In *Escherichia coli*, the Tat system does not translocate Tat signal sequence fused PhoA (RR-PhoA), as it requires disulfide formation for folding. Here we show that such a RR-PhoA construct can be efficiently targeted to the Tat translocon, but the transport is not completed. RR-PhoA is detectable in a 580-kDa TatBC-containing complex, which is the first substrate-bound TatBC complex detected in a bacterial system so far. A second TatBC complex near 440 kDa comprises most of the TatB and TatC but is devoid of RR-PhoA. The targeting of PhoA to the Tat translocon depends on the twin-arginine motif and results in severe growth defects. This physiological effect is likely to be due to proton leakage at the cytoplasmic membrane. The results point to mechanistic incompatibilities of the Tat system with unfolded proteins such as RR-PhoA. There does not exist an intrinsic quality control at the TatBC complex itself, although correct folding is inevitable for Tat-dependent translocation.

The Tat system is a transport machinery, which allows the translocation of folded proteins across biological membranes (1). This is in contrast to the Sec translocation apparatus, which cannot translocate fully folded proteins (2). The Tat system of *Escherichia coli* consists of three membrane-integral components, termed TatA, TatB, and TatC, which are encoded in one operon (3). A second TatA homolog, TatE, can functionally substitute for TatA and is encoded by a monocistronic gene (3). TatA(E) and TatB show sequence homology and are predicted to contain a N-terminal membrane anchor, whereas TatC is a polytopic membrane protein with six transmembrane domains (4—6). In *E. coli*, TatC appears to form a complex with TatB and TatA (7, 8). In analogy to the thylakoidal TatABC system, it seems that after substrate binding to the TatBC core complex, additional TatA is recruited in the presence of a twin-arginine motif and results in severe growth defects. This physiological data are presented, which strongly suggest that the Tat signal sequence binding chaperones are optimized to prevent proper folding prior to targeting of specific cofactor-containing Tat substrates, and this can be important for the formation of an active enzyme, as has been shown for *E. coli* hydrogenase-2 (15).

We used in this study a Tat signal sequence/PhoA fusion protein to address the question whether there exists a general selection for folded proteins at the Tat translocon. We show with unfolded PhoA for the first time in a bacterial system a direct and RR-specific substrate binding to the Tat translocon *in vivo*. The data indicate that TatB and TatC do not possess an intrinsic quality control for this binding event. Physiological data are presented, which strongly suggest that the Tat system attempts to translocate PhoA, which can become harmful for the cells. Therefore, albeit the translocon itself does not reject unfolded Tat substrates such as RR-PhoA, the folded state of Tat substrates is a very important criterion for successful translocation.

**MATERIALS AND METHODS**

**Strains and Growth Conditions**—*E. coli* MC4100 (16), with pBAD vectors, its arabinose-resistant derivative (17), was studied. *E. coli* XL1-Blue Mif’ Kan (Stratagene) was used for cloning, and *E. coli* CC118 was used for PhoA activity determinations (18). The bacteria were grown aerobically at 37 °C on LB medium (1% tryptone, 1% NaCl, 0.5% yeast extract) in the presence of the appropriate antibiotics (100 μg/ml ampicillin, 20 μg/ml chloramphenicol, 12.5 μg/ml tetracycline). For anaerobic growth, cells were grown on LB medium supplemented with 0.5% glycerol and 0.4% nitrate. TMAO was added to 1.1% for induction of torA expression.

**Plasmids and Genetic Methods**—For the fusion of the complete signal sequence of the Tat substrate HiPIP to PhoA, a BamHI restriction site was introduced into *hip* at codon 7 of the mature domain using QuikChange™ mutagenesis (Stratagene) with the primer pair *hip*-BamHI-F (5’-CGC TCG GAT CCG CAT GCA CGG GTA TCC-3’) and *hip*-BamHI-R (5’-CGG TCG CAT CGG TAT CCA CGG CAT TGG CG-3’), and pEXH5-tac as a template. pEXH5-tac is a tac-promoter derivative of the PET22b+- (Novagen)-based pEXH5 (19), produced by cloning of the XbaI/HindIII *hip*-containing fragment of pEXH5 into the XbaI/HindIII digested pTB-DG (17). PhoA was BamHI/HindIII excised from pTFD342 (20) and cloned into the corresponding plasmids.
sites of pEXH5-tac, resulting in pEX-hi

