YgfX (CptA) is a multimeric membrane protein that interacts with the succinate dehydrogenase assembly factor SdhE (YgfY)

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Serratia sp. strain ATCC 39006 produces the red-pigmented antibiotic prodigiosin. Prodigiosin biosynthesis is regulated by a complex hierarchy that includes the uncharacterized protein YgfX (DUF1434). The ygfX gene is co-transcribed with sdhE, an FAD assembly factor essential for the flavinylation and activation of the SdhA subunit of succinate dehydrogenase (SDH), a central enzyme in the tricarboxylic acid cycle and electron transport chain. The sdhEygfX operon is highly conserved within the Enterobacteriaceae, suggesting that SdhE and YgfX function together. We performed an extensive mutagenesis to gain molecular insights into the uncharacterized protein YgfX, and have investigated the relationship between YgfX and SdhE. YgfX localized to the membrane, interacted with itself, forming dimers or larger multimers, and interacted with SdhE. The transmembrane helices of YgfX were critical for protein function and the formation of YgfX multimers. Site-directed mutagenesis of residues conserved in DUF1434 proteins revealed a periplasmic tryptophan and a cytoplasmic aspartate that were crucial for YgfX activity. Both of these amino acids were required for the formation of YgfX multimers and interactions with SdhE but not membrane localization. Multiple cell division proteins were identified as putative interaction partners of YgfX and overexpression of YgfX had effects on cell morphology. These findings represent an important step in understanding the function of DUF1434 proteins. In contrast to a recent report, we found no evidence that YgfX and SdhE form a toxin–antitoxin system. In summary, YgfX functions as a multimeric membrane-bound protein that interacts with SdhE, an important FAD assembly factor that controls SDH activity.

INTRODUCTION

Oxidative phosphorylation, the predominant means of energy generation in both eukaryotic and prokaryotic organisms, is driven by the electron transport chain and the tricarboxylic acid cycle (Saraste, 1999; Smeitink et al., 2001). Succinate dehydrogenase (SDH, also known as Complex II or succinate:quinone oxidoreductase) is the only enzyme that functions in both the tricarboxylic acid cycle and the electron transport chain (Cecchini, 2003; Yankovskaya et al., 2003). SDH functions by coupling the oxidation of succinate to fumarate with the reduction of ubiquinone, resulting in the donation of electrons to the electron transport chain (Cecchini, 2003). SDH is also a major contributor to the establishment of infections caused by enterobacterial pathogens, such as Escherichia coli and Salmonella (Alteri et al., 2009; Bowden et al., 2010; Mercado-Lubo et al., 2008; Tchawa Yimga et al., 2006).

Essential to SDH function is the covalent attachment of the redox cofactor flavin adenine dinucleotide (FAD) to the flavoprotein subunit SdhA (Hao et al., 2009; Hederstedt, 1983; McNeil et al., 2012; Robinson et al., 1994). Recent work has shown that eukaryotes and the α, β and γ-proteobacteria share a conserved DUF339 protein (termed Sdh5 in eukaryotes and SdhE in bacteria) that is essential for the flavinylation of the SDH flavoprotein, Sdh1 and SdhA, respectively (Hao et al., 2009; McNeil et al., 2012; McNeil & Fineran, 2013). The loss of Sdh5/SdhE prevents FAD incorporation into Sdh1/SdhA and results in a non-functional SDH (Hao et al., 2009; McNeil et al., 2012). The process of flavinylation has traditionally been thought of as autocatalytic (Edmondson & Newton-Vinson, 2001; Heuts...
et al., 2009; Kim et al., 1995; Mewies et al., 1998). Consequently, SdhE was the first FAD assembly factor identified in bacteria (McNeil et al., 2012).

Within the Enterobacteriaceae, sdhE exists in a bicistronic operon with the gene encoding the membrane-bound conserved hypothetical protein YgfX (Fineran et al., 2005b; McNeil et al., 2012). In Serratia sp. ATCC 39006, both genes are involved in the transcriptional regulation of the biosynthetic genes (pigA–O) required for production of the secondary metabolite prodigiosin (pig) (McNeil et al., 2012). YgfX and SdhE lack DNA binding motifs, so this regulation is not likely to be via direct transcriptional control (Fineran et al., 2005b; Lim et al., 2005; McNeil et al., 2012). The regulation of pig biosynthesis in Serratia sp. ATCC 39006 involves a regulatory network that integrates signals from quorum sensing (Fineran et al., 2005b; Slater et al., 2003; Thomson et al., 2000), cyclic-di-GMP (Fineran et al., 2007; Williamson et al., 2008), phosphate availability (Gristwood et al., 2009; Slater et al., 2003) and carbon sources (Fineran et al., 2005a). Within this network, the master regulator, PigP, represses expression of the sdhE-ygfX operon (Fineran et al., 2005b). Previous genetic and bioinformatic analyses of SdhE and YgfX (McNeil et al., 2012) suggest these two proteins form a conserved system of functional importance amongst the Enterobacteriaceae. We previously demonstrated the role of SdhE and we have shown that YgfX is a major factor in pig production (Fineran et al., 2005b; McNeil et al., 2012). However, YgfX has not been examined in detail.

