Chapter 1

Introduction

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1. 1 History of *Bacillus subtilis* sporulation research

Various bacterial species show elaborate and sophisticated tactics to survive the harshest conditions. Members of the genera *Bacillus, Listeria* and *Clostridium* for instance, are able to form highly resistant endospores (from here on termed spores). These spores are extremely resistant to a great variety of stresses such as heat and cold stress, drought and UV-radiation (Driks, 2002). In fact, spores are considered some sort of 'time-capsules' and have been found to survive for over thousands, or even millions of years, e.g. tightly encapsulated in amber (Cano and Borucki, 1995). When conditions changes for the better, for example when the spore has been dispersed by wind or carried by another organism to a new niche, the spore can germinate and the cell resumes vegetative growth.

Of the sporulating bacteria, the Gram-positive *Bacillus subtilis* is by far the best studied bacterium, mainly because of its amenability to classical and reverse genetics. The interest in *Bacillus subtilis* dates back to the 19th century, to the times of Christian Gottfried Ehrenberg and Louis Pasteur. Initial work on endospores can be traced back to John Tydall, Ferdinand Cohn and Robert Koch, who observed that some preparations, such as the fruit juice solutions used by Pasteur, sometimes required long periods of boiling, sometimes hours, before they were completely sterilized. Cohn and Koch performed the first microscopic studies and discovered endospores in old *Bacillus* cultures (reviewed in Brock *et al*., 1984; Sonenshein *et al*., 2002). The use of *B. subtilis* as an experimental system for studying gene regulation and differentiation was spurred by the generation and identification of a strain that was found to be highly competent for natural transformation: *B. subtilis* strain 168 (Burkholder and Giles, 1947; Spizizen, 1958). This strain and its derivatives are still used in most *B. subtilis* laboratories and has become the paradigm for general research of Gram-positive bacteria. Having the ability to conveniently manipulate *B. subtilis* led to the mapping and elucidation in the 1970s of most genes important for spore formation (Hoch, 1971). In 1997, the complete genome sequence of *B. subtilis* was published, facilitating further molecular dissection and leading studies on this organism at the post-genomic level (Kunst *et al*., 1997).

1.2 Initiation of sporulation

Initiation of sporulation in *B. subtilis* can be triggered by multiple environmental signals, like nutrient deprivation and high cell densities, and causes a specific subpopulation of cells to switch on an elaborate genetic program resulting in the formation of spores (reviewed in Hoch, 1991; Sonenshein, 2000; Piggot and Losick, 2002; Errington, 2003) (Fig. 1).

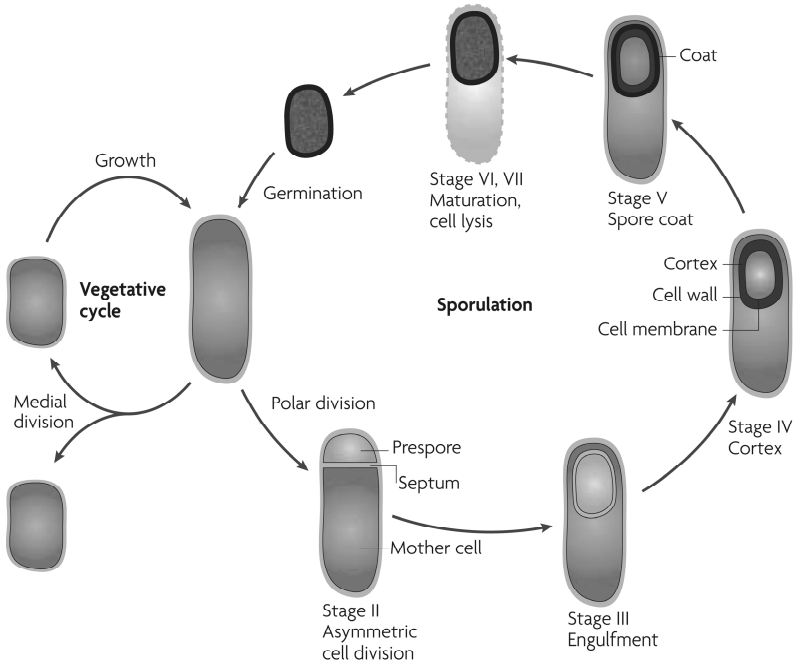

Figure 1. Sporulation of *Bacillus subtilis*. Taken from (Errington, 2003), with permission from the publisher and author.

The sporulation cycle begins by formation of an asymmetric septum. Next, the newly formed prespore gets engulfed and the endospore matures in a couple of hours. Finally, the mature and resistant spore is released by lysis of the mother-cell compartment.

In *B. subtilis*, the formation of spores influences the expression of more than 10% of all the genes in the genome (Fawcett *et al*., 2000). Thus, this last resort adaptive response is tightly regulated, since it is an energy intensive, time consuming and irreversible process. Initiation of sporulation is regulated by a so-called phosphorelay in which environmental signals are integrated resulting in the phosphorylation of the key sporulation regulator, Spo0A (Burbulys *et al*., 1991; Lewis *et al*., 2002). Spo0A is a typical response regulator and the capacity to bind DNA and activate or repress gene

transcription is stimulated upon phosphorylation. The multicomponent sporulation phosphorelay consists of five histidine kinases (KinA, KinB, KinC, KinD and KinE) and two phosphorelay proteins (Spo0F and Spo0B) (Perego and Hoch, 2002). The most important activator of the sporulation phosphorelay is KinA (Fujita and Losick, 2005). When environmental signals stimulate production and autophosporylation of KinA, KinA~P transfers its phosphoryl group to Spo0F. Spo0F~P in turn phosphorylates Spo0B which acts on the DNA-binding protein Spo0A. Phosphorylated Spo0A (Spo0A~P) is the active form and regulates expression of sporulation (related) genes (for a simplified regulatory scheme see Figure 1 of **Chapter 3**, page 44).

The output of the phosphorelay (in terms of intracellular Spo0A~P levels) is fine-tuned by multiple phosphatases (Perego and Hoch, 2002; Veening *et al*., 2005, **Chapter 3**). These phosphatases can be categorized in two groups on the basis of their targets. Sporulation related Rap phosphatases (e.g. RapA and RapE) act on the level of Spo0F~P, while the Spo0E-like phosphatases (including YnzD and YisI) directly remove the phosphoryl group from Spo0A~P. The phosphatase activity of Rap proteins is modulated by small-secreted peptides, so-called Phr peptides. Due to the temporal sequence of export, processing and import, it is assumed that the action of these pheromones act as a sporulation timing device (Perego, 1999; Veening *et al*., 2005, **Chapter 3**). A recent study has shown that the RapH protein, which expression is activated by the competence transcription factor ComK, exhibits a unique dual function since it acts both on ComA, that is also involved in competence development, and Spo0F~P. In fact, it was shown that RapH is crucial in the temporal separation of the two differentiation pathways (Smits *et al*., 2006a).

