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RESEARCH ARTICLE

Incorporation of FeoB into synthetic lipid vesicles enables functional GTPase activity in a cell-free artificial membrane system

[version 1; peer review: 3 approved with reservations, 1 not approved]

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Abstract

Background

Ferrous iron uptake is essential for the survival and virulence of many bacterial pathogens. The inner membrane protein FeoB is a key component of iron import, coupling ferrous iron transport to GTP hydrolysis through conserved G-protein domains. Despite the importance of this protein, studying FeoB in isolation remains challenging, as FeoB is an integral membrane protein whose function and stability depend on lipid membrane integration.

Methods

Full-length *E. coli* FeoB constructs fused to YFP were engineered and expressed in vitro. Purified FeoB was reconstituted into giant unilamellar vesicles composed of 1:2 POPC:POPG using a water-in-oil emulsion–transfer method. Bioinformatic analyses validated the domain architecture of FeoB. We assessed *in vitro* expression with both homemade cell-free extracts and the PURE system, evaluated protein encapsulation by confocal microscopy, and quantified GTPase activity using a luminescence-based assay under anaerobic conditions.

Results

Open Peer Review

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FeoB-YFP was successfully expressed and purified, with expression enhanced at lower temperatures. Bioinformatic analysis confirmed the presence of conserved G-protein motifs and transmembrane domains. Encapsulation of FeoB within synthetic vesicles resulted in robust YFP fluorescence, indicating successful membrane integration. Vesicle-encapsulated FeoB bound a fluorescent GTP analog, demonstrating active G-domain function. Luminescence-based assays revealed FeoB GTPase activity that was potentiated by ferrous iron and further enhanced by potassium ions.

Conclusions

We reconstituted and functionally characterized FeoB within controlled artificial membrane environments. The demonstration of active GTPase domains in reconstituted FeoB provides a foundation for dissecting iron transport mechanisms and for screening inhibitors targeting bacterial FeoB-mediated virulence pathways.

Keywords
FeoB, Ferrous-ion transport, giant unilamellar vesicles (GUVs), cell-free expression, membrane protein reconstitution, GTPase activity

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Introduction

Iron is a fundamental nutrient for nearly all living organisms, supporting processes including replication, metabolism, and oxidative stress management (Andrews et al., 2003; Udipi et al., 2012; Cassat and Skaar, 2013). In pathogenic bacteria, competition for iron at the host–pathogen interface is a critical determinant of virulence. Bacteria therefore deploy a wide range of iron uptake systems, including siderophores and dedicated transporters that allow efficient acquisition under iron-limited conditions while preventing toxicity during iron abundance (Clarke et al., 2001; Raymond et al., 2015; Page, 2019; Marlovits et al., 2002; Hagelueken et al., 2016).

The Feo system is one such transport mechanism that enables ferrous iron (Fe^{2+}) uptake under anaerobic or microaerobic conditions (Cartron et al., 2006; Brown et al., 2021). The central component of this system, FeoB, is an integral membrane protein comprising transmembrane helices and a cytosolic N-terminal GTP-binding domain (Kammler et al., 1993; Guilfoyle et al., 2009; Petermann et al., 2010). This G-domain contains conserved motifs and switch regions involved in nucleotide binding, hydrolysis, and conformational cycling (Eng et al., 2008; Marlovits et al., 2002; Ash et al., 2011a,b; Wittinghofer, & Vetter, 2011). GTP hydrolysis is believed to drive the transport of Fe^{2+} across the membrane (Lau et al., 2016; Seyedmohammad et al., 2016).

Although FeoB has been examined in bacterial cells, progress toward defining the mechanism is limited by the surrounding cellular milieu, uncharacterized cofactors, and variation in membrane composition. Reconstituting FeoB into synthetic lipid vesicles provides a means to examine intrinsic properties within a controlled environment. Here, we use cell-based and cell-free expression systems and synthetic membrane vesicles to produce, reconstitute, and functionally characterize full-length FeoB.

Materials and methods

Plasmid construction

FeoB-YFP fusion constructs were assembled using Gibson assembly into pUC57 with an incorporated T7 promoter and pGEX-6p1 with a *tac* promoter. A His-tagged variant (FeoB-YFP-His) was prepared in pGEX-6p1. All constructs were verified by sequencing. Concentrations were measured with a NanoDrop spectrophotometer (Thermo Fisher NanoDrop 2000).

Bioinformatic analysis

Domain architecture was analyzed using SMART (Schultz et al., 2000). Disorder prediction and binding-region identification were obtained using IUPred2A (Mészáros et al., 2018). Functional annotations were retrieved from UniProt.

Recombinant protein expression and purification

FeoB-YFP-His was expressed and purified from *Escherichia coli* BL21(DE3). Protein production was induced at an OD^{660} of ~0.4–0.5 by adding 1.0 mM isopropyl β-D-thiogalactoside (IPTG) (Merck Life Sciences S.r.l. 367931), followed by overnight incubation at 18 °C. Cells were harvested by centrifugation at 5,000 rpm for 30 min at 4 °C and resuspended in cell-resuspension buffer (50 mM HEPES (Merck Life Sciences S.r.l. 40340), 300 mM NaCl (Merck Life Sciences S.r.l. S7653), 2 mM $MgCl_2$ (Merck Life Sciences S.r.l. M2670), EDTA-free protease inhibitor tablet (Merck Life Sciences S.r.l. E5134), 0.1 mg/mL DNase I (Merck Life Sciences S.r.l. 10104159001), and lysozyme; pH 7.0 (Merck Life Sciences S.r.l. 10837059001). The suspension was sonicated and clarified by centrifugation at 10,000 rpm for 30 min at 4 °C.

The supernatant was transferred to ultracentrifuge tubes and centrifuged at 35,000 rpm for 50 min at 4 °C to pellet the membrane fraction. This pellet was resuspended in membrane-resuspension buffer (50 mM HEPES, 300 mM NaCl, 5% glycerol (Merck Life Sciences S.r.l., G5516), EDTA-free protease inhibitor; pH 7.0) and centrifuged again at 35,000 rpm for 50 min at 4 °C. The resulting membrane pellet was solubilized in the same buffer supplemented with 2% (w/v) *n*-dodecyl β-D-maltoside (DDM) (Merck Life Sciences S.r.l. 850520P) and incubated for 2 h at 4 °C with gentle shaking. Insoluble material was removed by ultracentrifugation at 35,000 rpm for 50 min at 4 °C.

