

Molecular Insights into How the Motions of the β -Barrel and POTRA Domains of BamA Are Coupled for Efficient Function

Corresponding Author: Professor Jean Francois Collet

Parts of this Peer Review File have been redacted as indicated to remove third-party material.

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

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Csoma et al. reported that the structural flexibility of BamA is important for its function as an assembly machinery through the analysis of a loss of function mutant that was accidentally obtained by introducing a thrombin site (LVPR) between the POTRA and β -barrel domains of BamA. The paper reports that the structural flexibility of BamA is important for its function as an assembly machinery.

Major points.

1. the molecular mechanism of Inside-Out Communication in the title is not clear. The authors propose the existence of that communication because the loss of function caused by the LVPR sequence inserted on the periplasmic (inner) side was suppressed by a further mutation (T434A) on the cell surface side (outer). However, it appears to me that this is solely due to the point mutation alleviating the fixation of the lateral gate to the inward-open state, which was accompanied by a reduction in flexibility due to the insertion of the LVPR. In other words, without a deeper insight into the molecular mechanism by which the flexibility of the hinge region is linked to the barrel domain and moves the lateral gate, it is not possible to show communication. It only shows the importance of flexibility. It is unchanged from the paper previously reported by the authors (Reference 31) and lacks novelty.

2. the authors show that LVPR sequence insertion causes loss of hinge flexibility, as predicted by Alpha fold and others (Fig. 1f), but there is a lack of direct experimental data on the extent to which flexibility is lost.

3. there is a lack of research to determine that the loss of function due to LVPR insertion is a loss of flexibility. We propose two other possibilities.

The first is the involvement of other lipoprotein subunits of the BAM complex: when comparing Fig. 3a (BamALVPR) and b (BamAGSGS), BamD appears to be closer to the β -barrel domain than BamAGSGS is to the LVPR; the same applies to BamE. BamD is an essential subunit that interacts with the substrate before BamA. In other words, it is very possible that this aparting of BamA and BamD affected the assembly efficiency. For example, would the loss of BamE, which destabilises the BAM complex, in combination with the inflexible LVPR insertion, suppress its effects?

The second is the effect of the interaction of the BAM complex with SurA chaperones. An increase in sigma E implies an accumulation of substrates in the periplasm, which could also be influenced by a reduction in the efficiency of substrate delivery from the SurA chaperone to the BAM complex. In fact, sigma E is induced even in flexible GSGS sequences, so it may be premature to assume that flexibility is the only effect on function. We suggest the authors confirm the influence of SurA and the BAM complex by HDX-MS experiments with the addition of SurA.

Minor point.

1. the lack of loading controls in Fig. 1c and Fig. 4c westerns makes it impossible to assess the increase or decrease in OMPs.

Reviewer #2

(Remarks to the Author)

One of the critical questions in the field is how the motions of the β -barrel coordinate with the five polypeptide transport associated (POTRA) domains in the BamA complex (and this is clearly stated in this manuscript in lines 29 and 30). The main results that emanate from this work are: 1) flexibility in the hinge region between the β -barrel and the POTRA domains is necessary for optimal functioning of the Bam machinery and 2) a point mutation from threonine to alanine at position 434 helps to rescue some of the lost functionality of the Bam complex through presumably some allosteric effect. Neither of these two findings however answer that critical question of how the motions of the β -barrel and the (POTRA) domains are coordinated. Nor do they provide insight into whether motion in the POTRA domains induces motion in the β -barrel or vice versa. But these two findings provide additional clues that could be exploited with additional work (from this group or others) that could lead to resolution of the question.

Overall, while this work is not groundbreaking, it still has a lot of value in providing additional insight into the mechanism of action for the BamA complex which would be useful to the scientific community when planning future experiments.

This work provides additional clues about how motions of the β -barrel and the (POTRA) domains are coordinated and so advances knowledge on how the Bam complex regulates outer membrane biogenesis and as such would be of interest in the quest to understand how gram-negative bacteria protect themselves from toxic compounds. The work itself, while novel, does not answer the question on how the motions in the β -barrel and the POTRA domains are coordinated. But the knowledge of flexibility that is required in the hinge region and the point mutation that can rescue functionality of the BamA complex can be exploited by others in the future to get a true mechanistic understanding of how the Bam complex functions.

The work described in this manuscript is quite thorough. The claims of reduced activity for the BAMA complex and subsequent membrane impairment are supported through several independent analyses, including studies investigating sensitivity to antimicrobial agents, folding assays (rate and yield) for OMPx and tOMP_a, and OMP and LptD expression yields from proteomics experiments. Separately, each of these analyses support the claim of reduced activity, but together provide a powerful argument that increasing rigidity in the hinge region is detrimental to the ability of the BamA complex to function optimally.

The single particle cryoEM experiments reinforce the claim that the flexibility of the hinge is critical to maintain activity of the BamA complex. For BamALVPR, POTRA domain 5 obscures the base of the β -barrel in what would nominally be an outward-open configuration when the lateral gate is closed in a nominally inward-open configuration. This is not the case for the wild type, nor for the complex with the flexible GS_{GS} linker.

The analyses for the T434A point mutation support the claim for partial recovery of foldase activity and the H-D exchange experiments to elucidate solvent accessibility are useful for highlighting which parts of the complex are affected by this mutation. The authors state a possible allosteric effect that is responsible for the recovery of activity without any specifics on mechanistically how this might happen. It is reasonable to suggest this since there are no experiments within this body of work that can unequivocally provide such information. Still, this conjecture provides some basis for potential future experiments to test.

There are no major flaws in the data analysis, interpretation and conclusions. Although there is some minor messaging that needs to be taken care of prior to publication of this work. One noticeable misleading statement is in lines 188-191, where it is claimed that there is 'approximately 100% folding efficiency with wild-type BamA'. I agree that this is the case for tOMP_a, but for OpmX, Figure 2b and Supplementary Table 1, the folding efficiency is 84%. I would not claim that 84% is approximately 100%. Also, for the 24-hour cases, it is stated that ~100% yield for both tOMP_a and OpmX is obtained, when in fact, it is 70-90%. Yes, this is better than the 3-hour cases, but not ~100%. Please rephrase.

Another, message that needs clarification relates to the statement in lines 102-103 in which the authors state 'maintaining flexibility in the hinge region is necessary for BAM activity'. There are examples in the manuscript in which BAMLVPR and BAMLVPR* do show protein folding (Supplementary Table 1, Fig 4) albeit much less efficiently. Suggest authors rephrase this statement along the lines of 'efficient' or 'optimal BAM activity'.

The methodology and approach are sound and the experiments described in this manuscript are to be expected for this investigation. For the experiments that I am most familiar with, there is enough detail to reproduce the experiments. For those that I am less familiar with, there is adequate detail in the methods section that make me believe that these too are reproducible.

General comments.

There are several grammatical errors in the manuscript, most of which are found in the Methods and Materials section. Just as a few examples: Line 436: 'work frame' should be 'framework', 454: 'LB agar or McConkey' should be 'LB or McConkey agar', 460: 'is lay flat' should be 'is laid flat', 463: 'incubated 37 C' should be 'incubated at 37 C'... I can go on but instead of that, I suggest authors run the text through a grammar and spell checker. Also, look for places where abbreviations are used but not yet defined. Example 'SDM' for 'site-directed mutagenesis', 'DDA' for 'data-dependent analysis', etc...

A few more specific suggestions:

Line 160, section title: 'Flexibility is required in the hinge region of BamA' for what?

For Figure 3d and e (and in Supplementary Figs. 2 and 3), instead of just labeling structures as Inward-open and Outward-open, label as the BAMWT Inward-open and BAMWT Outward-open. This additional labeling makes the figures easier to grasp.

I find it hard to distinguish 6LYU and BAMSGS in the supplementary Figs 2d and 2f. I recommend changing 6LYU to a color with better contrast to that of BAMSGS.

