nature portfolio

Peer Review File

Cryo-EM, biochemical and cellular characterization of the anydromuropeptide permease AmpG central to bacterial fitness and β-lactam antibiotic resistance

Corresponding Author: Professor Natalie Strynadka

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript by Sverak et al. presents structural and functional studies for the permease, AmpG, found in Gram-negative bacteria. This permease is linked to beta-lactam antibiotic resistance in pathogens such as Pseudomonas aeruginosa, Klebsiella pneumoniae and Enterobacter. Briefly, AmpG is a located in the inner membrane where it transports 1,6 anhydromuropeptides, generated during peptidoglycan remodeling, from the periplasm into the cytosol for recycling. One of the final products of this recycling is UDP-MurNAc, which is not only used for synthesizing new building blocks for the peptidoglycan, but also binds to a transcriptional regulator that block the production of AmpC, a beta-lactamase that confers resistance to numerous beta-lactam antibiotics. However, upon beta-lactam use, peptidoglycan remodeling is disrupted and AmpG necessarily transports larger numbers of 1,6 anhydromuropeptides into the cytosol. Here the recycling machinery is unable to keep up with the influx, resulting in some of the intermediate products displacing UDP-MurNac from the transcriptional regulator, resulting in AmpC production. This convoluted role of AmpG in beta-lactam resistance implies that inhibitors of the permease are predicted to synergize with beta-lactam antibiotics, thus enhancing their effectiveness in the treatment of Gram-negative infections.

Sverak et al. have elucidated the cryo-EM structure for AmpG from Escherichia coli to near atomic resolution. They complement this work with biophysical studies on cargo specificity, mutagenesis studies to elucidate mechanistic aspects, and modeling studies. Most notably the manuscript presents: 1) a molecular understanding for how AmpG recognizes 1,6 anhydromuropeptides for transport; 2) presents a model for how transport occurs across the inner membrane; and 3) explains how various conserved residues play a critical role in the transport process. Overall, the presented research provides a wealth of new information on how AmpG performs its task at a level of detail that opens new avenues for developing synergistic beta-lactam treatments with enhanced therapeutic efficacy.

In my assessment, there can be no real criticism on the performed research. The team assembled for this manuscript possesses ample expertise in structural biology, synthetic organic chemistry and molecular microbiology, and their combined effort has resulted in a thorough examination of how AmpG contributes to beta-lactam resistance in molecular detail. In fact, because of the scope of tools used to examine this permease, I believe the authors have effectively answered all the important questions for AmpG functioning. Two minor questions the authors were not able to definitively answer. Sverak et al. have not (yet) elucidated the inward conformation for AmpG, though their very creative use of AlphaFold3 has provided a very compelling model, that I suspect is likely to be correct. Also, while their structural and mutagenesis studies provide ample insight into how proton transport is linked to cargo transport, some details for the “charge-relay” are inevitably somewhat murky and will likely spark further studies. Perhaps molecular dynamics approaches can be exploited to computational examine some of the transport energetics. However, I would like to be clear that any such further experiments fall well outside the scope of this current manuscript.

While I have no real critique, I do have one question that I would like to ask the authors. The proposed model for the “inward conformation” produced by AlphaFold3 is supported by two sets of evolutionary coupled residues: V41 – A257 and L46 – E377, which are separated in the outward conformation but are proposed to come together in the inward state. However, the manuscript does not discuss what the structural basis for this evolutionary coupling is. I can speculate that for V41 – A257 the sidechains for the two residues directly interact in the inward conformation and that this interaction consists of

complementary Vander Waals surfaces, thus rationalizing their evolutionary linkage. However, for the L46 – E377 a simple rational for evolutionary coupling is not obvious to me. An explanation of why these residues are evolutionary coupled would further support the use of the AlphaFold3 model for the inward conformation.

