Escherichia coli Twin Arginine (Tat) Mutant Translocases Possessing Relaxed Signal Peptide Recognition Specificities*

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The twin arginine (Tat) secretion pathway allows the translocation of folded proteins across the cytoplasmic membrane of bacteria. Tat-specific signal peptides contain a characteristic amino acid motif (S/T)RRXFLK including two highly conserved consecutive arginine residues that are thought to be involved in the recognition of the signal peptides by the Tat translocase. Here, we have analyzed the specificity of Tat signal peptide recognition by using a genetic approach. Replacement of the two arginine residues in a Tat-specific precursor protein by lysine-glutamine resulted in an export-defective mutant precursor that was no longer accepted by the wild-type translocase. Selection for restored export allowed for the isolation of Tat mutant translocases still efficiently accepted the unaltered precursor protein, indicating that the substrate specificity of the translocases was not strictly changed; rather, the translocases showed an increased tolerance toward variations of the amino acids occupying the positions of the twin arginine residues in the consensus motif of a Tat signal peptide.

Transport of proteins across biological membranes is a fundamental process in all living cells. In almost all cases, proteins that have to exit the cytosol are initially synthesized as larger precursor proteins possessing an amino-terminal signal peptide (1). In eubacteria, the export of the vast majority of extracytosolic proteins is mediated by the general (Sec) secretion system. Powered by the translocation motor SecA, Sec-dependent proteins are translocated across the plasma membrane in a more or less unfolded state through a protein-conducting channel (SecYEG) (for a recent review, see Ref. 2). Besides the Sec system, many bacteria possess another protein export pathway for the translocation of a subset of proteins, which in many cases, contains a tightly bound cofactor (for reviews, see Refs. 3–6). This Sec-independent mechanism, which translocates its substrates in a fully folded or even oligomeric form across the plasma membrane, has been designated Tat (for twin-arginine translocation) due to the fact that a characteristic amino acid motif (S/T)RRXFLK including two consecutive arginine residues can be identified in the signal peptide of the respective precursor proteins (7, 8).

Various site-directed mutagenesis studies have manifested the importance of the two arginines for Tat-dependent export. In most cases the substitution of either arginine by other amino acids resulted in a complete block of transport or a severe reduction in the export efficiency (Ref. 9 and references therein). These results, together with their high conservation, strongly suggests that the two arginine residues are a crucial part of the signal by which Tat-dependent precursors are specifically recognized by one or more components of the Tat export machinery.

In Escherichia coli four genes (tata, tatb, tac, and tate) have been found that encode the membrane-integral components of the Tat translocation apparatus (8, 10). Based mostly on biochemical evidence from Escherichia coli (11) and the thylakoidal Tat (ΔPH) pathway (12), the current model of Tat-dependent protein translocation predicts that the precursor proteins are recognized by a complex consisting of TatB and TatC. After this initial recognition step, multiple copies of the pore component TatA are recruited to the TatBC-precursor complex, a step that is dependent on the presence of a transmembrane H+ (ΔPH) gradient. Subsequently, the substrate is translocated across the membrane, and after cleavage of the signal peptide, is released on the trans side of the membrane. Redissociation of TatA from TatBC resets the Tat system for further rounds of substrate recognition and translocation. The tatE gene encodes a paralogue of TatA that is expressed at a very low level and, due to this fact, is currently regarded as a cryptic gene duplication of tatA (13).

A detailed cross-linking study from E. coli in which a reactive amino acid residue was introduced at different positions into the signal peptide of the Tat substrate preSufI has shown that a hierarchy exists in the steps of substrate-translocase interactions (11). First, the precursor contacts the primary signal peptide receptor TatC by virtue of the region encompassing the highly conserved RR motif. Subsequently, the precursor is passed to TatB, which in addition to the RR consensus motif region also contacts the hydrophobic region of the signal peptide. No signal peptide-TatB/C cross-links were obtained when the two arginines of the RR motif were conservatively replaced.

