nature portfolio

Peer Review File

The role of the essential GTPase ObgE in regulating lipopolysaccharide synthesis in Escherichia coli

Corresponding Author: Professor Liselot Dewachter

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.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Reviewer summary:

The study by Dewachter et al aims to better understand the function of the GTPase ObgE by investigating the mechanism of cell death in mutants expressing obgE*. Using reversion analysis, the authors find that ObgE*-mediated cell death can be suppressed by mutations in lpxA, a gene involved in synthesis of the essential glycolipid LPS. The authors show that LPS levels are decreased in the ObgE* mutant and that the outer membrane is physically weak. In vitro data suggest that ObgE* interacts with LpxA, that this interaction is enhanced when ObgE* is bound to GTP, and that ObgE* inhibits LpxA activity. Mutations in lpxA that suppress ObgE* cell death prevent interaction between ObgE* and LpxA and restore LPS levels in the obgE* mutant. Based on these data, the authors propose that ObgE* kills cells by inhibiting LpxA and decreasing LPS biosynthesis.

The effect wildtype ObgE has on LPS biosynthesis is less clear. The authors show that wildtype ObgE can interact with LpxA, though this interaction is not affected by mutations lpxA that suppress ObgE*-mediated death. Overexpressing obgE increases sensitivity to the LPS inhibitor PF-04753299 and decreases fitness in cells with reduced expression of genes involved in envelope biogenesis. However, the authors show that wildtype ObgE does not inhibit LpxA activity in vitro and that LPS levels are not affected by obgE overexpression. Furthermore, overexpressing obgE allows cells to grow well as L-forms, a condition that requires an intact outer membrane.

Overall, this study has identified a novel mechanism for regulating LPS biosynthesis. While most studies have focused on regulation of LpxC, the enzyme that catalyzes the first committed step of LPS biosynthesis, the results of this study show that the rate of LPS biosynthesis can be altered by regulating activity of LpxA.

Reviewer comments:

Figure 2E: The authors should quantify the number of cells that show the two-stage lysis phenotype to show that this is a general phenomenon. The authors could do this by plotting the time of the first lysis event on the x axis and the second lysis event on the y axis for many individual cells.

Figure 3C: It would be helpful to highlight the active site in LpxA so that the reader can see where the active site of the protein is relative to the isolated mutations.

Line 317-319: How do LPS levels in the obgE* mutant compare to that of the lpxAR216C mutant? The authors argue that the obgE* mutant dies due to decreased levels of LPS. It is interesting that the lpxAR216C mutant suppresses obgE* cell death but also decreases LPS levels when obgE* is not expressed. Are LPS levels lower in the obgE* mutant than the lpxAR216C mutant? If not, there may be other changes in the obgE* mutant that are contributing to cell death.

Line 601 – 604: The way this is currently written, it seems as if it is the activation of the Rcs response that triggers cell death. However, the authors showed that disabling the Rcs response does not prevent cell death. These sentences should be reworded to clarify that the Rcs response is likely induced by the low LPS, but that the induction of Rcs response is not

responsible for cell death.

Line 625 – 629: The authors hypothesis could be strengthened by looking at cells with decreased ObgE expression or activity. If wildtype ObgE does indeed inhibit LpxA, decreasing ObgE expression should increase LPS levels.

Reviewer #2

(Remarks to the Author)
Summary:
In this manuscript [NCOMMS-24-04788-T] entitled “The essential GTPase ObgE regulates lipopolysaccharide synthesis in Escherichia coli". Authors Dewachter et al. set out to study the lethality of E. coli K-12 strain BW25113 with ectopic expression (increased gene dosage) of a dominant-negative variant of ObgE (referred as ObgE*). Results from transcriptomic analysis were first presented and from which genes for making colanic acid as well as related components for stress response sensing, rcs were reported to be upregulated. Next, using a bacterial L-form model, authors reported that the mutant ObgE* affected cell division, following up with lysis of OM and IM sequentially via time-lapse recording under microscope. These approaches provided inconclusive answers to the death mechanism of ObgE* expressing E. coli. To gain insight of the underline science of lethality created by ObgE*, authors went to study the suppressors of E. coli expressing ObgE*. Suppressor mutations were primarily found contained in LpxA (the enzyme catalyse the first step in making Lipid-A in Raetz pathway). Following saturated genome-wide CRISPR-Cas mutagenesis, frequent mutations in LpxA were identified to be contained in a region and were validated to rescue growth of the lethal strain, with additional evidence generated that rescue could also be achieved via increased expression of LpxA. This prompted authors then to further validate that the LPS production which was found to be reduced in the lethal strain with increased sensitivity to LpxC inhibitor (compound targets enzyme for committed step of LPS synthesis). Subsequently, interactions between LpxA-ObgE-WT were confirmed by bacterial two-hybrid assays and KD values of LpxA-ObgE-WT and LpxA-ObgE* were determined in the presence of nucleotides. Additionally, the molecular ratio for protein complex unit were determined in vitro only for the mutant ObgE*-LpxA. The ObgE*-nucleotide as a complex to inhibit LpxA was further studied. Lastly, authors performed genome-wide CRISPRi screening in strains overexpressing WT-ObgE, albeit with no further exact mechanism deciphered. Overall, this manuscript described a strong inhibitory effect of ObgE* (but not ObgE-WT) to LpxA by binding in high affinity in its nucleotide bound state, which partly elucidated the lethality in cells overexpressing ObgE*. The methodology employed in this work is appropriate which provides a relatively comprehensive data set. However, the reviewer feels that the main conclusion that GTPase ObgE regulates LPS synthesis in E. coli lacks support based on data accumulated in this work, thus seems to be overinterpreted. This is because although novel interactions between WT ObgE and LpxA were identified and further characterised, effects in LPS synthesis as well as LpxA activity were not observed in any with overexpression of WT ObgE. The main conclusion made seems to root from the observation based on the interaction between mutant ObgE* and LpxA, which was known to be lethal. The authors could not rule out the possibility that the interaction between mutant ObgE* and LpxA is a gain-of-function, hence not representing the WT ObgE function. Nevertheless, the report of the mechanism on novel ObgE* variant in negatively affecting LpxA in a dominant manner is of scientific interest, as it provided an additional investigation tool by genetic approach (in addition to chemical LpxC inhibitors) in studying LPS synthesis as well as bacterial cell envelope. It also pointed out a new drug target (LpxA) to be investigated in the future.

Overall, the manuscript was clearly written but parts of it are repetitive and verbose. Part of analysis especially those with negative results could be summarised in short and be combined with other sections as they provided limited information in gaining our understanding in the biological system. In addition, the results that authors reported, whether they are consequences of or causes of the ObgE* lethality model, needs to be carefully interpreted to make sound scientific conclusion. The reviewer feels that the conclusion drew from the interaction between mutant ObgE* and LpxA in explaining the lethal phenotype of overexpressing ObgE* in E. coli was supported. However, the claim that WT ObgE has a role in regulating LPS synthesis by interacting with LpxA is not backed up by the evidence presented and the biological role of ObgE in interacting with LpxA thus remains unclear.

Major Comments:
1. Rcs response is generally activated upon cell envelope stress including LPS defects, this could then through signalling pathways leads to the production of colanic acid (for which the biological role of its production is still not completely understood) (PMID: 16166540). This is normally the consequence of stress induced and is the consequence for many conditions with OM disruptions, rather than the cause of the problem. Thus, the presentation of analysing rcs and wca deletions are not necessary. Author used suppressor studies found LpxA, a target which was then confirmed to be affected by nucleotide-bound ObgE* and confirmed the LPS production defect. The reviewer thus feel that the text section of transcriptomic analysis could be significant reduced to improve the conciseness of the main story by combining this section with the suppressor study to support the impact on LpxA in making LPS by expressing ObgE*, which triggers broader envelope stress response.

2. Line 185-186: in a L-form model, ObgE* caused proliferation defect. It was reported that disruption of LPS synthesis by mutating LpxC with reduced LPS production (PMID: 7002913) also have defects in cell division (PMID: 4887513). Authors should combine the previous work and discuss. Nevertheless, these L-Form experiments provided limited mechanistic insight of the ObgE* caused lethality, thus should be combined with the LpxA suppressor study as supportive evidence for the reason stated above.

3. Line 331-345: An LpxC inhibitor exhibited synergistic growth inhibition for overexpression of both WT ObgE and mutant ObgE*, the CFU reduction is also observed when expressing WT ObgE (Figure 4C) Vs vector control. Therefore, author suggests that WT ObgE, like the mutant ObgE* negatively regulates LPS synthesis. However, overexpression of ObgE were discussed previously by the author (PMID: 28702018) have complicated growth defects like ObgE deletion strains. Importantly, there were no differences observed in LpxA activity change and LPS change upon WT-ObgE expression. Therefore, the reviewer feels that the conclusion of overexpression affects LPS synthesis based on the observation [overexpression of ObgE caused increased sensitivity to LpxC inhibitor] could be an indirect effect and therefore weak.

4. Figure 5F, the stoichiometry between ObgE* and LpxA were determined, have authors consider analysing the complex (if they form in solution) between WT ObgE and LpxA? The reviewer also suggests crosslinking experiments followed by SDS-PAGE immunoblot can be done to compare different complex to gain more information on the different contact. Limited proteolysis could also be performed to probe the different protein-protein interfaces.

5. In Figure B, mutant ObgE* inhibits LpxA activity when bound to nucleotide yet loses the inhibitory effect to LpxA variants found in suppressors. However, WT ObgE (Figure S6) in multiple experimental repeats had no effects on LpxA activity. Based on these results, it is unclear to what extent the observation made with the mutant ObgE* on LpxA activity applies to the biological role of WT ObgE. The abnormal high affinity between ObgE* to LpxA (Table 2) might be a gain of function of the ObgE*, and not for the WT ObgE. After all, the mutation causes lethality that is to be selected out. Therefore, the reviewer feels such a claim [WT ObgE interacts with LpxA to regulate LPS synthesis] is an overinterpretation and may be misleading. Examples of gain-of-function mutations in other proteins beyond their biological function could be found in other studies: 1) a mutation (F332S) in WaaL (O antigen Ligase) had been reported previously to ligate PG precursor onto Lipid-A-core and display PG on cell surface (PMID: 25551294). This does not apply to the biological relevant function of WaaL. 2), a dominant mutation in MlaA* disrupts cell envelope and causes cell death, which was due to gain-of-function in mis-positioning PL to the outer leaflet of OM rather than its biological relevant function in retrograde PL trafficking and OM asymmetry maintenance (PMID: 26929379). Therefore, the reviewer suggests authors to rewrite their manuscript to better reflect objectivity.

6. To gain insight in the biological relevance of studying this mutant, the reviewer suggests authors perform bioinformatic analysis on both ObgE and LpxA broadly in different strains/species to check if such mutations exist in both LpxA and ObgE.

7. The reviewer found the last section of the CRISPRi experiments could be moved earlier in the section where LpxA suppressors were described since these results provided with no further depth in our understanding in the biological significance of LpxA and ObgE interaction.

8. Line 622-631. As explained in 5, evidence generated does not conclusively support the claim that the WT ObgE-LpxA interaction observed here is for regulation of LPS production. The observation based on ObgE*-LpxA interaction does not necessarily apply for the WT ObgE-LpxA. LPS synthesis is mainly controlled at the committed step level targeting LpxC, regulated by FtsH, LapC, LapB etc. It is unclear on the importance of having another regulation system as such to control LPS synthesis at non-committed step LpxA.