Ni^{2+}–nitrilotriacetate (Ni-NTA) resin (Merck Life Sciences S.r.l., 70666) was equilibrated with binding buffer (50 mM HEPES, 300 mM NaCl, 5% glycerol, 0.2% DDM; pH 7.0), and the solubilized protein was incubated with the resin overnight at 4 °C. The resin was transferred to a gravity-flow column and was subsequently washed with 10 column volumes of binding buffer. FeoB-YFP-His was eluted with 50 mM HEPES, 300 mM NaCl, 5% glycerol, 250 mM imidazole (Merck Life Sciences S.r.l., I2399), 0.2% DDM; pH 7.0. Protein yield and purity were assessed by 10% SDS-PAGE. Fractions containing the target protein were pooled and concentrated at 4 °C.

GTPase assay

GTP hydrolysis was quantified using a modified GTPase-Glo assay (Promega, Madison, WI, USA; cat. V7681) under anaerobic conditions in nitrogen-purged glovebox. Reactions contained 10 μM FeoB (recombinantly expressed and purified from *E. coli* BL21(DE3)) in the absence of vesicles, 400 μM GTP, and iron and potassium cofactors where indicated. After a 10 min incubation, luminescence was measured using an EnSight Multimode Plate Reader.

Cell-free extract preparation

E. coli Rosetta 2(DE3) was grown overnight in 2 × YTP medium (Merck Life Sciences S.r.l., Y1375) supplemented with 34 μg/mL chloramphenicol at 37 °C and 220 rpm. The culture was then diluted to an OD^{600} of approximately 0.01 and incubated for an additional 3.5 h under the same conditions. Cells were harvested by centrifugation at 6000 rpm for 10 min at 4 °C.

Extract preparation followed a protocol adapted from the Noireaux laboratory (Marshall & Noireaux, 2019), with the modification of using Snakeskin dialysis membranes (10 kDa MWCO, Thermo Fisher 68100). Freshly prepared extract was aliquoted, flash-frozen in liquid nitrogen, and stored at −80 °C.

The amino acid solution was prepared according to Caschera and Noireaux to achieve equimolar concentrations at pH 7.86 (Caschera & Noireaux, 2014). Cell-free reaction mixtures contained 10 nM plasmid DNA, 33% (v/v) extract, 7.14% (v/v) energy solution, 1.5 mM amino acid solution, and 2% (v/v) PEG-8000 (Sun et al., 2013). Reactions were supplemented with 12 mM maltose for ATP regeneration. Magnesium glutamate (Merck Life Sciences S.r.l., S595179) was added at 5 mM for reactions using T7 RNA polymerase or at 10 mM when relying on the endogenous *E. coli* RNA polymerase.

Cell-free transcription-translation

Unless stated otherwise, reactions employing the cell-free extract were carried out in 10 μL volumes containing 10 nM plasmid DNA. All components were assembled on ice, and incubation at 30 °C initiated the reaction. Fluorescence was recorded continuously for 5 h using a Rotor-Gene Q 6plex system (Qiagen). The yellow channel was selected for YFP detection (excitation: 513 nm; emission: 527 nm). Each reaction was performed in at least three biological replicates.

Cell-free protein synthesis was also examined with the PURE system (PURExpress 2.0, Cosmo Bio). Reaction mixtures contained 5 μL Solution I, 0.5 μL Solution II, and 1 μL Solution III, prepared according to the manufacturer's instructions. FluoroTect™ Green Lys (1.0 μL, Promega) was included, together with plasmid DNA (0.25 to 1 nM). Reaction mixtures were incubated for 2 h and 4 h, respectively, in a thermal cycler at 37 °C. Products were then resolved by PAGE in a dark room and visualized using an Amersham™ Typhoon™ Biomolecular Imager.

Liposome preparation and encapsulation

Liposomes were prepared using a water-in-oil emulsion–transfer method. POPC (1-palmitoyl-2-oleoyl-sn-glycero-3--phosphocholine) and POPG [1-palmitoyl-2-oleoyl-sn-glycero-3-(phospho-*rac*-(1-glycerol))], each supplied in chloroform at 100 mg/mL (Avanti Polar Lipids), were combined in a round-bottom flask in a 1:2 molar ratio. The lipid mixture was rotary evaporated at room temperature for 3 h to yield a dry thin film. Mineral oil (500 μL) was added to the film and vortexed at high speed for 20 s. The suspension was heated in a 70 °C water bath for 1 min, vortexed for 30 s at room temperature, incubated again at 70 °C for 1 min, and vortexed vigorously for an additional 2–3 min until the mixture returned to room temperature.

An inner solution (20 μL; 2.4 mg Ficoll in 0.2 M sucrose) was introduced into the lipid–oil suspension and vortexed for 30 s, producing a translucent white emulsion. In a separate tube, 300 μL of an outer solution (0.2 M glucose) was prepared, and the oil emulsion was layered on top. The two-phase system was centrifuged at 10,000 g for 5 min at 20 °C. The mineral-oil layer and residual white debris at the interface were removed carefully with a pipette. Giant unilamellar vesicles (GUVs), collected from the bottom aqueous layer, were transferred gently to a fresh tube for subsequent experiments.

Cell-free transcription-translation in GUVs

Following formation of the lipid thin film, mineral oil was added to resuspend the lipids as described earlier. The cell-free reaction mixture and plasmid DNA were then incorporated together with 2.4 mg Ficoll and 2 M sucrose to generate the inner solution. The outer solution consisted of a cell-free reaction mixture supplemented with spermidine and 2.5 M glucose. Liposome preparation proceeded as described above, and the resulting pellet was incubated at 37 °C for 4 h. Protein synthesis within the vesicles was subsequently visualized by confocal microscopy. For experiments using a GTP analogue, 20 nM BODIPY FL–labeled GTP was added to the inner solution, and all subsequent steps were performed in the dark.

