Twin-Arginine Protein Translocation

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Abstract Twin-arginine protein translocation systems (Tat) translocate fully folded and co-factor-containing proteins across biological membranes. In this review, we focus on the Tat pathway of Gram-positive bacteria. The minimal Tat pathway is composed of two components, namely a TatA and TatC pair, which are often complemented with additional TatA-like proteins. We provide overviews of our current understanding of Tat pathway composition and mechanistic aspects related to Tat-dependent cargo protein translocation. This includes Tat pathway flexibility, requirements for the correct folding and incorporation of co-factors in cargo proteins and the functions of known cargo proteins. Tat pathways of several Gram-positive bacteria are discussed in detail, with emphasis on the Tat pathway of *Bacillus subtilis*. We discuss both shared and unique features of the different Gram-positive bacterial Tat pathways. Lastly, we highlight topics for future research on Tat, including the development of this protein transport pathway for the biotechnological secretion of high-value proteins and its potential applicability as an antimicrobial drug target in pathogens.

Abbreviations

Sec pathway	General Secretory pathway
Tat	Twin-arginine translocation
NMR	Nuclear magnetic resonance
Y2H	Yeast two-hybrid

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1 The Twin-Arginine Protein Translocation Pathway

The movement of substances across biological membranes is essential for the growth, replication and survival of all living cells. In order to translocate substances through these phospholipid bilayers a variety of diverse import and export systems have evolved. One class of compounds that need to be translocated across membranes are proteins. However, transporting proteins over a membrane is not enough to guarantee their proper function, as proteins require correct folding and often co-factors for full functionality. Consequently, there is an intimate relationship between the protein translocation process and protein folding. Protein translocation pathways ensure that substrates are folded either post-translocationally or pre-translocationally. In the case of the Sec pathway for protein secretion, the translocated proteins are folded after translocation (Tjalsma et al. 2000). In contrast, twin-arginine translocation (Tat) pathways are known specifically for the ability to move pre-folded and co-factor-containing proteins across membranes. Translocation of large globular and tightly folded proteins across a membrane is no small feat as the energy needed and the size of the membrane passage required is far greater than that needed for translocating a loosely folded polypeptide chain by the Sec pathway. Exactly how the Tat pathway is able to transport these globular proteins without breaking the cellular barrier or destroying transmembrane ion gradients (e.g. the proton-motive force) is perplexing and of great fundamental scientific interest.

The Tat pathway is evolutionarily conserved in all the kingdoms of life. It is present in 77 % of bacteria, in archaeal species and in the membranes of thylakoids in plants and cyanobacteria (Chaddock et al. 1995; Hutcheon and Bolhuis 2003; Simone et al. 2013). Remnants of the pathway have even been observed in sponges (Pett and Lavrov 2013a). The focus of this chapter is the Gram-positive bacterial

Tat pathway, where the folded proteins are moved from the cytoplasm into the membrane, cell wall or extracellular milieu. Tat systems have been most extensively studied in the Gram-negative bacterium *Escherichia coli*, but also in the Gram-positive *Bacillus* and *Corynebacterium* species, and in pea thylakoids [reviewed in (Palmer and Berks 2012; Goosens et al. 2014b; Patel et al. 2014)].

What has become evident from comparing the various Tat systems is that they are broadly conserved with a high degree of similarity between proteins and mechanisms. For this reason, this review builds on and refers to observations made in Tat systems from other species in addition to Gram-positive bacteria.

2 Tat-Dependent Cargo

The number of Tat-dependent cargo proteins ranges from over 100 in *Streptomyces* species, to only a few in *B. subtilis* and *Staphylococcus aureus* and none in, for example, *Lactobacillus* species (Schaerlaekens et al. 2001, 2004b; Widdick et al. 2006; Joshi et al. 2010; Yamada et al. 2007; Biswas et al. 2009; Goosens et al. 2013; Bolotin et al. 2001; Kleerebezem et al. 2003). These Tat-dependent cargo proteins include secreted proteins, lipoproteins, cell wall-associated proteins and proteins that form components of larger extracytoplasmic complexes on the membrane surface (Widdick et al. 2011; Keller et al. 2012; Monteferrante et al. 2012b; Goosens et al. 2013; Miethke et al. 2013).

Cargo may be destined for the Tat pathway for numerous reasons. Many Tat-dependent substrates are known to require complex co-factors for activity and these are incorporated into the protein in the cytoplasm prior to membrane translocation. Certain other proteins that bind divalent metal ions with affinity ranges lower down in the Irving Williams series, such as Mn, may use the Tat pathway to avoid competing ions with higher binding affinities, such as Zn (Tottey et al. 2008; Monteferrante et al. 2012b). Extremophiles and archaea may need to fold the proteins prior to secretion due to the harsh external milieus in which they live (Bolhuis 2002; Rose et al. 2002). Further, some Tat-destined proteins form multi-protein complexes that are translocated in a hitchhiker or piggyback manner (Rodrigue et al. 1999; Friedrich et al. 2000; Wu et al. 2000a).

