Live cell imaging shows reversible assembly of the TatA component of the twin-arginine protein transport system

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The twin-arginine translocation (Tat) machinery transports folded proteins across the cytoplasmic membrane of bacteria and the thylakoid membrane of chloroplasts. It has been inferred that the Tat translocation site is assembled on demand by substrate-induced association of the protein TatA. We tested this model by imaging YFP-tagged TatA expressed at native levels in living Escherichia coli cells in the presence of low levels of the TatA parologue TatE. Under these conditions the TatA-YFP fusion supports full physiological Tat transport activity. In agreement with the TatA association model, raising the number of transport-competent substrate proteins within the cell leads to an increase in the number of large TatA complexes present. Formation of these complexes requires both a functional TatBC substrate receptor and the transmembrane proton motive force (PMF). Removing the PMF causes TatA complexes to dissociate, except in strains with impaired Tat transport activity. Based on these observations we propose that TatA assembly reaches a critical point at which oligomerization can be reversed only by substrate transport. In contrast to TatA-YFP, the oligomeric states of TatB-YFP and TatC-YFP fusions are not affected by substrate or the PMF, although TatB-YFP oligomerization does require TatC.

Bacteria and archaea possess two parallel pathways for the routine export of proteins across the cytoplasmic membrane. The Sec (secretion) pathway threads unstructured proteins across the membrane through an aqueous channel (1), whereas the Tat (twin-arginine translocation) pathway transports folded proteins using a currently uncharacterized mechanism (2, 3). The Tat transport system has the greater mechanistic challenge, not only because folded proteins require a wider transport pathway, but because proteins have very different sizes and shapes when folded making it difficult to seal tightly around the protein during transport. Thus elucidating the unusual mechanism of Tat transport is of great interest. Substrate movement through the Sec transporter is driven by a combination of ATP hydrolysis and the transmembrane proton motive force (PMF). In contrast, Tat transport is energized solely by the PMF (4, 5). Sec and Tat pathways are conserved in the thylakoid membranes of chloroplasts where they mediate protein transport into the thylakoid lumen (6).

In bacteria the Tat pathway is important in diverse processes including energy metabolism, formation of the cell envelope, and nutrient acquisition (2, 7). The Tat system is also normally required for the virulence of bacterial pathogens (8). In plants the Tat pathway plays an essential role in the biogenesis of the photosynthetic electron transport chain (9).

The Tat system of Escherichia coli comprises the integral membrane proteins TatA, TatB, TatC, and TatE (10–13). TatA and TatE are homologous and functionally equivalent proteins (10). However, TatA expression is 50-fold higher than that of TatE, and so TatA is thought to be the dominant contributor to Tat function under physiological conditions (14).

Proteins are targeted to the Tat pathway by N-terminal signal peptides containing a consensus amino acid sequence motif that includes two consecutive arginine residues (15–17). Tat-signal peptides are recognized at the membrane by a TatBC receptor complex (18–23). Substrate protein binding to TatBC causes a PMF-dependent recruitment of TatA (and presumably TatE) to assemble the active translocation site (18, 24). Within the resultant TatABC complex, TatA is proposed to provide the pathway for substrate movement across the membrane bilayer (24). The association of TatA with TatBC is reversed when substrate is no longer available (24).

TatA recruitment to the TatBC–substrate complex is associated with a significant increase in the number of chemical cross-links that can be formed between TatA molecules, indicating that TatA undergoes a major structural reorganization in the translocation complex (25, 26). One possible interpretation of this reorganization is that TatA undergoes substrate-induced association. Such a model would solve the problem of maintaining the membrane permeability barrier between transport events because the TatA transporter element would be assembled only when a substrate protein is available. Substrate-induced association of TatA promoters could allow stepwise construction of a channel-like element around the substrate until the required size for transport is achieved (27). Alternatively, concentrating TatA might perturb the local membrane structure to allow substrate movement (25, 28–30). It also is possible that formation of a TatA oligomer stores conformational energy that is then used to drive the substrate across the membrane.

Despite the appeal of the substrate-induced TatA association model, cross-linking alone cannot demonstrate that the TatA

Significance

The twin-arginine translocation (Tat) pathway transports folded proteins across a membrane without significant ion leakage. The mechanism by which Tat is able to carry out this challenging feat is unclear. We used direct imaging of fluorescent protein-tagged Tat components in bacterial cells to show that the TatA element of the Tat system undergoes substrate- and proton motive force-dependent oligomerization. Thus the Tat transporter element is assembled on demand, avoiding the need to seal the transporter between translocation events.


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reorganization corresponds to TatA assembly rather than to TatA conformational change. To address this issue, we previously undertook direct imaging of YFP-labeled TatA (TatA-YFP) that had been expressed at native levels in living E. coli cells (31). Individual TatA complexes could be tracked by video-rate fluorescence microscopy, and their oligomeric state could be estimated from their photobleaching traces. This analysis indicated that TatA forms complexes of variable stoichiometry with a median size of 25 polypeptides. In the absence of TatBC, only small TatA oligomers were found (median size, four polypeptides). This observation is consistent with the prediction from the substrate-induced TatA association model that substrate-bound TatBC mediates TatA assembly. However, no influence of substrate or PMF on the TatA oligomeric state was detected. Furthermore, the TatA-YFP fusions used in these experiments supported only trace transport activity, raising the question of the physiological relevance of our observations.

Here we show that TatA-YFP fusions are able to support efficient Tat transport in the presence of the TatA parologue TatE. Using this experimental system, we demonstrate that formation of TatA complexes depends on the presence of substrate protein, the TatBC complex, and the PMF. These observations provide direct evidence for key tenets of the substrate-induced TatA-association model. Our data also indicate that disassembly of the TatA complex is a PMF-independent process.

Results

Coexpression with TatE Improves the Transport Activity of Cells Expressing TatA-YFP. Our previous studies using fluorescence microscopy to visualize TatA in E. coli cells suffered from the significant limitation that TatA-fluorescent protein fusions support only very low levels of Tat transport and so may not be accurate reporters of the behavior of native TatA (31). In an attempt to overcome this problem, we explored whether a TatA-YFP fusion protein could be integrated into a well-functioning Tat transport pathway by coexpression with either the native TatA protein or with the TatA parologue TatE.

We constructed E. coli strains in which a gene coding for a TatA-YFP fusion was placed under the control of the native tatA promoter and then integrated into the chromosome at the attA promoter and then integrated into the chromosome at the tatA-YFP fusion was placed under the control of the native proteins they produce, with TatA or TatE producing strains that are able to carry out physiological Tat transport. However, only in the strain coexpressing TatE is it certain that the fusion protein contributes to the activity of the pathway. For this reason we chose the strain coexpressing TatE to study the oligomeric state of TatA in an active Tat transport system. We have, however, confirmed our key observations with the strain coexpressing TatA (see below).

