molecular systems biology

The inoculum effect and band-pass bacterial response to periodic antibiotic treatment

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 30 April 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees agreed that these findings are potential interesting. They raise, however, a series of important concerns, which, I am afraid to say, preclude its publication in its present form.

Perhaps most importantly, the third reviewer felt that the direct necessity of the heat shock response for the inoculum effect had not yet been conclusively demonstrated, and worried that differences between bactericidal and bacteriostatic antibiotics complicated the interpretation of the existing data. This reviewer makes some suggestions for experiments that may be able to address this issue. The reviewers also felt that this work would benefit from the presentation and analysis of data from independent biological replicates, and from a wider range of inoculum concentrations, to help demonstrate the robustness of these conclusions.

When submitting your revised work, that editor asks that you also consider the following format and content points:

1. In addition to our capacity to host datasets in our supplementary information section, we provide a new functionality that allows readers to directly download the 'source data' associated with selected figure panels (e.g. <http://tinyurl.com/365zpej>). This sort of figure-associated data may be particularly appropriate for this work. Please see our Instructions of Authors for more details on preparation and formatting of figure source data (<http://www.nature.com/msb/authors/index.html#a3.4.3>).

2. Please supply a single pdf file containing both the Supplementary Information and the Supplementary Figures (with legends immediately below each figure). This file should begin with a Table of Contents that lists all Supplementary Materials included with this work.

3. Molecular Systems Biology strongly encourages authors to provide machine readable versions of mathematical models in a common format as supplementary material, and encourages the deposition of SBML versions of models in the BioModels database whenever appropriate.

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favorable.

Please note As part of EMBO Publications' Transparent Editorial Process, Molecular Systems Biology now publishes a Review Process File with each accepted manuscript. In the event of acceptance, your cover letter/point-by-point response will be included in this file, which will be available to the scientific community. Authors may opt out of the transparent process at any stage prior to publication (contact us at msb@embo.org). More information is available in our Instructions to Authors (http://tinyurl.com/79zy6ap).

Sincerely,
Editor - Molecular Systems Biology
msb@embo.org

Referee reports:

Reviewer #1 (Remarks to the Author):

The authors present experimental and theoretical analysis of the innoculum effect in E coli for a group of different antibiotics. Their paper is an excellent example of a study in which the models developed (supported by initial experiments) are subsequently used to design novel and not immediately obvious experiments that highlight new phenomena. Overall I strongly support publication of this paper; however, there are a few points with regards to how the experimental data is presented which could benefit from slight additional work.

Specific points:

P 8 / Fig 2:

Data should be drawn as dose response curves (either final OD or growth rate as a function of antibiotic concentration and innoculum).

It would also be useful to see a dose reponse curve of inhibition by Kan at 8 ug/ml as a function of innoculum size, to more completely map out the effect of innoculum size .

P11 / Fig 4D: Should expand on the effect of these knock outs lon and clpX. Also, it appears to be a very marginal effect based on the growth curves and dose response curves would be useful here as well.

P14: When discussing the band pass behavior, it would be interesting to study more realistic antibiotic decay functions to better understand how IE plays into the clinical setting. This should be done using the model (not necessary to do experimentally)

Minor point:

p4: "Taken these together, we ask whether IE can be explained by nonlinear dynamics resulting from the antibiotic-mediated inhibition of the ribosome. "

This sentence is quite abrupt and could benefit from further elaboration.

Reviewer #2 (Remarks to the Author):

General Comments

The authors take a scholarly approach to studying the inoculum effect. The experiments are generally conducted with the necessary controls, and thorough rationale for each experiment is provided. The article is written clearly and relatively concisely.

I recommend that the authors describe the initial mathematical model in more detail, especially how it relates to Figure 1D. Since the entirety of the manuscript is based on the model, and the model can be visually explained by the aforementioned figure, I believe that much more emphasis must be placed on explaining the relationship between the equation and its corresponding graphical interpretation. Furthermore, I suggest that the authors describe the derivation of their model (equation 1) in a manner that allows individuals of many backgrounds to understand. While the mathematical derivation provided in the supplementary materials is necessary, a more easily accessible explanation would help a wider audience appreciate the development of the theory.

