EMBO reports

A bacterial toxin-antitoxin system involved in an unusual response to genotoxic stress

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Dear Dr. Ensminger,

Thank you for the submission of your manuscript to EMBO reports. I have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of interest. However, they have several comments, concerns, and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all the referee concerns need to be addressed, I will not detail them here.

Given the constructive referee comments, I would like to invite you to revise your manuscript with the understanding that the concerns of the referees must be addressed in the revised manuscript and in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. Please contact me to discuss the revision (also by video chat) if you have questions or comments regarding the revision, or should you need additional time.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

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4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines:
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See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study. This section is mandatory. As indicated above, if no primary datasets have been deposited, please state this in this section

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We now request the publication of original source data with the aim of making primary data more accessible and transparent to the reader. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

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: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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15) Please make sure that all the funding information is also entered into the online submission system and that it is complete and similar to the one in the acknowledgement section of the manuscript text file.

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I look forward to seeing a revised form of your manuscript when it is ready.

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The ms by Lin & Ensminger describes the characterization of a particular toxin-antitoxin system (TA) in L. pneumophila that, upon genotoxic stress, trigger cell death. Under co-culture conditions, the TA-mutant cells enhance the survival of the wild-type cells through a mechanism that remains to be elucidated and requires cell-to-cell contact.

While I found the ms potentially interesting, there is a substantial number of questions and issues that must be resolved. Importantly, the GndRX system is homologous to the Pseudomonas aeruginosa NatRT system (Santi et al., EMBO J, 2004) (this information appears only in the discussion). Both systems share several characteristics: they are part of the core genome; the toxins are not toxic by themselves, and their activity is similar (depletion of NAD). I believe that this should be clearly stated early on in the introduction. The NatR antitoxin and NatT toxin should be included in the sequence and structure comparisons showed in figures 4 and S4.

The Jenal group showed that the NatR antitoxin appears to activate the NatT toxin, at specific levels. This should be tested with the GndRX system. Additionally, the construction of the gain-of-function mutation, NatTE29D, in the GndR toxin, assuming the conservation of the amino acid, and subsequent confirmation of the resulting phenotype, are recommended.

Figure 2. If I understand correctly, the authors are using 25 microgr/mL of CIP, which seems to be above the MIC (matching the guidelines to study persistence) - although the MIC of the 2 strains are not reported but can be inferred from figure S2. Classical MIC measurements should be performed.
Although the authors use the term 'persistence', the time-kill curve for the WT strain does not show a classical biphasic curve (the rate of killing seems to be constant). On the contrary, the delta TA mutant shows a biphasic curve, indicative of persistence. In figure 7, survival is measured at shorter time-points (up to 25h). In this graph, the curves are biphasic, and the survival is similar for the 2 strains. This is quite confusing and should be carefully interpreted. A possible interpretation is that the deletion of the TA system enhance survival during long-term treatment with CIP. The authors should provide the complete time-kill curve with appropriate timepoints; all along the duration of the experiments (Figure 2F and 7B bare difficult to compare due to different

Y scales).

Figure S2D. The authors tested another genotoxic. Mitomycin C appears to be significantly more potent than ciprofloxacin (time scale for killing the entire population is far shorter than with ciprofloxacin). No difference is detected in terms of survival between the 2 strains. How much is the MIC for mitomycin C? Is it similar for the 2 strains? In Figure S2E, the authors measure the OD at varying doses, however they should measure survival. I don't think they can conclude that there are dose-dependent differences in susceptibility only based on OD measurements.
Importantly, how the authors reconciliate the phenotype observed under ciprofloxacin and mitomycin C treatment? The HGA production appears to be higher for the WT strain in mitomycin C than in ciprofloxacin. Do the authors have some explanation?

The RNA-Seq data should be presented differently. For instance, in Figure 5, more 'dots' should be assigned to gene names, especially those are the most extreme. More useful information can certainly be extracted and presented.

Figure S5. The graphs presented in the B panel should be overlaid. It is quite difficult to see the differences between the 2 strains. How do the authors explain the absence of difference when cells are treated in stationary phase? It seems that there is not much difference in the 'early' condition either. Possibly related to that is the data regarding the 9:1 ratio between the WT and the mutant, where the mutant 'loses' the capacity to survive better than the WT. Is it a question of cell density? This hypothesis should be tested.

Figure 7A and figure S7A. The OD are quite different between the 2 experiments. Could the authors comment on that?