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TABLE 1

| Bacterial strains and plasmids used in this study                  | Relevant properties                                      | Source |
|-------------------------------------------------------------------|----------------------------------------------------------|--------|
| **E. coli strains**                                               |                                                          |        |
| DH5α                                                              | supE44, ΔlacU169(Φ80 lacZΔM15) hsdR17 recA1 endA1         | Ref. 20|
| GS1100                                                           | MC4100 × P1(MMI29)>Te<sup>6</sup> ΔmalE444 zfb729-Tn10  | Ref. 17|
| GS1101                                                           | DADE × P1(MMI29)>Te<sup>6</sup> ΔmalE444 zfb729-Tn10    | Ref. 17|
| **Plasmids**                                                      |                                                          |        |
| pHSG-TatABCE                                                      | pHSG575 derivative; carrying the tatABCE genes of *E. coli* | Ref. 18|
| pHSG-TatABCE-KQS100                                               | pHSG-TatABC(K96F)E                                        | This study |
| pHSG-TatABCE-KQS101                                               | pHSG-TatABC(A135T)E(K18M,P194S)E                          | This study |
| pHSG-TatABCE-KQS102                                               | pHSG-TatABC(N22I,V35A)E(K41T)                            | This study |
| pHSG-TatABCE-KQS103                                               | pHSG-TatABC(Q23R)C(K18E,Y195H,V198A,G238D)E              | This study |
| pHSG-TatABCE-KQS104                                               | pHSG-TatABC(K18M)E                                        | This study |
| pHSG-TatABCE-KQS105                                               | pHSG-TatABC(K18E)E                                        | This study |
| pHSG-TatABCE-KQS106                                               | pHSG-TatABC(N22I)E                                       | This study |
| pTorA(KK)-MalE                                                    | pBR1MC2-2 carrying the torA-malE fusion gene, Km<sup>R</sup> | Ref. 17|
| pTorA(KQ)-MalE                                                    | pTorA-MalE (R11K and R12Q)                              | This study |
| pTorA(KQ)-MalE                                                    | pTorA-MalE (R11K and R12Q)                              | This study |

Km<sup>R</sup>, kanamycin resistance; Cm<sup>R</sup>, chloramphenicol resistance; Tc<sup>R</sup>, tetracycline resistance

by lysine residues, stressing again the importance of these amino acids in substrate recognition.

In this work we have analyzed the specificity of signal peptide recognition by the Tat translocase using a genetic approach. Replacement of the two arginine residues in the signal peptide of a Tat-specific, selectable reporter protein (TorA-MalE) by a lysine-glutamine pair resulted in an export-defective precursor protein (TorA(KQ)-MalE) that is no longer accepted by the Tat translocase. Selection for restored export of the protein (TorA(KQ)-MalE) that is no longer accepted by the lysine-glutamine pair resulted in an export-defective precursor of a Tat-specific, selectable reporter protein (TorA-MalE) by a drastic alteration in the highly conserved twin arginine motif in the amino-terminal domain of TatB or the first cytosolic domain of Tat translocases, containing amino acid alterations in either the amino-terminal domain of TatB or the first cytosolic domain of TatC. Besides the finding that the mutant Tat translocases have gained the ability to handle a Tat precursor protein possessing a drastic alteration in the highly conserved twin arginine motif in the signal peptide, they fully retained their ability to recognize and translocate the unaltered TorA-MalE precursor. Our results strongly indicate that, in the mutant Tat translocases, the specificity of the twin arginine binding site in the TatBC substrate receptor complex has been significantly relaxed.

EXPERIMENTAL PROCEDURES

**Bacterial Strains, Plasmids, and Culture Conditions**—The bacterial strains and plasmids used in this study are listed in Table 1. Bacterial strains were grown at 37 °C in Luria Bertani medium (14), minimal medium (15) supplemented with 0.4% maltose, or MacConkey agar base medium (Difco) supplemented with 1% maltose. If required, isopropyl-β-D-thiogalactopyranoside was used at a 0.1 mM concentration. Antibiotic supplements were at the following concentrations: kanamycin, 50 mg/liter; chloramphenicol, 25 mg/liter; tetracycline, 15 mg/liter.