I also recommend that the authors repeat their growth experiments (to visualize inoculum effect) using more than two inoculum concentrations. Theoretically, increasing the initial bacterial density would proportionately increase the drug concentrations where the inoculum effect is observed. Particularly in Figure 2A/B, an exploration into the inoculum effect across a wide range of initial culture densities would provide further evidence supporting the generalizability of the model, and may provide insight into its limitations.

Specific Comments
1. Figure 1D red line. Description in body of manuscript shows =10-6, whereas in the figure it shows =10-5.
2. Figure S1C. A necessary control for comparison purposes is to show the effect of heat shock alone on the degradation of ribosomal protein L13-YFP. Theoretically, heat shock alone should act similarly to the Cm+HS sample. However, if the heat shock only control deviates from the Cm+HS sample, a theory to describe the chloramphenicol-induced deviation would be required. Whether or not this would change the overarching theory of the manuscript is unknown.
3. Figure S1E. For the same reason as above, a Cm+HS sample is required to analyze the degradation of rRNA. Likewise, the same samples and controls should be ran in both S1C and S1E, since they are closely related and attempt to explain two interrelated aspects of the same phenotype.
4. Figure 4C. To further test your model, it would be interesting to incubate cultures with chloramphenicol + heat + ser inh, cys inh, and asp inh. Theoretically, the addition of these inhibitors in combination with chloramphenicol and heat should result in the abolishment of the inoculum effect that is seen when chloramphenicol and heat are combined. Similarly, combining kanamycin with heat and these inhibitors should result in a phenotype similar to kanamycin alone.

Reviewer #3 (Remarks to the Author):

The authors model a proposed mechanism for the 'Inoculum Effect' (IE), whereby increased bacterial density decreases the efficacy of particular antibiotics. All antibiotics discussed within the context of this manuscript affect the ribosome, and the model proposed is similarly ribosome-centric. At its essence, this model assumes a positive feedback loop governing ribosomal production that is inhibited by antibiotic treatment, through both increased ribosomal degradation and decreased ribosomal synthesis. This model predicts that, given sufficiently high ribosomal degradation rates, ribosome concentration [and relatedly bacterial density] will determine whether the bacterial population will grow in the presence of a given antibiotic concentration. Although not explicitly required to satisfy their model, the authors take extensive efforts to argue the induction of the heat shock response (HSR) drives ribosome degradation. The authors also demonstrate that kanamycin pulses of intermediate duration and frequency result in decreased antibiotic efficacy, whereas, in the case of chloramphenicol, shorter, more-frequent pulses were more effective than longer, less-frequent treatment.
The authors describe the inoculum-effect in the case of kanamycin, for multiple bacterial species, as well as in E. coli with streptomycin, and puromycin and a handful of additional aminoglycoside

antibiotics. The authors also predict that an increased ribosomal degradation-rate would promote IE, demonstrate diminished RNA in the presence of kanamycin (but not chloramphenicol), and subsequently observe IE in kanamycin (and related antibiotics), but not chloramphenicol. This evidence affirms the core tenants of their model, but their further assertion that the HSR was necessary to produce IE, however, is not sufficiently supported by the data they provide.

Regarding the HSR: the molecular evidence used to link kanamycin, but not chloramphenicol, with HSR is weak (Fig. S1). Western blots against DnaK (Fig. S1A) were used to infer up-regulation of HSR in the case of treatment with kanamycin, but not chloramphenicol. No significance values were given when blot intensity was quantified, and the marginal differences in the two conditions (specifically in 'Trial 2') do not inspire confidence in the authors' data. The authors also show: (i) increased degradation of CFP fused to the ClpP-targeting SsrA-tag in the presence of kanamycin (10µg/ml) versus chloramphenicol (2µg/ml) and (ii) increased degradation of a YFP-L13 fusion protein in kanamycin (6µg/ml) versus chloramphenicol (1µg/ml). No explanation is given for the different antibiotic concentrations used in each experiment, and therefore the reader cannot be sure the results aren't simply due to a 5-6 fold higher dose of kanamycin, rather than a true antibiotic-specific response.

