Chapter 6

Single cell analysis of gene expression patterns of competence development and initiation of sporulation in *Bacillus subtilis* grown on chemically defined media

This chapter has been adapted from:

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Some pathogens are able to develop natural competence, which is a serious medical problem with the increased acquired multi-drug resistance of these organisms. Another adaptive microbial response is spore formation. Because of their heat resistance and hydrophobicity, spores of a variety of species are of major concern for the food industry. Using the model organism *B. subtilis*, we show that competence development and sporulation in a chemically defined medium are initiated in a bistable and sequential manner. We furthermore provide experimental evidence that both competence and sporulation can develop under conditions that normally do not trigger these processes. This finding suggests that the developmental switch may be noise-based, which has major implications for the control of unwanted differentiation processes in pathogenic and food-spoilage micro-organisms.

Introduction

Among the Gram-positive bacteria, many pathogens and food-spoilage species have been identified (for reviews see Jones *et al.*, 1999; Ohlsen *et al.*, 2004; Jespersen and Jakobsen, 1996). Some pathogens are able to develop natural competence and, as a consequence of this adaptive strategy, show increased antibiotic resistance (Claverys *et al.*, 2000; Lorenz and Wackernagel, 1994). Resistance to a wide-range of antibiotics poses a serious epidemic threat for the community (Walsh and Amyes, 2004; McCormick, 1998; Dobay *et al.*, 2004). Moreover, the occurrence of highly resistant spores at different stages in the production of food products, causes serious problems with regard to food preservation and safety (Brown, 2000; Raso and Barbosa-Canovas, 2003) and compromises medical hygiene as well.

Instead of trying to develop novel antibiotics to kill pathogenic or food-spoilage bacterium, one could try to identify compounds that inhibit the development of the unwanted differentiation process (*anti-differentics*). In the case of sporulation, for instance, a compound that would inhibit this process would render the species susceptible to normal methods of sterilisation. In the case of competence, *anti-differentics* could reduce the spread of drug-resistance. For this reason, more information is desired about the molecular basis underlying these developmental processes. To study the origin of competence development and spore formation, we employ the Gram-positive model organism *Bacillus subtilis*. This rod-shaped bacterium is one of the best-studied microbial organism that exhibits cellular differentiation. Following exponential growth, *B. subtilis* is able
Noise in cellular differentiation to secrete several proteins (e.g. proteases, antimicrobials and pheromones) into its extracellular environment, develop competence for DNA uptake and form highly resistant endospores (for reviews see Tjalsma et al., 2004; Chen and Dubnau, 2004; Errington, 1993). Competence in *B. subtilis* develops under specific nutritional conditions during late exponential and early stationary growth phase (Dubnau, 1991a). To initiate competence, the key regulator ComK needs to be expressed. This protein in turn activates expression of more than 100 genes, among which those required for DNA-uptake and recombination (Hamen et al., 2002).

Spore formation in *B. subtilis* is a last resort adaptive response to starvation (Sonenshein, 2000; Piggot and Losick, 2002). It is controlled by a multi-component phosphorelay in which several stimuli can be integrated and cause phosphorylation (activation) of the key sporulation regulator, Spo0A (Burbulys et al., 1991). Activation of Spo0A affects the expression of more than 10% of all the genes in the genome of *B. subtilis* and can eventually lead to the formation of a highly heat resistant spore (Fawcett et al., 2000).

