Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Microbiology

Persistence: a copacetic and parsimonious hypothesis for the existence of non-inherited resistance to antibiotics

Bruce R Levin[1], Jeniffer Concepción-Acevedo[1] and Klas I Udekwu[2]

We postulate that phenotypic resistance to antibiotics, persistence, is not an evolved (selected-for) character but rather like mutation, an inadvertent product of different kinds of errors and glitches. The rate of generation of these errors is augmented by exposure to these drugs. The genes that have been identified as contributing to the production of persisters are analogous to the so-called mutator genes; they modulate the rate at which these errors occur and/or are corrected. In theory, these phenotypically resistant bacteria can retard the rate of microbiological cure by antibiotic treatment.

Addresses
[1] Department of Biology, Emory University, Atlanta, GA 30322, USA
[2] Swedish Medical Nanoscience Center, Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden

Corresponding author: Levin, Bruce R (blevin@emory.edu)

Current Opinion in Microbiology 2014, **21**:18–21

This review comes from a themed issue on **Antimicrobials**

Edited by **James J Collins** and **Roy Kishony**

http://dx.doi.org/10.1016/j.mib.2014.06.016

Introduction

In the super-reductionist old days of bacterial genetics (between 1950 and 1970), words like 'leaky' were used to describe, albeit not explain, phenotypic heterogeneity among cells of the same genotype. In these more enlightened and somewhat less reductionist times, the mechanisms responsible for this phenotypic variation have been worked out for a number of cases, for example [1,2].

What may well be the first description of phenotypic heterogeneity within monoclonal populations of bacteria was the observation in 1942 that some 1% of '*Staphylococcus pyogenes*' exposed to penicillin survive and produce colonies [3]. Two years later, Joseph Bigger presented evidence that this resistance to the bactericidal effects of penicillin was phenotypic rather than inherited; when recovered colonies were re-cultured, they were as susceptible to penicillin-mediated killing as their ancestors [4]. Bigger called these survivors 'persisters'. Here we review evidence that supports the hypothesis that exposure to antibiotics contributes to the generation of persisters as well as reveals their existence. We postulate that persistence is analogous to mutation, an inadvertent consequence of different kinds of glitches and errors, rather than an evolved (selected-for) character. In this interpretation, the genes and processes that have been identified to contribute to the frequency of persisters are analogous to those that modify the rate of mutation, so-called 'mutator genes'. Using computer simulations, we explore the potential contribution of persistence to the microbiological course of antibiotic treatment.

Persistence can be attributed to a number of different mechanisms

Persistence appears to be a universal property of all bacterial species [5••]. When exposed to cytotoxic drugs fungi and neoplastic cells exhibit an analogous phenomenon, minority populations of phenotypically and presumable genetically susceptible cells survive [6]. Genome-wide mutant screening, and molecular genetic studies of persisters have identified two major classes of genes and mechanisms contributing to and/or modulating the frequency of persisters: one, changes in the expression of toxin or antitoxin genes; two, modifications and/or changes in effector protein concentrations that lead to changes in metabolism, for superb reviews see ([5••,7••,8••,9,10•]). Arguably, the genes responsible for competence for transformation in *Bacillus subtilis* also contribute to persistence; upon introduction to fresh media, replication is delayed for the small fraction of cells that are competent to acquire DNA [1].

What is clear and central to the following arguments is that multiple processes contribute to the generation and/or modulation of the fraction of genetically identical cells surviving exposure to antibiotics [11,12••]. Stated another way, there are multiple reasons why viable bacteria in an otherwise replicating populations do not divide or divide at a lower rate or have longer lag periods than the majority population. During these periods of arrested or slowed growth, these bacteria can be refractory to antibiotics and thereby persisters.

Antibiotics promote the generation of persisters

Persistence is not a convenient phenotype to work with; it is only manifest in a minority of a population and, at least

at the whole population level requires antibiotic exposure to be observed. Could it be that antibiotics not only reveal the presence of pre-exiting persisters but also contribute to their generation? Three lines of evidence support a positive answer to this question.