Tat substrates have been implicated in a wide range of cellular functions and in the case of pathogenic bacteria they have been associated with virulence, antibiotic resistance and antibacterial compounds (McDonough et al. 2005; De Buck et al. 2008; Weatherspoon-Griffin et al. 2011). Notably, certain industrially relevant proteins are difficult to produce due to co-factor—or disulphide-bond requirements and in some organisms, such as *E. coli* and *Corynebacterium glutamicum*, the Tat pathway has been successfully used for export of these types of proteins, including the alkaline phosphatase PhoA, carbohydrate oxidase, antibody fragments and human tissue plasminogen activator (DeLisa et al. 2003; Kim et al. 2005; Bruser 2007; Ribnicky et al. 2007; Panahandeh et al. 2008; Maurer et al. 2009; Matos et al. 2013; Scheele et al. 2013). Also, the Tat system of Gram-positive bacteria has been

used to secrete small enzymes (De Keersmaeker et al. 2006; Kikuchi et al. 2006, 2008; Scheele et al. 2013). However, biotechnological applications have not yet taken full advantage of the potential of Gram-positive bacterial Tat systems. Although the ability to secrete complex cargo directly into the fermentation broth is enticing, production has been hampered by low yields possibly due to yet unidentified quality control requirements.

In addition to a folded state, Tat cargo proteins also have a unique 'twin-arginine' signal peptide signature. In principle, the N-terminal signal peptides of Tat substrates have a similar tripartite structure as the N-terminal signal peptides of Sec substrates; they are made up of a polar N-region, a hydrophobic H-region in the middle and a polar C-region next to the signal peptidase cleavage site (Tjalsma et al. 2004). However, a distinguishing feature of Tat signal peptides is the presence of two-arginine residues in the N-terminal region that form part of the consensus motif SRRxFLK where x is a polar amino acid (Berks et al. 2000; Stanley et al. 2000; Tjalsma et al. 2004). Compared to Sec signal peptides, the Tat signal peptides also tend to be longer, their N-terminal region is more positively charged (Tjalsma et al. 2000), and their H-region is slightly less hydrophobic (Cristobal et al. 1999). While the twin-arginine residues are conserved, mutation studies have shown that changes in the motif result in a variation of phenotypes, ranging from completely blocked to slowed down translocation of the cargo (Chaddock et al. 1995; DeLisa et al. 2002; Mendel et al. 2008).

Several Tat prediction software programs are available, including TatFind and PRED-TAT (Rose et al. 2002; Bendtsen et al. 2005; Bagos et al. 2010). However, although the signal peptide region is important for translocation, amino acid sequence motifs and patterns are not always reliable predictors, especially since Tat cargo has also been associated with piggyback or hitchhiker mechanisms in organisms like *E. coli*. Here, proteins without a signal peptide of their own bind to the Tat substrate possessing the Tat signal peptide and are exported as a complex by the machinery (Rodrigue et al. 1999; Wu et al. 2000a). Further, Sec–Tat substrate overlap has been shown to occur and sequence ambiguity can lead to false-positive identifications (Tjalsma et al. 2000; Jongbloed et al. 2002; Kouwen et al. 2009; Keller et al. 2012; Goosens et al. 2013). Therefore, although bioinformatic tools are invaluable for lead finding, potential Tat substrates need to be confirmed experimentally.

3 Tat Components and Processes

Genes for the Tat system are observed in 77 % of sequenced microbial genomes. The respective organisms typically contain a TatA and TatC pair (Fig. 1), which are often encoded by genes found within a single operon (Wu et al. 2000b; Yen et al. 2002; Simone et al. 2013). Such *tatA-tatC* operons are occasionally located in the vicinity of genes for Tat cargo proteins (Jongbloed et al. 2000, 2004; Biswas et al. 2009).

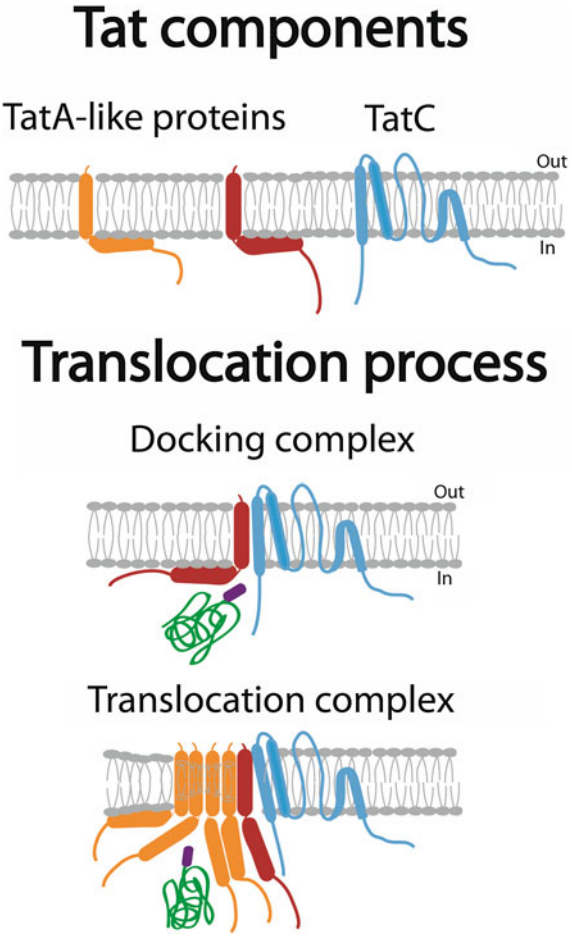

Fig. 1 Tat components and interactions of Tat complexes with their cargo. Tat protein translocases are essentially composed of two types of subunits, namely TatA-like proteins and TatC, which have distinct membrane topologies (*upper panel*). TatA-like proteins (indicated in *orange* and *red*) have an N_{out}–C_{in} topology and consist of two helical domains, one of which spans the membrane while the other one (amphipathic) is exposed to the cytoplasm. TatC is an integral membrane protein with six transmembrane domains and an N_{in}–C_{in} topology. The translocation process is believed to involve two major Tat complexes, namely a docking complex (*middle panel*) and a translocation complex (*lower panel*). The docking complex is composed of TatC and a TatA-like protein (*red*). In some organisms, such as *E. coli*, the latter TatA-like protein has a specialized docking function in which case it is referred to as TatB. Docking of cargo proteins (*green*) involves interaction of the twin-arginine signal peptide (*purple*) with TatC. Once the cargo protein has docked, a large number of TatA-like proteins (*orange*) are recruited to the translocation site, thereby forming the translocation complex

