

## An Essential Component of a Novel Bacterial Protein Export System with Homologues in Plastids and Mitochondria\*

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**Proteins are transported across the bacterial plasma membrane and the chloroplast thylakoid membrane by means of protein translocases that recognize N-terminal targeting signals in their cognate substrates. Transport of many of these proteins involves the well defined Sec apparatus that operates in both membranes. We describe here the identification of a novel component of a bacterial Sec-independent translocase. The system probably functions in a similar manner to a Sec-independent translocase in the thylakoid membrane, and substrates for both systems bear a characteristic twin-arginine motif in the targeting peptide. The translocase component is encoded in *Escherichia coli* by an unassigned reading frame, *yigU*, disruption of which blocks the export of at least five twin-Arg-containing precursor proteins that are predicted to bind redox cofactors, and hence fold, prior to translocation. The Sec pathway remains unaffected in the deletion strain. The gene has been designated *tatC* (for twin-arginine translocation), and we show that homologous genes are present in a range of bacteria, plastids, and mitochondria. These findings suggest a central role for TatC-type proteins in the translocation of tightly folded proteins across a spectrum of biological membranes.**

Numerous proteins are transported into the bacterial periplasmic space by means of N-terminal extensions, termed signal peptides, that direct their translocation across the plasma membrane by the Sec apparatus (reviewed in Ref. 1). The translocation of substrates by this system involves the participation of both cytoplasmic and membrane-bound components; the soluble components, SecB and SecA, serve to prevent folding of the protein until it is directed to the membrane-bound translocase, a complex of SecYEG together with several less well defined ancillary proteins. Translocation of a partially unfolded substrate protein through the SecYEG complex is

then driven by the ATPase function of the SecA protein.

The Sec apparatus recognizes signal peptides that contain three characteristic domains: an N-terminal charged domain (usually basic), a hydrophobic core domain and a more polar C-terminal domain (reviewed in Ref. 2). Similar signals have been shown to target proteins across the chloroplast thylakoid membrane (3), and it is now clear that a prokaryotic-like Sec system operates in this membrane, presumably inherited from the cyanobacterial-type progenitor of the chloroplast (4, 5). However, biochemical studies of thylakoid protein transport (reviewed in Ref. 6) have pointed to the existence of a parallel pathway that requires neither soluble factors nor ATP but that is instead completely reliant on the thylakoidal  $\Delta\text{pH}$  (7–10). Remarkably, the substrates on this pathway are synthesized with signal-type peptides (transfer peptides) that nevertheless direct translocation only by the  $\Delta\text{pH}$ -dependent pathway (11, 12). The dominant factor in this sorting process is the presence of a twin-arginine motif immediately upstream of the hydrophobic domain that is essential for translocation by the  $\Delta\text{pH}$ -dependent system (13). The structure of this Sec-independent system has been unclear for some time, but a recent study on a maize mutant has resulted in the cloning of a gene, *hcf106*, encoding the first component (14). The Hcf106 protein is localized in the thylakoid membrane and appears to comprise a single transmembrane span with the bulk of the protein exposed to the stromal phase.

There is now clear evidence for the existence of a similar system in prokaryotes. It has been pointed out (15) that a subset of exported proteins are synthesized with twin-Arg-containing presequences, and this applies particularly to proteins that bind any of a range of complex redox cofactors, such as iron-sulfur clusters or molybdopterin cofactors. These cofactors are apparently inserted in the cytoplasm (15), which may well require the folding of substantial sections of the protein and hence preclude translocation by the Sec machinery. Consistent with this idea, one such protein has been found to be exported in a Sec-independent manner in *Escherichia coli* (16). Finally, homologues of Hcf106 are encoded by previously unassigned open reading frames in the majority of eubacterial and archaeal genomes, and recent studies on *E. coli* have shown that these homologues are indeed involved in Sec-independent protein export. However, the precise role of these proteins has been the subject of some confusion. Weiner *et al.* (17) isolated an *E. coli* mutant defective in the export of  $\text{Me}_2\text{SO}$  reductase (a predicted substrate for this pathway) in which the mutation was found to lie in a previously unassigned gene, designated *mttA* (for membrane targeting and translocation). The product of this gene appeared to be homologous to Hcf106, and the gene was proposed to form an operon with two further genes, *mttB* and *mttC*. However, it now transpires that the operon structure is more complex than was at first apparent; we have recently shown that this operon comprises four distinct genes because of the presence of a stop codon in the gene identified as *mttA*. The first gene in this operon is homologous to *hcf106*, whereas the gene affected in the Weiner *et al.* (17) study lies in a separate gene unrelated to *hcf106* (18). Disruption of the authentic *hcf106* homologue was found to adversely affect the export of several cofactor-containing proteins, and a complete block in the export of four proteins was observed in a double mutant in

