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***Escherichia coli* tatC Mutations that Suppress Defective Twin-Arginine Transporter Signal Peptides**

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Abstract

In vitro studies have suggested that the TatBC complex serves as the receptor for signal peptides targeted for export *via* the twin-arginine translocation (Tat) pathway. Substitution of the hallmark twin-arginine dipeptide with two lysines abrogates export of physiological substrates in all organisms. We report the isolation and characterization of suppressor mutations that allow export of an ssTor(KK)-GFP-SsrA tripartite fusion. We identified two amino acid suppressor mutations in the first cytoplasmic loop of TatC. In addition, two other amino acids in the first cytoplasmic loop exhibit epistatic suppression. Surprisingly, we also identified a suppressor mutation predicted to lie within the second periplasmic loop of TatC, a region that is not expected to interact directly with the signal peptide. The suppressor mutations allowed export of the native *Escherichia coli* Tat substrate trimethylamine *N*-oxide reductase with a twin-lysine substitution in its signal sequence. The cytoplasmic suppressor mutations conferred SDS sensitivity and partial filamentation, indicating that Tat export of authentic substrates was impaired.

Keywords

twin-arginine translocase; TatC; signal peptide recognition; flow cytometry; suppressors

The bacterial twin-arginine (RR) translocation (Tat) pathway is responsible for the transport of cofactor-containing proteins and other folded polypeptides across the cytoplasmic membrane. Proteins exported *via* Tat contain long signal peptides featuring a conserved RR motif with a consensus sequence of S/T-R-R-x-F-L-K.¹ In *Escherichia coli*, the functional Tat translocon is composed of the membrane proteins TatA, TatB and TatC. Among Gram-positive bacteria, a few organisms can be found containing only two identifiable *tat* genes, encoding TatA and TatC homologues and lacking a *tatB* gene.^{2–5} In *Escherichia coli*, TatA is purified as a series of high molecular weight homo-oligomers that form pore-like structures of varying diameters. Based on these observations, Gohlke *et al.* proposed that TatA forms the protein-conducting pore that assembles on demand, the size of which is determined by the dimensions of the translocated protein substrate.⁶ Biochemical analyses have revealed that TatB copurifies in a complex with TatC and that this complex plays a key role in the targeting of Tat substrates to the translocon.^{7–9} Based on cross-linking of *in vitro* translocated proteins into inverted membrane vesicles, Alami *et al.* discovered that TatC in particular exhibits extensive contact with the signal peptide and recognizes the RR motif.⁷

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We sought to obtain genetic evidence that supports the role of TatC in the recognition of the RR motif and to further define the TatC epitopes that are important for this process. Substitution of the nearly invariant RR dipeptide by two lysines completely abolishes the export of all physiological Tat proteins tested so far.^{10,11} However, there is evidence for very low level export of heterologous proteins such as colicin V² and the maltose binding protein (MBP) when fused to the trimethylamine *N*-oxide reductase (TorA) signal peptide in which the RR motif had been mutated to a KK dipeptide (TorA(KK)).¹²

Here we report the isolation and characterization of TatC suppressor mutations that allow export of precursor proteins fused to a defective TorA(KK) signal peptide. These results provide additional genetic evidence that TatC serves as the receptor for Tat signal peptides. In addition, the suppressor mutations we have obtained help define regions in TatC that play a role in the recognition of the RR motif. As this manuscript was about to be submitted for publication, Kreutzenbeck *et al.* reported the isolation of suppressor mutations in a plasmid encoding the TatABCE protein conferring export of an ssTorA-MBP fusion in which the RR dipeptide had been substituted by a lysine-glutamine sequence.¹² Kreutzenbeck *et al.* presented evidence showing that the suppressor mutations permitted near wild-type (w.t.) level of export of ssTorA-MBP containing a w.t. RR dipeptide. In contrast, mutants we report here appear to represent a different class of suppressors that allow export of TorA(KK) signal peptides but impair translocation of native Tat proteins.

