Biosynthesis of selenate reductase in *Salmonella enterica*: critical roles for the signal peptide and DmsD

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*Salmonella enterica* serovar Typhimurium is a Gram-negative bacterium that is able to grow under a variety of environmental conditions (McClelland et al., 2001). As with most facultative anae-robics, *S. enterica* prefers to respire aerobically using short respiratory electron transport chains linked by quinones (Unden et al., 2014). Under anaerobic conditions, however, *S. enterica* can modify its respiratory chain specificity to cope with a range of alternative terminal electron acceptors such as thiosulfate (Stoffels et al., 2012), tetrathionate (Hensel et al., 1999) or nitrate (Lundberg et al., 2004). Indeed, the ability to utilize such a spectrum of electron acceptors has been implicated in the mechanism of infection and pathogenesis used by *S. enterica* (Lopez et al., 2015; Rowley et al., 2012; Winter et al., 2010, 2013).

One unusual terminal electron acceptor that *S. enterica* can utilize is selenate, which is a water-soluble oxidized form of selenium (Köhrl et al., 2000). *S. enterica* can reduce selenate to selenite, which is itself then further reduced to red elemental selenium (Guymer et al., 2009). The selenate reductase is encoded by the *ynfEFGH-dmsD* operon, which is predicted to be transcribed in response to anaerobiosis (Guymer et al., 2009). The YnfE protein is predicted to be the catalytic subunit of the enzyme and is a periplasmically oriented molybdenum-containing enzyme carrying an additional [4Fe–4S] cluster. YnfE is an orthologue of YnfE but does not contribute greatly to cellular selenate reductase activity (Guymer et al., 2009). YnfG is an electron transferring subunit that is predicted to link YnfE to the YnfH quinol oxidase in the inner membrane.

The ultimate step in the biosynthesis of the *S. enterica* selenate reductase is the transmembrane translocation of the YnfE catalytic subunit, most likely in tandem with the electron transferring subunit Ynflg (Guymer et al., 2009; Lubitz & Weiner, 2003). YnfE is synthesized as a precursor with an N-terminal twin-arginine signal peptide, which would direct the fully folded protein to the twin-arginine translocation (Tat) machinery for export (Palmer & Berks, 2012). Tat signal peptides share a common structure comprising a polar n-region and a central h-region separated by a conserved SRRxFLK twin-arginine motif (Palmer & Berks, 2012). The h-region is so named because it contains a stretch of 12–20 residues rich in glycine and hydrophobic side chains (Cristóbal et al., 1999). It is common in such complex multi-cofactor, multi-subunit Tat substrates for the cofactor insertion and membrane translocation steps to
Table 1. Bacterial strains and plasmids used and constructed in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. enterica</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LT2a</td>
<td>Wild strain (attenuated)</td>
<td>Lab. stock</td>
</tr>
<tr>
<td>DIG100</td>
<td>As LT2a, ΔtatABC</td>
<td>Guymer et al. (2009)</td>
</tr>
<tr>
<td>DIG103</td>
<td>As LT2a, ΔynfF (STM1498)</td>
<td>This work</td>
</tr>
<tr>
<td>KM01</td>
<td>As LT2a, ΔynfF ynfE L24Q</td>
<td>This work</td>
</tr>
<tr>
<td>KM02</td>
<td>As LT2a, ΔynfF ynfE A28Q</td>
<td>This work</td>
</tr>
<tr>
<td>KM03</td>
<td>As LT2a, ΔynfF ynfE L33Q</td>
<td>This work</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1655</td>
<td>F⁻ λ⁻ ivG⁻ rfb-50 rph-1</td>
<td>Blattner et al. (1997)</td>
</tr>
<tr>
<td>MAE01</td>
<td>As MG1655, ΔcyuA::Apra⁺</td>
<td>F. Sargent (unpublished)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUNI-PROM</td>
<td>As pT7.5, 103 bp <em>E. coli</em> tat promoter (Amp⁺)</td>
<td>Jack et al. (2004)</td>
</tr>
<tr>
<td>pUNI-DmsDst*</td>
<td>As pUNI-PROM, <em>S. enterica</em> dmsD</td>
<td>This work</td>
</tr>
<tr>
<td>pQE-80L</td>
<td>Overproduction vector (Amp⁰)</td>
<td>Qiagen</td>
</tr>
<tr>
<td>pQE-His-TEV-DmsDst</td>
<td>As pQE-80L, <em>S. enterica</em> dmsD</td>
<td>This work</td>
</tr>
<tr>
<td>pUT18</td>
<td>Encoding adenylate cyclase T18 domain (for N-t fusions), Amp⁺</td>
<td>Karimova et al. (1998)</td>
</tr>
<tr>
<td>pUT18-spYnfE†</td>
<td>Encoding spYnfE-T18 fusion</td>
<td>This work</td>
</tr>
<tr>
<td>pT25</td>
<td>Encoding adenylate cyclase T25 domain (for C-t fusions), Cm⁺²</td>
<td>Karimova et al. (1998)</td>
</tr>
<tr>
<td>pT25-DmsD‡</td>
<td>Encoding T25-DmsD fusion</td>
<td>This work</td>
</tr>
</tbody>
</table>

