translocation ATPase of *Escherichia coli*, allows ATP binding to its amino-terminal region. *J. Biol. Chem.,* **265,** 8760-8765.

Meijer, W.J., de Jong, A., Bea, G., Wisman, A., Tjalsma, H., Venema, G., Bron, S. & van Dijl, J.M. (1995). The endogenous *Bacillus subtilis (natto)* plasmids pTA1015 and pTA1040 contain signal peptidase-encoding genes: identification of a new structural module on cryptic plasmids. *Mol. Microbiol.,* **17,** 621-631.

Merchante, R., Pooley, H.M. & Karamata, D. (1995). A periplasm in *Bacillus subtilis*. *J. Bacteriol.,* **177,** 6176-6183.

Mori, H. & Cline, K. (2002). A twin arginine signal peptide and the pH gradient trigger reversible assembly of the thylakoid [Delta]pH/Tat translocase. *J. Cell Biol.,* **157,** 205-210.

Mori, H., Summer, E.J. & Cline, K. (2001). Chloroplast TatC plays a direct role in thylakoid (Delta)pH-dependent protein transport. *FEBS Lett.,* **501,** 65-68.

Müller, J., Walter, F., van Dijl, J.M. & Behnke, D. (1992). 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,** 89-96.

Müller, J.P., An, Z., Merad, T., Hancock, I.C. & Harwood, C.R. (1997). Influence of *Bacillus subtilis* phoR on cell wall

anionic polymers. *Microbiology,* **143,** 947-956.

Müller, J.P., Ozegowski, J., Vettermann, S., Swaving, J., van Wely, K.H. & Driessen, A.J. (2000). Interaction of *Bacillus subtilis* CsaA with SecA and precursor proteins. *Biochem. J.,* **348 Pt 2,** 367-373.

Müller, J.P. & Wagner, M. (1999). Localisation of the cell wall-associated phosphodiesterase PhoD of *Bacillus subtilis*. *FEMS Microbiol. Lett.,* **180,** 287-296.

Muren, E.M., Suciu, D., Topping, T.B., Kumamoto, C.A. & Randall, L.L. (1999). Mutational alterations in the homotetrameric chaperone SecB that implicate the structure as dimer of dimers. *J. Biol. Chem.,* **274,** 19397-19402.

Nikaido, H. & Hall, J.A. (1998). Overview of bacterial ABC transporters. *Methods Enzymol.,* **292,** 3-20.

Niviere, V., Wong, S.L. & Voordouw, G. (1992). Site-directed mutagenesis of the hydrogenase signal peptide consensus box prevents export of a beta-lactamase fusion protein. *J. Gen. Microbiol.,* **138 (Pt 10),** 2173-2183.

Nossal, N.G. & Heppel, L.A. (1966). The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. *J. Biol. Chem.,* **241,** 3055-3062.

Ochsner, U.A., Snyder, A., Vasil, A.I. & Vasil, M.L. (2002). Effects of the twin-arginine translocase on secretion of virulence factors, stress response, and pathogenesis. *Proc. Natl. Acad. Sci. U. S. A,* **99,** 8312-8317.

Oguro, A., Kakeshita, H., Takamatsu, H., Nakamura, K. & Yamane, K. (1996). The effect of Srb, a homologue of the mammalian SRP receptor alpha-subunit, on *Bacillus subtilis* growth and protein translocation. *Gene,* **172,** 17-24.

Oliver, D.B., Cabelli, R.J., Dolan, K.M. & Jarosik, G.P. (1990). Azide-resistant mutants of *Escherichia coli* alter the SecA protein, an azide-sensitive component of the protein export machinery. *Proc. Natl. Acad. Sci. U. S. A,* **87,** 8227-8231.

Palmer, T. & Berks, B.C. (2003). Moving folded proteins across the bacterial cell membrane. *Microbiology,* **149,** 547-556.

Pandey, A., Szakacs, G., Soccol, C.R., Rodriguez-Leon, J.A. & Soccol, V.T. (2001). Production, purification and properties of microbial phytases. *Bioresour. Technol.,* **77,** 203-214.

Pohlschröder, M., Dilks, K., Hand, N.J. & Wesley, R.R. (2004). Translocation of proteins across archaeal cytoplasmic membranes. *FEMS Microbiol. Rev.,* **28,** 3-24.

