Chapter 8

Summary and Discussion

Bistable cellular differentiation is widespread in nature and occurs in micro-organisms as well as in higher organisms such as mammals. In this thesis, the bistable differentiation process of bacterial spore formation in clonal populations of the model organism *Bacillus subtilis* was studied. Upon starvation, *B. subtilis* has the capacity to enter into the irreversible process of sporulation. Only a subpopulation of cells actually forms spores. Therefore, this developmental process has been described as exhibiting bistability. How genetically identical cells in the same environment differentiate into radically different cell fates has been the subject of considerable investigation; however the molecular mechanisms underlying phenotypic variation are only recently becoming understood. Sporulation in *B. subtilis* is governed by the so-called phosphorelay, which integrates multiple environmental signals such as cell density and nutrient status. To initiate sporulation, Spo0A, the master sporulation regulator, needs to be phosphorylated by the phosphorelay. This signal transduction cascade appeared to be a source for heterogenic responses. Many other adaptive responses in *B. subtilis*, like competence development, secretion and biofilm formation, have also integrated the sporulation phosphorelay into their regulatory circuitry. Therefore, this thesis not only focuses on bistability in spore formation, but also deals with the more general (heterogeneous) output from the phosphorelay (‘phosphorelay heterogeneity’).

Initiation of sporulation is heterogeneous

It was known for a long time that within isogenic *B. subtilis* cultures not all cells demonstrate the same phenotype and some cells sporulate, while others do not (Schaeffer *et al.*, 1965; Dawes and Thornley, 1970). In 1994, the first single cell study on sporulation heterogeneity was published (Chung *et al.*, 1994). This research was not seriously followed up, partially because it was argued that sporulation bistability was an artifact of the laboratory strain used and therefore not interesting. The laboratory strain *B. subtilis* 168 was initially selected for increased competence development (Burkholder and Giles, 1947; Spizizen, 1958) and this would suppress efficient sporulation. However, over the years many more examples of bistable expression patterns in bacteria (including *B. subtilis*) were reported, making the study of the molecular mechanisms underlying bistability certainly not trivial (Smits *et al.*, 2006b; Dubnau and Losick, 2006).

Another reason why phenotypic variability in *B. subtilis* was not intensively studied was the lack of proper molecular biological tools to study this phenomenon. The
discovery of the Green Fluorescent Protein (GFP) and its spectral derivatives (Cyan and Yellow Fluorescent Protein) changed this. The use of GFP, CFP and YFP allows single cell analysis. Unfortunately, CFP and YFP could not be used with high success in B. subtilis, due to a lack of sensitivity. Chapter 2 describes the construction of new variants of the genes encoding CFP and YFP which show an increased efficiency of translation in B. subtilis. A leader sequence of a native B. subtilis gene (comGA) was fused to the original cfp and yfp genes, which contain a strong bias for eukaryotic codons. This leader was shown to efficiently enhance translation (20- to 70-fold), and thereby overcome poor translation due to the eukaryotic codon bias. Fusions of these enhanced variants with the promoters of abrB (encoding a transition state regulator and indicator for cells in the exponential growth phase) and spoIIA (an early target of Spo0A~P and reporter for sporulation initiation) showed that cells initiating sporulation do not express abrB and vice versa (Veening et al., 2004). This technological advance made it possible to study and quantify heterogeneous differentiation in B. subtilis in detail.

Initiation of sporulation shows the hallmarks of a bistable switch

Theoretical modeling and experiments in both prokaryotic and eukaryotic model systems have demonstrated that positive feedback of a transcriptional regulator can lead to a bimodal probability distribution in expression (Hasty et al., 2000; Becskei et al., 2001; Isaacs et al., 2003). It is believed that stochastic fluctuations within the cell causes some cells to reach a certain threshold level to activate the feedforward loop, and these cells will end up in the ‘high expressing’ population. Other cells do not reach this threshold and remain in the ‘low expressing’ state (Ferrell, Jr., 2002; Rao et al., 2002). Previous studies have established that spo0A is autostimulated by Spo0A~P in a rather complex manner (see Chapter 3, Fig 1). Since autostimulation can be the basis of a bistable phenotype we wondered whether Spo0A-autoactivation is important for sporulation heterogeneity. To test this, we bypassed the phosphorelay using a constitutively active form of Spo0A (Spo0A*). Using this artificial system, it was shown that a threshold level of Spo0A* is required to activate cells in the high expressing state, thus initiating sporulation. At intermediate concentrations of Spo0A* a bistable population was generated, while at high levels most cells were in the high expressing state. Removing the autostimulation resulted in a graded response in which the expression of the reporter fusion increased linearly with increasing amounts of Spo0A*. Taken together, these experiments showed that the sporulation cascade has all the requirements to act as a bistable switch that results in a variable phenotype (Chapter 3).

