Salt Sensitivity of Minimal Twin Arginine Translocases

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Background: Minimal twin arginine preprotein translocases consist of TatA and TatC subunits.

Results: Here we show that various mutated and wild-type minimal Tat translocases are salt-sensitive.

Conclusion: Salt-sensitive electrostatic interactions seem to have critical roles in the preprotein translocation activity of minimal Tat translocases.

Significance: Our results represent first experimental evidence for an important role of salt-sensitive electrostatic interactions in Tat translocation.

Bacterial twin arginine translocation (Tat) pathways have evolved to facilitate transport of folded proteins across membranes. Gram-negative bacteria contain a TatABC translocase composed of three subunits named TatA, TatB, and TatC. In contrast, the Tat translocases of most Gram-positive bacteria consist of only TatA and TatC subunits. In these minimal TatAC translocases, a bifunctional TatA subunit fulfils the roles of both TatA and TatB. Here we have probed the importance of conserved residues in the bifunctional TatAy subunit of Bacillus subtilis by site-specific mutagenesis. A set of engineered TatAy proteins with mutations in the cytoplasmic hinge and served residues in the bifunctional TatAy subunit of E. coli. Nevertheless, these mutated TatAy proteins did assemble into TatAy and TatAyCy complexes, and they facilitated membrane association of twin arginine precursor proteins in E. coli. Interestingly, most of the mutated TatAyCy translocases were salt-sensitive in B. subtilis. Similarly, the TatAC translocases of Bacillus cereus and Staphylococcus aureus were salt-sensitive when expressed in B. subtilis. Taken together, our present observations imply that salt-sensitive electrostatic interactions have critical roles in the preprotein translocation activity of certain TatAC-type translocases from Gram-positive bacteria.

Bacterial, archaeal, and thylakoidal twin arginine translocation (Tat) pathways facilitate the passage of fully folded substrate proteins across biological membranes (1–8). There are two key prerequisites for the transport of substrate proteins via Tat. First, these proteins need to have an N-terminal signal peptide with a characteristic twin arginine (RR- motif (K/R)RX-FK (9–15)). This RR-signal peptide facilitates precursor targeting to the Tat translocase and initiation of the translocation process. Second, the substrate protein needs to be presented to the Tat translocase in a folded state (16, 17).

In bacteria, two types of Tat translocases have been identified. Gram-negative bacteria and a few Gram-positive bacteria, such as streptomycetes, have TatABC translocases (18–21). These translocases are composed of three subunits, TatA, TatB, and TatC, all of which are of critical importance for translocation activity. TatABC-type translocases are also present in the chloroplast thylakoidal membrane (22–24). In contrast, the vast majority of Gram-positive bacteria contain TatAC translocases that are composed only of TatA and TatC subunits (25–27). In this case the TatA subunit is bifunctional, fulfilling the roles of both TatA and TatB (28–30). Although the precise modes of action of TatABC and TatAC translocases have not yet been fully elucidated, the consensus mechanistic model envisions that the RR motif in signal peptides is recognized by a TatBC receptor complex in Gram-negative bacteria or a TatAC receptor complex in Gram-positive bacteria (29, 31–33). The TatBC or TatAC precursor complexes would subsequently associate with TatA complexes that co-exist as separate entities in the membrane. This in turn would lead to the formation of a flexible channel composed of multiple TatA subunits, facilitating membrane passage of substrate proteins that can range in diameter from ~20 to ~70 Å (33–36). The proton-motive force is believed to strengthen the signal peptide-receptor complex interaction and to drive protein translocation across the membrane (33, 34, 37, 38).

In certain bacteria, such as the Gram-positive bacterium Bacillus subtilis, multiple paralogues of TatA or TatC are present. Specifically, B. subtilis contains two TatAC systems named TatAdCd and TatAyCy that are encoded by the tatAdtatCd
and tatAy-tatCy operons (25, 28). The TatAdCd and TatAyCy translocases have different but overlapping specificities, and their usage is strongly dependent on growth conditions. Under conditions of phosphate starvation, the TatAdCd translocase is responsible for secretion of the phosphodiesterase PhoD, whereas TatAyCy is dispensable for this process (25, 39). Conversely, when cells are cultivated in the standard Luria-Bertani (LB) medium, the TatAyCy translocase secretes the Dyp-type peroxidase YwbN without any detectable involvement of TatAdCd (28, 40). Interestingly, both TatAC translocases from B. subtilis are capable of transporting hybrid precursors consisting of RR signal peptides from Escherichia coli fused to the green fluorescent protein (GFP) (42).

The structure-function relationships in the TatA and TatB subunits of the TatABC translocase from E. coli have been studied in great detail (43–49). In contrast, relatively little is known about the bifunctional TatA subunits that are part of the TatAC translocases of Gram-positive bacteria. An initial mutagenesis screen of several conserved residues in the N-terminal part of the TatAd protein has identified a number of residues that are important for its bifunctional activity when heterologously expressed in E. coli (50). This prompted us to assess the functions of conserved residues in the B. subtilis TatAy protein by site-specific mutagenesis. To monitor the activity of translocases with mutated TatAy subunits in B. subtilis, we used the secreted YwbN protein as a reporter. An additional advantage of using TatAy for mutagenesis studies is that TatAy-dependent YwbN secretion is required for achieving wild-type growth rates and survival upon entry in the stationary phase when B. subtilis cells are grown in LB medium lacking NaCl (40). This relates to a critical role of YwbN in the acquisition of iron under these conditions.

