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Interpreting phenotypic antibiotic tolerance and persister cells as evolution via epigenetic inheritance

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Abstract

Epigenetic inheritance is the transmission of nongenetic material such as gene expression levels, RNA, and other biomolecules from parents to offspring. There is a growing realization that such forms of inheritance can play an important role in evolution. Bacteria represent a prime example of epigenetic inheritance because a large array of cellular components are transmitted to offspring, in addition to genetic material. Interestingly, there is an extensive and growing empirical literature showing that many bacteria can form 'persister' cells that are phenotypically resistant or tolerant to antibiotics but most of these results are not interpreted within the context of epigenetic inheritance. Instead persister cells are usually viewed as a genetically encoded bet-hedging strategy that has evolved in response to a fluctuating environment. Here I show, using a relatively simple model, that many of these empirical findings can be more simply understood as arising from a combination of epigenetic inheritance and cellular noise. I therefore suggest that phenotypic drug tolerance in bacteria might represent one of the best-studied examples of evolution under epigenetic inheritance.

Keywords

antibiotic resistance; infectious disease; nongenetic; transgenerational inheritance; drug resistance; dormancy

Modern evolutionary biology is founded on the Mendelian-genetic model of inheritance but there is a growing appreciation that this model is incomplete (Jablonka et al., 1992; Jablonka and Lamb, 1995; Richards, 2006; Bossdorf et al., 2007; Youngson and Whitelaw, 2008; Bonduriansky and Day, 2009; Jablonka and Raz, 2009; Szyf, 2015). A considerable body of empirical evidence now shows that a wide spectrum of biological factors such as methylation patterns, cytoplasmic components, nutritional elements, and other biomolecules are often inherited alongside genetic material (Johannes et al., 2009; Jablonka and Raz, 2009; Heard and Martienssen, 2014). Furthermore, these nongenetic factors can have interesting and important evolutionary consequences (Lachmann and Jablonka, 1996; Uller, 2008; Jablonka and Raz, 2009; Day and Bonduriansky, 2011; Shea et al., 2011; Danchin et al., 2011; Bonduriansky et al., 2012).

Bacteria provide an excellent example of nongenetic inheritance because, by being unicellular and generally reproducing through binary fission, they transmit a large array of cellular components from parents to offspring in addition to genetic material (Veening et al., 2008a; van der Woude, 2011; Satory et al., 2011). There is a growing realization that this nongenetic material can cause genetically identical bacterial cells to differ from one another in wide variety of important ways (Ackermann, 2015), including their susceptibility to antibiotics. Several examples of nongenetic antibiotic resistance have been documented (Adam et al., 2008; El-Halfway and Valvano, 2012; Wakamoto et al., 2013; Herman et al., 2014; Sanchez-Romero and Casadesus, 2014; Calo et al., 2014), but the vast majority of research in this area has focused on so-called persister cells (Keren et al., 2004a; Gefen and Balaban, 2009; Lewis, 2007, 2010a,b; Wood et al., 2013; Helaine and Kugelberg, 2014).

When a population of bacteria is exposed to an antibiotic there is typically a very rapid phase of cell killing. This phase is then sometimes followed by a plateau in population size, with the remaining small number of cells being largely unaffected by the drug (Keren et al., 2004a; Dhar and McKinney, 2007; Gefen and Balaban, 2009; Lewis, 2010a,b). These unaffected cells were first described by Bigger (1944) who called them 'persisters'. Interestingly, when these persister cells are placed in fresh, drug free, media they replicate and re-establish a population that is just as susceptible to the antibiotic as was the original population (Bigger, 1944; Keren et al., 2004a; Lewis, 2010a,b). Thus the ability to survive antibiotics appears to be a transient, phenotypic, property of some bacterial cells rather than an genetically determined trait.

The phenomena of persister cells has been the subject of extensive research and this initial interpretation of persister cells as being in an alternative phenotypic state has been confirmed by detailed single-cell studies in some species (Balaban et al., 2004; Gefen and Balaban, 2009). It has been shown that persisters are non-replicating (or slowly replicating) cells and that this dormancy accounts for their reduced susceptibility to drugs (Shah et al., 2006; Hu and Coates, 2012; Wood et al., 2013). Moreover, normal cells can become persister cells stochastically, or in response to environmental triggers such as resource stress or the presence of the antibiotics themselves (Balaban et al., 2004; Dorr et al., 2009; Lewis, 2010a,b; Balaban, 2011; Vega et al., 2012; Cohen et al., 2013; Johnson and Levin, 2013; Helaine and Kugelberg, 2014; Helaine et al., 2014). And cells in this dormant state can re-enter the active replication state (Balaban et al., 2004). Thus the persister cell state is a phenotypic phenomenon.

Most of the research on persister cells is not discussed or interpreted within the context of the growing work on nongenetic inheritance in evolutionary biology. Instead, persister cells are usually viewed as a form of non-heritable drug tolerance because persister cells are not genetically distinct from normal cells, and because they can readily revert to drug sensitivity. Thus persister cell formation is instead viewed as an adaptive, genetically determined, bet-hedging strategy that has evolved in response fluctuating environmental conditions (Balaban et al., 2004; Kussell and Leibler, 2005; Kussell et al., 2005; Gefen and Balaban, 2009; Lewis, 2010a; Cohen et al., 2013). In effect, it is thought that individuals are hardwired to produce a small fraction of phenotypically dormant offspring in case environmental conditions become unfavourable.