9. It might be interesting to investigate the detailed interface between mutant ObgE* and LpxA to design short peptide derived therapeutics targeting LpxA in inhibiting bacterial growth.

Minor comments:

1. Line 68-69: The minimal structure of LPS is not KDO¬2-Lipid (IV)A, as waaA has been shown can be successfully deleted without suppressor mutations, albeit cell grow at lower temperature (PMID: 19346244), therefore minimal structure of LPS for the survival of Gram-negative bacteria is Lipid IV(A) precursors.

2. Line 117-118: RcsF-dependent triggered colanic production is temperature dependent, please specify the temperature under which the cells were grown for RNAseq analysis.

3. Line 272: LPS inhibitor PF-04753229 is first mentioned here, please move the explanation of its molecular target here from line 336.

4. Line 321-329: The reviewer suggests reducing the length of this section to improve conciseness. Since restoration of O antigen was not done in K-12, rather tested in another E. coli producing different Lipid-A-core and O antigen, hence modify the wording in line 328-329: therefore “likely” not explain the toxic ObgE* phenotype.

5. Line 351-352: Vector control was used as a baseline (normalized to be 1.00) to transform dataset therefore lost the data variation for Vector control group. However, statistics were performed with multiple comparisons test against the vector control group, which is inappropriate, as the standard deviation of this group is lost.

6. Figure 7A, light grey colour on white background is hard to read, please consider changing to give better contrast.

7. Line 528, This is misleading, these were observed when ObgE were ectopically over-expressed (Figure 4C). hence “this GTPase, was shown to interact with LpxA in vitro and when supplied in excessive amount, can sensitise cells to LPS inhibition.”

8. Line 598: 1,000-fold not 1.000-fold

Reviewer #3

(Remarks to the Author)
In the manuscript titled “The essential GTPase ObgE regulates lipopolysaccharide synthesis in Escherichia coli” Dewachter et. al. describe a novel regulatory interaction between LpxA, the first enzyme in LPS biosynthesis, and ObgE, a conserved GTPase. LPS biosynthesis is thought to be tightly regulated largely at the committed step in the pathway, which is catalyzed by the enzyme that acts immediately downstream of LpxA. Recent work, however, has highlighted the potential for LpxA to also serve as a regulatory node. Here, the Dewachter and colleagues characterize a previously isolated dominant lethal allele of ObgE, ObgE*, and provide compelling genetic and biochemical evidence that this variant inhibits the activity of LpxA to reduce LPS biosynthesis, thereby inhibiting growth.

Overall, the work presented by Dewachter et. al. convincingly demonstrates that ObgE has the capacity to regulate LPS biosynthesis at the level of LpxA, which is a critical enzyme in gram-negative bacteria whose regulation is poorly understood. However, all of the presented experiments are performed in the context of artificial overexpression of ObgE* and thus, the biological relevance of this regulation is unclear. The work would greatly benefit from some analysis or commentary on the phenotypes associated with deletion/depletion of obgE. Specifically, are there any phenotypes that would be expected to be associated with increased activity of LpxA (accumulation of LPS, increased resistance to PF-04753299, stabilization of LpxC)?

Aside from this major comment, I offer additional minor comments for the authors to consider:

1.Given the conservation of LpxA and ObgE, is there evidence that this mechanism of regulation could be conserved in other organisms? Does Alphafold predict an interaction between the proteins from other bacteria?
2.Line 185-186 – “ObgE* negatively affects L-form growth and strongly inhibits their proliferation”. Please define how “successful divisions” are defined in these experiments.
3.Fig S2AB – it is challenging to interpret the reduction in ROS levels in the ObgE* background upon treatment with ROS scavengers. This could be remedied by combining these two graphs and providing “N” values for each strain/condition.
4.Line 270 – “Similarly, LPS synthesis is significantly decreased in E. coli lpxAR216C..” Can the authors comment on why LPS levels appear reduced in the R216C mutant in fig S3C but not in S4A?
5.Table 2 and figure 5 seem redundant.
6.Table2/Figure 5 - Why was ObgE without any added nucleotide tested for its ability to interact with LpxA?
7.Figure S5 – please label the regions of the lDDT plots that correspond to the LpxA/ObgE sequences.
8.Line 417-418 – “Considering that in model 1 the G domain of ObgE*, which contains the K268I mutation, is involved in the interaction with LpxA, we regard the latter model as the most biologically relevant” Should this be the former model?
9.Lines 564-565 – “show that expression of only a small number of genes” this should be expression of a small number of sgRNAs or altered expression of a small number of genes. Similarly for lines 568-569.
10. Methods – oligo sequences used in the process of this study should be provided.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
As in the original report, Dewachter et al. provide good evidence that the toxicity caused by ObgE* is due to inhibition of LpxA. While the revisions have addressed many of my concerns, I still find evidence suggesting that wildtype ObgE inhibits the activity of LpxA to be conflicting. In support of the author's hypothesis, wildtype ObgE interacts with LpxA both in vivo and in vitro. Furthermore, overexpression of ObgE increases sensitivity to an LpxC inhibitor, and this sensitivity can be ameliorated by mutations that prevent interaction between ObgE* and LpxA (but not wildtype ObgE and LpxA). The authors also show that decreasing ObgE expression can sometimes increase LPS levels, albeit only slightly. However, the authors show that overexpressing wildtype ObgE does not decrease LPS levels and that wildtype ObgE does not inhibit LpxA activity in vitro. Furthermore, the results of the author's CRISPRi screen show that reducing expression of LPS biosynthesis genes actually improves fitness in cells overexpressing ObgE. This particular result directly contradicts what the authors observe with the LpxC inhibitor. Both the LpxC inhibitor and sgRNAs targeting LPS biosynthesis genes should decrease enzyme activity/expression, and one would expect these data to show similar results. The results would be more convincing if the authors could identify a condition where wildtype ObgE strongly and consistently affects LPS and LpxA, much like ObgE*. Otherwise, it is entirely possible that ObgE* is a neomorphic mutation that has gained new capabilities compared to the wildtype (much like MlaA* and WaaL15, as mentioned by another reviewer).

Major comments:

Line 497-498: The authors show that the lpxA suppressor mutations do not affect the interaction between wildtype ObgE and

LpxA. However, the lpxA mutations can rescue the ObgE overexpression strain from the LpxC inhibitor. Can the authors explain why they think the lpxA mutations rescue the sensitivity?

Line 506-507: The effect of decreasing obgE expression on LPS levels is weak and is observed with only 2 out of the 4 sgRNAs. Are obgE levels decreased to the same extent with all four sgRNAs or is there a greater decrease with the sgRNAs that show increased LPS levels? Would the authors expect similar results if targeting a known LPS inhibitor like LapB or FtsH?

Minor comments:

Line 69 - 70: Here, the authors state that LpxA is not thought to be regulated. In line 612, the authors mention that LpxA is regulated by RnhB and ppGpp.

Figure 7E: The authors show that the lpxA point mutations do not rescue cells overexpressing ObgE* from the LpxC inhibitor. In fact, two of these lpxA mutations appear to increase sensitivity. Can the authors explain why this may be the case?

Reviewer #2

(Remarks to the Author)
Upon revision, authors have provided more experiments in supporting their claim that wild-type ObgE interacts with LpxA affecting LPS synthesis. Two additional experimental results (FIGURE 7E and 7F) are critical for the scientific field in understanding this reported interaction between ObgE and LpxA. Figure 7E showed overexpression of ObgE in WT, but not in two ObgE* suppressors, sensitises towards lpxC inhibitor, suggesting that the sensitisation to LpxC inhibitor by ObgE overexpression is LpxA dependent, indirectly supporting the claim that WT ObgE interacts with LpxA to impact LPS synthesis. Figure 7F showed that altering ObgE levels have effects on LPS levels. Since these two experiments are critical in understanding ObgE function in regulating LPS synthesis by interacting with LpxA. The reviewer strongly suggests including raw data of these two experiments with the manuscript. This could be raw CFU counts for Figure 7E, as shown in Figure 7D across time points, or by serial dilution plating in the presence or absence of LpxC inhibitor.
Please also include raw LPS images used for LPS quantification in Figure 7E.

Reviewer #3

(Remarks to the Author)
The revised manuscript by Dewachter et. al. has added critical evidence to support their proposed model in which wt ObgE regulates LpxA and have addressed all of my other previous concerns.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I am satisfied with the additional experiments performed. These experiments provide further support for the authors arguments.

Reviewer #2

(Remarks to the Author)
Upon revision, the reviewer feels that previous requests have been addressed and additional MS data further supports the interactions between ObgE and LpxA. The reviewer however still feels that this WEAK interaction originated from mutant ObgE observations lacks biological significance/relevance and having a tendency to be interpreted as an artifact. Therefore, the reviewer suggests with no further experiments, but insists modifications to the title to: "The essential GTPase ObgE bare the potential to influence lipopolysaccharide synthesis in Escherichia coli", or similar to reflect their findings accurately. Where appropriate, main text as well.

RESPONSE TO REVIEWER COMMENTS

Reviewer #1

Reviewer summary:

The study by Dewachter et al aims to better understand the function of the GTPase ObgE by investigating the mechanism of cell death in mutants expressing obgE*. Using reversion analysis, the authors find that ObgE*-mediated cell death can be suppressed by mutations in lpxA, a gene involved in synthesis of the essential glycolipid LPS. The authors show that LPS levels are decreased in the ObgE* mutant and that the outer membrane is physically weak. In vitro data suggest that ObgE* interacts with LpxA, that this interaction is enhanced when ObgE* is bound to GTP, and that ObgE* inhibits LpxA activity. Mutations in lpxA that suppress ObgE* cell death prevent interaction between ObgE* and LpxA and restore LPS levels in the obgE* mutant. Based on these data, the authors propose that ObgE* kills cells by inhibiting LpxA and decreasing LPS biosynthesis.
The effect wildtype ObgE has on LPS biosynthesis is less clear. The authors show that wildtype ObgE can interact with LpxA, though this interaction is not affected by mutations lpxA that suppress ObgE*-mediated death. Overexpressing obgE increases sensitivity to the LPS inhibitor PF-04753299 and decreases fitness in cells with reduced expression of genes involved in envelope biogenesis. However, the authors show that wildtype ObgE does not inhibit LpxA activity in vitro and that LPS levels are not affected by obgE overexpression. Furthermore, overexpressing obgE allows cells to grow well as L-forms, a condition that requires an intact outer membrane.
Overall, this study has identified a novel mechanism for regulating LPS biosynthesis. While most studies have focused on regulation of LpxC, the enzyme that catalyzes the first committed step of LPS biosynthesis, the results of this study show that the rate of LPS biosynthesis can be altered by regulating activity of LpxA.

> We thank the reviewer for the time spent on our manuscript and the helpful comments provided. We understand that, in its original form, a role for wt ObgE in regulating LpxA activity and LPS synthesis may not have been sufficiently demonstrated yet. However, we believe that the additional experiments included in our revised manuscript strengthen this claim considerably, as also detailed in our responses to the reviewer comments below. Shortly, we now show that (1) the synergy found between overexpression of wt *obgE* and the LPS inhibitor PF-04753299 depends on LpxA as it can be counteracted using the LpxA variants that also provide resistance to ObgE*, and (2) depleting *obgE* expression by CRISPRi increases cellular LPS levels.

Reviewer comments:

Figure 2E: The authors should quantify the number of cells that show the two-stage lysis phenotype to show that this is a general phenomenon. The authors could do this by plotting the time of the first lysis event on the x axis and the second lysis event on the y axis for many individual cells.

