Protein secretion via the Twin-arginine translocation pathway of *Bacillus subtilis*

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Robyn T. Eijlander, Jan D. H. Jongbloed and Oscar P. Kuipers

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Chapter 4

Relaxed specificity of the *Bacillus subtilis* TatAdCd translocase in Tat–dependent protein secretion

Protein translocation via the Twin-arginine translocation (Tat) pathway is characterised by the translocation of prefolded proteins across the hydrophobic lipid bilayer of the membrane. In *Bacillus subtilis*, two different Tat translocases are involved in this process and both display a different substrate specificity; PhoD is secreted via TatAdCd, whereas YwbN is secreted via TatAyCy. It was previously assumed that both TatAy and TatCy are essential for the translocation of pre-YwbN. Through complementation studies we now show that TatAy can be functionally replaced by TatAd when the latter is offered to the cells in excess amounts. Moreover, under conditions of overproduction TatAdCd, in contrast to TatAyCy, shows an increased tolerance towards the acceptance of various Tat-dependent proteins.

Introduction

Translocation of proteins across hydrophobic membranes of cells is an essential mode of survival for all organisms. Several mechanisms have evolved to ensure this transport. Among these mechanisms, the Twin-arginine translocation (Tat) pathway has been shown to be capable of translocating fully folded proteins across the membrane. Proteins that depend on this translocase for their secretion are characterised by an R/K-R-X-φ-φ motif in their signal peptide, in which φ represents a hydrophobic residue. Protein translocation via this pathway has been extensively studied in mainly thylakoids of plants and the Gram-negative bacterium *Escherichia coli* (Lee et al., 2006a; reviewed in Robinson and Bolhuis, 2004; Berks et al., 2003). Details on the unique mechanism of activity of the Tat pathway remain to be elucidated, although several working models have been proposed that assist in the understanding of this complex translocation machinery (Westermann et al., 2006; Oates et al., 2005; Cline and Mori, 2001). In both *E. coli* and thylakoids the Tat core complex has been shown to consist of TatA, TatB and TatC. Of these, the TatB and TatC proteins are involved in initial substrate recognition and binding (Alami et al., 2003), whereas TatA is thought to form a protein translocation channel (Gohlke et al., 2005). These three proteins are known to interact with and be dependent on each other, ensuring the formation of a stable core complex (Lange et al., 2007; Mangels et al., 2005). Additionally, in *E. coli* a fourth Tat component (TatE) has been identified that shows high similarity with TatA. This protein is able to substitute for TatA in the translocation of several Tat substrates (Sargent et al., 1998).
The above described composition of the Tat machinery is fairly consistent for the majority of thylakoidal and Gram-negative Tat machineries. In Gram-positive microorganisms, however, some distinct differences in the composition of the Tat complex have been observed (Jongbloed et al., 2006). All Gram-positive Tat systems, with the exception of those in *Streptomyces* species, lack the TatB component. Moreover, most contain multiple paralogues of TatA and TatC. Recently, complementation studies with TatA of *Bacillus subtilis* showed a bifunctional activity of Gram-positive TatA, as it was able to restore secretion in both a tatA and a tatB mutant of *E. coli* (Barnett et al., 2008).

To obtain further insight in the working mechanism of the Gram-positive Tat secretion system, including differences and similarities with that of Gram-negative bacteria and thylakoids in chloroplasts, the Gram-positive soil bacterium *Bacillus subtilis* has been adopted as a model organism for these studies. Within *B. subtilis*, three variants of the TatA protein have been identified, denoted TatAd, TatAy and TatAc. TatAd has been shown to form an active complex with one of the two *B. subtilis* TatC proteins, namely TatCd. The genes encoding these proteins are located in the *phoD* operon of the *pho* regulon, which is only expressed during phosphate starvation growth conditions (Jongbloed et al., 2000). PhoD, a phosphodiesterase of which the corresponding gene is located directly upstream of the *tatAd* gene, is the only substrate identified so far as being dependent on TatAdCd for its secretion. The second TatC component, TatCy, forms an active complex with TatAy, and specifically translocates the iron-dependent peroxidase YwbN (Jongbloed et al., 2004). The function of the third TatA protein (TatAc) is unknown, although it has been established that this protein is not required for translocation of the two Tat-dependent substrates identified to this date (Jongbloed et al., 2004). Previous complementation studies in *E. coli* have already confirmed the difference in substrate specificity between the two Tat translocases of *B. subtilis*. In a tat deletion strain of *E. coli* the secretion of the Tat-dependent TMAO reductase TorA could only be restored when TatAdCd of *B. subtilis* was offered to the cell. Although shown to be active in the secretion of several other Tat-dependent proteins of *E. coli*, TatAyCy was unable to enable the secretion of TorA (Barnett et al., 2008).

