nature portfolio

Peer Review File

Advances in methods and concepts provide new insight into antibiotic fluxes across the bacterial membrane

Corresponding Author: Dr Jean-Marie Pages

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The review authored by Vergalli et. al. focuses on the relation between passive efflux and active influx of drug molecules across bacterial membrane. The authors have provided some insights into the key methodologies developed and in use to track the transport of drugs across bacteria to identify key parameters involved in their activities.

The authors fail to provide details or highlight on key advances in the role of antibiotic fluxes and its connection to efficient drug development. Here are the major concerns:
1) As mentioned in Page 6 first line, the authors have mentioned about the design of “faster diffusion” drug as a major objective to rationalise the current study. The authors have provided a schematic of the techniques associated with understanding the relation between drug in vivo drug concentrations and transporters. However, they have failed to put forward major insights or methodological approach on a rational drug design based on bacterial drug susceptibility phenotype (resistant, intermediate or susceptible) from an antibiotic/drug flux point of view.
2) Although the authors primarily focus on membrane transporters and their role in determining intracellular and extracellular drug concentrations via import and export, it is important to consider drug molecules that diffuse into cells independent of importers. Such drug molecules often depend on the pH of the environment (PMCID: PMC5198042). The authors should provide a detailed explanation of why understanding mechanisms involving uptake transporters and optimizing drugs based on these mechanisms would be more efficient in the future than developing drugs that can diffuse faster without relying on any importer.
3) On Page 5, Second paragraph the authors have provided a mechanistic detail of conformational changes upon substrate binding with RND efflux pump AcrAB-TolC. Moreover, In Figure 1 the authors have highlighted the mechanistic similarities between efflux and porin pumps and key gaps in understanding antibiotic transport through porins and RND. However, for a better and fluent understanding the authors are suggested to provide similar details on conformational changes and electrostatic interactions in major classes of porins found uniquely in Gram negative pathogens along with detailed figure.
4) Since the overall manuscript deals with the advances in the methods and concepts of detection of antibiotic fluxes, the authors must provide a comprehensive detail on all the currently used techniques for the same (As provided in Figure 2).
5) On Page 6, under in cellulo models the authors have mentioned about drug quantification based on flourimetry, LC-MS/MS, deep UV microscopy. The authors must provide a detailed insight into these techniques and their current use rather than providing just the working principle. Moreover, the authors have not mentioned about Flow cytometric analysis which is also an important in cellulo technique to determine drug accumulation and efflux in bacteria (PMCID: PMC6787898).
6) On page 13, in the last paragraph, the authors discuss the combination of LC/MS-MS and imaging techniques to determine in situ bacterial response. The authors should provide references supporting the application of such a technique.
7) Authors must provide references in certain sections for the information presented. For instance, on Page 5, first paragraph, the authors claim that early defense mechanism against drugs in bacteria limits internal drug concentrations; the authors must cite the exact mechanisms that exist in literature for such mode of actions.
8) The authors must provide line numbers in manuscript for the ease of the reviewers in providing the comments.

Reviewer #2

(Remarks to the Author)
In this manuscript Vergalli et al revisit recently developed methods and tools to track and
correlate the transport of drugs across the bacterial membrane. This topic is very important considering the pressing and global issue of antimicrobial resistance that applies not only to bacteria but microbes in general including fungi. The review is fairly comprehensive, however, some major points need to be addressed before this manuscript can be considered for publication.

Major issues:

- there are a series of typos that render reading and understanding the manuscript very difficult, e.g., merged should be emerged, antagonist should be antagonistic, mechanical mechanism should be mechanisms, in Fig. 1 driven force should be driving force etc. Please amend and proof read the manuscript before resubmission.