Importantly, we also showed that sporulation bistability is maintained by phosphatases that act on the phosphorelay. RapA is a phosphatase of Spo0F~P, a phosphorelay intermediate. Initially, all cells express rapA. Some cells initiate sporulation, but cells that do not initiate sporulation, continue to accumulate RapA.
More RapA means less Spo0A~P. Thus, cells that do not initiate sporulation are actively delayed by the accumulation of RapA. The activity of RapA is antagonized by PhrA, a small peptide, the gene of which lies in the same operon as rapA. PhrA needs to be secreted, processed, and taken up again before it can act on RapA. Therefore, the RapA-PhrA system acts as a sporulation timing device. Deletion of rapA increases the pressure to sporulate, and in liquid cultures most cells will initiate sporulation (Chapter 3).

Deletion of spo0E, a phosphatase that acts directly on Spo0A~P, also resulted in abolishment of the bistable expression pattern. Artificial induction of a heterologous Rap phosphatase restored heterogeneity in a rapA or spo0E mutant. These results demonstrated that with phosphatases, B. subtilis can use the phosphorelay as a tuner to modulate the bistable outcome of a sporulating culture.

A noisy development

Based on mathematical modeling and synthetic gene-regulatory networks, it has been found that stochasticity in gene expression (referred to as noise), when amplified by positive feedback, can be the generator of a bistable response (Kærn et al., 2005). Since all the evidence so far showed that both competence development and sporulation are subject to bistable switching (Smits et al., 2005b; Maamar and Dubnau, 2005; Fujita et al., 2006; Veening et al., 2005), one can ask the question whether both processes also suffer from a noisy output. 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). However, noise can be a useful phenomenon when present in adaptive pathways such as competence development or sporulation. The resulting non-genetic variability 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).

In Chapter 4 we examined the initiation of competence development and sporulation under a-typical, non-stimulatory conditions. The rationale of this study was that if competence and sporulation are subjected to accidental switching (noise), it would mean that some cells would enter one of these developmental routes regardless of the growth condition. We analyzed the noise, for both competence and sporulation, in liquid cultures and biofilms using flow cytometry, and were able to show that both competence development and initiation of sporulation show significant noise. This means that a small part of the population does not confine to the general rules of gene regulation and becomes competent or forms spores under conditions that normally should not trigger the initiation of these processes. This has major implications for the control of unwanted differentiation processes in for example pathogenic and food-
spoilage micro-organisms. Some pathogens are also able to develop natural competence, which is a serious medical problem with the increasing multi-drug resistance of these organisms, and spores of a variety of species are of major concern for the food industry. From our study, it becomes apparent that, although the number of expressing cells can significantly be reduced using specially developed media, there are always certain cells that behave atypically, due to the noisy character of the developmental routes. Therefore, future research must be aimed at localizing the noisiest regulatory components in developmental pathways and neutralizing these targets.

Aging and epigenetic inheritance in sporulation

Besides noise, intrinsic physiological parameters, such as the cell cycle and cell age, have been shown to contribute to phenotypic variability as well (Avery, 2006). Whether the outcome of a bistable cellular differentiation process is influenced by such physiological parameters, or whether it is purely a stochastic phenomenon is unknown. It is also unclear how far in advance of the appearance of the phenotypic change such decisions are made. In Chapter 5, we used time-lapse microscopy to follow the growth, division, expression and differentiation of individual B. subtilis cells in order to identify elements of cell history and ancestry that affect cell fate such as spore formation. This study revealed that B. subtilis undergoes aging, and experiences a reduced growth rate over subsequent divisions with increasing aging of the poles. It is interesting to note that the magnitude of this aging effect is nearly identical to that seen in E. coli, and similar to that of Caulobacter crescentus (Stewart et al., 2005; Ackermann et al., 2003). This observation extends the occurrence of aging beyond the proteobacteria, to which both E. coli and C. crescentus belong, to Gram positives, emphasizing the prevalence of aging throughout the tree of life. However, aging does not appear to determine the outcome of the bistable development that occurs in a sporulating B. subtilis culture. In fact, none of the physiological parameters (cell width, length, growth rate, birth time) that could be measured in our experimental set-up could be linked to a predisposed fate. Our experiments suggest that the original decision of this bistable regulation process has a stochastic origin, in line with the results of Chapter 4. However, we noticed that the ancestry of the cell is of considerable importance, and that epigenetic inheritance appears to influence cell fates over as many as six generations. This suggests that the trigger to sporulate is already present during the exponential growth phase, well before the nutrients become limiting. As dilution of a static signal can be up to 64-fold in such a case, it seems likely that an active system retains a ‘memory’ of the signal through cell growth and division. The presence of memory or hysteresis in the sporulation cascade is also exemplified by the fact that the bistable outcome is greatly determined by the history of the culture (i.e. the medium of the overnight culture). If for instance cells are grown overnight in a medium
designed for competence development and diluted in a medium used to induce sporulation, less cells will sporulate compared to a culture grown overnight in sporulation medium (Veening, unpublished results). Using 2D-proteomics, clear differences in proteome profiles were observed between exponentially growing cultures that were inoculated with stationary phase cells grown in competence- or sporulation medium (Veening, Hesseling, unpublished results). Future experiments should be aimed at identifying self-sustaining proteins that may be responsible for this memory or ‘hysteretic’ effect.