Supplementary Table 5: For pBAMALVPR* and pBAMt434A, the phrase 'substitution T for A at position 434' is misleading. This implies that 434 is A in the WT and is substituted with T. What is described in the manuscript is the opposite.

Reviewer #3

(Remarks to the Author)

I have been asked to comment on the HDX-MS and will limit my review to this. I see no problems with the authors methods and data processing. They've used Waters Corp off the shelf methods and analysis tools for HDX-MS, further data processing has been done with Deuterios which is a fairly standard package built off statistical analysis by Houde&Engen. Data are represented in Woods plots which looks fine and also projected onto structures using a binary protected/deprotected representation which is fairly common these days. The only thing I don't understand is the 4 degree C labelling temperature. This is unusual and I couldn't find text to justify it so I assume it's been done at this temp because its the coldest you can go to match cryo conditions? For HDX folks might want to justify that (unless you've already done this and I missed it). Why there's nothing wrong with this temp it does make the longest labelling time effectively 3 mins (compared to ambient and pH 8) and I wonder if the HDX is showing the whole picture as labelling is quite limited under these conditions (might want to comment on this). One more thing the difference in DDM concs between Cryo (0.05%) and HDX (0.02%) was not explained. It's probably not going to affect anything (both above the CMC) but could smooth out with a line of justification.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Review of Revised Manuscript

It seems the authors have put in effort to address our previous concerns, which is a positive development, no doubt. Let us now evaluate the revised manuscript.

Major Points

Our concerns regarding Major points 1 and 2 have been addressed satisfactorily. This resolves our previous issues on these aspects.

Regarding Major point 3:

The absence of disclosed structures for 9H84 and 9H85 somewhat impedes a fully comprehensive structural discussion. Nevertheless, we will proceed with the provided figures. Upon comparing Fig 3a and Fig 3b, in the LVPR variant (Fig 3a), based on previously reported structures, one can reasonably infer a spatial separation of at least 10 Å between the periplasmic interface of the BamA β-barrel domain and BamD. Conversely, in the GSGS variant (Fig 3b), a portion of BamD appears to directly interact with the BamA β-barrel domain.

It is somewhat perplexing that the authors emphasize an RMSD of 2.2 Å while seemingly overlooking local structural variations exceeding 10 Å. This, if you ask me, suggests a potentially biased interpretation of the data and a lack of thorough evaluation of the structural differences. Furthermore, the reasons for the small differences in the local resolution of the EM density maps warrant further clarification. It is plausible that these differences arise from variations in lipoprotein orientation, isn't it?

The authors state, "Generally, the lipoproteins are thought to shift BamA's conformational ensemble towards outward-open (at least in detergent)." However, many reported structures indicate that lipoprotein binding does not invariably lead to an outward-open conformation. For example, in structure 5D0O, which the authors themselves utilize for modeling within the text, all lipoproteins are bound, yet the conformation is not outward-open. This observation suggests that the authors' assertion may be based on a selective interpretation of limited data, potentially compromising scientific accuracy.

Regarding the discussion of SurA, we concur with the authors that dedicated experiments fall outside the scope of this manuscript. However, a recently published structure of BAM-SurA (Sci Adv. 11(14):eads6094.) demonstrates that SurA engages in extensive interactions extending beyond POTRA1 into the POTRA2-BamB region. Moreover, it has been

proposed that another chaperone, BepA, exhibits even more extensive interactions with BamD (Mol Microbiol. 106(5):760-776.). Consequently, a comprehensive consideration of these prior findings does not unequivocally support the authors' conclusion that reduced flexibility of the POTRA domain would not impact interactions with chaperones such as SurA.

In conclusion, the authors' current explanations do not adequately exclude the possibility that the observed functional impairment in the LVPR variant could stem from subtle alterations in lipoprotein interactions or changes in binding to chaperones like SurA. Therefore, the authors should broaden their discussion beyond a sole focus on lateral gate and hinge communication to include these alternative possibilities, thereby enriching the avenues for future research.

Minor Points

To be frank, the authors' response on this particular point is disappointing.

Firstly, no information whatsoever has been provided concerning cell morphology. Furthermore, a comparison of samples merely normalized by optical density is not "a widely accepted method in bacteriology." The inclusion of a loading control is essential. It is pertinent to note that the corresponding author has previously published work on GroEL (citation omitted), wherein a polyclonal rabbit anti-GroEL antibody was employed. Consideration should be given to utilizing this as a loading control for a cytosolic factor. Moreover, the evaluation must be performed on a single, uncut gel, unlike the presentation in their previous version. Moreover, recent research ethics workshops strongly recommend presenting data on a single, uncut gel, avoiding any unnecessary manipulation such as cutting and pasting. Do the authors have an alternative rationale for not improving this aspect of the data presentation?

Reviewer #2

(Remarks to the Author)

I have reviewed the revised manuscript and authors have adequately addressed the concerns and suggestions I made in my initial review. I have no further concerns at this point.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Comment For Major point 3

I understand the author's point about the major points well. No further discussion is necessary.

Comments for Minor points

We analyzed the bands in the gel data you provided here. Please see the attached figure. This time, we analyzed only the bands in the top left gel. We quantified and compared the green asterisk band and the two bands below it. The green asterisk bands do not appear to be different at first glance, but if lane 3 is 100%, lane 2 is only about 60%, and the evaluation of the results will be significantly different. In addition, if normalized with band 3 below, lane 4 is only about 46% if lane 2 is 100%, and this result does not guarantee that equal amounts of sample were run. In addition, there is no guarantee that this non-specific band is derived from the E. coli sample. Therefore, it cannot be denied that it is derived from contamination contained in the SDS-PAGE buffer in the author's lab, and this cannot be used to compare samples.

Next, we analyzed the data you provided for cell size (attached figure). We evaluated cell elongation based on these cell lengths, cell areas, and aspect ratios. As the authors show, no significant differences in cell size were observed in GSGS and LVPR, which are the focus of this study. However, we report that a significant difference was observed in T434 and a small amount of variance was observed in LVPR*. If cell morphology is affected, intracellular proteins have a significant impact, so we need to pay attention to even small variances.

It seems inevitable to present the amount of another E. coli protein in the same sample as a loading control. In particular, considering that some research misconduct has occurred recently from Western gels, shouldn't these be dealt with more carefully and appropriately?

I would like to thank you for your guidance on "a widely accepted method in bacteriology," including papers, as I am unfamiliar with the field of microbiology. I have reviewed and studied these papers in detail.

First, Combs and Silhavy, PNAS, 2021, which came up first. In this paper, as the Combs say, when comparing the amount of Skp in Fig. 2C, the non-specific band above it (shown with an X in the figure) was used as a loading control. However, in Fig. 2A, OmpA is used as a loading control. Furthermore, in C, the SDS-PAGE of the same sample as in A is checked with a different antibody. Therefore, this X is not a single loading control, but I think that a loading control is already presented with OmpA. Is this the same as the widely accepted method in bacteriology that you mentioned, which is to compare samples with the same cell number without a loading control? I don't think so.

Grim et al, PNAS, 2020, has blotting data in Fig. 1F, and the amounts are indeed compared without a loading control.

However, this is LPS, not a protein. Was it LPS that was being compared in this paper? LPS is an abbreviation for lipopolysaccharide, not a protein. This difference seems insufficient to prove a widely accepted method in bacteriology.

In Hart et al, J Bacteriol., 2019, MBP is presented as a loading control in Fig. 1A, Fig. 2A, B, Fig. 3B, and GroEL is presented as a loading control in Fig. 5A. Have I missed something?

In Dos Santos et al, PNAS, 2024, LptE is presented as a loading control in the lysate sample in Fig. 3E.

In Tomasek et al, Nature, 2020, RpoA and LptF are presented as loading controls in Fig. 1c and e, respectively.