- It is not immediately clear what is the novelty of this review compared with previous reviews e.g. https://pubs.acs.org/doi/10.1021/acs.chemrev.0c01137 that has not been cited

- An important recently developed area that is not covered in this review is the development and use of fluorescent antibiotic derivatives pioneered by the Blaskovich group to study antibiotic penetration and efflux at the scale of the whole bacterial cell, including the following key papers that should be included in this review:

https://pubs.rsc.org/en/content/articlehtml/2019/md/c9md00124g

https://www.nature.com/articles/s42003-023-04745-x

https://elifesciences.org/articles/74062

https://pubs.rsc.org/en/content/articlehtml/2020/cb/d0cb00118j

And similar approaches developed by the Blair's group using dyes that are substrates of efflux pumps:

doi: 10.3389/fmicb.2019.02319

https://doi.org/10.1128/mBio.02608-21

https://www.biorxiv.org/content/10.1101/2023.04.03.535035v2

The authors categorically state "conclusions based on assays that use modified compounds or efflux substrates without bacterial activity are biased and might have a very different kinetics than antibacterial compounds used for therapeutic purposes." Ignoring the facto all the knowledge about membrane transport that has been generated using the methods above and other techniques such as tradisort 10.1128/mBio.01200-16 that should be discussed in this review.

"Biases" have been observed also when using in vitro assays such as purified outer membrane proteins, or in silicon models, as rightly acknowledged by the authors. Yet, such methods, employed by both the authors of this review and by others, have revealed over the years a wealth of knowledge about antibiotic transport and therefore have been included in this review, and rightly so. The authors should equally discuss and acknowledge the alternative in cellulo approaches suggested above, together with their limitations, as they have done for the in vitro assays and in silico models.

- Can the authors please clarify the sentence "Diffusion then depends on a balance between alignment and translocation". This is a rather important concept but I did not find this concept has been clearly explained.

Minor points:

- I suggest rephrasing the following sentence in the abstract "However, despite intensive search for a general set of rules to describe the uptake the investigations were rather unsuccessful." It has a negative note and minimises the amazing work that have been done in this area, including the pioneering work of the authors of this review. I suggest changing this sentence to something like "Despite intensive theoretical and experimental investigations a general set of pathways underpinning antibiotic uptake has not been identified".

- clarify the meaning of membrane resistance in the abstract

- "for development more effective drugs" change to "for the development of more effective drugs"

- define what ECDC, RND etc stand for

- decide on the use of channel vs canal

- see "?A-?E" points described in Fig. 1 please amend ?

- "They aim to study" who is they?

- The following sentence "the mechanics of transport and the kinetic parameters which manage the sequence of functional processes of transporters - a molecular aspect by studying the dialogue between the drug and the transporter during key stages of transport - the relationship between concentration and antibacterial action at the first times of contact. The final

objective is to rationalize the design of a “faster diffusion” drug and to help the clinician in their choice of molecule.” is totally unclear

- reference 23 does not use deep UV microscopy, please amend this statement

Reviewer #3

(Remarks to the Author)
In this review, the authors provide a description of recently developed methods and tools that track the transport of drugs across the bacterial membrane. These are important given the general lack of correlation between susceptibility established by typical MIC testing and more accurate antibiotic activity that considers intracellular concentration and the role of the target. More specifically, the authors cover advances to address this gap including in vitro, in cellulo and in silico methods. The authors describe these tools in detail, highlighting their respective advantages and disadvantages. Based on these concepts, the authors provide new perspective for the future development of more effective antibiotics, which are needed to address rampant antibiotic resistance.

Overall, this is a well-organized manuscript with high-quality figures that effectively illustrate key knowledge gaps and methodologies. The review is a valuable resource for understanding the current state of tools and methods used for drug transport and their contribution to drug design.

However, the manuscript would greatly benefit from a comprehensive edit to enhance its clarity and readability, as it currently contains numerous grammatical and linguistic errors. This is especially true for the Introduction and the first section, “In and out transport is governed by key steps”.

The following section, “Understanding the drug transport across membrane with different models”, is clearer and well-organized. The reviewer suggests making Supplementary Table 1 a main Table in the manuscript for easier access, as it is both useful and comprehensive. The advantages and disadvantages of the various methods are well described, and the example of cephalosporins effectively illustrates the point of variability amongst methods.

The next section, “Understanding the dialogue between the porin/transporter and the drug to guide drug design”, lacks detail on how in vitro models/methods can contribute in this context. For the in cellulo part, a key citation is missing that goes beyond appending a primary amine and suggests that other positively-charged functional groups should be considered when designing antibiotics with Gram-negative activity (PMID 33228356).