The aims of this study were to dissect the functional motifs and domains of YgfX and to assess the relationship with SdhE. YgfX interacted with itself and with SdhE, whilst the transmembrane domains and several conserved amino acids were critical for the localization, function and interactions of YgfX. In contrast to a recent study (Masuda et al., 2012b), over-expression of YgfX in our study was not toxic in Serratia sp. ATCC 39006 or E. coli. However, in agreement with this recent study, YgfX interacted with multiple cell division proteins.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** Bacterial strains and plasmids are listed in Tables S1 and S2, available in Microbiology Online. Serratia sp. ATCC 39006 and E. coli strains were grown at 30 °C and 37 °C, respectively. Cultures were grown in Luria broth (LB: 5 g l⁻¹ yeast extract, 10 g l⁻¹ bacto tryptone and 5 g l⁻¹ NaCl) or M9 minimal medium with 0.5% glycerol and 0.2% Casamino acids (Sambrook et al., 1989) at 180 r.p.m., or on LB supplemented with 1.5% (w/v) agar (LBA) (Miller, 1972). Unless otherwise stated, all cultures were grown in 25 ml of media in 250 ml flasks. Antibiotics were used at the following concentrations: kanamycin 50 μg ml⁻¹ (Km), ampicillin 100 μg ml⁻¹ (Ap) and chloramphenicol 25 μg ml⁻¹ (Cm).

**DNA manipulation and sequencing analysis.** Molecular biology techniques, unless stated otherwise, were performed using standard techniques. Oligonucleotides are shown in Table S3. DNA sequencing was performed at the Allan Wilson Centre, New Zealand and analysed using Chromas and BLAST2. Membrane topology mapping was performed using Phobius (Käll et al., 2007) and displayed using TOPO2 (http://www.sacs.ucsf.edu/TOPO2/). YgfX homologues were identified using Pfam (Finn et al., 2010) and downloaded from Uniprot. Alignments were performed using Clustal W2 (Larkin et al., 2007) and outputs generated using Boxshade 3.21 (www.ch.embnet.org/software/BOX_form.html). Secondary structure analysis was performed using Jpred (Cole et al., 2008) with Serratia sp. ATCC 39006 YgfX as the input query.

**Plasmid construction.** The construction of plasmids expressing untagged or tagged full-length, truncated, MalF domain swap and alanine swap mutagenesis variants of YgfX is detailed in the supplementary methods. Plasmids constructed in this study were confirmed by sequencing and pQE-80LoriT derivatives were introduced into Serratia sp. ATCC 39006 by conjugation using either E. coli SM10 pir or S17-1 pir using minimal media to counter-select the donors and appropriate antibiotics to select for the transconjugants. Other plasmids were introduced by transformation.

**Cellular localization of His-tagged YgfX variants.** Soluble and membrane fractions of His-YgfX (pMIC2), His-YgfXΔ1–54 (pMIC3), His-MalF-YgfX (pJSC24), His-YgfXΔ1–54,MX (pMAT61), His-YgfX W34A (pMIC6) and His-YgfX D117A (pMIC25) expressed as previously (McNeil et al., 2012). Enzyme assays were performed as described previously on the cytoplasmic malate dehydrogenase (MDH) (Vasudevan et al., 1995) and membrane-bound SDH (McNeil et al., 2012) to determine the fractionation quality.

**Prodigiosin assays of YgfX variants.** For prodigiosin assays of YgfX derivatives, bacterial cultures were grown for 12 h at 30 °C from a starting OD₆₀₀ of 0.02. Plasmids were induced with 0.1 mM IPTG at 0 h unless otherwise stated. One millilitre samples were collected and centrifuged for 10 min at 13 000 r.p.m., and cell pellets were assayed for prodigiosin production as described previously (Gristwood et al., 2011; McNeil et al., 2012). Enzyme assays were performed as described previously (Slater et al., 2003) and expressed as A₅₃₄ ml⁻¹ OD₆₀₀⁻¹.