Even though the difference in substrate specificity between TatAdCd and TatAyCy has been clearly documented, it is so far unknown how these translocases recognise their substrates and whether substrate recognition and the formation of a functional translocase is restricted to the specific TatAdCd and TatAyCy combinations only. Therefore, to shed new light on the working mechanism and specificity of these two Tat translocases, complementation studies were performed in several *B. subtilis tat* mutant backgrounds and the effect of Tat component complementation on the translocation of YwbN and PhoD in these strains was monitored. Here we show that TatAd can functionally replace TatAy when offered in excess amounts to a tatAy mutant *B. subtilis* strain, suggesting that TatAd and TatCy can “cross-interact” and that the resulting translocase is able to transport YwbN to the extracellular medium. Furthermore, our results show that the overproduction of both TatAd and TatCd enables the secretion of YwbN in the absence of its specific TatAyCy translocase. In contrast, no secretion of TatAdCd-dependent PhoD was observed upon overproduction of TatAy and TatCy in the absence of TatAd or TatCd components. These
findings demonstrate that TatAdCd is more tolerant in the acceptance of Tat-dependent substrates when compared to TatAyCy and suggests that the TatA component plays a determining role in this process.

Results

TatAd can functionally replace TatAy
To study the level of functional compatibility between Tat components of *Bacillus subtilis*, the secretion of the TatAyCy-dependent substrate YwbN was monitored in several *tat* deletion strains, complemented by different *B. subtilis* Tat components expressed from a constitutive promoter (production increased approximately 5 to 10-fold, data not shown). For this purpose, the X-ywbN-myc cassette and plasmids encoding different Tat components and operons were introduced in *tat* mutant strains as described before (Jongbloed *et al.*, 2004). Next, overnight TY cultures of wild type and *tat* deletion strains, with or without complementation plasmids, were diluted 100-fold in fresh TY medium. Strains were grown to exponential growth phase, at which time YwbN-myc production was induced by the addition of 1% xylose. After three hours of induction cells were harvested by centrifugation, separating cellular fractions from extracellular medium fractions. To analyse the production and secretion of YwbN-myc, SDS PAGE samples were prepared as described in the Materials and methods section and analysed by SDS PAGE, Western Blotting, and immunodetection with antibodies raised against the c-Myc epitope. Importantly, analysis of the presence of this protein in cellular fractions demonstrated the production of YwbN-myc in all strains studied (data not shown). Notably, no significant accumulation of YwbN precursor was observed in these fractions.