**DNA Manipulations**—All of the DNA manipulations followed standard procedures (16). The replacements of the twin arginine within the n-region of the TorA signal peptide with a twin-lysine (RR → KK, resulting in plasmid pTorA(KK)-MalE) or a lysine-glutamine dipeptide (RR → KQ, resulting in plasmid pTorA(KQ)-MalE) were done using the QuikChange<sup>®</sup> site-directed mutagenesis kit (Stratagene) with pTorA-MalE (17) as a template and primers KK-for (5′-GAT CTC TTT CAG GCA TCA AAA AAA CGT TTT CTG GCA CAA CTC GCC-3′) and KK-rev (5′-GCC GAG TTG TGC CAG AAA ACG TTT TTT TGA TGC CTG AAA GAG ATC-3′) or KQ-for (5′-GCC GAG TTG TGC CAG AAA ACG CTG TTT TGT GTA CGT ATG GAA CTG C-3′) and KQ-rev (5′-GCC GAG TTG TGC CAG AAA ACG CTG TTT TGT GTA CGT ATG GAA ATC-3′), respectively, according to the manufacturer’s instructions. Likewise, the single amino acid substitutions in the TatC protein, K18M, K18E, and N22I, were introduced using the same procedure with pHSG-TatABCE (18) as a template and primers K18M-for (5′-GAT TGA GCT GCC TAT GAG TCT GCT GAA ATG C-3′) and K18M-rev (5′-GCA GTT CAG GAC CAT ACC ATG C-3′), K18E-for (5′-GCC TAT GAT TGA GCT GCC TAT GAG TCT GCT GAA ATG C-3′) and K18E-rev (5′-GCC GAA CTG CAA GCC ATG GAA ATG C-3′) or N22I-for (5′-CGT AAG CGT CTG CTA TGG CCG ATG GGA ATG C-3′) and N22I-rev (5′-CCG TAT GCC GAC CAT ACC ATG C-3′), respectively, using 2-aminopurine (causing GC → AT transitions) (14) or 5-azacytidine (causing GC → TA and GC → CG transversions) (19) as mutagenic agents. Approximately 1000–10000 cells of *E. coli* DH5α (20) containing pHSG-TatABCE were inoculated in 5 ml of Luria Bertani medium containing 700 μg/ml 2-aminopurine or, alternatively, 5-azacytidine in concentrations of 5, 10, 50, 70, or 100 μg/ml. The cells were incubated at 37 °C on a rotary shaker with 200 rpm for 24 h. Subsequently, plasmid DNA was prepared from the various pools of cells and used to transform GS1101 (pTorA(KQ)-MalE).

**Isolation of Tat Mutants**—Plasmid pHSG-TatABCE (18) was mutagenized in vivo using 2-aminopurine (causing GC → AT and AT → GC transitions) (14) or 5-azacytidine (causing GC → TA and GC → CG transversions) (19) as mutagenic agents. Approximately 1000–10000 cells of *E. coli* DH5α (20) containing pHSG-TatABCE were inoculated in 5 ml of Luria Bertani medium containing 700 μg/ml 2-aminopurine or, alternatively, 5-azacytidine in concentrations of 5, 10, 50, 70, or 100 μg/ml. The cells were incubated at 37 °C on a rotary shaker with 200 rpm for 24 h. Subsequently, plasmid DNA was prepared from the various pools of cells and used to transform GS1101 (pTorA(KQ)-MalE).

**In vitro mutagenesis** of pHSG-TatABCE using hydroxylation (causing GC → AT transitions) or using 5-azacytidine in concentrations of 5, 10, 50, 70, or 100 μg/ml. The cells were incubated at 37 °C on a rotary shaker with 200 rpm for 24 h. Subsequently, plasmid DNA was prepared from the various pools of cells and used to transform GS1101 (pTorA(KQ)-MalE).

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Twin Arginine Signal Peptide Recognition

Substitution of the Twin Arginine Residues in the Signal Peptide of a TorA-MalE Reporter Protein by Lysine-Glutamine Prevents Its Tat-dependent Membrane Translocation—Previously, we have established a sensitive Tat-specific reporter system (TorA-MalE) that allows an easy in situ detection of Tat-dependent protein export on indicative media (17, 18). The plasmid-encoded TorA-MalE reporter protein consists of the mature part of the periplasmic maltose-binding protein (MalE) fused to the signal peptide of the Tat-dependent periplasmic trimethylamine N-oxide reductase (TorA). The presence of MalE in the periplasm is absolutely required for growth of *E. coli* on minimal agar plates containing maltose as the sole carbon source (25) and, in addition, for the formation of red colonies on MacConkey agar plates containing maltose (26). When plasmid pTorA-MalE is transformed into GSJ101 (a malE-negative derivative of the *tat* deletion strain DADE (17, 27), growth on maltose minimal medium and red colonies on MacConkey maltose agar plates are only observed when plasmid pHSG-TatABCE (containing the known *tat* genes cloned in an operon-like fashion) but not when the empty vector pHSG575 is cotransformed into the same strain, showing that TorA-MalE export strictly requires the presence of a functional Tat system (Table 2) (17, 18).