3.1 TatA, TatA-like and TatC Proteins

TatA and TatA-like proteins are small membrane proteins with an N-out C-in topology. They have a small N-terminal extracytoplasmic domain, a single transmembrane helix and an amphipathic helix that lies on the membrane surface or is partially embedded in the membrane on the cytoplasmic side (Fig. 1) (Lange et al. 2007; Hu et al. 2010; Walther et al. 2010). In numerous species, *tatA* genes have undergone multiple duplication events forming the *tatA*-like genes, and these are found in the main *tat* operon or elsewhere in the genome (Wu et al. 2000b; Yen et al. 2002). Some TatA-like proteins can simply be duplicate TatA proteins, such as TatE or TatA2 in *Corynebacterium* species, *Streptomyces coelicolor*, *Salmonella enterica* and *E. coli* (Sargent et al. 1999; Ikeda and Nakagawa 2003; Kalinowski et al. 2003; Nishio et al. 2003; Baglieri et al. 2012). In *B. subtilis* and *C. glutamicum*, the expression of the duplicate proteins TatAc and TatE, respectively, has been shown to assist the activity of the primary TatA protein (Kikuchi et al. 2006; Goosens et al. 2015). However, in some instances, duplicate TatA-like proteins (often referred to as TatB) have further undergone sequence divergence and functional specialization. This seems to have occurred independently numerous times. TatA-like proteins with divergent functions, such as TatB have been studied in *E. coli*, *Streptomyces* species and in the thylakoids of plant chloroplasts (where they are referred to as Hcf106). In these organisms, both TatA and TatB are needed for full translocation activity (Sargent et al. 1999; Mori and Cline 2001; Schaerlaekens et al. 2001; De Keersmaeker et al. 2005a). Intriguingly, certain TatA proteins (e.g. the TatA proteins from *B. subtilis*) are able to functionally replace both *E. coli* TatA and TatB in the TatA-B-C system (Barnett et al. 2008). This is even more remarkable since TatA shares only 20 % sequence similarity with TatB although the *E. coli* TatA and TatB are structurally the same (Hicks et al. 2003; Lange et al. 2007; Hu et al. 2010; Walther et al. 2010). Nevertheless, minor changes to *E. coli* TatA allow it to complement for TatB (Blaudeck et al. 2005; Barrett et al. 2007). Therefore, no clear definition exists that allows one to properly differentiate TatA from TatB, and in many sequence annotations where a second TatA-like protein has been defined as TatB, this annotation may be erroneous. Importantly, over 50 % of the Tat-encoding genomes sequenced to date specify only a TatA-TatC component (Simone et al. 2013). Accordingly, the concept that a core Tat system composed of a core TatA-TatC pair with various assistant TatA-like proteins is gaining more support. This view is backed by studies in *B. subtilis,* where a third TatA protein (TatAc) was shown to assist TatAy in protein translocation, and in *Helicobacter pylori, Campylobacter jejuni,* and *C. glutamicum* where a second TatA-like protein was shown to be essential only under some conditions of Tat-dependent protein translocation but not all (Kikuchi et al. 2006; Benoit and Maier 2014; Liu et al. 2014; Goosens et al. 2015; Oertel et al. 2015). Thus, in many organisms, it is not necessary to have a TatB as is found in *E. coli* and thylakoids, which have TatA, TatB and TatC subunits, each with their own function.

Unlike TatA, TatC proteins are large integral membrane proteins with six membrane-spanning domains (Fig. 1). TatC is central to the Tat pathway as is evident by the multiple protein interactions in which this protein is involved. The TatC transmembrane regions interact with other TatC proteins (Buchanan et al. 2002; Punginelli et al. 2007), but also with the cargo protein (Behrendt et al. 2004; Frobel et al. 2012). Both the cargo protein and TatA(-like) proteins (e.g. *E. coli* TatB) have been shown to interact with the membrane-embedded region of TatC and its conserved cytoplasmic loop (Buchanan et al. 2002; McDevitt et al. 2006; Schreiber et al. 2006; Holzapfel et al. 2007; Strauch and Georgiou 2007; Frobel et al. 2011; Zoufaly et al. 2012; Ma and Cline 2013; Blümmel et al. 2015). Although the extracytoplasmic loops do not share high sequence similarity, random mutagenesis studies showed that the conserved secondary structure is vital (Strauch and Georgiou 2007; Kneuper et al. 2012). Further, the C-terminal tail of TatC has been shown to be essential for successful Tat-dependent protein translocation in *B. subtilis* (Eijlander et al. 2009b).

