Two minimal Tat translocases in *Bacillus*

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Summary

Activity of the Tat machinery for protein transport across the inner membrane of *Escherichia coli* and the chloroplast thylakoidal membrane requires the presence of three membrane proteins: TatA, TatB and TatC. Here, we show that the Tat machinery of the Gram-positive bacterium *Bacillus subtilis* is very different because it contains at least two minimal Tat translocases, each composed of one specific TatA and one specific TatC component. A third, TatB-like component is apparently not required. This implies that TatA proteins of *B. subtilis* perform the functions of both TatA and TatB of *E. coli* and thylakoids. Notably, the two *B. subtilis* translocases named TatAdCd and TatAyCy both function as individual, substrate-specific translocases for the twin-arginine preproteins PhoD and YwbN, respectively. Importantly, these minimal TatAC translocases of *B. subtilis* are representative for the Tat machinery of the vast majority of Gram-positive bacteria, *Streptomycetes* being the only known exception with TatABC translocases.

Introduction

The Twin-arginine translocation (Tat) machinery for protein transport has attracted particular interest, because it is capable of transporting fully folded proteins across membranes of Gram-negative and Gram-positive bacteria, archaea, and chloroplasts (Mori and Cline, 2001; Robinson and Bolhuis, 2001; Yen *et al*., 2002; Berks *et al*., 2003; Dilks *et al*., 2003). These translocases derive their name from the fact that they transport proteins carrying twin-arginine (RR)-signal peptides with the motif R/K-R-X-ϕ-ϕ (ϕ is a hydrophobic residue; Cristóbal *et al*., 1999). Originally, this motif was defined as (S/T)-R-R-X-F-L-K (Berks, 1996).

Tat translocases of *Escherichia coli* and chloroplast thylakoids are presently best characterized. In these systems, the key components are the integral membrane proteins TatA, TatB and TatC (Tha4, Hcf106 and cpTatC in thylakoids). Several models have been presented for Tat translocase functioning. The general view is that TatB and TatC serve in RR-signal peptide reception, while TatB and TatC in complex with multiple TatA components form a protein-conducting channel (Cline and Mori, 2001; Alami *et al*., 2003). Consistently, Tat components in the thylakoidal membrane are present in two subcomplexes: an Hcf106-cpTatC (TatB-TatC) preprotein receptor complex and a Tha4-Hcf106-cpTatC (TatA-TatB-TatC) complex that is formed after preprotein recognition. Association of Tha4 with the other components requires a functional precursor and a ΔpH (Mori and Cline, 2002). Similarly, TatB and TatC of *E. coli* form a functional and structural unit that interacts with RR-signal peptides before translocation, and subsequently recruits multiple TatA molecules to form an active translocase. Preprotein interactions with TatA occur only upon initiation of translocation (Alami *et al*., 2003). Most likely, TatA molecules are recruited by TatB, as TatA and TatB were shown to interact (Bolhuis *et al*., 2000; de Leeuw *et al*., 2002). Importantly, the translocation of authentic RR-preproteins into the thylakoid lumen, or the periplasm of Gram-negative bacteria, such as *E. coli*, requires TatA (or TatE), TatB and TatC.

Phylogenetic analyses showed that many bacteria and archaea contain genes for multiple TatA, TatB and/or TatC-like proteins. In this respect, it should be noted that TatA and TatB show limited structural similarity. While paralogous TatA/B proteins are found in Gram-positive and Gram-negative bacteria and archaea, paralogous TatC proteins were only identified in Gram-positive bacteria (two TatC proteins) and archaea (up to three TatC proteins; Yen *et al*., 2002; Dilks *et al*., 2003). Specifically, the Gram-positive bacterium *Bacillus subtilis* contains two TatC proteins (TatCd and TatCy) and three TatA/B-like proteins (TatAd, TatAy and TatAc; Jongbloed *et al*., 2000). Two of the *tatA* genes (*tatAd* and *tatAy*) are organized in

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an operon-like structure with a *tatC* gene (*tatCd* and *tatCy*). Interestingly, TatCd and TatCy have distinct functions, as only TatCd was required for secretion of the phosphodiesterase PhoD (Jongbloed *et al*., 2000). A specific role for TatCy in Tat translocation was, thus far, not demonstrated. Remarkably, certain Gram-positive bacteria, such as *B. subtilis*, as well as certain archaea, have *tatA* and *tatC* genes, but lack a *tatB* gene (Dilks *et al*., 2003). In fact, some have only one *tatA* and one *tatC* gene. This suggests that the minimal Tat translocase is composed of TatA and TatC molecules only.