*Seven derivatives carrying individual codon changes were prepared but not listed here (Fig. 2c).
†Thirty-nine derivatives carrying individual codon changes were prepared but not listed here (Fig. 1b).
‡Seven derivatives carrying individual codon changes were prepared but not listed here (Fig. 3).

be coordinated by signal peptide binding proteins (Chan et al., 2014; Sargent, 2007; Turner et al., 2004). The signal peptide binding chaperone that interacts with the selenate reductase (YnfE) signal peptide is called DmsD and it is encoded within the ynfEFGH-dmsD operon (Guymer et al., 2009).

*S. enterica* DmsD is a member of the TorD/DmsD/NarJ family of peptide binding proteins (Chan et al., 2014). One of the best characterized members of this family is *Escherichia coli* DmsD, which shares 77% overall sequence identity with *S. enterica* DmsD. *E. coli* DmsD is involved in the assembly of DMSO reductase (Oresnany & Clemons, 2001) and selenate reductase (Guymer et al., 2009) in that organism. Although the *E. coli* DmsD protein can form homo-oligomers in vitro (Sarfo et al., 2004), the monomeric form has been structurally characterized (Ramasamy & Clemons, 2009; Stevens et al., 2009, 2012). The *E. coli* DmsD protein is thought to bind to the h-region of the DmsA Tat signal peptide (Shanmugham et al., 2012; Winstone et al., 2013), but a precise binding epitope has not yet been established experimentally.

The *S. enterica* DmsD protein has been structurally characterized and is a 204 amino acid residue monomer consisting of 11 α-helices and no β-strands (Qiu et al., 2008). The purified protein binds tightly to the *S. enterica* YnfE signal peptide (Kₐ ~45 nM) (Guymer et al., 2009). In this work, genetic and biochemical approaches were taken to identify precisely the binding epitope for DmsD on the YnfE signal peptide. YnfE residues L24 and A28 were found to be involved in both DmsD recognition and selenate reductase biosynthesis. A YnfE A28Q variant could be rescued by providing DmsD in excess and this was used as a genetic screen to identify inactive variants of *S. enterica* DmsD. One key residue was identified as V16. Finally, a synthetic peptide containing the YnfE binding epitope could be chemically crosslinked to DmsD and signal peptide binding was found to prevent DmsD dimerization in vitro.

**METHODS**

**Growth and construction of *S. enterica* strains.** Naturally attenuated *S. enterica* serovar Typhimurium LT2a was used as the parental strain for this research (Jamieson et al., 1986). *S. enterica* was typically grown in 'low salt' LB medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl] and, where necessary, was transformed with plasmids using electroporation. Strains were grown microaerobically with shaking at 37°C in a volume of 5 ml in a 30 ml universal tube. The ΔynfE strain DIG103 (Guymer et al., 2009) was further modified to separately carry ynfE L24Q, ynfE A28Q and ynfE L33Q alleles (Table 1). Gene replacement on the chromosome was achieved through the use of the vector pMAK705 for homologous recombination (Hamilton et al., 1989). The genetic region ~500 base pairs upstream and downstream of the codons of interest was amplified from DIG103 genomic template using primers 5’-GGCGAAGCTTAGACATGTCATTATCAGAC-3’ and 5’-GGCGGAATTCGGTGGTGTCAGAACC-3’ and first
cloned into pBluescript KSI (Amp\(^\beta\)) as a BamHI–HindIII fragment as an intermediate to allow facile codon changes by the QuikChange PCR protocol (Stratagene). Mutant alleles were then transferred to pMAK705 and on to the chromosome of DIG103 (Hamilton et al., 1989) and verified by sequencing.