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which a second, unlinked *hcf106* homologue was also disrupted. The four genes in the above operon were designated *tatABCD* (for twin-arginine translocation pathway), and the unlinked *hcf106* homologue was designated *tatE*.

The components of the Sec-independent translocase analyzed to date (the Hcf106 homologues TatA and TatE) together with the gene product mutated in the Weiner *et al.* (17) study (TatB according to the above nomenclature) play important roles in the translocation process, but we have now addressed the question of whether additional components are involved. *tatA* and *tatB* form a transcriptional unit with two other unassigned reading frames including one that we have provisionally designated *tatC* (originally designated *yigU*). Because *tatC* homologues are present only in those prokaryotic genomes with genes for Hcf106-like proteins and are in many cases linked to such genes, we considered TatC a potential additional component of the Sec-independent protein export system. In this report we show that TatC plays a particularly crucial role in the translocation mechanism, and we show that homologues are present in a wide range of bacteria, plastids, and mitochondria.

#### EXPERIMENTAL PROCEDURES

**Mutant Construction**—A 590-base pair fragment covering the upstream region and the first three codons of *tatC* was amplified by PCR<sup>1</sup> using primers TATC1 (5'-GCGCTCTAGAGCGGATACGAATCAGGAACAGGC-3') and TATC2 (5'-GCGCGGATCCTACAGACATGTTTACG-GTTTATCACTC-3') with chromosomal DNA as template. The product was digested with *Xba*I and *Bam*HI and cloned into the polylinker of pBluescript (Stratagene) to give plasmid pFAT21. A 592-base pair fragment covering the last four codons of *tatC* and downstream DNA was amplified using primers TATC3 (5'-CGCATCGATACTGAAGAATAAA-TTCAACCGCCGTC-3') and TATC4 (5'-GCGCGGTACCTTCATCGC-AAACCAACCGGTAATGCC-3'), digested with *Cla*I and *Kpn*I and cloned into pFAT21 to give plasmid pFAT23. The deletion construct, pFAT23, would therefore encode a protein of 20 amino acids, of which the three N- and three C-terminal residues are derived from TatC, and the remainder of the residues specified by pBluescript polylinker DNA. The DNA covering the in-frame deletion of *tatC* was excised by digestion with *Xba*I and *Kpn*I and cloned into the polylinker of pMAK705 (19) to give the construct pFAT24. The mutant allele of *tatC* was transferred to the chromosome of strain MC4100 (20) as described (19). The mutant strain, B1LK0, obtained from this procedure was verified by PCR using primers TATC1 and TATC4, and the chromosomal PCR product was sequenced to ensure that no mismatched bases had been introduced during the procedure.

Construction of the TorA signal sequence-23K fusion was as follows. A 176-base pair fragment of chromosomal DNA was amplified with primers TorASS1 (5'-GCGGAATTCAAGAAGGAAGAAAAATAG-3') and TorASS2 (5'-GCGGAATTTCGGTACCGTACGTCGCGCTTG-3'). This covered DNA from 17 bases upstream of the TorA start codon to the sixth codon of the mature TorA sequence. The product was digested with *Eco*RI and cloned into the polylinker of pBluescript (Stratagene). A clone with the insert in the correct orientation was determined by digestion with *Kpn*I and designated pMW11. The gene encoding the mature region of the spinach 23-kDa oxygen evolving complex protein (23K) was excised from plasmid pOEC23mp (provided by R. B. Klösgen) by digestion with *Avi*III and *Sal*I, end filled with Klenow DNA polymerase, and cloned into *Eco*RV-digested pMW11 to give plasmid pMW18. The DNA covering the TorA-23K fusion was excised by digestion with *Sac*I and *Sal*I and cloned into pDHB5700 (kindly provided by J. Beckwith) to give pMW23. All clones constructed from PCR-amplified DNA were sequenced to ensure that no mismatches had been introduced during amplification.