Isolation of suppressor TatC variants by fluorescence-activated cell sorting

ssTorA fused to the fluorescent reporter GFP-SsrA allows the facile and quantitative detection of Tat export by flow cytometry.¹¹ The ssTorA-GFP-SsrA (TGS) fusion is encoded by the plasmid pTGS¹¹ under the control of the arabinose promoter. The presence of the SsrA tag on green fluorescence protein (GFP) ensures that any fusion protein that fails to be exported *via* the Tat pathway is degraded by ClpXP.¹³ Thus, cell fluorescence arises only from TGS that has been sequestered away from the proteolytic machinery following Tat-dependent export into the periplasmic space (Fig. 1).^{11,14} *E. coli* B11k0 (Δ tatC)¹⁵ cells carry a complete deletion of the *tatC* gene and therefore exhibit background fluorescence with GFP-SsrA; in addition, they are sensitive to anionic detergents such as SDS and form filaments. B11k0 cells were transformed with the plasmid pBR322-*tatC*, which encodes the *tatC* gene with its own ribosome-binding site downstream of the constitutive *tat* promoter. B11k0 pBR322-*tatC* cells also containing pTGS exhibited a sevenfold increase in GFP-SsrA fluorescence and normal cell morphology and were resistant to SDS (Fig. 1 and data not shown). Mutation of the RR dipeptide within TGS to a KK sequence reduced the cell fluorescence to the background level, but as expected did not have any effect on cell morphology or SDS resistance (Fig. 1).¹¹

The *tatC* gene was subjected to random mutagenesis by error-prone PCR.¹⁶ The resulting DNA was ligated into pBR322-*tatC* in place of the w.t. *tatC* gene. The ligation product was transformed into XL-1 blue cells (Stratagene) resulting in a library of 2×10^6 independent transformants. The plasmid preparation of mutagenized pBR322-*tatC* was then introduced into *E. coli* B11k0 cells transformed with a plasmid encoding ssTorA(KK)-GFP-SsrA (pKGS). Sequencing of six clones selected at random revealed a mutation rate of 0.6% nucleotide substitutions per gene.

B11k0 cells expressing the *tatC* alleles and KGS were then screened by flow cytometry and clones with increased fluorescence were isolated. No clones with fluorescence comparable to the wild type (around sevenfold higher than background) were obtained even after repeated attempts. However, four clones (B11k0 with pTatC-N6, pTatC-W10, pTatC-X19 and pTatC-Y2) that reproducibly exhibited about 2- to 2.5-fold higher fluorescence over background were obtained and studied further (see Fig. 1 and Figure ST2, Supplemental Data, for sequence

alignment). The elevated fluorescence phenotype was dependent on the expression of both the TatC variant and the KGS reporter protein, suggesting that the mutations act as suppressors of the export defect caused by the KK substitution in the ssTorA signal peptide.

With the exception of pTatC-W10, which contained the single amino acid substitution P142S, all isolated *tatC* genes contained multiple amino acid changes. Several of the respective single amino acid mutations (W92G, F94S, P97S, L99P, A160S, V167D, L172P, C179R and K192N) as well as the double amino acid substitution W92G/P97S were generated by site-specific mutagenesis and their ability to export KGS was analyzed by fluorescence-activated cell sorting (FACS). TatC carrying the amino acid substitutions F94S and L99P conferred cell fluorescence comparable to clones pTatC-X19 and pTatC-Y2, respectively. B11k0 carrying pTatC-N6 contained the amino acid substitutions W92G, P97S and P172L. While neither the amino acid change in pTatC-W92G nor pTatC-P97S displayed export of KGS above background, the double substitution variant W92G/P97S exhibited a fluorescence profile comparable to that of pTatC-N6 (Fig. 1C). None of the other tested single amino acid substitutions conferred fluorescence above background in cells co-transformed with pKGS. To investigate the effect of mutations on the substrate specificity for the w.t. TorA signal peptide, we analyzed the fluorescence of the mutants expressing TGS (Fig. 1C). We calculated the relative export efficiency after subtracting the background fluorescence of B11k0 cells transformed with pBR322-*tatC* and pKGS. The export efficiency of the w.t. signal peptide fusion for all suppressing variants except for the clone carrying pTatC-W10 (containing the single mutation P142S) was around the same amount as for KGS, indicating a partial impairment of export. The TatC P142S variant conferred around 65% transport efficiency of TGS compared to the w.t. TatC plasmid.