**Western blot analysis of YgfX proteins.** To assess the stability of His-YgfX derivatives, Western blotting was performed. Bacterial cultures were grown for 8 h at 30 °C from a starting OD₆₀₀ of ~0.04. IPTG (1 mM) was then added and cultures grown for a further 4 h. After this induction step, 1 ml samples were taken and normalized by OD₆₀₀ using 200 mM Tris/HCl and Western blotting was performed as described previously (Gristwood et al., 2011).

**Bacterial adenylate cyclase two-hybrid (BACTH) analyses.** The construction of bacterial two-hybrid plasmids using the vectors pUT18, pUT18C, pKT25 and pKNT25 is described in the supplementary methods. β-Galactosidase assays on the two-hybrid strains were performed as described previously and expressed as Miller units (Przybylski et al., 2011).

**Co-immunoprecipitation (Co-IP).** A pBAD30-based plasmid for the expression of FLAG-tagged YgfX and SdhE was already available (McNeil et al., 2012). A compatible His-tagged YgFX vector was generated by digesting pMIC2 with BamHI and SpHl and cloning this ygfX fragment into pMAT15 (a pQE-80L derivative with RP4 oriT and Cm), giving, plasmid pMAT8 (His-YgfX). Plasmids containing YgfXΔ1–54, YgfX W34A and D117A for Co-IP were constructed using an identical process, giving pMAT31, pMAT38 and pMAT39. Co-IP was performed by growing strains containing the appropriate plasmids to an OD₆₀₀ ~0.4, inducing with 1 mM IPTG and 0.1% arabinose. Cells were grown for an additional 4 h and then harvested by centrifugation at 10 000 r.p.m. for 10 min, with pellets frozen at −80 °C until required. Frozen pellets were thawed, resuspended in lysis buffer (Sigma) containing 3% Triton X-100 and lysed by sonication (6 × 10 s bursts). The anti-FLAG resin, following incubation with cleared cell lysates, was washed three times as recommended by the supplier (Sigma). Proteins were eluted using the 3 × FLAG
peptide and analysed by Western blotting. Bands were visualized as described above for cellular localization.

**Protein identification by mass spectrometry.** Proteins co-purifying with YgfX-FLAG expressed in WT *Serratia* sp. ATCC 39006 were identified as previously described for anti-FLAG Co-IP (McNeil et al., 2012). Briefly, lanes of interest (YgfX-FLAG purified on an anti-FLAG resin, or an empty vector control) were excised from SDS-PAGE gels and analysed on an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Scientific). For protein identification MS/MS data were searched against both a user-defined and a *Serratia* sp. ATCC 39006 amino acid sequence database (4417 sequence entries) using an in-house Mascot Server (http://www.matrixscience.com). The significance of identified protein matches was calculated using probability-based scoring, i.e. Mascot score (Matrix Science). The confidence of a significant match is proportional to the Mascot score.

**Microscopy.** Samples were collected at 2, 4, 6, 8 and 24 h of growth. Cells were heat fixed to microscope slides and stained by Gram staining. Cells were visualized using a Olympus BX-51 microscope. The lengths of 110 cells were measured using Image J and statistical analysis was performed using a t-test (unpaired, two tailed) in GraphPad Prism.

## RESULTS

**YgfX interacts with the FAD assembly factor SdhE**

Analysis of the RASTA-Bacteria database (Sevin & Barloy-Hubler, 2007), which predicts possible toxin–antitoxin (TA) systems, indicated that *sdhE*-*ygfY*-*ygfX* might encode a new TA locus, TA systems are widespread and have diverse roles (Cook et al., 2013). Currently, there are five types of TA systems: type I involves an RNA–RNA antisense inhibition (Gerdes & Wagner, 2007), type II involves a protein–protein complex (Gerdes et al., 2005), type III involves a toxin protein–antitoxin RNA interaction (Fineran et al., 2009), type IV involves the toxin and antitoxin having antagonistic effects on a target (Masuda et al., 2012a) and type V involves an antitoxin protein that cleaves the mRNA of the toxin (Wang et al., 2012).

To investigate if the SdhE and YgfX proteins formed a type II TA system, interactions between SdhE and YgfX were tested. N- and C-terminal FLAG- and His-tagged SdhE and YgfX plasmids were created. All SdhE plasmids except SdhE-His complemented the growth impairment of a Δ*sdhE* mutant (Fig. S1a, b). Furthermore, all YgfX plasmids activated pig production in a Δ*ygfX* strain (Fig. S1c, d), consistent with our previous results for the expression of untagged YgfX (McNeil et al., 2012). In contrast, only His-YgfX, YgfX-FLAG, His-SdhE and SdhE-FLAG were detected by Western blotting (Fig. S1e, f). Consequently, protein interactions were examined using Co-IP with His-YgfX, YgfX-FLAG, His-SdhE and SdhE-FLAG. Purification of SdhE-FLAG resulted in the co-purification of His-YgfX (Fig. 1a). The bait protein His-YgfX did not bind the anti-FLAG resin without SdhE-FLAG, demonstrating that His-YgfX specifically interacts with SdhE. In contrast, purification of YgfX-FLAG did not result in the co-purification of His-SdhE (data not shown), suggesting the tag orientation interferes with the interaction. In conclusion, YgfX interacts with SdhE.

