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Isolation and Characterization of Bifunctional *Escherichia coli* TatA Mutant Proteins That Allow Efficient Tat-dependent Protein Translocation in the Absence of TatB*

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In *Escherichia coli*, the Tat system promotes the membrane translocation of a subset of exported proteins across the cytoplasmic membrane. Four genes (*tatA*, *tatB*, *tatC*, and *tatE*) have been identified that encode the components of the *E. coli* Tat translocation apparatus. Whereas TatA and TatE can functionally substitute for each other, the TatB and the TatC proteins have been shown to perform distinct functions. In contrast to Tat systems of the ABC(E) type found in *E. coli* and many other bacteria, some microorganisms possess a TatAC-type translocase that consists of TatA and TatC only, suggesting that, in these systems, TatB is not required or that one of the remaining components (TatA or TatC) additionally takes over the TatB function. We have addressed the molecular basis for the difference in subunit composition between TatABC(E) and TatAC-type systems by using a genetic approach. A plasmid-encoded *E. coli* minimal Tat translocase consisting solely of TatA and TatC was shown to mediate a low level translocation of a sensitive Tat-dependent reporter protein. Suppressor mutations in the minimal Tat translocase were isolated that compensate for the absence of TatB and that showed substantial increases in translocation activities. All of the mutations mapped to the extreme amino-terminal domain of TatA. No mutations affecting TatC were identified. These results suggest that in TatAC-type systems, the TatA protein represents a bifunctional component fulfilling both the TatA and TatB functions. Furthermore, our results indicate that the structure of the amino-terminal domain of TatA is decisive for whether or not TatB is required.

Transport of proteins across biological membranes is a crucial process in all living cells. In eubacteria, the translocation of the vast majority of proteins across the plasma membrane is mediated by the general protein secretion (Sec) system, consisting of a protein-conducting channel (SecYEG) and a translocation motor (SecA). Sec-dependent proteins are threaded through the SecYEG pore in a more or less unfolded state and only fold after their release on the *trans*-side of the membrane (for a recent review, see Ref. 1).

In addition to the Sec machinery, many bacteria possess a second protein export system, the so-called Tat (twin arginine translocation) system, for the translocation of a subset of proteins. In marked contrast to the Sec system, the Tat machinery translocates its substrates in a fully folded or even oligomeric state (for reviews, see Refs. 2–5). The Tat export machinery consists of a surprisingly low number of components. In *Escherichia coli*, four genes (*tatA*, *tatB*, *tatC*, and *tatE*) have been identified that encode components of the Tat translocation apparatus (6, 7). TatA, TatB, and TatE are sequence-related proteins. TatA and TatE show more than 50% sequence identity and can partially substitute for each other functionally (7). However, because the *tatA* gene is expressed about 100 times higher than *tatE*, the latter gene is currently regarded as a cryptic gene duplication of *tatA* (8). In contrast, despite the fact that TatA and TatB show some weak sequence identity, genetic evidence suggests that they perform different functions (9). Therefore, TatA, TatB, and TatC seem to be the minimal components of the Tat export apparatus in *E. coli*. So far, very little is known about the mechanism of Tat-mediated protein transport. Biochemical evidence from *E. coli* (10) and from investigations of the homologous ΔpH pathway in plant thylakoids (11) suggests that Tat precursor proteins first bind to a complex consisting of TatB and TatC. After interacting with the primary recognition component TatC, the signal peptide seems to be transferred via TatB to the actual translocation pore, presumably consisting of multiple copies of TatA. Recruitment of TatA molecules to the TatBC complex is dependent on the presence of the transmembrane H^+ gradient (ΔpH). After transport of the substrate across the membrane plane, the substrate protein is released on the *trans*-side of the membrane and the pore component TatA redissociates from the TatB-TatC receptor complex, allowing the recycled components to catalyze further rounds of substrate binding and translocation.

As described above, the *E. coli* TatB seems to function as a mediator between substrate recognition by TatC and subsequent translocation events. Genetic evidence clearly showed that the presence of TatB is essential for efficient Tat-dependent protein translocation in *E. coli* (9). In contrast to Tat systems of the ABC(E) type, Tat systems of the AC type (found for example in *Rickettsia prowazekii*, *Bacillus subtilis*, and *Staphylococcus aureus*) lack TatB (2, 12–14), suggesting that, in these systems, TatB is not required or that one of the remaining components (TatA or TatC) additionally takes over the TatB function.

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TABLE I
Bacterial strains and plasmids used in this study
Km^R, kanamycin resistance; Cm^R, chloramphenicol resistance; Tc^R, tetracycline resistance.