The Tat system is capable of translocating folded proteins, such as cofactor-containing or even oligomeric enzymes (4). For some reason, the Tat system does not translocate unfolded proteins, such as apo-forms of c-type cytochromes or reduced alkaline phosphatase, when such proteins are N-terminally fused to Tat signal sequences (12, 13). We addressed this question and examined the targeting of a RR-PhoA construct, which contains the structurally well studied signal sequence of the Tat substrate HiPIP (23, 29). As a control we used KK-PhoA, which has the two consecutive arginines of the twin-arginine motif and their exchange in KK-PhoA. The n-, h-, and c-regions are indicated, as well as the two constructs were confirmed by sequencing.

Biochemical Methods—SDS-PAGE was carried out according to Laemmli (24), and blue native polyacrylamide gel electrophoresis (BN-PAGE) was conducted with 5–14.5% gradient gels according to Schagger and von Jagow (25). Proteins were semidry blotted on nitrocellulose (Laemmli gels) or polyvinylidene fluoride (BN gels) and developed using the ECL system. Antibodies were used against PhoA (Rockland), HiPIP (18), TatA, TatB (both donations from Tim Yahr, Iowa City, IA), or TatC (donation of Matthias Müller, Freiburg, Germany). For BN-PAGE analyses, membranes were prepared under thioreducing conditions with buffer containing 20% sucrose, 50 mM Bis-Tris HCl, pH 7.0, 10 mM MgCl₂, and 1 mM β-mercaptoethanol by French pressure cell passage (55 MPa), low (20,000 g, 10 min, 4 °C) and high speed (130,000 g, 30 min, 4 °C) centrifugation, and used at a protein concentration of 1.5 mg/ml for solubilization with 0.5% digitonin in the same buffer. Protein estimations were carried out according to the Lowry method (26). Periplasm, membranes, and cytoplasm were fractionated as described previously (19).

Inverted vesicles (INV) were prepared by a French press passage of the cells at 55 MPa in INV buffer (250 mM sucrose, 50 mM Tris acetate, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA), followed by a low speed centrifugation to remove cell debris, an ultracentrifugation to sediment the membranes, and resuspension of the INV to an A₂₈₀nm of 10 in 50 mM Tris acetate, pH 7.5, 250 mM sucrose, 5 mM MgCl₂. Fluorescence quenching experiments were carried out using the spectrofluorometer SFM25 (Kontron Instruments, Eching, Germany). The assays contained in a total volume of 2 ml of 50 mM Tris acetate, pH 7.75, 250 mM sucrose, 5 mM MgCl₂, 30 mM creatine phosphate, 100 μg/ml creatine kinase, 2 μM acridine orange, and 500 μg/ml INV protein. The pH gradient formation was initiated by addition of 1.25 mM ATP; the assay was terminated by addition of 100 μM carbonyl cyanide 3-chlorophenylhydra-

Periplasmic PhoA activity was assayed with whole cells by the method of Brickman and Beckwith (27). The sensitivity to SDS was quantified as described previously (17). The activity of TorA in the periplasmic fraction was visualized as described by Silvestro et al. (28).

RESULTS

The Tat system is capable of translocating folded proteins, such as cofactor-containing or even oligomeric enzymes (4). For some reason, the Tat system does not translocate unfolded proteins, such as apo-forms of c-type cytochromes or reduced alkaline phosphatase, when such proteins are N-terminally fused to Tat signal sequences (12, 13).

In initial experiments, we tested whether or not our RR-PhoA fusion protein was translocated by the Tat system. The phoA-mutant strain CC118 was used for all determinations of PhoA activity. We compared
the activities with natural PhoA, RR-PhoA, KK-PhoA, or the empty vector control (Fig. 1B). While PhoA with its own Sec signal sequence gave a high PhoA activity, the Tat signal sequence fused PhoA resulted in a reduction to about 6% of that activity. This low activity was not Tat-dependent, as a RR > KK exchange within the twin-arginine motif had no significant effect. The Tat independence of the activity suggests that some RR-PhoA or KK-PhoA is translocated via the Sec pathway. Such a minor Sec-dependent translocation is not unusual and has already been observed earlier for several other Tat signal sequences when fused to PhoA (13). The leakage of Tat signal fusions of Sec subunits via the Sec pathway is believed to be caused by Sec determinants on the mature domain, which can override the Tat determinants on the signal sequence (30). As the data indicated that the Tat signal sequence of HiPIP did not allow a Tat-dependent translocation of PhoA, the construct was used in the following to study the Tat system incompatibility of PhoA.

