The ferric citrate regulator, FecR, is translocated across the bacterial inner membrane via a unique Twin-arginine transport dependent mechanism.

FecR insertion into the cytoplasmic membrane

Authors

Ian J Passmore¹, Jennifer M. Dow¹, Francesc Coll¹, Jon Cuccui¹, Tracy Palmer², Brendan W. Wren¹#

¹Department of Infection Biology, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK

²Centre for Bacterial Cell Biology, Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle upon Tyne, NE2 4HH, UK

# Corresponding author

Email bredan.wren@lshtm.ac.uk
Abstract

In *Escherichia coli*, citrate-mediated iron transport is a key non-heme pathway for the acquisition of iron. Binding of ferric citrate to the outer membrane protein FecA induces a signal cascade that ultimately activates the cytoplasmic sigma factor FecI, resulting in transcription of the *fecABCDE* ferric citrate transport genes. Central to this process is signal transduction mediated by the inner membrane protein, FecR. FecR spans the inner membrane through a single transmembrane helix, which is flanked by cytoplasmic and periplasmic-orientated moieties at the N- and C- terminus. The transmembrane helix of FecR resembles a twin-arginine signal sequence, and substitution of the paired arginine residues of the consensus motif decouples the FecR-FecI signal cascade, rendering the cells unable to activate transcription of the *fec* operon when grown on ferric citrate. Furthermore, fusion of beta-lactamase C-terminal to the FecR transmembrane helix results in translocation of the C-terminal domain that is dependent on the twin-arginine translocation (Tat) system. Our findings demonstrate that FecR belongs to a select group of bitopic inner membrane proteins that contain an internal twin arginine signal sequence.

Importance

Iron is essential for nearly all living organisms due to its role in metabolic processes and as a cofactor for many enzymes. The FecRI signal transduction pathway regulates citrate-mediated iron import in many Gram-negative bacteria, including *Escherichia coli*. The interaction of FecR to outer membrane protein, FecA, and cytoplasmic anti-sigma factor, FecI, has been extensively studied. However, the mechanism by which FecR inserts into the membrane has not previously been reported. In this study, we demonstrate that targeting of FecR to the cytoplasmic membrane is dependent on the Tat system. As such, FecR
represents a new class of bitopic Tat-dependent membrane proteins with an internal twin arginine signal sequence.

Introduction

Iron is an essential element for virtually all organisms. Despite this, the toxicity, availability and solubility of iron present major challenges for bacteria, which require specialised iron transport systems. *Escherichia coli* K12 encodes two main non-heme pathways for the acquisition of iron from the environment. The first of these systems involves sequestering of iron via the siderophores enterobactin and ferrichrome that compete for Fe$^{3+}$ bound to host proteins(1), whereas the second system involves ferric citrate uptake(2). Ferric citrate transport is mediated via the Fec system, which is comprised of a TonB-dependent outer membrane protein (FecA), a periplasmic-binding protein (FecB), inner-membrane proteins (FecCD) and ATPase (FecE)(3). The *fecABCDE* transport genes are induced upon detection of ferric citrate via the FecR-FecI signal cascade. FecR is an inner membrane protein with both periplasmic and cytoplasmic globular domains either side of a single trans-membrane helix(4). Binding of ferric citrate to FecA induces a conformational change, which is detected by the periplasmic C-terminal domain of FecR (Figure 1A). The signal is transduced across the membrane whereupon the cytoplasmic N-terminal face of FecR activates and releases the sigma factor FecI, which in turn recruits RNA polymerase to the *fec* operon(5, 6). Signal transduction across the inner membrane is central to this process and a number of key residues on the periplasmic and cytoplasmic domains of FecR have been identified as essential for interaction with FecA and FecI, respectively(7, 8). However, the mechanism by which FecR is inserted into the cytoplasmic membrane has not previously been reported.
Targeting of proteins to the bacterial cytoplasmic membrane occurs via the action of Sec machinery, the YidC insertase or the twin-arginine translocation (Tat) translocation pathway (9, 10). While most Tat substrates are soluble proteins released into the periplasm, a few substrates remain anchored in the cytoplasmic membrane, usually by an uncleaved N-terminal signal peptide, or a single C-terminal transmembrane helix (11–13). Whereas Sec exported proteins fold post-export, the Tat system exports proteins that have folded and matured in the cytoplasm. Tat substrates often contain complex cofactors and may co-export bound partner proteins, or are utilised by bacteria in extreme environments (14–16). However, some Tat substrates also include a number of monomeric, cofactor-less proteins, and for example, some halophilic archaea secrete the majority of their proteins via the Tat system, which may be an adaptive response to the fast-folding kinetics of proteins in a highly saline environment (17, 18).