![Fig. 1](Image 344x255 to 515x343)

**Fig. 1.** YgfX interacts with SdhE and YgfX. (a) His-YgfX (pMAT8) and SdhE-FLAG (pMAT7) were co-expressed in WT *Serratia* and purified on anti-FLAG agarose. Input, expression levels prior to Co-IP; Wash, final wash; Co-IP, elution. For each lane 20 μl of OD600-adjusted culture, wash or Co-IP fraction was loaded except the input for the His antibody, which was diluted tenfold. Interactions between YgfX and itself were determined by (b) BACTH and (c) Co-IP analysis. (b) In BACTH, the positive control (+ve) consisted of T18 and T25 fused to two leucine zippers, whilst the negative controls (–ve) consisted of T25 (pKT25) and T18 (pUT18C) domains alone. Data shown are the mean ± SD. A one-way ANOVA with a Tukey’s post-hoc test was used to determine statistical differences between samples. *P*-value < 0.05 when compared with the negative control. (c) For Co-IP, His-YgfX (pMAT8) and YgfX-FLAG (pMAT3) were co-expressed in WT *Serratia* and purified on an anti-FLAG resin. Lane details are the same as for (a).
Fig. 2. YgfX is not part of a TA system in Serratia or E. coli. (a) Overexpression of SdhE (pTA71), YgfX (pTA72) or SdhE, YgfX (pTA73) in Serratia. Cultures were induced with 1 mM IPTG at OD$_{600}$ ~0.4. (b) Overexpression of SdhE (pTA71), YgfX (pTA72) and SdhE, YgfX (pTA73) in Serratia ΔsdhEygfX grown in LB and induced with 1 mM IPTG at 0 h. (c) Growth and pig production following YgfX-FLAG overexpression in Serratia ΔygfX grown in LB and induced with 0.2 % arabinose at 0 h. (d) WT Serratia grown in LB and YgfX-FLAG was overexpressed by inducing with 0.2 % arabinose at OD$_{600}$ ~0.4 and detected by Western blot. (e, f) E. coli YgfX (pMAT18) was overexpressed in E. coli BW25113 grown in LB (e) or M9 (f) (supplemented with 0.5 % (v/v) glycerol and 0.2 % Casamino acids) and induced with 1 mM IPTG at OD$_{600}$ ~0.4 (e) or OD$_{600}$ ~0.2 (f). In all experiments an empty vector (Vector) negative control was used; arrows denote the time of induction and the data presented are the mean ± SD of at least three independent experiments.
YgfX and SdhE do not function as a TA system

The interaction data, RASTA-Bacteria prediction and the conservation of sdhE/ygfX suggested that it may form a type II TA system. Therefore, a TA function of YgfX and SdhE was investigated in *Serratia*. To examine whether DUF1434 proteins were toxins, untagged YgfX was expressed from mid exponential phase (OD600 ~0.4) in WT *Serratia* grown in LB. There was no detectable difference in OD600 or c.f.u. ml⁻¹ (data not shown) following YgfX induction compared with a vector control, the expression of SdhE alone or both SdhE and YgfX (Fig. 2a). To rule out any suppression of a toxic phenotype by the chromosomally encoded putative antitoxin (sdhE), a strain lacking this operon was used. When expressed in a ΔsdhE/ygfX mutant, neither YgfX nor SdhE caused a reduction in OD₆₀₀ compared with the empty vector control when induced from the start of growth (Fig. 2b). Note that the ΔsdhE/ygfX mutant has reduced growth due to a non-functional SDH (McNeil et al., 2012). Expression of YgfX in both WT and ΔsdhE/ygfX backgrounds increased pig production, demonstrating functional protein expression (McNeil et al., 2012). To confirm protein expression and further test if YgfX expression is toxic, YgfX-FLAG was used. YgfX-FLAG complemented pig production in the ΔygfX mutant (Fig.
2c), yet did not cause a reduction in OD₆₀₀ (Fig. 2c). Furthermore, expression of YgfX-FLAG in WT Serratia was detected by Western blotting without causing a toxic phenotype (i.e. no reduction in OD₆₀₀) (Fig. 2d).