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The importance of the conserved twin arginine residues (RR) in the TorA signal peptide for Tat-dependent export of TorA-MalE was analyzed by replacing them with either two lysine (KK) residues or a lysine-glutamine (KQ) pair. Tat\(^+\) (but not Tat\(^-\)) cells expressing the TorA(KK)-MalE reporter were still able to form red colonies on MacConkey maltose plates and to grow on maltose minimal medium although at a slower rate when compared with Tat\(^+\) cells expressing the unaltered reporter (Table 2). These results show that the conservative replacement of RR by KK obviously reduced but not completely abolished the Tat-dependent export of TorA-MalE into the periplasm. In contrast, a less conservative change of RR to KQ resulted in an export-defective TorA(KQ)-MalE mutant precursor protein, since Tat\(^+\) cells expressing this reporter variant showed pale colonies on MacConkey maltose plates and did not grow on maltose minimal medium (Table 2).

Taken together, these results clearly demonstrate that the TorA signal peptide mediates Tat-dependent export of MalE into the periplasm of E. coli and, furthermore, underscores the importance of the conserved twin arginine residues for the productive recognition and membrane translocation of Tat precursor proteins by the Tat export machinery.

**Isolation of Tat Mutants Restoring Membrane Translocation of the Otherwise Export-defective TorA(KQ)-MalE Precursor Protein—** As shown in the previous chapter, the replacement of the conserved RR residues by KQ in the TorA signal peptide completely prevented recognition and/or membrane translocation of the corresponding TorA(KQ)-MalE precursor protein. Next, we asked whether mutant Tat translocases can be identified that possess a relaxed specificity with respect to the requirement for the RR amino acid residues in the consensuus motif of Tat signal peptides and, therefore, might allow export of TorA(KQ)-MalE. Plasmid pHSG-TatABCE was mutagenized either in vivo using 2-aminopurine or 5-azacytidine or in vitro using hydroxylamin as mutagens. The differently mutagenized pools of pHSG-TatABCE were transformed into GSJ101 containing pTorA(KQ)-MalE, and the resulting transformants were plated onto maltose minimal agar plates. After 2 days of incubation, the formation of single colonies could be observed. 14 randomly chosen colonies that after restreaking showed reproducible growth were selected for further analysis. In KQS100, isolated 13 times in independent experiments, the mutations present in the multiple mutants KQS101, KQS102, and KQS103 that allow suppression of the TorA(KQ)-MalE export defect also in a single context are indicated in bold.

**TABLE 3**

| Mutant translocate | Amino acid alterations |
|-------------------|-----------------------|
| KQS100            | TatC: K41T            |
| KQS101            | TatB: A135T           |
|                   | TatC: K18E, P194S     |
| KQS102            | TatC: N221, V35A      |
| KQS103            | TatE: K41T            |
|                   | TatB: Q23R            |
| KQS104            | TatC: K18M            |
| KQS105            | TatC: K18E            |
| KQS106            | TatC: N221            |
| KQS200            | TatB: E8K             |

**TABLE 2**

**Phenotype of bacterial strains on MacConkey maltose and maltose minimal medium**

Bacterial strains were streaked on minimal medium agar plates containing 0.4% maltose as the sole carbon source or on MacConkey agar plates containing 1% maltose and incubated at 37°C. +++, fast growth; ++, medium growth; +, slow growth; −, no growth.