As mentioned above, all of the papers you introduced had loading controls. However, there is no loading control in Fig. 1c of this paper. How is this a widely accepted method in bacteriology? Please explain in more detail to me, who does not understand. In addition, none of the papers you introduced involved cutting the gel between lanes, as in Fig. 1c.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Csoma et al. reported that the structural flexibility of BamA is important for its function as an assembly machinery through the analysis of a loss of function mutant that was accidentally obtained by introducing a thrombin site (LVPR) between the POTRA and β -barrel domains of BamA. The paper reports that the structural flexibility of BamA is important for its function as an assembly machinery.

Major points.

1. the molecular mechanism of Inside-Out Communication in the title is not clear. The authors propose the existence of that communication because the loss of function caused by the LVPR sequence inserted on the periplasmic (inner) side was suppressed by a further mutation (T434A) on the cell surface side (outer). However, it appears to me that this is solely due to the point mutation alleviating the fixation of the lateral gate to the inward-open state, which was accompanied by a reduction in flexibility due to the insertion of the LVPR. In other words, without a deeper insight into the molecular mechanism by which the flexibility of the hinge region is linked to the barrel domain and moves the lateral gate, it is not possible to show communication. It only shows the importance of flexibility. It is unchanged from the paper previously reported by the authors (Reference 31) and lacks novelty.

We acknowledge the need for further mechanistic insights into how communication between the inside (hinge region) and the outside controls BAM function. Nonetheless, the fact that the point mutation alleviates the fixation of the gate caused by insertion of LVPR clearly shows communication over a long distance, even though at this stage we cannot know precisely how it is enacted. In response, we have revised the manuscript to clarify that while our data strongly support the functional coupling between these two domains, further studies will be required to dissect the exact molecular basis of this coordination. Additionally, we have modified the title to more accurately reflect our findings.

2. the authors show that LVPR sequence insertion causes loss of hinge flexibility, as predicted by Alpha fold and others (Fig. 1f), but there is a lack of direct experimental data on the extent to which flexibility is lost.

We agree with the reviewer that we do not provide data quantifying the exact extent of the loss of flexibility. However, it is well documented in the literature that proline residues restrict protein conformational space^{1,2}, which supports the idea that the LVPR insertion rigidifies the hinge region. To further address this, we have now included a dihedral angle distribution analysis of the C-alpha atoms for NTGS (wild-type), LVPR, and GSGS sequences. The newly added figure demonstrates that the LVPR insertion results in a highly restricted dihedral angle distribution, indicating reduced conformational flexibility.

In contrast, NTGS and GSGS exhibit broad distributions, confirming that these sequences maintain flexibility in the hinge region. This additional data reinforces our conclusion that LVPR rigidifies the hinge region, impairing BAM function. The new figure (**Fig. 1g**) has been included in the revised manuscript. Further, the manuscript has been modified to better discuss the impact of inserting LVPR on the flexibility of the hinge region.

3. there is a lack of research to determine that the loss of function due to LVPR insertion is a loss of flexibility. We propose two other possibilities.

The first is the involvement of other lipoprotein subunits of the BAM complex: when comparing Fig. 3a (BamALVPR) and b (BamAGSGS), BamD appears to be closer to the β -barrel domain than BamAGSGS is to the LVPR; the same applies to BamE. BamD is an essential subunit that interacts with the substrate before BamA. In other words, it is very possible that this aparting of BamA and BamD affected the assembly efficiency. For example, would the loss of BamE, which destabilises the BAM complex, in combination with the inflexible LVPR insertion, suppress its effects?

The second is the effect of the interaction of the BAM complex with SurA chaperones. An increase in sigma E implies an accumulation of substrates in the periplasm, which could also be influenced by a reduction in the efficiency of substrate delivery from the SurA chaperone to the BAM complex. In fact, sigma E is induced even in flexible GSGS sequences, so it may be premature to assume that flexibility is the only effect on function. We suggest the authors confirm the influence of SurA and the BAM complex by HDX-MS experiments with the addition of SurA.

We thank the reviewer for these suggestions.

We have checked the relative positioning of BamD to BamA in BAM_{LVPR} and BAM_{GSGS} and they are very similar, indicated by the close structural overlay in **Fig. 3c** and the global RMSD of 2.2 Å between the two models. While the base of the barrel in BamA_{GSGS} is more expanded than that of BamA_{LVPR}, there is no structural evidence for significant rearrangement or alteration to the BamA-BamD interaction interface. The apparent difference between BamD in the two models is due to small differences in the local resolution of the EM density maps, not true domain motion (as shown by the fitted models). The interfaces of the other BamA interacting lipoproteins (BamB and BamE) to BamA are also unchanged between BAM_{LVPR} and BAM_{GSGS}, suggesting that structural rearrangements to lipoproteins (or their interactions) induced by the LVPR or GSGS insertion do not explain the altered activity. To clarify this, when describing the similarity of the BAM_{LVPR} and BAM_{GSGS} structures, the following phrase has been added (green, line 219):

‘The structures are similar, including the subunit interaction interfaces (fitted model RMSD: 2.2 Å, Fig. 3c), although the inserted hinge region and the base of the barrel around the insert (b1-3) are poorly resolved (Supplementary Fig. 3).’

The proposition that loss of BamE might suppress BamA_{LVPR} is intriguing – it is known that BamE is required for stabilising the BamA-BamD interaction, although the structural

consequences to the rest of the BAM complex are unclear³. Generally, the lipoproteins are thought to shift BamA's conformational ensemble towards outward-open (at least in detergent)⁴⁻⁶. While BAM_{LVPR} shows structural uncoupling between BamA's barrel and periplasmic domains, BamE and BamD are still interacting with the barrel. Loss of BamE would remove these (presumably) outward-open stabilising interactions on the barrel, further decoupling the barrel and the POTRA domains, possibly acting to strengthen the BamA_{LVPR} effect. As noted by the reviewer, this effect may be countered by destabilisation of the complex, although the magnitude of this effect is uncertain.

Regarding the second possibility, the role of SurA as a key chaperone for OMP biogenesis is well documented. Our recent structural insights⁷ demonstrate that SurA binds to the POTRA1 domain of BamA, facilitating substrate handover for OMP insertion. While we cannot entirely exclude the possibility, as suggested by the reviewer, that insertion in the hinge region could impact the SurA-POTRA1 interaction, this seems unlikely given the considerable distance between this domain and the hinge region, the overall flexibility of the periplasmic POTRA domains and data implicating POTRA1 as the key interaction site for SurA. We now explicitly consider this possibility in the discussion. However, we believe that assessing SurA's influence on BamA_{LVPR} and BamA_{GSGS} using HDX-MS is unlikely to yield insightful results into the efficiency of substrate delivery from the SurA chaperone to the BAM complex. To directly address that question (which is a good one) would require a whole new project on the rate of substrate delivery with methods and assays that are not yet developed, and addressing these questions is way beyond the scope of this work.

Minor point.

1. the lack of loading controls in Fig. 1c and Fig. 4c westerns makes it impossible to assess the increase or decrease in OMPs.

In our study, we normalized protein samples based on optical density (OD), a widely accepted method in bacteriology to ensure equal input. This information has been added to the legend of Figure 1c and 4c. Additionally, we verified that the cells had similar morphology, eliminating the risk of artifacts due to differences in cell shape.

Reviewer #2 (Remarks to the Author):

One of the critical questions in the field is how the motions of the β -barrel coordinate with the five polypeptide transport associated (POTRA) domains in the BamA complex (and this is clearly stated in this manuscript in lines 29 and 30). The main results that emanate from this work are: 1) flexibility in the hinge region between the β -barrel and the POTRA domains is necessary for optimal functioning of the Bam machinery and 2) a point mutation from threonine to alanine at position 434 helps to rescue some the lost functionality of the Bam complex through presumably some allosteric effect. Neither of these two findings however answer that critical question of how the motions of the β -barrel and the (POTRA) domains are coordinated. Nor do they provide insight into whether motion in the PORTA domains induces motion in the β -barrel or vice versa. But these two findings provide additional clues that could be exploited with additional work (from this group or others) that could lead to resolution of the question.