	Relevant properties	Source
E. coli strains		
DH5α	*supE44, ΔlacU169(Φ80 lacZΔM15) hsdR17 recA1 endA1 hsdR gyrA relA thi*	Ref. 21
MC4100	F^- *araD*139 Δ(*argF-lac*)U169 *rpsL*150 *relA*1 *fruA*25	Ref. 36
DADE	MC4100 Δ*tatA-tatD* Δ*tatE*	Ref. 27
GSJ100	MC4100 × P1.MM129>Tc^R Δ*malE*444 *zjb*729::Tn*10*	Ref. 18
GSJ101	DADE × P1.MM129>Tc^R Δ*malE*444 *zjb*729::Tn*10*	Ref. 18
Plasmids		
pBBR1MCS-2	*mob, rep*, *lacZa*$^+$; Km^R	Ref. 37
pACYCtac	Cm^R	R. Siewe, unpublished data
pHSG575	pSC101 replicon, *lacZa*$^+$; Cm^R	Ref. 38
pTatABC	pHSG575 derivative; carrying the *tatABC* genes of *E. coli*	Ref. 18
pHSG-TatABCE	pHSG575 derivative; carrying the *tatABCE* genes of *E. coli*	This study
pHSG-TatAC	pHSG575 derivative; carrying the *tatAC* genes of *E. coli*	This study
pHSG-TatAC-M1	pHSG-TatAC containing mutation M1 in TatA (Gly^2 → Ser; Gly^3 → Asp)	This study
pHSG-TatAC-M2	pHSG-TatAC containing mutation M2 in TatA (Gly^3 → Asp)	This study
pHSG-TatAC-M3	pHSG-TatAC containing mutation M3 in TatA (Gly^2 → Asp)	This study
pHSG-TatAC-M4	pHSG-TatAC containing mutation M4 in TatA (Ile^4 → Met)	This study
pHSG-TatAC-M5	pHSG-TatAC containing mutation M5 in TatA (Ile^6 → Ser)	This study
pTorA-MalE	pBBR1MCS-2 derivative; carrying the *torA-malE* fusion gene	Ref. 18

In the present study, we have addressed the molecular basis for the difference in subunit composition between TatABC(E) and TatAC-type Tat systems by using a genetic approach. A plasmid-encoded *E. coli* minimal Tat translocase consisting solely of TatA and TatC was found to allow some very low but nevertheless detectable residual translocation of a sensitive Tat reporter protein. Mutants of the minimal Tat translocase were isolated that showed substantial increases in translocation activities. All of the mutational alterations were found to be localized in the extreme amino-terminal domain of the TatA protein. No mutations affecting TatC could be identified. Our results strongly suggest that in Tat systems of the AC type, the TatA protein represents a bifunctional component fulfilling both the mediator activity of TatB as well as the pore-forming function of TatA. Furthermore, our results indicate that the structure of the amino-terminal domain of TatA is decisive whether a TatB mediator is required or not.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—The bacterial strains and plasmids used in this study are listed in Table I. Bacterial strains were grown at 37 °C in Luria Bertani medium (15); minimal medium (16) supplemented with 0.4% maltose, 0.4% glucose, or 0.4% glycerol; or MacConkey agar base medium (Difco) supplemented with 1% maltose. If required, isopropyl-β-D-thiogalactopyranoside was used at a 1 mM concentration. Antibiotic supplements were at the following concentrations: kanamycin, 50 μg/ml; chloramphenicol, 25 μg/ml; and tetracycline, 15 μg/ml. For anaerobic growth on the nonfermentable carbon source glycerol with trimethylamine *N*-oxide (TMAO)[1] as sole respiratory electron acceptor, minimal medium (16) was supplemented with 0.4% glycerol and 50 mM TMAO.

DNA Manipulations—All of the DNA manipulations followed standard procedures (17). For the construction of pHSG-TatABCE, plasmid pTatABC (18) was digested with BamHI and PstI, and the *tatABC* fragment was ligated into BamHI/PstI-digested pACYCtac,[2] resulting in pACYC-TatABC. The *tatE* gene was amplified by PCR using chromosomal DNA of MC4100 as a template and primers TatE-for (5′-CAG ACT GCA GTT GTG TGC GCG CGT CG-3′) and TatE-rev (5′-GAT CTA GAG GGA GCG TCC TGC TCG CCA CG-3′). Via these primers, restriction sites were introduced immediately upstream of the ribosome binding site of the *tatE* gene (PstI) and downstream of the stop codon of the *tatE* gene (XbaI). The amplified PCR fragment was cleaved with PstI and XbaI and ligated into PstI/XbaI-digested pACYC-TatABC, resulting in pACYC-TatABCE. The *tatABCE* fragment was excised with EcoRI and XbaI, and the sticky ends were filled in with Klenow enzyme and ligated into SmaI-digested pHSG575, resulting in pHSG-TatABCE. Plasmid pHSG-TatAC was constructed via cross-over PCR, using pHSG-TatABCE as a template. First, *tatA* was amplified by PCR using primers TatA-for (5′-CAG GAT CCA CAG AGG AAC ATG TAT GG-3′) and TatAC-rev (5′-GCG TGA TAA GCG GTT GAG TAT CTT CTA CAG ACA TGG ATT ACA CCT GCT CTT TAT CGT GGC GC-3′) with its 5′ region corresponding to the 5′ end of the *tatC* gene. In a second PCR, the *tatC* gene was amplified using primers TatC-rev (5′-GCC CTG CAG GGC GGT TGA ATT TAT TC-3′) and TatAC-for (5′-GCG CCA CGA TAA AGA GCA GGT GTA ATC CAT GTC TGT AGA AGA TAC TCA ACC GCT TAT CAC GC-3′) with the 5′ end of primer TatAC-for corresponding to the 3′ end of the *tatA* gene. In a final PCR using primers TatA-for and TatC-rev, an equimolar mixture of the PCR products derived from the two PCR reactions described before was used as the template to create an artificial *tatAC* operon, in which the start codon of *tatC* is localized at the same position as the start codon of *tatB* in the *tatABC* operon of *E. coli*. The resulting cross-over PCR product was cleaved with BamHI and PstI and ligated into BamHI/PstI-digested pHSG575, resulting in plasmid pHSG-TatAC. The correctness of all plasmid constructions was verified by DNA sequencing.