Lastly, in the “Future Perspective” section, more discussion on emerging technologies that could further enhance our understanding of drug transport mechanisms would strengthen the manuscript, including potential challenges/limitations these new methods may face, as well as strategies to overcome them. Emphasizing interdisciplinary collaborations and integrating insights from fields such as biophysics, computational biology, medicinal chemistry, etc., is needed to provide a more comprehensive outlook on the future development of effective antibiotics.

Responses to Reviewers (in blue)

Important point: This manuscript is not primarily focused on antibiotic resistance. Several comprehensive reviews on this topic have already been published and are cited within our manuscript. Instead, it aims to propose an original and integrated perspective on the drug transport and accumulation in Gram negative bacteria through a comprehensive analysis of recent methods, concepts and tools. The combined approach utilizes advanced techniques and technologies to monitor the kinetics of transport, accumulation and localization, as well as the activity of molecules, specifically targeting resistant clinical isolates. This approach is intended to provide a detailed depiction of the key steps involved in the action of an antibiotic, ultimately identifying the strategic structures that a molecule must possess to overcome membrane resistance.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The review authored by Vergalli et. al. focuses on the relation between passive efflux and active influx of drug molecules across bacterial membrane. The authors have provided some insights into the key methodologies developed and in use to track the transport of drugs across bacteria to identify key parameters involved in their activities.

The authors fail to provide details or highlight on key advances in the role of antibiotic fluxes and its connection to efficient drug development.

We thank the Reviewer for his criticism. Typically, analyses measuring the relationship between the structure of molecules and their antibacterial activity are based on MIC measurements. MIC-based approaches are carried out after long incubation periods and neglect critical intermediate steps, including penetration, accumulation, sensitivity to efflux pumps, and the half-life of the molecule close to the target. By focusing only on an overall perspective, it fails to capture the molecular and kinetic specifics of these key steps that modulate the antibiotic action. Our goal is to propose a new strategy to identify the pharmacophoric groups involved in these steps. This requires an integrated approach that combines various techniques and based on clinical isolates known to present challenges (see figure 3). Further steps to implement this corpus of information into a drug design procedure can be a task for future applications of ML/AI approaches, as described in ref. Mehla et al. (Mehla J, Malloci G, Mansbach R, et al. 2021. Predictive rules of efflux inhibition and avoidance in *Pseudomonas aeruginosa*. mBio 12:e02785-20. https://doi.org/10.1128/mBio.02785-20.)

Here are the major concerns:

1) As mentioned in Page 6 first line, the authors have mentioned about the design of “faster diffusion” drug as a major objective to rationalise the current study.

We thank the Reviewer for pointing this out. Indeed, 'diffusion' is misleading; we meant 'fast accumulation,' which results from influx and efflux. The sentence has been reworded.

The authors have provided a schematic of the techniques associated with understanding the relation between in vivo drug concentrations and transporters. However, they have failed to put forward major insights or methodological approach on a rational drug design based on bacterial drug susceptibility phenotype (resistant, intermediate or susceptible) from an antibiotic/drug flux point of view.

The primary goal of our study was to emphasize the critical importance of considering drug influx and efflux parameters in the design of future antibiotics. Given that the bacterial membrane acts as the

initial barrier to antibiotics, it is essential to incorporate these parameters for optimizing drug design. Indeed, any new insights into the accumulation of antibiotics inside bacteria should be considered crucial for drug design. We have made a preliminary attempt to trace the fate of a compound from its structure to its interaction with transporters. While this approach is still in its early stages, it sets the groundwork for future developments. However, we believe that the explanation of how the information reviewed in our manuscript can be applied to specific drug scaffolds falls outside the scope of our study, as our focus was not on detailing medicinal chemistry procedures.

We acknowledge the challenge of developing a comprehensive methodological approach that includes these parameters, particularly when aiming to improve drug design based on bacterial drug susceptibility phenotypes (resistant, intermediate, or susceptible). As stated in our manuscript, it is difficult to draw definitive conclusions about drug transport across membranes from multiparametric activity measurements or estimations that involve enzymes, target mutations, and other factors without isolating information on drug influx and efflux.