The activity of Spo0A is subject to multiple auto-stimulatory loops, both at the transcriptional level and at the level of phosphorylation (Strauch *et al*., 1993; Strauch *et al*., 1992; Fujita and Sadaie, 1998). First of all, Spo0A~P binds at its own promoter and is able to stimulate its own transcription (Strauch *et al*., 1992). An indirect autostimulatory loop is active *via* the transcriptional regulator AbrB. During exponential growth, AbrB represses various stationary phase processes, including the transcription of genes required for sporulation (e.g. *kinA*) (Strauch *et al*., 1989). Importantly, AbrB represses gene expression of the alternative RNA polymerase sigma-factor SigH that recognizes an alternative promoter upstream of *spo0A*, and in addition, activates genes required for phosphorylation of Spo0A such as *kinA* and *spo0F* (Lewandoski *et al*., 1986; Weir *et al*., 1991; Predich *et al*., 1992). When Spo0A is phosphorylated, it represses *abrB* expression (Robertson *et al*., 1989). Thus, alleviation of AbrB repression by Spo0A~P stimulates both transcription of *spo0A* and indirectly phosphorylation of Spo0A. A simplified scheme of the gene regulatory network governing sporulation in *B. subtilis* is depicted in Figure 2A.

Using chromatin immunoprecipitation in combination with DNA-microarray analysis it was shown that Spo0A~P directly regulates at least 121 genes (Molle *et al*., 2003). Importantly, it was shown that there are categories of genes responding to different thresholds of Spo0A~P. *abrB* and *spo0A* for instance, are part of the low-threshold

repressed and low-threshold activated genes, respectively, whereas most of the sporulation specific genes, like *spoIIA* encoding the sporulation specific sigma factor SigF (Fort and Piggot, 1984), belong to the category that requires high levels of Spo0A~P for activation. It was shown that a gradual increase of intracellular Spo0A~P is essential for successful spore formation, and it is assumed that a stepwise sensitivity of Spo0A regulated promoters ensures proper timing of activation or repression of sporulation related genes. (Fujita and Losick, 2005; Veening *et al*., 2006a; **Chapter 7**).

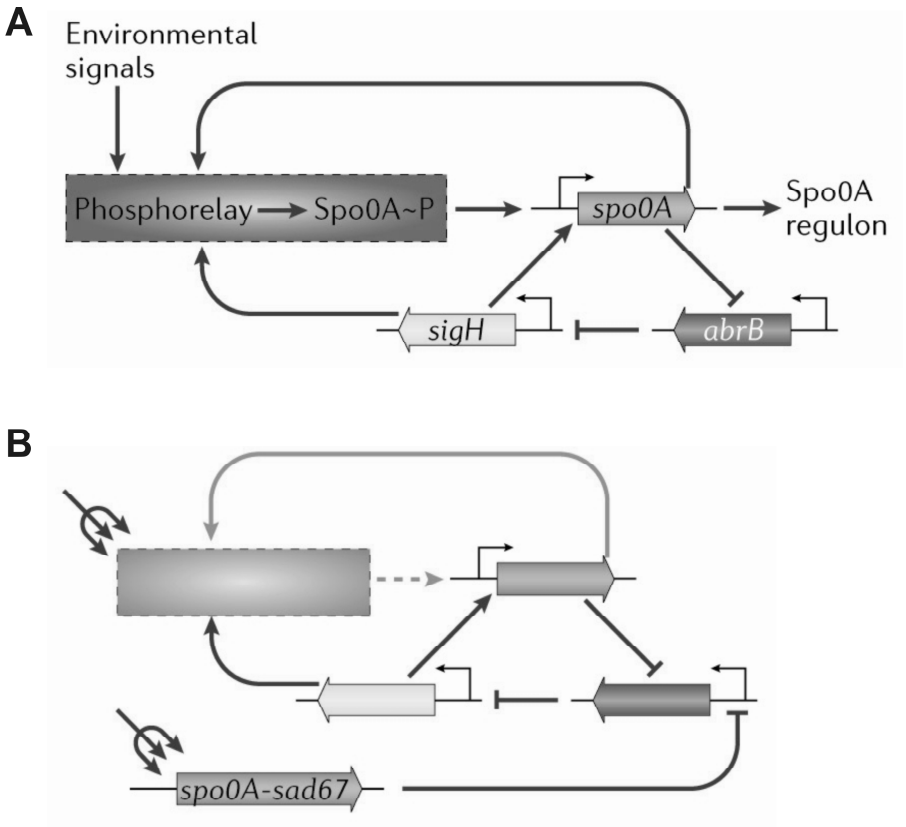

Figure 2. Initiation of sporulation in *Bacillus subtilis*. Arrows indicate the positive actions; blunt ends indicate negative actions. **(A)** Wildtype situation. **(B)** Artificial system used in **Chapter 5** to bypass the phosphorelay and demonstrate bistable switching within the sporulation pathway (see text below). Artificial gene induction is indicated with a trident. Taken from (Smits *et al*., 2006b) with permission from the publisher .

Directionality to the developmental program of spore formation is provided by the activation of a cascade of alternative sigma factors (Eichenberger *et al*., 2004). After the formation of an asymmetric septum at stage II, when the cytosol of prespore and mother cell becomes separated, the sporulation process becomes irreversible (Parker *et al*., 1996; Dworkin and Losick, 2005).

1.3 Interplay between sporulation and biofilm formation

In nature, bacteria are predominantly found in the form of multicellular communities known as biofilms (Davey and O'toole, 2000) (Fig. 3). Biofilms of *B. subtilis* have been defined as communities of cells embedded in a polymeric matrix, which can either be pellicles at an air-liquid interface or colonies grown on semi-solid agar surfaces (Branda *et al*., 2001; Veening *et al*., 2006a; Veening *et al*., 2006b; **Chapters 4** and **7**). The formation of these structures strongly depends on the growth conditions and is highly variable among strains. Unlike most laboratorium (cultured) strains, many undomesticated 'wild' *B. subtilis* strains are able to form complex colonies with elevated structures and fruiting bodies that preferentially produce spores at their tips (Branda *et al*., 2001; Shapiro, 1998). It is believed that the formation of spores at elevated structures within biofilms is some form of a dispersal strategy (Branda *et al*., 2005) (Fig. 3). The master regulator in *B. subtilis*, which governs the transition from free-living (planktonic) to sessile cells in a surface-associated biofilm, was found to be SinR (Kearns *et al*., 2005). A number of genes essential for biofilm formation, such as the *epsA-O* and *yqxM-tasA-sipW* operons, responsible for the synthesis of extracellular matrix components, are repressed by SinR (Branda *et al*., 2006; Kearns *et al*., 2005). This regulation is counteracted by the product of *sinI*, a small gene upstream of *sinR*, which binds to SinR, thereby releasing SinR from its target DNA (Bai *et al*., 1993).