TatAy was shown to be a key-component in the secretion of YwbN, as deletion of this vital component completely abolishes YwbN secretion (demonstrated in Figure 1A, lane ΔAy). Since the deletion of *tatCy* results in the same effect (Figure1B, lane ΔCy) and because the genes are organised in an operon, it has been suggested that these two components together form an active complex, in which both play a critical role in the secretion of YwbN (Jongbloed *et al.*, 2004). Notably, the absence of (pre-)YwbN in these fractions implicates that these *tat* mutant strains do not show an increased sensitivity to lysis as was also described previously for *B. subtilis* (Jongbloed *et al.*, 2002) and in contradiction to what was observed for *tat* mutants of *E. coli* (Stanley *et al.*, 2001). Moreover, analysis of the presence of the cytoplasmic protein DnaJ demonstrated the absence of this protein in medium fractions of wild type and the *tatAy* mutant strains indicating that *tat* mutant strains are not more sensitive to lysis (data not shown). To study putative complementation of *tatAy* or *tatCy* deletions, individual Tat components were introduced in the *tatAy* and *tatCy* mutant strains expressed from pGDL48 derivatives. YwbN secretion into the extracellular medium was detected using c-Myc specific antibodies.
As expected, YwbN secretion was restored in both the \( \text{tatAy} \) and the \( \text{tatCy} \) deletion strains by the production of plasmid-borne TatAy or TatCy, respectively (Figure 1A, lane Ay and Figure 1B, lane Cy), as well as by complementing the \( \text{tatAy} \) and \( \text{tatCy} \) deletion strains with the complete TatAyCy complex (Figure 1A and B, lane AyCy). Strikingly, plasmid-borne TatAd is also able to restore YwbN secretion in the \( \text{tatAy} \) deletion strain (Figure 1A, lane Ad). It should be noted that during these experimental growth conditions in rich TY medium the endogenous \( \text{tatAd} \) and \( \text{tatCd} \) genes are not expressed. Therefore, these observations suggest that TatAd is able to form an active complex with endogenous TatCy, resulting in secretion of YwbN at levels comparable to the situation of complementation with plasmid-borne TatAy. In addition, and as expected on the basis of the results with complementing TatAd, the production of TatAd and TatCd (Figure 1A, lane AdCd) also
efficiently restores YwbN secretion. In contrast to TatAd forming a functional Tat complex with TatCy, TatAy seems to be unable to form functional complexes with TatCd, as TatCd is unable to complement the deletion of tatCy (Figure 1B, lane Cd). However, introduction of TatAdCd in the tatCy mutant strain does restore YwbN secretion (Figure 1B, lane AdCd), suggesting that the TatAdCd complex is able to translocate YwbN. Notably, in this situation the TatAy component is still available for YwbN secretion as well. However, the assumption that TatAdCd alone is capable of YwbN transport is confirmed by the results shown in Figure 1C, lanes AdCd, demonstrating that even in the absence of both TatAy and TatCy (strain ΔtatAyCy) constitutive production of TatAdCd can restore the secretion of YwbN.

**TatAdCd can only aid in the secretion of YwbN when overproduced**

The above described phenomenon of TatAdCd being functional in the secretion of YwbN was only observed when TatAd and TatCd components were expressed from pGDL48 derivatives. Since the genes are constitutively expressed from the erythromycin promoter of pDGL48, the production of the TatAd and TatCd components is most likely increased. This is also demonstrated by the fact that introducing constitutively expressed TatAyCy in ΔtatAy and ΔtatCy mutant strains mostly results in an increase in YwbN secretion (Figures 1A, B and C, lane AyCy) compared to that in the parental strain (Figures 1A, B and C, lane wt). To examine whether TatAdCd is also able to complement tatAy and/or tatCy deletions at wild type expression levels of endogenous tatAd and tatCd, ΔtatAy and ΔtatCy mutant strains were grown in a low phosphate medium, thereby initiating the expression of tatAdCd from the chromosome (Antelmann et al., 2000). Cells were grown in the presence of 1% xylose for three hours after reaching exponential phase to induce expression of ywbN-myc and the secretion of YwbN was monitored. As is clearly shown in Figure 2, YwbN cannot be detected in the extracellular medium of cells in which the tatAy and/or tatCy genes are deleted (Figure 2, lanes ΔAy, ΔCy, ΔAyCy). This implicates that under growth conditions with wild type expression levels of tatAd and tatCd, the TatAdCd translocase is specifically dedicated to the export of PhoD and, putatively, other yet unknown TatAdCd-specific substrates. This is consistent with earlier findings by Jongbloed et al. (2004).

