EPIGENETIC STUDIES IN ECOLOGY AND EVOLUTION

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 is 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, dormancy, drug resistance, infectious disease, nongenetic, transgenerational inheritance

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Introduction

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 & Lamb 1995; Richards 2006; Bossdorf *et al.* 2007; Youngson & Whitelaw 2008; Bonduriansky & Day 2009; Jablonka & 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 (Jablonka & Raz 2009; Johannes *et al.* 2009; Heard & Martienssen 2014). Furthermore, these nongenetic factors can have interesting and important evolutionary consequences (Lachmann & Jablonka 1996; Uller 2008; Jablonka & Raz 2009;

Danchin *et al.* 2011; Day & Bonduriansky 2011; Shea *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; Satory *et al.* 2011; van der Woude 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 & Valvano 2012; Wakamoto *et al.* 2013; Calo *et al.* 2014; Herman *et al.* 2014; Sanchez-Romero & Casadesus 2014), but the vast majority of research in this area has focused on so-called persister cells (Keren *et al.* 2004a; Lewis 2007, 2010a, b; Gefen & Balaban 2009; Wood *et al.* 2013; Helaine & Kugelberg 2014).

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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 & McKinney 2007; Gefen & 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 a genetically determined trait.

The phenomenon 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 & Balaban 2009). It has been shown that persisters are nonreplicating (or slowly replicating) cells and that this dormancy accounts for their reduced susceptibility to drugs (Shah *et al.* 2006; Hu & 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 & Levin 2013; Helaine *et al.* 2014; Helaine & Kugelberg 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 nonheritable 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 to fluctuating environmental conditions (Balaban *et al.* 2004; Kussell *et al.* 2005; Kussell & Leibler 2005; Gefen & Balaban 2009; Lewis 2010a; Cohen *et al.* 2013). In effect, it is thought that individuals are hard-wired 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 nonheritable. As previous reviews have documented (Jablonka *et al.* 1992; Richards 2006; Youngson & Whitelaw 2008; Bonduriansky & Day 2009; Jablonka & Raz 2009; Heard & 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; Satory *et al.* 2011; van der Woude 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 & 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 *Escherichia coli*; Moyed & Bertrand (1983); Korch *et al.* (2003); Gefen & Balaban (2009); Lewis (2010a); Rotem *et al.* (2010); Vega *et al.* (2012); Wood *et al.* (2013); Helaine & 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; Satory *et al.* 2011; van der Woude 2011; Casadesus & Low

2013; Motta *et al.* 2015). The function $n(x, t)$ denotes the density of individuals in the population with expression level x at time t (i.e. $n(x, t)\Delta x$ is the number of individuals with expression level between x and $x + \Delta x$).

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

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 eqn (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 of toxin (and

$$\frac{\partial n(x, t)}{\partial t} = \underbrace{r(x, t)n(x, t)}_{\text{reproduction}} + \underbrace{\frac{\sigma\mu}{2}\frac{\partial^2}{\partial x^2}[n(x, t)b(x, t)]}_{\substack{\text{between-generation}\\\text{change}}} - \underbrace{\frac{\partial}{\partial x}[v(x)n(x, t)]}_{\substack{\text{within-generation}\\\text{directional change}}} + \underbrace{m\frac{\partial^2}{\partial x^2}n(x, t)}_{\substack{\text{within-generation}\\\text{noise}}} \qquad (eqn1)$$

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 eqn (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 I 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 eqn (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.

other toxin–antitoxin modules) has been implicated in some bacteria (Moyed & Bertrand (1983); Korch *et al.* 2003; Keren *et al.* 2004b; Gefen & Balaban 2009; Schumacher *et al.* 2009; Lewis 2010a; Rotem *et al.* 2010; Wood *et al.* 2013; Helaine & 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 & McKinney 2007; Zhang 2007; Joers *et al.* 2010; Allison *et al.* 2011; Hofsteenge *et al.* 2013; Johnson & Levin 2013; Levin *et al.* 2014; Sanchez-Romero & Casadesus 2014), and that there is no single genetic mechanism governing their dynamics (Dhar & 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

Table 1. Mathematical notation.