We verified that the TatA-YFP fusion in the strain coexpressing TatE (AyBCE) is stable and localized in the membrane fraction of broken cells (Fig. 1C). Coexpression of TatE was found to increase the level of TatA-YFP in the cells even though there is no change in the genomic context of the fusion-coding gene. We excluded the possibility that enhanced levels of TatA-YFP were responsible for the increased transport activity of this strain by showing that overproduced TatA-YFP does not support Tat transport in the absence of TatE (Fig. S1).
Fluorescence Imaging of TatA-YFP in a Transport-Active Background.

We used fluorescence microscopy to determine the effects of TatE coexpression on the oligomerization of the TatA-YFP fusion. In the absence of TatE the TatA-YFP fusion is located predominantly in bright, independently mobile foci that exhibit no preferred localization within the cell membrane (strain AyBC; Fig. 2A). Our earlier analysis of TatA-YFP foci in the same genetic background showed that the foci correspond to complexes containing tens of copies of the fusion protein (31). Coexpression with TatE caused a striking redistribution of fluorescence with the majority of the fluorescent proteins now present as a dispersed halo around the periphery of the cell with only occasional bright foci (strain AyBCE; Fig. 2A and Movie S1). Fluorescence of the membrane-associated TatA-YFP protein appears as a halo at the cell periphery due to the imaging geometry. At higher time resolution this halo is resolved as numerous, dim, fast-moving foci (Movie S2). Fluorescent halos were also seen if TatA-YFP was coexpressed with TatE instead of TatA (strain AAYBC; Fig. 2A). For convenience we will term the bright foci as TatA “assemblies” and the halos as “dispersed” TatA.

We developed an automated classification routine to determine the number of bright foci in individual cells (SI Materials and Methods). Across a population of cells this analysis confirmed that strain AyBC has a significantly higher number of foci (i.e., TatA assemblies) per cell than either strain AyBCE or AAYBC (Fig. 2B and C).

The oligomeric states of the TatA-YFP fusion proteins observed in transport-active cells can be interpreted using a simple model based on the TatA association hypothesis (Fig. 2D). In this model TatA can be in two interconverting states, either dispersed in the membrane or assembled with TatBC to form an active translocation site. TatA association is induced by substrate binding to TatBC in the presence of a PMF, whereas TatA dissociation occurs when substrate is no longer bound to the translocation complex. Our imaging experiments determine the steady-state distribution of TatA between the two states. This distribution depends on the relative rates of the TatA association and dissociation processes, which are influenced both by the availability of substrate to initiate TatA assembly events and by the rate of substrate removal from the translocation site by transport to trigger TatA dispersion.

In the AyBCE strain TatA-YFP is found predominantly in the dispersed state. The levels of endogenous Tat substrates in aerobically grown cells are expected to be low (14), resulting in a limited frequency of initiation of TatA assembly. At the same time, the Tat transport activity of this strain is high, leading to efficient completion of transport. Thus, the cells have excess transport capacity, and those sites that do assemble move rapidly through the transport cycle, releasing the translocated substrate and returning TatA-YFP to the dispersed membrane pool. The net result is that at any given time only a small proportion of the TatA-YFP present in the cell is part of an assembled translocation site.

By contrast, TatA-YFP accumulates in the assembled state in the absence of TatE (strain AyBC). This behavior can be explained by the low transport activity of this strain, because in our model slowing transport retards the release of the substrate protein from the translocation site and delays TatA dissociation.

**Fig. 2.** Fluorescence imaging of strains expressing TatA-YFP. (A) Fluorescence images of TatA-YFP in representative cells of the indicated strains. (Scale bar: 1 μm.) (B) The population distributions of the number of intense fluorescence spots per cell for the indicated strains. (C) The data in B presented as a box plot showing the median (thick black line), the interquartile range (IQR) (gray box), data within 1.5 IQR of the lower and upper quartiles (whiskers), and data points falling outside this range (●). The number of cells (N) examined in each population is shown above each distribution. The statistical significance of differences between the mean spot counts observed for selected pairs of strains was assessed by Welch’s t test with P < 0.05 taken as significant and marked with an asterisk. (D) Two-state mechanistic model for TatA oligomerization. In our experiments TatA is visualized in either a dispersed state (Left) or in an assembled state (Right). The interconversion between the states is controlled by binding of a substrate protein (orange) to the TatABC complex in the presence of the transmembrane PMF. Substrate protein is released from the TatABC complex after transport, leading to dissociation of the TatA oligomer. The numbers of TatA subunits and TatBC units shown in the assembled TatABC complex are arbitrary and were selected for illustrative convenience.

**Tat Substrates Induce TatA Association.** Our model predicts that an increase in the availability of substrate in strain AyBCE should increase the rate of TatA association and thereby increase the proportion of TatA-YFP molecules in the assembled state. Consistent with this hypothesis, overproduction of the *E. coli* Tat substrate CueO (34) from a plasmid caused a shift in the distribution of TatA-YFP from a diffuse to a punctate pattern (Fig. 3A and Movie S3). This effect requires a functional signal peptide, because no induction of spot formation was seen if the essential twin arginine residues of the CueO signal peptide were conservatively substituted with lysine residues (CueOKK) (Fig. 3A). Frequency analysis of the number of foci per cell shows a significant difference between the population of cells expressing CueO and the populations of cells that either do not overproduce CueO or that express the transport-inactive variant (Fig. 3B). Cell fractionation and immunoblotting demonstrated that the wild-type and variant CueO proteins were present at similar levels in these experiments and confirmed that only the wild-type protein was transported to the periplasm and underwent signal peptide cleavage (Fig. 3C).
Substrate-dependent TatA-YFP association was also seen when an alternative Tat substrate, the *Acidithiobacillus ferrooxidans* high-potential iron-sulfur protein (HiPIP) (35), was overproduced and TatA association was again dependent on a functional signal peptide (Fig. 3 A–C). Substrate-dependent TatA-YFP association was also observed if TatA-YFP was coexpressed with TatA instead of TatE (Fig. S2). Thus, TatA association is a generic response to the presence of transport-competent substrate proteins.

To further test the causal relationship between substrate expression and TatA-YFP association, we immobilized cells containing the CueO expression plasmid on a microscope slide and took time-lapse images after treatment with isopropyl β-D-thiogalactopyranoside (IPTG) to induce CueO expression. Over a time course of minutes the distribution of TatA-YFP changed from diffuse to punctate (Fig. 3D). Immuno blotting of the same strain after induction of CueO expression shows that TatA-YFP association is correlated with an increase in cellular CueO concentration (Fig. 3E).

**TatA-YFP Association Requires TatBC.** The substrate-induced TatA-association model predicts that substrate binding to the TatBC complex is required to form TatA assemblies (Fig. 2D). In agreement with this prediction, removal of TatB and TatC prevented formation of TatA-YFP assemblies even in the presence of overproduced CueO substrate (Fig. 4 A and B). However, substrate-induced TatA-YFP assemblies could be recovered when both TatBC and CueO were expressed from plasmids in this strain (Fig. 4 A and B). When examining the images in Fig. 4A, note that strains with a complete defect in Tat transport exhibit a cell-chaining phenotype because of the mislocalization of two periplasmic amides (36, 37).