The level of suppression conferred by the various single amino acid TatC variants may also be related to the expression level of the respective proteins. In pBR322-*tatC*, *tatC* expression occurs from the weak *tat* promoter and a pBR322 origin. Attempts to detect TatC produced from pBR322-*tatC* using Western blot analysis with existing polyclonal sera¹⁷⁻¹⁹ were unsuccessful, evidently because of the low level of *tatC* expression. To circumvent this problem, TatC was fused to an octahistidine tag (His8). Consistent with previous observations, we confirmed that a C-terminal His8 fusion does not interfere with the function of w.t. TatC or with that of the suppressor variants (data not shown).^{17,18} Western blot analysis of TatC variants in total cell lysate revealed that the level of expression of pTatC-F94S-His8 and pTatC-W10-His8 (P142S) was identical with that of w.t. pTatC-His8, whereas pTatC-L99P-His8 accumulated at somewhat lower levels (Figure S2). Thus, at least for two-thirds of the single amino acid TatC variants, the degree of suppression does not stem from differences in expression level.

Further, we examined whether the putative suppressors identified in the screen above could export a native *E. coli* protein having a KK substitution in the RR dipeptide. The enzyme TorA reduces trimethylamine *N*-oxide (TMAO) to trimethylamine. TorA contains a bismolybdopterin guanine dinucleotide cofactor, which is incorporated into the apo protein within the cytoplasm, a process that requires the chaperone TorD.^{20,21} Following cofactor incorporation, the TorA holoenzyme is exported into the periplasm by the Tat machinery.²² Reduction of TMAO has to occur on the periplasmic side of the inner membrane in order to assure electron transfer from TorC to TorA onto TMAO.²³

In *E. coli* GB22KK-C (*torA* R11K,R12K; Δ *dmsABC*:: Kan^R, Δ *tatC*::Spec^R) the RR dipeptide in the signal peptide of the chromosomal *torA* gene has been replaced with a KK sequence¹⁰ and there is a complete *tatC* deletion marked with a spectinomycin resistance cassette.¹⁷ Strain GBKK22-C also lacks *dsmABC* gene that encodes the only other enzyme that is known to reduce TMAO.¹⁰ The TorA protein in GBKK22-C cannot be exported, but it is competent for

cofactor assembly and, as a result, the active enzyme accumulates in the cytoplasm.¹⁰ GBKK22-C cells expressing the TatC variants encoded by pTatC-N6, pTatC-W10 (P142S), pTatC-X19, pTatC-Y2, the TatC single amino acid substitution construct pTatC-L99P or the double amino acid substitution pTatC-W92G/P97S were grown anaerobically for 22-24 h in Cohen and Rickenberg medium,²⁴ harvested, and fractionated by the osmotic shock procedure.²⁵ The efficiency of the fractionations was evaluated by monitoring the level of GroEL and DsbA as cytoplasmic and periplasmic markers, respectively. No GroEL could be detected by Western blot analysis in the periplasmic fractions, whereas DsbA was localized in the periplasmic fraction as expected (data not shown). Periplasmic and cytoplasmic proteins were then resolved by native electrophoresis and the gel was stained with methyl viologen in the presence of the reducing agent dithionite. Reduced methyl viologen served as an artificial electron donor for TMAO reductase, resulting in a readily detectable color change from blue to transparent upon oxidation of TMAO by the mature TorA enzyme²⁶ (Fig. 2). In *E. coli* MC4100 cells, about 50% of the active TMAO reductase was localized in the periplasmic fraction. No periplasmic TMAO reductase band was detected in *E. coli* GBKK22-C expressing w.t. TatC from pBR322-*tatC* (Fig. 2). Enzymatically active TMAO reductase was clearly detected in periplasmic fractions of cells co-expressing the TatC suppressor plasmids except for cells with pTatC-W10 (Fig. 2). However, we noticed that cells expressing the plasmid pTatC-W10 carrying the *tatC* P142S allele grew anaerobically with glycerol (0.6%) and TMAO (0.4%) as the final electron acceptor. Since periplasmic TMAO reductase activity is required for growth on TMAO, TatC P142S variants must be capable of exporting TorA having a KK mutation in its signal sequence. Consequently, it is possible that the failure to detect soluble TorA in the periplasmic fraction of these cells is due to the retention of the enzyme on the surface of the spheroplasts. Alternatively, it is possible that a very low level of exported TMAO that cannot be detected by the assay we used is still sufficient to allow growth under these conditions.