$n(x, t)$	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 $J(x) = -\frac{\partial}{\partial x}[mn(x, t)] + v(x)n(x, t)$ 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
a	'Attack' rate of resource by bacteria
ε	Conversion efficiency of resource into bacteria

Fig. 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 nonpersister 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.

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 $\int_{x^*}^\beta n(x, t)dx$ (Fig. 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 centred around a value that allows for replication. This is modelled by choosing the function $v(x)$ representing within-generation directional processes to have the qualitative form shown in Fig. 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
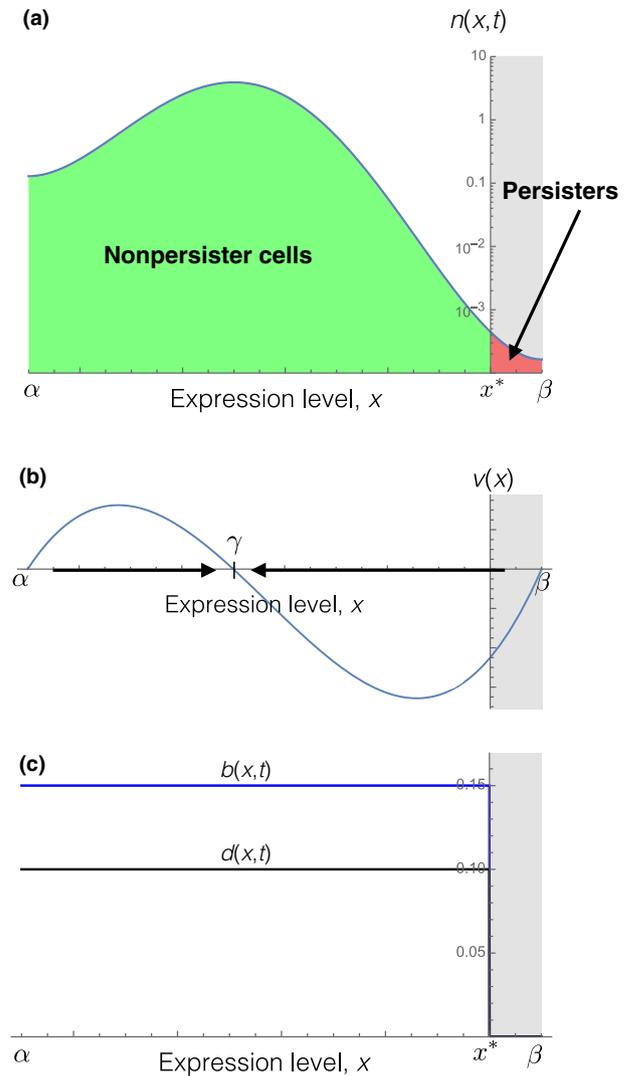
expression level is above the set point. At the same time, the expression level is also subject to stochastic noise in various cellular processes (Elowitz et al. 2002; Satory et al. 2011).

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 (Fig. 1c).

Much of the experimental data on persisters and drug tolerance in bacteria have 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 modelling a resource and its consumption by the bacterial population. The per capita birth rate of nonpersister microbes is assumed to depend on the amount of resource present according to the equation $b(x, t) = \varepsilon a R(t)$ 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 modelled as

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

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 nonpersisters 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 $v(x)$ or the amount of noise σ and m. In this way, mutants like the HipA mutant (Moyed & Bertrand 1983) might be viewed as genotypes with altered parameter values governing the nongenetic inheritance.