**Construction of plasmids for production of DmsD and its variants.** The _S. enterica dmsD_ gene was amplified by PCR and cloned as a BamHI–PstI fragment into the pUNI-PROM (Amp\(^\beta\)) vector, which carries the constitutive _E. coli_ tat promoter as an EcoRI–BamHI insert (Jack et al., 2004), to give pUNI-DmsDst. This plasmid was further mutagenized using the QuikChange PCR protocol (Stratagene) to yield V16Q, W91Q, R94Q, S96Q, G100Q, T103Q and G171Q derivatives. For high-level overexpression of _dmsD_ in _E. coli_, the _S. enterica dmsD_ gene was amplified with primers 5′-GGCGGGATCCGAAAACCTGTA TTTTGCGGATGACACTTTTTTACACCGGATG-3′, which would incorporate an in-frame tobacco etch virus (TEV) protease cleavage site sequence, and 5′-GGCGGACGTTTATTAACCGGAAAGGC TTTCACGGCCGC-3′ before being cloned as a BamHI–HindIII fragment into pQE-80L (Qiagen).

**Bacterial two-hybrid analysis.** The bacterial two-hybrid system is based on the reconstitution of an adenylate cyclase signal transduction pathway in _E. coli_ cyA\(^\beta\) mutant where two complementary fragments, T18 and T25, of a soluble adenylate cyclase domain from _E. coli_ (as MG1655 _cyaA_−) cells were transformed with relevant _E. coli_ _tat_ inhibitors (protease inhibitor cocktail set III, EDTA-free; Calbiochem), lysosyme and DNase were added before cells were lysed by three times passage at 15000 p.s.i. through an EmulsiFlex C3 high-pressure homogenizer. Cell debris was removed by centrifugation at 27 143 g at 4°C. The resulting supernatant was filtered through a 0.45 μm membrane filter before being loaded onto a 5 ml HisTrap HP column (GE Healthcare) pre-equilibrated with 25 mM Tris/HCl (pH 7.5), 25 mM imidazole and 250 mM NaCl at 10 μl g\(^{-1}\) cells (wet weight). Protease inhibitors (protease inhibitor cocktail set III, EDTA-free; Calbiochem), 1 mM phenylmethylsulfonyl fluoride and 20 μM leupeptin were added. Protein G-agarose beads were washed three times with wash buffer (25 mM Tris/HCl (pH 7.5), 250 mM NaCl and 0.5 mM imidazole) and the bound protein eluted in 20 μM imidazole and 250 mM NaCl at 0.5 ml min\(^{-1}\). Bound protein was eluted in the same buffer with an imidazole gradient of 25–500 mM imidazole over five column volumes. Fractions containing the protein of interest were identified by SDS-PAGE and were pooled and concentrated if required. To remove the affinity tag, purified protein was mixed with in-house TEV\(^{\text{HiP}}\) protease in a ratio of 10:1 (per milligram). This was dialysed for 16 h at 4°C against 25 mM Tris/HCl (pH 7.5), 25 mM imidazole, 250 mM NaCl and 1 mM DTT. Cleaved DmsD protein was isolated by reverse immobilized metal affinity chromatography with the unbound fraction retained for further experimentation.

**Chemical crosslinking in vitro.** DmsD and its variants were dialysed into 50 mM HEPES (pH 7.6) and 150 mM KCl (pH 6.8) pre-equilibrated with 25 mM Tris/HCl (pH 7.5), 25 mM imidazole and 250 mM NaCl at 0.5 ml min\(^{-1}\). Bound protein was eluted in the same buffer with an imidazole gradient of 25–500 mM imidazole over five column volumes. Fractions containing the protein of interest were identified by SDS-PAGE and were pooled and concentrated if required. To remove the affinity tag, purified protein was mixed with in-house TEV\(^{\text{HiP}}\) protease in a ratio of 10:1 (per milligram). This was dialysed for 16 h at 4°C against 25 mM Tris/HCl (pH 7.5), 25 mM imidazole, 250 mM NaCl and 1 mM DTT. Cleaved DmsD protein was isolated by reverse immobilized metal affinity chromatography with the unbound fraction retained for further experimentation.

**Protein analytical techniques.** Proteins were analysed by SDS-PAGE by the method of Laemmli (1970). Where stated, Western immunoblotting was carried out by transferring proteins previously separated by SDS-PAGE onto nitrocellulose (Dunn, 1986). Secondary antibodies

**Table 2. Synthetic peptides used in this study**

<table>
<thead>
<tr>
<th>Name</th>
<th>Amino acid sequence</th>
<th>Length (aa)</th>
<th>Molecular mass (kDa)</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide 1</td>
<td>SLALAAAGGVS LPFGMRK</td>
<td>17</td>
<td>1.675</td>
<td>Crosslinking</td>
</tr>
<tr>
<td>Peptide 2</td>
<td>SRRTLVKSALGSLAAGGVS LPFGMRK</td>
<td>29</td>
<td>2.915</td>
<td>Crosslinking</td>
</tr>
<tr>
<td>Peptide 3</td>
<td>SRRTLVKSALGSLAAGGVS LPFGMRK</td>
<td>28</td>
<td>2.787</td>
<td>Crosslinking</td>
</tr>
</tbody>
</table>
conjugated to horseradish peroxidase (Bio-Rad) were used allowing development of immunoblots by chemiluminescence.