In our manuscript, we have proposed several approaches that we believe can advance our understanding of the problems posed by antibiotic resistance. One of our key suggestions is the need to properly account for Membrane-Associated Mechanisms (MAM) during drug design, as well as to conduct investigations on clinical isolates. Our proposed prospective approach aims to address the remaining gaps in understanding drug transport across membranes, by using a combination of methods that provide valuable information (Figure 3). This information can be used by clinicians for selecting appropriate antibiotics or drug combinations, and by pharmacochemists for a rational synthesis of new molecules.

2) Although the authors primarily focus on membrane transporters and their role in determining intracellular and extracellular drug concentrations via import and export, it is important to consider drug molecules that diffuse into cells independent of importers. Such drug molecules often depend on the pH of the environment (PMCID: PMC5198042). The authors should provide a detailed explanation of why understanding mechanisms involving uptake transporters and optimizing drugs based on these mechanisms would be more efficient in the future than developing drugs that can diffuse faster without relying on any importer.

We agree with the Reviewer that many molecules penetrate through the outer membrane via diffusion. Indeed, porins serve "only" as facilitators for the influx of certain molecules. Thus, using accumulation assays allows for measuring the uptake of a drug as a whole.

It is also important to note that the most administered and still-developed antibacterial compounds in clinical settings are β-lactams, β-lactamase inhibitors, and β-lactam/β-lactamase inhibitor combinations, whose transport is dependent of porins. Porin-mediated influx generates a rapid increase in intracellular drug concentration at the initial stages of bacterial exposure to the antibiotic, a crucial window that determines the cell's fate (cell death, persistence, adaptation through the acquisition of new resistances).

Nevertheless, we want to convey the message that a drug's ability to accumulate within bacteria must be considered during drug design, which means that efflux, total influx, both porin-mediated and non-mediated have to be considered. This can be accounted for by using the accumulation methods we extensively discussed in the manuscript.

We recognize that pH, along with various *in situ* conditions, is a critical parameter influencing drug influx and efflux by affecting the drug charge and membrane physiology. With this in mind, we

emphasize the importance of directing research towards methodologies that closely mimic *in situ* conditions, thereby considering the influence of environmental parameters at infectious sites (see Figure 3). The text has been revised to highlight this perspective more prominently.

3) On Page 5, Second paragraph the authors have provided a mechanistic detail of conformational changes upon substrate binding with RND efflux pump AcrAB-TolC. Moreover, In Figure 1 the authors have highlighted the mechanistic similarities between efflux and porin pumps and key gaps in understanding antibiotic transport through porins and RND. However, for a better and fluent understanding the authors are suggested to provide similar details on conformational changes and electrostatic interactions in major classes of porins found uniquely in Gram negative pathogens along with detailed figure.

The paragraph on translocation through porins has been revised. Studies reporting the involvement of the electrostatic field in this transport have been cited (DOIs: 10.1021/acsnano.6b08613; 10.1088/1361-648X/aa543b; 10.1038/s41579-019-0294-2; 10.1093/jac/dkae265). Figure 1 has also been updated to include a schematic representation of the loop 3/anti-loop3 residues involved in the electrostatic forces at the porin eyelet.

4) Since the overall manuscript deals with the advances in the methods and concepts of detection of antibiotic fluxes, the authors must provide a comprehensive detail on all the currently used techniques for the same (As provided in Figure 2).

Our goal is to highlight the strengths and limitations of the relevant methods in their ability to investigate the key interactions between drugs and porins/efflux pumps, particularly focusing on the electrostatic interactions and their role in drug accumulation (as discussed in relation to the water-associated screening effects in AcrB), rather than providing a redundant overview of all existing methods. Indeed, many of these techniques have already been extensively detailed in other reviews (DOIs 10.1021/acs.chemrev.0c01137, 10.1128/mBio.00840-16, 10.1038/s41596-021-00598-y, 10.1016/j.resmic.2017.11.005). We believe this targeted approach provides valuable insights into the mechanisms underlying antibiotic resistance, which could be overlooked in a broader survey.

However, we recognize that some techniques may not have been discussed in sufficient detail. In response to the reviewer's feedback, we have expanded our discussion on the referenced methods and incorporated additional relevant studies that further explain or utilize these techniques.

