The twin arginine translocation (Tat) pathway of bacteria and plant chloroplasts mediates translocation of essentially folded proteins across the cytoplasmic membrane. The detailed understanding of the mechanism of protein targeting to the Tat pathway has been hampered by the lack of screening or selection systems suitable for genetic analysis. We report here the development of a highly quantitative protein reporter for genetic analysis of Tat-specific export. Specifically, export via the Tat pathway rescues green fluorescent protein (GFP) fused to an SsrA peptide from degradation by the cytoplasmic proteolytic ClpXP machinery. As a result, cellular fluorescence is determined by the amount of GFP in the periplasmic space. We used the GFP-SsrA reporter to isolate gain-of-function mutants of a Tat-specific leader peptide and for the genetic analysis of the “invariant” signature RR dipeptide motif. Flow cytometric screening of trimethylamine N-oxide reductase (TorA) leader peptide libraries resulted in isolation of six gain-of-function mutations that conferred significantly higher steady-state levels of export relative to the wild-type TorA leader. All the gain-of-function mutations occurred within or near the (S/T)RR consensus motif, highlighting the significance of this region in interactions with the Tat export machinery. Randomization of the consensus RR dipeptide in the TorA leader revealed that a basic side chain (R/K) is required at the first position whereas the second position can also accept Gln and Asn in addition to basic amino acids. This result indicates that twin arginine translocation does not require the presence of an arginine dipeptide within the conserved sequence motif.

Recent findings have established that protein translocation across the cytoplasmic membrane of Gram-negative bacteria is mediated by at least four distinct pathways: the general secretory (Sec) pathway (2, 3), the signal recognition particle (SRP)-dependent pathway (4), the recently discovered YidC-dependent process (5), and the twin arginine translocation (Tat) system (6). The first three of these pathways involve a threading mechanism whereby the polypeptide chain is translocated across the membrane in a largely unfolded state. Protein export through these pathways also requires the participation of the SecYEG translocon and is driven by the hydrolysis of ATP (7). Protein translocation via the Tat pathway is fundamentally different from the Sec pathway in that Tat substrates are at least partially folded prior to export. Moreover, the driving force for protein translocation is not provided by the hydrolysis of ATP but instead by the asymmetric distribution of protons across the membrane (∆pH) (8).

The Tat pathway was first discovered in the thylakoid membranes of photosynthetic organisms and subsequently in bacteria (9, 10). It derives its name from the signature Arg-Arg motif found in the leader peptides of proteins that are engaged in this mode of export from the cytoplasm. Estimates based on proteomic and bioinformatic analyses indicate that 5–8% of secreted proteins in bacteria such as Escherichia coli and Bacillus subtilis are translocated via the Tat pathway (11). Many of these natural substrates are proteins that have to fold in the cytoplasm to acquire a range of cofactors such as FeS centers or molybdopterin. However, proteins that do not require cofactors have also been reported to traverse the cytoplasmic membrane via the Tat pathway, perhaps because they fold too rapidly or tightly for Sec-dependent transport (6, 11).

How large molecules are allowed to pass through the membrane without perturbing the cell remains a mystery. Nonetheless, large proteins of molecular mass up to 120 kDa have been documented to be exported through the Tat pathway. A combination of homology searches and biochemical studies have so far established that the integral membrane proteins TatABC are essential components of the Tat translocone in E. coli (10, 12) and, in conjunction with TatE, form a translocation pore (13). The TatA and TatB proteins are predicted to contain one transmembrane α-helix, while the TatC protein has six predicted transmembrane helices and has been proposed to function both as the principal component of the translocation channel and as a receptor for preproteins (11, 14, 15). Mutagenesis of either TatB or TatC completely abolishes export (10, 12, 14), and purification of the Tat complex from solubilized E. coli membranes was found to contain only TatABC (16). In support of this finding, in vitro reconstitution of the translocation complex demonstrated a minimal requirement for TatABC and an intact membrane potential (17).

