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Coordinating assembly and export of complex bacterial proteins

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The *Escherichia coli* twin-arginine protein transport (Tat) system is a molecular machine dedicated to the translocation of fully folded substrate proteins across the energy-transducing inner membrane. Complex cofactor-containing Tat substrates, such as the model (NiFe) hydrogenase-2 and trimethylamine *N*-oxide reductase (TorA) systems, acquire their redox cofactors prior to export from the cell and require to be correctly assembled before transport can proceed. It is likely, therefore, that cellular mechanisms exist to prevent premature export of immature substrates. Using a combination of genetic and biochemical approaches including gene knockouts, signal peptide swapping, complementation, and site-directed mutagenesis, we highlight here this crucial 'proofreading' or 'quality control' activity in operation during assembly of complex endogenous Tat substrates. Our experiments successfully uncouple the Tat transport and cofactor-insertion activities of the TorA-specific chaperone TorD and demonstrate unequivocally that TorD recognises the TorA twin-arginine signal peptide. It is proposed that some Tat signal peptides operate in tandem with cognate binding chaperones to orchestrate the assembly and transport of complex enzymes.

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Introduction

Targeting of proteins to their sites of physiological function is an important feature of all biological systems. In bacteria, the generation of energy by electron transfer chains involves cofactor-containing enzymes often embedded in the cytoplasmic membrane or located entirely on the extracytoplasmic side. Thus, bacterial growth and survival in many environments depends upon the controlled targeting and transport of redox enzymes to and across this membrane. Studies of *Escherichia coli* have established that a subset of cofactor-containing exported proteins are synthesised with N-terminal signal peptides containing an SRR×FLK 'twin-arginine' amino-acid sequence motif (reviewed by Berks *et al*, 2003). Preproteins bearing twin-arginine signal peptides are translocated post-translationally across the cytoplasmic membrane by the twin-arginine transport (Tat) system (Berks *et al*, 2003). Biochemical studies have shown the integral membrane proteins TatA, TatB, and TatC to form the core components of the *E. coli* Tat system. The TatBC unit is believed to form the twin-arginine signal peptide recognition module (Alami *et al*, 2003), while TatA forms a very large oligomeric ring structure presumed to be the protein-conducting channel itself (Berks *et al*, 2003). Most remarkably, substrates of the Tat translocase are required to be fully folded before successful translocation can occur (e.g. DeLisa *et al*, 2003). Key Tat-dependent components of the respiratory chain, (NiFe) hydrogenase and trimethylamine *N*-oxide (TMAO) reductase, have been shown to acquire their redox cofactors, and even oligomerise, prior to the transport event (Santini *et al*, 1998; Rodrigue *et al*, 1999). Recently, however, a number of cofactor-less Tat substrates have been uncovered in *E. coli* (e.g. Bernhardt and de Boer, 2003), implicating the Tat pathway as a more general export route specifically evolved for folded substrates.

Tat transport is the ultimate point-of-no-return in periplasmic enzyme biosynthesis. It is important that export of cofactor-containing, and in many cases multimeric, Tat-substrate proteins is not performed or attempted until all assembly processes are complete. Mechanisms are likely to exist, therefore, to coordinate the cofactor-insertion and export processes, to prevent wasteful export of immature substrates, or to curb competition between immature and mature proteins for the transporter. Such 'proofreading' of Tat substrate protein 'maturity' may operate at different levels on the export pathway. There is good evidence that the Tat translocase cannot transport unfolded proteins and this has been interpreted as a 'sensing' of substrate folded state by the Tat translocase itself (DeLisa *et al*, 2003). We demonstrate here, however, that transport of complex Tat substrates can be regulated at an earlier stage on the Tat pathway by dedicated cytoplasmic chaperones that recognise specific twin-arginine signal peptides. Swapping of twin-arginine signal peptides between the unrelated hydrogenase-2 and the TMAO reductase (TorA) systems established the first practicable assay for chaperone-mediated proofreading activity, and we provide evidence that TorD is a twin-arginine signal peptide-binding protein.

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Results and discussion

A specific chaperone and the native signal peptide are required for hydrogenase-2 assembly

E. coli (NiFe) hydrogenase-2 is encoded by the *hybOABCDEFG* operon and the core enzyme consists of a membrane-bound heterodimer of an Fe–S cluster-binding subunit (HybO),

together with a partner subunit that binds the Ni–Fe active site cofactor (HybC). The HybOC dimer is targeted to the Tat translocon as a pre-formed unit by a twin-arginine signal peptide located on only one subunit (HybO) (Rodrigue *et al*, 1999). The HybO Tat signal peptide is typical of all twin-arginine signals in that it contains a polar 'n-region' preceding the highly conserved twin-arginine motif, which itself is followed by a relatively hydrophobic 'h-region' and punctuated by a more polar (often positively charged) 'c-region' that contains the signal peptidase-I AxA cleavage site. Following successful transport, the HybO Tat signal is cleaved off and plays no other role in enzyme structure or function. HybC is also proteolytically processed during the assembly process, but at the C-terminus prior to transport and immediately following the cofactor-loading event (reviewed by Vignais and Colbeau, 2004). HybC itself contains no obvious targeting signals. Following Tat transport, the HybOC catalytic dimer is anchored at the periplasmic side of the cytoplasmic membrane by a single transmembrane segment located at the C-terminus of HybO (Hatzixanthis *et al*, 2003).

Biosynthesis of the core HybOC dimer requires careful coordination of cofactor loading and subunit recruitment before protein transport can proceed. It is crucial that the signal-bearing HybO subunit is not exported before Fe–S cluster insertion is complete or the HybC partner subunit has docked. A recent two-hybrid study implicated the HybE accessory protein as a key player in this 'proofreading' or 'quality control' process (Dubini and Sargent, 2003).

Hydrogenase-2 activity can be specifically measured in whole cells using the redox-dye benzyl viologen (BV) as an artificial electron acceptor (Figure 1A): activity is abolished in a strain lacking only the hydrogenase-2 Tat-dependent subunit HybO (Figure 1A). A Δ*hybE* strain (FTD673) demonstrates very low hydrogenase-2 activity (Figure 1A). Western analysis revealed that HybO and HybC were synthesised at normal levels in the Δ*hybE* background (Figure 1B); however, while the HybO subunit could be visualised in the membrane fraction (Figure 1B), HybC was no longer tightly associated and was instead located in the cytoplasm (Figure 1B). Targeting of HybO in the Δ*hybE* strain remained Tat-dependent since co-inactivation of the Tat translocase resulted in the loss of the HybO antigen from the membrane fraction (Figure 1B) and indeed completely destabilised the HybO protein. Note that HybC was C-terminally processed in the Δ*hybE* strain, indicating that the nickel cofactor had been incorporated (Figure 1C).

These data point to defects in proofreading activity on the hydrogenase-2 assembly pathway rather than protein targeting *per se*. The normally tight control of assembly and transport has apparently been lost, resulting in premature Tat-dependent targeting of HybO before HybC attachment. The most obvious route to preventing protein export while enabling cofactor insertion and subunit recruitment to proceed would be to mask the HybO signal peptide itself. Taken together with our previous two-hybrid study in which HybE was shown to recognise specifically the signal-bearing HybO precursor (Dubini and Sargent, 2003), these data suggest HybE has a role in coordinating the assembly and export of hydrogenase-2.