RESULTS

Genetic evidence for the DmsD binding epitope on the S. enterica YnfE signal peptide

In order to understand further the role of the YnfE signal peptide in selenate reductase activity, the first step was to identify the binding epitope for DmsD on the signal peptide. To do this, a glutamine scanning mutagenesis approach was taken combined with a bacterial two-hybrid genetic screen based on reconstitution of adenylate cyclase activity. When the T18 and T25 fragments of B. pertussis toxin adenylate cyclase domain are co-expressed as separate polypeptides, they do not interact. However, adenylate cyclase activity can be restored to an E. coli ΔcytA host strain if T18 and T25 are brought close together by fusing each to interacting proteins (Karimova et al., 1998).

In this work, the 42-residue YnfE Tat signal peptide (spYnfE) was placed at the N-terminus of T18. Next, a plasmid was constructed that encoded a T25-DmsD fusion protein, with S. enterica DmsD covalently attached to the C-terminus of T25. Co-expression of both plasmids in the appropriate reporter strain demonstrated a strong interaction between spYnfE and DmsD (Fig. 1). Scanning

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**Fig. 1.** Genetic analysis of the YnfE signal peptide and DmsD interaction. (a) Primary amino acid sequences of the signal sequences of the three binding partners of DmsD from both E. coli and S. enterica aligned using CLUSTALW (http://embnet.vital-it.ch/software/ClustalW.html), and highlighted using BoxShade (http://embnet.vital-it.ch/software/BOX_form.html). The hydrophobic region of the signal peptides is labelled. Arrows indicate positions of S. enterica YnfE L24, A28, V31, L33 and F35. Note that the gene sequence of S. enterica ynfE suggests two possible adjacent translation initiation sites. The nomenclature used here assumes that a single methionine is at the N-terminus of YnfE. (b) E. coli strain MAE01 (ΔcytA::Apra) was co-transformed with the vectors pT25-DmsD and pUT18-spYnfE (and mutant versions) before strains were cultured aerobically and β-galactosidase was measured as an indicator of protein–protein interactions. β-Galactosidase activities are displayed relative to native activity generated by the spYnfE–DmsD interaction (WT). Cells containing both empty vectors were used as the negative control (labelled ‘UT18+T25’). In this data set, the positive control (100%) was recorded as 1646±160 Miller units and the negative control was recorded as 166±30 Miller units. Data expressed relative to the positive control as means ± standard error of means (n=3).
mutagenesis of the YnfE sequence was then performed, involving the substitution of each individual amino acid (from E3 to A42, but excluding Q6 and Q7) of the signal peptide with glutamine. Glutamine is a polar amino acid rarely present in Tat signal peptides (Cristobal et al., 1999), and therefore is an ideal amino acid for attempting to disrupt Tat signal peptide function. In the case of YnfE Q6 and Q7, these side chains were substituted with tryptophan.

Using the site-directed mutagenesis approach, the amino acids of the highly conserved twin-arginine motif were found to be not involved in chaperone binding in this assay (Fig. 1). This was also the case regarding the entire n-region of the YnfE signal peptide (Fig. 1). Similarly, the polar c-region of the signal peptide was found to be not critical for the interaction (Fig. 1). However, the data clearly highlighted the hydrophobic stretch of the signal peptide as important in interactions with DmsD (Fig. 1). The spYnfE L24Q, A28Q, V31Q, L33Q and F35Q variants all displayed reduced levels of β-galactosidase activity – approximately 10% of that observed for the native signal peptide (Fig. 1), which were in fact similar values to the background levels observed for the negative controls (Fig. 1).

