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A Tat ménage à trois — The role of *Bacillus subtilis* TatAc in twin-arginine protein translocation

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ABSTRACT

The twin-arginine translocation system (Tat) is a protein transport system that moves fully folded and cofactor-containing proteins across membranes of bacteria, archaea and thylakoids. The minimal Tat pathway is composed of two subunits, TatA and TatC. In some organisms TatA has been duplicated and evolved to form a third specialized subunit, TatB. The *Bacillus subtilis* genome encodes two TatC subunits (TatCd and TatCy) and three TatA subunits (TatAd, TatAy and TatAc). These subunits combine to form two parallel minimal pathways, TatAy-TatCy and TatAd-TatCd. The purpose and role of the third TatA component, TatAc, has remained ambiguous. In this study we examined the translocation of two natively expressed TatAy-TatCy-dependent substrates, EfeB and QcrA, in various Tat-deficient genetic backgrounds. More specifically, we examined the ability of different mutated TatAy subunits to complement for the absence of wild-type TatAy. We further detailed a graded growth phenotype associated with the functional translocation of EfeB. We found that in various instances where specific amino acid substitutions were made in TatAy, a definite TatAc-associated growth phenotype occurred in genetic backgrounds lacking TatAc. Altogether, our findings show that TatAy and TatAc interact and that this TatAy–TatAc interaction, although not essential, supports the translocation of the Tat substrate EfeB when TatAy function is compromised. This implies that the third TatA-like protein in *B. subtilis* could represent an intermediate evolutionary step in TatA-TatB specialization.

1. Introduction

The Twin-arginine translocation system (Tat) is found in thylakoids, archaea, Gram-positive and Gram-negative bacteria where it facilitates the transport of fully folded and cofactor-attached proteins across membranes [1–3]. The proteins targeted to this system contain a defined N-terminal signal peptide with a characteristic twin-arginine (RR) motif [3,4]. The basic Tat system is composed of TatC and TatA-like proteins, where TatC is a relatively large integral membrane protein with six transmembrane domains (Fig. 1) [5,6]. In contrast, TatA-like proteins are smaller with a single transmembrane-spanning domain followed by a hinge region, an amphipathic helix and a densely charged C-terminal tail [7–11]. According to the current consensus model of Tat-dependent protein translocation, the translocation process is initiated when a substrate with the correct RR-signal peptide interacts with a docking complex composed of TatC and a TatA-like protein [12,13]. The docking complex that interacts specifically with the signal peptide [6,14–17], has been implicated in proofreading [3,18], and inserts the substrate into the membrane [19]. The docking complex then recruits the pore-forming TatA components [17,20] and the proton-motive force is used as an energy source for translocation [14,21,22].

Phylogenetic comparisons between bacterial, archaeal and thylakoidal Tat pathways have shown that the Tat system is most often composed of TatC and two sequence-diverged TatA-like proteins [23,24]. The two TatA-like components are a result of gene duplication and have, in some instances, diverged drastically [23,25]. In *Escherichia coli* the diverged evolution of TatA is well illustrated as the TatA-like paralogs have evolved different functions, namely substrate docking (TatB) or pore formation (TatA or TatE) [17,18,23,26–28]. It is important to note that in many species with multiple TatA proteins, not all of these have diverged functionality [29]. For example, in the Gram-positive bacterium *Bacillus subtilis* the TatAd and TatAy proteins can perform both substrate docking and pore formation functions [7,30–32].

B. subtilis is a soil bacterium with a high capacity to secrete proteins [33,34]. The core Tat pathway in *B. subtilis* has been defined as a single TatA and TatC pair, namely TatAy-TatCy [32,74]. However, genes for two TatC (TatCd and TatCy) and three TatA components (TatAd, TatAy and TatAc) have been identified on the *B. subtilis* genome [75]. These Tat components can combine to form two separate TatA-TatC translocases, TatAy-

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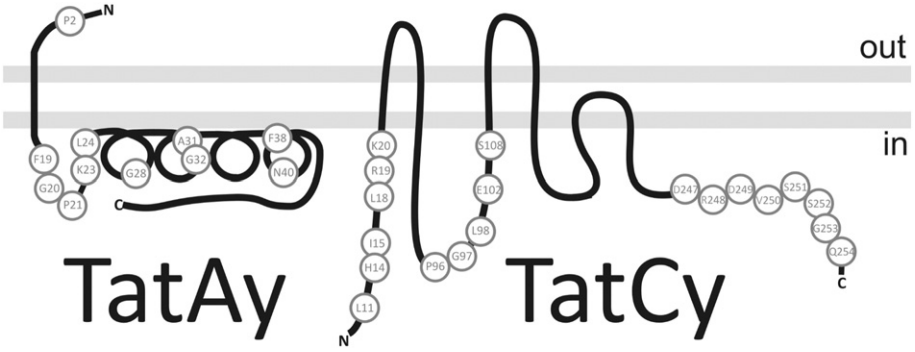

Fig. 1. Graphical representation of TatAy and TatCy and the amino acid residues substituted in this study. Mutated amino acid residues are illustrated. In, cytoplasmic side of the membrane; out, cell wall-exposed side of the membrane; N, N-terminus; C, C-terminus.

TatCy (TatAyCy) and TatAd-TatCd (TatAdCd). Each pathway works independently of the other, translocating its own substrates [32,35]. The *tatAyCy* and *tatAdCd* pairs are expressed operonically. Large-scale expression studies [36,37] have shown that the TatAdCd translocase is expressed only under conditions of phosphate limitation, corresponding to the expression of its one known substrate, the phosphodiesterase PhoD [35,38]. In contrast, TatAyCy is expressed constitutively over many tested conditions and exports more substrates, including the Dyp-type peroxidase EfeB (YwbN), the Rieske iron-sulfur protein QcrA and the alkaline phosphatase YkuE [32,39,40].