| Bacterial strain                        | Growth on maltose minimal medium | Color of colonies on MacConkey maltose |
|-----------------------------------------|----------------------------------|---------------------------------------|
| GSJ101                                  | −                                | Pale                                  |
| GSJ101 (pTorA-MalE)                     | −                                | Pale                                  |
| GSJ101 (pTorA-MalE, pHSG575)            | −                                | Pale                                  |
| GSJ101 (pTorA-MalE, pHSG-TatABCE)       | ++                               | Red                                   |
| GSJ101 (pTorA(KK)-MalE, pHSG575)        | ++                               | Red                                   |
| GSJ101 (pTorA(KK)-MalE, pHSG-TatABCE)   | ++                               | Red                                   |
| GSJ101 (pTorA(KQ)-MalE, pHSG-TatABCE)   | ++                               | Red                                   |
| GSJ101 (pTorA(KQ)-MalE, pHSG-TatABCE-KQS100) | + (++)                           | Lightred(pink)                        |
| GSJ101 (pTorA(KQ)-MalE, pHSG-TatABCE-KQS101) | +                               | Lightred(pink)                        |
| GSJ101 (pTorA(KQ)-MalE, pHSG-TatABCE-KQS102) | +                               | Lightred(pink)                        |
| GSJ101 (pTorA(KQ)-MalE, pHSG-TatABCE-KQS103) | +                               | Lightred(pink)                        |
| GSJ101 (pTorA(KQ)-MalE, pHSG-TatABCE-KQS104) | +                               | Lightred(pink)                        |
| GSJ101 (pTorA(KQ)-MalE, pHSG-TatABCE-KQS105) | +                               | Lightred(pink)                        |
| GSJ101 (pTorA(KQ)-MalE, pHSG-TatABCE-KQS106) | +                               | Lightred(pink)                        |
| GSJ101 (pTorA(KQ)-MalE, pHSG-TatABCE-KQS200) | +                               | Lightred(pink)                        |
Corresponding mutations at these positions are, in fact, the ones that exert the suppressing activity in the multiple mutants, we separately introduced these mutations into the tatC gene by site-directed mutagenesis, resulting in mutant translocases KQS104 (TatC(K18M)), KQS105 (TatC(K18E)), and KQS106 (TatC(N22I)).

Next, the amounts of the proteins TatA, TatB, and TatC in the membrane fraction of the cells expressing the mutant Tat translocases were equal or slightly lower when compared with the amounts of Tat proteins in the membrane fraction of the cells expressing a Tat wild-type translocase. This finding excludes that the observed effects are due to increased amounts of Tat proteins in the strains expressing the Tat mutant translocases and are indeed caused by the corresponding amino acid alterations.

**The Mutant Tat Translocases Mediate Export of TorA(KQ)-MalE into the Periplasm to Various Degrees**—Next, TorA(KQ)-MalE export in the strains expressing the various mutant Tat translocases was analyzed (i) indirectly by plate assays and (ii) directly by determining the amount of MalE in the periplasm. As shown in Table 2, GSJ101(pTorA(KQ)-MalE, pHSG-TatABCE) did not grow on maltose minimal medium and formed pale colonies on MacConkey agar plates containing maltoose. In contrast, GSJ101 (pTorA(KQ)-MalE, pKQS100) showed efficient growth on maltose minimal medium and the formation of red colonies on MacConkey maltose agar plates. Significant, but somewhat slower growth and the formation of pink colonies was observed with the strains expressing the other KQS mutant Tat translocases. Interestingly, the strains expressing TorA(KQ)-MalE in combination with the single mutant Tat translocases KQS104, KQS105, and KQS106, generated by site-directed mutagenesis, all showed identical behavior in the plate assays as the strains expressing the multiple mutant translocases KQS101, KQS102, and KQS103, from which they were derived, indicating that the amino acid alterations present in the cytosolic extreme amino-terminal part of TatC, are in fact the ones that are primarily responsible for the suppressing activity of the multiple mutated Tat translocases.

Because the plate assays only indirectly reflect the presence of MalE in the periplasm in a semiquantitative manner, we directly and quantitatively analyzed the export of TorA(KQ)-MalE in the corresponding cells by determining the subcellular localization of MalE-derived polypeptides after EDTA-lysosome spheroplasting (18). The fractions encompassing C/M and P, respectively, were separated by SDS-PAGE followed by Western blotting using MalE-specific antibodies. As a control for the quality of the fractionation experiments, the subcellular distribution of the cytoplasmic enzyme transaldolase B was ana-
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FIGURE 3. Subcellular localization of TorA(KQ)-MalE-derived polypeptides. Cells were fractionated into a P fraction (A) and a combined C/M fraction (B) by EDTA-lysozyme spheroplasting as described under “Experimental Procedures.” The samples corresponding to an identical amount of cells were subjected to SDS-PAGE and immunoblotting using anti-MalE antibodies. The positive control was E. coli GSJ101 containing plasmids pTorA-MalE and pHSG-TatABCE (lane 1). All other samples correspond to GSJ101 containing plasmid pTorA(KQ)-MalE and in addition one of the pHSG-TatABCE plasmids encoding the Tat translocases indicated above the lanes. p, TorA-MalE/TorA(KQ)-MalE precursor in the C/M fraction; m, mature form of MalE in the P fraction; asterisks, positions of TorA-MalE/TorA(KQ)-MalE degradation products in the C/M fraction. C, relative export efficiency of TorA(KQ)-MalE in strains expressing wild-type or mutant Tat translocases. The amount of exported MalE protein in the P fractions of strains GSJ101 containing plasmid pTorA(KQ)-MalE in addition to plasmid pHSG-TatABCE (wild-type tat genes) or one of the various pHSG-TatABCE-KQS plasmids (KQS mutant translocases) was determined in three different independent experiments via quantification of the chemiluminescence signals. The signals were recorded by a CCD camera and subsequently analyzed by the program AIDA 2.41 (Raytest). The relative export efficiency of the positive control GSJ101 (pTorA-MalE, pHSG-TatABCE) was set to 100%.