Fluctuation experiments [13]

If the persisters in a given population are all generated before antibiotic exposure, as with random mutation, there would be considerable variation in the number of persisters among independent cultures of the same size. While this is the case for *Staphylococcus aureus*, there is roughly the same amount of variation in the number of persisters among cultures derived from mixtures of these independent cultures [12••]. The latter is what would be anticipated if the selective agent, the antibiotic, also contributes to the production of persisters.

Correlation between the number of persisters produced by exposure to different drugs

If persisters were a single class of nondividing or slowly dividing cells produced during the course of growth, there would be a correlation in the number of cells surviving exposure to different antibiotics, even if these antibiotics differed in their ability to kill persisters. To test this hypothesis, we used methods described in [12••] to estimate the number of cells surviving after 22 hours of exposure to 20× MIC ciprofloxacin and 20× MIC gentamicin for 10 independent cultures of *S. aureus* Newman. The estimated correlation coefficient $r = 0.27$ for this experiment is not significantly different from 0, ($p \sim 0.44$).

SubMIC exposure increases the frequency of persisters

For both, *Escherichia coli* and *S. aureus*, the fraction of persisters is greater when these bacteria are grown at low (subMIC) concentrations of antibiotics than antibiotic-free media [12••,14].

We want to emphasize that while these observations support the hypothesis that antibiotics promote the generation of persisters, they do not reject the alternative. There may well be persister cells present before the population is exposed to antibiotics as well as those generated after exposure.

For *E. coli* and ciprofloxacin, a mechanism has been suggested to account for how exposure to this drug increases the level of persistence; the induction of an SOS response and thereby an increase in the TisB toxin [14]. Other than vague phrases like 'increasing stress' it is not clear how exposure of *S. aureus* to ciprofloxacin or other antibiotics increases persistence levels [12••]. Also requiring a mechanistic explanation is the great variance in the number of persisters produced by different cultures and in different experiments [11,12••,15] and the associated frustrating problem of quantitatively replicating results. For example, although it is clear that exposure to subMIC concentrations of antibiotics increases the levels of persistence to multiple drugs, the magnitude of this contribution varies between experiments, at least in our experience.

An evolutionary perspective

Persistence has been described as a bet-hedging mechanism [16,17••], a way for populations of bacteria to survive periodic confrontations with agents, like antibiotics, that kill growing cells. Consistent with this bet-hedging perspective are the results of mathematical modeling studies [12••,16,18] showing that when periodically exposed to agents that kill growing cells, bacteria that produce higher frequencies of persisters have an advantage over those that produce lower frequencies. This was observed in experiments with competing populations of genetically competent *B. subtilis* (*com*+) and mutants that cannot produce competent cells (*com*−). In the absence of these episodes where the population is exposed to agents that kill growing cells the *com*− have an advantage over the *com*+. If, however, the cultures are periodically exposed to penicillin, the *com*+ are favored [18].

Even if persisters provide a way for populations to survive episodes where they encounter agents that kill growing cells, this 'episodic selection' [18] need not be the force responsible for the evolution and maintenance of persistence, no more than penicillin binding proteins evolved to bind to this antibiotic. Indeed, our models suggest that even if the generation of persisters could be attributed to single genes, those genes would only have an advantage when they are common [12••]. When rare, the frequency of the phenotype generated by these genes, persisters, would be too low to provide the producing population an advantage and that subpopulation would be eliminated when the population at large is confronted with agents that kill growing cells.

Persisters and biofilms

In addition to the persisters generated in planktonic populations of bacteria, which have been the main object of study of this phenotypic resistance since Bigger's 1944 paper, it has been suggested that the antibiotic refractory cells in biofilms should also be considered persisters [19,20]. Why not? After all, there are many mechanisms by which these phenotypically antibiotic resistant cells are generated. The fraction of non-replicating genetically susceptible bacteria in a biofilm with actively replicating cells would be phenotypically refractory to antibiotics and thereby persisters in the Bigger definition.

