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Programmed Heterogeneity: Epigenetic Mechanisms in Bacteria

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Josep Casadesús[‡1] **and David A. Low**[§2]

From the [‡]*Departamento de Genética, Facultad de Biología, Universidad de Sevilla, 41080 Seville, Spain and the* [§]*Department of Molecular, Cellular, and Developmental Biology, University of California, Santa Barbara, California 93106*

Contrary to the traditional view that bacterial populations are clonal, single-cell analysis reveals that phenotypic heterogeneity is common in bacteria. Formation of distinct bacterial lineages appears to be frequent during adaptation to harsh environments, including the colonization of animals by bacterial pathogens. Formation of bacterial subpopulations is often controlled by epigenetic mechanisms that generate inheritable phenotypic diversity without altering the DNA sequence. Such mechanisms are diverse, ranging from relatively simple feedback loops to complex self-perpetuating DNA methylation patterns.

The term "epigenesis" was introduced into contemporary biology by Conrad Waddington, a British visionary embryologist, to describe how cell lineages are formed during the development of multicellular eukaryotes (1, 2). During differentiation of eukaryotic tissues, genetically identical cells diversify into distinct lineages by inheritable changes in gene expression without loss or alteration of the DNA sequence. Many decades after Waddington, a universally accepted definition of epigenetics remains to be agreed upon. However, a tentative definition may be that epigenetics addresses the study of cell lineage formation by non-mutational mechanisms.

Most textbooks and reviews on epigenetic gene regulation concern only eukaryotes. One reason may be the enormous success of eukaryotic epigenetics and its implications for human disease. In addition, bacteria have been traditionally viewed as clonal populations of genetically identical cells with phenotypes merely reflecting their genetic constitution. This view is, however, naïve. Certain bacterial genera undergo complex developmental programs that involve cell differentiation. Spore formation by *Bacillus subtilis* (3), differentiation of *Rhizobium* into nitrogen-fixing bacteroids (4), asymmetric cell division in *Caulobacter* (5), formation of fruiting bodies by *Myxococcus* (6), heterocyst formation in cyanobacteria (7), and biofilm formation in many bacterial species (8, 9) are well known examples of bacterial development. In all of these phenomena, bacterial cells with distinct morphological and physiological properties are formed while the genome DNA sequence remains intact.

Formation of phenotypically distinct cells in populations made of genetically identical bacteria is not restricted to developmental programs. In the last few decades, the introduction of single-cell analysis in bacteriology has revealed many examples of subpopulation formation. For instance, clonal populations of bacteria can sometimes bifurcate into two distinct states, a phenomenon known as bistability (10, 11). Reversible bistability, traditionally known as phase variation, is also common (12). Transition at high frequency between two or more phenotypic states (13) can occur through mutations at genomic repeat sequences (14, 15) or via site-specific recombination (16–19). In other cases, bistability and phase variation are controlled by epigenetic mechanisms with strikingly different levels of complexity, from the propagation of simple feedback loops to the formation of DNA methylation patterns reminiscent of chromatin modification in eukaryotic cells (20–22).

Subpopulation formation can often be observed in the laboratory. However, it may be especially relevant in natural environments, either as an adaptive strategy (*e.g.* to evade the immune system and other host defenses during bacterial infection) or as a bet-hedging strategy that may facilitate survival if environmental changes occur (23). Relevant examples of phenotypic heterogeneity in natural environments are the formation of "persisters" (dormant bacterial cells resistant to antibiotics) (24, 25), the formation of lineages during *Salmonella* colonization of animals (26–28), and the bistable expression of extracellular matrix genes during biofilm formation by *B. subtilis* (9).

Even though subpopulation formation can be seen as the execution of intrinsic bacterial programs, it often involves stochastic events. For instance, random fluctuations in gene expression, a phenomenon known as "noise," can establish cell-to-cell differences in an isogenic population of bacteria (29). These quantitative differences can become qualitative (30) in the sense that expression above a critical threshold will provide a distinct signal, and expression below the threshold will provide a different signal (21, 31). Propagation of these signals by feedback loops enables the formation of epigenetic lineages (Fig. 1).

