The twin-arginine translocation (Tat) system mediates the transport of proteins across the bacterial plasma membrane and chloroplast thylakoid membrane. Operating in parallel with Sec-type systems in these membranes, the Tat system is completely different in both structural and mechanistic terms, and is uniquely able to catalyze the translocation of fully folded proteins across coupled membranes. TatC is an essential, multispanning component that has been proposed to form part of the binding site for substrate precursor proteins. In this study we have tested the importance of conserved residues on the periplasmic and cytoplasmic face of the *Escherichia coli* protein. We find that many of the mutations on the cytoplasmic face have little or no effect. However, substitution at several positions in the extreme N-terminal cytoplasmic region or the predicted first cytoplasmic loop lead to a significant complete loss of Tat-dependent export. The mutated strains are unable to grow anaerobically on trimethylamine N-oxide minimal media and are unable to export trimethylamine-N-oxide reductase (TorA). The same mutants are completely unable to export a chimeric protein, comprising the TorA signal peptide linked to green fluorescent protein, indicating that translocation is blocked rather than cofactor insertion into the TorA mature protein. The data point to two essential cytoplasmic domains on the TatC protein that are essential for export.

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1 The abbreviations used are: Tat, twin-arginine translocation; lsLB, low salt Luria broth; TMAO, trimethylamine N-oxide; IPTG, isopropyl-β-D-thiogalactopyranoside; GFP, green fluorescent protein.

### EXPERIMENTAL PROCEDURES

**Bacterial Strains, Plasmids, and Growth Conditions—** *E. coli* strain MC4100 (20) was the parental strain; Δ*tatAE*, Δ*tatB*, Δ*tatC*, and Δ*statABCDE* have been described before (14, 15, 21), and arabinose-resistant derivatives were used as described (22). Plasmid pBAD-ABC has been described before (22). *E. coli* was aerobically grown at 37 °C in modified low salt Luria broth (lsLB) (23). *E. coli* was grown anaerobi-
Site-specific mutagenesis was used to generate a vector that encoded the tat operon within pBAD-ABC with point mutations in the tatC gene, using the QuikChange™ mutagenesis system (Strategene) according to the manufacturer’s instructions. For studies on the effects of these mutations on green fluorescent protein (GFP) export, these tatABC sequences were removed from pBAD-ABC using XhoI and XbaI, and cloned into the pEXT22 vector (25) using the XbaI site, generating pEXT-ABC and mutant derivatives which are compatible with pJDT1 encoding TorA-GFP (see text below).

**RESULTS AND DISCUSSION**

**Structures of TatC Proteins**—An alignment of several bacterial TatC proteins and the chloroplast TatC from *Arabidopsis thaliana* is shown in Fig. 1, with the predicted membrane-spanning regions shaded and identical residues denoted by asterisks. The figure also illustrates 9 residues (also shaded) that are conserved in at least 30 of the 31 bacterial and plastid TatC sequences available in the data base. The predicted topology of the protein is illustrated in Fig. 2. Given that chloroplast TatC appears to play a role in binding of precursor proteins, we reasoned that a cytoplasmic domain(s) in TatC may mediate this process in *E. coli*.

TatC proteins are not particularly highly conserved but analysis of Fig. 1 shows that two cytoplasmically exposed loops contain a number of highly conserved residues, whereas periplasmic loops contain few invariant residues. A few conserved residues are present in the transmembrane spans, but these were not examined in this study. The extreme cytoplasmic N-terminal region and the (predicted) first cytoplasmic loop, in particular, contain a range of conserved charged residues; these are indicated as cytoplasmic domains 1 and 2 (CD1 and CD2) in Fig. 1. These are more highly conserved than is the positively charged twin-arginine signal peptide of TorA, and we sought to remove the negative charge but retain the side chain properties as far as possible.

**Fig. 1. Alignment of bacterial and Arabidopsis TatC proteins.** The alignment was performed using the TatC sequences from *E. coli* (E.c.), *Hemophilus influenzae* (H.i.), *Rickettsia prowazekii* (R.p.), *H. pylori* (H.p.), *A. thaliana* (A.t.), the chloroplast sequence. Predicted transmembrane (TM) spans are indicated and shown shaded, identical residues are denoted by asterisks, and conserved residues by dots. The figure also highlights 9 residues that are either absolutely conserved, or nearly so, in an alignment of 31 bacterial and plastid TatC proteins (see text). Residues mutated in this study are shown in bold and the two conserved cytoplasmic domains (CD1 and CD2) are indicated by *boxes*.

**Fig. 2. Predicted topology of E. coli TatC.** The diagram shows the 6-span topology of the *E. coli* TatC protein predicted using the TopPred II algorithm (32) and the positions of the residues targeted for analysis in this study. *Shaded* residues represent those that exhibit major defects in Tat-dependent export when mutated as described in this study.

in Fig. 2. Most of the mutations were to Ala but Glu15 and Glu103 were changed to Gln. The reason for this strategy was that these residues were considered as candidates for binding the positively charged twin-arginine signal peptide of TorA, and we sought to remove their negative charge but retain the side chain properties as far as possible.

