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Growth feedback as a basis for persister bistability

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A small fraction of cells in many bacterial populations, called persisters, are much less sensitive to antibiotic treatment than the majority. Persisters are in a dormant metabolic state, even while remaining genetically identical to the actively growing cells. Toxin and antitoxin modules in bacteria are believed to be one possible cause of persistence. A two-gene operon, HipBA, is one of many chromosomally encoded toxin and antitoxin modules in *Escherichia coli* and the HipA7 allelic variant was the first validated high-persistence mutant. Here, we present a stochastic model that can generate bistability of the HipBA system, via the reciprocal coupling of free HipA to the cellular growth rate. The actively growing state and the dormant state each correspond to a stable state of this model. Fluctuations enable transitions from one to the other. This model is fully in agreement with experimental data obtained with synthetic promoter constructs.

As far back as the 1940s, it was known that a small fraction of a bacterial population can survive even when exposed to prolonged antibiotic treatment (1, 2). This phenomenon is termed persistence and members of the surviving subpopulation are called persisters. It has been estimated that the frequency of persisters in normal wild-type populations is extremely small, perhaps of order 10^{-5}–10^{-6} (3). Although the number of persisters is tiny, they are often the main obstacle to attempts to completely eradicate infection.

Remarkably, there is no apparent change in the persisters' DNA sequence; i.e., their survival is not due to mutation (4). Already in 1944, Bigger suggested that persisters are phenotypically different, in a dormant state instead of an actively growing state (1). The dormant state is presumably better able to deal with common antibiotics, which typically target only actively growing cells. Bigger's assumption was confirmed by a later study (3). In this study, Balaban et al. investigated the persistence of a single cell of *Escherichia coli* by using a microfluidic device. They showed that individual persisters do not always remain in the dormant state. Instead, they stochastically transit into an actively growing state and these newly transited cells are indistinguishable from other normally growing cells. Conversely, normal cells can transit into the persistent state. Thus, bacterial persistence at the population level is governed by a single-cell "phenotypic switch." The precise workings of this switch have to date remained unclear.

In the 1980s, Moyed and Bertrand identified the first high-persistence mutant, HipA7, having a persister frequency that is near 10^{-2} (4). The discovery of HipA7 facilitated the study of bacterial persistence due to its relatively high proportion of persisters. It was found that HipA7 is formed by a two-residue substitution in the HipA protein. This protein acts as a toxin in a toxin–antitoxin (TA) module (5, 6), where the *hipB* gene is coexpressed with *hipA* and the corresponding protein binds to and neutralizes HipA toxicity. To date, HipA is one of only a few molecules that are validated tolerance factors (7).

There have already been several models proposed for the Hip system and its connection to persistence. Two modeling groups have claimed that fluctuations cause the apparent coexistence of these two phenotypes, growing and dormant, even though there may or may not be any formal bistability. They were partially driven to this conclusion by their inability to find bistability in their assumed dynamics. The pioneering model of Rotem et al. (8) did not consider the dimerization of HipB and the repression by the HipB dimer of the hip promoter. In the alternate formulation of Koh and Dunlop (9), the HipA-dependent reduction of the translation rate and the growth rate is not included. Thus, both these works claim that bistable states are not necessarily the mechanism underlying persister formation. However, models with a single stable state invariably predict fast transitions between persisters and normally growing cells. For example, simulations in ref. 9 show that transitions from persisters to normally growing cells typically happen within 1 h. In contrast, a sizeable number of persisters can survive even when the antibiotic treatment is maintained for longer than 1 d. If cells stay in a persister state only for less than 1 h, and the persister becomes fragile when it transits into the normally growing state, they would not survive much longer than the other normal cells. The correct picture must include a long-time duration of the persister state.

One model has indeed suggested that bistability is the key to the formation of persisters (10). This model made some assumptions now known to be inaccurate, for example that free HipA undergoes dimerization and that the binding of the HipA-HipB complex to the hip promoter is independent from the binding between the HipB dimer and the hip promoter. (Actually they compete with each other in binding to the same operator sites.) However, this model does explain an interesting observation, that often persisters are formed much more readily in stationary phase and in fact persisters seen in normal exponential phase are often just the remnant of persisters formed at a different growth stage. This pattern has been called type I persistence (3) and is the type seen in the HipA7 mutant. As we will see, this occurs due to the fact that the range of bistability can depend on the growth condition. A different issue is that this model is fully deterministic and hence cannot address stochastic effects such as transitions between the two stable states.