Substrates are addressed to the Tat pathway via N-terminal signal peptides with a distinctive tripartite structure consisting of a basic n-region containing a conserved S/T-R-R-x-F-L-K “Tat motif”, a hydrophobic h-region, and a polar c-region harbouring the signal peptidase cleavage site (19). They frequently also contain one or more positive charges in the c-region that are not required for Tat transport but act as a Sec-avoidance motif (20, 21). Recently the polytopic Rieske protein of *Streptomyces coelicolor* was shown to be an unusual Tat substrate because it utilises the Sec machinery for insertion of its first two transmembrane helices and has an internal Tat signal sequence that forms the third transmembrane domain and that mediates export of the folded cofactor-containing domain across membrane (22, 23). Since this initial study, other families of polytopic inner membrane proteins that are simultaneously targeted to the Sec and Tat pathways have recently been described (21).
Here we demonstrate that FecR uses an internal Tat-targeting sequence for export of the 22 kDa C-terminal domain to the periplasm, whilst leaving a 9 kDa N-terminal domain in the cytoplasm. This is the first example of a class of biotopic Tat-dependent membrane proteins with an internal twin-arginine signal sequence.

Results

An internal Tat-motif is conserved amongst FecR orthologues

Previous studies have shown that *E. coli* K12 FecR spans the cytoplasmic membrane(4), but the mechanism by which it is inserted into the bilayer has not been described. FecR does not contain a classical signal sequence within its cytoplasmically orientated N-terminal domain (residues 1-75). However, a twin-arginine motif with a good match to the Tat consensus sequence immediately precedes a region of hydrophobicity that corresponds to the transmembrane domain (Figure 1B). Both features are conserved characteristics of Tat signal peptides. Similar to other Tat-dependent inner membrane proteins, FecR contains no predicted cleavage site (Figure 1B). The amino acid sequences of putative FecR orthologues derived from the eggNOG database(24) were aligned to assess conservation of this putative Tat-targeting sequence (Figure S1). Out of the 95 predicted orthologues analysed, 93.7% contained an internal twin-arginine motif. Those without Tat motifs were among those that displayed the lowest sequence amino acid similarity to *E. coli* K12 FecR (Table S1) and clustered independently from the other orthologues (Table S1, Figure S2A), suggesting that these may not represent true FecR orthologues. Sequence motifs were generated using Weblogo.3(25) with and without these outliers (Figure 1C and Figure S2B, respectively). These alignments show clear conservation of the twin arginines, indicating that they may be
required for function. Furthermore, a high frequency of serine/threonine (-1 position), leucine (+3 position) and lysine residues (+4 position) was also noted, which are hallmarks of Tat signal sequences(26). FecR also contains two basic residues adjacent to the c-region that are known to act as Sec avoidance motifs (Figure 1B)(20, 21). This feature was also conserved amongst putative FecR orthologues (Figure 1D).

**Periplasmic translocation of the FecR C-terminal domain is Tat-dependent**

To determine whether membrane integration of FecR is dependent on the Tat export machinery, we constructed a fusion protein that could be deployed as a reporter for periplasmic translocation of the C-terminal domain (Figure 2A). The reporter FecR-BlaM was constructed by fusing the N-terminal domain and membrane-spanning region (amino acid residues 1-115) of *E. coli* FecR to β-lactamase (BlaM). This reporter was expressed under the transcriptional control of an arabinose inducible promoter in a tat+ *E. coli* K12 strain (10β). β-lactamase fusions serve as ideal reporters for periplasmic export because they must be trafficked beyond the bacterial inner membrane to effectively protect the cell from β-lactam antibiotics. Furthermore, many β-lactamases can be translocated across the inner membrane via either Sec or Tat systems and are used as a reporter for both pathways(27, 28).