Results

Expression and purification of FeoB-YFP

FeoB-YFP was expressed in *E. coli* using both T7- and tac-driven constructs. Lower expression was observed from the T7-driven FeoB-YFP construct compared to the tac-driven construct (Figure 1). Expression improved markedly at reduced temperatures, consistent with known folding constraints for membrane proteins. Purification via Ni-NTA

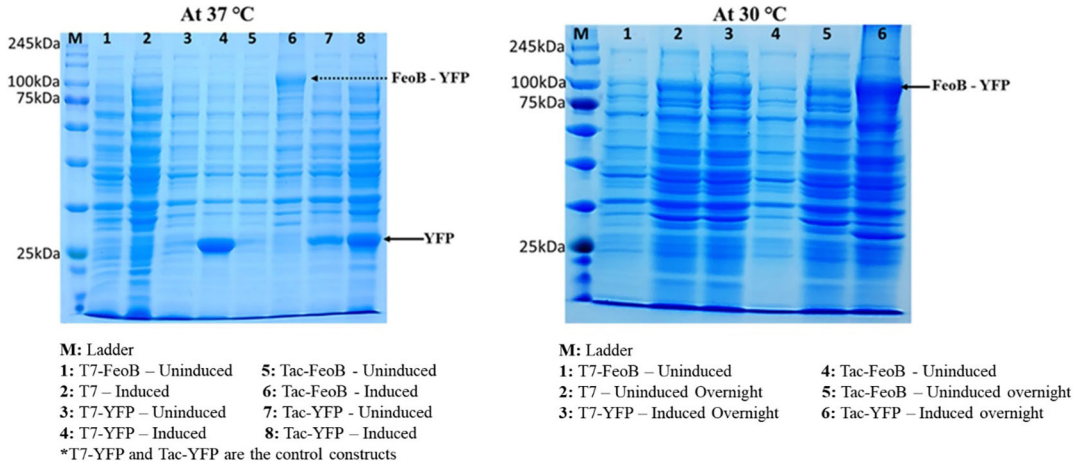

Figure 1. SDS–PAGE analysis of recombinant FeoB–YFP expressed in *E. coli* BL21 (DE3). The left panel (37 °C) shows protein expression from T7- and Tac-driven constructs. Lanes 1 and 5: uninduced T7-FeoB–YFP and Tac-FeoB–YFP; lanes 2 and 6: induced T7-FeoB–YFP and Tac-FeoB–YFP; lanes 3 and 7: uninduced samples (replicates); lanes 4 and 8: induced samples (replicates). The band corresponding to FeoB–YFP is highlighted. Control constructs (T7-YFP and Tac-YFP) show strong YFP overexpression independent of promoter type. The right panel shows expression profiles of the same constructs at 30 °C. The PageRuler Prestained Protein Ladder (Thermo Scientific, USA) is indicated as M.

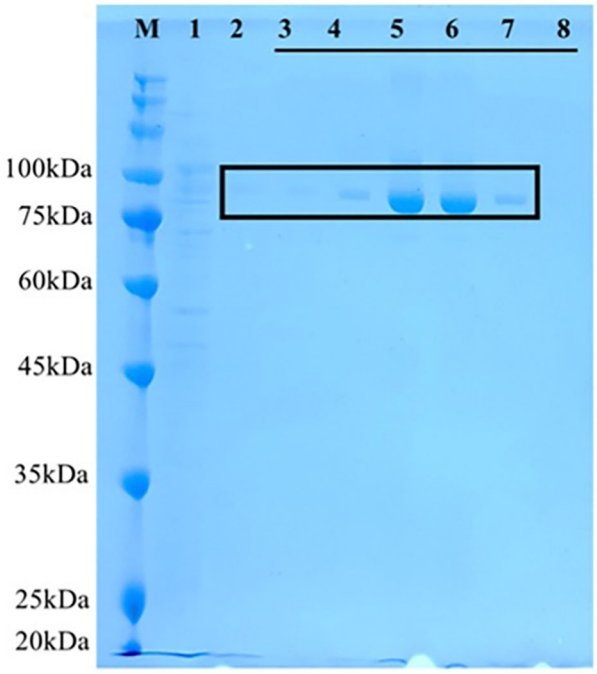

Figure 2. Purification of FeoB by Ni–NTA affinity chromatography. The boxed region highlights elution fractions containing FeoB (~84.5 kDa). Elution fractions in lanes 5 and 6 were pooled for downstream analysis. Lane M: PageRuler Prestained Protein Ladder (Thermo Scientific™, USA); lane 1: lysate flowthrough; lane 2: binding/wash flowthrough; lanes 3–8: successive elution fractions containing FeoB.

chromatography yielded FeoB at the expected molecular weight (~85 kDa) with Coomassie-stained polyacrylamide gels confirming successful enrichment (Figure 2).

Bioinformatic validation of FeoB architecture

SMART, IUPred2A, and UniProt analyses confirmed that the FeoB sequence contains canonical G-protein motifs, switch regions, ordered/disordered segments, and the predicted seven transmembrane helices (Figure 3). These results validated the integrity of the FeoB constructs used for downstream assays.

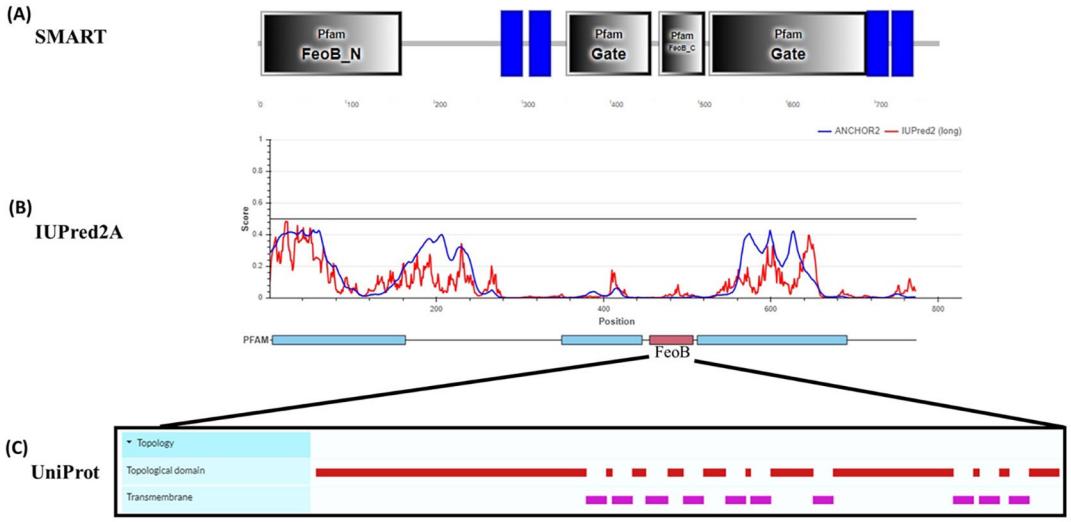

Figure 3. Bioinformatic analysis of the FeoB sequence. (A) Visualization of predicted functional domains. (B) Amino acid sequence and homology predictions. (C) Functional annotations of the FeoB sequence.

