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Crystal Ball

Are we really studying persister cells?

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Although persistence may be the most prevalent growth state for prokaryotes, since almost all bacteria and archaeal cells are starving, the persister field is muddled. This has occurred by the widespread publication of reports that include the term 'persister' but have little to do with persistence. Instead, these reports are usually related to the normal stress response of actively metabolizing cells. For example, it was recently claimed that compounds that slow metabolism increase persister killing when combined with traditional antibiotics (Mohiuddin *et al*., 2020). However, analysis of their data shows this group was more likely studying the stress response of the stationary-phase cells they began with, not persister cells, and these stationary-phase cells were dying from the application of two sequential antimicrobials. These kinds of reports perpetuate the classic error of confusing slowly growing cells with persister cells, usually as a result of mistaking stationary-phase cells or cells stressed by a metabolic shift, with persister cells (Amato *et al*., 2013; Amato and Brynildsen, 2014; Amato and Brynildsen, 2015; Orman and Brynildsen, 2015; Radzikowski *et al*., 2016).

To avoid these kinds of errors, perhaps it is best to start with a definition (Wood *et al*., 2013; Kim and Wood, 2017). Persistence is the phenomenon of transient tolerance to myriad stresses as a result of metabolic dormancy; these stresses kill their metabolically active counterparts and kill in the same manner the progeny of the persisters after they resuscitate. Persistence differs from resistance since persisters form without mutation, and resistant bacteria grow in the presence of the stress after mutation. Persister cells merely survive the stress without growth, and without much death (in the presence of the stress and in the absence of a carbon source). Also, persistence differs from tolerance in that the whole population of stationary-phase cells become tolerant to many antibiotics due to a reduction in growth (e.g., stationary-phase cells) whereas the persister sub-population is small (<1%), and persisters withstand stress even better than the slowly growing tolerant cells.

Recently an attempt was made to make these distinctions (Balaban *et al*., 2019); however, it goes astray in considering 'persistence may arise without dormancy'. In fact, the lack of growth of persister cells was the cornerstone of the original studies in which persistence was discovered (Hobby *et al*., 1942; Bigger, 1944). Moreover, taking shortcuts in generating 'persister cells,' in an attempt to increase the persister population to have a large enough cell population to study, has led to many of the errors in the field (Amato *et al*., 2013; Amato and Brynildsen, 2014; Amato and Brynildsen, 2015; Orman and Brynildsen, 2015; Radzikowski *et al*., 2016). However, robust techniques exist for creating *bona fide* persister cells including stopping translation (Kwan *et al*., 2013), production of toxins (Hong *et al*., 2012; Chowdhury *et al*., 2016), and pretreatment with non-lethal stress such as acid and hydrogen peroxide (Hong *et al*., 2012). For example, the pretreatment method of stopping transcription to cease translation to create persister cells has been vetted eight ways (Kim *et al*., 2018b) and this approach has been used by 11 groups to date to study persistence (Kwan *et al*., 2013; Grassi *et al*., 2017; Cui *et al*., 2018; Narayanaswamy *et al*., 2018; Sulaiman *et al*., 2018; Tkhilaishvili *et al*., 2018; Pu *et al*., 2019; Rowe *et al*., 2020; Sun *et al*., 2020; Yu *et al*., 2020; Zhao *et al*., 2020).

Building on the research of Hobby and Bigger, persister cell formation is an elegantly regulated response to environmental conditions (Kwan *et al*., 2013). Critically, by increasing the fraction of persister cells so that they may be studied directly, it has been discovered that inactivation of ribosomes via dimerization is the likely general mechanism leading to persistence (Kim *et al*., 2018a; Kim *et al*., 2018b; Wood *et al*., 2019; Wood and Song, 2020; Yamasaki *et al*., 2020; Song and Wood, 2020a, 2020b). Therefore, actively growing, even exponentially growing cells, can become persistent, if challenged with stress and a threshold level of ribosomes dimerize (Kwan *et al*., 2013; Wood