Effects of *tatC* suppressor alleles on SDS resistance and cell separation

In *E. coli*, the *N*-acetylmuramoyl-L-alanine amidases AmiA and AmiC are exported by Tat.² These enzymes cleave the peptide moiety of the *N*-acetylmuramic acid in peptidoglycan. Mutations that block the Tat pathway and prevent the export of AmiA and AmiC result in sensitivity to SDS² and partially inhibit cell division.²⁷ In *amiC*-deficient strains, the daughter cells separate poorly and, as a result, up to 30% of the population forms chains.²⁸ *amiA* mutants exhibit a milder defect in septation with only 5-10% of the cell population forming short chains. *amiA* and *amiC* double mutants show a much more pronounced cell-chain phenotype than *amiC* mutants.²⁷⁻²⁹ Light microscopy of stationary-phase cells expressing the *tatC* suppressor genes in B11k0 (Δ *tatC*) revealed different degrees of cell-chain formation (Fig. 3). The TatC P142S variant gave the least defect in cell separation with several short chains (3-4 cells) observed in addition to a few single cells. B11k0 cells containing pTatC-N6 showed a higher percentage of longer chains (>4 cells per chain). The TatC W92G/P97S substitutions that recapitulate the cell fluorescence and TMAO activity phenotypes of cells carrying pTatC-N6, which contains these two amino acid changes next to the substitution P172L, showed a lower percentage of long chains. The TatC variants, encoded by pTatC-X19, pTatC-Y2, pTatC-F94S and pTatC-L99P, showed severe impairment in cell division. Collectively, these data indicate that none of the suppressor mutations completely restored a normal cell morphology, suggesting that normal Tat export of proteins involved in cell division, presumably AmiA and AmiC, is at least partially impaired.

A functional Tat export system is required for normal cell envelope formation as determined by the ability of the cells to grow in the presence of the anionic detergent SDS. W.t. *E. coli* cells are almost unaffected by the presence of 2% SDS. The *tatC* strain B11k0 fails to form colonies on agar plates with 2% SDS, but upon transformation with pBR322-*tatC* completely

restored SDS resistance. However, cells expressing the *tatC* suppressor alleles failed to form colonies on LB plates with 2% SDS. The only exception was the TatC P142S substitution, which showed normal colony formation on 2% SDS (data not shown). These observations provide further evidence that the TatC suppressor mutations do not support the export of the native Tat proteins that are responsible for detergent resistance.

Discussion

Substitution of the prototypical RR sequence found in Tat signal peptides by a lysine dipeptide prevents translocation *via* the Tat pathway in all organisms examined so far, presumably because this mutation inhibits the interaction of the signal peptide with *tatC*.⁷ A search for mutations in the *tatC* gene that allow export of KGS, conferring increased cell fluorescence, ultimately led to the isolation of two different types of suppressor *tatC* alleles having a single amino acid substitution each. Two single amino acid suppressor mutations located on the cytoplasmic site of TatC conferred 20-25% of the cell fluorescence relative to the level observed with the w.t. *tatC* regardless of the signal peptide (RR or KK) used. We also identified two mutations, W92G and P97S, that acted in an epistatic manner to suppress the export defect resulting from the substitution of the RR dipeptide by KK. Neither of these two amino acid substitutions alone conferred export of signal peptides with a KK dipeptide. In addition, the two single amino acid suppressors and the W92G/P97S allele were shown to confer export of TMAO reductase (TorA) fused to a signal peptide containing the KK substitution. Finally, the cells displayed defects in septation and in SDS sensitivity. On the other hand, the amino acid substitution P142S constitutes a different kind of suppressor in that it confers around 15% relative export efficiency for KGS but a significantly higher relative export efficiency for TGS (~65%). This finding and the cell's higher resistance to SDS suggest that export of Tat substrates containing w.t. signal peptides is not greatly impaired by the TatC P142S mutation. While we could not detect TMAO activity in the periplasmic space of GB22KK-C with the TatC P142S variant encoded by pTatC-W10, the finding that these cells can grow anaerobically with TMAO as a final electron acceptor, a process that depends on the presence of TMAO reductase in the periplasm, revealed that export of the enzyme was taking place.

The topology of TatC within the membrane has been determined experimentally using numerous reporter fusions (Fig. 4).³⁰⁻³² According to this model, the first types of suppressor mutations, F94S, L99P and W92G/P97S, are located at the end of the second transmembrane domain and at the beginning of the first cytoplasmic loop of TatC, respectively. The first cytoplasmic loop, also referred to as the second cytoplasmic domain of TatC, corresponds to a highly conserved region in the bacterial and chloroplast TatC family and plays a critical role in Tat export.^{17,33} In earlier studies, it was reported that the mutation L99A severely reduces the export of authentic Tat substrates to less than 5%. However, the effect of this mutation with respect to KK export had not been tested.¹⁷ Similarly it has been reported that an F94A substitution completely abolishes export.