Although it is now clear that the distinction between normal and persister cells is nongenetic, this does not imply that such drug tolerance is necessarily non-heritable. As previous reviews have documented (Jablonka et al., 1992; Richards, 2006; Youngson and Whitelaw, 2008; Bonduriansky and Day, 2009; Jablonka and Raz, 2009; Heard and Martienssen, 2014) there are many instances of heritability that are underlain by nongenetic mechanisms. And as already mentioned, bacteria are a prime example of this because virtually all of the cellular material, in addition to genetic material, is transmitted from parent to offspring (e.g., Veening et al. (2008b); van der Woude (2011); Satory et al. (2011)). Thus, it might be expected that nongenetic inheritance would be involved in at least some aspects of the persister cell phenomenon (Veening et al., 2008a; Casadesus and Low, 2013; Motta et al., 2015).

What can be gained from thinking about phenotypic resistance and persister cells in the context of evolution via nongenetic inheritance? Here I use a model for the inheritance of nongenetic material such as gene expression patterns or biochemical concentrations (collectively referred to as epigenetic inheritance) to show that such inheritance, coupled with cellular noise, can provide a simple explanation for many of the observed properties of persister cells. Thus I suggest that phenotypic antibiotic tolerance and the persister cell phenomenon might well represent one of the best-studied empirical examples of evolution via epigenetic inheritance.

The Model

Unlike genetically based inheritance which involves the transmission of discrete gene variants, many examples of nongenetic inheritance are better described by a continuous variable. For instance, gene expression levels, patterns of flux through a biochemical network, or concentrations of RNA might all be inherited, and these are all naturally described by continuous variables. Likewise, some persister cell formation is thought to be affected by the expression level or concentration of certain biomolecules like toxins or signalling molecules (e.g., HipA toxin in *E. coli*; Moyed and Bertrand (1983); Korch et al. (2003); Gefen and Balaban (2009); Lewis (2010a); Rotem et al. (2010); Vega et al. (2012); Wood et al. (2013); Helaine and Kugelberg (2014)). This too might be transmitted from parent to offspring and is best described by a continuous variable. Therefore, I begin by developing a general equation for the evolution of such quantitative epigenetic traits.

I use x to denote the value of the quantitative trait and, for ease of terminology, I will refer to it simply as an individual's 'expression level'. I assume that the expression level must lie between α and β , and that it can be passed from parent to offspring during reproduction with some fidelity (Veening et al., 2008a; Casadesus and Low, 2013; Motta et al., 2015; van der Woude, 2011; Satory et al., 2011). The function $n(x, t)$ denotes the density of individuals in the population with expression level x at time t (i.e., $n(x, t)$ is the number of individuals with expression level between x and $x + dx$).

Four main processes affect the density of individuals having a given expression level: (i) faithful transmission of expression level during reproduction, (ii) alteration of the expression level between generations during the process of reproduction, (iii) within-generation

directional or deterministic change in expression level, and (iv) within-generation noise or unbiased random perturbations in expression level.

Appendix A derives the following equation that accounts for the dynamical consequences of the above four processes:

$$\frac{\partial n(x, t)}{\partial t} = \underbrace{r(x, t) n(x, t)}_{\text{reproduction}} + \underbrace{\frac{\sigma \mu}{2} \frac{\partial^2}{\partial x^2} [n(x, t) b(x, t)]}_{\text{between- generation change}} - \underbrace{\frac{\partial}{\partial x} [v(x) n(x, t)]}_{\text{within- generation directional change}} + \underbrace{m \frac{\partial^2}{\partial x^2} n(x, t)}_{\text{within- generation noise}} \quad (1)$$

where $r(x, t)$ is the net per capita rate of reproduction of individuals with expression level x at time t (i.e., $r(x, t) = b(x, t) - d(x, t)$ where b and d are the birth and death rates), μ is the probability of a change in expression level during reproduction, σ is the variance in this change in expression level, $v(x)$ is the rate of directional change in expression level within a generation, and m is a measure of the unbiased noise in expression level within a generation (see Table 1 for notation).

The first and second terms of equation (1) account for processes occurring between generations. The first term gives the total rate of reproduction of individuals with expression level x (i.e., the per capita rate $r = b - d$ multiplied by the density of individuals n). Some of these reproductive events will result in offspring having a different expression level than that of their parents, however, because the transmission of expression level is not perfectly faithful. This ‘mutational’ effect during reproduction is accounted for by the second term. Appendix A provides an equation that allows for a more general form of such change. It should also be noted that a lack of heritability of expression level can be accounted for in this model as a special case.

The third and fourth terms of equation (1) account for processes occurring within a generation. The third term represents how the density of individuals with expression level x changes as a result of directional or deterministic change from one expression level to another. This within-generation change might be due to physiological or metabolic homeostatic processes operating within an individual, but it can also account for any environmentally induced plastic changes in expression. The function $v(x)$ represents the rate of change of individuals with expression level x towards higher levels. The fourth term accounts for how the density of individuals with expression level x changes as a result of noise giving rise to unbiased stochastic changes in expression level.

Phenotypic Drug Tolerance and Persister Cells

I now consider equation (1) in the context of phenotypic antibiotic tolerance and persister cells, focusing on the case where persister cells are dormant or nonreplicating. The physiological pathway through which these cells are formed is not completely understood. As mentioned above, the concentration HipA toxin (and other toxin-antitoxin modules) have been implicated in some bacteria (Moyed and Bertrand, 1983; Korch et al., 2003; Keren et al., 2004b; Schumacher et al., 2009; Gefen and Balaban, 2009; Rotem et al., 2010; Lewis, 2010a; Wood et al., 2013; Helaine and Kugelberg, 2014). For example in *E. coli* persister

cell formation appears to be regulated, in part, by whether the cellular concentration of this molecule exceeds a certain threshold (Rotem et al., 2010). However, it is increasingly believed that persister cells form a heterogeneous group (Dhar and McKinney, 2007; Zhang, 2007; Joers et al., 2010; Allison et al., 2011; Hofsteenge et al., 2013; Johnson and Levin, 2013; Sanchez-Romero and Casadesus, 2014; Levin et al., 2014), and that there is no single genetic mechanism governing their dynamics (Dhar and McKinney, 2007; Balaban, 2011; Hofsteenge et al., 2013; Willenborg et al., 2014; Germain et al., 2015; Mok et al., 2015). This viewpoint is consistent with the notion that persister cells lie on some biochemical continuum, from ‘shallow’ persisters that readily leave the dormant state to ‘deep’ persisters that take longer to reactivate (Zhang, 2007; Joers et al., 2010; Ma et al., 2010).