Our previous studies, involving proteomic approaches to identify Tat-dependent extracellular proteins, revealed that the *B. subtilis* Tat pathway is highly selective (Jongbloed *et al*., 2002); only one out of ~100 identified extracellular proteins, PhoD, was shown to be secreted *via* Tat. Therefore, we used controlled gene expression and epitope-tagging to identify additional Tat-substrates. Thus, the newly identified YwbN protein was shown to be secreted Tat-dependently. Moreover, using various *tat* mutant strains and the RR-proteins PhoD and YwbN as substrates, substrate specificities of the *B. subtilis* TatA and TatC translocase components were studied. Our findings demonstrate that two minimal Tat translocases are active in *B. subtilis*, each composed of specific TatA and TatC molecules.

Results

TatCy and TatAy-dependent YwbN secretion

In an approach to identify Tat-dependent *B. subtilis* preproteins other than PhoD, the 46 kDa YwbN preprotein was tagged with the c-Myc epitope (EQKLISEEDLN), as described in *Supplementary material*. YwbN was selected, because its signal peptide conforms to the most stringent criteria for predictions of genuine RR-signal peptides of *E. coli* (Jongbloed *et al*., 2000; 2002). The YwbN-myc encoding gene was placed under control of the xylose-inducible *xylA* promoter, and the resulting *xylA-ywbN-myc* cassette (X-*ywbN*) was integrated into the *amyE* gene of *B. subtilis* 168. To test YwbN-myc production, an overnight culture of the resulting strain 168 X-*ywbN* was diluted in fresh medium, grown for 3 h without and, subsequently, for 3 h with 1% xylose. As revealed by Western blotting and immunodetection with c-Myc specific antibodies, YwbN-myc was produced at detectable levels (Fig. 1). Both precursor and mature forms of YwbN-myc were identified in cells, and mature YwbN-myc was present in the medium (Fig. 1A). An unidentified, extracellular protein (protein X), migrating close to mature YwbN-myc during SDS-PAGE, cross-reacts with c-Myc specific antibodies. The detection of protein X depends on the batch of c-Myc antibody used.

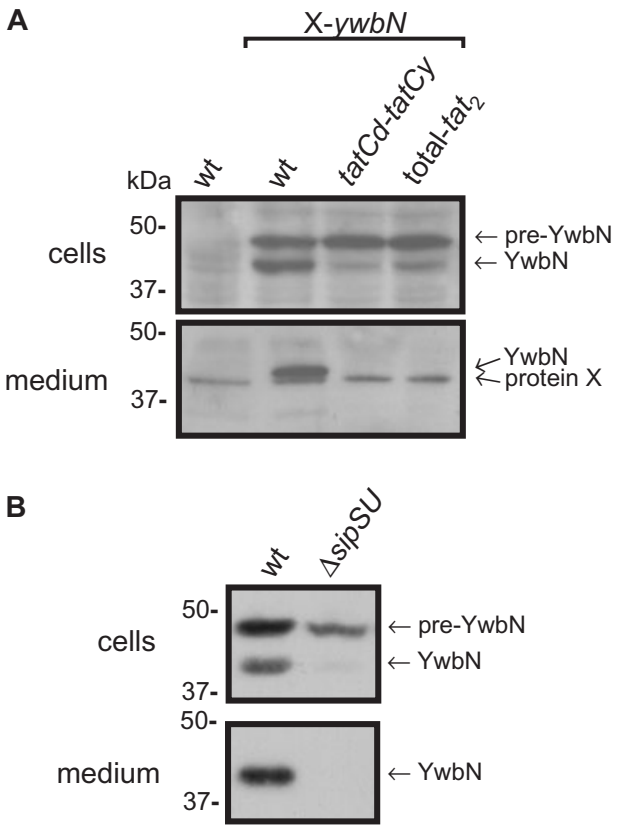

Fig. 1. Tat-dependent YwbN secretion. *B. subtilis* strains 168 (wt), 168 X-*ywbN*, *tatCd-tatCy* X-*ywbN*, and total-*tat*$_2$ X-*ywbN* (A), and strains 8G5 X-*ywbN* (wt) and Δ*sipSU* X-*ywbN* (B) were analysed as described in the *Experimental procedures* section. Precursor and mature forms of YwbN-myc are indicated. Protein X, extracellular *B. subtilis* protein, which is cross-reactive with c-Myc specific antibodies.