A **Twin-arginine-specific Effect of RR-PhoA at Wild Type as Well as at Increased Tat Levels**—When we compared the growth of strains expressing either RR-phaA or KK-phaA from an arabinose-inducible promoter in a wild-type tat background, the induction of RR-phaA caused a growth inhibition which was significantly higher than with KK-phaA (Fig. 2A). As the KK-phaA control indicated any possible negative effect which could be caused by the expression system, the significantly reduced growth of the strain expressing RR-phaA clearly reflected a toxic effect that was solely due to the presence of the twin-arginine motif in the signal sequence. This finding was intriguing, as it strongly pointed to a Tat system interaction of the unfolded RR-PhoA.

RR-PhoA obviously harmed the cells at wild type Tat levels, and thus we wondered whether this effect would be larger when the Tat system was more abundant. This was indeed the case. When we increased the abundance of the Tat translocon about 20-fold by normally, as did the empty vector control strain. As the growth inhibition was clearly dependent on the presence of the RR-motif in the signal sequence and as the effect was strongly enhanced when the Tat translocon was more abundant, it was very likely that the effect was due to a

**FIGURE 2. The presence of RR-PhoA affects growth at wild type and at higher abundant Tat levels.**

A, growth curves of E. coli MC4100 with pBAD-hip^−^phaA (solid lines) or pBAD-hip^−^KK-phaA (dotted lines), either without (thin lines) or with (thick lines) arabinose induction (0.5%). The Δ indicates the difference between the induction effects of RR-PhoA and KK-PhoA. B, growth curves of E. coli MC4100 with pBAD22 (empty vector control (thin line)), pBAD-hip^−^phaA (thick line), or pBAD-hip^−^KK-phaA (dotted line), in the presence of pABS-tatABC, and thus of higher TatABC levels. Induction of pBAD expressions was with 0.5% arabinose at the time point indicated by an arrow. Standard deviations are indicated and are based on three parallel independent cultures. Again, the Δ indicates the difference between the induction effects of RR-PhoA and KK-PhoA.

**FIGURE 3. The RR-PhoA effect is suppressed by a decrease of the plasmid copy number.**

A, the growth arrest is suppressed during prolonged growth. The growth of a strain carrying pBAD-hip^−^phaA in MC4100 with pABS-tatABC was measured. Induction of pBAD-expressions was with 0.5% arabinose at the time point indicated by an arrow. Standard deviations are indicated and are based on three parallel independent cultures. Note that after 10 h the cultures recover from their growth arrest. B, detection of TatA (at 18 kDa), TatB (at 30 kDa), TatC (at 28 kDa), and PhoA (upper band at 55 kDa) by specific antisera (Western blot after SDS-PAGE) and of Ncol-restricted plasmids (ethidium bromide-stained agarose gel). The strains contain pABS-tatABC, which in all lanes gives rise to the upper band at 7142 bp, and in addition the following vectors: pBAD22 (vector control without insert, 4542-bp fragment), pBAD-hip^−^phaA (RR-PhoA, 5119-bp fragment), or pBAD-hip^−^KK-phaA (KK-PhoA, 5119-bp fragment). The suppressor strain is labeled "RR-PhoA supp." Note that the suppressor strain has significantly reduced levels of TatA, TatB, TatC, and RR-PhoA as a result of a plasmid copy number decrease.
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**FIGURE 4. Membrane stability is affected by RR-PhoA.** A, fluorescence quenching shows that RR-PhoA-containing membranes as prepared from MC4100 with pABS-tatABC and pBAD-his^6^-phoA build up a diminished proton gradient. ATP-induced proton gradient formation is followed by quenching of acridine orange fluorescence. CCCP addition abolishes the proton gradient. Membranes were used from a vector-only control strain (thin line), a RR-PhoA strain (thick line), and a KK-PhoA strain (dotted line). For experimental details, see “Materials and Methods.” B, comparison of the fluorescence quenching results. The extent of fluorescence quenching was measured at the CCCP addition step. The results are from three independent assays and include their standard deviations. C, comparison of membrane proteins in samples used for fluorescence quenching. Upper part, equal amounts of membrane proteins (10 μg) were separated on a 15% SDS-PAGE gel and Coomassie-stained. Lower part, detection of TatA after Western blotting demonstrates equal amounts of Tat system in the respective membrane fractions.