Results

A formal mathematical analysis of eqn (1) will be presented elsewhere, but it is relatively easy to understand the general qualitative behaviour of the model by con-

sidering the effects of the four processes embodied by this equation. Reproduction (the first term of eqn 1) tends to increase the density of individuals with expression levels below the threshold x^* (Fig. 2a). However, lack of fidelity in expression level during reproduction (the second term of eqn 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 eqn 1), also tending to equalize the density of individuals with different expression levels (Fig. 2b). Finally, within-generation homeostasis
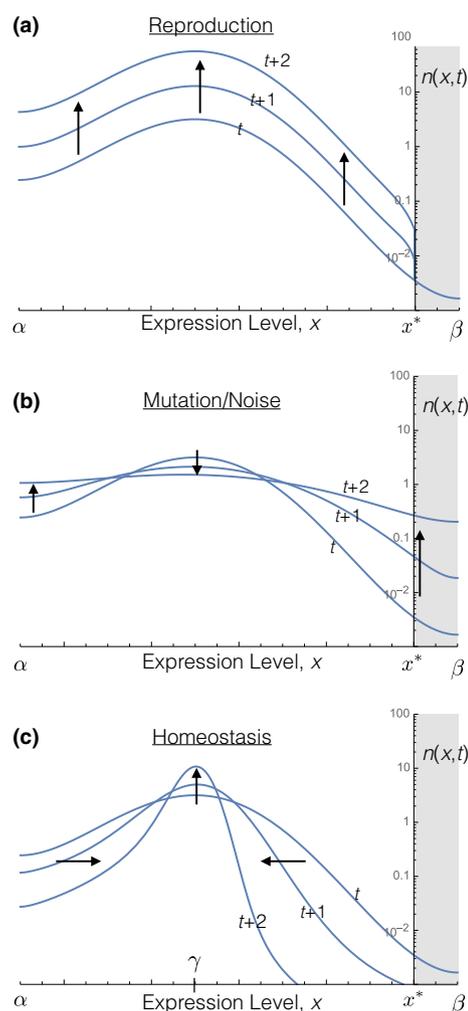

Fig. 2. Processes embodied by eqn (1). The constants α and β are the lower and upper bounds on expression level. Expression level x^* is the value above which a cell becomes a persister. Grey shading indicates expression levels for which persister formation occurs. All plots show density of cells with different expression levels at three different time points. (a) When resources are abundant, reproduction increases the density of nonpersister 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.

(the third term of eqn 1) tends to concentrate the density of individuals around the homeostatic set point (Fig. 2c). All results presented below arise from differences in the relative strengths of these processes.

I begin by first illustrating the typical dynamical behaviour 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 (Fig. 3a). Over time the phenotypic distribution of the
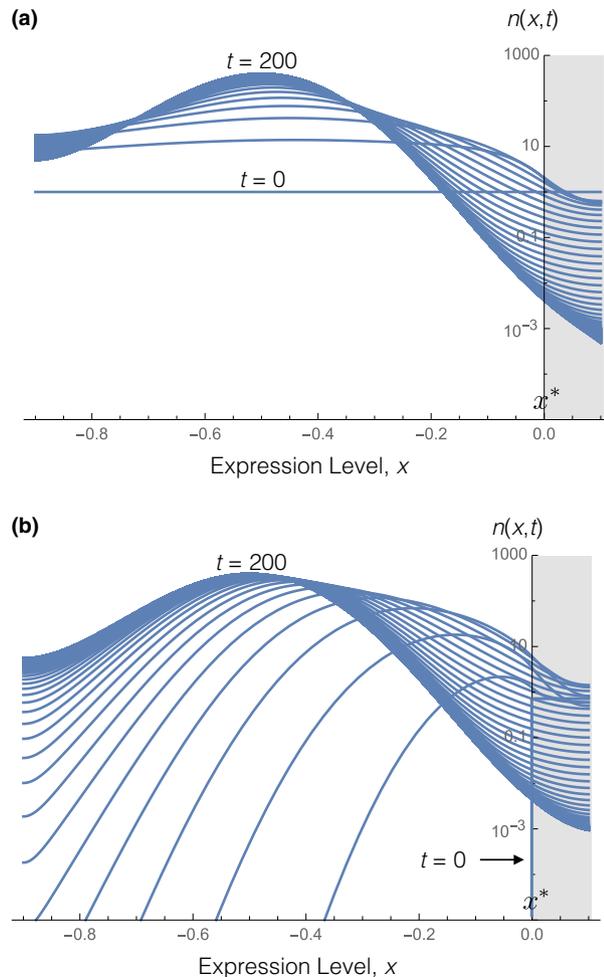