> We have followed the reviewer's suggestion and have included Figure S2C (also shown below). This figure shows that 88% of all lysis events of *obgE**-expressing L-forms proceed via two distinct steps. Also the timing of these different stages of lysis is shown. This information has also been added at appropriate places in the text (results, materials & methods, and figure legend).

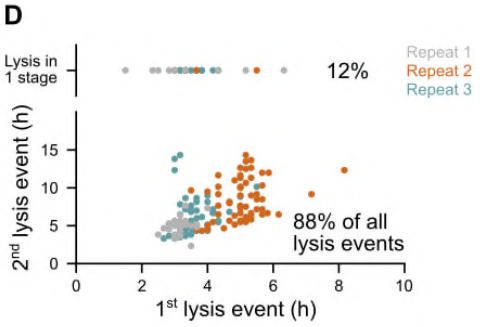
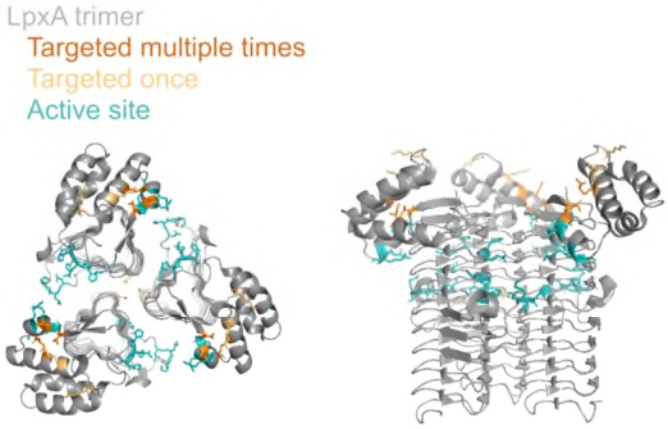


Figure 3C: It would be helpful to highlight the active site in LpxA so that the reader can see where the active site of the protein is relative to the isolated mutations.

We have followed this suggestion and have now also highlighted the protein active site in Figure 3C (also shown below).



Line 317-319: How do LPS levels in the obgE* mutant compare to that of the lpxAR216C mutant? The authors argue that the obgE* mutant dies due to decreased levels of LPS. It is interesting that the lpxAR216C mutant suppresses obgE* cell death but also decreases LPS levels when obgE* is not expressed. Are LPS levels lower in the obgE* mutant than the lpxAR216C mutant? If not, there may be other changes in the obgE* mutant that are contributing to cell death.

When expressed relatively compared to a wt background, the LPS content in the *lpxA*$_{R216C}$ mutant (no *obgE** expression, Figure S3C) is reduced to 79% of the wt. This information was added to the manuscript at line 231. This reduction is statistically significant but smaller than the reduction caused by ObgE* in the wt background which leads to LPS levels that are 66% of the wt vector control (Figure 6D). It is possible that this difference in LPS content allows *lpxA*$_{R216C}$ mutants to survive and grow, while wt cells expressing *obgE** die.

However, we also want to stress that these numbers are not directly comparable. Whereas the *lpxA*$_{R216C}$ mutation is continuously present and influences LPS production throughout the experiment and also in the preculture, this is not the case for ObgE*. Expression of *obgE** is induced for only 2 hours prior to measuring LPS levels. At this time, we already detect very strong ObgE* toxicity when CFUs/ml are measured (see below, panel A). However, when evaluating toxicity with a more instantaneous read-out, i.e. by using the membrane-impermeable propidium iodide (PI) dye, it is clear that at this time point a large fraction of the population is still alive/intact (see below, panels B-C). We therefore suspect that the 66% LPS content found in *E. coli* wt

expressing *obgE** at this time is not a stable endpoint and may further deteriorate, while this is not the case for *E. coli* $lpxA_{R216C}$. This may also explain the strong toxicity detected for *E. coli* wt expressing *obgE** and not *E. coli* $lpxA_{R216C}$.

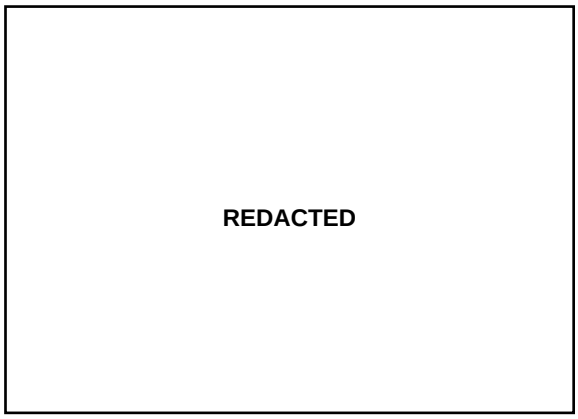


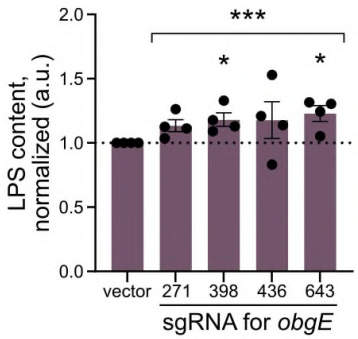
Figure from Dewachter *et al.* (2017) Front in Microbiol. https://doi.org/10.3389/fmicb.2017.01193

Line 601 – 604: The way this is currently written, it seems as if it is the activation of the Rcs response that triggers cell death. However, the authors showed that disabling the Rcs response does not prevent cell death. These sentences should be reworded to clarify that the Rcs response is likely induced by the low LPS, but that the induction of Rcs response is not responsible for cell death.

We agree with the reviewer that this might be confusing. We changed the text accordingly. Line 547: "This LPS deficit triggers the Rcs cell envelope stress response and also causes cell death independently of Rcs activation."

Line 625 – 629 (now line 569-571): The authors hypothesis could be strengthened by looking at cells with decreased ObgE expression or activity. If wildtype ObgE does indeed inhibit LpxA, decreasing ObgE expression should increase LPS levels.

Following the reviewer's suggestion, we have decreased *obgE* expression using CRISPRi and measured LPS levels. We have used 4 different sgRNAs that target *obgE* and found that, after 4h of CRISPRi induction, LPS levels are significantly increased for 2 of them (sgRNA 398 and 643). For the other two sgRNAs, the observed increase was not statistically significant. When results from all sgRNAs are combined, LPS levels are significantly increased. These data are now shown in Figure 7F (also shown below) and further support the involvement of wt ObgE in the regulation of LPS synthesis in *E. coli*.



Reviewer #2

Summary:
In this manuscript [NCOMMS-24-04788-T] entitled “The essential GTPase ObgE regulates lipopolysaccharide synthesis in Escherichia coli". Authors Dewachter et al. set out to study the lethality of E. coli K-12 strain BW25113 with ectopic expression (increased gene dosage) of a dominant-negative variant of ObgE (referred as ObgE*). Results from transcriptomic analysis were first presented and from which genes for making colanic acid as well as related components for stress response sensing, rcs were reported to be upregulated. Next, using a bacterial L-form model, authors reported that the mutant ObgE* affected cell division, following up with lysis of OM and IM sequentially via time-lapse recording under microscope. These approaches provided inconclusive answers to the death mechanism of ObgE* expressing E. coli. To gain insight of the underline science of lethality created by ObgE*, authors went to study the suppressors of E. coli expressing ObgE*. Suppressor mutations were primarily found contained in LpxA (the enzyme catalyse the first step in making Lipid-A in Raetz pathway). Following saturated genome-wide CRISPR-Cas mutagenesis, frequent mutations in LpxA were identified to be contained in a region and were validated to rescue growth of the lethal strain, with additional evidence generated that rescue could also be achieved via increased expression of LpxA. This prompted authors then to further validate that the LPS production which was found to be reduced in the lethal strain with increased sensitivity to LpxC inhibitor (compound targets enzyme for committed step of LPS synthesis). Subsequently, interactions between LpxA-ObgE-WT were confirmed by bacterial two-hybrid assays and KD values of LpxA-ObgE-WT and LpxA-ObgE* were determined in the presence of nucleotides. Additionally, the molecular ratio for protein complex unit were determined in vitro only for the mutant ObgE*-LpxA. The ObgE*-nucleotide as a complex to inhibit LpxA was further studied. Lastly, authors performed genome-wide CRISPRi screening in strains overexpressing WT-ObgE, albeit with no further exact mechanism deciphered. Overall, this manuscript described a strong inhibitory effect of ObgE* (but not ObgE-WT) to LpxA by binding in high affinity in its nucleotide bound state, which partly elucidated the lethality in cells overexpressing ObgE*. The methodology employed in this work is appropriate which provides a relatively comprehensive data set. However, the reviewer feels that the main conclusion that GTPase ObgE regulates LPS synthesis in E. coli lacks support based on data accumulated in this work, thus seems to be overinterpreted. This is because although novel interactions between WT ObgE and LpxA were identified and further characterised, effects in LPS synthesis as well as LpxA activity were not observed in any with overexpression of WT ObgE. The main conclusion made seems to root from the observation based on the interaction between mutant ObgE* and LpxA, which was known to be lethal. The authors could not rule out the possibility that the interaction between mutant ObgE* and LpxA is a gain-of-function, hence not representing the WT ObgE function. Nevertheless, the report of the mechanism on novel ObgE* variant in negatively affecting LpxA in a dominant manner is of scientific interest, as it provided an additional investigation tool by genetic approach (in addition to chemical LpxC inhibitors) in studying LPS synthesis as well as bacterial cell envelope. It also pointed out a new drug target (LpxA) to be investigated in the future. Overall, the manuscript was clearly written but parts of it are repetitive and verbose. Part of analysis especially those with negative results could be summarised in short and be combined with other sections as they provided limited information in gaining our understanding in the biological system. In addition, the results that authors reported, whether they are consequences of or causes of the ObgE* lethality model, needs to be carefully interpreted to make sound scientific conclusion. The reviewer feels that the conclusion drew from the interaction between mutant ObgE* and LpxA in explaining the lethal phenotype of overexpressing ObgE* in E. coli was supported. However, the claim that WT ObgE has a role in regulating LPS synthesis by interacting with LpxA is not backed up by the evidence presented and the biological role of ObgE in interacting with LpxA thus remains unclear.

We thank the reviewer for carefully considering our manuscript. Based on the provided comments we have improved our manuscript, as also detailed in our responses to the comments below. Of note, we have shortened our manuscript and provided additional evidence for the involvement of wt ObgE in the regulation of LPS synthesis through LpxA. Shortly, we now show that (1) the synergy found between overexpression of wt *obgE* and the LPS inhibitor PF-04753299 depends on LpxA as it can be counteracted using the LpxA variants that also provide resistance to ObgE*, and (2) depleting *obgE* expression by CRISPRi increases cellular LPS levels.

Major Comments:

1. Rcs response is generally activated upon cell envelope stress including LPS defects, this could then through signalling pathways leads to the production of colanic acid (for which the biological role of its production is still not completely understood) (PMID: 16166540). This is normally the consequence of stress induced and is the consequence for many conditions with OM disruptions, rather than the cause of the problem. Thus, the presentation of analysing rcs and wca deletions are not necessary.

