Phenotypic variation in bacteria

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Phenotypic variation in bacteria: the role of feedback regulation

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Abstract | To survive in rapidly changing environmental conditions, bacteria have evolved a diverse set of regulatory pathways that govern various adaptive responses. Recent research has reinforced the notion that bacteria use feedback-based circuitry to generate population heterogeneity in natural situations. Using artificial gene networks, it has been shown that a relatively simple 'wiring' of a bacterial genetic system can generate two or more stable subpopulations within an overall genetically homogeneous population. This review discusses the ubiquity of these processes throughout nature, as well as the presumed molecular mechanisms responsible for the heterogeneity observed in a selection of bacterial species.

Phenotypic variation
Cells within an isogenic population that show variable expression patterns.

Epigenetic
Any heritable change in gene expression that is not caused by a change in DNA sequence.

Multistationarity
The possibility of the existence of two (or more) stationary states of gene expression between which individual cells can switch.

Bistability
Situation in which two stable states coexist among cells within a population.

Multistability
The existence of two (or more) distinct phenotypes within an isogenic population owing to multistationarity.

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The ability of a bacterium to survive in different niches and in the presence of various stresses strongly depends on its genetic content. However, these survival strategies are often only used by part of a bacterial population. This response heterogeneity could help a bacterium to utilize different niches within an ecosystem, and even has the potential to increase the overall fitness of the species[1]. Various processes can contribute to this variability within a bacterial population. In this article, we do not discuss the population heterogeneity that results from genetic rearrangement, as is the case in phase variation, or DNA modification, such as methylation — these subjects have already been reviewed thoroughly[2,3]. Instead, we focus on phenotypic variation based on the feedback architecture of genetic networks (BOX 1). This type of variability is epigenetic in nature, relies on direct or indirect autostimulation of pivotal transcription factors, and is observed in various bacterial systems.

The prerequisites for variable output from a genetic network (multistationarity), without the necessity of genetic reorganization or modification, have been determined by several groups using modelling and synthetic gene circuits (reviewed in REFS 4–9). It was shown that specific feedback in combination with a nonlinear response within a network can generate a bistable or multistable output: the co-occurrence of two or more phenotypically distinct subpopulations in a culture (BOX 2). Nonlinearity can occur, for instance, when multimerization is required for a transcription factor to be active, or when cooperativity is observed in DNA binding. The use of single-cell analytical techniques is becoming common practice among microbiologists (BOX 3;TABLE 1) and, through the use of these techniques, it has been shown that the heterogeneous output of several bacterial processes can be traced back to the feedback-based wiring of the network involved[10–13]. Therefore, it seems that feedback-based multistability (FBM) is a common and widespread feature exploited by many bacteria, especially in their adaptive responses to changes in the environment and in the stresses encountered. This hypothesis is further strengthened by the observation that adaptive phenotypes of natural isolates often disappear quickly (too fast to account for them simply by mutational loss) when cultured under laboratory conditions, indicating an epigenetic effect[14]. In this review, we discuss the molecular mechanisms of some of the natural bistable systems that have been identified in bacteria.

Natural bistable systems in bacteria

***Lactose utilization in* Escherichia coli.** In 1957, a groundbreaking study by Novick and Weiner showed that the genetic programme which regulates lactose utilization in *Escherichia coli* — encoded by the genes of the *lac* operon — is initiated in only a subpopulation of an isogenic culture[15]. They showed that when the population is induced at low levels and diluted to the single-cell level, re-culturing yielded a population of either high- or non-*lac*-expressing cells. Further characterization of this system revealed that with specific sugars in the growth medium, and depending on the history of the inoculum, a fraction of the bacterial cell population highly expressed

Box 1 | Definition of feedback-based multistability

Phenotypic variation is a widespread phenomenon that occurs in prokaryotes, and the molecular mechanisms that underlie this variation are similarly diverse. Variation can originate from genetic changes, as with some phase-variable phenotypes including genomic inversion (for example, *Escherichia coli fim*[109] and *Salmonella enterica* serovar Typhimurium *hix*[110]) and strand-slippage mechanisms (for example, *Neisseria* spp. *opa*[111] and *Bortedella pertussis bvg*[112]). Alternatively, the regulation of phenotypic variation can be epigenetic in nature and not be accompanied by changes in DNA sequence. Epigenetic processes are found, for example, in the *pap* and antigen 43 (Ag43) phase-variable phenotypes of *E. coli*, in which phenotypic variation depends on methylation of certain DNA sequences (reviewed in REFS 113,114). In contrast to the mechanisms described above, and providing the focus of the current review, some epigenetic traits depend on the presence of positive or double-negative feedback loops in the regulatory networks that determine the activity of key regulators. This multistationarity at the cellular level can generate multistable bacterial populations. Phenotypic variation based on this type of network architecture is referred to as feedback-based multistability, and seems to be a common feature of adaptive processes in the bacterial realm.

the *lac* operon, whereas the remainder of the cells did not[16]. As early as 1961, it was hypothesized that the heterogeneity in lactose utilization might be attributed to multistationarity[17], although it was not seen as an example of bistability in prokaryotes at that time. Research on the *lac* operon has continued ever since, and the system is now well characterized[18] (FIG. 1).

The polycistronic *lac* operon comprises three genes that are required for the uptake and catabolism of lactose: *lacZ*, encoding β-galactosidase; *lacY*, encoding lactase permease; and *lacA*, encoding a transacetylase. Expression of the *lac* operon is negatively regulated by a repressor (LacI), which in turn is inhibited by allolactose. LacI is also positively regulated by the cyclic-AMP receptor protein (CRP). CRP is activated by cyclic AMP under low sugar availability. Both LacI and CRP act as multimers.

In the lactose-utilization network, high levels of lactose lead to the accumulation of high intracellular levels of allolactose by the action of β-galactosidase. The allolactose, in turn, inhibits the activity of the LacI repressor, leading to an increase in expression of the *lac* operon, which also includes the structural gene for the lactose permease, *lacY*, and therefore constitutes a feedback loop. However, as LacZ can metabolize lactose and allolactose, autostimulation is interrupted. Therefore, in the natural situation the system behaves as a graded response. However, bistability is established when cells in which the *lac* operon is not induced are subjected to a suboptimal concentration of a gratuitous inducer and the population consists of cells with either high or low expression of *lac*[12]. Switching from one state to the other requires an induction, or relief of inducer, greater than that required for the reverse transition, and this phenomenon is responsible for the observed memory (or locking) of either induced or uninduced cells (hysteresis). This can be followed through the abundance and stability of the LacY permease. When little permease is present, the concentration of inducer required to trigger the stimulatory loop is high. By contrast, when the level of permease is high (mostly corresponding to an already induced state), cells need little inducer to maintain high levels of *lac* expression (FIG. 1b).

Importantly, by modelling and single-cell analyses of the parameters within the natural *lac* operon, Ozbudak and colleagues showed that a binary (bistable) response can be converted to a graded response[12], providing a framework for the unexplained observation that both states can occur[19]. The *E. coli lac* operon remains a paradigm for bistable enzyme induction, although similar systems have been described for the arabinose-utilization operon in *E. coli*[20,21] and the lactose operon in *Salmonella enterica* serovar Typhimurium (*S. typhimurium*)[22].