Direct RR-PhoA interaction with the Tat translocon. This was interesting in the light of the fact that PhoA neither can fold properly in the reducing cytoplasm nor can it be translocated by the Tat system. Importantly, the number of colony-forming units per 1 OD_{600 nm} remained constant (2.5 × 10^8 colony-forming units/ml).

The growth inhibition lasted for about 8 h. Thereafter, the cultures recovered to normal growth, which was enabled by a drastic decrease of the plasmid copy number, resulting in low TatABC and RR-PhoA levels (Fig. 3). Retransformed plasmids from such suppressor strains showed a normal copy number, indicating that the decrease of the copy numbers was not due to mutations on the plasmids (data not shown). Together, these results indicate that RR-PhoA was targeted to the Tat translocon by an unusual Tat substrate binding pathway. This targeting had negative physiological effects and thus led to a growth arrest.

**RR-PhoA Targeting Affects the Trans-membrane Proton Gradient—**One possible explanation for the growth retardation was a proton leakage, which could be caused by stable RR-PhoA/Tat system interactions, such as some dead-end assembly of the Tat system around RR-PhoA or alternatively a release of unfolded PhoA into the membrane. This was tested with inverted membrane vesicles (INV), which build up a proton gradient when ATP is added, as the reversible ATP synthase pumps protons into the vesicles under these conditions. This proton gradient can be conveniently measured by acridine orange fluorescence quenching (31). The proton gradient formation with INV from the RR-PhoA-containing growth-inhibited strain was compared with the gradient formation with INV from a strain with KK-PhoA or with INV from a strain without recombinant Tat substrates. As shown in Fig. 4A, the proton gradient was significantly reduced with INV from the RR-PhoA-containing strain when compared with the gradient formed with INV from the KK-PhoA-containing strain or the strain carrying the empty vector. As calculated from the step-like increase of fluorescence upon addition of the uncoupler CCCP, the fluorescence quenching with vesicles from the RR-PhoA-containing strain was below 50% of the control values (Fig. 4B). The membrane protein pattern was identical in all three strains and the amount of Tat system was the same in vesicles from RR-PhoA or KK-PhoA-containing strains, as can be seen in the detection of TatA by Western blotting (Fig. 4C). Together, the results suggest that the expression of RR-PhoA affects membrane permeability.

**Direct Binding of RR-PhoA to the Tat Translocon—**To obtain a more direct evidence for an interaction of RR-PhoA with the Tat translocon, we used the BN-PAGE method, which has been applied successfully with the plant thylakoid Tat apparatus to show Tat substrate-Tat translocon interactions (32). BN-PAGE has been established recently as a useful tool also for the analysis of Tat complexes of bacteria. So far, no bacterial Tat substrate-Tat translocon complex could be demonstrated by BN-PAGE, but a stable TatABC complex has been detected (8).

In our analyses, TatA formed a ladder (Fig. 5A), which was already observed recently by the group of Colin Robinson (8), and TatB and TatC comigrated at about 440 and 580 kDa, indicating TatBC complexes of these sizes (Fig. 4, B and C). The 440-kDa TatBC complex, which most likely corresponds to the 370 kDa complex observed previously (8), was so abundant that it was detectable with anti-TatB serum even at wild type levels without tatABC overexpression, which gives also an idea about the relative expression level achieved with pABS-tatABC (see left lanes in Fig. 5B). In addition to the 440 kDa TatBC complex, we detected TatB and TatC in a band at about 580 kDa. This 580-kDa TatBC complex was always present in lower amounts than the 440-kDa complex. Further bands were observed below 440 kDa, which most likely reflected some complex dissociation upon solubilization, and some material did not enter the gels, possibly due to some aggregation after solubilization. No distinct TatA signal could be attributed to a TatBC-containing complex (Fig. 5A).