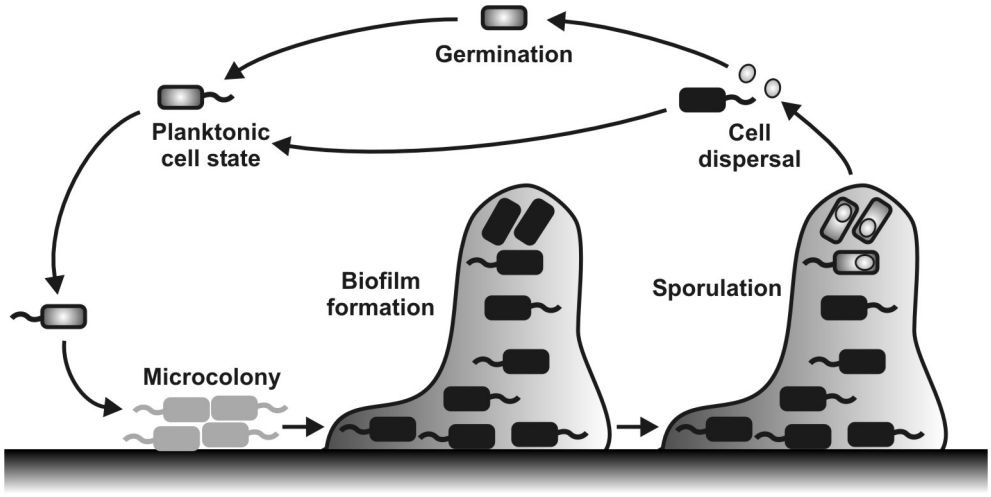

Figure 3. Schematic representation of biofilm formation and sporulation in *B. subtilis.*

The expression of *sinI* is activated by Spo0A~P (Shafikhani *et al*., 2002) and repressed by the pleiotropic regulator AbrB (Strauch, 1995b). This renders the process of biofilm formation highly intertwined with initiation of sporulation. Genes involved in biofilm formation, such as *abrB* and *sinI* respond to lower levels of Spo0A~P than sporulation genes, and this led to the hypothesis that biofilm formation occurs prior to

sporulation. Indeed, coordinated timing of the biofilm and sporulation pathways, was shown to be crucial for maximal spore resistance (Veening *et al*., 2006a; **Chapter 7**).

1.4 Bistability in spore formation: some cells sporulate, others do not

How cells develop and differentiate from one cell type into another is an important question in biology. Most of the genes and proteins involved in spore formation in *B. subtilis* have been identified and have been studied for the past three decades (see above and for current reviews see Piggot and Losick, 2002; Perego and Hoch, 2002; Errington, 2003; Hilbert and Piggot, 2004; Barak and Wilkinson, 2005). One of the fundamental questions that remain, and which is the main topic of this thesis research, is the question why some cells within a clonal population differentiate while others do not.

When a *B. subtilis* culture sporulates not all cells enter this process, and this leads to two clearly distinguishable cell types; sporulating and non-sporulating cells (Fujita *et al*., 2005; Chung *et al*., 1994; Veening *et al*., 2005). Therefore, this system was described as 'exhibiting bistability' (Fujita *et al*., 2005; Veening *et al*., 2005; Smits *et al*., 2006b; Dubnau and Losick, 2006). Using flow cytometric analyses, Chung *et al.* suggested that, before sporulation is initiated, a threshold level of Spo0A~P needs to be reached (Chung *et al*., 1994). Mutations within the phosphorelay, leading to lowered concentrations of intracellular Spo0A~P, caused a change in the outcome of the bistable response, and a smaller population of cells to initiate sporulation. On the other hand, mutations that leads to an increased concentration of Spo0A~P results in more cells initiating sporulation (Veening et al 2005). Furthermore, Fujita and co-workers showed, using an inducible Spo0A construct, that spores are only formed at a certain threshold level of Spo0A, indicating a non-linear response (Fujita *et al*., 2005). Work presented in **Chapter 5** demonstrated that the complex autostimulatory Spo0A cascade can be considered as a classical auto-stimulatory loop with a bistable outcome (Veening *et al*., 2005). By employing a constitutively active variant of Spo0A and thereby bypassing the phosphorelay, we showed that sporulation bistability is abolished when Spo0A-autostimulation was replaced by a graded induction (Fig. 2B).

In 1970, Dawes and Thornley described that some cells sporulated even at very high dilution rates when grown in chemostats (conditions that do not trigger sporulation) (Dawes and Thornley, 1970). These observations suggest that there is a stochastic and noisy basis for sporulation bistability; a supposition which has been supported by two recent studies (Maughan and Nicholson, 2004; Veening *et al*., 2006b, **Chapter 4**). In addition, we have shown that the decision to sporulate is not dependent on cell age, cell length or timing of cell division, providing another strong argument for a stochastic origin in this cell fate decision (Veening *et al*., 2006c, **Chapter 5**). However, more experimental work needs to be done to determine the exact role of noise on

sporulation heterogeneity. The role of noise in generating phenotypic variability is discussed in more detail below.

Interestingly, Losick and coworkers discovered that sporulating cells are able to produce a killing factor to which non-sporulating cells are sensitive (Gonzalez-Pastor *et al*., 2003; Ellermeier *et al*., 2006). Since Spo0A is already phosphorylated at an early stage during spore formation (before formation of the asymmetric septum), Spo0A-active cells can utilise the nutrients released from the dead cells to delay the decision to commit to spore formation. Direct evidence that cells actually profit from their dead relatives was shown in studies using time-lapse microscopy (Veening *et al*., 2006c; **Chapter 5**). Based on this, we propose that the bistable differentiation that occurs in clonal *B. subtilis* populations can be regarded as a form of 'bet-hedging', whereby a population of cells differentiates to maximize survival of at least part of the population in the face of unpredictable future conditions (Veening *et al*., 2006c, **Chapters 5 and 8**). Part of the population (the spore formers) will be protected in case of a sudden catastrophic event, while the vegetative cells will be ready to rapidly resume growth in case nutrients reappear. Theoretical studies support the notion that phenotypic variation based on bistable switching is an optimal strategy for coping with fluctuating environments (Thattai and van Oudenaarden, 2004; Kussell and Leibler, 2005).