The present studies identify several residues that are critical for TatAy activity when B. subtilis cells are grown in the standard LB medium with 1% NaCl. The inactive mutated TatAy proteins were all capable of assembling into TatAy and TatAyCy complexes when produced in E. coli, and in this organism most of them facilitated membrane association (though not full translocation) of a hybrid RR precursor protein. These findings indicated that the respective mutations did not primarily interfere with TatAyCy complex formation but rather with the translocation activity of TatAyCy complexes. All mutated TatAy proteins facilitated at least low levels of YwbN secretion in B. subtilis cells grown in LB medium without added NaCl. The latter experiment revealed that most of the respective mutated TatAyCy translocases were salt-sensitive. Equally surprising, additional experiments revealed that the TatAC translocases from Bacillus cereus and Staphylococcus aureus were also salt-sensitive when expressed in B. subtilis. Taken together, our findings imply that certain TatAC translocases are intrinsically salt-sensitive, which suggests critical roles for electrostatic interactions in their preprotein translocation activity.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Bacterial Strains, and Media—**Strains and plasmids are listed in Table 1. The standard LB medium that was also used in all our previous studies contained Bacto Tryptone (1%), Bacto yeast extract (0.5%), and NaCl (1%). For some experiments NaCl was omitted from the LB medium (“no NaCl”). When required, media for E. coli were supplemented with ampicillin (100 \( \mu \)g ml\(^{-1} \)), kanamycin (20 \( \mu \)g ml\(^{-1} \)), or spectinomycin (100 \( \mu \)g ml\(^{-1} \)); media for B. subtilis were supplemented with erythromycin (2 \( \mu \)g ml\(^{-1} \)), kanamycin (20 \( \mu \)g ml\(^{-1} \)), or spectinomycin (100 \( \mu \)g ml\(^{-1} \)).

**DNA Techniques—**Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of E. coli were carried out as described (51). Enzymes were from Roche Molecular Biochemicals or New England Biolabs. B. subtilis was transformed as described (52). PCR was carried out with Pwo DNA polymerase. All constructs were sequenced to confirm correctness.

**Construction of tatAy Mutations and tatAyCy Complementation Plasmids—**Particular codons in tatAy were replaced by site-directed mutagenesis using PCR primers with one or more nucleotide changes. Supplemental Table 1 lists the primers used in this study. In a first PCR the 5’ part of tatAy was amplified using the forward primer JW01Ay2 and a reverse primer specifying one or two nucleotide changes in tatAy. The resulting PCR product was purified using the Roche Molecular Biochemicals PCR purification kit. This PCR product was then used as a forward primer with JW04Cy2 as the reverse primer to create a second PCR product, specifying the complete tatAyCy operon with a mutant tatAy gene. Mutant tatAyCy operons were cloned in pUC18, and the correctness of the amplified sequences was verified. For expression and activity studies in B. subtilis, the mutant tatAyCy operons were excised from pUC18 using HindIII and EcoRI and ligated into the Smal and EcoRI restriction sites of the E. coli-B. subtilis shuttle vector pHB201. To study TatAyCy complex formation in E. coli, mutant tatAyCy operons were transferred to pBAD24. For this purpose, the mutant tatAyCy operons were amplified by PCR with a 3’ primer specifying a Strep-II tag that is coupled to the C terminus of TatCy. The resulting PCR products were cleaved with BsmBI and XbaI and ligated to pBAD24 that had been cleaved with BsmBI and NcoI. To study TatAyCy-facilitated export of a hybrid precursor composed of the RR signal peptide of E. coli MdoD fused to the green fluorescent protein (MdoD-GFP) in E. coli, the low expression vector pEXT22 was used. To this end, the mutant tatAyCy operons were amplified by PCR, cleaved with HindIII and EcoRI, and ligated to HindIII-EcoRI-cleaved pEXT22.

**Cloning of the B. cereus, Listeria monocytogenes, and S. aureus tatAC Genes—**Primers for amplification of the tatAC genes from B. cereus, L. monocytogenes EGD, and S. aureus RN4220 were designed from the respective genome sequences as deposited in the NCBI data base (www.ncbi.nlm.nih.gov; supplemental Table 1). The amplified PCR products were cloned in pUC18 or pTOPO (Invitrogen) and sequenced. For expression in B. subtilis, correctly amplified tatAC genes from B. cereus and S. aureus were cloned in the EcoRI site of pHB201. The fragment with the L. monocytogenes tatAC operon was cleaved with BamHI and XhoI and ligated to pHB201 cleaved with the same enzymes.
Export Assays for YwbN and MdoD-GFP—B. subtilis strains lacking the tatAyCy genes were transformed with pH2B01 plasmids carrying tatAyCy genes from B. subtilis (wild type or mutant), B. cereus, S. aureus, or L. monocytogenes. To monitor the activity of pH2B01-encoded TatAC translocases, the XywbN-Myc cassette was integrated in the amyE locus, allowing xylose-inducible expression of Myc-tagged XywbN. Secretion of XywbN-Myc was assessed by transferring cells from an overnight culture to fresh medium. After 3 h of growth in fresh medium at 37 °C, XywbN-Myc expression was induced by the addition of 1% xylose and 0.5 M NaCl to the growth medium. YwbN-Myc expression was monitored by Western blotting and immunodetection. The presence of YwbN-Myc in the different fractions was monitored by Western blotting and immuno-detection.