Formation of Epigenetic Lineages by a Positive Feedback Loop

Bistable gene expression occurs when a unimodal pattern of gene expression becomes bimodal, bifurcating into two distinct patterns (10, 32). Bistability can be generated either by a positive feedback loop or by a double-negative feedback loop (22, 33). A classical example of bistability generated by a positive feedback loop was described in the *Escherichia coli lac* operon (34). When added at high concentrations, the gratuitous inducer isopropyl β-D-thiogalactopyranoside (IPTG)[3] fully derepresses the *lac* operon. At low concentrations, however, IPTG is unable to induce a naïve (uninduced) culture. However, if a fully induced culture is transferred to medium containing

[1] To whom correspondence may be addressed. E-mail: casadesus@us.es.
[2] To whom correspondence may be addressed. E-mail: david.low@lifesci.ucsb.edu.

[3] The abbreviation used is: IPTG, isopropyl β-D-thiogalactopyranoside.

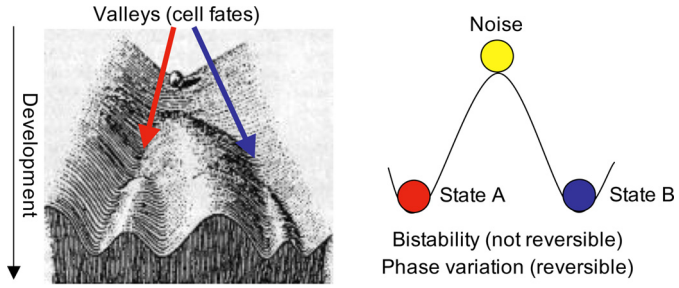

FIGURE 1. *Left panel*, Waddington's artistic drawing of an "epigenetic landscape" as a ball that falls to stable valleys from unstable ridges (adapted from Ref. 1). *Right panel*, bistability viewed as the fall of a ball from an unstable state on a ridge to a stable state in a valley. In phase variation, the valley state is metastable, and the ball periodically returns to the ridge.

low concentrations of IPTG, a subpopulation of cells is able to maintain the fully induced state (34). Maintenance occurs because fully induced cells have a high level of β-galactoside permease in their membrane. The permease transports IPTG, providing a high internal concentration of inducer, which maintains full induction (32, 34). The positive feedback loop in this system is that a high level of permease is required to concentrate IPTG in the cell, and high internal IPTG levels are required for high levels of permease synthesis (34). In other cells, however, a decrease in the internal concentration of inducer will reduce permease synthesis, which in turn will cause further reduction in the internal concentration of IPTG, driving the cell toward the repressed state via binding of the LacI repressor. The overall consequence is that a fully induced population bifurcates into two bistable states: fully induced and uninduced (repressed) (32–34).

Errors made during transcription can also provide signals for epigenetic switching in the *E. coli lac* operon (35). An increased error rate during transcription, caused either by mutations that reduce transcription fidelity of RNA polymerase or by the absence of transcription fidelity factors GreA and GreB, increases switching of the *lac* operon from the off state (uninduced) to the on state (induced) (35). The interpretation is that errors in *lacI* mRNA synthesis cause a transient decrease in the Lac repressor level, which permits switching to the on state (35, 36). Note that an uninduced *E. coli* cell contains ~10 molecules of the Lac repressor, an amount small enough to make the system noisy and therefore metastable. Perturbation of this delicate equilibrium by transcription inaccuracy can switch the system to the on state. Even though the decrease in the Lac repressor concentration is transient, synthesis of permease will generate a positive feedback loop that will maintain in the on state in certain cells (34). Lac bistability is not observed in cells containing a 10-fold higher Lac repressor level, consistent with the hypothesis that switching occurs only under conditions in which repressor levels are subsaturating.

Another classical example of bistability occurs in *B. subtilis*. Upon entry into stationary phase, a fraction of *B. subtilis* cells acquire the capacity to take up DNA, a phenomenon known as competence (10). A crucial factor for competence development is accumulation of ComK, which activates genes required for DNA uptake as well as the *comK* gene itself (37). During exponential growth, ComK is synthesized but degraded. When the culture approaches stationary phase, a quorum sensing-related

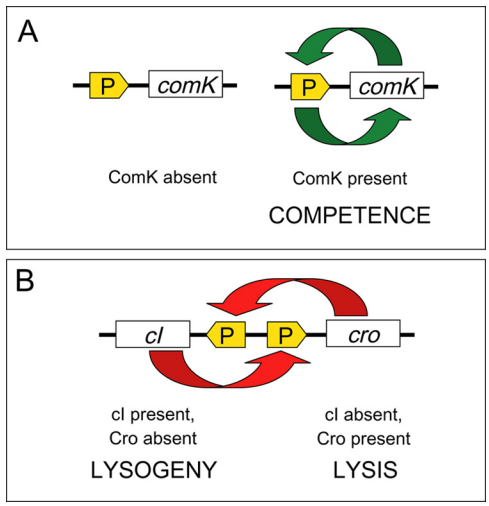

FIGURE 2. *A*, competence development in *B. subtilis*, an example of bistability created by a positive feedback loop. *B*, the lysis/lysogeny decision in bacteriophage λ, an example of bistability created by a double-negative feedback loop.