3.2 Translocation Process

The mechanism of translocating cargo proteins via the Tat pathway has not been concretely defined. However, what is currently agreed upon is that there are at least two Tat complexes and at least three steps to the process (Cline and Mori 2001). The first step, the formation of a docking complex, is initiated when cargo proteins interact with a TatC and TatA(-like) complex at the membrane (Fig. 1) (Bolhuis et al. 2001; Whitaker et al. 2012). In *E. coli* and thylakoids, this is where the (TatA-like) TatB proteins perform their specialized functions. The signal peptide of the cargo protein directly interacts with TatC in the docking complex and is inserted into the membrane after proofreading (Cline and Mori 2001; Alami et al. 2003; Papish et al. 2003; Robinson et al. 2011; Frobel et al. 2012). In the second step, the translocation complex is formed. This occurs once the cargo protein has 'docked', and a large number of TatA proteins are recruited to the translocation site in a manner that is dependent on the proton-motive force, thereby forming the translocation complex (Fig. 1) (Mori and Cline 2002; Alami et al. 2003; Dabney-Smith et al. 2006). The final step is the translocation itself, but how this occurs is not clear. A recent study by Blümmel et al. (2015) indicates that TatBC oligomers can assemble into closed intramembrane substrate-binding cavities, where TatB monomers would form dome-like structures that are surrounded by TatC monomers. These TatBC complexes would bind the N-termini of TatA protomers facilitating contacts with TatB and membrane-inserted cargo proteins.

There are two popular translocation models, which are both speculative: the pore and the membrane-weakening models [reviewed in (Berks 2015; Patel et al. 2014)]. Both models are supported by data and, depending on their interpretation, some results are used to reinforce either. The pore model was conceived based upon single particle electron microscopy studies that showed TatA and TatA-like proteins

self-assemble to form cup-like structures or pores with varying diameters (Gohlke et al. 2005; Oates et al. 2005; Beck et al. 2013). Consistent with this model, the *E. coli* TatA complexes have been shown to form ladders of multiple sizes in native gels (Gohlke et al. 2005; Oates et al. 2005; Beck et al. 2013) giving rise to the hypothesis that a pore made up of TatA proteins adapts its diameter to the globular cargo by varying the amount of TatA components (Fig. 1). The theory goes that TatA-cargo protein interactions within the cup-like TatA structure allow for translocation by the folding-in (trap-door mechanism) or twisting (iris mechanism) of the amphipathic helix of TatA up into the membrane (Berks et al. 2000; Gouffi et al. 2004; Gohlke et al. 2005; Walther et al. 2013). In contrast, the membrane-weakening model predicts that the TatA complexes observed by electron microscopy do not form a pore, but that the aggregates of TatA proteins form destabilised membrane regions that permit cargo passage (Bruser and Sanders 2003). Data that support this include the length of the TatA transmembrane region, which is too short to span the lipid bilayer (Rodriguez et al. 2013). Also, the *B. subtilis* TatAd complexes observed by single particle electron microscopy were structurally too small to represent pores that can accommodate a substrate (Beck et al. 2013). Other evidence not consistent with the pore model is that the large size variation and laddering effect seen in *E. coli* TatA complexes have not been convincingly observed for other TatA and TatA-like proteins (Baglieri et al. 2012; Monteferrante et al. 2012a; Walther et al. 2013). Moreover, NMR studies suggest that, because the TatA amphipathic helix is not flexible (Walther et al. 2010), the movement of the amphipathic helix into the membrane would have to be sudden and, most likely, disruptive. Another piece of evidence that seems to support the membrane-weakening model is the involvement of the phage shock protein PspA in Tat-dependent protein transport. For example, PspA has been implicated in the stabilization of the membrane under stress conditions (Darwin 2005; Vrancken et al. 2008) and in suppressing proton leakage (Kobayashi et al. 2007). PspA binds both *E. coli* TatA (Mehner et al. 2012) and phospholipids (Kobayashi et al. 2007) forming scaffold-like structures in the membrane (Standar et al. 2008). The possible involvement of PspA in Tat-dependent protein transport suggests that the translocation event induces stress. Importantly, expression of PspA has been shown to improve Tat-dependent protein secretion in both *S. lividans* and *E. coli* and, hence, its role in suppressing proton leakage that may occur in the Tat export process may be conserved in both Gram-positive and Gram-negative bacteria (DeLisa et al. 2004; Vrancken et al. 2007).

4 Cargo protein Processing and Quality Control

A defining feature of Tat is its inability to translocate incorrectly folded proteins. Although a small amount of flexibility has been described for small synthetic peptides (Hynds et al. 1998; Richter et al. 2007; Rocco et al. 2012), the system is known to have strict folding requirements regarding its native substrates. If a

protein is not sufficiently folded or does not have its co-factors inserted, the translocation is prevented and the protein is degraded (Jack et al. 2004; Kolkman et al. 2008; Matos et al. 2008). Protein folding, co-factor insertion and quality control prior to Tat complex interactions are therefore considered important for cargo translocation. The quality control step has been shown to occur at the docking complex (Buchanan et al. 2008; Panahandeh et al. 2008; Frobel et al. 2012; Rocco et al. 2012). Further evidence of quality control prior to docking complex formation has been clearly described in *E. coli*, where a number of substrate-specific chaperones have been identified (Oresnik et al. 2001; Jack et al. 2004). However, homologous chaperones have not been characterized in other organisms, and it remains unclear which factors may be involved in pre-translocational protein folding and quality control prior to docking-complex interactions in Gram-positive bacteria. Nonetheless, in *B. subtilis* the Tat-dependent QcrA protein was shown to undergo quality control at two subcellular locations, in the cytoplasm and membrane, and on two different pre-QcrA intermediates. While neither of the pre-QcrA proteins were translocated, pre-QcrA quality control occurred both at the membrane, where the Tat-docking complex is shown to perform proofreading functions, and in the cytoplasm via an as-yet-unknown mechanism (Goosens et al. 2014a). Quantitative proteomic studies have implicated the membrane-targeting chaperone DnaJ and co-factor assembly protein SufS with the Tat pathway in *B. subtilis* (Albrecht et al. 2011; Goosens et al. 2013; Castanie-Cornet et al. 2014). However, functional studies are still required to confirm these links. What has clearly been shown is a direct interaction between the *B. subtilis* TatAd protein and the soluble chemoreceptor HemAT, and between TatAd and the putative pentose transporter CsbC within the membrane. Not only do HemAT and CsbC individually interact with TatAd, but they are also essential for the secretion of the TatAd-specific cargo protein PhoD (Monteferrante et al. 2013). Exactly what the roles of these proteins in the PhoD quality control and Tat-dependent export pathway are remained unclear.

