



RESEARCH ARTICLE

REVISED Crystallization and preliminary X-ray diffraction analysis of YejM from *Salmonella typhimurium*: an essential inner membrane protein involved in outer membrane directed cardiolipin transport [version 2; referees: 1 approved, 1 not approved]

Previously titled: Towards understanding the molecular mechanism of cardiolipin transport in *Salmonella typhimurium*: interactions between an essential inner membrane protein YejM and its newly found ligand, YejL

Uma Gabale, Gene Qian, Elaina Roach, Susanne Ressler

Molecular and Cellular Biochemistry Department, Indiana University Bloomington, Bloomington, IN, 47405, USA

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Abstract

Salmonella typhimurium is responsible for over 35% of all foodborne illness related hospitalizations in the United States. This Gram-negative bacterium possesses an inner and an outer membrane (OM), the latter allowing its survival and replication within host tissues. During infection, OM is remodeled by transport of glycerophospholipids across the periplasm and into the OM. Increased levels of cardiolipin in the OM were observed upon PhoPQ activation and led to the discovery of YejM; an inner membrane protein essential for cell growth involved in cardiolipin binding and transport to the OM. Here we report how YejM was engineered to facilitate crystal growth and X-ray diffraction analysis. Successful structure determination of YejM will help us understand how they interact and how YejM facilitates cardiolipin transport to the OM. Ultimately, *yejM*, being an essential gene, may lead to new drug targets inhibiting the pathogenic properties of *S. typhimurium*.

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Referee Status:

	Invited Referees	
	1	2
REVISED		
version 2 published 11 Dec 2017		
version 1 published 02 Jun 2016	report	report

- 1 **William T. Doerrler**, Louisiana State University, USA
- 2 **Yihua Huang**, Chinese Academy of Sciences, China

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Comments (0)

Corresponding authors: Uma Gabale (ugabale@indiana.edu), Susanne Ressler (suessl@indiana.edu)

Author roles: **Gabale U:** Conceptualization, Data Curation, Formal Analysis, Methodology, Validation, Writing – Original Draft Preparation, Writing – Review & Editing; **Qian G:** Data Curation; **Roach E:** Data Curation; **Ressler S:** Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Supervision, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

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REVISED Amendments from Version 1

The reviewers' comments motivated us to redo our experiments and perform additional ones. The results are summarized in new [Supplementary Figure 1–Supplementary Figure 3](#), provided with detailed figure legends. Although being an interesting possibility, we decided that with the current body of work, our suggested interaction between YejL and YejM, is too speculative. For the purpose of transparency, we provide all additional body of work that we performed in response to the reviewers' critique in [Supplementary File 1](#). We hope that this in-detail reply clarifies our decision and motivation to have made this revision of our manuscript. We have also revised [Figure 1](#) and removed [Figure 2](#).

See referee reports

Introduction

Salmonella typhimurium is a Gram-negative bacterium responsible for over 35% of all foodborne illness-related hospitalizations in the United States ([Painter et al., 2013](#)). *S. typhimurium* possesses an additional outer membrane (OM) with an asymmetric lipid composition, that serves as a barrier to the environment allowing its survival and replication within host tissues ([Dalebroux & Miller, 2014](#); [Needham & Trent, 2013](#); [Pagès et al., 2008](#)). Mechanisms for the transport and assembly of OM lipopolysaccharides, proteins, and exopolysaccharides have been defined ([Dong et al., 2006](#); [Dong et al., 2014](#); [Hagan et al., 2011](#); [Whitfield & Trent, 2014](#)); however, the transport of glycerophospholipids across the periplasm and insertion into the inner-leaflet of the OM is not well understood.

Interestingly, increased levels of cardiolipin in *S. typhimurium* OM were observed upon PhoPQ regulator activation ([Dalebroux et al., 2014](#)). Recently the inner membrane protein YejM (also known as PbgA) was shown to bind cardiolipin and be involved in OM formation ([Dalebroux et al., 2015](#)). Furthermore, YejM is known to be an essential gene in *E. coli* and was shown to be involved in intrinsic multidrug resistance ([De Lay & Cronan, 2008](#); [Duo et al., 2008](#)). YejM is comprised of 586 amino acids forming five predicted N-terminal transmembrane helices, followed by an arginine-rich periplasmic random coil linker region, and a C-terminal periplasmic domain ([Figure 1A](#)), and it was shown that YejM associates as a tetramer in solution ([Dalebroux et al., 2015](#)). The arginine-rich linker region and periplasmic globular domain of YejM were demonstrated to bind cardiolipin and are required for OM remodeling and cell growth ([Dalebroux et al., 2015](#)). The molecular mechanism of the interplay between PhoPQ system and YejM, and how cardiolipin molecules

are transported to the OM, need further structural and functional investigation.