Western Blotting and Immunodetection—The presence of the TatAy, YwbN-Myc, or GroEL proteins in cellular and growth medium fractions of B. subtilis was monitored by Western blotting. Protein samples for SDS-PAGE were prepared with loading buffer containing reducing agent (Invitrogen). After separation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) by semidyey blotting (1 h and 15 min; 1 mA cm⁻²). For detection of YwbN-Myc, monoclonal Myc–specific antibodies from Gentaur were used. Antibody detection was performed with fluorescent IgG secondary antibodies (IRDye 800 CW goat anti-rabbit or goat anti-mouse from LiCor Biosciences) in combination with ECL detection kit (Amersham Biosciences). Densitometric image analysis to quantify relative protein amounts as detected by Western blotting was performed with the program ImageJ (rsbweb.nih.gov).

The presence of TatAy and GFP in subcellular fractions of E. coli was monitored by Western blotting with anti-TatAy (Eurogentec) and GFP (Promega, Living Colors). Bound antibodies were visualized with goat anti-rabbit IgG horse radish peroxidase (HRP) conjugate and the ECL detection kit (Amersham Biosciences). Likewise, the presence of TatCy-Strep-II was detected by Western blotting using a Streptactin-HRP conjugate (Tut fur Bioanalytik) and the ECL detection kit.

# TABLE 1

**Strains and plasmids**

| Strains and plasmids | Relevant properties | Reference |
|----------------------|---------------------|-----------|
| E. coli DH5α         | supE44 ΔlacZΔ800 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 | (51)       |
| E. coli MC4100        | F’ ΔlacU169 araD139 rpsL150 relA1 ptsF ΔF redB8530 | (63)       |
| E. coli MC4100 ΔtatABCDE | Deletion of the tatABCDE operon | (53)       |
| B. subtilis 168      | trpC2               | (41)       |
| B. subtilis 168 tatAyCy X-ywbN | trpC2, tatAy-tatCy:Sp', amyE-xyiA-ywbN-c-myc | (28)       |

**Export Assays for YwbN and MdoD-GFP**

**Expression E. coli**

| Expression | Relevance | Reference |
|------------|-----------|-----------|
| pBAD-MdoD-GFP | pBAD24-derivative containing the MdoD-GFP gene hybrid; Ap’ | (42)       |
| pBAD-A2/28B  | pBAD24-derivative vectors containing the tatAy-tatCy-Strept-II tag operon; 5.5 kb; Ap’ | This work |
| pBAD-A2/28B  | pBAD24-derivative vectors containing the tatAy-tatCy-Strept-II tag operon. The respective tatAy genes specify single A to B amino acid substitution in residues 2–28 of TatAy; 5.5 kb; Ap’ | This work |
| pEXT-AyCy    | pEXT22-derivative containing the B. subtilis tatAy-tatCy; 7.1 kb; Km’ | (42)       |
| pEXT-A2/28B  | pEXT22-derivative vectors containing the tatAy-tatCy operon. The respective tatAy genes specify single A to B amino acid substitution in residues 2–28 of TatAy; 7.1 kb; Km’. Specifically these vectors were used to express the following mutated TatAy proteins: P2A, P2D, G20A, P21A, K22A, K23A, L24A, P25A, G28A, A31G, G32A, F38A, N40A. | This work |
| pHB-SDM-A2/40B| pHB201-derivative vectors containing the tatAy-tatCy operon. The respective tatAy genes specify single A to B amino acid substitutions in residues 2–40 of TatAy; 7.6 kb; Em’, Cm’. Specifically these vectors were used to express the following mutated TatAy proteins: P2A, P2D, F19A, G20A, P21A, K22A, K23A, L24A, P25A, G28A, A31G, G32A, F38A, N40A. | This work |
| pHB-AyCy     | pHB201-derivative containing the tatAy-tatCy operon; 7.6 kb; Em’, Cm’ | This work |
| pHB-Bce      | pHB201 derivative containing the B. cereus tatA-tatC operon; 7.6 kb; Em’, Cm’ | This work |
| pHB-Sau      | pHB201-derivative containing the S. aureus tatA-tatC operon; 7.6 kb; Em’, Cm’ | This work |
| pHB-Lmo      | pHB201-derivative containing the L. monocytogenes tatA-tatC operon; 7.6 kb; Em’, Cm’ | This work |
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**Growth Experiments**—*B. subtilis* strains were precultured in LB medium containing 1% NaCl and subsequently diluted in LB medium with no NaCl or 1% NaCl to an optical density at 600 nm (A<sub>600</sub>) of ~0.01. Growth was continued in a 96-well microtiter plate (Nunc) that was incubated in a Biotek Synergy 2 plate reader (37 °C, variable shaking). A<sub>600</sub> readings were recorded for 15 h.