Competence and sporulation are subjected to multiple regulatory mechanisms and share many regulators in their gene-circuits (e.g. AbrB, SinR, DegU, CodY and Spo0A; reviewed in Hamoen et al., 2003b; Msadek, 1999). Certain conditions that stimulate competence development have a negative effect on sporulation and vice versa. However, other inputs stimulate or repress both gene-regulatory networks (Hamen et al., 2003b; Msadek, 1999). Fascinatingly, when proper conditions are applied to a *B. subtilis* culture, only part of the population becomes competent or forms spores (Cahn and Fox, 1968; Hadden and Nester, 1968; Chung et al., 1994). Since, in both cases, there are clearly two distinguishable cell types, the systems were described as exhibiting bistability (Fujita et al., 2005; Maamar and Dubnau, 2005; Smits et al., 2005b; Veening et al., 2005). We have previously shown that the observed bistability is the result of a non-linear auto-stimulation on *comK* and *spo0A*, respectively (Smits et al., 2005b; Veening et al., 2005). This was demonstrated using promoter-GFP fusions (Green Fluorescent Protein) combined with microscopic and flow cytometric analyses. However, single cell studies on competence development and initiation of sporulation have so far only been performed in complex, undefined media and never within the same (isogenic) strain. To determine whether competence and sporulation are processes that are mutually exclusive or simultaneously initiated, we make use of a chemically defined medium (CDM), in which both processes are initiated. This may resemble the conditions in industrially relevant processes, where chemically defined media are often used (e.g. Huang et al., 2004; Fang and Demain, 1989; Vierheller et al., 1995). Therefore, we set out to perform single cell analyses on the expression patterns of both competence development and initiation of sporulation in chemically defined media. To further extend this, we composed a chemically defined medium in which both processes are initiated.

While previous studies were performed in liquid media, bacterial contaminations observed in industry and hospitals are often derived from dense biofilms. Therefore, we set out to determine the single cell gene expression patterns of competence and sporulation in a variety of biofilms. The mechanism that decides, within an isogenic and homogeneous population, which cell actually reaches the threshold level necessary to activate auto-stimulation and become competent or to form a spore remains elusive. It was shown that stochastic fluctuations (or noise) of regulatory components within certain gene-regulatory pathways, can cause bistability (Hasty et al., 2000; Isaacs et al., 2003). In the establishment of this kind of bistability, noise in gene expression is supposed to play a key role. We quantitatively analyzed the noise, for both competence and sporulation, in both liquid cultures and biofilms. We 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.

Results

Competence and sporulation are sequential processes

Competence development is initiated when a threshold level of ComK is reached and ComK auto-activation is triggered (Smits et al., 2005b; Maamar and Dubnau, 2005). ComK in turn activates the expression of the comG operon, which encodes factors required for the uptake of exogenous DNA from the environment (Chung and Dubnau, 1998). Previously, it was shown that the promoter of the comG operon fused to the gfp gene is a good reporter to identify cells that have initiated competence development (Smits et al., 2005b). Since this reporter was so far only used in B. subtilis 8G5, a strain that poorly sporulates (Bron and Venema, 1972), we transformed this construct to the completely sequenced and sporulating B. subtilis strain 168 (Kunst et al., 1997), resulting in strain comG-gfp (P_{comG-gfp}). To study initiation of sporulation at the single cell level, we made use of the previously published IIA-gfp strain (B. subtilis 168 P_{spoIIA-gfp}) (Veening et al., 2005). The spoIIA operon comprises spoIIAA, spoIIAB and sigF, a forespore-specific sigma factor essential for sporulation. The operon is activated at a high threshold level of activated Spo0A and was shown to be a good reporter for cells that initiate sporulation (Chung et al., 1994; Fujita et al., 2005; Veening et al., 2005).

Previous single cell analysis using these two reporters were performed in complex undefined media that are optimized to either study competence or sporulation (Smits et al., 2005b; Veening et al., 2005). To examine whether one of these media or a combination of both is suited for the analysis of both competence and sporulation and to examine whether the processes are mutually exclusive or happen simultaneously, flow cytometric analyses (Figure 1) of strains comG-gfp and IIA-gfp grown in dedicated media were published before (Smits et al., 2005b; Veening et al., 2005). Furthermore, when the two media are mixed in a range of ratios, either competence or sporulation development is poor. Interestingly, there is a low level of expression of spoIIA-gfp when cells were grown in MM (Figure 1A, right panel). This is most likely caused by repression (or activation) of low-threshold Spo0A genes (see Discussion).