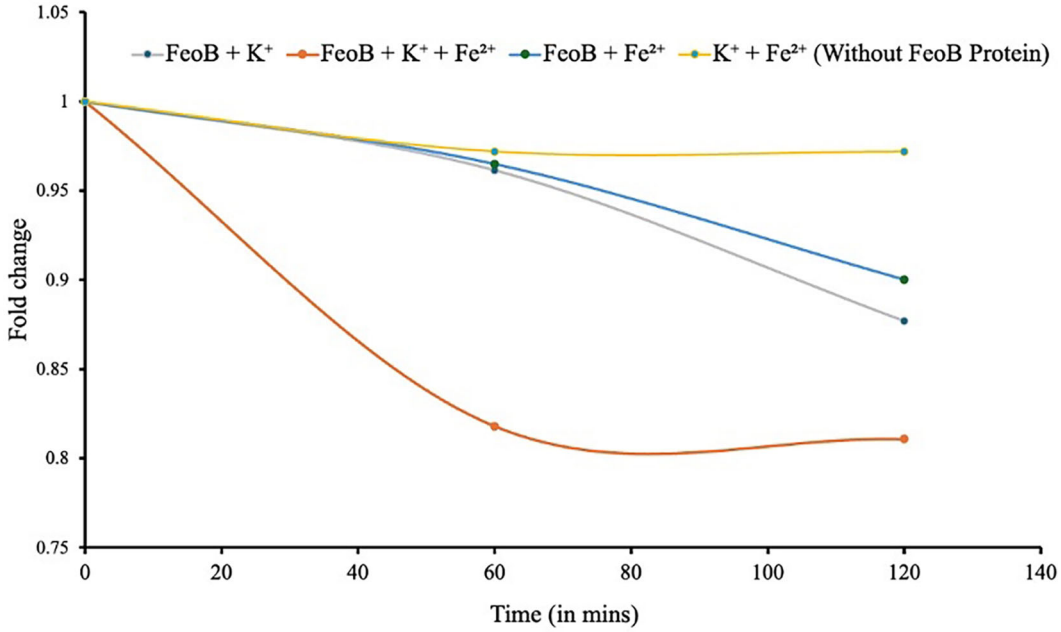

Figure 4. GTPase activity of FeoB measured using a luminescence-based assay. A 10 μM FeoB sample exhibited measurable GTPase activity in the presence of 1 mM Fe^{2+}, and this activity increased further upon addition of 200 mM K^{+}. No detectable GTPase activity was observed in the absence of FeoB. Lower values indicate increased GTPase activity.

GTPase activity is enhanced by iron and potassium

To test whether the GTPase domain retained activity, a luminescence-based GTPase assay was used to monitor time-dependent GTP hydrolysis by the purified protein. The activity of FeoB was significantly increased in the presence of Fe^{2+} and further enhanced upon addition of K^+ under anaerobic conditions (Figure 4). Control reactions lacking FeoB showed no activity. These findings align with earlier reports showing potassium-dependent activation of G-protein domains in FeoB. (Ash et al., 2010 (c)).

In vitro expression using cell-free systems

To facilitate reconstitution in lipid membranes, we probed whether FeoB-YFP could be expressed via in vitro transcription-translation. With *E. coli* Rosetta 2(DE3) cell extract, reactions expressing FeoB-YFP showed temperature-dependent profiles (Figure 5). At 37 °C, expression was limited; however, robust YFP fluorescence emerged at 25–30 °C. The production of protein was confirmed by repeating the reactions with fluorescently labeled Lys residues with the PURE system and resolving the protein on an SDS-PAGE (Figure 6).

Encapsulation of FeoB in lipid vesicles

In vitro transcription-translation in the presence of GUVs revealed the formation of GUVs containing FeoB-YFP integrated into the membrane, as determined by confocal fluorescence microscopy (Figure 7). Fluorescent signal