**Protein Methods**—Cells were cultured anaerobically in the medium (CR) of Cohen and Rickenberg (21) supplemented with glycerol together with the electron acceptor appropriate to the reductase to be analyzed or fumarate for experiments with hydrogenases. Cells were fractionated, and the fractions were analyzed by rocket immunoelectrophoresis and activity staining as described previously (22–25). For pulse-chase

experiments, *E. coli* MC4100 cells and the mutant strain were grown overnight in LB medium and then diluted 1:75 in CR minimal medium supplemented with ammonium molybdate/potassium selenite (1  $\mu$ M each), thiamine (0.001%), MgCl<sub>2</sub> (1 mM), glucose (0.4%), and sodium nitrate (40 mM). The culture was grown anaerobically at 37 °C until midlog phase ( $A_{600} = 0.4$ ). Cells were then harvested and resuspended in CR medium lacking peptone and casamino acids but supplemented with a methionine-free amino acid mixture (0.2 mg/ml each amino acid). After growth for 1 h, expression of TorA-23K was induced by the addition of isopropyl-1-thio- $\beta$ -D-galactopyranoside (0.04 mM) for 30 min. 5-ml aliquots of the culture were then incubated with 30  $\mu$ Ci of [<sup>35</sup>S]methionine for 1 min, after which cold methionine was added to a concentration of 0.5 mg/ml. Where appropriate, spheroplasts were formed by collection at 14,000 rpm for 2 min, resuspension in ice-cold buffer (40% w/v sucrose, 33 mM Tris, pH 8.0), and incubation with lysozyme (5  $\mu$ g/ml in 1 mM EDTA) for 15 min on ice. Aliquots of the spheroplasts were incubated on ice for 1 h in either the presence or the absence of 0.3 mg/ml proteinase K. At the end of this period, phenylmethylsulfonyl fluoride was added (final concentration, 0.33 mg/ml), and the samples were precipitated with trichloroacetic acid (5%). The precipitate was pelleted, resuspended in 10 mM Tris/2% SDS, and immunoprecipitated with antiserum to OmpA (kindly provided by G. von Heijne) or 23K.

#### RESULTS AND DISCUSSION

**The Export of Five Different Cofactor-containing Proteins Is Blocked in a  $\Delta$ tatC Mutant**—To test the role of the *tatC* gene product we constructed a strain in which the *tatC* gene was inactivated by an in-frame deletion as described under "Experimental Procedures." The deletion strain is viable under aerobic respiratory or fermentative growth conditions, indicating that the gene does not encode an essential protein. However, most of the proposed (15) substrates for the Sec-independent translocase in *E. coli* are components of anaerobic respiratory pathways. We therefore tested the effects of the TatC mutation on the localization of five such proteins.

Trimethylamine *N*-oxide (TMAO) reductase (TorA) is a soluble periplasmic enzyme containing a molybdopterin guanine dinucleotide (MGD) cofactor. Me<sub>2</sub>SO reductase is a membrane-bound enzyme in which the DmsA subunit binds the active site MGD cofactor and is synthesized with a twin-arginine transfer peptide (15). The  $\Delta$ tatC mutant fails to grow on the nonfermentable carbon source glycerol with either TMAO or Me<sub>2</sub>SO as sole terminal electron acceptor, indicating a defect in respiration involving these oxidants. Analysis of the mutant strain cultured on a fermentable carbon source shows that the TMAO and Me<sub>2</sub>SO reductase activities are both mislocalized to the cytoplasmic compartment (Table I). Whereas the vast majority (83%) of TorA is found in the periplasmic fraction in the wild-type cells, over 96% of the enzyme activity is cytoplasmically located in the  $\Delta$ tatC mutant. The localization of Me<sub>2</sub>SO reductase is affected to a similar extent; the enzyme is almost exclusively located in the membrane fraction in wild-type cells, whereas over 94% is found in the cytoplasm in the  $\Delta$ tatC strain. These data indicate a severe defect in the export of these enzymes.