The Rcs stress response is indeed activated in response to a variety of cell envelope disruptions. However, in contrast to other stress responses, high levels of Rcs activation are detrimental rather than helpful to the cell and were shown to cause cell death (doi: 10.1038/s41579-019-0199-0). When presenting this story to people in the field, we often got asked about the possible causal nature of Rcs activation to cell death. We would therefore prefer to keep these data in the manuscript. Similarly, it has been shown that the occurrence of dead-end intermediates in colanic acid synthesis can lead to peptidoglycan defects (doi 10.1128/JB.00550-16) which in turn can contribute to Rcs activation and toxicity. We therefore felt it was important to show that colanic acid synthesis is unrelated to ObgE* toxicity by completely eliminating this pathway using Δ*wcaE* and Δ*wcaJ* mutants. Nonetheless, we agree with the reviewer that these data are not highly relevant to our main story line which is why we choose to put them in a supplementary figure. In addition, we have now shortened the text describing these experiments and findings.

Author used suppressor studies found LpxA, a target which was then confirmed to be affected by nucleotide-bound ObgE* and confirmed the LPS production defect. The reviewer thus feel that the text section of transcriptomic analysis could be significant reduced to improve the conciseness of the main story by combining this section with the suppressor study to support the impact on LpxA in making LPS by expressing ObgE*, which triggers broader envelope stress response.

Based on the reviewer's suggestion, we have considerably shortened the suggested sections of our manuscript. These include the first results section detailing the RNA-seq experiment, the section on L-forms (see next comment) and the section where we isolate *lpxA* suppressors. Following our own personal preference, we have kept the original structure of our results section and have not integrated all experiments – which we see as different research lines – into the same section.

2. Line 185-186 (now line 157-159): in a L-form model, ObgE* caused proliferation defect. It was reported that disruption of LPS synthesis by mutating LpxC with reduced LPS production (PMID: 7002913) also have defects in cell division (PMID: 4887513). Authors should combine the previous work and discuss. Nevertheless, these L-Form experiments provided limited mechanistic insight of the ObgE* caused lethality, thus should be combined with the LpxA suppressor study as supportive evidence for the reason stated above.

Whereas we recognize the scientific value of the suggested papers that report the phenotypic effects of reduced LPS production on *E. coli* cell division, we believe it does not directly relate to

the lack of L-form proliferation we observe. There are huge mechanistic differences in the proliferation of L-forms versus walled *E. coli* cells. Whereas walled *E. coli* cell division relies on septum formation by the divisome which is recruited by the FtsZ protein that forms a ring at midcell, this is not true for *E. coli* L-forms. In fact, *ftsZ* has become dispensable for L-form proliferation (doi: 10.1038/nature08232, doi: 10.1038/nmicrobiol.2016.91). Instead, L-forms divide by random membrane fission events, meaning that excess membrane synthesis promotes L-form proliferation (doi: 10.1016/j.cell.2013.01.043). Since the suggested papers do not contain information that is related to the amount of membrane synthesis (as a whole, not just LPS production), we believe that incorporating these references will not contribute to a correct interpretation of our phenotypes. We therefore prefer not to include them.

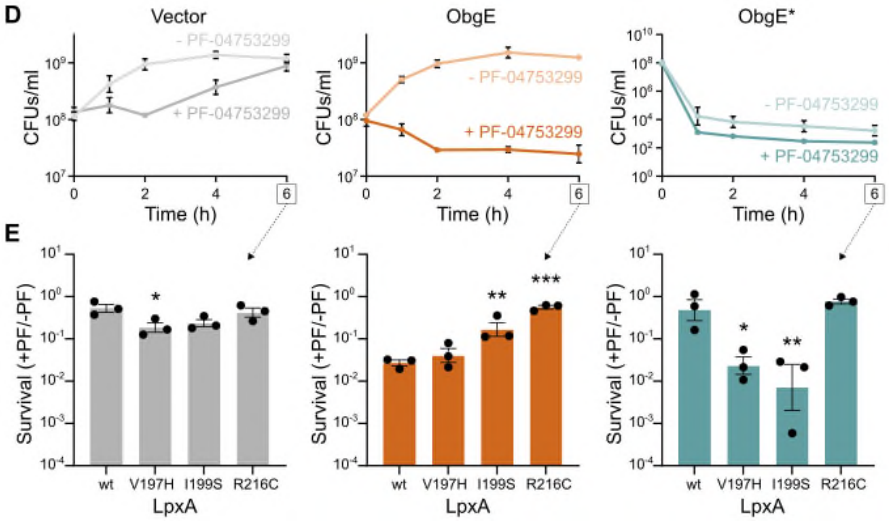
While we agree that our L-form experiments provide limited mechanistic insight into the lethality of ObgE*, we believe these experiments are very interesting in their own right which is why we chose to show them in a separate main text figure. We here successfully use L-forms as a research tool (rather than a life form to be studied), which we believe may be useful to other cell envelope related research projects as well. In addition, we describe an interesting L-form specific phenotype caused by LPS deficiency which will be of interest to both L-form and LPS researchers to interpret phenotypes they may encounter at some point.

However, we agree with the reviewer that this experimental section of our paper is quite lengthy. Together with several other sections, we have shortened it considerably, as also stated in our response to the previous comment.

3. Line 331-345 (now line 492-501): An LpxC inhibitor exhibited synergistic growth inhibition for overexpression of both WT ObgE and mutant ObgE*, the CFU reduction is also observed when expressing WT ObgE (Figure 4C) Vs vector control. Therefore, author suggests that WT ObgE, like the mutant ObgE* negatively regulates LPS synthesis. However, overexpression of ObgE were discussed previously by the author (PMID: 28702018) have complicated growth defects like ObgE deletion strains. Importantly, there were no differences observed in LpxA activity change and LPS change upon WT-ObgE expression. Therefore, the reviewer feels that the conclusion of overexpression affects LPS synthesis based on the observation [overexpression of ObgE caused increased sensitivity to LpxC inhibitor] could be an indirect effect and therefore weak.

To more convincingly show that the synergy between wt *obgE* overexpression and the LPS inhibitor PF-04753299 is due to a direct effect of ObgE on LpxA and LPS synthesis, we also performed this experiment using our *lpxA* suppressor mutants. Importantly, we find that the synergy of ObgE and PF-04753299 is significantly weakened by two out of the three tested LpxA variants. Because this synergy is LpxA-dependent, it strongly points towards an effect of wt ObgE on LpxA activity and LPS synthesis. This experiment, which is shown in our manuscript in Figure 7E (also included below), further supports a role for wt ObgE in regulating LpxA activity and LPS synthesis.

We have also collected additional support for the involvement of ObgE in the regulation of LPS synthesis by downregulating ObgE through CRISPRi, which we found leads to increased LPS levels. These findings are discussed in more detail in one of the next comments.

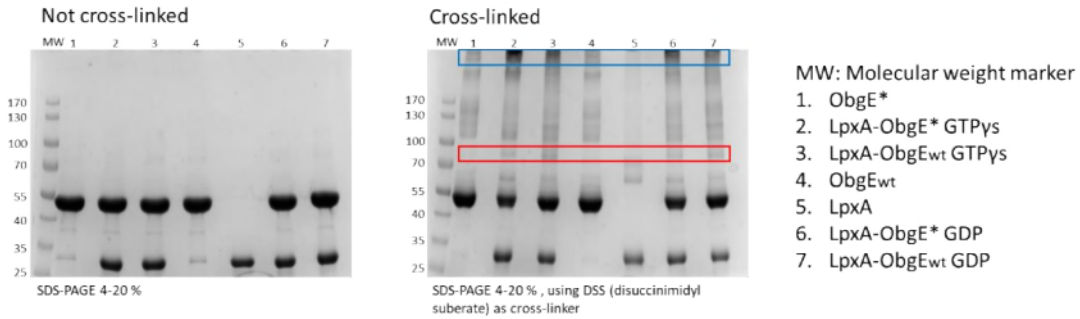


4. Figure 5F (now Figure 4F), the stoichiometry between ObgE* and LpxA were determined, have authors consider analysing the complex (if they form in solution) between WT ObgE and LpxA? The reviewer also suggests crosslinking experiments followed by SDS-PAGE immunoblot can be done to compare different complex to gain more information on the different contact. Limited proteolysis could also be performed to probe the different protein-protein interfaces.

The determination of the stoichiometry of the ObgE*-LpxA complex in presence of GTPγS was performed using SEC-MALS, thus requiring the complex to remain stably bound during the entire period of the size-exclusion chromatography (SEC). This was made possible because of the high affinity of this complex (K_D = 8 nM as determined using MST). Indeed, the stability of the complex during SEC is crucially dependent on the off-rate (k_{off}) and the closely related half-life ($t_{1/2}$) of the complex. If we assume a typical k_{on} for the ObgE*-LpxA- GTPγS complex of 10^{-6} $M^{-1}s^{-1}$, a K_D of 8 nM would correspond to a $t_{1/2}$ = 87 sec (assuming no rebinding), which was sufficient to observe the complex on the small HPLC SEC column. However, the affinity of ObgE wild type for LpxA was determined via MST to be in the range of 50-100 µM, corresponding to a $t_{1/2}$ = 14 msec, using the same assumptions, thus making this complex too transient to be observed on SEC-MALS. Correspondingly, we were not able to detect the complex peak for ObgE-LpxA on SEC-MALS.

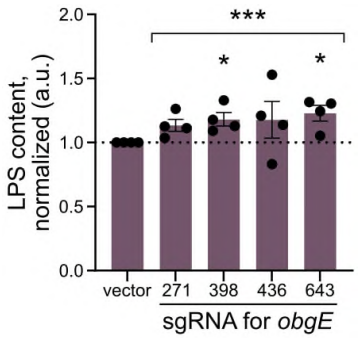
As the referee suggested we next attempted to use cross-linking as yet another method to confirm binding of ObgE to LpxA, besides the Bacterial-2-hybrid and the MST methods that already clearly showed this interaction in our paper. In this experiment we included cross-linking (using DSS) between LpxA and ObgE wild type and ObgE*, in presence of both GDP and GTPγS. Cross-linking of the individual proteins was used as a control and the non-cross-linked samples were used as reference. The results of this experiment are shown below.

The analysis of these results is complicated by the internal cross-linking between the subunits of the LpxA trimer and by non-specific cross-linking of ObgE. Nevertheless, some bands are observed that are unique or more pronounced in the samples containing a mixture of LpxA and ObgE(*). The bands that are highlighted in red, would correspond to the molecular weight of 1 ObgE(*) subunit linked to 1 LpxA subunit. Also the protein bands at the top of the gel (indicated by a blue square) are more intense in the samples containing the complex. However, surprisingly, the intensity of these bands seems to be independent of the nucleotide state and ObgE* mutation. As the outcome of this experiment does not add new information to our conclusions, we decided not to include it in the manuscript.