To confirm that TatA-YFP association was the result of a productive interaction between substrate proteins and TatBC, we expressed both CueO and TatBC from plasmids in strain AyE, but this time we used a TatC variant containing the substitutions F94A and E103A that are known to block signal peptide binding (21, 38). These cells failed to produce TatA-YFP assemblies, demonstrating that TatBC must be capable of binding substrate proteins for TatA association to occur (Fig. 4 A and B). Immunoblotting confirmed that TatB and the TatC variant had been successfully expressed in this experiment (Fig. 4C).

**Essential TatA Residue Phe39 Is Not Required for TatA Association.** The structure of *E. coli* TatA consists of a transmembrane helix followed by an amphipathic helix and then a natively unstructured tail (30). Phenylalanine 39 at the end of the amphipathic helix is invariant and essential for TatA function (39, 40). We confirmed that a TatA<sup>F39A</sup> variant is transport inactive when expressed from the chromosome under the control of the native *tatA* promoter (Fig. 5A, strain A<sup>F39ABCE</sup>). However, in contrast to the dominant-negative phenotype reported for the TatA<sup>F39A</sup> variant when expressed from a multicopy plasmid (39, 40), the chromosomally expressed TatA<sup>F39A</sup> variant did not affect the activity of wild-type TatA or TatE proteins (strains A<sup>F39ABCE</sup> and A<sup>F39ABCE</sup>; Fig. 5A).

The F39A substitution was also found to be inactive in a strain in which TatA-YFP association was impaired by a TatA<sup>F39A</sup> variant in the absence of other Tat substrates (strain AyF39A; Fig. 5B). In this situation, the absence of transport-competent substrate proteins suppressed the expression of TatA-YFP association, while the transport-active TatA<sup>F39A</sup> variant did not affect the activity of wild-type TatA or TatE proteins (strains A<sup>F39ABC</sup> and A<sup>F39ABCE</sup>; Fig. 5A).

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The presence of TatA-YFP assemblies in strain AyF39ABCE indicates that the inactivating F39A substitution does not prevent TatA association but inhibits a later step in Tat transport.

**TatA Association, but Not Dissociation, Requires the PMF.** Protein transport by the Tat system is energized by the transmembrane PMF (4, 5). To explore whether the oligomeric state of TatA-YFP is sensitive to the PMF, we used the protonophore carbonyl cyanide-m-chlorophenyl hydradine (CCCP) to dissipate the cytoplasmic membrane PMF of AyBCE cells expressing either CueO or HiPIP (Fig. 6). We considered that cells treated with CCCP and exhibiting TatA-YFP dispersion might retain an unmeasurable Δψ of this magnitude. We also observed TatA-YFP disassembly with cells that had been treated with the ionophores valinomycin and nigericin in the presence of potassium ions (Fig. 6 B and C). This combination dissipates both the Δψ and ΔpH components of the PMF and is reported to block completion of Tat transport at low Δψ (41). TatA-YFP assemblies were likewise dispersed by addition of the pore-forming colicin E1, which depolarizes the E. coli cytoplasmic membrane (Fig. 6 B and C) (42). Thus, TatA-YFP disassembly can occur in the absence of a Δψ.

To investigate the timescale of TatA-YFP complex association and dissociation, we took time-lapse images of a single cell as it was treated successively with CCCP and then with β-mercaptoethanol (Fig. 6D). Dissociation and reassociation of TatA-YFP complexes occurred within 1 min of the addition of CCCP or β-mercaptoethanol, respectively. We analyzed the disassembly and assembly processes with higher time resolution and found that TatA-YFP complexes dissociate within 5–15 s after CCCP is added and reform within 20–120 s after β-mercaptoethanol is added. Representative experiments are shown in Fig. 6 E and F and in Movies S4 and S5.

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**Fig. 4.** Assembly of TatA-YFP complexes requires functional TatBC. (A) Representative fluorescence images of TatA-YFP in strain AyE producing, variously, the Tat substrate protein CueO from plasmid pQE80-CueO (pCueO), TatBC from plasmid p101C*TatBC (pBC), or an inactive TatBC variant from plasmid p101C*TatBCEA (pBCEA). (B) Distribution of the number of TatA-YFP complexes per cell within each experimental population. The presentation of the distribution data and the statistical tests of the significance of differences between populations are as in Fig. 2C. (C) Immunoblot showing production of TatB and TatC in membranes isolated from the indicated strains.

**Fig. 5.** TatA Phe39 is not required for TatA association. (A) Growth of the indicated strains on LB/glycerol/TMAO medium under anoxic conditions. Error bars represent the SEM (n = 4). (B) Fluorescence images of TatA-F39A-YFP in representative cells of the indicated strains. (Scale bar: 1 μm.) (C) Distribution of the number of TatA-YFP complexes per cell within each experimental population. The presentation of the distribution data and the statistical tests of the significance of differences between populations are as in Fig. 2C.

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The presence of TatA-YFP assemblies in strain AyF39A-BCE indicates that the inactivating F39A substitution does not prevent TatA association but inhibits a later step in Tat transport.

**TatA Association, but Not Dissociation, Requires the PMF.** Protein transport by the Tat system is energized by the transmembrane PMF (4, 5). To explore whether the oligomeric state of TatA-YFP is sensitive to the PMF, we used the protonophore carbonyl cyanide-m-chlorophenyl hydradine (CCCP) to dissipate the cytoplasmic membrane PMF of AyBCE cells expressing either CueO or HiPIP (Fig. 6 A and C). With either substrate protein the steady-state distribution of TatA-YFP shifts from the assembled to the dispersed form after the addition of the protonophore. This change was reversible, because subsequent treatment with β-mercaptoethanol to inactivate CCCP led to the reappearance of TatA-YFP assemblies at pre-CCCP levels (Fig. 6 A and C). This last observation shows that the PMF is required for TatA complex assembly, as predicted by the substrate-induced TatA association model.

It has been inferred previously that completion of Tat transport requires a membrane potential (Δψ) that is below the detection limit of indicator dyes (41). We considered that cells treated with CCCP and exhibiting TatA-YFP dispersion might retain an unmeasurable Δψ of this magnitude. However, we also observed TatA-YFP disassembly with cells that had been treated with the ionophores valinomycin and nigericin in the presence of potassium ions (Fig. 6 B and C). This combination dissipates both the Δψ and ΔpH components of the PMF and is reported to block completion of Tat transport at low Δψ (41). TatA-YFP assemblies were likewise dispersed by addition of the pore-forming colicin E1, which depolarizes the E. coli cytoplasmic membrane (Fig. 6 B and C) (42). Thus, TatA-YFP disassembly can occur in the absence of a Δψ.