During the course of this research a study in E. coli demonstrated that YgfX (termed CptA) caused reduced growth (OD₆₀₀) but that co-expression with SdhE (YgfY) alleviated this effect (Masuda et al., 2012). To examine a TA role in E. coli, native E. coli YgfX was overexpressed in E. coli strain BW25113 grown in both LB and M9 minimal media supplemented with 0.5% glycerol and 0.2% Casamino acids [the experimental conditions of Masuda et al. (2012)]. No toxic phenotype was observed following the induction of E. coli YgfX in mid exponential phase (Fig. 2e, f) or from time 0 in LB (Fig. S2). E. coli YgfX complemented pig production in ΔygfX Serratia, demonstrating that at this level of induction E. coli YgfX is expressed and functional (Fig. S3). Furthermore, E. coli YgfX plasmids from cultures grown in M9 media for 24 h were sequenced and contained no mutations. In conclusion, we could find no evidence that YgfX and SdhE form a TA system in Serratia sp. ATCC 39006 or E. coli under the conditions tested in this study.

YgfX interacts with itself in the membrane

An alternative hypothesis for the YgfX–SdhE interaction is that it forms a signal transduction system, with YgfX as a sensor. A common feature of membrane-bound signalling proteins is the ability to form multimers (Krell et al., 2010; Mascher et al., 2006). To investigate YgfX multimerization, a BACTH method was used, based on complementation of two fragments (T18 and T25) of the Bordetella pertussis adenylate cyclase. T18 and T25 are non-functional unless two fragments (T18 and T25) of the

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Fig. 4. The TMHs of YgfX are required for self-interactions. BACTH of (a) YgfXΔ¹⁵–⁵⁴ and (b) MalF-YgfX variant interactions with WT YgfX and themselves. In (a) and (b) YgfX is represented as X relative to the orientation of the T18 and T25 domains. The positive control (+ve) consisted of T18 and T25 fused to two leucine zippers, whilst the negative controls (–ve) consisted of 25 (pKT25) and T18 (pUT18C) domains alone. Data shown are the mean ± SD of three biological replicates. For (a) and (b) statistical differences between samples were determined as described for Fig. 1b. *P-value <0.05 when compared with the negative control. (c) For Co-IP, His-YgfXΔ¹⁵–⁵⁴ (pMAT31) or His-MalF-YgfX (pMAT37) was co-expressed with YgfX-FLAG (pMAT3) in WT Serratia and purified on an anti-FLAG resin. Input, expression levels prior to Co-IP. For each lane 20 μl of OD₆₀₀ adjusted culture, wash or Co-IP fraction was loaded, except the input for the His antibody, which was diluted tenfold.

The transmembrane helices of YgfX are required for function

YgfX is predicted to have a short N-terminal cytoplasmic domain followed by two transmembrane helices (TMHs) separated by a short periplasmic loop and finally, a larger

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C-terminal cytoplasmic domain (Fig. 3a). We previously demonstrated that YgfX is membrane associated (McNeil et al., 2012). However, it is unknown whether the membrane localization of DUF1434 proteins (e.g. YgfX) is important for function. To assess the different regions of YgfX, two truncation variants were generated that lacked amino acids 1–13 (YgfX<sub>D</sub>1–13; lacks cytoplasmic N terminus) or 1–54 (YgfX<sub>D</sub>1–54; lacks N terminus and both TMHs) (Fig. 3b). A
second set of these truncation variants was generated with N-terminal His-tags to assess their stability. His-YgfX<sub>A1–13</sub> was not detected by Western blot, whereas His-YgfX<sub>A1–54</sub> was highly expressed (data not shown). His-YgfX was solely present in the membrane (Fig. 3c), but truncation of the two TMHs (YgfX<sub>A1–54</sub>) resulted in localization to soluble and membrane fractions (Fig. 3c). To confirm the cell fractionation, the activities of cytoplasmic (MDH) and membrane (SDH) enzymes were determined, showing negligible cross contamination between the fractions (Fig. 3c). Interestingly, given the link between SdhE and SDH activity, the overexpression of WT or variant YgfX proteins did not alter the level of detectable SDH activity relative to WT with an empty vector (data not shown).

To assess the effects of the TMH on YgfX, the ability of the YgfX variants to activate pig production in a ygfX mutant was compared with full-length YgfX. Untagged and His-YgfX<sub>A1–54</sub> were non-functional (Fig. 3d, e), indicating that the TMHs were necessary. His-tagged and native YgfX<sub>A1–13</sub> both caused a small increase in pig production in the ygfX mutant, in agreement with partial functionality and poor stability (Fig. 3d, e).