Fig. 3. Density of cells with different expression levels over time, $n(x, t)$. Each curve represents a particular point in time. Expression levels are arbitrarily bound between $\alpha = -0.9$ and $\beta = 0.1$ with $x^* = 0$ being the threshold for persister formation. Grey shading indicates expression 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: $\theta = 100$, $\eta = 1$, $\varepsilon = 0.1$, $m = 0.001$, $\sigma = 0.0015$, $\mu = 0.00075$, $\gamma = -0.5$, $v(x) = 0.5(x-\alpha)(x-\beta)(x-\gamma)$, $a = 0.15$, $d = 0.1$. Model solved numerically for 200 time units using *Mathematica*.

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 Fig. 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 nonpersister 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 (Fig. 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 & 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 cause 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 (Fig. 3b). 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 Fig. 2a will dominate the other processes in Fig. 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 Fig. 4a becomes constant). Consequently, the number of persister cells does not increase much during the exponential growth phase (Fig. 4c) and so their frequency initially declines (Fig. 4d). As carrying capacity is reached (i.e. as the

upward movement of the density curve in Fig. 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; Fig. 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 Fig. 4b becomes constant before it increases much in height). In this case, there is then always nearly a con-
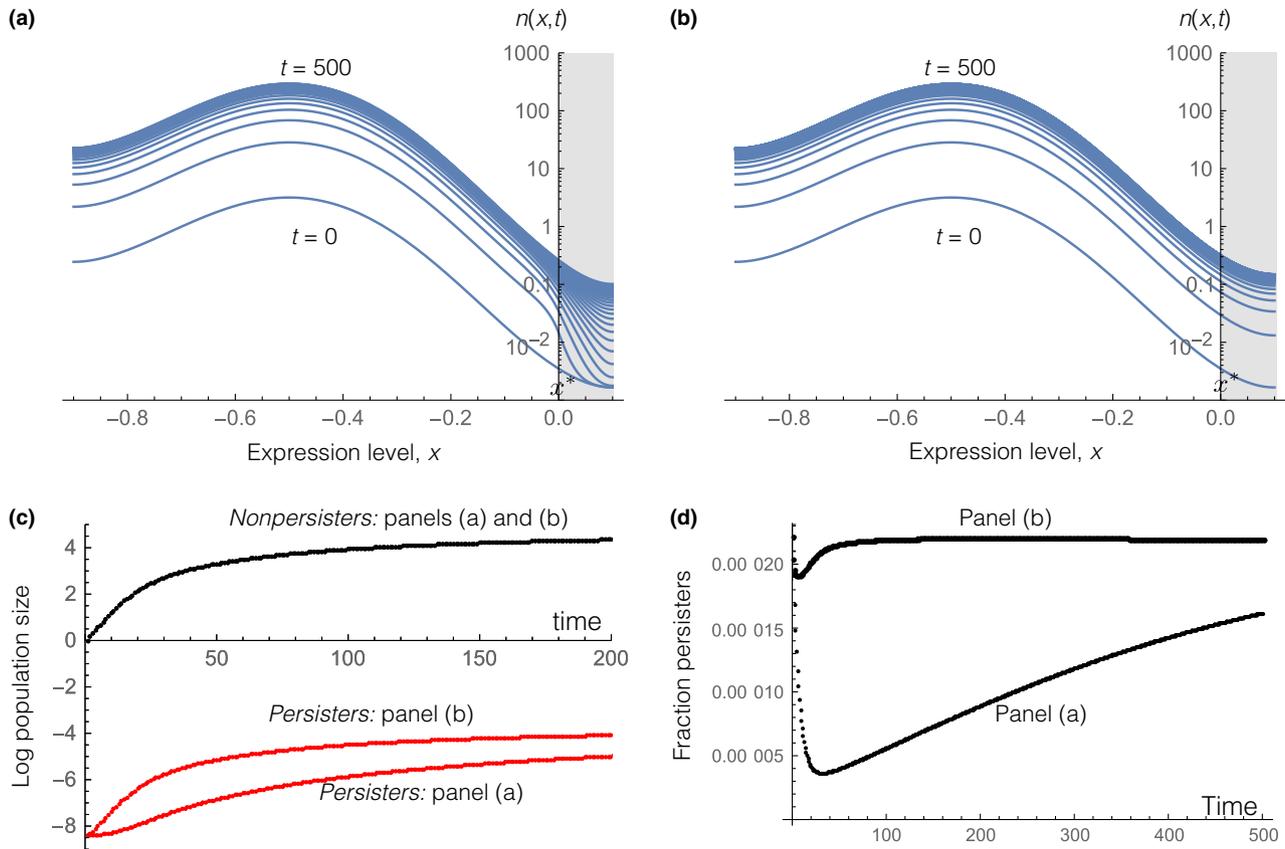