Pooley, H.M., Merchante, R. & Karamata, D. (1996). Overall protein content and induced enzyme components of the periplasm of *Bacillus subtilis*. *Microb. Drug Resist.,* **2,** 9-15.

Pop, O., Westermann, M., Gerlach, R. & Müller, J. (2004). Dual localisation of homo-multimeric TatA elucidates its function in Tat-dependent mediated targeting. *submitted to Journal of Molecular Biology,* **JMB-D-04-00516** .

Pop, O., Martin, U., Abel, C. & Müller, J.P. (2002). 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,** 3268-3273.

Pop, O.I., Westermann, M., Volkmer-Engert, R., Schulz, D., Lemke, C., Schreiber, S., Gerlach, R., Wetzker, R. & Müller, J.P. (2003). Sequence-specific binding of prePhoD to soluble $TatA_d$ indicates protein-mediated targeting of the Tat export in *Bacillus subtilis*. *J. Biol. Chem.,* **278,** 38428-38436.

Porcelli, I., de Leeuw, E., Wallis, R., van den Brink-van der Laan, de Kruijff, B., Wallace, B.A., Palmer, T. & Berks, B.C. (2002). Characterization and membrane assembly of the TatA component of the *Escherichia coli* twin-arginine protein transport system. *Biochemistry,* **41,** 13690-13697.

Poritz, M.A., Bernstein, H.D., Strub, K., Zopf, D., Wilhelm, H. & Walter, P. (1990). An *E. coli* ribonucleoprotein containing 4.5S RNA resembles mammalian signal recognition particle. *Science,* **250,** 1111-1117.

Prinz, A., Behrens, C., Rapoport, T.A., Hartmann, E. & Kalies, K.U. (2000). Evolutionarily conserved binding of ribosomes to the translocation channel via the large ribosomal RNA. *EMBO J.,* **19,** 1900-1906.

Robinson, C. & Bolhuis, A. (2001). Protein targeting by the twin-arginine translocation pathway. *Nat. Rev. Mol. Cell Biol.,* **2,** 350-356.

Robinson, C. & Klosgen, R.B. (1994). Targeting of proteins into and across the thylakoid membrane-a multitude of mechanisms. *Plant Mol. Biol.,* **26,** 15-24.

Rose, R.W., Brüser, T., Kissinger, J.C. & Pohlschröder, M. (2002). Adaptation of protein secretion to extremely high-salt

conditions by extensive use of the twin-arginine translocation pathway. *Mol. Microbiol.,* **45,** 943-950.

Samuelson, J.C., Chen, M., Jiang, F., Moller, I., Wiedmann, M., Kuhn, A., Phillips, G.J. & Dalbey, R.E. (2000). YidC mediates membrane protein insertion in bacteria. *Nature,* **406,** 637-641.

Santini, C.L., Ize, B., Chanal, A., Müller, M., Giordano, G. & Wu, L.F. (1998). A novel sec-independent periplasmic protein translocation pathway in *Escherichia coli*. *EMBO J.,* **17,** 101-112.

Sargent, F., Bogsch, E.G., Stanley, N.R., Wexler, M., Robinson, C., Berks, B.C. & Palmer, T. (1998). Overlapping functions of components of a bacterial Sec-independent protein export pathway. *EMBO J.,* **17,** 3640-3650.

Sargent, F., Gohlke, U., de Leeuw, E., Stanley, N.R., Palmer, T., Saibil, H.R. & Berks, B.C. (2001). Purified components of the *Escherichia coli* Tat protein transport system form a double-layered ring structure. *Eur. J. Biochem.,* **268,** 3361-3367.

Saurin, W. & Dassa, E. (1994). Sequence relationships between integral inner membrane proteins of binding protein-dependent transport systems: evolution by recurrent gene duplications. *Protein Sci.,* **3,** 325-344.

Schiebel, E., Driessen, A.J., Hartl, F.U. & Wickner, W. (1991). Delta mu H+ and ATP function at different steps of the catalytic cycle of preprotein translocase. *Cell,* **64,** 927-939.

Scotti, P.A., Urbanus, M.L., Brunner, J., de Gier, J.W., von Heijne, G., van Der, D.C., Driessen, A.J., Oudega, B. & Luirink, J. (2000). YidC, the *Escherichia coli* homologue of mitochondrial Oxa1p, is a component of the Sec translocase. *EMBO J.,* **19,** 542-549.