Additionally, the authors did not demonstrate that IE was a specific result of antibiotic-induced HSR (as they imply throughout the manuscript), rather than a product of treatment with bactericidal (almost exclusively aminoglycosides) versus bacteriostatic antibiotics. Although mentioned in the discussion, the authors did nothing to address the alternative hypothesis that reactive oxygen species, or some other cell-death related process, caused the increased deterioration of RNA, or is otherwise responsible for the inoculum-effect. Antibiotics that do not complex with the ribosome (e.g. beta-lactams [bactericidal] or trimethoprim [bacteriostatic]) may offer an opportunity to test whether the IE is a more general phenomenon of bactericidal antibiotics.

Of interest is the observation that kanamycin pulses of intermediate length and frequency are less effective than pulses of short-length/rapid-frequency or long-length/slow-frequency. This is in contrast with chloramphenicol treatment, where increasing pulse-length at the expense of frequency always reduced efficacy. These observations matched model predictions, lending credence to the model. While not formally required for this particular manuscript, it would have been great to see data with any additional antibiotics (e.g. puromycin, beta-lactams, tetracycline, trimethoprin), to be convinced the effect is more general.

Furthermore, if only antibiotics displaying IE exhibit this "band-pass" response to periodic treatment, one would expect chloramphenicol at 42C (for which IE was observed) to exhibit this response whereas chloramphenicol treatment alone would not. I strongly encourage the authors' to perform and report on this modest experiment. Not only would this provide excellent validation of the model, it would eliminate the variables not accounted for with all other bactericidal vs bacteriostatic comparisons, lending credence to their assertion the HSR is required for IE.

Other specific criticisms:

Transitioning between kanamycin and streptomycin (e.g. page 11 text, Figures 4D, S2H) without explicit justification makes the referee wonder whether results using the former antibiotic (and the choice antibiotic for most experiments) simply didn't produce the desired results. The referee had trouble interpreting whether a strain with decrease H2S production can still produce a robust IE phenotype: the data was very low resolution (three data points), used a different antibiotic than was standard elsewhere in the manuscript, and the 'IE' phenotype was minor.

No significance values were given in any of the data. Without these values, it is difficult to interpret meaningful differences in the data. This referee recommends performing at least some replicate analysis, obtaining standard deviations, and reporting the resulting p-values on bar-charts as well as on X-Y plots (when appropriate).

Supplementary table 2, note I should include a reference to figure S4E. This is where the authors show the chloramphenicol + heat-shock data. It is not mentioned previously, referencing its existence without pointing to the data was frustrating.

We wish to submit a revised manuscript, "**The inoculum effect and band-pass bacterial response to periodic antibiotic treatment"**, for publication in *Molecular Systems Biology* as a research article.

We greatly appreciate the efforts by you and the reviewers to evaluate our manuscript. The reviewers have pointed out the potential significance of our study but have raised some technical issues. Before addressing these comments and suggestions in detail, we would like to clarify the scope and key conclusions of our study. Briefly, our study has analyzed the generation of IE by antibiotics that target ribosomes, as well as the consequence of IE during periodic antibiotic treatment. In this context, we found that:

a. A critical requirement for generating IE is sufficiently fast turnover of ribosomal components, which can be induced by certain antibiotics that cause the heat shock response.

b. Indeed, antibiotics that cause heat shock response can generate IE.

c. Antibiotics that do not cause heat shock response cannot generate IE by themselves, but can do so when coupled with direct heat shock treatment. Moreover, the generated IE can then be abolished by inhibiting the proteases that target ribosomal components.

d. IE, when coupled with a delayed recovery time induced by antibiotic treatment, can lead to the generation of band-pass response by bacteria to periodic antibiotic treatment.

All of our data, including those from the new experiments suggested by the reviewers, fully support our conclusions and are consistent with the predictions by our mathematical models (for the generation of IE itself and for the generation of band-pass response). Taken together, our results and analysis have defined a previously unknown mechanism to generate IE, as well as the counter-intuitive consequences of IE.

We found the reviewers' comments and suggestions to be highly constructive. To address them, we have carried out additional experiments and data analysis, and revised the main text and supplemental information for clarification. Major revisions are highlighted in red in the manuscript.

