Signal peptide etiquette during assembly of a complex respiratory enzyme

Martyn J. James, Sarah J. Coulthurst, Tracy Palmer and Frank Sargent*
College of Life Sciences, University of Dundee, Dundee DD1 5EH, Scotland, UK.

Summary

*Salmonella enterica* serovar Typhimurium is a Gram-negative pathogen capable of respiration with a number of terminal electron acceptors. Tetrathionate reductase is important for the infection process and is encoded by the *ttrBCA* operon where TtrA and TtrB are metallocofactor-containing proteins targeted to the periplasmic side of the membrane by two different Tat targeting peptides. In this work, the inter-relationship between these two signal peptides has been explored. Molecular genetics and biochemical approaches reveal that the processing of the TtrB Tat signal peptide is dependent on the successful assembly of its partner protein, TtrA. Inactivation of either the TtrA or the TtrB Tat targeting peptides individually was observed to have limited overall effects on assembly of the enzyme or on cellular tetrathionate reductase activity. However, inactivation of both signal peptides simultaneously was found to completely abolish physiological tetrathionate reductase activity. These data suggest both signals are normally active during assembly of the enzyme, and imply a code of conduct exists between the signal peptides where one can compensate for inactivity in the other. Since it appears likely that tetrathionate reductase presents itself for export as a multi-signal complex, these observations also have implications for the mechanism of the bacterial Tat translocase.

Introduction

*Salmonella* species are some of the most common opportunistic bacterial pathogens associated with food-borne illness in adults living in developed countries (de Bruyn, 2000) and are responsible for about 20–30% of food-related deaths (Mead et al., 1999). *Salmonella enterica* serovar Typhimurium (hereafter referred to as ‘*Salmonella*’) causes gastroenteritis in humans and Typhoid fever in the mouse (McClelland et al., 2001; Thiennimitr et al., 2012). *Salmonella* is a Gram-negative bacterium and a member of the γ-Proteobacteria group and, because of its diverse life cycle that involves time spent in animal hosts, as well as in plant, water and soil environments, *Salmonella* is capable of growing and surviving under a huge range of environmental conditions, including in the complete absence of oxygen (McClelland et al., 2001). To cope with changing environmental conditions, *Salmonella* has the capability to produce many alternative respiratory enzymes (Richardson, 2000).

One interesting feature of *Salmonella* physiology is the ability to use sulphur compounds such as tetrathionate (3OS-S-S-SO3− or S4O62−) and thiosulphate (S−SO3− or S2O32−) as terminal electron acceptors during anaerobic respiration (Hensel et al., 1999; Hinsley and Berks, 2002; Stoffels et al., 2012). Indeed, this activity has historically formed the basis of diagnostic phenotypic tests for *Salmonella* species (King and Metzger, 1968). Recently, however, a new role for tetrathionate respiration in pathogenesis has been unearthed (Winter et al., 2010). Work in a mouse model has identified that the gut inflammation response, induced initially by *Salmonella* itself, causes the generation of tetrathionate in the gut mucosa (Winter et al., 2010). This tetrathionate can then be used by the infecting *Salmonella* as a respiratory electron acceptor in order to boost growth and survival relative to other gut microbiota that lack tetrathionate reductase activity (Winter et al., 2010). Moreover, this tetrathionate-dependent respiratory activity was found to be critical to the success of the *Salmonella* infection process (Winter et al., 2010).

The *Salmonella* tetrathionate reductase is encoded at the *ttrRSBCA* locus (Fig. 1A) located on pathogenicity island 2 (Hensel et al., 1999). The complete tetrathionate reductase complex most likely comprises three subunits where TtrA is the catalytic subunit and is predicted to contain an iron-sulphur cluster, as well as a molybdenum cofactor in the form of bis-molybdopterin guanine dinucleotide (MGD). TtrB is an electron transferring subunit predicted to contain four iron-sulphur clusters, while TtrC most likely acts as a membrane anchoring subunit and quinol dehydrogenase. Such an arrangement would require close interaction between the three gene products following...
assembly of the complex. Genes for similar tetrathionate reductases can be identified in other bacterial pathogens, for example Proteus mirabilis, Bordetella parapertussis and Serratia marcescens, thus utilization of this unusual compound may be a common trait exhibited by opportunistic pathogens.

The subcellular localization of the enzyme is critical to its physiological function since the substrate, tetrathionate, is produced by the animal host outside the bacterial cell and is membrane impermeable. Extracellular tetrathionate is first detected in the environment by the periplasmic domain of a membrane-bound sensor histidine kinase (TtrS) from a classical two-component system. Autophosphorylation of TtrS leads to the subsequent phosphorylation of a DNA-binding response regulator, TtrR, which then activates transcription of ttrBCA (Hensel et al., 1999). Synthesis of the three proteins is followed by cofactor insertion into TtrA and TtrB and then the final, critical, act of positioning these proteins at the periplasmic face of the membrane must occur. As a result, both TtrA and TtrB are predicted to be located at the periplasmic side of the membrane and inspection of the primary sequences corroborates this since both are synthesized with N-terminal Tat targeting peptides (Fig. 1B and C) (Hensel et al., 1999; Hinsley et al., 2001). Tat signal peptides target proteins to the twin-arginine translocation system, which transports folded proteins across the inner membrane (Palmer and Berks, 2012). The Tat system is well known to transport molybdo- and iron-sulphur-enzymes related to Salmonella tetrathionate reductase, including heterodimeric complexes such as the DmsAB subunits of dimethyl sulphoxide reductase and the FdnGH subunits of formate dehydrogenase (Sargent, 2007); however, Salmonella tetrathionate reductase is distinctive for several reasons. First, the TtrB subunit contains a non-canonical, but active, Tat signal peptide (Hinsley et al., 2001). Bacterial Tat signal peptides exhibit a tripartite structure comprising a positively charged ‘N-region’ followed by a hydrophobic ‘h-region’, which is usually followed by a conserved proline residue and a more polar ‘C-region’ containing an AxA signal peptide cleavage site (Palmer and Berks, 2012). At the interface of the N- and h-regions is the (S/T)RRxA motif where the RR dipeptide is almost completely invariant and essential for the transport process (Palmer and Berks, 2012). Unusually, the Salmonella TtrB protein contains a KR dipeptide within its Tat motif (Fig. 1C), which in this case does not compromise the targeting activity of the signal peptide (Hinsley et al., 2001). Furthermore, for Salmonella tetrathionate reductase both periplasmic subunits of the enzyme (TtrA and TtrB) are expected to have active Tat signal peptides, since TtrA bears a canonical twin-arginine motif (Fig. 1B). This is unusual for such Tat-dependent homodimeric complexes since the Tat translocase is capable of transporting heterodimers in which only one of the two subunits bears a signal peptide (e.g. dimethyl sulphoxide reductase, formate dehydrogenase, [NiFe] hydrogenase, and indeed probably both Archaeoglobus fulgidus and S. marcescens tetrathionate reductases where TtrB is signal-less). However, the ‘multi signal’ arrangement remains conserved in other bacteria that encode tetrathionate reductases, for example both TtrA and TtrB from Wolinella succinogenes have canonical

© 2013 The Authors. Molecular Microbiology published by John Wiley & Sons Ltd., Molecular Microbiology, 90, 400–414
N-terminal twin-arginine signal peptides, while the *P. mirabilis*, *B. parapertussis*, as well as some other *Serratia* species, produce TtrB subunits with similar ‘KR’ signal motifs as found in the *Salmonella* TtrB protein.

In this work, *Salmonella* was used as a genetically tractable model system to ascertain the roles of the different Tat signal peptides in tetrathionate reductase assembly and activity. It is demonstrated that both the TtrA and TtrB Tat targeting signals are fully functional, and that the TtrA signal peptide is not processed and so serves as an N-terminal signal anchor. Moreover, the data reported here suggest a system where TtrA and TtrB are not transported separately to the periplasm, but in one where they form a multi-signal heterodimer that is co-transported across the cytoplasmic membrane. Truncation of the TtrA molybdenoprotein, or prevention of its correct assembly by negation of molybdenum cofactor biosynthesis, blocked processing of the signal peptide of its partner protein, TtrB. Inactivation of the TtrB signal peptide alone was found to be not sufficient to impair assembly of the tetrahionate reductase. Likewise, inactivation of the TtrA signal peptide alone was not sufficient to impair assembly either. Instead, both signal peptides must be modified simultaneously in order to abolish physiological tetrahionate reductase activity. A mechanism is proposed where both signal peptides are active for translocation but can either cooperate with, or compensate for, each other as circumstances dictate. It is possible that co-translocation of multi-signal complexes is a common event catalysed by the Tat translocase.