Figure 7B. The survival is similar for both strains. However, the WT is lysing and about half of the population is PI-stained, while only 2% of the mutant cells are PI stained. It would be helpful to show the non-treated conditions to have a better sense of the gating used. Also, the LacZ test revealed that the mutant is lysing as well. Hower the OD remains constant. How to reconcile these observations? That OD measurement is a poor proxy?

Figure 7D. The intracellular concentration of NAD drastically drops in the WT cells, suggesting that they are metabolically inactive ('dormant'). However, the authors claim in the discussion (L483) that the mutant cells are in a dormant state that helps them to survive. It's a bit confusing. What is the definition of dormancy and the evidence supporting the hypothesis that dormancy helps to survive?

On an evolutionary point of view, what would be the advantage of selecting a TA system that is deleterious, integrating it in the host regulatory network and maintaining it? This system seems to be widespread in bacteria. This should be discussed.

Minor comments:

L62. Add references 44 and 45.

L105. Why is the repertoire 'reduced'? Not all the bacterial strains have 'a lot' of TA systems. E.g. The E. coli lab strain has 10 identified type II TA systems.

The phylogenetic tree presented in Figure S4 is unreadable.

Referee #2:

The manuscript describes the role of Legionella toxin-antitoxin systems in regulation of cell physiology. The experiments indicate unexpected effects: deletion of one of the TA systems decreases sensitivity to ciprofloxacin; the effect is regulated by cell to cell contact. The results are of high interest for the colleagues in the field and are also important for wider audience. The experiments are well planned and performed, the manuscript reads well.

There is one piece of experimental evidence that I find missing: what happens to NAD levels if you overexpress gndR? It is speculated in the manuscript that overexpression of gndR might influence NAD levels to the extent that does not effect growth. This would be relatively easy experiment to perform, considering that the authors have the experimental system set up.

As a very minor note, symbols are not visible in Fig 3B left. Please find a more clear graphical solution. In addition, the Fig.3B left has smaller variability of data when compared to the other datasets. Was the same number of replicates performed correctly?

I find the Discussion lengthy. The authors might consider shortening.

Referee #3:

The manuscript by Lin and Ensminger investigates toxin-antitoxin (TA) systems of Legionella pneumophila, the causative agent of Legionnaires' disease. The study documents the construction of a marker-less "pan-TA" L. pneumophila deletion strain lacking all 7 annotated TA systems and the characterization of stress responses of this mutant strain. Intriguingly, one of the 7 TAs, TA2 (Lpg1604-1605, GndRX), is activated specifically by genotoxic stress (ciprofloxacin, mitomycin C, UV) and promotes bacterial cell death rather than survival. The deletion of gndRX does not affect bacterial growth, host cell infection, or survival during translation and cell wall stress. However, a ΔgndRX mutant strain shows increased production of the metabolite homogentisic acid (HGA) and greatly enhanced survival (in a dormant state). These features are conferred to wild-type bacteria in a contact-dependent manner and are affected by the ratio of ΔgndRX mutant to wild-type bacteria. In contrast to canonical TA systems, the overproduction of GndR or GndX is not toxic for L. pneumophila or E. coli. GndR interacts with GndX (as shown by yeast two hybrid), likely binds NAD+ through its conserved RES (arginine, glutamine, serine) domain and promotes cellular NAD+ depletion upon the exposure to genotoxic stress. By promoting contact-dependent survival, GndRX reveals an interesting new function for bacterial TA systems.

The study represents an exciting analysis of a novel bacterial TA system with an unprecedented function. The thorough investigation comprises many appropriate controls, including an assessment of a "fixed" ΔgndRX mutant strain. The story unfolds in a straightforward, logical manner, and the well-written manuscript is actually a pleasure to read. A few minor amendments would further strengthen the study.

1) Fig. 4D: The asterisk mentioned in the figure legend (supposedly highlighting the conserved serine) is actually an arrow. Please fix.

2) Statistics: the calculation of the SEM should include at least 3 independent experiments. This is apparently not the case for Fig. 2DE, 6DE, 7B, S2ABD, S3ADE, S4DE, S5BC, and S7AB. Please show individual data points. Also, please indicate the overall number of experiments performed for Fig. 2G, S2C, S3A, and S5A.

3) Line 378 or Discussion section: please discuss "VBNC" L. pneumophila in more detail, e.g. PMID: 23968544, PMID: 39810465.

4) Line 445: possibly also cite PMID: 34314090.

We wish to thank the editor and reviewers for their thoughtful, constructive comments and careful consideration of our manuscript.