Here we report crystal growth and present successful crystallization conditions for full-length YejM from *Salmonella typhimurium* using lipidic cubic phase crystallization and how the periplasmic domain of YejM was engineered to facilitate crystallization, and present preliminary X-ray diffraction analysis of the construct YejM241-586. Future successful structure determination of YejM will help us to understand cardiolipin transport to the OM and may lead to new drug targets inhibiting the pathogenic properties of *S. typhimurium*.

Results

Full-length YejM crystallized using lipidic cubic phase

Full-length YejM was expressed and purified ([Figure 1B](#), first lane) as described in ([Dalebroux et al., 2015](#)), and concentrated to 15 mg/ml in the presence of 0.01% DDM. Micro crystals appeared in many conditions after two weeks. [Figure 1C](#) shows examples of LCP boli with micro crystals as judged by UV microscopy. Further work on full-length YejM will be aimed to optimize current crystal conditions and optimize detergents for LCP and regular vapor diffusion crystallization.

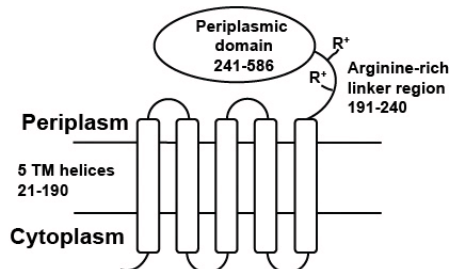
Specific construct development was needed to achieve YejM crystal growth

We expressed and purified periplasmic construct YejM191-586 as described in ([Dalebroux et al., 2015](#)). The original construct YejM191-586 failed to crystallize and showed a degradation product after electrophoresis in the SDS polyacrylamide gel ([Figure 1B](#), second lane). To prevent degradation, reduce flexible protein parts and remove positively charged arginine clusters, and increase the chance for crystal growth, we deleted the linker region A191 to E240 in the YejM191-586 construct, resulting in YejM241-586 ([Figure 1B](#), third lane). Both constructs eluted as well-defined peaks when applied to size exclusion chromatography (SEC) ([Figure 1D](#)). A clear shift in size between both constructs was observed in SEC when performed timely after affinity chromatography. When compared to the standards, both constructs eluted at later volumes than expected for their size ([Figure 1D](#)). Initial crystals of YejM241-586 appeared after one week incubation at 18°C under different conditions; e.g. needle clusters in 2.8 M sodium acetate trihydrate pH 7.0, 0.1 M BIS-TRIS propane pH 7.0 (Hampton SaltRx condition A2, Hampton Research), needle clusters in 2.8 M sodium acetate (Hampton Index condition B12, Hampton Research), and rhombohedral crystals appeared in 3.5 M sodium formate pH 7.0 (Hampton Index HR conditions C1, Hampton Research). These

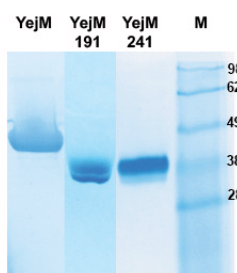
early hit crystals diffracted poorly, only up to 6 Å and optimization and up-scaling of the crystallization setup from a 96 well format to a 24 well format did not improve the diffraction quality. Further screening using (Hampton PEGRx HT screen, Hampton Research) resulted in new crystal forms grown in condition C6 (0.1 M HEPES pH 7.5, 12% w/v polyethylene glycol 3,350) and C4 (0.1 M Citric acid pH 3.5, 25% w/v polyethylene glycol

3,350). Screening around these two conditions led to crystals in a condition consisting of (0.1 M citric acid pH 4, 18% w/v polyethylene glycol 3,350 (Figure 1C). YejM241-586 crystals grown in condition C6 diffracted well, up to 1.6 Å (Figure 1E). Data indexing and scaling with XDS (Kabsch, 2010) and further analysis with AIMLESS (Evans, 2006) resulted in a data set up to 1.8 Å resolution and good overall statistics (Table 1).