We assessed resistance to the β-lactam antibiotic ampicillin using M.I.C.Evaluator strips and by spotting serial dilutions on ampicillin plates (Figure S3). Table 1 and Figure 2B demonstrate that cells producing the FecR-BlaM reporter grew to a concentration of >256 μg ml⁻¹, indicating effective translocation of the β-lactamase to the periplasm. Removal of arabinose from the plate rendered this strain fully sensitive to ampicillin. To test whether the twin arginine motif was important for recognition of FecR by the Tat pathway, we
constructed substitutions of the arginine pair to twin alanine (radical) or twin lysine (conservative). Cells producing the mutated fusion proteins demonstrated dramatically increased sensitivity to ampicillin (MIC = 16 μg ml⁻¹) suggesting that almost no β-lactamase had now been translocated to the periplasm. To further explore the Tat-dependence of this fusion, the FecR-BlaM reporter was expressed in the Tat-deficient strain, HS3018-ΔtatABC. Consistent with the notion that insertion of the FecR transmembrane helix was Tat-dependent, this strain demonstrated markedly increased ampicillin sensitivity relative to the isogenic wild type (Figure 2C). Translation and membrane localisation of the fusion proteins were not negatively affected by mutation of the twin-arginine motif or deletion of the Tat system (Figure 3B and C), suggesting that ampicillin sensitivity was the consequence of ineffective β-lactamase translocation.

**Mutation of the twin-arginine motif does not prevent membrane interaction**

Next, we determined whether substitutions of the paired arginine residues R79/80 influenced localisation of the FecR and the FecR-BlaM reporter (Figure 3A and B). Comparison of the relative proportion of FecR and FecR-BlaM in the soluble (Figure 3A) and membrane fractions (Figure 3B) revealed that the R79/80 substitutions did not prevent membrane association of the proteins. We also expressed the FecR-BlaM reporter in the wild type (HS3018-A) and tat mutant (HS3018-ΔtatABC) strains (Figure 3C). Similarly, FecR-BlaM localised to the membrane in the absence of a functional Tat system. To determine whether FecR-BlaM was fully integrated into the bilayer, membranes of the wild type and tat strains producing FecR-BlaM were washed with either 0.2M Na₂CO₃ or 4M urea (which can displace peripheral membrane proteins by disrupting ionic interactions and disrupting hydrogen bonding). Fig 3D shows that while carbonate washing had little effect on the
membrane localisation of FecR-BlaM in either strain, urea washing displaced FecR-BlaM from the membrane fraction of the tat mutant strain but not the wild type. These results confirm that FecR-BlaM behaves like an integral membrane protein in the wild type strain, but not is not correctly membrane integrated in the absence of the Tat machinery.

Mutation of the twin-arginine residues results in downregulation of the fecABCDE operon

No marked difference in growth rate was observed between HS3018-A and the tat mutant when grown with ferric citrate as a sole iron source (Figure S4), which is consistent with observations made by Ize et al., 2004(29). Previous characterisation of the Fec system in E. coli has been performed using strains with an aroB mutation (E. coli strain AA93), which are unable to synthesise the siderophore enterobactin (which may compensate for the lack of Fec-mediated ferric iron import)(30, 31). Although these studies have been performed using different genetic backgrounds, these data indicate that some strains of E. coli K12 can acquire ferric citrate in the absence of functional FecR.

To explore whether fecR RR to KK mutation influenced recognition of ferric citrate in iron-limiting conditions, transcription of the fecABCDE genes was determined by qPCR analysis. A strain deficient in the fecR gene (E. coli strain BW25113) was complemented in trans with either wild-type fecR or fecR R79-80K substitution, which were grown in media supplemented with 2’2’-dipyridyl and 1 mM sodium citrate. Cells were harvested at early stationary phase and we observed no difference in final optical density between the strains tested, suggesting that the fecR mutant could grow using ferric citrate as a sole iron source.

We observed statistically higher expression of fecABCD (but not fecE) in the fecR mutant relative to the fecR R79-80K, indicating that there is low level of transcription of the fec genes in this strain. Crucially, we observed significantly increased expression of the

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Operon in strain complemented with wild type fecR relative to fecR R79-80K and the fecR mutant (Figure 4). This is consistent with the notion that the arginine to lysine mutation prevents periplasmic translocation of the C-terminal domain, which abrogates FecR binding to citrate-loaded FecA and decouples the FecIR signal cascade.