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A different response to protonophore addition was observed in strains that assemble TatA-YFP but do not support significant Tat transport activity. When strain AyBC was treated with CCCP, no dissociation of TatA complexes was observed (Fig. 6 G and H).
Similarly, when CCCP was added to strain Ay^E39A^BCE, numerous TatA-YFP assemblies remained visible (Fig. 6 G and H) and were significantly more abundant than in strains with dispersed TatA-YFP such as AyBCE. Thus, TatA complex disassembly is correlated with an active Tat system.

**TatB-YFP and TatC-YFP Form Oligomers That Are Unaffected by Substrate Concentration or the PMF.** Having established that TatA undergoes changes in oligomerization state, we investigated whether other Tat components also show such changes under similar conditions. We generated strains expressing either TatB-YFP or TatC-YFP by inserting an operon carrying tatA-tatBYP-tatC or tatA-tatB-tatCyfp at the att site of a ∆tatABC∆tatE strain. Although immunoblotting of cell membranes showed that strain AByC had nearly wild-type levels of all three Tat proteins, the three Tat proteins were present at low or undetectable levels in strain ABCy, possibly because of mRNA instability (Fig. S3A). To overcome this problem, we constructed low-copy-number plasmids that permit the expression of either TatB-YFP or TatC-YFP at nearly native levels (Fig. S3). The AByC strain and the plasmids expressing TatB-YFP and TatC-YFP fusions all supported full transport activity as assessed by anaerobic growth on LB/glycerol/TMAO medium (Fig. S3B).

TatB-YFP and TatC-YFP both exhibited multiple, mobile fluorescent foci when plasmid-expressed in the presence of the other three Tat components (Fig. 7 A and C and Movies S6 and S7) or when chromosomally expressed in strain AByC (Fig. S3C). No significant change in the fluorescence distribution of either fusion was found after overproduction of the substrate protein CueO or upon the addition of the protonophore CCCP (Fig. 7). Taken together, these data show that the oligomeric states of TatB and TatC do not change under conditions that lead to TatA complex assembly or disassembly.

TatC-YFP was still able to oligomerize in the absence of TatB (strain AE pCy; Fig. 7 C and D). By contrast, cells expressing TatB-YFP without TatC showed a clear shift to a more dispersed pattern of fluorescence, indicating that TatB oligomerization requires TatC (strain AE pBy; Fig. 7 A and B).

**Discussion**

TatA fluorescent protein fusions are unable to functionally substitute for the wild-type TatA protein (31), and this limitation has made it difficult to use fluorescence-based methods to probe TatA behavior. We now show that a TatA-YFP fusion can be made to support physiological Tat transport activity in E. coli by expression alongside the endogenous TatA paralogue TatE. We have used this experimental system to test the substrate-induced TatA association model in the context of a functional Tat system.

For TatA-YFP to be a useful reporter of TatA behavior, the fusion protein must be incorporated into active translocation sites. Our data strongly suggest this incorporation occurs, because the otherwise inactive TatA-YFP fusion markedly enhances the Tat transport activity supported by TatE. The changes in Tat-YFP oligomer state seen in response to transport-relevant perturbations were the same whether Tat-YFP had been coexpressed with native TatA or TatE. This observation indicates that it is the absence of a fusion domain, and not some special feature of TatE, that allows functional cooperation with TatA-YFP. More specifically, because TatA-YFP expressed without TatA or TatE is unable to sustain transport but still forms large assemblies, we can infer that the YFP domain interferes with a step in Tat transport that occurs only after TatA association.

The substrate-induced TatA-YFP assemblies observed in the presence of TatE probably contain only a very small proportion of TatE protomers because of the low level of TatE expression relative to TatA expression (14) (indeed, the TatE concentration present in our experiments is observed to be transport limiting; Fig. 1 A and B). A low TatE:TatA-YFP ratio suggests that TatA-YFP can be functionally tolerated at most, but not all, positions within the assembled TatA complex. This notion in turn raises the possibility that TatA is present in at least two different structural environments within the active translocation site. A plausible scenario would have a limited number of TatA protomers in intimate contact with the TatBC complex, nucleating the oligomerization of the bulk of the TatA subunits into a structure extending out into the surrounding phospholipid bilayer. In this model TatA-YFP fusions are able to function at the “bulk” but not at the “intimate” positions. Such a model would be consistent with observations that some TatA molecules associate with the resting TatBC complex in E. coli as judged by site-specific photoaffinity cross-linking (43), FRET studies (44), or copurification related to the Tat transport complex but not to the TatA subunit.
ification (45–47). As an alternative, a limited number of TatE proteomers interdigitated among the TatA-YFP subunits may provide sufficient conformational flexibility to relieve the steric effects of the YFP domains.

Our imaging experiments capture the steady-state distribution of TatA-YFP between large assemblies and a pool of small units. When only endogenous levels of substrate proteins are present, the TatA assemblies are rare. However, when substrate proteins are overproduced, the number of TatA assemblies present in the cell increases dramatically, as expected if TatA association is triggered by substrate availability. This phenomenon is likely to be general, because it was seen with the two very different substrates, CueO, a large native E. coli Tat substrate (5–6 nm in diameter) that acquires copper ion cofactors in the periplasm, and A. ferrooxidans HiPIP, a small heterologous Tat substrate (2.5–3 nm in diameter) that has an iron-sulfur cluster inserted in the cytoplasm before transport.

Induction of TatA assembly required the substrate protein to have a functional Tat signal peptide, showing that formation of TatA complexes is a consequence of a specific interaction of the substrate with the Tat system. Complex formation was also absolutely dependent upon the presence of TatBC, as expected if TatA is assembled by a substrate-activated TatBC complex. Importantly, TatA association requires the TatBC complex to be competent for substrate binding, excluding the possibility that TatBC is a passive scaffold with which TatA is permanently associated.

A key feature of the substrate-induced TatA association model is that the formation of TatA complexes is driven by the transmembrane PMF, as indeed we observed in our transport-active experimental system. Specifically, we found that TatA assemblies reform upon restoration of the PMF to previously de-energized cells. In the presence of inducing levels of substrate, the TatA subassemblies were rebuilt in as little as 20 s, suggesting that PMF-driven TatA association is sufficiently fast to be part of the Tat transport mechanism. The kinetics of TatA complex formation after the induction of substrate overproduction were considerably slower (ca. 20 min to completion) than those observed in the PMF restoration experiment. This difference indicates that it requires a significant period after induction for the substrate to reach a concentration that affects the steady-state level of TatA complexes present.