Formate dehydrogenase-N (Fdn) is a third MGD-dependent enzyme with a twin-arginine transfer peptide on the catalytic (FdnG) subunit. Analysis of this membrane-bound enzyme system is complicated by the presence of two other formate dehydrogenase activities in *E. coli*, and so the enzyme is more readily identified by rocket immunoelectrophoresis using an antiserum raised against the whole formate dehydrogenase complex. Fig. 1 shows that the Fdn protein is found in the membrane fraction of wild-type cells (Fig. 1A, lane 3), in which the enzyme can be visualized using an activity stain (Fig. 1B, lane 3). In the *tatC* mutant, however, substantial quantities of Fdn-immunoreactive protein accumulate in the cytoplasmic fraction (Fig. 1A, lane 6) in an enzymatically inactive form (see Fig. 1B).

*E. coli* hydrogenases-1 (Hya) and -2 (Hyb) are membrane-

<sup>1</sup> The abbreviations used are: PCR, polymerase chain reaction; TMAO, trimethylamine *N*-oxide; MGD, molybdopterin guanine dinucleotide; Fdn, formate dehydrogenase-N.

TABLE I  
Enzyme activities in a *tatC* mutant

Oxidoreductase activities are expressed as substrate-dependent benzyl viologen oxidations (units are  $\mu\text{mol}$  benzyl viologen oxidized/min) (29–32). Acid phosphatase activities were determined as  $\mu\text{mol}$  *p*-nitrophenyl phosphate hydrolyzed/min (33).

Enzyme	Fraction	Activity	
		MC4100 parent strain	B1LK0 <i>ΔtatC</i>
<i>units/g cells</i>			
TMAO reductase	periplasm	157	4.5
	membrane	3.1	0.63
	cytoplasm	34	113
Me <sub>2</sub> SO reductase	periplasm	<0.01	<0.01
	membrane	1.7	0.12
	cytoplasm	0.03	2.1
Nitrate reductase	periplasm	1.0	1.4
	membrane	28	59
	cytoplasm	1.6	2.7
Fumarate reductase	periplasm	<0.01	<0.01
	membrane	1.3	2.5
	cytoplasm	0.05	0.18
Acid phosphatase	periplasm	4.1	3.1
	membrane	<0.01	<0.01
	cytoplasm	<0.01	<0.01

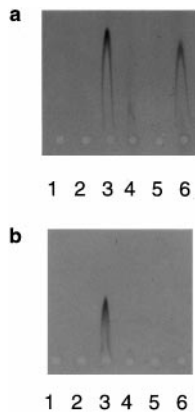


FIG. 1. **Fdn accumulates as an inactive cytosolic form in the *ΔtatC* strain.** Samples of wild-type and *ΔtatC* cells were analyzed by roelet immunoelectrophoresis. *a* is stained for total protein with Coomassie Brilliant Blue-R, and *b* is stained for Fdn activity (25). *Lanes 1*, MC4100 (parent strain), periplasmic fraction; *lanes 2*, *ΔtatC* periplasmic fraction; *lanes 3*, MC4100, Triton X-100 solubilized membrane fraction; *lanes 4*, MC4100, cytosolic fraction; *lanes 5*, *ΔtatC*, Triton X-100 solubilized membrane fraction; *lanes 6*, *ΔtatC*, cytosolic fraction. All samples represent the same proportion (0.2%) of total protein present in each fraction.

bound enzymes containing cofactor-binding subunits bound to the periplasmic face of the plasma membrane. In each enzyme a large catalytic subunit (HyaB and HybC) binding a Ni-Fe cofactor is partnered by a small subunit (HyaA and HybO) containing iron-sulfur clusters (26, 27). The small subunits are synthesized as precursors with twin-arginine transfer peptides (15). The localization of these hydrogenase isoenzymes in the mutant strain was also investigated by immunological methods (Fig. 2). In wild-type cells both enzymes are found exclusively in the membrane fraction (*lanes 3*) as expected. Neither hydrogenase is correctly targeted in the *ΔtatC* mutant strain, and each accumulates instead in enzymatically active form in the cytoplasm (Fig. 2, *a* and *b*, *lanes 6*).