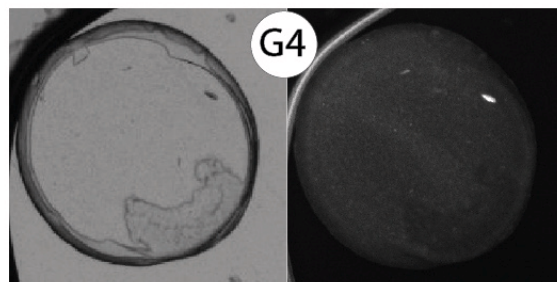
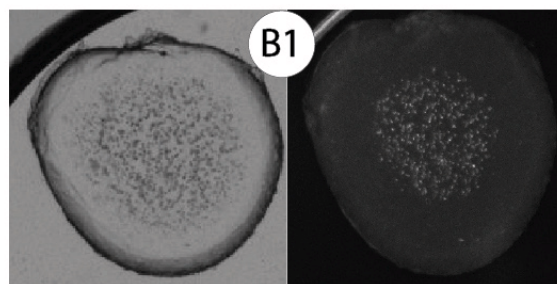
A YeJM domain architecture



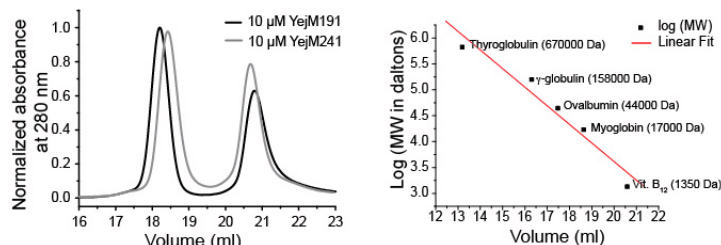
B SDS-PAGE analysis



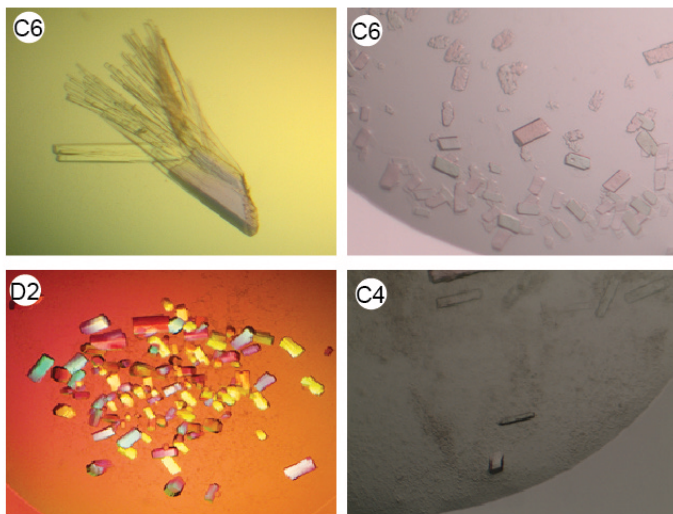
C Lipidic cubic phase crystallization



D Size Exclusion Chromatography



E Vapor diffusion crystallization



E X-ray Crystal Diffraction

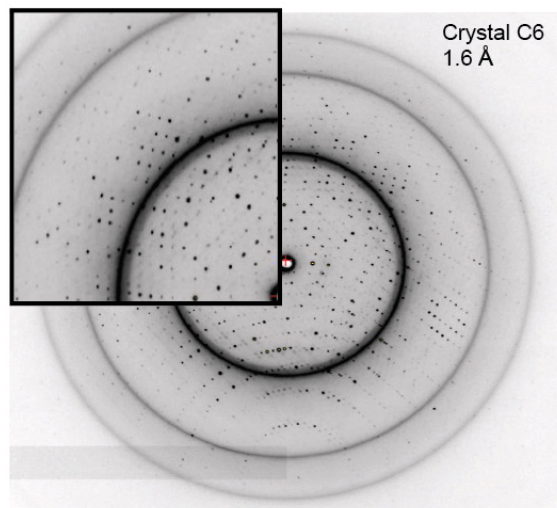


Figure 1. YeJM architecture, purification, crystallization and diffraction data. **A)** YeJM protein domain architecture. **B)** SDS-PAGE analysis of purified protein samples. **C)** LCP crystallization droplets of full length YeJM mixed with Monoolein. Crystals are shown in light microscopy image (left) and corresponding UV image (right). B1 and G4 refer to conditions from MemGold crystal screen 1. **D)** Crystals of YeJM241 under various conditions. **E)** Diffraction image of a YeJM241-586 crystal from condition C6.