The role of the third *B. subtilis* TatA component, TatAc, has remained ambiguous [29]. Although it is constitutively expressed [37], *tatAc* mutant strains have not shown any phenotype [32,41,42]. Further, TatAc was unable to compensate for the absence of TatAy or TatAd in the translocation of EfeB or PhoD, respectively [43]. Nevertheless, TatAc has shown functionality in *E. coli*, as active TatAc-TatCd or TatAc-TatCy complexes of *B. subtilis* translocated the *E. coli* Tat substrates AmiA, AmiC and TorA [44]. Also in *E. coli*, TatAc was able to compensate for the absence of native TatA and TatB [45]. Protein–protein interaction studies using the Yeast two-hybrid (Y2H) technique have shown that TatAc not only interacts directly with itself, with TatAd and TatAy, but also with the HemAT protein recently implicated in PhoD secretion [46]. Combined, these recent studies have implicated TatAc in protein translocation, but a precise role for TatAc in vivo in *B. subtilis* Tat-dependent translocation had yet to be defined.

The natively expressed TatAyCy substrate EfeB is localized at the outside of the cytoplasmic membrane where it forms part of the EfeUOB iron transport system [47]. In addition, processed EfeB is TatAyCy-dependently secreted into the growth medium [32,41,42,47]. Under nutrient-rich growth conditions no major phenotypes associated with the Tat system have been observed [32]. However, under conditions of limited iron or NaCl availability clear growth phenotypes are observed [48]. These phenotypes are directly associated with the TatAyCy-dependent EfeB export and its role in ferric iron uptake [47,48]. In the present study, advantage was taken of the drastic lysis phenotype of *tatAyCy* or *efeB* mutant cells under low-salt growth conditions [48] to assess the importance of particular amino acid residues within TatCy or TatAy with regards to the translocation of EfeB. Importantly, we were able to grade the defects in EfeB translocation by the severity of the growth phenotypes observed, in the presence and in the absence of TatAc. In doing so, a novel role for TatAc in the active translocation of EfeB was uncovered, which suggests that this third TatA-like protein in *B. subtilis* might represent an intermediate evolutionary step in TatA-TatB specialization. Additionally, comparisons between the translocation profiles of EfeB and a second TatAyCy substrate, QcrA, revealed variations at certain TatAy amphipathic helix residues, thereby suggesting that these residues in TatAy are associated with specific substrate interactions.

2. Materials and methods

2.1. Bacterial strains, plasmids, media and basic growth conditions

Bacterial strains and plasmids used in this study are listed in Supplementary Table S1. Basic Lysogeny Broth (LB) was composed of 1% tryptone and 0.5% yeast extract. It was used either without NaCl supplementation (0% LB), or supplemented with 0.17 M NaCl (1% LB; i.e. the standard LB medium) or 1.02 M NaCl (6% LB). If not specified, bacteria were grown in 1% LB broth at 37 °C under vigorous shaking, or on 1% LB agar plates incubated at 37 °C. When appropriate, the cultures were supplemented with 100 μM $FeCl_3$ or antibiotics: *E. coli* cultures with 100 μg/ml ampicillin (Ap) and *B. subtilis* cultures with 2 μg/ml erythromycin (Em), 5 μg/ml chloramphenicol (Cm), 10 μg/ml tetracycline (Tc), 100 μg/ml spectinomycin (Sp), or 20 μg/ml kanamycin (Km). *B. subtilis* cells were grown to competence in Paris Medium and transformations were performed as previously described [49].

2.2. Cloning and DNA techniques

Cloning and ligation reactions were performed as described previously [50] using reagents from New England Biolabs. PCR reactions were performed using the Phusion polymerase (New England Biolabs). Previously constructed functional *tatAcCd* and *tatAcCy* operons [44] were amplified by PCR with primers NL-AcCdy and NR-AcCdy (Supplementary Table S1), and cloned into the *E. coli–B. subtilis* shuttle vector pHB201 for complementation analyses [51]. Previously published mutant *tatAy* genes encoding TatAy proteins with specific amino acid replacements were amplified by PCR with primers VJGAyL and VJGAyR (Supplementary Table S1), and cloned into vectors pBDU-C1 and pGAD-C1 for Y2H analyses [55,56].

2.3. Growth assay associated with iron scavenger deficiency

Phenotypes associated with the absence of active EfeB were monitored by optical density readings at 600 nm in microtiter plate readers (Biotek Synergy 2) using an adapted methodology described previously [37]. Strains were grown overnight in LB medium with 1% NaCl and appropriate antibiotics. Cultures were then diluted 50-fold into fresh LB with 1% NaCl in 96-well microtiter plates and grown till the mid-exponential growth phase (approximately 3 h). Cultures were then re-diluted 50-fold in LB without NaCl, but supplemented with freshly prepared 100 μM $FeCl_3$ and grown till the mid-exponential growth phase. A final 50-fold dilution was made into LB without NaCl and without iron supplementation, and growth was monitored for 14 h. Each strain was grown in triplicate within each experiment and each experiment was repeated five to ten times.

2.4. Crude cell fractionations

B. subtilis cultures were grown to early stationary phase in LB media, unless specified in the text. Culture aliquots (2 ml) were supplemented with the complete protease inhibitor cocktail without EDTA (Roche), and cells were pelleted by centrifugation. The growth medium fraction (1.5 ml) was removed and the extracellular proteins in this fraction were precipitated with trichloroacetic acid (TCA; final concentration 10%) overnight. The TCA-precipitated proteins were then washed with acetone and resuspended in 50 μl lithium dodecyl sulfate (LDS) gel loading buffer and reducing agent (NuPAGE, Invitrogen). The cell pellet was resuspended in 100 μl LDS gel loading buffer and reducing agent. Cells were subsequently disrupted by bead-beating three times with glass beads at 6500 rpm for 3 s with 30 s intervals (Precellys 24 lysis & homogenization, Bertin Technologies). Samples were heated at 95 °C and, if necessary, stored at −20 °C. Crude cell extract aliquots of 10 μl and growth medium aliquots of 20 μl corresponding to 2 OD_{600} units were used for NuPAGE and Western blotting.