The PASH hypothesis

To us, the most parsimonious and copacetic explanation for the existence of persisters and the diversity of mechanisms responsible for their generation is that these phenotypically resistant cells are the product of different kinds of glitches and errors in cell division, Persistence As Stuff Happens (PASH) [12••]. In accord with this

hypothesis, persistence is analogous to mutation, an inadvertent product of errors rather than an evolved (selected for) character. What has evolved and has been elucidated and described in the recommended reviews [5••,7••,8••,9,10•] are genes that modify the rate at which persisters are generated and/or correct the errors responsible for their generation and are thereby analogous to mutator genes.

Potential clinical implications

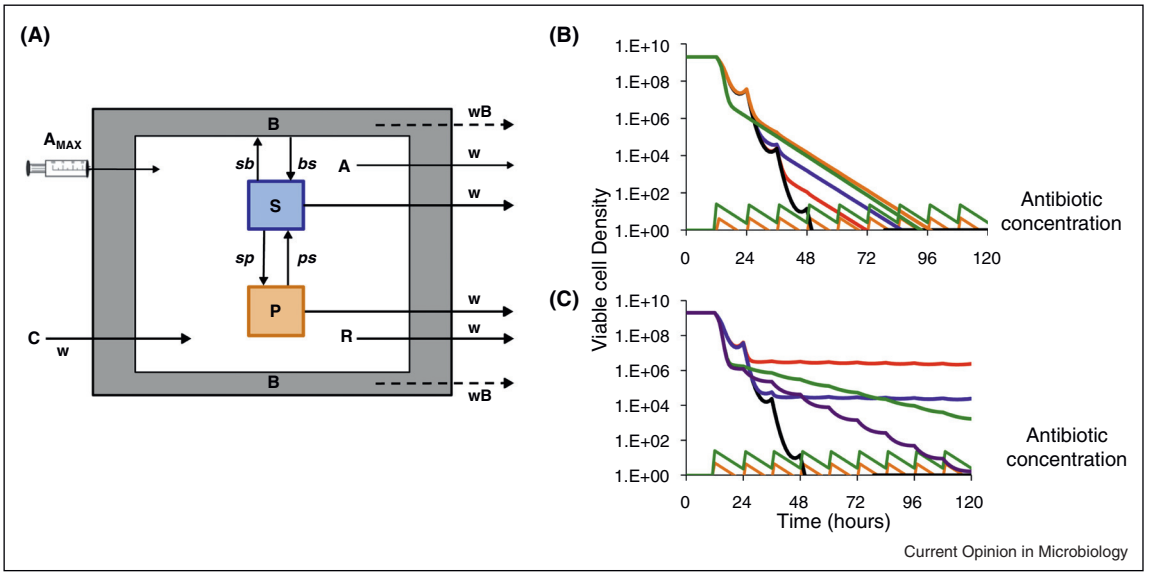
As interesting as phenotypic variation may be academically, much of the motivation and justification for studying persistence are its potential clinical implications, the contribution of this non-inherited form of resistance to the microbiological course of antibiotic treatment and the generation of genetically resistant bacteria during treatment. Whether persistence evolved by natural selection or like mutation is an inadvertent product of errors, phenotypically resistant subpopulations are clearly not a good thing for either of these aspects of antibiotic treatment [21]. How important persistence is to antibiotic therapy, however, is not at all clear. The results of our theoretical studies of the pharmacokinetics and pharmaco-dynamics and population dynamics of antibiotic treatment suggest that the clinical consequences of persistence depend on the physical nature of these phenotypically resistant subpopulations: whether the persisters are planktonic or reside in sub-habitats like biofilm, which are not only refractory to antibiotics but are removed at lower rates from the overall habitat than the planktonic cells from whence they are derived.

To illustrate this we use computer simulations of a model similar to that in [22] (Figure 1a). The bacteria are of three states, susceptible and persister planktonic cells, S and P, respective, or B in sub-habitat that is flowing out at a lower rate as planktonic populations. S, P and B are the densities as well as the designations of these populations. Relative to S, the P and B populations are refractory to the antibiotic and replicate at a lower rate. Resources flow into a habitat of unit volume at a constant rate, w per hour, which is the same rate at which the planktonic cells, unused resources, R, and antibiotics, A are removed. The B population is removed from habitat at lower rate than either S or P, $wb < w$. The bacteria change states in the directions and rates noted in the figure, respectively S → P, P → S, S → B and B → S, *sp*, *ps*, *sb*, and *bs* per cell per hour.