To test if membrane localization and/or other sequences within the TMHs were required, a hybrid MalF–YgfX fusion protein was generated (Fig. 3b). The first two TMHs of <i>E. coli</i> MalF (residues 1–59) were fused to the cytoplasmic domain of YgfX<sub>A1–54</sub>, which restored membrane localization (Fig. 3c) but did not function to native (Fig. 3d) and His-tagged versions (Fig. 3e). It remained possible that the short N-terminal cytoplasmic portion of YgfX was important for function and not the TMHs. As described above, deletion of the first 13 residues of YgfX (i.e. YgfX<sub>A1–13</sub>) produced an unstable partially functional protein. To test the role of the N-terminal amino acids in an alternative way, the first 13 residues of YgfX were used to replace the first 13 residues of MalF-YgfX (Fig. 3b). The fusion variant, termed YgfX<sub>A1–13-MX</sub> (specifically YgfX<sub>A1–13-MalF<sub>1–59</sub>-YgfX<sub>55–143</sub></sub>), was stable and correctly localized to the membrane (Fig. 3c), but both native (Fig. 3d) and His-tagged (Fig. 3e) versions were non-functional. In conclusion, the TMHs of YgfX are required for correct localization and YgfX function.

Transmembrane domains are required for YgfX multimerization

To investigate if the TMHs or membrane localization are necessary for YgfX–YgfX interaction, the YgfX<sub>A1–54</sub> and MalF-YgfX variants were cloned into BACTH vectors and tested for interactions. Both YgfX<sub>A1–54</sub> (Fig. 4a) and MalF-YgfX (Fig. 4b) variant proteins were unable to self-interact or interact with the WT YgfX. To validate the loss of interaction, Co-IP was performed using WT YgfX-FLAG as bait and either His-YgfX<sub>A1–54</sub> or His-Malf-YgfX as prey. Both YgfX variants failed to co-purify with WT YgfX-FLAG (Fig. 4c), validating the BACTH data. Taken together, we conclude that YgfX forms dimers or larger multimer in the membrane and that the two TMHs are required.

Secondary structure analysis and site-directed mutagenesis of YgfX

Since there was no structural or functional information available for DUF1434 proteins, sequence analyses were performed. Of the 323 DUF1434 protein sequences in Pfam (accessed 10 April 2012), most (306) are in the Enterobacteriaceae, Shewanellaceae, Sutterellaceae and Citrobacteraceae (<i>accessed 10 April 2012</i>), most (306) are in the Enterobacteriaceae, Shewanellaceae, Sutterellaceae and Citrobacteraceae (16 are in other <i>Vibrionaceae</i>, <i>Shewanellaceae</i> and <i>Coxiellaceae</i>) and one is in <i>β</i>-proteobacteria. Therefore, DUF1434 proteins are predominantly, but not exclusively, present in <i>Enterobacteriaceae</i>. Using representative strains from <i>Enterobacteriaceae</i>, an alignment was performed (Fig. 5a). The alignment highlighted residues that are conserved across DUF1434
members, including a large number of tryptophan residues (7/143 residues), consistent with DUF1434 proteins being transmembrane proteins (Schiffer et al., 1992). A secondary structure analysis was performed, resulting in the prediction of α-helices in the two N-terminal TMHs (α1 and α2) and a cytoplasmic domain composed of five β-strands (β1 to β7) and a C-terminal α-helix (Fig. 5a). Tertiary structure predictions using PUGUE (Shi et al., 2001) and Phyre (Kelley & Sternberg, 2009) yielded no significant structural homologues (data not shown).

Based on the alignments, 26 amino acids were selected and mutated to alanine (Figs 3a and 5a). The function of these site-directed variants was assessed in a ygfx mutant by comparing activation of pig production by WT His-YgfX with the YgfX variants (Fig. 5b, c). Seven variants were not different from the WT YgfX control (P35A, L45A, R55A, S56A, I60A, E66A and W89A) (Fig. 5b, c). The remaining 19 variants were impaired and could only stimulate pig production by 5–60% compared with WT YgfX (Fig. 5b, c). Of interest were the W34A and D117A variants, which were almost completely inactive. These mutations mapped to the periplasmic loop (W34A) and the cytoplasmic domain (D117A) (Fig. 3a) and these protein variants were stably expressed (Fig. 5d). All other variants were detectable by Western blotting. The levels of a few variant proteins (e.g. G65A, W77A, W82A, L99A and R125A) appeared lower than the YgfX controls (Fig. 5d). All other variants were undetectable by Western blotting. The levels of a few variant proteins (e.g. G65A, W77A, W82A, L99A and R125A) appeared lower than the YgfX controls (Fig. 5d), which may have contributed to their reduced activity. Furthermore, all YgfX variants were overexpressed in WT Serratia to test for any dominant negative phenotypes via disruption of YgfX–YgfX interactions. None of the variants caused pig production to decrease in the WT (data not shown). In summary, YgfX is anchored in the membrane via two TMHs linked by an important tryptophan (W34) and has a mainly β-sheet cytoplasmic domain containing a critical aspartate (D117) in the predicted β7–α3 loop.