**Pulse-Chase Analysis of a *TorA*-23K Construct**—The above data clearly show that multiple twin-arginine precursor proteins are mislocalized in the *ΔtatC* strain. To directly demonstrate that the mutation affects the kinetics of twin-Arg precursor export and processing, we carried out pulse-chase tests. However, the twin-arginine precursors examined above are

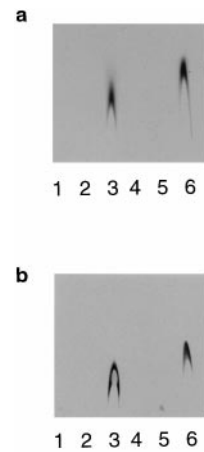


FIG. 2. **Hydrogenase 1 and hydrogenase 2 accumulate as active cytosolic forms in the *ΔtatC* mutant.** *a* is performed with anti-hydrogenase 1 serum, and *b* is performed with anti-hydrogenase 2 serum. Both *a* and *b* are stained for hydrogenase activity (23). All lanes are as follows: *Lanes 1*, MC4100 (parent strain), periplasmic fraction; *lanes 2*, *ΔtatC* periplasmic fraction; *lanes 3*, MC4100, Triton X-100 solubilized membrane fraction; *lanes 4*, MC4100, cytosolic fraction; *lanes 5*, *ΔtatC*, Triton X-100 solubilized membrane fraction; *lanes 6*, *ΔtatC*, cytosolic fraction. All samples represent the same proportion (0.2%) of total protein present in each fraction.

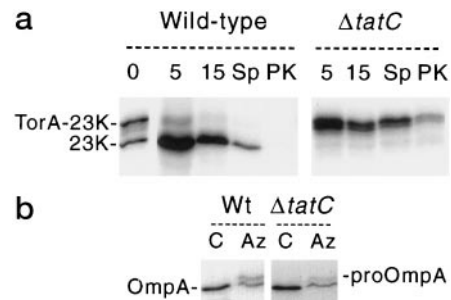


FIG. 3. **Export of a *TorA*-23K construct is blocked in the *ΔtatC* strain.** *a*, wild-type *E. coli* MC4100 cells or the *ΔtatC* strain expressing a chimeric *TorA*-23K construct were pulse-chased with [<sup>35</sup>S]methionine, after which samples were immunoprecipitated after 0, 5, or 15 min (as indicated) using an anti-23K antiserum. Cells chased for 15 min were also converted to spheroplasts (*Sp*) and samples incubated with proteinase K (*PK*). 23K, mature 23K protein. *b*, wild-type and *ΔtatC* cells were pulse-labeled under control conditions (*lanes C*, carried out as in *a*) or in the presence of 2 mM sodium azide (*lanes Az*) and samples immunoprecipitated after a 1-min chase using an antiserum to *OmpA*. Mobilities of *OmpA* and pro-*OmpA* are indicated.

either membrane-associated, which complicates the analysis of export, or are relatively large proteins for which it is difficult to detect presequence processing by a change in electrophoretic mobility. For these reasons we carried out the pulse-chase experiments on a simplified construct, *TorA*-23K, in which the transfer peptide of *TorA* is fused to the mature 23-kDa protein (23K) of the plant photosystem II oxygen-evolving complex. 23K is targeted exclusively by the Sec-independent pathway in chloroplasts (9, 11, 12) and was predicted to be tolerated by the corresponding system in bacteria. *TorA*-23K expression was placed under the control of an inducible promoter, and Fig. 3*a* shows that induction of synthesis results in efficient export of the protein. A mixture of precursor protein and mature 23K is apparent immediately after pulse-labeling of wild-type cells (*lane 0*), but only mature 23K is detected after chase times of 5 and 15 min. When spheroplasts were generated from these cells (*lane Sp*) a substantial proportion of the mature protein is lost, suggesting a periplasmic location, and this is confirmed by the finding that the residual mature 23K is completely sensitive to added proteinase K. In contrast, no mature protein

whatsoever can be detected in the *tatC* deletion strain, even after a 15-min chase, and we observe instead only the precursor protein, which runs as a doublet, possibly indicating partial degradation. The precursor protein is resistant to proteinase K digestion, indicative of a cytoplasmic location. We conclude that export of the TorA-23K construct is completely blocked in the *tatC* deletion strain.