**Figure 2**  
Secretion of YwbN in several tat mutant backgrounds when grown in LPDM. Cells were grown in Low Phosphate Depletion Medium (LPDM) as described in the Materials and methods section. The expression of ywbN-myc was induced by the addition of 1% xylose. YwbN-myc in the extracellular medium (indicated on the right) was visualised using specific antibodies against the C-terminal myc-tag. The size of the protein is indicated by marker bands on the left (in kDa). ΔAy, *B. subtilis* 168 ΔtatAy X-ywbN; ΔCy, *B. subtilis* 168 ΔtatCy X-ywbN; ΔAyCy, *B. subtilis* 168 ΔtatAyCy X-ywbN; ΔAdCd, *B. subtilis* 168 ΔtatAdCd X-ywbN; Δtotal, *B. subtilis* 168 Δtat total X-ywbN.
TatAyCy components are unable to complement the absence of TatAd and TatCd

The results described above implicate that certain Tat components can “cross-interact” to form an active translocase (i.e. TatAd with TatCy) and that a certain degree of substrate tolerance, at least by *B. subtilis* TatAdCd translocases, is accepted. This possibility was further investigated by performing complementation studies in ΔtatAd and ΔtatCd mutant backgrounds. *B. subtilis* strains 168 ΔtatAd, ΔtatCd and Δtat total were grown in a Low Phosphate Depletion Medium (LPDM) to induce the expression of the *phoD* operon, including *phoD*, *tatAd* and *tatCd*. Shortly after reaching transition phase, cells were separated from the growth medium by centrifugation. Next, the presence of PhoD in cellular fractions of the above mentioned strains was verified by analysis of these samples by SDS PAGE and Western Blotting and subsequent immunodetection using specific antibodies against PhoD (data not shown). The secretion of PhoD to the extracellular medium was monitored by applying a similar approach. As shown in Figure 3, PhoD is only secreted to the medium when the absence of TatAd and TatCd components is restored by re-introduction of the respective TatAd and/or TatCd components expressed from a
plasmid (Figures 3A and B, lanes Ad, Cd and AdCd). In contrast, the introduction of plasmid-borne TatAy, TatCy or TatAyCy does not result in PhoD secretion (Figures 3A and B, lanes Ay, Cy and AyCy). Although the complementation by plasmid-borne TatAd in a ΔtatAd mutant background is less efficient when compared to wild type secretion levels (Figure 3A, lane Ad), these experiments were repeated several times and showed a reproducible secretion pattern. In none of the cases complementation by TatAy was observed. These results show that, unlike previous observations for TatAdCd, the TatAyCy complex is not able to complement the absence of TatAdCd components and suggest that TatAyCy is less substrate tolerant when compared to TatAdCd.

**TatAc does not actively participate in Tat-dependent protein translocation**

The third tatA gene of *B. subtilis*, tatAc, is monocistronic and has no TatC counterpart. So far no functional activity for TatAc has been documented. Since the tatAc gene is not associated with any tatC gene on the chromosome, it seems unlikely that this third TatA component of *Bacillus subtilis* is involved in the secretion of Tat-dependent proteins. This hypothesis is confirmed by the inability of TatAc to complement the absence of TatAy in the secretion of YwbN or of TatAd in the secretion of PhoD (Figure 1A, lane Ac and Figure 3A, lane Ac).

**Discussion**

The two Tat translocases of *Bacillus subtilis* are known to display different substrate specificities: YwbN is specifically secreted via TatAyCy and PhoD via TatAdCd (Jongbloed et al., 2004). To obtain more insight in specificity determinants of Tat-dependent protein secretion in *B. subtilis*, the ability of plasmid-borne Tat components (solely or in combination, i.e. TatAdCd or TatAyCy) to complement different tat deletions was studied. Which specific molecular interactions determine substrate specificity remains to be elucidated.