Signal peptide amino acid substitutions affect selenate reduction in vivo

Bacterial two-hybrid analysis implicated five YnfE residues L23, A28, V31, L33 and F35 as possibly playing a role in DmsD interactions. To explore the physiological roles of these YnfE residues, the next task was to incorporate the mutant alleles onto the native copy of ynfE on the S. enterica chromosome. To do this, strain DIG103, which contains an in-frame deletion of the gene coding for YnfE, which shares 71% sequence overall identity with YnfE, was selected since this strain produces only YnfE as its sole selenate reductase catalytic subunit (Guymer et al., 2009). Using technology based on homologous recombination, it was possible to construct three mutant strains (Table 1). These were KM01 (ΔynfF, ynfE L24Q), KM02 (ΔynfF, ynfE A28Q) and KM03 (ΔynfF, ynfE L33Q) (Table 1).

A direct biochemical assay for selenate reductase activity has not been developed for the S. enterica enzymes, most likely because of poor expression levels and poor reactivity with redox dyes (Guymer et al., 2009); however, it is possible to detect selenate reduction by a facile growth test (Guymer et al., 2009). Incubation of cells in the presence of 10 mM selenate leads to initial reduction of selenate to selenite by selenate reductase and then further reduction of selenite to a red elemental selenium precipitate. In this case, the three mutant strains KM01 (ynfE L24Q), KM02 (ynfE A28Q) and KM03 (ynfE L33Q) were cultured in rich medium containing selenate (Fig. 2a). Strains producing the spYnfE variants L24Q and A28Q were clearly devoid of selenate reductase activity (Fig. 2a), while the spYnfE variant L33Q retained the ability to reduce selenate in vivo (Fig. 2a).

Overproduction of DmsD rescues selenate reductase activity in the YnfE A28Q variant

The implication of spYnfE L24 and A28 in DmsD binding (Fig. 1), together with the selenate reductase-minus phenotypes of KM01 (spYnfE L24Q) and KM02 (spYnfE A28Q) (Fig. 2), immediately suggested the possibility of designing a second-site suppressor positive screen. This would involve constructing a random mutant library for dmsD that could be screened for those able to recognize the variant signal peptides and rescue selenate reductase activity. To begin to explore this idea, the KM01 (spYnfE L24Q) and KM02 (spYnfE A28Q) strains were transformed with pUNI-DmsDst, encoding native S. enterica DmsD, and the empty vector pUNI-PROM. Following overnight growth with sodium selenate, KM01 (spYnfE L24Q) containing either pUNI-PROM or pUNI-DmsDst remained devoid of selenate reductase activity (Fig. 2b). However, it was clear that the KM02 (spYnfE A28Q) with pUNI-DmsDst was restored for in vivo selenate reductase activity (Fig. 2b).

The KM01 (spYnfE L24Q) strain was deemed suitable for screening a dmsD mutant library. This was prepared by error-prone PCR where the final resultant error rate was 1.5% (4–18 errors across 615 bp of dmsD). The PCR products were cloned into vector pUNI-PROM resulting in around 300,000 individual clones. Screening the library involved transformation of KM01 (spYnfE L24Q) and plating directly onto LB agar plates containing 10 mM sodium selenate and appropriate antibiotic. The plates were then incubated for 36 h under anaerobic conditions with colonies then being picked for further analysis according to colour. For KM01 (spYnfE L24Q), red colonies were desired as this would indicate reconstitution of selenate reductase activity. Unfortunately, no selenate reductase-positive clones were identified in this screen.

A negative screen for isolation of DmsD variants affecting selenate reductase activity

The colorimetric plate test was next adapted for the isolation of inactive variants of S. enterica DmsD. In this case, the behaviour of KM02 (spYnfE A28Q) when transformed with pUNI-DmsDst was exploited (Fig. 2b). The hypothesis to be tested was that since the combination of the YnfE A28Q substitution and increased levels of active DmsD allowed red selenium deposits to be observed, then this assay may also be used to select for inactive variants of DmsD that do not induce selenium precipitation.

The dmsD mutant library was used in the transformation of KM02 (spYnfE A28Q), with cells plated directly onto LB (ampicillin) agar. Individual colonies were then picked onto fresh solid medium plates containing 10 mM sodium selenate and appropriate antibiotic. The plates were then incubated for 36 h under anaerobic conditions and colonies that remained white (and so contained no selenate reductase activity) were used to inoculate 30 ml anaerobic liquid cultures. Aliquots of cells were then harvested, whole cell proteins
separated by SDS-PAGE and Western immunoblotting carried out with an anti-DmsD (E. coli) serum to screen for clones producing full-length DmsD (Fig. S1, available in the online Supplementary Material).