Tat-specific leader peptides exhibit several distinct differences compared with sequences capable of directing proteins to the Sec or SRP pathways (18, 19). Specifically, Tat signals are longer because of the extended n-region, have a significantly less hydrophobic h-region and display more basic residues in the c-region (18). A hallmark of both plant and prokaryotic Tat leader peptides is the presence of an (S/T)RRXFLK sequence...
motif at the n-region/h-boundary of the leader (6). Earlier studies have indicated that the twin arginine dipeptide is an essential determinant for targeting to the Tat pathway (18). However, Stanley et al. (20) reported that for the E. coli SuFI protein, substitution of an arginine for a lysine allowed Tat transport to occur albeit at a slower rate. Furthermore, a naturally occurring Tat leader peptide containing a Lys residue at the first position of the dipeptide has been reported in *Salmonella enterica* (21).

The analysis of the salient features of Tat-specific leader peptides and, for that matter of the biochemical components involved in the export pathway, have been hampered by the absence of suitable protein reporters useful for genetic analysis. Commonly used reporter proteins such as alkaline phosphatase and β-galactosidase, which had proved invaluable for the genetic dissection of the Sec pathway cannot be employed for the analysis of the Tat pathway (2, 22–24). Specifically, the former cannot fold within the bacterial cytoplasm thereby precluding Tat export (Ref. 22), whereas the β-galactosidase tetramer is presumably too large to be accommodated by the Tat translocase.

We have developed a facile and quantitative protein reporter system suitable for the genetic analysis of the Tat pathway, and here we report its application to the mutational analysis of the *E. coli* trimethylamine N-oxide reductase (Tara) leader peptide. Fusion of green fluorescent protein (GFP) to the Tara leader peptide (but not to a Sec-specific leader peptide) results in accumulation of correctly folded, fluorescent GFP in the *E. coli* periplasmic space (25, 26). However, in bacteria expressing Tara-GFP about 50% of the protein fails to be exported and remains localized in the cytoplasm in an active, fluorescent form. The whole cell fluorescence is the sum of the intensity of exported Tara-GFP and that of TorA-GFP trapped in the cytosol. The whole cell fluorescence is the sum of the intensity of the exported GFP and that of TorA-GFP about 50% of the protein fails to be exported and remains localized in the cytoplasm in an active, fluorescent form. The whole cell fluorescence is the sum of the intensity of the exported GFP and that of TorA-GFP trapped in the cytoplasm, and therefore it is not useful as a reporter of increase in single cell fluorescence. In the present work, we show that the TorA-GFP remaining in the cytoplasm could be eliminated by appending a C-terminal SsrA peptide, which targets the protein for rapid degradation by the ClpAP proteolytic machinery (27). Only export from the cytoplasm via the Tat pathway can rescue the TorA-GFP-SsrA from proteolysis and thus confer increased cell fluorescence (Fig. 1A). The level of single cell fluorescence in a population can be conveniently measured by flow cytometry, and desired clones can be readily isolated by sorting (28). Here the GFP-SsrA reporter was exploited to isolate the first ever gain-of-function mutants of a Tat-specific leader and also to define genetically the range of permissible amino acid substitutions within the signature twin arginine dipeptide.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—All strains and plasmids used in this study are listed in Table I. *E. coli* strain XL1-Blue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac) was used for all library experiments unless otherwise noted. Strain XL1-tatB was constructed using pFAT166 (29) and was made using standard protocols (30). All plasmids constructed in this study were subcultured into fresh LB medium with chloramphenicol and induced with 0.2% arabinose in mid-exponential phase growth. After 6 h, cells were washed once with phosphate-buffered saline, and 5 µl of washed cells were diluted into 1 ml of phosphate-buffered saline prior to analysis using a BD Pharmingen FACSort.

**Flow Cytometric Analysis**—Overnight cultures of XL1-Blue cells harboring GFP-based plasmids were subcultured into fresh LB medium with chloramphenicol and induced with 0.2% arabinose in mid-exponential phase growth. After 6 h, cells were washed once with phosphate-buffered saline, and 5 µl of washed cells were diluted into 1 ml of phosphate-buffered saline prior to analysis using a BD Pharmingen FACSort.