So what exactly is bistability or multistability?

1.5 Feedback-Based Multistability (FBM)

Phenotypic variation is a widespread phenomenon in prokaryotes, and the molecular mechanisms that underlie this variation are similarly diverse. Variation can originate from genetic changes, including reversible genomic inversion (*e.g.* phase-variation in *Escherichia coli fim* (Abraham *et al*., 1985) and *Salmonella enterica* serovar *typhimurium hix* (Zieg *et al*., 1977)) and strand-slippage mechanisms (e.g. *Neisseria* spp. *opa* (Meyer *et al*., 1990) and *Bortedella pertussis bvg* (Stibitz *et al*., 1989)). 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 van der Woude *et al*., 1996; Owen *et al*., 1996). In contrast to the mechanisms described above, 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 (FBM), and seems to be a common feature of adaptive processes in the bacterial realm (Smits *et al*., 2006b).

The prerequisites for variable outputs from a genetic network (multistationarity), without the necessity of genetic reorganization or modification, have been determined

by several groups using mathematical modelling and synthetic gene circuits (reviewed in Thomas, 1998; Gardner *et al*., 2000; Hasty *et al*., 2002; Rao *et al*., 2002; Ferrell, Jr., 2002; Kobayashi *et al*., 2004). 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. Nonlinearity can occur, for instance, when multimerization is required for a transcription factor to be active, or when cooperativity is observed in DNA binding.

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 (for review see Brehm-Stecher and Johnson, 2004). Therefore, the use of single-cell analytical techniques is becoming common practice among microbiologists (Table 1). 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 regulatory network involved (Smits *et al*., 2005b; Veening *et al*., 2005; Ozbudak *et al*., 2004; Guespin-Michel *et al*., 2004). Thus, it seems that 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 when cultured under laboratory conditions (Guespin-Michel, 2001).

1.6 Characteristics of bistable systems

Figure 4 shows 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 cooperative multimerization of the protein causes the nonlinear dynamics of the system. Therefore, the production of **A**, $\boldsymbol{f}(a_p)$, 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, $\boldsymbol{f}(a_d)$. 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.

Table 1. Techniques suitable for single-cell analyses in bacterial populations.

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.	(Brehm-Stecher and Johnson, 2004)
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.	(Brehm-Stecher and Johnson, 2004; **Chapters 3-7**)
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 conjunction with standard biochemical techniques or one of the single-cell techniques listed here.	(Cahn and Fox, 1968; Nishino *et al*., 2003; **Chapter 6**)
Single-cell β-gal 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.	(Chung *et al*., 1994; Russo-Marie *et al*., 1993)
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.	(Krimmer *et al*., 1999; Lee *et al*., 1999)
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.	(Tolker-Nielsen *et al*., 1998; Lange *et al*., 2000)
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 GFP (green fluorescent protein) 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.	(Tsien, 1998; Southward and Surette, 2002; **Chapters 2-7**)
Bio-luminescent 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.	(Greer and Szalay, 2002)
Immuno-fluorescence	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.	(Davey and Kell, 1996)
Micro-electro-phoresis	Can study individual cells with respect to their surface charge or zeta-potential.	(Glynn, Jr. *et al*., 1998)

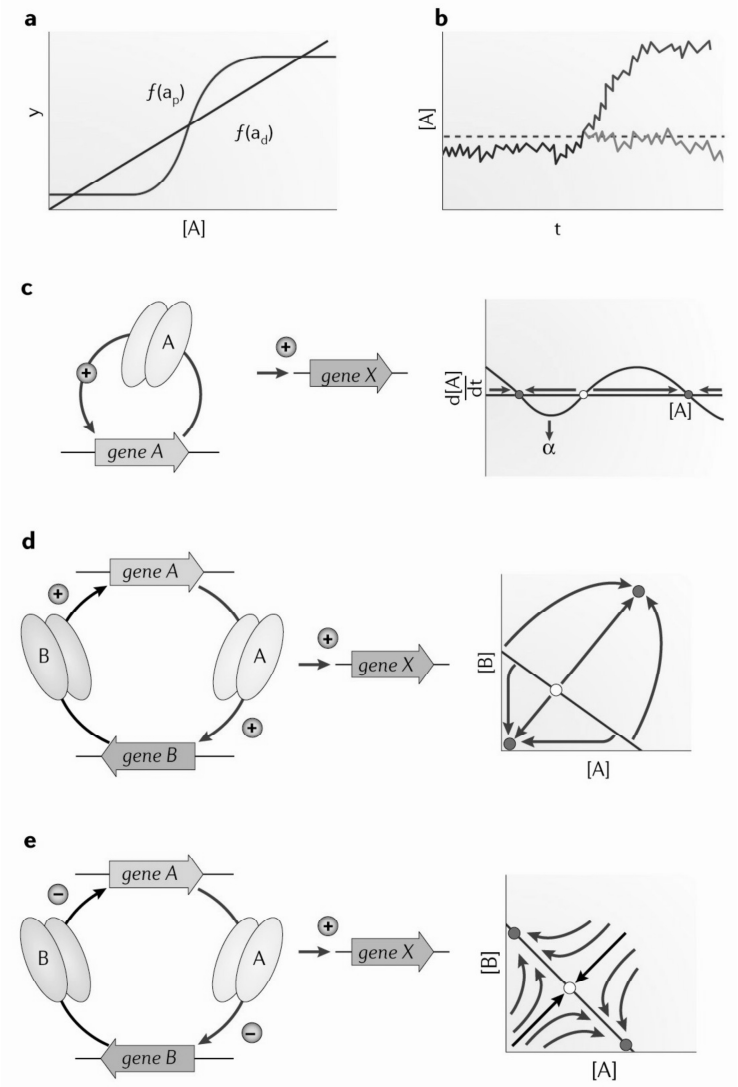

Fig. 4. (**a**) Plot of the functions ***f***(a_p) and ***f***(a_d) within the same graph. From this graph, it becomes apparent that there are three intersection points, indicating the steady states in which dA/dt is equal to 0. (**b**) The level of the protein fluctuates over time. When the level exceeds a threshold (indicated by the dotted line), cells accumulate high levels of the activator when positive or double-negative feedback is present. 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. 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. 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 (Angeli *et al*., 2004), and bistability is impossible if one of the components acts too strongly or too weakly compared to the other (Isaacs *et al*., 2003). Also, a combination of three negative

feedback loops can cause a gene network to oscillate between states (Elowitz and Leibler, 2000). Taken from (Smits *et al*., 2006b) with permission from the publisher.