After the bacteria are at their equilibrium densities, antibiotics are introduced at a concentration, A_{MAX} at defined intervals, d hours. For the pharmacodynamics of the antibiotics and bacteria, we assume Hill functions [23]. More details about this model and the values of the parameters used can be seen in the Supplemental Material. The Berkeley Madonna^{TM} program used for these simulations can be obtained from www.eclf.net.

While planktonic persisters will increase the time before clearance to an extent proportional to the rate at which they

Figure 1



Simulation of antibiotic treatment. **(a)** Diagram of the model (details and equations in the Supplemental Material). Changes in the viable cell densities and antibiotic concentrations. **(b)** Planktonic persisters: Black-persister-free control. Red — $sp = ps = 10^{-6}$, Blue — $sp = ps = 10^{-4}$, Green — $sp = ps$ = 1E−3, sp = ps = 10^{-3} with high antibiotic dose. **(c)** Refuge (biofilm) persister population, Black — persister-free control, Blue — sb = bs = 10^{-6}, Red — sp = bs = 10^{-4}, Green — sp = bs = 10^{-4} with high dose standard antibiotic, Purple — sp = bs = 10^{-4} with a high does antibiotic that's effective for treating 'biofilms'. For other parameter values see Supplemental Material.

are generated, since these antibiotic refractory cells are washed out, they will not prevent clearance (Figure 1b). Unless it specifically kills planktonic persisters, increasing the dose of the antibiotic will have little effect on the course of treatment. If, however, the persisters are an antibiotic refractory subpopulation that is not turning over at a lower rate than the planktonic population, as in a biofilm, in the absence of other processes, like the host immune defenses [24] they can prevent clearance (Figure 1c). Because in this simulation the B population is replicating and is somewhat susceptible to the drug, increasing the concentration of the antibiotic can lead to a decline in the density of viable cells. This is particularly so, if as in [25••] the drug is active against persisters and including those in biofilms.

Conclusion

While persisters may be products of errors and glitches rather than an evolved (selected for) character, at least in theory they can substantially retard the rate of microbiological cure by antibiotic treatment. Elucidating the mechanisms responsible for modulating the rates at which these phenotypically resistant cells are produced and why they are refractory to these drugs may lead to the identification and development of procedures to better treat infections with persistent subpopulations.

Acknowledgements

Support for this endeavor has been provided by the US National Institutes of General Medical Science, GM098175 (BRL) and the Hesselman Fund (travel) and a Swedish Research Council junior investigator grant, VR # 2012-3564, (KU). We wish to thank Mark P. Brynildsen and the reviewers for helpful comments and suggestions and those who developed, maintain and support Google Hangouts, which greatly facilitated this international collaboration.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/ j.mib.2014.06.016.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Dubnau D, Losick R: **Bistability in bacteria**. *Mol Microbiol* 2006, **61**:564-572.

2. Gonzalez-Pastor JE, Hobbs EC, Losick R: **Cannibalism by sporulating bacteria**. *Science* 2003, **301**:510-513.

3. Hobby GL, Meyer K, Chaffee E: **Observations on the mechanism of action of penicillin**. *Exp Biol Med* 1942, **50**:281-285.

4. Bigger JW: **Treatment of Staphylococcal infections with penicillin**. *Lancet* 1944, **244**:497-500.

5. •• Cohen NR, Lobritz MA, Collins JJ: **Microbial persistence and the road to drug resistance**. *Cell Host Microbe* 2013, **13**:632-642.
A compelling, broad-based review of different processes responsible for persistence and the diversity of these mechanisms among organisms.

6. Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S, McDermott U, Azizian N, Zou L, Fischbach MA *et al.*: **A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations**. *Cell* 2010, **141**:69-80.

7. •• Gerdes K, Maisonneuve E: **Bacterial persistence and toxin–antitoxin loci**. *Annu Rev Microbiol* 2012, **66**:103-123.
A well-written and thorough review of the contribution of toxin–antitoxin genes to the frequency of persisters.