Because persister cell formation appears to involve many different cellular processes I attempt to capture the main qualitative empirical findings mentioned above by viewing x as some generic physiological state that I will interpret as the propensity of a cell to become a persister. This could simply be the concentration or expression level of some relevant biomolecule but I will treat it more generally, and view it as the expression level of the persister phenotype. High values of expression level correspond to a high propensity for becoming a persister cell and vice versa. However, to keep things as simple as possible, I assume that an individual cell becomes a persister if its expression level exceeds a threshold x^* . Thus, at time t the total number of cells is $\int_{\alpha}^{\beta} n(x, t) dx$ and the number of persister cells is $\int_{x^*}^{\beta} n(x, t) dx$ (Figure 1a). In this way, individuals with expression levels far exceeding the threshold x^* can be viewed as ‘deep’ persisters while those closer to the threshold are ‘shallow’ persisters.

I suppose that cellular homeostatic mechanisms tend to keep the expression level x of an individual centered around a value that allows for replication. This is modeled by choosing the function $v(x)$ representing within-generation directional processes to have the qualitative form shown in Figure 1b. This function is positive below the homeostatic set point γ and negative above it, meaning that cellular homeostatic processes tend to push the expression level of each individual upward if it is currently below the set point and downward if the expression level is above the set point. At the same time, the expression level is also subject to stochastic noise in various cellular processes (Elowitz et al., 2002; Satory et al., 2011).

To model replication I set $b(x, t) = d(x, t) = 0$ for expression levels above x^* because such expression levels correspond to persister cells and these cells are assumed dormant (allowing persister cells to continue dying at a small rate does not qualitatively alter the results). For simplicity I also suppose that the birth and death rates of individuals with expression levels below x^* are independent of x (Figure 1c).

Much of the experimental data on persisters and drug tolerance in bacteria has been collected during both exponential population growth as well as when the population approaches stationary phase. I therefore include such density-dependent growth by explicitly modeling a resource and its consumption by the bacterial population. The per capita birth rate of non-persister microbes is assumed to depend on the amount of resource present according to the equation $b(x, t) = eaR(t)$ for all values of expression level below the

threshold x^* . Here $R(t)$ is the amount of the resource at time t , a is the ‘attack rate’ of the resource, and ϵ is the conversion efficiency of resource into bacterial cells. The time dynamics of $R(t)$ are modeled as

$$\frac{dR(t)}{dt} = \theta - \eta R(t) - aR(t) \int_{\alpha}^{x^*} n(y, t) dy$$

where θ is the resource inflow rate and η is the per capita loss rate of the resource. For simplicity I assume that the death rate of non-persisters is a constant d .

At this point it should also be noted that the model developed here for density dependence assumes an effectively continuous culture with resource influx rather than a batch culture as is used in most experiments. Thus the stationary phase of population growth in the model arises from a balance between births and deaths rather than the exhaustion of free resources as might occur in batch culture. This is common in many mathematical models of bacterial growth and the results presented below should provide a reasonable approximation to stationary phase in batch culture so long as, when dealing with stationary populations, we restrict attention to durations of time over which bacteria would still maintain some type of physiological activity in batch culture.

Finally, although the dynamics of all persister cells are governed by the inheritance of the nongenetic expression level x in the above model, this does not preclude the possibility that genetic factors influence the persister phenomenon as well. For example, genetic factors might determine quantities like the threshold level x^* , the strength of homeostasis $\nu(x)$, or the amount of noise σ and m . In this way, mutants like the HipA mutant (Moyed and Bertrand, 1983) might be viewed as genotypes with altered parameter values governing the nongenetic inheritance.

Results

A formal mathematical analysis of equation (1) will be presented elsewhere but it is relatively easy to understand the general qualitative behavior of the model by considering the effects of the four processes embodied by this equation. Reproduction (the first term of equation (1)) tends to increase the density of individuals with expression levels below the threshold x^* (Figure 2a). However, lack of fidelity in expression level during reproduction (the second term of equation (1)) tends to smooth out the density profile across all expression levels because it spreads the offspring produced more randomly across the possible values of x . Within-generation noise has a similar effect (the fourth term of equation (1)), also tending to equalize the density of individuals with different expression levels (Figure 2b). Finally, within-generation homeostasis (the third term of equation (1)) tends to concentrate the density of individuals around the homeostatic set point (Figure 2c). All results presented below arise from differences in the relative strengths of these processes.

I begin by first illustrating the typical dynamical behavior of the model. Suppose the population is initialized with a small number of individuals distributed uniformly across all expression levels. Over time the total population size (i.e., the total area under the density

curve) grows, eventually reaching carrying capacity (Figure 3a). Over time the phenotypic distribution of the population (i.e., the shape of the density curve) changes as well, eventually reaching a steady state that reflects a balance among the processes described in Figure 2. The number of persister cells in the population at any time is given by the area under the density curve for values of expression greater than the threshold x^* .