1.7 Natural bistable systems in bacteria

As described above, sporulation in *B. subtilis* is a typical example of a phenotypic variation as a result of feedback regulation. There are many more bistable phenotypes described for microorganisms. To get an appreciation of the ubiquity of phenotypic variability by FBM, a few examples are described below.

Lactose utilization in Escherichia coli. In 1957, a groundbreaking study by Novick and Weiner showed that the genetic program for lactose utilization in *E. coli* (*lac* operon) is initiated in only a subpopulation of an isogenic culture (Novick and Weiner, 1957). 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 the *lac* operon, whereas the remainder of the cells did not (Cohn and Horibata, 1959). As early as 1961, it was hypothesized that the heterogeneity in lactose utilization might be attributed to multistationarity (Monod and Jacob, 1961), 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 (Muller-Hill, 1996).

The polycistronic *lac* operon comprises three genes that are required for the uptake and catabolism of lactose: *lacZ*, encoding β-galactosidase; *lacY*, encoding lactose permease; and *lacA*, encoding a transacetylase. Expression of the *lac* operon is negatively regulated by the LacI repressor, which in turn is inhibited by allolactose. Allolactose is an isomer of lactose that is converted from lactose by β-galactosidase in the alternative reaction to the hydrolytic one. LacI is positively regulated by the cyclic-AMP receptor protein (CRP), which 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 (Figure 5). However, as β-galactosidase metabolizes 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 (compound that inactivates a repressor without being metabolized by the induced enzymes) and the population

consists of cells with either high or low expression of *lac* (Ozbudak *et al*., 2004). Switching from one state to the other requires either an induction or a 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, see below). 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. Importantly, by mathematical 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 (Ozbudak *et al*., 2004), providing a framework for the unexplained observation that both states can occur (Biggar and Crabtree, 2001). Similar bistable enzyme induction systems have been described for the arabinose-utilization operon in *E. coli* (Khlebnikov *et al*., 2002; Morgan-Kiss *et al*., 2002) and the lactose operon in *Salmonella typhimurium* (Tolker-Nielsen *et al*., 1998).

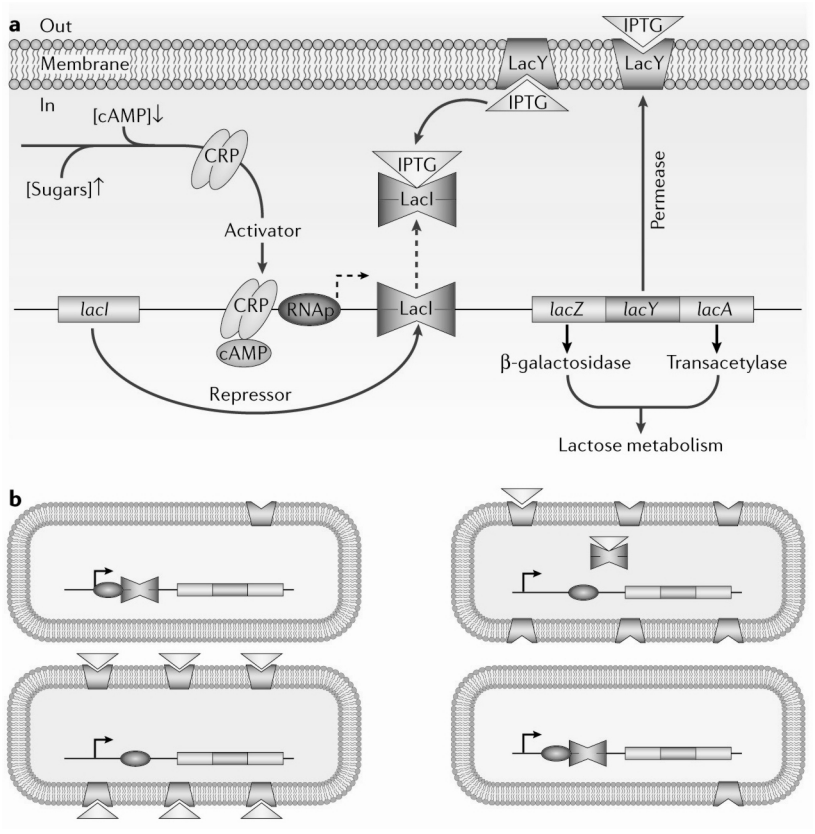

Figure 5. Bistability in the lactose-utilization network. **(a)** The *Escherichia coli lac* operon comprising *lacZ*, *lacY* and *lacA* is under catabolite control through the cyclic AMP (cAMP) receptor protein (CRP). High levels

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. 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). Taken from (Smits *et al*., 2006b) with permission from the publisher.

Lysis or lysogeny of bacteriophage λ. Bacteriophage λ is a virus that infects *E. coli* and, either reproduces quickly, thereby killing the host and releasing phage particles (lytic pathway), or integrates into the genome of the host and remains dormant (lysogenic pathway). The developmental program that controls the type of infection has become a paradigm for developmental switches (reviewed in Gottesman, 1999; Ptashne, 2005). By 1976, it was suggested, using mathematical modeling, that positive feedback within the gene network is responsible for the bistability observed (Thomas *et al*., 1976). Later research supported this (Shea and Ackers, 1985; Arkin *et al*., 1998). 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 particles 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 of 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. 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 (Arkin *et al*., 1998). With certain stimuli, such as ultraviolet radiation, the dormant phage is induced and viral particles are produced (Gottesman, 1999; Ptashne, 2005; Herskowitz and Hagen, 1980), however this process involves proteolytic activity of RecA, and is independent of the CI/Cro switch.

Mucoidy and cytotoxicity of Pseudomonas aeruginosa. P. 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. The regulatory cascade of alginate synthesis involves 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 by the same operon and antagonizes AlgU (Schurr *et al*., 1994; Schurr *et al*., 1993). With the identification of mutations in the *mucB* gene that resulted in a stable mucoid phenotype (Schurr *et al*., 1994), the possibility of FBM in *P. aeruginosa* has been overlooked. However, many strains of *P. aeruginosa* display unstable mucoid phenotypes, and experimental evidence also suggests a level of hysteresis, a common characteristic of bistable systems. Theoretical modeling 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 (Guespin-Michel *et al*., 2004; Guespin-Michel and Kaufman, 2001). 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 (Guespin-Michel *et al*., 2004). Genes that are essential for cytotoxicity require the regulator ExsA, an auto-activating protein, for expression (Dacheux *et al*., 2001). One of the activated operons encodes an inhibitor of ExsA, termed ExsD (McCaw *et al*., 2002). 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*.