Discussion

In this study we have addressed the membrane integration pathway for the bitopic membrane protein FecR. Analysis of the transmembrane domain of FecR homologues demonstrates that it is preceded by a conserved twin arginine motif, and that several positive charges are located close to the C-terminal end, a feature which is known to act as a Sec-avoidance motif. Consistent with this, replacement of the C-terminal extracellular domain of FecR with beta-lactamase resulted in beta-lactamase translocation that was dependent on both the twin arginines and the Tat pathway. The Tat system is known to integrate several classes of membrane protein, including monotopic proteins that are anchored by a single N- or C-terminal transmembrane domain, and polytopic proteins where only the final transmembrane domain is Tat-dependent(13, 21, 22, 32, 33). FecR constitutes a new class of bitopic Tat-dependent membrane proteins with an internal, uncleaved twin arginine signal sequence that separates two globular domains.

The polytopic Rieske protein of Streptomyces coelicolor, which require concerted action of Sec and Tat pathways for membrane integration, contains more than one transmembrane helix and have an odd number of transmembrane helices before the twin arginine residues. Given that FecR only contains a single helix, it seems unlikely that it is targeted to the membrane by a similar dual-action mechanism. Our data indicate that FecR associates with the membrane in the absence of a functional Tat system (Figure 3), which could suggest co-
operation with another pathway for its insertion. However, a study by Gray et al., (2011) demonstrated that FecR membrane localisation was unperturbed in a yidC mutant(10). The E. coli Tat substrate, SufI, and some thylakoid proteins have been shown to bind to the membrane before interaction with the Tat or in the absence of functional Tat machinery(34–37). Although our data clearly demonstrate that FecR is a Tat substrate, we do not rule out the possibility that it targets to the membrane via another pathway.

The mechanism by which the Tat system recognises this internal signal sequence is unclear, but it should be noted that the related thylakoid Tat system is capable of translocating the substrate protein pOE17 even after deliberate fusion of a large polypeptide domain N-terminal to the Tat signal peptide(38). This indicates that integration of bitopic proteins is likely to be a common feature of the Tat pathway from different organisms.

Interestingly, some complex Tat substrates have signal peptides that contain greatly extended n-regions prior to the twin-arginine motif(39). Such extensions are almost invariably found on substrates that bind redox cofactors and/or partner proteins prior to export, and they appear to serve as binding sites for dedicated chaperones that co-ordinate folding and assembly(40–44). FecR is distinct from these Tat substrates since it does not contain any redox cofactor, and its signal sequence n-region is considerably longer than other Tat signal peptide n-regions. It is not clear why FecR should be a Tat substrate, although feasibly it may be energetically favourable for FecR to fold in the cytoplasm prior to transport through the Tat machinery. Alternatively, it is conceivable that the FecR N-terminal domain binds FecI, driving cytoplasmic folding before its integration into the membrane. In conclusion, FecR joins an expanding list of inner membrane proteins that contain a non-N-terminal Tat signal sequence.
Materials and Methods

Strains and plasmids

Bacterial strains and plasmids used in this study are listed in Tables S2 and S3 respectively. The ampicillin resistance cassette of plasmid pEC415 was exchanged for a kanamycin cassette by Gibson assembly (NEBuilder HiFi Assembly master mix, NEB) using the primers pET28akanF/R (pET28a as template) and pEC415kanF/R (pEC415 as template) to generate the plasmid pEC415K.

Plasmid pECfecR-blaM was constructed by Gibson assembly using the primers fecRF/fecRR and pEC415KfecRF/pEC415KfecRR with E. coli genomic DNA and pEC415K as template. Amino acid residues 79 and 80 were mutated from arginine to alanine or lysine using the primers fecR R7980A F/ fecR R7980 R and fecR R7980K F/fecR R7980 R, respectively. 5’-phosphorylated primers fecR-blaM His F and R were used to introduce a C-terminal His-tag into fecR-blaM and fecR-R7980K-blaM using pECfecR-blaM and pECfecR-R7980K-blaM as templates, generating plasmid pECfecR-blaM-His and pECfecR-R7980K-blaM-His, respectively. Similarly, primers fecR His F and R were used to generate C-terminally His-tagged fecR using pECfecR and pECfecR R7980K as templates to generate pECfecR-His and pECfecR R7980K-His.