When the presence of RR-PhoA was analyzed, a sharp signal was reproducibly detectable at 580 kDa, which is the size of the larger TatBC complex (Fig. 5D). Strikingly, RR-PhoA was not part of the 440-kDa complex. The signal was not detectable when the Tat system was low abundant, i.e. without coexpression of tatABC. The clear dependence of the RR-PhoA signal from tatABC coexpression indicates that the 580-kDa TatBC complex binds RR-PhoA. The KK-PhoA derivative did not bind to the 580-kDa TatBC complex, demonstrating the twin-arginine specificity of the observed targeting (right lane in Fig. 5D), which is in agreement with the twin-arginine specific growth inhibition described above. In addition, a signal smear was generally observed, which was not
twin-arginine-specific, as all of these signals were also detectable with KK-PhoA. As shown in Fig. 5E, the amount of PhoA was very similar in all membrane fractions, even with KK-PhoA, which was not associated with the Tat translocon. Thus, the occurrence of the KK-PhoA and probably of most of the RR-PhoA in the membrane fraction might be explained by membrane interactions that precede a translocon interaction, as have been described earlier (23, 32, 33). Some PhoA degradation bands were reproducibly detected, which indicate that PhoA was sensitive toward proteases (Fig. 5E).

Importantly, the 580-kDa TatBC-containing complex was also seen in the absence of RR-PhoA. This suggested that this Tat complex was not caused by the PhoA constructs (Fig. 5, B and C). To address this, the Tat complexes were analyzed from strains which did not contain RR- or KK-PhoA. In all cases, the TatBC complexes as detected by BN-PAGE were identical, indicating that indeed the 580-kDa complex is not caused by the PhoA expression system (Fig. 6). Importantly, these blots were the best in sensitivity, which we obtained so far, and we could detect here also the 580-kDa TatBC complex at wild type expression level. The ratio of the two complexes was not altered at wild type level. Clearly, both complexes, the 440- and the 580-kDa complex, are naturally formed and not induced by the expression system. In contrast, bands at lower molecular mass (<440 kDa) were likely to be caused by disassembly processes upon solubilization.

**RR-PhoA Targeting Does Not Block the Tat Translocon but Inhibits Translocation Indirectly When the Proton Gradient Is Affected**—The above data show that RR-PhoA caused a strong growth inhibition. As this effect depends on the RR-motif and the Tat system, the data indicate that the targeting of RR-PhoA to the Tat-translocon is the cause of the phenotype. We now asked the question whether translocon-targeted RR-PhoA blocked the translocation of other Tat substrates. If the
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Tat system would be blocked by RR-PhoA, this would indicate a long lasting translocon interaction, which could be the cause of the proton leakage. If, on the other hand, the translocation of normal Tat substrates would not be blocked despite a RR-phoA overexpression, toxic effects would have to be explained differently. Therefore, the knowledge about the effect of RR-PhoA targeting on the translocation of other Tat substrates could provide valuable evidence for explanations of the described RR-PhoA phenotype. A block of translocation should be more severe when only wild type levels of Tat components are present. As described below, we observed the opposite; there was little effect at wild type Tat levels, whereas the translocation of Tat substrates was markedly reduced when the Tat components were coexpressed.

The first evidence for an ongoing translocation of other Tat substrates during expression of RR-phoA came from measurements of the SDS-phenotype, which relates to the translocation efficiency of the amidases AmiA and AmiC, two Tat substrates that are involved in cell division (34). Without coexpression of the tat genes, the SDS sensitivity was slightly but significantly affected by RR-PhoA expression, especially at lower SDS concentrations (Fig. 7A). Like in the case of the growth phenotype, the SDS phenotype was markedly increased by higher Tat levels. The empty vector control and the KK-PhoA control were unaffected (Fig. 7B). As RR-PhoA affected the translocation of AmiA and AmiC more at higher than at lower TatABC abundance, these data suggest that the effects of RR-PhoA on Tat-dependent translocation are indirect. A reduction of the membrane proton gradient, such as has been detected in the above described experiments (Fig. 3), is therefore the most likely reason for a lowered translocation efficiency and consequently the observed increased SDS sensitivity.