factor stabilizes ComK (38, 39). At that moment, a competition is initiated between several repressors and ComK for binding to regulatory regions of the *comK* promoter (40, 41). Binding of ComK initiates a positive feedback loop, leading to increased synthesis of ComK and subsequent transcription of competence genes. Binding of the repressors inhibits *comK* expression and prevents competence. A crucial property for bifurcation of the population into two subpopulations is that the level of ComK in individual cells fluctuates, generating stochastic noise. When the ComK level reaches a threshold in a *B. subtilis* cell, a quantitative difference becomes qualitative: the ComK positive feedback loop will be activated, and competence will develop (42–44). Development of competence thus occurs in cells that undergo a small but critical increase in ComK concentration (Fig. 2). In turn, *comK* will be repressed in cells in which the ComK level remains below the threshold, and they will not develop competence (Fig. 2) (43).

Formation of Epigenetic Lineages by a Double-negative Feedback Loop

Infection of *E. coli* by bacteriophage λ can follow two developmental programs. One is lysis of the bacterial cell; the other is lysogeny, a symbiosis-like association in which the phage enters a dormant state. Although the lysis/lysogeny decision is influenced by the physiological state of the cell and by environmental factors, the fate of individual infections is unpredictable and may be considered stochastic (33, 45, 46). Phage λ has two repressors, cI and Cro, each of which represses expression of the other. At the onset of infection, both repressors are produced, and the lysis/lysogeny decision may be viewed as a repressor race: the repressor that first occupies specific regulatory DNA sites in λ DNA will repress synthesis of its antagonist (45). If the winner is Cro, synthesis of cI will be repressed, and λ will lyse the host cell (Fig. 2). If the winner is cI, synthesis of Cro will be repressed, and λ will lysogenize the cell (Fig. 2) (45). Note that the outcomes of a positive feedback loop and a double-negative feedback loop are analogous (22, 33). In the case of λ,