It has been suggested that the Tat-associated quality control is linked to a pool of cytoplasmic TatA (Pop et al. 2003; De Keersmaeker et al. 2005a, b; Schreiber et al. 2006; Westermann et al. 2006; De Keersmaeker et al. 2007; Frielingsdorf et al. 2008). In this model, the cytoplasmic TatA of *B. subtilis*, *Streptomyces lividans* or thylakoids interacts with cargo prior to translocation and guides it to the docking complex in the membrane. Also, overexpressed TatA molecules have been observed to form distinct tubes in the cytoplasm (Berthelmann et al. 2008). However, since the cytoplasmic TatA-cargo protein interaction has only been observed under induced circumstances and the presence of TatA in the cytoplasm has not been shown consistently under all experimental methodologies, future studies will need to verify the possible quality control function of cytoplasmic TatA (Wexler et al. 2000; Barnett et al. 2008; Leake et al. 2008; Barnett et al. 2009; Ridder et al. 2009).

Other steps in the quality control of Tat-dependent cargo proteins occur during or shortly after membrane translocation. In particular, these include the removal of

the twin-arginine signal peptide by signal peptidases, which has been widely observed (Jongbloed et al. 2004; Luke et al. 2009; Widdick et al. 2011; Goosens et al. 2013). In addition to signal peptidases, extracytoplasmic proteases have also been shown to effect the Tat-dependent cargo protein EfeB in *B. subtilis.* EfeB directly interacts with and requires the cell wall-bound protease WprA for processing (Monteferrante et al. 2013), but is degraded by extracellular proteases in the growth medium (Krishnappa et al. 2012). EfeB forms part of a membrane-bound complex with the EfeU and EfeO proteins, and the association with extracellular proteases is an indication of a possible assembly proofreading mechanism (Monteferrante et al. 2013).

5 Flexibility of the Tat System Between Different Organisms

Expression of *B. subtilis* Tat components in *E. coli* leads to Tat-dependent export and functionally replaces the Tat pathway in *E. coli* (Barnett et al. 2008, 2009; Monteferrante et al. 2012a; van der Ploeg et al. 2012). Single *B. subtilis tatA* genes are able to functionally replace both the *E. coli* TatA and TatB proteins (Barnett et al. 2008; Monteferrante et al. 2012a; Beck et al. 2013). However, when similar interspecies experiments were performed in a *B. subtilis* background, complementation was not that simple. Although the Tat systems from *Bacillus cereus* and *Listeria monocytogenes* were functional in *B. subtilis,* the Tat system from *S. aureus* was barely active in *B. subtilis* (Barnett et al. 2008, 2009; van der Ploeg et al. 2011a, 2012). These differences suggest that the Tat pathway alone is not enough to ensure complete translocation and possible chaperone or quality control mechanisms in *E. coli* and *S. aureus* do not match up with those in *B. subtilis*.

Interspecies variations have also been observed with regard to the export of cargo proteins. The addition of a Tat signal peptide to a cargo protein has allowed for heterologous Tat-dependent translocation in many cases, but this does not always equally prove successful in all genetic backgrounds and with all cargo (Thiemann et al. 2006; Meissner et al. 2007; Kikuchi et al. 2008; Widdick et al. 2008; Scheele et al. 2013). Environmental salt (i.e. NaCl) conditions also affected the Tat-dependent export of cargo that was heterologously expressed in *B. subtilis* suggesting external conditions and media may affect Tat-dependent secretion (van der Ploeg et al. 2011a, 2012). However, the environmental salt concentration did not significantly affect the amount of Tat-dependently translocated QcrA in *B. subtilis* (Goosens et al. 2015). The influence of salt on the translocation of other cargo is therefore not necessarily an intrinsic Tat effect.

6 Monoderm Gram-Positive Bacterial Tat Systems

Bacterial phyla are broadly defined by the physical properties of the outer layer of their cell structure. In most cases, bacteria are classified by the outcome of so-called Gram staining. The Gram staining procedure was developed in the late nineteenth century and works by interaction of the stain with the peptidoglycan of the cell wall. The stain is either retained by the peptidoglycan, giving cells a purple colour, or washed out. Accordingly, this gave rise to the common nomenclature of Gram-positive bacteria where the stain is retained, or Gram-negative bacteria where the stain is not retained. The Gram-positive bacteria have a single plasma membrane surrounded by a thick outer cell wall composed of peptidoglycan (i.e. a monoderm cell envelope). In contrast, Gram-negative bacteria have a double membrane with a peptidoglycan layer in between (i.e. a diderm cell envelope). Although the Gram-staining-based nomenclature is generally a good indicator of the physical properties of the outer layer of cells, it can be ambiguous. Some bacteria stain positive, but do in fact have a diderm cell envelope structure. Such bacteria include mycobacteria, corynebacteria, rhodococci and nocardiae. The Tat systems of these diderm Gram-positive bacteria will not be detailed here, as they have been reviewed previously (Goosens et al. 2014b) and not much new information has become available since this review was published.