Reviewer #2

(Remarks to the Author)

The paper by Sverah H and colleagues provides a well-integrated study of AmpG, combining cryo-EM, biochemical, and cellular analyses, offering a thorough understanding of the protein's structure and function. The cryo-EM structure determination of AmpG in an outward-open conformation provides valuable insights into its substrate-binding cavity and the unique hydrophobic vestibule structure, elucidating the mechanism of substrate binding and transport. The explanation of the proton-mediated transport mechanism, including the role of conserved residues in substrate binding and proton relay, is supported by mutational data and evolutionary coupling analyses. The study links the function of AmpG to β-lactamase induction and antibiotic resistance in clinically relevant pathogens, highlighting the potential of targeting AmpG for developing new antimicrobial therapies. Overall, this paper improves our understanding of AmpG structure and function, particularly its potential role in antibiotic resistance. Despite some limitations in complexity and speculative aspects, the largely multidisciplinary approach offers valuable insights into AmpG's mechanistic role in PG fragment transport.
Strengths
• The integration of cryo-EM, biochemical, and cellular analyses offers a comprehensive understanding of AmpG's structure and function.
• Determination of the cryo-EM structure of AmpG in an outward-open conformation provides valuable insights into its substrate-binding cavity and unique hydrophobic vestibule structure, elucidating the mechanism of substrate binding and transport.
• The successful solubilization of AmpG in N-dodecyl-β-D-maltopyranoside (DDM) and incorporation into MSP1D1 nanodiscs with E. coli polar lipids are commendable, especially since these proteins are notoriously difficult to handle. These conditions likely mimic the native environment, aiding in maintaining the protein's functional conformation.
• The confirmation of purity and monodispersity using SDS-PAGE and negative-stain TEM validates the successful isolation of the protein in a suitable form for structural studies.
• The inclusion of a BRIL tag at the C-terminus and the use of the synthetic antibody BAG2 facilitated structure determination. Although the tag or BAG2 was not observed in the 3D-classification, it did not appear to disrupt overall visualization of AmpG.
• The detailed explanation of the proton-mediated transport mechanism, supported by mutational data and evolutionary coupling analyses, enhances the understanding of conserved residues in substrate binding and proton relay.
• The study highlights the potential of targeting AmpG to develop new antimicrobial therapies by linking its function to β-lactamase induction and antibiotic resistance in clinically relevant ESKAPE pathogens.
• The structure was complemented with detailed structure-functional studies to boost the mechanistic understanding from the structure.
Weaknesses
• The validation of the AmpG-BRIL/BAG2 complex formation primarily through negative-stain TEM and glycerol gradient centrifugation is adequate. However, additional biophysical techniques such as size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) or analytical ultracentrifugation could provide more robust evidence of complex formation and stability.
• While the authors mention that the BRIL tag did not have a deleterious effect on structure, it would be beneficial to include more functional assays to confirm that the tagged protein retains its native activity. This would ensure that the structural data correspond to the protein's functional state.
• The paper is dense and could be challenging for readers who are not specialists in the field. Simplifying some sections or providing a clearer summary of key findings could improve accessibility. For example, Figure 1 and supplementary figures 2 and 3 were dense and difficult to visualize, with some aspects of the figures and labeling only visible after extreme magnification.
• The figure legends were dense and read like duplications of the results section.
• While the structural and binding data are comprehensive, the paper could benefit from more concise in vivo functional discussion to validate the proposed mechanisms and their physiological relevance.
• Some mechanistic proposals, such as the role of ordered lipids in substrate-induced signaling, are speculative. While the reviewer agrees with some of the claims, they should be toned down or supported with additional evidence through assays or other supporting studies.

Version 1:

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Reviewer #1

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My previous assessment of the manuscript by Sverak et al. only raised one minor comment, namely the lack of a rational for

evolutionary coupled residues. More specifically, the authors reported that L46 and E377 were linked, which was to support the modeled inward conformation. Unfortunately, a sound explanation was not provided for the linkage distracting from the proposed mechanism for cargo transport across the lipid bi-layer. In this revised manuscript, the authors have fully addressed this comment as they have revisited the evolutionary linkage analysis and noted that E377 is actually evolutionary linked to L46 AND K47. Of these two linkages the K47-E377 combination is readily explainable and now strongly supports the inward conformation model. This is mostly clearly shown in Figures 5c and 7a. With this minor comment now addressed I have no further concerns.