Now suppose that an antibiotic is applied to this hypothetical population and that it affects only non-persister cells. All microbes having an expression level less than x^* will therefore be killed, leaving only those with expression levels greater than x^* (i.e., the persisters) (not shown). If this remaining persister population is then reintroduced into fresh, drug-free, medium then the model predicts that individuals will again begin to replicate as they stochastically leave the persister state (Figure 3b). Over time the very same equilibrium population is again established, and so the fraction of the population that is made up of persister cells at this point will be identical to that of the population prior to the drug exposure.

As discussed in the introduction, the above dynamic, in which the same fraction of persisters is produced both before and after exposure to a drug, is characteristic of experimental studies of persisters (Keren et al., 2004a; Gefen and Balaban, 2009; Lewis, 2010a,b; Willenborg et al., 2014). Indeed this is sometimes taken as a defining feature of phenotypic drug tolerance. This pattern is often described as demonstrating a lack of heritability of the persister phenotype but the model makes it clear that such a pattern does not imply a lack of heritability *per se*. In the model the expression level, and thus the persister phenotype, can be perfectly heritable (i.e., $\mu = 0$) and the very same re-establishment of a susceptible population occurs. Heritability quantifies the relationship between the phenotype of parents and that of their offspring and so perfect heritability simply means that offspring always have the same expression level as their parents (i.e., the same propensity to become a persister cell). Even in this case, however, cellular noise and homeostasis within a generation causes the phenotypic composition of the population to change as regrowth occurs, ultimately giving rise to the same distribution of expression levels, and thus the same number of persister cells as was present before drug exposure (Figure 3,b). And this is true regardless of the number of times the drug has been applied. In effect the phenotypic distribution of the population rapidly evolves through epigenetic inheritance, noise, and homeostasis to attain the same equilibrium state.

One might guess that the way in which the equilibrium population state is reached, and the time it takes for this to occur, will depend on the amount of within-generation noise and the heritability of the expression level. This is indeed the case. For example, suppose that the processes of within-generation noise in expression level and homeostasis are weak (and heritability is high) relative to the process of reproduction during the exponential growth phase. In this case the process of reproduction in Figure 2a will dominate the other processes in Figure 2b,c until carrying capacity is approached. As a result, the total population size (i.e., $\int_{\alpha}^{\beta} n(x, t) dx$) reaches carrying capacity before the homogenizing effects of between-generation change/noise, and the directional effects of homeostasis, equalize the expression levels (i.e., before the *shape* of the density curve in Figure 4a becomes constant).

Consequently, the number of persister cells does not increase much during the exponential growth phase (Figure 4c) and so their frequency initially declines (Figure 4d). As carrying capacity is reached (i.e., as the upward movement of the density curve in Figure 4a starts to slow) the distribution of expression levels continues to equalize, eventually resulting in an increase in the number of persister cells even though the total population is nearly constant by this time (i.e., it is in stationary phase; Figure 4c).

On the other hand, if the processes of within-generation noise in expression level and homeostasis are strong (and heritability is low) relative to the process of reproduction during the exponential growth phase, then the homogenizing effects of noise and homeostasis will equalize the expression levels before much change in population size occurs (i.e., the shape of the density curve in Figure 4b becomes constant before it increases much in height). In this case there is then always nearly a constant fraction of the population that is made up of persisters throughout the exponential growth and stationary phases (Figure 4b,c).

Interestingly, the contrasting behavior in Figure 4a versus Figure 4b has been observed experimentally (e.g., Keren et al. (2004a); Balaban et al. (2004)). This difference has been taken to imply the existence of two qualitatively distinct kinds of persister cells (Balaban et al., 2004; Gefen and Balaban, 2009; Willenborg et al., 2014). So-called Type I persisters are thought to be produced in response to stresses like the reduced resource level that occurs as the population approaches stationary phase whereas so-called Type II persisters are instead thought to be produced stochastically at a constant rate throughout the entire growth cycle of the population. However, Figure 4c shows that these dynamical patterns might also arise for other reasons. In particular, even if the formation of persister cells is never induced by the environment, the processes of replication coupled with different levels of heritability and cellular noise can generate these very same patterns. As before this is because epigenetic inheritance allows the phenotypic distribution of the population to rapidly evolve in response to changing conditions.

Along similar lines it has also been shown experimentally that persister cells can sometimes be reduced to below detectable levels by maintaining the bacterial culture in a state of early exponential growth via serial dilution (Keren et al., 2004a). The explanation for this finding has been that these persister cells are of Type I and therefore no new persisters are formed under these resource rich conditions. As a result, any persister cells already present will be lost through serial dilution. The model developed here shows that this finding might actually be expected, even in the absence of environmentally induced changes in the rate of persister formation. For example, under exponential growth the asymptotic frequency distribution of expression levels is always skewed towards low expression levels and thus towards a low frequency of persister cells. This occurs simply because it is the replicating cells (i.e., those with low expression levels) that dominate the population under these resource rich conditions (Figure 5). Moreover, if the replication rate is large relative to the amount of noise in expression level and the strength of homeostasis (i.e., if the process in Figure 2a is strong relative to that in Figures 2b,c) then the fraction of the population that is made up of persister cells becomes negligible (the 'high' case in Figure 5). Again this has nothing to do with environmentally induced changes in persister formation but instead is a dynamic

consequence of the evolutionary shift in the phenotypic composition of the population through epigenetic inheritance when resources are abundant.