Lysis or lysogeny of bacteriophage λ. Bacteriophage λ is a virus that infects *E. coli* and, soon after infection, either reproduces quickly, thereby killing the host and releasing phage particles (lytic pathway), or integrates in the genome of the host and remains dormant (lysogenic pathway). The developmental programme that controls the type of infection has become a paradigm for developmental switches (reviewed in REFS 23,24). By 1976, it was suggested, using mathematical modelling, that positive feedback within the gene network is responsible for the bistability observed[25]. Later research supported and expanded these findings[26,27]. The fraction of λ-infected cells that becomes lysogenic is influenced by environmental conditions, including the nutritional state of the host cell and the number of phage present at the time of infection. Key components in the switch between lysis and lysogeny are the Cro and CI transcriptional regulators, which bind as multimers to the same operator sites in a divergent phage promoter. CI represses genes that are required for phage propagation and activates its own transcription at high levels. Cro also represses one of the two promoters driving the expression of *cI*. Therefore, when intracellular CI levels are high, the level of CI remains high owing to the positive feedback, and the phage remains dormant within the genome of the host bacterial cell. With certain stimuli, such as ultraviolet radiation, the dormant phage is induced and viral particles are produced[23,24,28]. McAdams and Arkin argued that the inherent stochasticity of the biochemical reactions that govern gene expression (transcription and translation) can lead to fluctuations in the levels of proteins. They have shown that the delicate balance between the regulators of bistable switching (Cro and CI) is affected by these fluctuations, and that this mechanism has an important role in the 'choice' between the lytic and lysogenic pathways under normal growth conditions[27]. It is important to realize that after ultraviolet irradiation, induction of the dormant phage depends on a different process, involving proteolytic activity of RecA.

***Mucoidy and cytotoxicity of* Pseudomonas aeruginosa.** *Pseudomonas aeruginosa* is an opportunistic pathogen and an important cause of mortality in cystic fibrosis (CF) patients, owing to the secretion of alginate (mucus), which increases respiratory difficulties in CF patients. The regulatory cascade of alginate synthesis involves

Multistability
The existence of two (or more) distinct phenotypes within an isogenic population owing to multistationarity.

Feedback-based multistability
(FBM). The existence of two or more distinct subpopulations in a culture, based on the presence of positive or negative feedback in the underlying regulatory network.

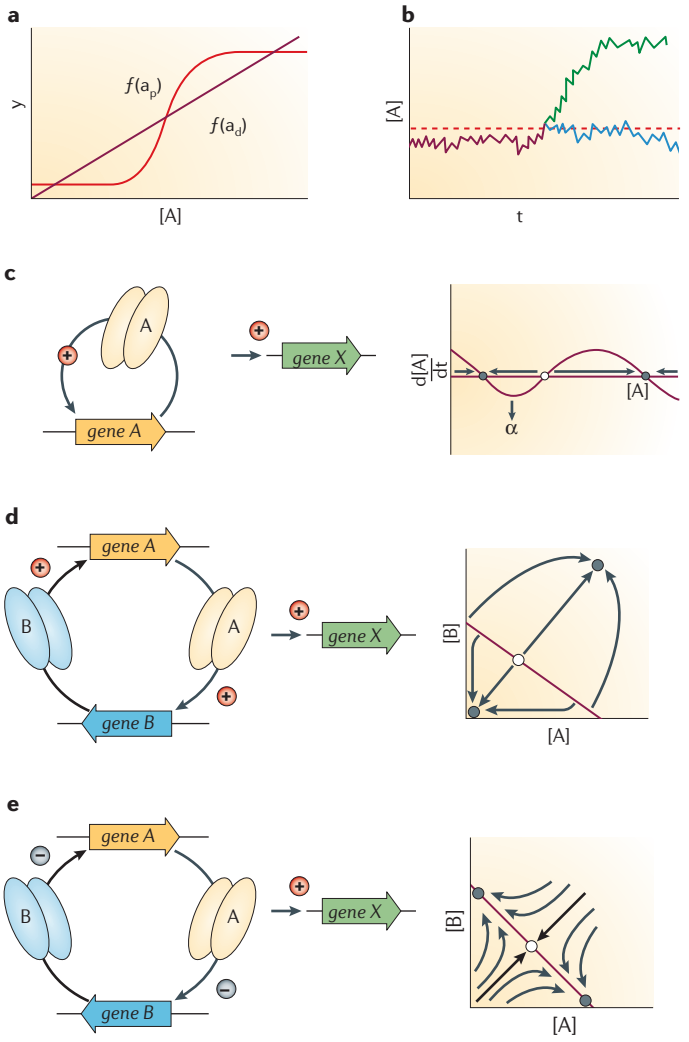
Graded response
Expression of a gene in direct correlation with the intracellular levels of its regulator.

Gratuitous inducer
Compounds that bind to, and inactivate, a repressor without being metabolized by the induced enzymes.

Hysteresis
Situation in which the switch from one state to the other requires a force unequal to that required for the reverse transition.

Binary
Exhibiting an 'on' and 'off' state.

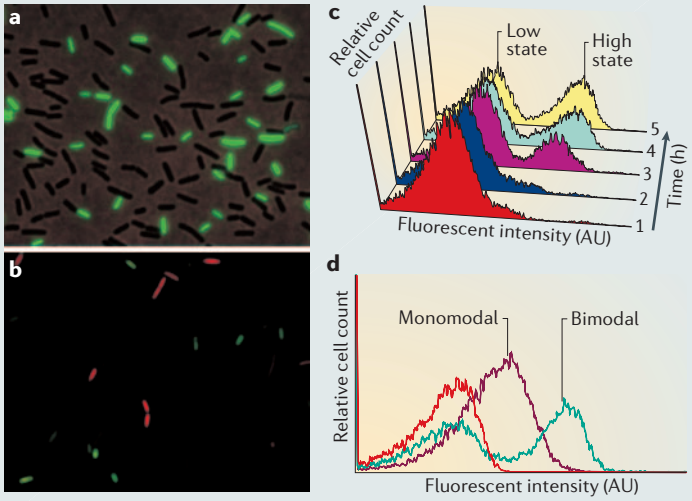
Box 2 | **Characteristics of bistable systems**