lyzed in parallel. As expected, transaldolase B was found exclusively in the C/M fraction of all cells examined (data not shown).

As shown in Fig. 3B, various MalE-derived polypeptides can be detected in the C/M fraction of cells coexpressing the unaltered TorA-MalE together with the wild-type Tat translocase (lane 1). As described previously (18), these bands correspond to the unprocessed precursor protein and various cytosolic degradation products of it. In the P fraction (Fig. 3A, lane 1) of the same cells, a mature-sized MalE-derived polypeptide is present that corresponds to the MalE protein that has been translocated across the cytoplasmic membrane in a Tat-dependent manner (18). In the C/M fractions of the strains expressing the TorA(KQ)-MalE reporter in combination with either the wild-type or the KQS mutant Tat translocases, a similar pattern of bands and a pronounced accumulation of unprocessed precursor is observed (Fig. 3B, lanes 2–10). As expected from the plate assays, no mature MalE is visible in the P fraction of GSJ101 (pTorA(KQ)-MalE; pHSG-TatABCE), directly demonstrating that the TorA(KQ)-MalE mutant reporter is practically not exported by the wild-type Tat translocase (Fig. 3A, lane 2). In contrast, various amounts of mature MalE can be detected in the P fraction of the strains expressing TorA(KQ)-MalE together with the KQS mutant translocases (Fig. 3A, lanes 3–10). Quantification revealed that the amount of MalE translocated into the periplasm of GSJ101 (pTorA(KQ)-MalE; pHSG-TatABCE) is extremely low (~0.8%) compared with the positive control GSJ101 (pTorA-MalE; pHSG-TatABCE) that was set to 100%. In the strains expressing the mutant KQS Tat translocases, values between 2.9% (KQS103) and 44% (KQS100) were observed (Fig. 3C). Together with the results obtained from the plate assays, these results also directly show that, in contrast to the wild-type translocase, the KQS mutant translocases have gained the ability to recognize and translocate the normally export-defective TorA(KQ)-MalE precursor to various degrees.

The KQS Mutant Tat Translocases Still Allow Efficient Membrane Translocation of the Unaltered TorA-MalE Reporter—Subsequently, we asked whether the KQS mutant translocases have completely changed their substrate specificity or whether they are still able to accept the unaltered TorA-MalE precursor. Plating of GSJ101 coexpressing TorA-MalE together with the KQS mutant translocases on maltose minimal medium showed that, in all cases, growth of the corresponding strains was indistinguishable from growth of GSJ101 (pTorA-MalE, pHSG-TatABCE). Furthermore, all strains formed red colonies on MacConkey maltose agar plates (Table 2). The results from the plate assays, already indicative for efficient export of MalE into the periplasm, were directly confirmed by cell fractionation experiments. As described above, the cells were fractionated by EDTA-lysozyme spheroplasting (18), and the fractions encompassing C/M and P, respectively, were separated by SDS-PAGE followed by Western blotting using MalE-specific antibodies. As a control for the quality of the fractionation experiments, the subcellular distribution of the cytoplasmic enzyme transaldolase B was analyzed in parallel also in these experiments. Transaldolase B was found exclusively in the C/M fraction of all cells examined (not shown). As shown in Fig. 4B, a similar pattern of MalE-derived bands, corresponding to the unprocessed precursor and cytosolic degradation products of it, were detected in the C/M fraction of all strains. Whereas no mature MalE was detectable in the P fraction of the negative control GSJ101 expressing the export-defective TorA(KQ)-MalE together with the wild-type Tat translocase (Fig. 4A, lane 2), similar amounts of mature MalE protein are present in the periplasmic fractions of the strains coexpressing the unaltered TorA-MalE precursor together with either the wild-type or the KQS mutant Tat translocases (Fig. 4, A, lanes 1 and 3–10, and C). Taken together, these results clearly show that the KQS mutant Tat translocases have acquired a broadened substrate specificity with respect to the nature of the amino acid residues