This approach identified three clones that produced full-length recombinant DmsD but were unable to rescue selenate reductase activity in KM02 (ynfE A28Q). Gene sequencing of the three clones revealed that one encoded a single W91R substitution in DmsD, one carried two mutations encoding R94H and G100S and the third contained four mutations coding for a DmsD variant with V16G, S96G, T103I and G171R substitutions. Next, each of the seven identified residues was individually targeted and a bank of seven vectors was prepared, encoding variant DmsD proteins carrying glutamine substitutions at positions 16, 91, 94, 96, 100, 103 and 171. The KM02 (spYnfE A28Q) strain was transformed individually by each of the seven plasmids and cultured in the presence of 10 mM selenate (Fig. 2c). It is clear that V16Q, W91Q and G100Q versions of DmsD are unable to coordinate selenate reductase biosynthesis in S. enterica (Fig. 2c).

For quantification of the ability of the identified DmsD variants to interact with the signal sequence of YnfE, the bacterial two-hybrid system was utilized (Fig. 3).
experiment, the seven dmsD mutations identified in the random screen were moved on to plasmid pT25-DmsD. Interactions of these DmsD variants were tested against the spYnfE-T18 fusion through use of the β-galactosidase reporter system (Fig. 3). The results show that DmsD W91Q, R94Q, S96Q, G100Q, T103Q and G171Q typically displayed β-galactosidase activity levels close to or in excess of native levels (Fig. 3). These were therefore considered to be essentially unimpaired in signal peptide recognition. However, the DmsD V16Q variant clearly differed in its β-galactosidase activity, with levels almost identical to that of the empty vector negative control (Fig. 3).

Taken altogether, these experiments suggest that YnfE signal peptide residue A28 is specifically involved in the DmsD interaction as part of a minimum binding epitope covering the C-terminal half of the h-region. The YnfE A28Q variant cannot be impaired in Tat transport per se, since it can be rescued by simply increasing cellular levels of DmsD. In addition, these experiments identified DmsD V16 as playing a critical role in the interaction with the YnfE signal peptide and, interestingly, two other DmsD variants (W91Q and G100Q) were isolated that retain signal peptide recognition capabilities but are unable to restore selenate reductase activity to a mutant strain.

Using chemical crosslinking to study the spYnfE–DmsD interaction

The genetic experiments (Figs 1 and 2) have helped us to identify the potential binding epitope for DmsD on the YnfE signal peptide. The 16 residue sequences between YnfE S23 and R38 contain all of the critical amino acids for DmsD binding (Fig. 1). In order to biochemically characterize binding by this epitope, with the long-term view of obtaining structural information on the binary complex, synthetic peptides were synthesized covering this region

![Image](https://microbiologyresearch.org/dmsD.png)

**Fig. 3.** DmsD variant V16Q is unable to recognize the YnfE signal peptide. *E. coli* strain MAE01 (ΔcygA::Apra) was co-transformed with vectors pUT18-spYnfE and pT25-DmsD plus variants and β-galactosidase was measured as an indicator of protein–protein interactions. β-Galactosidase activities are displayed relative to native spYnfE–DmsD activity (WT). Cells containing both empty vectors were used as the negative control (UT18+T25). In this data set, the positive control (100 %) was recorded as 1718±212 Miller units and the negative control was recorded as 108±10 Miller units. Data expressed relative to the positive control as means±SEM (n=3).

![Image](https://microbiologyresearch.org/chemical_crosslinking.png)

**Fig. 4.** DSS or formaldehyde, but not EDC, can crosslink a peptide ligand to DmsD. Purified DmsD (2 µM) and synthetic peptide 2 (Table 2; 100 µM) were incubated together in a final volume of 100 µl prior to the addition of chemical crosslinkers [1 mM DSS; 1 mM EDC; 1 % (v/v) formaldehyde, final concentrations]. Reactions were stopped after 30 min with 50 mM Tris/HCl (pH 8.0) before 10 µl samples were analysed by SDS-PAGE [17 % (w/v) acrylamide gel] and Western immunoblotting. Antibodies used were anti-*E. coli* DmsD (1 : 20,000) with a horseradish peroxidase-conjugated anti-rabbit IgG secondary (1 : 10,000). The asterisk marks the position of peptide crosslinked DmsD.
addition of 50 mM Tris/HCl (pH 8) before 10 volume of 100

The reaction was stopped after 30 min with the addition of 50 mM Tris/HCl (pH 8) before 10 µl samples were analysed by SDS-PAGE [17 % (w/v) acrylamide gel] and Western immunoblotting. Antibodies used were anti-DmsD (1 : 20 000) and an anti-rabbit IgG secondary (1 : 10 000). The positions of the DmsD monomer (DmsD), dimer (2×DmsD) and peptide crosslinked forms (*) are indicated.