Isolation of Tat Mutants—Plasmid pHSG-TatAC was mutagenized *in vivo* using 2-aminopurine (causing G-C → A-T and A-T → G-C transitions) (19) or 5-azacytidine (causing G-C → T-A and G-C → C-G transversions) (20) as mutagenic agents. Approximately 1000–10000 cells of *E. coli* DH5α (21) containing pHSG-TatAC 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 the *tat* deletion mutant GSJ101 (18) (see below). *In vitro* mutagenesis of pHSG-TatAC using hydroxylamine (causing G-C → A-T transitions) as a mutagenic agent was performed as described by Humphreys *et al.* (22) with slight modifications. 2 μg of pHSG-TatAC were incubated for 30–90 min at 70 °C in 200 μl of 1 M hydroxylamine, 50 mM Tris-HCl, pH 8.0, 0.25 mM EDTA. Subsequently, the DNA was precipitated with 2.5 volumes of ethanol and 70 mM NaCl (final concentration) and washed several times with 70% ethanol. The mutagenized plasmid pools were independently transformed into the *tat* deletion mutant GSJ101 by electroporation. The transformed cells were plated on minimal medium agar plates containing 0.4% glycerol and 50 mM TMAO and incubated anaerobically (gas-generating kit; Oxoid) at 37 °C for up to 5 days. Single mutant colonies that appeared on the selection plates were restreaked on the same medium, and 18 isolates that showed reproducible anaerobic growth were chosen for further characterization. From these isolates, plasmid pHSG-TatAC was isolated, and the *tatAC* gene fragment was transferred into fresh, nonmutagenized pHSG575 vector to eliminate possible mutations that might have occurred in the vector part of pHSG-TatAC. The resulting pHSG-TatAC plasmids were subsequently used for DNA sequence analysis and further functional characterizations.

Miscellaneous Procedures—Fractionation of cells into a fraction containing the cytosol and membranes (C/M) and a periplasmic fraction (P) by using an EDTA-lysozyme spheroplasting method has been described previously (18). For the preparation of membranes, the cells were grown overnight in 10 ml of Luria Bertani medium. The cells were harvested

[1] The abbreviations used are: TMAO, trimethylamine *N*-oxide; C/M, cytosol and membranes; P, periplasmic.
[2] R. Siewe, unpublished observations.

TABLE II
Phenotype of bacterial strains on MacConkey maltose, maltose minimal medium, and glycerol/TMAO agar plates

Bacterial strain	Growth on maltose minimal medium[a]	Color of colonies on MacConkey maltose[a]	Anaerobic growth on glycerol/TMAO[b]
GSJ100	−	Pale	+
GSJ101	−	Pale	−
GSJ101 (pHSG-TatABCE)	−	Pale	+
GSJ101 (pHSG-TatAC)	−	Pale	−
GSJ100 (pTorA-MalE)	+++	Red	+
GSJ101 (pTorA-MalE)	−	Pale	−
GSJ101 (pTorA-MalE, pHSG575)	−	Pale	−
GSJ101 (pTorA-MalE, pHSG-TatABCE)	+++	Red	+
GSJ101 (pTorA-MalE, pHSG-TatAC)	+	Light red (pink)	−
GSJ101 (pTorA-MalE, pHSG-TatAC-M1)	+++	Red	+
GSJ101 (pTorA-MalE, pHSG-TatAC-M2)	++	Red	+
GSJ101 (pTorA-MalE, pHSG-TatAC-M3)	+++	Red	+
GSJ101 (pTorA-MalE, pHSG-TatAC-M4)	+++	Red	+
GSJ101 (pTorA-MalE, pHSG-TatAC-M5)	+++	Red	+

[a] 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.
[b] Bacterial strains were streaked on glycerol/TMAO agar plates and incubated anaerobically at 37 °C. +, growth; −, no growth.

by centrifugation, washed once with 10 mM Tris-HCl, pH 7.5, and subsequently broken by ultrasonification (UP 200 S; Dr. Hielscher GmbH, Teltow, Germany; amplitude, 60%, cycle, 0.6). After removal of residual cell debris by a low speed centrifugation step (15 min at 4 °C at 21,000 × *g*), the membranes were isolated by ultracentrifugation (1 h at 4 °C at 200,000 × *g*). The membrane pellet was washed once with 1 ml of 1 M potassium acetate and subsequently resuspended in 200 μl of 1% Triton X-100, 10 mM Tris-HCl, pH 7.5. Protein concentrations in the samples were determined by the method of Bradford (23). SDS-PAGE and Western blotting using anti-TatA, anti-TatC, or anti-TalB antibodies were done as described earlier (24). Western blotting using anti-MalE was performed by using the ECL Western blotting detection kit (Amersham Biosciences) according to the instructions of the manufacturer. The chemoluminescent protein bands were recorded and quantified using the CCD camera and image analyzing system Fujifilm LAS-1000 (Fuji Photo Film) together with the software AIDA 2.41 (Raytest).

