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RESEARCH ARTICLE

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 [version 1; peer review: 1 approved, 1 not approved]

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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. Another protein that might be playing a role in cardiolipin transport is YejL, as its gene is localized upstream of *yejM* on the same operon. Here we report how YejM was engineered to facilitate crystal growth and X-ray diffraction analysis. Furthermore, we present for the first time that YejL is a ligand for YejM. Successful structure determination of YejM and YejL 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*.

Keywords

Salmonella typhimurium, cell growth, outer membrane, membrane protein, YejM, YejL, cardiolipin

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Any reports and responses or comments on the article can be found at the end of the article.

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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 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 shown to bind cardiolipin and are

A. YejM and YejL operon and domain architecture

B. SDS-PAGE analysis

C. Vapor diffusion crystallization

D. X-ray Crystal Diffraction

Figure 1. YejM and YejL architecture, purification, crystallization and diffraction data. **A)** Operon architecture containing genes *yejL* and *yejM*, YejM protein domain architecture, YejL structure (PDB ID: 2JRX) showing electrostatic surface pattern, black arrows indicating negative charged areas, and in cartoon representation the two intertwined dimer (chain A in green and chain B in blue) forming a four-helix bundle **B)** SDS-PAGE analysis of purified protein samples. **C)** Crystals of YejM241 under various conditions. **D)** Diffraction image of YejM241 crystal from condition C6.

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.

Interestingly the gene *yejL* is localized upstream of *yejM* on the same operon (Figure 1A), and encodes for the 8.5 kDa protein, YejL, that forms a homodimer (pdb ID: 2JRX) (Figure 1A). The function of YejL is not known; however based on the co-location on the operon and YejL's negatively charged areas shown in the homodimer NMR structure, we hypothesize that YejL is a ligand to YejM. We further hypothesize that YejL binds to the same site on YejM as cardiolipin and therefore may be a regulator of YejM-mediated cardiolipin transport.

Here we report how YejM was engineered to facilitate crystal growth and present successful crystallization conditions with preliminary X-ray diffraction analysis. We further report for the first time that YejL is a ligand of YejM. Future successful structure determination of YejM alone and in complex with YejL 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

Specific construct development was needed to achieve YejM crystal growth

We purified full-length YejM and periplasmic constructs 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, middle lane). To prevent degradation, reduce flexible protein parts and remove positively charged arginine clusters, we deleted the linker region A191 to E240 in the YejM191-586 construct, resulting in YejM241-586. These modifications were made to increase the chance of crystal growth. 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 (data not shown) 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 1D). 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).

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)

YejL is a ligand of YejM

The gene *yejL* is localized upstream on the same operon as *yejM*. *yejL* encodes for a small (8.5 kDa) protein, YejL, that forms an intertwined four-helix bundle (PDB code: 2JRX) and has negatively charged regions located at the “tail” ends of the four-helix bundle (Figure 1A). The biological function of YejL is unknown, however, its presence on the same operon as YejM suggests that it may interact with YejM and function as a regulatory element. More specifically, we hypothesize that the negatively charged regions of YejL could be involved in specific interaction with the positively charged arginine-rich linker region of YejM. Notably, this arginine-rich linker region is proposed to bind cardiolipin and may be a crucial region for cardiolipin translocation by YejM (Dalebroux *et al.*, 2015). To test our hypothesis that YejL is a ligand of YejM, we purified and mixed both proteins in different stoichiometric ratio and conducted Blue Native PAGE (BNE) and size-exclusion chromatography (SEC) for detection of population of higher molecular weight oligomers/complexes in the mixtures. Our SEC experiments showed that, increasing concentrations of YejL fraction into YejM191-586 or YejM241-586 fractions, respectively (Figure 2A) resulted in a shift in the apparent molecular weight that suggested formation of a higher molecular weight complex. In BNE, we observed a clear shift in position of YejL towards YejM191-586 and YejM241-586, which indicated binding between YejL and YejM191-586 or YejM241-586, respectively (Figure 2B). Our data do not show an apparent difference between

Figure 2. YeJL is a ligand of YeJM. A) SEC chromatograms showing controls and mixtures of YeJM191-586 with YeJL (left) and YeJM241-586 with YeJL (right). **B)** BNE showing controls and mixtures of YeJM191-586 with YeJL (left) and YeJM241-586 with YeJL (right), BSA was used as size reference.

YeJL binding to YeJM191-586 that includes the arginine-rich linker region, and YeJM241-586 that lacks the linker region. Further analysis using isothermal titration calorimetry and/or microscale thermophoresis will be used to determine the exact binding affinity and potential changes thereof between the two YeJM constructs and YeJL.

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 will use the current 1.8Å dataset (Table 1) for structure determination. We also aim to solve the co-crystal structure of YeJL and/or cardiolipin bound to YeJM. Ultimately these structures will help in understanding: i) where

and how YeJL and/or cardiolipin bind to YeJM, ii) the role of YeJL, iii) whether YeJM's architecture is that of a transporter or channel, and iv) the molecular mechanism of cardiolipin translocation to the OM of *S. typhimurium*.