**Generation of a Reporter Combinatorial Libraries**—A library of random mutants was constructed by error-prone PCR of the *torA* gene sequence using 0.5 mM MnCl₂, 3.32 or 4.82 mM MgCl₂ (33, 34), *E. coli* BL21-geneomic DNA, and the torASsrA and torAXbaI primers. Nucleotide concentrations were skewed as follows: 0.22, 0.20, 0.34, and 2.36 mM and 0.12, 0.5, 0.35, and 3.85 mM dATP, dCTP, dGTP and dTTP, respectively, for 0.5 and 1.5% error-rate libraries, respectively. Directed nucleotide mutations were performed using standard PCR protocols and NNK degenerate primers where N represents any nucleotide and K represents guanine or thymine. A library targeting the two consensus arginine residues was constructed using plasmid pGTF as template DNA, the torAXbaI primer and the following degenerate primer: RRtoXX

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GGATGATGCTTCGGGATGCGGCTTGCGCCGC-3
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Sequence reactions were electrophoresed into electrophoretic XLI-Blue cells (Stratagene), and serial dilutions were plated on selective plates to determine the number of independent transformants.

**Library Screening**—Transformants were grown at 37 °C in LB medium with chloramphenicol, induced with 0.2% arabinose for 6 h, and diluted 20-fold in 1 ml of phosphate-buffered saline. FACS gates were set based upon FSC/SSC and FL1/FL2. Prior to sorting, the library cell population was labeled with propidium iodide for preferential labeling of non-viable cells (33). Typically, ~3 x 10⁶ cells were examined in 30 min, and 250–1000 events were collected. The collected solution was sterile-filtered (0.45 µm, Millipore), and the filters were placed on LB plates for chloramphenicol selection. After 12 h of incubation, individual colonies were inoculated into LB with chloramphenicol in triplicate 96-well plates. Following 12 h of growth at 37 °C, cells were similarly subcultured into triplicate 96-well plates containing LB with chloramphenicol and 0.2% arabinose and grown 6 h at 37 °C. Individual clones were screened via flow cytometry and a fluorescent plate reader (BioTek FL600, Bio-Tek Instruments, Winooski, VT) for verification of fluorescent phenotype.

**Cell Fractionation**—The fraction of periplasmic proteins was obtained by spheroplasting bacteria by lysozyme-EDTA treatment under isotonic conditions according to the procedure of Kaback (35). Specifically, cells were collected by centrifugation and resuspended to an OD₆₀₀ of 10 in a buffer containing 100 mM Tris-Cl (pH 8.0), 0.5 mM sucrose, and 1 mM NaEDTA. Lysozyme (Sigma) was added to 50 µg/ml, and cells were incubated for up to 1 h at room temperature to generate spheroplasts. The latter were pelleted by 15 min of centrifugation at 3,000 x g, and the supernatant containing periplasmic proteins was collected for electrophoretic analysis. The pellet containing spheroplasts was resuspended in 10 ml of TE (10 mM Tris-Cl, pH 7.5, 2.5 mM NaEDTA) and homogenized in a French press cell (Carver) at 2,000 psi. Following homogenization, the homogenate was collected by centrifugation in 10 ml of TE followed by subject to French press homogenization was performed.

**Western Blotting Analysis**—Western blotting was according to Chen et al. (36). The following primary antibodies were used: monoclonal rabbit anti-GFP (CLONTECH) diluted 1:5,000, monoclonal rabbit anti-DsbA (gift from John Joly, Genentech) diluted 1:10,000, and monoclonal...
rabbit anti-GroEL (Sigma) diluted 1:10,000. The secondary antibody was 1:10,000 goat anti-mouse and goat anti-rabbit horseradish peroxidase. Membranes were first probed with anti-GFP and anti-DsbA antibodies and, following development, were stripped in Tris-buffered saline/2% SDS/0.7M \( \beta \)-mercaptoethanol. Stripped membranes were reblocked and probed with anti-GroEL antibody.