RESULTS

An E. coli TatAC Minimal Translocase Possesses Residual Translocation Activity—In a previous study, we have established a Tat-specific reporter system (TorA-MalE) that is based on the periplasmic maltose-binding protein (MalE) and allows an easy *in situ* detection of Tat-dependent protein export on indicative media (18). MalE is part of the uptake system for maltose, and its localization in the periplasm is absolutely required for growth of *E. coli* on minimal medium agar plates containing maltose as the sole carbon source (25). Furthermore, the red color of *E. coli* cells on MacConkey agar plates containing maltose (indicative for acid production) is only observed when MalE is exported across the plasma membrane (26). The TorA-MalE reporter consists of the mature part of the normally Sec-dependent periplasmic maltose-binding protein fused to the signal peptide of the Tat-dependent periplasmic trimethylamine *N*-oxide reductase (TorA). Export of the TorA-MalE hybrid precursor has been shown to be strictly dependent on the presence of a functional Tat system (18).

In the present study, the gene encoding the TorA-MalE reporter as well as the *tat* genes under examination were cloned on two different plasmids (Table I) and subsequently cotransformed into the *tat* deletion mutant GSJ101, a *malE*-negative derivative of the strain DADE (27) in which all the known *tat* genes are deleted (18). GSJ101 containing solely the plasmid pTorA-MalE could not grow on minimal medium containing maltose and showed a pale color on MacConkey plates containing maltose (Table II). The same phenotype was observed with GSJ101 (pTorA-MalE) cotransformed with the pHSG575 empty vector. In contrast, cells of GSJ101 containing plasmids pTorA-MalE and pHSG-TatABCE (a pHSG575 derivative containing the genes *tatA*, *tatB*, *tatC*, and *tatE* cloned in an operon like fashion under the regulatory control of the *lac* promoter) efficiently grew on maltose minimal medium and showed a red color on MacConkey plates containing maltose (Table II). These results confirm our previous findings that TorA-MalE is exported in a strictly Tat-dependent fashion (18).

Next, we analyzed whether the presence of TatB is required for membrane translocation of the TorA-MalE protein. For this purpose, plasmid pHSG575-TatAC was constructed that contains the *tatA* and *tatC* genes cloned under the control of the *lac* promoter. To verify expression of the two genes, plasmids pHSG-TatABCE and pHSG-TatAC were transformed into the *tat* deletion strain GSJ101, and the membrane fractions of the respective strains were analyzed by Western blotting using TatA- and TatC-specific antibodies. As shown in Fig. 1, the amounts of TatA were identical for strain GSJ101 (pHSG-TatAC) (Fig. 1, *upper panel*, *lane 6*) and GSJ101 (pHSG-TatABCE) (Fig. 1, *upper panel*, *lane 8*). In contrast, the amount of TatC was somewhat increased in GSJ101 (pHSG-TatAC) (Fig. 1, *lower panel*, *lane 6*) when compared with GSJ101 (pHSG-TatABCE) (Fig. 1, *lower panel*, *lane 8*), which might be due to the fact that the *tatC* gene in plasmid pHSG-TatAC is positioned closer to the *lac* promoter than it is in plasmid pHSG-TatABCE or to an altered mRNA stability. Alternatively, because *tatB* initiates with GTG and thus translational efficiency falls dramatically after *tatA* translation in the *tatABCE* construct and because *tatC* is translationally coupled to *tatB*, the translation of *tatC* is also low. In pHSG-TatAC, the ATG start codon of the *tatC* gene has been retained (and not changed to GTG) and, therefore, translation of *tatC* is likely to be much higher. Next, pHSG-TatAC was transformed into GSJ101 (pTorA-MalE), and the resulting strain GSJ101 (pTorA-MalE, pHSG-TatAC) was tested for MalE export on indicative media. As shown in Table II, GSJ101 (pTorA-MalE, pHSG-TatAC) was able to grow on maltose minimal medium, although at a significantly lower rate when compared with GSJ101 (pTorA-MalE, pHSG-TatABCE). On MacConkey plates containing maltose, GSJ101 (pTorA-MalE, pHSG-TatAC) formed light red (pink) colonies. From these results we conclude that low levels of TorA-MalE can be exported in the absence of TatB and that the components TatA and TatC are sufficient to form a functional, although inefficient minimal translocase in *E. coli*.

Isolation of Tat Mutants Allowing Export of TorA in the Absence of TatB—TMAO reductase (TorA) is a periplasmic molybdoenzyme that allows *E. coli* to use TMAO as a respiratory final electron acceptor. TorA is exported in a Tat-dependent manner, and it has been shown that TatB is required for TorA membrane translocation and for anaerobic growth of

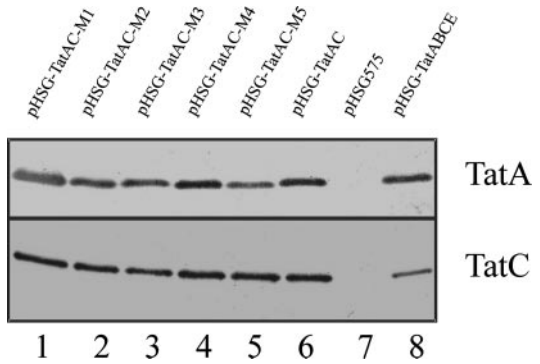

FIG. 1. **Expression levels of TatA and TatC proteins.** Membrane preparations corresponding to identical amounts of cells were subjected to SDS-PAGE and immunoblotting using TatA (*upper panel*) or TatC (*lower panel*) antibodies. The samples correspond to *E. coli* GSJ101 containing plasmids pHSG-TatAC-M1 (*lane 1*), pHSG-TatAC-M2 (*lane 2*), pHSG-TatAC-M3 (*lane 3*), pHSG-TatAC-M4 (*lane 4*), pHSG-TatAC-M5 (*lane 5*), pHSG-TatAC (*lane 6*), pHSG575 (*lane 7*), and pHSG-TatABCE (*lane 8*).