Overall, while this work is not groundbreaking, it still has a lot of value in providing additional insight into the mechanism of action for the BamA complex which would be useful to the scientific community when planning future experiments.

This work provides additional clues about how motions of the beta-barrel and the (POTRA) domains are coordinated and so advances knowledge on how the Bam complex regulates outer membrane biogenesis and as such would be of interest in the quest to understand how gram-negative bacteria protect themselves from toxic compounds. The work itself, while novel, does not answer the question on how the motions in the beta-barrel and the POTRA domains are coordinated. But the knowledge of flexibility that is required in the hinge region and the point mutation that can rescue functionality of the BamA complex can be exploited by others in the future to get a true mechanistic understanding of how the Bam complex functions.

The work described in this manuscript is quite thorough. The claims of reduced activity for the BAMA complex and subsequent membrane impairment are supported through several independent analyses, including studies investigating sensitivity to antimicrobial agents, folding assays (rate and yield) for OMPx and tOMP_A, and OMP and LptD expression yields from proteomics experiments. Separately, each of these analyses support the claim of reduced activity, but together provide a powerful argument that increasing rigidity in the hinge region is detrimental to the ability of the BamA complex to function optimally.

The single particle cryoEM experiments reinforce the claim that the flexibility of the hinge is critical to maintain activity of the BamA complex. For BamALVPR, POTRA domain 5 obscures the base of the beta-barrel in what would nominally be an outward-open configuration when the lateral gate is closed in a nominally inward-open configuration. This is not the case for the wild type, nor for the complex with the flexible GSGS linker.

The analyses for the T434A point mutation support the claim for partial recovery of foldase activity and the H-D exchange experiments to elucidate solvent accessibility are useful for highlighting which parts of the complex are affected by this mutation. The authors state a possible allosteric effect that is responsible for the recovery of activity without any specifics on mechanistically how this might happen. It is reasonable to suggest this since there are no experiments within this body of work that can unequivocally provide such information. Still, this conjecture provides some basis for potential future experiments to test.

Thank you; we agree and we state in our discussion that the molecular and atomistic details of any allosteric network within BAM remain to be discovered, and this will certainly form the basis of future work.

There are no major flaws in the data analysis, interpretation and conclusions. Although there is some minor messaging that needs to be taken care of prior to publication of this work. One noticeable misleading statement is in lines 188-191, where it is claimed that there is 'approximately 100% folding efficiency with wild-type BamA'. I agree that this is the case for tOmp_A, but for OpmX, Figure 2b and Supplementary Table 1, the folding

efficiency is 84%. I would not claim that 84% is approximately 100%. Also, for the 24-hour cases, it is stated that ~100% yield for both tOmpA and OpmX is obtained, when in fact, it is 70-90%. Yes, this is better than the 3-hour cases, but not ~100%. Please rephrase.

We thank the reviewer for pointing this out. We have revised the paragraph to accurately reflect the folding efficiencies reported in Figure 2b and Supplementary Table 1.

Another, message that needs clarification relates to the statement in lines 102-103 in which the authors state ‘maintaining flexibility in the hinge region is necessary for BAM activity’. There are examples in the manuscript in which BAMLVPR and BAMLVPR* do show protein folding (Supplementary Table 1, Fig 4) albeit much less efficiently. Suggest authors rephrase this statement along the lines of ‘efficient’ or ‘optimal BAM activity’.

The sentence has been modified to: *“In contrast, inserting a flexible GSGS sequence had no impact on bacterial growth and only minor effects on BAM function, confirming that maintaining flexibility in the hinge region is necessary for optimal BAM activity.”*

The methodology and approach are sound and the experiments described in this manuscript are to be expected for this investigation. For the experiments that I am most familiar with, there is enough detail to reproduce the experiments. For those that I am less familiar with, there is adequate detail in the methods section that make me believe that these too are reproducible.

General comments.

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We apologize for these grammatical errors. We have carefully revised the manuscript, particularly the Methods and Materials section, to correct grammatical and spelling mistakes.

A few more specific suggestions:

Line 160, section title: ‘Flexibility is required in the hinge region of BamA’ for what?

The title of the section has been changed to “Flexibility is required in the hinge region of BamA for optimal activity”.

For Figure 3d and e (and in Supplementary Figs. 2 and 3), instead of just labeling structures as Inward-open and Outward-open, label as the BAMWT Inward-open and BAMWT Outward-open. This additional labeling makes the figures easier to grasp.

The Figure was modified as requested.

I find it hard to distinguish 6LYU and BAMGSGS in the supplementary Figs 2d and 2f. I recommend changing 6LYU to a color with better contrast to that of BAMGSGS.

The Figure has been modified as requested.

Supplementary Table 5: For pBAMALVPR* and pBAMt434A, the phrase ‘substitution T for A at position 434’ is misleading. This implies that 434 is A in the WT and is substituted with T. What is described in the manuscript is the opposite.

This has been corrected in the table.

Reviewer #3 (Remarks to the Author):

I have been asked to comment on the HDX-MS and will limit my review to this. I see no problems with the authors methods and data processing. They've used Waters Corp off the shelf methods and analysis tools for HDX-MS, further data processing has been done with Deuterios which is a fairly standard package built off statistical analysis by Houde&Engen. Data are represented in Woods plots which looks fine and also projected onto structures using a binary protected/deprotected representation which is fairly common these days. The only thing I don't understand is the 4 degree C labelling temperature. This is unusual and I couldn't find text to justify it so I assume it's been done at this temp because its the coldest you can go to match cryo conditions? For HDX folks might want to justify that (unless you've already done this and I missed it). Why there's nothing wrong with this temp it does make the longest labelling time effectively 3 mins (compared to ambient and pH 8) and I wonder if the HDX is showing the whole picture as labelling is quite limited under these conditions (might want to comment on this). One more thing the difference in DDM concs between Cryo (0,05%) and HDX (0.02%) was not explained. It's probably not going to affect anything (both above the CMC) but could smooth out with a line of justification.

We chose to perform our HDX analysis at 4°C to slow the rate of exchange relative to room temperature experiments, as we have previously published⁸. Indeed, this is consistent with current practice for manual HDX experiments, where most perform the exchange ‘on ice’. With respect to the DDM concentrations used, this was because we wanted to use conditions that were consistent with our previous HDX studies of BAM⁸. As the reviewer identifies, both concentrations used for cryoEM and HDX-MS are above the CMC and so we do not envisage any adverse effects from this difference. We do not believe that additional HDX-MS experiments would afford any further useful information, and we have now cited our previous work in the Methods section to clarify that the conditions used have been described previously.

References:

- 1 MacArthur, M. W. & Thornton, J. M. Influence of proline residues on protein conformation. *J Mol Biol* **218**, 397-412, doi:10.1016/0022-2836(91)90721-h (1991).
- 2 Schimmel, P. R. & Flory, P. J. Conformational energies and configurational statistics of copolypeptides containing L-proline. *J Mol Biol* **34**, 105-120, doi:10.1016/0022-2836(68)90237-4 (1968).
- 3 Kumar, S. & Konovalova, A. BamE directly interacts with BamA and BamD coordinating their functions. *Mol Microbiol* **120**, 397-407, doi:10.1111/mmi.15127 (2023).
- 4 Albrecht, R. *et al.* Structure of BamA, an essential factor in outer membrane protein biogenesis. *Acta Crystallogr D Biol Crystallogr* **70**, 1779-1789, doi:10.1107/S1399004714007482 (2014).
- 5 Morgado, L., Zeth, K., Burmann, B. M., Maier, T. & Hiller, S. Characterization of the insertase BamA in three different membrane mimetics by solution NMR spectroscopy. *J Biomol NMR* **61**, 333-345, doi:10.1007/s10858-015-9906-y (2015).
- 6 White, P. *et al.* The role of membrane destabilisation and protein dynamics in BAM catalysed OMP folding. *Nat Commun* **12**, 4174, doi:10.1038/s41467-021-24432-x (2021).
- 7 Fenn, K. L. *et al.* Outer membrane protein assembly mediated by BAM-SurA complexes. *Nat Commun* **15**, 7612, doi:10.1038/s41467-024-51358-x (2024).
- 8 Schiffrin, B. *et al.* Dynamic interplay between the periplasmic chaperone SurA and the BAM complex in outer membrane protein folding. *Commun Biol* **5**, 560, doi:10.1038/s42003-022-03502-w (2022).