To study both processes simultaneously and get a better idea of the timing and possible co-occurrence of the developmental pathways, we examined the expression pattern of strains comG-gfp and IIA-gfp in chemically defined competence medium (CDCM; Smits et al., 2005b), chemically defined sporulation medium (CDSM; Hageman et al., 1984) and a mix of both media in a 1:1 ratio (50/50). As shown in Figure 1B, both competence (left panel) and sporulation (right panel) are initiated in a bistable manner in the aforementioned chemically defined media. With increasing concentrations of CDSM, more cells initiate competence development as well as sporulation, although the timing of sporulation differs slightly between the media (data not shown). For further analysis we chose to continue with the 50/50 medium, since under these conditions both proc-
Esses seem to be initiated discretely. To better visualize the timing of both processes, an overlay of the expression patterns of strains comG-gfp and IIA-gfp was made in which samples were taken of both strains at identical points in their growth phase (Figure 1C).

For this image, flow cytometric histograms were taken from cultures independent of the cultures used for Figure 1B. As shown in this figure, competence development and sporulation are sequentially initiated; first a subpopulation of cells becomes competent and later a subpopulation initiates sporulation. Interestingly, competence is already initiated during late-logarithmic growth, in contrast to growth in complex medium where expression of \textit{comG} is generally not observed until two hours after entry into stationary growth phase (Smits et al. 2005b). Furthermore, sporulation is initiated from approximately two hours after entry into the stationary growth phase, while in SM, this occurs already at transition point (Veening et al. 2005). Overall, these results indicate that in the 50/50 CDM, both developmental processes occur but appear to differ in timing. Furthermore, competence bistability is clearly reversible, whereas sporulation bistability seems to be a unidirectional process under the conditions tested.

Competent cells can enter sporulation

To answer whether or not the two developmental pathways are mutually exclusive (can competent cells sporulate?), we added an integrative plasmid DNA containing an antibiotic resistance marker to the culture in the timeframe where fluorescence was observed for the \textit{comG-gfp} reporter strain. Incubation was continued until 24h after the start of growth and cells were plated on selc-
tive and non-selective plates (to determine transformation frequency and viable count, respectively). Furthermore, at this time-point samples were treated with chloroform to kill vegetative cells and the remaining chloroform resistant spores were plated on selective and non-selective plates (to determine the transformed, and non-transformed spore counts, respectively). As shown in Table 2, some spores were found to be resistant to spectinomycin. This establishes that competent cells are not limited to this developmental pathway, but can enter the sporulation pathway at a later stage. Since the optical density of a culture in stationary phase does not increase further, but rather decreases, and competent cells are arrested in growth (Haijema et al., 2001), it is highly unlikely that cells which have been competent at the end of the logarithmic growth phase divide again and subsequently sporulate. More likely, cells first become competent, and subsequently have the ability to sporulate. However, using the comG-gfp reporter we show that competence is already initiated during late exponential growth (Figure 1). This could mean that cells that become competent during logarithmic growth, could have some time to resume division and/or ‘reset’ and later initiate sporulation. Whether such a ‘resetting’ is required to initiate sporulation remains to be investigated. Time-lapse and/or double-labeled fluorescent microscopy could serve to address this hypothesis, and is currently under investigation in our laboratory.