The systems described here are the simplest forms in which a gene network could potentially demonstrate multistationarity resulting in a bistable output at the population level. For all hypothetical systems depicted here, protein A represents a key regulator in a signal-transduction cascade that is only active when present as a multimer. In this situation, the multimerization of the protein causes the nonlinear dynamics of the system. Therefore, the production of A, $f(a_p)$, can be described by a Hill-type function. In general, protein production has a plateau, meaning that the feedback systems are not allowed to increase protein levels to infinity. Furthermore, A has a certain deactivation rate, which can be described by a linear-type function, $f(a_d)$. The change of A over time (dA/dt) can be described by a differential equation in which the production of A is combined with the deactivation function. Without trying to find a numerical solution for such a differential equation, one can obtain some qualitative information by looking for equilibriums or points where the derivative is zero and determine whether the function moves towards or away from these points. Therefore, we have made phase-plane sketches that could represent the dynamics of the bistable systems that are described. Note that these phase-plane sketches are purely hypothetical and are merely shown to indicate the putative behaviour of the feedback systems in an intuitive manner. Closed and open circles depict stable and unstable steady states, respectively. (**a**) Plot of the functions $f(a_p)$ and $f(a_d)$ within the same graph. From this graph, it becomes apparent that there are three intersection points, indicating the steady states in which dA/dt is equal to 0. (**b**) The concentration of activator A over time. The level of the protein fluctuates over time. When the level exceeds a threshold (indicated by the red dotted line), cells accumulate high levels of the activator when positive or double-negative feedback is present (green line). Bistability is observed when some cells reach the threshold and others do not. In the absence of a feedback loop, cellular levels of the regulator do not markedly increase (blue line). In this situation no bistability is observed. (**c**) Bistable switch by single-positive feedback (left panel) based on the characteristics depicted in **a**. A phase-plane analysis is shown in the right panel. The curve in which the change of A is equal to 0 is plotted against the concentration of A (nullcline). If at a certain moment in time the cell has an intracellular concentration of A=**α**, there is more deactivation than production of A, and the concentration of A will drop until production and deactivation are in equilibrium (stable steady state). The directions of the arrows in such a plot indicate the movement to the equilibrium. If two arrows move away from each other, this represents an unstable steady state. Therefore, this system has the theoretical potential to demonstrate three distinct subpopulations. In practice, however, it will only show two expressing states, as there are two stable steady states: one in which the levels of A are low (and *gene X* is not transcribed) and one in which the levels of A are high and *gene X* is activated. (**d**) Bistability by a two-component positive feedback loop. In this circuit, protein B activates the expression of *gene A* and protein A activates expression of *gene B*. A phase-plane plot is shown in the right panel. There can only be a stable steady state with either both A and B on (and *gene X* activated), or A and B off (and *gene X* not activated), but never with A on and B off or vice versa. (**e**) Bistability by a double-negative feedback loop. In this feedback system, activator A also acts as a repressor of *gene B*, and protein B represses *gene A* (left panel). As shown from the phase-plane plot (right panel), a stable steady state is only present when the levels of B are high and A low, and when the levels of A are high and B low. Therefore, *gene X* is only activated in the latter steady state. Note that combinations of different types of feedback can, in some cases, also generate bistability[116], and bistability is impossible if one of the components acts too strongly or too weakly compared to the other[95]. Also, a combination of three negative feedback loops can cause a gene network to oscillate between states[117].

Phase plane
Solution of a system of differential equations plotted in a two-dimensional plane.

Box 3 | **Single-cell analysis and bistability**



An increased awareness of the fact that population-wide reporter studies might overlook important aspects of development and physiology has led to the development and use of techniques that can discriminate subpopulations within isogenic bacterial cultures (TABLE 1). The quantification of fluorescence through fluorescence microscopy, or in an automated fashion using flow cytometry, is the principal technique used to evaluate gene-expression patterns in individual cells over time (for review see REF. 115). Panel **a** shows a fluorescent microscopic analysis of a culture in which only a subpopulation expresses the green fluorescent protein (GFP). Using the cyan and yellow fluorescent proteins, the expression of two genetic pathways can be visualized simultaneously within the same isogenic strain (panel **b**). Flow-cytometric analysis allows rapid investigation of a single-cell-expression pattern. Depending on the flow cytometer and the settings used, the fluorescence of more than 1,000 individual cells per second can easily be determined, making it an extremely valuable technique for time-series analyses. As shown in panel **c**, with increasing time, a fraction of the population begins expressing GFP and a clear bistable expression pattern is established. An 'on' and an 'off' population is observed when the low state corresponds to non-fluorescent cells. Note that bistability can also be observed when the low state corresponds to weakly fluorescent cells. Flow cytometry, owing to its high sensitivity, also has the power to discriminate a heterogeneous, but monomodal, pattern from a truly bistable distribution, as depicted in panel **d**. The red peak is the background fluorescence of a non-expressing culture. The purple line represents the expression pattern of a culture in which cells express at both a low and high level but in a monomodal fashion. By contrast, the green line represents a typical bistable expression pattern. AU, arbitrary units.

direct positive autoregulation of the sigma (σ) factor AlgU, which binds to its own promoter, and negative autoregulation through an anti-σ factor (MucB) that is encoded in the same operon and which antagonizes the action of AlgU[29,30]. With the identification of mutations in the *mucB* gene that result in a stable mucoid phenotype[29], the possibility of FBM in *P. aeruginosa* has been overlooked. However, many strains of *P. aeruginosa* display unstable mucoidy, and experimental evidence also suggests a level of hysteresis, a common characteristic of bistable systems. Theoretical modelling of the molecular origin of mucoidy by Guespin and co-workers has shown that multistationarity might be possible on the basis of the feedback architecture in the network that governs AlgU expression[13,31]. This makes the occurrence of bistability in the system probable. Interestingly, they have also extended their hypotheses to the phenomenon of *P. aeruginosa* cytotoxicity, in which the bacterium injects toxins into the target cell or culture medium[13]. Genes that are essential for cytotoxicity require the regulator ExsA, an auto-activating protein, for expression[32]. One of the activated operons encodes an inhibitor of ExsA, termed ExsD[33]. As such, the regulatory network that governs cytotoxicity in *P. aeruginosa* has a similar architecture to the network that underlies mucoidy. Both studies presented experimental set-ups to verify these hypotheses. However, this work has not yet been done, and it remains to be established to what extent FBM contributes to these variable phenotypes of *P. aeruginosa*.

ComK
Master regulator for competence development in *Bacillus subtilis*.

Competence for genetic transformation. Competence is the ability of bacteria to take up exogenous DNA and incorporate it into the genome. The development of competence is an adaptive process that has been identified in at least 40 bacterial species[34], and it has been proposed to function as a source of templates for DNA repair, to enhance genetic diversity through homologous recombination or to serve as a source of nutrients, especially phosphate[35]. It has been known for a long time that competence is a physiological state that does not involve the whole population[36,37]. However, recently, the observation that only part of an isogenic culture develops competence has been revisited with bistability in mind.

