THE EMBO JOURNAL

Role of endopeptidases in peptidoglycan synthesis mediated by alternative cross-linking enzymes in Escherichia coli

Henri Voedts, Delphine Dorchêne, Adam Lodge, Waldemar Vollmer, Michel Arthur, and Jean-Emmanuel HUGONNET
DOI: 10.15252/embj.2021108126

Corresponding author: Jean-Emmanuel HUGONNET (jean-emmanuel.hugonnet@crc.jussieu.fr)

Review Timeline:

Submission Date:	26th Feb 21
Editorial Decision:	12th Apr 21
Revision Received:	8th Jun 21
Editorial Decision:	15th Jul 21
Revision Received:	22nd Jul 21
Accepted:	24th Jul 21

Editor: Ieva Gailite / David del Alamo

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 12th Apr 2021

Dear Dr. Hugonnet,

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received three referee reports on your manuscript, which are included below for your information.

As you will see from the comments, all reviewers appreciate the work and the quality of the data. However, they also indicate a number of issues that would have to be addressed and clarified in the revised manuscript. Based on these positive evaluations, I would like to invite you to submit a revised version of your manuscript in response to reviewers' comments.

I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic, and I would be happy to discuss the revision in more detail via email or phone/videoconferencing.

We have extended our 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. This means that competing manuscripts published during revision period will not negatively impact on our assessment of the conceptual advance presented by your study. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess. Please also see the attached instructions for further guidelines on preparation of the revised manuscript.

Please feel free to contact me if you have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to receiving the revised manuscript.

Best regards,

Ieva Gailite

Ieva Gailite, PhD
Scientific Editor
The EMBO Journal
Meyerhofstrasse 1
D-69117 Heidelberg
Tel: +4962218891309
i.gailite@embojournal.org

When submitting your revised manuscript, please carefully review the instructions below and include the following items:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures

and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. Please note that the Data Availability Section is restricted to new primary data that are part of this study. If no data deposition in external databases is needed for this paper, please then state in this section: This study includes no data deposited in external repositories.
*** Note - All links should resolve to a page where the data can be accessed. ***

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

9) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure

S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labelled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

10) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors:
https://www.embopress.org/page/journal/14602075/authorguide

Please click on the link below to submit the revision online. The resubmission deadline is currently set to 11th Jul 2021. Please contact us if you would need an extension.

Link Not Available

Referee #1:

The manuscript by Voedts et al. presents a thorough genetic analysis of an E. coli mutant that has gained resistance against beta lactam antibiotics by switching from beta lactam sensitive 4,4 to resistant 3,3 crosslinks. The authors identify the basic repertoire of endopeptidases required for growth in this strain and report the surprising finding that annotated D, D endopeptidases can also exhibit activity on L,D crosslinks. As such, this is an important contribution to the field and illuminates the biology of 3,3 crosslinks, which is as of yet poorly-understood. The experiments are overall well-designed and carefully controlled. I have some concerns about data presentation. Please see below for detailed comments.

Major points

I am confused about MepK vs. MepM. It seems that MepK is required for growth of the 3,3 strain (which is expected), while MepM is exclusively required for beta lactam resistance in this background (unless it is the only EP remaining, in which case it is required for growth). This is not specifically discussed, but suggests that despite both EPs' ability to cleave 3,3 crosslinks, they function differently. Have they ever looked at these 3,3 mutants cells growing with vs. without beta lactam antibiotics? Morphology might give clues as to the mode of action of these endopeptidases. At the very least, the relationship between MepM and MepK should be discussed in more detail and perhaps incorporated into their model.

Line 302 - maybe there is something fundamental I am missing here, but is the observation that mepM overexpression is toxic in the presence of ceftriaxone (Fig. 6) not a direct contradiction to Fig. 4, where they show that mepM overexpression is required for cef resistance?

Lines 534 - 535 - I do not see any data supporting this claim (might be a missing figure reference?).

I have some concerns about Fig. 11B and interpretation. For example, line 540 states "YcbB induction [...] was dispensable for beta lactam resistance", while the data simply show that YcbB overexpression might not confer resistance in this background (not the same as "dispensable"). This zone of inhibition experiment should be repeated and an average zone diameter given (the zone looks smaller in the +IPTG image, so perhaps YcbB does confer some resistance here? Quantification would allow you to make a statement about this). Another (minor) issue: Why was a ΔrelA strain used for the lower panel? Also, line 540 makes reference to a "wt and its ΔsltY derivative" but Fig. 11B only shows data on ΔsltY backgrounds.

It is not clear how L,D TPs relate to the aPBPs/Rod system, respectively. Have they done experiments using specific inhibitors of PBP2,3, or aPBPs? Is the 3,3 strain resistant against mecillinam, aztreonam and/or cefsulodin? To be clear - this is not an experiment required to support their conclusions, but it is an easy one that would add a lot of information.