The nature of the sporulation signal transduction system provides an attractive model of how this epigenetic inheritance may occur. It has been proposed that autophosphorylating kinases have the potential to store memory. In this scenario, a specific stimulus activates the kinase and due to its autocatalytic properties the kinase stays in its active state, regardless of the presence or absence of the stimulus (Lisman, 1985). As a result, the progeny of cells in the ‘ON-state’ will also be in the ‘ON-state’ because the activated kinase is passed on to the offspring. Maturation of Xenopus oocytes, for example, was shown to be governed by such a bistable positive-feedback memory module (Xiong and Ferrell, Jr., 2003). In fact, using artificial bistable gene regulatory circuits in both *E. coli* and *Saccharomyces cerevisiae*, it was shown that autostimulatory regulation systems can function as memory devices in microorganisms as well (Gardner et al., 2000; Becskei et al., 2001). Thus, the signal to sporulate in *B. subtilis* could be ‘memorized’ by the positive feedback architecture of the Spo0A activation circuitry, which also includes autophosphorylating kinases (e.g. KinA and KinB). The mere presence of positive feedback regulation alone does not automatically result in a memory response. For instance, for the development of genetic competence, another bistable differentiation process in *B. subtilis* that depends on positive feedback, it was found that cells were not significantly more likely to become competent if their sibling became competent (Suel et al., 2006). In our lab however, we have observed subfamilies of competent cells in a *mecA* mutant, indicating that epigenetic inheritance may play a role in competence development as well (Smits, Hamoen, unpublished results).

The benefits of epigenetic inheritance within a sporulating culture are puzzling. For cold shock adaptation in bacteria, it was shown that cells that are pre-treated by a mild cold shock ‘memorize’ this stress and are better prepared for a harsher cold shock, which would otherwise be lethal (Goldstein et al., 1990). In analogy with this, it can be envisaged that propagation of the sporulation signal from the mother cell to its descendants helps the progeny to be prepared for potential nutritional limitations in the future in such a way that they can rapidly respond and commit to spore formation when required. However, there might be another function for epigenetic inheritance of *B. subtilis* cell fates. In our study we looked at microcolonies; a sessile community of bacterial cells. In such social macrostructures, epigenetic inheritance affects those cells that are geographically grouped, in contrast to cells within planktonic cultures. It has been shown that sporulation primarily takes place at elevated structures (fruiting bodies).
bodies and bundles) within biofilms (Branda et al., 2001; Veening et al., 2006a, Chapter 7). The formation of biofilms requires systematic cell differentiation, generally including cell lysis (Webb et al., 2003). In fact in B. subtilis, multicellular 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, Chapters 3 and 7). Therefore we postulate that, in contrast to genetic inheritance, the more flexible, yet still orderly action of non-genetic inheritance might be crucial for the formation of socially organized structures such as biofilms and fruiting bodies. Future research should examine whether epigenetic inheritance is important for B. subtilis multicellular structure formation.

Sporulation bistability as a bet-hedging strategy

Bet-hedging is a strategy to diversify phenotypes with the aim to increase fitness in temporally variable conditions (Bradshaw, 1965; Stearns, 1976). Under challenging environments, an organism may produce offspring with variable phenotypes to ensure that at least one will be appropriate (fit) under a given situation (Cohen, 1966). This is a risk spreading or bet-hedging strategy, because not every offspring will be optimally suited for the future environment. However, the overall fitness of the genotype will increase since some offspring will have the proper adaptation. The classic bet-hedging example is seed dormancy in annual plants where the germination of seeds is spread out over a number of years, because not every year sustains successful growth and reproduction (Cohen, 1966).