**Expression, Purification, and Gel Filtration Analysis of TatAyCy and TatAy Complexes**—*E. coli* ΔtatABCDE cells containing the mutant tatAyCy operons on the pBAD24 plasmid were grown aerobically to mid-exponential phase with induction of the tatAyCy genes using 0.5 mM arabinose. Cells were fractionated into membrane and cytosolic components as described previously, and the membranes were solubilized in 2% digitonin (32). To analyze TatAyCy complexes, the solubilized membranes were incubated with 2 µg/ml avidin to block any biotin-containing proteins before application to an equilibrated 4-ml Streptactin affinity column (Institut fur Bioanalytik). The column was washed with 8 column volumes of equilibration buffer containing Tris-HCl, pH 8.0, 2% glycerol, 150 mM NaCl, and 0.1% digitonin. Bound protein was eluted from the column in 6 × 2.0 ml fractions using the same buffer as above but containing 3 mM desthiobiotin (Sigma). For gel filtration experiments, affinity-purified TatAyCy was concentrated to 250 µl using Vivaspin-4 centrifugal concentrators (molecular weight cutoff 10,000; Vivascience). The concentrated sample was loaded onto a Superose-6HR gel filtration column (Amersham Biosciences) and eluted with the equilibration buffer described above (32). Peak elution fractions (15–35) were used for TatCy-Strep detection with a Streptactin-HRP conjugate or immunoblotting with specific anti-TatAy antibodies. To analyze TatAy complexes, Streptactin column flow-through and wash fractions containing solubilized TatAy complexes were also subjected to gel filtration chromatography, and peak elution fractions (10–35) were immunoblotted with specific anti-TatAy antibodies. Residual amounts of TatCy-Strep in these fractions were detected with a Streptactin-HRP conjugate. Molecular mass markers for gel filtration were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa).

**RESULTS**

**Site-specific Mutagenesis of TatAy**—The roles of conserved residues in TatAy were assessed by site-specific mutagenesis. The mutated residues included Pro-2 in the extracytoplasmic N-terminal section, a series of residues in the “hinge” region at the end of the transmembrane span, and several conserved residues in the cytoplasmic amphipathic helix (Fig. 1). Several of these conserved residues had previously been shown to be important for TatA function in *E. coli* (43–49). Residues in TatAy were substituted with Ala, except for Pro-2, which was also replaced with Asp, and Ala-31, which was replaced with Gly. The resulting mutated proteins were constitutively expressed from plasmid pHB201, which results in the expression of TatAy at levels that are comparable with the regular TatAy expression levels in *B. subtilis* strain 168 grown in LB medium with 1% NaCl (data not shown).

**Stability of Mutated TatAy Proteins**—Because amino acid substitutions can have detrimental effects on protein stability, we investigated the production of TatAy mutated proteins by Western blotting using cells from overnight cultures in LB medium with 1% NaCl. As shown in Fig. 2A, only the P2D mutated protein was produced at very low levels, whereas all other mutated TatAy proteins were readily detectable, albeit at slightly varying levels. *B. subtilis* strains producing the K22A- or P25A-mutated TatAy proteins showed growth defects (data not shown), suggesting that the expression of these mutated proteins has pleiotropic detrimental effects on cell physiology. Interestingly, the G20A mutation resulted in a significantly elevated cellular TatAy level, whereas the A31G mutation resulted in a significantly decreased level of TatAy (Fig. 2A). These findings indicate that the P2D- and A31G-mutated proteins are more prone to degradation than other mutated TatAy proteins or wild-type TatAy (Table 2). In contrast, the G20A protein seems to be more stable than the wild-type TatAy as the levels of this mutated protein are relatively high.