Settles, A.M. & Martienssen, R. (1998). Old and new pathways of protein export in chloroplasts and bacteria. *Trends Cell Biol.,* **8,** 494-501.

Settles, A.M., Yonetani, A., Baron, A., Bush, D.R., Cline, K. & Martienssen, R. (1997). Sec-independent protein translocation by the maize Hcf106 protein. *Science,* **278,** 1467-1470.

Shiozuka, K., Tani, K., Mizushima, S. & Tokuda, H. (1990). The proton motive force lowers the level of ATP required for the in vitro translocation of a secretory protein in *Escherichia coli. J. Biol. Chem.,* **265,** 18843-18847.

Simonen, M. & Palva, I. (1993). Protein secretion in *Bacillus species*. *Microbiol. Rev.,* **57,** 109-137.

Smeekens, S., Weisbeek, P. & Robinson, C. (1990). Protein transport into and within chloroplasts. *Trends Biochem. Sci.,* **15,** 73-76.

Stanley, N.R., Findlay, K., Berks, B.C. & Palmer, T. (2001). *Escherichia coli* strains blocked in Tat-dependent protein export exhibit pleiotropic defects in the cell envelope. *J. Bacteriol.,* **183,** 139-144.

Stanley, N.R., Palmer, T. & Berks, B.C. (2000). The twin arginine consensus motif of Tat signal peptides is involved in Sec-independent protein targeting in *Escherichia coli*. *J. Biol. Chem.,* **275,** 11591-11596.

Stanley, N.R., Sargent, F., Buchanan, G., Shi, J., Stewart, V., Palmer, T. & Berks, B.C. (2002). Behaviour of topological marker proteins targeted to the Tat protein transport pathway. *Mol. Microbiol.,* **43,** 1005-1021.

Swaving, J., van Wely, K.H. & Driessen, A.J. (1999). Preprotein translocation by a hybrid translocase composed of *Escherichia coli* and *Bacillus subtilis* subunits. *J. Bacteriol.,* **181,** 7021-7027.

Takemura, K., Mizuno, M. & Kobayashi, Y. (1996). A *Bacillus subtilis* gene cluster similar to the *Escherichia coli* phosphate-specific transport (*pst*) operon: evidence for a tandemly arranged *pstB* gene. *Microbiology,* **142 (Pt 8),** 2017-2020.

Tam, R. & Saier, M.H., Jr. (1993). Structural, functional, and evolutionary relationships among extracellular solute-binding receptors of bacteria. *Microbiol. Rev.,* **57,** 320-346.

Tani, K., Shiozuka, K., Tokuda, H. & Mizushima, S. (1989). In vitro analysis of the process of translocation of OmpA across the *Escherichia coli* cytoplasmic membrane. A translocation intermediate accumulates transiently in the absence of the proton motive force. *J. Biol. Chem.,* **264,** 18582-18588.

Tani, K., Tokuda, H. & Mizushima, S. (1990). Translocation of ProOmpA possessing an intramolecular disulfide bridge into membrane vesicles of *Escherichia coli*. Effect of membrane energization. *J. Biol. Chem.,* **265,** 17341-17347.

Teter, S.A. & Theg, S.M. (1998). Energy-transducing thylakoid membranes remain highly impermeable to ions during protein translocation. *Proc. Natl. Acad. Sci. U. S. A,* **95,** 1590-1594.

Thanassi, D.G. & Hultgren, S.J. (2000). Multiple pathways allow protein secretion across the bacterial outer membrane. *Curr. Opin. Cell Biol.,* **12,** 420-430.

Thomas, J.D., Daniel, R.A., Errington, J. & Robinson, C. (2001). Export of active green fluorescent protein to the periplasm by the twin-arginine translocase (Tat) pathway in *Escherichia coli. Mol. Microbiol.,* **39,** 47-53.

Tjalsma, H., Bolhuis, A., Jongbloed, J.D., Bron, S. & van Dijl, J.M. (2000). Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome. *Microbiol. Mol. Biol. Rev.,* **64,** 515-547.

Tjalsma, H., Bolhuis, A., van Roosmalen, M.L., Wiegert, T., Schumann, W., Broekhuizen, C.P., Quax, W.J., Venema, G., Bron, S. & van Dijl, J.M. (1998). Functional analysis of the secretory precursor processing machinery of *Bacillus subtilis:* identification of a eubacterial homolog of archaeal and eukaryotic signal peptidases. *Genes Dev.,* **12,** 2318-2331.