An important general conclusion presented in Chapter 5 is that during microcolony development, B. subtilis uses a bet-hedging strategy, whereby some cells sporulate whereas others utilize alternative metabolites to continue growth (and can pursue other survival tactics). Cells that chose to follow the pathway of diauxic growth contribute the most in terms of total offspring (Chapter 5, Table 1). In fact, the B. subtilis laboratory strain used in this study is a poor sporulator when compared to some natural isolates (Maughan and Nicholson, 2004). Since this strain has been propagated in the laboratory for many decades it appears that it is evolutionary optimized to rapidly grow and colonize from vegetative cells. While this condition is potentially a response to human-imposed conditions, natural environments with fluctuating nutrient levels may result in the evolution of similar distributions of the bistable states.

Another benefit of the simultaneous presence of both pathways is the optimal use of resources. Even in a population with complete sporulation efficiency, mother cells will lyse to release the endospores. Consequently, cellular components that could be used as a nutrient source are released as well. Thus, heterogeneity in the timing of spore formation allows utilization of these resources that would otherwise be lost.
Altruism in stationary phase *B. subtilis* cultures?

Since most gene expression experiments in the stationary growth phase are based on cell cultures and not on individual cells, little is known about gene expression in the non-sporulating subpopulation. In Chapter 6 the transcriptome profiles of both spore formers and non-spore formers within a *B. subtilis* culture were determined. This analysis revealed additional levels of heterogeneity, which was confirmed using different GFP-reporter fusions. Part of this heterogeneity can be explained by the fact that in sporulating cells, $\sigma^A$, the major housekeeping RNA polymerase sigma-factor, loses its activity (Linn et al., 1973). Since only part of the population sporulates, this fraction will demonstrate reduced gene expression from $\sigma^A$ dependent promoters and in this way generate heterogeneity. Interestingly, reporter strains of two members of the DegU regulon, *bpr* and *aprE*, were only expressed in part of the non-sporulating sub-population. Bpr (bacillopeptidase) and AprE (subtilisin) 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). Single cell analyses showed that after overnight growth in liquid medium, three distinct cell types could be identified: highly expressing *aprE* or *bpr* cells, cells that do not express *aprE* or *bpr*, and sporulating cells (that do not, or not anymore, express *aprE* or *bpr*). Further genetic dissection showed that *aprE* expression depends on the sporulation phosphorelay, since it requires de-repression of a number of transcription factors under the control of Spo0A~P. Because only part of the population reaches the Spo0A~P levels that are required to relieve the *aprE* promoter from its negative regulators, only part of the population is primed to activate *aprE* gene expression. A prerequisite for *aprE* expression is the presence of the phosphorylated form of the response regulator DegU. We show that activation and phosphorylation of DegU is limited to a sub-population of cells (Chapter 6). Thus, only cells that have both high levels of Spo0A~P AND DegU~P will express *aprE*. A mathematical model that integrates the conditions required to activate *aprE* was indeed successful in generating a bistable output (data not shown). This model predicts that the observed *aprE* heterogeneity could be the result of a relative simple logic AND-gate system. From an evolutionary perspective, it makes sense to interlink two-regulatory pathways (of which at least one displays bistable behavior) to generate multistability, because it does not involve a complex switch with more than two steady states. Again, this study demonstrates the versatility of the sporulation phosphorelay to integrate signals and generate a heterogeneous output.

Only part of the population expresses *aprE* and *bpr*. Thus, cells that produce and secrete these proteases do not only help themselves, but all the cells within the local (liquid) growth medium. This could reflect a simple form of altruism; a behavior that decreases the fitness of the altruistic individual while benefiting others (West et al., 2006). Altruism can only be explained when the cooperation is directed towards individuals who are genetically similar (Kin selection) (Hamilton, 1964; Maynard Smith,
1964). By this definition, it is easy to understand the social behavior of *B. subtilis* observed in the current study. Heterogeneity in gene expression ensures that not all cells commence into the costly production of Bpr and AprE, but all cells within the clonal population benefit from the activity of these extracellular proteases. Future research should be aimed at examining whether this is true altruism, where the actors suffer from their behavior and the whole population benefits, or whether it is cooperative and that both the actor as well as the recipient benefits (mutual benefit). Alternatively, it can be envisaged that these proteases attack cell-wall attached proteins on living cells (instead of chewing up dead cells and cell debris or degrading complex media components). Future research is required to figure out which scenario is happening to determine whether cells that express extracellular proteases are either altruists or cannibals.