5) On Page 6, under in cellulo models the authors have mentioned about drug quantification based on flourimetry, LC-MS/MS, deep UV microscopy. The authors must provide a detailed insight into these techniques and their current use rather than providing just the working principle. Moreover, the authors have not mentioned about Flow cytometric analysis which is also an important *in cellulo* technique to determine drug accumulation and efflux in bacteria (PMCID: PMC6787898).

We have added a paragraph explaining the use of these methods (DOIs: 10.1038/nature22308, 10.1038/s42003-022-04035-y, 10.1038/s41598-017-08775-4, 10.1093/jac/dky396) and cited works that detail these techniques (DOIs: 10.1038/s41596-021-00598-y, 10.1038/nprot.2018.036,). The use of flow cytometry has also been included as a method to visualize heterogeneity of responses within a population.

6) On page 13, in the last paragraph, the authors discuss the combination of LC/MS-MS and imaging techniques to determine in situ bacterial response. The authors should provide references supporting the application of such a technique.

Currently, various mass spectrometry techniques offer promising opportunities for studying membrane associated mechanisms of resistance, including LC-MS/MS, which is discussed in detail in this manuscript, as well as other mass spectrometry imaging methods. In response to the Reviewer's suggestions, references to examples of the use of imaging techniques based on mass spectrometry have been included. The combination of these different techniques – which, previously applied in separate studies, will require technical adjustments - will allow for the study of both the spatial distribution and quantification of antibiotic concentrations within samples from the same tissue.

7) Authors must provide references in certain sections for the information presented. For instance, on Page 5, first paragraph, the authors claim that early defense mechanism against drugs in bacteria limits internal drug concentrations; the authors must cite the exact mechanisms that exist in literature for such mode of actions.

Regarding the paragraph in question, we were referring to the outer membrane of cells, which acts as a barrier to antibiotic penetration, as well as to the efflux pumps that expel antibiotics out of the cell. We have revised this paragraph to clarify the message.

8) The authors must provide line numbers in manuscript for the ease of the reviewers in providing the comments.
Line numbers have been added in the revised manuscript.

Reviewer #2 (Remarks to the Author):

In this manuscript Vergalli et al revisit recently developed methods and tools to track and correlate the transport of drugs across the bacterial membrane. This topic is very important considering the pressing and global issue of antimicrobial resistance that applies not only to bacteria but microbes in general including fungi. The review is fairly comprehensive, however, some major points need to be addressed before this manuscript can be considered for publication.

Major issues:

1- there are a series of typos that render reading and understanding the manuscript very difficult, e.g., merged should be emerged, antagonist should be antagonistic, mechanical mechanism should be mechanisms, in Fig. 1 driven force should be driving force etc. Please amend and proof read the manuscript before resubmission.

The manuscript has been reviewed and the errors pointed out by the reviewer have been corrected. We hope that the corrections made improve the readability and understanding of the text.

2- It is not immediately clear what is the novelty of this review compared with previous reviews e.g. https://pubs.acs.org/doi/10.1021/acs.chemrev.0c01137 that has not been cited

We agree that the topics of the cited review and our own are aligned. We have added this reference, which, indeed, contributes significantly to the topic. It is a remarkable work that lists numerous methods for studying transport and presents conclusions mainly focused on the calculations and estimations of transport kinetics. We aim to provide a more synthetic view of the importance of studying drug influx and efflux through a combination of methods that offer insights at both mechanistic and molecular levels, with potential applications in drug design.

Our intention was not to provide an exhaustive survey of methods and conclusions on the subject, but rather to offer a comprehensive reflection on the advantages and disadvantages with respect to the key interactions between drugs and porins/efflux pumps and to the reality of *in vivo* conditions, thereby integrating our perspectives into a more clinical context. Indeed, the general approaches previously used did not consider intermediate steps such as penetration, accumulation, sensitivity to efflux pumps, half-life of the molecule near the target, among others, and instead relied solely on a global outlook. Our objective is to propose a novel strategy to identify which pharmacophoric groups are involved in these respective steps through an integrated approach based on problematic clinical isolates. The final figure illustrates a potential combined strategy.

