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Expression and purification of functionally active ferrous iron transporter FeoB from *Klebsiella pneumoniae*

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ABSTRACT

The acquisition of ferrous iron (Fe^{2+}) is an important virulence factor utilized by several hospital-acquired (nosocomial) pathogens such as *Klebsiella pneumoniae* to establish infection within human hosts. Virtually all bacteria use the ferrous iron transport system (Feo) to acquire ferrous iron from their environments, which are often biological niches that stabilize Fe^{2+} relative to Fe^{3+}. However, the details of this process remain poorly understood, likely owing to the few expression and purification systems capable of supplying sufficient quantities of the chief component of the Feo system, the integral membrane GTPase FeoB. This bottleneck has undoubtedly hampered efforts to understand this system in order to target it for therapeutic intervention. In this study, we describe the expression, solubilization, and purification of the Fe^{2+} transporter from *K. pneumoniae*, *Kp*FeoB. We show that this protein may be heterologously overexpressed in *Escherichia coli* as the host organism. After testing several different commercially-available detergents, we have developed a solubilization and purification protocol that produces milligram quantities of *Kp*FeoB with sufficient purity for enzymatic and biophysical analyses. Importantly, we demonstrate that *Kp*FeoB displays robust GTP hydrolysis activity (k_{cat}^{GTP} of ~10^{-1} s^{-1}) in the absence of any additional stimulatory factors. Our findings suggest that *K. pneumoniae* may be capable of using its Feo system to drive Fe^{2+} import in an active manner.

1. Introduction

Iron is an essential and versatile element for virtually every living organism and has been adopted to serve in some of the most important biological processes [1,2]. However, iron-based life represents a double-edged sword for most organisms, as the reduced form of iron (Fe^{2+}) is highly reactive yet bioavailable, whereas the oxidized form of iron (Fe^{3+}) is relatively inert but somewhat intractable [3]. Accordingly, every organism that utilizes iron employs biological pathways to obtain this element from its environment, to regulate its bioavailable concentration, and to sequester its excess [1,4]. Prokaryotic iron uptake has been best studied in *Escherichia coli*, but several pathways common to many bacteria have been identified and characterized, such as siderophore-based Fe^{3+} acquisition [1], and heme acquisition [5,6]. Divergent from these systems, nearly all bacteria also possess an Fe^{2+} uptake system. Emerging evidence suggests Fe^{2+} import represents an important route of bacterial iron uptake during infection, but much less is known about this process by comparison to bacterial Fe^{3+} uptake [1,4,7–11].

The ferrous iron transport (Feo) system is encoded by the *feo* operon (Fig. 1) and is the major prokaryotic Fe^{2+} import route [1,7]. In several pathogenic bacteria, this region is under control of iron-responsive transcriptional regulators, such as the fumarate and nitrate reductase (FNR) regulator, and/or the ferric uptake regulator (FUR) [7,8]. The archetypal *feo* operon encodes for two soluble, cytoplasmic proteins (FeoA and FeoC) and an integral membrane protein (FeoB) (Fig. 1) [7,12]. FeoA is strongly conserved across species, whereas FeoC is chiefly found in only γ-proteobacteria [7,8]. FeoB is the main component of the Feo system and is present in virtually all bacteria [7,8]; the only notable exceptions to FeoB conservation are those species that may couple a P_{1B}-ATPase to

Abbreviations: ATP, adenosine triphosphate; $C_{12}E_8$, octaethylene glycol monododecyl ether; CD, circular dichroism; CHAPSO, 3-([3-cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate; DM, *n*-decyl-β-D-maltopyranoside; DDM, *n*-dodecyl-β-D-maltopyranoside; Feo, ferrous iron transport system; GAP, G-activating protein; GTP, guanosine triphosphate; GTPBD, GTP-binding domain; IPTG, isopropyl β-D-*l*-thiogalactopyranoside; LDAO, lauryldimethylamine oxide; NFeoB, N-terminal G-protein-like domain of FeoB; OG, *n*-octyl-β-D-glucoside; SDS, sodium dodecyl sulfate.

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Fig. 1. The arrangement of the *feo* operon in *K. pneumoniae* (strain 342). The *K. pneumoniae* (strain 342) *feo* operon encodes for the soluble proteins FeoA and FeoC, and the integral membrane protein FeoB.

FeoA/C utilization [13], but this unique genomic arrangement is uncommon. The roles of FeoA and FeoC in Fe^{2+} import remain enigmatic, but it is clear that FeoB is the major protein that facilitates ferrous iron uptake across a lipid bilayer [4,7,8].

FeoB is an inner membrane protein that comprises an N-terminal G protein-like soluble domain tethered to ~8–12 transmembrane helices (Fig. 2) [7,8,14]. It was initially thought that FeoB was an ATPase based on the misidentification of the N-terminal nucleotide-binding domain as an adenosine triphosphate (ATP)-binding site. It is now clear that this domain is capable of hydrolyzing guanosine triphosphate (GTP) and bears a strong structural resemblance to eukaryotic G proteins [15]. This structural similarity is based on several X-ray crystal structures from numerous bacterial species of the isolated, soluble N-terminal G protein-like domain (commonly termed NFeoB) in the presence and/or absence of GTP analogs [16–20]. *In vivo* studies have indicated that intact NFeoB is necessary for bacterial Fe^{2+} uptake, but *in vitro* studies have indicated that the isolated NFeoB domain hydrolyzes GTP too slowly to be suited for active transport without any additional stimulatory factors [15,17,19]. Consequently, reconciling the slow rate of NFeoB GTP hydrolysis with the strong metabolic requirements for iron of most bacteria has remained difficult. To circumvent a similar problem with slow GTP hydrolysis rates, many eukaryotes utilize G-activating proteins (GAPs) that increase the rate of GTP hydrolysis [21–23], but no such protein has been identified in bacteria for FeoB stimulation. Thus, it remains unclear how functionally related FeoB is to its structurally similar eukaryotic counterparts.