In order to investigate further the inter-relationships between *E. coli* twin-arginine signal peptides and their cognate passenger proteins, we designed a fusion protein. An *E. coli*

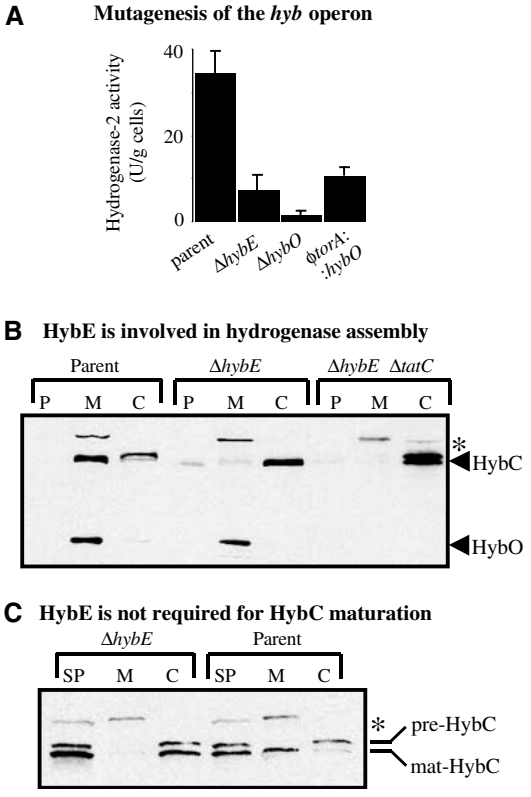

Figure 1 HybE is required for hydrogenase-2 assembly and activity. (**A**) Hydrogenase-2 activities of mutant strains. Strains MC4100 ('parent'), FTD673 ('Δ*hybE*'), RJ608 ('Δ*hybO*'), and RJ603 ('ϕ*torA*::*hybO*'), which produce a HybO protein bearing the TorA signal peptide, were grown anaerobically in CR medium containing glycerol and fumarate. Washed whole cells were assayed for hydrogen::BV oxidoreductase activity with units as μmol BV reduced/min/g cells. (**B**) Western blot analysis of the core hydrogenase-2 αβ dimer HybOC. Strains MC4100 ('parent'), FTD673 ('Δ*hybE*'), and RJ503 ('Δ*hybE*, Δ*tatC*') were cultured anaerobically in CR medium supplemented with glycerol and fumarate. Cells were fractionated into periplasm (P), total membranes (M), and cytoplasm (C) proteins, separated by SDS–PAGE (14% w/v acrylamide), blotted, and challenged with an antihydrogenase-2 serum. The location of the hydrogenase-2 61 kDa α-subunit (HybC) and 35 kDa β-subunit (HybO) are indicated. (**C**) Western analysis of the hydrogenase-2 α-subunit HybC. Strains MC4100 ('parent') and FTD673 ('Δ*hybE*') were cultured in CR medium supplemented with glycerol and fumarate. Sphaeroplasts were prepared from whole cells (SP) and further fractionated into membrane (M) and cytoplasm (C). Proteins were separated by SDS–PAGE (10% w/v acrylamide), blotted, and challenged with an antihydrogenase-2 serum. The location of the precursor form of the α-subunit ('pre-HybC') and the C-terminally processed mature form ('mat-HybC') are indicated. The *asterisks* denote a nonspecific immunoreactive band.

strain (RJ603) was constructed in which a signal peptide with a fundamentally different overall primary structure to (NiFe) hydrogenase systems had been swapped for the Tat signal peptide from HybO (Figure 2A). The coding region for the well-characterised *E. coli* TMAO reductase (TorA) twin-arginine signal peptide (Figure 2A) was precisely fused to the *hybO* gene at the native *hyb* locus on the chromosome, thus generating a ϕ*torA*::*hybO* chimera. Substitution of the Tat signal peptide of the dimeric Fe–S/nickel protein HybOC with that of the monomeric molybdoprotein TorA resulted in very low levels of periplasmic hydrogenase-2 activity (Figure 1A). In order to corroborate these findings, we repeated the

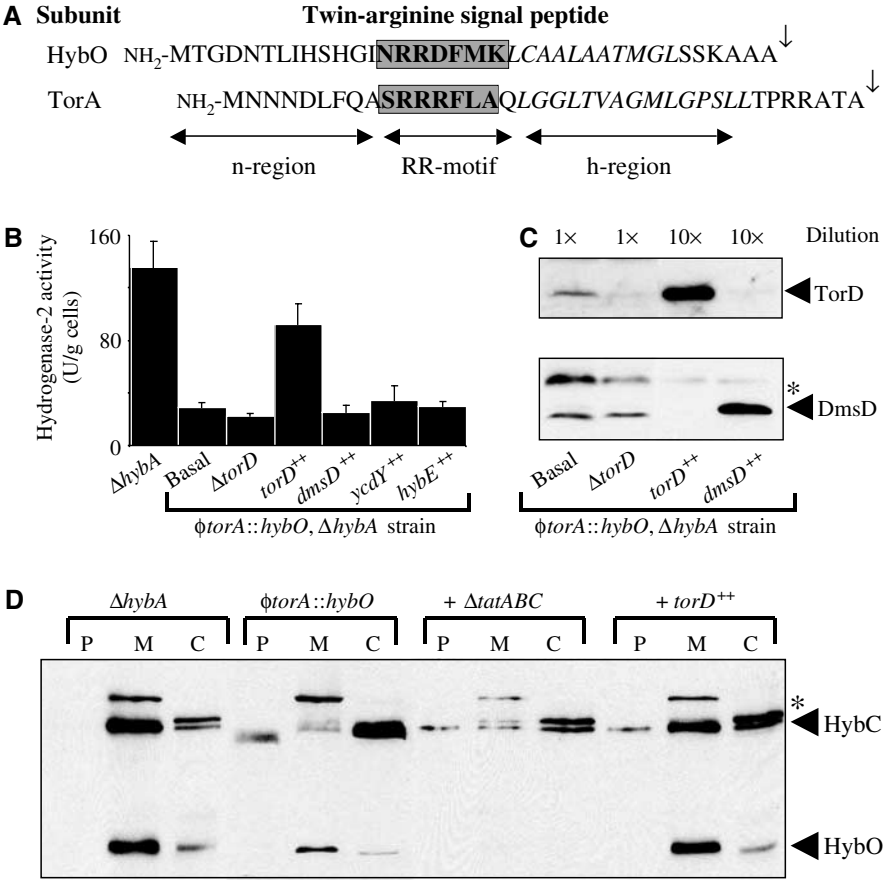

Figure 2 Signal-swapping implicates TorD as a signal peptide chaperone. (**A**) Amino-acid sequences of the twin-arginine signal peptides from the *E. coli* hydrogenase-2 β-subunit (HybO) and the TMAO reductase (TorA). The twin-arginine motifs are boxed, the hydrophobic h-regions are shown in italics, and the signal peptidase-I cleavage sites are indicated by the arrows. (**B**) Hydrogenase-2 activities in mutant strains. Strains RJ606 ('Δ*hybA*'), RJ607 ϕ*torA*::*hybO*, Δ*hybA*, that produces a HybO protein bearing the TorA signal peptide, and RJ607-D ('Δ*torD*') were grown anaerobically in CR medium containing glycerol and fumarate. In addition, RJ607 was transformed with the pSU series of plasmids that constitutively overproduce TorD ('$torD^{++}$'), DmsD ('$dmsD^{++}$'), YcdY ('$ycdY^{++}$'), or HybE ('$hybE^{++}$'), and are grown under identical conditions. Washed whole cells were assayed for hydrogen::BV oxidoreductase activity with units as μmol BV reduced/min/g cells. (**C**) Western analysis of TorD and DmsD. Strains RJ607 (ϕ*torA*::*hybO*, Δ*hybA*), RJ607-D ('Δ*torD*'), and RJ607 transformed with plasmids overexpressing either *torD* ('$torD^{++}$') or *dmsD* ('$dmsD^{++}$') were cultured anaerobically in CR medium supplemented with glycerol and fumarate. Cells were harvested and resuspended to a concentration of 100 mg (wet weight)/ml ('1 ×'), and a sample diluted to 10 mg/ml ('10 ×'). Identical volumes of protein samples were separated by SDS–PAGE (14% w/v acrylamide), blotted, and challenged with either anti-TorD (top panel) or anti-DmsD (bottom panel) serum at 1:10 000 dilution. (**D**) Western analysis of the hydrogenase-2 αβ-dimer HybOC. Strains RJ606 ('Δ*hybA*'), RJ607 ('ϕ*torA*::*hybO*'), RJ607-T (ϕ*torA*::*hybO*, Δ*hybA*, Δ*tatABC*::Kan^R; '+ Δ*tatABC*') and RJ607 following transformation with plasmid pSU-*torD* overexpressing *torD* (labelled '+ $torD^{++}$') were cultured anaerobically in the presence of glycerol and fumarate. Cells were fractionated in periplasm (P), total membranes (M), and cytoplasm (C), proteins separated by SDS–PAGE (14% w/v acrylamide), blotted, and challenged with an anti-hydrogenase-2 serum. The location of the hydrogenase-2 α-subunit (HybC) and β-subunit (HybO) are indicated. The *asterisks* denotes a nonspecific immunoreactive band.