3- An important recently developed area that is not covered in this review is the development and use of fluorescent antibiotic derivatives pioneered by the Blaskovich group to study antibiotic penetration and efflux at the scale of the whole bacterial cell, including the following key papers that should be included in this review:

https://pubs.rsc.org/en/content/articlehtml/2019/md/c9md00124g

https://www.nature.com/articles/s42003-023-04745-x

https://elifesciences.org/articles/74062

https://pubs.rsc.org/en/content/articlehtml/2020/cb/d0cb00118j

And similar approaches developed by the Blair's group using dyes that are substrates of efflux pumps:

doi: 10.3389/fmicb.2019.02319

https://doi.org/10.1128/mBio.02608-21

https://www.biorxiv.org/content/10.1101/2023.04.03.535035v2

The authors categorically state "conclusions based on assays that use modified compounds or efflux substrates without bacterial activity are biased and might have a very different kinetics than antibacterial compounds used for therapeutic purposes." Ignoring the facto all the knowledge about membrane transport that has been generated using the methods above and other techniques such as tradisort 10.1128/mBio.01200-16 that should be discussed in this review.

"Biases" have been observed also when using in vitro assays such as purified outer membrane proteins, or in silicon models, as rightly acknowledged by the authors. Yet, such methods, employed by both the authors of this review and by others, have revealed over the years a wealth of knowledge about antibiotic transport and therefore have been included in this review, and rightly so. The authors should equally discuss and acknowledge the alternative in cellulo approaches suggested above, together with their limitations, as they have done for the in vitro assays and in silico models.

We do not dispute the advances in antibiotic transport achieved using fluorescent antibiotic derivatives or dyes. Our aim was not to create an exhaustive list of methods but to provide a perspective on the various approaches, envisioning a near future where these methods will best mimic the interactions between a 'real' antibiotic (commonly used in clinic) and the bacterial membrane in its natural environment. However, we acknowledge that we may have been overly concise, overlooking the insights gained from using fluorescent antibiotics or dyes, even though some of these methods have been well presented by Blair's group (DOI 10.1128/mBio.00840-16). Following the reviewer's suggestions, we have added paragraphs discussing the potential offered by fluorescent antibiotics and methods using dye efflux substrates.

4- Can the authors please clarify the sentence “Diffusion then depends on a balance between alignment and translocation”. This is a rather important concept but I did not find this concept has been clearly explained.

The sentence has been reworded.

Minor points:

- I suggest rephrasing the following sentence in the abstract “However, despite intensive search for a general set of rules to describe the uptake the investigations were rather unsuccessful.” It has a negative note and minimises the amazing work that have been done in this area, including the pioneering work of the authors of this review. I suggest changing this sentence to something like “Despite intensive theoretical and experimental investigations a general set of pathways underpinning antibiotic uptake has not been identified”.

We have modified the sentence according to the reviewer's recommendations.

- clarify the meaning of membrane resistance in the abstract

The meaning of membrane resistance has been clarified.

- “for development more effective drugs” change to “for the development of more effective drugs”

The sentence has been modified.

- define what ECDC, RND etc stand for

The meaning of acronyms has been added to the text.

- decide on the use of channel vs canal

We have favoured the use of channel.

- see “?A-?E” points described in Fig. 1 please amend ?

The ? have been removed.

- “They aim to study” who is they?

The sentence has been modified to clarify it.

- The following sentence “the mechanics of transport and the kinetic parameters which manage the sequence of functional processes of transporters - a molecular aspect by studying the dialogue between the drug and the transporter during key stages of transport - the

relationship between concentration and antibacterial action at the first times of contact. The final objective is to rationalize the design of a “faster diffusion” drug and to help the clinician in their choice of molecule.” is totally unclear

The sentence has been reworded, in the hope that this will make the message clearer.

- reference 23 does not use deep UV microscopy, please amend this statement

Reference 23 referred to the heterogeneity of cell behaviour within a population and not to the use of DUV microscopy. We understand that this was not explicit, so we have removed this part.