Table 1. Diffraction data statistics of crystal grown in condition C6 (Figure 1C).

Diffraction source	ALS BL 4.2.2
Wavelength (Å)	1.001
Temperature (K)	100
Detector	CMOS
Crystal-to-detector distance (mm)	200
Rotation range per image (°)	0.106
Total rotation range (°)	190
Space group	P 1 2 1
a, b, c (Å)	82.58, 86.85, 89.03
α , β , γ (°)	90.00, 115.39, 90.00
Resolution range (Å)	59.01–1.8 (1.9–1.8)
Total No. of reflections	315601 (30716)
No. of unique reflections	94930 (9801)
Completeness (%)	91.5 (64.8)
Multiplicity	3.3 (3.1)
$\langle I/\sigma(I) \rangle$	11.2 (2.2)
R_{meas}	8.7 (80.4)

Conclusions

Here we report successful crystallization using protein engineered specifically to enable crystal growth of YejM. Our initial X-ray data analysis of YejM241-586 crystals suggests a dimer assembly of the periplasmic domain, therefore the membrane domain is very likely needed to form the YejM tetramer. We also aim to solve the crystal structures of YejM alone and with bound cardiolipin. Ultimately these structures will help in understanding where and how cardiolipin binds to YejM, and whether YejM's architecture is that of a transporter or channel.

Material and methods

Cloning, expression and purification of YejM

Initial clones of full-length YejM 1-586 (YejM) (Uniprot ID P40709) in pBAD24 and the periplasmic domain of YejM 191-586 in pET28a plasmid are described in (Dalebroux *et al.*, 2015). We used forward primer YejM241-586 5'-ccgcgccgcagccat-atggctagcgcggtctccgttcagtagccg- 3' and reverse primer YejM241-586 5'-gcggtactgaacggagaccgcgtagccatagctgcccgcgg- 3' to create a shorter construct of the periplasmic domain lacking the linker region resulting in YejM 241-586. Purification of YejM, YejM 191-586, and YejM 241-586 was performed as described previously (Dalebroux *et al.*, 2015). Samples used for subsequent crystallization experiments were further purified by SEC using a Superose 6 increase 10/300 GL column (GE Healthcare) in buffer containing 50 mM Tris pH 8.0, and 150 mM NaCl. SEC buffer for YejM contained 20 mM HEPES, pH 7.5, 150 mM NaCl, and 0.02% Dodecyl- β -D-Maltopyranoside (DDM). The

concentration of DDM was kept right above the critical micelle concentration throughout all subsequent experiments. YejM and YejM241-586 SEC peak fractions were pooled and concentrated with 30 kDa NMWL centricon (Millipore) to 12 mg/ml and up to 50 mg/ml, respectively. The purity of the samples was judged by polyacrylamide gel electrophoresis (Figure 1B).

Crystallization of full-length YejM using lipidic cubic phase crystallization

YejM (15 mg/ml) and Monoolein (Hampton Research) were mixed at a 1:1.5 ratio at 23°C in a coupled Hamilton syringe using a LCP Mixer Station (ARI) until LCP mixture appeared clear. LCP crystallization screens were set up using a Gryphon robot (ARI) pipetting 50 nl LCP boli and 500–800 nl precipitant solution of the following screens: MemGold 1 and 2 (Molecular Dimensions), MembFac (Hampton Research), and JBScreen Membrane (Jena Bioscience). LCP crystallization was set up using LaminexTM plates (Molecular Dimensions).

Crystallization of the periplasmic domain of YejM

Vapor diffusion crystallization of YejM 241-586 (5–50 mg/ml) was set up using 96-well crystallization plates (Hampton Research) with a Phoenix robot (ARI). Various sparse matrix screens were used to set up sitting drops with a drop size between 500 nl to 1 μ l. Crystallization plates were incubated at 20°C and monitored for crystal growth in a MinstrelTM HT crystal imaging and detection tower (Rigaku). Optimal crystal growth was obtained at a protein concentration of 4mg/ml.

Data collections and processing of YejM 241-586 crystals

Crystals were harvested using Litho loops (Molecular Dimensions) and Nylon loops (Hampton Research), submerged into paraffin and blotted until no phase separation was visible between paraffin and the excess crystallization solvent. Diffraction data of YejM241-586 crystals were collected at the Advanced Light Source beamline 4.2.2 in Berkeley CA at 100K, using an oscillation of 0.1–0.2° per image. Diffraction data were processed using iMosflm (Powell *et al.*, 2013) or XDS (Kabsch, 2010) and scaled with Scala (Evans, 2006).