Minor points

Line 29 - substituted is the wrong word. "outfitted"? "modified"?

Fig. S1 - Only one possible model is presented. Another possibility is that an entire strand of old PG is removed during this process. Since this is an open question in the field, better be agnostic.

Line 103 - I know what they mean, but "polymerization" typically suggests glycosyltransferase activity, not crosslinking. Maybe just change to "crosslinking"?

Lines 305 - 344 would benefit from not being broken up into subheadings. One of the headings only has two sentences (316 - 318), it would read easier if this was all subsumed under one heading.

There should be at least some discussion as to why beta lactam resistance differs between liquid broth and solid medium.

Line 528 - why was ampicillin used here while all other experiments were done with ceftriaxone? Did they try to select for ceftriaxone-resistant mutants but failed? If so, this would be informative, especially if there is any published information as to the differential PBP inhibitory activity of these different beta lactams.

Line 565 - I sort of agree with this statement ("beyond any required experimental demonstration"), but still feel it should be softened. In the absence of any direct evidence, no matter how logical or intuitive an explanation might be, one should tread a little more lightly, e.g. "it seems clear that insertion of new...".

Line 626 - I am not sure this statement is true. Whether or not aPBPs can crosslink external strands is not well-established.

Line 630 - should be "repair", not "reparation"

Referee #2:

Voedts et al. examined the roles of endopeptidases in E. coli growth involving Dap-Dap crosslinks. Multiple knockouts and overexpression strains were used to identify essential endopeptidases under these conditions, and biochemical studies revealed the specificities of the endopeptidases for different crosslinked dimers. The manuscript is tour de force on E. coli endopeptidases.

Major points.
1. Thanks to the authors for a well-written manuscript.
2. There are no major deficiencies in the manuscript.

Minor points.
1. Fig. 5. Add number labels in the lower part of Fig. 5A (as in the upper part) to help the reader identify the diminished peaks.
2. Table 1. How many times were these experiments repeated? Are differences between strains significant?
3. The manuscript is a bit E. coli - centric. To broaden the significance a bit, the authors should mention studies where endopeptidases were found to be important in infections, such as those required for cell shape in H. pylori, NOD agonist release in N. gonorrhoeae, or persistence in M. tuberculosis.

Referee #3:

Voedts and colleagues present evidence for the essential role of different endopeptidases to sustain E. coli growth when its cell wall machinery is driven exclusively by LD-transpeptidases instead of the more classical DD-transpeptidases. In general, the work is well done and the genetic studies are well designed and cleaver.
However, there is major caveat to the work. Evidence for the different essential roles of endopeptidases comes from genetic complementation experiments without validation that proteins are being produced. So, when there is complementation, it is clear that the given endopeptidase has to be produced in significant levels to support growth. However, lack of complementation is not evidencing that a given endopeptidase is not able to perform its function without further proof that the corresponding protein is actually produced. Hence, the authors conclusions are valid only under the conditions tested and not a general conclusion for each endopeptidase tested. Consequently, the importance of the work is undermined by this major limitation. The message would be much stronger if the authors could provide evidence for protein production and stability in their complementation assays.
Otherwise, the work is technically valid and I have no criticism on the presented data.

Response to the referees' comments

Referee #1:

The manuscript by Voedts *et al*. presents a thorough genetic analysis of an E. coli mutant that has gained resistance against beta lactam antibiotics by switching from beta lactam sensitive 4,4 to resistant 3,3 crosslinks. The authors identify the basic repertoire of endopeptidases required for growth in this strain and report the surprising finding that annotated D, D endopeptidases can also exhibit activity on L,D crosslinks. As such, this is an important contribution to the field and illuminates the biology of 3,3 crosslinks, which is as of yet poorly-understood. The experiments are overall well-designed and carefully controlled. I have some concerns about data presentation. Please see below for detailed comments.

Answer

We thank the reviewer for his positive comments about the importance of our contribution and the design of our experiments. Below we address the reviewer's concern.

Major points

Referee 1, comment 1.

I am confused about MepK vs. MepM. It seems that MepK is required for growth of the 3,3 strain (which is expected), while MepM is exclusively required for beta lactam resistance in this background (unless it is the only EP remaining, in which case it is required for growth). This is not specifically discussed, but suggests that despite both EPs' ability to cleave 3,3 crosslinks, they function differently. Have they ever looked at these 3,3 mutants cells growing with vs. without beta lactam antibiotics? Morphology might give clues as to the mode of action of these endopeptidases. At the very least, the relationship between MepM and MepK should be discussed in more detail and perhaps incorporated into their model.

Answer to comment 1.