These synthetic peptides do not contain terminal amino or carboxyl groups; however, because the genetic data described here point to no role for the Tat motif and a binding epitope at the extreme C-terminus of the YnfE signal, an extra lysine residue was incorporated in the peptide (Table 2). These synthetic peptides do not contain terminal amino or carboxyl groups; however, because the genetic data described here point to no role for the Tat motif and a binding epitope at the extreme C-terminus of the YnfE signal, an extra lysine residue was incorporated in the peptide...
experimentally, a third synthetic peptide (peptide 3) was synthesized that contained a single internal lysine close to the twin-arginine motif (Table 2). When Western immunoblotting was used, crosslinked complexes between peptide 3 and DmsD could be weakly detected (Fig. 6); however, the efficiency of crosslinking was clearly weaker in comparison with experiments using peptide 2 (Figs 1 and 5) with incubation in 100× peptide 3 producing only a weak complex signal (Fig. 6). Taken together, these data indicate that all three peptides tested can form interactions with DmsD to differing extents in vitro and the binary complex can be chemically crosslinked using DSS.

DISCUSSION

A semi-conserved DmsD binding epitope on Tat signal peptides

The S. enterica DmsD protein binds directly to at least three Tat signal peptides in the cell, including those of DmsA, YnfE and YnfF (Guymer et al., 2009). These three Tat signal peptides already contain obvious stretches of sequence conservation (Fig. 1a). However, the n-region of the YnfE signal peptide, which precedes the conserved Tat motif, is very different in primary structure from that of DmsA and YnfF (Fig. 1a). This immediately suggests that, if the DmsD recognition sequence is to be similar among the three signal peptides, the n-region is not involved in chaperone binding. The genetic data presented here appear to corroborate that hypothesis, with signal peptide-DmsD interactions recorded for all variants constructed between YnfE G3 and V10 (Fig. 1a).

These data also point to no role for the twin-arginine motif in binding S. enterica YnfE by DmsD (Fig. 1a). In this regard, the behaviour of S. enterica DmsD is similar to E. coli TorD, which does not rely on the Tat motif for signal peptide recognition (Buchanan et al., 2008). Note, however, that, for Archaeoglobus fulgidus TtrD, which is related to DmsD, and E. coli NapD, which is from a different protein family to DmsD (Turner et al., 2004), the twin-arginine motif, or residues close to it, was found to be part of the chaperone binding epitope (Coulthurst et al., 2012; Grahl et al., 2012). There is also some biochemical evidence that E. coli DmsD does interact with the twin-arginine motif to some extent (Winstone & Turner, 2015), while the hydrophobic h-region contains the major drivers for specificity (Shanmugham et al., 2012; Winstone et al., 2013).

In this work, certain key side chains in the YnfE signal peptide h-region were found to be critical for observation of a clear interaction with S. enterica DmsD in vivo (Fig. 1b). The bacterial two-hybrid data suggested that five residues could be important – YnfE L24, A28, V31, L33 and F35 (Fig. 1b). Of these, the A28 residue was established here as being important for DmsD recognition and biosynthesis of selenate reductase. Based on the two-hybrid data, three mutant strains were constructed encoding YnfE L24Q, YnfE A28Q and YnfE L33Q. Although L33 is completely conserved in all target peptides for DmsD (this is equivalent to L37 in E. coli DmsA), a strain carrying an L33Q mutation in the ynfE gene was still able to correctly assemble selenate reductase (Fig. 2a). On the other hand, strains producing YnfE L24Q or A28Q were devoid of selenate reductase activity. The equivalent of L24 is completely conserved in all DmsD target peptides (Fig. 1a); however, it is most likely that this residue has a dual role in both DmsD binding and Tat translocation. This is because no suppressors whatsoever were found in the dmsD mutant library screen. The YnfE A28Q variant, however, could be rescued by supplying extra native DmsD to the cell, which would be expected if the YnfE A28Q substitution reduced the affinity of the signal peptide for DmsD but was not important for final Tat translocation following enzyme assembly. Interestingly, the tolerance of the YnfE signal peptide to an A28Q substitution may have been predictable. In other DmsD-dependent systems, this residue is often a polar serine side chain (Fig. 1a).