Reviewer #2

(Remarks to the Author)
The authors have thoroughly addressed all my concerns. The paper is exceptionally well-presented, and I have no additional useful comments or suggestions.

REVIEWER COMMENTS

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The manuscript by Sverak et al. presents structural and functional studies for the permease, AmpG, found in Gram-negative bacteria. This permease is linked to beta-lactam antibiotic resistance in pathogens such as Pseudomonas aeruginosa, Klebsiella pneumoniae and Enterobacter. Briefly, AmpG is a located in the inner membrane where it transports 1,6 anhydromuropeptides, generated during peptidoglycan remodeling, from the periplasm into the cytosol for recycling. One of the final products of this recycling is UDP-MurNAc, which is not only used for synthesizing new building blocks for the peptidoglycan, but also binds to a transcriptional regulator that block the production of AmpC, a beta-lactamase that confers resistance to numerous beta-lactam antibiotics. However, upon beta-lactam use, peptidoglycan remodeling is disrupted and AmpG necessarily transports larger numbers of 1,6 anhydromuropeptides into the cytosol. Here the recycling machinery is unable to keep up with the influx, resulting in some of the intermediate products displacing UDP-MurNac from the transcriptional regulator, resulting in AmpC production. This convoluted role of AmpG in beta-lactam resistance implies that inhibitors of the permease are predicted to synergize with beta-lactam antibiotics, thus enhancing their effectiveness in the treatment of Gram-negative infections.

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We thank the reviewer for their positive comments. In response to the query about the evolutionary coupled residues, specifically L46 and E377, we agree this is an apparent less than ideal pairing for a complementary interaction between a hydrophobic and charged side chain. However, in terms of the predicted 3D structure for the inward-open state, the leucine side chain packs alongside the hydrophobic region of the glutamate while the carboxylate forms a salt bridge with the neighbouring lysine (K47). Indeed, K47 has a similar EV score with E377 compared to L46 – as can be seen in Supplementary Fig. 8 – both of which are amongst the most significant EV couplings. We do agree with the reviewer and to make this analysis and justification more intuitive, we have changed the pair here to K47 and E377 and updated in Fig. 5 and Fig. 7.

Reviewer #2 (Remarks to the Author):

The paper by Sverak H and colleagues provides a well-integrated study of AmpG, combining cryo-EM, biochemical, and cellular analyses, offering a thorough understanding of the protein's structure and function. The cryo-EM structure determination of AmpG in an outward-open conformation provides valuable insights into its substrate-binding cavity and the unique hydrophobic vestibule structure, elucidating the mechanism of substrate binding and transport. The explanation of the proton-mediated transport mechanism, including the role of conserved residues in substrate binding and proton relay, is supported by mutational data and evolutionary coupling analyses. The study links the function of AmpG to β-lactamase induction and antibiotic resistance in clinically relevant pathogens, highlighting the potential of targeting AmpG for developing new antimicrobial therapies. Overall, this paper improves our understanding of AmpG structure and function, particularly its potential role in antibiotic resistance. Despite some limitations in complexity and speculative aspects, the largely multidisciplinary approach offers valuable insights into AmpG's mechanistic role in PG fragment transport.
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• The study highlights the potential of targeting AmpG to develop new antimicrobial therapies by linking its function to β-lactamase induction and antibiotic resistance in clinically relevant ESKAPE pathogens.
• The structure was complemented with detailed structure-functional studies to boost the mechanistic understanding from the structure.

We thank the reviewer for their positive comments and constructive suggestions. We have updated the manuscript and provided additional data as requested, see below.

Weaknesses
• The validation of the AmpG-BRIL/BAG2 complex formation primarily through negative-stain TEM and glycerol gradient centrifugation is adequate. However, additional biophysical techniques such as size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) or analytical ultracentrifugation could provide more robust evidence of complex formation and stability.