The referee has correctly summarized our conclusions on the role of MepM and MepK. As recommended we have more clearly described these roles and incorporated MepK in our model. Changes are as follow:

(i) Fig. 12. We have added MepK to the biosynthetic scheme and two sentences to the legend (lines 958 to 960 of the revised manuscript): “Production of YcbB leads to a requirement for an additional endopeptidase, MepK. This function of MepK can be bypassed by overproduction of MepS.”

(ii) A concluding statement inspired by the comment of the referee has been added (lines 439 to 443 of the revised manuscript): “In conclusion, MepK is specifically required for growth if the peptidoglycan contains a large proportion of 3→3 cross-links, while MepM is additionally required for β-lactam resistance, unless it is the only endopeptidase remaining, in which case it is also required for growth in the absence of the drug.”.

Regarding the 2nd part of the comment, time-lapse microscopy was used to monitor cell morphology defects associated with induction of *ycbB* in a Δ*mepK* background. BW25113 Δ*mepK* pHV63(*ycbB*) was grown in BHI broth supplemented with 20 µg/ml chloramphenicol to late exponential phase (OD_{600} = 2.0), diluted a 100 fold in BHI broth, and a sample was deposited at the surface of a BHI agarose pad containing 20 µg/ml chloramphenicol and 1% L-arabinose to induce *ycbB*. Bacteria were imaged using a Nikon TI microscope equipped with a 100x plan apo objective and an incubation chamber. Images were taken at 37° every 5 min for 3h. Production of YcbB in the absence of MepK was found to lead to an increase in the size of the bacteria, affecting both the diameter and the length of the cells, and to an apparent thickening of the cell wall. Lysis occurred in more than 90% of the cells at the end of the experiment. Cells were frequently bursting with formation of bulges prior to lysis. Thus, induction of *ycbB* in the absence of MepK did not specifically block formation of the side walls or septum, which would be expected to lead to growth as spheres or filaments, respectively. Rather, the peptidoglycan was synthesized but did not fulfill its osmoprotective role. Bulges were frequently located at the septum and at midcell suggesting that preseptal peptidoglycan was preferentially affected. Growth of BW25113(*ycbB, relA'*) in the presence of ceftriaxone was not observed in the agarose pad conditions perhaps due to a low oxygen pressure. It was therefore not possible to analyze the impact of the *mepM* deletion in the presence of the drug. These data are preliminary, and we prefer not including them in the main manuscript. We acknowledge Arnaud Gutierrez for his help in the time-lapse microscopy analysis of the BW25113 Δ*mepK* pHV63(*ycbB*).

Figure for reviewers removed

Referee 1, comment 2.

Line 302 - maybe there is something fundamental I am missing here, but is the observation that mepM overexpression is toxic in the presence of ceftriaxone (Fig. 6) not a direct contradiction to Fig. 4, where they show that mepM overexpression is required for cef resistance?

Answer to comment 2.

We are sorry for the confusion and have now clarified this point. This apparent contradiction comes from overproduction of MepM using different translation initiation signals (TIS1 or TIS2). By combining an ATG initiation codon and an RBS with extensive complementarity to 16S rRNA, TIS1 causes a high production of MepM which is toxic. For TIS2, the production level of MepM is expected to be lower because TIS1 combines a TTG initiation codon to limited complementarity to 16S rRNA (these translation initiation signals are described in detail in lines 171 to 176 of the revised manuscript).

Since this point was not clear in the first version of the manuscript we introduce the following modifications in the revised version:

(i) In the legend to Fig 6, we indicated that the endopeptidases were produced under the control of TIS1 (line 891 of the revised manuscript).

(ii) A sentence was added to clarify the basis for the toxicity of MepM (lines 235 to 238 of the revised manuscript): "Importantly, MepM toxicity was only observed when its production was under the control of the effective TIS1 translation initiation signal, but not of the weaker TIS2 signal used for the experiment described in Fig. 4."

Referee 1, comment 3.

Lines 534 - 535 - I do not see any data supporting this claim (might be a missing figure reference?).

Answer to comment 3.

The growth curves for the Δ*sltY* strain and the control parental strain have been included in the revised version of Fig. 11A (reference to Fig. 11A has been included line 384 of the revised manuscript, and legend to Fig. 11A has been modified lines 943 to 946 of the revised manuscript).

Referee 1, comment 4.

I have some concerns about Fig. 11B and interpretation. For example, line 540 states "YcbB induction [...] was dispensable for beta lactam resistance", while the data simply show that YcbB overexpression might not confer resistance in this background (not the same as "dispensable"). This zone of inhibition experiment should be repeated and an average zone diameter given (the zone looks smaller in the +IPTG image, so perhaps YcbB does confer some resistance here? Quantification would allow you to make a statement about this). Another (minor) issue: Why was a ΔrelA strain used for the lower panel? Also, line 540

makes reference to a "wt and its ΔsltY derivative" but Fig. 11B only shows data on ΔsltY backgrounds.