To test whether YwbN is secreted Tat-dependently, the X-*ywbN* cassette was introduced into *B. subtilis* strains *tatCd-tatCy*, lacking both *tatC* genes, and total-*tat*$_2$, lacking all *tat* genes. To study the effects of these mutations, cells of strains 168 X-*ywbN*, *tatCd-tatCy* X-*ywbN*, and total-*tat*$_2$ X-*ywbN* were analysed as described above. Western blotting showed significantly reduced levels of mature YwbN-myc in cellular fractions of *tat* mutants lacking both TatC or all known Tat components (Fig. 1A). Moreover, YwbN-myc was absent from the medium. These observations show that YwbN secretion is dependent on a functional Tat pathway.

To verify whether processing of preYwbN-myc to the mature form was catalysed by signal peptidases (SPases) that are required for the processing of genuine secretory proteins, the X-*ywbN* cassette was introduced in *B. subtilis* strains lacking one or more of the five chromosomal SPase-encoding *sip* genes. Single *sip* mutations did not affect processing of preYwbN-myc and secretion of mature YwbN-myc into the medium (not shown). However, mature YwbN-myc was completely absent from the

medium of a strain lacking SipS and SipU (Fig. 1B). Moreover, cellular fractions showed reduced levels of a mature product. These results show that translocation of pre-YwbN-myc across the membrane and release of the mature protein into the medium depends on a functional Tat pathway and either SipS or SipU.

Previous analyses showed that TatCd is specifically required for PhoD secretion (Jongbloed *et al.*, 2000). Therefore, we investigated whether one or both TatC components are involved in YwbN secretion. For this purpose, the X-*ywbN* cassette was introduced into *B. subtilis* strains carrying *tatCd* or *tatCy* mutations. The resulting strains were analysed as described above. Like the double *tatC* and total-*tat* mutants, the *tatCy* mutant did not secrete mature YwbN-myc into the medium, and only small amounts of mature YwbN-myc were detectable in cells (Fig. 2A). In contrast, significant amounts of mature YwbN-myc were present in cellular and medium fractions of the *tatCd* mutant and the parental strain 168. These observations show that TatCy, but not TatCd, has a critical role in YwbN secretion.

To investigate whether one or more TatA components are involved in YwbN secretion, single and multiple *tatA* mutants were constructed. The X-*ywbN* cassette was introduced into the resulting mutants (Table 1; see: *Supplementary material*, Table S2), and YwbN-myc secretion analysed. As shown by Western blotting, YwbN-myc was absent from the media of all strains lacking *tatAy* (Fig. 2B; note that small variations in extracellular YwbN levels of TatAyCy-containing strains can occur). In contrast, *tatAc* and *tatAd* single or double mutants secreted significant amounts of YwbN-myc into the medium. Consistently, *tatAy* mutant cells contained relatively small amounts of mature YwbN-myc compared to cells with an intact *tatAy*