In *Bacillus subtilis*, the development of natural competence is governed by the competence transcription factor ComK[38]. This tetrameric protein activates more than 100 genes, including those essential for DNA binding and uptake[39–41]. Regulation of ComK activity is controlled through proteolytic degradation, quorum sensing and transcriptional control[42] (FIG. 2a). ComK binds to its own promoter and is required for its own expression[43,44]. The competence regulatory network therefore contains a positive feedback loop. Two groups independently reached the conclusion that this autoregulation is required for competence bistability. Furthermore, there seems to be a system of two mutually repressing transcription factors. Rok is a protein that negatively regulates competence by binding to the *comK* promoter, and *in vivo* and *in vitro* data indicate that ComK is similarly able to repress *rok*

Table 1 | **Techniques suitable for single-cell analyses**

Technique	Description	Example references
Fluorescence microscopy (FM)	One of the most common techniques. Cells are fixed on a glass slide and visualized by a phase-contrast microscope. Specific filters allow excitation of fluorophores and detection of fluorescent signals at certain wavelengths. Images can be captured using digital cameras.	115
Flow cytometry (FC)	Can analyse a large number of cells in a short period of time. Particle size and fluorescence intensity of individual cells can be measured when individual cells pass an intense light source (for example, a laser) combined with appropriate filters. Preferred method to assess bistability (see BOX 3).	115
Density centrifugation	Can discriminate differences in both growth phase and cellular differentiation, if accompanied by differences in cellular buoyant density. To confirm the different identities of the subpopulations, used in conjugation with standard biochemical techniques or one of the single-cell techniques listed here.	36,37,77, 78,118,119
Single-cell β-galactosidase activity assays	Using a fluorigenic substrate for β-galactosidase, can monitor the activity of the classical LacZ –reporter protein in single cells when combined with FM or FC.	59,64
Fluorescent *in situ* hybridization	Fluorescently labelled nucleic-acid probes in conjunction with FM or FC are primarily used to qualitatively identify specific bacterial species at the single-cell level in a complex mixture. Can also be used, for example, to detect phase-variable genomic regions, and therefore help discriminate phase-variation and feedback-based multistability.	120,121
In situ RT-PCR	Allows quantitative analysis of gene expression, using a reverse transcription (RT) reaction with fluorescent nucleotides on messenger RNAs in individual cells. Depends on FM or FC for detection.	22,122
Fluorescent protein reporters	In combination with FM and FC, provides the means to study both gene expression and localization of proteins in time in individual living cells. Modified variants, such as the cyan and yellow fluorescent proteins, have made it possible to study co-localization and interactions of proteins, as well as expression of multiple genes in the same cell.	123,124
Bioluminescent reporters	*lux*-based systems rarely used for single-cell analyses, because of the relatively low resolution. Primary applications are in biosensors, studies of host–pathogen interactions and oscillatory gene expression in populations.	125
Immunofluorescence	Using a specific antibody in combination with a second fluorescently labelled antibody (or a single labelled antibody), localization of proteins within a single cell can be visualized using FM. Fluorescently labelled cells can also be subjected to FC analyses.	126
Microelectrophoresis	Can study individual cells with respect to their surface charge or zeta-potential.	127

expression[45]. Both single-positive feedback and double-negative feedback could lead to bistability (BOX 2). Smits and co-workers[10] based their argument that ComK autostimulation is responsible for bistability on two lines of research (FIG. 2b). First, a strain of *B. subtilis* was created in which the known regulatory inputs of competence development were systematically removed, with the exception of ComK autostimulation[10]. In the resulting mutant strain, fluorescence from a competence-specific reporter (*comG–gfp*, green fluorescent protein) was observed to still occur in a subpopulation of cells. Second, the removal of the autostimulatory loop by deletion of the native *comK* locus and the introduction of an inducible copy in an ectopic locus led to a monomodal distribution in fluorescence of the competence reporter, and to a graded response after higher induction. These results are consistent with those obtained with artificial gene-regulatory networks in *Saccharomyces cerevisiae*[46]. Similarly, Maamar and Dubnau[47] evaluated the fluorescence of a *comK–gfp* reporter in a strain with and without the autostimulatory loop, using a different inducible system (FIG. 2c). By inducing at a time point preceding normal competence development, they bypassed the growth-phase-dependent regulatory mechanisms that are usually important in competence development. The data strongly indicate a crucial role for ComK autostimulation, but do not exclude the putative toggle switch, as this is also affected when the autostimulatory loop is removed. Maamar and Dubnau elegantly addressed this problem by introducing a *rok* mutation in the strain with the inducible ComK. A bistable gene-expression pattern was observed both in the presence and absence of *rok*, but only in the presence of ComK autostimulation, excluding a toggle-switch-like mechanism involving ComK and Rok.

Interestingly, *B. subtilis* is not the only organism in which competence is associated with subpopulations of a genetically homogeneous bacterial culture. Steinmoen and colleagues reported that competent cells induced cell lysis and DNA release, leading to a 'donor' and an 'acceptor' population in *Streptococcus pneumoniae*[48,49]. Recently, it was reported that competent *Streptococcus mutans* cells show concomitant development of competence and production of a bacteriocin in a competence-dependent manner[50]. This mechanism is similar to the cannibalism described for sporulating *Bacillus* cells[51], discussed later in this review. There are also some interesting parallels with allolysis, the release of cytotoxic factors by non-competent cells, triggered by competent cells[52]. To our knowledge, no single-cell analysis using competence reporters has been carried out in *Streptococcus* species, and therefore the system might reflect both inter-species and intraspecies competition. However, these results indicate that the occurrence of a subpopulation of competent cells might not be limited to *B. subtilis*.

Sporulation. Various species of bacteria use elaborate survival tactics to cope with the harshest of environmental conditions. For instance, members of the genera

Monomodal
Demonstrating a single Gaussian distribution.

Toggle switch
A bistable switch formed by double-negative feedback.

Bacteriocin
Bacterially produced, small, heat-stable peptide that is active against other bacteria and to which the producer has a specific immunity mechanism. Bacteriocins can have a narrow or broad target spectrum.