By monitoring the secretion of epitope-tagged YwbN during the complementation in tatAy and tatCy deletion strains by different Tat components, we were able to show that the absence of TatAy can be complemented by plasmid-borne TatAd (Figure 1A, lane Ad). In contrast, the absence of tatCy could not be complemented by tatCd expressed from the pCCd plasmid (Figure 1B, lane Cd). Nevertheless, it seems that YwbN translocation can be facilitated by a TatAdCd complex in the absence of both TatAy and/or TatCy components, since YwbN was shown to be secreted in ΔtatCy and ΔtatAyCy(Ac) mutant strains that are complemented by the overproduction of the TatAd and TatCd components (Figure 1B, lane AdCd and Figure 1C, lanes AdCd). In previous studies, it was shown that TatAdCd is able to translocate the *E. coli* Tat substrate TorA in the *E. coli* tat null mutant strain ΔABCDE (Barnett et al., 2008). Notably, complementation by TatAdCd in *E. coli* as well as in *B. subtilis* was only observed when excess amounts of TatAd and TatCd components were offered to the cells (Figures 1 & 2 of this study and Barnett et al., 2008). It is therefore
feasible that the TatAdCd complex may have a low affinity for the YwbN substrate when produced at wild type levels, which cannot be detected with the techniques used in this study. The overexpression of TatAd and TatAdCd can overcome this low affinity, which results in a relaxed substrate specificity. Taken together, these results provide new insights in the substrate specificity and complex formation of Tat components of \textit{B. subtilis}. The fact that TatAd can complement the absence of TatAy for the secretion of YwbN suggests that it is likely that TatAd forms an active complex with TatCy, provided that TatAd is present in excess amounts. In contrast, a TatAdCy complex seems to be unable to enable the secretion of PhoD, as the overproduction of TatCy in a \textit{ΔtatCd} mutant background (in which TatAd is still produced) does not result in the presence of PhoD in the extracellular medium of this strain (Figure 3B, lane Cy). Likewise, TatAy is unable to form an active complex with TatCd for the secretion of YwbN, even when TatCd is overproduced (Figure 1B, lane Cd) or of PhoD (Figure 3A, lane Ay). This ambiguous behaviour of Tat components can only be explained by yet to be identified specificity determinants in the amino acid sequence of the separate Tat components and/or Tat substrate signal peptides that promote successful Tat protein interactions, substrate recognition and pre-protein transport in some cases (export of YwbN by TatAdCy) but not in other cases (export of PhoD by TatAdCy or export of YwbN by TatAyCd).

In previous studies it was shown that the third \textit{B. subtilis} TatA component, TatAc, is not required for the translocation of YwbN or PhoD (Jongbloed \textit{et al.}, 2004). Accordingly, we now show that TatAc, despite its production in excess amounts, is unable to complement for the absence of TatAy or TatAd in the secretion of YwbN or PhoD, respectively (Figure 1A, lane Ac and Figure 3A, lane Ac). It is conceivable that TatAc does not play any part in Tat-dependent protein translocation. The fact that TatAc does not have a TatC counterpart supports that theory. On the other hand, we cannot exclude the possibility that TatAc, like TatE of \textit{E. coli}, provides an additional pool of TatA molecules that might be needed under conditions of high Tat production. Moreover, also the existence of TatAc-specific substrates can still not be excluded. In that case, the transport of these putative substrates has to be accomplished in conjunction with TatCd and/or TatCy.

With respect to the working mechanism of the Tat machinery of \textit{E. coli} and thylakoids, it has been proposed that a TatBC complex is involved in the initial recognition and binding of Tat-dependent substrates (Alami \textit{et al.}, 2003; Bolhuis \textit{et al.}, 2001; Cline and Mori, 2001) and that subsequently TatA proteins are recruited to form the translocation channel (Berks \textit{et al.}, 2000). Recent results of studies concerning Gram-positive Tat complex formation in \textit{E. coli} suggest a similar model for the activity of \textit{B. subtilis} Tat complexes (Barnett \textit{et al.}, 2008, Chapter 2 of this thesis). Considering the fact that \textit{B. subtilis} does not contain a TatB analogue, it would be plausible to assume that TatCy is involved in the recognition and binding of pre-YwbN, and that subsequently a separate TatAy complex is recruited that will be responsible for the translocation of the YwbN protein. This proposed working model for YwbN secretion by TatAyCy differs from the recently proposed working model for TatAdCd-dependent translocation of PhoD by Westermann \textit{et al.} (2006) and Schreiber \textit{et al.} (2006). These authors favour a model in which cytosolic TatAd is involved in the initial
B. subtilis Tat complementation studies