Recent work has also shown that, in some instances, the number of persister cells remaining after exposure to a drug increases with the amount of time the population spends in stationary phase in batch culture prior to the drug being applied (Luidalepp et al., 2011). One interpretation of this finding is again that adaptive persister formation continually occurs in stationary phase as a means of dealing with resource deprivation. Although the model developed here does not explicitly account for stationary phase in batch cultures it does predict that the longer the population remains at carrying capacity after exponential growth, the more even the distribution of expression levels will become because cellular noise then has had more time to equalize this distribution. As a result, we would expect to see a positive relationship between the number of persister cells and the amount of time spent at carrying capacity (e.g., Figure 4c), even if there were not environmentally-induced changes in persister formation.

The results presented so far have shown that many of the observed empirical features of persister cells can be explained by evolution via epigenetic inheritance and cellular noise. Moreover, these features are an expected consequence of these processes even in the absence of environmentally-induced changes in persister formation. That said, there are several studies that show how the environment, including the presence of antibiotics and other stresses, can affect the likelihood of persister cell formation (Dorr et al., 2009; Lewis, 2010a,b; Balaban, 2011; Vega et al., 2012; Cohen et al., 2013; Johnson and Levin, 2013; Helaine and Kugelberg, 2014; Helaine et al., 2014). As shown next, the model of evolution under epigenetic inheritance can explain these results as well, if cellular noise increases with environmental stress.

It seems plausible that stress, such as the presence of antibiotics or the absence of resources, might lead to an increased noise in expression level (and perhaps a decrease in the fidelity of transmission of expression level from parents to offspring). If so, such stress would tend to further equalize the distribution of expression levels in the population (i.e., it would increase the relative strength of the process in Figure 2b). As Figure 6 illustrates, this flattening of the density curve increases the number of cells with expression levels greater than the threshold x^* , and as a result the fraction of persister cells increases. Notice that this environmentally-induced increase in persister cell formation is not an evolved adaptive response to stress, but instead is again a dynamic consequence of the evolutionary shift in the phenotypic composition of the population due to the altered balance of the processes described in Figure 2.

Finally, it is worth emphasizing that including the above stress-induced change in cellular noise does not qualitatively alter any of the previous conclusions (Day unpublished results). For example, in Figure 3, if a decline in resource abundance causes stress-induced noise, then at equilibrium the density curve is simply a bit more flat (and there is a greater frequency of persister cells as a result). The same is true for Figure 4, and stress-induced noise then also enhances the increase in the rate of appearance of persister cells as carrying capacity is reached in Figure 4c. And for Figure 5, because there is no resource depletion,

including stress-induced noise has no effect. Thus, a model of evolution via epigenetic inheritance (with cellular homeostasis and stress-induced noise) provides a simple alternative hypothesis to the view that persister cell dynamics reflect an adaptive, genetically-encoded, response to environmental fluctuations.

Discussion

The modeling results presented here suggest that many of the empirically documented features of phenotypic antibiotic tolerance and the formation of bacterial persister cells can be explained by evolution via epigenetic inheritance. This contrasts the predominate view in the literature that persister cells represent a genetically encoded form of bet-hedging or adaptive plasticity (Balaban et al., 2004; Kussell and Leibler, 2005; Kussell et al., 2005; Gefen and Balaban, 2009; Lewis, 2010a; Cohen et al., 2013). Given the extensive experimental work that has been conducted on phenotypic antibiotic tolerance, it therefore might well represent one of the best-studied examples of the role of epigenetic inheritance in evolution.

The current empirical observations that can potentially be explained by the model of epigenetic inheritance and evolution include that: (i) the persister cells that survive an antibiotic go on to re-establish a population that is just as susceptible to the drug as was the original population (Figure 3); (ii) sometimes persister cells arise only as the population reaches stationary phase (so-called Type I persisters) whereas in other cases they arise continuously throughout the growth cycle (so-called Type II persisters; Figure 4); (iii) cultures maintained in early exponential growth phase through serial dilution show a marked decrease in persister cells (Figure 5); (iv) the time a culture spends in stationary phase is positively related to the number of persister cells that form (Figure 4c); and (v) stressors like antibiotics or resource depletion can increase the formation of persister cells (Figure 6).

The above five findings have been explained previously as resulting from adaptive bet-hedging or adaptive phenotypic plasticity. For example, if bacterial cells have evolved (genetically) to switch back and forth randomly between dormant and active states as an adaptive response to environmental variation, then such populations will display the patterns seen in finding (i) above (Balaban et al., 2004; Kussell et al., 2005). These bacteria would represent so-called Type II persister cells. On the other hand, Type I persister cells have previously been explained as resulting from bacteria evolving a (genetically encoded) form of adaptive phenotypic plasticity. In essence, bacteria have evolved to more readily switch into the dormant state when conditions become stressful. This would also explain findings (ii)-(v).

The main novelty of the results presented here is to show that these same five findings can instead be readily explained as a result of a combination of epigenetic inheritance and cellular noise. During replication parents transmit their expression level (i.e., the propensity to become a persister) to the descendant cells with some level of fidelity. This transmission process is noisy, however, and there is also assumed to be within-generation noise in the maintenance of the expression level. As a result, the phenotypic distribution of the

population is very malleable and so can readily evolve in response to changing conditions through epigenetic inheritance.

When resources are abundant the population grows exponentially and the phenotypic distribution evolves a skew towards low expression levels because these are the cells with the greatest growth rate (finding (iii)). As stationary phase is approached, resources become more scarce and the phenotypic distribution relaxes as noise evens out the abundance of different expression levels. As a result, the number of persister cells increases. If resource depletion also causes a stress-induced increase in cellular noise then this pattern is further enhanced. However, the way in which the number of persister cells increases, and how quickly this happens, depends on the rate of exponential growth relative to the amount of noise in expression level (findings (ii) and (iv)). Moreover, this evolutionary dynamic recurs upon repeated exposure of the population to antibiotics (finding (i)).