Data availability

Raw diffraction data images were uploaded to the Coherent X-ray Imaging Data Bank (<http://cxidb.org/id-42.html>) and are available under CXIDB ID 42, DOI 10.11577/1252489 (Gabale *et al.*, 2016).

Author contributions

U.G. expressed and purified proteins, performed SEC and BNE, cell fractionation, SEC-MALS, analyzed data and wrote manuscript, G.Q and E.R expressed, purified, crystallized and performed BNE, S.R. collected and analyzed data, designed experiments and wrote manuscript.

Competing interests

No competing interests were disclosed.

Grant information

The author(s) declared that no grants were involved in supporting this work.

Acknowledgments

We thank: Prof. Samuel I. Miller and Dr. Zachary D. Dalebroux for constructs of full-length YejM and periplasmic domain of

YejM191-586, Richard Pfuetzner for stimulating discussions, Jonathan T. Siler and Jennifer Wong for help with initial protein purification and crystallization experiments, Dr. Ardian Soca Wibowo for his excellent service at the macromolecular crystallization facility at Indiana University Bloomington, and Dr. Jay Nix for his excellent support at Molecular Biology Consortium Beamline 4.2.2.2 at the Advanced Light Source (Berkeley, CA).

Supplementary material

Supplementary File 1. Supplementary Text and Experiments

[Click here to access the data.](#)

Supplementary Figure 1. Cellular fractionation.

[Click here to access the data.](#)

Supplementary Figure 2. Native PAGE showing controls and mixtures of A: YejM191-586 with YejL and B: YejM241-586 with YejL.

[Click here to access the data.](#)

Supplementary Figure 3. SEC-MALS of YejM241-586 and YejL.

[Click here to access the data.](#)

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Current Referee Status:



Version 1

Referee Report 06 July 2016

doi:10.5256/f1000research.9303.r14811



Yihua Huang

National Laboratory of Biomacromolecules, National Center of Protein Science-Beijing, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China

In this manuscript, Gabele *et al.* reported the structure of YejL and preliminary X-ray crystallographic characterization of the periplasmic domain (Residues 241-586) of YejM, an inner membrane protein potentially responsible for transport of glycerophospholipid cardiolipin. The authors also tested interaction between YejL and YejM using blue native gel electrophoresis and size exclusion chromatography. In general, it is an interesting study, yet the following issues need to be addressed before accepted for indexation:

1. It is not proper to claim that YejL is a ligand of YejM. "Ligand" is dedicated for "receptors" only. Also, there is no strong genetic evidence to show that YejL is involved in cardiolipin transport.
2. The authors claimed that YejM is a dimer based on its periplasmic domain, and probably, also because of YejL forming a dimer. This is not clear as the authors did not study the oligomerization state of the full-length YejM. Other than this, when running SEC on superpose 6, the authors did not include a standard marker for reference. It is hard to judge if the periplasmic domain of YejM forms a dimer in solution.
3. The author believed that the negative charge surfaces of YejL might bind with the arginine-rich loop of YejM. Why the fragment of YejM (241-586) that seems to not include the arginine-rich loop still binds YejL? Please explain.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 28 Nov 2017

Susanne Ressler, Indiana University at Bloomington

- 1.) We apologize for the incorrect use of terminology and would rather term YejL as potential interaction partner of YejM.
- 2.) We carefully checked within the manuscript what section/sentence could have caused this

reviewer's understanding that we claimed YejM to be a dimer. We found the following sentence in the "Conclusions" section of our originally submitted manuscript: "*Our initial X-ray data analysis of YejM241-586 crystals suggests a dimer assembly of the periplasmic domain, therefore the membrane domain is very likely needed to form the YejM tetramer*".

In this sentence, we suggested the existence of a periplasmic domain YejM241-586 dimer based on our analysis of the crystallographic unit. In this analysis the content of the asymmetric unit that builds the crystal resulted in a dimer of YejM 241-586, and therefore we proposed it to be the likely physiologically relevant state of the periplasmic domain alone. From the SEC-MALS studies in (Dalebroux *et al.* 2015) that full-length YejM forms a tetramer when purified with the detergent DDM. Therefore we reasoned in the above stated sentence that further oligomerization into a tetramer must be facilitated by the trans-membrane domain. Whether it exists as a dimer or a monomer either in the native membrane, in the presence of specific lipids or purified using a different detergent is a matter of current investigation by other laboratories (Miller and Dalebroux labs).