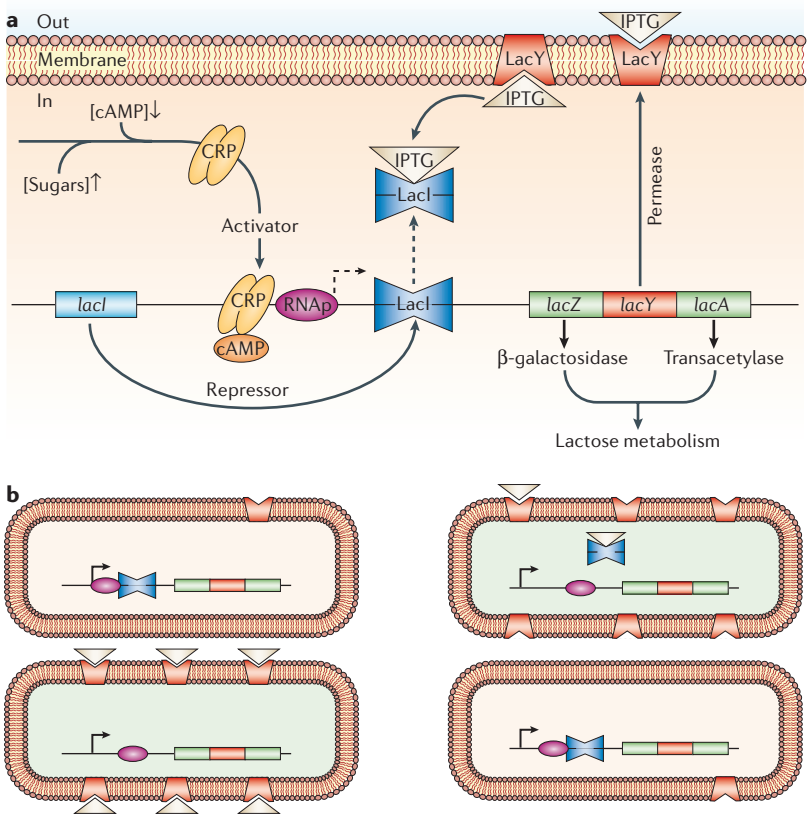


Figure 1 | **Bistability in the lactose-utilization network. a** | The *Escherichia coli lac* operon comprising *lacZ*, *lacY* and *lacA* is under catabolite control through the cyclic AMP (cAMP) receptor protein (CRP). High levels of sugars, such as glucose, galactose or lactose, generate a drop in cAMP which in turn binds to CRP, causing transcriptional activation and modulation of gene expression in the *lac* operon[12]. LacI binds to the promoter and represses transcription by RNA polymerase (RNAp). Repression is relieved when the natural inducer allolactose or a gratuitous inducer such as isopropylthio-β-galactoside (IPTG) binds to LacI and LacI dissociates from the promoter. **b** | Bistability can be observed when a gratuitous inducer such as IPTG is present at suboptimal levels, meaning that the concentration is just enough to reach the threshold to relieve LacI from the promoter. As shown in this sketch, spatial variation of the inducer can contribute to the observed bistability. Two cells have reached this threshold and consequently produce high levels of the LacY permease. This facilitates the uptake of more IPTG, generating more LacY. This positive feedback loop ensures that cells have the *lac* operon in the active state. The upper-right cell had previously been induced and, owing to hysteresis, can reach the active state at a concentration of IPTG that is insufficient to trigger a previously uninduced cell (upper left).

Bacillus, *Myxococcus* and *Clostridium* can form highly resistant endospores. Initiation of sporulation can be triggered by environmental signals such as nutrient deprivation or high cell densities, and causes a specific subpopulation of cells to switch on an elaborate genetic programme that results in the formation of spores (reviewed in REFS 53,54). Spores can remain dormant for many years and resume growth and division after germination when conditions become favourable again.

In *B. subtilis*, the formation of spores involves the expression of more than 10% of all the genes in the genome. Therefore, this last-resort adaptive process is tightly regulated, as it is energy intensive and time consuming[55]. Initiation of sporulation is regulated by a so-called phosphorelay, a series of consecutive reactions catalysed by phosphotransferases. Environmental signals can be integrated by phosphorylating or dephosphorylating components of the phosphorelay by external pathways. Eventually, the cascade leads to phosphorylation of the key sporulation regulator, Spo0A[56]. Furthermore, to be active, Spo0A needs to be present as a dimer[57]. The activity of Spo0A is subject to several autostimulatory loops, both at the transcriptional level and at the level of activation[53]. A simplified scheme of the gene-regulatory network that governs sporulation in *B. subtilis* is depicted in FIG. 2d. When conditions that trigger sporulation are applied to a *B. subtilis* culture, not all cells enter this process, leading to two clearly distinguishable cell types: sporulating and non-sporulating cells[11,58,59]. Gonzalez-Pastor and co-workers showed that sporulating cells can produce a killing factor to which the non-sporulating cells are sensitive[51]. The Spo0A-active cells can use the nutrients that are released from the dying non-sporulating population, and delay further commitment to sporulation. Using single-cell flow-cytometric analyses, Chung *et al.* suggested that, before sporulation is initiated, a threshold level of active Spo0A needs to be reached[59]. Mutations within the phosphorelay, leading to lower concentrations of intracellular Spo0A~P, caused a change in the outcome of the bistable response, with a smaller population of cells initiating sporulation.

Recently, two research groups advanced our understanding of sporulation heterogeneity in *B. subtilis*. Fujita and co-workers showed, using an inducible Spo0A construct, that spores are only formed when a certain threshold level of Spo0A is reached, indicating a non-linear response[58]. Furthermore, they showed that there are categories of genes within the Spo0A regulon[60] that respond to different thresholds of Spo0A[58]. Veening *et al.* combined previous knowledge of sporulation with more recent advances in our understanding of synthetic gene-regulatory systems and showed, using flow cytometry in combination with promoter–*gfp* fusions, that the complex autostimulatory Spo0A cascade can be considered as a classical autostimulatory loop with a bistable outcome[11]. By using a constitutively active variant of Spo0A and thereby bypassing the need for activation of Spo0A by the phosphorelay, they could show that sporulation bistability is abolished when Spo0A autostimulation was replaced by a graded induction (FIG. 2e). These experiments clearly establish sporulation bistability as a typical (but complex) example of FBM.

In 1970, Dawes and Thornley observed that some sporulating cells formed even at high dilution rates when grown in chemostats (conditions that normally do not trigger sporulation)[61]. Although not recognized as such at the time, this suggests a stochastic and noisy basis for sporulation bistability; a supposition which was supported by two later studies[62,63]. However, more experimental work needs to be carried out to determine the exact role of noise and other inputs (such as AbrB, CodY, RacA, Soj/Spo0J and SinR) on sporulation heterogeneity.

Other bistable systems in bacteria. Based on fluorescent reporters, several other bacterial systems were found to have an expressing and a non-expressing

Spo0A
Master regulator for sporulation in *Bacillus subtilis*.

Flow cytometry
A technique that measures the fluorescence of individual cells as they pass through a laser beam.

Noise
Part of a signal or parameter that is a deviation from the true value.