A point-by-point response to previous reviews is provided below:

Referee #1:

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While I found the ms potentially interesting, there is a substantial number of questions and issues that must be resolved. Importantly, the GndRX system is homologous to the Pseudomonas aeruginosa NatRT system (Santi et al., EMBO J, 2004) (this information appears only in the discussion). Both systems share several characteristics: they are part of the core genome; the toxins are not toxic by themselves, and their activity is similar (depletion of NAD). I believe that this should be clearly stated early on in the introduction. The NatR antitoxin and NatT toxin should be included in the sequence and structure comparisons showed in figures 4 and S4.

We thank the reviewer for their careful reading of our manuscript and constructive feedback, which has helped us to revise our work with additional experiments and analyses. We feel this has resulted in a much improved and more complete story. We provide point-by-point responses below.

The NatRT paper was published shortly before our manuscript was submitted and thus, we did not provide a more in-depth comparison in our initial submission. Our revised manuscript now includes the NatRT system for both the sequence and structure comparisons in Figures 4 and S4, as well as functional comparisons between GndRX and NatRT (detailed in the next section). We show that among the RES-Xre TA systems, GndRX is most closely related to the NatRT system, including the N-terminal ‘flap’ motif in the toxin protein, and both systems share little sequence similarity with the other RES-Xre systems. However, despite their overall structural similarity GndRX and NatRT are quite divergent homologs and we propose that critical functional differences underlie the distinct physiological roles that these systems perform.

Finally, we now include in-depth comparisons of the NatRT and GndRX systems in both the results and discussion sections on pages 9, 10, and 20.

The Jenal group showed that the NatR antitoxin appears to activate the NatT toxin, at specific levels. This should be tested with the GndRX system. Additionally, the construction of the gain-of-function mutation, NatTE29D, in the GndR toxin, assuming the conservation of the amino acid, and subsequent confirmation of the resulting phenotype, are recommended.

We thank the reviewer for recommending these experiments, as they have yielded key insights that we feel distinguish the GndRX and NatRT systems in a way that reflects the underlying differences in the physiological roles of these systems. To address the reviewer’s suggestions, we constructed a two-inducer co-expression system in *L. pneumophila* to compare the effect of varying the ratios of GndR and GndX on cell growth. This was done in the same manner as was used for NatR and NatT and showed only a minor effect on cell growth at the highest tested expression levels of GndR. These results are presented in a revised Figure 4.

Furthermore, the E29 residue is indeed conserved in GndR (E32) and we tested both ectopic expression and ciprofloxacin survival of the analogous mutation (E32D) in *L. pneumophila*. Surprisingly, in neither instance did we observe an effect on system function, suggesting that while the GndRX system is most closely related to NatRT, it has undergone additional functional divergence which supports our hypothesis of domestication of an ancestral TA system. We now present these experimental findings in revised Figures 4 and EV4.

Figure 2. If I understand correctly, the authors are using 25 microgr/mL of CIP, which seems to be above the MIC (matching the guidelines to study persistence) - although the MIC of the 2 strains are not reported but can be inferred from figure S2. Classical MIC measurements should be performed.

We have performed these experiments and provide the results in a new figure panel (Figure EV2B), as well as mentioning these values in the text on lines Page 6 (Lines 142-144):

"Consistent with this, we confirmed that the minimum inhibitory concentration (MIC) of ciprofloxacin was the same across strains (Figure EV2B) and that our treatment concentration (25 µg/mL) far exceeded the MIC (>100x)."

Although the authors use the term 'persistence', the time-kill curve for the WT strain does not show a classical biphasic curve (the rate of killing seems to be constant). On the contrary, the delta TA mutant shows a biphasic curve, indicative of persistence.
In figure 7, survival is measured at shorter time-points (up to 25h). In this graph, the curves are biphasic, and the survival is similar for the 2 strains. This is quite confusing and should be carefully interpreted. A possible interpretation is that the deletion of the TA system enhance survival during long-term treatment with CIP. The authors should provide the complete time-kill curve with appropriate timepoints; all along the duration of the experiments (Figure 2F and 7B bare difficult to compare due to different Y scales).

We thank the reviewer for these insightful observations and appreciate that the presentation of our time-kill assay data across two separate plots may have led to some confusion. Our interpretation is that both the WT and ∆7TA strains exhibit a classical biphasic kill curve along the conventional timescale of several hours. Subsequent to this, and after prolonged incubation with antibiotics, the strains diverge in their survival/viability. We are curious as to whether this is due to differences in cellular resuscitation from dormancy, for example, however the exact mechanisms underlying this fascinating phenomenon remain unclear. As requested by the reviewer, we now include experimental data from time-kill assays performed with appropriate time points across the full duration of the experiment in a panel Figure EV2A.