**Results**

**TtrA and TtrB contain active Tat targeting signals**

The *Salmonella* ttrBCA operon is predicted to encode a single heterotrimeric molybdoenzyme complex; however, somewhat unusually for this broad family of respiratory enzymes, both TtrA and TtrB are predicted to bear N-terminal Tat targeting sequences (Fig. 1B and C). The Tat targeting peptide of TtrB is known to be transport-active when used in a Tat translocation assay with a heterologous reporter protein (Hinsley *et al*., 2001), while the Tat transport activity of the isolated TtrA signal sequence has not been reported.

In order to test directly the ability of the TtrA N-terminus to act as an active twin-arginine signal sequence a positive reporter system developed by Ize *et al.* (2009) was utilized. *Escherichia coli* contains two Tat-dependent periplasmic amidases (AmiA and AmiC) and if their transport to the periplasm is blocked, by inactivating either respective signal peptides or the Tat system itself, the result is a serious cell wall defect that renders mutant strains sensitive to the presence of SDS in the growth medium (Ize *et al.*, 2003). This growth phenotype on SDS-containing media can be rescued by expressing fusion proteins between the AmiA mature domain and heterologous Tat signal sequences in trans (Ize *et al.*, 2009).

First, constructs encoding AmiA fusions to the predicted Tat targeting signals of *Salmonella* TtrA and TtrB were prepared. Expression of both constructs in an *E. coli* reporter strain revealed that each could complement the mutant phenotype and rescue growth on media containing SDS (Fig. 1D). This provides the first direct evidence that TtrA contains an active twin-arginine signal sequence, and corroborates previous work that TtrB contains an active Tat targeting peptide (Hinsley *et al.*, 2001). Next, the TtrA–AmiA fusion was modified such that the conserved arginines of the signal peptide Tat motif where substituted with glutamine residues (Fig. 1B). The R6Q:R7Q variant of the TtrA signal sequence was unable to rescue growth of the *E. coli* reporter strain on SDS (Fig. 1D), and established that this derivative of the TtrA signal peptide is inactive as a signal peptide. Similarly, an R6Q variant of the TtrB Tat targeting signal was also shown to be inactive in this assay (Fig. 1D).

**Epitope tagged tetrathionate reductase retains enzymatic activity**

Having established that TtrA and TtrB Tat signal sequences were both transport-active in isolation, attention turned next to the native *Salmonella* tetrahionate reductase complex. In order to follow the targeting and assembly of the TtrA and TtrB proteins under native conditions they were differentially epitope tagged. First, the *ttrA* gene was modified at its native chromosomal locus by incorporation of a DNA sequence encoding a hexa-Histidine tag at its 3’ end to give strain MJJ001 (Table 1). The MJJ001 (*ttrAHis*) strain was able to grow anaerobically with tetrathionate as sole terminal electron acceptor and demonstrated slightly increased MV-linked tetrathionate reductase activity in a whole-cell assay (Fig. 2A). Next, the *ttrB* gene was modified at its native chromosomal locus by incorporation of a DNA sequence encoding a nine-residue haemagglutinin (HA) tag (NH2-YPYDVPDYA-COOH) at its 3’ end to give strain MJJ002 (Table 1). The *ttrBHA* strain also demonstrated the ability to respire anaerobically with tetrathionate and retained dye-linked tetrathionate reductase activity (Fig. 2A). Finally, a strain was constructed, MJJ100 (*ttrAHis, ttrBHA*), carrying both epitopes combined (Table 1). The MJJ100 (*ttrAHis, ttrBHA*) double epitope strain was able to grow anaerobically with tetrathionate as sole electron acceptor and tetrathionate reductase activity was unimpaired (Fig. 2A). These growth tests, taken together with the use of membrane-impermeable methyl viologen and tetrathionate in the assays, demonstrate that the subunits of the reductase have been correctly targeted to the...
Table 1. Salmonella strains used in this study.

| Strain       | Relevant genotype | Reference       |
|--------------|-------------------|-----------------|
| LT2a         | Wild type         | Lab stocks      |
| DIG100       | As LT2a, ΔtatABCD | Guymer et al. (2009) |
| MJJ001       | As LT2a, ttrA<sup>His</sup>, ttrB<sup>HA</sup> | This work       |
| MJJ002       | As LT2a, ttrB<sup>HA</sup> | This work       |
| MJJ100       | As LT2a, ttrA<sup>His</sup>, ttrB<sup>HA</sup>, ΔtorD::Apra<sup>R</sup> | This work       |
| MJJ101       | As LT2a, ttrA<sup>His</sup>, ttrB<sup>HA</sup>, ΔmoaA::Apra<sup>R</sup> | This work       |
| MJJ102       | As LT2a, ttrA<sup>His</sup>, ttrB<sup>HA</sup>, ΔttrC::Apra<sup>R</sup> | This work       |
| MJJ103       | As LT2a, ttrA<sup>His</sup>, ttrB<sup>HA</sup>, ΔttrC::Apra<sup>R</sup> | This work       |
| MJJ104       | As LT2a, ttrA<sup>His</sup>, ttrB<sup>HA</sup>, ΔttrC::Apra<sup>R</sup> | This work       |
| MJJ105       | As LT2a, ttrA<sup>His</sup>, ttrB<sup>HA</sup>, ΔttrC::Apra<sup>R</sup> | This work       |
| MJJ106       | As LT2a, ttrA<sup>His</sup> R6Q, ttrB<sup>HA</sup> | This work       |
| MJJ107       | As LT2a, ttrA<sup>His</sup> R7Q, ttrB<sup>HA</sup> | This work       |
| MJJ108       | As LT2a, ttrA<sup>His</sup> R6Q:R7Q, ttrB<sup>HA</sup> | This work       |
| MJJ109       | As LT2a, ttrA<sup>His</sup> (frameshift Δnt924), ttrB<sup>HA</sup> | This work       |
| MJJ110       | As LT2a, ttrA<sup>His</sup>, ttrB<sup>HA</sup> KSR | This work       |
| MJJ111       | As LT2a, ttrA<sup>His</sup>, ttrB<sup>HA</sup> R6Q | This work       |
| MJJ112       | As LT2a, ttrA<sup>His</sup>, ttrB<sup>HA</sup> KSR:R6Q | This work       |
| MJJ113       | As LT2a, ttrA<sup>His</sup> R6Q:R7Q, ttrB<sup>HA</sup> KSR | This work       |
| MJJ114       | As LT2a, ttrA<sup>His</sup> R6Q:R7Q, ttrB<sup>HA</sup> R6Q | This work       |
| MJJ115       | As LT2a, ttrA<sup>His</sup> R6Q:R7Q, ttrB<sup>HA</sup> KSR:R6Q | This work       |
| MJJ116       | As LT2a, ttrA<sup>His</sup>, ttrB<sup>HA</sup> KSR, ΔtatABC::Apra<sup>R</sup> | This work       |

periplasmic side of the membrane in all of the strains tested (Hensel et al., 1999; Hinsley et al., 2001; Hinsley and Berks, 2002).

The MJJ100 (ttrA<sup>His</sup>, ttrB<sup>HA</sup>) double epitope strain was used to follow production of the TrrA and TtrB proteins under various conditions. As expected (Hensel et al., 1999), enzyme production was shown to be dependent upon exogenous tetrathionate (Fig. 2B), with as little as 125 μM able to induce synthesis of detectable amounts of TtrB (Fig. 2B), demonstrating that the addition of the epitope tags did not disrupt the natural expression characteristics of the system. Since the HA epitope tag is located at the C-terminus of TtrB, Western immunoblotting can be used to observe both the full-length precursor form (28 105 Da including HA tag) and the mature form of TtrB following signal processing (predicted to be 25 291 Da). Indeed, both forms of TtrB<sup>HA</sup> could be resolved during the whole-cell expression tests (Fig. 2B).