Finding (v) can be explained by the idea that drug exposure or resource depletion are stressors that increase the within-generation noise in expression level and/or decrease the fidelity of transmission from parent to offspring. In this case, in the absence of the stressor, the expression levels of individuals will be clustered around the homeostatic set point. If the population is then exposed to a stressor then the distribution of expression levels will become more uniform, generating an increase in the number of persister cells.

The above-described process of noise-induced persistence is effectively a variation on the ‘persistence as stuff happens’ or PaSH hypothesis put forward by Levin and colleagues (Johnson and Levin, 2013; Levin et al., 2014). They argued that persister cells arise from random errors and glitches due to antibiotic-induced stress and, as such, are effectively like deleterious mutations. The noise-related process outlined here is similar in that antibiotic-induced stress causes the physiological state of individual cells to deviate from the homeostatic set point. This too can be viewed as deleterious although it represents a deviation of physiological state within the range of normal variation rather than a deleterious mutation *per se*. Furthermore, stress-induced noise would increase the density of cells with expression levels lower than the set point, in addition to increasing the number of persister cells (i.e., those with very high expression levels).

The predominate view of persister cells in the literature is that they represent a genetically-encoded bet-hedging strategy and/or a form of adaptive phenotypic plasticity. Consequently, considerable research effort has been devoted to identifying the genes that underly this strategy. The consensus to date is that many different genetic pathways appear to play some role in the formation of persister cells but no major genetic control mechanism has been identified (Lewis, 2010a; Shan et al., 2015). For example, genetic knock-out studies in *E. coli* have failed to produce genotypes that no longer generate persister cells (Hansen et al., 2008), suggesting that there are many pathways involved in their formation. Interestingly this has also been interpreted as evidence for the strong selective advantage of bet-hedging because we might then expect many redundant genetic pathways to evolve as backups. As Levin and colleagues have argued, however, the lack of singular genetic control is exactly what would be expected under their PaSH hypothesis (Johnson and Levin, 2013). It is also exactly what is expected here as well. If persister cell formation involves the type of

epigenetic inheritance described here, then they should continue to be formed almost irrespective of the underlying genotype. At the same, however, many different genetic mutations would likely alter the processes described in Figure 2, and so would alter the way in which persister cells are formed and their abundance.

Whether or not the formation of persister cells through environmental induction is adaptive remains an interesting and important open question. In fact, although the results presented here show that stress-induced noise alone can increase the formation of persister cells, this response might still be adaptive. For example, if such plastic, environmentally-induced, persister formation is selectively advantageous, then a noise-related process like that described here might provide a simple mechanism through which it could operate. Interestingly, the model also suggests one way in which these two possibilities might be distinguished experimentally.

Suppose one could measure the physiological propensity of a cell to become a persister (i.e., measure the expression level x of a molecule whose concentration is known to have a positive effect on persister cell formation). Furthermore, suppose it was possible to measure the within-generation stochastic change in this expression level. Now imagine conducting an experiment in which one group of bacterial cells was subjected to a stress while a control group was maintained under normal conditions. The distribution of within-generation changes in expression level for each group could then be compared. Under the stress-induced noise hypothesis outlined above, the distribution of expression level changes in the group subjected to the stress should have the same mean as that of the control group but a higher variance, because stress simply increases the noise. More persisters would nevertheless be formed in the stressed group because such unbiased noise would result in a net change towards higher expression levels as it equalizes the distribution (i.e., as in Figure 6). Such a finding would thus be consistent with the hypothesis that persister cell formation is not adaptive but instead is the result of epigenetic inheritance and noise.

On the other hand, if the distribution of within-generation changes in expression level in the stressed group had a higher mean than that of the control group then, regardless of any difference between the groups in variance, this would reveal a deterministic or directional change in expression that was induced by the environment. In terms of the model such an effect would be reflected by the function $v(x)$ changing sign as a result of the stress. In other words, there would be a stress-induced *directional* change in expression and thus a deterministic induction of the persister phenotype. This would provide much more compelling evidence that the induced response was adaptive and would thereby call into question the idea that stress-induced noise alone is sufficient to explain persister cell formation.

The model explored here assumes that the expression level is free to change in an unbiased fashion, aside from homeostasis tending to push it towards a set point value. There is some suggestion in the literature that, in fact, a positive feedback mechanism might operate that tends to hold persister cells in their dormant state as well (Lou et al., 2008; Feng et al., 2014). In effect, a continuous variable like expression level somehow underlies a bistable cellular ‘switch’ between the persister and non-persister states (Satory et al., 2011). This can

also be included in the present model by specifying a slightly different function $v(x)$ so that there are then two different homeostatic set points, one for non-persister cells and one for persisters.

It has also been noted recently that the formation of persister cells is sometimes dependent on the concentration of the antibiotic used, and that higher concentrations sometimes ultimately result in fewer persister cells remaining after the antibiotic has been applied (zur Wiesch et al., 2015; Regoes et al., 2004). Recent work has also shown how this pattern can be explained by simple reaction kinetics underling the drug action (zur Wiesch et al., 2015).

Here I simply note that this phenomenon is also consistent with the current hypothesis. For example, if higher drug concentrations result in faster killing of non-persister cells, then the net flow of persister cells into the active state will be higher over this time period, essentially because the gradient driving this ‘diffusion’ will be steeper. As a result, more persister cell will be killed over a fixed period of time. I also note, however, that relaxing the simplifying assumption that the birth and death rates of cells are step functions of expression level, and that persister formation obeys a strict threshold, further allows for more nuanced patterns of persister formation. In either case however the same conclusion remains - evolution via epigenetic inheritance can explain a wide variety of the empirical observations about phenotypic drug tolerance and persister cell formation.