Answer to comment 4.

We agree that Fig. 11B was not complete and that the controls supporting the conclusions were not explicitly described. The following modifications were added:

(i) We revised the end of the Results section to clarify the resistance phenotypes (lines 385 to 393 and 399 to 406 of the revised manuscript).

(ii) We added a Table (Table 3) showing the diameter of the inhibition zones for the full sets of strains and inducing conditions (lines 978 to 985 of the revised manuscript).

(iii) We performed three biological repeats of the disk diffusion assay and provided median values.

(iv) We explored the basis for the small decrease in the diameter of the inhibition zone around the disk containing ceftriaxone upon induction of *ycbB* only in BW25113(*ycbB*, *relA'*) Δ*sltY* (Table 3; last paragraph of Results section of the revised version). These additional results show that induction of YcbB conveys a moderate level of resistance to ceftriaxone in the absence of relA'.

(v) There was an ambiguity in the nomenclature of the strain due to the use of the BW25113 (*ycbB*, *relA*') abbreviation. The full designation of this strain, BW25113 Δ*relA* pKT2(*ycbB*) pKT8(*relA'*), was introduced into panel B of Fig. 11 to make clear that this strain harbors the Δ*relA* mutation.

(vi) Regarding the significance of differences, we have repeated the determination of the inhibition zones using the same batches of culture medium and of disks containing the antibiotics to minimize variations. We inserted a modified version of Table 3 (below, for review only) to provide the ranges of diameter values in order to assess the variability of the technique. Significant differences discussed in the text relate to a resistant *versus* susceptible phenotype leading to differences between the diameters of the inhibition zones ≥ 9 mm for all individual values obtained for the same strain. An intermediate phenotype was observed for one strain and one antibiotic, namely BW25113 Δ*relA* Δ*sltY* pKT2(*ycbB*) pKT8(*relA'*) in the presence of IPTG. This result indicates that induction of *ycbB* in the absence of *relA*' induction decreases susceptibility to ceftriaxone in the Δ*sltY* background. The *relA'* gene was fully dispensable for the intermediate phenotype since BW25113 Δ*relA* Δ*sltY* harboring pKT2(*ycbB*) and pHV7 [the vector used to construct pKT8(*relA'*)] was as resistant as BW25113 Δ*relA* Δ*sltY* pKT2(*ycbB*) pKT8(*relA'*) to ceftriaxone in conditions of induction of *ycbB* by IPTG (25 mm *versus* 26 mm).

Modified Table 3, for review only. YcbB-mediated β-lactam resistance in BW25113(*ycbB*, *relA'*) and its derivative obtained by deletion of *sltY*.

Host / Plasmid	Inducer[b]	Inhibition zones (mm)[a]		
		Mec	**Amp**	**Cro**
Susceptible (range of medians)		(20-25)	(16-22)	(32-36)
Resistant (range of medians)		(<8-9)	(<8-<8)	(9-15)
Intermediary		NA	NA	(25-26)
BW25113 Δ*relA*				
pKT2(*ycbB*) pKT8(*relA'*)	None	20 (20-22)	18 (18-19)	34 (34-35)
	IPTG (*ycbB*)	21 (19-21)	16 (16-18)	32 (30-32)
	Ara (*relA'*)	9 (9-10)	18 (17-18)	35 (35-35)
	IPTG + Ara	< 8 (<8-<8)	< 8 (<8-<8)	15 (15-16)
BW25113 Δ*relA* Δ*sltY*				
pKT2(*ycbB*) pKT8(*relA'*)	None	21 (21-22)	21 (21-22)	33 (33-33)
	IPTG (*ycbB*)	22 (22-22)	18 (18-18)	26 (26-26)
	Ara (*relA'*)	< 8 (<8-<8)	< 8 (<8-<8)	9 (9-10)
	IPTG + Ara	< 8 (<8-<8)	< 8 (<8-<8)	12 (11-12)
BW25113 Δ*relA* Δ*sltY*				
pHV6[c] pKT8(*relA'*)	None	20 (20-22)	22 (20-23)	34 (33-35)
	IPTG	22 (21-22)	22 (21-22)	34 (33-34)
	Ara (*relA'*)	< 8 (<8-9)	21 (21-22)	36 (36-37)
	IPTG + Ara	< 8 (<8-10)	20 (20-20)	35 (34-36)
BW25113 Δ*relA* Δ*sltY*				
pKT2(*ycbB*) pHV7[d]	None	23 (22-25)	21 (21-22)	34 (33-35)
	IPTG (*ycbB*)	25 (25-26)	18 (17-18)	25 (25-26)
	Ara	25 (25-25)	21 (20-22)	34 (33-35)
	IPTG + Ara	24 (24-25)	17 (17-18)	26 (25-26)

[a] The diameter of inhibition zones was determined by the disk diffusion assay around disks containing 10 µg mecillinam (Mec), 10 µg ampicillin (Amp), or 30 µg ceftriaxone (Cro). Data are the medians from three experiments.