2.5. Gel electrophoresis and Western blotting

Proteins were separated using NuPAGE gels (Invitrogen) and transferred onto nitrocellulose membranes (Protran, Schleicher&Schuell) by semi-dry blotting. Polyclonal rabbit antibodies specific for EfeB, QcrA and LipA have been described previously [39,40,54]. Bound antibodies were detected with fluorescent IgG secondary antibodies (IRDye 800 CW goat anti-rabbit from LiCor Biosciences) and visualized at 700 or 800 nm with the Odyssey Infrared Imaging System (LiCor Biosciences).

2.6. Yeast two-hybrid assay

Mutant *tatAy* genes were cloned into the bait vector pGBDU-C1 (URA3) and the prey vector pGAD-C1 (LEU2) as detailed in the Cloning and DNA techniques section above. This created translational fusions to the C-termini of the GAL4 DNA-binding domain (BD) and the GAL4 activation domain (AD), respectively [55]. The resulting bait and prey constructs, and similar bait and prey constructs with the wild-type *tatAy*, *tatAc*, *tatAd*, *tatCy* or *tatCd* genes from a previous study [46], were then introduced into the *S. cerevisiae* haploid strains PJ69-4a and PJ69-4α, respectively. Assays to detect direct interactions between the Tat fusions were performed according to a previously described mating strategy [56]. Matings with empty pGBDU and pGAD vectors were performed as controls for self-activation of the fusion proteins. Interaction phenotypes were scored by replica plating the diploids onto plates selecting for the expression of the interaction reporters (His + and Ade +). All interactions were reproduced at least two times and with two independent clones of the haploid bait and prey strains.

3. Results

3.1. Mutations in TatCy affect translocation of active EfeB and result in graded growth phenotypes

Growth defects of *B. subtilis* due to the absence of EfeB and/or TatAyCy have been reported for conditions of iron limitation or low salt [48]. In particular, it was shown that strains lacking TatAyCy or EfeB displayed a strong lysis phenotype in LB medium without NaCl. This lysis phenotype was fully complemented by flooding the system with ferric iron or by reintroducing TatAyCy or EfeB (Supplementary Figure S1a and [48]). The lysis phenotype is thus directly related to the block in TatAyCy-dependent translocation of EfeB from the cytoplasm to the outer surface of the cytoplasmic membrane, where it oxidizes ferrous iron to ferric iron [47]. Here, we further investigated whether this lysis phenotype could act as a read out for active translocation of EfeB by examining the impact of different *tat* mutations on growth in the absence of NaCl. If so, this would inform us further as to which TatAyCy amino acid residues are vital for EfeB translocation. For the purpose of this study, all strains were first pre-cultured in LB without NaCl supplemented with 100 μM $FeCl_3$. This allowed the strains to pre-adapt to a low salt environment so that any growth effects observed would correlate to the deficiency in the export of active EfeB via TatAyCy. Subsequently, the lysis phenotype of different *tat* mutant strains in LB medium without NaCl was monitored by optical density readings, as readout for the amount of active EfeB translocated. This approach made it possible to observe and categorize new phenotypes associated with site-specific TatCy or TatAy mutations as detailed in the following sections. The lysis phenotype was first investigated in strains with mutated TatCy.

The effect of amino acid replacements in TatCy on active EfeB translocation was assessed in a strain devoid of both chromosomal *tatC* genes, but constitutively expressing a plasmid pHB201-borne mutated *tatCy* copy. The *tatCy* gene was altered to generate 13 different mutant proteins, where the mutated residues were previously chosen based on their predicted importance [53]. These *tatCy* mutations resulted in 11 TatCy proteins with individual amino acid substitutions, and two TatCy proteins with C-terminal truncations of 5 or 8 residues, respectively (Fig. 1) [53]. Strains expressing TatCy proteins with individual H14A, L18A, K20A or L98A mutations, or with C-terminal truncations displayed growth and/or lysis phenotypes (Fig. 2a). The growth/lysis phenotype of each strain was very consistent between repeated experiments and phenotypes could be clearly categorized depending on the ability of the mutant strains to recover: a mild growth phenotype where the strain was able to recover quickly, a severe lysis/growth phenotype where the optical density dropped drastically and growth resumed between 200 and 300 min after the start of the experiment, and a very severe phenotype where strains were only able to recover after 300 min or longer (Fig. 2b). Importantly, growth phenotypes were only observed for strains with TatCy mutations that caused EfeB export defects as assessed by Western blot (see Supplementary Table S2 and [53]). Specifically, a mild growth phenotype was observed for the strain expressing TatCy-H14A. In contrast, the strain expressing TatCy-K20A showed a severe phenotype, and strains expressing TatCy proteins with the L18A, L98A, C-5 or C-8 mutations showed very severe growth phenotypes (Fig. 2a, Supplementary Table S2). Despite their growth defects, all strains were able to recover within 14 h, implying an adaptive response. This adaptation was not sustained because, upon re-inoculation of the strains after recovery, they showed similar growth phenotypes as before when grown on low salt (Supplementary Figure S1b).