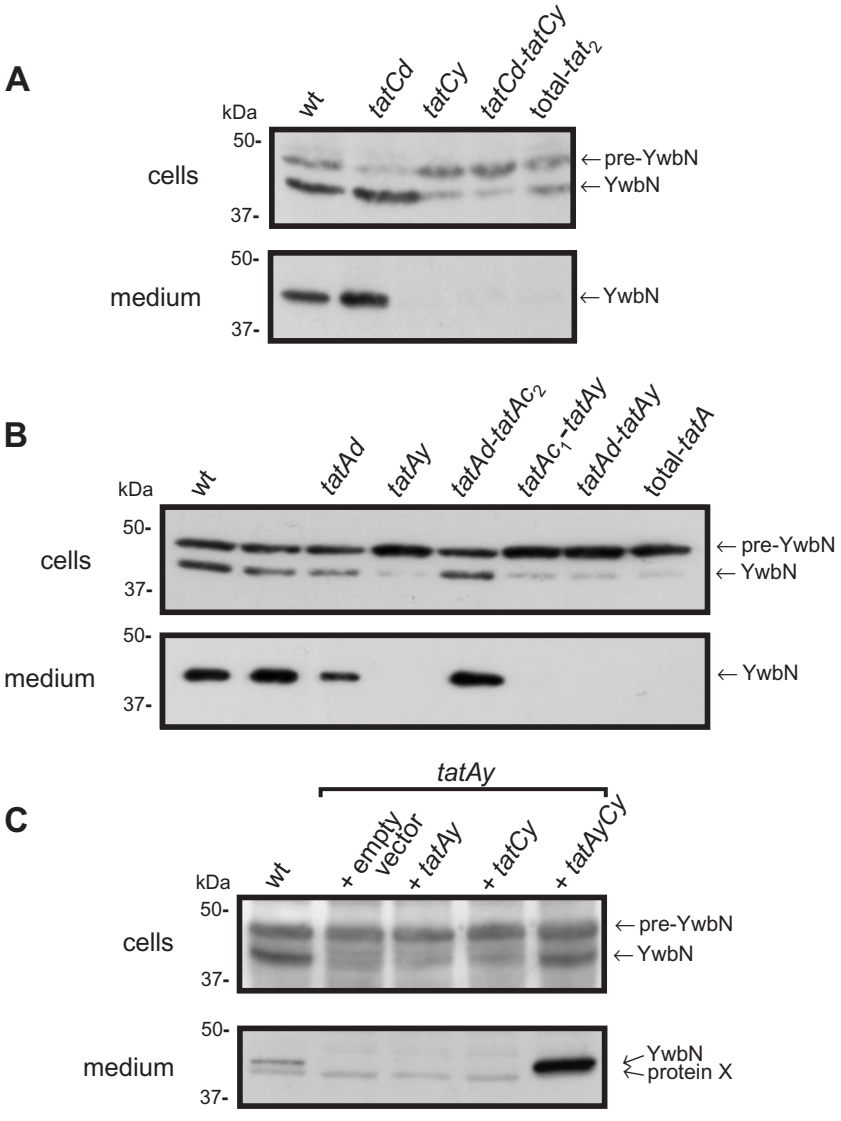

Fig. 2. TatAy and TatCy-dependent YwbN secretion. *B. subtilis* strains 168 (wt), *tatCd*, *tatCy*, *tatCd-tatCy*, and total-tat_2 (A), 168 (wt), $tatAc_1$, *tatAd*, *tatAy*, *tatAd-*$tatAc_2$, $tatAc_1$-*tatAy*, *tatAd-tatAy*, and total-*tatA* (B), and 168 (wt), *tatAy* (pGDL48; empty vector), *tatAy* (pCAy; *tatAy*), *tatAy* (pCCy; *tatCy*), and *tatAy* (pCACy; *tatAyCy*) (C), all containing the X-*ywbN* cassette, were analysed as described in the *Experimental procedures* section. Precursor and mature forms of YwbN-myc are indicated. Protein X, extracellular *B. subtilis* protein.

Table 1. Plasmids and strains.