Growth conditions

E. coli strains were cultured in LB broth or agar (Merck, Millipore) at 37°C and supplemented, when required, with 50 µg ml⁻¹ kanamycin and 100 µg ml⁻¹ ampicillin.
Susceptibility of the *E. coli* strains harbouring FecR-BlaM reporter to ampicillin was investigated by determining the Minimum Inhibitory Concentration (MIC) that prevented growth. Overnight cultures of each strain was diluted to OD$_{600}$ 0.5 and a bacterial lawn was grown on LB plates (supplemented with kanamycin) by swabbing. M.I.C Evaluator strips (Oxoid) were placed on the plates, which were grown overnight at 37°C. Three independent replicates were performed and representative images are shown.

For transcript and growth kinetic analysis cells were grown at 37°C in Nutrient Broth (Merck) supplemented with 50 µM 2,2'-dipyridyl and 1 mM citrate.

**Membrane extractions**

*E. coli* cells harbouring the plasmid pECfecR-blaM were grown overnight at 37°C in media supplemented with 0.2% w/v L-arabinose and 50 µg ml$^{-1}$ kanamycin. Cells were pelleted by centrifugation (3200 x g) and resuspended in 20 mM Tris-HCl (pH7.5), 200 mM NaCl. Cells were lysed using a FastPrep homogeniser (MPBio) and unlysed cells and large cell debris was removed by centrifugation (7000 x g). The resulting clarified lysate was pelleted by ultracentrifugation (1 hour 150,000 x g) to separate membrane and soluble fractions.

Membrane pellets were resuspended in 50 mM Tris HCl (pH 7.5), 5 mM MgCl$_2$, 10% (v/v) glycerol. For membrane interaction assays, cells were resuspended in 50 mM Tris HCl (pH 7.5), 10% (v/v) glycerol and lysed as described above. Crude lysate was treated with either 0.2M Na$_2$CO$_3$ or 4M urea for 1 hour, at 4°C, followed by ultracentrifugation at 150,000 x g. Membrane pellets were resuspended in 50 mM Tris HCl (pH 7.5), 5 mM MgCl$_2$, 10% (v/v) glycerol.
Immunoblot analysis

Total protein concentration of each sample was quantified by Bradford assay and normalised to equal concentrations. Proteins were resolved by SDS-PAGE with Mini-PROTEAN TGX Stain-Free gels (BioRad) and transferred to nitrocellulose membranes using the iBlot 2 dry blotting system (ThermoFisher). Prior to transfer, gels were imaged using BioRad ChemiDoc MP imaging system to determine total protein content loaded in each well. Primary antibody, Mouse anti-6xHis (Invitrogen, UK, used at 1:10000 dilution), was suspended in PBS and 0.1% (v/v) Tween 20 and incubated with the membrane for 1 hour. Membranes were washed three times with PBS and incubated for 45 min with a secondary goat anti-mouse IgG IRDye680 antibody (LI-COR Biosciences, UK, both at 1:10000 dilution). Fluorescent signal was detected with the Odyssey LI-COR detection system (LI-COR Biosciences, UK).

qPCR

Cells were grown to early stationary phase. All cells were harvested and stored in RNAlater (Ambion) at 4°C overnight. Cells were sedimented by centrifugation and RNA was extracted using the Monarch Total RNA Miniprep kit (NEB) according to the manufacturer’s instruction. The resulting RNA was used as a template for reverse transcription and conversion into cDNA using Superscript IV reverse transcriptase (Invitrogen). qPCR was performed on the cDNA using Power SYBR green (Thermo) according to the manufacturer’s instructions, with 10 pmol of the appropriate primers (see Table S4). Amplification was performed using an ABI PRISM 7500 real-time PCR system, and fluorescence data was processed using SDS software (ABI). Relative gene expression was determined using gyrA.
and rpoS as controls. Three independent biological replicates were performed for each strain and growth condition.

**Bioinformatic analysis**

Sequences of theoretical FecR orthologues were retrieved from the eggNOG database of orthologous groups and functional annotations (24). Sequence alignments were constructed using ClustlW and ESPript 3.0 (45). Phylogenetic trees were generated using Interactive Tree of Life software (46). Weblogo sequence motifs were generated using weblogo3 (25).

**Acknowledgments**

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**Competing interests**

The authors declare no competing interests.