The mutation P142S is predicted to be located in the second periplasmic loop, raising the question of how it is able to affect the recognition of the signal peptide, since this event is expected to take place or at least be initiated in the cytoplasm. However, in a search for *tatC* mutants that allow export of a defective ssTorA(KQ)-MBP fusion, Kreutzenbeck *et al.* reported the isolation of the amino acid substitution E8K located at the N terminus of TatB, a domain that is predicted to be localized in the periplasm.¹² The finding that mutations in periplasmic residues in TatC or in TatB can impact the recognition of the signal sequence highlights the complexity of the organization of the Tat translocon. As has been noted by Kreutzenbeck *et al.*, mutations in residues exposed to the periplasmic side could result in a reorganization of the tertiary structure of TatC or TatB. For the thylakoid Tat pathway, signal peptide binding was reported to occur in a binding pocket deep inside the membrane domains of the TatB/TatC

(Hfc106/cpTatC) receptor complex.⁸ Thus, it is conceivable that mutations in a periplasmic domain may affect recognition events that occur either deep within the membrane or perhaps in proximity of the periplasmic site. Alternatively, we cannot rule out the possibility that during the translocation cycle TatB and also some domains of TatC may undergo dramatic conformational changes, as has been proposed to be the case for TatA.³⁴

The *tatC* mutations we identified in the present study exhibit different phenotypes relative to those reported recently by Kreutzenbeck *et al.*¹² The latter were isolated based on their ability to confer growth of cells expressing ssTorA(KQ)-MBP on maltose plates but they were also shown to allow efficient recognition or even higher membrane translocation rates of w.t. ssTorA-MBP. We utilized a more stringent screen in which export could be quantitatively determined based on cell fluorescence and isolated mutants that suppressed the export defect of KGS. We found two different types of suppressors: one type of suppressor variants exhibited an ~80% reduction in the export of TGS and reduced ability to export authentic Tat substrates as manifested by septation defects and SDS sensitivity. These suppressor mutations, namely, substitutions F94S, L99P and W92G/P97S, are located in the first cytoplasmic loop of TatC, unlike the majority of Kreutzenbeck's mutations, which localized within the first few N-terminal residues of the protein. In addition, we isolated the mutation P142S, which is located within a putative periplasmic loop and has a more moderate effect on the export of authentic substrates, thus leading to SDS resistance but partial defects in septation.

The suppressors we characterized in the present study, F94S, L99P, W92G/P97S and P142S, lie in the center of the protein sequence at opposite sides of the third transmembrane domain. All suppressor mutations resulted in nonconservative substitutions that are likely to have a significant effect on protein conformation. Interestingly, the TatC single point mutations identified by Kreutzenbeck *et al.* and the ones reported in the present study were all located within the N-terminal half of the protein.¹² This suggests that the N-terminal of TatC is likely to be particularly important for the recognition and interactions with the signal peptide. In agreement with these observations, recent *in vitro* cross-linking studies also highlighted the significance of the N terminus of TatC for signal peptide recognition.³⁵

As was the case with the mechanistic elucidation of the Sec export pathway, the synergism between genetic and biochemical analysis is expected to lead to deep insights that would be unlikely to arise solely by either approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

Tat, twin-arginine translocation
MBP, maltose-binding protein
GFP, green fluorescence protein
FACS, fluorescence-activated cell sorting
KGS, ssTorA(KK)-GFP-SsrA

TGS, ssTorA-GFP-SsrA
 pKGS, plasmid encoding ssTorA(KK)-GFP-SsrA
 pTGS, plasmid encoding ssTorA-GFP-SsrA
 KK, twin-lysine
 RR, twin-arginine
 TorA, trimethylamine *N*-oxide reductase
 TMAO, trimethylamine *N*-oxide
 w.t., wild type