3.2. A TatAy-supporting role for TatAc in EfeB translocation

To determine the effects of TatAy mutations on growth in LB without NaCl, we employed 12 previously generated TatAy mutant proteins with site-specific amino acid substitutions [52]. A synthetic operon composed of mutated *tatAy* and wild-type *tatCy* was reintroduced into different *tatAy* mutant strains via the expression plasmid pHB201. The amino acid substitutions in TatAy span the whole protein (Fig. 1); two substitutions were generated in the N-terminal region (P2A and P2D), four were located in the hinge region (F19A, G20A, P21A, K23A), and six were in the amphipathic helix region (L24A, G28A, A31G, G32A, F38A, N40A) [52].

Growth effects of the expression of mutant TatAy proteins were observed in various *tat* mutant backgrounds, which included strains where an individual Tat pathway was removed (*tatAyCy*), both Tat pathways were removed (*tatAdCd-tatAyCy*), the TatAyCy pathway was removed in combination with TatAc (*tatAc-tatAyCy*), or all Tat components were removed ('total-*tat*'). As described for the TatCy mutant proteins, growth/lysis phenotypes of graded severity were observed for strains expressing mutant TatAy proteins (Table 1 and Supplementary Figure S2) and, upon re-inoculation of these strains after recovery, they showed similar growth phenotypes as before (Supplementary

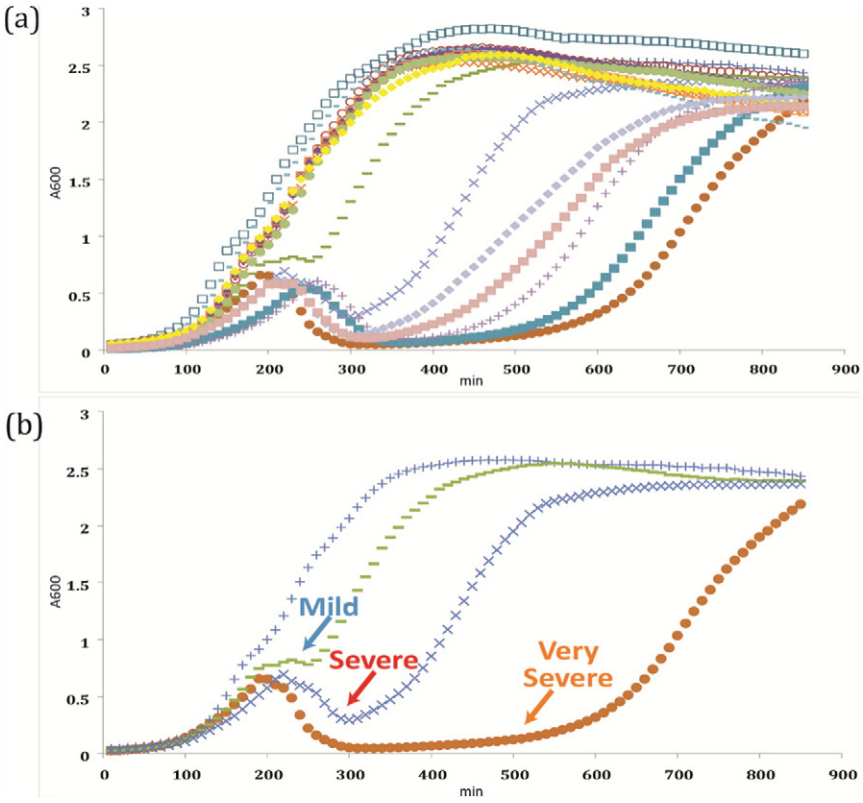

Fig. 2. Growth of strains expressing mutant TatCy proteins in LB without NaCl. (a) Growth of strains expressing site-specifically mutated TatCy proteins grown in LB without NaCl. A summary of growth phenotypes is presented in Supplementary Table S2. Strains are represented as follows: *B. subtilis* 168 (□), *tatCd-tatCy* mutant background (●), *tatCd-tatCy* mutant background with pHB-*tatCy*$^{wild\text{-}type}$ (+), pHB-*tatCy*^{L11A} (○), pHB-*tatCy*^{H14A} (▬), pHB-*tatCy*^{I15A} (◆), pHB-*tatCy*^{L18A} (■), pHB-*tatCy*^{R19A} (×), pHB-*tatCy*^{K20A} (×), pHB-*tatCy*^{P96A} (□), pHB-*tatCy*^{G97A} (●), pHB-*tatCy*^{L98A} (+), pHB-*tatCy*^{E102A} (▬), pHB-*tatCy*^{S108A} (◆), pHB-*tatCy*$^{C\text{-}5}$ (◆), or pHB-*tatCy*$^{C\text{-}8}$ (■). (b) Simplified graphical representation of the graded severity of growth phenotypes. The blue arrow illustrates a mild growth phenotype, which was defined as a dip at 200 min with immediate recovery. The red arrow illustrates the severe lysis phenotype, which was defined by a strong drop in optical density with a recovery between 200 and 300 min, and the orange arrow illustrates the very severe lysis phenotype where growth did not recover well over 300 min into the assay.

Table 1
Summary of growth phenotypes and protein secretion phenotypes in various *tat* mutant strains.