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'-ccgcgccgcagccatattggctagcgcgtctccgttcagttaccgg- 3' and reverse primer YeJM241-586 5'-gcggtactgaacggagaccgcgctagccatattggctgccgcgcgg- 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 YeJL 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).

Expression and purification of YeJL

YeJL construct (ID ER309-21.7), an *E. coli* homolog, was obtained from the Northeast Structural Genomics (NESG) consortium (<http://www.nesg.org>) with the vector ID: pET21_NESG. The pET21-YeJL plasmid was transformed into *E. coli* BL21(DE3) (Novagen) competent cells. Overnight saturated cultures of transformed bacteria in Luria Bertani (LB) medium (EMD Chemicals Inc.) with 50 μ g/ml ampicillin (Dot Scientific Inc.) were grown at 37°C and diluted 1:200 in fresh Terrific Broth (TB) medium (Dot Scientific Inc.) with 50 μ g/ml ampicillin and further grown at 37°C at 200 rpm. The cultures were induced with 0.1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) (Teknova) when OD₆₀₀ reached 0.5–0.8. The expression was carried out at 37°C for three hours. All further steps were performed at 4°C unless noted otherwise. Cells were harvested by centrifugation and resuspended in a buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, and 5 mM MgCl₂. 10 μ g/ml DNase (Sigma Aldrich), 1 mg/ml lysozyme (Sigma Aldrich), protease inhibitor cocktail (Roche), and 0.1 mM Phenylmethane sulfonyl fluoride (PMSF) (Dot Scientific Inc.) were freshly added to the resuspended cells. Cells were lysed by passing several times through a microfluidizer (Divech Equipment Company) and the cell debris was removed by centrifugation.

The clear cell lysate was bound to Ni-NTA resin (Qiagen) in batch mode and incubated on a rotarod at 4°C for 30 – 60 minutes. The slurry was loaded into a column and the flow through was collected under gravity. The column was washed first with 30 column volumes of Wash buffer 1 (25mM Tris pH, 8.0, 300mM NaCl and 25mM Imidazole); then with two column volumes of Wash buffer 2 (25mM Tris pH 7.5–8, 150mM NaCl, 75mM Imidazole). The bound protein was eluted from the column with elution buffer (25 mM Tris, pH 8.0, 150 mM NaCl, and 300 mM Imidazole) in 200–500 μ l increments. Elutions were monitored for protein content by Bradford test. YeJL was further purified by SEC using a Superose 6 increase 10/300 GL column (GE Healthcare) equilibrated in a buffer containing 50 mM Tris, pH 8.0 and 150 mM NaCl. Peak fractions of YeJL were consolidated and concentrated using 3 kDa NMWL centricon (Millipore).

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 sizes between 500 nl to 1 μ l.

Crystallization plates were incubated at 20°C and monitored for crystal growth in a Minstel™ HT crystal imaging and detection tower (Rigaku). Optimal crystal growth was obtained at a protein concentration of 4mg/ml.

Blue Native PAGE

10 μ M of YeJM191-586 or YeJM241-586 was mixed with varying concentrations (2.5, 5, 10, and 15 μ M) of YeJL separately. Samples were incubated at 4°C for minimum 1 hr, mixed with sample buffer (5% Coomassie Brilliant Blue G250, 100mM Bis-Tris pH 7.5, 0.5M 6-aminocaproic acid (Biorad) for a total individual sample volume of 30 μ L. The samples were loaded on Mini-protean TGX Any-kD precast gels (Biorad) and electrophoresis was carried out at 4°C at 100 V for 60–90 minutes. The gels were destained in a solution containing 5% ethanol and 7.5% acetic acid.

Size Exclusion Chromatography of protein mixtures of YeJL with YeJM191 and YeJL with YeJM241

10 μ M of YeJM191-586 and YeJM241-586 were each mixed with varying concentrations (2.5, 5, 10, and 15 μ M) of YeJL separately. Each protein mixture was incubated overnight at 4°C and subsequently purified by size-exclusion chromatography using Superose 6 Increase 10/300 GL gel-filtration column (GE Healthcare) equilibrated with 50 mM Tris pH 8.0, 150 mM NaCl. The UV absorption profiles at 280 nm of each run were normalized and compared.

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://cxiidb.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, analyzed data and wrote manuscript, G.Q. and E.R. expressed, purified, crystallized and performed BNE, S.R. expressed, purified, crystallized, collected and analyzed data, designed experiments and wrote manuscript.

Competing interests

No competing interests were disclosed.

Grant information

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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 at the Advanced Light Source (Berkeley, CA).

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Yihua Huang

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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 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.

Author Response 28 Nov 2017

Susanne Ressler, Indiana University Bloomington, Bloomington, USA

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.

Reviewer Report 03 June 2016

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William T. Doerrler

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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 crystalize 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 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.

Author Response 28 Nov 2017

Susanne Ressler, Indiana University Bloomington, Bloomington, USA

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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