Control experiments demonstrate that the periplasmic localization of acid phosphatase, a protein with a standard Sec signal peptide, is not affected in the *ΔtatC* strain (Table I). Furthermore, Fig. 3*b* shows that the export and processing of a typical Sec substrate, pro-OmpA, is unaffected in the mutant strain. Only a single OmpA band is detected under standard pulse-chase conditions, and this can be confirmed as mature size because the precursor protein becomes apparent when azide is included during the pulse-chase to inhibit ATP hydrolysis by SecA (28) and hence block Sec pathway export (*lanes Az*). Export and processing of pro-OmpA are equally affected in the wild-type and *ΔtatC* strains. The *tatC* deletion thus has no measurable effect on export via the Sec pathway.

We have also been able to exclude the possibility that the *tatC* mutation causes a defect in cofactor insertion, rather than in export. The enzymes nitrate reductase-A and fumarate reductase contain cytoplasmically located subunits binding cofactors (MGD and iron-sulfur clusters) of the type found in twin-arginine transfer peptide-dependent periplasmic proteins. We demonstrated that the activities of these enzymes are undiminished in the mutants (Table I). Furthermore, hydrogenases 1 and 2 and TorA accumulate in the cytoplasm of the *tatC* mutant in active forms, indicating that cofactor insertion has taken place. Finally, export of the TorA-23K is blocked even though this construct does not bind cofactors.

**Severity of the *ΔtatC* Phenotype**—These data confirm an essential and specific role for the TatC protein in the translocation of proteins with twin-arginine signal peptides. Our previous study (18) on the roles of TatA and TatE (the two Hcf106 homologues) indicated that these proteins play important roles because deletion strains are severely affected in Sec-independent protein export. However, neither strain is totally defective in this export pathway. A complete block in the export of four proteins is observed when both genes are disrupted, suggesting overlapping functions for the two gene products, but the membrane localization of hydrogenase-2 is still not completely blocked even in this strain. In contrast, in this report we have shown that the *tatC* deletion leads to a complete block in the export of all five proteins tested indicating a critical role in the translocation process.

***tatC* Homologues Are Also Present in Plastids and Mitochondria**—TatC homologues are present in all fully sequenced prokaryotic genomes, including the archaeon *Archaeoglobus fulgidus*, that code for proteins with twin-arginine signal peptides, strongly suggesting a central role in the Sec-independent export of proteins in these species. TatC homologues are furthermore present in the plastid genomes of the eukaryotic algae *Porphyra purpurea* and *Odontella sinensis*. Although these genes are absent from the plastid genomes of higher plants, it is highly likely that such genes are present in the nuclear DNA of these species because the plastid genomes of these algae are notable for containing genes (including, for example, *secY* and *secA*) that have been transferred to the nucleus in all higher plant species analyzed to date. The presence of TatC homologues in chloroplasts is not unexpected given the presence of a Sec-independent thylakoid import system in these organelles. Much more intriguing is the presence of TatC-like proteins coded by the mitochondrial genomes of four higher plants including *Arabidopsis thaliana*, the liver-

wort *Marchantia polymorpha*, and the nonphotosynthetic protist *Reclinomonas americana*. The function of these mitochondrial homologues is not yet clear, but it may be involved in either the import of folded proteins and/or their export from the matrix into the intermembrane space. We have been unable to identify potential substrates for this system that contain a twin-Arg presequence, but the system may well differ in certain respects from the bacterial/plastid systems, and the targeting signal may also have been modified during the course of evolution. The predicted topological organization of the TatC homologues is the same in all cases with six predicted transmembrane helices arranged such that the N terminus of the proteins is at the N-side (that is, cytoplasmic in prokaryotes) of the membrane.

In summary, we have identified a key component of a novel bacterial Sec-independent export system that may be used primarily for the translocation of folded proteins. The available evidence suggests that TatC-dependent systems operate also in chloroplasts and mitochondria, raising the possibility that this type of system may be almost ubiquitous in nature.

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