[b] The *ycbB* and *relA'* genes carried by plasmid pKT2 and pKT8 were induced with 40 µM IPTG and 1% L-arabinose (Ara), respectively.

[c] pHV6 is the vector used for construction of pKT2(*ycbB*).

[d] pHV7 is the vector used for construction of pKT8(*relA'*).

NA, not applicable

Referee 1, comment 5.

It is not clear how L,D TPs relate to the aPBPs/Rod system, respectively. Have they done experiments using specific inhibitors of PBP2,3, or aPBPs? Is the 3,3 strain resistant against mecillinam, aztreonam and/or cefsulodin? To be clear - this is not an experiment required to support their conclusions, but it is an easy one that would add a lot of information.

Answer to comment 5.

These aspects have been specifically addressed in our previous publication (Hugonnet *et al*. eLife 2016;5:e19469 DOI: 10.7554/eLife.19469). In this previous analysis, we generated deletions of the *mrcA* (encoding PBP1a) or *mrcB* (PBP1b) genes (and of genes encoding the associated LpoA and LpoB lipoproteins) and showed that *mrcB* (and *lpoB*) but not *mrcA* (and *lpoA*) were required for resistance. We also generated a gene encoding a transpeptidase-defective derivative of PBP1b by replacing the catalytic Ser by Ala. These analyses showed that the glycosyltransferase activity of PBP1b, but not its transpeptidase activity, was required for resistance. We preferred to use this approach rather than testing PBP-specific β-lactam inhibitors since these inhibitors may indirectly interfere with the L,D-transpeptidation pathway by inhibiting the D,D-carboxypeptidases involved in the supply of the tetrapeptide donor substrate of the YcbB L,D-transpeptidase. In parallel, we also showed in Hugonnet *et al*. that activation of the L,D-transpeptidation pathway results in high-level resistance to the PBP3-specific inhibitor aztreonam and to the PBP2-specfic inhibitor mecillinam. Thus, these drugs cannot be used to study the impact of inactivation of the transpeptidase domain of specific PBPs since the D,D-transpeptidase activity of PBPs do not contribute to peptidoglycan cross-linking in BW25113(*ycbB*, *relA′*) in the presence of the drugs.

In the current manuscript, Fig. EV3 provides the resistance phenotypes to cefsulodin and aztreonam, which specifically inactivate class A PBPs (preferentially PBP1a) and class B PBP3, respectively. We have indicated in the legend to this figure that bypass of the transpeptidase activity of PBPs by that of YcbB was previously shown to lead to high-level resistance to ampicillin, cefsulodin, and aztreonam and cited the appropriate reference.

A sentence was added to the revised version of the manuscript to draw the attention of the reader on our previous publication (lines 1019 to 1022 of the revised manuscript):

"It was previously shown that ampicillin, cefsulodin, and aztreonam do not inhibit peptidoglycan cross-linking in BW25113(*ycbB*, *relA′*) due to full bypass of the D,D-transpeptidase activity of PBPs by the L,D-transpeptidase activity of YcbB, which is not inactivated by these drugs (Hugonnet et al. 2016)."

Regarding mecillinam resistance, the information was provided lines 496 to 498 of the first version of the manuscript, *i.e*. induction of *relA′* is sufficient for resistance to this drug (now lines 354 to 356 of the revised manuscript).

Minor points

1. Line 29 - substituted is the wrong word. "outfitted"? "modified"?

Answer: The sentence has been rephrased (lines 33 to 34 of the revised manuscript): “PG is assembled from a disaccharide-peptide subunit consisting of *N*-acetylglucosamine (GlcNAc), *N*-acetylmuramic acid (MurNAc), and a stem pentapeptide linked to MurNAc”

2. Fig. S1 - Only one possible model is presented. Another possibility is that an entire strand of old PG is removed during this process. Since this is an open question in the field, better be agnostic.

Answer: There is a consensus that the model presented in panel Fig. EV1 applies to the incorporation of PG into the side walls (our own unpublished data and De Jonge *et al*. J Bacteriol. 1989 Nov;171(11):5783-94. DOI: 10.1128/jb.171.11.5783-5794.1989). We have added this reference to the legend that support this conclusion. We agree that other models may apply, in particular for the septum. However, comparing models is beyond the scope of the current study as all models predict that endopeptidases are required. Two sentences were added at the end of the legend to supplementary Fig. EV1 to mention the diversity of the peptidoglycan synthesis models (lines 1002 to 1004 of the revised manuscript): “(De Jonge et al., 1989). There are other models in which whole glycan strands are removed (J. V. Höltje and Heidrich, 2001). All models predict that amide bonds should be cleaved for extension of the peptidoglycan network.”