Point-to-point responses to specific comments:

Reviewer #1 (Remarks to the Author):

1. The authors present experimental and theoretical analysis of the inoculum effect in E coli for a group of different antibiotics. Their paper is an excellent example of a study in which the models developed (supported by initial experiments) are subsequently used to design novel and not immediately obvious experiments that highlight new phenomena. Overall I strongly support publication of this paper; however, there are a few points with regards to how the experimental data is presented which could benefit from slight additional work.

We thank the reviewer for recognizing the significance of our study and for the constructive comments, which we have fully addressed in the revised manuscript.

Specific points:
2. P 8 / Fig 2: Data should be drawn as dose response curves (either final OD or growth rate as a function of antibiotic concentration and inoculum). It would also be useful to see a dose response curve of inhibition by Kan at 8 ug/ml as a function of inoculum size, to more completely map out the effect of inoculum size.

As suggested by the reviewer, we have plotted the main results in Fig. 2C & D in the form of dose-response curves. We note that the different way of presenting our data does not change our conclusions. Furthermore, we have collected data for four inoculum sizes (50X, 500X, 5000X, & 50000X) for both chloramphenicol and kanamycin (Fig. S2I & J). The data are consistent with our original results.

3. P11 / Fig 4D: Should expand on the effect of these knock outs lon and clpX. Also, it appears to be a very marginal effect based on the growth curves and dose response curves would be useful here as well.

We have expanded the results as suggested by the reviewer. Specifically, we have updated the results in Fig. 4D and included the original data in Fig. S4H-J. We note that these data are fully consistent with conclusions presented in the original submission.

4. P14: When discussing the band pass behavior, it would be interesting to study more realistic antibiotic decay functions to better understand how IE plays into the clinical setting. This should be done using the model (not necessary to do experimentally).

We thank the reviewer for the suggestion. We would like to note that our model has taken into account of antibiotic profiles implicitly. Specifically, our model assumed that bacteria will not grow when antibiotic concentration is above a certain threshold and will recover with a time delay when antibiotic concentration decreased below a certain threshold. Therefore, based on our model, band-pass would still occur with antibiotic decay. We have updated the text to clarify this and have included a figure of the modeling results (Fig. S6C&D).

Minor point:
5. p4: "Taken these together, we ask whether IE can be explained by nonlinear dynamics resulting from the antibiotic-mediated inhibition of the ribosome. " This sentence is quite abrupt and could benefit from further elaboration.

We have reorganized this sentence and parts of the introduction to make this sentence clearer. Furthermore, we have elaborated on our model as suggested.

Reviewer #2 (Remarks to the Author):
General Comments
1. The authors take a scholarly approach to studying the inoculum effect. The experiments are generally conducted with the necessary controls, and thorough rationale for each experiment is provided. The article is written clearly and relatively concisely.

We thank the reviewer for acknowledging both the quality and quantity of control experiments that were carried out.

2. I recommend that the authors describe the initial mathematical model in more detail, especially how it relates to Figure 1D. Since the entirety of the manuscript is based on the model, and the model can be visually explained by the aforementioned figure, I believe that much more emphasis must be placed on explaining the relationship between the equation and its corresponding graphical interpretation. Furthermore, I suggest that the authors describe the derivation of their model (equation 1) in a manner that allows individuals of many backgrounds to understand. While the mathematical derivation provided in the supplementary materials is necessary, a more easily accessible explanation would help a wider audience appreciate the development of the theory.
I also recommend that the authors repeat their growth experiments (to visualize inoculum effect) using more than two inoculum concentrations. Theoretically, increasing the initial bacterial density would proportionately increase the drug concentrations where the inoculum effect is observed. Particularly in Figure 2A/B, an exploration into the inoculum effect across a wide range of initial culture densities would provide further evidence supporting the generalizability of the model, and may provide insight into its limitations.

We thank the reviewer for making very helpful comments with respect to both data presentation and model clarification. These comments are indeed consistent with those by reviewer 1, which we have addressed as described above.

Specific Comments
3. Figure 1D red line. Description in body of manuscript shows γ=10-6, whereas in the figure it shows γ=10-5.