**W34A and D117A YgfX variants localize correctly but cannot interact**

It was of interest to determine whether the W34A and D117A variants are defective in membrane localization or YgfX multimerization. Firstly, localization experiments demonstrated that both variants are still membrane associated (Fig. 6a). To investigate the protein–protein interactions W34A and D117A were cloned into BACTH vectors. YgfX W34A weakly interacted with WT YgfX (Fig. 6b), whilst YgfX D117A could not interact with WT YgfX (Fig. 6b). Both W34A and D117A variants were significantly impaired for self-interactions or interactions with each other (Fig. 6b). In conclusion, the defective W34A and D117A variants localize to the membrane but are either severely reduced or unable to interact and form YgfX dimers or multimers. This indicates that the protein–protein interactions are important for the function of DUF1434 proteins.

**Fig. 6.** W34A and D117A YgfX variants localize correctly but cannot interact. (a) Western blot of His-tagged YgfX W34A and D117A variants in the membrane (M) or soluble (S) fraction in a ygfX mutant background (HSPIG46). Thirty micrograms of total protein was analysed in each lane. (b) BACTH analysis of the ability of YgfX W34A and D117A variants to interact with WT YgfX, themselves and each other. Positive (+ve) and negative (−ve) controls are as described in (Fig. 1b). Data shown are the mean ± SD of three biological replicates. A one-way ANOVA with a Tukey’s post-hoc test (comparing all data with each other) was used to determine statistical differences between samples. *P*-value <0.05 when compared with the negative control. (c, d) Co-IP of YgfX W34A and D117A variants to interact with WT SdhE. His-YgfX [W34A (pMAT38) and D117A (pMAT39)] and SdhE-FLAG (pMAT7) were co-expressed in WT Serratia and purified on an anti-FLAG resin. Input, expression levels prior to Co-IP; Wash, final wash; Co-IP, elution. For each lane 20 μl of OD600-adjusted culture, wash or Co-IP fraction was loaded except the input for the His antibody, which was diluted tenfold. Plasmid details are listed in Table S2.
YgfX variants are not able to interact with SdhE

We predicted that YgfX–YgfX interactions, mediated in part via the W34 and D117 residues, are required for interactions with SdhE. To examine this, YgfX variants (W34A and D117A) were tested for co-purification with SdhE using Co-IP. Unlike WT His-YgfX (Fig. 1a), both His-tagged W34A and D117A YgfX variants as prey failed to co-purify with SdhE-FLAG as bait (Fig. 6c, d). The loss of the interaction between SdhE and YgfX SDM variants also demonstrates that the interaction detected between WT SdhE and YgfX proteins is specific. These results demonstrate that non-functional YgfX variants are unable to interact with SdhE and that W34 and D117 are essential for this process, possibly via the formation of dimeric or multimeric forms of YgfX.

A short list of putative interaction partners of YgfX

To further investigate the function of YgfX we identified additional putative interaction partners of YgfX. YgfX-FLAG was purified and co-eluting proteins were identified by MS that were absent in a co-purification control lacking YgfX-FLAG. Proteins required for pig biosynthesis (e.g. PigC, PigE, PigA, PigH, PigB and PigI) were identified (Table 1) (Williamson et al., 2005). Furthermore, proteins involved in cell division co-purified with YgfX-FLAG, including MreB, FtsZ, FtsH, MinD and MreC (Table 1). In support of these putative interactions, Masuda et al. (2012b) recently reported that interactions between YgfX and FtsZ/MreB impair FtsZ/MreB polymerization and produce morphological defects in E. coli. Although all of these putative YgfX binding partners have yet to be validated by other techniques, the data in Table 1 provide a short list for future validation.

Under the conditions tested in this study, we could not demonstrate that YgfX-SdhE functioned as a TA system in either E. coli or Serratia sp. ATCC 39006. Typically, in TA systems the antitoxin cannot be deleted, since toxicity caused by the toxin alone causes cell death. Interestingly, the proposed antitoxin sdhE has been deleted in multiple species of Enterobacteriaceae, including Serratia sp. ATCC 39006 (McNeil et al., 2012), E. coli (Baba et al., 2006) and Salmonella enterica serovar Typhimurium (Langridge et al., 2009). These results support that YgfX is not a toxin. Finally, in a recent shotgun cloning study to identify novel TA systems from a range of microbial genomes, YgfX was not identified as being toxic (Kimelman et al., 2012; Sberro et al., 2013). These independent lines of experimental investigation provide further evidence that YgfX-SdhE is not a TA system.