3.) Indeed, the arginine-rich linker region of YejM would be the most obvious binding region for YejL. However, we cannot exclude the possibility that YejL binds to solvent-accessible positively charged residues on the YejM241-586 surface.

Competing Interests: No competing interests.

Referee Report 03 June 2016

doi:10.5256/f1000research.9303.r14132



William T. Doerrler

Department of Biological Sciences, Louisiana State University, Baton Rouge, LA, USA

Here, the authors report the expression, purification and isolation of diffractable crystals of the periplasmic domain of the essential salmonella protein YejM, putatively involved in transport of cardiolipin to the outer membrane. They also report interaction between the periplasmic domain of YejM and the small protein YejL produced from a gene immediately upstream and likely cotranscribed with YejM.

YejM was identified by a screen for mutants defective in PhoPQ mediated outer membrane remodeling and was renamed PbgA (PhoPQ barrier gene A). The regions of the protein and their proposed functions are as follows: 1-190, transmembrane domain of the inner membrane; 191-240, arginine-rich linker that binds to cardiolipin and 240-586, the periplasmic domain seemingly required for interaction with OM. The authors report isolation of crystals of the YejM241-596 periplasmic domain and interaction of YejL with YejM241-586 and YejM191-586 truncated mutants. Sadly, the inability to crystallize YejM191-586 will forestall attempts to examine cardiolipin-YejM interactions.

This work represents a preliminary attempt to understand at the molecular level an extremely interesting process required for Salmonella virulence namely OM lipid remodeling. YejM/PbgA represents a novel virulence factor and potential drug target. The importance of its identification cannot be understated.

Major issues:

1. This reviewer questions the physiological relevance of the interaction between YejL and the YejM periplasmic domains. YejL is a 75 amino acid protein encoded by the gene immediately upstream

of YejM. It is not predicted to contain a secretion signal and the PDB itself predicts it to be a soluble cytoplasmic protein. It is difficult to envision how it would interact with the periplasmic domain of YejM in a living cell. Cellular fractionation studies showing a periplasmic localization of YejL would need to accompany the data in Figure 2 in order for it to be meaningful. Genetic studies may also be useful- for example, does the YejL mutant show alterations in PhoPQ mediated survival or YejM tetramer formation?

2. The BNE gels are not convincing. Why does YejL migrate at such a large molecular weight? YejM migrates as a smear in both gels when loaded by itself. Adding YejL reduces the smear but it cannot be said that YejL alters the MW of YejM in either gel.

Minor issues:

1. Why not call YejM by its new name PbgA throughout? It would cause less confusion since the senior author was associated with the publication reporting the discovery last year.
2. Figure 1A, the membrane domain in the figure should be labeled 0-190 not 191-240. Also the “j” in “YejL structure” is partially obscured.
3. I take issue with describing YejL as a ‘ligand’. To me and most biochemists a ligand is a small molecule that interacts with a target protein (receptor) causing some kind of conformational change and usually generation of a signal of some sort. What they are describing here is a protein-protein interaction and should be referred to as such, i.e. “YejL interacts with the periplasmic domain of YejM”.

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Competing Interests: No competing interests were disclosed.

I have read this submission. I 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.

Author Response 28 Nov 2017

Susanne Ressler, Indiana University at Bloomington

Major issues:

- 1.) We performed cellular fractionation studies based on the reviewer's suggestions and present the results in the supplemental section in the updated version of the manuscript. The suggested genetic studies are beyond our laboratory's main focus and scope of revised manuscript; however these studies are being currently performed in the laboratory of Dr. Dalebroux (personal communication).
- 2.) We improved the quality of our BNE experiments and show the results in the supplemental section of the updated version of the manuscript.

Minor issues:

- 1.) PbgA is not yet officially listed as an alternative name for YejM. Uniprot lists entries for PbgA as

proteins that are Phospho-beta-glucosidases. To prevent confusion, we chose to use the official Uniprot database nomenclature of YejM (<http://www.uniprot.org/uniprot/P40709>).

2.) This figure is not included in the updated version of the manuscript.

3.) We agree that this was not the correct term to use and would rather term YejL as potential interaction partner of YejM.

We thank both reviewers for their helpful critique.

Competing Interests: No competing interests.

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