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 (Lorenz and Wackernagel, 1994), and it has been proposed to function in DNA repair, to enhance genetic diversity or to serve as a source of nutrients, especially phosphate (Chen and Dubnau, 2004). It has been known for a long time that competence in *B. subtilis* is a physiological state that does not involve the whole population (Hadden and Nester, 1968; Cahn and Fox, 1968). However, recently, the observation that only part of an isogenic culture develops competence has been revisited with bistability in mind.

In *B. subtilis*, the development of natural competence is governed by the competence transcription factor ComK (van Sinderen *et al*., 1995). This tetrameric protein activates more than 100 genes, including those essential for DNA binding and uptake (Hamoen *et al*., 2002; Ogura *et al*., 2002; Berka *et al*., 2002). Regulation of ComK activity is controlled through proteolytic degradation, quorum sensing and transcriptional control (Dubnau, 1991; Dubnau and Lovett JR, 2002) (Fig. 6A). ComK binds to its own promoter and is required for its own expression (van Sinderen and Venema, 1994; Hamoen *et al*., 1998). The competence regulatory network therefore

contains a positive feedback loop. Two groups independently reached the conclusion that this autoregulation is required for competence bistability (Smits *et al*., 2005b; Maamar and Dubnau, 2005; Fig. 6).

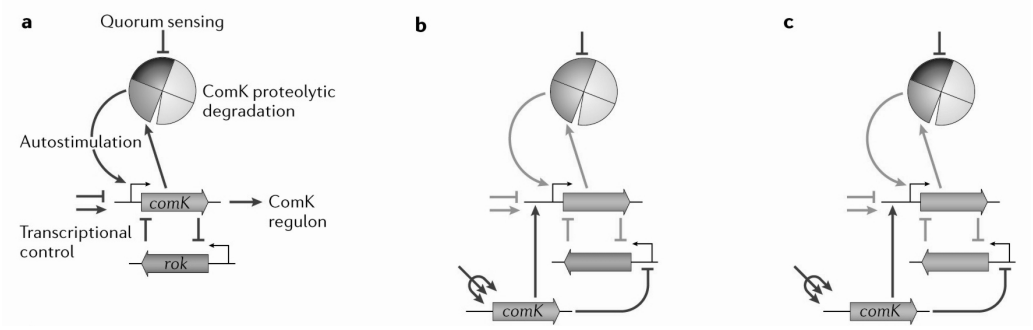

Figure 6. Bistability in competence development. (**a**) Simplified schema depicting the development of competence. At a specific quorum of competence pheromones, the proteolytic degradation of the key regulator ComK (red) is inhibited. The subsequent release of ComK enables *comK* auto-activation, and 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. 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*. Taken from (Smits *et al*., 2006b) with permission from the publisher.

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* (Steinmoen *et al*., 2002; Steinmoen *et al*., 2003). Recently, it was reported that competent *Streptococcus mutans* cells show concomitant development of competence and production of a bacteriocin in a competence-dependent manner (Kreth *et al*., 2005). This mechanism is similar to the cannibalism described for sporulating *Bacillus* cells (Gonzalez-Pastor *et al*., 2003), discussed earlier. There are also some interesting parallels with allolysis, the release of cytotoxic factors by non-competent *Streptococcus* cells, triggered by the competent cell fraction (Guiral *et al*., 2005). To our knowledge, no single-cell analysis using competence reporters has been carried out in *Streptococcus* species, and therefore the system might reflect both interspecies and intraspecies competition.

Other bistable systems in bacteria. Based on fluorescent reporters, several other bacterial systems were found to have an expressing and a non-expressing population of certain genes. For instance, in *Myxococcus xanthus* 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 (Russo-Marie *et al*., 1993; Thony-Meyer and Kaiser, 1993). It was postulated that negative autoregulation of the *devRS* locus is not responsible for this phenomenon, because a transposon mutant of this locus still showed bistability. This is in line with the model that predicts that a single-negative feedback cannot cause multistationarity (Thomas, 1981). 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 (Burchard *et al*., 1977), 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 is only expressed in 3% of the population (as judged by fluorescence microscopy) (Mulec *et al*., 2003). 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 (Kuhar *et al*., 2001). However, the ratio of *cka*-expressing and non expressing cells did not change significantly in a strain that does not produce ppGpp (Mulec *et al*., 2003). More experiments are required to elucidate the mechanism of bistable *cka* expression, but it is most likely that it is caused by some sort of negative feedback process acting on LexA, or on a positive regulator that activates *cka* expression. In the same study, the authors showed that the immunity gene *cki* was expressed in all cells, protecting the whole colicinogenic population from the bacteriocin. This observation shows that related functions need not be similarly bistable.

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 (Hautefort *et al*., 2003). 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 that might harbour the feedback loops necessary for bistable switching, 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 (Sentchilo *et al*., 2003b; Sentchilo *et al*., 2003a). 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 (Sentchilo *et al*., 2003b), but possible bistable characteristics of either of these regulatory mechanisms have not been documented. 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 harbors a small subpopulation of surviving cells (around 5%) when shifted to high osmolarity (Booth, 2002). Re-culturing of these survivors again yielded two subpopulations, indicating that the modification is non-genetic in nature (Booth, 2002). 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 (Balaban *et al*., 2004; Kussell *et al*., 2005). This phenomenon also occurs in pathogens including *Staphylococcus aureus*, *Streptococcus pyogenes, P. aeruginosa* and *Mycobacterium tuberculosis* (Levin, 2004). The origin of the phenotypic switch is unknown (Balaban *et al*., 2004), but it is tempting to speculate that a feedback-based mechanism could contribute to the persistence phenotype.

Furthermore, subpopulations showing differential gene-expression patterns or physiological properties were identified using density centrifugation in *E. coli* (Makinoshima *et al*., 2002), *Vibrio haemolyticus* (Nishino *et al*., 2003) and *P. aeruginosa* (Vidal-Mas *et al*., 2001). But this variability could be attributed to cells in different stages of the cell cycle. Last, bacteria growing in biofilms show a high degree of spatial and temporal heterogeneity (Branda *et al*., 2005, **Chapter 7**). 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 gene expression 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 (Smits *et al*., 2005b; Veening *et al*., 2005; Muller-Hill, 1996; Ptashne, 2005; Maamar and Dubnau, 2005).

1.8 Translating the Noise

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 (Rao *et al*., 2002; Paulsson, 2004; Kærn *et al*., 2005). This phenomenon is believed to be an important factor in bistability.