6.1 Bacillus subtilis

Bacillus subtilis is the major Gram-positive model organism with an extensive array of genetic tools, including in-depth genomic, transcriptomic and proteomic insights (Kunst et al. 1997; Tjalsma et al. 2000; Eymann et al. 2004; Wolff et al. 2007; Buescher et al. 2012; Nicolas et al. 2012). A number of *Bacillus* species are biotechnologically relevant. *B. subtilis, Bacillus licheniformis* and *Bacillus amyloliquefaciens*, for example, have the ability to secrete large titres of proteins, qualify for the Qualified Presumption of Safety (QPS) status of the European Food Safety Authority, and many of their products have a Generally Recognized As Safe (GRAS) status from the US Food and Drug Administration. Furthermore, *B. subtilis* becomes naturally competent, thereby allowing for easy genetic modification (Tjalsma et al. 2000; van Dijl et al. 2002; Graumann 2011).

The *B. subtilis* Tat system is one of the most extensively studied Tat systems within the field, because it has unique characteristics in particular relating to gene duplication. As indicated above, duplication of TatA and TatA-like proteins is a common feature in most Tat systems. However, the duplication of TatC proteins is rare except in *Bacillus* species, where multiple isoforms of TatC have been observed (Jongbloed et al. 2000; Yen et al. 2002; Simone et al. 2013).

The core progenitor operon in *B. subtilis* is *tatAy-tatCy* (Simone et al. 2013). This operon has been duplicated and, consequently, there is a second separate *tat*

operon named *tatAd-tatCd.* Thus, *B. subtilis* Tat is specified by two separate operons, which are expressed at different times and, under normal conditions, do not share substrate specificity (Jongbloed et al. 2004; Eijlander et al. 2009a; Nicolas et al. 2012; Goosens et al. 2013). The predominant Tat pathway is TatAy-TatCy and, although an early bioinformatics analysis has predicted up to 69 potential Tat-dependent substrates, only three substrates have been confirmed to be strictly TatAy-TatCy-dependent, namely EfeB, QcrA and YkuE (Tjalsma et al. 2000; Jongbloed et al. 2002, 2004; Monteferrante et al. 2012b; Goosens et al. 2013). The genes for the second *B. subtilis* Tat pathway, TatAd-TatCd, are found next to the gene for its only known substrate, the phosphate acquisition protein PhoD. Accordingly, the *tatAd-tatCd* operon is only expressed under low-phosphate conditions (Eder et al. 1996; Jongbloed et al. 2000, 2004; Nicolas et al. 2012).

Apart from duplicating the whole *tatAy-tatCy* operon, the Tat system of *B. subtilis* has a further *tatA* duplication, namely *tatAc*. This third *tatA* gene is located elsewhere on the chromosome, and it is expressed constitutively under numerous conditions (Nicolas et al. 2012). Although it was investigated in several studies, a physiological role for TatAc has, until recently, remained enigmatic (Tjalsma et al. 2000; Jongbloed et al. 2002, 2004; Eijlander et al. 2009a; Nicolas et al. 2012). TatAc is unable to form an active translocon when paired with TatC proteins in *B. subtilis* (Eijlander et al. 2009a; Goosens et al. 2015). However, when expressed in *E. coli,* TatAc formed functional translocases with either *E. coli* TatBC, *B. subtilis* TatCd or *B. subtilis* TatCy (Monteferrante et al. 2012a; Beck et al. 2013). This difference illustrates the interpathway flexibility of *E. coli* and further suggests potentially different quality control or chaperone activities in the Tat pathways of *B. subtilis* and *E. coli*. Yeast two-hybrid (Y2H) protein–protein interaction studies have shown that not only does TatAc interact with itself and the *B. subtilis* TatA proteins, but it also directly interacts with HemAT (Monteferrante et al. 2013). HemAT was in turn shown to be essential for the Tat-dependent secretion of PhoD, which therefore suggested a functional role for TatAc in *B. subtilis* (Monteferrante et al. 2013). A functional role for TatAc as a Tat-assistance protein was confirmed when it was shown to permit the translocation of EfeB in cells with an impaired TatAy function, despite the fact that TatAc was unable to replace TatAy (Goosens et al. 2015). It thus seems that TatAc, the third TatA-like protein of *B. subtilis*, reflects an intermediate evolutionary step in TatA-TatB specialization. In this scenario, the presently available data suggest that the defective TatAy protein has a role that is comparable to that of *E. coli* TatB, while TatAc has a role similar to that of *E. coli* TatA. Altogether, it can be concluded that the core Tat translocon in *B. subtilis* is composed of a TatAy-TatCy pair and that the TatAc protein has a non-essential assistant role in translocation. For example, TatAc could allow for more efficient cargo-Tat protein–protein interactions, and it might improve the overall efficiency of the Tat pathway (Goosens et al. 2015).