We thank the reviewer for the detailed and careful reading of our manuscript. We have corrected the typographical error as indicated by the reviewer.

4. Figure S1C. A necessary control for comparison purposes is to show the effect of heat shock alone on the degradation of ribosomal protein L13-YFP. Theoretically, heat shock alone should act similarly to the Cm+HS sample. However, if the heat shock only control deviates from the Cm+HS sample, a theory to describe the chloramphenicol-induced deviation would be required. Whether or not this would change the overarching theory of the manuscript is unknown.

We have now included the control experiment in the figure. We note that both heat shock treatment alone and heat shock coupled with chloramphenicol resulted in a similar decrease in L13-YFP levels (Fig. S1C, HS & Cm+HS).

5. Figure S1E. For the same reason as above, a Cm+HS sample is required to analyze the degradation of rRNA. Likewise, the same samples and controls should be ran in both S1C and S1E, since they are closely related and attempt to explain two interrelated aspects of the same phenotype.

We have now included this control in the figure. Specifically, treatment with 0.7μg/ml of chloramphenicol leads to an increase in rRNA levels. However, when this treatment is coupled with heat shock, the total amount of rRNA decreases. We note that this concentration of chloramphenicol lies within the IE region (when cells are treated with chloramphenicol and heat shock) and is thus fully consistent with our original results.

6. Figure 4C. To further test your model, it would be interesting to incubate cultures with chloramphenicol + heat + ser inh, cys inh, and asp inh. Theoretically, the addition of these inhibitors in combination with chloramphenicol and heat should result in the abolishment of the inoculum effect that is seen when chloramphenicol and heat are combined. Similarly, combining kanamycin with heat and these inhibitors should result in a phenotype similar to kanamycin alone.

We thank the reviewer for suggesting this interesting experiment. Indeed, when we treated cells with chloramphenicol, heat shock, and all three protease inhibitors (20μg/mL), the inoculum effect was not observed (Fig. 4C, Cm+42^{o}C+Inh). This experimental condition resulted in an MIC of 2μg/ml of chloramphenicol, which is consistent with the MIC of chloramphenicol when the cells were grown at 37^{o}C and without inhibitors. Furthermore, when we treated cells with kanamycin, heat shock, and all three protease inhibitors, the IE region shifted to 5μg/ml and shrunk (Fig. 4C, Kan+42^{o}C+Inh), which is consistent with our observation using cells treated with both kanamycin and the serine protease inhibitor (Fig. 4C, Kan+Ser Inh).

Reviewer #3 (Remarks to the Author):

1. The authors model a proposed mechanism for the 'Inoculum Effect' (IE), whereby increased bacterial density decreases the efficacy of particular antibiotics. All antibiotics discussed within the context of this manuscript affect the ribosome, and the model proposed is similarly ribosome-centric. At its essence, this model assumes a positive feedback loop governing ribosomal production that is inhibited by antibiotic treatment, through both increased ribosomal degradation and decreased ribosomal synthesis. This model predicts that, given sufficiently high ribosomal degradation rates, ribosome concentration [and relatedly bacterial density] will determine whether the bacterial population will grow in the presence of a given antibiotic concentration. Although not explicitly required to satisfy their model, the authors take extensive efforts to argue the induction of the heat shock response (HSR) drives ribosome degradation. The authors also demonstrate that kanamycin pulses of intermediate duration and frequency result in decreased antibiotic efficacy, whereas, in the case of chloramphenicol, shorter, more-frequent pulses were more effective than longer, less-frequent treatment.

The authors describe the inoculum-effect in the case of kanamycin, for multiple bacterial species, as well as in E. coli with streptomycin, and puromycin and a handful of additional aminoglycoside antibiotics. The authors also predict that an increased ribosomal degradation-rate would promote

IE, demonstrate diminished RNA in the presence of kanamycin (but not chloramphenicol), and subsequently observe IE in kanamycin (and related antibiotics), but not chloramphenicol. This evidence affirms the core tenants of their model, but their further assertion that the HSR was necessary to produce IE, however, is not sufficiently supported by the data they provide.