3. Line 103 - I know what they mean, but "polymerization" typically suggests glycosyltransferase activity, not crosslinking. Maybe just change to "crosslinking"?

Answer: The text was modified as suggested (line 97 of the revised manuscript).

4. Lines 305 - 344 would benefit from not being broken up into subheadings. One of the headings only has two sentences (316 - 318), it would read easier if this was all subsumed under one heading.

Answer: The section presenting data on the complementation of Δ*mepM* by the eight endopeptidase genes extend beyond lines 305 to 344 of the first version of the manuscript. We believe that the subheadings are critical for the clarity of the text and would like to maintain the current presentation.

5. There should be at least some discussion as to why beta lactam resistance differs between liquid broth and solid medium.

Answer: We do not have any insights regarding the difference observed between the broth and agar BHI media. There are multiple parameters differing between the two conditions including the oxygen availability and osmotic pressure, which is ill defined within a colony. We think that speculations on the basis of the difference between the two growth media

would make the text more cumbersome without providing any valuable hypothesis. We would therefore like to maintain the first version regarding this matter.

6. Line 528 - why was ampicillin used here while all other experiments were done with ceftriaxone? Did they try to select for ceftriaxone-resistant mutants but failed? If so, this would be informative, especially if there is any published information as to the differential PBP inhibitory activity of these different beta lactams.

Answer: We have tested many mutants from the previous (Hugonnet *et al*. 2016) and current studies, and unpublished work, and have never observed a difference in resistance phenotype to ampicillin and ceftriaxone. Either the strains were sensitive to both β-lactams or resistant to both. This is not surprising because (i) ampicillin and ceftriaxone inhibit all PBPs except PBP5/6 (Kocaoglu & Carlson, 2015 May;59(5):2785-90 DOI: 10.1128/AAC.04552-14) and (ii) neither ampicillin nor ceftriaxone effectively inhibit L,D-transpeptidases (Triboulet *et al*. PLoS One. 2013 Jul 4;8(7):e67831 DOI: 10.1371/journal.pone.0067831). Historically, we started to use ampicillin as a selector. We then shifted to cephalosporin since we were initially concerned that (partial) resistance could result from activation of the chromosomal *ampC* gene, which encodes a β-lactamase potentially hydrolyzing ampicillin but not ceftriaxone. Given that all strains tested showed no difference in response to ampicillin or ceftriaxone, we can exclude that AmpC affects our results. Hence, there is no benefit in testing the susceptibility of all strains to both β-lactams.

7. Line 565 - I sort of agree with this statement ("beyond any required experimental demonstration"), but still feel it should be softened. In the absence of any direct evidence, no matter how logical or intuitive an explanation might be, one should tread a little more lightly, e.g. "it seems clear that insertion of new...".

Answer: The text has been modified as suggested (line 412 of the revised manuscript).

8. Line 626 - I am not sure this statement is true. Whether or not aPBPs can crosslink external strands is not well-established.

Answer: Our conclusion was based on the difference in the requirement of YcbB and PBPs for tetrapeptide and pentapeptide donor substrates, respectively. We agree that the general statement appearing line 626 of the first version of the manuscript was not fully justified. We have rephrased the corresponding section for improving clarity and providing a more specific statement (lines 473 to 478 of the revised manuscript): "In contrast, the D,D-transpeptidase activity of PBPs exclusively requires pentapeptide-containing donors. Analyses of peptidoglycan structure indicate that D-Ala^5 is rapidly cleaved off from pentapeptide stems by D,D-carboxypeptidases if they are not used for formation of 4→3 cross-links (Glauner *et al*, 1988). These observations suggest that the transpeptidase activity of PBPs exclusively relies on *de novo* synthesis and translocation of pentapeptide-containing subunits."

9. Line 630 - should be "repair", not "reparation"

Answer: The text has been modified as suggested (line 480 of the revised manuscript).

Referee #2:

Voedts *et al*. examined the roles of endopeptidases in *E. coli* growth involving Dap-Dap crosslinks. Multiple knockouts and overexpression strains were used to identify essential endopeptidases under these conditions, and biochemical studies revealed the specificities of the endopeptidases for different cross-linked dimers. The manuscript is tour de force on *E. coli* endopeptidases.

Major points.

1. Thanks to the authors for a well-written manuscript.
2. There are no major deficiencies in the manuscript.

Answer:

We thank the reviewer for their positive comment about our work on the endopeptidases and the manuscript.

Minor points.

1. Fig. 5. Add number labels in the lower part of Fig. 5A (as in the upper part) to help the reader identify the diminished peaks.