It is plausible that the slow rate of GTP hydrolysis of NFeoB is a result of missing interactions provided from the membrane components that comprise the majority of FeoB. Unfortunately, perhaps owing to its recalcitrant nature as a large, complex membrane protein, only a few studies have shown that full-length FeoB may be expressed heterologously, solubilized from bacterial membranes and purified [24–26]. Only one FeoB construct from *Pseudomonas aeruginosa* has been demonstrated to successfully hydrolyze GTP

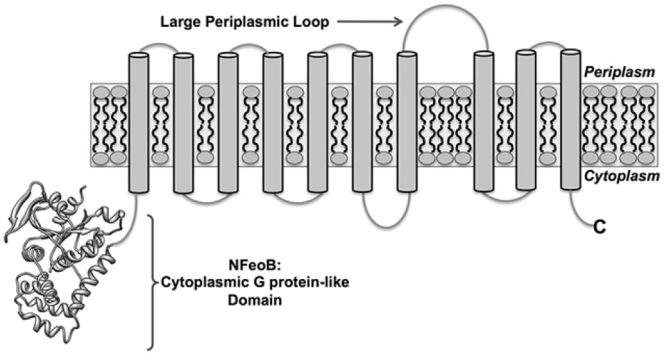

Fig. 2. Predicted structure and topology of *Kp*FeoB. At the N-terminus of *Kp*FeoB is the G protein-like domain (PDB ID 2WIA, NFeoB). Transmembrane helical predictions, utilizing the TMHMM server [43], suggest the core of *Kp*FeoB comprises 10 transmembrane helices, with a large periplasmic loop between transmembrane helices 7 and 8, as the peptide chain progresses towards the C-terminus.

[24,25], but this catalytic rate constant still remained below the regime accepted for active transport. Venter and coworkers have postulated that FeoB may function as a GTP-gated channel, and that a slow GTP hydrolysis rate may regulate the gating of this channel and subsequently Fe^{2+} import [25]. Thus, the available biochemical data raise questions towards the most fundamental nature of FeoB-mediated ferrous iron transport: *i.e.*, is the process active, facilitated, or passive?

Notwithstanding these unanswered questions, it remains clear that ferrous iron import appears to be a key route of iron uptake for pathogens living in several biological niches. FeoA and FeoB knockouts in model bacteria have decreased or abrogated growth of several strains [27–31]. Furthermore, gene knockouts of the *feo* operon native to several human pathogens have either reduced [32,33] or wholly prevented [9] colonization of these pathogens within mouse models, emphasizing the importance of this uptake pathway to bacterial infection in mammals. Despite the significance of this iron uptake pathway in virulence, additional questions regarding the mode of iron binding and the mechanism of iron uptake mediated by the Feo family of proteins remain unanswered. This paucity of fundamental mechanistic information is likely due (in part) to the few expression and purification systems existing that can produce milligram quantities of the protein FeoB for biophysical characterization [24–26].

We report herein the isolation, purification, and functional characterization of FeoB from *Klebsiella pneumoniae* (*Kp*FeoB), the causative agent of a number of hospital-acquired (nosocomial) infections [34,35]. We demonstrate that *Kp*FeoB may be heterologously overexpressed in *E. coli*, solubilized in several non-denaturing detergents, and purified using metal-affinity chromatography to yield milligram quantities of enzyme active towards GTP hydrolysis. Importantly, we demonstrate that *Kp*FeoB is the first intact FeoB to be characterized that displays a rate of GTP hydrolysis within the active regime, and this catalytic rate is sensitive to the nature of the detergent used. Combined, these results suggest that FeoB may be generally capable of importing Fe^{2+} in an active manner by deriving energy from GTP hydrolysis within bacteria.

2. Materials and methods

2.1. Materials

The pET-21a(+) expression plasmid was purchased from EMD-Millipore (MilliporeSigma). Modified forms of the BL21(DE3) and C43(DE3) *E. coli* expression cell lines in which the gene for the multidrug exporter AcrB (a common contaminant from *E. coli* membranes) had been deleted (BL21(DE3) Δ*acrB* and C43(DE3) Δ*acrB*, respectively) were generous gifts of Prof. Edward Yu (Iowa State University). All materials used for buffer preparation, protein expression, and protein purification were purchased from RPI, MilliporeSigma, and/or VWR and were used as received. Detergents and Na_2GTP were purchased from Sigma-Aldrich, stored at −20 °C, and used as received.

2.2. Cloning, expression, and purification of full-length KpFeoB

DNA encoding for the gene corresponding to FeoB from *Klebsiella pneumoniae* (strain 342) (Uniprot identifier B5XTS7) was commercially synthesized by GenScript (Piscataway, NJ), with an additionally engineered DNA sequence encoding for a C-terminal TEV-protease cleavage site (ENLYFQS). This gene was subcloned into the pET-21a(+) expression plasmid using the *Nde*I and *Xho*I restriction sites, encoding for a C-terminal $(His)_6$ affinity tag when read in-frame. The complete expression plasmid was transformed