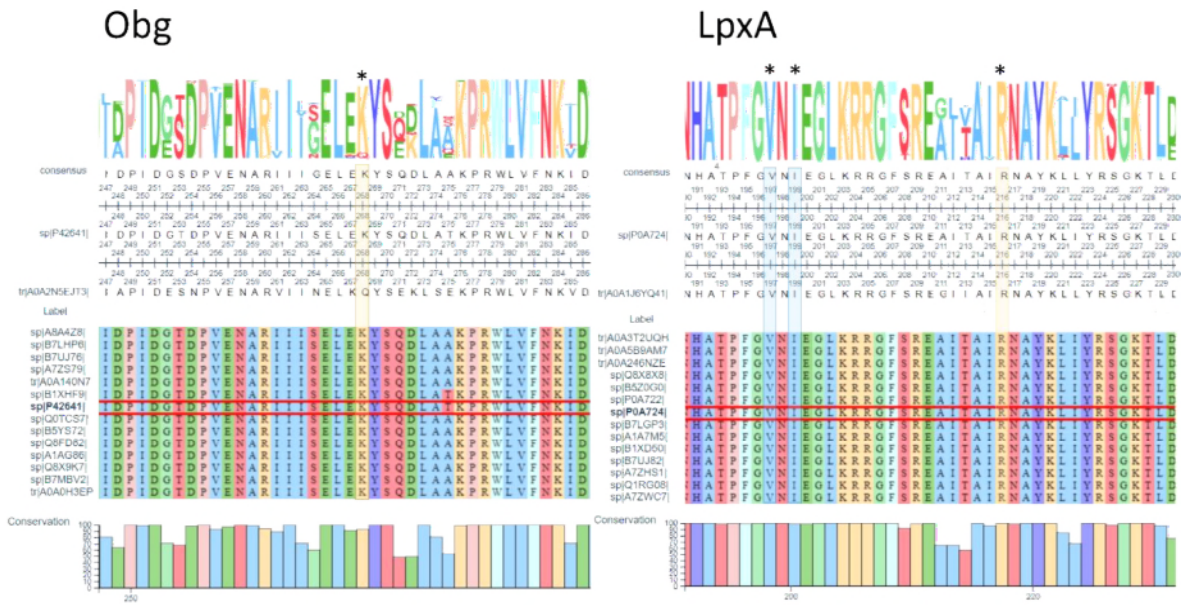
5. In Figure 7B (now Figure 6B), mutant ObgE* inhibits LpxA activity when bound to nucleotide yet loses the inhibitory effect to LpxA variants found in suppressors. However, WT ObgE (Figure S6) in multiple experimental repeats had no effects on LpxA activity. Based on these results, it is unclear to what extent the observation made with the mutant ObgE* on LpxA activity applies to the biological role of WT ObgE. The abnormal high affinity between ObgE* to LpxA (Table 2) might be a gain of function of the ObgE*, and not for the WT ObgE. After all, the mutation causes lethality that is to be selected out. Therefore, the reviewer feels such a claim [WT ObgE interacts with LpxA to regulate LPS synthesis] is an overinterpretation and may be misleading. Examples of gain-of-function mutations in other proteins beyond their biological function could be found in other studies: 1) a mutation (F332S) in WaaL (O antigen Ligase) had been reported previously to ligate PG precursor onto Lipid-A-core and display PG on cell surface (PMID: 25551294). This does not apply to the biological relevant function of WaaL. 2), a dominant mutation in MlaA* disrupts cell envelope and causes cell death, which was due to gain-of-function in mis-positioning PL to the outer leaflet of OM rather than its biological relevant function in retrograde PL trafficking and OM asymmetry maintenance (PMID: 26929379). Therefore, the reviewer suggests authors to rewrite their manuscript to better reflect objectivity.

To further strengthen our claim that wt ObgE is involved in the regulation of LpxA activity and LPS synthesis, we have measured LPS levels upon decreasing *obgE* expression using CRISPRi. We have used 4 different sgRNAs that target *obgE* and found that, after 4h of CRISPRi induction, LPS levels are significantly increased for 2 of them (sgRNA 398 and 643). For the other two sgRNAs, the observed increase was not statistically significant. When results from all sgRNAs are combined, LPS levels are significantly increased. These data are now shown in Figure 7F (also shown below). Together with our additional data on the LpxA-dependency of the synergy between wt ObgE and LPS inhibitor PF-04753299, these findings further demonstrate the involvement of wt ObgE in the regulation of LPS synthesis in *E. coli*. We believe that, with these additional experiments, our claim on the role of wt ObgE in LPS synthesis is now sufficiently supported.



6. To gain insight in the biological relevance of studying this mutant, the reviewer suggests authors perform bioinformatic analysis on both ObgE and LpxA broadly in different strains/species to check if such mutations exist in both LpxA and ObgE.

We performed a sequence alignment of Obg and LpxA representatives in *Gammaproteobacteria*, specifically focusing on the region surrounding the position of the "Obg* mutation" (K268) in the G domain of ObgE and the cluster of resistance-conferring residues of LpxA (V197, I199, R216). This shows that these positions are very highly conserved in *Gammaproteobacteria* (see below). This is not surprising, since it would be highly unlikely that bacteria would select for a mutation that is highly toxic to the cell (Obg*) and that can only be neutralized by mutations in a highly conserved region of the essential *lpxA* gene. We therefore do not mean to claim that the ObgE* mutant is biologically relevant in and of itself. Rather, we have used it as a tool to gain insight into the functions of wild-type ObgE. As detailed in our revised manuscript and the responses to reviewer comments, we believe that we now provide sufficient evidence to implicate wild-type ObgE in the regulation of LPS synthesis.



Sequence conservation in the region surrounding the position of the "Obg* mutation" (K268) in the G domain of ObgE (*left*) and the cluster of resistance-conferring residues of LpxA (V197, I199, R216) (*right*). K268 in ObgE and V197, I199 and R216 In LpxA are indicated with *. The multiple sequence alignments were performed with BLAST, using *E. coli* ObgE (UniProt code: P42641) and LpxA (Uniprot code: P0A722 P0A722) as queries to obtain at least 100 non-identical protein sequences. The conservation analysis was performed using the iBIS2Analyzer webserver (Oteri et al., 2022, NAR).

7. The reviewer found the last section of the CRISPRi experiments could be moved earlier in the section where LpxA suppressors were described since these results provided with no further depth in our understanding in the biological significance of LpxA and ObgE interaction.

We indeed restructured our results section, also to more logically include all additional experiments performed. We now put all results on the link between wt ObgE and the cell envelope together (CRISPRi screen, sensitivity to PF-04753299 and ObgE depletion experiments). This section starts at line 449: "Wt ObgE is involved in the regulation of cell envelope synthesis through LpxA".

8. Line 622-631 (now line 566-575). As explained in 5, evidence generated does not conclusively support the claim that the WT ObgE-LpxA interaction observed here is for regulation of LPS production. The observation based on ObgE*-LpxA interaction does not necessarily apply for the WT ObgE-LpxA. LPS synthesis is mainly controlled at the committed step level targeting LpxC, regulated by FtsH, LapC, LapB etc. It is unclear on the importance of having another regulation system as such to control LPS synthesis at non-committed step LpxA.

We agree with the reviewer that this part of our discussion section is speculative. Nonetheless, we still want to highlight the striking fact that – although all possible LpxA variants were interrogated – we failed to obtain suppressor variants in which the interaction between ObgE and LpxA is abolished. This observation definitely opens up the possibility of this interaction being important for viability. Further research will have to confirm or reject this hypothesis but so far all our experiments, including the additional assays performed as part of the revisions (outcome: ObgE and PF-04753299 synergy was found to be dependent on LpxA and ObgE depletion increases LPS levels), allow for this possibility. To make the reader fully aware of the speculative nature of this paragraph, we included the phrasing "Although further investigation is necessary to draw strong conclusions, it is tempting to speculate that…".

9. It might be interesting to investigate the detailed interface between mutant ObgE* and LpxA to design short peptide derived therapeutics targeting LpxA in inhibiting bacterial growth.

The possibility of inhibiting LpxA activity by mimicking the interaction with GTP-bound ObgE* is highly interesting. We have therefore initiated work on this subject and will, in a first step, indeed characterize the structure of the complex and try to create short peptides that block LpxA activity in a way similar to GTP-bound ObgE*. However, this is a substantial amount of work that requires a lot of testing and optimization and therefore constitutes a study on its own. We therefore believe that this interesting suggestion is out of scope of the current manuscript.

Minor comments:

1. Line 68-69: The minimal structure of LPS is not KDO¬2-Lipid (IV)A, as waaA has been shown can be successfully deleted without suppressor mutations, albeit cell grow at lower temperature (PMID: 19346244), therefore minimal structure of LPS for the survival of Gram-negative bacteria is Lipid IV(A) precursors.

We have added this information at line 57: "The minimal LPS structure that is required for E. coli survival under standard growth conditions is the Kdo-modified lipid IVA (Brabetz et al., 1997; Whitfield and Trent, 2014), while at low temperatures, E. coli can survive with only lipid IVA (Klein et al., 2009)."

2. Line 117-118: RcsF-dependent triggered colanic production is temperature dependent, please specify the temperature under which the cells were grown for RNAseq analysis.

As stated in our Materials & Methods section, all experiments were performed at 37°C unless mentioned otherwise. We have now also explicitly repeated this information in the RNA-seq methods section at line 687.

3. Line 272: LPS inhibitor PF-04753229 is first mentioned here, please move the explanation of its molecular target here from line 336.

We have moved this information to the suggested location, which is now line 233.

4. Line 321-329: The reviewer suggests reducing the length of this section to improve conciseness. Since restoration of O antigen was not done in K-12, rather tested in another E. coli producing different Lipid-A-core and O antigen, hence modify the wording in line 328-329: therefore "likely" not explain the toxic ObgE* phenotype.

We shortened this section and have included the word "likely" as suggested by the reviewer. In our revised manuscript, this section can be found at line 413.

5. Line 351-352: Vector control was used as a baseline (normalized to be 1.00) to transform dataset therefore lost the data variation for Vector control group. However, statistics were performed with multiple comparisons test against the vector control group, which is inappropriate, as the standard deviation of this group is lost.

We have now changed our analysis to one-sample t tests to check which samples produce a relative LPS content that significantly deviates from one. Importantly, this correction of our statistical analysis did not change our conclusions as *obgE** expression still significantly affects LPS content in an *E. coli* wt background but not in selected *lpxA* mutants. This information has been corrected in the legends of Figure 6 and S7.

6. Figure 7A, light grey colour on white background is hard to read, please consider changing to give better contrast.

We have changed this figure panel, which is now Figure 6A, and switched to a much darker shade of grey to increase the contrast.

7. Line 528, This is misleading, these were observed when ObgE were ectopically over-expressed (Figure 4C). hence "this GTPase, was shown to interact with LpxA in vitro and when supplied in excessive amount, can sensitise cells to LPS inhibition."

We have changed the text accordingly. Line 451: "this GTPase was shown to interact with LpxA (both *in vitro* and upon overexpression *in vivo*) and can sensitize cells to LPS inhibition"

8. Line 598 (now line 543): 1,000-fold not 1.000-fold

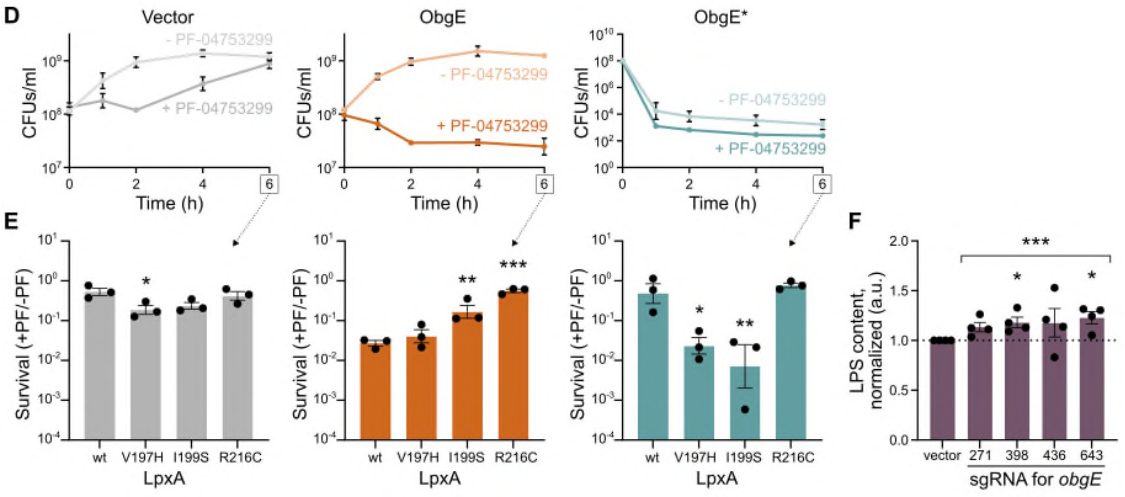
We have corrected this error.