**The Hinge Region and Amphipathic Helix of TatAy Are Important for YwbN Secretion**—To test the functionality of the mutated TatAy proteins, the secretion of Myc-tagged YwbN (YwbN-Myc) by cells grown in standard LB with 1% NaCl was monitored by Western blotting (Fig. 2B). Strains producing the K22A- or P25A-mutated TatAy proteins were excluded from this analysis because of their growth defects. The YwbN protein was efficiently secreted into the medium by wild-type TatAyCy (lane labeled AyCy), whereas several amino acid substitutions in TatAy resulted in drastically reduced secretion of YwbN (Fig. 2B). Specifically, no YwbN secretion was detectable for strains producing the P2A-, P2D-, G20A-, P21A-, L24A-, or G28A-mutated TatAy proteins. Compared with strains producing the wild-type TatAyCy protein, reduced levels of YwbN secretion were observed for strains producing the K23A, A31G, G32A, F38A, or N40A proteins. Overall, these results show that the targeted conserved residues in the hinge and amphipathic regions of TatAy are very important for effective YwbN secretion (Table 2). The sole exception is the F19A mutation, which unexpectedly resulted in an increase of YwbN secretion by about 30% (Fig. 2B). It should be noted here that the production of the wild-type or mutated TatAyCy translocases had no detectable qualitative or quantitative effects on the overall secretion of proteins by *B. subtilis* as deduced from the patterns of extracellular proteins on SDS-PAGE (supplemental Fig. 1). This is consistent with the fact that the vast majority of *B. subtilis* proteins are secreted via the Sec pathway (26). Furthermore, most fractions contained equal amounts of the cytoplasmic lysis marker protein GroEL (Fig. 2C), except for the strain carrying the empty pHB201 plasmid. This indicates that the plasmid-directed expression of wild-type or mutated TatAyCy translocases induced a certain (low) degree of cell lysis.

**Mutated TatAy Proteins Facilitate Membrane Binding of MdoD-GFP in *E. coli***—To further assess the functionality of the P2D-, P2A-, G20A-, P21A-, K22A-, L24A-, P25A-, and G28A-mutated TatAy proteins, the respective mutant tatAy-tatCy operons were expressed in *E. coli*, and their activity was monitored with a substrate comprising GFP fused to the RR signal peptide of the *E. coli* MdoD protein. This hybrid MdoD-GFP
precursor was previously shown to be effectively translocated when the \textit{B. subtilis} TatAyCy translocase was co-expressed in an \textit{E. coli} tatABCDE deletion mutant (42) and was thus considered to be a good candidate substrate for the TatAyCy system. Notably, the heterologously expressed K22A- and P25A-mutated proteins do not cause growth defects in \textit{E. coli}, and they were, therefore, included in this analysis.

To study MdoD-GFP export to the periplasm of \textit{E. coli} by mutated TatAyCy translocases, the \textit{mdoD-gfp} gene fusion was expressed from pBAD24, and the different mutant tatAy-tatCy operons were co-expressed from the compatible pEXT22 vector. Periplasmic, membrane, and cytoplasmic cell fractions were analyzed by Western blotting using GFP-specific antibodies. No mature GFP was detected in the periplasmic fractions of strains producing the P2D or G28A mutated TatAy proteins (Fig. 3). Strains producing the P2A-, G20A-, P21A-, K22A-, L24A-, or P25A-mutated TatAy proteins contained minute amounts of GFP in the periplasm, reflecting a severely impaired translocation activity (Fig. 3). Interestingly, strains producing the P2A-, G20A-, P21A-, K22A-, L24A-, or P25A-mutated TatAy proteins contained relatively high amounts of MdoD-GFP in the membrane that in most cases was at least partially processed to the mature form. The membrane-associated MdoD-GFP was not detectable when no Tat proteins were produced (Fig. 3). Together, these findings indicate that the respective mutated TatAyCy translocases were able to interact with the MdoD-GFP precursor and to promote its binding to the membrane. However, these mutated translocases were unable to facilitate effective transport of MdoD-GFP to the periplasm. Strains producing the P2D TatAy protein did not contain membrane-associated MdoD-GFP (Fig. 3), which seems to be due to the production of insufficient amounts of P2D TatAy as was also the case in \textit{B. subtilis} (Fig. 1). This provides additional support for the view that the TatAyCy system is responsible for pre-MdoD-GFP binding to the membrane and not nonspecific interactions. Furthermore, the strain producing the L24A-mutated TatAy protein contained an abundant GFP degradation product in the membrane fraction. Similarly

\begin{figure}
\centering
\includegraphics{fig1}
\caption{The \textit{B. subtilis} TatAy protein. A, secondary structure prediction of TatAy indicating the location of the transmembrane (TM), hinge, and amphipathic helix regions is shown. B, shown is sequence alignment of TatAy with TatA proteins from \textit{B. subtilis} (Bsu), \textit{L. monocytogenes} (Lmo), \textit{S. aureus} (Sau), and \textit{B. cereus} (Bce) with TatA and TatB from \textit{E. coli}. The arrows at the top highlight residues that were selected for replacement with alanine (aspartic acid in the case of Pro-2 or glycine in the case of Ala-31). C, shown is a schematic representation of the generally proposed N\textsubscript{out}-C\textsubscript{in} membrane topology of TatAy. The positions of replaced residues are indicated with arrows. It should be noted, however, that some studies provide support for the existence of an N\textsubscript{in}-C\textsubscript{out} topology of TatAy (not shown) that might, for example, exist transiently during the protein translocation reaction (61, 62). D, a helical wheel projection of the amphipathic helix of TatAy is shown.}
\end{figure}
sized GFP degradation products were also detectable in the membrane fractions of strains producing other mutated TatAy proteins (e.g. P2A), albeit at much lower amounts (Fig. 3). Such degradation products were not detectable when the wild-type TatAyCy translocase was produced, suggesting that MdoD-GFP was prone to specific degradation when the P2A- or L24A-mutated TatAy proteins were produced.