The drawbacks of these models have motivated us to construct a more precise and comprehensive stochastic model for the HipBA system. A recent paper revealing the structure of HipA and its binding has helped guide us to correct the assumptions in the previous bistable model (11). We show that our approach can consistently account for different classes of experimental data and hence can form a framework for continuing analysis of this important survival strategy for wide classes of bacteria.

Significance

The phenomenon of persistence is important both at a fundamental level in serving as a striking example of adaptive phenotypic variability and from the applied perspective as it contributes to the antibiotic resistance of bacteria in general and biofilms in particular. Our paper presents a unique quantitatively successful model of persistence in *Escherichia coli* and helps explain many puzzling observations in the literature. It will serve as a guide for further work, both experimental and theoretical. The primary molecular actors in our approach are a toxin–antitoxin pair HipBA, and we use very recent structural data to formulate a comprehensive approach to this problem and to guide further work. Finally, our effort is consistent with recent ideas regarding the fact that many toxin–antitoxin pairs may contribute in a parallel manner to the persister state.

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Genetic Circuit of the HipBA System

The HipBA operon is one of many chromosomally encoded TA modules identified in *E. coli*. The HipBA operon encodes two molecules, HipA and HipB. HipA is a 50-kDa protein, which has a bacterial serine/threonine protein kinase that can phosphorylate the essential translation factor, EF-Tu. Therefore, it has been suggested that HipA places the cell into a dormant state by inhibition of protein synthesis (11, 12). The *hipA* gene is preceded in the operon by the *hipB* gene, which encodes a 10-kDa protein. HipB forms a complex with HipA, leading to its deactivation (13). Typically, the toxin is relatively stable and the antitoxin unstable; this means that turning off the operon will typically result in growth arrest due to residual toxin. The HipB dimer can in fact bind to one of four operator sites on the hip promoter and repress the expression of both *hipA* and itself. A recent structural study showed that two HipA molecules bind to opposite sides of a HipB dimer and such interaction increased the binding affinity between the HipB dimer and DNA (11) (Fig. 1).

We include in our model dimerization of HipB as well as cooperative binding of two HipA monomers to the HipB dimer, resulting in the deactivation of the toxin activity. These reactions are both more rapid processes than the slower transcription and translation; we thus assume they are always are in equilibrium. This leaves us with the algebraic relations

$$\begin{aligned} B_f^2 &= K_B \cdot B_2 \\ A_f^2 \cdot B_2 &= K_{A_2B_2} \cdot A_2B_2 \\ A_T &= A_f + 2A_2B_2 \\ B_T &= B_f + 2B_2 + 2A_2B_2, \end{aligned}$$

where the concentrations of interest are total HipA or HipB denoted as A_T or B_T, free HipA or HipB denoted as A_f or B_f, HipB dimer B_2, and the HipA-HipB complex A_2B_2. K_B is the dissociation constant of the B dimer and $K_{A_2B_2}$ is the dissociation constant of A_2B_2. This then leaves us with two dynamic equations for the production of the two proteins; in the deterministic limit, these are

$$\begin{aligned} \frac{d[A_T]}{dt} &= \frac{\alpha\beta}{1+\left(\frac{B_2+\theta A_2B_2}{K_{B_2}}\right)^4} f_1(A_f) - \gamma_1 A_f - f_2(A_f) A_T d \\ \frac{d[B_T]}{dt} &= \frac{\alpha\beta}{1+\left(\frac{B_2+\theta A_2B_2}{K_{B_2}}\right)^4} f_1(A_f)\cdot N - \gamma_2 B_f - 2\gamma_3 B_2 - f_2(A_f) B_T d. \end{aligned} \qquad [1]$$

The first term in the two equations combines transcription and translation. It takes into account the fact that the HipB dimer either by itself or bound to HipA represses transcription, albeit with slightly different rates. Here K_{B_2} is the number of free B_2 to reach half-maximal repression on transcription of A and B. The Hill coefficient is 4 because HipB binds cooperatively to four operators upstream of *hipBA*. N is used for describing the fact that the number of B is approximately five times the number of A in normally growing cells (13). The function f_1 arises via translation repression due to free HipA and is assumed to be of the Hill function form

$$f_1(A_f) = \frac{1}{1+\left(\frac{A_f}{K_{A_{21}}}\right)^{n3}},$$

where $K_{A_{21}}$ is number of free A to reach half-maximal repression on translation. $n3$ is the Hill coefficient, and we assume it to be 2 to observe a wide bistable region (*Results*).