To further characterize the AmpG-BRIL/BAG2 complex, we have added affinity pull down and cross-linking data (Supplementary Fig. 2d and e) showing co-purification and complex formation. We have also directly determined the dissociation constant using surface plasmon resonance (SPR), showing a 9.6 nM Kd (Supplementary Fig. 3e). These results are presented on page 4, lines 113-117.

"The high affinity interaction between AmpG-BRIL and BAG2 was verified with surface plasmon resonance (SPR) with a binding constant (K_d) of 9.6 nM (Supplementary Fig. 3e). AmpG-BRIL in DDM was incubated with BAG2 at a 1:1 molar ratio and complex formation was validated by glycerol gradient centrifugation, pull-down assay, native PAGE, and negative-stain TEM (Supplementary Fig. 2c-h)"

Together, we believe these data clearly demonstrate the formation of a high affinity complex. We also stress that BAG2 was absolutely required in our hands for successful structure determination and all attempts to determine the structure without this tool failed.

• While the authors mention that the BRIL tag did not have a deleterious effect on structure, it would be beneficial to include more functional assays to confirm that the tagged protein retains its native activity. This would ensure that the structural data correspond to the protein's functional state.

We have carried out additional binding experiments (microscale thermophoresis) demonstrating that both AmpG-BRIL and the AmpG-BRIL/BAG2 complex bind the GlcNAc-1,6-anhMurNAc substrate with comparable, albeit slightly diminished, affinity to the wild-type protein (Page 6, lines 178-180; Supplementary Fig. 3d).

*"Binding of compound **1** to AmpG-BRIL and the AmpG-BRIL BAG2 complex used for structure determination was also assayed showing comparable binding to the wild-type protein (Supplementary Fig. 3d)."*

We suggest that this slight reduction (although still close to the error of the MST experiments) is due to the additional mass around the surface of the protein, potentially interfering with the free diffusion and access of substrate to the substrate binding cavity. Nonetheless, the comparable binding supports the presented structure of AmpG-BRIL/BAG2 as representative of the native protein structure.

• The paper is dense and could be challenging for readers who are not specialists in the field. Simplifying some sections or providing a clearer summary of key findings could improve accessibility. For example, Figure 1 and supplementary figures 2 and 3 were dense and difficult to visualize, with some aspects of the figures and labeling only visible after extreme magnification.

We have made an effort to make the writing and figures more succinct. We have extensively edited the manuscript, reducing the length of the manuscript to ~5500 words. We have also updated the figures, especially Fig. 1, with the aim to make them simpler and the text/images not too small. For Fig. 1 specifically, we acknowledge there is a lot of information; however, we believe that much of it is helpful to the reader in order to better understand the roles that AmpG plays both physiologically and pathophysiologically. With that in mind, we have ensured all the images and text are of sufficient size and simplified the figure where possible.

• The figure legends were dense and read like duplications of the results section.

We have updated the figure legends throughout the manuscript.

• While the structural and binding data are comprehensive, the paper could benefit from more concise in vivo functional discussion to validate the proposed mechanisms and their physiological relevance.

The structure-informed point mutations reported in the manuscript show the perturbation of key substrate-facing residues. This leads to increased antibiotic sensitivity in two different species with measurable phenotypes, which are now shown in a main figure in the text for improved clarity (Figure 6). The MST

data at lines 309-311 and 389-392 shows comparable reduced binding, providing a rationale for in vivo loss of function, which is outlined in a more succinct discussion.

• Some mechanistic proposals, such as the role of ordered lipids in substrate-induced signaling, are speculative. While the reviewer agrees with some of the claims, they should be toned down or supported with additional evidence through assays or other supporting studies.

We acknowledge the speculative nature of some claims but believe they are compelling and warrant some discussion. We have toned done these throughout the manuscript especially the discussion of the role of the observed lipid, see page 15, lines 451-459.