recognition and binding of the double arginine-containing signal peptide of pre-PhoD, after which this TatAd-prePhoD complex is targeted to a membrane-associated TatCd complex. This complex then assists in the membrane insertion of the cytosolic TatAd-prePhoD complex and subsequent pre-PhoD translocation. Taken together, these two models either contradict or supplement each other. Our complementation data suggest that the two Tat translocases of B. subtilis operate in a different manner, at least at the level of Tat substrate specificity. This hypothesis is illustrated in Figure 4. Since we were able to show that TatAd can be involved in the secretion of both PhoD and YwbN, it is conceivable that this component is responsible for the tolerant character of the TatAdCd translocon during overexpression conditions in rich medium. To explain the observations described for our complementation studies, we propose, in concordance to earlier findings of Westermann et al. (2006), that TatAd can be actively involved in the recognition and binding of random Tat signal sequences, after which it can interact with membrane-bound TatCd. In contrast to TatAd, TatCd is very specific in complex formation with TatAd only: TatAy was shown to be unable to form an active complex with TatCd, at least for the secretion of YwbN. However, TatAd is not restricted to its own counterpart TatCd, but is also capable of forming an active complex with TatCy. Since this was only observed for the secretion of

Figure 4  Tat component interactions and activities. The Tat proteins studied are represented by rectangles in a membrane, connected by intracellular and extracellular loops. TatA has a single membrane-spanning domain with a predicted outside N terminus and inside C terminus. TatC has six transmembrane-spanning domains and a predicted in-in topology. TatAy and TatCy are represented by black rectangles, whereas TatAd and TatCd are represented by light-grey rectangles. The naturally present TatAyCy complex is specific in the secretion of YwbN (hexagon) harbouring an RR consensus motif in the signal peptide. In the absence of TatAy, complementation with plasmid-borne TatAd results in secretion of YwbN to the extracellular environment. In contrast, results indicative of a TatAyCd complex that is active in the secretion of either YwbN or PhoD could not be obtained (indicated with a black cross). Finally, overproduction of the TatAdCd complex in a rich medium can facilitate the secretion of YwbN in B. subtilis and TorA (octagon) in E. coli (Barnett et al., 2008). Additionally, the at wild type levels produced as well as overproduced TatAdCd complexes secrete PhoD (pentagon) in B. subtilis.
YwbN, and not for PhoD, we propose that TatCy is the specificity determining factor in the TatAyCy complex and thus responsible for recognising the signal sequence of YwbN (in a TatAd-preYwbN complex or separately). After recognising and binding pre-YwbN, TatCy will subsequently form an active translocation complex with either TatAd or TatAy. This model is only applicable when there are excess amounts of TatAd present, since lower (wild type) production levels (i.e. under the conditions of phosphate depletion) of TatAd do not lead to a YwbN-exporting TatAdTatCy machine. Under these conditions a clear substrate specificity of both Tat complexes is observed: TatAyCy being specifically involved in YwbN secretion and TatAdCd being specifically active in PhoD secretion.

Topology predictions of TatCy show that this protein most likely forms four intracellular loops and three extracellular loops, that are connected by six transmembrane domains. Which part of the TatCy protein is involved in the recognition and binding of YwbN is so far unknown. Studies with *E. coli* TatC provided some clues concerning the role of conserved residues in the first two cytoplasmic loops in substrate recognition, since conservative substitutions of these residues resulted in a complete block in protein translocation (Holzapfel et al., 2007; Allen et al., 2002). It is possible that the same regions, or even residues, within the TatCy protein are involved in recognition of and/or binding to pre-YwbN. We are currently pursuing this line of research further.

Finally, the specific cross-interactive nature of TatAdCd when produced in excess amounts leads to the hypothesis that the TatAdCd translocase is less specific in its affinity for Tat substrates than TatAyCy. This could consequently result in a preference of the TatAdCd translocase over the TatAyCy translocase as a candidate for the commercial production of pre-folded heterologous proteins.