The next set of terms in the equations describes decay of the proteins. Finally, the last term arises via the diluting effect of cell growth, parameterized by the "bare" dilution rate d simply related to cell growth in the absence of any free toxin; this rate is assumed to be modulated by the toxin concentration by another Hill function,

$$f_2(A_f) = \frac{1}{1+\left(\frac{A_f}{K_{A_{22}}}\right)^{n4}},$$

where $K_{A_{22}}$ is the number of free A to reach half-maximal repression on growth. For a similar purpose to that for $n3$, we assume it to be 4.

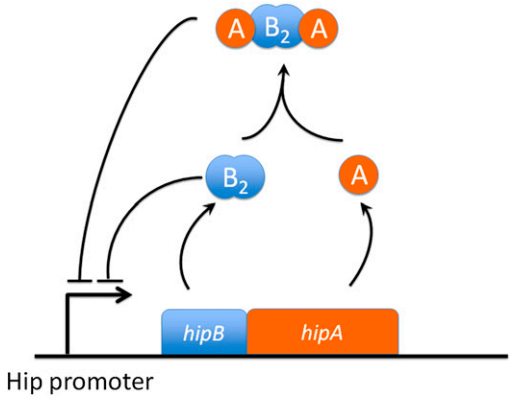

Fig. 1. Schematic representation of the genetic circuit of the HipBA system.

Methods

Our approach is to study both the solution of this deterministic system and the stochastic process defined by assuming each of the (slow) reactions occurs stochastically with rates given by the various individual terms. We obtain simulation results by conducting Monte Carlo simulations, using the Gillespie algorithm (details in *SI Text*).

The parameters are given in Table S1. In our simulations, whether a cell is labeled a persister or a normally growing cell is determined by the level of total HipA (details in *SI Text*).

For the experiments in which *hipA* and/or *hipB* is expressed from artificial promoters, we remove the transcription regulation factor in the first term of the first and/or the second term of Eq. **1** and replace the fixed constant β with constants β_1 and/or β_2. Finally, the effect of the mutant allele hipA7 is modeled by a dramatic reduction in the affinity factor governing formation of the inactive complex.

Results

First, we present a typical dynamic simulation of the HipBA system as predicted by our model (Fig. 2*A*). From this curve, we can see the stochastic transition from a low HipA state (normally growing state) to a high HipA state (persister state) in a cell, as well as the transition from persister back to normal state. Note that the cell remains in the persister state for over 50 h, which could explain the sizable survivor population under prolonged antibiotic treatment. Long-time simulation shows that the whole population could be divided into two distinct subpopulations. (Fig. 2*B*). Such a bimodal distribution has been observed in an experiment by a sorting method based on cell growth rate (14). The long transition time from one stable state to the other is presumably partly caused by the considerable distance between these two stable states.

Because the total number of HipB molecules is approximately the same in both states, the key variable determining the growth state of the cell is the total number of HipA molecules. We found that this is most affected by the dilution rate. This is because we assumed, in line with what is known in general about TA modules, that the intrinsic decay of HipA is negligibly small. Hence, it is bare dilution rate (i.e., the rate at vanishing HipA) that determines the level of HipA. Indeed, as we increased the

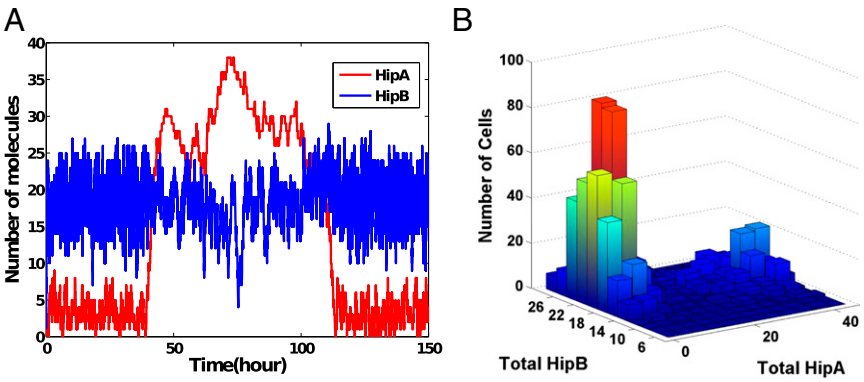

Fig. 2. (*A*) Dynamic curve of the HipBA system. Red line: total HipA number in the cell. Blue line: total HipB number in the cell. (*B*) Distribution of molecular numbers. Starting from 1,000 empty cells, we ran the simulation for a long time to let the system reach equilibrium. Then we determined the distribution of molecule numbers within these cells. Here we present an extreme example in which the frequency of persisters is huge so that we can see the persister state more clearly.

dilution, fewer cells were found in the high HipA persister state (Fig. 3). In other words, a higher dilution rate results in a lower persister fraction. The persister fraction can in fact become quite low, depending on exactly where the system sits in the phase diagram.