The TtrA protein is not processed and contains an N-terminal signal anchor

The TtrC protein is predicted to be an inner membrane protein with nine transmembrane domains adopting an N-out, C-in conformation (Rothery et al., 2008). The protein is not predicted to contain any cofactors but is assumed to be the location of quinol oxidation for the tetrathionate reductase as well as acting as a membrane anchor for TtrB and/or TtrA (Hensel et al., 1999; Hinsley and Berks, 2002; Rothery et al., 2008). In this work, strains were constructed that lacked the ttrC gene. A ΔttrC strain demonstrated a high level of methyl viologen-linked enzymatic activity, which indicated that at least TrrA was successfully targeted to the periplasmic side of the membrane in the absence of TtrC (Fig. 3A). However, consistent with the predicted role of TtrC as a quinol dehydrogenase subunit, cells devoid of ttrC were unable to grow on tetrathionate (Fig. 3A). This demonstrates that the methyl viologen assay involves electron transfer via active TtrAB but is not dependent on a functional TtrC. Interestingly, Western immunoblotting and fractionation (Fig. 4) experiments with a ΔttrC strain revealed that both TtrA<sup>His</sup> and TtrB<sup>HA</sup> remained tightly membrane bound in the absence of TtrC (Fig. 4). Moreover, TtrB was also clearly proteolytically processed in the absence of ttrC (Figs 3 and 4). These data suggest that TtrC is not the sole membrane anchor for the other tetrathionate reductase subunits.

Inspection of the TtrA and TtrB sequences suggests neither contains obvious transmembrane domains; however, both TatFIND (Rose et al., 2002) and TatP (Bendtsen et al., 2005) bioinformatics programs suggest that the TtrA twin-arginine signal peptide may not contain a canonical signal peptidase cleavage site. It is therefore possible that the TtrA signal peptide acts as an N-terminal signal anchor domain. In order to explore this hypothesis the engineered hexa-Histidine tag on TtrA was exploited.

A Salmonella strain lacking ttrC but producing both TtrA<sup>His</sup> and TtrB<sup>HA</sup> was cultured anaerobically in the presence of tetrathionate. Total membranes were prepared and dispersed in detergent before insoluble material and debris were removed by ultracentrifugation. The resultant detergent-soluble membrane fraction was found to contain TtrA<sup>His</sup> and TtrB<sup>HA</sup> by Western immunoblotting (Fig. 5A). Upon application to an IMAC column both TtrA<sup>His</sup> and...
TtrB bound to the column and were eluted with imidazole (Fig. 5A). Concentration of the eluted IMAC pool allowed the identification of a protein band with an apparent molecular mass of \( \sim 110 \) kDa by SDS-PAGE (Supplementary Fig. S1). Attempts at direct Edman N-terminal sequencing of this protein failed, suggesting the terminal amino group of protein may be modified, which could further indicate that the formyl-Methionine initiator residue remained present on this protein. Next, tryptic peptide, and chymotryptic peptide, mass fingerprinting analysis of the \( \sim 110 \) kDa protein excised from the SDS-PAGE experiment (Supplementary Fig. S2) was carried out. This revealed that the \( \sim 110 \) kDa protein was indeed TtrA and, most importantly, identified peptide masses that would correspond to the h-region of the TtrA twin-arginine signal sequence (Supplementary Fig. S2). This purification experiment therefore provides definitive evidence that TtrA does not have a cleavable twin-arginine signal peptide, but instead has a non-processed twin-arginine signal anchor sequence analogous to those observed for prokaryotic and chloroplast Rieske Fe-S proteins (Keller et al., 2012).

The TtrB<sup>HA</sup> protein was observed to co-purify with TtrA<sup>His</sup> throughout this experiment (Fig. 5A), providing direct evidence that TtrA and TtrB interact. Tryptic peptide...
mass fingerprinting of the co-purifying TtrB<sup>HA</sup> protein also confirmed the Tat targeting signal was proteolytically processed (Supplementary Fig. S2). A revised model for the structure of the TtrABC tetrathionate reductase is given in Fig. 5B.

**Processing of TtrB is intimately linked to assembly of its partner, TtrA**

The components of the Tat translocase are essential for targeting of all Tat substrates (Palmer and Berks, 2012) and processing of cleavable Tat-targeting signals is a post-transport event that is catalysed by a membrane-bound signal peptidase with their active sites at the extracytoplasmic side of the membrane (Lüke et al., 2009). The MJJ100<sup>Δ<sup>tatABCD</sup></sup> double epitope strain was further modified by the addition of a Δ<sup>tat</sup>A<sub>100</sub>Δ<sup>ttrC</sup> allele that would...
prevent Tat transport. The resultant MJJ102 (ttrA<sup>His</sup>, ttrB<sup>His</sup>, Δtat) strain was devoid of dye-linked tetrathionate reductase activity in whole cells (Fig. 3A), indicating the catalytic subunits were not correctly targeted, and was consequently unable to grow anaerobically on tetrathionate (Fig. 3A). In addition, Western immunoblotting revealed that the HA-tagged TtrB protein was detectable and clearly present as the full-length, unprocessed, precursor form in the tat mutant (Fig. 3B). In some instances, a TtrB<sup>His</sup> band with an intermediate mass between that of mature and precursor form was observed in the Δtat background (Fig. 4A). It is not clear what the molecular basis of this is, but it is either a result of non-specific proteolysis, or a result of some other post-translational modification, of the mislocalized precursor. Any non-specific proteolysis must be confined to the N-terminus of TtrB<sup>His</sup> within the signal peptide since the protein is detected by a C-terminal epitope tag in these experiments (Fig. 4A). It is also noticeable that the TtrA<sup>His</sup> and TtrB<sup>His</sup> proteins are predominantly associated with the membrane fraction in the Δtat background (Fig. 4B). It is not uncommon for mis-localized Tat substrates to associate with the membranes, for example the NapA nitrate reductase from both E. coli and Ralstonia eutropha behaves in a similar fashion (Weiner et al., 1998; Bernhard et al., 2000). Moreover, there is reasonable evidence that Tat substrates in general interact with the lipid bilayer before engaging the Tat translocase as part of the natural Tat transport cycle (Shanmugham et al., 2006; Bageshwar et al., 2009).

Next, a ΔmoaA allele was introduced into the MJJ100 (ttrA<sup>His</sup>, ttrB<sup>His</sup>) double epitope strain. This new strain – MJJ103 (ttrA<sup>His</sup>, ttrB<sup>His</sup>, ΔmoaA) – is unable to synthesize the molybdenum cofactor necessary for TtrA activity (Hensel et al., 1999) and as a result no tetrathionate reductase activity could be detected in whole cells and the strain was unable to grow with tetrathionate as sole electron acceptor (Fig. 3A). Very surprisingly, however, the iron-sulphur protein TtrB was found to be unprocessed in the ΔmoaA strain (Fig. 3B), despite it not containing such a cofactor, and was observed to migrate during SDS-PAGE in an identical fashion to the precursor form of TtrB. To determine whether this observation was as a direct result of the incorrect assembly of TtrA, the strain MJJ109 was constructed carrying a frameshift mutation at base-pair 924 in ttrA that would result in premature termination of ttrA translation. Western immunoblotting of this strain reveals that TtrB<sup>His</sup> remains unprocessed in the specific absence of TtrA (Fig. 6B, lane 9). The ttrA strain is also devoid of tetrathionate reductase activity and unable to respire tetrathionate in vivo (Fig. 6A). Taken together, these data give the initial indication that Tat-dependent targeting and processing of TtrB is reliant upon the successful assembly of its TtrA partner protein.

