Relaxed Specificity of the \textit{Bacillus subtilis} TatAdCd Translocase in Tat-Dependent Protein Secretion\textsuperscript{\textdagger}

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Protein translocation via the twin arginine translocation (TAT) pathway is characterized by the translocation of prefolded proteins across the hydrophobic lipid bilayer of the membrane. In \textit{Bacillus subtilis}, two different Tat translocases are involved in this process, and both display different substrate specificities: 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 the YwbN precursor. 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 toward the acceptance of various Tat-dependent proteins.

The translocation of proteins across the 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 characterized by an R/K-R-X-\textdagger-

Protein translocation via this pathway has been extensively studied mainly in the thylakoids of plants and the gram-negative bacterium \textit{Escherichia coli} (reviewed in references 5, 20, and 25). 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 (9, 24, 33). In both \textit{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 (1), whereas TatA is thought to form a protein translocation channel (10, 19). These three proteins are known to interact with and be dependent on each other, ensuring the formation of a stable core complex (21). Additionally, in \textit{E. coli}, a fourth Tat component (TatE) has been identified that shows high similarity to TatA. This protein is able to substitute for TatA in the translocation of several Tat substrates (27).

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 (17). All gram-positive Tat systems, with the exception of those in \textit{Streptomyces} species, lack the TatB component. Moreover, most contain multiple paralogues of TatA and TatC. Recently, complementation studies with TatA of \textit{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 \textit{E. coli} (4).

To obtain further insight into the working mechanism of the gram-positive Tat secretion system, including differences from and similarities to the secretion systems of gram-negative bacteria and thylakoids in chloroplasts, the gram-positive soil bacterium \textit{Bacillus subtilis} has been adopted as a model organism for these studies. Within \textit{B. subtilis}, three variants of the TatA protein have been identified, TatAd, TatAy, and TatAc. TatAd has been shown to form an active complex with one of the two \textit{B. subtilis} TatC proteins, namely, TatCd. The genes encoding these proteins are located in the \textit{phoD} operon of the \textit{pho} regulon, which is only expressed during phosphate starvation growth conditions (16). PhoD, a phosphodiesterase of which the corresponding gene is located directly upstream of the \textit{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 (15). The function of the third TatA protein (TatAc) is unknown, although it has been established that this protein is not required for the translocation of the two Tat-dependent substrates identified to date (15). Previous complementation studies with \textit{E. coli} have already confirmed the difference in substrate specificity between the two Tat translocases of \textit{B. subtilis}. In a \textit{tat} deletion strain of \textit{E. coli}, the secretion of the Tat-dependent trimethylamine N-oxide reductase TorA could only be restored when TatAdCd of \textit{B. subtilis} was offered to the cell. Although shown to be active in the secretion of several other Tat-dependent proteins of \textit{E. coli}, TatAyCy was unable to facilitate the secretion of TorA (4).

Even though the difference in substrate specificity between TatAdCd and TatAyCy has been clearly documented, it is so far unknown how these translocases recognize their substrates.
and whether substrate recognition and the formation of a functional translocase are restricted to the specific TatAdCd and TatAyCy combinations only. Therefore, to shed new light on the working mechanisms and specificities of these two Tat translocases, complementation studies were performed in several B. subtilis tat mutant backgrounds, and the effect on 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 facilitates the secretion of YwbN in the absence of its specific TatAyCy translocase. In contrast, no secretion of TatCd-dependent PhoD was observed upon the absence of its specific TatAyCy translocase. In this study, no TatAyCy translocase is able to transport YwbN to the extracellular medium. Furthermore, our results show that the overproduction of TatAyCy combinations only. Therefore, to shed new light on the working mechanisms and specificities of these two Tat translocases, complementation studies were performed in several B. subtilis tat mutant backgrounds, and the effect on 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 facilitates the secretion of YwbN in the absence of its specific TatAyCy translocase. In contrast, no secretion of TatCd-dependent PhoD was observed upon the absence of TatAy and TatCy in the absence of the TatAdCd component. These findings demonstrate that TatAdCd is more tolerant in the acceptance of Tat-dependent substrates than TatAyCy and suggest that the TatA component plays a determining role in this process.