experiment in a *hybA* strain that has enhanced hydrogenase-2-dependent hydrogen::BV oxidoreductase activity (Dubini *et al*, 2002). Again, swapping of the HybO signal peptide for that of TorA in the Δ*hybA* background significantly reduced hydrogenase-2 activity (Figure 2B). Western analysis of the ϕ*torA*::*hybO*, Δ*hybA* strain showed very little HybC present in the membrane, while its HybO partner was targeted correctly in a Tat-dependent manner (Figure 2D).

TorD operates in tandem with the TorA twin-arginine signal peptide

Clearly, native Tat signal peptides have more far-reaching roles than simply guiding their cognate passenger proteins to the Tat translocon. One possibility is that swapping of the hydrogenase-2 signal peptide has the knock-on effect of also removing any specific signal-binding chaperones, for example, HybE, from the biosynthetic process. Indeed, while the loss of HybE has a slightly more marked effect on hydrogenase activity than the signal swap (Figure 1), probably because this protein may contact HybC and other biosynthetic factors as well as HybO during hydrogenase assembly (Dubini and Sargent, 2003), it is notable that the Δ*hybE* mutant and the ϕ*torA*::*hybO* phenotypes are broadly similar. In order to explore this hypothesis further, we considered the possibility that TorA might also employ a signal-binding specific chaperone during its biosynthesis.

In *E. coli*, TorA is encoded by the *torCAD* operon, where TorC is a membrane-bound quinol dehydrogenase and *torD* encodes a cytoplasmic protein (Méjean *et al*, 1994). In the current literature, TorD has been characterised as a 'cofactor chaperone' required for efficient molybdopterin guanine dinucleotide (MGD) cofactor insertion into TorA (Pommier

et al, 1998; Ilbert *et al*, 2003, 2004). A crystal structure for a TorD homodimer from *Shewanella massilia* has recently been described (Tranier *et al*, 2003), although TorD may also exist as a 22.5 kDa monomer in solution (Tranier *et al*, 2002). It is clear that TorD makes extensive protein–protein contacts with TorA during the cofactor-loading process (Pommier *et al*, 1998); however, details of recognition site(s), stoichiometry of binding, or energetic requirements remain ambiguous. Sequence analysis highlighted that TorD is related to an uncharacterised *E. coli* protein, YcdY, and intriguingly, to the only twin-arginine signal peptide-binding protein described to date, DmsD (Oresnik *et al*, 2001; Ilbert *et al*, 2004).

In order to investigate the action of TorD on our ϕTorA::HybOC chimera, we introduced a multi-copy plasmid constitutively expressing *torD* into our ϕ*torA*::*hybO*, Δ*hybA* (RJ607) strain (Figure 2B). Very interestingly, the low hydrogenase activity phenotype of RJ607 can be significantly rescued by introduction of our pSU-*torD* plasmid overproducing TorD (Figure 2B). Furthermore, co-targeting of HybO and HybC was also shown to be restored by co-expression of *torD* in the ϕ*torA*::*hybO* strain (Figure 2D). Western analysis suggests that introduction of our pSU-*torD* plasmid to fumarate-grown cells increases the cellular levels of TorD ~90 times (Figure 2C). This induction of TorD synthesis suppresses the assembly defects caused by attachment of the TorA signal peptide to hydrogenase-2. The most plausible explanation for these data is that TorD is forced to recognise our engineered ϕTorA::HybO chimera and that this event mimics the native hydrogenase-2 proofreading event so closely that assembly is rescued. The basal level of hydrogenase-2 activity in the RJ607 strain (ϕ*torA*::*hybO*, Δ*hybA*) is TorD independent, since deletion of the *torD* gene in this background had no further deleterious effects (Figure 2B).

The restoration of hydrogenase assembly in this system is entirely specific to the TorD protein. Overproduction of the TorD homologs DmsD (confirmed by Western blot in Figure 2C) or YcdY, or the hydrogenase chaperone HybE, in our ϕ*torA*::*hybO*, Δ*hybA* background, had no significant effect on hydrogenase activity (Figure 2B). These data are consistent with a proofreading process in which a specific signal-recognising protein regulates export of a Tat substrate until assembly is complete. Since (NiFe) hydrogenase-2 is not at all related to any known molybdoproteins, these data must point very strongly to a previously unidentified role in TorA signal peptide recognition for the TorD protein. This hydrogenase-based assay cannot be biased by the molybdenum cofactor-insertion activity of the TorD protein (e.g. Ilbert *et al*, 2003). Thus, we have successfully uncoupled a previously submerged Tat proofreading activity from the cofactor-insertion activity, and our assay can be exploited to study the molecular mechanism of proofreading performed by TorD.

Further evidence that TorD binds the TorA signal peptide

In an independent but complementary set of experiments, we took a further genetic approach to assess the physiological role of TorD. Protein–protein interactions can be detected using a bacterial two-hybrid system devised by Karimova *et al* (1998) based on reconstitution of *Bordetella pertussis* adenylate cyclase in an *E. coli cya* mutant. We employed this method to screen a random *E. coli* genomic library for gene products that could interact with TorD.

TorD was fused to the C-terminus of *B. pertussis* adenyate cyclase T25 fragment (pT25-TorD) and this fusion was used as 'bait' in a blind screen of a random library expressing fusions to the N-terminus of *B. pertussis* adenyate cyclase T18 fragment. We performed six independent rounds of screening and isolated 15 colonies expressing possible interacting partners for TorD. In all, 5/15 isolates harboured identical 1007 bp fragments covering codon 147 of *torC* through codon 92 of *torA* (*clone 1*; Figure 3A). A further 8/15 isolates harboured identical 264 bp fragments covering codon 5–92 of the *torA* gene (*clone 2*; Figure 3A). The remaining 2/15 isolates proved to be 'false positives' containing out-of-frame fusions to small fragments of chromosome.

It is striking that, using TorD as bait, 13/15 possible interacting clones expressed in-frame fusions of the TorA N-terminus, including twin-arginine signal peptide, to the T18 fragment. *Clone 1* produces a T18 fusion to the intact TorA signal peptide plus 53 amino acids of the mature protein. *Clone 2* produces a chimeric T18 protein in which 16 residues derived from the pUT18 vector are fused to the TorA signal (though the first four residues of the n-region are missing) and the identical 53 amino acids from the mature TorA polypeptide (Figure 3A). Subsequent β-galactosidase measurements (reconstitution of adenylate cyclase induces transcription of the *lac* operon in the reporter strain) confirmed that TorD interacts with both fusions (Figure 3B). However, given the possibility that fusion protein expression levels may differ between *clones 1* and *2*, and so influence the β-galactosidase activities measured, we decided to deliberately construct N-terminal T18 fusions to TorA using a strategy that would equalise transcription and translation levels as much as possible.