Figure S2D. The authors tested another genotoxic. Mitomycin C appears to be significantly more potent than ciprofloxacin (time scale for killing the entire population is far shorter than with ciprofloxacin). No difference is detected in terms of survival between the 2 strains. How much is the MIC for mitomycin C? Is it similar for the 2 strains? In Figure S2E, the authors measure the OD at varying doses, however they should measure survival. I don't think they can conclude that there are dose-dependent differences in susceptibility only based on OD measurements. Importantly, how the authors reconciliate the phenotype observed under ciprofloxacin and mitomycin C treatment? The HGA production appears to be higher for the WT strain in mitomycin C than in ciprofloxacin. Do the authors have some explanation?

We again thank the reviewer for the suggested experiments as we feel that the data from them has strengthened our manuscript. We now include MIC, broth growth, and survival measurements during mitomycin C (MMC) treatment for both the WT and Δ7TA strains in revised Figure panels EV2G/H/I. These data demonstrate that while the MIC for MMC is the same for both strains, growth and survival at or above the MIC concentration (as measured by paired growth curves and CFU counts) is not. This suggests that these two strains may differ in how they tolerate DNA stress rather than their intrinsic susceptibility to it. We feel that this new dose-dependent survival data is much more definitive than the OD measurements we showed previously, as the reviewer noted.

Regarding the relative HGA production for the WT strain between ciprofloxacin and MMC, we do not have a strong explanation for why this is occurring. If we were to speculate, perhaps it is the consequence of the difference in killing kinetics between these two drugs and thus the severity of DNA stress being experienced by the WT during MMC treatment relative to ciprofloxacin. In the case of the Δ*gndRX* genotype, HGA production is associated with enhanced cell survival and reduced NAD depletion. It may be the case that WT cells treated with MMC adopt a predominantly dormant rather than dying state (as with ciprofloxacin) or undergo less NAD depletion or reactive oxygen species production, the outcome of which is increased HGA production. However, future work will be required to establish a clear mechanistic understanding of how DNA stress is connected to HGA production in these strains.

The RNA-Seq data should be presented differently. For instance, in Figure 5, more 'dots' should be assigned to gene names, especially those are the most extreme. More useful information can certainly be extracted and presented.

We have now included more gene labels for the dots with the strongest signal on the volcano plot in Figure 5. Unfortunately, the *L. pneumophila* genome is not well annotated and there is limited functional information at the individual gene level for many of the most differentially enriched genes. Thus in many cases we can only include gene identifiers, however these will be of use to those in the field that are familiar with *Legionella* genetics. Furthermore, we have added the following lines in the results section on Page 12 (lines 303-308):

“Many of the differentially enriched genes are uncharacterized however, such as *lpg1327*, which showed the largest fold change during genotoxic stress but has no predicted function. Interestingly, the gene upstream of *gndRX* (*lpg1603*) was also highly enriched in the wild type relative to the deletion strain, suggesting a possible transcriptional coupling between these genes. The protein product Lpg1603 is a predicted phospholipase but is otherwise uncharacterized, thus it remains unclear whether this protein has any functional relationship with GndRX.”

Figure S5. The graphs presented in the B panel should be overlaid. It is quite difficult to see the differences between the 2 strains. How do the authors explain the absence of difference when cells are treated in stationary phase? It seems that there is not much difference in the 'early' condition either. Possibly related to that is the data regarding the 9:1 ratio between the WT and the mutant, where the mutant 'loses' the capacity to survive better than the WT. Is it a question of cell density? This hypothesis should be tested.

We had initially chosen to display the differences within each strain across growth phases, however we appreciate that like the reviewer, other colleagues may be most interested in the comparison between strains at each growth phase. We have therefore adapted Figure EV5B to now display this data as suggested.

Our findings do indeed show the largest magnitude of survival difference between strains in mid-exponential growth phase and no difference in stationary phase. One explanation for this is that cells in stationary phase are typically highly tolerant to antibiotics due to their progression toward a dormant state and the bias of antibiotics toward dividing cells (Luidalepp et al. 2011, J Bacteriol). With regard to early exponential phase, one possibility is that cells at this time point may also not be rapidly dividing and thus the metabolic consequences of genotoxic stress are reduced relative to exponential growth. In our opinion, these growth phase-dependent differences in survival are likely distinct from the density-dependent survival differences we observed when altering the ratio of WT:Δ*gndRX* cells, which may instead be the consequence of the dilution of some protective factor of Δ*gndRX* cells, such as a membrane protein. In summary, cells that are rapidly dividing are the most susceptible to antibiotics and we speculate that the differences in survival between the WT and Δ*gndRX* strains are most apparent at this time of maximum damage.