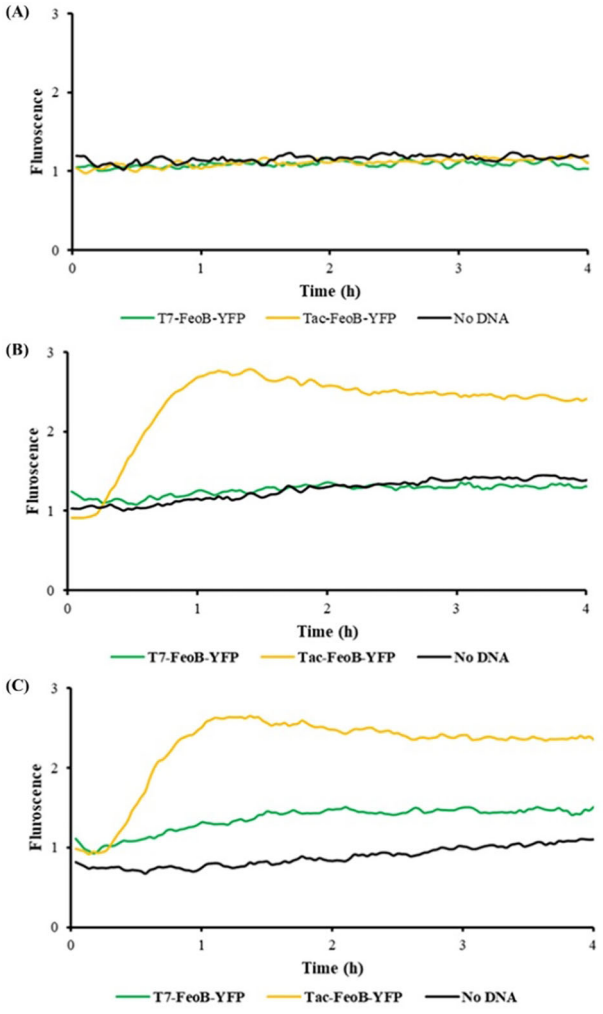

Figure 5. Fluorescence profiles of *in vitro*–expressed constructs T7-FeoB-YFP and Tac-FeoB-YFP at different temperatures using the *E. coli* Rosetta 2(DE3) cell-free system. (A) Expression of the YFP-tagged FeoB driven by either the T7 or *tac* promoter was undetectable at 37 °C. (B) Lowering the temperature to 30 °C resulted in enhanced expression with a *tac* promoter. (C) At 25 °C, both constructs expressed protein, with a greater amount of detectable protein synthesized with the *tac* promoter. Yellow, T7 transcriptional promoter; green, *tac* transcriptional promoter; black, no template DNA.

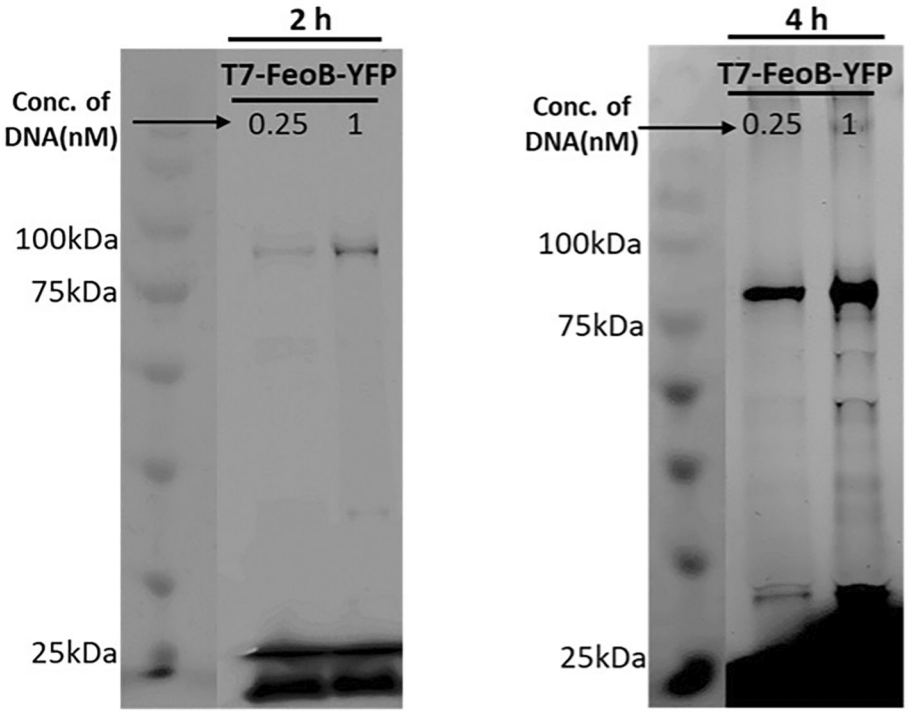

Figure 6. Detection of synthesized FeoB by fluorescent labeling. Transcription-translation was with the PURE system, and the translated product was labeled with FluoroTect™ GreenLys. Reaction mixtures were resolved on a 12% SDS–PAGE gel, and fluorescence was detected with a laser scanner. FeoB synthesis was evident at both time points, with markedly stronger signal observed after 4 h. Aliquots were treated with RNaseA prior to loading on the gel.

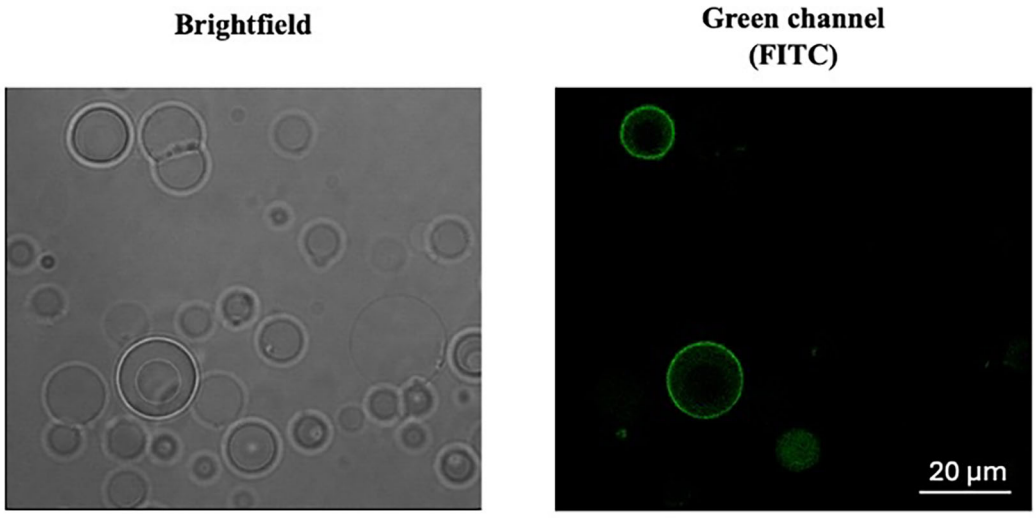

Figure 7. Confocal microscopy of 1:2 molar ratio POPC:POPG vesicles reveals a heterogeneous population of vesicle sizes containing FeoB–YFP after transcription–translation reaction with a tac-driven construct. Encapsulated transcription-translation was with the *E. coli* Rosetta 2(DE3) cell extract. Reactions were run for 4 h at 37 °C. The left panel shows vesicles in brightfield phase contrast, enabling visualization of overall morphology and structural integrity. The right panel displays the corresponding green fluorescence, demonstrating the presence of FeoB–YFP within the vesicle lumen and confirming successful encapsulation of the fluorescent fusion protein.