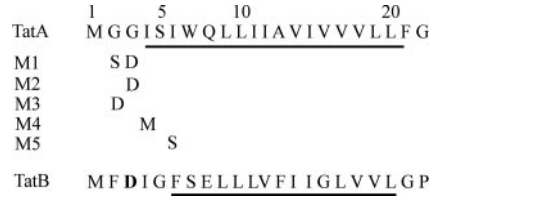

FIG. 2. **Amino acid alterations present in the TatA mutant proteins.** *TatA*, the primary sequence corresponding to the short amino-terminal periplasmic region and the proposed transmembrane domain (*underlined*) of the wild type TatA protein is shown. *M1* to *M5*, TatA mutant proteins possessing the indicated amino acid alterations. For a comparison, also the corresponding homologous region of TatB is shown. An aspartate residue frequently occurring in this region of TatB proteins is indicated in *bold type*.

E. coli on the nonfermentable carbon source glycerol with TMAO as sole respiratory electron acceptor (9). Consistent with these results, we found that strain GSJ101 containing pHSG-TatABCE, but not pHSG-TatAC, was able to grow anaerobically on minimal medium containing glycerol and TMAO (Table II).

We therefore asked whether mutations in the *E. coli* TatAC minimal translocase could be isolated that would allow for TorA translocation despite the absence of TatB, thereby converting a barely functional core of a TatABC(E)-type system into an efficient translocase of the TatAC type. To allow a broad spectrum of mutagenesis events, plasmid pHSG-TatAC was subjected either to *in vivo* mutagenesis using 2-aminopurine (19) or 5-azacytidine (20) or to *in vitro* mutagenesis using hydroxylamine (22) as mutagens. After introduction of the differently mutagenized pools of pHSG-TatAC into the *tat* deletion strain GSJ101 by electroporation, the corresponding transformed cells were plated onto TMAO/glycerol minimal medium and incubated under anaerobic conditions. Depending on the nature and concentration of the mutagen used, the formation of up to 200 single colonies could be observed after 5 days of incubation. After restreaking of the colonies on TMAO/glycerol medium, 18 randomly selected clones from independent mutagenesis approaches that showed reproducible growth under anaerobic conditions were chosen for further characterization. To test whether mutations in the plasmid-localized *tatAC* genes were responsible for the growth behavior, pHSG-TatAC was isolated from the mutant bacteria, and the corresponding *tatAC* gene regions were recloned into fresh, nonmutagenized pHSG575 vector. Subsequently, the reconstructed pHSG-TatAC plasmids were transformed into GSJ101, and the resulting transformants were retested for growth on TMAO/glycerol medium under anaerobic conditions. In all cases, the corresponding cells were able to grow, indicating that in fact mutational alterations in the *tatAC* genes must be responsible for the observed phenotype. Because anaerobic growth with TMAO as the sole electron acceptor requires the successful transport of TorA into the periplasm, these findings indicate that the mutational alterations must have created a TatAC minimal translocase that, in contrast to the unaltered TatAC translocase, now allows for significant TorA membrane translocation despite the absence of TatB.

The Alterations Compensating for the Lack of TatB Localize to the Amino-terminal Domain of TatA—DNA sequencing of the mutant *tatAC* genes revealed that, in the 18 mutant clones analyzed, five different mutations (M1 to M5) could be identified. Interestingly, all of these mutations were confined to the six amino-terminal amino acid residues of TatA, corresponding to the short periplasmic domain and the beginning of the single transmembrane segment (Fig. 2). No alterations affecting the *tatC* gene were found. In mutant M1 (isolated three times in independent experiments), a double mutation (GGT GGT → AGT GAT) was present that altered the two glycine residues at positions 2 and 3 to serine-aspartate. In contrast to the double mutation in M1, only a single amino acid change was found in the other four mutant classes. In mutant M2 (isolated six times), the glycine residue at position 3 was changed to aspartate (GGT → GAT), whereas in mutant M3 (isolated three times), a glycine to aspartate alteration had occurred at position 2 (GGT → GAT). Interestingly, an aspartate residue is present in the TatB protein at a similar position (position 3). In mutant M4 (isolated five times), the isoleucine at position 4 was changed to methionine (ATC → ATG), whereas in mutant M5 (isolated once), the isoleucine at position 6 was replaced by a serine residue (ATT → AGT). Our findings that all of the suppressor mutations compensating for the absence of TatB mapped to TatA and that no suppressing mutations were found in TatC strongly suggest that it is the TatA component (and not TatC) that exerts both the TatA and TatB functions in the TatAC-type systems.

The TatAC Mutant Translocases Allow Improved Membrane Translocation of the TorA-MalE Reporter Protein—As a first step in the characterization of the TatAC mutant translocases, the amounts of the two Tat proteins present in the membrane fractions of the respective strains were analyzed by Western blotting using TatA- and TatC-specific antibodies. As shown in Fig. 1, comparable amounts of TatC are found in strains GSJ101 (pHSG-TatAC) (Fig. 1, *lower panel*, *lane 6*) and GSJ101 (pHSG-TatAC-M1 to -M5) (Fig. 1, *lower panel*, *lanes 1–5*). Likewise, the amounts of TatA were found to be identical between GSJ101 (pHSG-TatAC) (Fig. 1, *upper panel*, *lane 6*) and GSJ101 (pHSG-TatAC-M4) (Fig. 1, *upper panel*, *lane 4*), whereas a slight reduction in the TatA amounts was observed in the GSJ101 strains expressing the TatAC mutant translocases M1, M2, M3, and M5 (Fig. 1, *upper panel*, *lanes 1–3* and *5*). Importantly, however, in all of the mutant strains, the TatA and TatC proteins were present in amounts equal or even slightly lower when compared with the strain expressing the unaltered TatAC translocase, excluding the possibility that the observed effects are due to increased amounts of the two Tat proteins.