The intact TorA signal peptide, the TorA signal peptide minus the entire n-region, and the region of mature TorA identified in the original screen, were fused separately to the T18 fragment (Figure 3A). The ability of each fusion to be recognised by TorD expressed from pT25-TorD was assessed (Figure 3C). Significant β-galactosidase activity, indicative of strong *in vivo* protein–protein interaction, was measured only when the clone expressing the full-length TorA signal peptide was co-expressed with the TorD fusion (Figure 3C). No interactions between TorD and either a signal peptide lacking the n-region (Figure 3A and C), or the region of TorA mature protein identified by the random screen (Figure 3A and C), could be detected by this system. These data demonstrate unequivocally that TorD binds directly to the TorA twin-arginine signal peptide. Moreover, this assay suggests that recognition of the TorA signal peptide by TorD is dependent on a completely intact signal n-region. Note that the signal peptide twin-arginine motif itself is so highly conserved between all Tat substrates that this is unlikely to be the principle feature that a TorA-specific chaperone like TorD would recognise. The TorA signal n-region is unique to TorA and may enable TorD to specifically recognise this signal above all others.

Taken together with our studies of the ϕTorA::HybOC fusion protein, this work points directly to TorD as a TorA signal peptide-binding protein that operates to harmonise assembly and export of Tat-dependent proteins.

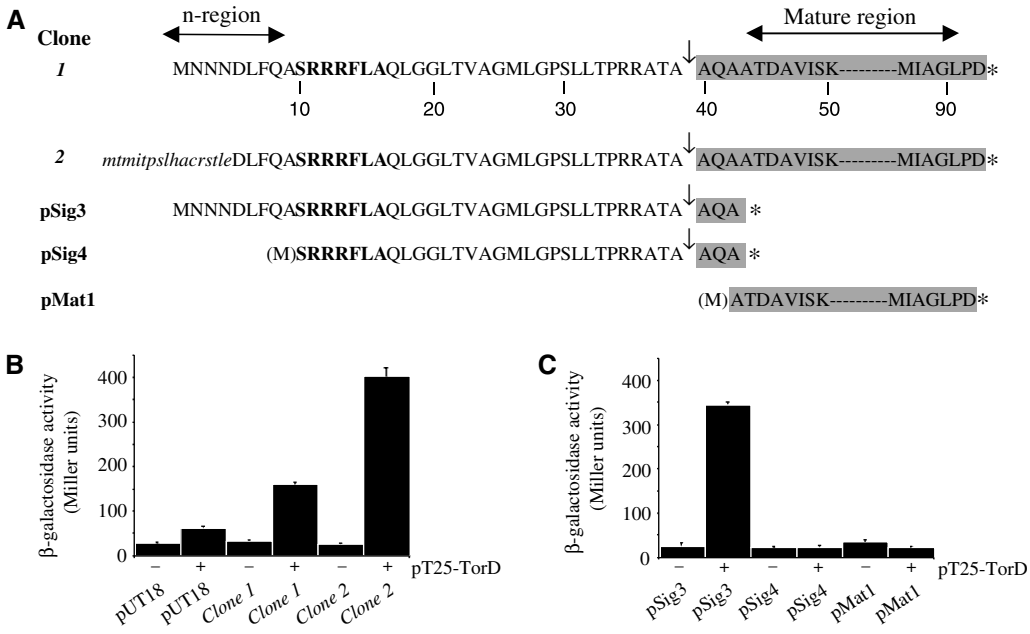

Figure 3 TorD interacts with the TorA signal peptide. (**A**) Amino-acid sequences of N-terminal TorA fusions to the adenylate cyclase T18 fragment. All TorA-specific residues are shown in upper case and the twin-arginine motif is shown in upper bold case. Residues are numbered from the extreme N-terminus of *clone 1*. For the *clone 2* sequence, residues derived from the *lacZ*′ gene on the vector are shown in lower case italics. Signal peptidase cleavage sites are indicated by the arrows. Fusion junctions with the T18 fragment are indicated by the asterisks. Sequences corresponding to the mature TorA protein (signal peptide cleaved) are shaded. (**B**) TorD interacts with the N-terminus of TorA. Interactions were measured between pT25 plasmid ('−') or pT25-TorD expressing the TorD fusion ('+') and either pUT18 or the library isolates *clone 1* and *clone 2* in the reporter strain BTH101. (**C**) TorD recognises the TorA signal peptide. Interactions were measured between pT25 plasmid ('−') or pT25-TorD expressing the TorD fusion ('+') and the engineered fusions to the complete signal peptide, *pSig3*, the signal peptide lacking n-region, *pSig4*, and 49 residues of the mature TorA protein, *pMat1*, in the reporter strain BTH101.

Role of TorD-family proteins in TMAO reductase activity

The twin-arginine signal peptide-binding function of TorD compelled us to re-visit the role of TorD in TorA assembly. Western analysis shows that TorD is a cytoplasmic protein (Figure 4A) and is therefore not part of the periplasmic TMAO reductase system *per se*. We next constructed single in-frame deletion mutants in the *torD* and *dmsD* genes, a *torD/dmsD* double deletion strain, and a *torD/dmsD/ycdY* triple deletion mutant. Only strains carrying *torD* deletions showed any marked effect on periplasmic TMAO reductase activity (Figure 4B), and only the addition of a plasmid expressing TorD was able to rescue the mutant phenotype of the *torD/dmsD/ycdY* strain (not shown). Our data therefore agree precisely with those of others (Pommier *et al*, 1998; Ilbert *et al*, 2004). The loss of TorD causes a significant decrease, but does not abolish, in TorA activity and does not impair targeting of the enzyme to the periplasm. Interestingly, all TorA activity in *torD* mutants is located in the periplasm (Ilbert *et al*, 2004), a phenotype unlike that observed for defects in MGD insertion into other enzymes (e.g. Blasco *et al*, 1998), or Tat transport (e.g. Sargent *et al*, 1998).

Evidence for a second TorD-binding site on the TorA precursor

In order to test the role of the native twin-arginine signal peptide in TorA enzyme assembly, we next swapped exactly the TorA signal peptide for that of the (NiFe) hydrogenase-2 subunit HybO. The RJ600 strain expressing the ϕHybO::TorA chimera displayed periplasmic TMAO reductase activity indistinguishable from that of the parent strain (Figure 4C). The TMAO reductase activity stemming from the ϕHybO::TorA enzyme is not being boosted by hydrogenase-specific chaperones since inactivation of either the *hya* operon, encoding the Tat-dependent hydrogenase-1 (strain RJ601; ϕ*hybO*::*torA*, Δ*hya*) or the *hyb* operon (strain RJ602; ϕ*hybO*::*torA*, Δ*hyb*), did not modulate TMAO reductase activity (not shown). Furthermore, deletion of *torD* in the ϕ*hybO*::*torA* strain (yielding strain RJ600-D) resulted in a significant drop in periplasmic TMAO reductase activity—but again of about 50%, precisely that observed for a *torD* deletion in a strain expressing native *torA* (Figure 4C). As the TorA signal peptide is not present in these experiments, the phenotype of the ϕ*hybO*::*torA*, Δ*torD* (RJ600-D) strain indicates that the reduced TMAO reductase activity observed in other *torD* strains (Figure 4) is as a result of inefficient MGD insertion rather than a loss of proofreading activity. Thus, when the native TorA/TorD system is studied, the dominant cofactor-insertion activity eclipses the Tat proofreading activity of TorD. Indeed, previous *in vitro* and *in vivo* studies of MGD insertion into TMAO reductases had already indicated that the signal peptide was not required for this process (Temple and Rajagopalan, 2000; Ilbert *et al*, 2004). Given that TorA is a single-subunit enzyme binding a single cofactor, and that transport should be allowed to proceed as soon as cofactor loading is complete, it is perhaps understandable that in the course of evolution the cofactor-insertion role of TorD has taken precedence over the Tat proofreading role. In multi-subunit and multi-cofactor systems like hydrogenase, however, the requirement for an overriding Tat proofreading system remains strong. This again highlights the importance of our unique TorA::Hydrogenase-2 fusion experiments, which allow the twin-arginine signal