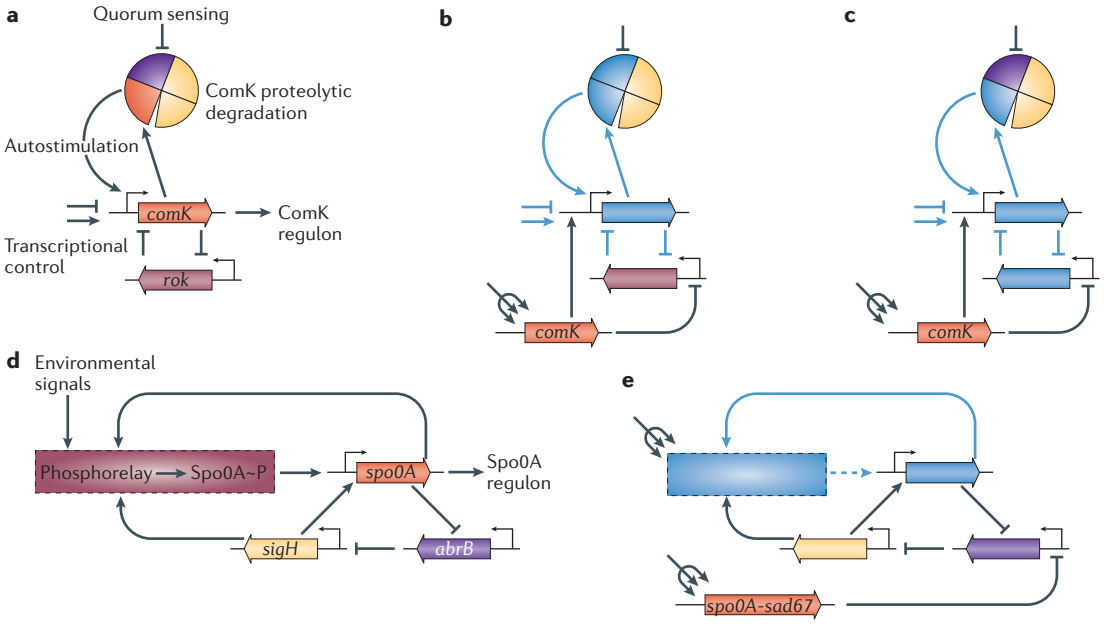


Figure 2 | **Bistability in competence and sporulation of *Bacillus subtilis*.** In all studies described above, promoter–GFP (green fluorescent protein) fusions were used as single-cell reporters for gene expression. Modifications made to investigate the mechanism underlying bistability are indicated in light blue (when removed) or black (when introduced) in panels **b**, **c** and **e**. Blunt arrows indicate repression, arrows indicate activation or production of protein. Induction is depicted with a trident. **a** | Simplified schema depicting the development of competence. At a specific quorum of competence pheromones, the proteolytic degradation of the key regulator ComK (red) is inhibited. The subsequent release of ComK enables *comK* auto-activation, and the genes required for competence development are expressed. Transcriptional control either enhances or inhibits competence development. **b** | In a strain in which all regulatory modules except ComK autostimulation have been deleted, this positive feedback is sufficient to generate competence bistability[10]. This was substantiated by the observation that a graded response was achieved upon induction of an ectopic copy of ComK in the absence of autostimulation. **c** | Competence bistability does not result from a putative toggle switch that could be present in the double-negative feedback between ComK and the major repressor of *comK*, Rok. Similar to **b**, competence bistability is caused by ComK auto-activation and is independent of the presence of *rok*[47]. **d** | Simplified schema depicting the initiation of sporulation. Environmental signals are integrated into the phosphorelay and result in the phosphorylation of Spo0A. Spo0A~P directly and indirectly auto-activates its own transcription and phosphorylation, for example, through depression of AbrB-regulated genes such as the sporulation-specific σ factor SigH. At high thresholds of Spo0A~P, genes required for efficient spore formation are activated[58]. **e** | An inducible, constitutively active form of Spo0A (*spo0A-sad67*) can be introduced ectopically, bypassing the autoregulation[11]. Depending on the presence of auto-activation, either a graded or bistable sporulation response is observed, showing that the naturally occurring bistable response in sporulation is due to Spo0A auto-activation.

population for certain genes. In *Myxococcus xanthus*, for instance, fruiting-body formation requires the expression of the gene *devR*, which encodes a developmental regulator. Using a fluorescent substrate for β-galactosidase activity, it was reported that *devR–lacZ* shows a bistable distribution in fluorescence[64,65]. It was postulated that negative autoregulation of the *devRS* locus is not responsible for this phenomenon, because a transposon mutant of the *dev* locus, incapable of negative autoregulation, still showed bistability. This is in line with the model that predicts that a single-negative feedback cannot cause multistationarity[66]. However, there might be other, unknown, factors in the upstream regulatory cascade that cause the heterogeneity in *devRS* expression. Interestingly, *M. xanthus* displays more variable phenotypes. Some of these, such as the tan/yellow switch in colony phenotype[67], have never been attributed to genomic inversion and might be revisited in light of the recent developments with respect to FBM.

In *E. coli*, it was observed that *cka* — the structural gene encoding the bacteriocin colicin K — fused to *gfp* is only expressed in 3% of the population (as judged by fluorescence microscopy)[68]. Expression of *cka* is strongly repressed by the action of LexA, and in a LexA-knockout strain almost 100% of the cells express *cka*. The expression of *cka* is indirectly regulated by guanosine 3′,5′-bispyrophosphate (ppGpp), the so-called stress alarmone[69], but the ratio of *cka*-expressing and *cka*-non-expressing cells did not change significantly in a strain that does not produce ppGpp[68]. More experiments are required to elucidate the exact mechanism of the bistable expression of *cka*, but it is most likely that it is caused by some sort of negative feedback process acting on LexA, or on an unknown positive regulator that activates *cka* expression. In the same study, the authors showed that the immunity gene *cki*, which is transcribed in the opposite direction, was expressed in all cells, protecting the whole colicinogenic population from the bacteriocin. This observation shows that related functions need not be similarly bistable.

Alarmone
Signalling molecule produced in response to nutritional and/or physicochemical stress

Using single-copy *gfp* fusions in combination with flow cytometry, Hautefort and colleagues showed that the *prgH* gene of *Salmonella* species is only expressed in a subpopulation of cultured cells in a bistable manner[70]. This gene encodes a basal component of the needle complex of the *Salmonella* pathogenicity island I (SPI1) type III secretion machinery, which is induced by several forms of stress and in a growth-phase-dependent manner. Regulation of the invasive phenotype of *S. typhimurium* involves elaborate regulatory cascades, but these were not evaluated with respect to the bistable expression of *prgH*.

In *Pseudomonas* sp. strain B13, Sentchilo and colleagues reported that an integrase (*intB13*) that is part of the so-called *clc* genomic island is expressed in maximally 15% of the population[71,72]. The *clc* element, in contrast to many other genomic islands that have a role in pathogenicity, seems to have an ecological or catabolic function. Its expression is growth-phase dependent and is increased in the presence of 3-chlorobenzoate. Interestingly, the genes on the genomic island might encode enzymes for the breakdown of this compound. The genomic island is thought to be both positively and negatively regulated[71], but the bistable characteristics of either of these regulatory mechanisms is undocumented. The observed bistable pattern in gene expression of *intB13* could also apply to other genomic islands, resulting in phenotypic variation in pathogen populations.

There are several systems in which the occurrence of bistability has been postulated based on experiments other than single-cell analyses. For instance, Booth reported that a culture of an *E. coli* strain lacking two major mechano-gated channels consistently harbours a small subpopulation of around 5% of surviving cells when shifted to high osmolarity[73]. Re-culturing of these survivors again yielded two subpopulations, indicating that the modification is non-genetic in nature[73]. These findings are reminiscent of the persistence phenotype observed in various bacterial species, such as *E. coli*. Persistence refers to a subpopulation of bacterial cells that grow slowly and are resistant to antimicrobial compounds, or cells that induce a state of slow metabolism in response to nutrient starvation. Persistence is believed to be an epigenetic phenomenon because the survivor population is still sensitive to antimicrobials upon reculturing[74,75]. This phenomenon also occurs in pathogens including *Staphylococcus aureus*, *Streptococcus pyogenes*, *P. aeruginosa* and *Mycobacterium tuberculosis*[76]. The origin of the phenotypic switch is unknown[74], but it is tempting to speculate that a feedback-based mechanism could contribute to the variability.

Furthermore, subpopulations showing differential gene-expression patterns or physiological properties were identified using density centrifugation in *E. coli*[77], *Vibrio haemolyticus*[78] and *P. aeruginosa*[79]. Last, bacteria growing in biofilms show a high degree of spatial and temporal heterogeneity[80]. This heterogeneity might be due to the micro-environment and does not necessarily reflect FBM. In fact, for some of the processes mentioned above it is unclear whether the differential geneexpression profiles are the result of asynchronous growth or whether there is a molecular mechanism maintaining the differences, as is the case for the better-characterized examples[10,11,18,24,47].