Interestingly, His-YgfX^{41–54} localized to both the membrane and soluble fraction in a ygfX mutant, suggesting that the cytoplasmic domain might interact with other membrane proteins. Recently, the N-terminal region (residues 32–55) of E. coli FtsZ was reported to interact with YgfX, whilst the cytoplasmic domain of YgfX inhibited the polymerization of FtsZ/MreB (Masuda et al., 2012b). Our study in Serratia, using co-purification and MS, validated the interaction between YgfX-FtsZ and MreB previously observed in E. coli. Furthermore, our work has generated a short list that extends the repertoire of cell division proteins that YgfX potentially interacts with to
include FtsH (Akiyama et al., 1995), MinD (de Boer et al., 1991) and MreC (Kruse et al., 2005). Further validation is required to confirm that YgfX interacts with these putative interaction partners. Both our study and that of Masuda et al. (2012b) observed effects on cell length with the over-expression of YgfX, demonstrating that YgfX interferes with correct cell division. Furthermore, we have previously shown that sdhE deletion mutants have a reduced cell size (McNeil et al., 2012), suggesting that the interaction between YgfX and SdhE may influence the YgfX-dependent effects on cell morphology. In conclusion, the results presented here consolidate previous findings demonstrating that YgfX interacts with and inhibits the polymerization of cell division proteins, consequently affecting cellular morphology.

Numerous proteins involved in Pig biosynthesis also co-purified with YgfX. Pig is ultimately produced from the condensation of the terminal products from the MAP and MBC biosynthetic pathways (Williamson et al., 2005, 2006). Interestingly, PigB is an FAD-containing inner-membrane protein that catalyses the terminal reaction in MAP biosynthesis, whilst PigC is responsible for the final condensation reaction (Williamson et al., 2005). PigN catalyses the terminal step in MBC synthesis and is also predicted to be membrane bound (Williamson et al., 2006). It is possible that, in addition to indirect transcriptional activation of pigA–O, YgfX might facilitate the formation of a terminal pig biosynthetic complex consisting of PigB and PigC. Consequently, the overexpression of YgfX would increase the formation of this terminal pig biosynthetic complex, leading to increased pig production, whilst the loss of YgfX would prevent the formation of the terminal complex and decrease pig production. This concept is supported by in vivo data, with DygfX producing 90% less (McNeil et al., 2012) and the overexpression of YgfX producing 500% more pig than WT Serratia sp. ATCC 39006. However, caution is required since the co-purification of these Pig proteins may be an artefact associated with the upregulation of pig production associated with the overexpression of YgfX rather than direct interactions with YgfX.

W34 and D117 were essential for YgfX–YgfX interactions, for interaction with SdhE and for YgfX function. We propose that the formation of YgfX multimers is required to enable interactions with SdhE. When the non-Enterobacteriaceae (Rickettsiella grylli, Shewanella baltica and Photobacterium
Genomic conservation, co-expression (McNeil et al., 2012) and protein–protein interactions suggest that YgfX and SdhE function in the same pathway in the Enterobacteriaceae. The direction of information flow is unclear (i.e. YgfX to SdhE, SdhE to YgfX or bi-directional). The overexpression and loss of YgfX (McNeil et al., 2012) has no effect on SDH activity under currently examined lab conditions. Therefore, the interaction between YgfX and SdhE may function independently of SDH. Although this scenario requires further analysis, it is supported by the observation that the loss of sdhE affects many phenotypes, which might be independent of SDH activity (McNeil et al., 2012).

Alternatively, YgfX activity could be influenced by SdhE in response to the cell’s metabolic state (i.e. via FAD and/or SDH). Further investigations are required to determine the physiological role of the interaction between YgfX and SdhE. Consistent with previous work, YgfX interacted with cell division proteins and affected morphology when over-expressed. The interactions between YgfX, SdhE and cell division proteins might act as a regulatory link between metabolism and cell division, either to control SDH in response to cell division or vice versa. Finally, the involvement of YgfX-SdhE in strongly regulating pig production demonstrates that the YgfX-SdhE pathway can also have strain-specific functions. In conclusion, in this study we have clearly demonstrated for the first time, to our knowledge, that DUF1434 proteins are multimeric membrane-bound proteins that interact with the cytoplasmic SDH assembly factor SdhE.

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Molecular dissection of YgfX


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