References

1. Berks BC. A common export pathway for proteins binding complex redox cofactors? *Mol. Microbiol* 1996;22:393–404. [PubMed: 8939424]
2. Ize B, Gerard F, Zhang M, Chanal A, Voulhoux R, Palmer T, et al. *In vivo* dissection of the Tat translocation pathway in *Escherichia coli*. *J. Mol. Biol* 2002;317:327–335. [PubMed: 11922668]
3. Jongbloed JD, Antelmann H, Hecker M, Nijland R, Bron S, Airaksinen U, et al. Selective contribution of the twin-arginine translocation pathway to protein secretion in *Bacillus subtilis*. *J. Biol. Chem* 2002;277:44068–44078. [PubMed: 12218047]
4. Pop O, Martin U, Abel C, Muller JP. The twin-arginine signal peptide of PhoD and the TatAd/Cd proteins of *Bacillus subtilis* form an autonomous Tat translocation system. *J. Biol. Chem* 2002;277:3268–3273. [PubMed: 11719524]
5. Yen MR, Tseng YH, Nguyen EH, Wu LF, Saier MH Jr. Sequence and phylogenetic analyses of the twin-arginine targeting (Tat) protein export system. *Arch. Microbiol* 2002;177:441–450. [PubMed: 12029389]
6. Gohlke U, Pullan L, McDevitt CA, Porcelli I, de Leeuw E, Palmer T, et al. The TatA component of the twin-arginine protein transport system forms channel complexes of variable diameter. *Proc. Natl. Acad. Sci. USA* 2005;102:10482–10486. [PubMed: 16027357]
7. Alami M, Luke I, Deitermann S, Eisner G, Koch HG, Brunner J, Muller M. Differential interactions between a twin-arginine signal peptide and its translocase in *Escherichia coli*. *Mol. Cell* 2003;12:937–946. [PubMed: 14580344]
8. Gerard F, Cline K. The thylakoid proton gradient promotes an advanced stage of signal peptide binding deep within the Tat pathway receptor complex. *J. Biol. Chem* 2007;282:5263–5272. [PubMed: 17172598]
9. Dabney-Smith C, Mori H, Cline K. Oligomers of Tha4 organize at the thylakoid Tat translocase during protein transport. *J. Biol. Chem* 2006;281:5476–5483. [PubMed: 16407186]
10. Buchanan G, Sargent F, Berks BC, Palmer T. A genetic screen for suppressors of *Escherichia coli* Tat signal peptide mutations establishes a critical role for the second arginine within the twin-arginine motif. *Arch. Microbiol* 2001;177:107–112. [PubMed: 11797051]
11. DeLisa MP, Samuelson P, Palmer T, Georgiou G. Genetic analysis of the twin arginine translocator secretion pathway in bacteria. *J. Biol. Chem* 2002;277:29825–29831. [PubMed: 12021272]
12. Kreutzenbeck P, Kroger C, Lausberg F, Blaudeck N, Sprenger GA, Freudl R. *Escherichia coli* twin arginine (Tat) mutant translocases possessing relaxed signal peptide recognition specificities. *J. Biol. Chem* 2007;282:7903–7911. [PubMed: 17229735]
13. Levchenko I, Seidel M, Sauer RT, Baker TA. A specificity-enhancing factor for the ClpXP degradation machine. *Science* 2000;289:2354–2356. [PubMed: 11009422]
14. Tullman-Ercek D, DeLisa MP, Kawarasaki Y, Iranpour P, Ribnicky B, Palmer T, Georgiou G. Export pathway selectivity of *Escherichia coli* twin arginine translocation signal peptides. *J. Biol. Chem* 2007;282:8309–8316. [PubMed: 17218314]
15. Bogsch EG, Sargent F, Stanley NR, Berks BC, Robinson C, Palmer T. An essential component of a novel bacterial protein export system with homologues in plastids and mitochondria. *J. Biol. Chem* 1998;273:18003–18006. [PubMed: 9660752]
16. Fromant M, Blanquet S, Plateau P. Direct random mutagenesis of gene-sized DNA fragments using polymerase chain reaction. *Anal. Biochem* 1995;224:347–353. [PubMed: 7710092]