### 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 Bacto tryptone (1% [wt/vol]), Bacto yeast extract (0.5% [wt/vol]), and NaCl (1% [wt/vol]), unless indicated otherwise. The induction of a phosphate depletion response was achieved by overnight growth in high-phosphate depletion medium and dilution and subsequent growth in low-phosphate depletion medium (LPDM). Both media were prepared as described by Müller et al. (23). When required, media were supplemented with erythromycin (5 μg/ml), kanamycin (10 μg/ml), chloramphenicol (5 μg/ml), tetracycline (6 μg/ml), and spectinomycin (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 the transformation of E. coli were performed as described by Sambrook et al. (26). The transformation of B. subtilis subtilis was performed as described by Bron and Venema (8). All enzymes were from Roche Molecular Biochemicals or Fermentas Life Sciences. The PCR was performed using Expand DNA polymerase (Roche) as previously described by van Dijl et al. (31).

#### Construction of complementation plasmids pCAd, pCCd, pCACd, and pAac.
To construct plasmid pCAd, the tatAd gene was amplified from the B. subtilis 168 chromosome by PCR using primers JW05Ad2 (5'-CAC CGG GTG GTA CG-3') and EcoRI and cloned into the corresponding sites of pGDL48 (22), resulting in pCAd. To construct plasmid pCCd, the tatCd gene was amplified by PCR using primers CdfwdSal (5'-ACA CGT CGA CAA GGA GAG GGG CTT TTT TG-3') and CdfwdSal (5'-ACA CGT CGA CAA GGA GAG GGG CTT TTT TG-3') and cloned into the corresponding sites of pGDL48, resulting in pCCd. To construct plasmid pCACd, the tatCd gene was amplified by PCR using primers CAC CGG GTG GTA CG-3') and ClmwdCac (5'-GGA ATT CCG GTG TCT GCC TCA GC-3') and ClmwdCac (5'-GGA ATT CCG GTG TCT GCC TCA GC-3') and into the corresponding sites of pGDL48, resulting in pCACd. To construct plasmid pCAdC, the tatAc gene was amplified by PCR with primers JW09Ac2 (5'-CAC CGG GTG GTA CG-3') and ClmwdCac (5'-GGA ATT CCG GTG TCT GCC TCA GC-3') and ClmwdCac (5'-GGA ATT CCG GTG TCT GCC TCA GC-3') and into the corresponding sites of pGDL48, resulting in pCAdC.

#### TABLE 1. Plasmids and strains used in this study

<table>
<thead>
<tr>
<th>Plasmids and strains</th>
<th>Relevant properties</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>pGDL48</td>
<td>pGDL41 derivative lacking the sipS gene and containing an mcs; 7.5 kb; Km'</td>
<td>22</td>
</tr>
<tr>
<td>pCAy</td>
<td>pGDL48 derivative containing the tatAy gene; 7.0 kb; Ap'; Km'</td>
<td>15</td>
</tr>
<tr>
<td>pCCy</td>
<td>pGDL48 derivative containing the tatCy gene; 7.5 kb; Ap'; Km'</td>
<td>15</td>
</tr>
<tr>
<td>pCACy</td>
<td>pGDL48 derivative containing the tatAy-tatCy operon; 7.7 kb; Ap'; Km'</td>
<td>15</td>
</tr>
<tr>
<td>pCCd</td>
<td>pGDL48 derivative containing the tatCd gene; 7.5 kb; Ap'; Km'</td>
<td>This study</td>
</tr>
<tr>
<td>pCACd</td>
<td>pGDL48 derivative containing the tatAd-tatCd operon; 7.7 kb; Ap'; Km'</td>
<td>This study</td>
</tr>
<tr>
<td>pCAd</td>
<td>pGDL48 derivative containing the tatAd gene; 7.0 kb; Ap'; Km'</td>
<td>This study</td>
</tr>
<tr>
<td>pDG646</td>
<td>Plasmid carrying the Em resistance cassette for Bacillus; 4.3 kb; Ap'; Em'</td>
<td>12</td>
</tr>
<tr>
<td>pDG1664 (Promotes ectopic integration into the thrC locus)</td>
<td>6.0 kb; Em'; Sp'</td>
<td>11</td>
</tr>
<tr>
<td>pTHR-P(tatAc)</td>
<td>pDG1664 derivative containing a p_tatAc-tatCy fusion; 7.5 kb; Em'; Sp'</td>
<td>This study</td>
</tr>
<tr>
<td>pJAd1</td>
<td>pJAd1 derivative containing the tatAd gene; Ap'</td>
<td>15</td>
</tr>
<tr>
<td>pJRAd</td>
<td>pJAd1 derivative containing flanking regions of tatAd disrupted by a terminator-less Em resistance cassette; 5.8 kb; Ap'; Em'</td>
<td>This study</td>
</tr>
</tbody>
</table>

a Ap', ampicillin resistance; Km', kanamycin resistance; Em', erythromycin resistance; Sp', spectinomycin resistance; Cm', chloramphenicol resistance.
3'). The amplified fragment was cleaved with Sall and EcoRI and cloned into the corresponding sites of pGDL48, resulting in pCAc.