**Fig. 6.** Mutagenesis of the TtrA signal anchor alone. 

A. Tetrathionate reductase activity assays in intact bacterial cells. The strain MJJ100 (TtrA<sup>His</sup>, TtrB<sup>His</sup>) and its derivatives MJJ106 (TtrA<sup>His</sup>R6Q, TtrB<sup>His</sup>R6Q), MJJ107 (TtrA<sup>His</sup>R7Q, TtrB<sup>His</sup>R7Q), MJJ108 (TtrA<sup>His</sup>R6Q:R7Q, TtrB<sup>His</sup>R6Q:R7Q), MJJ109 (ttrA<sup>His</sup>, TtrB<sup>His</sup>) were grown anaerobically in the presence of tetrathionate, washed and assayed for tetrathionate-dependent MV oxidation activity. Activities are presented as percentages relative to the parental strain [biological replicates n = 3, one-way analysis of variance (Holm-Sidak method) P < 0.001 (***)]. Inset: *Salmonella* strains grown anaerobically on minimal media glycerol/tetrathionate plates: 1 – TtrA<sup>His</sup>, TtrB<sup>His</sup>; 2 – DIG100 Δtat strain; 3 – TtrA<sup>His</sup>R6Q, TtrB<sup>His</sup>R6Q, 4 – TtrA<sup>His</sup>R7Q, TtrB<sup>His</sup>R7Q, 5 – TtrA<sup>His</sup>R6Q:R7Q, TtrB<sup>His</sup>R6Q:R7Q, and 6 – ttrA<sup>His</sup>, TtrB<sup>His</sup>. B. Strains MJJ100 (TtrA<sup>His</sup>, TtrB<sup>His</sup>), MJJ106 (TtrA<sup>His</sup>R6Q, TtrB<sup>His</sup>R6Q), MJJ107 (TtrA<sup>His</sup>R7Q, TtrB<sup>His</sup>R7Q), MJJ108 (TtrA<sup>His</sup>R6Q:R7Q, TtrB<sup>His</sup>R6Q:R7Q) and MJJ109 (ttrA<sup>His</sup>, TtrB<sup>His</sup>) were cultured in rich media with 0.5% (w/v) glycerol and in the presence or absence of 10 mM potassium tetrathionate. Whole-cell samples were taken after 24 h incubation and analysed by Western immunoblotting. A blot against maltose-binding protein (MBP) was used as a loading control.

One active Tat targeting signal is sufficient for assembly of tetrathionate reductase 

To test directly the role of the Tat signal peptides in assembly of the tetrathionate reductase, a programme of mutagenesis was initiated. First, the MJJ100 (ttrA<sup>His</sup>, ttrB<sup>His</sup>) double epitope strain was modified to give three new strains where the ttrA<sup>His</sup> gene would encode either R6Q, R7Q or R6Q:R7Q variants of the TtrA<sup>His</sup> protein. Surprisingly, although the R6Q:R7Q variant of the TtrA signal...
sequence is known to be transport inactive (Fig. 1), modification of the TtrA twin-arginine motif had no effect on the ability of the bacterium to grow anaerobically with tetrathionate (Fig. 6A). Quantification of the dye-linked tetrathionate reductase activity in intact cells demonstrated that the introduced point mutations had little effect on the cellular levels of the enzyme (Fig. 6A) and Western immunoblotting confirmed that TtrAHis was stable in all cases (Fig. 6B). In addition, the HA-tagged TtrB protein was clearly detectable and processed in the TtrA signal peptide variants (Fig. 6B).

Attention turned next to the TtrB. The transport-inactive ttrB R6Q allele (Fig. 1) was introduced into the MJJ100 (ttrAHis, ttrBΔ) double epitope strain to yield strain MJJ111 (ttrAHis, ttrBΔ R6Q). Interestingly, the ttrBΔ R6Q strain was able to grow anaerobically with tetrathionate and retained essentially native levels of tetrathionate reductase activity (Fig. 7A). However, despite the essentially normal levels of enzymatic activity observed (Fig. 7A), Western immunoblotting suggested that the inactive TtrBΔ R6Q was not correctly processed in the MJJ111 (ttrAHis, ttrBΔ R6Q) strain (Fig. 7B, lane 5). The full-length precursor form and intermediate-sized bands were evident (Fig. 7B).

Further amino acid substitutions at the unusual ‘KR’ motif within the TtrB Tat signal involved Replacement of K5 with arginine in strain MJJ110 (ttrAHis, ttrBΔ K5R), MJJ110 (ttrAHis, ttrBΔ KSR), MJJ116 (ttrAHis, ttrBΔ KSR, Δtat), thus restoring a canonical twin-arginine motif. The TtrB K5R substitution noticeably reduced cellular levels of TtrBΔ (Fig. 7B, lane 3) and therefore reduced cellular tetrathionate reductase activity as a result (Fig. 7A).

Simultaneous inactivation of both Tat signals abolishes tetrathionate reductase activity

Since mutagenesis of the TtrA signal peptide alone had little effect on cellular tetrathionate reductase activity (Fig. 6), and mutagenesis of the TtrB signal peptide did not completely abolish assembly and activity (Fig. 7), it was decided to combine the signal peptide substitutions into a single strain. In this case, dramatic effects on tetrathionate

© 2013 The Authors. Molecular Microbiology published by John Wiley & Sons Ltd., Molecular Microbiology, 90, 400–414
Two active Tat targeting sequences are required for tetrathionate reductase assembly.

A. Tetrathionate reductase activity assays in intact bacterial cells. The strain MJJ100 (TtrA\textsuperscript{HA} TtrB\textsuperscript{HA}) and its derivatives MJJ108 (TtrA\textsuperscript{HA} R6Q:R7Q), MJJ113 (TtrA\textsuperscript{HA} R6Q:R7Q, TtrB K5R), MJJ114 (TtrA\textsuperscript{HA} R6Q:R7Q, TtrB HA) and MJJ115 (TtrA\textsuperscript{HA} R6Q:R7Q, TtrB\textsuperscript{K5R:R6Q}) were grown anaerobically in the presence of potassium tetrathionate, washed and assayed for tetrathionate-dependent MV oxidation activity. Activities are presented as percentages relative to the parental strain [biological replicates \( n = 3 \), one-way analysis of variance (Holm-Sidak method) \( P < 0.001 (***) \)]. Inset: Salmonella strains grown anaerobically on minimal media glycerol/tetrathionate plates: 1 – TtrA\textsuperscript{HA} TtrB\textsuperscript{HA}; 2 – TtrA\textsuperscript{HA} R6Q:R7Q; 3 – TtrA\textsuperscript{HA} R6Q:R7Q, TtrB\textsuperscript{K5R:R6Q}; 4 – TtrA\textsuperscript{HA} R6Q:R7Q, TtrB\textsuperscript{HA} R6Q; 5 – TtrA\textsuperscript{HA} R6Q:R7Q, TtrB\textsuperscript{HA} K5R:R6Q; and 6 – TtrA\textsuperscript{HA}.

B. Strains MJJ100 (TtrA\textsuperscript{HA} TtrB\textsuperscript{HA}), MJJ108 (TtrA\textsuperscript{HA} R6Q:R7Q), MJJ113 (TtrA\textsuperscript{HA} R6Q:R7Q, TtrB K5R), MJJ114 (TtrA\textsuperscript{HA} R6Q:R7Q, TtrB\textsuperscript{HA} R6Q) and MJJ115 (TtrA\textsuperscript{HA} R6Q:R7Q, TtrB\textsuperscript{K5R:R6Q}) were cultured in rich media with 0.5% (w/v) glycerol and in the presence or absence of 10 mM potassium tetrathionate. Whole-cell samples were taken after 24 h incubation and analysed by Western immunoblotting. A blot against maltose-binding protein (MBP) was used as a loading control. The asterisk marks the intermediate sized band (neither precursor nor mature) adopted by TtrB\textsuperscript{HA}.

Fig. 8. Two active Tat targeting sequences are required for tetrathionate reductase assembly.