8. •• Zhang Y: **Persisters, persistent infections and the Yin–Yang model**. *Emerg Microbes Infect* 2014, **3**:e3.
An intriguing and broad-ranging perspective on phenotypic and inherited antibiotic resistance.

9. Lewis K: **Persister cells**. *Annu Rev Microbiol* 2010, **64**:357-372.

10. • Amato SM, Fazen CH, Henry TC, Mok WW, Orman MA, Sandvik EL, Volzing KG, Brynildsen MP: **The role of metabolism in bacterial persistence**. *Front Microbiol* 2014, **5**:70.
Highlights the role of metabolic conditions to the production of persisters and as potential target for agents to treat infections with persister subpopulations.

11. Allison KR, Brynildsen MP, Collins JJ: **Heterogeneous bacterial persisters and engineering approaches to eliminate them**. *Curr Opin Microbiol* 2011, **14**:593-598.

12. •• Johnson PJ, Levin BR: **Pharmacodynamics, population dynamics, and the evolution of persistence in *Staphylococcus aureus***. *PLOS Genet* 2013, **9**:e1003123.
A jointly theoretical and experimental study that provides evidence for antibiotics augmenting the rates of generation of persisters and the failure of selection to favor rare persister generating cell lines.

13. Luria SE, Delbruck M: **Mutations of bacteria from virus sensitivity to virus resistance**. *Genetics* 1943, **28**:491-511.

14. Dorr T, Vulic M, Lewis K: **Ciprofloxacin causes persister formation by inducing the TisB toxin in *Escherichia coli***. *PLoS Biol* 2010, **8**:e1000317.

15. Wiuff C, Zappala RM, Regoes RR, Garner KN, Baquero F, Levin BR: **Phenotypic tolerance: antibiotic enrichment of noninherited resistance in bacterial populations**. *Antimicrob Agents Chemother* 2005, **49**:1483-1494.

16. Kussell E, Kishony R, Balaban NQ, Leibler S: **Bacterial persistence: a model of survival in changing environments**. *Genetics* 2005, **169**:1807-1814.

17. •• Wakamoto Y, Dhar N, Chait R, Schneider K, Signorino-Gelo F, Leibler S, McKinney JD: **Dynamic persistence of antibiotic-stressed mycobacteria**. *Science* 2013, **339**:91-95.
Compelling evidence for isoniazid associated persistence being a dynamic state of a balance between cell division and death.

18. Johnsen PJ, Dubnau D, Levin BR: **Episodic selection and the maintenance of competence and natural transformation in *Bacillus subtilis***. *Genetics* 2009, **181**:1521-1533.

19. Lewis K: **Persister cells and the riddle of biofilm survival**. *Biochemistry (Mosc)* 2005, **70**:267-274.

20. Wang X, Wood TK: **Toxin–antitoxin systems influence biofilm and persister cell formation and the general stress response**. *Appl Environ Microbiol* 2011, **77**:5577-5583.

21. Levin BR, Rozen DE: **Non-inherited antibiotic resistance**. *Nat Rev Microbiol* 2006, **4**:556-562.

22. Levin BR, Udekwu KI: **Population dynamics of antibiotic treatment: a mathematical model and hypotheses for time-kill and continuous-culture experiments**. *Antimicrob Agents Chemother* 2010, **54**:3414-3426.

23. Regoes RR, Wiuff C, Zappala RM, Garner KN, Baquero F, Levin BR: **Pharmacodynamic functions: a multiparameter approach to the design of antibiotic treatment regimens**. *Antimicrob Agents Chemother* 2004, **48**:3670-3676.

24. Ankomah P, Levin BR: **Optomizing the treatment of acute, self-limiting infections: exploring the collaboration between antibiotics and the immune response**. *PNAS* 2014, **111**:8331-8338.

25. •• Conlon BP, Nakayasu ES, Fleck LE, LaFleur MD, Isabella VM, Coleman K, Leonard SN, Smith RD, Adkins JN, Lewis K: **Activated ClpP kills persisters and eradicates a chronic biofilm infection**. *Nature* 2013, **503**:365-370.
A promising application of the acyldepsipeptide antibiotic (ADEP4) to kill persisters and other non-replicating populations of bacteria.