	Relevant properties	Reference
Plasmids		
pX	Vector for the integration of genes in the *amyE* locus of *B. subtilis*; integrated genes transcribed from the *xylA* promoter; carries the *xylR* gene; 7.5 kb; Ap^r; Cm^r	Kim *et al.* (1996)
pXYNm1	pX-derivative; carries *ywbN-myc* downstream of the *xylA* promoter; 8.9 kb; Ap^r; Cm^r	This work
pGDL48	Contains multiple cloning site to place genes under the control of the erythromycin promoter; 6.8 kb; Ap^r; Km^r	Tjalsma *et al.* (1998)
pCAy	pGDL48-derivative containing the *tatAy* gene; 7.0 kb; Ap^r; Km^r	This work
pCCy	pGDL48-derivative containing the *tatCy* gene; 7.5 kb; Ap^r; Km^r	This work
pCACy	pGDL48-derivative containing the *tatAy-tatCy* operon; 7.7 kb; Ap^r; Km^r	This work
Strains		
B. subtilis		
168	*trpC2*	Kunst *et al.* (1997)
tatAc	*trpC2*; *tatAc*::Em; Em^r, previously referred to as Δ*tatAc*	Jongbloed *et al.* (2002)
$tatAc_1$	*trpC2*; *tatAc*::Km; Km^r	This work
$tatAc_2$	*trpC2*; *tatAc*::Tc; Tc^r	This work
tatAd	*trpC2*; *tatAd*::Km; Km^r	This work
tatAy	*trpC2*; *tatAy*::Em; Em^r	This work
tatAd-tatAc$_2$	*trpC2*; *tatAd*::Km; *tatAc*::Tc; Km^r; Tc^r	This work
$tatAc_1$-*tatAy*	*trpC2*; *tatAc*::Km; *tatAy*::Em; Km^r; Em^r	This work
tatAd-tatAy	*trpC2*; *tatAd*::Km; *tatAy*::Em; Km^r; Em^r	This work
total-*tatA*	*trpC2*; *tatAd*::Km; *tatAc*::Tc^r; *tatAy*::Em^r; Km^r; Tc^r; Em^r	This work
tatCd	*trpC2*; *tatCd*::Km; Km^r; previously referred to as Δ*tatCd*	Jongbloed *et al.* (2000)
tatCy	*trpC2*; *tatCy*::Sp; Sp^r; previously referred to as Δ*tatCy*	Jongbloed *et al.* (2000)
tatCd-tatCy	*trpC2*; *tatCd*::Km; *tatCy*::Sp; Km^r; Sp^r; previously referred to as Δ*tatCd*-Δ*tatCy*	Jongbloed *et al.* (2000)
tatAdCd	*trpC2*; *tatAd-tatCd*::Km; Km^r	This work
tatAyCy	*trpC2*; *tatAy-tatCy*::Sp; Sp^r; previously referred to as Δ*tatAyCy*	Jongbloed *et al.* (2002)
tatAdCd-tatAc$_2$	*trpC2*; *tatAd-tatCd*::Km; *tatAc*::Tc; Km^r; Tc^r	This work
tatAc-tatAyCy	*trpC2*; *tatAc*::Em; *tatAy-tatCy*::Sp; Em^r; Sp^r; previously referred to as Δ*tatAc*-Δ*tatAyCy*	Jongbloed *et al.* (2002)
tatAyCy-tatAc$_2$	*trpC2*; *tatAy-tatCy*::Sp; *tatAc*::Tc; Sp^r; Tc^r	This work
total-tat_2	*trpC2*; *tatAc*::Em; *tatAy-tatCy*::Sp; *tatAd-tatCd*::Km; Em^r Sp^r; Km^r	This work
8G5	like 168; *tyr*; *his*; *nic*; *ura*; *rib*; *met*; *ade*	Tjalsma *et al.* (1998)
Δ*sipSU*	like 168; *tyr*; *his*; *nic*; *ura*; *rib*; *met*; *ade*; *sipS; sipU*; previously referred to as ΔSU	Tjalsma *et al.* (1998)
all strains designated X-*ywbN*	*amyE*::*xylA-ywbN-myc*; Cm^r	This work

gene. These findings indicate that TatAy, but not TatAc or TatAd, is required for YwbN secretion.

As *tatCy* is located downstream of *tatAy*, the replacement of the latter might have polar effects on *tatCy* transcription and thus TatCy production. To test which gene(s) of the *tatAy-tatCy* region can restore YwbN export in the *tatAy* mutant, a *trans* complementation analysis was performed with the *tatAy*, *tatCy*, or *tatAy* plus *tatCy* genes. As shown in Fig. 2C, introduction of the plasmid-borne *tatAy-tatCy* region in the *tatAy* mutant did not only restore secretion of YwbN-myc, but also resulted in significantly increased extracellular levels of this protein (compare also the relative levels of YwbN and protein X with those in Fig. 1A). In contrast, plasmid-borne copies of *tatAy* or *tatCy* did not restore YwbN-myc secretion, showing that the expression of *tatCy* is affected in *tatAy* mutants. These findings show that TatAy and TatCy are indispensable for YwbN secretion.