For example, the monomeric trimethylamine N-oxide reductase (TMAR) interacts with the TorD chaperone via its signal peptide and at another nearby site (Dow et al., 2013), and the Tat signal peptide of the monomeric periplasmic nitrate reductase is bound tightly by its NapD chaperone before translocation (Grahl et al., 2012). Perhaps a closer analogy to Salmonella TtrAB assembly is biosynthesis of the core DmsAB dimer of the DMSO reductase, where a single signal peptide on the DmsA subunit interacts directly with the DmsD chaperone before transport (Chan et al., 2008). It is notable that in every example it is the protein bearing the molybdendium cofactor that also sports the signal peptide onto which the chaperones bind. This might suggest that any chaperone-dependent proofreading activity might be focused on the Salmonella TtrA protein. Indeed, the twin-arginine signal peptide of a putative TtrA-like protein from the archaeon *Archaeglobus fulgidus* has recently been shown to interact directly with a TorD-like chaperone (Coulthurst et al., 2012). In this work, TtrA and TtrB are intimately linked.

Discussion

The subcellular localization of tetrathionate reductase at the periplasmic side of the cytoplasmic membrane is critical for its physiological function. Because TtrA and TtrB each contain their own signal peptides it may have been assumed that each would be transported separately to the periplasm where they would individually associate with the periplasmic loops of TtrC to form the active enzyme. Data presented here, however, has dispelled that assumption and has clearly shown that Tat transport of TtrA and TtrB is intimately linked.

Communication between TtrA and TtrB

A key observation from this work is that the iron-sulphur protein TtrB is not processed in the absence of its molybdoprotein partner TtrA (Figs 3 and 6). How does TtrB ‘sense’ that TtrA is absent and so delay translocation? There are very well-characterized examples from *E. coli* where Tat-dependent molybdoenzymes interact directly with ‘Tat proofreading chaperones’ prior to export during the cofactor insertion and subunit oligomerization events. For example, the monomeric trimethylamine N-oxide (TMAR) reductase interacts with the TorD chaperone via its signal peptide and at another nearby site (Dow et al., 2013), and the Tat signal peptide of the monomeric periplasmic nitrate reductase is bound tightly by its NapD chaperone before translocation (Grahl et al., 2012). Perhaps a closer analogy to Salmonella TtrAB assembly is biosynthesis of the core DmsAB dimer of the DMSO reductase, where a single signal peptide on the DmsA subunit interacts directly with the DmsD chaperone before transport (Chan et al., 2008). It is notable that in every example it is the protein bearing the molybdendium cofactor that also sports the signal peptide onto which the chaperones bind. This might suggest that any chaperone-dependent proofreading activity might be focused on the Salmonella TtrA protein. Indeed, the twin-arginine signal peptide of a putative TtrA-like protein from the archaeon *Archaeglobus fulgidus* has recently been shown to interact directly with a TorD-like chaperone (Coulthurst et al., 2012). In this work, TtrA and TtrB are intimately linked.
the role of *Salmonella* TorD in tetrathionate reductase activity and assembly was investigated. The MJJ100 (ttrAHis, ttrBHA) double epitope strain was modified by the introduction of a ΔtorD allele; however, processing, assembly and activity of the *Salmonella* tetrathionate reductase was unaffected by this mutation (Fig. 3). To date, we have been unable to identify a chaperone for the *Salmonella* TtrA signal peptide (unpublished).

The observed behaviour of TtrB is reminiscent of that exhibited by the small subunit of [NiFe] hydrogenases, which are themselves iron-sulphur proteins synthesized as precursors with a Tat signal peptide. The hydrogenase small subunits must await attachment of signal-less partner proteins (the nickel-containing large subunits of the hydrogenases) before transport can proceed (Schubert *et al*., 2007). In the case of [NiFe] hydrogenases it is also believed that system-specific Tat proofreading chaperones, such as the HyaE/HoxO, HyaF/HoxQ or HybE/HoxT families, interact with the small subunit to prevent its premature export (Schubert *et al*., 2007). However, there is currently no evidence for genes encoding these types of proteins to be linked to those encoding tetrathionate reductases, or for related gene products to be important in tetrathionate reductase activity. Future work will focus on understanding how transport of TtrB is suppressed in the absence of TtrA and establishing if there are other proteins involved.

**Translocation of a multi-signal complex**

The data presented here suggest a ‘cytoplasmic assembly model’ for the TtrA and TtrB subunits of tetrathionate reductase (Fig. 9). In this model, TtrA and TtrB associate prior to export and then engage the Tat translocase together, probably via an initial membrane-binding step (Fig. 9). The evidence for this is strong. Clearly both signal peptides are transport active (Figs 1, 6 and 7) with only simultaneous inactivation of both preventing enzyme assembly (Fig. 8). It is notable, however, that the inactive R6Q variants of TtrB are not proteolytically processed in the correct way as observed for their transport-active counterpart (Figs 7 and 8). In the case of the MJJ114 (ttrAHis R6Q:R7Q, ttrBHA R6Q) double mutant (Fig. 8) there is no possibility of transmembrane translocation and the enzyme would therefore become mis-localized in the cell as if it was produced in a Δtat genetic background. Indeed, similar non-specific proteolysis is seen for native TtrB when it is produced in a tat mutant (Fig. 4). More intriguing is the aberrant processing of TtrBHA R6Q observed in a strain with a fully functional TtrA partner protein and a fully functional Tat system (Fig. 7). In this case tetrathionate reductase activity is largely unaffected (Fig. 7) indicating that TtrAB is correctly positioned at the periplasmic side of the membrane, and that TtrBHA R6Q must have been transported through the Tat channel. Why, then, is TtrBHA R6Q apparently not correctly processed by leader peptidase? One plausible explanation could stem from the possibility that the TatC protein is responsible for correct positioning of Tat signal peptides in the membrane to allow processing by leader peptidase. Crystal structures for TatC have recently been determined (Rollauer *et al*., 2012; Ramasamy *et al*., 2013). TatC is the initial contact point for the twin-arginine motif of Tat signal peptides, and the arginines themselves are crucial for this interaction (Rollauer *et al*., 2012). Moreover, it is possible to model an extended signal peptide structure into a hydrophobic groove on TatC that
neatly positions the twin-arginine motif at the cytoplasmic side, and the signal peptidase cleavage site at the periplasmic side, of the membrane (Ramasamy et al., 2013). Thus, the correct interaction between TatC and a signal peptide may be important in ensuring correct processing following translocation. In the case of the TrbR<sup>B</sup> R6Q signal peptide, it is unlikely that this inactive signal will interact with TatC (Rollauer et al., 2012). It is likely that TrbR<sup>R</sup> R6Q is simply pulled through the Tat translocation channel by the Tat-active TrbA<sup>R</sup> protein to which it is bound and so is never positioned correctly in the membrane by TatC in order to be correctly processed by signal peptidase. The result is an active, periplasmic enzyme complex but bearing a largely non-processed, or spuriously degraded, N-terminus of TrbB.

Taken altogether, these observations suggest each pre-export TrbAB complex contains two active signal peptides – but is it feasible that a single Tat translocase could transport such a complex? Recent work on the chloroplast Tat system would seem to support this hypothesis (Ma and Cline, 2010). Using in vitro transport assays with isolated thylakoid membranes Ma and Cline (2010) were able to show that covalently linked dimers or tetramers of Tat substrates (thus displaying two or four active signal peptides) could bind to a single Tat translocase, which itself is thought to contain upwards of seven copies of the TatC signal-peptide-binding subunit (Tarry et al., 2009; Ma and Cline, 2010), and then be efficiently translocated in unison as a complex (Ma and Cline, 2010). The Salmo-nella tetrathionate reductase described here is therefore a naturally occurring multi-signal Tat substrate that adds extra weight to the observations of Ma and Cline (2010).