All confirmed *B. subtilis* Tat-dependent cargo proteins are known to contain co-factors, thereby emphasizing their need for the Tat pathway (Schneider and Schmidt 2005; Monteferrante et al. 2012b; Miethke et al. 2013; Rodriguez et al. 2014). Further, QcrA contains a disulphide bond in addition to its iron-sulphur

cluster (Iwata et al. 1996; Link et al. 1996; Schmidt and Shaw 2001; Hunsicker-Wang et al. 2003). Of note, QcrA has been observed to be a Tat-dependent substrate in a wide-range of organisms (Molik et al. 2001; Bachmann et al. 2006; De Buck et al. 2007; Goosens et al. 2013; Pett and Lavrov 2013b; Oertel et al. 2015). Both QcrA and EfeB form part of larger extracytoplasmic complexes, where the QcrA-B-C proteins form the cytochrome bc_1 complex, whereas the EfeU-O-B proteins form an iron uptake system. This organization of Tat-dependent substrate proteins into larger complexes further suggests that the Tat pathway assists in protein complex assembly (Yu et al. 1995; Schneider and Schmidt 2005; Miethke et al. 2013; Sousa et al. 2013). The extracellular protease WprA also directly affects EfeB and indirectly influences YkuE (Monteferrante et al. 2013). The action of WprA may thus be associated with this complex maturation.

Most of the observed phenotypes associated with Tat-deficiencies have been linked directly to the known substrates, i.e. PhoD is required under phosphate starvation and EfeB is required under conditions of iron deficiency and low salt (Jongbloed et al. 2000; van der Ploeg et al. 2011b). However, quantitative proteomic studies revealed that numerous proteins associated with motility and biofilm formation were decreased in *tatAy-tatCy* deficient strains, leading to the identification of an, as-yet, not-well-understood Tat-associated delayed biofilm formation phenotype (Goosens et al. 2013). Most studies investigating Tat have used Western blotting techniques to validate Tat-dependency of substrates. Although this remains the golden standard and a powerful tool, it does not give an indication of whether the protein is correctly folded and active. In the *B. subtilis* studies, the activity of cargo proteins was determined using the alkaline phosphatase activity of YkuE (Monteferrante et al. 2012b) and the ferric iron uptake to assess EfeB activity (Miethke et al. 2013; Goosens et al. 2015). EfeB is a hemoprotein that oxidizes ferrous iron to ferric iron for uptake via EfeU and EfeO. For this reason, EfeB stimulates growth under microaerobic conditions where ferrous iron is more abundant. In addition, EfeB was shown to have an important role in the protection against cell envelope stress through the elimination of reactive oxygen species that are generated in the presence of ferrous iron (Miethke et al. 2013).

6.2 *Streptomyces*

Streptomyces species are found naturally in the soil where they often form mycelia. These bacteria have become workhorses for industry as they can be used for the high-level production of various antibiotics and secreted proteins (Anne et al. 2012). The Tat system is a major contributor to overall protein secretion in these species with numbers of potential substrates ranging between 100 and 189 (Widdick et al. 2006; Joshi et al. 2010; Palmer and Berks 2012). The Tat system in *Streptomyces* species is composed of at least three Tat components, where the genes for a minimal TatA-TatC system are found clustered and the gene for an extra

TatA-like protein (depending on the species, these are called TatB or TatA2) is located elsewhere on the chromosome (Schaerlaekens et al. 2001; Palmer and Berks 2012). Crosstalk between the Sec and Tat pathways has been observed in the sense that Sec-dependent secretion was enhanced by a mutated Tat system or reduced by an overexpressed Tat system (Schaerlaekens et al. 2004a; De Keersmaeker et al. 2006).

In *S. lividans,* optimal secretion of the Tat-dependent substrates xylanase C and tyrosinase occurred when all three Tat components were present. Secretion did still occur when a single TatA or TatB protein was paired with TatC, albeit at lowered efficiency. It was therefore concluded that the TatA-like proteins in *Streptomyces* were unable to fully functionally replace each other and that each must have a specialized function (De Keersmaeker et al. 2005a). A very interesting observation made in *S. lividans* was that both TatA and TatB proteins were detected in the cytoplasm under native conditions (De Keersmaeker et al. 2005b), and when expression was induced these TatA-like proteins apparently interacted with Tat-dependent cytoplasmic pre-proteins (De Keersmaeker et al. 2005a, 2007).

6.3 Staphylococcus

Not all *Staphylococcus* species have a Tat pathway. However, this pathway has been identified in *Staphylococcus haemolyticus*, *Staphylococcus carnosus, Staphylococcus lugdunensis* and *S. aureus* (Biswas et al. 2009). The staphylococcal Tat pathway is composed of a single TatA–TatC pair. This Tat pathway has been investigated for biotechnological applications in *S. carnosus* and, although shown to secrete heterologous proteins, it was considered inadequate for the required applications (Thiemann et al. 2006; Meissner et al. 2007). To date, only one native Tat-dependent staphylococcal substrate, FepB, has been confirmed, and inactivation of the Tat system did not show any global changes in protein secretion profiles (Yamada et al. 2007; Biswas et al. 2009). FepB is an iron-dependent peroxidase encoded by the *fepABC* operon, and the corresponding complex is very similar to the iron-scavenging EfeUOB complexes in *E. coli, B. subtilis* and *L. monocytogenes* (Biswas et al. 2009; Miethke et al. 2013; Turlin et al. 2013). Interestingly, in a mouse kidney abscess model, the bacterial load of *tat* or *fepB* mutant strains was shown to be decreased, thereby pointing at a physiologically relevant role of Tat-dependent export of FepB in staphylococcal disease (Biswas et al. 2009).

6.4 Listeria monocytogenes

Listeria monocytogenes is a saprophytic bacterium that, once it has entered the food chain, becomes a dangerous food-borne pathogen. The *Listeria* Tat system is composed of a TatA–TatC pair (Desvaux and Hebraud 2006; Machado et al. 2013).