**References**


428 Figures legends

429 Figure 1

A- Schematic representation of FecR-mediated signal transduction. Binding of ferric citrate to the outer membrane protein, FecA, initiates a signal that is transmitted across the cytoplasmic membrane by FecR. B- Sequence alignment of the putative internal Tat motif of FecR (residues 68-109) with the N-terminal Tat motif of TorA (residues 1-52), displayed on
the figure. The Tat motif is boxed in red, TorA cleavage site is boxed in orange. Region of hydrophobicity/membrane spanning region indicated green. Positively charged residues adjacent to the FecR c-region are indicated by arrows and putative Sec avoidance (which correspond to weblogo shown in Figure 1D) boxed in purple. C- Consensus Tat sequence motif of predicted FecR eggNOG-derived orthologues, excluding the six sequences that do not contain a consensus twin-arginine motif. Amino acid position relative to the twin-arginine motif is denoted below. D- Sequence alignment consensus logo of the c-region of the predicted FecR eggNOG-derived orthologues. Charged amino acid residues are coloured in blue.

**Figure 2**

Mutation of twin arginine residues inhibits periplasmic translocation of a FecR-BlaM fusion.

A- Domain architecture of the FecR-BlaM reporter. Ampicillin sensitivity of the FecR-BlaM reporter with RR to AA and KK substitutions (E. coli strain, 10β) (B) and the FecR-BlaM reporter expressed in a Tat-deficient strain (E. coli strain, HS3018-A and HS3018-AΔtatABC) (C) determined using M.I.C.Evaluator strips. Representative images of three biological replicates are shown.

**Figure 3**

Cell localisation of FecR-Bla-His reporter and FecR-His with RR to KK substitutions (10β) (A and B) or FecR-Bla-His expressed in a Tat-deficient strain (HS3018-AΔtatABC) (C). Soluble and membrane fractions were resolved by SDS-PAGE, transferred to nitrocellulose membranes probed with an anti-6xHis antibody. All gels were imaged prior to transfer to determine total protein loaded in each well (shown in lower panel). A- Soluble fractions, Lane 1: FecR-Bla-His; lane 2: FecR-Bla-His R79/80A; lane 3: FecR-His; lane 4: FecR-His
R79/80K. B-Membrane fractions- Lane 1: FecR-Bla-His; lane 2: FecR-Bla-His R79/80A; lane 3: FecR-His; lane 4: FecR-His R79/80K. C- Lane 1: HS3018-A FecR-Bla-His, soluble fraction; lane 2: HS3018-A∆tat FecR-Bla-His, soluble; lane 3 HS3018-A FecR-Bla-His, membrane fraction; lane 4: HS3018-A∆tat FecR, FecR-Bla-His, membrane fraction. D- Crude cell extracts were washed with either 0.2M Na$_2$CO$_3$ or 4M urea prior to membrane sedimentation. Lane 1: HS3018-A FecR-Bla-His, membrane fraction, Na$_2$CO$_3$; lane 2: HS3018-A∆tat FecR-Bla-His, membrane fraction, Na$_2$CO$_3$; lane 3 HS3018-A FecR-Bla-His, membrane fraction, urea; lane 4: HS3018-A∆tat FecR-Bla-His, membrane fraction, urea.

**Figure 4**

Relative gene expression of the fecABCDE operon and fecR in iron-limiting conditions.

Quantitative real time PCR of a fecR mutant (strain BW25113) expressing either wild type fecR or fecR R79/80K substitution and an empty plasmid control was performed on RNA/cDNA extracted from cell cultures grown in media supplemented with 2’2’-dipyridyl and 1 mM sodium citrate. Error bars represent the standard deviation from mean derived from three biological replicates.

**Table 1**

Effect of amino acid substitutions and a functional Tat system on periplasmic translocation of the FecR-BlaM fusion and the ability to support growth on ampicillin. Determination of M.I.C was performed in triplicate and representative images are shown in Figure 2.

<table>
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<tr>
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</tbody>
</table>
Figure 2

A

Plasmid control

B

Δtat fecR- βlac WT fecR- βlac

C

FecR 1-115

N C

BlaM
Figure 4

**fecR**

fold change

FecR TAT signal

fecA

fecB

fecC

fecD

Model

**fecR**

fold change

FecR TAT signal

fecA

fecB

fecC

fecD

Model