	Graded growth phenotype — active EfeB					Western blot translocation profiles			
	tatAyCy	*tatAdCd-tatAyCy*	*tatAc-tatAyCy*	total-*tat*	*tatAc-tatAyCy* + pC-Ac	EfeB in total-*tat* or *tatAyCy*	EfeB-myc in *tatAyCy* [52]	QcrA in *tatAyCy*	QcrA in total-*tat*
Δ	Very severe	Very severe	Very severe	Very severe	Very severe	–	–	–	–
Δ + Ay-WT	WT	WT	WT	WT		WT	WT	WT	WT
Δ + Ay-P2A	Severe	Severe	Severe	Severe	WT	<WT	–	<WT	–
Δ + Ay-P2D	WT*	WT*	Mild	Mild	WT	<WT	–	<WT	–
Δ + Ay-F19A	WT	WT	WT	WT		WT	WT	WT	~WT
Δ + Ay-G20A	Very severe	Very severe	Very severe	Very severe	WT	–	–	–	–
Δ + Ay-P21A	Severe	Severe	Very severe	Very severe	WT	–	–	–	–
Δ + Ay-K23A	WT	WT	WT	WT		~WT	<WT	WT	~WT
Δ + Ay-L24A	Severe	Severe	Severe	Severe	WT	<WT	–	–	–
Δ + Ay-G28A	WT	WT	WT	WT	WT	<WT	–	–	–
Δ + Ay-A31G	WT*	WT*	Severe	Severe	WT	<WT	<WT	<WT	<WT
Δ + Ay-G32A	WT	WT*	Very severe	Very severe	Very severe	–	<WT	–	–
Δ + Ay-F38A	WT	WT	WT	WT		~WT	WT	~WT	<WT
Δ + Ay-N40A	WT	WT	WT	WT		WT	WT	WT	<WT
Δ + Ac-Cd	Very severe	Very severe	Very severe	Very severe	Very severe				
Δ + Ac-Cy	Very severe	Very severe	Very severe	Very severe	Very severe				

B. subtilis strains lacking particular chromosomal *tat* genes as indicated in the second row of this Table were complemented with site-specifically mutated TatAy proteins plus a wild-type TatCy protein. This complementation was achieved by introducing plasmid pHB201 with a synthetic *tatAy-tatCy* operon, including the mutated *tatAy* gene and a wild-type *tatCy* gene. The specific TatAy amino acid mutations within the synthetic operon as well as the expression of synthetic TatAc-TatCd or TatAc-TatCy operons are indicated in the far left column. The Δ indicates that the respective strain lacks certain *tat* genes as indicated in the second row of the Table. The readout of active EfeB and growth phenotypes for strains lacking different chromosomal *tat* genes and expressing particular plasmid-borne *tat* genes relate to the growth curves on LB without salt as presented in Supplementary Figure S2, and they are summarized in accordance with the classification presented in Fig. 2B: WT: growth equivalent to the 168 wild-type strain. WT*: in some assays a very mild phenotype was observed, however this was not consistent in every assay. Mild: a phenotype was observed as a small dip at 200 min with immediate recovery. Severe: a phenotype that showed a strong drop in optical density and a recovery between 200 and 300 min into the assay. Very severe: a phenotype where growth did not recover well over 300 min into the assay. Western blotting profiles of the translocation of QcrA, EfeB and EfeB-myc were analyzed using specific antibodies against QcrA, EfeB and the C-myc epitope, respectively. The columns indicate the antibody used and the *tat* genetic background in individual assays. Protein detection levels are summarized as follows: WT: wild-type levels of protein detected as in wild-type *B. subtilis* 168; <WT, lowered levels of protein detected in the growth medium; – : no protein detected in the growth medium. The corresponding Western blots for EfeB and QcrA are presented in Supplementary Figure S4 and quantified in Supplementary Table S3. The data for EfeB-myc were previously published [52].

Figure S3). In *tatAyCy* or *tatAdCd-tatAyCy* genetic backgrounds the growth phenotypes observed for particular TatAy mutant proteins were very similar. Specifically, strains producing TatAy-P2A, TatAy-P21A or TatAy-L24A together with wild-type TatCy showed severe growth phenotypes, and strains producing TatAy-G20A showed a very severe growth phenotype.

Intriguingly, expression of particular mutant TatAy proteins *plus* wild-type TatCy in *tatAc*-deficient backgrounds (i.e. the *tatAc-tatAyCy* or total-*tat* mutants) showed a number of important differences relative to the *tatAc*-proficient backgrounds (i.e. the *tatAyCy* or *tatAdCd-tatAyCy* mutants) (Table 1, Supplementary Figure S2 a–d). Strains lacking the chromosomal *tatAyCy* or *tatAdCd-tatAyCy* genes and expressing the P2D, A31G or G32A TatAy mutant proteins *plus* wild-type TatCy showed extremely mild growth phenotypes if any, while the growth defect of cells expressing the TatAy-P21A mutant protein was graded as severe. However, when *tatAyCy* and *tatAc* were absent, the growth defect of cells expressing the TatAy-P2D mutant protein was graded as mild, the growth defect of cells expressing the A31G mutant was graded as severe, and the growth defects of cells expressing the P21A or G32A mutant proteins as very severe. This implied that the malfunction of these TatAy mutant proteins was exacerbated in the absence of TatAc, and thus that TatAc could at least partially complement the TatAy defects. To further investigate a possible complementing role of TatAc, plasmid pC-Ac expressing TatAc was introduced into strains with the chromosomal *tatAc-tatAyCy* mutation and containing plasmid pHB-TatAy^{aa}Cy$^{wild\text{-}type}$ for expression of a modified *tatAyCy* operon [43,75]. Next, growth assays were performed using LB without NaCl. The results of these assays are shown in Supplementary Figure S2e and summarized in Table 1. Notably, the ectopic expression of TatAc from plasmid pC-Ac restored wild-type growth in nearly all cases, even in cells expressing TatAy-P2A or -L24A, which showed severe growth phenotypes in all *tat* mutant backgrounds. The only exception was observed for the strain producing TatAy-G32A, where the very severe growth phenotype was not complemented upon pC-Ac introduction. Together, these observations show that TatAc was able to compensate for the functional defects of all TatAy mutant proteins except the defect of TatAy-G32A. In this respect, it is noteworthy that TatAc was probably overexpressed from pC-Ac since the equivalent plasmid pC-Ay, which carries *tatAy* instead of *tatAc*, was shown to result in ~5-fold overexpression of TatAy [40].