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 (Elowitz *et al*., 2002; Ozbudak *et al*., 2002; Swain *et al*., 2002). 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 (Elowitz *et al*., 2002; **Chapter 3**). It was found that both intrinsic and extrinsic noise contribute to phenotypic variability using the model organisms *E. coli* (Elowitz *et al*., 2002; Swain *et al*., 2002) and *S. cerevisiae* (Raser and O'Shea, 2004). 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 (Rosenfeld *et al*., 2005). 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 Kærn *et al*., 2005). Based on mathematical modeling, it was predicted that noise is most dependent on the translation rate, but is independent of the transcription rate (Thattai and van Oudenaarden, 2001). Ozbudak and colleagues substantiated these findings experimentally through the use of GFP as a reporter for protein production in *B. subtilis* (Ozbudak *et al*., 2002). 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. In contrast, for the eukaryotic organism *S. cerevisiae*, it was reported that transcriptional efficiency does have a role in noise generation (Blake *et al*., 2003). The authors suggest that this difference might be due to 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 (Rao *et al*., 2002). One of the most ubiquitous noise-attenuating mechanisms is negative feedback (reviewed in Rao *et al*., 2002; Smolen *et al*., 2000; Heinrich and Schuster, 1998). 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, thus reducing noise. Indeed, it was reported that negative autoregulation is predominant for housekeeping functions of *E. coli* (Thieffry *et al*., 1998).

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 (Ninfa and Mayo, 2004). A neat analogy that describes memory by hysteresis was sketched in a review by Casadesus and D'Ari (Casadesus and D'Ari, 2002). Microbiological agar is a polymer solution that at 60 ºC can be either liquid or solid. If the agar is melted by heating it to 100 ºC and then cooled down to 60 ºC, it will remain liquid, whereas if solid agar at room temperature is warmed up to 60 ºC, it will remain solid. Thus the state of agar at 60 ºC is a memory of its past history.

The origin of hysteresis 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 of *E. coli* (Ozbudak *et al*., 2004). 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 (Bren and Eisenbach, 2001).

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 (Isaacs *et al*., 2003; Pedraza and van Oudenaarden, 2005; Hooshangi *et al*., 2005). 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 (Hooshangi *et al*., 2005).

1.9 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 demonstrating a (multistable) phenotype depends on conditions that are set (such as the threshold level of a regulator required for auto-activation).

For instance, in case of the *E. coli lac* operon, the output of the bistable response is modulated by the activity of the activator CRP (Ozbudak *et al*., 2004). In *B. subtilis* 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 (Smits *et al*., 2005b). 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 (Veening *et al*., 2005; Chung *et al*., 1994; **Chapter 3**). 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 (Schaeffer *et al*., 1965).

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 (Hasty *et al*., 2002). However, *in vivo*, some FBM systems act as unidirectional switches, such as oocyte differentiation in Xenopus (Ferrell, Jr., 2002; Ferrell, Jr. and Machleder, 1998) and spore formation in *B. subtilis* (Veening *et al*., 2005; Piggot and Losick, 2002). This suggests that the escape time of such a system is effectively infinite: the chance that switching occurs during a cell's lifetime is negligible (Hasty *et al*., 2002). The locking of a bistable switch might originate from the architecture of the gene network, but can also be mediated by environmental signaling. Sporulation in *B. subtilis*, for instance, relies on a cascade of alternative σ factors which provide directionality to the developmental programme (Eichenberger *et al*., 2004), 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.

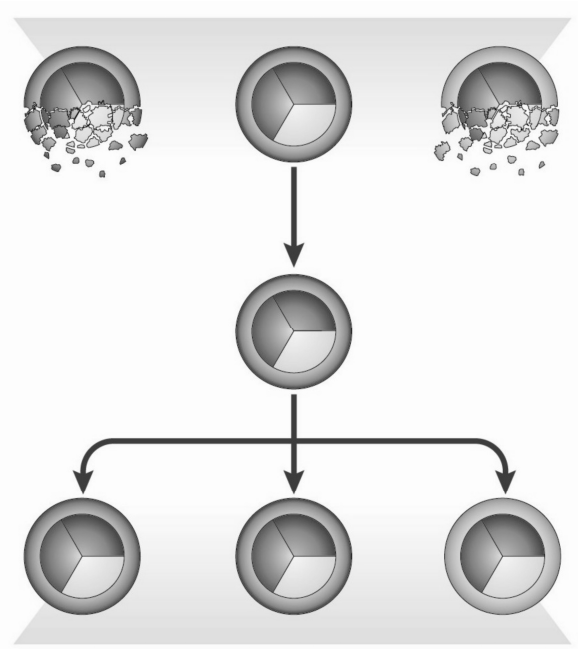

Figure 7. Survival of variable phenotypes under selective pressure. Three genetically identical cells are depicted in the upper part of the figure. The triangular parts depicted in red, yellow and blue represent the genetic content. The outer ring depicts the (variable) phenotype that each cell displays. 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. Taken from (Smits *et al*., 2006b) with permission from the publisher. Available in color at http://molgen.biol.rug.nl/publication/epi_data/).

1.10 Evolutionary benefits of FBM

Based on modelling and mutant analysis, it has been suggested that noise is an evolvable trait (Fraser *et al*., 2004). 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 (Rao *et al*., 2002; Paulsson, 2004; Vilar *et al*., 2002; Barkai and Leibler, 2000). 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 (Fraser *et al*., 2004). 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 positive feedback, potentially leading to FBM (Ferrell, Jr., 2002; Hasty *et al*., 2000). 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 (Thattai and van Oudenaarden, 2004; Hallet, 2001; Rao *et al*., 2002; Arkin *et al*., 1998; Korobkova *et al*., 2004). For instance, the switching of phage populations from a lytic to lysogenic pathway is thought to have evolved as an adaptation to changes in their environment (the host cell) (Mittler, 1996; Stumpf and Pybus, 2002; Wolf *et al*., 2005). 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 generating genetic diversity and DNA repair, and the drawbacks such as growth arrest, possible illegitimate recombinations, 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 (Thattai and van Oudenaarden, 2004; Wolf *et al*., 2005). 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 (Figure 7).

For adaptive phenotypes, it is important that the response to stress is quick and efficient. As discussed above, FBM can quickly generate phenotypic variability 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* (Sung and Yasbin, 2002).

1.11 Scope of this thesis

Phenotypic variation displayed within isogenic (clonal) populations is a fascinating phenomenon and potentially very important for the fitness of the lineage (Fig. 7). In this thesis work, we have used sporulation of *B. subtilis* as a model to study bistable differentiation. Some cells within a clonal population form spores, while others do not and the mechanisms that underlie this phenotypic variation were unknown.