Reviewer #3

In the manuscript titled "The essential GTPase ObgE regulates lipopolysaccharide synthesis in Escherichia coli" Dewachter et. al. describe a novel regulatory interaction between LpxA, the first enzyme in LPS biosynthesis, and ObgE, a conserved GTPase. LPS biosynthesis is thought to be tightly regulated largely at the committed step in the pathway, which is catalyzed by the enzyme that acts immediately downstream of LpxA. Recent work, however, has highlighted the potential for LpxA to also serve as a regulatory node. Here, the Dewachter and colleagues characterize a previously isolated dominant lethal allele of ObgE, ObgE*, and provide compelling genetic and biochemical evidence that this variant inhibits the activity of LpxA to reduce LPS biosynthesis, thereby inhibiting growth.

Overall, the work presented by Dewachter et. al. convincingly demonstrates that ObgE has the capacity to regulate LPS biosynthesis at the level of LpxA, which is a critical enzyme in gram-negative bacteria whose regulation is poorly understood. However, all of the presented experiments are performed in the context of artificial overexpression of ObgE* and thus, the biological relevance of this regulation is unclear. The work would greatly benefit from some analysis or commentary on the phenotypes associated with deletion/depletion of obgE. Specifically, are there any phenotypes that would be expected to be associated with increased activity of LpxA (accumulation of LPS, increased resistance to PF-04753299, stabilization of LpxC)?

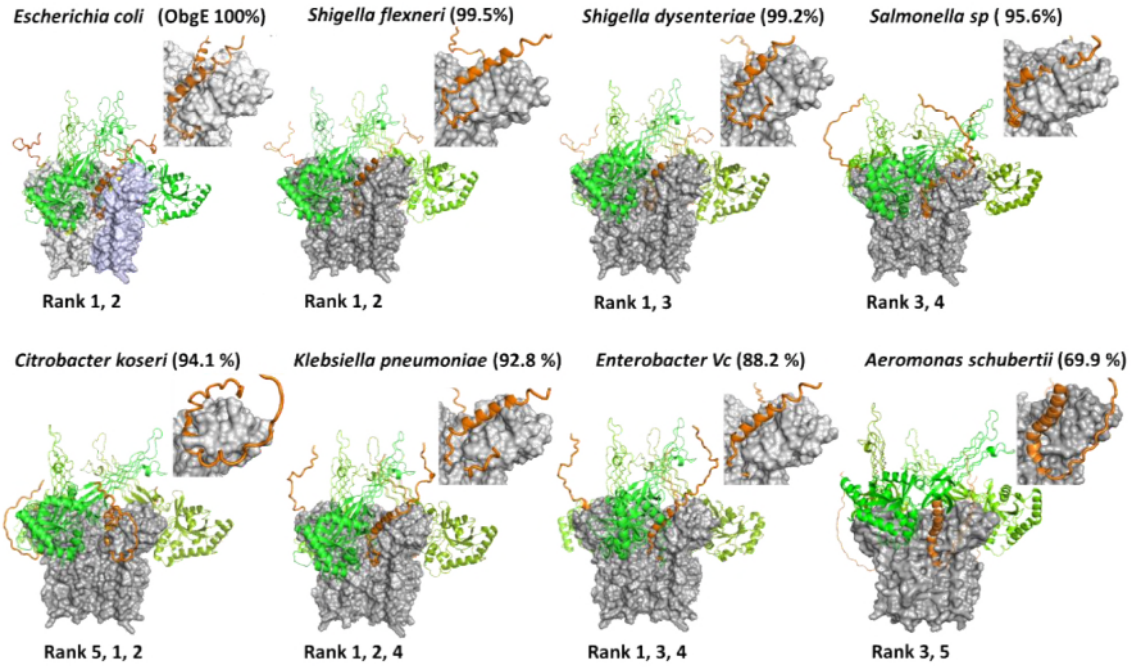
We want to thank the reviewer for the time dedicated to our manuscript and the helpful suggestions. To further demonstrate the biological relevance of our findings, our revised manuscript now contains some additional experiments that strongly support a role for wt ObgE in regulating LPS synthesis through LpxA *in vivo*. First, we show that the synergy we previously observed between overexpression of wt *obgE* and the LPS inhibitor PF-04753299 depends on LpxA as it can be counteracted using the LpxA variants that also provide resistance to ObgE*. Moreover, we have depleted *obgE* expression by CRISPRi using 4 different sgRNAs that target *obgE* and found that, after 4h of CRISPRi induction, LPS levels are significantly increased for 2 of them (sgRNA 398 and 643). For the other two sgRNAs, the observed increase was not statistically significant. These data are now shown in Figure 7E and F (also shown below) and further support the involvement of wt ObgE in the regulation of LPS synthesis in *E. coli*.



Aside from this major comment, I offer additional minor comments for the authors to consider:

1.Given the conservation of LpxA and ObgE, is there evidence that this mechanism of regulation could be conserved in other organisms? Does Alphafold predict an interaction between the proteins from other bacteria?

As suggested by the referee, an additional AlphaFold multimer modeling was performed of complexes formed between several representatives of homologues of Obg and LpxA belonging to the class of the *Gammaproteobacteria*. This analysis shows that in all tested cases a structure very close to Model 1 is obtained among the top 5-ranked predictions. This suggests that the binding of Obg to LpxA is more widely conserved among the *Gammaproteobacteria*. These data are now included in a new supplementary figure:



UNIPROT codes for all sequences used.

	Obg	LpxA
Escherichia coli	P42641	P0A722
Shigella flexneri	Q0T0A1	Q0T828
Shigella dysenteriae	Q32BF0	Q32JS8
Salmonella sp	WP_192513500.1	B4TYE1
Citrobacter koseri	A0A381GXD2	A0A381H7L8
Klebsiella pneumoniae	A6TEK2	A6T4Y3|
Enterobacter Vc	WP_220267381.1	WP_220267606.1
Aeromonas schubertii	A0A0W7TWA3	A0A0W7U3W8

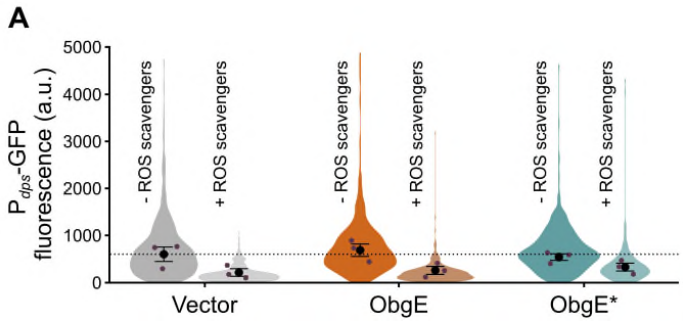
Figure S5: AlphaFold models of the complexes formed between representatives of Obg and LpxA from the class of *Gammaproteobacteria*. The sequence identity between the respective Obg proteins and the *E. coli* ObgE is indicated in between brackets. In all cases, binding modes that are very similar to Model 1 are obtained among the top 5 ranked models (the rank of the models similar to Model 1 are indicated below each structure model). The insets show a close-up view of the interaction between the C-terminal tail of Obg and LpxA.

2.Line 185-186 – “ObgE* negatively affects L-form growth and strongly inhibits their proliferation”. Please define how “successful divisions” are defined in these experiments.

This important information is now added to our materials & methods section at line 749: “A successful L-form division was defined as a fission event where at least one of the progeny L-forms was able to survive for minimum 20 min after the division event.”

3.Fig S2AB – it is challenging to interpret the reduction in ROS levels in the ObgE* background upon treatment with ROS scavengers. This could be remedied by combining these two graphs and providing “N” values for each strain/condition.

We have followed this suggestion and have plotted the data with and without ROS scavengers onto the same graph (Figure S2A, see also below). We believe this visualization clearly highlights the difference in P_{dps} fluorescence found in the presence and absence of ROS scavengers across the different conditions. We have adjusted the figure legend and any references in the results section accordingly.



4.Line 270 (now line 231) – “Similarly, LPS synthesis is significantly decreased in E. coli lpxAR216C..” Can the authors comment on why LPS levels appear reduced in the R216C mutant in fig S3C but not in S4A?

Because of the growth defects, changes in morphology and increased sensitivity to PF-04753299 and vancomycin, we are convinced that the *lpxA*$_{R216C}$ mutation indeed leads to subtle defects in LPS synthesis, as Figure S3C shows. However, we have also noticed the discrepancy with results shown in Figure S7A (previously Figure S4A). Unfortunately, we currently don’t know where this difference comes from.

All data points shown in Figure S3C and S7A stem from different biological repeats that were prepared and sampled on different days (although afterwards, samples were sometimes analyzed simultaneously by running on the same gel or on different gels at the same time). We are therefore confident that the observed differences are not due to carelessness or faulty experimental design on our part. We can, however, not exclude that they are due to noise and random chance.

Alternatively, it is possible that small differences between the samples and experimental protocols can explain our observations. An obvious difference between both samples is that when assessing the influence of *obgE(*)* expression on LPS levels (Figure S7A), cells contain the pBAD33Gm plasmid and are induced with arabinose for 2 hours. In contrast, data presented in Figure S3C are obtained using cultures that were never exposed to arabinose and do not carry any plasmids. However, if and how the pBAD33Gm plasmid and/or arabinose would influence LPS synthesis in the *lpxA*$_{R216C}$ mutant is unclear.

5.Table 2 and figure 5 seem redundant.

All information displayed in Table 2 is indeed also captured in Figure 4 (previously Figure 5). We have therefore removed Table 2 from our revised manuscript.

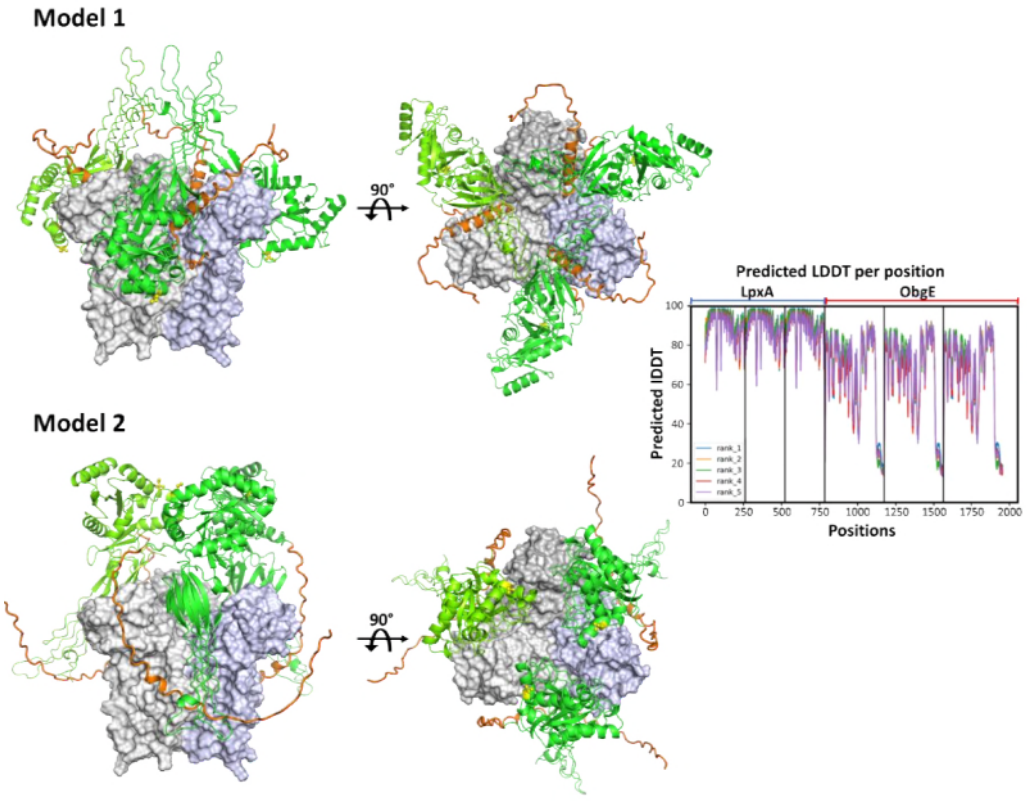
6.Table2/Figure 5 - Why was ObgE without any added nucleotide tested for its ability to interact with LpxA?