Noise in gene expression

The production of a specific protein in genetically identical cells in an essentially identical environment can differ among cells owing to stochastic fluctuations (or noise) during transcription and translation, leading to differences in protein levels[7,81,82]. This phenomenon is believed to be an important factor in multistationarity, and therefore FBM.

An important factor that contributes to the origin of noise is the so-called finite number effect. In essence, this hypothesis predicts that noise is more abundant for processes that involve limited numbers of molecules. This was shown experimentally by fluorescent-reporter studies[83–85]. This conclusion is of importance to FBM, as transcription and translation are supposed to be infrequent events when compared with, for instance, protein–protein interactions, and transcription factors are often present in low abundance.

Noise can be inherent to the biochemical process of gene expression (intrinsic noise) or originate from fluctuations in other factors that influence gene expression (extrinsic noise). The two types of noise can be discriminated using a system of distinguishable cyan and yellow fluorescent proteins expressed from the same promoter at different chromosomal locations. It was found that that both intrinsic and extrinsic noise contribute to phenotypic variability using the model organisms *E. coli*[83,85] and *S. cerevisiae*[86]. Importantly, in the latter it was found that noise can be both gene specific and independent of the regulatory pathway or the rate of expression. Using time-lapse fluorescence microscopy, it was shown that intrinsic noise in gene expression fluctuates rapidly, whereas extrinsic noise can occur over longer periods of time[87]. This trade-off between speed and accuracy in cellular transcriptional responses has implications for FBM, as it implies that fast-acting networks (such as positive autoregulation) are more sensitive to noise.

The origin of noise has been addressed in several theoretical and experimental studies (reviewed in REF. 82). Based on mathematical modelling, it was predicted that noise is most dependent on the translation rate, but is independent of the transcription rate[88]. Ozbudak and colleagues substantiated these findings experimentally through the use of GFP as a reporter for protein production in *B. subtilis*[84]. To measure the contribution of transcriptional and translational efficiency on noise, mutations within the promoter region and ribosomal-binding site of a single copy of *gfp* were introduced. The study showed that noise in *B. subtilis* primarily increased with increasing translational efficiency. By contrast, for the eukaryotic organism *S. cerevisiae*, it was reported that transcriptional efficiency does have a role in noise generation[89]. The authors suggest that this difference might be due to

Finite number effect
The effect that fewer molecules lead to increased noise levels.

Intrinsic noise
Noise inherent to the biochemical process of gene expression (transcription and translation).

Extrinsic noise
Noise due to fluctuations in other cellular components required for gene expression in a cell (such as polymerase and regulators).

Transcriptional reinitiation
Repeated rounds of transcription from a single mRNA, without dissociation of the transcription machinery.

transcriptional reinitiation, a process that presumably does not occur in prokaryotes.

Despite its stochastic origin, noise can be controlled by several mechanisms. One of the most obvious ways to reduce noise, considering the finite number effect, is to increase the concentrations of the relevant molecules, so that fluctuations in the levels of one of the components do not significantly impinge on the network. However, this strategy is costly for cells and, in natural situations, other tactics are usually adopted[7]. One of the most ubiquitous noise-attenuating mechanisms is negative feedback (reviewed in REFS 7,90,91). If the concentration of a component increases, a negative feedback loop ensures downregulation of the production of this component, therefore limiting the range over which the concentrations of components within the network fluctuate and reducing noise. Indeed, it was reported that negative autoregulation is predominant for housekeeping functions of *E. coli*[92].

Another important noise-control mechanism relevant to FBM is hysteresis. Hysteresis reflects a situation in which the switch from one state to another requires a force unequal to the reverse transition[93]. The origin of this phenomenon can, for instance, lie in the stability of one or more of the components of the bistable network, as is seen for the permease in the lactose-utilization network[12]. The unequal force essentially acts as a buffer, so that the phenotypic switch is robust in relation to noise and the possibility of accidental switching between states is minimized. For example, hysteresis is responsible for reducing the accidental switching of the direction of flagellar rotation in bacterial chemotaxis[94].

Developmental pathways are in general regulated by complex regulatory cascades resulting in the production of a pivotal transcription factor. Therefore, the mechanism of noise propagation in a network, and how this affects multistability, is important for our understanding of FBM. Recent research has shown that longer signal-transduction cascades can amplify noise and that upstream regulatory events can have a bigger effect on the variability of gene expression than the intrinsic noise of the gene itself[95–97]. Interestingly, however, Hooshangi and co-workers also showed that signal-transduction cascades might act as a filter to dampen the short-lived fluctuations of an input signal, because of the time required to transmit a signal through the network[97].

Modulation of bistability

Noise might be important in establishing bistability, but when exploited by bacteria to generate phenotypic variability, regulatory processes are also involved. Developmental processes are frequently primed by environmental signals. In effect, the output of a multistationary switch can often be modulated by these signals. This means that the fraction of cells in a specific subpopulation of a multistable phenotype depends on conditions that are set (such as the threshold level of a regulator required for auto-activation).

For instance, in the case of the *lac* operon, the output of the bistable response is modulated by the activity of the activator CRP[12]. The bacterial quorum-sensing pathway that controls the proteolytic degradation of the key transcription factor required for competence also affects the fraction of bacterial cells that becomes competent[10]. Some regulators, such as Spo0A in *B. subtilis*, must be phosphorylated to be active. As such, dedicated kinases or phosphatases of such regulators can have a considerable influence on the ratio of cells present in one of the two regulated states[11,59]. Furthermore, as discussed above, different strategies can be used by bacteria to alter noise levels within the FBM circuit, thereby affecting bistability. Using any of the mechanisms described, the ratio of bacterial cells in a particular subpopulation is fine-tuned to suit the prevailing environmental conditions that the bacterial population is subject to. This notion is reinforced by early experimental evidence that shows, for instance, that the fraction of cells that sporulate strongly depends on growth conditions[98].

In principle, most FBM systems are reversible, allowing individual cells to switch between states. The time that is necessary for the switch to occur is termed the escape time[6]. However, *in vivo*, some FBM systems act as unidirectional switches, such as oocyte differentiation in *Xenopus*[8] and spore formation in *B. subtilis*[11,53]. This suggests that the escape time of such a system is effectively infinite[6] — the chance that switching occurs during a cell's lifetime is negligible. The locking of a bistable switch might originate from the architecture of the gene network, but can also be mediated by environmental signalling. Sporulation in *B. subtilis*, for instance, relies on a cascade of alternative σ factors which provide directionality to the developmental programme[99], and is therefore different from an intrinsically irreversible epigenetic switch.

To determine the individual contributions of different regulatory mechanisms on the bistable output of an FBM system is a major challenge for future research and requires real-time analysis of multiple components in a single cell.

Evolutionary benefits of FBM

Based on modelling and mutant analysis, it has been suggested that noise is an evolvable trait[100]. For gene-regulatory pathways that govern cellular homeostasis, such as genetic and metabolic networks, noise is undesirable as it can be detrimental to the fitness of the species[7,81,101,102]. Therefore, one would predict that essential genes would be subject to considerably lower levels of noise when compared to non-essential genes. This hypothesis was examined using a computational approach in which noise levels of all the genes of *S. cerevisiae* were calculated[100]. It was shown that essential genes did indeed exhibit lower levels of noise compared to most other genes.