RESULTS

Establishing a Genetic Screen for the Tat Export Pathway—A cDNA encoding the complete amino acid sequence (amino acids 1–46) of the \( E. \ coli \) TorA leader peptide plus the first eight residues of mature TorA was fused in-frame to the mutant2 green fluorescent protein variant (GFPmut2, Ref. 32), downstream from a pBAD arabinose-inducible promoter. In agreement with earlier reports (25, 26) 40–50% of the total fluorescence emission at 509 nm (and of the GFP protein as determined by Western blotting) was localized in the periplasmic fraction of \( E. \ coli \) XL1-Blue cells. In contrast, 95% of the total cell fluorescence and of the GFP protein band on Western blots was found in the cytoplasm of \( E. \ coli \) XL1-tatB in which the \( tatB \) gene, required for protein translocation via the Tat pathway (29), has been inactivated (Fig. 1B).

To generate a protein reporter for Tat-specific export, we sought to reduce GFP fluorescence arising from TorA-GFP that remains in the cytoplasm (Fig. 1A). Previous reports have demonstrated that the C-terminal fusion of the SsrA sequence (AANDENYALAA) to GFP targets the protein to rapid and efficient degradation by the cytoplasmic proteases, ClpXP and ClpAP (27, 37). Preliminary experiments revealed that the level of accumulation of GFP-SsrA is highly dependent on the plasmid copy number and promoter used; at high levels of expression, e.g. from a \( Trc \) promoter or from high copy number plasmids, the intracellular concentration of GFP-SsrA saturates the SsrA-dependent proteolytic machinery resulting in a high cell fluorescence.3 Fig. 1B shows the fluorescence histograms of cells expressing GFP-SsrA, TorA-GFP-SsrA, and TorA-GFP from cultures grown and induced under identical conditions. A comparison of the mean fluorescence intensities (\( M \)) conferred by GFP-SsrA or TorA-GFP-SsrA expressed in either wild-type \( E. \ coli \) or in a \( tatB \)-null mutant strain shows that export via the Tat pathway rescues a portion of the SsrA-tagged GFP from proteolysis resulting in 4–6-fold higher fluorescence signal. Importantly, replacing the twin arginines with twin lysine residues (TorA(R11K:R12K)), amino acid substitutions that had been reported to completely block export (18), results in non-fluorescent cells. The mean fluorescence for TorA-GFP was 556 au but as noted above, 50% of the fluorescence signal is due to the periplasmic GFP, whereas the rest is contributed by translocation-incompetent protein in the cytoplasm. A mutation that renders all TorA-GFP-SsrA translo-
tion-competent and allows complete rescue from SsrA-mediated degradation would be expected to result in a level of fluorescence comparable with that observed by TorA-GFP (i.e. 556 au) (assuming that the periplasmic and cytoplasmic GFP fluorescence comparable with that observed by TorA-GFP (Fig. 2A)). Cell fractionation and detection of GFP by Western blotting further established that the increased fluorescence was accompanied by greater accumulation of GFP in the periplasmic space. Representative GFP subcellular localization data for clones B7 and E2 are shown in Fig. 2B. Consistent with earlier findings (17), GFP in the periplasmic fraction accumulates as a doublet presumably due to incomplete processing of the TorA leader. Virtually no detectable GFP protein was seen in the spheroplast fractions of any cells (Fig. 2B), indicating that degradation of TorA-GFP-SsrA in the cytoplasm is highly efficient. Similarly, nearly all the GFP fluorescence signal was found associated with the periplasmic fraction whereas the spheroplast fraction exhibited only background fluorescence (data not shown). In these experiments the efficiency of cell fractionation was established by monitoring the subcellular distribution of GroEL as a cytoplasmic marker and of the periplasmic oxidase DsbA. For reasons that are not clear, B7 but none of the other mutants reproducibly reduced the level of DsbA.