We are glad that the reviewer noted that our experimental evidence "affirms the core tenants of the model". As mentioned above (page 1), the critical requirement to generate IE for this class of antibiotics is the fast turnover of the ribosomal components. Generation of HSR by certain antibiotics serves as a mechanism to trigger faster turnover of ribosomal components and in turn causes IE for appropriate concentrations of antibiotics. Indeed, our experimental results are fully consistent with these points.

For example, we have demonstrated that kanamycin causes fast degradation of ribosomal protein and rRNA, whereas chloramphenicol does not. We then demonstrated that chloramphenicol coupled with heat shock results in IE and degradation of ribosomal protein and rRNA. Furthermore, we have demonstrated that we can perturb IE regions using heat shock and inhibitors that specifically target clpX and lon proteases, both of which have been implicated in degrading ribosomes.

We do not claim HSR is *the necessary condition* to generate IE. As noted by the reviewer, our results alone cannot exclude the possibility that mechanisms other than HSR could also cause faster turnover of ribosomal components, and thus contribute to generation of IE. Indeed, our model for the generation of IE does not assume the generation of HSR. For the antibiotics and experimental conditions we tested, however, HSR is a *sufficient condition* to generate IE, by causing faster turnover of ribosomal components. We have revised the manuscript to further clarify this issue.

2. Regarding the HSR: the molecular evidence used to link kanamycin, but not chloramphenicol, with HSR is weak (Fig. S1). Western blots against DnaK (Fig. S1A) were used to infer up-regulation of HSR in the case of treatment with kanamycin, but not chloramphenicol. No significance values were given when blot intensity was quantified, and the marginal differences in the two conditions (specifically in 'Trial 2') do not inspire confidence in the authors' data.

In light of the reviewer's comment, we repeated the Western blot in Figure S1 and have included a statistical analysis of our quantification. Indeed, the amount of DnaK produced by kanamycin is significantly higher than that produced by chloramphenicol. We note that, as described in our initial submission, kanamycin, as well as several other antibiotics examined in our study, has been observed previously to elicit the heat shock response at both the protein (Vanbogelen & Neidhardt, PNAS 1990) and transcript level (Kohanski et al., Cell 2007). Along the same line, chloramphenicol and tetracycline have been previously observed to not elicit a heat shock response (Vanbogelen & Neidhardt, PNAS 1990).

3. The authors also show: (i) increased degradation of CFP fused to the ClpP-targeting SsrA-tag in the presence of kanamycin (10ug/ml) versus chloramphenicol (2ug/ml) and (ii) increased degradation of a YFP-L13 fusion protein in kanamycin (6ug/ml) versus chloramphenicol (1ug/ml). No explanation is given for the different antibiotic concentrations used in each experiment, and therefore the reader cannot be sure the results aren't simply due to a 5-6 fold higher dose of kanamycin, rather than a true antibiotic-specific response.

We apologize for not having clearly described the justification for choosing these antibiotic concentrations. They were chosen because they represented the MIC (i) and ~0.5 MIC (ii) of kanamycin and chloramphenicol.

In light of the reviewer's comment, we further examined the degradation rate of the L13-YFP construct using 10μg/mL kanamycin and 2μg/mL chloramphenicol. At these concentrations, which are consistent with the concentrations of antibiotics in Figure S1B, kanamycin caused rapid degradation of L13-YFP while chloramphenicol did not. We note that this trend is fully consistent with the results presented in our original manuscript.

4. Additionally, the authors did not demonstrate that IE was a specific result of antibiotic-induced HSR (as they imply throughout the manuscript), rather than a product of treatment with bactericidal

(almost exclusively aminoglycosides) versus bacteriostatic antibiotics. Although mentioned in the discussion, the authors did nothing to address the alternative hypothesis that reactive oxygen species, or some other cell-death related process, caused the increased deterioration of RNA, or is otherwise responsible for the inoculum-effect. Antibiotics that do not complex with the ribosome (e.g. beta-lactams [bactericidal] or trimethoprim [bacteriostatic]) may offer an opportunity to test whether the IE is a more general phenomenon of bactericidal antibiotics.