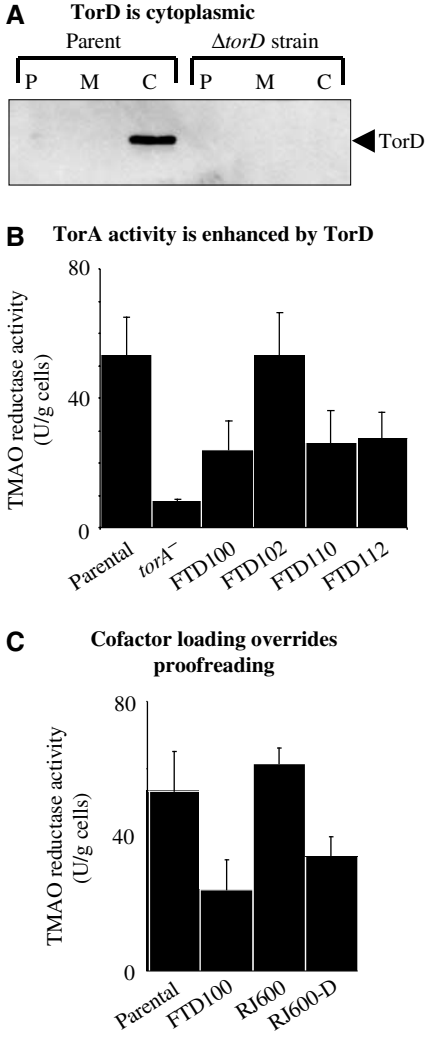

Figure 4 TorD is a cytoplasmic protein with a complex role in TorA assembly. (**A**) Western analysis of TorD. Strains MC4100 ('parent') and FTD100 ('Δ*torD*') were cultured anaerobically in CR medium with glycerol and TMAO. Cells were fractionated into periplasm (P), total membranes (M), and cytoplasm (C), proteins separated by SDS–PAGE (14% w/v acrylamide), blotted, and challenged with anti-TorD serum. (**B**) TMAO reductase activity. Strains MC4100 ('parent'), LCB628 (*torA*$^{-}$), FTD100 (Δ*torD*), FTD102 (Δ*dmsD*), FTD110 (Δ*torD*, Δ*dmsD*), and FTD112 (Δ*torD*, Δ*dmsD*, Δ*ycdY*) were grown anaerobically in CR medium containing glycerol and TMAO. Washed intact cells were assayed for TMAO::BV oxidoreductase activity with units as μmol BV reduced/min/g cells. (**C**) TMAO reductase activity. Strains MC4100 ('parent'), FTD100 (Δ*torD*), RJ600 (ϕ*hybO*::*torA*) that produces a TorA protein bearing the HybO signal peptide, and RJ600-D (ϕ*hybO*::*torA*, Δ*torD*::Kan^{R}) were grown anaerobically in CR medium containing glycerol and TMAO. Whole cells were assayed.

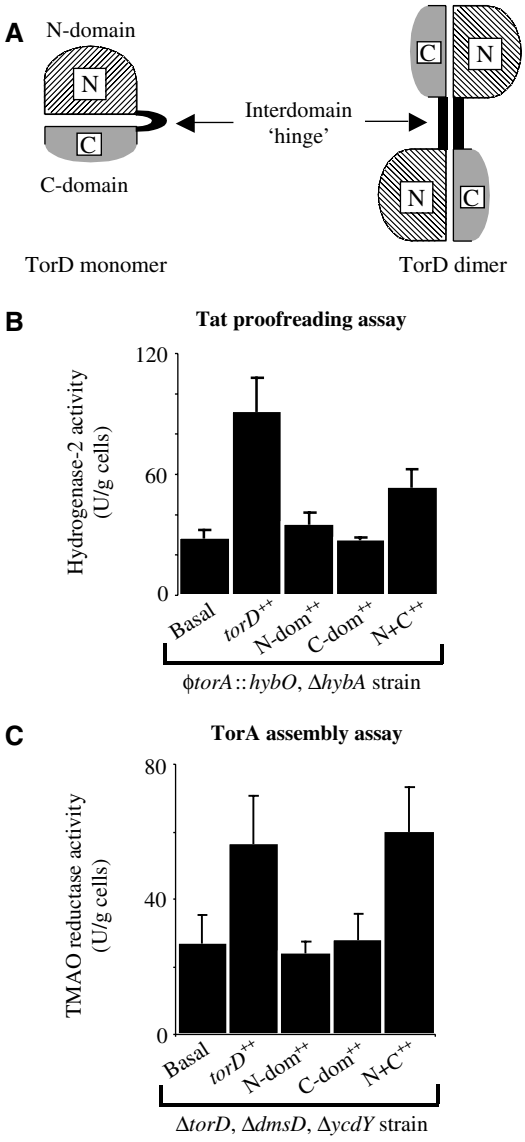

Figure 5 Dissection of TorD activity. (**A**) Cartoon representation of the predicted structures of TorD-family proteins based on the crystal structure of a TorD dimer from *S. massilia*. An N-terminal domain (hatched) is separated from a C-terminal domain (shaded) by a short 'hinge' region. 'Domain swapping' results in the formation of the TorD homodimer. (**B**) Hydrogenase-2 activity. Strain RJ607 (ϕ*torA*::*hybO*, Δ*hybA*) was grown anaerobically in CR medium containing glycerol and fumarate. In addition, the RJ607 strain was transformed with plasmids that constitutively overproduce TorD from pSU-*torD* ('*torD*$^{++}$'), the TorD N-terminal domain from pUNI-NDOM ('N-dom^{++}'), the TorD C-terminal domain from pSU-CDOM ('C-dom^{++}'), or both separated domains together from plasmids pUNI-NDOM and pSU-CDOM ('N + C^{++}'), and hydrogenase-2 activity assayed in intact cells. (**C**) TMAO reductase activity. Strain FTD112 (Δ*torD*, Δ*dmsD*, Δ*ycdY*) was grown anaerobically in CR medium containing glycerol and TMAO. In addition, the FTD112 strain was transformed with plasmids that constitutively overproduce TorD from pSU-*torD* ('*torD*$^{++}$'), the TorD N-terminal domain from pUNI-NDOM ('N-dom^{++}'), the TorD C-terminal domain from pSU-CDOM ('C-dom^{++}'), or both separated domains together from plasmids pUNI-NDOM and pSU-CDOM ('N + C^{++}'), and TMAO reductase activity assayed in intact cells.

peptide-binding proofreading activity of TorD to be completely uncoupled from the overlapping MGD cofactor-loading events. Our data taken together with that of others point towards the existence of at least two TorD-binding sites on the TorA protein: one within the twin-arginine signal peptide and a second within the 'mature' portion of the protein.

Molecular dissection of TorD activity

We next considered how it was possible that TorD could recognise two different regions of the TorA polypeptide and participate in two different assembly processes. Structural studies of a TorD-family protein have revealed that each protomer consists of two distinct domains (an N-terminal domain and a C-terminal domain) linked by a short

conserved 'hinge' region (Tranier *et al*, 2003). The TorD protein from *S. massilia* can be purified as mixtures of stable monomer and dimer (Tranier *et al*, 2002). Dimerisation is driven by 'domain swapping' (Tranier *et al*, 2003) in which the N domain from one protomer interacts with the C domain of the second protomer (Figure 5A). It is not yet clear which forms exist *in vivo*, or what the functional significance of each form might be. We therefore considered that each domain may play a separate role in TorA assembly.