Effects of a comK mutation on sporulation bistability

ComK is the master regulator for competence development. The protein activates more than 100 genes, amongst which those necessary for the uptake of DNA from the environment and integration into the genome (Hamoen et al., 2002). To date, no effects of ComK on sporulation have been reported. However, as mentioned before, these studies were mainly performed in media

<table>
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<tr>
<th>Fraction</th>
<th>Percentage</th>
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<tr>
<td>Viable count</td>
<td>100</td>
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<td>Spore count</td>
<td>12</td>
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<td>Transformed cells</td>
<td>$60 \times 10^{-7}$</td>
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<td>Transformed spores</td>
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Table 1. Competent cells are able to sporulate. To determine competence and sporulation efficiencies, cells were plated onto TY agar plates as described in the Materials and Methods section. After overnight incubation at 37°C, colony-forming units were counted and percentages were determined by normalizing on basis of the viable count.
Noise in cellular differentiation

designed for efficient competence or sporulation and mutational effects were only studied on the level of population averages. The fact that in 50/50 medium competence development and initiation of sporulation are sequentially initiated, prompts the question whether competent cells actively compete with the initiation of sporulation in this medium. To test this hypothesis, we introduced a \( \text{comK} \) mutation in our sporulation reporter strain. The resulting strain, IIA-gfp/\( \text{comK} \) (\( P_{\text{spoIIA}} \)-gfp, \( \Delta \text{comK} \)), was grown in 50/50 medium and cells were analyzed by flow cytometry. As shown in Table 3, a mutation in \( \text{comK} \) has no significant effect on the final bistable sporulation outcome. However, sporulation seems to initiate somewhat earlier in the \( \text{comK} \) mutant. This might be caused by the slight increase in growth rate that is observed for the \( \text{comK} \) mutant in the 50/50 CDM (data not shown). We are currently looking into the reasons of this increase.

### Competence and sporulation in biofilms

Above, it was demonstrated that in a chemically defined medium, competence and sporulation are still initiated in a bistable manner. The single cell analyses of both developmental processes have so far been limited to those performed in liquid media (Chung et al., 1994; Maamar and Dubnau, 2005; Maamar and Dubnau, 2005; Smits et al., 2005b; Veening et al., 2005). In practice, bacterial infections and contaminations are often observed in the form of dense biofilm-like structures (such as colonies on solid surfaces and pellicles on liquids). Under these circumstances, the expression patterns may be significantly different from those observed during growth under laboratory circumstances in which cells are homogeneously shaken and quorum sensors are more likely to be distributed equally throughout the culture. In biofilms however, quorum sensing plays a pivotal role in the initiation of adaptive responses such as competence and sporulation (see for instance Ren et al., 2004; Stanley et al., 2003; Branda et al., 2001). Therefore, we were interested to see the flow cytometric profiles of the reporter strains under these conditions. Strains \( \text{comG-gfp (P}_{\text{comG}} \)-gfp) and IIA-gfp (\( P_{\text{spoIIA}} \)-gfp) were inoculated on solid agar plates con-

| Percentage of cells in the high \( \text{spoIIA} \) state |
|--------------|-----------|-----------|
| Time | wildtype | \( \Delta \text{comK} \) |
| 1 | 0.1 ± 0.0 | 0.1 ± 0.0 |
| 2 | 2.5 ± 0.4 | 5.2 ± 0.7 |
| 3 | 22.6 ± 1.4 | 25.8 ± 1.1 |
| 4 | 39.6 ± 1.8 | 40.3 ± 2.2 |

| Percentage of highly fluorescent cells * |
|--------------|-----------|-----------|-----------|
| Strain | Biofilms | Liquid cultures |
| | MM † | SM ‡ | MM | SM |
| 168 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| IIA | 0.70 ± 0.18 | 21.06 ± 0.90 | 0.06 ± 0.00 | 91.30 ± 0.74 |
| comG | 13.02 ± 0.48 | 0.07 ± 0.01 | 4.58 ± 0.07 | 0.03 ± 0.00 |

Table 2. Competence does not significantly affect sporulation bistability. Cells with a fluorescence level higher than channel 650 were considered to be in the high \( \text{spoIIA} \) state, as described before (Veening et al., 2005). An average of the percentage of highly fluorescent cells (related to the total cell count) of four independent experiments are shown; ± indicates the standard error in these data. Time is given in hours relative to the transition between exponential and stationary growth phase (T0).