FIGURE 4. Membrane translocation of the unaltered TorA-MalE reporter. As described above, the cells were fractionated by EDTA-lysozyme spheroplasting (18), and the fractions encompassing C/M and P, respectively, were separated by SDS-PAGE followed by Western blotting using MalE-specific antibodies. As a control for the quality of the fractionation experiments, the subcellular distribution of the cytoplasmic enzyme transaldolase B was analyzed in parallel also in these experiments. Transaldolase B was found exclusively in the C/M fraction of all cells examined (not shown). As shown in Fig. 4B, a similar pattern of MalE-derived bands, corresponding to the unprocessed precursor and cytosolic degradation products of it, were detected in the C/M fraction of all strains. Whereas no mature MalE was detectable in the P fraction of the negative control GSJ101 expressing the export-defective TorA(KQ)-MalE together with the wild-type Tat translocase (Fig. 4A, lane 2), similar amounts of mature MalE protein are present in the periplasmic fractions of the strains coexpressing the unaltered TorA-MalE precursor together with either the wild-type or the KQS mutant Tat translocases (Fig. 4, A, lanes 1 and 3–10, and C). Taken together, these results clearly show that the KQS mutant Tat translocases have acquired a broadened substrate specificity with respect to the nature of the amino acid residues
Twin Arginine Signal Peptide Recognition

In this study we have found that replacement of RR by KK in the TorA signal peptide reduced but not abolished membrane translocation of the very sensitive Tat reporter protein TorA-MalE. A similar finding was reported by Ize et al. (28) using a likewise very sensitive reporter system that is based on the bactericidal effect of colicin V (ColV), which is observed only when the colicin gains access to the plasma membrane from the periplasmic side. Replacement of RR by KK in the signal peptide of the TorA-ColV hybrid precursor significantly reduced but not completely abolished its Tat-specific membrane translocation. Together, these combined results suggest that an arginine residue at either position of the RR residues in Tat signal peptides is not absolutely required for precursor recognition by the Tat translocase and that a KK pair allows a weak binding of the TorA signal peptide to the TatBC substrate receptor. To completely block export of TorA-MalE, a more drastic alteration (i.e. RR to KQ) was required. In this case binding of the corresponding TorA(KQ)-MalE precursor to the TatBC substrate receptor most likely is too weak to establish a productive interaction. So far, almost nothing is known about the nature and the number of contacts that are required for the productive interaction of Tat signal peptides with the Tat translocase. A possible scenario would be that the Tat consensus motif in Tat signal peptides plays a crucial role in substrate recognition specificity and makes several contacts to a signal peptide binding pocket in the TatBC receptor complex. We have addressed this possibility by genetic means. If the export-defect of the TorA(KQ)-MalE precursor is indeed caused by an inability of the Tat translocase to recognize the altered signal peptide, then mutations in the translocase components might exist that restore the defective recognition step. In fact, several mutations in the tat genes that suppress the export defect of the TorA(KQ)-MalE precursor could be identified.

In the strain expressing the strongest suppressor translocase (KQS100) together with TorA(KQ)-MalE, the amount of present at the twin arginine position in the consensus motif of the TorA signal peptide.

**DISCUSSION**

In the present work we have shown that the replacement of the conserved twin arginine residues in the TorA signal peptide by a lysine-glutamine pair completely abolished Tat-dependent membrane translocation of a TorA(KQ)-MalE reporter protein. Mutant Tat translocases were identified that showed a restoration of TorA(KQ)-MalE export to various degrees. Because the mutant Tat translocases still efficiently accepted the TorA-MalE reporter protein possessing the unaltered TorA(RR) signal peptide, the substrate specificity of the translocases was not entirely changed from RR to KQ; rather, the translocases showed an increased tolerance toward variations of the amino acids occupying the positions of the twin arginine residues in the consensus motif of a Tat signal peptide.

So far, the exact role of the highly conserved RR residues in the consensus motif of Tat signal peptides is not clear. Using an extensive cross-linking approach, Alami et al. (11) showed that the Tat precursor protein preSufl first binds to the primary substrate receptor TatC in an RR-dependent manner. Subsequently, the precursor is handed over to TatB that, in addition to the RR region, also closely contacts the hydrophobic region of the signal peptide. No cross-links of preSufl to the TatBC receptor complex were observed when the RR residues in preSufl were replaced by KK. Based on these findings, it has been proposed that the initial step in precursor binding to the Tat translocase occurs via the specific recognition of the RR consensus motif by the receptor TatBC.