Because of the high cellular concentration of guanine nucleotides *in vivo* throughout all different growth phases, GTPases such as ObgE are thought to always be associated with nucleotides and to adjust their conformation and activity based on the bound nucleotide (GTP, GDP, ppGpp). Because of these reasons, we have not tested apo ObgE in the assays shown in Figure 4 (previously Figure 5).

To test the effect of ObgE and ObgE* on LpxA activity, we initially optimized our assays in the absence of any nucleotides. We realize now that showing these results is inconsistent with the conditions used to test the interaction and may be confusing to the reader. We have therefore omitted the effects of apo-ObgE and ObgE* on LpxA activity from Figures 6 and S6 and now only show how nucleotide-bound ObgE and ObgE* influence enzymatic activity.

7.Figure S5 – please label the regions of the lDDT plots that correspond to the LpxA/ObgE sequences.

Figure S4 (previously Figure S5) has been changed accordingly:



8.Line 417-418 – “Considering that in model 1 the G domain of ObgE*, which contains the K268I mutation, is involved in the interaction with LpxA, we regard the latter model as the most biologically relevant” Should this be the former model?

We thank the reviewer for spotting this mistake. We have now adjusted the text. Line 325: “we consider model 1 also as the most biologically relevant”

9.Lines 564-565 – “show that expression of only a small number of genes” this should be expression of a small number of sgRNAs or altered expression of a small number of genes. Similarly for lines 568-569.

To be more correct, we have adjusted the text at both locations. Line 480-481: “only a small number of sgRNAs are depleted. These few sgRNAs correspond to genes whose expression is important for proliferation when ObgE is present in excess.” Line 484-486: “On the other hand, a large number of sgRNAs was found to be significantly enriched, meaning that inhibiting the expression of corresponding genes increases fitness upon *obgE* overexpression.”

10. Methods – oligo sequences used in the process of this study should be provided.

A supplementary table (Table S3) containing oligo sequences used in this study is now included.

RESPONSE TO REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

As in the original report, Dewachter et al. provide good evidence that the toxicity caused by ObgE* is due to inhibition of LpxA. While the revisions have addressed many of my concerns, I still find evidence suggesting that wildtype ObgE inhibits the activity of LpxA to be conflicting. In support of the author's hypothesis, wildtype ObgE interacts with LpxA both in vivo and in vitro. Furthermore, overexpression of ObgE increases sensitivity to an LpxC inhibitor, and this sensitivity can be ameliorated by mutations that prevent interaction between ObgE* and LpxA (but not wildtype ObgE and LpxA). The authors also show that decreasing ObgE expression can sometimes increase LPS levels, albeit only slightly. However, the authors show that overexpressing wildtype ObgE does not decrease LPS levels and that wildtype ObgE does not inhibit LpxA activity in vitro. Furthermore, the results of the author's CRISPRi screen show that reducing expression of LPS biosynthesis genes actually improves fitness in cells overexpressing ObgE. This particular result directly contradicts what the authors observe with the LpxC inhibitor. Both the LpxC inhibitor and sgRNAs targeting LPS biosynthesis genes should decrease enzyme activity/expression, and one would expect these data to show similar results. The results would be more convincing if the authors could identify a condition where wildtype ObgE strongly and consistently affects LPS and LpxA, much like ObgE*. Otherwise, it is entirely possible that ObgE* is a neomorphic mutation that has gained new capabilities compared to the wildtype (much like MlaA* and WaaL15, as mentioned by another reviewer).

We thank the reviewer for their careful consideration of our revised manuscript and the valid points they raise. Indeed, we realize that our results obtained with wt ObgE are not as straightforward to interpret. Like the reviewer, we too would have expected that our CRISPRi screen results would point to additional fitness defects when LPS biosynthesis genes are depleted while *obgE* is overexpressed. While we currently do not fully understand these findings, we do want to highlight the fact that also KEGG pathways “fatty acid biosynthesis” and “peptidoglycan biosynthesis” were identified as hits for which sgRNAs are significantly enriched (i.e. downregulating these pathways is beneficial upon *obgE* overexpression in stationary phase). LpxA is known to act as a hub between fatty acid, peptidoglycan and LPS biosynthesis. Affecting its activity may therefore have complex influences on all three pathways. We hope to further elucidate these intricate connections (and the role that ObgE may play in them) in the future.

An important step towards this goal would be to identify a condition in which wt ObgE strongly affects LPS and LpxA, like the reviewer suggests. However, so far we have remained unsuccessful (overexpression in exponential and stationary phase, and depletion in exponential phase). Our *in vitro* data do not show an influence of wt ObgE on LpxA activity. A possible explanation could be that, given the significantly lower binding affinity of wt ObgE to LpxA, we cannot reach sufficiently high ObgE concentrations in the *in vitro* assay to observe inhibition. Alternatively, an additional *in vivo* (protein) factor may be needed to manifest the *in vivo* effects we have observed so far (CRISPRi screen results, wt ObgE + PF-04753299 effect neutralized by *lpxA* suppressors, and CRISPRi depletion of ObgE increases LPS levels). We hope to exploit our CRISPRi screening results to identify this cellular factor in the future, but we would like to argue that this is not feasible to include in a reasonable timeframe in the current manuscript.

Nonetheless, we believe that with the additional data we provide in our revised manuscript, we have again strengthened the support for a role for wt ObgE in regulation of LpxA activity and LPS synthesis (see below). We therefore believe that it is justified to highlight this potential role of ObgE in our manuscript, although we also cautiously inform the reader that future work is needed to further firmly establish wt ObgE as a regulator of LpxA activity.

Major comments:

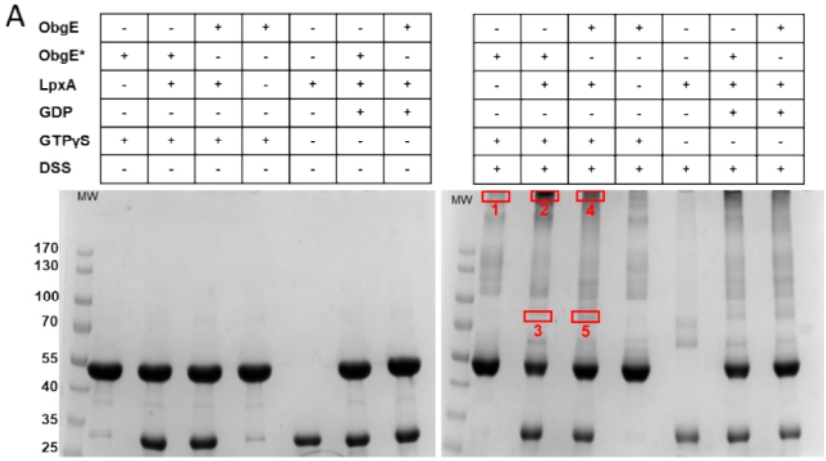
Line 497-498: The authors show that the lpxA suppressor mutations do not affect the interaction between wildtype ObgE and LpxA. However, the lpxA mutations can rescue the ObgE overexpression strain from the LpxC inhibitor. Can the authors explain why they think the lpxA mutations rescue the sensitivity?

To address the concern of the referee about the interaction between wild-type ObgE and LpxA, and the influence of the LpxA mutants on this interaction, we performed two additional experiments.

First, we again confirmed that LpxA does interact with both ObgE* and wild-type ObgE using a crosslinking experiment followed by MS^2 analysis of the crosslinked protein bands. The results of this experiment are shown in the figure below, which is also inserted as a Supplementary Figure S4 in the revised manuscript. As can be seen on this figure, SDS-PAGE analysis of samples after crosslinking of LpxA with either ObgE* or wild-type ObgE (in presence of either GDP or GTPγS) with the lysine-reactive crosslinker DSS shows the appearance of a number of bands that are not observed in controls without crosslinker or in controls where only one of the two proteins is present. The fact that multiple bands are observed after crosslinking is due to the homotrimeric nature of LpxA, allowing either one, two or three copies of ObgE to crosslink with the internally crosslinked LpxA trimer. Further tandem MS analysis of the bands indicated with a red box indeed confirms the presence of both ObgE(*) and LpxA as the major species in these bands.

In the revised manuscript the following text is added (line 280-283): "The ObgE-LpxA interaction that was detected *in vivo* by BACTH was subsequently confirmed *in vitro* via chemical crosslinking mass spectrometry (XL-MS) using the purified LpxA and ObgE or ObgE* proteins and using the amine-reactive crosslinker disuccinimidyl suberate (DSS) (Figure S4), and was subsequently quantified via Microscale Thermophoresis (MST) (Figure 4C-F)."

A



B

Protein composition	MW [kDa]	#Peptides	SC* [%]	FragCov** [%]
Band 1				
ObgE	45,4	19	51	18,3
Band 2				
ObgE	45,4	14	38,5	13,7
LpxA	30,2	9	31,2	2,8
Band 3				
ObgE	45,4	16	40,2	23,2
LpxA	30,2.	5	21,3	2,5
Band 4				
ObgE	45,4	21	50,5	23,2
LpxA	30,2	12	35,8	2,5
Band 5				
ObgE	45,4	19	46,8	22,9
LpxA	30,2	8	28,4	3,9

*Sequence Coverage
** Fragment Coverage

Figure S4: Crosslinking and MS analysis of the ObgE-LpxA and ObgE*-LpxA complexes. A) SDS-PAGE analysis of the complexes formed between LpxA and either ObgE or ObgE* after crosslinking with disuccinimidyl suberate (DSS). The analysis was performed with ObgE/ObgE* either bound to GDP or GTPγS. Crosslinking of the individual LpxA, ObgE and ObgE* proteins was used as a control. As a reference also the corresponding non-crosslinked samples were analyzed on SDS-PAGE (left gel). The bands indicated with a red box were excised for MS analysis. B) Table summarizing the results of the MS analysis of the bands excised from the SDS-PAGE shown in panel A (red boxes). The bands were excised, and the proteins digested with trypsin according to the proteaseMax manufacturers protocol and analyzed with MALDI-TOF/TOF.