Table 3. Noise of competence and sporulation. Cells with a fluorescence level higher than channel 650 were considered highly fluorescent. An average of the percentage of highly fluorescent cells (related to the total cell count in which at least 10^5 cells were counted) of four independent experiments are shown; ± indicates the standard error in these data. MM is minimal medium which is commonly used to induce competence development (Leskela et al., 1996). SM is sporulation medium which is generally used to induce sporulation (Schaeffer et al., 1965).
taining CDCM, 50/50 and CDSM and high-cell density multicellular structures in the form of colonies were allowed to form overnight at 37°C. The resulting colonies were homogenized and analyzed by flow cytometry as described in the Materials and Methods. As shown in Figure 2A, in contrast to growth in liquid media, competence is poorly initiated when grown on solid chemically defined media (on solid MM competence is well initiated; see Table 4). Sporulation however, is still well initiated when grown on solid chemically defined media (Figure 2B). With increasing concentrations of CDSM, more cells produce GFP expressed from the \( \text{spoIIA} \) promoter. However, the pattern is clearly less bistable when compared to initiation of sporulation in liquid chemically defined media. This suggests that sporulation is stronger induced in dense biofilms compared to growth in liquid shaken cultures under the conditions tested. To determine the competence and sporulation expression patterns in liquid-air pellicle-like biofilms (as described by (Branda et al., 2001), a standing 50 ml Greiner tube was filled with 10 ml of CDCM, 50/50 and CDSM, respectively, and strains comG-gfp and IIA-gfp were allowed to grow in these tubes for 72h at 37°C without shaking. Cells were extracted from both the pellicle (liquid-air interface) and the liquid-phase (planktonic cells) and homogenized and analyzed by flow cytometry as described in the Materials and Methods. As shown in Figure 3 (left panels), competence development is poorly initiated in both the pellicle and standing liquid culture in all chemically defined media tested. While sporulation (Figure 3, right panels) is poorly initiated in CDCM (Figure 3A) and 50/50 (Figure 3B) media, it is efficiently initiated in CDSM (Figure 3C). In all cases however, sporulation is more efficiently initiated in the pellicle.
Noise in cellular differentiation

Recently, it was demonstrated that the bistability in gene expression that is observed in complex liquid media, requires the presence of a positive feedback loop involving the key-regulators of competence and sporulation (Maamar and Dubnau, 2005; Smits et al., 2005b; Veening et al., 2005). This type of bistability is thought to originate from noise in the expression of the key-regulators. However, limited experimental studies have been performed to quantify this. Maamar and Dubnau postulated a role for noise in competence development based on the occasional observation of cells expressing the reporter under atypical conditions (Maamar and Dubnau, 2005). Dawes and Thornley studied sporulation in *B. subtilis* using continuous chemostat cultures (Dawes and Thornley, 1970). They observed that even at very high dilution rates, conditions that should not trigger sporulation, some cells sporulate. We propose that this may well be contributed to noise within the sporulation network. Motivated by these previous findings, we were interested in extending this work and quantitatively analyze the noise, if present, in both competence and sporulation in both liquid cultures and biofilms. We set out to quantify noise in our system, by looking at cells that initiate sporulation and competence under non-stimulatory conditions. To this end, we adapted the conditions for flow cytometric measurements, to count large numbers of cells (at least 100,000), and to filter out false negative signals by rigorous gating, which removes particles with a different size than a single cell from the analysis and only counts cells with a very high fluorescence level (*noise-measurement*). This strategy eliminates background signals and false-positives, but may also reduce the amount of true positives, and thus effectively gives an under-representation of the fluorescent signals. To obtain significant ‘noise’ data, it is essential to measure many single cells within a population. The use of flow cytometry in combination with promoter-GFP fusions as described in this work greatly facilitates such studies. Strains comG-gfp and IIA-gfp were grown on solid and liquid MM and SM, respectively. To allow single cell analyses, colonies were homogenized and analyzed by flow cytometry as described in the Materials and Methods. Cells from liquid cultures were immediately analyzed on a flow cytometer. For each culture condition, 4 individual colonies or cultures were measured. As mentioned in the first paragraph of this study, no clear development of competence was observed on complex sporulation medium (Figure 1A), nor sporulation on a complex medium dedicated to induce competence (Figure 1B) using standard flow cytometric settings. However, using *noise-measurements*, it becomes clear that under all conditions tested, there are some cells that demonstrate development of competence or sporulation, despite the lack of triggers for these processes (Table 4). Importantly, a negative control (a strain without fluorescent reporter) gives no fluorescent signals at all under the conditions tested using *noise-measurements*. Furthermore, it seems that the sporulation pathway is noisier than the competence pathway. Interestingly, both developmental processes show a higher degree of noise in dense-biofilms compared to liquid cultures. Overall, by quantifying the atypical initiation of the developmental pathways for the first time, these observations strengthen the hypothesis that noise contributes to the establishment of heterogeneity for competence and sporulation. In addition, it suggests that the levels of noise differ between different cellular processes.
Discussion