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and Song, 2020). Furthermore, increasing the fraction of persister cells through pretreatment along with single-cell studies led to the discovery that most persister cells revive rapidly by activating ribosomes and chemotaxis by reducing ppGpp and cAMP (Yamasaki *et al*., 2020). Also, there is heterogeneity in waking (Kim *et al*., 2018b); those cells that fail to wake in the presence of a carbon source are likely dead, even if they have an intact membrane as indicated by a lack of staining by propidium iodide (Kim *et al*., 2018a). Therefore, propidium iodide will correctly indicate cells as dead when they have compromised membranes but it underestimates the number of dead cells since it fails to indicate the dead cells that lack cytosol but have intact membranes; these dead ghost cells can be the dominant particle after nutrition stress (Kim *et al*., 2018a). Similarly, counting cell particles by fluorescence-activated cell sorting (FACS) is not appropriate for determining the number of viable cells; instead, transmission electron microscopy should be used to ensure particles contain dense cytosol since many cells die but have intact membranes (Kim *et al*., 2018a). Hence, plate counts will indicate the number of persister cells and this number may be far less than the number of particles seen via FACS and less than the number estimated by propidium iodide staining.

For study in the persister field, nothing can be discerned about persistence unless you study persister cells. For example, the Noji group claimed to show *Mycobacterium smegmatis* persisters have metabolic activity by using single-cell D_2O Raman imaging spectroscopy (Ueno *et al*., 2019). Unfortunately, this group treated with an antibiotic to generate persister cells but did not show they had treated long enough to convert the cells into the persister state. To confirm persistence, you need to demonstrate you have a sub-population of cells that are more stress tolerant than the original population and show increasing the stress has little effect on the number of persister cells. A quick way to create persister cells for a study is to overproduce a toxin from a plasmid, this invariably creates persister cells (Chowdhury *et al*., 2016), and these kinds of cells may be used to confirm your results for persisters generated in another manner.

Once you are convinced you have persisters for your species, there are a few additional guidelines. First, you should be very hesitant about writing about 'mutations' and 'persisters'. For example, many whole transcriptome, proteome and mutagenesis studies have failed to identify a coherent persistence mechanism, primarily since it makes little sense to search for a transcriptome or proteome response in a quiescent cell. Along these lines, a recent report claimed that the HipA/HipB toxin/antitoxin system 'mediate persistence in *Caulobacter crescentus*;' however, the data of the authors based on mutations of *hipBA* do not indicate HipA/HipB have a role in persistence since this part of the work showed the death of active cells, rather than the death of persister cells (e.g., Fig. 5) (Huang *et al*., 2020). Moreover, several studies have demonstrated toxin/antitoxin systems have little to do with persistence (Goormaghtigh *et al*., 2018; Pontes and Groisman, 2019; Svenningsen *et al*., 2019). Of course, when toxin HipA is *overproduced* in *C. crescentus*, persisters cells were generated (Huang *et al*., 2020), but this only shows HipA is toxic since the production of almost any toxin makes cells dormant/persistent (Chowdhury *et al*., 2016). Mutations probably do not make sense for persister cells unless they affect the rate of ribosome dimerization. For example, the *rpoS spoT* mutations that eliminate ppGpp in *E. coli* reduce persistence dramatically (Chowdhury *et al*., 2016) since ppGpp actively promotes ribosome dimerization (Song and Wood, 2020b).

Moreover, persister resuscitation work is probably not meaningful unless single-cell methods are used (Kim *et al*., 2018b). The primary reason is that if resuscitation is monitored by changes in culture turbidity or plate counts, all the interesting short time events are missed since persister waking is heterogeneous (Kim *et al*., 2018b).

Just as researchers rushed to claim (incorrectly through errors with FACS) that persister cells are metabolically active (Orman and Brynildsen, 2013), the latest disturbing trend is to claim resistance stems from persistence. For example, the Brynildsen group claimed a single exposure to the fluoroquinolone ofloxacin led to accelerated resistance in persister cells (Barrett *et al*., 2019). Unfortunately, the authors used stationary-phase cells, which are primarily non-persister cells that were clearly dying at appreciable rates during their experiments, rather than using *bona fide* persister cells (Fig. S1a of Barrett *et al*. shows 5% of the stationary-phase population was used, but persisters are usually far <1% of the original culture). Hence, they were likely studying resistance in stressed, non-persister cells undergoing a normal stress (filamentation) response in their work which invalidates their conclusions related to persister cells. Clearly, the authors should have pretreated with an antibiotic for longer times, i.e., waited until a true plateau was reached, so that persister cells would have been utilized (Barrett *et al*., 2019). Moreover, dormant cells do not mutate so they cannot, by definition, become resistant; resistance is heritable based on mutation, whereas persistence is a non-heritable phenotype. Of course, any time a cell survives antibiotic treatment, its progeny, when they begin growing, undergo mutation and become resistant like normal cells, because resuscitated persisters are identical to normal cells and grow identically (Kim *et al*., 2018b). Similarly, it was claimed