Answer: As requested, labels have been added to the peaks of the lower chromatogram.

2. Table 1. How many times were these experiments repeated? Are differences between strains significant?

Answer: We have repeated the determination of the inhibition zones using the same batches of culture medium and of disks containing the antibiotics to minimize variations. We have indicated in footnote a to Table 1 that data are the medians from three experiments (line 968 of the revised manuscript). Significant differences discussed in the text relate to a resistant *versus* susceptible phenotype leading to difference in the diameter of the inhibition zones ≥ 9 mm for all individual values obtained for the same strain. We inserted a modified version of Table 1 (below, for review only), which includes the range of values to substantiate this claim. This analysis also shows that there is no overlap between the sizes of the inhibition zones assigned to the susceptible and resistant phenotypes. This justifies the assignment of the phenotypes into two classes only.

Modified Table 1, for review only. YcbB-mediated β-lactam resistance in BW25113 derivatives harboring all endopeptidase genes or only *mepM* and *mepK* (Δ6EDs)

Host		Inhibition zones (mm)[a]		
Plasmid	Inducer[b]	Mec	Amp	Cro
Susceptible (range of medians)		(19-22)	(16-22)	(32-37)
Resistant (range of medians)		(<8-9)	(<8-10)	(15-18)
BW25113 Δ*relA*				
pKT2(*ycbB*) pKT8(*relA'*)	None	20 (20-22)	18 (18-19)	34 (34-35)
	IPTG (*ycbB*)	21 (19-21)	16 (16-18)	32 (30-32)
	Ara (*relA'*)	9 (9-10)	18 (17-18)	35 (35-35)
	IPTG + Ara	< 8 (<8-<8)	< 8 (<8-<8)	15 (15-16)
BW25113 Δ*relA* Δ6EDs				
pKT2(*ycbB*) pKT8(*relA'*)	None	19 (19-22)	22 (21-22)	37 (37-38)
	IPTG (*ycbB*)	20 (19-24)	19 (19-20)	35 (35-36)
	Ara (*relA'*)	< 8 (<8-<8)	10 (10-10)	18 (18-19)
	IPTG + Ara	< 8 (<8-<8)	< 8 (<8-<8)	16 (15-16)
BW25113 Δ*relA* Δ6EDs				
pHV6[c] pKT8(*relA'*)	None	22 (21-23)	18 (18-19)	35 (34-35)
	IPTG	22 (22-23)	18 (18-18)	35 (34-35)
	Ara (*relA'*)	10 (9-10)	18 (18-18)	37 (37-37)
	IPTG + Ara	9 (9-10)	17 (17-18)	37 (36-37)

[a] The diameter of inhibition zones was determined by the disk diffusion assay around disks containing 10 µg mecillinam (Mec), 10 µg ampicillin (Amp), or 30 µg ceftriaxone (Cro). Data are the medians from three experiments. Examples of the original results are presented in supplementary Fig. EV4. Ranges are indicated in brackets.
[b] The *ycbB* and *relA'* genes carried by plasmid pKT2 and pKT8 were induced with 40 µM IPTG and 1% L-arabinose (Ara), respectively.
[c] pHV6 is the vector used for construction of pKT2(*ycbB*).

3. The manuscript is a bit *E. coli* - centric. To broaden the significance a bit, the authors should mention studies where endopeptidases were found to be important in infections, such as those required for cell shape in *H. pylori*, NOD agonist release in *N. gonorrhoeae*, or persistence in *M. tuberculosis*.

Answer: As suggested by the reviewer, we added a sentence in the introduction to introduce known roles of endopeptidases in bacteria other than *E. coli* (lines 85 to 88 of the revised

manuscript): "Various roles have been assigned to endopeptidases in bacteria other than *E. coli* such as the determination of cell shape in *Helicobacter pylori* (Bonis et al., 2010), release of NOD agonist in *Neisseria gonorrhoeae* (Lenz et al., 2017) or persistence in *Mycobacterium tuberculosis* (Healy et al., 2020)."

Referee #3:

Voedts and colleagues present evidence for the essential role of different endopeptidases to sustain *E. coli* growth when its cell wall machinery is driven exclusively by LD-transpeptidases instead of the more classical DD-transpeptidases. In general, the work is well done and the genetic studies are well designed and cleaver.

Answer:

We appreciate the positive comments by the reviewer about our work.

However, there is major caveat to the work. Evidence for the different essential roles of endopeptidases comes from genetic complementation experiments without validation that proteins are being produced. So, when there is complementation, it is clear that the given endopeptidase has to be produced in significant levels to support growth. However, lack of complementation is not evidencing that a given endopeptidase is not able to perform its function without further proof that the corresponding protein is actually produced. Hence, the authors conclusions are valid only under the conditions tested and not a general conclusion for each endopeptidase tested. Consequently, the importance of the work is undermined by this major limitation. The message would be much stronger if the authors could provide evidence for protein production and stability in their complementation assays. Otherwise, the work is technically valid and I have no criticism on the presented data.