The mutant TatC proteins were expressed after induction with arabinose and their levels were analyzed by immunoblotting using antibodies to TatA, TatB, and the Strep II tag on TatC. Fig. 3 shows a representative immunoblot of cell extracts from approximately half of the mutants, which illustrates that the various strains all contain similar levels of Tat components,
indicating that the tatABC operon is induced to similar extents. Similar findings were made using the other mutants (data not shown). When induced in this manner, the pBAD24 system leads to an about 10-fold overproduction of TatABC (17) which means that the Tat components are effectively present in excess. The effects of the mutations on Tat-dependent export were assessed using several criteria as explained below.

Mutations in TatC That Block Anaerobic Growth on TMAO Minimal Media—A standard means of assaying for Tat-dependent export in E. coli is to test for anaerobic growth on TMAO minimal media (11–14). The periplasmic molybdopterin-containing protein TMAO-reductase (TorA) is a known Tat substrate (6, 11) and tat mutants are accordingly unable to grow using TMAO as an electron acceptor during anaerobic respiration. It should be emphasized, however, that this assay selects only for a complete lack of functional Tat system. Null mutations in tatAE, tatB, or tatC are completely unable to grow but we found that the pBAD-ABC plasmid in the ΔtatABCDE strain supported anaerobic growth even without induction, when levels of TatABC were almost undetectable (17, 22). Clearly, even very low levels of the Tat apparatus are sufficient to support growth on TMAO minimal medium.

Table I shows the results of this analysis, together with a summary of the export capabilities of these strains (see below). In fact, our results show that very few of these highly conserved residues are essential for Tat function, and the majority of these strains grow at normal rates. However, substitution of either Arg$^{17}$ led to a complete loss of growth, suggesting an important function of this (predicted) cytoplasmically exposed region as shown in Fig. 2. In view of the apparent importance of the CD1 region we tested the effects of deleting Leu$^{20}$ to Asn$^{22}$, and this mutant (Δ20–22) was again completely unable to grow under these conditions.

Perhaps surprisingly, a series of other highly conserved residues are not essential for Tat function. His$^{12}$, Glu$^{15}$, and Arg$^{19}$, for example, are all present in cytoplasmic domain 1 and are essentially invariant throughout bacterial and plastid TatC sequences, yet their substitution by Ala (or Gln in the case of Glu$^{15}$) has no detectable effect. In cytoplasmic domain 2, while substitution of Arg$^{104}$ does inhibit growth, substitution of Arg$^{105}$ by Ala has no detectable effect on growth even though a basic residue is present at this position in the vast majority of bacterial and plastid TatC sequences (although this mutation is affected in export of torA-GFP; see below). Even more surprisingly, Pro$^{97}$ is one of the few invariant residues but its substitution again fails to impair anaerobic growth. Relatively few residues on the periplasmic side were targeted for mutagenesis in this study, but it is notable that substitution of Pro$^{16}$ blocks growth entirely, suggesting an important function for this residue.

Sever tatC Mutants Are Unable to Export Either TorA or TorA-GFP—To analyze more directly the consequences of the tatC mutations, we next analyzed the distribution of a known Tat substrate, TorA, in cells expressing the various mutant forms. This was achieved using native gels in which the TorA activity is visualized directly in the polyacrylamide gel, using a methyl viologen-linked assay. Fig. 4 shows that the vast majority of active TorA is localized in the periplasm in wild-type cells expressing the pBAD-ABC vector (pBAD-ABC panel) as shown previously (22). The results obtained with the mutant strains, in general, agree with the data from the anaerobic growth analysis. Those tatC mutants that fail to grow anaerobically (R17A, Δ20–22, and P48A) are also unable to export TorA and the activity is found almost exclusively in the cytoplasmic fraction. The cytoplasmic TorA has a reduced mobility in this gel system (denoted TorA*), possibly due to the presence of the presequence but more likely due to other effects on folding or binding to other factors, since the presequence is not detectable in this gel system (denoted TorA*), possibly due to the presence of the presequence. Accumulation of the cytoplasmic TorA species is also evident with two other mutants, L16A and R105A. Both residues are in the conserved cytoplasmic domains and, in the case of R105A as discussed above, a charged residue is highly conserved among bacterial TatC proteins. These mutations do, therefore, affect TatC function.