Figure 7A and figure S7A. The OD are quite different between the 2 experiments. Could the authors comment on that?

These differences are due to the path length differences of a cuvette and 96-well plate when performing spectrophotometric measurements. To assist others in the interpretation of these data, we have included a note in each figure legend indicating this.

Figure 7B. The survival is similar for both strains. However, the WT is lysing and about half of the population is PI-stained, while only 2% of the mutant cells are PI stained. It would be helpful to show the non-treated conditions to have a better sense of the gating used. Also, the LacZ test revealed that the mutant is lysing as well. Hower the OD remains constant. How to reconcile these observations? That OD measurement is a poor proxy?

The reviewer makes excellent observations, and we agree that these orthogonal readouts are complicated to interpret, which is why we chose to include all of them to complement each of their respective limitations. We now include a revised supplementary Figure S2C which shows the untreated controls (live untreated and heat-killed cells) used in the gating for Figure 7C. Furthermore, our interpretation is indeed that absorbance is a coarse measurement in this instance, as it can only capture cell death dynamics if lysis occurs. Thus we chose to use turbidity to measure cell replication kinetics, the LacZ to measure lysis, flow cytometry to monitor the state of cell damage, and CFUs to measure the persister subpopulation. Our interpretation is that many Δ*gndRX* cells in the bulk population continue to divide (or at least attempt to) during the first 24h of drug treatment (Figure 7A shows an increase in turbidity for this strain). We hypothesize that this is the consequence of not having the GndRX system and thus maintaining higher levels of NAD after stress exposure. However, these cells are still being incubated with large amounts of drug and thus during this time a subset of cells also undergo lysis. These concurrent phenomena result in the OD appearing to remain relatively constant while also producing a signal in the LacZ assay. During this time, the persister subfraction (which is a small number of cells relative to the bulk population) declines equivalently for each strain as these are likely quasi-dormant cells that are unaffected by the death kinetics of the broader population.

Figure 7D. The intracellular concentration of NAD drastically drops in the WT cells, suggesting that they are metabolically inactive ('dormant'). However, the authors claim in the discussion (L483) that the mutant cells are in a dormant state that helps them to survive. It's a bit

confusing. What is the definition of dormancy and the evidence supporting the hypothesis that dormancy helps to survive?

We appreciate this feedback from the reviewer and have refined the writing of our manuscript to be more specific when describing cells as dormant. The term “dormancy” is complex and nebulous, and likely encompasses many different cell states along a continuum of growth arrest (Walker et al. 2024, Trends Microbiol). While there currently isn’t a consensus in the field on what dormancy specifically describes (McDonald et al. 2024, Trends Microbiol), we generally view it as a cell state of highly reduced metabolism and a reversible cessation of growth and replication. The adoption of a dormant or non-replicative state can enhance cell survival to diverse stresses (Lennon et al. 2011, Nat Rev Microbiol.), such as antibiotics, due in part to the targeting of replicating cells. In our experimental system, we observed that reduction in cellular NAD is coincident with cell death. This is consistent with the activity of the other RES toxins, which deplete or consume NAD, and as a direct or indirect consequence, this leads to cell death. Thus we consider NAD depletion to be a property of dying cells rather than dormant cells, which likely require the retention of some level of critical factors (like NAD) to remain viable even if they are not dividing. We refer to cells in the mutant population as dormant because they are not dead or dying, retain higher levels of NAD, and likely represent a characterized state of dormancy referred to as the viable but non-culturable (VBNC) state. VBNC cells have been studied in *L. pneumophila* previously, including with the methodologies we use to detect these populations (flow cytometry and CFU quantification). As dormancy is indeed a complex term associated with many different cell states, we have modified our text to refer specifically to the VBNC state.

On an evolutionary point of view, what would be the advantage of selecting a TA system that is deleterious, integrating it in the host regulatory network and maintaining it? This system seems to be widespread in bacteria. This should be discussed.