We found that TatA-YFP assemblies dissociate rapidly upon dissipation of the PMF even though substrate molecules are still present. A possible interpretation of this observation is that the oligomer equilibrium is strongly biased in favor of the assembled state when substrate and a PMF are both present, but that with removal of the PMF the direction of this equilibrium is reversed, leading to dissociation of TatA oligomers. However, the situation is more complex than this, because TatA assemblies are able to persist in the absence of a PMF in strains that can assemble TatA but are incompetent for transport (strains AyBC and AyE3657ABCE), showing that it is possible for TatA oligomers to reach a state that is stable to removal of the PMF. Although it is conceivable that the TatA-YFP oligomers in these transport-incompetent strains experience a nonphysiological stabilization against reversal of assembly, such an enhancement of subunit interactions within the oligomer would be a surprising consequence of the removal of a mechanistically important component or residue. Instead, these strains are most likely to be defective in catalyzing a transport step that takes place after TatA assembly. If so, our observations can be explained by suggesting that there is a critical stage in TatA assembly at which TatA oligomerization becomes irreversible. Such a critical point could correspond, for example, to closure of a TatA ring or to a conformational change associated with completion of TatA assembly. Only on release of the substrate protein through transport is the TatABC complex able to re-enter a state in which TatA oligomerization changes (i.e., disassembly) are again possible. Because

The transport-inactive strains are unable to expel the bound substrate protein by translocation; they accumulate TatA-YFP in the PMF-insensitive assembled state. This model is set out in Fig. 8.

Because a PMF will always be present in living cells, substrate availability controls the direction of the TatA assembly/disassembly process. When substrate proteins are scarce, each transport event will be associated with a complete cycle of TatA assembly and disassembly. However, during transport at high substrate concentrations, binding of the next substrate molecule to the TatBC complex might occur before TatA dissociation has started or before it has been fully completed. Under these conditions it is implicit in our model that TatA sites can be reused, as recently suggested (20), or regenerated from a partially disassembled state by reversing the direction of the disassembly equilibrium upon substrate binding (Fig. 8). Similar considerations apply if multiple substrate-binding sites within the TatBC complex are able to access the same TatA assembly (20, 48).

In contrast to TatA, we find that the oligomeric states of both TatB and TatC are unaffected by either raising substrate concentrations or dissipating the PMF. This finding is consistent with previous observations that these two proteins copurify in detergent solution as a large TatBC complex and that the size and composition of the complex are not significantly altered by substrate binding (19, 22, 45, 49, 50). It is also consistent with the observation that TatBC complexes do not exhibit the exchange of TatC subunits that would be expected if TatC undergoes assembly cycles (23). We find that the TatB protein has a lower oligomer state if TatC is absent but that TatC oligomerization is independent of TatB. These observations are compatible with the previous characterization of these proteins in detergent solution where, in the absence of a partner protein, TatC still forms a distinct multimeric complex but TatB is reported to be in small or heterogeneous oligomers (49, 50).

In summary, our TatA imaging experiments in E. coli verify the key predictions of the substrate-induced TatA association model that was derived from cross-linking experiments in plant thylakoids: that TatA can exist in both dispersed and high-
omer states; that the number of TatA assemblies is a function of substrate availability; that TatA association requires the substrate to be able to bind to the TatBC complex; and that TatA association occurs only in the presence of the transmembrane PMF. Our experiments extend the TatA association model by suggesting that transport requires progression through distinct assembled states that can be distinguished by their sensitivity to removal of the PMF.

Materials and Methods

Strain Construction, Cell Growth and Analytical Methods. Construction of plasmids and strains, preparation of cells for microscopy, and analytical methods are described in SI Materials and Methods. The plasmids and stains used in this work are listed in Tables S1 and S2.

Fluorescence Microscopy. Cells were imaged using an inverted microscope (TI-E; Nikon) illuminated by a 532-nm DPSS Nd:YAG laser (Compass 215M; Coherent Inc.). The excitation light was focused at the back aperture of an oil immersion objective lens (100x Plan Apo N.A. 1.4; Nikon) to provide glancing illumination of the coverslip surface as in Hilos or pseudo total internal reflection fluorescence imaging (S1, S2). Typically, Hilos illumination images a sample to a depth of 5 μm but offers improved signal as compared with absolute epifluorescence (S2). The power entering the back of the objective was 0.04 mW (power density 75; Semrock) transmitted through dichroic (C67195; Nikon) and emission (BrightLine 582/75; Semrock) filters. Images were recorded using an EMCCD camera with exposure times between 4 and 20 ms (iXon+ DV860E; Andor Technology). For figure composition, image stacks were imported into MATLAB (MathWorks). Image stacks averaged over 40 ms were scaled to display 1,500 arbitrary units (a.u.) for each channel as the minimum (black) and 7,500 a.u. as the maximum (white) unless exposure times between 4 and 20 ms (iXon+ DV860E; Andor Technology).

Automated Spot Counting. An algorithm was devised to discriminate effectively between TatA-YFP present as quickly moving, dim, diffuse spots and the slower-moving but bright foci observed upon Tat activation. By averaging together frames in our image sequence, we trade time resolution for an improved signal-to-noise ratio. In doing so, the fast-moving diffuse background no longer appears as individual, poorly resolved spots but as a diffuse halo at the cell periphery. This method provides clear discrimination between the two populations and forms the basis of our algorithm.

ACKNOWLEDGMENTS. We thank Holger Kneuper for providing plasmid pTatBC101, Violaine Bonnefoy for providing the A. ferrooxidans HiPIP gene, Colin Kleanthous for providing the colicin E1 protein, and Trevor Lithgow for providing BamA antibodies. This work was supported by the Biotechnology and Biological Sciences Research Council through Grants H018050 and D004578.

Supporting Information

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SI Materials and Methods

Strain Construction. The Escherichia coli strains used in this work are listed in Table S1.

To create a chromosomal deletion of tatABC (ΔABC-A), the apramycin-resistant ΔtatABC::apra’ allele from strain BIF030 (1) was transferred to strain MC4100-A (2) by P1-mediated transduction using standard protocols (3). To create a chromosomal deletion of tatBC (ΔBC), a tatBC deletion allele was assembled by digesting plasmid pFAT23 (4) with ClaI and KpnI to release the downstream part of the tatC deletion allele and then cloning this fragment into pFAT164 containing an analogously assembled tatB deletion allele (5) that had been digested with the same enzymes. The resultant tatBC deletion allele was released by digestion with XbaI and KpnI and was cloned into similarly digested pMAK705 (6). The tatBC allele subsequently was moved onto the chromosome of MC4100 as described previously (6).

All insertions into the E. coli phage lambda attachment site (att) were delivered using plasmid pRS552 (7).