Point-by-point response to the comments

Regarding Major point 3:

The absence of disclosed structures for 9H84 and 9H85 somewhat impedes a fully comprehensive structural discussion. Nevertheless, we will proceed with the provided figures. Upon comparing Fig 3a and Fig 3b, in the LVPR variant (Fig 3a), based on previously reported structures, one can reasonably infer a spatial separation of at least 10 Å between the periplasmic interface of the BamA β -barrel domain and BamD. Conversely, in the GSGS variant (Fig 3b), a portion of BamD appears to directly interact with the BamA β -barrel domain.

It is somewhat perplexing that the authors emphasize an RMSD of 2.2 Å while seemingly overlooking local structural variations exceeding 10 Å. This, if you ask me, suggests a potentially biased interpretation of the data and a lack of thorough evaluation of the structural differences. Furthermore, the reasons for the small differences in the local resolution of the EM density maps warrant further clarification. It is plausible that these differences arise from variations in lipoprotein orientation, isn't it?

We apologise if Figure 3a and 3b has led to confusion and would like to confirm to the editor and the referee that we have considered all of our structures in detail for each resolved region of the map, and in reference to other previously published structures. We are happy to enclose the maps and models for the referee to consider. Additionally, cryoEM reconstructions and corresponding coordinates have been deposited in the Electron Microscopy Data Bank (EMDB) and the Protein Data Bank (PDB).

It is important to note, that in our text we chose to focus our discussion on major differences between 9H84 and 9H85 and other published structures. The referee is correct to note that there could be other small differences (for example in residues 98-112 of BamD which interact with BamA and which we think the referee is referring to), but these differences are difficult to interpret as they are within the resolution limit of the EM maps. We have added some remarks to clarify this in our revised text (lines 245-253, lines 384-388). We hope that having seen the maps, models and above text, you will agree that there is no biased interpretation of the data, and we acknowledge that small differences, that may be important but are beyond the local resolution, cannot be interpreted with confidence.

The authors state, "Generally, the lipoproteins are thought to shift BamA's conformational ensemble towards outward-open (at least in detergent)." However, many reported structures indicate that lipoprotein binding does not invariably lead to an outward-open conformation. For example, in structure 5D0O, which the authors themselves utilize for modeling within the text, all lipoproteins are bound, yet the conformation is not outward-open. This observation suggests that the authors' assertion may be based on a selective interpretation of limited data, potentially compromising scientific accuracy.

We are fully aware of the many BAM structures from our lab and others which were solved by cryoEM and, as in the case of 5D0O, crystallography. For all cryoEM structures of apo-BAM in DDM the structure is outward-open, and hence our finding that BamA's barrel domain in 9H84 and 9H85 more closely resembles the inward-open state is surprising. We have clarified in the text that our discussion was referring to cryoEM structures that are obtained in similar solution conditions (line 233) and removed the single sentence where we discussed the lipoproteins in the context of their role in modulating BamA conformation, which we agree was misleading. We would like to emphasise here that the most surprising finding in our structures is the uncoupling of the motion of the lateral

gate to the motions of the POTRA domains and their associated BamB, C, D, E lipoproteins which we hope is clear in our text. We have added text to lines 234-240 to make this point clear.

Regarding the discussion of SurA, we concur with the authors that dedicated experiments fall outside the scope of this manuscript. However, a recently published structure of BAM-SurA (Sci Adv. 11(14):eads6094.) demonstrates that SurA engages in extensive interactions extending beyond POTRA1 into the POTRA2-BamB region. Moreover, it has been proposed that another chaperone, BepA, exhibits even more extensive interactions with BamD (Mol Microbiol. 106(5):760-776.). Consequently, a comprehensive consideration of these prior findings does not unequivocally support the authors' conclusion that reduced flexibility of the POTRA domain would not impact interactions with chaperones such as SurA.

We agree with the reviewer that our data does not exclude the possibility of altered interactions of BAM with its partners (e.g. SurA, BepA). Indeed, we do not state anywhere in our manuscript that reduced flexibility does not impact such interactions. This point can only be answered by experiments in the future, and we hoped that we had addressed this point in our previous revisions. We have now added a further discussion about this in our second revised manuscript where we discuss the known structural basis of both SurA and BepA interactions with BAM (line 391-405).

In conclusion, the authors' current explanations do not adequately exclude the possibility that the observed functional impairment in the LVPR variant could stem from subtle alterations in lipoprotein interactions or changes in binding to chaperones like SurA. Therefore, the authors should broaden their discussion beyond a sole focus on lateral gate and hinge communication to include these alternative possibilities, thereby enriching the avenues for future research.

We have addressed these two comments in our responses above. In particular, we have broadened the discussion section to include details of the key areas highlighted by the reviewer. Given that the text is entirely speculative we have kept it to a length that we hope makes the point, stimulating further ideas and options, without making it too verbose (lines 391-405).

Minor Points

To be frank, the authors' response on this particular point is disappointing.

Firstly, no information whatsoever has been provided concerning cell morphology. Furthermore, a comparison of samples merely normalized by optical density is not "a widely accepted method in bacteriology." The inclusion of a loading control is essential. It is pertinent to note that the corresponding author has previously published work on GroEL (citation omitted), wherein a polyclonal rabbit anti-GroEL antibody was employed. Consideration should be given to utilizing this as a loading control for a cytosolic factor. Moreover, the evaluation must be performed on a single, uncut gel, unlike the presentation in their previous version. Moreover, recent research ethics workshops strongly recommend presenting data on a single, uncut gel, avoiding any unnecessary manipulation such as cutting and pasting. Do the authors have an alternative rationale for not improving this aspect of the data presentation?

We understand the reviewer's concern. For this reason, we have provided the original, uncut, and unprocessed gel images as supplementary information in the data source Excel file linked to the

paper and referenced in Fig. 1. These clearly show additional, unidentified bands whose consistent intensity across lanes serves as internal evidence of equivalent protein loading (Fig. 1 below, bands marked with a green asterisk). The use of cross-reactive proteins as loading controls has been previously employed by others (Combs and Silhavy, PNAS, 2021 (<https://doi.org/10.1073/pnas.2114997119>)).