A previous study showed that TatAc is capable of forming an active translocon with TatCy and TatCd in *E. coli* [44]. The ability of TatAc to form an active translocon in *B. subtilis* with TatCy or TatCd in a Tat-deficient background was therefore investigated. We constructed plasmids expressing the functional synthetic operons encoding TatAc-TatCy or TatAc-TatCd. Although TatAc-TatCy was able to partially complement the *tatCd-tatCy* background, the TatAc-TatCd and TatAc-TatCy operons were unable to compensate for TatAy in *tatAy*-deficient backgrounds (Table 1 and Supplementary Figure S2f). We therefore conclude that, in *B. subtilis*, TatAc is not capable of forming a fully active translocon, neither with TatCy nor with TatCd. Nonetheless, there are several instances where TatAc can complement TatAy with severe amino acid mutations.

3.3. Differential effects of certain mutations in TatAy and TatCy on the export of EfeB and QcrA

In parallel to the lysis assays detailed above, Western blot assays with polyclonal antibodies were performed to monitor the TatAyCy-dependent extracellular accumulation of natively expressed EfeB. As a secondary TatAyCy-dependent readout, the extracellular accumulation of processed QcrA (QcrA*) was also assessed [39]. Here it should be noted that QcrA is an 18-kDa membrane protein with N_{in}-C_{out} topology [39] that forms part of the Cytochrome bc_1 complex on the outside of the membrane [57]. The *B. subtilis* QcrA protein undergoes cleavage by signal peptidases resulting in the release of a 14-kDa processed form (i.e. QcrA*) into the growth medium [39]. This processed QcrA* is therefore a valid indicator for QcrA translocation [58].

The effects of the site-specific mutations in TatAy or TatCy on export profiles were investigated using cells grown in LB without NaCl supplemented with 100 μM $FeCl_3$ and in LB with 1% NaCl. The EfeB and QcrA* export profiles of strains expressing mutated TatCy proteins in a *tatCd-tatCy* deficient background, correlated well with the results of the lysis assays and with previously described export profiles for the ectopically expressed EfeB-myc protein [53], as summarized in Supplementary Table S2.

As expected, the EfeB secretion profiles of strains expressing mutated TatAy proteins in different *tatA* deficient backgrounds were generally consistent with the lysis phenotypes of the respective strains (Table 1 and Supplementary Figures S2 and S4a–c). Nevertheless, strains producing the P2A or L24A mutant TatAy proteins did secrete some EfeB while showing a strong phenotype in the lysis assay, suggesting that the EfeB secreted by these mutant strains is not fully active. Furthermore, certain notable variations in the EfeB and QcrA* secretion profiles were observed (Table 1; Supplementary Figure S4, a–c). Firstly, it was found that in a total-*tat* background TatAy-P2A and -P2D mutations had greater effects on the secretion of QcrA* than on the secretion of EfeB. Secondly, these P2A and P2D mutations affected the secretion of QcrA* more strongly in the total-*tat* mutant than in the *tatAyCy* mutant background. This observation supports the idea that TatAc assists TatAyCy in translocation. Thirdly, the L24A and G28A mutations in the amphipathic helix of TatAy had a significant impact on QcrA*, while EfeB secretion was relatively mildly affected (Table 1; Supplementary Figure S4 and Table S3). In addition, varying effects on QcrA* relative to EfeB secretion were observed for the amphipathic helix mutation A31G, depending on the salt concentration in the growth medium (Supplementary Figure S4 and Table S3). The latter differences in the secretion profiles of QcrA* and EfeB imply that the L24, G28 and A31 residues of TatAy are important for substrate-specific interactions. Lastly, previous studies showed that the secretion of some Tat substrates, especially EfeB, was influenced by the NaCl concentrations in the growth medium [48,52,59]. However, except for the afore-mentioned variations observed for the TatAy-A31G mutant, this effect was not evident for QcrA* (Supplementary Figure S4, c–e).

3.4. Mutations in TatAy affect the interaction with TatAc

Our results from both the growth analyses and the protein secretion assays showed that TatAc facilitated protein translocation by the TatAyCy translocase, thereby suggesting that TatAc may interact with subunits of the TatAy-TatCy machinery. Therefore, a Y2H analysis was performed to visualize TatAc interaction with the various Tat components, including particular TatAy mutant proteins with distinct phenotypes. We also investigated the interactions of mutant TatAy proteins with themselves and with wild-type Tat components (i.e. TatAy, TatAc, TatAd, TatCy and TatCd). Interaction phenotypes were scored after replica-plating the diploids onto -LUA medium to identify the strongest interactions and onto the less stringent -LUH medium to visualize weaker interactions as described previously [56].

Previous Y2H studies have shown that the *B. subtilis* TatA proteins interact with each other and themselves ([46], Table 2 and Supplementary Figure S5). Therefore, interactions between the wild-type Tat components and the selected TatAy mutants were examined in both bait-pray and pray-bait orientations. A total of seven TatAy mutants were examined. These included two TatAy proteins with amino acid substitutions in the hinge region (G20A and P21A) and three with substitutions in the amphipathic region (G28A, A31G and G32A), because these mutations resulted in EfeB and/or QcrA translocation phenotypes (Table 1). The two remaining amino acid substitutions examined served as controls for each region (K23A in the hinge, and N40A in the amphipathic helix) as the mutation of K23 and N40 only had a relatively minor impact on EfeB or QcrA translocation (Table 1). The protein–protein

Table 2
Representation of the Y2H matrix with TatAy amino acid mutants.