Are there other examples of multi-signal Tat substrates outwith the tetrathionate reductase family? Certainly there are many examples of bacterial Tat substrates that are known to exist as homo- and hetero-oligomers in the post-export mature form, for example glucose-fructose oxidoreductase is a homotramer (Nurizzo et al., 2001); many [NiFe] hydrogenases dimerize (Ballantine and Boxer, 1986; Volbeda et al., 2012); and formate dehydrogenase forms a homo-trimer (Jormakka et al., 2002). Moreover, the E. coli [NiFe] hydrogenase-2, for which Salmonella has a homologue, can be isolated as a complex that contains the Tat-signal-containing HybO subunit, its partner subunit HybC that has no signal peptide, and the Tat-signal-containing HybA protein (Lukey et al., 2010). While previous discussions have focused on how the cell could prevent such multimerization of Tat substrates prior to export (Sargent et al., 2002; Sargent, 2007), it should now be considered that pre-export oligomerization of substrates into multi-signal complexes may be the norm and that the Tat translocase may actually have evolved to cope with multi-signal Tat substrates with consummate ease. Indeed, the physiological requirement to transport tight protein complexes bearing multiple active signal peptides may be one of the evolutionary drivers that necessitated the polymerization of TatBC into large structures containing multiple signal peptide binding sites relatively close together.

Different roles for the tetrathionate reductase signal sequences

Finally, the experiments described here have shed some light on the overall architecture of tetrathionate reductase. In the absence of TrbC, the remainder of the tetrathionate reductase remains membrane-bound and is not soluble in the periplasm, as has been observed in analogous experiments where membrane subunits have been genetically removed (Stanley et al., 2002). This suggests strongly that TrbAB is attached by additional means to the membrane. The TrbB protein sequence contains a conserved 60-residue C-terminal extension following the motif for the fourth and final Fe-S cluster. In Tat-dependent formate dehydrogenases and [NiFe] hydrogenases this region contains an obvious hydrophobic transmembrane domain (Sargent et al., 2002), however TrbB clearly is not predicted to have such a helix. Instead, the TrbAB heterodimer is anchored to the membrane by the TrbA signal sequence, which is not proteolytically processed and so acts as an N-terminal signal anchor. This observation may provide an additional reason for the tetrathionate reductase retaining two Tat signal peptides: while TrbB is purely a targeting signal, and so ultimately cleaved off, the TrbA signal sequence also behaves as an unprocessed transmembrane domain and thus has an additional important structural role. Indeed, it is notable from the data presented here that inactivation of the TrbB signal peptide has much more deleterious effect on tetrathionate reductase activity than inactivation of the TrbA signal anchor alone. Thus there may be strong evolutionary pressure to keep the efficient TrbB signal peptide for assembly reasons, and retain the TrbA signal anchor for structural reasons. The inferred structure and topology of tetrathionate reductase would make it a new addition to the ever-growing examples of Tat-dependent integral membrane proteins.

Concluding remarks

Understanding the activity, physiological roles and biosynthesis of molybdenum-dependent enzymes is an increasingly important area with medical implications. Bacterial molybdenum enzymes are central to bacterial pathogenesis (Contreras et al., 1997; Winter et al., 2010; 2013) and genetic defects in the assembly of related human enzymes result in fatal neurodegenerative diseases (Schwarz, 2005). This work has provided fresh insight into the interrelationship between the molybdenum-dependent
tetrahionate reductase and the bacterial Tat system, which central to its assembly and biosynthesis. In the correct physiological context two apparently separate signal peptides can be seen to operate in close cooperation.

**Experimental procedures**

**Reporter plasmids and the amidase-based export assay**

Ize *et al.* (2009) described a sensitive reporter system designed to show activity of Tat signal sequences. The system was based on the observation that an *E. coli amiA, amiC* double mutant was sensitive to killing by exogenous SDS, but that the phenotype could be rescued by heterologous Tat signal peptides genetically fused to the mature domain of AmiA (Ize *et al.*, 2009). Here, DNA encoding the predicted twin-arginine signal sequence of TtrA was amplified with oligonucleotides TtrAssfor (5′-tagtgcgatgcagaatttaacccagcagcagtggc-3′) and TtrAssrev (5′-tagtgcgatgcagaatttaaaccgacagcg-3′) using *Salmonella* LT2 chromosomal DNA as template. The product was digested with BamHI and Xbal and cloned into similarly digested pAmiA(-SP) (KanR) plasmid (Ize *et al.*, 2009). Here, DNA encoding the signal peptide of TtrB was amplified with TtrBssfor (5′-ggcgcggatccatggacagcagtaaacg-3′) and TtrBssrev (5′-ggcgcggatccatggacagtaa acagcaatttctcc-3′) using *Salmonella* LT2 as template. The product was digested with BamHI and Xbal and cloned into similarly digested pAmiA(-SP) to give pSU-ssTtrBKQSt-AmiA. DNA encoding a variant of the TtrA signal peptide with the twin arginines substituted was amplified with oligonucleotides TtrAssfor (5′-tagtgcgatgcagaatttaacccagcagcagtggc-3′) and TtrAssrev, and strain MJJ108 as template. The product was digested with BamHI and Xbal and cloned into similarly digested pAmiA(-SP) to give pSU-ssTtrAQQSt-AmiA.

DNA encoding the signal peptide of TtrB was amplified with oligonucleotides TtrBssfor (5′-tagtgcgatgcagaatttaaccgacagggcgcggc-3′) and TtrBssrev (5′-tagtgcgatgcagaatttaaaccgacagcg-3′) using *Salmonella* LT2 as template. The product was digested with BamHI and Xbal and cloned into similarly digested pAmiA(-SP) to give pSU-ssTtrBKQSt-AmiA. DNA encoding a variant of the signal peptide with the Arg 6 codon substituted by a lysine codon was amplified with TtrBssKQfor (5′-ggcgcggatccatggacagcagtaaacg-3′) and TtrBssrev using strain MJJ111 as template. The product was digested with BamHI and Xbal and was again cloned into similarly digested pAmiA(-SP) to give pSU-ssTtrBKKSt-Bla.

SDS sensitivity assays were performed using *E. coli* strains MCDSSAC (as MC4100, amiAΔ2−33, amiCΔ2−32; Ize *et al.*, 2003) and the isogenic Tat mutant MCDSSACdtatABC (Keller *et al.*, 2012) harbouring either of pSU-ssTrtRASt-AmiA, pSU-ssTrtAQQSt-AmiA, pSU-ssTrrBSt-AmiA, pSU-ssTrrBKQSt-AmiA or the pSU40 vector (Bartolomé *et al.*, 1991) as a control. A single colony of each strain/plasmid combination was suspended in 100 μl of sterile water and 5 μl aliquots were replica- spotted onto solid LB medium supplemented with kanamycin, and solid LB medium supplemented with kanamycin plus 2% (v/v) SDS. Plates were incubated for 12 h at 30°C before being photographed.

**Bacterial growth conditions**

The *S. enterica* serovar Typhimurium LT2a strains used in this study are listed in Table 1. Starter cultures of 4 ml LB ‘low salt’ (5 g l−1 NaCl) with or without antibiotics were inoculated with the required colony and cultured overnight with shaking (200 r.p.m.) for at least 16 h at 37°C. For 24 h microaerobic cultures, 16 μl of the starter culture was added to 1.6 ml of pre-warmed LB containing 0.5% (v/v) glycerol and 0−10 mM potassium tetrahionate in a 1.5 ml microfuge tube. For 1 h cultures, 160 μl of starter culture was added to a 1.5 ml microfuge tube and centrifuged for 20 s at 13 000 r.p.m. Following aspiration of the supernatant, the bacterial pellet was resuspended in pre-warmed 1.6 ml of LB containing 0.5% (v/v) glycerol and 0−10 mM potassium tetrahionate. For both 24 and 1 h cultures, the microfuge tubes were sealed and cultured for the required time at 37°C without agitation. Processing of cultures involved pelleting the samples for 20 s at 13 000 r.p.m., aspiration of supernatants followed by resuspension of cell pellets in 150 μl Laemmli disaggregation buffer containing 10% (v/v) β-mercaptoethanol. Samples were boiled for 4 min.

Anaerobic growth of strains on solid minimal media tetrahionate plates containing M9 salts, tetrahionate (25 mM final concentration) and 0.5% (v/v) glycerol was carried out under strict anaerobic conditions for 3−4 days at 37°C using airtight jars and Anaerogen sachets (Oxoid).