17. Buchanan G, Leeuw E, Stanley NR, Wexler M, Berks BC, Sargent F, Palmer T. Functional complexity of the twin-arginine translocase TatC component revealed by site-directed mutagenesis. *Mol. Microbiol* 2002;43:1457–1470. [PubMed: 11952898]
18. de Leeuw E, Granjon T, Porcelli I, Alami M, Carr SB, Muller M, et al. Oligomeric properties and signal peptide binding by *Escherichia coli* Tat protein transport complexes. *J. Mol. Biol* 2002;322:1135–1146. [PubMed: 12367533]
19. Punginelli C, Maldonado B, Grahl S, Jack R, Alami M, Schroder J, et al. Cysteine scanning mutagenesis and topological mapping of the *Escherichia coli* twin-arginine translocase TatC component. *J. Bacteriol* 2007;189:5482–5494. [PubMed: 17545291]
20. Ilbert M, Mejean V, Giudici-Orticoni MT, Samama JP, Iobbi-Nivol C. Involvement of a mate chaperone (TorD) in the maturation pathway of molybdoenzyme TorA. *J. Biol. Chem* 2003;278:28787–28792. [PubMed: 12766163]
21. Pommier J, Mejean V, Giordano G, Iobbi-Nivol C. TorD, a cytoplasmic chaperone that interacts with the unfolded trimethylamine *N*-oxide reductase enzyme (TorA) in *Escherichia coli*. *J. Biol. Chem* 1998;273:16615–16620. [PubMed: 9632735]
22. Santini CL, Ize B, Chanal A, Muller M, Giordano G, Wu LF. A novel sec-independent periplasmic protein translocation pathway in *Escherichia coli*. *EMBO J* 1998;17:101–112. [PubMed: 9427745]
23. Gon S, Giudici-Orticoni MT, Mejean V, Iobbi-Nivol C. Electron transfer and binding of the c-type cytochrome TorC to the trimethylamine *N*-oxide reductase in *Escherichia coli*. *J. Biol. Chem* 2001;276:11545–11551. [PubMed: 11056172]
24. Cohen GN, Rickenberg HV. Not available. *Ann. Inst. Pasteur. (Paris)* 1956;91:693–720. [PubMed: 13395009]
25. Stanley NR, Palmer T, Berks BC. The twin arginine consensus motif of Tat signal peptides is involved in Sec-independent protein targeting in *Escherichia coli*. *J. Biol. Chem* 2000;275:11591–11596. [PubMed: 10766774]
26. Silvestro A, Pommier J, Giordano G. The inducible trimethylamine-*N*-oxide reductase of *Escherichia coli* K12: biochemical and immunological studies. *Biochim. Biophys. Acta* 1988;954:1–13. [PubMed: 3282544]
27. Heidrich C, Templin MF, Ursinus A, Merdanovic M, Berger J, Schwarz H, et al. Involvement of *N*-acetylmuramyl-l-alanine amidases in cell separation and antibiotic-induced autolysis of *Escherichia coli*. *Mol. Microbiol* 2001;41:167–178. [PubMed: 11454209]
28. Bernhardt TG, de Boer PA. The *Escherichia coli* amidase AmiC is a periplasmic septal ring component exported *via* the twin-arginine transport pathway. *Mol. Microbiol* 2003;48:1171–1182. [PubMed: 12787347]
29. Priyadarshini R, Popham DL, Young KD. Daughter cell separation by penicillin-binding proteins and peptidoglycan amidases in *Escherichia coli*. *J. Bacteriol* 2006;188:5345–5355. [PubMed: 16855223]
30. Drew D, Sjostrand D, Nilsson J, Urbig T, Chin CN, de Gier JW, von Heijne G. Rapid topology mapping of *Escherichia coli* inner-membrane proteins by prediction and PhoA/GFP fusion analysis. *Proc. Natl. Acad. Sci. USA* 2002;99:2690–2695. [PubMed: 11867724]
31. Ki JJ, Kawarasaki Y, Gam J, Harvey BR, Iverson BL, Georgiou G. A periplasmic fluorescent reporter protein and its application in high-throughput membrane protein topology analysis. *J. Mol. Biol* 2004;341:901–909. [PubMed: 15328603]
32. Behrendt J, Standar K, Lindenstrauss U, Bruser T. Topological studies on the twin-arginine translocase component TatC. *FEMS Microbiol. Lett* 2004;234:303–308. [PubMed: 15135537]
33. Allen SC, Barrett CM, Ray N, Robinson C. Essential cytoplasmic domains in the *Escherichia coli* TatC protein. *J. Biol. Chem* 2002;277:10362–10366. [PubMed: 11781311]
34. Gouffi K, Gerard F, Santini CL, Wu LF. Dual topology of the *Escherichia coli* TatA protein. *J. Biol. Chem* 2004;279:11608–11615. [PubMed: 14701831]
35. Holzapfel E, Eisner G, Alami M, Barrett CM, Buchanan G, Luke I, et al. The entire N-terminal half of TatC is involved in twin-arginine precursor binding. *Biochemistry* 2007;46:2892–2898. [PubMed: 17300178]
36. Sawers RG, Ballantine SP, Boxer DH. Differential expression of hydrogenase isoenzymes in *Escherichia coli* K-12: evidence for a third isoenzyme. *J. Bacteriol* 1985;164:1324–1331. [PubMed: 3905769]