To start studying heterogeneity at the single cell level, we needed to generate the proper tool set for *B. subtilis*. For this, the ability to examine and be able to visualize

multiple gene activities within single cells is of great value. For *E. coli*, the successful use of the distinguishable cyan and yellow fluorescent proteins has been described (Elowitz *et al*., 2002). However, these variants of GFP were shown to be of poor use in *B. subtilis* due to the eukaryotic codon bias present in these genes. **Chapter 2** describes the generation of a new set of vectors carrying improved variants of *cfp* and *yfp* which can be successfully used in *B. subtilis*. These new variants were shown to demonstrate a significant increase in translation efficiency (Veening *et al*., 2004).

Inspired by work done in eukaryotic model systems and synthetic gene-networks, we designed and utilized an artificial system that demonstrated bistable switching characteristics for the sporulation pathway (**Chapter 3**). Furthermore, we showed that the bistable situation, in which some cells sporulate and others do not, is actively maintained by phosphorelay phosphatases.

Since sporulation and competence development seemed to comply with the general rules found to dictate bistable networks, which are often driven by noise, we examined whether sporulation and competence demonstrate stochastic switching behavior. As outlined in **Chapter 4**, both competence and sporulation are initiated in some cells under atypical growth conditions, providing indirect evidence that the initiations of these processes are indeed noise-based.

The results so far gave a good idea of how sporulation bistability is generated, but the question still remained why certain cells decide to sporulate while others do not and whether this decision can be traced back to any physiological parameter or is purely stochastic. To gain some insight on this, we performed time-lapse microscopy of single *B. subtilis* cells growing into microcolonies containing up to 600 cells (**Chapter 5**). Lineage analyses demonstrated that the decision to sporulate is not dependent on cell pole age, its previous growth rate, cell length, or birth point in time. Again, this indicated that the initial decision to sporulate is a stochastic one. Cellular aging has been described for *E. coli* and *Caulobacter crescentus*, both proteobacteria (Stewart *et al*., 2005; Ackermann *et al*., 2003). *B. subtilis* cells inheriting old-poles, more often demonstrated a significant aging effect, and displayed lower growth rates. This demonstrates the presence of aging for the first time in bacteria outside the proteobacteria. While this observation extends the apparent universality of aging, it does not appear to be important in cell fate decisions. However, our main finding is that cell fates, such as spore formation or cell lysis, are epigenetically inherited. The last common ancestor where the original bias in cell fate appeared to have been established occurred as much as six generations before the phenotypic appearance of the eventual cell fate. This discovery, in relation to bistable differentiation, provides considerable new insight into the development of multicellular structures such as biofilms. The importance of epigenetic inheritance of cell fates may be based on the effect it has on neighbouring cells. In bacterial colonies, sessile communities of cells, epigenetic inheritance affects those cells that are geographically grouped, in contrast to cells within planktonic cultures. The formation of biofilms requires systematic cell differentiation, including cell lysis (Webb *et al*., 2003). In fact, in *B. subtilis* multi-cellular

structure formation and sporulation are coordinated and intertwined by the action of Spo0A (Branda *et al*., 2001; Veening *et al*., 2006b; Veening *et al*., 2006a, **Chapter 7**). Thus, the more flexible, yet still orderly action of non-genetic inheritance might be crucial in the formation of socially organized structures such as biofilms and fruiting bodies.

In addition, we discovered that during microcolony development, a subpopulation of cells used alternative metabolites to continue growth, instead of following the expected cellular differentiation into sporulation. The consequences of this, in terms of reproductive advantage, and the related bet-hedging strategy that the bacterium appears to follow, provided important evolutionary insights.

In **Chapter 6** the genome-wide expression profiles of the non-sporulating subpopulation are examined. This study revealed additional levels of stationary phase heterogeneity in *B. subtilis*. It was found that two members of the DegU regulon (*bpr* and *aprE*), which encode extracellular proteases, are expressed during the stationary phase only in a subpopulation of non-sporulating cells. Importantly, we show that this bistable activation, can be explained by a logic 'AND gate' circuitry involving both the DegU phosphorylation pathway and the Spo0A-phosphorelay. Cells will only express *aprE* when both a certain level of DegU~P and Spo0A~P are reached. AprE and Bpr are scavenging proteins that are secreted into the growth medium and degrade (large) proteins into smaller peptides which can be taken up and used as an alternative nutrient source (Msadek, 1999). As we report, only part of the population expresses *aprE* and *bpr*. Thus, cells that produce and secrete AprE and Bpr do not only help themselves, but all the cells within the liquid growth medium. This could reflect a simple form of altruism; a behaviour that decreases the fitness of the altruistic individual while benefiting others (West *et al*., 2006).

Finally, in **Chapter 7** the role of the sporulation phosphorelay in multicellular structure formation is determined. It is becoming more and more obvious that in natural habitats, bacteria are predominantly present in dense biofilms. The ability to form complex multicellular structures, however, is often lost in laboratory strains (Branda *et al*., 2001). The genetic program that regulates biofilm formation has been elucidated in some detail in *Bacillus subtilis* (Branda *et al*., 2001; Hamon and Lazazzera, 2001; Stanley *et al*., 2003; Hamon *et al*., 2004; Branda *et al*., 2004; Kearns *et al*., 2005). It was shown that there is a shared use of regulators, governing biofilm- and spore formation. In these reports, *B. subtilis* strains were chosen that are genetically accessible and show complex colony morphology. In **Chapter 7** it is shown that the sequenced and commonly used laboratory strain, *B. subtilis* 168 1A700 (Kunst *et al*., 1997), is able to form elevated structures (bundles) with complex architecture, but lacks the ability to swarm or form fruiting bodies. Time-lapse fluorescence microscopy studies on GFP-reporter strains show that bundles are preferential sites for spore formation, while flat structures surrounding the bundles contain vegetative cells. The elevated bundle structures are formed prior to sporulation, in agreement with a genetic developmental program in which these processes are sequentially activated.

Perturbations of the phosphorelay by disruption and overexpression of genes that lead to an increased tendency to sporulate, result in the segregation of sporulation mutations and decreased heat-resistance of spores in biofilms. These results stress the importance of a balanced control of the phosphorelay for biofilm and spore development.

In summary, this thesis work shows the versatility and importance of the *B. subtilis* sporulation phosphorelay in regulating many stationary phase processes including microcolony development (**Chapter 5**), biofilm formation (**Chapter 7**), competence development (**Chapter 4**), extracellular protease production (**Chapter 6**) and spore formation (**Chapter 3**). Moreover, it is demonstrated that the phosphorelay acts as a tuner for these processes and allows for a heterogeneous or bistable output which governs phenotypic variation in *B. subtilis*.