Appendix: Resuscitation dynamics reveal persister partitioning after antibiotic treatment

Xin Fang and Kyle R. Allison*

Wallace H. Coulter Department of Biomedical Engineering, Emory University and Georgia Institute of Technology, Atlanta, GA, USA; Department of Medicine/Division of Infectious Diseases, Emory University School of Medicine, Atlanta, GA, USA

*Corresponding author: Kyle R. Allison; email: <u>kyle.r.allison@emory.edu</u>; phone: (404) 727-6974

Table of Contents

| Appendix Table S1 | 3 |
|-----------------------|---|
| Appendix Figure S1 | 4 |
| Appendix Figure S2 | 5 |
| Appendix Figure S3 | 3 |
| Appendix Figure S4 | 7 |
| Appendix Figure S5 | 3 |
| Appendix Figure S6 | 9 |
| Appendix Figure S710 | C |
| Appendix Figure S811 | 1 |
| Appendix Figure S9 | 3 |
| Appendix Figure S1014 | 4 |
| Appendix Figure S1115 | 5 |
| Appendix Figure S1216 | 3 |
| Appendix Figure S1317 | 7 |
| Appendix Figure S1418 | 3 |
| Appendix Figure S1519 | 9 |
| Appendix Figure S1620 |) |
| Appendix Figure S1721 | 1 |
| Appendix Figure S1822 | 2 |

| Appendix Figure S19 | 23 |
|-----------------------|----|
| Appendix Figure S20 | |
| Appendix Figure S21 | 25 |
| Appendix Figure S22 | 26 |
| Appendix Figure S23 | 27 |
| Appendix Figure S24 | |
| Appendix Figure S25 | 29 |
| Appendix Figure S26 | 30 |
| Appendix Figure S27 | 31 |
| Appendix Figure S28 | 32 |
| Appendix Figure S29 | 33 |
| Appendix Figure S30 | 34 |
| Appendix Figure S31 | 35 |
| Appendix Figure S32 | 36 |
| Appendix Figure S33 | 37 |
| Appendix Figure S34 | 38 |
| Additional References | 39 |

| Strains/plasmids | Genotype/description | Source |
|--------------------------|---|-------------------------|
| MG1655 | E. coli K-12 F- , λ-, ilvG, rfb-50, rph-1 | ATCC |
| hipA7 | MG1655 hipA7 zde264::Tn10 dapA6 | (Korch <i>et al</i> , |
| | | 2003) |
| BW25113 | <i>E.</i> coli K-12 <i>F-,</i> Δ(araD-araB)567, ΔlacZ4787(::rrnB- | Yale strain |
| | 3), λ-, rph-1, Δ(rhaD-rhaB)568, hsdR514 | stock |
| toIC | BW25113 ΔtolC (Kan cassette removed) | Keio |
| | | Collection(Baba |
| | | <i>et al</i> , 2006) |
| tolC | MG1655 Δto/C (Kan cassette removed) | This study |
| | | |
| lexA3 | <i>E. coli</i> K-12 F- , λ <i>-, ilvG, rfb-50, rph-1 lexA3</i> (Ind-) | This study |
| pUA66 pompC::gfp | pUA66 derivative containing the <i>gfpmut2</i> gene under | (Zaslaver et al, |
| | control of the ompC promoter | 2006) |
| pUA66 ppbpG::gfp | pUA66 derivative containing the <i>gfpmut</i> 2 gene under | (Zaslaver et al., |
| | control of the <i>pbpG</i> promoter | 2006) |
| pUA66 precA::gfp | pUA66 derivative containing the <i>gfpmut2</i> gene under | (Zaslaver et al., |
| | control of the recA promoter | 2006) |
| pEB2-mScarlet-I | pEB2 derivative containing the mScarlet-I coding | (Balleza <i>et al</i> , |
| | sequence under constitutive promoter | 2018) |
| pCP20 | Thermosensitive helper plasmid encoding the FLP | (Datsenko & |
| | recombinase | Wanner, 2000) |
| Pseudomonas | <i>laclq</i> + Δ (lacZ)M15+ <i>tetA</i> + <i>tetR</i> + (ATCC 47085) | (Pestrak <i>et al</i> , |
| aeruginosa PAO1 | | 2018) |
| S. enterica serovar | ATCC 700720 | ATCC |
| Typhimurium LT2 | | |
| Klebsiella | ATCC 43816 | ATCC |
| pneumoniae | | |
| UTI <i>E.coli</i> strain | Clinical isolate stock from Emory hospital | This study |

Appendix Table S1: Bacterial strains and plasmids used in this work



Appendix Figure S1. Survival of MG1655 and MG1655 with pUA66 *pompC::gfp* grown 16 hours in LB then treated in fresh LB with 100 μ g/mL ampicillin. At the designated time points, samples were serially diluted and spot plated on LB agar to determine colony-forming units (CFUs). Survival data are plotted as the mean across three biological replicates and error bars indicate standard deviation (SD).