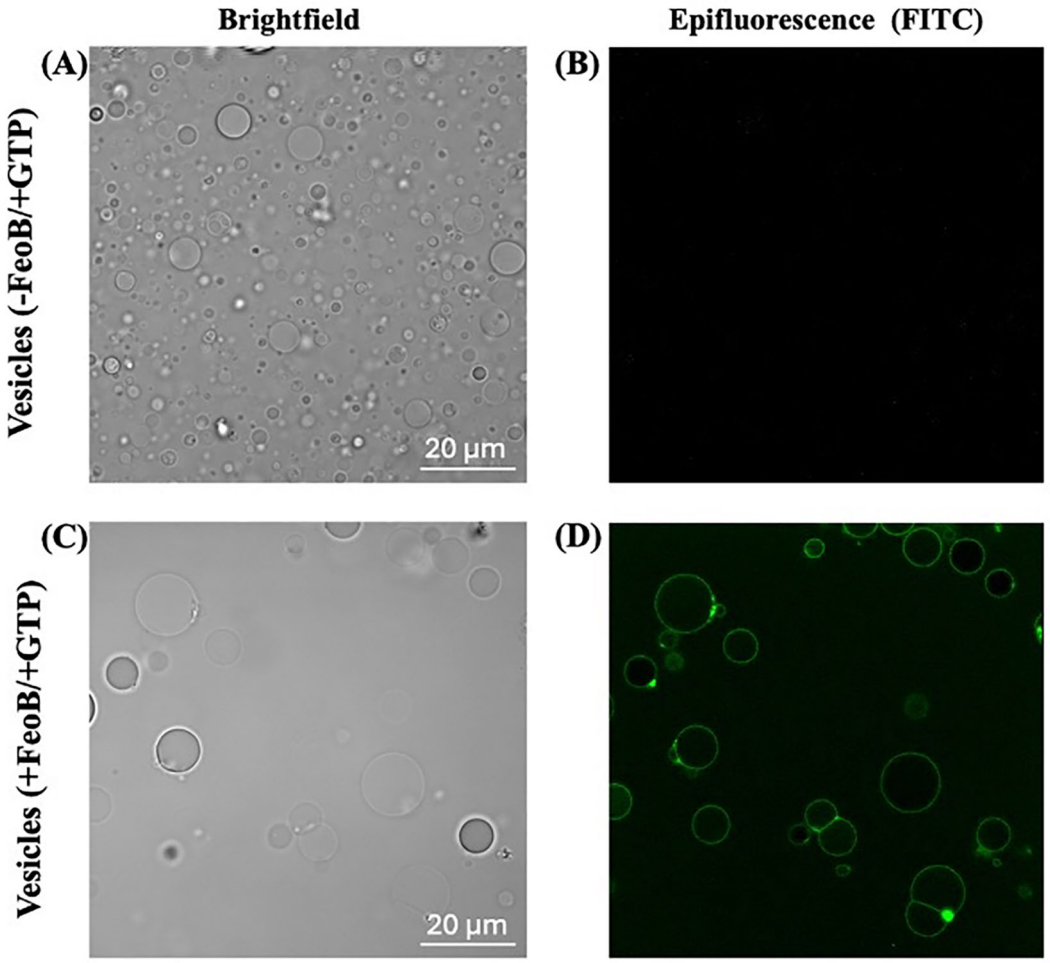

Figure 8. Functional assessment of FeoB using a fluorescent GTP analog. In the upper panels (A, B), the brightfield image (left) shows the vesicle population, while the corresponding fluorescence image (right) demonstrates that green fluorescent BODIPY-labeled GTP (BODIPY FL GTP) does not associate with vesicles lacking protein. In contrast, the lower panels (C, D) reveal that vesicles reconstituted with FeoB exhibit clear binding of the fluorescent GTP analog, as indicated by the green BODIPY FL GTP signal.

localized to vesicle membranes, indicating membrane integration and/or association. Osmotic balancing prevented vesicle rupture, improving reproducibility.

Functional assessment using GTP analogs

Fluorescent BODIPY-GTP did not associate with protein-free vesicles but specifically bound to vesicles containing FeoB that had been encapsulated with BODIPY FL–labeled GTP (Figure 8). This selective interaction indicates functional nucleotide binding by the G-domain within the reconstituted membrane environment.

Discussion

We established a platform to express, purify, and functionally characterize FeoB outside the native cellular context. Using complementary cell-based and cell-free approaches, we generated full-length FeoB and reconstituted the protein within synthetic 1:2 molar ratio POPC:POPG vesicles. This controlled environment enabled direct interrogation of FeoB's biochemical properties, independent of cellular complexity.

Our observations confirm that the G-protein domain of FeoB remains active following reconstitution, binding fluorescent GTP analogs and catalyzing GTP hydrolysis. The enhancement of activity by Fe^{2+} and K^{+} is consistent with models proposing ion-dependent modulation of FeoB during ferrous iron transport (Yamanaka et al., 2000; Scrima, & Wittinghofer, 2006). These results support the view that FeoB employs GTP hydrolysis in a tightly regulated manner, potentially involving additional cofactors such as FeoA *in vivo* (Cartron et al., 2006).

The artificial membrane system developed here can serve as a modular platform to dissect mechanistic features of FeoB, including pore formation, nucleotide cycling, and ion coordination. Moreover, such systems provide a highly controllable environment for screening small-molecule inhibitors targeting FeoB-mediated iron acquisition—an attractive strategy for anti-virulence therapies.

Conclusions

We demonstrate that full-length FeoB can be successfully expressed, purified, and functionally reconstituted within synthetic lipid vesicles using cell-free systems. The resulting proteoliposomes retain GTP-binding and GTPase activity, modulated by iron and potassium ions. This work highlights the potential of minimal, cell-free membrane systems to uncover mechanistic details of complex membrane proteins and to support the rational development of FeoB-targeted inhibitors.

Ethics and consent

Ethical approval and consent were not required.

Data availability

All data supporting the findings of this study will be made available in an open repository upon publication (https://doi.org/10.5281/zenodo.20503086) (Rajanya, B. 2026). This includes raw fluorescence data, gel images, GTPase measurements, and plasmid sequences.