The modeling results presented here suggest several avenues for future research, both empirical and theoretical. For example, it might prove useful to couple the epigenetic model developed here with a model of genetic inheritance. Although phenotypic antibiotic tolerance is now a widely appreciated phenomenon, it has also been suggested that genetically determined resistance sometimes follows after phenotypic resistance has been established (Cohen et al., 2013). Exactly when and how this might occur however is not completely understood. Exploring this possibility might not only be important from a medical standpoint, but it also has the potential to provide an excellent example of how genetic and nongenetic forms of inheritance interact in evolution more broadly.

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Appendix 1 - Derivation of Equation 1

The number of individuals with expression level between x and $x + \Delta x$ at time $t + \Delta t$ is approximately

$$n(x, t + \Delta t) \Delta x = (1 - d(x, t) \Delta t) n(x, t) \Delta x + (1 - \mu) n(x, t) \Delta x b(x, t) \Delta t + \mu \int_{\alpha}^{\beta} n(y, t) \Delta x b(y, t) \Delta t p(x; y) dy + J(x) \Delta t - J(x + \Delta x) \Delta t$$

(see Table 1 for notation). The second and third terms account for reproduction when between-generation change does not occur and when it does occur respectively. The third

term adds up all of the ways that offspring with expression level x can be produced by parents as a result of between-generation change during reproduction. Rearranging gives

$$\frac{n(x,t+\Delta t)-n(x,t)}{\Delta t} = -d(x,t)n(x,t) + (1-\mu)n(x,t)b(x,t) + \mu \int_{\alpha}^{\beta} n(y,t)b(y,t)p(x;y)dy - \frac{J(x+\Delta x)-J(x)}{\Delta x}$$

or

$$\frac{\partial n(x,t)}{\partial t} = r(x,t)n(x,t) - \mu n(x,t)b(x,t) + \mu \int_{\alpha}^{\beta} n(y,t)b(y,t)p(x;y)dy - \frac{\partial J(x)}{\partial x}$$

Using the equation for $J(x)$ from Table 1, this simplifies to

$$\frac{\partial n(x,t)}{\partial t} = r(x,t)n(x,t) - \mu n(x,t)b(x,t) + \mu \int_{\alpha}^{\beta} n(y,t)b(y,t)p(x;y)dy - \frac{\partial}{\partial x} [v(x)n(x,t)] + m \frac{\partial^2}{\partial x^2} n(x,t)$$

This last equation is the main result. However, the integral term can be difficult to work with and so it is worth considering how we might simplify this under certain assumptions.

Suppose that the distribution of jumps specified by $p(x;y)$ is identical for all y .

Mathematically, suppose that $q(\delta)$ is a probability density function for the jump size $\delta = x - y$ for all values of y . Thus we can write $p(x;y) = q(x - y)$. Then the second and third terms of the above equation are

$$-\mu n(x,t)b(x,t) + \mu \int_{-\infty}^{\infty} n(y,t)b(y,t)q(x-y)dy = -\mu n(x,t)b(x,t) + \mu \int_{-\infty}^{\infty} n(x-\delta,t)b(x-\delta,t)q(\delta)d\delta$$

Now suppose further that q is ‘narrow’ and has zero mean. Defining $F(x-\delta,t) = \mu(x-\delta)n(x-\delta,t)b(x-\delta,t)$ and expanding it in a Taylor series in δ near zero gives

$$\begin{aligned} & -\mu n(x,t)b(x,t) \\ & + \int_{-\infty}^{\infty} \left[F(x,t) - F_x(x,t)\delta + F_{xx}(x,t)\frac{\delta^2}{2} + \dots \right] q(\delta)d\delta \approx \\ & -\mu n(x,t)b(x,t) \\ & + F(x,t) \int_{-\infty}^{\infty} q(\delta)d\delta \\ & - F_x(x,t) \int_{-\infty}^{\infty} \delta q(\delta)d\delta \\ & + F_{xx}(x,t) \int_{-\infty}^{\infty} \frac{\delta^2}{2} q(\delta)d\delta \\ & = F_{xx}(x,t) \int_{-\infty}^{\infty} \frac{\delta^2}{2} q(\delta)d\delta \\ & = \frac{\sigma\mu}{2} \frac{\partial^2}{\partial x^2} [n(x,t)b(x,t)] \end{aligned}$$

where $\sigma = \int_{-\infty}^{\infty} \delta^2 q(\delta)d\delta$ is the variance in jump size.

Thus the full equation is

$$\frac{\partial n(x, t)}{\partial t} = r(x, t) n(x, t) + \frac{\sigma \mu}{2} \frac{\partial^2}{\partial x^2} [n(x, t) b(x, t)] - \frac{\partial}{\partial x} [v(x) n(x, t)] + m \frac{\partial^2}{\partial x^2} n(x, t)$$

Even with this approximation, however, one can mimic the effects of low heritability by taking σ large relative to the other parameter values.

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Figure 1.

Schematic of model assumptions. The constants α and β are the lower and upper bounds on expression level. Expression level x^* is the value above which a cell becomes a persister. Grey shading indicates expression levels for which persister formation occurs. (a) Density of cells with different expression levels, $n(x, t)$. Green area represents total number of non-persister cells. Red area represents total number of persister cells. (b) Qualitative form of function $v(x)$, giving the within-generation rate of directional change in expression level as result of physiological homeostasis within a cell. The constant γ is the homeostatic set point

expression level. (c) Birth and death rates, $b(x, t)$ and $d(x, t)$, as a function of expression level.

Figure 2.