Overall, this section of work identified a short stretch of hydrophobic residues between YnfE L24 and F35 as being the location of the DmsD binding epitope. When plotted on a helical wheel, the L24, A28, V31 and F35 side chains align perfectly onto one face of the α-helix (Fig. S3). Interestingly, L33 is an outlier, being predicted to locate on the opposite side of the helix to the L24/A28/V31/F35 face (Fig. S3). This, taken together with the positive selenate reductase activity in a YnfE L33Q strain, may suggest that the L33Q result in the bacterial two-hybrid study was a ‘false negative’. The data uncover a semi-conserved DmsD binding site at the C-terminal end of the signal peptide h-region for YnfE, YnfF and DmsA.

Signal peptide binding by DmsD

The second strand of this study involved the biochemical properties of DmsD itself. A random genetic screen identified three residues that may be critical for the function of S. enterica DmsD. The first of these is DmsD V16, which appears to be important for signal peptide binding but has not been implicated previously in other studies of chaperone function. The S. enterica DmsD V16 residue lies close to G18 of E. coli DmsD implicated in peptide binding by NMR (Stevens et al., 2012). The other interesting DmsD variants identified in this work (W91Q and G100Q) highlight the possibility that DmsD may play a biochemical role in the cell beyond peptide recognition and binding. Both S. enterica DmsD variants were capable of peptide binding using a genetic test, but were impaired in assembly of an active selenate reductase.

The E. coli DmsD protein has been extensively mutagenized and peptide binding of the variant proteins analysed in vitro (Chan et al., 2008; Winstone & Turner, 2015). A key residue for efficient binding of the E. coli DmsA signal peptide by E. coli DmsD is W87, which has a direct equivalent in S. enterica DmsD and is distinct from W91 identified here.
While the physiological function of *E. coli* DmsD W87S and W87Y variants has not been reported, the identification of an *S. enterica* variant in this region by a completely random screen reinforces the critically important nature of this part of DmsD to its function.

**Signal peptide binding prevents dimerization of DmsD in vitro**

The chemical crosslinking experiments described here introduce a hitherto unexplored approach to studying signal peptide binding by Tat proofreading chaperones. The main conclusions to be drawn are that amine-specific crosslinkers such as DSS and formaldehyde are an appropriate choice for studying chaperone–Tat signal peptide interactions, and that the self-dimerization of DmsD frequently observed *in vitro* can be impaired by peptide binding. The latter was the most surprising observation from the crosslinking experiments. DmsD-like proteins are known to form dimers and higher oligomers *in vitro* (Guymer et al., 2010; Sarfo et al., 2004; Tranier et al., 2002). In this work, dimer formation can be captured by the addition of DSS or formaldehyde as a crosslinker (Figs 4 and 5). Most interestingly, however, the formation of DmsD dimer can be prevented by co-incubation with signal peptide (Figs 4 and 5). This suggests that the dimerization interface comprises, or is close to, the signal peptide binding site on the surface of DmsD.

Another interesting observation was the possible generation of two different DmsD–peptide crosslinked species under some circumstances (Figs 4 and 5). By Western immunoblotting, a clearly slower-migrating complex matching closely the predicted molecular mass of a DmsD–peptide 2 combination was seen after DSS treatment (Fig. 4) but a weaker band of even larger mass, possibly indicative of two peptides crosslinked to one DmsD protein, was also observed (Fig. 4). It should be noted, however, that this larger DmsD–peptide complex was only seen with peptide 2 (which contains two lysine side chains) and previous *in vitro* calorimetric analysis identified only one high-affinity peptide binding site per DmsD (Guymer et al., 2009). It is possible that, under the conditions used, a pool of peptide 2–peptide 2 self-crosslinks are being generated that retain a free lysine side chain available for crosslinking to DmsD. Taken altogether, the crosslinking experiments and other published evidence suggest that DmsD dimerization is prevented by signal peptide binding and that only one signal peptide binds per DmsD monomer.

In conclusion, this work adds new knowledge to the function of Tat signal peptides and their relationship with their cognate Tat proofreading chaperones. The *S. enterica* selenate reductase is a useful model system for understanding the biosynthesis of complex metalloenzymes and it has here helped to define the binding epitope for DmsD on a Tat signal peptide. The work also reinforces the tangled relationship between two different biochemical processes carried out by the YnfE signal peptide and DmsD. Clearly, the Tat transport activity of the signal peptide overlaps with its chaperone binding function, with the YnfE L24 side chain implicated in both. This finding may also add to the hypothesis that DmsD may have a role in protein targeting (Kostecki et al., 2010; Kuzniatsova et al., 2016), though there is also evidence against that (Ray et al., 2003). On the other hand, this work has not only identified DmsD residues that may be important for signal peptide recognition but also identified others that are involved in an as yet undefined biochemical process outwith peptide binding.

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