Tjalsma, H., Noback, M.A., Bron, S., Venema, G., Yamane, K. & van Dijl, J.M. (1997). *Bacillus subtilis* contains four closely related type I signal peptidases with overlapping substrate specificities. Constitutive and temporally controlled expression of different sip genes. *J. Biol. Chem.,* **272,** 25983-25992.

Tjalsma, H., van den, D.J., Meijer, W.J., Venema, G., Bron, S. & van Dijl, J.M. (1999). The plasmid-encoded signal peptidase SipP can functionally replace the major signal peptidases SipS and SipT of *Bacillus subtilis. J. Bacteriol.,* **181,** 2448-2454.

Ullah, A.H., Sethumadhavan, K., Mullaney, E.J., Ziegelhoffer, T. & Austin-Phillips, S. (2002). Cloned and expressed fungal *phyA* gene in alfalfa produces a stable phytase. *Biochem. Biophys. Res. Commun.,* **290,** 1343-1348.

Valent, Q.A., de Gier, J.W., von Heijne, G., Kendall, D.A., Hagen-Jongman, C.M., Oudega, B. & Luirink, J. (1997). Nascent membrane and presecretory proteins synthesized in *Escherichia coli* associate with signal recognition particle and trigger factor. *Mol. Microbiol.,* **25,** 53-64.

Valent, Q.A., Scotti, P.A., High, S., de Gier, J.W., von Heijne, G., Lentzen, G., Wintermeyer, W., Oudega, B. & Luirink, J. (1998). The *Escherichia coli* SRP and SecB targeting pathways converge at the translocon. *EMBO J.,* **17,** 2504-2512.

van Dalen, A., Killian, A. & de Kruijff, B. (1999). Delta psi stimulates membrane translocation of the C-terminal part of a signal sequence. *J. Biol. Chem.,* **274,** 19913-19918.

van der Wolk J., Klose, M., Breukink, E., Demel, R.A., de Kruijff, B., Freudl, R. & Driessen, A.J. (1993). Characterization of a *Bacillus subtilis* SecA mutant protein deficient in translocation ATPase and release from the membrane. *Mol. Microbiol.,* **8,** 31-42.

van der Wolk, J.P., de Wit, J.G. & Driessen, A.J. (1997). The catalytic cycle of the *Escherichia coli* SecA ATPase comprises two distinct preprotein translocation events. *EMBO J.,* **16,** 7297-7304.

van Dijl, J.M., Braun, P.G., Robinson, C., Quax, W.J., Antelmann, H., Hecker, M., Müller, J., Tjalsma, H., Bron, S. & Jongbloed, J.D. (2002). Functional genomic analysis of the *Bacillus subtilis* Tat pathway for protein secretion. *J. Biotechnol.,* **98,** 243-254.

van Dijl, J.M., de Jong, A., Vehmaanpera, J., Venema, G. & Bron, S. (1992). Signal peptidase I of *Bacillus subtilis*: patterns of conserved amino acids in prokaryotic and eukaryotic type I signal peptidases. *EMBO J.,* **11,** 2819-2828.

Voelker, R. & Barkan, A. (1995). Two nuclear mutations disrupt distinct pathways for targeting proteins to the chloroplast thylakoid. *EMBO J.,* **14,** 3905-3914.

von Heijne, G. (1985). Signal sequences. The limits of variation. *J. Mol. Biol.,* **184,** 99-105.

von Heijne, G. (1986). Net N-C charge imbalance may be important for signal sequence function in bacteria. *J. Mol. Biol.,* **192,** 287-290.

von Heijne, G. (1998). Life and death of a signal peptide. *Nature,* **396,** 111, 113.

von Heijne, G., Steppuhn, J. & Herrmann, R.G. (1989). Domain structure of mitochondrial and chloroplast targeting peptides. *Eur. J. Biochem.,* **180,** 535-545.

Walker, M.B., Roy, L.M., Coleman, E., Voelker, R. & Barkan, A. (1999). The maize *tha4* gene functions in Sec-independent protein transport in chloroplasts and is related to *hcf106, tatA,* and *tatB*. *J. Cell Biol.,* **147,** 267-276.