We thank the reviewer for this insightful question and agree that the presence of this system across bacterial clades seems counterintuitive. Our hypothesis is that this system could have selective benefits during stress conditions (such as phage infection or general genotoxic stress) whereby total population fitness is enhanced by the eradication of infected or irreversibly damaged cells. This could allow for healthy kin cells to access limiting nutrients or prevent the spread of infectious agents and deleterious mutations in the population. Conversely, this system may also provide a means for growth arrest to facilitate repair—rather than lead to outright cell death—under physiological stress conditions rather than the extreme stresses of the laboratory environment. Given the fascinating and seemingly paradoxical conservation of this system across bacteria, we speculate about its evolutionary success in the discussion starting on page 21 (lines 546-563):

“These observations therefore raise the question: why have this system in the first place? The rapidly expanding catalogue of bacterial immune systems has revealed both the prevalence of NAD+ depletion as a mode of defense (Boyle & Hatoum-Aslan, 2023) and similar instances of TA-based systems that rely on both proteins for toxic activity (Burman et al, 2024). Perhaps, GndRX may function as one component of a larger response pathway and serves an intermediary role in converting a DNA damage signal into the depletion of a critical cellular metabolite, thereby acting as a homeostatic sensor rather than an autonomous immune element. Furthermore, it is possible that cell death is in fact an exacerbated consequence of the magnitude and duration of genotoxic stress exposure we utilized, and this system would otherwise produce a bacteriostatic effect under physiological conditions. For example, when experiencing acute DNA stress, it may be advantageous for cells to restrict growth to reduce

oxidative damage and await improving conditions for repair to proceed. Alternatively, as not all wild-type cells die even after prolonged damage, activation of GndRX may instead be a means of heterogeneity generation and cellular altruism. As vacuolar compartments and biofilm communities are inherently spatially constrained environments with finite resources, it could be advantageous for damaged or genotypically compromised cells to undergo growth restriction or programmed cell death to liberate replicative resources for kin cells. Finally, it is possible that GndRX is indirectly activated upon DNA stress induction and performs a secondary function, which under conditions of prolonged and irreparable genotoxic stress, leads to cell death."

Minor comments:

L62. Add references 44 and 45.

Done.

L105. Why is the repertoire 'reduced'? Not all the bacterial strains have 'a lot' of TA systems. E.g. The E. coli lab strain has 10 identified type II TA systems.

We used the term reduced because we were referring specifically to genetically tractable bacteria studied in the laboratory and not the overall distribution of TA systems across bacteria. *L. pneumophila* has a relatively small set of TA systems compared to typical models (*E. coli*, *Salmonella*, *Mtb*) in that it has both fewer type II systems and no predicted systems of other types. So compared with these systems, in which the majority of TA research has been conducted, the total number of TA systems in *L. pneumophila* is much fewer than the total number in these other model bacteria. For clarity, we have reworded "reduced" to "limited".

The phylogenetic tree presented in Figure S4 is unreadable.

We have simplified the phylogeny in Figure EV4 by excluding taxonomic information at the Class level and now only show the Phylum level. Our intention is only to convey the broad distribution of GndR homologs across bacterial phyla rather than specific details about taxonomic associations.

Referee #2:

The manuscript describes the role of Legionella toxin-antitoxin systems in regulation of cell physiology. The experiments indicate unexpected effects: deletion of one of the TA systems decreases sensitivity to ciprofloxacin; the effect is regulated by cell to cell contact. The results are of high interest for the colleagues in the field and are also important for wider audience. The experiments are well planned and performed, the manuscript reads well.

There is one piece of experimental evidence that I find missing: what happens to NAD levels if you overexpress gndR? It is speculated in the manuscript that overexpression of gndR might influence NAD levels to the extent that does not effect growth. This would be relatively easy experiment to perform, considering that the authors have the experimental system set up.

We thank the reviewer for their positive feedback on our manuscript and this very helpful experimental suggestion. We have performed the experiments as requested and now include these data in a revised Figure 7E. We show that expression of GndR and GndR+GndX both result in the depletion of cellular NAD, though not to levels seen during even the 6h of genotoxic

stress in WT cells. Additionally, we compared NAD depletion across both exponential and post-exponential growth phases to confirm that the strains grew normally and could reach saturating growth. These findings therefore support the hypothesis that both GndR and GndR+GndRX can deplete cellular NAD when expressed, and that this activity does not cause sufficient toxicity to the cell such that growth is arrested of death occurs.

As a very minor note, symbols are not visible in Fig 3B left. Please find a more clear graphical solution. In addition, the Fig.3B left has smaller variability of data when compared to the other datasets. Was the same number of replicates performed correctly?

We have switched and dispersed the symbols in Figure 3B left to make this figure more accessible to the reader. The same number of independent experiments (ie biological replicates) was performed to generate the data in Fig 3B as with other experiments, however in this instance the variation was very small between the Δ*gndRX*, Δ*gndR*, and Δ*gndX* strains which resulted in unavoidable overlap between the data points on the plot.