It is known that the dissociation rate between the HipB dimer and HipA in the HipA7 strain is higher than that in the wild-type strain (8). Thus, the dissociation rate between the HipB dimer and HipA should also be a critical parameter for the persister fraction. The reason is straightforward: There is more free HipA (or more precisely HipA7) in the cells due to a lower binding affinity between HipA7 and HipB. Hence more persisters are formed.

We can plot a bifurcation diagram to explore the influence of these two critical parameters on the formation of persisters (Fig. 3*C*). Imagine cells in a batch culture: In early exponential phase, the growth rate of cells is relatively high due to the favorable environment. [Note that the dilution rate is directly determined by this growth rate, $d = ln2$ (1/cell cycle period)]. Suppose the state of a cell is located near point C; at this point, there can be no persister formation because point C is located in a region where the only stable state is the normally growing state. Then as the size of the population grows, the growth rate (dilution rate) of cells decreases due to the increasingly adverse environment and the state of cells moves from point C to point A. In the process the parameters governing the cell would cross the border of the bistable region (normal cell and persister coexistence) (Fig. 4). Once the cell lies in the bistable region, any individual has the chance to become persistent and the lower the dilution rate, the higher this chance would be (Fig. 3 *A* and *B*).

This phenomenon is exactly what happens in what has been called type I bacterial persistence (Fig. 3*D*). Two different persistence types were defined in previous studies (3, 15). In type I persistence, persisters always spontaneously switched to normally growing cells, but were formed only in late exponential phase or stationary phase. In type II persistence, the switching occurred spontaneously in both directions during all growing stages. From the discussion above, we find that the reason for the different possibilities of persister formation in selected growth phases is the different dilution rate (10). In other words, our model predicts that it is quite likely that restoring rapid growth will move the system out of the bistable range and hence the original persisters will relatively rapidly decay to normal cells. This can be directly shown in a simulation of our model (Fig. S1). Using our bifurcation graph here, we could move one step further: If we shift the state of cells sufficiently far rightward, cells in all growth phases could be located within the bistable region (as the solid double arrow in Fig. 3*C* indicates). That suggests that a much higher dissociation rate of the HipBA complex could be an explanation for the appearance of type II persistence. Note that *hipA7* still exhibits type I persistence and hence is not "far enough" to the right of the phase diagram.

There is another conclusion we can obtain from this bifurcation graph. If we imagine that the state of the *hipA7* strain settles at point A, we should expect that the *hipA* wild-type strain is located over to the left, due to the lower dissociation rate between the HipB dimer and HipA (this is based on the assumption that HipA7 has the same toxicity as HipA) (*Discussion*). As the bifurcation graph shows, a leftward shift resulted in a smaller persister fraction or sometimes even no persister fraction (Fig. 3*C*, point D). In the latter case, it suggests that the formation of persisters for the *hipA* genotype may not in fact be always caused by the HipBA system. This latter possibility is in fact consistent