**Materials and methods**

*Bacterial strains, plasmids and media*

All strains and plasmids used in this study are listed in Table 1. All strains were grown in TY (tryptone/yeast extract) medium, consisting of Bactotryptone (1%; w/v), Bacto yeast extract (0.5%; w/v) and NaCl (1%; w/v), unless indicated otherwise. Induction of a phosphate depletion response was achieved by overnight growth in HPDM (high phosphate depletion medium) and dilution and subsequent growth in LPDM (low phosphate depletion medium). Both media were prepared as described by Müller et al., (1997). When required, media were supplemented with erythromycin (Em; 5 μg/ml), kanamycin (Km; 10 μg/ml), chloramphenicol (Cm; 5 μg/ml) tetracyclin (Tc; 6 μg/ml) and/or spectinomycin (Sp; 100 μg/ml). To ensure the production of YwbN-myc, the expression of the corresponding gene was induced by the addition of 1% xylose to cells after entering the exponential growth phase.

*DNA cloning techniques*

All cloning techniques and transformation of *E. coli* were performed as described (Sambrook et al., 1989). Transformation of *Bacillus subtilis* was performed as described (Bron and Venema, 1972). All enzymes used were from Roche Molecular Biochemicals, or Fermentas Life Sciences. The Polymerase Chain Reaction (PCR) was performed using Expand DNA polymerase (Roche) as previously described (van Dijl et al., 1995).
### Table 1: Plasmids and strains

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<td>pGDL48</td>
<td>pGDL41 derivative lacking the sipS gene; 7.5 kb; Km'</td>
<td>(Meijer et al., 1995)</td>
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<td>pCAy</td>
<td>pGDL48-derivative containing the tatAy gene; 7.0 kb; Ap'; Km'</td>
<td>(Jongbloed et al., 2004)</td>
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<td>pCCy</td>
<td>pGDL48-derivative containing the tatCy gene; 7.5 kb; Ap'; Km'</td>
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<td>(Guérout-Fleury et al., 1995)</td>
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<td>168</td>
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<tr>
<td>168 X-ywbN</td>
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<td>ΔtatAyCy-</td>
<td>X- trpC2; tatAyCy::Sp; amyE::xylA-ywbN-myc; Sp'; Cm'</td>
<td>This study</td>
</tr>
<tr>
<td>tatAc- X-ywbN</td>
<td>trpC2; tatAc::Cm; amyE::xylA-ywbN-myc; Sp'; Cm'</td>
<td>(Jongbloed et al., 2004)</td>
</tr>
<tr>
<td>Δtat total X-ywbN</td>
<td>X-ywbN trpC2; tatAyCy::Sp; tatAc::Em; tatAdCd::Km amyE::xylA-ywbN-myc; Sp'; Em'; Cm'</td>
<td>(Jongbloed et al., 2004)</td>
</tr>
<tr>
<td>ΔtatAd</td>
<td>trpC; tatAd::Em; Em'</td>
<td>(Jongbloed et al., 2002)</td>
</tr>
<tr>
<td>ΔtatCd</td>
<td>trpC2; tatCd::Cm; previously referred to as ΔtatCd(Cm)</td>
<td>(Jongbloed et al., 2002)</td>
</tr>
<tr>
<td>Δtat total</td>
<td>trpC2; tatAyCy::Sp; tatAc::Em; tatAdCd::Cm; Sp'; Ery'; Cm'; previously referred to as total-tat</td>
<td>(Jongbloed et al., 2002)</td>
</tr>
</tbody>
</table>
Construction of complementation plasmids pCAd, pCCd, pCACd and pCAc

To construct plasmid pCAd, the tatAd gene was amplified from the *B. subtilis* 168 chromosome by PCR using primers JW05Ad2 (5’-ACG CGT CGA CGA ATT AAG GAG TGG-3’) and JW06Ad2 (5’-GGA ATT CCG GTG TCT GCC TCA TCA GC-3’). The amplified fragment was cleaved with Sall and EcoRI, and cloned into the corresponding sites of pGDL48 (Meijer et al., 1995), resulting in pCAd. To construct plasmid pCCd, the tatCd gene was amplified by PCR using primers CdfwdSal (5’-ACG CGT CGA CGA AAG GGA GGG CTT TTT TG-3’) and CdrevEco (5’-GGA ATT CGA AGT CAC CGG GTG GTA CG-3’). The amplified fragment was cleaved with SalI and EcoRI, and cloned into the corresponding sites of pGDL48, resulting in pCCd. To construct plasmid pCACd, the tatAd-tatCd region was amplified by PCR using primers JW05Ad2 (5’-ACG CGT CGA CGA ATT AAG GAG TGG-3’) and CdrevEco (5’-GGA ATT CGA AGT CAC CGG GTG GTA CG-3’). The amplified fragment was cleaved with Sall and EcoRI, and cloned into the corresponding sites of pGDL48, resulting in pCACd.