The cassette Ppat-A-EAK-eyfp′2206k was used to construct Ay strains. TatA was amplified from pUNITAT2 (8) with primers TatAPROM1 (5-GGCCGATATCCTGCGTGAGAACGCAAGCCCA-ACACGCCGT-3′) and TatANostopREV (5′-GCGCGATATCCTGCGTGAGAACGCAAGCCCA-CCCGGCTTATTCTTCAGTTTTTTCGCTTTCTGC-3′) and was cloned into pBluescript KS (Stratagene) digested with the same enzymes to create pTatAnosNSTOP. DNA encoding an α-helical linker [amino acid sequence (EAAAK)]₉ (9) was synthesized (Genscript) with an NsiI site at the 5′ end and a BsiWI site followed by a BamHI site at the 3′ end. The linker was excised with NsiI and BamHI and then was cloned into pTatAnosNSTOP-digested with the same enzymes to produce pTatAnosF. The δerminase-suppressing substitution A206K (10) was introduced into the EYFP-coding gene using Quikchange mutagenesis (Stratagene) of plasmid pYFPF1 (11) and primers A206KF (5′-CTACGCTGATCTGACAGCTATGGAGCATCGTAC-3′) and A206KR (5′-GGTTGGGTTTTGGATCTCGTATTCTGTGGACTGCT-3′), resulting in plasmid pYFP206K. The resulting eyfp′2206k allele was amplified using primers EYFPBsiFOR (5′-GGCGCGATATCCTGCGTGAGAACGCAAGCCCA-CCCGGCTTATTCTTCAGTTTTTTCGCTTTCTGC-3′) and EYFP-BamHI REV (5′-GGCGCGATATCCTGCGTGAGAACGCAAGCCCA-CCCGGCTTATTCTTCAGTTTTTTCGCTTTCTGC-3′), then the product was digested with BamHI and XbaI and was cloned into pGEM4-BsiWI and pGEM4-BamHI, and was then cloned into pTatAnosF digested with the same enzymes to produce pTatAnosF. This plasmid then was digested with EcoRI and BamHI, and the released Ppat-A-EAK-eyfp′2206k cassette was cloned into the same sites in pRS552 to allow integration into the chromosome.

For A′35A’ strains the phc39ala mutation was introduced into tatA-A-EAK-eyfp in plasmid pBSTatAry by site-directed mutagenesis using primers TatAF39AR (5′-GGTTGGGTTTTGGATCTCGTATTCTGTGGACTGCT-3′) and TatAF39AR (5′-CCATGCTGATCTGACAGCTATGGAGCATCGTAC-3′) and a BsiWI and BamHI fragment was assembled in pTatBCyfp. Finally, the entire insert was subcloned into pRS552 using BamHI and EcoRI to give p552ABCyf. p552ABCyf was used for integration of Ppat-A-EAK-eyfp′2206k into the chromosome of strain DADE.

To verify the construction of att-directed integrant strains, the tat and att loci were amplified from genomic DNA using primers UbiBdown (5′-TCTGATGGATGGTGGTTC-3′) and SphlTatCR (5′-CCCCGATCTGTTATCTGCTTTTGGTTCGCTTCG-3′) or 552F (5′-AAACTGCGAGAATTTG-3′) and 5522R (5′-GATGTTGGTACCGCC-3′), respectively, and were sequenced.

Plasmid Construction. The plasmids used in this work are listed in Table S2.

pQEO-CueOΔ4‘ was created by site-directed mutagenesis of pQE80-CueO (11) with primers CueOKKF (5′-GAAAAATATGACGATATGCTGTTAA-3′) and CueOKKR (5′-AGTTGGTACGAGGAG-3′), then the product was digested with BamHI and EcoRI, and the released Ppat-A-EAK-eyfp′2206k cassette was cloned into the same sites in pRS552 to allow integration into the E. coli chromosome.

For A′35A’ strains the phc39ala mutation was introduced into tatA-A-EAK-eyfp in plasmid pBSTatAry by site-directed mutagenesis using primers TatAF39AR (5′-GGTTGGGTTTTGGATCTCGTATTCTGTGGACTGCT-3′) and TatAF39AR (5′-CCATGCTGATCTGACAGCTATGGAGCATCGTAC-3′) and an XhoI-XbaI fragment was assembled in pTatBCyfp. Finally, the entire insert was subcloned into pRS552 using BamHI and EcoRI to give p552ABCyf. p552ABCyf was used for integration of Ppat-A-EAK-eyfp′2206k into the chromosome of strain DADE.

For A′35A’ strains the phc39ala mutation was introduced into tatA in plasmid pKSUnia (12) using primers TatAF39AR and TatAF39AR. An EcoRI-BamHI fragment was assembled in pTatBCyfp. Then the product was digested with BamHI and EcoRI, and the released Ppat-A-EAK-eyfp′2206k was then subcloned into pRS552 for delivery onto the chromosome.

To construct strain DADE-ΔABCyF, the tatB gene was amplified from pUNITAT2 with primers TatBFOR (5′-GCGCGGATATCCTCGTATTCTGCTTTAGCG-3′) and TatB-
To achieve approximately wild-type levels of tatBC expression, plasmid p101C*BC was constructed as follows: First the tatBC genes were amplified from pTAT101 (16) using primers tatBC2 primer set (5'-GGAGATCTTGATCCCGATC-3') and TatBC primer set (5'-AGCTTATACAGCG-3'). The amplicons were digested with BamHI and PstI and then was cloned into the same sites in pTAT101 to give pTatBC101. This construction places tatA immediately downstream of the tatA promoter in the vector. To construct plasmid p101C*TatCy, pPstIorf was subcloned as an EcoRI-PstI fragment from pTatBC101 into pTH19Cr (17) to make p101C*TatBC. To reduce the level of tatA expression from this plasmid, the ribosome-binding site was altered by site-directed mutagenesis with primers TatBrBS'F (5'-CATCATCT-TACCAGACGAGATCGTGTGTTG-3') and TatBrBS'R (5'-CAACACCGATCTCTGCTGTAATGAGTG-3') generating p101C*TatA. Plasmid p101C*TatA FEA was created by two rounds of site-directed mutagenesis with primers TatBrBS'F (5'-CTATCATGTTGGGACGCTAT-CGCCACCGCTGCT-3') and TatBrBS'R (5'-CAGCTGCTG-GGGGATAGTGCCACCTGATAG-3') and primers TatCE103AF (5'-CGCTGTATAGCATGCACGCCTGG-3') and TatCE103AR (5'-CACCAGGCGCTGATGCCTGTCATACCCGG-3').

To construct plasmid p101C*TatCy, the tatCy-yfpA260K gene was amplified from p552ABCy using primers BamHITatBF (5'-ATAGGATCCATGTCTGTAGAAGATACTCAACC-3') and SphIYFPR (5'-TTAGCATGCTTACTTGTACAGCTCGTCC-3'). The amplicon was cloned behind the tatA promoter by insertion between the BamHI and SphI sites of p101C*TatA, which was altered by site-directed mutagenesis with primers TatCrBS'F (5'-CATCTACACAGAGACGAGTTGCTGTTAGAAAGATCACC-3') and SphIYFPR and then was digested with BamHI and SphI. pTatBC101 was digested with these enzymes to release the tatBC insert (but not the tatA promoter) from this plasmid, which was then replaced with the tatCy-yfpA260K PCR product.