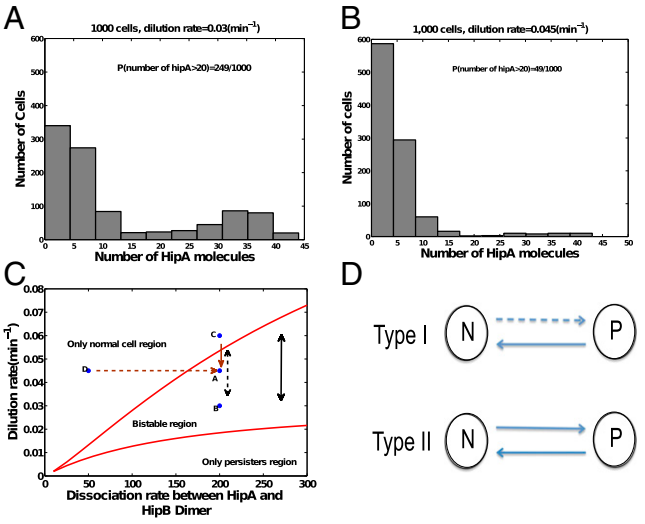

Fig. 3. (*A*) The 2D distribution graph in Fig. 2 reduced to a distribution of HipA numbers. (*B*) Distribution of HipA numbers under a higher dilution rate. (*C*) Bifurcation graph. The whole graph can be divided into three parts: the top region where normal state is the only stable state, the middle region where both normal state and persister state are stable states, and the bottom region where the persister state is the only stable state. Point A corresponds to the *Upper Right* graph (*B*), whereas point B corresponds to the *Upper Left* graph (*A*). Point C represents a cell state in early exponential phase. Points A, B, and C are for the HipA7 strain whereas point D is for the wild-type strain. The brown dashed arrow shows the mutation from wild-type HipA to HipA7. The brown solid arrow describes the cell in the early exponential phase gradually entering stationary phase. The black dashed double arrow shows a range that can generate a persister fraction near 10^{-2}. The black solid double arrow tells how type II persistence is formed. (*D*) A scheme for two different types of bacterial persistence. The solid line shows transition happens in all conditions, whereas the dashed line shows transition happens only in the slow-growth state.

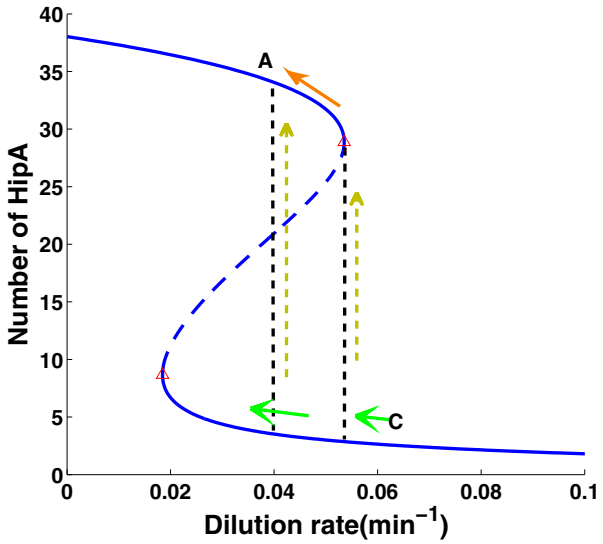

Fig. 4. Transition from single stable state to bistable states: C and A here correspond to point C and point A in Fig. 3C. The dashed arrow indicates the transition from the normal state to the persister state. The solid blue line describes the steady state of HipA vs. dilution rate. The segment between two red triangles (blue dashed line) correspond to an unstable state that cannot be observed.

with the fact that it has been hard to demonstrate that deletion of this module significantly changes persister fraction (16). Under either assumption, we can explain the much smaller persister fraction observed in the wild-type strain.

One key parameter that determines the level of fluctuations in the system is the typical number of molecules in the various states. These numbers are not known experimentally. To see what difference this might make, we rescaled our system to make the number of both HipA and HipB molecules three times as large as before, keeping fixed all of the parameters that enter into the dynamics in the deterministic limit. In Fig. 5, we plot the dynamics of persister formation at two different levels of noise, starting from the no persister state. Reducing the noise (Fig. 5*B*) causes the system to require a much longer time to reach equilibrium. In fact, when HipA and HipB are abundant in the cell, it is likely that the frequency of persisters never gets to equilibrium. In stationary phase, the frequency keeps on increasing [e.g., Fig. 3*A* showed the steady-state value of this frequency could reach 25% under the same dilution rate $(0.03\ \text{min}^{-1})$] and the observed number would depend strongly on the precise details of the experimental protocols. It is of course also possible that the dynamics in the truly stationary phase become altered, which could explain why we observe only a very low frequency of persisters.

The above simulation addressed how the frequency of persisters changes starting from normal state cells; to do this we set the number of HipA and HipB molecules to zero initially. The reverse process is equally interesting: If we start with all persisters in the system, how fast would the transition back to the normally growing state be? In the experiment, when adding antibiotics to a bacterial culture, the killing curve is well described by double-exponential kinetics (Fig. 6*A*) (3). A simple explanation for this curve is that after all normally growing cells die, the population is primarily composed of persisters, which gives rise to a much lower rate of decrease of the population size. However, there exist two possibilities: Persisters could die at a much slower rate or persisters could essentially survive the antibiotic treatment but die once they stochastically convert back into normal cells. To investigate this issue, we started from 10,000 persisters and measured the transition rate from persisters to normal cells. (Fig. 6*B*). It turned out that even for model parameters where the number of HipA and HipB molecules was relatively scarce, the transition rate was extremely slow. Hence our model is more consistent with the former explanation: Persisters still die under antibiotic treatment but at a much slower rate than normally growing cells. Note that this prediction is not contradicted by data showing that sensitivity to phage infection scaled directly with transitions back to normal growth (17). In that experiment, the system was placed in fresh medium, and hence the stability of the persister state was lost, and there was rapid reversion (Fig. S1). This could be very different from the case of a population exposed to antibiotics where long-term survival (tens of hours) means that there must be a barrier to the disappearance of the persistence state.