We cloned DNA encoding the TorD N domain (from Methionine-1 through Histidine-125) and C domain (from Methionine-113 through Arginine-199) into our vectors. When expressed separately, neither domain could rescue the mutant phenotypes of either the *torD/dmsD/ycdY* triple mutant (FTD112) or the ϕ*torA*::*hybO*, Δ*hybA* strain (RJ607) (Figure 5C and B, respectively). However, when the separate TorD domains were co-expressed together in the *torD/dmsD/ycdY* mutant, periplasmic TMAO reductase activity could be restored close to the levels observed in the parent strain (Figure 5C). This clearly indicates that each separate domain is functional and need not be covalently attached in order to enhance the assembly of native TorA. In the case of our hydrogenase-based Tat proofreading assay, co-expression of the separate TorD domains did not significantly rescue the defect in hydrogenase-2 assembly (Figure 5B). Thus, separated TorD domains are essentially incapable of performing a Tat proofreading function (Figure 5B).

We pursued this line of investigation further with a programme of site-directed mutagenesis. Analysis of TorD-family proteins highlighted four conserved polar residues: Serine-95 in the N-terminal domain; Aspartate-124, and Histidine-125 in the interdomain 'hinge'; and Glutamate-142 in the C-terminal domain (Tranier *et al*, 2003—*E. coli* numbering applied), and we separately substituted each with alanine. Western immunoblotting established that the variant TorD proteins were stable and produced at levels equivalent to native TorD in the same system (Figure 6A).

In order to study only the MGD cofactor-insertion activities of the mutant proteins without interference from the Tat proofreading activity, we utilised our ϕ*hybO*::*torA*, Δ*torD* (RJ600-D) strain that expresses TorA with the HybO signal peptide. Complementation analysis of the ϕ*hybO*::*torA*, Δ*torD* (RJ600-D) strain with our TorD S95A, D124A, H125A, and E142A variants revealed that the residues located in the inter-domain hinge (D124 and H125) are not required for efficient cofactor insertion into TorA (Figure 6C). However, N-domain residue S95 and C-domain residue E142 are required to catalyse the enhancement of cofactor insertion (Figure 6C). These data corroborate our experiments with separated TorD domains (Figure 5) and confirm that residues located in both N and C domains are involved in the cofactor-insertion process.

We next employed our hydrogenase-based proofreading assay and introduced the variant TorD proteins to our ϕ*torA*::*hybO*, Δ*hybA* (RJ607) strain. In this case, TorD

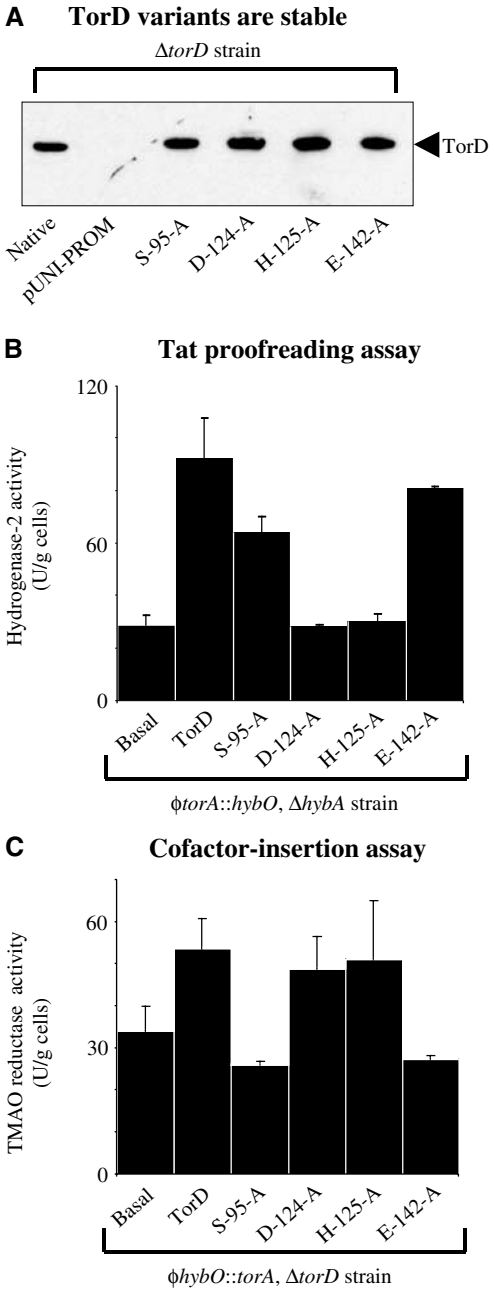

Figure 6 Site-directed mutagenesis of the TorD protein. (**A**) Expression, production, and relative stability of mutant TorD proteins. The FTD100 strain (Δ*torD*) was transformed with the original cloning vector pUNI-PROM (Amp^R), pUNI-*torD* ('native'), and the four pUNI-*torD* derivatives expressing the mutant *torD* genes (as indicated). Cultures were grown anaerobically in CR medium supplemented with glycerol and TMAO. Cell pellets were harvested, washed, and resuspended to 100 mg (wet weight)/ml. Whole-cell proteins were separated by SDS–PAGE (14% w/v acrylamide), blotted, and challenged with anti-TorD. Identical proportions of cellular protein were loaded in each lane. (**B**) Hydrogenase-2 activity. Strain RJ607 (ϕ*torA*::*hybO*, Δ*hybA*) was grown anaerobically in CR medium containing glycerol and fumarate. The RJ607 strain was transformed with plasmids that constitutively overproduce TorD from pUNI-*torD* ('$torD^{++}$') or the four pUNI-*torD* derivatives expressing the mutant *torD* genes (as indicated). Whole cells were assayed for hydrogen::BV oxidoreductase activity with units as μmol BV reduced/min/g cells. (**C**) TMAO reductase activity. Strain RJ600-D (ϕ*hybO*::*torA*, Δ*torD*::Kan^R) was grown anaerobically in CR medium containing glycerol and TMAO. The RJ600-D strain was transformed with plasmids that constitutively overproduce TorD from pUNI-*torD* ('$torD^{++}$') or the four pUNI-*torD* derivatives producing TorD variants (as indicated). Whole cells were assayed for TMAO::BV oxidoreductase activity with units as μmol BV reduced/min/g cells.

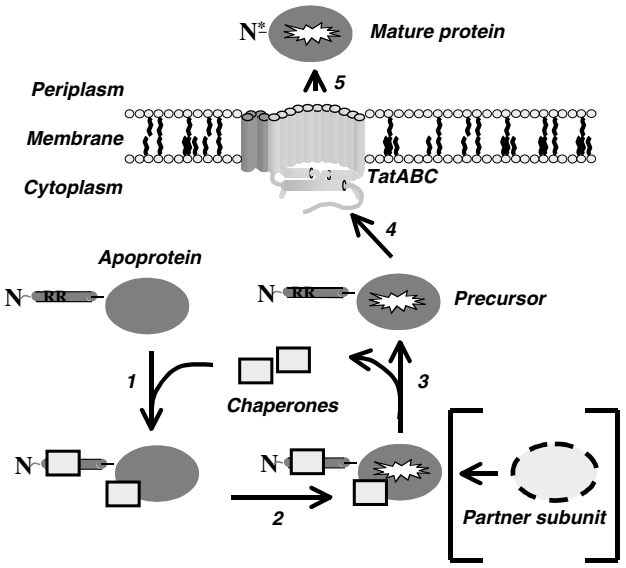

Figure 7 A model for chaperone-mediated proofreading. An apoprotein bearing a twin-arginine signal peptide is released from the ribosome. Step 1: In order to prevent premature export, a proofreading chaperone binds to the twin-arginine signal peptide. In some cases, specific or general chaperones also bind to the mature portion of the protein at this stage. Step 2: Cofactor loading into the mature portion of the protein proceeds. At this stage, binding of partner subunits would also occur, if applicable. Step 3: All chaperones are released. Step 4: Targeting of the precursor to the Tat translocon proceeds. Step 5: Following Tat transport, the signal peptide is cleaved ('N*') and the mature protein is released to the periplasm. TatABC model is from Palmer *et al* (2004).

variants D124A and H125A were found to be completely inactive in our proofreading assay (Figure 6B), demonstrating that the hinge region is critical either for TorA signal recognition *per se* or for a downstream biochemical process normally triggered by signal recognition. Interestingly, the domain-located residues S95 and E142 essential for cofactor loading into TorA (Figure 6C) are dispensable in the TorD-dependent rescue of proofreading activity (Figure 6B). Thus, amino-acid requirements for Tat proofreading by TorD are exactly the opposite to those required for cofactor insertion.