I find the Discussion lengthy. The authors might consider shortening.

We thank the reviewer for this feedback and have edited the discussion section for brevity while retaining all the core points.

Referee #3:

The manuscript by Lin and Ensminger investigates toxin-antitoxin (TA) systems of Legionella pneumophila, the causative agent of Legionnaires' disease. The study documents the construction of a marker-less "pan-TA" L. pneumophila deletion strain lacking all 7 annotated TA systems and the characterization of stress responses of this mutant strain. Intriguingly, one of the 7 TAs, TA2 (Lpg1604-1605, GndRX), is activated specifically by genotoxic stress (ciprofloxacin, mitomycin C, UV) and promotes bacterial cell death rather than survival. The deletion of gndRX does not affect bacterial growth, host cell infection, or survival during translation and cell wall stress. However, a ΔgndRX mutant strain shows increased production of the metabolite homogentisic acid (HGA) and greatly enhanced survival (in a dormant state). These features are conferred to wild-type bacteria in a contact-dependent manner and are affected by the ratio of ΔgndRX mutant to wild-type bacteria. In contrast to canonical TA systems, the overproduction of GndR or GndX is not toxic for L. pneumophila or E. coli. GndR interacts with GndX (as shown by yeast two hybrid), likely binds NAD+ through its conserved RES (arginine, glutamine, serine) domain and promotes cellular NAD+ depletion upon the exposure to genotoxic stress. By promoting contact-dependent survival, GndRX reveals an interesting new function for bacterial TA systems.

The study represents an exciting analysis of a novel bacterial TA system with an unprecedented function. The thorough investigation comprises many appropriate controls, including an assessment of a "fixed" ΔgndRX mutant strain. The story unfolds in a straightforward, logical manner, and the well-written manuscript is actually a pleasure to read. A few minor amendments would further strengthen the study.

We thank the reviewer for their thorough reading and positive assessment of our manuscript.

1) Fig. 4D: The asterisk mentioned in the figure legend (supposedly highlighting the conserved serine) is actually an arrow. Please fix.

We thank the reviewer for noticing this and have changed the asterisk to an arrow.

2) Statistics: the calculation of the SEM should include at least 3 independent experiments. This is apparently not the case for Fig. 2DE, 6DE, 7B, S2ABD, S3ADE, S4DE, S5BC, and S7AB. Please show individual data points. Also, please indicate the overall number of experiments performed for Fig. 2G, S2C, S3A, and S5A.

We have made the requested changes by adding the number of experiments performed to all figure captions and we now include individual data points for all of the main figures except for Figure 7B, which was made too difficult to read when all replicate data were included. This was similarly the case for the figures in the supplement, thus for the purposes of readability and not expanding many of the already large figures with more panels, we have retained the summary statistics in the supplemental plots. However, we have provided all the raw data values for the main figures in the Source Data Files.

3) Line 378 or Discussion section: please discuss "VBNC" L. pneumophila in more detail, e.g. PMID: 23968544, PMID: 39810465.

We have added sections on the VBNC state in *L. pneumophila* to the results and discussion:

(page 16, lines 410-411)
"In this state, *L. pneumophila* VBNC cells have been shown to retain membrane potential and metabolic activity but are unable to be resuscitated using conventional culturing (Schmid & Hilbi, 2025)."

(page 16, lines 415-418)
"Given that *L. pneumophila* VBNC cells are characterized by distinct transformations in their ultrastructure (Al-Bana et al, 2014), an important goal of future work will be to determine whether Δ*gndRX* cells adopt similar morphological changes."

(page 18, lines 460-464)
"Within *Legionella* biology, VBNC cells are a common developmental form adopted in response to diverse environmental stresses and are critical to the bacterium's capacity for prolonged survival during harsh extracellular conditions (Robertson et al, 2014). Thus, by regulating the transition to either death or the VBNC state, GndRX appears to play an important role in the bacterium's life cycle as opposed to mere selfish parasitism."

4) Line 445: possibly also cite PMID: 34314090.

Done.

Dear Prof. Ensminger

Thank you for the submission of your revised manuscript. I have now received the reports from the two referees that I asked to re-evaluate the study, you will find below. As you will see, both referees now support the publication of your study.

Before we can proceed with formal acceptance, I have these editorial requests I ask you to address in a final revised manuscript:

- Please provide the abstract written in present tense throughout.

- Please order the manuscript sections like this, using (only) these names:
Title page - Abstract - Keywords - Introduction - Results - Discussion - Methods - Data availability section - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

- Please remove now the referee tokens from the data availability section and make sure that all deposited datasets are public latest upon online publication of the manuscript.