Gain-of-function Mutations in the TorA Leader Peptide—The protein flux through the Tat pathway is relatively low compared with export via the Sec-dependent pathway (11). Recent evidence indicates that the protein flux can be modulated by substitutions within the consensus motif (20) and may be limited by the initial interaction of the leader peptide with the Tat export apparatus (13). We examined whether mutations in the TorA leader could increase export and result in a higher steady-state accumulation of GFP in the periplasmic space. The TorA leader peptide was mutagenized by error-prone PCR under conditions that result in expected mutation frequencies of 0.5, 1.5, or 3.5% nucleotide substitutions per gene (33, 34). Actual error rates of 0.4, 1.2, and 3.1, respectively, were determined by sequencing 20 randomly selected library clones. Libraries between 10^{6} and 10^{7} transformants were obtained and screened by FACS to isolate clones exhibiting a higher fluorescence than cells expressing the wild-type TorA-GFP-SsrA. A total of six highly fluorescent clones were obtained from the more heavily mutagenized libraries (three from the 1.5% library and three from the 3.5% library). The high fluorescence phenotype was shown to be plasmid-encoded and was completely lost in a tatB mutant strain where export via the Tat pathway is impaired (Table II). DNA sequencing of the entire TorA-GFP-SsrA gene revealed that mutations in all six clones occurred only within or adjacent to the (S/T)RRXFLK consensus motif sequence (Table II).4

The six mutant leader peptides conferred between 3- and 5-fold greater mean cell fluorescence compared with the wild-type TorA (Fig. 2A). Cell fractionation and detection of GFP by Western blotting further established that the increased fluorescence was accompanied by greater accumulation of GFP in the periplasmic space. Representative GFP subcellular localization data for clones B7 and E2 are shown in Fig. 2B. Consistent with earlier findings (17), GFP in the periplasmic fraction accumulates as a doublet presumably due to incomplete processing of the TorA leader. Virtually no detectable GFP protein was seen in the spheroplast fractions of any cells (Fig. 2B, lanes 4–6) indicating that degradation of TorA-GFP-SsrA in the cytoplasm is highly efficient. Similarly, nearly all the GFP fluorescence signal was found associated with the periplasmic fraction whereas the spheroplast fraction exhibited only background fluorescence (data not shown). In these experiments the efficiency of cell fractionation was established by monitoring the subcellular distribution of GroEL as a cytoplasmic marker and of the periplasmic oxidase DsbA. For reasons that are not clear, B7 but none of the other mutants reproducibly reduced the level of DsbA.

TABLE I

| Strain or plasmid | Relevant genotype/phenotype | Source |
|-------------------|-----------------------------|--------|
| XL1-Blue          | recA1 endA1 gyrA96 thi-1 hisD17 supE44 relA1 lacF proAB lacY1 ZAM15 Tn10 (Tet') | Stratagene |
| XL1-tatB          | as XL1-Blue, ΔtatB           | This study |
| Plasmids          |                             |        |
| pFAT166           | pMAK705 carrying tatB deletion allele | Ref. 29 |
| pGFP              | Signal sequenceless GFP in pBAD33 | This study |
| pGS               | Signal sequenceless GFP tagged with C-terminal SsrA tag in pBAD33 | This study |
| pTG               | TorA leader peptide fused to GFP in pBAD33 | This study |
| pTGS              | TorA leader peptide fused to SsrA-tagged GFP in pBAD33 | This study |
| pTR11K-R12KGS     | pTGS with R11K, R12K mutation in leader | This study |
| pH2G               | PhoA leader peptide fused to GFP-SsrA in pBAD33 | This study |
| pBG6G and pB6GS   | Clone B6 leader cloned into pGFP or GFP-SsrA | This study |
| pB7G and pB7GS    | Clone B7 leader cloned into pGFP or GFP-SsrA | This study |
| pE2G and pE2GS    | Clone E2 leader cloned into pGFP or GFP-SsrA | This study |
| pF1G and pF1GS    | Clone F1 leader cloned into pGFP or GFP-SsrA | This study |
| pF11G and pF11GS  | Clone F11 leader cloned into pGFP or GFP-SsrA | This study |
| pH2G and pH2GS    | Clone H2 leader cloned into pGFP or GFP-SsrA | This study |
| pTR12QG          | pTG with R12Q mutation in leader | This study |
| pTR12QGS         | pTGS with R12Q mutation in leader | This study |
| pTA9TG           | pTG with A9T mutation in leader | This study |
| pPA9TGS          | pTG with A9T mutation in leader | This study |