Appendix Figure S2. Fraction of cells leaving persistent state after ampicillin treatment. Histogram of remaining cells transitioning out of the persistent state ($-\Delta P/P$) as a function of time (*t*) (n = 228 cell lineages, same dataset as Fig. 1C).



Appendix Figure S3. Residuals analysis for the stochastic **(A)** and exponential **(B)** models versus directly observed resuscitation times. The residual sum of squares (RSS) for both models is indicated on plots.



Appendix Figure S4. Imputation of t_R (resuscitation time) from cells per colony. The resuscitation time (t_R) was imputed from the numbers of cell per colony identified computationally and the growth equation displayed in the figure.



Appendix Figure S5. Computational segmentation and clustering. Illustration of endpoint fluorescence microscopy for determination of resuscitation time of persistent bacteria. After ampicillin treatment, persistent bacteria harboring a constitutively-expressed fluorescent protein were grown at 37°C in the absence of antibiotic on fresh medium and imaged after 3 hours. Tiled images have been acquired and stitched. The Laplacian of Gaussian method was applied to images for edge detection and cell identification, and cells were enumerated and clustered computationally. The same image was used in Figure 2D.



Appendix Figure S6. Fraction of cells leaving persistent state from fluorescence data. Histogram of imputed remaining cells transitioning out of the persistent state ($-\Delta P/P$) as a function of time (*t*) (n = 645 cell lineages).



Appendix Figure S7. Imputed resuscitation time (t_R) predicts directly observed t_R values. (A) Example micrographs of *E. coli* MG1655 persister carrying pUA66 *pompC::gfp* reporter resuscitation after ampicillin (100 µg/mL) treatment. Microcolony doubling time after resuscitation was ~30 minutes. (B) Scatter plots of imputed versus observed t_R . The imputed t_R was calculated from cells per colony and the growth equation displayed in Appendix Fig S4. (C) The residuals between imputed and observed t_R .



Appendix Figure S8. Density dependence of resuscitation after ampicillin treatment. **(A)** Representative micrographs of ampicillin persister resuscitation on LB agarose pad with different cell densities. Wild-type ampicillin persisters containing either green (GFP) or red (mScarlet, depicted in magenta) fluorescent proteins were mixed at a range of ratios and allowed to resuscitate. The Red:Green ratio (*e.g.*, "4X") is indicated on each micrograph and the scale bar represents 50 µm. **(B)** Histograms of resuscitation times of GFP labelled persisters, with different Red:Green ratios (1X, n=85; 2X, n=136; 4X, n=123; 8X, n=85; 16X, n=115; 32X, n=101). **(C)** Mean and standard resuscitation times for the distributions in **(B)**. **(D)** α -values computed by fitting the exponential model to the data in **(B)**. Plot represents $\alpha \pm 95\%$ confidence intervals.



Appendix Figure S9. Persister resuscitation dynamics of wild-type and *HipA7* E. coli after treatment with 100 μ g/mL ampicillin. Cells in persistent state (*P*) over time (*t*) for wild-type and *hipA7* strains (data binned into 10 time points; n = 131 and 212 for wild-type and *hipA7*, respectively) with exponential model fit curves.



Appendix Figure S10. Persister resuscitation dynamics (log-scale) of wild-type and *HipA7* E. coli after treatment with 100 μ g/mL ampicillin. Cells in persistent state (*P*) over time (*t*) for wild-type and *hipA7* strains (data binned into 10 time points; n = 131 and 212 for wild-type and *hipA7*, respectively) with exponential model fit curves.



Appendix Figure S11. Persister resuscitation dynamics of *S. enterica* after treatment with 100 μ g/mL ampicillin. Cells in persistent state (*P*) over time (*t*) for wild-type *S. enterica* strains with both stochastic and exponential model fit curves. Insert depicts linear-scale of the same data.