Fig. 4. (a) and (b). Density of cells with different expression levels over time, $n(x, t)$. Each curve represents a particular point in time. Expression levels are arbitrarily bound between $\alpha = -0.9$ and $\beta = 0.1$ with $x^* = 0$ being the threshold for persister formation. Grey shading indicates 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 nonpersister (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: $\theta = 100$, $\eta = 1$, $\varepsilon = 0.1$, $\sigma = 0.00015$, $\mu = 0.000075$, $\gamma = -0.5$, $a = 0.15$, $d = 0.1$. Model solved numerically for 500 time units using *Mathematica*.

stant fraction of the population that is made up of persisters throughout the exponential growth and stationary phases (Fig. 4b, c).

Interestingly, the contrasting behaviour in Fig. 4a vs. Fig. 4b has been observed experimentally (e.g. Balaban *et al.* 2004; Keren *et al.* 2004a.). This difference has been taken to imply the existence of two qualitatively distinct kinds of persisters cells (Balaban *et al.* 2004; Gefen & 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, Fig. 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 (Fig. 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 Fig. 2a is strong relative to that in Fig. 2b, c), then the fraction of the population that is made up of persister cells becomes negligible (the 'high' case in Fig. 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.
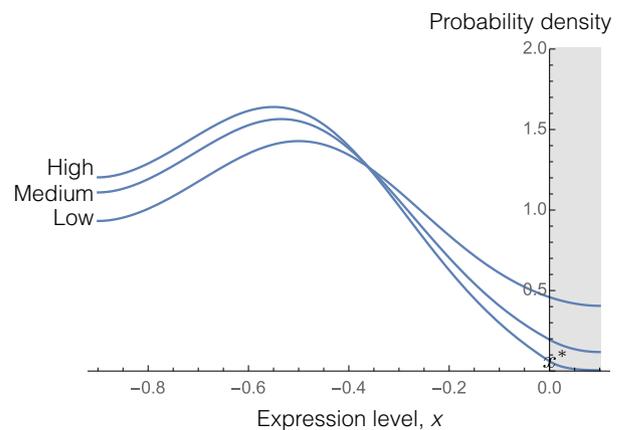

Fig. 5. Asymptotic *probability density* of cells with different expression levels during exponential population growth (i.e. the area under the curve is 1). Curves correspond to different exponential growth rates (i.e. resource abundances). Expression levels are arbitrarily bound between $\alpha = -0.9$ 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 nonpersister 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.