| Clone ID | Amino Acid Sequencea | + SsrA wild-typeb | + SsrA tatB nullb | − SsrA wild-typeb |
|----------|----------------------|-------------------|-----------------|------------------|
| wt       | MNNNDLFQASRRRFLAQLGLGVTAGMLGFSLITPRRAAT AAQAATDA | 44    | 12   | 556   |
| B6       | ---DFQASRRRFLAQL----- | 237   | 7    | 1811  |
| B7       | ---DFQASRRRFLAQL----- | 268   | 17   | 1900  |
| E2       | ---DFQASRRRFLAQL----- | 186   | 8    | 1486  |
| F1       | ---ELFQASRRRFLAQL----- | 120   | 6    | 1196  |
| F11      | ---DFQASRRRFLAQL----- | 185   | 14   | 1777  |
| H2       | ---DFQASRRRFLAQL----- | 145   | 6    | 1531  |

a Twin-arginine consensus motif is indicated by underlined amino acids; first 8 residues of mature TorA protein are indicated by italics; mutations in TorA leader peptide are indicated by bold letters.

b Mean fluorescence (M) is the average of three replicate experiments (standard error, <5%).

4 Additional silent mutations were detected in some clones and were independently shown not to contribute to enhanced export (data not shown).
This reduction in the level of DsbA was not observed when the SsrA tag of B7 was removed (Fig. 2C).

The above results strongly argue that the mutations in the TorA leader peptide facilitate export via the Tat pathway. However it was possible that the leader peptide mutations resulted in greater localization of GFP in the periplasm simply because they interfered with degradation of TorA(mt)-GFP-SsrA in the cytoplasm thus increasing the total amount of protein available for export. To evaluate this hypothesis, the SsrA tag was deleted from all the mutants, and the whole cell fluorescence and subcellular localization of the resulting TorA-GFP fusions was determined (Table II and Fig. 2C). Cells expressing GFP fused to the mutant TorA leader peptides exhibited 3–4-fold greater mean fluorescence compared with export via the wild-type leader. Similarly, the total amount of GFP reacting bands was increased accordingly in whole cell lysates of B7 compared with wild-type cells. The increase was due to the presence of a substantially greater amount of mature GFP that occurred as a result of the gain-of-function mutations. The increased amount of mature GFP was found exclusively in the periplasmic fraction. In contrast, the amount of TorA-GFP remaining in spheroplasts was comparable for the B7 mutant and the wild type. It should be noted that the same amount of total protein per lane was loaded on the SDS-PAGE shown in Fig. 2C, and therefore the intensity of the GFP band in the periplasmic fraction was significantly greater. Comparison of Fig. 2, B versus C reveals that removal of the SsrA tag results in higher accumulation of mature GFP in the periplasm. Mechanisms for the degradation of SsrA-tagged proteins, although not nearly as efficient as the ClpXP machinery (27), also exist in the periplasm and may be partly responsible for the lower than expected amount of GFP-SsrA.

Finally, to address whether the increased fluorescence of the gain-of-function mutants was due to greater export efficiency or enhanced accumulation of total cellular GFP, the TorA(wt)-GFP and TorA(mt)-GFP fusions were expressed in the tatB background. Comparison of total cellular GFP levels for the wild-type, TorAB7, and TorAE2 leaders fused to GFP but lacking the SsrA tag indicated that all fusion constructs accumulated at approximately the same level in an export-deficient tatB background (Fig. 3). In fact, clone E2 accumulated at a slightly lower level than both the wild-type and TorAB7 leader. Monitoring of DsbA protein levels confirmed that equivalent amounts of protein were loaded in each lane. Mean fluorescence measurements were in close agreement with the above Western blot data. These data demonstrate that the TorA(mt) leader peptides do not affect the steady-state level of the GFP fusion proteins. Thus, the higher amount of mature GFP protein observed in Fig. 2C reflects the more efficient export of the protein rather than a change in protein synthesis level.