Construction of *Bacillus subtilis ΔtatAy X-ywbN*

To construct *B. subtilis ΔtatAy X-ywbN*, the promoter region of tatAy was fused to the tatCy gene and the resulting cassette was introduced into the thrC locus of *B. subtilis* strain ΔtatAyCy X-ywbN (Jongbloed et al., 2004). Amplification of the upstream region of tatAy was realised using primers REAy-up-BamHI-F (5’-CGC GGA TCC GGA AAA CGC TTG ATC AGG ATG-3’) and REAy-up-Esp3I-R (5’-CGC GTC TCG ATT TGG GCT CCT CCT TTC CC-3’). The tatCy gene was amplified using primers PAy-Cy-Esp3I-F (5’-CGC GTC TCG AAA TAT GAC ACG AAT GAA AGT GAA TTC-3’) and RECy-down-HindIII-R (5’-CCC AAG CTT CTT TGC CGT AGG GTG CAT C-3’). The amplified fragments were cleaved with Esp3I. Cleavage of the tatCy PCR product with Esp3I created a 5’ overhang compatible with the 3’ overhang of the Esp3I-cleaved PCR product carrying the tatAy promoter region, allowing the production of a fusion of the respective promoter region with the tatCy gene. The resulting product was cleaved with BamHI and HindIII and cloned into the corresponding sites of pDG1664 (Guérout-Fleury et al., 1996), resulting in pTHR-P_{AytatCy}. Finally, strain ΔtatAy X-ywbN was obtained by ectopic integration of the P_{AytatCy} cassette into the thrC locus of *B. subtilis* 168 ΔtatAyCy X-ywbN via a double crossover event. Correct integration of the P_{AytatCy} cassette was verified by analysing growth on minimal medium (Bran and Venema, 1972) with or without the addition of threonine.

Construction of *Bacillus subtilis ΔtatAd*

To construct the ΔtatAd strain, a terminatorless erythromycin resistance cassette was amplified from pDG646 with primers Pery-Hind-F (5’-CCC AAG CTT CTC TAG AGG ATC CTT TAA CTC TGG C-3’) and Ery-Hind-R (5’-CGG AAG CTT TTA CTT ATT AAA TTA TTT ATA GCT ATT GAA AAG AG-3’). The resulting PCR fragment was cleaved with HindIII and ligated into the corresponding site of pJAd1 (Jongbloed et al., 2004). The resulting plasmid pJRad was checked for correct orientation of the erythromycin cassette using a SacI restriction analysis and used for transformation of *B. subtilis* 168. Finally, *B. subtilis* 168 ΔtatAd strain was obtained by a double crossover recombination event between the erythromycin-disrupted tatAd gene on pJRad and the chromosomal tatAd gene. Consequently, the downstream tatCd gene was placed under the control of the P_{ery} promoter.

Protein techniques

To detect YwbN-myc and PhoD, medium and cellular fractions were prepared as described previously (Jongbloed et al., 2000). After separation by SDS-polyacrylamide gel electrophoresis, proteins were transferred to a polyvinylidene-difluoride (PVDF) membrane (Molecular Probes Inc.) as described (Towbin et al., 1979). YwbN was detected with specific antibodies against the C-
terminal myc epitope (Clontech Laboratories, Inc.) and horse radish peroxidase-conjugated sheep anti-mouse antibodies (Amersham Biosciences) according to the manufacturer's instructions. PhoD was detected with specific antibodies against PhoD (kindly provided by J Müller) and horse radish peroxidase-conjugated donkey anti-rabbit antibodies (Amersham Biosciences) according to the manufacturer's instructions.

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