As outlined above, the TatAC mutant translocases have been isolated using a selection for anaerobic growth on TMAO as sole electron acceptor, being indicative for improved export of TorA. To analyze their membrane translocation activities in more detail, export of the TorA-MalE reporter protein by the mutant translocases was assayed (i) indirectly by plate assays and (ii) directly by measuring the amount of exported MalE

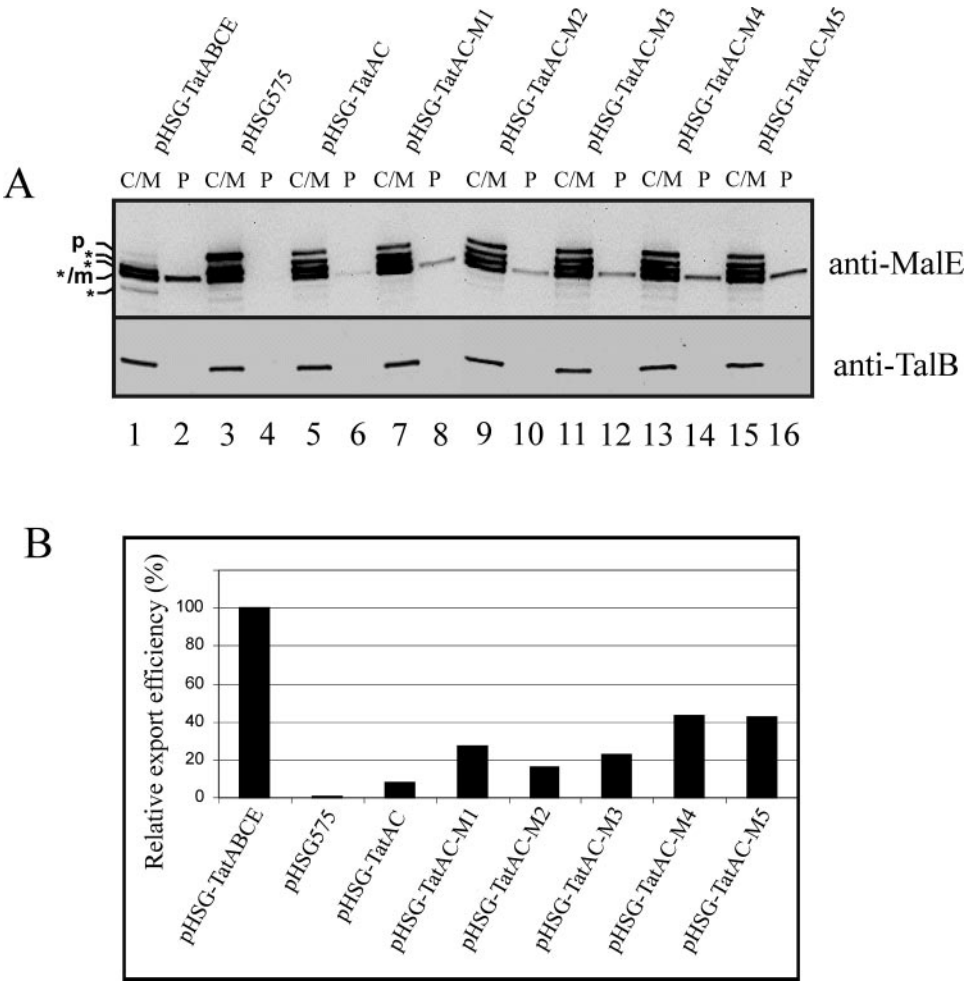

FIG. 3. *A*, subcellular localization of MalE polypeptides. The cells were fractionated into a C/M fraction (*odd-numbered lanes*) and a P fraction (*even-numbered lanes*) by EDTA-lysozyme spheroplasting as described previously (18). The samples were subjected to SDS-PAGE and Western blotting using anti-MalE antibodies (*upper panel*) or, as a control, antibodies directed against the cytoplasmic protein TalB (*lower panel*). The MalE-derived protein bands were visualized using the ECL Western blotting system (Amersham Biosciences). The samples correspond to *E. coli* GSJ101 (pTorA-MalE) containing in addition plasmid pHSG-TatABCE (*lanes 1* and *2*), pHSG575 (*lanes 3* and *4*), pHSG-TatAC (*lanes 5* and *6*), pHSG-TatAC-M1 (*lanes 7* and *8*), pHSG-TatAC-M2 (*lanes 9* and *10*), pHSG-TatAC-M3 (*lanes 11* and *12*), pHSG-TatAC-M4 (*lanes 13* and *14*), or pHSG-TatAC-M5 (*lanes 15* and *16*). *p*, TorA-MalE precursor; m, position of mature form of TorA-MalE in the P fraction; *asterisks*, positions of TorA-MalE degradation products in the C/M fraction. *B*, relative export efficiencies of Tat translocases. The amounts of exported MalE protein present in the P fractions (shown in Fig. 3*A*) of strains GSJ101 (pTorA-MalE) containing the indicated Tat plasmids were quantified via their chemoluminescence signals. The signals were recorded by a CCD camera and subsequently analyzed with the program AIDA 2.41 (Raytest). The relative amount of MalE in the P fraction of GSJ101 (pTorA-MalE, pHSG-TatABCE) was set to 100%.