FIGURE 4. Subcellular localization of TorA-MalE-derived polypeptides. Cells were fractionated into a P fraction (A) and a combined C/M fraction (B) by EDTA-lysozyme spheroplasting as described under “Experimental Procedures.” The samples corresponding to an identical amount of cells were subjected to SDS-PAGE and immunoblotting using anti-MalE antibodies. Positive control, E. coli GSJ101 containing plasmids pTorA-MalE and pHSG-TatABCE (lane 1). The negative control was GSJ101 containing plasmids pTorA(KQ)-MalE and pHSG-TatABCE (lane 2). All other samples correspond to GSJ101 containing plasmid pTorA-MalE and in addition one of the pHSG-TatABCE plasmids encoding the Tat translocases indicated above the lanes, p, TorA-MalE/TorA(KQ)-MalE degradation products in the C/M fraction; m, mature form of MalE in the P fraction; asterisks, positions of TorA-MalE/TorA(KQ)-MalE degradation products in the C/M fraction. C, relative export efficiency of TorA-MalE in strains expressing wild-type or mutant Tat translocases. The amount of exported MalE protein in the P fractions of strains GSJ101 containing plasmid pTorA-MalE in addition to plasmid pHSG-TatABCE (wild-type Tat translocase) was determined above the lanes, p. TorA-MalE/TorA(KQ)-MalE precursor in the C/M fraction; m, mature form of MalE in the P fraction; asterisks, positions of TorA-MalE/TorA(KQ)-MalE degradation products in the C/M fraction. C, relative export efficiency of TorA-MalE in strains expressing wild-type or mutant Tat translocases. The amount of exported MalE protein in the P fractions of strains GSJ101 containing plasmid pTorA-MalE in addition to plasmid pHSG-TatABCE (wild-type Tat translocase) was determined above the lanes, p. TorA-MalE/TorA(KQ)-MalE precursor in the C/M fraction; m, mature form of MalE in the P fraction; asterisks, positions of TorA-MalE/TorA(KQ)-MalE degradation products in the C/M fraction.
The KQS translocase mutations either directly or indirectly might alter the signal peptide binding pocket in a way that a stronger binding of amino acids at a non-RR position in the extended twin arginine motif or elsewhere in the signal peptide compensates for the weakened or missing binding of the altered amino acid residues at the RR position. Thereby, a productive recognition of the TorA(KQ) signal peptide without negatively affecting recognition of the TorA(RR) wild-type signal peptide would be allowed.

Such a phenotype is reminiscent of the phenotype of the so-called prl alleles of various sec genes that have been isolated by genetic selections that were basically similar to the selection that has been performed in this study. These prl alleles were also isolated as suppressors that allow export of signal peptide-defective Sec-dependent precursor proteins (Ref. 29; for reviews, see Refs. 30–31). prl alleles of the genes secA, secY, secE, and secG result in Sec translocases that, besides being still proficient of accepting wild-type Sec signal peptides, allow the recognition and membrane translocation of proteins containing different mutations in their signal peptides or even of proteins that entirely lack a signal peptide (32–34). Originally, it was reasoned that the Prl mutations in the Sec components would act by restoring defective signal peptide-translocase interactions. Because of a lack of allele specificity and the fact that even signal-less proteins could be exported in a prl strain background, this hypothesis was abandoned, and it was suggested that the Sec translocase possesses a proofreading activity that rejects proteins lacking a functional signal peptide and which is reduced in the Prl mutant translocases. However, recent modeling of the effects of prl mutations on the structure of the E. coli SecYEG complex suggests that the Prl variants of the channel components SecY, SecE, and SecG most likely act by destabilizing the closed state of the protein-conducting SecYEG channel or by stabilizing its open form, thereby allowing channel opening to occur without the triggering event of signal peptide binding that is required to open the channel in a wild-type SecYEG complex (35). Therefore, at present it cannot be entirely excluded that the KQS mutant translocases, isolated in this study, exert their suppressing activity by means other than restoring a defect in signal peptide recognition via the conserved twin arginine motif. Nevertheless, the mutations analyzed in this communication represent the first gain-of-function mutations described for the Tat system so far and, like the prl mutations affecting the Sec translocase components, might significantly contribute to the understanding of the molecular mechanism of Tat-mediated protein translocation across biological membranes.

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