Appendix Figure S12. Persister resuscitation dynamics after treatment with 100 μ g/mL ampicillin for varying times. **(A)** Cells in persistent state (*P*) over time (*t*) after initial antibiotic treatment for 3, 4.5, or 6 hours. Data binned into 10 time points with exponential-model fit curves (n = 334, 181, and 153 for 3, 4.5, and 6 hour treatments, respectively). **(B)** Cells in persistent state (*P*) over time (*t*) after initial antibiotic treatment for 4.5 (n=181) or 12 hours (n=431). Exponential model curves are fit to data.



Appendix Figure S13. Persister resuscitation dynamics after treatment with varying concentrations of ampicillin. Cells in persistent state (*P*) over time (*t*) after initial treatment with different ampicillin concentrations ([A]_{treatment}) (data were binned into 10 time points; n = 279, 261, 214, and 178 microcolonies for 50, 100, 150, and 200 μ g/mL, respectively) with exponential-model fit curves.



Appendix Figure S14. Role to *tolC* in persister resuscitation dynamics after treatment with 100 μ g/mL ampicillin. Cells in persistent state (*P*) over time (*t*) in wild-type (BW25113) and efflux-impaired Δ *tolC* strains (data binned into 10 time points; n = 330 and 122 microcolonies for wild type and Δ *tolC*, respectively) with exponential-model fit curves.



Appendix Figure S15. Inhibiting efflux delays persister resuscitation after ampicillin treatment. Cells in persistent state (*P*) over time (*t*) with differing concentrations of efflux inhibitor phenylalanine-arginine β -naphthylamide ([PA β N]) (data binned into 10 time points; n = 121, 171, 186, and 107 microcolonies for 0, 50, 100, and 200 µg/mL, respectively) with exponential-model fit curves. PA β N was only added during resuscitation and was not present during antibiotic treatment.



Appendix Figure S16. PA β N does not affect number of resuscitating persisters. **(A)** % survival determined by CFU/mL with PA β N concentrations of 0, 50, 100, and 200 µg/mL in LB agar plates. **(B)** Example on LB agar of colonies formed from persisters after 16 hours growth in the presence of PA β N. Colonies appear later in the presence of PA β N, but equivalent number of colonies are observed.



Appendix Figure S17. Effect of $\Delta tolC$ mutation on persister survival. **(A)** Persistence as a function of growth. Cultures were grown for 12, 15, 18, 21 hours then treated with 100 µg/mL ampicillin for 3 hours to determine survival. **(B)** Representative images showing the delayed growth of $\Delta tolC$ persister colonies after resuscitation compared to wild-type (MG1655) persisters. Images of persisters derived from 12- and 15-hour overnight culture were taken around 8 hours (early) and 21 hours (late) after plating.



Appendix Figure S18. The effect of efflux activity on stationary-phase *E. coli* regrowth in fresh LB. (A) Impact of 100 µg/mL PA β N on lag time distributions of MG1655 cell-regrowth in fresh LB from stationary-phase culture (n=221 and 189 for control and PA β N, respectively). (B) Resuscitation curves for stationary phase MG1655 grown with or without 100 µg/mL PA β N in the fresh LB (n=221 and 189 for control and PA β N, respectively). (C) Effect of *tolC* mutation on lag time distributions of BW25113 cell-regrowth in fresh LB from stationary-phase culture (n=249 and 311 for wild-type BW25113 and Δ *tolC*, respectively). (D) Resuscitation curves for stationary phase wild-type (BW25113; n = 249) and efflux-impaired Δ *tolC* (n = 311) strains in fresh LB.



Appendix Figure S19. Antibiotic damage in persister progeny. Example microcolonies deriving from single persister cells after resuscitation from 100 μ g/mL ampicillin treatment. Red arrows indicate cells with structural defects consistent with ampicillin mode of action.



Appendix Figure S20. *pbpG* expression during persister resuscitation from 100 µg/mL ampicillin treatment. Example time courses of resuscitating persisters carrying a *pbpG*-GFP transcriptional reporter (pUA66 *ppbpG::gfp*). Scale and time are indicated on images. Dashed line indicates microcolony boundary. Scale bar: 8 µm.