Reviewer #3 (Remarks to the Author):

In this review, the authors provide a description of recently developed methods and tools that track the transport of drugs across the bacterial membrane. These are important given the general lack of correlation between susceptibility established by typical MIC testing and more accurate antibiotic activity that considers intracellular concentration and the role of the target. More specifically, the authors cover advances to address this gap including in vitro, in cellulo and in silico methods. The authors describe these tools in detail, highlighting their respective advantages and disadvantages. Based on these concepts, the authors provide new perspective for the future development of more effective antibiotics, which are needed to address rampant antibiotic resistance.

Overall, this is a well-organized manuscript with high-quality figures that effectively illustrate key knowledge gaps and methodologies. The review is a valuable resource for understanding the current state of tools and methods used for drug transport and their contribution to drug design.

We thank the reviewer for these comments and their attention to our work.

However, the manuscript would greatly benefit from a comprehensive edit to enhance its clarity and readability, as it currently contains numerous grammatical and linguistic errors. This is especially true for the Introduction and the first section, “In and out transport is governed by key steps”.

The manuscript has been reviewed and some grammatical and linguistic errors have been corrected. We hope it has been improved the readability and comprehensiveness of the text.

The following section, “Understanding the drug transport across membrane with different models”, is clearer and well-organized. The reviewer suggests making Supplementary Table 1 a main Table in the manuscript for easier access, as it is both useful and comprehensive. The advantages and disadvantages of the various methods are well described, and the example of cephalosporins effectively illustrates the point of variability amongst methods.

We have followed the reviewer's suggestion and moved Supplementary Table into the main text.

The next section, “Understanding the dialogue between the porin/transporter and the drug to guide drug design”, lacks detail on how in vitro models/methods can contribute in this context. For the in cellulo part, a key citation is missing that goes beyond appending a primary amine and suggests that other positively-charged functional groups should be considered when designing antibiotics with Gram-negative activity (PMID 33228356).

We have included the suggested reference which indeed provides new insights into functional groups that favor accumulation in Gram-negative bacteria.

Lastly, in the “Future Perspective” section, more discussion on emerging technologies that could further enhance our understanding of drug transport mechanisms would strengthen the manuscript, including potential challenges/limitations these new methods may face, as well as strategies to overcome them. Emphasizing interdisciplinary collaborations and integrating insights from fields such as biophysics, computational biology, medicinal chemistry, etc., is needed to provide a more comprehensive outlook on the future development of effective antibiotics.

The example of using mass spectrometry imaging methods has been included, emphasizing the integration of data from multiple techniques.
The concept of a strategy based on combining insights from various disciplines (as illustrated in Figure 3) has also been strengthened.

Responses to Reviewers (in blue)

Your manuscript entitled "Advances in methods and concepts provide new insight into antibiotic fluxes across the bacterial membrane" has now been screened by our editors. A few final editorial recommendations and suggestions are given below and especially formatting requests in the attached accept in principle table. In light of this, I am delighted to say that we are happy, in principle, to publish a suitably revised version in Communications Biology.

The authors thank the editors for their attention to our manuscript and for their suggestions, which have helped to improve it.

** Since you mention AlphaFold in the in silico part: Please include some lines on AlphaFold 2 (and include as reference) and include in table 1.

A few lines have been added regarding the contributions of AlphaFold 2 and 3, including the corresponding references.

** We noticed some bulk citations, these parts could be enhanced by focusing/narrowing down to less main/most important references or referencing separately after specific statements, Line 42; Line 333 (might include more recent reviews and/or narrowing down to the best/main reviews)

Only the most relevant or recent references have been retained.

** You mentioned reference vs. clinical strains in L428-431: Could you give examples in text or add a list/table of the most important ones (including references for both options)?

We revised this paragraph to better clarify our message and mentioned the standard collections of reference strains, ATCC and NCTC.

** Given the recent advance in spatial biology, could you add some lines/references, e.g. spatial metabolomics, if this is (or might be, in the future) important for the field?
** Are there any references on in situ applications out already? Either cite or, if not, please note.

We thank the editor for this suggestion. Indeed, spatial biology, and particularly spatial metabolomics, offers very interesting perspectives in this field, although we have not found any applications related to antibiotics. Therefore, we have added a paragraph on this topic in the perspectives section.