We thank the reviewer for this comment. We have indeed considered performing the suggested experiments, but ruled them out due to several reasons. The main objective of this manuscript is to identify the mechanism that gives rise to IE for antibiotics that interact with ribosomes. By constraining the objective to ribosomes-targeting antibiotics, we were able to model the interactions specifically and predicted results of perturbation accurately. As such, our model cannot be directly used to describe the interaction of antibiotics with other cell components, such as beta-lactams.

Within this context (for antibiotics that inhibit ribosomes), we have indeed carried out extensive control experiments and analysis to examine other well-established mechanisms (SI). We have also demonstrated that, in the presence of heat shock, chloramphenicol, a bacteriostatic antibiotic, generated IE in a manner fully consistent with our central hypothesis. Furthermore, we have performed additional perturbation experiments as suggested by both reviewers 2 & 3; in particular:

(a) Inhibition of proteases during heat shock treatment shrunk and shifted the IE region as predicted by our model (see Point 6 in the responses to Reviewer 2).

(b) Chloramphenicol coupled with heat shock led to band-pass response with periodic antibiotic treatment, as predicted by our model (see Point 6 below).

These new results, along with the data in the original submission, are fully consistent with the main points as outlined above (Page 1). Nonetheless, as noted above (Response to Point 1), our study does not intend to rule out other potential mechanisms that may enhance degradation of ribosomal components. Instead, based on well-established literature, we have identified one major pathway that contributes to the enhanced degradation of both rRNA and r-protein. This conclusion is supported by our perturbation experiments in Fig. 4. As such, we conclude that HSR is one way, but not necessarily the only way, in which ribosome degradation can occur. We have clarified this in the main text.

5. Of interest is the observation that kanamycin pulses of intermediate length and frequency are less effective than pulses of short-length/rapid-frequency or long-length/slow-frequency. This is in contrast with chloramphenicol treatment, where increasing pulse-length at the expense of frequency always reduced efficacy. These observations matched model predictions, lending credence to the model. While not formally required for this particular manuscript, it would have been great to see data with any additional antibiotics (e.g. puromycin, beta-lactams, tetracycline, trimethoprin), to be convinced the effect is more general.

We thank the reviewer for acknowledging the significance of our results. While the reviewer did not formally request that we perform the above experiments using an additional antibiotic, we have performed additional experiments with puromycin. Specifically, we observed that, using 80μg/ml puromycin (within the IE region of puromycin), growth is highest at intermediate pulse periods of 120 & 240 minutes (Figure S7C). We note that growth in the band-pass region was reduced as compared to kanamycin, which likely due to differences in recovery time of bacteria from antibiotic treatment.

These results are consistent with our original submission and they add to the generality of the observation presented in the manuscript.

6. Furthermore, if only antibiotics displaying IE exhibit this "band-pass" response to periodic treatment, one would expect chloramphenicol at 42C (for which IE was observed) to exhibit this response whereas chloramphenicol treatment alone would not. I strongly encourage the authors' to perform and report on this modest experiment. Not only would this provide excellent validation of the model, it would eliminate the variables not accounted for with all other bactericidal vs bacteriostatic comparisons, lending credence to their assertion the HSR is required for IE.

We thank the reviewer for suggesting this interesting experiment. We have set up a new system to perform the suggested experiment because 42°C is beyond the operating range of our original equipment (fluorescence microscope). Indeed, we observed bandpass response using 10μg/ml chloramphenicol coupled with heat shock at 42°C (Figure S7D). As acknowledged by the reviewer, this result provided further support to our central points.

Furthermore, we have clarified in our main text the requirements to generate a band-pass response to antibiotics. We note that, in addition to the inoculum effect, a sufficient difference in recovery time is also required to generate a band-pass response.

Other specific criticisms:
7. Transitioning between kanamycin and streptomycin (e.g. page 11 text, Figures 4D, S2H) without explicit justification makes the referee wonder whether results using the former antibiotic (and the choice antibiotic for most experiments) simply didn't produce the desired results.