Fig. 1. FACS analysis of cells expressing reporter fusions. (a) Schematic analysis of the screen for the isolation of suppressors. (b) Fluorescence histograms of *E. coli* Blk0 cells expressing either TGS (pTGS) or KGS (pKGS). *tatC* with its own promoter sequence was amplified from pFAT417¹⁷ using primers EMS21 (GCGGCGGACGTCTCTAGAGGAAGTGCAGCCGCAACTGG) and EMS22 (GCGGCGCCTGCAGGGTCGACTTATTCTTCAGTTTTTCGCTTTCTGC) and then was inserted into pBR322 between the AatII and SalI sites. A 0.6% nucleotide substitution library of *tatC* was generated by error-prone PCR¹⁶ using primers TatB2¹⁷ and EMS22; the mutagenized gene product was used to replace *tatC* gene in the pBR322-*tatC* construct. For

immunostaining, an octahistidine tag was added by PCR amplification with the primers TatB2 and EMS23 (CGCCGCGTCGACTTAATGGTGATGGTGATGGTGTTCTTCAGTTTTTTCGCTTTCTGC). Cells were cultivated in LB after subculturing 1/20 from saturated overnight cultures. Cultures were induced in mid-logarithmic phase with 0.02% arabinose, and after a 5-h induction period, cells were washed once with PBS, diluted 100 times and analyzed by flow cytometry on a BD FACSTM Sorter or FACS Aria. The fluorescence histograms of single amino acid suppressors and a reconstructed double amino acid substitution exporting KGS are indicated; data shown were acquired by FACS Aria (Gm, geometric mean fluorescence). (c) Average relative export efficiencies of isolated variants and single point mutations exporting KGS and the w.t. TorA signal peptide fused to GFP-SsrA. The geometric mean fluorescence of the negative control (B11k0 pBR322-*tatC* with pKGS) was subtracted and the results were normalized by comparing the values obtained to the fluorescence in B11k0 pBR322-*tatC* expressing pTGS. Relative export efficiency was the average of three independent measurements from three separate cultures for each clone; error bars represent the standard deviation of the relative export efficiency.

Fig. 2.

Distribution of active TMAO reductase in cytoplasmic and periplasmic fractions of cells expressing *tatC* suppressors. Cell cultures (15 ml) were grown for 22-24 h anaerobically in Cohen and Rickenberg medium²⁴ as previously described³⁶ with 0.4% glucose and 0.4% TMAO (Sigma-Aldrich). Cells were normalized based on OD₆₀₀ to a total of OD₆₀₀ 5 and then fractionated by the cold osmotic shock procedure.²⁵ Briefly, cells were resuspended in fractionation buffer (30 mM Tris-HCl, pH 8.0, 20% (w/v) sucrose, 1 mM EDTA, pH 8.0), incubated at room temperature for 10 min, and then after centrifugation were resuspended in ice-cold MgSO₄ (5 mM). Spheroplasts were sonicated. Of the obtained fractions, 12.5% were loaded, and proteins were separated under native condition on a 7.5% Tris gel at pH 6.5. Gels were incubated with 20 ml of 100 mM sodium phosphate buffer (pH 7); 1 mM methyl viologen was added and reduced by an excess of a 1% dithionite solution in 0.1 mM NaOH. Gels were incubated under mild shaking until stained blue; 10 ml of a 2 M TMAO in the same phosphate buffer was added to visualize TorA activity as can be seen in the transparent lanes. MC4100 and B11k0 (Δ *tatC*) containing pBR322-*tatC* were used as positive controls. GB22KK-C cells contain the gene encoding TorA(R11K,R12K) in the chromosome and the deletion of *dmsABC::kan*.¹⁷

Fig. 3. Morphology of TatC suppressors. Light microscopy photographs of stationary phase of B11k0 transformed with plasmids expressing the indicated *tatC* suppressor alleles. Cells were grown in LB medium at 37 °C.

Fig. 4. Predicted location of the TatC suppressor mutations. The topological model of TatC was obtained from Buchanan *et al.*,¹⁷ Behrendt *et al.*,³² and Ki *et al.*³¹