Additionally, we also optimized the MST analysis to measure the interaction between wild-type ObgE and the LpxA I199S and R216C mutants. Considering the low binding affinity, we had to increase the LpxA concentration that was used in the titration. The results of this experiments are shown in the figure below, which is now also added as an additional panel of Figure 4 in the revised manuscript. Similar to what was found for ObgE*, this analysis shows that both LpxA mutations negatively impact the binding affinity for wild-type ObgE. While the effect of the LpxA mutations on wild-type ObgE binding is much less pronounced than for ObgE*, a similar trend is observed, with the I199S mutation having a smaller impact than the R216C mutation. This forms another very strong indication that wild-type ObgE and ObgE* bind in a similar fashion to LpxA.

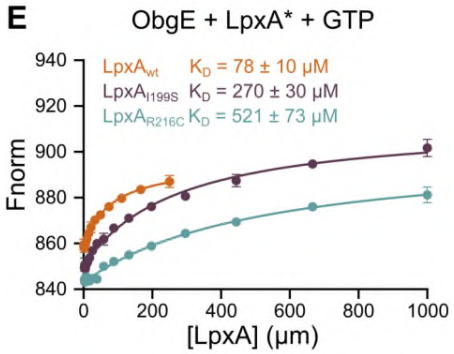


Figure 4E: Microscale thermophoresis (MST) was performed to measure binding affinities between wt ObgE and mutant LpxA proteins in the presence of GTPγS

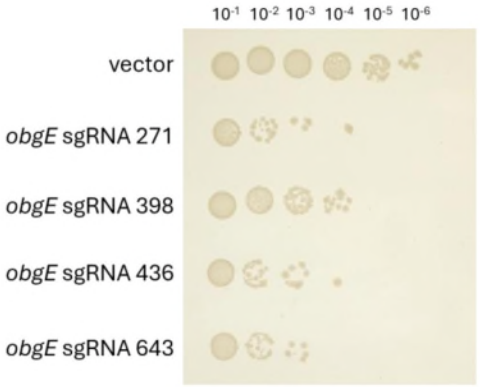
In the revised manuscript the following text is added (line 297-299): "A similar trend in the effect of the LpxA mutations is observed for the interaction with wt ObgE, albeit much less pronounced. For GTPγS-bound wt ObgE the K_D value increases from 78 ± 10 µM for wt LpxA to 270 ± 30 µM for LpxA$_{I199S}$ and 521 ± 73 µM for LpxA$_{R216C}$."

Line 506-507: The effect of decreasing obgE expression on LPS levels is weak and is observed with only 2 out of the 4 sgRNAs. Are obgE levels decreased to the same extent with all four sgRNAs or is there a greater decrease with the sgRNAs that show increased LPS levels? Would the authors expect similar results if targeting a known LPS inhibitor like LapB or FtsH?

sgRNAs targeting *obgE* were selected based on fitness results from an unrelated CRISPRi screening (see below). We selected sgRNAs that were strongly and significantly depleted from the population, indicating that expression of the essential ObgE protein is severely hampered.

sgRNA	Log_2 Fold Change	Adjusted p-value
obgE 271	-4,13601	5,31E-39
obgE 398	-5,22383	1,30E-65
obgE 436	-4,58124	2,92E-70
obgE 643	-4,44858	2,10E-22

We next verified that these sgRNAs indeed affect viability – and therefore likely deplete ObgE – with a spot assay on medium carrying the inducer of the CRISPRi system (aTc 100 ng/ml for *dcas9* expression) prior to using them for our LPS-related assays (see below).

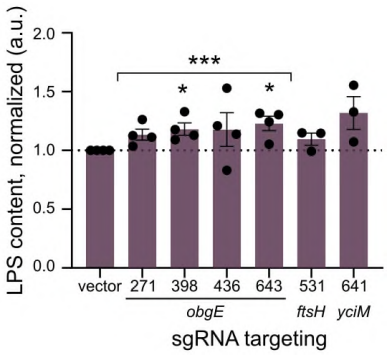


Based on these assays, it appears as though all sgRNAs affect *obgE* expression similarly (measured as effects on fitness and survival). Whereas sgRNA 398 allows for slightly more colony formation on plate (spot assay), it is depleted more strongly (Log2FC CRISPRi screen) and is therefore likely more or less equally effective.

As suggested, we have now also measured LPS levels upon CRISPRi depletion of known LPS inhibitors FtsH and LapB/YciM. To do so, we have used the same conditions (induction of *dcas9* for 4h after allowing cultures to enter exponential phase). Hereto, we have selected sgRNAs targeting these genes that were shown to be highly effective:

sgRNA	Log_2 Fold Change	Adjusted p-value
ftsH 531	-8,34572	7,09E-14
lapB/yciM 641	-4,11817	2,10E-13

The levels of LPS measured are displayed below and are also incorporated into our revised manuscript (Figure 7F). As can be seen, the changes detected upon depletion of the known LpxC inhibitors FtsH and YciM/LapB are very much in line with what is observed for depletion of ObgE.



This information has also been added to the text at line 514: "Under the same conditions (activation of CRISPRi for 4 hours starting in exponential phase), targeting the known LPS inhibitors FtsH or YciM leads to increases in LPS levels that are highly similar to what is observed for *obgE* depletion (Figure 7F). Because decreasing cellular ObgE levels appears to increase LPS production, these results indicate that wt ObgE might be capable of negatively regulating LPS production like ObgE* does. Collectively, our results therefore support a role for wt ObgE in the regulation of LPS

synthesis through modulating LpxA activity, although further research is needed to firmly establish this type of LPS regulation."

We realize that the effects of depletion of ObgE on LPS levels are relatively weak, but want to point out that they are highly similar to what is observed for well-established LpxC inhibitors FtsH and YciM. We believe that LPS levels are not more strongly influenced because of the relatively short period of depletion. However, we are very hesitant to extend the time of depletion for *obgE* because we know from personal experience and literature that a severe shortage of ObgE leads to a variety of pleiotropic phenotypes (with effect on DNA replication, chromosome segregation, stringent response, ribosome maturation and activity, dormancy, etc.). In addition, because of the essentiality of ObgE function, suppressor mutations often arise after extended periods of depletion. Because of these reasons, we believe that the 4 hour time point of CRISPRi depletion shown here and included in our manuscript, represents the most appropriate conditions to examine the effect of ObgE depletion on LPS synthesis.

Minor comments:

Line 69 - 70: Here, the authors state that LpxA is not thought to be regulated. In line 612, the authors mention that LpxA is regulated by RnhB and ppGpp.

We have now removed this inconsistency and already mention the recent study on the effect of RnhB and ppGpp on LpxA activity in our introduction. Line 69: "LpxA, on the other hand, was widely believed not to be subjected to any kind of regulation. However, recent work demonstrated that LpxA activity can be altered by RnhB and ppGpp, thereby indicating that additional levels of regulation in LPS synthesis exist."

Figure 7E: The authors show that the lpxA point mutations do not rescue cells overexpressing ObgE* from the LpxC inhibitor. In fact, two of these lpxA mutations appear to increase sensitivity. Can the authors explain why this may be the case?

The CFU results for *obgE** expression in *lpxA* wt cells in the presence of the LpxC inhibitor are slightly lower than those without inhibitor, pointing to synergy. However, we believe that the true synergistic effect of ObgE* with PF-04753299 in the *lpxA* wt background is far stronger, but cannot be accurately measured because the survival rate is lower than the mutation rate. When plating cells after induction of *obgE**, most colonies that are obtained contain promoter or stop codon mutations that prevent *obgE** expression. The formation of these mutant colonies are unaffected by the addition of sub-MIC levels of PF-04753299 and therefore distort the outcome of this assay. Because CFUs upon *obgE** expression are drastically increased in the *lpxA* mutant backgrounds, mutations that prevent *obgE** expression are irrelevant and the actual level of synergy can become apparent. Although we have no way of accurately measuring the level of synergy between ObgE* and PF-04753299 in *lpxA* wt cells, we expect that it is stronger than what is obtained for the *lpxA* suppressor mutants.
To make the reader aware of these confounding effects we have added a cautionary sentence at line 500: "As expected, the addition of PF-04753299 increases toxicity caused by ObgE*, albeit only slightly likely because the detection of synergy is limited by the emergence of suppressor mutants that no longer express *obgE** (i.e. frameshifts, promoter and/or stop codon mutations)."

Reviewer #2 (Remarks to the Author):

Upon revision, authors have provided more experiments in supporting their claim that wild-type ObgE interacts with LpxA affecting LPS synthesis. Two additional experimental results (FIGURE 7E and 7F) are critical for the scientific field in understanding this reported interaction between ObgE and LpxA. Figure 7E showed overexpression of ObgE in WT, but not in two ObgE* suppressors, sensitises towards lpxC inhibitor, suggesting that the sensitisation to LpxC inhibitor by ObgE overexpression is LpxA dependent, indirectly supporting the claim that WT ObgE interacts with LpxA to impact LPS synthesis. Figure 7F showed that altering ObgE levels have effects on LPS levels. Since these two experiments are critical in understanding ObgE function in regulating LPS synthesis by interacting with LpxA. The reviewer strongly suggests including raw data of these two experiments with the manuscript. This could be raw CFU counts for Figure 7E, as shown in Figure 7D across time points, or by serial dilution plating in the presence or absence of LpxC inhibitor.
Please also include raw LPS images used for LPS quantification in Figure 7E.

> We want to thank the reviewer for their careful evaluation of our work, leading to these final suggestions. Regarding the first one (include raw CFU counts for data presented in Figure 7E), we opted to display relative CFU counts (i.e. survival) in our figure because, in our opinion, these are easier to interpret. The message we want to convey with this figure is that the synergistic effect of wt *obgE* overexpression and the LPS inhibitor PF-04753299 is counteracted by some *lpxA* suppressor mutations. We therefore compare the CFUs obtained in the presence of the PF-04753299 compound to the appropriate control without PF-04753299. Because CFU values for the control (without PF-04753299) vary between conditions, we feel that it is more difficult to interpret these absolute CFU values (see below).
> However, we are very much in favor of sharing all raw data. These CFU values (and all other data related to this manuscript) are therefore included in our source data file that is submitted to Nature Communications and that will be made available to all readers upon publication.
> Likewise, the gel images used for LPS quantification, including those presented in Figure 7E, are also part of our source data. We apologize if anything went wrong with uploading this source data file for evaluation by the reviewers.

Reviewer #3 (Remarks to the Author):

The revised manuscript by Dewachter et. al. has added critical evidence to support their proposed model in which wt ObgE regulates LpxA and have addressed all of my other previous concerns.

> We want to thank the reviewer for their valuable input that helped to improve the quality of our work to the level where it is today.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

I am satisfied with the additional experiments performed. These experiments provide further support for the authors arguments.

> Again we want to express our gratitude to the reviewers who have helped to strengthen our manuscript. We understand that all concerns have been addressed.

Reviewer #2 (Remarks to the Author):

Upon revision, the reviewer feels that previous requests have been addressed and additional MS data further supports the interactions between ObgE and LpxA. The reviewer however still feels that this WEAK interaction originated from mutant ObgE observations lacks biological significance/relevance and having a tendency to be interpreted as an artifact. Therefore, the reviewer suggests with no further experiments, but insists modifications to the title to: "The essential GTPase ObgE bare the potential to influence lipopolysaccharide synthesis in Escherichia coli", or similar to reflect their findings accurately. Where appropriate, main text as well.

> In correspondence with the suggestion of the reviewer and editor, we have changed the title of our manuscript. Throughout our manuscript, we have tried to make it clear that further research is needed to firmly implicate ObgE in the regulation of LpxA activity under physiologically relevant conditions. We understand that by doing so, all concerns have now been addressed.