As a second approach, we tested the RR-PhoA effect on the translocation of the Tat substrate TMAO reductase. E. coli cultures were shifted to anaerobic growth on LB supplemented with glycerol and nitrate, and the production of TMAO reductase was induced by addition of TMAO 1 h prior to harvest. Translocation was then monitored by an activity stain of the periplasmic fraction (Fig. 7C). TMAO reductase activity can be detected in native gels by TMAO-dependent oxidation of reduced (blue) methyl viologen, resulting in stainless bands on a dark background. This simple assay can detect strong differences between activities in samples. Without induction, no TMAO reductase activity was detectable in the periplasm. This proves that the inducible TMAO reductase, which is TorA, was responsible for the detected activity. When TMAO was added, TorA was induced and transported into the periplasm, resulting in an active protein which stained in native gels. With wild type TatABC levels, equal activity of TMAO reductase was present in strains containing RR-PhoA, KK-PhoA, or the empty vector. However, when tatABC were coexpressed, the amount of TMAO reductase was markedly reduced by RR-PhoA. The presence of KK-PhoA or the empty vector had no effect. These results clearly show that (i) TorA translocation into the periplasm is not blocked by RR-PhoA in a wild type TatABC background, and (ii) when tatABC are coexpressed, RR-PhoA affects TorA translocation efficiency.

These data, together with the SDS sensitivity experiments, are strong evidence against a general block of the Tat translocon by RR-PhoA and support the view that the targeting of RR-PhoA to the Tat translocon results in a reduced proton gradient, which thereby indirectly affects the translocation of Tat substrates, such as AmiA, AmiC, or TorA.

DISCUSSION

Protein substrates of the Tat translocon have to be folded prior to translocation, at least in the well studied E. coli system (13). It has been shown recently that there exist substrate specific signal sequence bind-

![Image](https://example.com/image.png)

**FIGURE 7.** The translocon targeting of RR-PhoA does not result in a block of Tat-dependent translocation. A, analysis of SDS sensitivity of E. coli MC4100 carrying either the empty vector pJOE2702 (control), pJOE-hip^+^phoA (RR-PhoA), or pJOE-hip^+^KK-PhoA (KK-PhoA). The hamirosone promoter is advantageous for these experiments, as arabinose seems to effect translocation efficiency of some Tat substrates (37). The ratio of the optical density (OD$_{600}$) after 3-h growth in LB, 0.01% rhamnose with the indicated SDS concentrations toward the OD$_{600}$ of a culture grown without SDS was plotted against the given SDS concentration. B, analyses as described for A, but with coexpression of tatABC from pABS-tatABC in all strains. C, TorA activity stain in 10% native PAGE gels. Aerobic precultures were induced with 0.01% rhamnose. After 2 h of induction, equal amounts of cells were shifted to anaerobic growth on LB, 0.5% glycerol, 0.4% nitrate, 0.01% rhamnose with or without 1.1% TMAO for induction of TorA. After 1 h, equal amounts of cells were harvested and subjected to periplasm fractionation and native PAGE for the activity stain. One culture was not induced (left lane) to confirm the induction of the activity band by TMAO. The strains were the same as in A or B, where wt indicates wild type levels of tatABC, and tatABC^−^ indicates the coexpression of tatABC from pABS-tatABC. Note that RR-PhoA is the only construct that inhibits TorA translocation when tatABC are coexpressed.

ing chaperones, which support folding of the respective Tat substrates in the cytoplasm (15). In addition, also Sec substrates can be accepted by the Tat system, provided that a Tat signal sequence is fused to the protein and that the cytoplasmic folding of the substrate is allowed (12, 13). In the case of cytochromes, a recombinant mitochondrial heme ligase allowed cofactor assembly in the cytoplasm and this was a prereq-
uisite for Tat-dependent targeting of the holoprotein (12). A second example is alkaline phosphatase, which could be translocated by the Tat system in a thioredoxin reductase- and glutathione reductase-deficient mutant strain, thus under conditions that allowed cytoplasmic disulfide formation and folding (13). The fact that Tat-dependent targeting of Tat signal fused Sec substrates was achievable only when proper cytoplasmic folding was provided led to the idea of a general quality control mechanism for the folded state of Tat substrates (13, 14). For unknown reasons, the system seemed to distinguish between folded and unfolded, even with recombinant and overexpressed substrates for which naturally no cytoplasmic folding had to be achieved, such as cytochromes or PhoA.