We have highlighted key differences in the biochemical requirements for TorD-dependent Tat proofreading and TorD-dependent cofactor insertion. Signal recognition/proofreading clearly requires an intact TorD protein that would undoubtedly result in placement of the hinge residues (D124 and H125) in the correct orientation to fulfil their function, while domain-located side chains are important in the TorD-dependent cofactor-insertion process. TorD-family proteins can be isolated both in monomeric and dimeric forms, and it remains a possibility that one isoform is predominantly involved in proofreading while the other governs cofactor insertion. In the *S. massilia* TorD homodimer, the D124 and H125 hinge-located residues seem to be intimately intertwined in the structure (Tranier *et al*, 2003). The D124 equivalent in *S. massilia* (D134) is hydrogen-bonded to a histidine sidechain in the opposite protomer (Tranier *et al*, 2003), perhaps implicating it in dimer formation. This histidine residue is not conserved in the *E. coli* protein, however. The H125 equivalent in the *S. massilia* TorD (H135) is buried at the dimer interface and may be hydrogen-bonded to Tyr93 (which is conserved in the *E. coli* protein). Again, H125 could be implicated in influencing dimer formation; however, both D124 and H125 are part of an extensively intertwined stretch of nearly 30 consecutive sidechains from each protomer (Tranier *et al*, 2003), and single amino-acid substitutions would therefore not be expected to disrupt this structure. *E. coli* S95 (also S95 in the *S. massilia* TorD structure) probably contributes to a 'polar patch' within the N domain on the surface of the molecule, close to the inter-domain hinge (Tranier *et al*, 2003). E142 (E151 in *S. massilia* TorD) is a surface-exposed sidechain in the C-terminal domain (Tranier *et al*, 2003). Neither S95 nor E142 appear to be intimately involved with TorD dimer formation.

Concluding remarks

In the current work, we have shed light on an aspect of Tat-dependent protein transport that has puzzled researchers from the outset: what prevents premature targeting of cofactor-containing proteins before cofactor loading is complete, and what coordinates export of multi-protein complexes where one protein 'piggy-backs' on another? A system of 'proofreading' or 'quality control' governing these processes has been postulated since the bacterial Tat system was first conceived (e.g. Santini *et al*, 1998), and here we provide the first experimental evidence for the existence of a wide-ranging chaperone-mediated proofreading mechanism. Previous work had demonstrated that some signal peptides were both organism- and enzyme-specific (Sambasivarao *et al*, 2000; Blaudeck *et al*, 2001); however, our work suggests that specialisation can be extended to specific chaperones that operate hand-in-glove with signal peptides harmonising assembly and export processes. A model of how such a system may operate is shown in Figure 7. In *E. coli*, this system is in operation for TorA (with TorD as chaperone) and hydrogenase-2 (with HybE as chaperone). Biochemical, genetic, and sequence analyses suggest other cofactor-containing and multi-subunit enzymes could utilise similar systems during biosynthesis (Oresnik *et al*, 2001; Gross and Simon, 2003). Indeed, this work describes an assay that could, in principle, be used to identify signal peptide/chaperone pairs from any system.

Chaperone-mediated proofreading is an alternative concept of 'quality control' as previously documented by DeLisa *et al* (2003). DeLisa *et al* (2003) demonstrated by utilising alkaline phosphatase fusions that only folded proteins could be transported by the Tat translocase, and postulated that the Tat machinery itself performed a final quality control check before transport (essentially at *step 4* in Figure 7). The system of chaperone-mediated proofreading described here would be in operation at a much earlier stage on the Tat pathway, preventing premature targeting and ensuring that only fully assembled substrates are presented to the Tat translocase. Such a system would greatly increase the efficiency of Tat transport by decreasing the incidence of abortive engagements, and reducing competition, with substrates that are not ready for export.

We have shown that the *E. coli* TorA protein employs a proofreading system involving its cognate signal peptide and a specialised chaperone termed TorD. Most remarkably, the TorA signal peptide/TorD system can be isolated and transposed onto a completely unrelated enzyme, hydrogenase, where it continues to operate almost unabated.

Table I List of *E. coli* strains featured in this study

Strain	Relevant genotype	Source
MC4100	F-, Δ*lacU169*, *araD139*, *rpsL150*, *relA1*, *ptsF*, *rbs*, *flbB5301*	Lab Stocks
RJ606	Δ*hybA*	This work
RJ608	Δ*hybO*	This work
FTD673	Δ*hybE*	This work
RJ503	Δ*hybE*, Δ*tatC*::Spec^R	This work
RJ603	ϕ*torA*::*hybO* (produces TorA signal peptide on HybO mature protein)	This work
RJ607	ϕ*torA*::*hybO*, Δ*hybA*	This work
RJ607-D	ϕ*torA*::*hybO*, Δ*hybA*, Δ*torD*::Kan^R	This work
RJ607-T	ϕ*torA*::*hybO*, Δ*hybA*, Δ*tatABC*::Kan^R	This work
FTD100	Δ*torD*	This work
FTD101	Δ*torD*::Kan^R	This work
FTD102	Δ*dmsD*	This work
FTD110	Δ*torD*, Δ*dmsD*	This work
FTD112	Δ*torD*, Δ*dmsD*, Δ*ycdY*	This work
LCB628	*torC*::Ω Spec^R	V Méjean
RJ600	ϕ*hybO*::*torA* (produces HybO signal peptide on TorA mature protein)	This work
RJ600-D	ϕ*hybO*::*torA*, Δ*torD*::Kan^R	This work
RJ601	ϕ*hybO*::*torA*, Δ*hya*::Kan^R	This work
RJ602	ϕ*hybO*::*torA*, Δ*hyb*::Kan^R	This work
BTH101	F-, *cya99*, *araD139*, *galE15*, *galK16*, *rpsL1*(Str^R), *hsdR2*, *mcrA1*, *mcrB1*	D Ladant

Hydrogenases are not at all related to TMAO reductases: the primary structures of the enzymes are unrelated; the subunit composition is dissimilar; the cofactors used are different; and the accessory proteins are not homologous. The fact that the TorA/TorD proofreading system 'works' for *E. coli* HybOC suggests that the native hydrogenase-2 system is functionally very similar. This perhaps points to a converging of functionality between unrelated systems rather than a common evolutionary origin. The exchangeability of the TorA/TorD system with the hydrogenase system not only raises questions about the exquisite specificity of recognition (step 1 in Figure 7) but also about what triggers release of the chaperone following assembly (step 3 in Figure 7). It is possible that bound chaperones are simply dislodged by the folding substrate protein, though it should be noted that most chaperone–protein interactions are much more tightly controlled (e.g. Slepenkov and Witt, 2002). This work establishes the existence, and lays the foundation for detailed molecular analysis, of a chaperone-mediated proofreading system operating on the bacterial Tat pathway.

Materials and methods

Bacterial strains and growth conditions

Strains used are based on MC4100 (Table I). Most unmarked deletion mutant strains were constructed by cloning the mutant alleles into pMAK705 (Cm^R) as previously described (Sargent *et al*, 1998) and transferring onto the chromosome by the method of Hamilton *et al* (1989). In short, deletion alleles carry 500–600 bp of DNA upstream of the target gene, linked to a similar amount of downstream DNA to form a plasmid-borne in-frame deletion preserving translation stop signals and Shine–Dalgarno motifs for adjacent sequences. Marked deletions (e.g. Kan^R) were prepared by cloning resistance cassettes between the upstream and downstream segments of the deletion alleles.