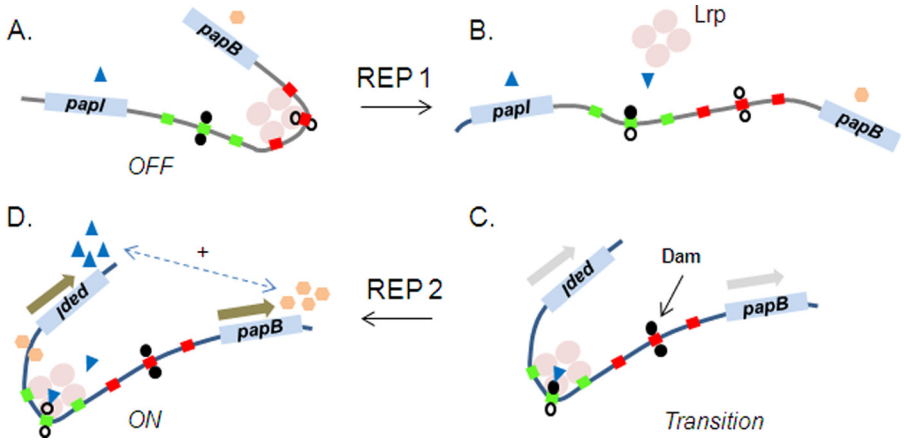

FIGURE 3. **Model for the *pap* off-to-on state transition, an example of a bistable switch controlled by DNA methylation patterns.** *A*, in the phase off state, an octamer of Lrp (a tetramer of dimers; only one tetramer is depicted) binds cooperatively to promoter-proximal sites 1–3 (*red boxes*). Lrp binding to sites 1–3 inhibits further binding of Lrp to sites 4–6 (*green boxes*) by mutual exclusion. *B*, immediately following passage of the replication fork (*REP 1*), the two daughter chromosomes become hemimethylated. Only the daughter chromosome methylated on the top strand is shown (*filled circle* above Lrp-binding site 5). *C*, two stochastic events occur in which PapI facilitates Lrp binding to sites 4–6, and Dam (DNA adenine methylase) methylates both strands of the proximal GATC site. Binding of Lrp at sites 4–6 reduces the affinity of Lrp for sites 1–3 by mutual exclusion and facilitates activation of *pap* transcription via cAMP-catabolite gene activator protein/RNA polymerase binding (not shown). Methylation of $GATC^{prox}$ reduces the affinity of PapI/Lrp for sites 1–3 and is required for transition to the on phase (98). *D*, one additional round of DNA replication (*REP 2*) completes transition to the phase on state, in which $GATC^{dist}$ is fully unmethylated. The on phase is self-perpetuating due to a bidirectional feedback loop between PapB and PapI (*dashed arrow*). The PapB level rises due to activation of transcription of the first gene of the *pap* operon, *papB*. PapB binds near the *papI* promoter, increases the PapI level via activation of *papI* transcription, and helps maintain the on state via binding of PapI/Lrp to sites 4–6.

preventing the synthesis of Cro by cI is equivalent to positive autoregulation of cI and vice versa.

Phase Variation via DNA Methylation Patterns

A common epigenetic mechanism to regulate switches involves the formation of DNA methylation patterns (47, 48). This occurs when a methylation sequence on DNA overlaps the binding site for a protein, and methylation of that sequence is blocked (49, 50). For example, most GATC sites in the *E. coli* chromosome are fully methylated except for a short time following DNA replication, in which they are hemimethylated. However, a few sites are stably unmethylated due to binding of proteins at sites that overlap or are adjacent to a GATC site, competing with Dam for binding and blocking methylation (47, 48, 51). Two such GATC sites in the *pap* (pyelonephritis-associated pili) operon of uropathogenic *E. coli* orchestrate Pap pilus phase variation (52, 53). The core of the Pap switch consists of two sets of binding sites, 1–3 and 4–6, within the *pap* promoter region for the global regulator known as the leucine-responsive regulatory protein, Lrp (54). Lrp appears to be predominantly a tetramer of dimers (octamer), with three Lrp dimers binding to three *pap* sites, leaving one dimer unbound (Fig. 3) (55–57).

A GATC site is present within site 2 ($GATC^{prox}$) and site 5 ($GATC^{dist}$); methylation of these sites affects Lrp binding, as discussed below. Lrp binds cooperatively to a set of three *pap* sites, but occupancy of all six Lrp sites occurs infrequently due to a mutual exclusion mechanism that requires negative DNA writhe (supercoils) (58). Lrp binding to sites 1–3 (Fig. 3, *red boxes*) blocks methylation of $GATC^{prox}$ and also blocks *pap* transcription because the RNA polymerase σ^{70}-binding site is in this region (Fig. 3*A*) (59). In contrast, binding of Lrp to sites 4–6 (Fig. 3, *green boxes*) blocks methylation of $GATC^{dist}$ and helps to activate *pap* transcription (60). The role of Lrp in activating transcription may be to bend DNA, facilitating binding of catabolite gene activator protein to the RNA polymerase α-subunit (61).

Transition from the phase off state to the phase on state requires two *pap*-encoded regulators, PapI and PapB. PapI increases the affinity of Lrp for *pap* sites 2 and 5 via an ACGATC sequence present in each site (52, 56, 62). PapB, the product of the first gene of the *pap* operon, binds near the *papI* promoter and activates *papI* transcription, forming a positive feedback loop (Fig. 3*D*, *dashed arrow*) (63). Methylation of $GATC^{prox}$ is required for the off-to-on transition because it lowers the affinity of PapI/Lrp for site 2, increasing the probability that PapI/Lrp will bind to sites 4–6 and initiate transition to the on phase (58). For this to occur, Lrp bound at sites 1–3 in off phase cells must dissociate to allow methylation of $GATC^{prox}$ by Dam. This likely occurs as the replication fork passes through the *pap* regulatory region, and a hemimethylated $GATC^{dist}$ site is generated (Fig. 3*B*). The affinity of PapI/Lrp for hemimethylated *pap* sites 4–6 is significantly higher than for the fully methylated DNA (52, 56). If PapI/Lrp binds to site 5 before Dam methylates the daughter strand, cooperative binding of Lrp/PapI to sites 4–6 will occur to initiate transition to the phase on state. Evidence indicates that a dimer of Lrp and a monomer of PapI bind to *pap* site 5 (56). This transition is also dependent on dissociation of Lrp from sites 1–3 and methylation of $GATC^{prox}$: increasing the off rate (k_{dis}) of Lrp at sites 1–3 increases the off-to-on rate (64).