To explore the mechanism behind bacterial persistence, Rotem et al. (8) created a synthetic system where *hipA* was removed from its normal promoter and instead put under the control of the tet promoter. This construct is depicted in Fig. 7*A*. In this way, the expression level of *hipA* could be directly controlled. In the experiment, they first induced the expression of *hipA* in cells for 3 h and then plated them under conditions in which the *hipA* expression was repressed. The growth arrest time was defined as the time duration from plating to the appearance of a colony. The dependence of growth arrest time on HipA expression level is reproduced in Fig. 7. We can directly model this experiment if we remove the transcription regulation of HipA production from our equation, replacing it with an externally controlled induction rate.

A distinguishing feature of the growth arrest time vs. HipA expression level curve is the existence of a relatively smooth growth of the arrest time above threshold. For the model presented in ref. 8, simulations sometimes exhibited a very sharp threshold instead of the smooth change seen in the measured curve (inset in their figure 7b). This is presumably due to their weaker nonlinearity that ultimately is the same feature that led to the lack of a bistable region. Also, they did not attempt to fit their model parameters to reproduce the actual timescale of the experiment. In contrast, our simulation results (Fig. 7) are consistent with experiment results both in the shape of the curve and in the physical values of the growth arrest times. If in addition we add to our calculation a reasonable caricature of measurement

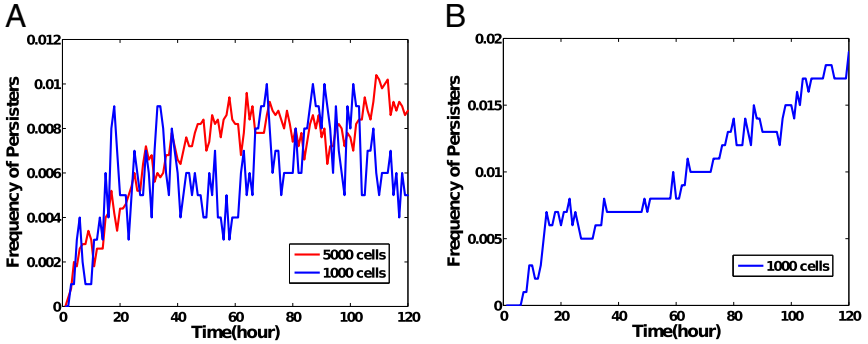

Fig. 5. Dynamic curve of frequency of persisters at two different scales. Start from empty cells and count the frequency of persisters at each point. (*A*) Dynamic curve when the number of molecules is small. Considering the fluctuation is relatively large when counting 1,000 cells, we increase the sample size to 5,000 cells and we can clearly see the frequency of persisters gets to equilibrium after 120 h (dilution rate $=0.04\ \text{min}^{-1}$). (*B*) Dynamic curve when the number of molecules is two times larger. The frequency of persisters keeps increasing even after 120 h. We choose a lower dilution rate $=0.03\ \text{min}^{-1}$ to get an equivalent frequency to that in *A* within 120 h.

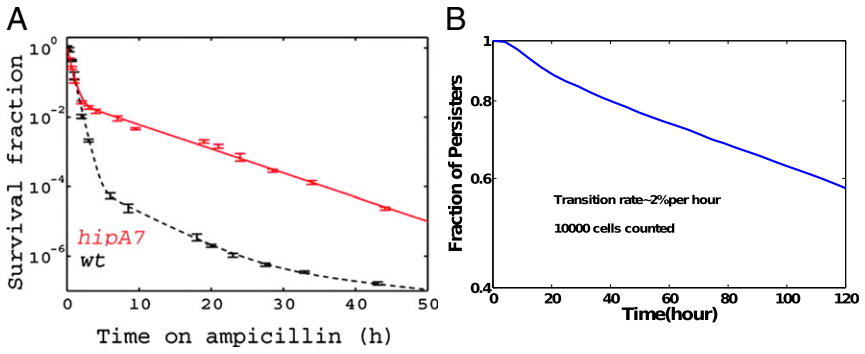

Fig. 6. (*A*) Experiment: killing curve of wild-type strain and *hipA7* mutant. (*B*) Simulation: transition curve from persister to normal cells. A sizable population still stays in the persister state after 120 h. *A* is reproduced with permission from the work of Balaban et al. (3).

error (Fig. 7*D*), the computational result becomes nearly identical to that seen in experiments.