Construction of Bacillus subtilis ΔtatAy X-erbB. To construct B. subtilis ΔtatAy X-erbB, the promoter region of X-erbB was fused to the tatCy gene, and the resulting cassette was introduced into the thrC locus of the B. subtilis ΔtatCy X-erbB strain (15). The amplification of the upstream region of tatAy was realized using primers REAy-esp3I-F (5'-CGG TCG ATT TGG CTT CCT CCC C-3') and REAy-esp3I-R (5'-GGT GTC TCG TTG CCT CTC CTT TCC-3'). The amplified fragment was cloned with Esp3I. Cleavage of the tatAy 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 tatAy gene. The resulting product was cloned with BamHI and HindIII and cloned into the corresponding sites of pDG1664 (11), resulting in pTHR-P-plexo-Cy. Finally, the TatAy X-erbB strain obtained was by the ectopic integration of the P_erm-tatCy cassette into the thrC locus of B. subtilis 168 ΔtatAy X-erbB via a double crossover event. The correct integration of the P_erm-tatCy cassette was verified by analyzing its growth on minimal medium (8) with or without the addition of threonine.

Construction of Bacillus subtilis ΔtatAd. To construct the ΔtatAd strain, a terminator-less erythromycin resistance cassette was amplified from pDG646 with primers Pery-Hind-F (5'-CCG AAT CTT TCA TAG AGT ATT CTA CTT TAA-3') and REy-Hind-R (5'-CGG TAG ACC TAG AAA AAG GAG GTT CCG ACC GGA AAA AAG AG-3'). The resulting PCR fragment was cleaved with HindIII and ligated into the corresponding site of pBAD15. The resulting plasmid, pRyAD, was checked for the correct orientation of the Em' cassette using a SacI restriction analysis and used for the transformation of B. subtilis 168. Finally, the B. subtilis 168 ΔtatAd strain was obtained by a double crossover recombination event between the Em'-disrupted tatAd gene on pBAD and the chromosomal tatAd gene. Consequently, the downstream tatCd gene was placed under the control of P_erm.

Protein techniques. To detect YwbN-myc and PhoD, the medium and cellular fractions were prepared as described previously (16). After separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred to a polyvinylidene difluoride membrane (Molecular Probes, Inc.) as described previously (30). YwbN was visualized with specific antibodies against PhoD (kindly provided by J. Müller) and horseradish peroxidase-conjugated goat anti-mouse antibodies (Amersham Biosciences) according to the manufacturer's instructions. PhoD was visualized with specific antibodies against PhoD (kindly provided by J. Müller) and horseradish peroxidase-conjugated goat anti-rabbit antibodies (Amersham Biosciences) according to the manufacturer's instructions.

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 bearing operons were introduced into tat mutant strains as described before (15). Next, overnight TY cultures of wild-type and tat deletion strains, with or without complementation plasmids, were diluted 100-fold in fresh TY medium. The strains were grown to exponential growth phase, at which time YwbN-myc production was induced by the addition of 1% xylose. After 3 h of induction, the cells were harvested by centrifugation, separating the cellular fractions from the extracellular medium fractions. In order to analyze the production and secretion of YwbN-myc, SDS-PAGE samples were prepared as described in Materials and Methods and analyzed by SDS-PAGE, Western blotting, and immunodetection with antibodies raised against the c-Myc epitope. Importantly, an analysis of the presence of this protein in the cellular fractions demonstrated the production of YwbN-myc in all the strains studied (data not shown). Notably, no significant accumulation of the YwbN precursor (pre-YwbN) was observed in these fractions.

TatAy has been shown to be a key component in the secretion of YwbN, as deletion of this vital component completely abolishes YwbN secretion (Fig. 1A, lane ΔAy). Since the deletion of tatCy results in the same effect (Fig. 1B, lane ΔCy) and because the genes are organized 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 (15). Notably, the absence of (pre-)YwbN in these fractions implies that these tat mutant strains do not show an increased sensitivity to lysis, as was also described previously for B. subtilis (14) and in contradiction to what was observed for tat mutants of E. coli (29). Moreover, an analysis of the presence of the cytoplasmic protein DnaJ demonstrated the absence of this protein in medium fractions of wild-type and tatAy mutant strains, indicating that tat mutant strains are not more sensitive to lysis (data not shown). To study the putative complementation of tatAy or tatCy deletions, individual Tat components were introduced into the tatAy and tatCy mutant strains expressed from pGDL48 derivatives. YwbN secretion into the extracellular medium was visualized using c-Myc-specific antibodies.