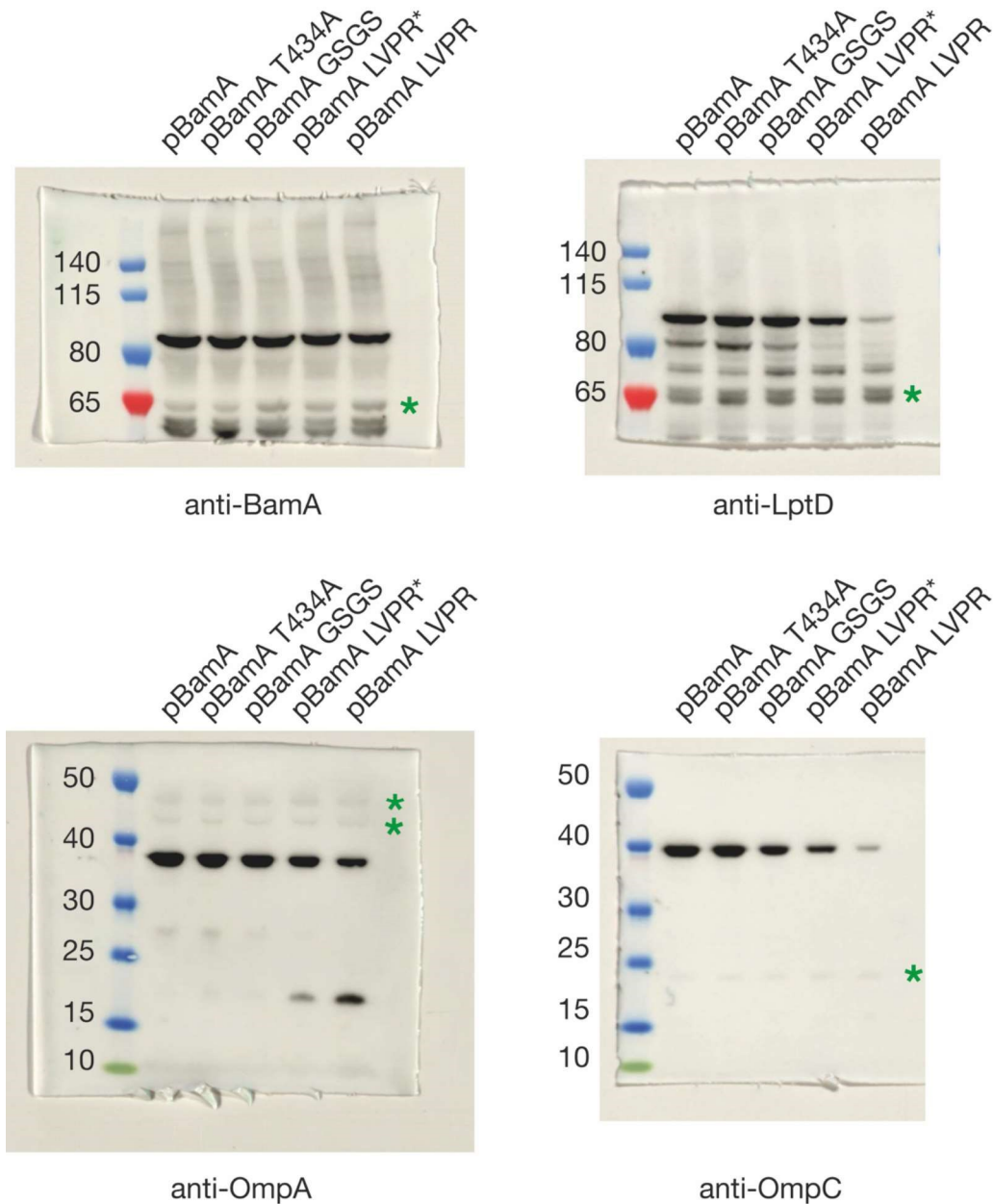


Figure 1: Uncut membranes corresponding to the results shown in Figures 1 and 4 of the main text. Images are also included in the source data file. Overlay images of chemiluminescence and molecular weight marker were obtained using a CCD camera. Western blot analysis of trichloroacetic acid (TCA) precipitates from *bamA::kan* cells expressing BamA variants from a plasmid. Cells were grown in LB medium at 30 °C until mid-log phase. Nitrocellulose membranes were probed with primary antibodies targeted against the indicated proteins. Cross-reactive bands, which can be used as loading controls, are annotated with a green asterisk.

In addition, we have imaged the cells by microscopy (Figure 2 below) and found them to be of comparable size across conditions. This observation supports the validity of using OD₆₀₀ as a normalization method in our experiments. We respectfully disagree with the reviewer; normalization by optical density remains a commonly employed method in the bacteriology literature, notably in recent publications by leading groups in the field, including that of Thomas Silhavy (Grimm et al, PNAS, 2020 (<https://doi.org/10.1073/pnas.2015556117>); Hart et al, J Bacteriol., 2019 (<https://doi.org/10.1128/jb.00745-18>)), Natividad Ruiz (Kumar et al, PNAS, 2024 (<https://doi.org/10.1073/pnas.2321512121>)) and Daniel Khane (Dos Santos et al, PNAS, 2024 (<https://doi.org/10.1073/pnas.2409672121>); Tomasek et al, Nature, 2020 (<https://doi.org/10.1038/s41586-020-2370-1>)).

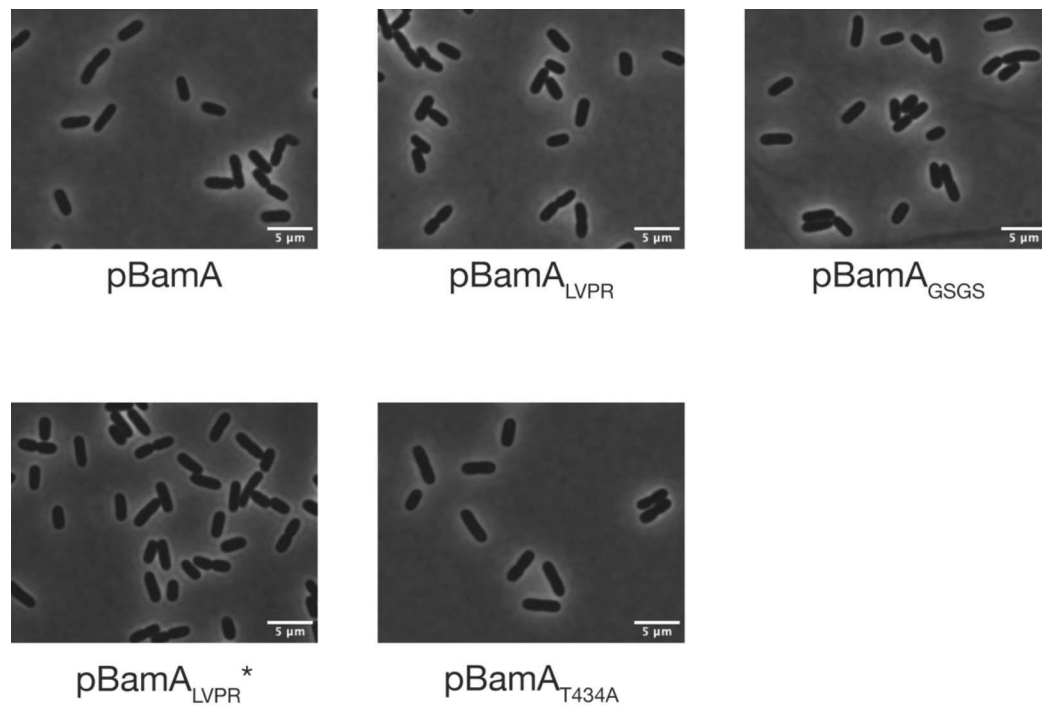


Figure 2: Cells harboring different variants of BamA exhibit similar cell morphology. *bamA::kan* cells expressing BamA variants from a plasmid were grown at 30 °C until mid-log phase. Two microliters of cell culture were spotted onto agar pads. Cells were imaged at 100× magnification using a Nikon Eclipse Ti2 microscope.

Reviewer #1 (Remarks to the Author):

Comment For Major point 3

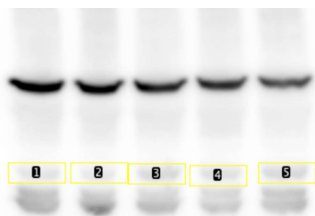
I understand the author's point about the major points well. No further discussion is necessary.

Comments for Minor points

We analyzed the bands in the gel data you provided here. Please see the attached figure. This time, we analyzed only the bands in the top left gel. We quantified and compared the green asterisk band and the two bands below it. The green asterisk bands do not appear to be different at first glance, but if lane 3 is 100%, lane 2 is only about 60%, and the evaluation of the results will be significantly different. In addition, if normalized with band 3 below, lane 4 is only about 46% if lane 2 is 100%, and this result does not guarantee that equal amounts of sample were run. In addition, there is no guarantee that this non-specific band is derived from the E. coli sample. Therefore, it cannot be denied that it is derived from contamination contained in the SDS-PAGE buffer in the author's lab, and this cannot be used to compare samples.