**Mutated TatAy Proteins Are Assembled into TatAyCy and TatAy Complexes in E. coli**

To investigate whether mutated TatAy proteins, which do not facilitate YwbN secretion, do assemble into TatAyCy and TatAy complexes as was previously shown for wild-type TatAy (42), the respective mutant tatAy-tatCy operons were expressed in a tatABCDE mutant strain of E. coli using the plasmid pBAD24. It should be noted that complex formation had to be studied in E. coli because TatAy and TatCy could not be produced in sufficiently high amounts in B. subtilis to allow detection of complexes by affinity purification or blue native PAGE (data not shown).

To characterize the TatAyCy complexes expressed in E. coli by affinity chromatography and subsequent gel filtration as previously described (42), a Strep-II tag was fused to the C terminus of TatCy. Next, membranes of strains producing P2A-, G20A-, P21A-, K22A-, L24A-, P25A-, or G28A-mutated TatAy proteins, and TatCy-Strep were isolated, solubilized in digitonin, and loaded on a Streptactin column for affinity purification. This resulted in the effective purification of TatAyCy complexes as exemplified in Fig. 4 for the wild-type TatAyCy complex. Importantly, only barely detectable amounts of TatCy-Strep were present in the flow-through and wash fractions (Fig. 4, upper panel). By contrast, the flow-through and wash fractions contained huge amounts of the wild-type or mutated TatAy proteins as was shown by Western blotting with antibodies against TatAy (Fig. 4, lower panel). It should be noted here that the TatAy proteins are not tagged, and therefore, they will not bind directly to the Streptactin columns that were used to obtain the TatAyCy complexes containing the Strep-II-tagged TatCy.

After elution from the Streptactin column, the TatAyCy complexes were concentrated and loaded on a Superose-6HR
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Salt-sensitive Mutated TatAyCy Translocases—B. subtilis strains lacking functional tatAyCy genes show a reduced exponential growth rate plus a lysis phenotype in the post-exponential growth phase when cultivated in LB medium without NaCl (Fig. 6A). This is due to a requirement for YwbN secretion in the acquisition of iron under these low salinity growth conditions (40). The fact that the low salinity growth phenotype of tatAyCy mutants can be rescued by introducing plasmid-borne copies of the wild-type tatAyCy genes provides a facile growth assay for the activity of mutated TatAy proteins (Fig. 6B). Intriguingly, all mutated TatAy proteins generated in this study were able to complement the growth defect of a tatAyCy-deficient strain when co-expressed with TatCy (Fig. 6C and supplemental Fig. 2). As previously shown, no growth complementation was observed when TatCy was expressed without the co-expression of TatAy (40). This suggested that the mutated TatAy proteins would assemble into translocation-competent TatAy and TatAyCy complexes when the producing cells were grown in LB without salt. This idea was tested by studying the secretion of YwbN-Myc in tatAyCy mutant cells with plasmid-borne copies of a mutant tatAy gene plus a wild-type tatCy gene. Indeed, all strains producing the P2A-, P2D-, P21A-, K22A-, L24A-, or G28A-mutated TatAy proteins secreted low but detectable amounts of YwbN when grown in LB medium without salt (Fig. 6D). YwbN secretion was also observed for the strains producing the K23A-, A31G-, G32A-, F38A-, or N40A-mutated TatAy proteins, and in this case, the respective YwbN secretion levels in LB medium without NaCl were actually comparable with the levels of YwbN secretion upon cultivation in LB medium with 1% NaCl (compare Figs. 2B and 6D). The observed YwbN secretion thus confirms the conclusion from the growth complementation experiments that the mutated TatAy proteins can form active TatAyCy translocases when cells are grown in LB medium without NaCl. Quite unexpectedly, this even applies to the unstable P2D mutated protein. These findings furthermore reveal that the TatAyCy translocases formed by the P2A-, P2D-, P20A-, P21A-, L24A-, or G28A-mutated TatAy proteins are salt-sensitive. In contrast, the TatAyCy translocases containing the K23A-, A31G-, G32A-, F38A-, or N40A-mutated TatAy proteins are apparently not affected by NaCl. Intriguingly, the translocase with the F19A-mutated TatAy protein seems to require NaCl for optimal activity. This can be inferred from the observation that cells growing on LB without NaCl and expressing the TatAyCy translocase with the F19A-mutated TatAy protein secreted less YwbN than the cells producing the wild-type TatAyCy translocase (Fig. 6D), whereas the opposite was true when these cells were grown in LB with 1% NaCl (Fig. 2B).