	Control	G20A	P21A	K23A	G28A	A31G	G32A	N40A	TatAy	TatAc	TatAd	TatCy
Control												
TatAy				x	–			x	x	**X**	x	x
TatAc		–	–	x	–			x	x	x	x	
TatAd		–	–	x	–			x	x			
TatCy					–							
TatCd												
G20A										x	x	
P21A										x	x	
K23A									–	–	–	
G28A				**X**				**X**	**X**	**X**	**X**	x
A31G												
G32A												
N40A								x	x	x	x	x

Bait vectors are represented in columns while the prey vectors are represented in rows. The control represents an empty bait. Crosses represent positive interactions in LUH medium, crosses in bold represent positive interactions in LUA and LUH medium, dash indicates the absence of an interaction in either bait/pray if the inverse analysis showed an interaction, and an empty cell represents the absence of interactions in both orientations.

interactions of the wild-type TatAy and the TatAy mutants were compared as summarized in Table 2 and shown in the Supplementary Figures S5 and S6. Importantly the TatAy mutations that barely affected EfeB and QcrA translocation, namely TatAy-K23A and N40A, were barely affected in their abilities to interact with the TatA components. In contrast, changes in protein–protein interactions of the other TatAy mutants were observed. The most drastic changes were observed for mutant TatAy proteins with the A31G and G32A substitutions in the amphipathic region. Further changes were observed for the TatAy proteins with a mutated hinge region (G20A and P21A) as these showed positive interactions with TatAc and TatAd, but not with wild-type TatAy. Interactions with TatAy-G28A were affected to a much lesser extent. Combined, these findings show that mutations in TatAy that seriously interfered with EfeB or QcrA* secretion (G20A, P21A, G28A, A31G, and G32A) also altered the ability of these TatAy proteins to self-interact, which would possibly lead to defects in oligomerization and pore formation. Further, two of these mutations (A31G, and G32A) affected the ability of TatAy to interact with other Tat components, at least in the Y2H assay system.

4. Discussion

In this study we examined the ability of a panel of mutated TatAy and TatCy proteins to complement for wild-type TatAy or TatCy in various *tat*-deficient genetic backgrounds of *B. subtilis*. Previous studies investigating the TatAyCy-dependent export of xylose-induced EfeB-myc showed, by Western blotting, that a number of residues are important for EfeB translocation (Table 1, Supplementary Table S2, [52,53]). However, results generated by overexpressing a substrate, while informative, do not reveal whether these proteins are fully mature or active. Here, we addressed this by developing a lysis assay associated with the active translocation of EfeB. This lysis assay allowed a distinction of growth phenotypes that were graded as mild, severe or very severe. This grading uncovered a role of TatAc in assisting TatAy in protein translocation. By complementing the results from the lysis assay with those generated by Western blot profiles of natively expressed EfeB and QcrA the importance of specific residues of TatAy and TatCy was further defined. Lastly, Yeast two-hybrid interaction studies showed that inter- and intra-Tat interactions were affected by amino acid substitutions in TatAy that interfered with effective secretion of EfeB and QcrA.

The lysis assay described in this study served as an active readout of EfeB translocation. It did not relate to an inability of the Tat pathway to function normally, as QcrA translocation was not significantly affected by the environmental salt concentration. Further, the grading of the growth phenotypes implied that the quality of translocation varied between strains with different *tat* mutations and suggested different efficiencies in EfeB maturation and translocation. All strains were eventually able to recover from the growth/lysis defects, and this recovery was not retained upon re-culturing. Hence, recovery likely relates to an adaptive response regarding iron uptake, or iron metabolism rather than the Tat system directly [47,52]. In cases where amino acid substitutions resulted in very severe phenotypes, a complete blockage of active EfeB translocation may occur. This is supported by the very severe phenotypes observed in the 'bare' mutant background strains without complementing plasmids. In contrast, in strains with 'merely' severe phenotypes the amino acid mutations resulted in inefficient EfeB translocation, but did not completely block it. These strains adapted to the environment faster than those with the very severe phenotype. This could correspond to a change in the quality or quantity of active translocated EfeB, potentially due to a severe delay in proofreading, translocation or release of EfeB. The milder growth phenotypes suggest that the amino acids mutated play roles in translocation, but that this was easily compensated for. The lysis assay therefore gave a direct representation of the EfeB translocation process and allowed for novel insights into the Tat translocation system of *B. subtilis*.

Individual TatCy amino acid residues showed comparable importance with regard to EfeB and QcrA secretion when examined by Western Blot and lysis phenotyping. Further, these secretion/lysis phenotypes associated with TatCy amino acid mutations strongly corresponded to the regions already shown to be important in *E. coli* TatC, namely the N-terminal region (H14, L18 and K20) [6,60–62], the first cytoplasmic loop (L98) [53,60,63–65], and the C-terminal tail (Fig. 1) [53].

For several mutant TatAy proteins, the lysis phenotypes were exacerbated in strains devoid of TatAc. Specifically, this applied to the TatAy-P2D, -P21A, -A31G or -G32A mutations (Table 1). When TatAc was re-introduced it fully restored growth to wild-type rates indicating wild-type EfeB translocation and activity (with the exception of the TatAy-G32A mutant, discussed below). This strongly suggests TatAc associates with TatAy with regards to translocating active EfeB, and that the P2, P21, A31 and G32 residues are of particular importance for this interaction. Markedly, TatAc must have an assistant role, as TatAc on its own [32,43] or combined with TatCy/TatCd was unable to compensate for the complete absence of TatAy. Unlike the panel of TatCy mutations, diverse phenotypes caused by certain TatAy amino acid mutations were observed, especially when comparing the EfeB and QcrA secretion profiles and lysis phenotypes.

The TatAy protein contains a number of defined structural regions including a short extracellular N-terminal domain, a single transmembrane domain, a short flexible hinge region and an amphipathic helix that is thought to lie flat against the membrane, as schematically represented in Fig. 1 [8,9,11]. The amphipathic helix region leads onto a second flexible end region containing a large number of densely charged

residues [10]. These structural regions are important as they all have roles in translocation and particular residues have been shown to be vital for this process [12,30,31,52,66–70].