Acknowledgements

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Version 1

Reviewer Report 17 July 2026

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Jan Steinkühler
Kiel University, Kiel, Germany

Banerjee et al. present their results on FeoB, which is discussed to drive the transport of Fe^{2+} across the membrane through GTP hydrolysis. Their paper focuses on the expression of FeoB in *E. coli*, crude *E. coli* extract, and the PURE system. The PURE system is also used to enable insertion of the protein into GUV membranes following encapsulation by an emulsion-transfer method. The work demonstrates protein expression and, in part, protein function using standard methods, including a GTP hydrolysis assay, fluorescent fusion proteins, and SDS-PAGE. The claim that FeoB in vesicles was obtained seems justified, but several aspects require clarification, additional controls, and better contextualization within the existing literature.

1. The work touches on multiple topics but cites the relevant literature only partially. This issue was correctly pointed out by another reviewer regarding the FeoB literature. The same applies to the GUV experiments. Although the specific results may be novel, PURE reactions in GUVs have now been demonstrated by several groups, and the relevant studies should be cited (e.g. see references in https://doi.org/10.1146/annurev-biophys-100620-114132, https://doi.org/10.1021/acssynbio.0c00433)
2. What does “fold change” mean in Figure 4? Are the observed differences statistically significant? Given that a signal is also present in the control samples, additional replicates and a statistical analysis appear necessary. Error bars should also be included or cleared stated that only single repeats were considered.
3. Were the crude-extract cell-free reactions performed with added lipids? This should be clearly stated in the Methods. Native vesicles might be sufficient and the relevant literature is https://doi.org/10.1038/s41467-021-22329-3.
4. Are raw relative fluorescence units shown in Figure 5? Fluorescence increases of only two- to threefold relative to the no-DNA control are very low. For comparison, (https://pubs.acs.org/doi/10.1021/acssynbio.3c00357) reports signals 4 to six orders of magnitude above the control for MscL–GFP expression in the PURE system. Can the authors provide a YFP expression control to demonstrate the fidelity and activity of their homemade crude extract? Again, error bars are missing, which is particularly important given the apparently small difference from the control.
5. The y-axis in Figure 5 should specify the fluorescence units. “Fluorescence” should also be

spelled consistently throughout the manuscript.

6. Figure 6 appears to show truncated products. Because the authors study FeoB–YFP, these products are fluorescently labelled and therefore visible. The possible formation of truncated products through ribosome stalling should be discussed and supported by the relevant literature (https://pubs.acs.org/doi/10.1021/acssynbio.3c00357).
7. Figure 7 shows multiple giant vesicles. In the phase-contrast image, two vesicles stand out because they have substantially higher contrast, visible as a dark ring, than the other vesicles. Interestingly, these are also the vesicles with the clearest FeoB–YFP signal. These vesicles may be multilamellar and therefore better called giant vesicles (GV). Are these images representative?
8. In contrast, the experiments using a fluorescent GTP analogue appear to show FeoB binding to vesicles that resemble unilamellar giant vesicles. This seems inconsistent with the results shown in Figure 7 and should be discussed. How was the orientation of FeoB in the membrane determined, and how does its orientation affect the accessibility and interpretation of the BODIPY FL GTP signal?
9. Figure 7 caption states “Confocal microscopy of 1:2 molar ratio POPC:POPG vesicles reveals a heterogeneous population of vesicle sizes containing FeoB–YFP after transcription–translation reaction with a **tac-driven construct**” Which is likely a typo because the tac prompter would not be expected functional in the PURE system as it only includes T7 polymerase.
10. The authors write that FeoB was “localized to vesicle membranes, indicating membrane integration and/or association” and that “osmotic balancing prevented vesicle rupture, improving reproducibility.” However, the phase-contrast images suggest that not all GUVs maintained membrane integrity, as indicated by the loss or strong variation of internal contrast. This issue could be addressed by using more cautious wording or, preferably, by quantifying GUV contrast, membrane-associated YFP fluorescence, vesicle integrity, and the fraction of vesicles showing membrane localization. Overall, the encapsulation experiments would benefit from improved quantification and independent experimental replicates.
11. The authors state: “These results support the view that FeoB employs GTP hydrolysis in a tightly regulated manner, potentially involving additional cofactors such as FeoA in vivo.” How is the conclusion regarding tight regulation or the involvement of FeoA supported by the presented results?

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Is the work clearly and accurately presented and does it cite the current literature?

No

Is the study design appropriate and does the work have academic merit?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cell-free expression, GUVs, membrane proteins

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 09 July 2026

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Aaron T Smith
University of Maryland Baltimore County, Baltimore, Maryland, USA

The manuscript by Banerjee and coworkers aims to incorporate the Escherichia coli ferrous iron transporter FeoB into synthetic lipid vesicles composed of two lipids, POPC and POPG, and to analyze its function in these vesicles. FeoB is a critical iron transporter utilized by bacteria to establish virulence under anaerobic/micro-aerobic conditions and much remains unknown about FeoB structure and function. This manuscript shows the expression of a YFP-tagged form of E. coli FeoB, assesses its GTP hydrolysis function in a detergent, and demonstrates the formation of FeoB-proteoliposomes through synthetic means and through a cell-free system. While the results here are worthwhile, there are several issues that should be addressed before this work could be indexed.

Major:

1. The introduction does not adequately cover FeoB function and is missing contextualized citations to several prior publications. For example, this new manuscript is not the first to describe FeoB in a lipid environment. Germane to this work, FeoB from P. aeruginosa was previously prepared in inside-out vesicles in PMC4847171 while FeoB from V. cholerae has been prepared in styrene maleic acid copolymers with native bacterial membranes in PMC11725443, but neither of these studies are mentioned.
2. The introduction describes FeoB as a GTP-hydrolyzing protein, but the current view in the field is that FeoB is a promiscuous NTPase (see PMC6410867, PMC7769900, PMC9841765, and PMC11406355). Were nucleotides other than GTP tested for hydrolysis in this work?
3. The introduction describes FeoB as using energy from NTP hydrolysis to drive ferrous iron uptake, but there is no consensus on this mechanism. The fastest NTP hydrolysis rates have been observed in PMID 28941825, but these rates are still likely too slow to drive uptake. The current consensus seems to be that FeoB is an NTP-gated channel, as described in PMC8980893, but that mechanism is still unclear and remains to be explored further.
4. Figure 4 does not actually show GTPase activity but merely “fold change” (of what, this reviewer is unsure). The kinetics of FeoB-YFP catalyzed GTP hydrolysis should be rigorously characterized, the data should be shown, and it should be clarified why a lower fold change is a faster rate. Also, it is surprising that there was no observed GTP hydrolysis in the presence of Mg (II) but the absence of FeoB, as GTP is known to hydrolyze in water (albeit slowly) in the presence of Mg (II) and to be affected by other divalent metals, such as Mn (II) and Fe (II). Were the kinetics corrected for self-hydrolysis in the presence of Mg (II) and/or Fe (II)?
5. Figure 6 has gels or sections of gels stitched together including a molecular weight ladder, which is not appropriate. Either the full gels should be shown, or the panels should be separated to make it clear to the reader that the gels were cropped.