**Construction of Salmonella strains**

In-frame deletion strains were generated by using the λRED method (Datsenko and Wanner, 2000), while epitope tags and point mutations were introduced by the pMAK705 method (Hamilton *et al.*, 1989). Briefly, segments of the *ttrA* and *ttrB* genes were cloned into pKS+ Bluescript by PCR designed to incorporate appropriate restriction sites and coding sequences for epitope tags. The different segments of the *ttr* genes were then joined into single pKS-TtRA-His and pKS-TtRB-HA constructs before being subcloned into pMAK705 and integrated into the *Salmonella* genome via homologous recombination. Missense mutations in the *ttrA* and *ttrB* genes were generated by Quikchange (Stratagene) on the original pKS-TtRA-His and pKS-TtRB-HA plasmids. Strain construction was verified by DNA sequencing.

**Isolation of TtrA**

The MJJ104 (*ttrAΔSacI, ttrBΔSacI, ΔttrC::ApraR*) strain was grown anaerobically for 16 h at 37°C in 15 l of LB supplemented with 0.5% (v/v) glycerol, 0.4% (w/v) fumarate and 0.2% (w/v) tetrahionate. The cells were harvested by centrifugation (~ 6 g wet weight was recovered) before being suspended in 20 ml 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, and protease inhibitors (Calbiochem). A crystal of DNase I and lysozyme was added before cells were lysed by three passages through a French pressure cell at 8000 psi (Thermo Scientific). Unbroken cells and debris were removed by centrifugation resulting in a ‘crude extract’. Total membranes were prepared by centrifuging the crude extract at 100 000 g in a Beckman Optima XL-100K ultracentrifuge. The supernatant was retained as ‘soluble fraction’ while the membrane pellet was taken up in 16 ml Tris-HCl (pH 7.5) and vesicles dispersed by the addition of 4 ml 20% (v/v) Triton X-100. Following incubation at 4°C for 30 min aggregated proteins, and other insoluble material, were removed by centrifugation at 100 000 g. The supernatant was considered the ‘membrane
fraction’ and was immediately loaded onto a 5 ml TALON immobilized metal affinity chromatography (IMAC) column (GE Healthcare) at a flow rate of 0.5 ml min⁻¹. The column was equilibrated in 50 mM Tris·HCl (pH 7.5), 0.05% (v/v) Triton X-100 and bound proteins were eluted with a 40 ml gradient of 0–1 M imidazole in the same buffer (1 ml min⁻¹). Fractions judged to contain TtrA His gradient of 0–1 M imidazole in the same buffer (1 ml min⁻¹). Fractions pooled and concentrated to 500 μl in a filtration device (10 kDa molecular weight cut-off) before being loaded onto a Superdex200 10/30 size exclusion chromatography (SEC) column (GE Healthcare) that has been equilibrated in 50 mM Tris·HCl (pH 7.5), 0.05% (v/v) Triton X-100. Fractions containing TtrA His were pooled and concentrated.

**Subcellular fractionation**

Separation of periplasmic, total membranes and cytoplasmic fractions was performed essentially as outlined in Osborn and Munson (1974). Briefly, anaerobic 500 ml cultures in rich media supplemented with 0.5% (v/v) glycerol and 10 mM tetrathionate were incubated for 24 h at 37°C. Cells were harvested by centrifugation at 16 000 g and resuspended at 1 g cells per 10 ml buffer in 50 mM Tris·HCl pH 8.0, 40% (w/v) sucrose. EDTA was added to 5 mM before fresh lysozyme was added to 0.6 mg ml⁻¹ (final) together with a crystal of DNase I. Following a 20 min incubation at 37°C the spheroplasts were recovered by centrifugation and the supernatant retained as the periplasmic fraction. Spheroplasts were further fractionated into cytoplasmic and total membrane fractions following sonication and ultracentrifugation steps.

**Western immunoblotting**

Standard SDS-PAGE gel electrophoresis (Laemmli, 1970) was performed with 9% (w/v) polyacrylamide gels for TtrA His and SufI detection, and 12.5% (w/v) polyacrylamide gels for TtrB His and maltose-binding protein (MBP). Proteins were then transferred to Immobilon-P (Millipore) using Towbin buffer (Towbin et al., 1979) containing 15% (v/v) methanol. Membranes were blocked overnight in TBS-Tween (0.05% v/v) buffer plus 5% (w/v) non-fat milk powder. Primary and secondary antibodies were incubated separately each for 1 h at room temperature in TBS-Tween plus 1% (w/v) non-fat milk powder. Signal was detected by ECL reagent (Millipore). Antibody concentrations used were as follows: anti-HA-HRP (Sigma) 1/3000; anti-pentaHis (Qiagen) 1/2000; anti-MBP (NEB) 1/10 000; anti-SufI (in house) 1/4000; anti-mouse-HRP (Bio-Rad) 1/25 000; anti-rabbit-HRP (Bio-Rad) 1/10 000; and anti-HA-HRP (Helma) containing 1.6 ml of N₂-saturated 100 mM sodium phosphate buffer pH 7 before 10 μl of 200 mM methyl viologen (Sigma) was added to the cuvette. Sodium dithionite was added until a steady A600 of approximately 2.5 was reached. Prior to addition of substrate, the rate of change of A600 was calculated before 10 μl of freshly prepared 2% (w/v) potassium tetrahionate was added. Protein concentrations were estimated by the Lowry method following methanol/chloroform precipitation (Lowry et al., 1951). Biological replicates were performed by culturing three independent microaerobic cultures from one starter aerobic culture.

**Acknowledgements**

This research was funded in the UK by Medical Research Council award G1100142 and Biotechnology and Biological Sciences Research Council award BB/D018986/1 to F.S. T.P. is the recipient of a Royal Society Wolfson Research Merit Award. We are grateful to the expert proteomic services provided by ‘Fingerprints Proteomics’ within the College of Life Sciences, University of Dundee.

**References**

Bageshwar, U.K., Whitaker, N., Liang, F.C., and Musser, S.M. (2009) Interconvertibility of lipid- and translocon-bound forms of the bacterial Tat precursor pre-SufI. Mol Microbiol 74: 209–226.

Ballantine, S.P., and Boxer, D.H. (1986) Isolation and characterisation of a soluble active fragment of hydrogenase isozyme 2 from the membranes of anaerobically grown *Escherichia coli*. Eur J Biochem 156: 277–284.

Bartolomé, B., Jubete, Y., Martinez, E., and de la Cruz, F. (1991) Construction and properties of a family of pACYC184-derived cloning vectors compatible with pBR322 and its derivatives. Gene 102: 75–78.

Bendtsen, J.D., Nielsen, H., Widdick, D., Palmer, T., and Brunak, S. (2005) Prediction of twin-arginine signal peptides. BMC Bioinformatics 6: 167.

Bernhard, M., Friedrich, B., and Siddiqui, R.A. (2000) *Ralstonia eutropha* TF93 is blocked in Tat-mediated protein export. J Bacteriol 182: 581–588.

de Bruyn, G. (2000) Infectious disease: diarrhea. West J Med 172: 409–412.

Chan, C.S., Winstone, T.M., Chang, L., Stevens, C.M., Workentine, M.L., Li, H., et al. (2008) Identification of residues in DmsD for twin-arginine leader peptide binding, defined through random and bioinformatics-directed mutagenesis. Biochemistry 47: 2749–2759.

Contreras, I., Toro, C.S., Troncoso, G., and Mora, G.C. (1997) *Salmonella typhi* mutants defective in anaerobic respiration are impaired in their ability to replicate within epithelial cells. Microbiology 143: 2665–2672.

Coulthurst, S.J., Dawson, A., Hunter, W.N., and Sargent, F. (2012) Conserved signal peptide recognition systems across the prokaryotic domains. Biochemistry 51: 1678–1686.

Datsenko, K.A., and Wanner, B.L. (2000) One-step
inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97: 6640–6645.

Dow, J.M., Gabel, F., Sargent, F., and Palmer, T. (2013) Characterisation of a pre-export enzyme–chaperone complex on the twin-arginine transport pathway. *Biochem J* 452: 57–66.

Gralh, S., Maillard, J., Pronk, C.A., Vuister, G.W., and Sargent, F. (2012) Overlapping transport and chaperone-binding functions within a bacterial twin-arginine signal peptide. *Mol Microbiol* 83: 1254–1267.