Does There Exist a Folding Quality Control in E. coli?—Two explanations for the incompatibility of unfolded proteins with the Tat system were conceivable: either (i) there exists a general quality control mechanism which does not allow effective binding of unfolded Tat substrates to the Tat translocon, and/or (ii) unfolded Tat substrates cannot be translocated simply due to their structural incompatibility with the translocon mechanism.

The results of this study support the second explanation. We could not obtain any evidence for a quality control at the Tat translocon or prior to translocon binding. Instead, the data show that unfolded proteins can interact with the translocon and that this interaction is harmful to the cells. The interaction with a TatBC complex of 580 kDa could be directly demonstrated and thus it can be concluded that the TatBC complex can bind unfolded Tat substrates. A quality control is therefore not an intrinsic property of TatA or TatC, the two components that have been shown to recognize the substrate and initiate its translocation (9, 10). A targeting of unfolded proteins to the translocon may occur more frequently under conditions of recombinant production of Tat substrates and may in part be responsible for the well known saturation of Tat-dependent translocation by recombinant Tat substrates (35). As chaperones have been identified that support the folding of many E. coli Tat substrates (4, 15), this may be sufficient under natural conditions to prevent toxic effects of incorrectly folded Tat substrates being targeted to the Tat translocon. A general folding quality control would mean that the quality of Tat substrates is assessed after folding. Chaperones, which are only involved in folding per se, decrease the amount of targeted unfolded Tat substrates, but they do not represent a general quality control system. If incorrectly folded or unfolded Tat substrates are rarely targeted to the Tat translocon, it is likely that proteases help to avoid any toxic effects.

Our results show that no quality control takes place at the TatBC complex. Furthermore, as outlined below, the translocon most likely attempts to translocate PhoA and therefore probably there is no quality control after binding to TatBC, for example by TatA. Although unlikely, we cannot exclude that at wild type Tat substrate levels a quality control takes place prior to the interaction with TatBC. However, in that context we would like to point out that the experimental basis for the proposed folding quality control has been obtained solely with overexpressed recombinant Tat substrates (12, 13).

How May RR-PhoA Targeting Result into the Observed Phenotype If the Translocon Interaction Is Transient?—Although the toxic effect depends on the RR signal sequence and the Tat translocon, it is possible that it is not caused by a strong and persistent translocon binding. The Tat system still works with other Tat substrates when RR-PhoA is targeted to it, indicating that at least a significant amount of Tat translocons is not blocked. However, translocation efficiency of natural Tat substrates is reduced by RR-PhoA targeting, especially when the Tat components are more abundant (Fig. 7). As it could be shown in vitro that the membrane potential is affected under the same conditions, the effect on Tat substrate translocation is most likely indirect and due to a decrease of the membrane potential in vivo.

We interpret our data with the following working model: RR-PhoA binds to a 580-kDa complex, which is also present without PhoA expression and thus likely to be a natural complex (Fig. 6). As the unfolded state is not rejected and the substrate is bound, there is no argument against an attempt of the translocon to transport RR-PhoA. As the translocon is not blocked (Fig. 7), the attempt to translocate RR-PhoA results most likely in its release from the translocon. However, this release apparently damages the membrane integrity in a way that is manifested even after vesicle preparation (Fig. 4). Therefore, the release of RR-PhoA most likely artificially results in a membrane-spanning PhoA.