Processes embodied by equation (1). The constants α and β are the lower and upper bounds on expression level. Expression level x^* is the value above which a cell becomes a persister. Grey shading indicates expression levels for which persister formation occurs. All plots show density of cells with different expression levels at three different time points. (a) When resources are abundant reproduction increases the density of non-persister cells only. (b) Between-generation change/noise tends to equalize the density across expression levels. (c) Homeostasis tends to concentrate expression levels around the set point γ .

Figure 3.

Density of cells with different expression levels over time, $n(x, t)$. Each curve represents a particular point in time. Expression levels are arbitrarily bound between $\alpha = -0.9$ and $\beta = 0.1$ with $x^* = 0$ being the threshold for persister formation. Grey shading indicates expression levels for which persister formation occurs. (a) Initial density is $n(x, 0) = 1$ (i.e., uniform across all expression levels). (b) Initial density is $n(x, 0) = 1$ if $x > x^*$ and $n(x, 0) = 0$ otherwise (i.e., only persister cells present). Parameter values: $\theta = 100$, $\eta = 1$, $\epsilon = 0.1$, $m =$

$0.001, \sigma = 0.0015, \mu = 0.00075, \gamma = -0.5, \nu(x) = 0.5(x - \alpha)(x - \beta)(x - \gamma), a = 0.15, d = 0.1$.
Model solved numerically for 200 time units using *Mathematica*.

Figure 4.

(a) and (b). Density of cells with different expression levels over time, $n(x, t)$. Each curve represents a particular point in time. Expression levels are arbitrarily bound between $\alpha = -0.9$ and $\beta = 0.1$ with $x^* = 0$ being the threshold for persister formation. Grey shading indicates expression levels for which persister formation occurs. (a) Between-generation change/noise and homeostasis are weak relative to reproduction when resources are abundant ($m = 0.00025$, $v(x) = 0.075(x - \alpha)(x - \beta)(x - \gamma)$). Phenotypic distribution (i.e., the shape of the density curve) equalizes slowly (particularly for values of $x > x^*$), and not until after carrying capacity is approached. (b) Between-generation change/noise and homeostasis are strong relative to reproduction when resources are abundant ($m = 0.05$, $v(x) = 15(x - \alpha)(x - \beta)(x - \gamma)$). Phenotypic distribution equalizes quickly and then simply increases until carrying capacity is reached. (c) Total population size of non-persister (black) and persister (red) cells over time for the numerical results in panels (a) and (b). (d) Fraction of population consisting of persister cells as a function of time for the numerical results in panels (a) and (b). All other parameter values: $\theta = 100$, $\eta = 1$, $\epsilon = 0.1$, $\sigma = 0.00015$, $\mu = 0.000075$, $\gamma = -0.5$, $a = 0.15$, $d = 0.1$. Model solved numerically for 500 time units using *Mathematica*.

Figure 5.

Asymptotic *probability density* of cells with different expression levels during exponential population growth (i.e., the area under the curve is 1). Curves correspond to different exponential growth rates (i.e., resource abundances). Expression levels are arbitrarily bound between $\alpha = -0.9$ and $\beta = 0.1$ with $x^* = 0$ being the threshold for persister formation. Grey shading indicates expression levels for which persister formation occurs. Parameter values: $m = 0.01$, $\sigma = 0.0015$, $\mu = 0$, $\gamma = -0.5$, $v(x) = 0.5(x - \alpha)(x - \beta)(x - \gamma)$, $d = 0.1$. Birth rate for non-persister cells was set to a constant value of either $b = 0.15$ (low), $b = 1$ (medium), or $b = 10$ (high) to generate different exponential growth rates.

Figure 6.

Density of cells with different expression levels over time, $n(x, t)$. Curves correspond to different levels of within-generation noise in expression level. Expression levels are arbitrarily bound between $\alpha = -0.9$ and $\beta = 0.1$ with $x^* = 0$ being the threshold for persister formation. Grey shading indicates expression levels for which persister formation occurs. Parameter values: $\sigma = 0.0015$, $\mu = 0$, $\gamma = -0.5$, $v(x) = 5(x - \alpha)(x - \beta)(x - \gamma)$, $d = 0.1$, and birth rate for non-persister cells set to balance death rate (i.e., $b = 0.1$). Noise levels are $m = 0.01$ (low) and $m = 0.02$ (high).

Table 1

$n(x, t)$ - density of individuals with expression level x at time t
α, β - lower and upper limits of expression level
x^* - threshold expression level above which a cell becomes a persister
$b(x, t)$ - birth rate of individuals with expression level x at time t
$d(x, t)$ death rate of individuals with expression level x at time t
$r(x, t) = b(x, t) - d(x, t)$ - net rate of reproduction of individuals with expression level x at time t
$J(x)$ - flux, at expression level x , of individuals towards higher levels of gene expression as a result of noise and within-generation directional change. It is given by Fick's law as
$J(x) = - \frac{\partial}{\partial x} \left[mn(x, t) \right] + v(x)n(x, t)$
where m is the 'rate of movement' or 'noisiness' of expression level x and $v(x)$ is the rate of increase of expression level as a result of directional change.
μ - the probability that, when an individual reproduces, the expression level of the offspring is different from that of the parent (i.e., 'mutation' occurs).
$p(x; y)$ - the probability density of the offspring's expression level x , given that the parent has expression level y and a 'mutation' occurs during reproduction
σ - mutational variance in expression level during reproduction; $\sigma = \int_{\alpha}^{\beta} (x - y)^2 p(x, y) dx$
$R(t)$ - resource abundance at time t
θ - rate of resource inflow
η - per capita loss rate of resource
α - 'attack' rate of resource by bacteria
ϵ - conversion efficiency of resource into bacteria