Dam methylase is highly processive, such that ~130 Dam molecules can efficiently methylate ~20,000 genomic GATC sites (65). Thus, when Dam methylates $GATC^{prox}$, it should have a high propensity to methylate the adjacent $GATC^{dist}$ site before dissociating from DNA. This would block PapI/Lrp binding to site 5 and block transition to the on phase (60). Recent work has shown that the presence of a poly(A) tract 5′ to the two *pap* GATC sites decreases the processivity of methyla-

tion by reducing the rate of methyl transfer (k_{chem}) (66). This may be necessary to allow PapI/Lrp to compete with Dam for access to hemimethylated $GATC^{dist}$ sites following DNA replication (67).

The phase on-to-off transition, which occurs at an ~100-fold higher rate than the off-to-on transition (47), has not been analyzed in detail. Following DNA replication, cells in the phase on state contain a hemimethylated $GATC^{dist}$ site and a fully unmethylated $GATC^{prox}$ site. If Dam methylates $GATC^{dist}$, binding of PapI/Lrp will be inhibited, providing an opportunity for Lrp binding at sites 1–3 due to release of mutual exclusion. Notably, binding of Lrp to site 2 is unaffected by methylation of $GATC^{prox}$ (52); therefore, the key step must be competition of Dam and PapI/Lrp for binding at site 5. Formation of the phase off DNA methylation pattern requires two rounds of DNA replication to convert a fully methylated $GATC^{prox}$ site to a fully unmethylated site.

The on and off *pap* transcription states are each self-perpetuating and heritable. In the off state, $GATC^{dist}$ is fully methylated, preventing PapI/Lrp binding to sites 4–6 (Fig. 3*A*). Conversely, in the on state, PapI expression is high due to the PapB positive regulatory feedback, and $GATC^{prox}$ is fully methylated, preventing PapI/Lrp binding to sites 1–3 (Fig. 3*D*). In addition, it is likely that both the off and on states are stabilized by mutual exclusion (58).

The *pap* switch is modulated by additional transcription factors that are environmentally responsive, including H-NS, RimJ, and CpxR. Transcription of *pap* is blocked at 23 °C by H-NS, which binds to the *pap* regulatory region and blocks GATC methylation (68). H-NS also modulates Pap switching at 37 °C in response to high osmolarity and other environmental conditions (69, 70). This may occur by altering PapI/Lrp binding to *pap* regulatory sites, but the mechanistic details are unknown. RimJ, which acetylates ribosomal protein S5, inhibits transition to the on state in response to temperature and other environmental conditions by an unknown mechanism (71). The CpxAR two-component regulatory system responds to cell envelope stress by phosphorylation of CpxR. Phosphorylated CpxR binds specifically to the *pap* regulatory region, competes with Lrp, and blocks *pap* transcription, which may protect cells from further cell envelope damage (72–74).

Other Switches Regulated by DNA Methylation Patterns

Many methylation-dependent phase variation systems have been identified since the initial discovery of the Pap system. Some of these systems, such as *foo*, *clp*, and *pef*, which all encode pili, are designed similarly to the *pap* switch (75–77). Remarkably, the latter two systems have a reversed architecture in which the PapI homologs ClpI and PefI act as negative regulators. Other methylation-controlled switches use DNA-binding proteins other than Lrp, including OxyR and Fur. The best characterized system is *agn43*, which controls the expression of antigen 43 (78, 79), an outer membrane protein that plays a role in biofilm formation and pathogenesis (80, 81). OxyR binds three GATC sites in the *agn43* regulatory region. Binding of OxyR blocks methylation of the three GATC sites and inhibits *agn43* transcription, forming the off phase. Transition to the on phase occurs following DNA replication if Dam can methylate both strands of the three GATC sites before OxyR rebinds to the sites (50, 82, 83). Notably, the poly(A) tracts adjacent to the GATC sequences in *pap* and its relatives are not present in *agn43*, and thus, Dam should processively methylate the three *agn43* GATC sites if they are not bound by OxyR (84). The on-to-off switch can occur after DNA replication, when the three GATC sites are hemimethylated. OxyR has a higher affinity for *agn43* DNA containing hemimethylated GATC sites *versus* fully methylated GATC sites (84, 85). Thus, if OxyR binds to the GATC region before Dam fully methylates the GATC sites, a phase off intermediate state will ensue, and after one more round of replication to convert the hemimethylated GATC sites to fully unmethylated sites, the phase off transition will be complete. On-to-off transition is affected by the local concentration of OxyR; the addition of three or more OxyR-binding sites upstream of *agn43* biases cells toward the off phase (84).