Guymer, D., Maillard, J., and Sargent, F. (2009) A genetic analysis of in vivo selenate reduction by *Salmonella enterica* serovar Typhimurium LT2 and *Escherichia coli* K12. *Arch Microbiol* 191: 519–528.

Hamilton, C.M., Aidea, M., Washburn, B.K., Babitzke, P., and Kushner, S.R. (1989) New method for generating deletions and gene replacements in *Escherichia coli*. *J Bacteriol* 171: 4617–4622.

Hensel, M., Hinsley, A.P., Nikolaus, T., Sawers, G., and Berks, B.C. (1999) The genetic basis of tetrathionate respiration in *Salmonella typhimurium*. *Mol Microbiol* 32: 275–287.

Hinsley, A.P., and Berks, B.C. (2002) Specificity of respiratory pathways involved in the reduction of sulfur compounds by *Salmonella enterica*. *Microbiology* 148: 3631–3638.

Hinsley, A.P., Stanley, N.R., Palmer, T., and Berks, B.C. (2001) A naturally occurring bacterial Tat signal peptide lacking one of the ‘invariant’ arginine residues of the consensus targeting motif. *FEBS Lett* 497: 45–49.

Ize, B., Stanley, N.R., Buchanan, G., and Palmer, T. (2003) Role of the *Escherichia coli/Tat* pathway in outer membrane integrity. *Mol Microbiol* 48: 1183–1193.

Ize, B., Coulthurst, S.J., Hatzixanthis, K., Caldelari, I., Buchanan, G., Barclay, E.C., et al. (2009) Remnant signal peptides on non-exported enzymes: implications for the evolution of prokaryotic respiratory chains. *Microbiology* 155: 3992–4004.

Jormakka, M., Tomroth, S., Byrne, B., and Iwata, S. (2002) Molecular basis of proton motive force generation: structure of formate dehydrogenase-N. *Science* 295: 1863–1868.

Keller, R., de Keyzer, J., Driessen, A.J., and Palmer, T. (2012) Co-operation between different targeting pathways during integration of a membrane protein. *J Cell Biol* 199: 303–315.

King, S., and Metzger, W.I. (1968) A new plating medium for the isolation of enteric pathogens. I. Hektoen enteric agar. *Appl Microbiol* 16: 577–578.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265–275.

Lüke, I., Handford, J.I., Palmer, T., and Sargent, F. (2009) Proteolytic processing of *Escherichia coli* twin-arginine signal peptides by LepB. *Arch Microbiol* 191: 919–925.

Lukey, M.J., Parkin, A., Roessler, M.M., Murphy, B.J., Harmer, J., Palmer, T., et al. (2010) How *Escherichia coli* is equipped to oxidize hydrogen under different redox conditions. *J Biol Chem* 285: 3928–3938.

Ma, X., and Cline, K. (2010) Multiple precursor proteins bind individual Tat receptor complexes and are collectively transported. *EMBO J* 29: 1477–1488.

McClelland, M., Sanderson, K.E., Spieth, J., Clifton, S.W., Latreille, P., Courtney, L., et al. (2001) Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature* 413: 852–856.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., et al. (1999) Food-related illness and death in the United States. *Emerg Infect Dis* 5: 607–625.

Nurizzo, D., Halbig, D., Sprenger, G.A., and Baker, E.N. (2001) Crystal structures of the precursor of glucose-fructose oxidoreductase from *Zymomonas mobilis* and its complexes with bound ligands. *Biochemistry* 40: 13857–13867.

Osborn, M.J., and Munson, R. (1974) Separation of the inner (cytoplasmic) and outer membranes of Gram-negative bacteria. *Methods Enzymol* 31: 642–653.

Palmer, T., and Berks, B.C. (2012) The twin-arginine translocation (Tat) protein export pathway. *Nat Rev Microbiol* 10: 483–496.

Ramasamy, S., Abrol, R., Suloway, C.J., and Clemens, W.M., Jr (2013) The glove-like structure of the conserved membrane protein TatC provides insight into signal sequence recognition in twin-arginine translocation. *Structure* 21: 777–788.

Richardson, D.J. (2000) Bacterial respiration: a flexible process for a changing environment. *Microbiology* 146: 551–571.

Rollauer, S.E., Tarry, M.J., Graham, J.E., Jaaskelainen, M., Jager, F., Johnson, S., et al. (2012) Structure of the TatC core of the twin-arginine protein transport system. *Nature* 492: 210–214.

Rose, R.W., Bruser, T., Kissinger, J.C., and Pohlschroder, M. (2002) Adaptation of protein secretion to extremely high-salt conditions by extensive use of the twin-arginine translocation pathway. *Mol Microbiol* 45: 943–950.

Rothery, R.A., Workun, G.J., and Weiner, J.H. (2008) The prokaryotic complex iron-sulfur molybdoenzyme family. *Biochim Biophys Acta* 1778: 1897–1929.

Sargent, F. (2007) Constructing the wonders of the bacterial world: biosynthesis of complex enzymes. *Microbiology* 153: 633–651.

Sargent, F., Berks, B.C., and Palmer, T. (2002) Assembly of membrane-bound respiratory complexes by the Tat protein-transport system. *Arch Microbiol* 178: 77–84.

Schubert, T., Lenz, O., Krause, E., Volkmer, R., and Friedrich, B. (2007) Chaperones specific for the membrane-bound [NiFe]-hydrogenase interact with the Tat signal peptide of the small subunit precursor in *Ralstonia eutropha* H16. *Mol Microbiol* 66: 453–467.

Schwarz, G. (2005) Molybdenum cofactor biosynthesis and deficiency. *Cell Mol Life Sci* 62: 2792–2810.

Shannugham, A., Wong Fong Sang, H.W., Bollen, Y.J., and Lill, H. (2006) Membrane binding of twin arginine preproteins as an early step in translocation. *Biochemistry* 45: 2243–2249.

Stanley, N.R., Sargent, F., Buchanan, G., Shi, J., Stewart, V., Palmer, T., and Berks, B.C. (2002) Behaviour of topological

© 2013 The Authors. Molecular Microbiology published by John Wiley & Sons Ltd., Molecular Microbiology, 90, 400–414
marker proteins targeted to the Tat protein transport pathway. *Mol Microbiol* **43**: 1005–1021.

Stoffels, L., Krehenbrink, M., Berks, B.C., and Unden, G. (2012) Thiosulfate reduction in *Salmonella enterica* is driven by the proton motive force. *J Bacteriol* **194**: 475–485.

Tarry, M., Schäfer, E., Chen, S., Buchanan, G., Greene, N.P., Lea, S.M., et al. (2009) Structural analysis of substrate binding by the TatBC component of the twin-arginine protein transport system. *Proc Natl Acad Sci USA* **106**: 13284–13289.

Thiennimitr, P., Winter, S.E., and Baumler, A.J. (2012) *Salmonella*, the host and its microbiota. *Curr Opin Microbiol* **15**: 108–114.

Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4354.

Volbeda, A., Amara, P., Darnault, C., Mouesca, J.M., Parkin, A., Roessler, M.M., et al. (2012) X-ray crystallographic and computational studies of the O₂-tolerant [NiFe]-hydrogenase 1 from *Escherichia coli*. *Proc Natl Acad Sci USA* **109**: 5305–5310.

Weiner, J.H., Bilous, P.T., Shaw, G.M., Lubitz, S., Frost, L., Thomas, G.H., et al. (1998) A novel and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins. *Cell* **93**: 93–101.

Winter, S.E., Thiennimitr, P., Winter, M.G., Butler, B.P., Huseby, D.L., Crawford, R.W., et al. (2010) Gut inflammation provides a respiratory electron acceptor for *Salmonella*. *Nature* **467**: 426–429.

Winter, S.E., Winter, M.G., Xavier, M.N., Thiennimitr, P., Poon, V., Keestra, A.M., et al. (2013) Host-derived nitrate boosts growth of *E. coli* in the inflamed gut. *Science* **339**: 708–711.

**Supporting information**

Additional supporting information may be found in the online version of this article at the publisher’s web-site.