In a subsequent experiment, the upstream promoter of *hipB* was substituted for as well. In this situation, the expression levels of *hipA* and *hipB* could be controlled simultaneously. If we further delete the influence of the HipB dimer and the HipBA complex on the expression of *hipB*, our model can then be applied to this experiment as well. Once again, the simulation is in excellent agreement with the experimental findings (Fig. 8).

Discussion

We have presented a quantitative model of the HipBA system, which can help explain both the nature of the persister state and the dynamics responsible for transitions into and out of that state. Our model employs a number of assumptions, some of which need to be tested in future experiments. For example, we assumed that the binding of the two HipA toxins to opposite sides of the HipB dimer was cooperative. In practice, we neglected the population of singly bound intermediates. Relaxing this assumption can have an effect on the range of parameters over which bistability is predicted to occur, as it will significantly alter the percentage of inactivated HipA at small overall HipA concentrations. Similarly, we have ignored the recently reported ability of HipA to autophosphorylate and hence inactivate itself. This effect also can in principle reduce the range of bistability. It is important to note, though, that the basic phenomenology of the model remains unchanged even if we include these additional effects in our description.

Deletion of the TA module HipBA was reported to have no easily detectable effect on persister formation in a growing culture (18). From the bifurcation graph we find that the lower the dissociation rate between HipA and HipB is, the lower the growth rate (dilution rate) that is required for the formation of persisters. Because the dissociation rate is lower in wild-type *hipA* than in *hipA7*, the HipBA module contributes to the formation of persisters only under a slower growth rate. That is why the deletion of the HipBA module did not affect the frequency of persisters in populations during exponential phase (maybe even in early stationary phase); there was no difference whether the HipBA module was deleted or not. However, for cells in sufficiently adverse environments, the dilution rate of cells may become slow enough to let the HipBA module become involved in the formation of persisters. Of course, there can be many other TA modules that contribute to persister formation and hence it might be hard to single out the effects of this one specific molecular system.

HipA7 is a high-persistence mutant, which contains two substitutions, G22S and D291A (19). Here we have made the assumption that these two mutations change only the binding strength between HipA and HipB but otherwise retain the full function of HipA (i.e., the same toxicity). Thus, because less HipB would bind to HipA7, free HipA7 would be available to phosphorylate the elongation factor, Ef-Tu and cause cell stasis. There is one set of reports by Korch et al. that claimed that HipA was toxic whereas HipA7 was not (19, 20), but this claim is problematic for several reasons. First of all, Korch and Hill

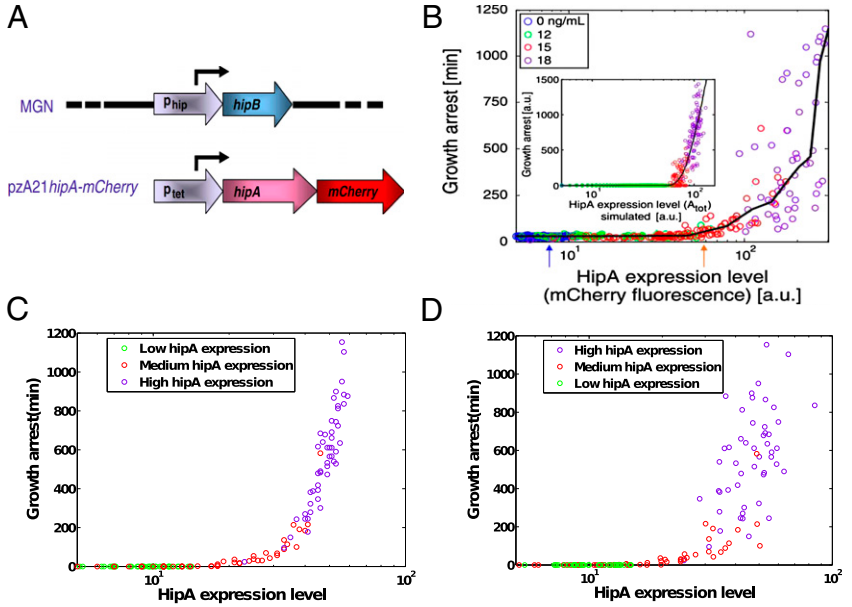

Fig. 7. (*A*) Schematic representation of the strain used for the experiments shown. (*B*) HipA was induced (in hipb$^+$) with anhydrous tetracycline (atc) to the indicated level for 3 h before plating on LB plates. The appearance time of colonies was continuously monitored by the automated scanner system. Growth arrest was defined as the time duration from plating to the appearance of the colony. *Inset* shows a Monte Carlo simulation result based on a model without bistability. (*C*) Monte Carlo simulation result based on our model. (*D*) To take into account measurement error in the experiment, we added a Gaussian distribution noise to the HipA expression level of each point. Here we assumed the variance of the noise of each point is 20% of its HipA expression level. *A* and *B* are reproduced with permission from the work of Rotem et al. (8).