As expected, YwbN secretion was restored in both the tatAy and the tatCy deletion strains by the production of plasmid-borne TatAy or TatCy, respectively (Fig. 1A, lane Ay, and Fig. 1B, lane Cy), as well as by complementing the tatAy and tatCy deletion strains with the complete TatAyCy complex (Fig. 1A and B, lane Ay/Cy). Strikingly, plasmid-borne TatAd is also able to restore YwbN secretion in the tatAy deletion strain (Fig. 1A, lane Ad). It should be noted that during these experimental growth conditions in rich TY medium, the endogenous tatAd and tatCd genes are not expressed. Therefore, these observations suggest that TatAd is able to form an active complex with endogenous TatCy, resulting in the secretion of YwbN at levels comparable to those during 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 (Fig. 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 (Fig. 1B, lane Cd). However, the introduction of TatAdCd into the tatCy mutant strain does restore YwbN secretion (Fig. 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 Fig. 1C, lanes AdCd, demonstrating that even in the absence of both TatAy and TatCy (the ΔtatCyC strain), the constitutive production of TatAdCd can restore the secretion of YwbN.

TatAdCd can only aid in the secretion of YwbN when over-produced. 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 Em' promoter of pGDL48, the production of the TatAd and TatCd components is most likely increased. This is also demonstrated by the fact that introducing constitutively expressed TatAyCy into the \( \Delta \)tatAy and \( \Delta \)tatCy mutant strains mostly results in an increase in YwbN secretion (Fig. 1A, B, and C, lanes wt). To examine whether TatAdCd is also able to complement strain (Fig. 1A, B, and C, lanes AyCy) compared to that in the parental strains mostly results in an increase in YwbN secretion (Fig. 1A, B, and C, lanes wt). (A) Bacillus subtilis \( \Delta \)tatAy (lane \( \Delta \)Ay) with xylose-induced YwbN-myc complemented by plasmid-borne TatAyC (lane AyCy), TatAd (lane Ad), TatAdCd (lane AdCd), and TatAc (lane Ac). (B) Bacillus subtilis \( \Delta \)tatCy (lane \( \Delta \)Cy) with xylose-induced YwbN-myc complemented by plasmid-borne TatCy (lane Cy), TatAyCy (lane AyCy), TatCd (lane Cd), and TatAdCd (lane AdCd). (C) Bacillus subtilis \( \Delta \)tatAyCy (\( \Delta \)AyCy) and \( \Delta \)tatAyCy-tatAc (\( \Delta \)AyCyAc) with xylose-induced YwbN-myc (X-ywbN) complemented by plasmid-borne TatAyC (lane AyCy) or TatAdCd (lane AdCd). Noncomplemented strains are indicated with a - symbol.

FIG. 1. YwbN secretion in several \( \Delta \)tat mutant backgrounds, complemented by various Tat components. The secretion of YwbN-myc (indicated on the right) into the extracellular medium was visualized using specific antibodies against the C-terminal myc tag. The size of the protein is indicated by marker bands on the left (in kDa). The secretion of YwbN-myc in the B. subtilis 168 X-ywbN-myc parental strain is shown in the first lane (wt). (A) Bacillus subtilis \( \Delta \)tatAy (lane \( \Delta \)Ay) with xylose-induced YwbN-myc complemented by plasmid-borne TatAyC (lane AyCy), TatAd (lane Ad), TatAdCd (lane AdCd), and TatAc (lane Ac). (B) Bacillus subtilis \( \Delta \)tatCy (lane \( \Delta \)Cy) with xylose-induced YwbN-myc complemented by plasmid-borne TatCy (lane Cy), TatAyCy (lane AyCy), TatCd (lane Cd), and TatAdCd (lane AdCd). (C) Bacillus subtilis \( \Delta \)tatAyCy (\( \Delta \)AyCy) and \( \Delta \)tatAyCy-tatAc (\( \Delta \)AyCyAc) with xylose-induced YwbN-myc (X-ywbN) complemented by plasmid-borne TatAyC (lane AyCy) or TatAdCd (lane AdCd). Noncomplemented strains are indicated with a - symbol.