However, noise can be a useful phenomenon and can be amplified by, for example, positive feedback, potentially leading to FBM[8,103]. Non-genetic variability arising from FBM can be beneficial for the population and, as a consequence, it has been suggested that some gene networks are more noisy than others[1,2,7,27,104]. For instance, the switching of phage populations from a lytic to lysogenic pathway is

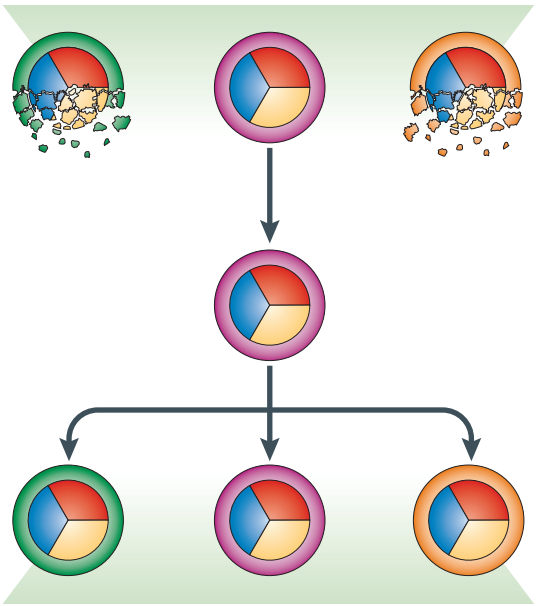


Figure 3 | **Survival of variable phenotypes under selective pressure.** Three genetically identical cells are depicted in the upper part of the figure. The triangular parts depicted in red, yellow and blue represent the genetic content. The outer ring depicts the (variable) phenotype that each cell displays. Upon a specific selective stress condition, only cells with a certain phenotype can survive. After selection, the survivor cell can regenerate the same phenotypic variation without altering its genetic content. One can envisage that in natural situations, bacterial populations encounter more than a single type of stress or fluctuations in environmental conditions. Feedback-based multistability systems can quickly generate phenotypic variability, potentially leading to the survival of a genotype.

thought to have evolved as an adaptation to changes in their environment (the host cell)[105–107]. Variable phenotypes in pathogens might help them to evade the immune responses of their hosts. Alternatively, the variation might serve to balance the benefits and disadvantages of a certain phenotype. In the case of competence, there might be a trade-off between the benefits of the generation of genetic diversity and repair through homologous recombination, and the drawbacks such as the possibility of illegitimate recombination, growth arrest and lysis owing to the sensitivity of competent cells.

The importance of FBM lies in the ability of a small proportion of the population to survive environmental stresses that kill the majority[1,107]. When multiple phenotypes coexist in a culture, the population as a whole remains viable even under fluctuating environmental conditions that result in the death of some of the subpopulations. Crucial to this view is the fact that the traits that are selected are not genetically determined. This ensures that upon outgrowth of the survivors, the cell population retains the ability to regenerate all the phenotypes that were present in the original culture (FIG. 3).

For adaptive phenotypes, it is important that the response to stress is quick and efficient. As discussed in this review, FBM can generate phenotypic variability within an isogenic culture, combined with a quick response owing to positive autostimulation. This might explain why FBM is a preferred mechanism for the bistable adaptive responses that have been identified in bacteria to date. At first glance, enzymatic bistability (as observed with the *lac* operon) might not seem relevant to adaptation processes. However, it was shown recently that individual persister cells of an *S. pyogenes* culture have different metabolic profiles that seem genetically fixed, as they are stably inherited. More importantly, it was found that the coexistence of several metabolic variants is required for outgrowth (B. Buttaro, personal communication). It can be envisaged that in early stationary phase of bacterial growth, metabolic differences reflect FBM, and that this variability is subsequently fixed by accumulating mutations. In that respect, it is striking that competence, a bistable process, and hypermutation seem to be linked in *B. subtilis*[108].

Concluding remarks

Single-cell analysis in a given bacterial population has only recently spurred an interest in non-genetic individuality and the molecular mechanisms responsible for this phenomenon. Prior to this, many heterogeneous processes might have gone undetected owing to investigation using culture-wide reporter studies. Also, the phenotypic variability observed in many biological processes might have been discarded as an artefact of the methods used in the analysis. To properly investigate these heterogeneous processes, there is a strong argument for the use of single-cell analyses over culture-wide assays. Only by evaluating the expression of genes in single cells will it be possible to discern between population heterogeneity with an underlying monomodal signal distribution (in which the supposed 'off' population reflects only the tail of the distribution below the detection limit of the system) and true multistability (BOX 3). In processes where true multistability has been established, it is necessary to investigate whether the phenotypic variation is epigenetic and, subsequently, whether it is due to feedback architecture in the regulatory network governing the process.

The notion that bistability in naturally occurring gene-expression patterns in bacteria can be the result of feedback architecture will have a big impact on the *in silico* investigation of gene networks. First of all, it might be possible to predict positive or double-negative autoregulation, and so identify processes that are prone to multistationarity which could result in a multistable population. It was shown mathematically that the number of possible independent stationary states depends on the number of unrelated positive circuits[4]. A prerequisite of such an analysis is that genome-wide prediction of transcription-factor-binding sites is possible and that information regarding the nature of the regulators is available. Second, once multistability has been established experimentally, it might be possible to reconstruct the upstream regulatory network and predict missing elements.

Another interesting question is whether or not there is significant interplay between the different subpopulations of a bacterial culture. For competence, it has been postulated that cultures consist of a donor population, releasing DNA through lysis or active transport, and a receptor population[48]. Similarly, it has been shown that sporulating bacterial cells can cannibalize non-sporulating cells[51]. Are these phenotypic differences a consequence of the same bistable response, or do they reflect differences in developmental timing between cells which have arisen during growth? And, related to this, are multiple bistable systems mutually exclusive, or can a subpopulation of cells be part of more than one bistable response? Positive regulators, such as those present in the ubiquitous two-component signalling systems, might have the ability to generate bistability as some of the requirements for multistationarity, such as cooperative binding and autoregulation, are met. Although the presence of feedback has been firmly established as a prerequisite for bistable gene-expression patterns, it is an oversimplification to ascribe phenotypic heterogeneity simply to the presence of noise within the biochemical reactions of a cell. Although noise might be the inherent source of bistability, prokaryotes use elaborate regulatory mechanisms to modulate the bistable output. Some of the components of these networks might be more prone to concentration fluctuations, and therefore contribute more to the final ratio between the high- and low-expressing populations. The identification of these components is an exciting challenge for future research.

Logic dictates that essential processes in cells are non-heterogeneous, a notion that is supported by *in silico* analyses[100]. The regulatory networks that govern these processes should, therefore, include noise-minimizing strategies, such as negative feedback. By contrast, it might be a common feature of adaptive phenotypes to use positive feedback, to ensure a rapid response to stresses encountered and to generate phenotypic variability to enhance fitness. The different examples of natural bistable processes that have been identified so far strengthen this view. It will be of great interest to see whether the occurrence of bistability is indeed predominant in adaptive rather than essential processes.

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The authors declare no competing financial interests.

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