Although all attempts to distinguish a membrane-inserted RR-PhoA from unspecific membrane binding of RR-PhoA and KK-PhoA failed so far (data not shown), we would like to point out that it is quite usual that Tat substrates can contain C-terminal extensions, which span the membrane and thus are likely to be released from the Tat translocon after incomplete translocation of the N-terminal globular domains (36). For example, E. coli has four of those naturally occurring C-terminally anchored Tat substrates, among which two are formate dehydrogenase small subunits that are cotranslocated together with their corresponding large subunits (FdhN and FdoH), and two are hydrogenase small subunits (HyaA and HybO), which carry their own Tat signal sequence (4). If therefore the N terminus of an extended substrate is translocated without completion, the disassembly of the translocon may occur naturally, resulting in a release of a membrane-spanning species into the lipid bilayer, which can be harmful in cases such as PhoA, which natively is not membrane spanning.

As an alternative explanation for our observations, one cannot exclude that there exist different populations of Tat translocons, and RR-PhoA may block one of these translocon populations without affecting the translocation of TorA, AmiA, or AmiC. We are currently investigating these alternatives.

Why Are the Physiological Effects of RR-PhoA Significant but Only Small at Wild Type Tat Levels?—The effects of RR-PhoA on growth and SDS sensitivity were small but significant already at wild type levels of Tat translocons. The data obtained with RR-PhoA indicate that a targeting of unfolded proteins to the Tat translocon can take place under physiological conditions and argue against a quality control at the Tat translocon itself at wild type Tat level. Certainly, an "intrinsic" quality control can also be excluded at overexpressed TatBC complexes. As outlined above, a quality control prior to translocon-binding cannot be excluded with this approach, but recombinant RR-PhoA overcomes any quality control that might exist.

The increase of the RR-PhoA effects at higher Tat abundance can be best explained with effects on the membrane proton gradient. If the above outlined interpretation is correct and the release of RR-PhoA into the cytoplasmic membrane or a block of a subpopulation of Tat translocons results in proton leakage, it should be a matter of degradation kinetics whether the membrane potential is significantly affected or not. In the light of this consideration, it is expected that the effect of RR-PhoA is significantly increased when the translocon is higher abundant, as targeting is more rapid with higher abundant Tat systems. This is exactly what we observed.

In this context, it is interesting to know that upon prolonged growth, the bacteria can overcome the toxic effect by lowering the plasmid copy numbers (Fig. 3). This indicates that the selective pressure is strongly forcing the bacteria to produce less Tat translocons and less unfolded protein.
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Tat substrates. These data further confirm that the toxicity of RR-PhoA translocon targeting depends on the number of targeting events, and they support the view that the folded state of the Tat substrate is crucial for Tat functionality and important for bacterial growth.

A Novel 580-kDa TatBC Complex That Can Bind Tat Substrates—In the course of this work, a new TatBC-containing complex was identified, which migrates at 580 kDa in blue native gel electrophoresis (Figs. 5 and 6). RR-PhoA was detected as part of this 580-kDa complex but not of a 440-kDa TatBC complex. It is not clear whether distinct translocon assemblies exist for different classes of substrates or which influence the binding of Tat substrates has on the composition or structure of Tat complexes.

The binding of Tat substrates to the bacterial translocon has not been demonstrated before in vivo, but in vitro translated proteins have been reported to interact with the Tat translocon, as well as a synthetic signal sequence of SulI, notably in the absence of any folded or unfolded mature domain (7, 10). The assembly of TatA subunits to substrate-bound TatBC complexes has been demonstrated in the thylakoid system to be dependent on the transmembrane proton gradient (9), and photo crosslinking experiments have indicated that the situation is similar in the E. coli system (10). The absence of TatA in the TatBC-substrate complex at 580 kDa may thus be due to a dissociation of TatA from the complex upon solubilization of the membrane and dissipation of a proton gradient. The 440- and 580-kDa complexes are also detectable at wild type levels of Tat systems and in the absence of any PhoA construct, indicating that both complexes are naturally occurring. One could imagine that the 580-kDa complex represents simply a substrate shifted form of the 440-kDa complex. Complex analyses after ribosomal translation inhibition could not resolve this hypothesis (data not shown), as both complexes were still detectable, albeit in low amounts. Clearly, dedicated future studies will have to show whether the two complexes are somehow interconvertible or not.

Why is the folded state of Tat substrates crucial for the function of the Tat system? Are extended substrates too long to be compatible with the system? We currently address these important aspects and hope that these studies will help to understand the translocation mechanism.

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