**The phosphorelay and biofilm formation**

In nature, bacteria predominantly occur in the form of multicellular communities known as biofilms (Davey and O’toole, 2000). In Chapter 7, we define *B. subtilis* biofilms as communities of cells embedded in a polymeric matrix, which can either be pellicles at an air-liquid interface or colonies with vein-like structures (bundles) grown on semi-solid agar surfaces. In line with the current model for biofilm formation, we demonstrate that overproduction of the phosphorelay components KinA and Spo0A stimulates bundle formation, while overproduction of the transition state regulators AbrB and SinR leads to repression of formation of elevated bundles. Time-lapse fluorescence microscopy studies with *B. subtilis* GFP-reporter strains show that bundles are the preferential sites for spore formation, while flat structures surrounding the bundles contain primarily 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. Specific perturbations of the phosphorelay 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. Again, these data indicate a versatile role for the sporulation-phosphorelay directing multiple stationary-phase phenomena including biofilm formation and sporulation. Furthermore, this work shows that, initially, the levels of Spo0A–P need to accumulate gradually to direct ordered biofilm formation prior to sporulation. In line with this, it was recently shown that the gradual increase in Spo0A protein and activity plays a critical role in triggering sporulation (Fujita and Losick, 2005).

In light of bistability, these results may indicate that autostimulation of Spo0A is only initiated after an initial gradual increase of Spo0A–P (Fujita *et al.*, 2005). Controversially, it could be that in natural situations, Spo0A autostimulation is not the mechanism for sporulation bistability, but that cells rather demonstrate a long-tailed
Gaussian distribution of Spo0A~P levels. Besides noise in transcription of any of the phosphorelay components (including spo0A), the differences in Spo0A~P-binding affinities of the regulated genes of the Spo0A-regulon could generate this tailed distribution. Once a cell has reached a certain threshold level of Spo0A~P and activates the Spo0A-high regulon, the unidirectional switch is set by activation of the alternative sigma factors leading to asymmetric septum formation. This hypothesis, however, does not explain the presence of memory and epigenetic inheritance within the sporulation pathway.

The most likely mode of action is that Spo0A autoactivation is already triggered at an early stage, generating a subpopulation of cells in the Spo0A-ON state and a subpopulation in the Spo0A-OFF state. Spo0A~P levels in the Spo0A-ON cells however, do no increase exponentially but rather in a gradual manner. This gradual increase would be controlled by the negative control elements present in the sporulation phosphorelay cascade (e.g. the phosphorelay phosphatases Spo0E and RapA, see Chapter 3).

Conclusions and future research

The occurrence of highly resistant bacterial spores at different stages in the production of food products causes serious problems with regard to food preservation, conservation, safety and medical hygiene. Bacillus spores are common contaminants on vegetables and fruits and they may cause food spoilage (B. subtilis, B. coagulans, B. licheniformis) or act as food pathogens (B. cereus). A notorious food-spoilage organism is Bacillus cereus, a close relative of Bacillus anthracis, the causative agent of anthrax. During its vegetative life style, B. cereus is able to produce an array of toxins. Importantly, spores of some B. cereus strains have the ability to germinate under psychrotropic conditions, which makes this species a notorious food spoiler with regard to the shelf-life of products that need to be refrigerated (e.g. ‘fresh foods’) (Abee et al., 2004). Food preservation is often confronted with the fact that heat inactivation of spore containing products or processes is complicated by the presence of both heat sensitive and heat resistant spores within the same isogenic population. The reason for this heterogeneity in spore resistance is poorly understood. The research described in this thesis revealed that different growth conditions during vegetative growth result in spore crops with different resistance properties (Veening et al., 2006a). Spores isolated from biofilms for instance, were shown to be significantly harder and more heat resistant than spores isolated from liquid cultures (Chapter 7). Furthermore, in this thesis I show that already during the earliest stages of sporulation in B. subtilis, a great degree of heterogeneity exists, and that part of this variability can be accounted to epigenetic mechanisms (Chapters 2-6). These findings give valuable clues to the mechanisms behind the origin of variability in stress resistance of bacterial spores, and provide useful leads for future research. An assessment of the relation between...
heterogeneous development and actual variability in spore resistance should be made, as this could reveal molecular mechanisms driving variability in spore resistance. To identify gene products that are directly involved in variability in heat resistance of isogenic *Bacillus* spores, a genomic and proteomic approach is necessary (Brul *et al.*, 2006). The outcome of such a comprehensive project will generate a detailed understanding of the origin of variability in stress resistance of *Bacillus* spores. Importantly, it will allow the food industry to predict measures that will help to ensure microbial food stability and safety.