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. Fig. 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 & Levin 2013; Helaine *et al.* 2014; Helaine & Kugelberg 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 form 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 Fig. 2b). As Fig. 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 Fig. 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 (T. Day, unpublished results). For example, in Fig. 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 persis-
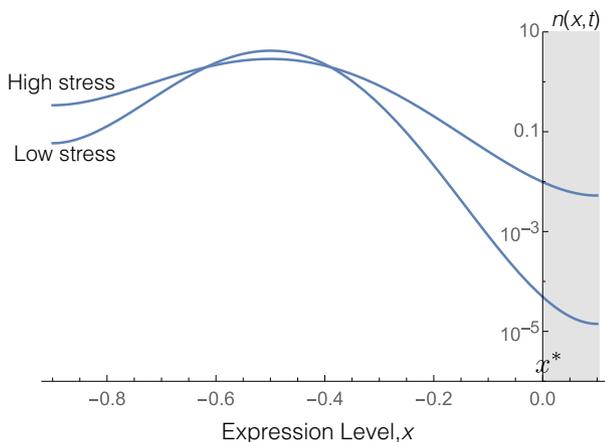
ter cells as a result). The same is true for Fig. 4, and stress-induced noise then also enhances the increase in the rate of appearance of persister cells as carrying capacity in reached in Fig. 4c. And for Fig. 5, because there is no resource depletion, including stress-induced noise has no effect. 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 modelling 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 *et al.* 2005; Kussell & Leibler 2005; Gefen & 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 (Fig. 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; Fig. 4); (iii) cultures maintained in early exponential growth phase through serial dilution show a marked decrease in persister cells (Fig. 5); (iv) the time a culture spends in stationary phase is positively related to the number of persister cells that form (Fig. 4c); and (v) stressors like antibiotics or resource depletion can increase the formation of persister cells (Fig. 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

Fig. 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 nonpersister cells set to balance death rate (i.e. $b = 0.1$). Noise levels are $m = 0.01$ (low) and $m = 0.02$ (high) .

(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 is a stressor that increases the within-generation noise in expression level and/or decreases 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 & 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 predominant 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 & 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 probably alter the processes described in Fig. 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 Fig. 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 nonpersister 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 nonpersister 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 Regoes *et al.* 2004; Wiesch *et al.* 2015). 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 phe-nomenon is also consistent with the current hypothesis. For example, if higher drug concentrations result in faster killing of nonpersister 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 modelling 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

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

(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

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

or

$$
\begin{aligned}
\frac{\partial n(x, t)}{\partial t} = {} & r(x, t)n(x, t) - \mu n(x, t)b(x, t) \\
& + \mu \int_{\alpha}^{\beta} n(y, t)b(y, t)p(x; y)\mathrm{d}y - \frac{\partial J(x)}{\partial x}.
\end{aligned}
$$

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

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

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

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

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

$$-\mu n(x,\,t)b(x,\,t) + \int_{-\infty}^{\infty} \left[F(x,\,t) - F_x(x,\,t)\delta + F_{xx}(x,\,t)\frac{\delta^2}{2} + \cdots \right] q(\delta)\mathrm{d}\delta$$

$$\approx -\mu n(x,\,t)b(x,\,t) + F(x,\,t)\int_{-\infty}^{\infty} q(\delta)\mathrm{d}\delta - F_x(x,\,t)\int_{-\infty}^{\infty} \delta q(\delta)\mathrm{d}\delta$$

$$+ F_{xx}(x,\,t)\int_{-\infty}^{\infty}\frac{\delta^2}{2} q(\delta)\mathrm{d}\delta = F_{xx}(x,\,t)\int_{-\infty}^{\infty}\frac{\delta^2}{2} q(\delta)\mathrm{d}\delta = \frac{\sigma\mu}{2}\frac{\partial^2}{\partial x^2}[n(x,\,t)b(x,\,t)]$$

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

Thus, the full equation is

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

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