TatAy and TatCy are sufficient for YwbN secretion

We next raised the question whether TatAy and TatCy are sufficient for the export of YwbN. To answer this question, *tatAd-tatCd* double and *tatAd-tatCd tatAc* triple mutants were constructed. As controls, *tatAy-tatCy* double and *tatAy-tatCy tatAc* triple mutants were constructed. Next, the X-*ywbN* cassette was introduced in these strains and YwbN-myc secretion was analysed. As shown by Western blotting, the simultaneous disruption of *tatAd-tatCd* (not shown), or *tatAd-tatCd* and *tatAc* (Fig. 3), did not block YwbN-myc secretion into the medium. Consistently, cells of the *tatAd-tatCd* (not shown) and *tatAd-tatCd tatAc* mutants contained significant amounts of mature YwbN-myc (Fig. 3). As expected, the *tatAy-tatCy* double and the *tatAy-tatCy tatAc* triple mutants did not secrete YwbN-myc (not shown). These results show that TatAy and TatCy are sufficient for YwbN secretion.

TatAd and TatCd are sufficient for PhoD secretion

Previous studies showed that TatCd is of major importance for PhoD secretion (Jongbloed *et al.*, 2000), and that TatAd also fulfils a role in membrane targeting and/or secretion of this protein (Pop *et al.*, 2002; 2003). To address the question whether TatAd and TatCd are sufficient for PhoD secretion, the export of PhoD by the *tatAy-*

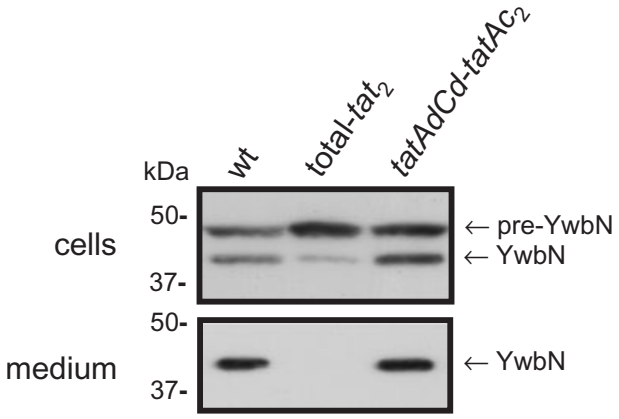

Fig. 3. TatAc, TatAd and TatCd are dispensable for YwbN secretion. *B. subtilis* strains 168 X-*ywbN* (wt), total-tat_2 X-*ywbN*, and *tatAdCd-tatAc*$_2$ X-*ywbN* were analysed as described in the *Experimental procedures* section. Precursor and mature forms of YwbN-myc are indicated.

tatCy tatAc triple mutant was studied. The mutant was grown under conditions of phosphate starvation, inducing *phoD* expression, and PhoD secretion was analysed by 2D gel electrophoresis. Figure 4 shows that mature PhoD was present in the media of both the *tatAy-tatCy tatAc* mutant and the parental strain 168. Moreover, the secretion of proteins lacking (genuine) RR-signal peptides was not affected by the *tat* mutation, as exemplified by the 'control spots' of WprA, LytD, XynD, YnfF, YwtD and Pel. This shows that TatAd and TatCd are sufficient for PhoD secretion. In conclusion, our present observations show that minimal Tat translocases of *B. subtilis* are composed of specific TatA and TatC molecules.

Discussion

All Tat systems characterized so far consist of TatA, TatB and TatC. Although phylogenetic analyses suggested that one TatA and one TatC component might be sufficient for Tat-dependent translocation (Yen *et al*., 2002; Dilks *et al*., 2003), the present studies on the *B. subtilis* Tat machinery demonstrate for the first time that minimal Tat translocases composed of specific TatA and TatC proteins are functional in this organism.