Answer:

The plasmid constructs used for complementation provided positive results for MepA (Fig. 9B and 10A), MepH (Fig. 6 and 8B), MepK (Fig. 9B and 10), MepM (Fig. 3, 6, and 8B), MepS (Fig. 6, 8B, 9B, and 10), and PBP7 (Fig. 8B). For these six endopeptidases, positive complementation has been obtained in at least one experiment indicating that the level of production of the protein and its stability were sufficient for identification of the function of the protein, as suggested by the reviewer. The reviewer is also right that we did not demonstrate that the remaining endopeptidases (PBP4 and AmpH) were present in the complemented strains. To address this point, we used a fluorescent penicillin to detect PBP4 and AmpH and showed that they are produced upon induction of gene expression. We added these additional results as a new panel of Fig. EV3 (panel B, lines 1022 to 1027 of the revised manuscript). We also added a paragraph to the Material and Methods section to describe the fluorescence assay used to detect PBP4 and AmpH (lines 592 to 601 of the revised manuscript).

1st Revision - Editorial Decision 15th Jul 2021

Dear Dr Hugonnet,

Thank you for the submission of your revised manuscript to The EMBO Journal. As you will see below, your article has been seen by the original referees, who now consider that you have properly dealt with all of their concerns. Before I can accept your article for publication, there are a couple of editorial details that need to be solved:

- Please include callouts for figures 10A, 10B, 12A, 12B and 12C in the main text as appropriate.
- The URL provided under the Data Availability Section links to the home page of the ENA database, and must link to the specific database you have uploaded.

Please let me know if you have any further questions regarding any of these points. Thank you again for giving us the chance to consider your manuscript for The EMBO Journal and congratulations!

I look forward to receiving the final version of your manuscript with these minor changes included.

Yours sincerely,

David del Alamo
Editor
The EMBO Journal

Further information is available in our Guide For Authors:
https://www.embopress.org/page/journal/14602075/authorguide

Please click on the link below to submit the revised version of your manuscript:

Link Not Available

Referee #1:

My comments have all been addressed in admirable detail, either by making the required text changes or by providing reasonable arguments against incorporating them. I now endorse publication.

Referee #2:

I am satisfied with the revised manuscript, and all of my issues have been addressed. Furthermore, the additional data supplied as requested by other reviewers has significantly strengthened the manuscript.

Referee #3:

The authors have answered convincingly to all the reviewers comments and have modified their manuscript accordingly.
I support the publication of this manuscript by EMBO J.

2nd Authors' Response to Reviewers 22nd Jul 2021

The authors performed the requested editorial changes.

2nd Revision - Editorial Decision 24th Jul 2021

Dear Dr. Hugonnet,

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for your contribution to The EMBO Journal.

Yours sincerely,

David del Alamo
Editor
The EMBO Journal

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here:
https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Your manuscript will be processed for publication in the journal by EMBO Press. Manuscripts in the PDF and electronic editions of The EMBO Journal will be copy edited, and you will be provided with page proofs prior to publication. Please note that supplementary information is not included in the proofs.

Please note that you will be contacted by Wiley Author Services to complete licensing and payment information. The 'Page Charges Authorization Form' is available here:
https://www.embopress.org/pb-assets/embo-site/tej_apc.pdf

Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Jean-Emmanuel Hugonnet

Journal Submitted to: Ieva Gailite

Manuscript Number: EMBOJ-2021-108126

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor
http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo
http://datadryad.org
http://figshare.com
http://www.ncbi.nlm.nih.gov/gap
http://www.ebi.ac.uk/ega
http://biomodels.net/
http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA: no animal used in this study
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA: no animal used in this study
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA: no animal used in this study
For animal studies, include a statement about randomization even if no randomization was used.	NA: no animal used in this study
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA: no animal used in this study
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA: no animal used in this study
5. For every figure, are statistical tests justified as appropriate?	NA
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	Antibiotic susceptibility data were medians of three independant experiments which is standard practice to determine resistance phenotypes.

Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	NA: no antibody used in this study
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA: no cell line used in this study

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA: no animal used in this study
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA: no animal used in this study
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA: no animal used in this study

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	We added a Data Availability section at the end of the manuscript: "DATA AVAILABILITY SECTION Whole genome sequencing raw data of sltY mutants (M1.1 to M1.4) are available on the European Nucleotide Archive database (ENA, accession number ERP129644, https://www.ebi.ac.uk/ena).
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA; All data were generated with a non-pathogenic strain of the model bacterium Escherichia coli.