FIG. 2. Secretion of YwbN in several \( \Delta \)tat mutant backgrounds when grown in LPDM. Cells were grown in LPDM as described in Materials and Methods to induce the expression of the phoD operon, including phoD, tatAd, and tatCd. Shortly after reaching transition phase, the cells were separated from the growth medium by centrifugation as described in Materials and Methods. Next, the presence of PhoD in the cellular fractions of the above-mentioned
strains was verified by an 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 into the extracellular medium was monitored by applying a similar approach. As shown in Fig. 3, PhoD is only secreted into the medium when the absence of the TatAd and TatCd components is restored by the reintroduction of the respective TatAd and/or TatCd components expressed from a plasmid (Fig. 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 (Fig. 3A and B, lanes Ay, Cy, and AyCy). Although the complementation by plasmid-borne TatAd in a ΔtatAd mutant background is less efficient compared to wild-type secretion levels (Fig. 3A, lane Ad), these experiments were repeated several times and showed a reproducible secretion pattern. In none of the cases was complementation by TatAy observed. These results show that, unlike previously described for TatAdCd, the TatAyCy complex is not able to complement the absence of TatAdCd components and suggest that TatAyCy is less substrate tolerant than TatAdCd.

TatAc does not actively participate in Tat-dependent protein translocation. The third tat 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 (Fig. 1A, lane Ac, and Fig. 3A, lane Ac).

DISCUSSION

The two Tat translocases of Bacillus subtilis are known to display different substrate specificities, in which YwbN is specifically secreted via TatAyCy and PhoD via TatAdCd (15). To obtain more insight into the 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 supplementation in the 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 (Fig. 1A, lane Ad). In contrast, the absence of TatCy could not be complemented by tatCd expression from the pCCd plasmid (Fig. 1B, lane Cd). Nevertheless, it seems that YwbN translocation can be facilitated by a TatAdCd complex in the absence of the TatAy and/or TatCy components, since YwbN was shown to be secreted in ΔtatCy and ΔtatAyCy(Ad) mutant strains that are complemented by the overproduction of the TatAd and TatCd components (Fig. 1B, lane AdCd, and Fig. 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 (4). Notably, complementation by TatAdCd in E. coli as well as in B. subtilis was only observed when excess amounts of the TatAd and TatCd components were offered to the cells (Fig. 1 and 2) (4). 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 into the substrate specificity and complex formation of Tat components of 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 facilitate the secretion of PhoD, as the overproduction of TatCy in a ΔtatCd mutant background, in which TatAd is still produced, does not result in the presence of PhoD in the extracellular medium of this strain (Fig. 3B, lane Cy). Likewise, TatAy is unable to form.

FIG. 3. PhoD secretion in several tat mutant backgrounds, complemented by various Tat components. Cells were grown in LPDM to induce the expression of phoD and tatAdCd. The secretion of PhoD (indicated on the right) into the extracellular medium was visualized using specific antibodies against PhoD. The size of the protein is indicated by marker bands on the left (in kDa). Secretion of PhoD in the parental strain Bacillus subtilis 168 is indicated in the first lane (wt). (A) B. subtilis 168 ΔtatAd (lane Ad) complemented by plasmid-borne TatAd (lane Ad), TatAy (lane Ay), TatAc (lane Ac), or TatAdCd (lane AdCd). (B) B. subtilis 168 ΔtatCd and B. subtilis 168 Δtat total complemented by plasmid-borne TatCd (lane Cd), TatCy (lane Cy), TatAdCd (lane AdCd), or TatAyCy (lane AyCy). Noncomplemented strains are indicated with a − symbol.
Recent results of studies concerning gram-positive Tat complexes are recruited to form the translocation channel (6). Tat-dependent substrates (1, 7, 9) and that subsequently TatA complex is involved in the initial 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 TatAyC differs from the recently proposed working model for the TatAdCd-dependent translocation of PhoD by Westermann et al. (33) and Schreiber et al. (28). These authors favor a model in which cytosolic TatAd is involved in the initial recognition and binding of the double arginine-containing signal peptide of the PhoD precursor (pre-PhoD), after which this TatAd-pre-PhoD complex is targeted to a membrane-associated TatCd complex. This complex then assists in the membrane insertion of the cytosolic TatAd-pre-PhoD 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 translocase subunits of B. subtilis operate in a different manner, at least at the level of Tat substrate specificity. This hypothesis is illustrated in Fig. 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. In order to explain the observations described for our complementation studies, we propose, in concordance with the earlier findings of Westermann et al. (33), 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, since 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 YwbN, and not for PhoD, we propose that TatCy is the specificity determining factor in the TatAyCy complex and thus responsible for recognizing the signal sequence of YwbN (in a TatAd-pre-YwbN complex).
complex or separately). After recognizing 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 TatAd-TatCy 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 (2, 13). It is possible that the same regions, or even residues, within the TatCy protein are involved in the 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. This could consequently result in a preference of the TatAdCd translocase over the TatAyCy

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REFERENCES