A number of phase variation switches appear to be regulated by mechanisms reminiscent of *agn43*. These include the *gtr* switch on the P22 bacteriophage (86) and the chromosomal switch locus *STM2209-STM2208* (*opvAB*) (87), each controlling modification of cell surface lipopolysaccharide of *Salmonella*, both of which are controlled by OxyR and Dam. In enteroaggregative *E. coli*, the *sci1* type VI secretion system is controlled by a phase switch in which the iron regulatory protein Fur blocks Dam methylation of *sci1* GATC sites, forming phase off and on methylation patterns (88).

Phasevarions: Formation of Epigenetic Lineages by Phase Variation of DNA Methylase Synthesis

Certain restriction-modification systems show phase variation, and a common mechanism for switching between off and on states is expansion and contraction of nucleotide repeats (89). Phase variation of restriction-modification systems may generate subpopulations of bacterial cells differing in their susceptibility to phage infection and in their ability to acquire foreign DNA. In addition, DNA adenine methylation by certain phase-variable restriction-modification systems regulates expression of specific genes (90). These systems, known as "phasevarions," conserve their restriction-modification activity but have additionally acquired epigenetic regulatory capacity (91, 92). In some phasevarions, the gene encoding the restriction enzyme is inactivated by mutation, whereas the modification gene (*mod*) remains active. Hence, in these mutant type III restriction-modification systems, the Mod enzyme is a functional analog of solitary methyltransferases (*e.g.* Dam).

In the human pathogens *Haemophilus influenzae*, *Neisseria meningitidis*, and *Neisseria gonorrhoeae*, DNA adenine methylation by Mod enzymes has been shown to regulate gene expression, and the loci under Mod control include genes with roles in envelope structure, virulence, and stress responses (92). Because synthesis of Mod DNA methylase is phase-variable, isogenic subpopulations contain two types of bacterial cells. One population contains N^6-methyladenine in the genome, whereas the other subpopulation does not. As a consequence, each lineage shows a distinct pattern of gene expression that affects all DNA methylation-sensitive loci.

Whereas individual phase variation systems, such as *pap* and *agn43*, generate heterogeneity of a single phenotypic trait, cell

lineages under phasevarion control differ in multiple phenotypic traits. The capacity of phasevarions to generate bacterial lineages may be further extended in bacterial species that contain multiple *mod* alleles, each with slightly different DNA-binding domains (92). Independent switching in the synthesis of several Mod proteins can be expected to generate multiple gene expression patterns, thus increasing the phenotypic heterogeneity of the population.

Hierarchical Epigenetic Networks

Phase variation of certain genetic loci causes bistable expression of other genes, extending phenotypic heterogeneity to cell functions encoded outside the phase variation locus. An example of this kind occurs in the *Salmonella enterica std* operon, which encodes fimbriae for attachment to the intestinal mucosa (93). Transcription of *std* is controlled by a LysR-like regulator known as HdfR and by two products of the *std* operon, StdE and StdF (94). Production of Std fimbriae in isogenic populations of *Salmonella* is subject to phase variation; the switching mechanism remains to be deciphered. However, it is well established that the StdE and StdF gene products regulate expression of genes outside the *std* operon, including the cluster of virulence genes known as *Salmonella* pathogenicity island 1, SPI-1 (95). Because SPI-1 expression is prevented by StdE/StdF, cells that produce Std fimbriae do not synthesize the SPI-1-encoded apparatus and vice versa (95). One may thus predict that phase variation of the *std* operon in the animal intestine will split *Salmonella* populations into two lineages, one able to invade the intestinal mucosa (causing acute disease) and one able to attach to the intestinal epithelium (causing latent infection). Depending on the host physiological conditions and the host response, one of the two subpopulations will be able to colonize the animal, whereas the other will be eliminated. Whatever the outcome, bet-hedging will increase the chances that a fraction of the *Salmonella* population survives. This model fits well with the view that colonization of animals by *Salmonella* involves subpopulation formation at several stages (26–28), and the same may be true for other human pathogens (25, 96, 97). Subpopulations may differ in their susceptibility to antibacterial drugs, thus explaining why certain bacterial infections are difficult or impossible to eradicate.

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