To construct plasmid pGTGArCy, an EcoRI-BamHI fragment encompassing Pnad-tatA-EAK-ysfA260K was excised from pBSTatA byC and was subcloned into the same sites in p101C*TatA, which was then cut with SphI to release the tatBC insert (but not the tatA promoter) from this plasmid, which was then digested with SphI and BamHI and then was ligated into p101C*BC. This plasmid was then transformed into the E. coli strain Strain 155 (Pt 12), which was then transferred to a microplate. The cells were then grown in LB medium to an OD of 0.5, and then were transferred to a microplate and incubated at 37 °C for 30 min. Cells were then prepared for microscopy as described above except for the addition of 1 mM IPTG and 0.1 mg/mL ampicillin to all wash buffers.

For depolarization of cells by colicin E1, purified, lyophilized ColE1 (a gift from Colin Kleanthous, University of Oxford, Oxford, UK) was dissolved in 25 mM Tris HCl (pH 7.4) at 1 mg/mL. This solution was added directly to M9/glucose medium at a final concentration of 10 μg/mL. Immobilized cells were washed with the CoE1-containing medium and then were incubated for at least 3 min before image capture.

For nigericin/valinomycin treatment, cells were first permeabilized by treatment with M9/glucose medium containing 5 mM EDTA for 10 min at room temperature. Cells were then resolated by centrifugation, suspended in fresh M9/glucose medium, and immobilized on tunnel slides as described above. Nigericin was dissolved in ethanol at 5 mM to give a 100X stock solution, and valinomycin was dissolved in DMSO at 8 mg/mL to give a 200X stock solution. The two ionophores then were diluted into M9/glucose medium containing 400 mM potassium acetate. A mock buffer of M9/glucose medium containing the same concentrations of potassium acetate, ethanol, and DMSO was also prepared. Immobilized EDTA-treated cells were washed with either the buffer containing valinomycin/nigericin or the mock buffer before image capture.


Fig. S1. Assessment of Tat activity in a strain with elevated levels of TatA-YFP. (A) Whole-cell lysates of the indicated strains were separated by SDS PAGE and immunoblotted with TatA antibodies. (Upper) pGTGAryBC refers to the Δtat strain DADE carrying a plasmid which expresses TatA-YFP, TatB, and TatC. (Lower) A Ponceau stain of the same membrane as a loading control. (B) Growth of the indicated strains on LB/glycerol/TMAO medium under anoxic conditions. Each data point represents the mean of two independent experiments.

Fig. S2. Substrate-induced TatA-YFP assembly in strain AAyBC. (A) Fluorescence images of TatA-YFP in representative cells of strain AAyBC either without (Left) or with (Right) overproduction of CueO. (B) Distribution of the number of TatA-YFP complexes per cell within each experimental population. The presentation of the distribution data and the statistical tests of the significance of differences between populations are as in Fig. 2C.
Fig. S3. Activity and fluorescence distribution in cells expressing tatB-yfp and tatC-yfp fusions. (A) (Left) Immunoblot showing the levels of TatA, TatB, and TatC in membranes isolated from cells carrying chromosomal (lanes 2 and 5) or plasmid-based (lanes 3 and 6) tatB-eyfp<sup>A206K</sup> (lanes 2 and 3) or tatC-eyfp<sup>A206K</sup> (lanes 5 and 6) relative to the wild-type strain (ABCE) (lanes 1 and 4). BamA levels were determined as a loading control. (Right) Because TatC-YFP is not recognized by our α-TatC antibodies, TatC-YFP levels were determined relative to TatB-YFP by α-GFP immunoblot. (B) Growth of the indicated strains on LB/glycerol/TMAO medium under anoxic conditions. Error bars represent the SEM (n = 4). (C) Fluorescence images of TatB-YFP in strain AByC. Images were scaled to display 1,000 arbitrary units (a.u.) as the minimum (black) and 2,500 a.u. as the maximum (white). (Scale bar: 1 μm.)
Fig. S4. Histograms showing the population distributions of the number of intense fluorescence spots per cell for the indicated strains.
### Table S1. E. coli strains used in this study

<table>
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<tr>
<th>Strain name</th>
<th>Abbreviation</th>
<th>Genotype</th>
<th>Source</th>
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<tr>
<td>MC4100-A*</td>
<td>ABCE</td>
<td>F−, ΔlacU169, araD139, rpsL150, relA1, ptsF, rbsR, flbB5301</td>
<td>(1)</td>
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<tr>
<td>ELV16-A*</td>
<td>BCE</td>
<td>MC4100 ΔtatA</td>
<td>(2)</td>
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<tr>
<td>J1M1</td>
<td>ABC</td>
<td>MC4100 ΔtatE</td>
<td>(3)</td>
</tr>
<tr>
<td>JARV16-A*</td>
<td>BC</td>
<td>MC4100 ΔtatA ΔtatE</td>
<td>(1)</td>
</tr>
<tr>
<td>DADE-A*</td>
<td>Δtat</td>
<td>MC4100 ΔtatABCD ΔtatE</td>
<td>(4)</td>
</tr>
<tr>
<td>MΔBC</td>
<td>AE</td>
<td>MC4100 ΔtatBC</td>
<td>This work</td>
</tr>
<tr>
<td>BOD</td>
<td>ABE</td>
<td>MC4100 ΔtatB</td>
<td>(2)</td>
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<tr>
<td>B1LK0</td>
<td>ABE</td>
<td>MC4100 ΔtatC</td>
<td>(5)</td>
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<tr>
<td>JARV16-A*</td>
<td>ΔAry</td>
<td>AyBC</td>
<td>MC4100 ΔtatA ΔtatE, attB::PtatA-EAK-eyfp&lt;sub&gt;A206K&lt;/sub&gt; (kan&lt;sup&gt;+&lt;/sup&gt;)</td>
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<tr>
<td>ELV16-A*</td>
<td>ΔAry</td>
<td>AyBCE</td>
<td>MC4100 ΔtatA, attB::PtatA-EAK-eyfp&lt;sub&gt;A206K&lt;/sub&gt; (kan&lt;sup&gt;+&lt;/sup&gt;)</td>
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<td>MΔABC-A*</td>
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<td>AyE</td>
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<td>AAYBC</td>
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<tr>
<td>JARV16-A*</td>
<td>ΔA F39A</td>
<td>A&lt;sup&gt;F39A&lt;/sup&gt;BC</td>
<td>MC4100 ΔtatA ΔtatE, attB::PtatA&lt;sup&gt;F39A&lt;/sup&gt; (kan&lt;sup&gt;+&lt;/sup&gt;)</td>
</tr>
<tr>
<td>ELV16 λA F39A</td>
<td>ΔA&lt;sup&gt;F39A&lt;/sup&gt;BCE</td>
<td>MC4100 ΔtatA, attB::PtatA&lt;sup&gt;F39A&lt;/sup&gt; (kan&lt;sup&gt;+&lt;/sup&gt;)</td>
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<tr>
<td>J1M1-ΔA F39A</td>
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<td>MC4100 ΔtatE, attB::PtatA&lt;sup&gt;F39A&lt;/sup&gt; (kan&lt;sup&gt;+&lt;/sup&gt;)</td>
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<td>ELV16 λA F39A</td>
<td>ΔA&lt;sup&gt;F39A&lt;/sup&gt;BCE</td>
<td>MC4100 ΔtatA, attB::PtatA&lt;sup&gt;F39A&lt;/sup&gt; (kan&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>This work</td>
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<tr>
<td>DADE-ΔAry F39A</td>
<td>ΔA&lt;sup&gt;F39A&lt;/sup&gt;BCE</td>
<td>MC4100 ΔtatABCD ΔtatE, attB::PtatA&lt;sup&gt;F39A&lt;/sup&gt;-EAK-eyfp&lt;sub&gt;A206K&lt;/sub&gt; (kan&lt;sup&gt;+&lt;/sup&gt;)</td>
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<tr>
<td>DADE-ΔAByC</td>
<td>ABCy</td>
<td>MC4100 ΔtatABCD ΔtatE, attB::PtatA&lt;sup&gt;F39A&lt;/sup&gt;-EAK-eyfp&lt;sub&gt;A206K&lt;/sub&gt;-C (kan&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>This work</td>
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<tr>
<td>DADE-ΔABCy</td>
<td>ABCy</td>
<td>MC4100 ΔtatABCD ΔtatE, attB::PtatA&lt;sup&gt;F39A&lt;/sup&gt;-EAK-eyfp&lt;sub&gt;A206K&lt;/sub&gt; (kan&lt;sup&gt;+&lt;/sup&gt;)</td>
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*All strains designated “-A” are arabinose-resistant derivatives.