Appendix Figure S21. Cellular fluorescence for resuscitating colonies after treatment with 100 μ g/mL ampicillin or no treatment. Transient *pbpG* induction for colonies derived from a single ampicillin persister. Colony *pbpG*-GFP intensity over time for microcolony derived from MG1655 ampicillin persisters or resuscitating untreated stationary phase cells. Values represent mean ± SD, n=6, biological replicates



Appendix Figure S22. *pbpG* expression and persister partitioning after 100 μ g/mL ampicillin treatment. Example time courses of resuscitating persisters carrying a *pbpG*-GFP transcriptional reporter. Scale and time are indicated on images. Dashed line indicates microcolony boundary. Scale bar: 8 μ m.



Appendix Figure S23. Representative micrographs of "failed" persisters carrying *pbpG*-GFP reporter after 100 μ g/mL ampicillin treatment. Scale and time are indicated on images. Scale bar: 8 μ m.



Appendix Figure S24. Pie chart depicting the proportion of resuscitated BW25113 persisters that are healthy, damaged, or fail after 100 μ g/mL ampicillin treatment.



Appendix Figure S25. Pie chart depicting the proportion of resuscitated MG1655 persisters that are healthy, damaged, or fail after 200 μ g/mL ampicillin treatment.



Appendix Figure S26: Representative micrographs of microcolonies deriving from single persister cells taken several hours after resuscitation from 100 μ g/mL ampicillin treatment (top: DIC; bottom: fluorescence). Microcolonies can harbor multiple cells with structural defects, indicated with white arrows. Scale is indicated on images. Scale bar: 6 μ m.



Appendix Figure S27. Representative micrographs of "failed" persisters carrying *recA*-GFP reporter after 5 μ g/mL norfloxacin treatment. Scale and time are indicated on images. Scale bar: 8 μ m.



Appendix Figure S28. Pie chart depicting the proportion of resuscitated MG1655 persisters that are healthy, damaged, or fail after 10 μ g/mL norfloxacin treatment.



Appendix Figure S29. Norfloxacin induces *recA* expression in MG1655. Expression of *recA*-GFP reporter in individual MG1655 cell undergoing norfloxacin treatment compared with that of untreated cell. Values represent mean \pm SD, n=10, biological replicates.



Appendix Figure S30. Representative micrographs of *recA* induction and persister partitioning after 5 μ g/mL norfloxacin treatment. Example time courses of resuscitating persisters carrying a *recA*-GFP transcriptional reporter. Scale and time are indicated on images. Dashed line indicates microcolony boundary. Images of damaged persister (upper panel) are used in Figure 7A. Scale bar: 8 μ m.



Appendix Figure S31. Comparing damaged and failed persisters after 5 μ g/mL norfloxacin treatment. **(A)** Max cell fluorescence (*recA*:GFP) of individual norfloxacin persister or failed persister as a function of cell size during resuscitation. persister: n=63, 'failed' persister: n=23. **(B)** Maximum cell fluorescence (*recA*-GFP) of individual norfloxacin persister or failed persister as a function of initial cell fluorescence during resuscitation. persister: n=63, 'failed' persister: n=23.



Appendix Figure S32. *lexA3* mutants produced higher fraction of failed persister after norfloxacin treatment. (A) Pie chart depicting the proportion of resuscitated persisters that are healthy, damaged, or fail after 5 μ g/mL norfloxacin treatment. Left: wild-type MG1655; Right: *lexA3* mutant. (B) Column chart depicting the proportion of resuscitated persisters that are healthy, damaged, or fail after 5 μ g/mL norfloxacin treatment. Black column: wild-type MG1655; Grey column: *lexA3* mutant. The same pie chart for wild-type control is used in Figure 6B.



Appendix Figure S33: Persister partitioning during resuscitation in an *E. coli* strain isolated from a urinary tract infection. **(A)** Stationary phase UTI-isolate *E. coli* carrying pUA66 *pompC::gfp* reporter were inoculated into fresh LB and treated with 100 μ g/mL ampicillin, 20 μ g/mL imipenem, or 5 μ g/mL ciprofloxacin (n=3, biological replicates). **(B)** Illustrative example microcolonies each deriving from a single damaged persister. Dashed circles indicate partitioned damaged cells. Antibiotic and scale are indicated on micrographs. Scale bar: 6 μ m.



Appendix Figure S34: Persister partitioning in gram-negative pathogens. Example microcolonies deriving from single resuscitated persister cells of *S. enterica*, *P. aeruginosa* and *K. pneumonia* after treatment with imipenem or quinolone antibiotics. White arrows indicate persister with defects. Scale bar: 3 µm.

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