**Fig. 2. Analysis of gain-of-function mutants by flow cytometry and Western blotting.** A, fluorescence histograms of cells expressing TorA(mt)-GFP-SsrA. Cultures were grown and induced identically. B, Western blotting analysis of the subcellular fractions obtained from TorAB7-GFP-SsrA- and TorAE2-GFP-SsrA-expressing cultures. Lanes 1–3 represent periplasmic fractions (obtained by lysozyme-EDTA treatment of cells as outlined under “Experimental Procedures”) for wild type, TorAB7, and TorAE2 respectively, whereas lanes 4–6 show the spheroplast fractions. C, gain-of-function mutant TorAB7 generated by removal of the SsrA tag and analyzed by Western blotting analysis of the subcellular fractions. Lanes 1 and 2 show periplasmic fractions, lanes 3 and 4 show spheroplast fractions and lanes 5 and 6 show whole cell lysates for wild-type TorA versus TorAB7 constructs. All were loaded with the same amount of total protein. GroEL and DsbA were used as fractionation markers for the spheroplast and periplasmic fractions, respectively.
with the isolation of the same amino acid substitution in the TorAB7 mutant discussed above. Consistent with earlier reports (18), an R11K/R12K mutant showed no detectable GFP in the periplasmic fraction and no fluorescence signal by FACS (Fig. 1Bc).

**DISCUSSION**

We have developed of a facile and quantitative system for the genetic analysis of the Tat pathway. Our system is based on the observation that fluorescent GFP can accumulate in the periplasm only when it is exported via the Tat pathway (25, 26). Earlier attempts to produce active, periplasmic GFP using the Sec pathway have been unsuccessful (38). In *E. coli* expressing GFP with a Tat leader, a significant percentage of the whole cell fluorescence arises from export-incompetent but correctly folded GFP in the cytoplasm. Therefore to develop a system in which the cell fluorescence is dependent only on the efficiency of export via the Tat transporter we sought to eliminate the fluorescence signal arising from the export-incompetent GFP by appending an SsrA degradation tag. Under conditions where the expression of GFP-SsrA does not overwhelm the ClpXP proteolytic system, the half-life of this protein is of the order of 2 min (37), which is compatible with the time scale for Tat export. Consequently, secretion via the Tat pathway can rescue the short-lived TorA-GFP-SsrA chimera from cytoplasmic proteolysis, conferring a fluorescence signal in proportion to the amount of exported protein.

The data shown in Fig. 1B reveal that the GFP-SsrA reporter protein provides a satisfactory signal to noise ratio and affords a potentially large dynamic range for detecting mutations that facilitate export. If desired, the sensitivity of the assay can be further increased by employing strains that overexpress ClpXP by using other degradation tags or by using GFP mutants whose fluorescence emission does not overlap with cellular autofluorescence. However, we have found that the system described here is satisfactory for genetic analyses.

Here we have employed the GFP-SsrA reporter: (i) to show that gain-of-function mutations in the TorA leader peptide can be isolated and (ii) to explore genetically what amino acids could be tolerated within the signature RR dipeptide of Tat-specific leaders. Screening of a total of >10⁸ random mutants by FACS led to isolation of six highly fluorescent TorA mutants. We showed that the increased fluorescence phenotype was completely dependent on a functional tatB gene and was not affected by the removal of the SsrA tag. These results together with the cell fractionation data (Fig. 2, B and C) support the notion that the six mutants we isolated represent gain-of-function alleles of the TorA leader peptide.

Earlier Simmons and Yansura (39) reported that mutations in the Sec-specific PhoA leader peptide that result in higher accumulation of protein in the periplasmic space modulate the frequency of translation initiation and thus the rate of protein synthesis. In contrast, the TorA mutations isolated here did not appear to affect the total level of protein synthesis. Indeed, expression of the TorA-GFP clones in a *tatB* background resulted in the same level of whole cell fluorescence for all the mutants. Notably most TorA mutations are located >20 nucleotides downstream of the AUG codon and hence are unlikely to have any effect on the frequency of translation initiation.

It is striking that all the gain-of-function mutations occurred within or near the (S/T)RRXXFLK conserved motif of Tat-specific leader peptides. The concentration of mutations in that region did not arise because the sequence encoding the conserved motif represent a mutagenic hotspot because sequencing of random clones in the present revealed the presence of nucleotide substitutions throughout the TorA sequence. Rather, the localization of the gain-of-function mutations around the conserved sequence motif underscores the significance of this region in protein sorting and the initiation of export. Buchanan et al. (40) have proposed that Tat-dependent export may require signal peptide recognition at multiple stages during translocation. Accordingly, mutations that enhance export probably result in improved interactions with the sorting components in the pathway.