We thank the reviewer for pointing out the gap between the transition between kanamycin and streptomycin. The knockout strains used for both Fig. 4D and Fig. S2H were created with kanamycin resistance markers. Therefore, we could not test the strains using kanamycin. Instead, we have used streptomycin that was demonstrated to produce IE. Despite the use of a different antibiotic, we note that the robustness of our model predictions was corroborated with the experiments using streptomycin.

8. The referee had trouble interpreting whether a strain with decrease H2S production can still produce a robust IE phenotype: the data was very low resolution (three data points), used a different antibiotic than was standard elsewhere in the manuscript, and the 'IE' phenotype was minor.

We have performed this experiment using additional concentrations of streptomycin. Indeed, IE is observed over a range of streptomycin concentrations (21-25μg/ml), suggesting that knocking out *sse*A does not abolish IE.

9. No significance values were given in any of the data. Without these values, it is difficult to interpret meaningful differences in the data. This referee recommends performing at least some replicate analysis, obtaining standard deviations, and reporting the resulting p-values on bar-charts as well as on X-Y plots (when appropriate).

We would like to clarify that we did include statistical analysis and values for several of the plots included in the Supplementary Material (section “Pulse-chase type assays” in SI). In light of the reviewer’s comment, we have now included additional statistical analysis on Figure S1A S1C, and S1E. A p-value has been explicitly included in each figure legend, where appropriate.

10. Supplementary table 2, note I should include a reference to figure S4E. This is where the authors show the chloramphenicol + heat-shock data. It is not mentioned previously, referencing its existence without pointing to the data was frustrating.

We apologize to the reviewer for not including this reference in our original submission. We have made this change in supplementary Table 2.

Again, we thank you and the reviewers for evaluating our manuscript. With these new data and clarifications, we hope you and the reviewers will now find the revised manuscript ready for publication at *Molecular Systems Biology*. Please do not hesitate to contact me should you have any questions. We look forward to hearing your editorial comments and decision.

2nd Editorial Decision 20 August 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the two referees who agreed to evaluate your revised study. These reviewer felt that the revisions made had satisfied their main concerns and they are now supportive of publication. We have however some minor remaining issues regarding data, format and content, which we ask you to address in a final revision of the present work.

1. Molecular Systems Biology generally requires that authors provide all key experimental data as supplementary information. To make supplementary experimental data more accessible to readers, we provide a new functionality that allows readers to directly download the 'source data' associated with selected figure panels (e.g. <http://tinyurl.com/365zpej>). Please see our Instructions of Authors for more details on preparation and formatting of figure source data (<http://www.nature.com/msb/authors/index.html#a3.4.3>). We ask that you provide source data files for the experimental results presented in this work, particularly Figures 2, 3, 5 & 7.

2. In addition, Molecular Systems Biology also asks that authors provide machine readable version of mathematical models when possible, ideally in a community standard format.

3. Please supply the main manuscript Figures as individual image files in EPS or TIFF format (EPS is preferred and will probably produce much smaller file sizes). Powerpoint is unfortunately somewhat notorious for poor image conversion to other formats -- you may need to remake the figures in a vector graphics program like Illustrator or Inkscape (direct PDF outputs from these programs are also acceptable).

Please contact us if any of the points above will be problematic. Thank you for submitting this paper to Molecular Systems Biology.

Yours sincerely,

Molecular Systems Biology
msb@embo.org

Referee reports:

Reviewer #1 (Remarks to the Author):

I think the authors have adequately addressed the points raised by the reviewers and that the manuscript is ready for publication.

Reviewer #3 (Remarks to the Author):

THe authors' have adequately addressed reviewer concerns in their revised manuscript, and I support publication of the paper in its current form.

2nd Revision - authors' response 28 August 2012

Thank you for accepting our manuscript, “The inoculum effect and band-pass bacterial response to periodic antibiotic treatment”, for publication in Molecular Systems Biology.

We have now addressed all editorial requests in a final revision of our manuscript. Specifically, we have submitted the following items:

1. Source data of Fig. 2, 3, 5C, and 7C&D in the MS Excel format.
2. High quality images of all main figures.
3. Matlab files for Fig. 1B-D and a SBML file for Fig. 1D.
4. Manuscript text files without color highlights.

Please feel free to let us know if additional changes are required.