that *M. smegmatis* persister cells mutate due to hydroxyl radicals to form resistant bacteria (Swaminath *et al*., 2020); however, Fig. 1 of Swaminath *et al*. shows that the resistant bacteria that arise do so as the antibiotic-stressed culture is dying, rather than arising from persister cells that formed later. Therefore, the resistant bacteria once again are derived from stressed cells rather than from persisters. Together, these two examples illustrate an alarming trend in persister research: groups are using dying cells rather than using true persister cells. Finally, the Michiels group also claimed persistence promotes antibiotic resistance/mutation (Windels *et al*., 2019); however, to get a correlation for mutation and persistence for their 20 environmental isolates, they eliminated 20% of the data (Fig. S2 of Windels *et al*.). Also, since they studied mutation over 20 days on rich medium agar plates + antibiotic, and they present no data indicating the persister population over the 20 days on plates, it is not clear how they calculated a mutation rate for the persister cells of the environmental isolates (without knowing the number of persister cells as a function of time). Critically, over the 20 days in which resistant colonies appeared on plates, persisters are resuscitating and then are killed by the antibiotics; during this period between persister resuscitation and cell death, resistance may occur so there is no clear evidence that it was not the resuscitated (i.e., normal cells) that mutated rather than the persister cells. Finally, their time-kill kinetics (Fig. S8 of Windels *et al*.) show persisters were never formed on the plates for the *hipA7*cells so its mutation rate cannot be calculated (but one was reported) (Windels *et al*., 2019). In summary, there appears to be no credible evidence showing persister cells mutate, but, instead, the actual problem is that persister cells give rise to actively growing cells that mutate and are selected like all other known bacteria. Clearly, '*de novo* mutation in non-growing cells' (Windels *et al*., 2019) is a non-sequitur.

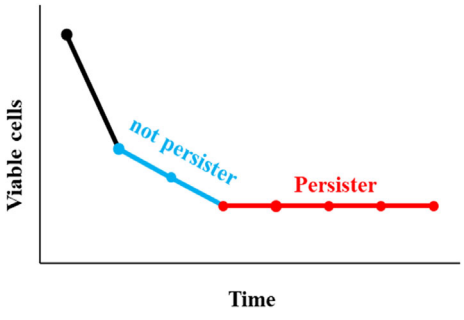

Fig 1. Source of error in many persister experiments. It is common for the rate of cell death to change over the course of stress treatment, so the presence of a change in death rate (blue) of a 'biphasic curve' is not sufficient to ensure persister cells are present. Instead, experiments involving persistence should only utilize cells that have truly plateaued and no longer show appreciable change with time (red).

For the experiments that are discussed herein that led to erroneous conclusions, the common theme is that non-persister cells were studied unintentionally; i.e., experiments were conducted with stressed, but metabolically active cells, and the results were attributed inappropriately to persister cells. Strikingly, it is not enough to use concentrations greater than the minimum inhibitory concentration for the cases of antibiotic stress and assume persister cells are formed just because a biphasic curve is seen for cell killing. Instead, one must wait for the cells to become quiescent and truly plateau in regard to cell temporal killing in order to discern anything about persister cells (Fig. 1). If the cells are dying appreciably, even though the rate of death is reduced compared to the initial rate, the cells are not persisters, since they are not 'persisting' (they are dying). In other words, a biphasic curve with appreciable cell death is not indicative of persistence, but a biphasic curve where the remaining cells are nearly completely tolerant to the stress is indicative of persistence (Fig. 1). Hence, as we move forward in persister-related research, we should ask ourselves, 'Are we really studying persister cells'.

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