- Please check again that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends. Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment' but clearly state if these were biological or technical replicates. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. If n<5, please show single datapoints for diagrams.

Presently, it seems that no statistical testing was performed for any of the diagrams (with n>2) shown in the main, EV or Appendix figures. Please do that and also add a paragraph to the methods section explaining the statistical methods used. See also:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

- Please add to each legend (main, EV and Appendix figures, where applicable) a 'Data Information' section (or name the provided section like this) explaining the statistics used or providing information regarding replicates and scales. See:

https://www.embopress.org/page/journal/14693178/authorguide#figureformat

- Please add the information provided in the Appendix Tables S1, S2 and S3 directly to the Reagents and Tools Table. Then please remove the tables from the submission system and also remove their legends from the Appendix file. Please also add callouts to the table where appropriate.

- Please add Appendix Table S4 into the Appendix file nest to its legend. Do not upload this separately.

- Appendix Tables S5, S6 and S7 are datasets. Please name and upload these as Dataset EV5, Dataset EV6 and Dataset EV7 in all places (source file names, titles in the submission system and their callouts). Please remove their legends from the Appendix. Instead, please put the respective legend on the first TAB of the corresponding Excel file.

In addition, I would need from you uploaded separately:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short (!) bullet points highlighting the key findings of your study (two lines each).
- a schematic summary figure as separate file that provides a sketch of the major findings (not a data image) in jpeg or tiff format (with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Best,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

I am statisfied with the revised manuscript. The authors answered all my questions/concerns.

Referee #2:

Authors have carefully considered the reviews. In my opinion the manuscript is now ready for publication.

Biochemistry
UNIVERSITY OF TORONTO

July 16, 2025

Dear Dr. Breiling,

Thanks again for the timely and productive review of, "**A bacterial toxin-antitoxin system involved in an unusual response to genotoxic stress.**" We thank you and the reviewers for the constructive feedback and suggestions.

We have completed the requested revisions and now return to you our revised manuscript.

One point that we would like to note is regarding the request to show raw data for all plots where n=2. In our previous revised manuscript, we changed all main figure panels with n=2 to show the raw data (as this request was made by one of our reviewers) except for panels 4F and 7B. This was because neither plot is conducive to showing non-averaged data, as 4F is a heatmap and 7B has a large number of data points even with averaging. Therefore, we chose to present these data as averages to maximize clarity for the readers of the paper, and additionally because the raw data are provided in the source data files.

There are several panels in the Extended View figures that are also plotted from n=2 data and we have again opted to keep these figures with averaged data. This is because many of these plots are composed of dozens of data points even after averaging, and consequently displaying the raw data would either render them unreadable or lead to individual data points being obscured due to the density of points being plotted. Our concern is that in this instance it would risk making the data we are presenting uninterpretable to readers. Please advise us as to whether this is acceptable as including each datapoint for these Extended View figures may require us to explore alternative ways of presenting the data.

We thank you again for your continued support of our manuscript and are happy to discuss the revisions further if you should have any concerns or additional requests.

Warm regards,

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Dear Prof. Ensminger,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship 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 $\chi 2$ 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Materials and Methods, subsection "Strains and plasmids" now includes: All strains and plasmids are available upon request.

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Not Applicable	

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	All primer sequences are included in Appendix Table S3.

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Methods section "U937 growth assays" includes this information "U937 (American Tissue Culture Collection, CRL-1593.2)"
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	All strains are described in the "Strains and plasmids" section of the methods as well as in Appendix Tables S1 and S4.

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	The Acknowledgements section includes the sentence "Illumina RNA-seq was performed by the Microbial Genome Sequencing Center (Pittsburgh, USA). Routine plasmid sequencing was performed at The Centre for Applied Genomics, Hospital for Sick Children (Toronto, Canada)."

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Sample sizes (ie biological and experimental replicate numbers) are indicated in the figure legend of all figures.
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	The only statistical testing utilized is differential enrichment analysis for the RNA-seq data in Figure 5. The software used (edgeR) does not assume a normal distribution for the count data and is designed to incorporate and control for variance intrinsic to RNA-seq datasets.

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	This information is included in all figure legends.
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	This information is included in all figure legends.

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

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The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	The Data Availabilities Section states that "DNA sequencing data has been deposited in the SRA under the BioProject accession PRJNA1168146. RNA sequencing data has been deposited in the NCBI Gene Expression Omnibus under the accessions GSE278701 (genotoxic stress experiments) and GSE278702 (growth phase experiments)."
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	