When substituting the proline residue in the N-terminal extracytoplasmic region of TatAy with either alanine or aspartic acid, two different phenotypes were observed. In the lysis assay the P2A substitution was not accepted in any genetic background and severe lysis was observed. However an aspartic acid at this position was tolerated. After the re-introduction of TatAc into a *tatAc-tatAyCy* mutant background, EfeB translocation and growth phenotypes reverted to wild-type. Further differences were seen on the Western blots of EfeB and QcrA*, where the effect of mutated P2 on the secretion profile depended on the genetic background. This proline residue is conserved in the TatA-like proteins of Gram-positive bacteria, but not in Gram-negative bacteria and a mutation in the equivalent residue of TatAd of *B. subtilis* (TatAd-P8A) was shown to be particularly important with regards to functionally replacing TatB in *E. coli* [30]. Further, electron microscopy and advanced atomic force microscopy approaches have shown that the TatAy-P2A protein formed super-complexes with TatCy where the TatAy:TatCy ratio was largely increased [71]. The combined data suggested that the initial formation of a docking complex by TatAy-P2A and TatCy, was followed by an uncontrolled addition of TatAy-P2A complexes. This would imply that TatAy-P2A is impaired in pore formation rather than docking complex formation, and that this defect in pore formation can be rescued by TatAc over-expression.

Two TatAy mutations in the hinge region at G20 and P21 caused drastic phenotypes under all conditions tested. These lysis phenotypes were similarly compensated for by the reintroduction of ectopically expressed TatAc. Therefore, ectopically expressed TatAc compensated for vital TatAy amino acids in the N-terminal and hinge regions. Of note, phenotypes caused by mutated residues in the amphipathic helix as reflected in the lysis assays and secretion profiles were dissimilar. The L24A and G32A mutations prevented both QcrA and EfeB translocation, while the phenotype caused by the A31G mutation was EfeB-specific, and G28A resulted in an absence of QcrA* secretion only. That Gly28 of TatAy was uniquely vital for QcrA* secretion suggests that this amino acid residue or interactions with this residue at the membrane-cytoplasm interface [52] may be more important for QcrA translocation than for EfeB translocation or may be involved in substrate specificity. The EfeB-associated growth phenotypes caused by the L24A and A31G substitutions were reversed when TatAc was reintroduced via the multicopy plasmid pC-Ac, but not the G32A substitution. Within the amphipathic helix, the residues L24 and A31 both lie against the membrane, while the hydrophilic G32 residue faces the cytoplasm [52]. The equivalent substitution of TatAy-G32A in TatAd is G34A. It has been suggested that the TatAd G34 residue is located in a region of the amphipathic helix involved in intramolecular salt bridges and self-assembly of the TatAd protein [10]. Hence the inability of ectopically (over-)expressed TatAc to compensate for TatAy-G32A could be due to a disruption of intramolecular salt bridges. A second theory as to why ectopically expressed TatAc is unable to compensate for TatAy-G32A is that the G32 residue may be needed in specific substrate recognition or quality control performed by the TatAy-TatCy docking complex and that the (over-)expressed TatAc was unable to compensate for the TatAy-G32A mutant protein in the docking complex. It is thus conceivable that the G32A mutation disrupts an essential *B. subtilis* docking complex function that cannot be rescued by TatAc, or that pore formation by TatAy-G32A depends on the presence of TatAc at just the right level.

Essential to the translocation process is the ability of TatAy to form complexes with itself and other Tat components, and Y2H studies have already confirmed these interactions [46]. The previous Y2H results have been particularly clear with regard to interactions between the TatA proteins of *B. subtilis*. Here we show that Tat protein–protein interactions were affected by amino acid substitutions in TatAy. This was especially the case for the A31G and G32A mutations introduced in the amphipathic helix. Although interactions of these and other mutated TatAy proteins were not detectable in the current Y2H analyses, they are likely to exist in *B. subtilis* cells that produce TatAc as evidenced by mild or absent growth phenotypes and relatively moderate translocation defects. This relates to the fact that, in general, not all existing protein–protein interactions are detectable with the Y2H approach. Nevertheless, combining our present Y2H results with the ability of ectopically expressed TatAc to compensate for most mutants in the lysis assay, it appears that TatAc stabilizes the TatA interactions. TatAc could thus compensate for TatAy in a pore formation capacity, but its inability to interact with TatCy suggests that it cannot perform docking complex activities in *B. subtilis*.

Phylogenetic analyses comparing the Tat proteins from all kingdoms have shown that in most cases the Tat system is composed of two TatA-like components and one TatC component [23–25]. Recent studies in *Helicobacter pylori* and *Campylobacter jejuni* have highlighted that of the two TatA-like proteins present only one is essential for functional translocation [72,73]. In contrast, *E. coli* has two essential TatA-like proteins (TatA and TatB), but both of these proteins can be functionally replaced by a single *B. subtilis* TatA protein [30,44]. Here we demonstrate that in *B. subtilis* TatAc can functionally compensate for defective TatAy. Nevertheless, TatAc is unable to replace TatAy. In this *Bacillus* scenario it seems that the defective TatAy protein takes on a role that is similar to the role of *E. coli* TatB, while TatAc functions similar to *E. coli* TatA. This implies that the bifunctional TatA-like proteins in *B. subtilis* represent an intermediate evolutionary step in TatA-TatB specialization. Accordingly, in *B. subtilis* one TatA-like protein (TatAc) could assist in forming the pore protein complex, while the second bifunctional TatA-like protein (TatAd or TatAy) forms part of both the pore and the docking complex with TatC. TatAc could therefore allow for faster cargo processing and improve the overall performance of the Tat pathway, thereby giving a role to the conserved multiplicity of TatA-like proteins in the Tat system.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2015.07.022.

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