As a final test we examined the export of a chimeric protein comprising the presequence of TorA linked to GFP. Previous

**Table I**

| Mutant | Anaerobic growth | TorA export | GFP export |
|--------|------------------|-------------|------------|
| Wild-type | ++++ | ++++ | ++++ |
| H12A | ++++ | ++++ | ++++ |
| L13A | ++++ | ++++ | ++++ |
| E15Q | ++++ | ++++ | ++++ |
| L16A | ++++ | ++++ | ++++ |
| R17A | None | None | None |
| R19A | ++++ | ++++ | ++++ |
| L20A | ++++ | ++++ | ++++ |
| Δ20–22 | None | None | None |
| P48A | + | + | + |
| K73A | ++++ | ++++ | ++++ |
| L99A | ++++ | ++++ | ++++ |
| E103Q | ++++ | ++++ | ++++ |
| R104A | ++++ | ++++ | ++++ |
| R105A | ++++ | ++++ | ++++ |
| Y154S | ++++ | ++++ | ++++ |
| L178A | ++++ | ++++ | ++++ |
| G182A | ++++ | ++++ | ++++ |
studies (23, 28) have shown this construct to be exported exclusively by the Tat pathway and GFP represents a much simpler export substrate than TorA, because it does not acquire an additional cofactor before export. Another important point is that the export of this protein is examined over a much shorter time scale. One disadvantage of the TorA assay is that only steady-state levels can be analyzed, and this can mean that defects in the TatC operation can be masked because the cells have sufficient time to export the protein even when the Tat system is functioning suboptimally. In contrast, the TorA-GFP is rapidly induced at fairly high levels, after which the Tat system is expressed and the cells are fractionated within a few hours. Under these conditions, effects on export rates are more likely to be apparent. (Ideally, the GFP would be analyzed using pulse-chase techniques which give a much better picture of the export kinetics. However, we have been unable to apply this technique to GFP export because the immunoprecipitation is ineffective, for unknown reasons.) To examine the export of this construct, we used a growth regime in which expression of the operon; panel pEXT-ABC) and the remaining panels represent ΔtatABCDE cells containing the same vector with mutated tatC genes. Export of TorA-GFP was induced using arabinose, after which expression of TatABC was induced using IPTG as detailed under “Experimental Procedures.” Cells were fractionated to yield cytoplasmic (C), membrane (M), and periplasmic (P) samples and immunoblotted using antibodies to GFP. Mature-size GFP and the TorA-GFP precursor protein are indicated.

FIG. 4. Localization of TMAO reductase activity in cells expressing TatC mutants. E. coli MC4100 cells containing the pBAD24 vector, or ΔtatABCDE cells expressing mutations in the tatC within pBAD-ABC were grown anaerobically and then fractionated to generate cytoplasmic and periplasmic fractions (C and P) as detailed under “Experimental Procedures.” The samples were analyzed by native gel electrophoresis and TMAO reductase activity was visualized in the gel. Mature-size TorA is indicated and TorA* denotes lower-mobility cytoplasmic form.

FIG. 5. Effects of tatC mutations on export of TorA-GFP. All strains contained plasmid pJDT1 encoding TorA-GFP in the pBAD24 vector (23). As a control, wild-type MC4100 cells contained in addition plasmid pEXT-ABC (encoding the wild-type tatABC operon; panel pEXT-ABC) and the remaining panels represent ΔtatABCDE cells containing the same vector with mutated tatC genes. Expression of TorA-GFP was induced using arabinose, after which expression of TatABC was induced using IPTG as detailed under “Experimental Procedures.” Cells were fractionated to yield cytoplasmic (C), membrane (M), and periplasmic (P) samples and immunoblotted using antibodies to GFP. Mature-size GFP and the TorA-GFP precursor protein are indicated.

In this report we have aimed to provide a first dissection of the E. coli TatC protein to begin mapping the important regions. The TatC family is in fact remarkably poorly conserved in terms of primary sequence, and the actual sequence data provide very few clues as to its role in Tat-dependent protein export. This lack of sequence conservation is all the more surprising given the highly conserved nature of the translocation mechanism as well as the targeting signals that are recognized by this system. For example, chloroplast thylakoids can recognize and import E. coli Tat substrates as efficiently as those of their cognate substrates (29, 30). While the precise role of TatC still requires detailed study, the available data indicate that (a) it functions together with TatB (or Hcf106 in chloroplasts) and (b) it may form part of the initial binding site for precursor proteins (17, 19).

Relatively few TatC residues are invariant even among eu- bacteria and in this study we have mutated the majority of conserved residues located in either the cytoplasmic or periplasmic loop regions. In fact, many of these mutations have no drastic effect on TatC function, although it must be stressed
that kinetic analyses on export rates may reveal minor effects that are not evident from the types of analysis used here. However, it is notable that a high proportion of these highly conserved residues are located in the two cytoplasmic domains described in this report, and we have now shown that mutations in several of these residues lead to an absolute block in Tat function. None of the mutations affect Tat subunit stability to any detectable extent, and we therefore propose that these data point to the presence of two essential cytoplasmic domains in the TatC protein. Of course, it is possible that these two areas of the protein function together as a single functional domain. We emphasize that the data do not indicate how these domains function and, while they may well form a critical binding site for incoming precursor proteins, it is also possible that these mutations destabilize the TatABC complex and hence affect translocation in a more indirect manner.

These data now pave the way for more detailed studies on the roles of the individual domains in TatC function. A recent report (31) has described an in vitro assay for Tat-dependent import into inverted E. coli inner membrane vesicles, and studies on the thylakoid system (19) have shown that Tat substrates can bind to the Tat complex under appropriate conditions. It should therefore be possible to use such techniques to determine in detail the function of individual residues and domains in TatC, in terms of either substrate binding or the subsequent translocation mechanism.

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