Weiner, J.H., Bilous, P.T., Shaw, G.M., Lubitz, S.P., Frost, L., Thomas, G.H., Cole, J.A. & Turner, R.J. (1998). A novel

and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins. *Cell,* **93,** 93-101.

Wexler, M., Sargent, F., Jack, R.L., Stanley, N.R., Bogsch, E.G., Robinson, C., Berks, B.C. & Palmer, T. (2000). TatD is a cytoplasmic protein with DNase activity. No requirement for TatD family proteins in sec-independent protein export. *J. Biol. Chem.,* **275,** 16717-16722.

Wu, L.F., Ize, B., Chanal, A., Quentin, Y. & Fichant, G. (2000). Bacterial twin-arginine signal peptide-dependent protein translocation pathway: evolution and mechanism. *J. Mol. Microbiol. Biotechnol.,* **2,** 179-189.

Wu, X.C., Lee, W., Tran, L. & Wong, S.L. (1991). Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. *J. Bacteriol.,* **173,** 4952-4958.

Xu, Z., Knafels, J.D. & Yoshino, K. (2000). Crystal structure of the bacterial protein export chaperone SecB. *Nat. Struct. Biol.,* **7,** 1172-1177.

Yahr, T.L. & Wickner, W.T. (2001). Functional reconstitution of bacterial Tat translocation *in vitro*. *EMBO J.,* **20,** 2472-2479.

Yamada, H., Matsuyama, S., Tokuda, H. & Mizushima, S. (1989). A high concentration of SecA allows proton motive force-independent translocation of a model secretory protein into *Escherichia coli* membrane vesicles. *J. Biol. Chem.,* **264,** 18577-18581.

Yen, M.R., Tseng, Y.H., Nguyen, E.H., Wu, L.F. & Saier, M.H., Jr. (2002). Sequence and phylogenetic analyses of the twin-arginine targeting (Tat) protein export system. *Arch. Microbiol.,* **177,** 441-450.

Young, J. & Holland, I.B. (1999). ABC transporters: bacterial exporters-revisited five years on. *Biochim. Biophys. Acta,* **1461,** 177-200.

Zimmermann, R. & Wickner, W. (1983). Energetics and intermediates of the assembly of Protein OmpA into the outer membrane of *Escherichia coli. J. Biol. Chem.,* **258,** 3920-3925.

Selbständigkeitserklärung

Hiermit erkläre ich an Eides statt, daß ich due vorliegende Arbeit selbständig verfaßt und keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe.

Jena, 27 August 2004

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Current scientific interests :

Protein translocation in bacteria, particularly, Tat-dependent protein export in *Bacillus subtilis*.

Tat export of heterologous proteins - biotechnological applications.

Publications :

- **O, Pop,** Martin, U., Abel, C. and J. Müller. 2002. The twin-arginine signal peptide of PhoD and the $TatA_d/C_d$ proteins of *Bacillus subtilis* form an autonomous Tat translocation system. J. Biol. Chem. 277(5): 3268-73.

- **O. Pop**, M. Westermann, R. Volkmer-Engert, Daniela Schulz, C. Lemke, S. Schreiber, R. Gerlach, R. Wetzker, J. Müller. 2003. Sequence specific binding of prePhoD to soluble TatAd indicates protein mediated targeting of the Tat export in *Bacillus subtilis*. J Biol Chem. 278(40): 38428-36.

- R. Gerlach, **O. Pop**, J. Müller. 2004 Tat-dependent export of *E. coli* phytase AppA by using the PhoD-specific transport system of *Bacillus subtilis*, Journal of Basic Microbiology. 44 (2004) 5, 351–359.

- **O. Pop**, M. Westerman, J. Müller**.** Homo-multimerisation of *B. subtilis* $TatA_d$ in the cytosol and membrane elucidates its function in Tat-dependent protein targeting. submitted for publication to J. of Molecular Biology; ref. code: JMB-D-04-00516 from July 2004.

- S. Schreiber, R. Stenger, **O. Pop**, J. P. Müller. The receptor function of $TatC_d$ in *B. subtilis* – in preparation.

- **O. Pop**, S. Schreiber, J. P. Müller, J. M., van Dijl, O. Kuipers, S. Bron, J. Jongbloed. The role of type I signal peptidases for secretion of proteins via the Tat pathway in *B. subtilis*. – in preparation.

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