TatAC Translocases from B. cereus and S. aureus Are Salt-sensitive When Produced in B. subtilis—The observation that several mutated TatAyCy translocases are salt-sensitive opened up the possibility that this is a more general feature of TatAC-type translocases from Gram-positive bacteria. We, therefore, investigated whether TatAC translocases from B. cereus, S. aureus, and L. monocytogenes might be salt-sensitive when heterologously expressed in B. subtilis. To this end, the corre-
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A

B

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FIGURE 6. Activity of mutated TatAy proteins produced by B. subtilis cells grown in LB medium without salt. B. subtilis tatAyCy mutant cells transformed with the empty pH201 vector (A, open squares), pH201 carrying the wild-type tatAyCy genes (B, open squares), or pH201 carrying the mutant P2D tatAy gene plus a wild-type tatAy gene (C, open squares) were grown in LB medium without added salt. Growth was monitored for 15 h by optical density readings at 600 nm (A600). Growth of the parental strain 168 is indicated by filled diamonds. D, YwbN-Myc secretion by tatAyCy mutant strains producing the indicated plasmid-encoded mutated TatAy plus wild-type TaCy was monitored by Western blotting as in Fig. 2.

DISCUSSION

Very little is currently known about the structure-function relationships in bifunctional TatA subunits of TatAC translocon from Gram-positive bacteria. In the present investigation we have, therefore, focused attention on residues of the bifunctional TatAy subunit of B. subtilis that are important for the specific activities of the encoded TatAy proteins.

FIGURE 5. Complex formation by mutated TatAy proteins. A, mutant tatAy-tatCy-StrepII operons were expressed in a tatABCDE mutant strain of E. coli. Isolated membranes were solubilized in digitonin and loaded on a Streptactin column for affinity purification as in Fig. 4. After elution from the column, the proteins were concentrated and loaded on a Superose-6HR gel filtration column. The collected gel filtration fractions were analyzed by Western blotting with specific antibodies against TatAy. Immunoblots of all elution fractions of membrane-localized TatAy that was co-purified with TatCy-Strep were analyzed by densitometry. The column was calibrated using a set of protein standards of known molecular mass, namely thyroglobin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa) as indicated with filled squares. Intensities of the bands were plotted against fraction number. The column was calibrated using a set of protein standards of known molecular mass, namely thyroglobin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa) as indicated with filled squares. Intensities of the bands were plotted against fraction number. Each graph shows the wild-type TatAy complexes (filled squares) and complexes obtained for a particular mutated TatAy protein (empty circles).
assembly and activity of the corresponding TatAyCy translocase. Previous studies have shown that TatA of E. coli is a remarkably flexible protein that can tolerate many changes in its structure (45, 46, 49). Fortunately, the TatAy protein also proved to be stable upon site-specific mutagenesis, which allowed us to study the functionality of most mutated TatAy proteins. The only mutation in TatAy resulting in a highly unstable TatAy protein was the N-terminal P2D substitution. This finding suggests that Pro-2 may have a role in stabilizing the transmembrane helix of TatAy at the extracytoplasmic side of the membrane (Fig. 1) and that the negatively charged side chain of Asp somehow interferes with helix stability. A P2A substitution did not cause TatAy instability, showing that the small and hydrophobic side chain of Ala can be tolerated at this position. Nevertheless, the P2A substitution in TatAy rendered the TatAyCy translocase inactive, showing that the Pro-2 residue is not only important for TatAy stability but also for TatAy function. Notably, the equivalent G3D substitution in E. coli TatA rendered this protein bifunctional (54), which underscores the view that there are important functional differences between the TatA subunits of TatAC and TatABC translocases.

Recent structural analyses on TatAd of B. subtilis have confirmed the previously postulated L-shaped structure of this protein, which is formed by the transmembrane helix and the C-terminally located amphipathic helix (Fig. 1C and Refs. 55–58). Furthermore, functional analyses suggested that the conserved hinge region connecting both helices might be of structural importance for TatAd (50). Indeed, the present results clearly show that five highly conserved residues of the TatAy hinge region are of major importance for TatAy function as their substitution with Ala rendered the TatAyCy translocase either more active (F19A), severely impaired in activity (K22A, K23A), or completely inactive (G20A, P21A) in B. subtilis and/or E. coli. Most likely, these substitutions change the flexibility of the hinge region such that translocase activity is impaired. Especially, the G20A substitution will make the hinge less flexible as Gly residues generally contribute to structural flexibility of polypeptide chains. Conversely, the P21A substitution might make the hinge more flexible. Likewise, replacement of the positively charged Lys-22 or Lys-23 residues with Ala may cause an increased flexibility of the hinge, as these two Lys residues may engage in interactions with negatively charged phospholipids in the membrane (Fig. 1C). These findings are largely consistent with studies on E. coli TatA, although substitution of Phe-20 (equivalent to Phe-19 in TatAy) strongly reduced the activity of the E. coli TatABC translocase, whereas the activity of the TatAyCy translocase was actually enhanced by the F19A mutation in the present study (supplemental Table 2). Unfortunately, the roles of conserved residues in TatB of E. coli have been studied less extensively, so comparisons of the effects of mutations in TatB and TatAy are only possible for a few residues. Clearly, there are differences, as a substitution in Gly-21 of TatB (equivalent to Gly-20 of TatAy) merely reduced TatB activity (supplemental Table 3), whereas the corresponding mutation G20A in TatAy completely inactivated TatAyCy function. Similarly, major differences in the effects on activity were observed for Arg-24 or Leu-25 substitutions in TatB and equivalent substitutions in TatAy.