Epitope-tagging and controlled gene expression allowed the identification of YwbN as a novel Tat substrate of *B. subtilis*. Interestingly, gene expression studies performed within the *Bacillus* Systematic gene Function Analysis (BSFA) programme showed that *ywbN* is expressed at very low levels in the post-exponential growth phase (http://locus.jouy.inra.fr/cgi-bin/genmic/madbase_home.pl). This explains why YwbN was not previously identified on the extracellular proteome of *B. subtilis* 168. Consistent with the presence of a conserved RR-motif in its signal peptide ($R_{13}R_{14}D_{15}I_{16}L_{17}$), YwbN is secreted in a strictly Tat-dependent manner (for additional information on the YwbN protein: see *Supplementary material*, Fig. S1, Table S1). The release of mature YwbN into the medium requires activity of SipS or SipU. This shows for the first time that secretion of Tat-dependent proteins can depend on SPase activity, similar to proteins secreted *via* the general Sec pathway. The reason why some cell-associated mature YwbN is formed in the absence of a functional Tat machinery is unknown. It has been reported that low-level targeting of certain RR-precursors to the Sec pathway of *E. coli* and thylakoids can result in the initiation of their translocation and processing (Koussevitzky *et al*., 1998; Sanders *et al*., 2001). This does not seem to be the case for pre-YwbN, because the processing of this precursor in absence of a functional Tat machinery is not inhibited by the SecA inhibitor azide (our unpubl. obs.). Most likely, non-translocated preYwbN is subject to alternative cleavage by cytoplasmic proteases, resulting in cellular accumulation of mature YwbN.

Strikingly, *B. subtilis* contains two minimal TatA-TatC translocases with distinct specificities for the RR-substrates PhoD and YwbN. Previously, we showed that TatCd, but not TatCy, is required for PhoD secretion (Jongbloed *et al*., 2000). The present studies show that TatCy, but not TatCd, is required for YwbN secretion. Recent *in vitro* cross-linking studies with *E. coli* vesicles containing the *E. coli* Tat system indicate that TatC is the initial receptor for proteins with RR-signal peptides (Alami *et al*., 2003). It is therefore tempting to speculate that Tat pathway specificity in *B. subtilis* is determined by RR–signal peptide interactions with TatCd or TatCy. A completely new finding is that each of these TatC components requires a specific TatA component to form an active translocase: TatCd requires TatAd, and TatCy requires TatAy. This is different from the situation in *E. coli,* where the paralogous TatA and TatE interact with TatB-TatC units to form active translocases (Sargent *et al*., 1998). It is presently not known whether, under specific conditions, certain TatA and TatC proteins of *B. subtilis* 'cross-react' to form active translocases (e.g. TatAd with TatCy, or TatAy with TatCd). The fact that the *tatAc* gene is constitutively transcribed

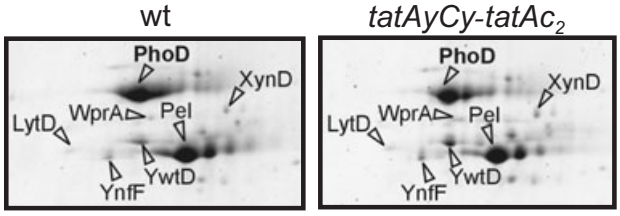

Fig. 4. TatAc, TatAy and TatCy are dispensable for PhoD secretion. *B. subtilis* strains 168 X-*ywbN* (wt) and *tatAyCy-tatAc*$_2$ X-*ywbN* were analysed as described in the *Experimental procedures* section. Only the relative positions of the PhoD, LytD, Pel, WprA, XynD, YnfF and YwtD spots are shown.

(not shown) implies that active TatAc-TatCy translocases and/or TatAc-TatCd translocases can be formed. Importantly, the present evidence for the activity of specific TatA-C translocases in *B. subtilis*, combined with previously documented evidence for an RR-receptor function of TatB-C in thylakoids and *E. coli* (Mori and Cline, 2002; Alami *et al*., 2003), suggests that particular TatA and TatC molecules form specificity determining RR-receptors of *B. subtilis* Tat translocases.