We appreciate the reviewer’s effort to reanalyze the gel data and acknowledge their concern regarding sample loading and interpretation of Western blot signals. However, we respectfully disagree with the implication that the differences observed in our data could be artefactual or derived from contamination.

First, we note that band quantification based on an overlay image—combining the chemiluminescence signal with the colorimetric molecular weight marker—is not appropriate. Such composite images are useful for presentation but not reliable for densitometric analysis, as they often involve signal compression. Chemiluminescence-only images, which allow accurate quantification, are provided in the Source Data file. Using ImageJ, we quantified the bands indicated by the reviewer (green asterisk on the BamA blot) and obtained the following values (see Table below). As shown, the small variations between lanes do not alter the interpretation of the data.



Lane	Quantification	Normalised
1-WT	39348.986	0.94
2-T434A	39457.451	0.94
3-GSGS	38864.744	0.93
4-LVPR*	40290.451	0.96
5-LVPR	41889.329	1

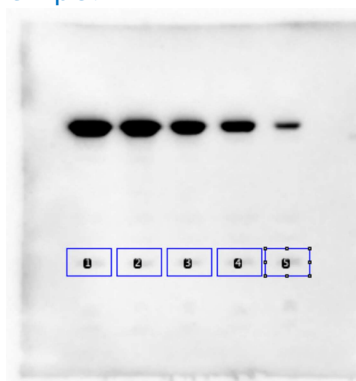
We also quantified the aspecific bands on the blots probed with the anti-OmpA and the anti-OmpC antibodies (see Tables below).

OmpA:



Lane	Quantification	Normalised
1-WT	32443.986	1
2-T434A	31406.865	0.97
3-GSGS	30413.108	0.94
4-LVPR*	29784.279	0.92
5-LVPR	30119.693	0.92

OmpC:



Lane	Quantification	Normalised
1-WT	6771.439	0.79
2-T434A	6516.924	0.76
3-GSGS	6981.167	0.81
4-LVPR*	8537.217	0.99
5-LVPR	8609.217	1

We observe slightly more variation in the intensity of the aspecific band on the OmpC blot (range: 0.76–1.00). However, this does not affect the overall conclusions. The first two lanes (BamA_{WT} and BamA_{T434A}) show comparable OMP expression levels. BamA_{GSGS} (lane 3) has a milder effect than BamA_{LVPR} (lane 5), and BamA_{LVPR*} (lane 4) partially restores OMP expression compared to BamA_{LVPR}.

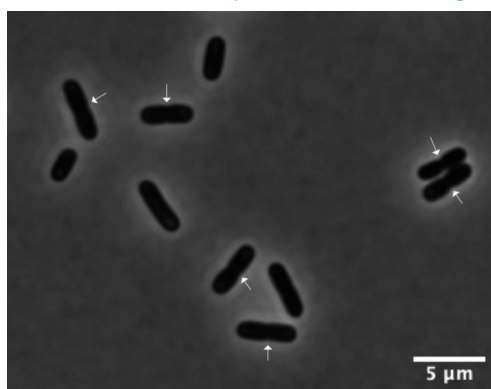
Finally, we disagree with the suggestion that the non-specific bands used for internal normalisation might result from buffer contamination. The fact that these bands differ across blots probed with different antibodies—despite being generated under similar buffer conditions—suggests that they result from cross-reactivity with endogenous bacterial proteins rather than from a persistent contaminant in the SDS-PAGE buffer. This supports the use of these bands as reliable internal references for normalisation.

We also note that Western blotting in this study was used as a complementary approach to support trends observed through independent and quantitative methods. Specifically, we provide quantitative MS data comparing BamA_{WT} and BamA_{LVPR} in minimal medium (Fig. 1e), as well as BamA_{WT} and BamA_{T434A} in LB (Supplementary Fig. 8). In both cases, the differences measured by MS are consistent with those observed in the Western blots (Supplementary Fig. 1a and Fig. 1c, respectively). Furthermore, these results align with additional phenotypic assays, including spot tests, disk diffusion assays, and σ^E activation measurements, strengthening the overall conclusions.

Next, we analyzed the data you provided for cell size (attached figure). We evaluated

cell elongation based on these cell lengths, cell areas, and aspect ratios. As the authors show, no significant differences in cell size were observed in GSGS and LVPR, which are the focus of this study. However, we report that a significant difference was observed in T434 and a small amount of variance was observed in LVPR*. If cell morphology is affected, intracellular proteins have a significant impact, so we need to pay attention to even small variances.

Based on your analysis, normalisation by OD₆₀₀ appears valid for strains expressing BamA_{WT}, BamA_{GSGS}, BamA_{LVPR}, and BamA_{LVPR*}. We note that the cells expressing BamA_{T434A} frequently exhibit a visible mid-cell constriction (see white arrow), indicating that many of them are in the process of division. This provides a straightforward explanation for the slight increase in average cell length observed in your analysis. Importantly, for the BamA_{T434A} strain, we also provide quantitative MS data, which independently confirms the trends observed by Western blotting.



It seems inevitable to present the amount of another E. coli protein in the same sample as a loading control. In particular, considering that some research misconduct has occurred recently from Western gels, shouldn't these be dealt with more carefully and appropriately?

We believe that the points detailed above fully justify the validity of our data and the appropriateness of their interpretation.

I would like to thank you for your guidance on "a widely accepted method in bacteriology," including papers, as I am unfamiliar with the field of microbiology. I have reviewed and studied these papers in detail.

First, Combs and Silhavy, PNAS, 2021, which came up first. In this paper, as the Combs say, when comparing the amount of Skp in Fig. 2C, the non-specific band above it (shown with an X in the figure) was used as a loading control. However, in Fig. 2A, OmpA is used as a loading control. Furthermore, in C, the SDS-PAGE of the same sample as in A is checked with a different antibody. Therefore, this X is not a single loading control, but I think that a loading control is already presented with OmpA. Is this the same as the widely accepted method in bacteriology that you mentioned, which is to compare samples with the same cell number without a loading control? I don't think so.

First, it is clearly stated in the Materials and Methods that sample loading was normalized based on OD₆₀₀ (see below):

[Redacted]

We agree with the reviewer that an aspecific band (X) is shown as a loading control in Figure 2C. However, we respectfully disagree with the interpretation that the samples in Figures 2A and 2C are identical. The authors state that the same assay conditions were used, but do not indicate that the samples themselves are the same. While a loading control is shown in both panels (OmpA in 2A and protein X in 2C), it is not used for normalisation when quantifying the LptD bands in Figures 2B and 2D. This further supports our point that normalisation by OD₆₀₀ is commonly used to compare protein levels across strains in the field.

[Redacted]

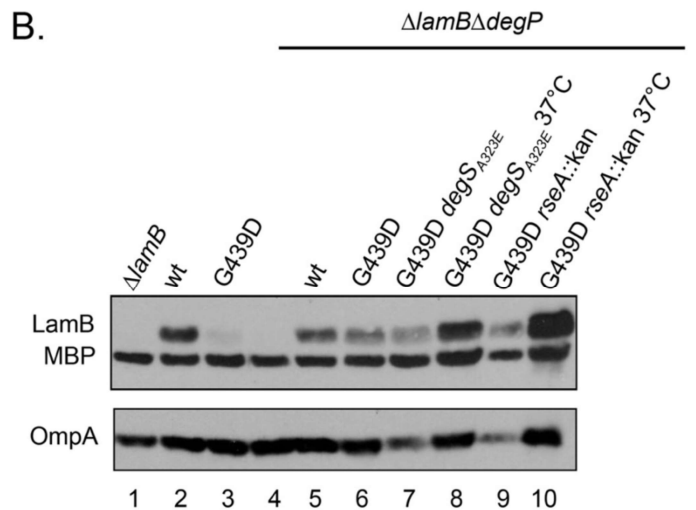
Grim et al, PNAS, 2020, has blotting data in Fig. 1F, and the amounts are indeed compared without a loading control. However, this is LPS, not a protein. Was it LPS that was being compared in this paper? LPS is an abbreviation for lipopolysaccharide, not a protein. This difference seems insufficient to prove a widely accepted method in bacteriology.