Our site-directed mutagenesis studies seem to divide the cytoplasmic amphipathic helix of TatAy into an essential N-terminal part and a less important C-terminal part, although it should be noted that not all residues of the amphipathic helix were substituted in the present studies. Clearly, the tested substitutions of residues in the N-terminal moiety of the amphipathic helix (Leu-24, Pro-25, Gly-28, Ala-31, and Gly-32) had drastic effects on translocation activity, whereas this was not the case for the C-terminal helix residues Phe-38 and Asn-40. These findings are consistent with the results of mutagenesis studies on TatA of E. coli, except for substitutions in Phe-39 (equivalent to Phe-38 of TatAy), which rendered the TatABC translocase of E. coli inactive (supplemental Table 2). By contrast, mutations in E. coli TatB that are equivalent to the presently tested mutations in TatAy had very little effect on TatB function (supplemental Table 3). The only exception is an L39C substitution in TatB, which resulted in strongly reduced translocation activity of the E. coli TatABC translocase, unlike the equivalent F38A mutation in TatAy, which had a relatively mild effect on TatAyCy function (supplemental Table 3). These findings clearly underscore the view that E. coli TatA and B. subtilis TatAy have functionally equivalent roles in RR precursor translocation. In this respect it is noteworthy that the tested mutated TatAy proteins appear not to cause defects in precursor binding.
by TatAyCy, as can be deduced from the studies on MdoD-GFP translocation in *E. coli*. This is consistent with the currently entertained model that a TatAy-TatCy subcomplex serves in precursor reception (29) like the TatB-TatC subcomplexes of the *E. coli* and thylakoidal TatABC translocases (31–33). Indeed, TatAyCy complex formation was not significantly affected by most TatAy mutations tested and, in those cases where effects were observed on the mass distribution of TatAyCy complexes (e.g. complexes with K22A- or P25A-mutated TatAy proteins), there were still substantial amounts of complexes with wild-type mass properties detectable (Fig. 5A).

Interestingly, most of the tested mutations in TatAy resulted in the formation of heterogeneously sized TatAy complexes. This suggests that the respective mutations, in particular the P2A, P21A, L24A, and G28A substitutions, may cause defects in the specific interactions needed for transfer of precursors from the presumed TatAy-TatCy-RR receptor complex to the translocation channel that is probably formed by TatAy protomers. If this view is correct, it implies that the interactions between the P2A-, P2D-, G20A-, P21A-, L24A-, or G28A-mutated TatAy complexes and the respective TatAyCy precursor complexes in the membrane are salt-sensitive, as these mutated TatAy proteins did assemble into translocation-competent TatAyCy complexes when cells were grown in medium without NaCl. As confirmed by the observed salt sensitivity of the TatAC translocases from *B. cereus* and *S. aureus* upon expression in *B. subtilis*, salt sensitivity may be an intrinsic property of certain TatAC translocases. If this interpretation is true, the observed salt sensitivity may reflect the existence of important electrostatic interactions during the formation of active TatAC channels for the transfer of proteins from the TatAC receptor complex into the TatA channel. Similarly, the apparent NaCl requirement for optimal activity of TatAyCy translocases containing the F19A-mutated TatAy protein could reflect the need for particular electrostatic interactions during TatAyCy-dependent protein translocation. Our present data would thus represent the very first experimental support for the hypothesis of Walther et al. (56) that the amphiphilic region of TatA could form intra- or even intermolecular salt bridges with potentially important roles in pore assembly (56). It is presently not clear why some wild-type translocases (such as TatAC of *B. cereus* and *S. aureus*) are salt-sensitive, whereas other wild-type translocases (such as TatAyCy of *B. subtilis* and TatAC of *L. monocytogenes*) do not share this property. However, this difference may relate to the high salt tolerance that has evolved in the latter organisms (40, 59). In any case, the finding that cells expressing wild-type TatAC translocases of *B. subtilis* or *L. monocytogenes* showed no altered YwbN secretion in response to changes in the salt content of the growth medium indicates that the salt-sensitive YwbN secretion by the TatAC translocases of *B. cereus* or *S. aureus* relates to properties of the respective translocases rather than salt-induced changes in the folding or co-factor assembly of the YwbN substrate protein.

In conclusion, the present finding that certain wild-type and mutated TatAC translocases are salt-sensitive provides novel leads for further mechanistic, structural, and applied studies on these intriguing systems for protein translocation. For future studies it will be interesting to investigate whether mutations in TatC, as were recently published by Eijlander et al. (60), will render TatAC-type translocases salt-sensitive as well or whether this is an exclusive feature of certain mutated TatA proteins. The identification of mutations (e.g. G20A) that stabilize TatAy may be of particular use in structural analyses, whereas mutations that enhance TatAC activity (e.g. F19A) may facilitate future biotechnological applications of engineered Tat pathways. Last, our present studies set the stage for follow-up experiments in which dominant negative mutant proteins are used to better understand the assembly and function of a minimal Tat system.

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