Strain RJ603 (as MC4100, ϕ*torA*::*hybO*), in which DNA encoding the TorA signal peptide *exactly replaced* that encoding the HybO signal peptide within the native *hyb* operon, was constructed by cloning DNA encoding the TorA signal peptide into pBluescript (Stratagene Europe) to yield plasmid pRAT7. Upstream and downstream sequences flanking the native HybO signal-coding region was incorporated into pRAT7, thus generating a plasmid-borne ϕ*torA*::*hybO* construct (pRAT46) which was subcloned into pMAK705 before transfer to the chromosome of MC4100. Strain RJ607 (ϕ*torA*::*hybO*, Δ*hybA*) was constructed by incorporating the Δ*hybA* deletion allele used to construct RJ606 (as MC4100, Δ*hybA*) into RJ603 by the method of Hamilton *et al* (1989). RJ600 (as MC4100 ϕ*hybO*::*torA*), in which DNA encoding the HybO signal peptide *exactly replaced* that encoding the TorA signal peptide at the native *tor* locus, was constructed using identical methodology employed to make RJ603. Strains RJ600-D (ϕ*hybO*::*torA*, Δ*torD*::Kan^R) and RJ607-D (ϕ*torA*::*hybO*, Δ*hybA*Δ*torD*::Kan^R) were generated by P1 transduction (Russell and Sambrook, 2001) of the Δ*torD*::Kan^R allele from FTD101 into RJ600. Strains RJ601 (ϕ*hybO*::*torA*, Δ*hya*::Kan^R) and RJ602 (ϕ*hybO*::*torA*, Δ*hya*::Kan^R) were constructed following P1 transduction of the appropriate alleles from HDJ123 (as MC4100, Δ*hya*::Kan^R, Δ*hyb*::Kan^R, Δ*hyc*::Cm^R; A Böck) into RJ600. The *tat* mutation used to construct RJ607-T (ϕ*torA*::*hybO*, Δ*hybA*, Δ*tatABC*::Kan^R) was made using the method of Datsenko and Wanner (2000).

During all genetic manipulations, cultures were grown in LB medium (Russell and Sambrook, 2001) with the appropriate antibiotics. For hydrogenase-2 assays, cultures were grown anaerobically in CR medium (Sargent *et al*, 1998) containing 0.5% (v/v) glycerol and 0.4% (w/v) fumarate. For TMAO reductase assays, cultures were grown in 0.5% (v/v) glycerol and 0.4% (w/v) TMAO.

Plasmids

Plasmid-expressed chaperone constructs used in this work are driven from the *tat* promoter to give constitutive transcription (Jack *et al*, 2001). They carry the *tatA* Shine–Dalgarno motif engineered close to the *Bam*HI site within the polylinker which, when a *Bam*HI is placed immediately upstream of the target gene AUG by PCR, can be used to align all heterologous start codons exactly with the native *tatA* AUG codon. The constructs were based on two different vectors:

(a) pSU-PROM (Kan^R): An engineered 100 bp PCR fragment covering the *tatA* promoter up to the *tatA* AUG was digested with *Eco*RI (5′ end) and *Bam*HI (3′ end) into vector pSU40 (Kan^R). Plasmids pSU-*hybE*, pSU-*torD*, pSU-*dmsD*, and pSU-*ycdY* (encoding *hybE*, *torD*, *dmsD*, and *ycdY* respectively) were constructed following amplification of the respective PCR fragments, digestion with *Bam*HI–*Xba*I and cloning into pSU-PROM. For site-directed

mutagenesis, the pSU-*torD* plasmid was used as template and the Quickchange (Stratagene) protocol was employed. Four independent mutations were incorporated into *torD*, resulting in pSU-S95A, pSU-D124A, pSU-H125A, and pSU-E142A in which the codons indicated were replaced by alanine codons. DNA encoding the C-terminal domain of TorD (from Methionine-113 through to the native *torD* stop codon) was amplified by PCR and cloned as a *Bam*HI–*Xba*I fragment into pSU-PROM to give pSU-CDOM.

(b) pUNI-PROM (Amp^R) was constructed by cloning the identical 100 bp *Eco*RI–*Bam*HI *tatA* promoter fragment into plasmid vector pT7.5 (Amp^R). The *hybE* gene, the *torD*-family genes, and the four site-specific mutants of *torD* were moved into pUNI-PROM. DNA encoding the N-terminal domain of TorD (from codon Methionine-1 through Histidine-125) and the C-terminal domain of TorD (above) were cloned as *Bam*HI–*Xba*I fragments into pUNI-PROM to give pUNI-NDOM and pUNI-CDOM, respectively.

Bacterial two-hybrid system

Direct protein interactions were detected using the method of Karimova *et al* (1998). The *torD* gene was incorporated downstream of the coding region of the N-terminal T25 fragment of adenylate cyclase on plasmid pT25 (Cm^R) to give pT25-TorD. An *E. coli* genomic library was prepared on plasmid pUT18 (Amp^R) encoding the C-terminal T18 fragment of adenylate cyclase. MC4100 chromosomal DNA was isolated and partially digested with *Sau*3AI. Fragments between 0.2 and 2 kbp were extracted from agarose and ligated into *Bam*HI-digested pUT18. Approximately 40 000 independent transformants were harvested and plasmid DNA stored as the *Sau*3AI library. For library screening, BTH101 was co-transformed with pT25-TorD and the *Sau*3AI library, and plated onto MacConkey-maltose at 30°C. Red colonies (indicating possible interactions) were selected after 48 h, plasmids isolated, and inserts sequenced.

Derivatives of pUT18 engineered to carry DNA fragments encoding the TorA signal peptide (pSig3 and pSig4) and the TorA mature protein (pMat1) were constructed as follows: DNA encoding the complete TorA signal, the TorA signal minus N-region, and the TorA mature protein from codons 43–92 were cloned as *Bam*HI–*Xba*I fragments into pUNI-PROM. Clones were then moved onto the pUT18 vector between the *Hin*dIII and *Eco*RI sites using PCR primers that preserved the optimised ribosome-binding site and placed a stop codon immediately upstream of the fragments of interest to prevent read-through translation from the vector *lacZ* gene. Protein interactions were estimated by measurement of β-galactosidase activity from BTH101 grown to mid-log phase at 30°C as described (Karimova *et al*, 1998).

Protein methods

During genetic manipulations, strains were grown aerobically in Luria–Bertani (LB) medium (Russell and Sambrook, 2001). For biochemical characterisations, strains were cultured anaerobically in Cohen–Rickenberg (CR) medium containing 0.5% (v/v) glycerol and 0.4% (w/v) fumarate or 0.4% (w/v) TMAO (Sargent *et al*, 1998). Subcellular fractionation of cell pellets was performed by the lysozyme/EDTA protocol (Hatzixanthis *et al*, 2003).

Antisera to TorD and DmsD was provided as a service by Sigma-Genosys. Hydrogenase-2 antiserum has been described previously (Sargent *et al*, 1998).

SDS–PAGE and immunoblotting analyses were by Lämmli (1970) and Towbin *et al* (1979). Immunoreactive bands were detected by ECL (Amersham) and intensities were estimated using a densitometer and MacBas version 2.0 software. Hydrogen- and TMAO-BV oxidoreductase activities were measured as described (Sargent *et al*, 1998; Dubini *et al*, 2002; Stanley *et al*, 2002). For intact-cell assays, pellets were washed twice in 20 mM Tris–HCl (pH 7.6) and resuspended at 0.1 g cells (wet weight) ml^{-1}.

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