protein present in the periplasm. As described before, GSJ101 (pTorA-MalE, pHSG-TatAC) showed slow growth on minimal maltose plates and formed light red (pink) colonies on MacConkey maltose agar plates (Table II). In contrast, strains GSJ101 (pTorA-MalE, pHSG-TatAC-M1 to -M5) showed fast growth on minimal maltose plates that was comparable with the growth of GSJ101 (pTorA-MalE, pHSG-TatABCE) containing the entire Tat translocase and formed deep red colonies on MacConkey maltose agar plates (Table II).

The subcellular localization of MalE polypeptides in the corresponding strains was directly analyzed by EDTA-lysozyme spheroplasting (18), yielding the C/M and P fractions. The fractions were subsequently analyzed by SDS-PAGE and Western blotting using anti-MalE antibodies (Fig. 3*A*, *upper panel*). As a control for the quality of the fractionation procedure, the distribution of the cytosolic enzyme transaldolase B (TalB) (28) was analyzed in parallel. As expected, TalB was found exclusively in the C/M fraction in all cases (Fig. 3*A*, *lower panel*). As described previously for the *tat* wild type strain GSJ100 containing plasmid pTorA-MalE (18), a faint band corresponding to the unprocessed precursor and various cytosolic degradation products of it can be detected in the C/M fraction of strain GSJ101 (pTorA-MalE, pHSG-TatABCE) expressing the Tat components from a plasmid (Fig. 3*A*, *upper panel*, *lane 1*). A similar pattern of bands is observed in the C/M fraction of all the other strains examined (Fig. 3*A*, *upper panel*, *odd-numbered lanes*). A mature-sized MalE polypeptide is detected in the P fraction of GSJ101 (pTorA-MalE, pHSG-TatABCE), corresponding to MalE protein that has been exported across the cytoplasmic membrane (Fig. 3*A*, *upper panel*, *lane 2*). In the absence of the *tat* genes (strain GSJ101(pTorA-MalE, pHSG575)), no exported MalE is present in the P fraction (Fig. 3*A*, *upper panel*, *lane 4*), confirming that a functional Tat system is required for export of the TorA-MalE reporter protein. GSJ101 (pTorA-MalE, pHSG-TatAC) expressing the unaltered TatAC minimal translocase shows very small amounts of exported MalE in the P fraction (Fig. 3*A*, *upper panel*, *lane 6*), being in perfect agreement with the slow growth of this strain on maltose minimal medium and the formation of light red (pink) colonies on MacConkey maltose agar plates. Compared with this strain, the amount of exported MalE in the P fractions is significantly increased to various degrees in the

strains expressing the mutant TatAC translocases (GSJ101 (pTorA-MalE, pHSG-TatAC-M1 to -M5) (Fig. 3*A*, *upper panel*, *lanes 8*, *10*, *12*, *14*, and *16*)). Quantification revealed that the amount of MalE exported to the P fraction by the unaltered TatAC minimal translocase was approximately 8% relative to that exported by GSJ101 (pTorA-MalE, pHSG-TatABCE) expressing all four Tat components. In the strains expressing the mutant TatAC translocases, values of 27% (M1), 16% (M2), 22% (M3), 43% (M4), and 43% (M5) of exported MalE were determined (Fig. 3*B*). Taken together, our results clearly show that an *E. coli* minimal TatAC translocase possesses a low translocation activity and, furthermore, that this inefficient minimal translocase could be converted by mutation into efficient translocases of the TatAC-type, possessing activities of up to 43% of the complete TatABCE translocase.

DISCUSSION

In the present work, we have shown that an *E. coli* minimal Tat translocase, consisting of TatA and TatC only, allows a low but nevertheless detectable membrane translocation of a sensitive reporter protein. Furthermore, the characterization of mutant TatAC translocases has revealed that the absence of TatB can significantly be compensated by alterations in the amino-terminal region of TatA, thereby converting a barely functional TatAC core translocase of a TatABC(E)-type system into an efficient translocase of the TatAC-type.

In *E. coli*, the TatB component has been shown to possess a pivotal role in Tat-dependent protein translocation. Inactivation of the *tatB* gene resulted in a block of the export of seven endogenous Tat substrates (including TorA), indicating that TatB is functionally distinct from TatA (and TatE) (9). Our finding that GSJ101 (pHSG-TatAC) was unable to grow anaerobically on glycerol/TMAO medium is in full agreement with these previous results, confirming that the absence of TatB does not allow the translocation of TorA in amounts sufficient for growth under these conditions. However, a low level of translocation by the TatAC minimal translocase could be detected for the sensitive TorA-MalE reporter protein. A similar finding has been obtained by Ize *et al.* (29) using a very sensitive reporter system that is based on the bactericidal effect of colicin V (ColV), which is exerted only when the colicin gains access to the plasma membrane from the periplasmic side. Membrane translocation of the strictly Tat-dependent TorA-ColV hybrid precursor was fully blocked in a Δ*tatC* mutant but only partially affected in *tatB* mutant strains. Likewise, residual export of the Tat-dependent xylanase C was observed in a *tatB* but not in a *tatC* deletion mutant of *Streptomyces lividans* (30). Together, these combined results suggest that TatB is not absolutely essential in a mechanical sense but rather is an important factor with respect to the efficiency of the translocation process in bacteria possessing a TatABC(E)-type translocase.