In strains where *tat* genes were deleted, no significant changes in cell viability or virulence have been described (Machado et al. 2013; Halbedel et al. 2014). Bioinformatic analysis predicted two potential Tat-dependent substrates, namely FepB (Lmo0367) and a FabF-like protein (Lmo2201) (Desvaux and Hebraud 2006). However, when these proteins were tagged and expressed neither was Tat-dependent (Halbedel et al. 2014). Nonetheless, the FepB signal peptide was shown to confer Tat-dependent secretion in the *S. lividans* agarase reporter assay, and both a *tatC* and *fepB* mutant strain displayed decreased overall ferric reductase activity (Widdick et al. 2008; Tiwari et al. 2015). Hence, there is evidence of a FepB-Tat association, but none of the currently available data confirms a direct Tat-dependency. The *fepB* gene is co-transcribed in an iron-induced *fepCAB* operon, which is also Fur-regulated (Ledala et al. 2010; Tiwari et al. 2015). Although the *tat* operon is transcribed in the early exponential phase in rich medium, it is Fur-regulated and highly induced under iron starvation conditions (Ledala et al. 2010; Machado et al. 2013; Tiwari et al. 2015). Therefore, it is conceivable that, in order to detect the possible Tat-dependency of FepB, environmental conditions with low iron availability may be required, or as in *B. subtilis,* other environmental conditions such as low salt (van der Ploeg et al. 2011b; Goosens et al. 2015). In fact, the *Listeria fepCAB* operon is highly reminiscent of the Tat-associated *fepABC* and *efeUOB* operons in *S. aureus* and *B. subtilis*, respectively. Accordingly, there appears to be a conservation of the Tat requirement in these iron-scavenging complexes.

6.5 *Streptococcus*

The majority of *Streptococcus* species studied have no identifiable Tat components. However, genes encoding a TatA and TatC protein have been identified in *Streptococcus sanguinis* and *Streptococcus thermophilus*. Intriguingly, in both *S. sanguinis* and *S. thermophilus* these *tat* genes are localized in close genomic proximity to three genes that resemble the *efeUOB*, *fepCAB* and *fepABC* operons of *B. subtilis*, *Listeria* and *Staphylococcus*, respectively. Further, in both *Streptococcus* species, the EfeB-like iron-dependent peroxidase contains a twin-arginine motif in the signal peptide. In the facultative anaerobe *S. thermophilus*, EfeB was shown to be translocated by the Tat system, and mutation of *efeB* or *tatC* resulted in decreased growth under aerobic conditions, suggesting that the respective proteins have a role in protecting the cell against oxidative stress (Xu et al. 2007; Zhang et al. 2015).

7 Conclusion

The investigations on the Tat pathways of Gram-positive bacteria, as described in this review, suggest a general association between the Tat pathway and iron-scavenging complexes, phosphate acquisition and respiratory complexes. Intriguingly, phylogenetic analyses of TatC showed that 89 % of species that have TatC are either facultative aerobes, or facultative or obligate anaerobes, while only 11 % are obligate aerobes (Simone et al. 2013). Thus, the majority of organisms with a Tat system find themselves in anaerobic environments. Of note, anaerobic bacteria and anaerobic growth are, over all, relatively poorly characterized. It thus seems likely that the full potential of the Tat pathway and the spectrum of biological functions that it fulfils are currently substantially underappreciated. With this in mind, an important challenge for future Tat-related research could be the exploration of this pathway in the microbiota of the human gut. Here bacteria, many of which are *Firmicutes*, need to thrive and survive in a challenging anaerobic environment that continuously changes depending on the ingestion of different nutrients by the host, continuous flow-through and influx of oxygen from the gut epithelium (Khan et al. 2012). Indeed, sequence analyses have shown that various dominant gut microbes do contain *tat* genes, and it would be interesting to explore their functions and find out whether they are conditionally essential.

The Tat system is known to be essential in only a few bacteria (Palmer and Berks 2012), including *Mycobacterium tuberculosis* where Tat has been shown to be important for drug resistance and virulence (Raynaud et al. 2002; McDonough et al. 2005). Yet, gene essentiality is often condition-dependent and this also applies to some *tat* genes as exemplified in *B. subtilis*, where the absence of the *tatAy-tatCy* operon leads to severe growth impairment in salt- or iron-depleted environments. This conditional essentiality implies that Tat is a potentially druggable target in notoriously drug-resistant pathogens, such as *M. tuberculosis*.

Today, there are various areas in the Tat field that merit further research, some of which have been touched upon in the present review. For example, this applies to the condition-dependent regulation of *tat* gene expression, including possible roles of antisense RNAs and small non-coding regulatory RNAs. In this respect, it is worth mentioning recent studies, showing that the non-coding RNA Mcr7 of *M. tuberculosis* modulates TatC expression, thereby serving as an intriguing new Tat secretion control mechanism (Solans et al. 2014). Other major knowledge gaps concerning the Tat pathways of Gram-positive bacteria relate to the chaperones that guide cargo folding and cofactor insertion, quality control of cargo prior to membrane translocation, the actual mechanism of Tat-mediated translocation of cargo across the membrane, and post-translocational cargo processing and quality control. Research into these areas will be important, not only to enrich our fundamental understanding of protein translocation mechanisms, but also to open up the enigmatic Tat pathway for the biotechnological secretion of high-value proteins, and to explore the potential of Tat as an antimicrobial drug target.

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