Minor:

1. Introduction: FeoB is described as an inner membrane protein. This is true in Gram-negative bacteria, but FeoB is also present in Gram-positive bacteria that only have a single lipid bilayer.
2. What is the predicted molecular weight of the FeoB-YFP fusion? Why does the fusion appear as >100 kDa in molecular weight in the expression trials (Fig. 1) but <100 kDa in molecular weight for the purified protein?
3. The bioinformatics on E. coli FeoB are fine, but many other groups have studied E. coli FeoB and E. coli NFeoB and have solved the structure of the latter (see PMC4919421). No mention of how these prior results compare to the bioinformatics data is provided.
4. Figure 7 is confusing based on the legend stating that the data are “demonstrating the presence of FeoB–YFP within the vesicle lumen” – lumen means inside the vesicle, but FeoB should be in the bilayer, not the lumen (maybe the YFP is sticking in the lumen?). The image looks like the fusion is in the bilayer—please clarify the figure legend.
5. In the discussion, it is stated that “We established a platform to express, purify, and functionally characterize FeoB outside the native cellular context.” – purification of FeoB outside the native cellular context has been done by many other groups, thus the results should be contextualized with those prior findings.
6. A couple of the statements in the discussion need to be clarified. For example: a) “These

results support the view that FeoB employs GTP hydrolysis in a tightly regulated manner, potentially involving additional cofactors such as FeoA in vivo" – how do the results presented support this view? FeoA is not studied in this manuscript, and nucleotide regulation is also not explored in this manuscript; b) "Such systems provide a highly controllable environment for screening small-molecule inhibitors targeting FeoB-mediated iron acquisition—an attractive strategy for anti-virulence therapies." – This statement is true, but how do the results in this work address this strategy?

7. The references should be carefully checked. For example, the Brown et al. paper is incorrectly referenced and should be PMC8979593.

Is the work clearly and accurately presented and does it cite the current literature?
No

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Structural biology, Biochemistry, Bioinorganic Chemistry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Reviewer Report 09 July 2026

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Małgorzata Ostrowska
University of Wrocław, Wrocław, Poland

The manuscript entitled „Incorporation of FeoB into synthetic lipid vesicles enables functional GTPase activity in a cell-free artificial membrane system" present an interesting approach for the expression, purification, and reconstitution of the bacterial FeoB transporter into synthetic lipid vesicles. Overall, the manuscript is well organized and clearly written. The topic is timely and relevant, particularly considering the growing interest in minimal cell systems and membrane protein reconstruction. Although the work is generally of good quality, several minor issues should be addressed to improve the clarity of data presentation. Therefore, I recommend acceptance after minor revision.

1. Quantitative characterization of GTPase activity is insufficient. Figure 4 appears to present only representative traces. The manuscript lacks: the number of biological replicates, error bars, statistical analysis, and significance testing. It is difficult to evaluate the robustness of the reported activation of Fe(II) and K(I). The authors should provide quantitative data presented as means +/- SD together with appropriate statistical analysis.
2. The specificity of the measured GTPase activity would be considerably strengthened by including appropriate negative controls.
3. More detailed enzymatic characterization is desirable. Only one experimental condition is investigated. A more biochemical characterization could include: Michaelis-Menten kinetics, determination of Km and Vmax, dependence on Fe/K concentration.
4. The manuscript does not report the purification yield of FeoB.
5. Additional details regarding maintenance of anaerobic conditions during GTPase measurements would improve reproducibility.
6. The manuscript occasionally shifts between past and present tense when describing experimental results.
7. Temperature notation should be used consistently throughout the manuscript.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Bioinorganic chemistry, coordinaton chemistry, biochemistry, enzymatic

characterization, metal ions transporters

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 08 July 2026

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Elka R Georgeiva
Texas Tech University, Lubbock, Texas, USA

The manuscript “Incorporation of FeoB into synthetic lipid vesicles enables functional GTPase activity in a cell-free artificial membrane system” reports the successful expression, purification, and incorporation of the prokaryotic iron transporter FeoP into lipid membranes.

The study appears carefully conducted, and the results are clearly presented.

This work represents an important step toward characterizing a transporter that is critical for bacterial viability and should support future structural and functional studies. Its main strengths are the production of highly pure, active detergent-solubilized FeoP and the cell-free expression of FeoP, which was successfully incorporated into POPC/POPG liposomes and shown to bind a fluorescently labeled GTP analogue.

However, it is unclear why the authors did not reconstitute the detergent-solubilized, GTPase-active FeoP into POPC/POPG liposomes and assess its activity in that system. Such experiments would strengthen the significance of the work, since reconstituted, highly purified membrane transporters provide valuable model systems for studying protein structure, conformational dynamics, and function. They would also allow a more precise evaluation of FeoP activity under near-native conditions, because both the protein concentration and protein-to-lipid ratio would be defined.

Could the authors provide additional experimental details, such as the number of histidine residues in the His-tag and the rationale for using a buffer at pH 7?
A more detailed discussion of previous efforts to purify FeoP would also help clarify the significance of this study. In particular, why has FeoP not previously been recombinantly produced and purified, despite the fact that many prokaryotic membrane transporters have been successfully produced and characterized?

There are no error bars in Figure 4.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and does the work have academic merit?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Memebrane proteins, protein-protein complexes, virus-host interactions

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.