With the exception of TorAF1, which notably confers the weakest increase in GFP fluorescence (Table II), all the other TorA gain-of-function mutants contained two amino acid substitutions each. In the case of the TorAB7 leader peptide at least, the effect of the two mutations was found to be synergistic. The substitution A9T alone did not result in higher whole cell fluorescence relative to the control (not shown). Thus, it appears that enhanced interaction of TorA with the Tat apparatus requires the coordinated action of two (or perhaps even more) amino acid substitutions. This may be the reason why most gain-of-function mutants were isolated only from hypermutated libraries with ≥1.5% nucleotide substitutions per gene.

An unexpected feature of clone TorAB7 was the presence of an R12Q mutation in the second position of the twin arginine dipeptide. Isolation of the R12Q mutation prompted an investigation to determine which amino acids could be tolerated within the signature RR dipeptide. A conservative lysine substitution in the first position (Arg-11) was found to confer reduced export. This finding is in accord with the earlier observation that single substitutions of Arg with Lys can be tolerated in the context of Tat-mediated export of *E. coli* SufF (20). Similarly, Palmer and coworkers (40) recently found that a KR dipeptide in the TorA leader conferred low but detectable levels of correctly folded periplasmically localized trimethylamine N-oxide reductase. Surprisingly, it was found that the second position of the invariant RR dipeptide is much more tolerant to amino acid substitutions at least with regards to the export of GFP. Glutamine, asparagine, as well as lysine at position 12 of the TorA leader gave a level of export comparable with the wild type. This result was unanticipated because

### Table III

**Summary of variants isolated from RR to XX library screening**

| Motif     | + SsrA wild-typea | + SsrA tatB-nullb | Frequency |
|-----------|-------------------|------------------|-----------|
| RR        | 44                | 9                | 29/80     |
| RK        | 36                | 11               | 33/80     |
| RQ        | 33                | 7                | 10/80     |
| RN        | 58                | 12               | 6/80      |
| R         | 34                | 8                | 2/80      |

a Mean fluorescence data are the average of three replicate experiments (S. E. <5%).
earlier studies had indicated that an R12K mutation abolishes export of TorA (40) and an R12Q substitution blocks export of Zymomonas mobilis glucose-fructose oxidoreductase (GFOR) (41). However, unlike GFP, TorA and GFOR require the attachment of a cofactor prior to (or concomitantly to) export. On the other hand, export of substrates that do not contain cofactors appear to be more amenable to substitutions in the second arginine position. This is best illustrated in work by Stanley et al. (20) who found that while Lys substitutions in the RR dipeptide allowed slow export of SufI export, identical substitutions were not tolerated in the export of YacK, an enzyme with 37% amino acid sequence similarity to SufI but known to acquire a multicopper cofactor. Taken together these results indicate that an RR dipeptide, while perhaps critical for the correct assembly of cofactor-containing proteins, is not essential for export via the Tat pathway. Further, our genetic analysis showed that at least in the context of GFP export, (R/R/N/Q/K) or a KR motif can substitute for the invariant RR consensus motif is occupied by arginine. It was recently shown that an R11K TorA mutant had identical export capabilities as Arg-11 or Arg-12. Thus, our observation that substitutions by Asn, Gln, R13 could not compensate for mutations in either Arg-11 or Arg-12. In closing we note that the availability of a protein reporter system for Tat export can greatly facilitate the dissection of the sorting mechanism and of the components that recognize specific features in the leader peptide. The genetic system we have developed is also significant for the potential use of the Tat pathway in biotechnology both for protein expression and for combinatorial library screening applications. Specifically the ability to enhance protein export via gain-of-function mutations in the leader peptide as shown here or via other mechanisms may ultimately enable the expression of Tat-secreted proteins at levels comparable with what has been obtained by secretion via the Sec pathway.

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