In some microorganisms (such as *R. prowazekii*, and *S. aureus*), only a single protein of the TatA/B family is present in addition to TatC. Based on phylogenetic analyses, these proteins have been classified as TatA homologues (2, 12, 31). However, it should be noted that sequence analysis alone might not always be sufficient to assign a protein to the TatA or the TatB family (32). Nevertheless, the presence of only one TatA/B homologue in these organisms suggests that either TatA or TatB is dispensable or that the properties of TatA and TatB are combined in a single polypeptide (33). Our findings that single amino acid substitutions in TatA from a TatABC(E)-type system can substantially compensate for the absence of TatB might be taken as evidence for the latter possibility.

Biochemical evidence from *E. coli* and the thylakoidal ΔpH pathway has shown that, in these ABC(E)-type Tat systems, the precursor is recognized by a complex of TatB and TatC. Subsequently to precursor binding, TatA is recruited to the TatBC-precursor complex (10, 11, 34). The characterization of Tat complexes isolated from overproducing *E. coli* strains has shown that at least two types of high molecular mass complexes were present in detergent solution (35). One complex consisted mainly of TatA with small amounts of TatB present, consistent with the relative abundance of these two proteins in the native membrane. In the other complex, mainly TatB and TatC (but also some TatA) were identified. These studies clearly demonstrated that TatB is capable of interacting with TatA even in the absence of TatC and, on the other hand, can form stable complexes with TatC. Because TatC (and not TatB) has been shown to be the primary substrate receptor in the TatBC complex (10), it is tempting to speculate that TatB might act as a physical connecting link between the primary substrate receptor TatC and the TatA pore component. If so, then in the absence of TatB, the TatA pore component might only inefficiently assemble with the TatC-precursor complex, allowing for only very low levels of protein translocation to occur. In contrast, the alterations present in our TatA mutant proteins might alter the conformation of TatA such that an efficient direct binding of the mutant TatA proteins to the TatC-precursor complex is possible without the need for a connecting link.

Cross-linking experiments, in which a reactive amino acid residue was introduced at various positions in the preSufI signal peptide, have revealed a hierarchy in the steps of interaction between Tat-dependent precursor proteins and the various Tat components (10). First, the precursor is recognized by virtue of the RR consensus motif in the signal peptide by the primary substrate receptor TatC. In a subsequent step, the precursor is passed to TatB that, in addition to the RR consensus motif region, also contacts the hydrophobic core (h-region) of the signal peptide. In the presence of a membrane potential, the signal peptide is further passed on to the pore component TatA. Interestingly, both TatB and TatA (but not TatC) seem to come into close contact with the entire signal peptide (*i.e.* the RR consensus motif and the h-region) (10). From these results it might be speculated that the signal peptide is positioned similarly when bound to TatB and to TatA and that no major rearrangement of the signal peptide is necessary upon transfer from TatB to TatA. In fact, such a scenario could provide a basis for an explanation of (i) why our bifunctional TatA mutant proteins can suppress the absence of TatB and (ii) the dual function of TatA in naturally occurring TatAC-type systems. We propose the following model: in TatABC(E)-type systems, TatB but not TatA (and most likely also not TatE) can efficiently interact physically with the substrate receptor TatC. Because of our finding that all of the identified mutations in TatA map to the short periplasmic region and the beginning of the transmembrane segment, it seems that differences in the amino acid composition or conformation of these regions between TatA and the homologous region of TatB are responsible for the different affinities of these components for TatC. Furthermore, our results make it a likely possibility that this region in TatB (and in TatA of TatAC-type systems) is a major site of contact between these proteins and TatC. In the TatA suppressor mutants, the corresponding region must have been altered such that its overall conformation has become to some extent more "TatB-like" and, most likely, one of the TatA mutant proteins present in the membrane can now assemble to the TatB-binding site of TatC, thereby substituting for the missing TatB component. Upon transfer of the precursor from TatC to TatB, from TatC to a bifunctional TatA mutant protein in the absence of TatB, or from TatC to the TatA protein in naturally occurring TatAC-type systems, the signal peptide is positioned

in a way that is ready to be passed on to additional TatA molecules without any further major rearrangements. Therefore, TatB might be nothing else than a specialized TatA molecule, whose sole function is that of a physical connecting link between the "real" TatA molecules and TatC. Because the *tatB* gene is thought to be the result of a duplication of the *tatA* gene, the acquisition of a specialized adaptor during evolution must have been of some advantage for the respective organisms, although our results as well as the natural occurrence of TatAC-type systems clearly show that TatB is not strictly essential in a mechanical sense.

In mutants M1, M2, and M3, aspartate residues were introduced at positions 2 and 3 of TatA. Interestingly, such an aspartate residue is present in TatB of *E. coli* (and many other TatB homologues; data not shown) at position 3. From this finding, it might be speculated that the presence or absence of an aspartate in the short periplasmic region is decisive for whether or not the TatA/B protein can assemble to TatC. However, no introduction of an aspartate in this region is found in the strongest TatA suppressor variants isolated in this study (M4 and M5), indicating that the overall conformation, rather than the precise amino acid sequence of this region, determines whether the binding affinity of the respective TatA or TatB proteins to the TatC substrate receptor is high or low.

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