### Table S2. Plasmids used in this study

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<td>pQE-80</td>
<td>Expression vector.</td>
<td>Qiagen</td>
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<tr>
<td>pQE80-CueO</td>
<td>Synthesis of <em>E. coli</em> CueO with a C-terminal his&lt;sub&gt;6&lt;/sub&gt; tag.</td>
<td>(11)</td>
</tr>
<tr>
<td>pQE-CueO&lt;sup&gt;Δhis&lt;/sup&gt;</td>
<td>Synthesis of <em>E. coli</em> CueO with a C-terminal his&lt;sub&gt;6&lt;/sub&gt; tag. RR→KK substitution in signal peptide. This work</td>
<td></td>
</tr>
<tr>
<td>pQE-HiPIP</td>
<td>Synthesis of <em>A. ferrooxidans</em> HiPIP with a C-terminal his&lt;sub&gt;6&lt;/sub&gt; tag. This work</td>
<td></td>
</tr>
<tr>
<td>pQE-HiPIP&lt;sup&gt;Δhis&lt;/sup&gt;</td>
<td>Synthesis of <em>A. ferrooxidans</em> HiPIP with a C-terminal his&lt;sub&gt;6&lt;/sub&gt; tag. RR→KK substitution in signal peptide. This work</td>
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</tr>
<tr>
<td>pTH19Kcr</td>
<td>Very low-copy vector, kan-resistant.</td>
<td>(17)</td>
</tr>
<tr>
<td>pTH19Kcr</td>
<td>Very low-copy vector, cam-resistant.</td>
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</tr>
<tr>
<td>pTatBC101</td>
<td>pTH19Kcr derivative. Expression of tatBC from the tatA promoter.</td>
<td>This work</td>
</tr>
<tr>
<td>p101TatBC</td>
<td>pTH19Kcr derivative. Expression of tatBC from the tatA promoter with a modified RBS.</td>
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<td>pTatBC with F94A and E103A mutations in tatC This work</td>
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<td>p101C&lt;sup&gt;ΔtatC&lt;/sup&gt;TatCy</td>
<td>pTH19Kcr derivative. Expression of tatC-eyfp&lt;sub&gt;A206K&lt;/sub&gt; from the tatA promoter with a modified RBS and start codon. This work</td>
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<td>p101KTatBy</td>
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<td>pGTGAryBC</td>
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Movie S1. Strain AyBCE. The movie shows the first 20 frames of an image stack of an AyBCE cell, with an initial halo of diffuse fluorescence. Pixels below 1,000 a.u. are shown as black, and those above 9,000 a.u. are shown as white. Playback has been slowed 10× (original: 50 frames at 10-ms exposure time). The area displayed is 8.8 μm².

Movie S1

Movie S2. Strain AyBCE. As Movie S1 but showing frames 30–50 of the image stack, with adjusted brightness scale. Because the cell fluorescence is partially bleached, it is possible to discern individual fluorescent foci diffusing rapidly around the cell. Pixels below 1,000 a.u. are shown as black, and those above 2,500 a.u. are shown as white. Playback has been slowed 10× (original 50 frames at 10-ms exposure time). The area displayed is 8.8 μm².

Movie S2
Movie S3.  Strain AyBCE expressing substrate protein CueO from plasmid pQE80-CueO (pCueO). TatA-YFP assembles into large, mobile spots in the presence of high levels of substrate. The mean diffusion coefficient for the TatA-YFP complexes in this video is $0.22 \pm 0.19 \mu m^2/s$ ($n = 5$). Pixels below 1,000 a.u. are shown as black, and those above 6,000 a.u. are shown as white. Playback has been slowed 20x (20 frames at 5-ms exposure time). The area displayed is 8.8 $\mu m^2$.

Movie S3

Movie S4.  Time-lapse imaging of CCCP-induced dissipation of TatAy spots in an AyBCE pCueO cell (Fig. 6E). CCCP (50 $\mu M$) was introduced at 15 s (frame 4). Pixels below 1,000 a.u. as are shown as black, and those above 7,000 a.u. are shown as white. Each frame in the video represents a single 20-ms exposure, with a 5-s delay between each exposure. Playback is at five frames/s (25x speed). The area displayed is 8.8 $\mu m^2$.

Movie S4
Movie S5. Time-lapse imaging of bME-induced recovery of TatAy spots in an AyBCE pCueO cell after CCCP dissipation (Fig. 6F). βME (0.05%) was introduced at 10 s (between frames 1 and 2). Pixels below 1,000 a.u. are shown as black, and those above 7,000 a.u. are shown as white. Each frame in the video represents a single 20-ms exposure, with a 20-s delay between exposures. Playback is at five frames/s (100× speed). The area displayed is 8.8 μm².

Movie S6. Strain ACE pB8y. In the presence of TatC, TatB-YFP forms bright, mobile foci. Pixels below 1,000 a.u. are shown as black, and those above 7,000 a.u. are shown as white. Playback has been slowed 5x (20 frames at 20-ms exposure time). The area displayed is 8.8 μm².

Movie S6
Movie S7. Strain ABE pCy. TatC-YFP forms mobile fluorescent foci. Pixels below 1,000 a.u. are shown as black, and those above 4,000 a.u. are shown as white. Playback has been slowed 10x (20 frames at 20-ms exposure time). The area displayed is 8.8 μm².

Movie S7