As stated in the figure legend (see below), samples were normalized by OD₆₀₀, and the bands were quantified without reference to a loading control. While we agree that LPS is not a protein, the detection method—SDS-PAGE followed by immunoblotting—is identical. If OD-based normalisation is acceptable for quantifying LPS levels on a blot, the same principle applies when the target is a protein.

[Redacted]

In Hart et al, J Bacteriol., 2019, MBP is presented as a loading control in Fig. 1A, Fig. 2A, B, Fig. 3B, and GroEL is presented as a loading control in Fig. 5A. Have I missed something?

There are no loading controls shown for the trimeric LamB blots. These samples were run on separate gels from those displaying monomeric LamB and MBP. According to accepted guidelines, a loading control is only meaningful when run on the same gel as the sample being analysed. In Figure 3B, MBP is indeed used as a loading control, but clear differences in signal intensity are still present between samples (e.g., lanes 8 and 10 compared to the others). Despite this variation, the authors proceed to compare LamB and OmpA expression levels. This further illustrates that minor differences in internal reference signals do not preclude comparative analysis, especially when overall trends are supported by multiple datasets—as it is the case in our study. Moreover, the Materials and Methods section clearly states that sample normalisation was performed based on OD_{600} .



Western blot analysis. Overnight cultures were normalized by optical density at 600 nm (OD_{600}). Samples were resuspended in the same volume of sample buffer containing β -mercaptoethanol (β -ME). For oxidized blots, the sample buffer lacked β -ME. Samples were boiled for 10 min and subjected to electrophoresis through an SDS-PAGE gel (10% for LamB blots and 8% for LptD blots). Proteins were transferred to a nitrocellulose membrane (GE Healthcare, Amersham). Immunoblotting was performed using rabbit polyclonal antisera that recognize LamB/OmpA/MBP (1:25,000), trimeric LamB (1:16,500), LptD (1:25,000), and GroEL (1:10,000). Donkey anti-rabbit IgG–horseradish peroxidase secondary antibody (GE Healthcare) was used at 1:10,000 dilution for all immunoblots.

Trimeric LamB sample preparation. Overnight cultures were normalized by OD_{600} . Cells were resuspended in Bugbuster (Millipore), protease cocktail inhibitor (1:100; Sigma-Aldrich), benzonase (1:100; Sigma-Aldrich), and 1 M $MgCl_2$ (1:100). Samples were lysed for 10 min at room temperature. Laemmli sample buffer (Bio-Rad) supplemented with β -mercaptoethanol was added to dilute the sample volume 1:2. Samples were electrophoresed and analyzed as described above.

In Dos Santos et al, PNAS, 2024, LptE is presented as a loading control in the lysate sample in Fig. 3E.

LptE is not an appropriate loading control in this context, as it forms a complex with LptD—the primary focus of the study—and its levels are likely influenced by the LptD mutations analysed. Moreover, no loading controls are presented in several key experiments, including the site-specific photocrosslinking assays (Figs. 2C and 2E) and the disulfide crosslinking and pulldown experiments (Figs. 4 and 5). As stated in the Materials and Methods, all samples were normalized based on OD_{600} , in line with standard practice.

In Tomasek et al, Nature, 2020, RpoA and LptF are presented as loading controls in Fig. 1c and e, respectively.

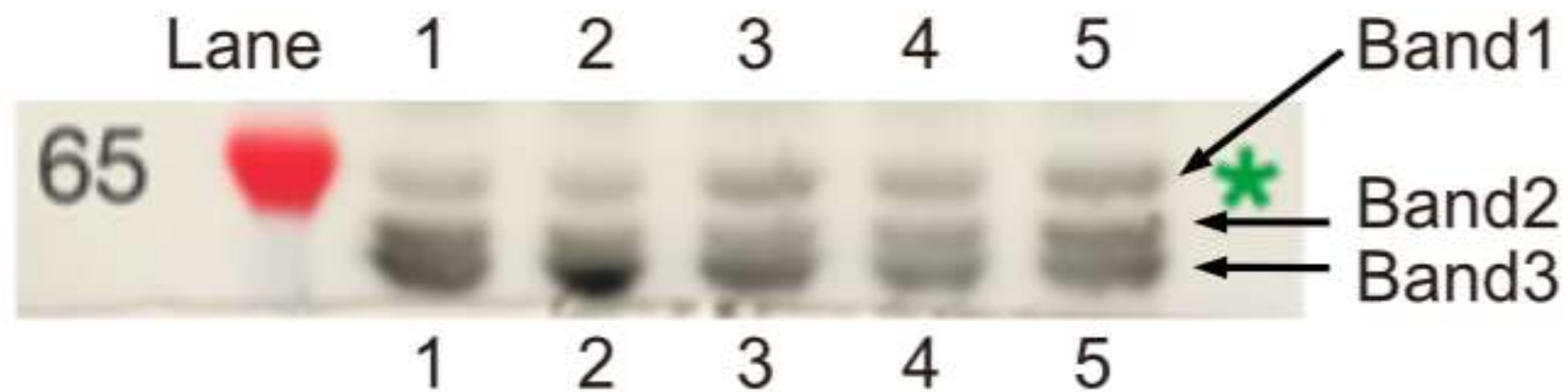
We agree with the reviewer that loading controls are shown in Figures 1c and 1e. However, for the site-specific photocrosslinking experiments (Figure 1f), the authors explicitly state in the Methods section that sample normalisation was based on OD_{600} . In addition, they present a BamA^M Western blot to confirm comparable protein levels across samples. This BamA^M construct is expressed from a plasmid, just as our various BamA variants are. The approach is therefore directly comparable to ours.

MC4100 *degP::cam* strains were generated, each containing pSup-BpaRS-6TRN(spec^R), pJL77 and one of the substrate-encoding plasmids. These strains were grown overnight in LB supplemented with 50 μ g/ml carbenicillin, 50 μ g/ml kanamycin, 50 μ g/ml spectinomycin and 0.2% (w/v) glucose (37 °C, 220 rpm). These overnight cultures were diluted 1:100 into 100 ml of fresh LB containing the same additives minus glucose but supplemented with 0.8 mM pBPA (Bachem), and were grown (37 °C, 220 rpm) to an OD_{600} of about 0.35. After normalization by optical density, each culture was split in half, with one half used directly for irradiation with UV light at 365 nm for 10 min (on ice). Photo-crosslinking was performed by using a UVP Blak-Ray B-100AP high-intensity UV lamp with a 100-W spot bulb. All cells were then pelleted by centrifugation (5,000g, 10 min, 4 °C).

As mentioned above, all of the papers you introduced had loading controls. However, there is no loading control in Fig. 1c of this paper. How is this a widely accepted method in bacteriology? Please explain in more detail to me, who does not understand. In addition, none of the papers you introduced involved cutting the gel between lanes, as in Fig. 1c.

In response to this comment, we have revised Figures 1 and 4 to avoid cutting the gel between lanes. The relevant panels have been merged into a single composite now shown in Figure 1c. In addition, we have included the results from three independent biological replicates—cells grown in separate test tubes and inoculated from individual colonies—to illustrate the reproducibility of the data (see Source Data file). We trust that this revised presentation addresses the reviewer’s concern and further reinforces the robustness of our findings.

We thank the reviewer for their detailed analysis and comments. We have revised the manuscript accordingly and provided clarifications where necessary.



	1	2	3	4	5
Band1	67.9	62.2	100	80.5	87.6
Band2	100	76.7	67.6	68.5	78.5
Band3	75.1	100	66.8	46.3	57.9

Comparison with the lane with the darkest band set to 100%