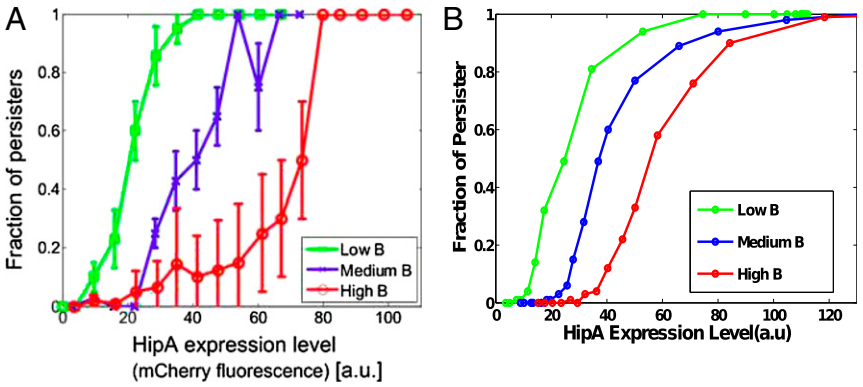

Fig. 8. The dependence of the threshold on HipB. (*A*) Experiments: HipA expression was induced from pBAD33A-mCherry at a fixed level, and the number of persisters was measured by microscopy for different levels of HipB from plasmid pZS21B in MGN. Green, low HipB (61 cells); blue, medium HipB (179 cells); red, high HipB (201 cells). (*B*) Monte Carlo simulation: the fraction of persisters was calculated by the proportion of cells in which the free HipA is over a threshold A_0. *A* is reproduced with permission from the work of Rotem et al. (8).

predicted that the ability of wild-type *hipA* to generate persisters did not contribute to its inhibition of macromolecular synthesis (20). However, in an experiment, inhibition of macromolecular synthesis is indeed observed in the persister state. If this claim is correct, this would lead to a series of difficult questions: Why does a persister have slow macromolecular synthesis? Why does a persister have multidrug tolerance, etc.? In addition, G22S and D291A seem to be far from the active site of HipA, which suggested a relatively low likelihood of affecting its kinase activity. In early work, Korch et al. reported that HipA could induce filamentation of cells (19), but this turned out to be an artifact (20). Finally, we did not find other papers to support Korch's hypothesis. Hence, we continue to believe that the toxicity of HipA is simply due to its capability of causing cell stasis.

It has been reported that gene expression level can be dependent on growth rate of the cell (21), but we did not include such a dependence in our model for two reasons. The main reason is that the extremely low growth rate of persisters is far beyond the range of growth rates that have been probed. Besides, if we include such dependence in our model, the simulation result in Fig. 7 will become less consistent with experimental results whereas our other simulations seem to be insensitive to such dependence. Future work should probe the relation between gene expression and growth rate in the near-zero growth rate region.

In summary, we have constructed a quantitative stochastic model and demonstrated that bistability can be the mechanism behind the formation of persisters. We show that our model can generate results consistent with a series of experiments involving induction of either *hipA* expression or both *hipA* and *hipB* expression. Furthermore, the model can explain several of the striking features of the basic persistence phenomenon, such as the existence of type I and type II strains and the existence of high-persistence mutants. Further study of the function of HipA is needed to test various aspects of our approach. To determine the typical number of molecules involved in the HipBA system, further experiments are needed, focusing on the dependence of persister frequency on the length of time spent in stationary phase. Also, our results suggest that persisters do in fact die under antibiotic treatment as do normally growing cells, but at a much slower rate. Finally, we suggest that persisters in the wild-type strain may not be caused solely by the HipA toxin but that there may be additional routes to this dormant phenotype. After completion of this manuscript, a paper appeared (22) that supports many of our ideas (e.g., the role of bistability, the importance of growth rate, and most specifically the possible involvement of many TA pairs) but does not attempt to quantitatively explain the artificial promoter experiments, which is one of the main points of our work.

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