Finally, our present observation that minimal translocases composed of specific TatA and TatC molecules are active, suggests that a TatB-like component is not required for Tat translocase activity in *B. subtilis*. It thus appears that TatA proteins of *B. subtilis* perform the functions of both the TatA and TatB components of *E. coli* and thylakoids, although the involvement of a, yet unidentified, TatB analogue in *B. subtilis* can formally not be excluded. In this respect it is interesting to note that *B. subtilis* TatA proteins contain both the phenylalanine residue which is strictly conserved in TatA/E proteins of Gram-negatives (Phe20 in *E. coli* TatA), as well as the proline residue which is strictly conserved in TatB proteins of these organisms (Pro22 of *E. coli* TatB; Jongbloed *et al.* 2000; Robinson and Bolhuis, 2001; Barrett *et al*., 2003; Hicks *et al*., 2003). These properties make the *B. subtilis* Tat machinery different to that of *E. coli* and thylakoids, both at the structural and functional levels. Interestingly, *Streptomycetes* appear to be the only Gram-positive bacteria with a Tat machinery composed of TatA, TatB and TatC (Schaerlaekens *et al*., 2001; 2004). The vast majority of other Gram-positives, including important pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus* and *Mycobacterium tuberculosis* seem to contain minimal TatA-TatC systems as now identified in *B. subtilis* (Yen *et al*., 2002; Dilks *et al*., 2003). To date, the role of the Tat machinery of these pathogens in the secretion of virulence factors has not been assessed experimentally, but their genomes encode a variety of typical virulence factors with RR-signal peptides. Consequently, the Gram-positive Tat machinery appears to be an attractive potential target for novel anti-infectives. This makes the analysis of TatA-TatC translocases not only interesting from a fundamental scientific, but also from a biomedical point of view. For further progress in this area, the *B. subtilis* system provides excellent possibilities because its two minimal Tat translocases with distinct specificities will allow detailed analyses of determinants for Tat substrate specificity and critical interactions between specific TatA and TatC components.

Experimental procedures

Plasmids, bacterial strains and media

Table 1 lists plasmids and bacterial strains used. TY medium contained Bacto tryptone (1%), Bacto yeast extract (0.5%) and NaCl (1%). High phosphate (HPDM) and low phosphate (LPDM) defined media were prepared as described (Antelmann *et al*., 2000). When required, media for *E. coli* were supplemented with erythromycin (Em; 100 μg ml^{-1}), kanamycin (Km; 40 μg ml^{-1}), chloramphenicol (Cm; 5 μg ml^{-1}), tetracyclin (Tc; 12 μg ml^{-1}), or spectinomycin (Sp; 100 μg ml^{-1}); media for *B. subtilis* were supplemented with Em (1 μg ml^{-1}), Km (10 μg ml^{-1}), Cm (5 μg ml^{-1}), Tc (6 μg ml^{-1}) or Sp (100 μg ml^{-1}).

DNA techniques

Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of *E. coli* were carried out as described (Jongbloed *et al*., 2000). Enzymes were from Roche Molecular Biochemicals. *B. subtilis* was transformed as described (Tjalsma *et al*., 1998). PCR was carried out with Pwo DNA polymerase. Detailed descriptions of strain constructions are presented as *Supplementary material* (Fig. S2 and Table S2).

Protein techniques

To detect YwbN-myc, cells were separated from the medium by centrifugation. Proteins in the medium were concentrated 20-fold upon precipitation with trichloroacetic acid (TCA) and samples for SDS-PAGE were prepared as described (Jongbloed *et al*., 2000). After separation by SDS-PAGE, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Molecular Probes), and YwbN-myc was visualized with c-Myc specific antibodies (CLONTECH Laboratories) and horseradish peroxidase-conjugated goat antimouse antibodies (Amersham Biosciences).

Extracellular proteome analysis was performed as described (Antelmann *et al*., 2000). Briefly, *B. subtilis* strains were grown at 37°C under vigorous agitation in synthetic medium containing 0.16 mM KH_2PO_4 to induce a phosphate starvation response. After 1 h of post-exponential growth, cells were separated from the medium by centrifugation. Secreted proteins in the medium were TCA-precipitated, collected by centrifugation, and separated by 2D gel electrophoresis.

Acknowledgements

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Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi4341/mmi4341sm.htm

Appendix S1. The YwbN protein.

Appendix S2. Strain constructions.

Fig. S1. The *ywbLMN* operon. Schematic representation of the chromosomal organization of conserved *ywbLMN* oper-

ons from *B. subtilis, S. coelicolor* and *E. coli*. Homologous genes in the different operons are represented by arrows with identical levels of gray shading.

Fig. S2. Construction of *tatA* mutant strains of *B. subtilis*.

Table S1. Twin-arginine signal peptides of YwbN and YwbN-like proteins.

Table S2. Plasmids and strains.

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