Competence for genetic transformation and sporulation form a major problem for food and medical industry because they render bacteria insusceptible to conventional methods of sterilization and treatment. To effectively counter these bacterial adaptive strategies, one approach would be to specifically inhibit the unwanted developmental process. For this reason, detailed knowledge is required about the molecular mechanisms governing the development of competence and sporulation. Most studies so far have been performed under conditions in shaken batch cultures in complex media. These conditions may not reflect industrial or medical circumstances.

In literature, some investigations of competence and sporulation in chemically defined media are available. However, all the studies evaluate gene expression patterns at the population level instead of looking at single cell gene expression patterns (Hageman et al., 1984; Murayama et al., 2004). Here, we present the first single cell analysis of competence development and the initiation of sporulation in the same genetic background, in a chemically defined medium, and under biofilm-promoting conditions.

Our results demonstrate that also in chemically defined liquid media both competence and sporulation show a bistable distribution in gene expression (Figure 1). Though the ratio between high- and low-expressing cells seems to vary depending on culturing conditions, bistability is independent of media.

As shown in Figure 1A right panel, the expression of spoIIA-gfp is higher than the background fluorescence and all cells are in this so-called low state (Veening et al., 2005). Recently, it was found that, for instance abrB, is repressed by Spo0A at a low threshold level (Fujita et al., 2005). AbrB represses the gene encoding sigH, a gene encoding an alternative sigma factor (Weir et al., 1991). Since the spoIIA operon is expressed from a sigma-H dependent promoter (Wu et al., 1991), the low level of expression observed in the right panel of Figure 1B might be caused by the relief of AbrB-repression on the sigH promoter. For a high activation of spoIIA however, high levels of Spo0A are required (Fujita et al., 2005; Bongers et al., 2005). This observation might also explain why competence development is stimulated in MM, since it was shown that to initiate competence, Spo0A needs to downregulate abrB and does this already at a low threshold level (Hahn et al., 1995b; Fujita et al., 2005). Interestingly, not only the ratio between both populations seems to vary depending on the media, also the timing of the processes seems to differ between complex media and chemically defined media. Competence, for instance, is normally initiated in stationary growth phase in complex medium, whereas in the CDM, comG was already expressed during the late exponential growth phase (Figures 1B and 1C). This difference could be due to the fact that in the CDM, cultures reach a much higher optical density than in complex competence medium. Since a key step in the development of competence is regulated through quorum sensing, the threshold for the quorum sensing response may be reached earlier. Consistent with this hypothesis, it was reported that overproduction of a downstream component of the quorum sensing event, ComS, leads to precocious and increased competence development (Maamar and Dubnau, 2005). Next, it was found that competence and sporulation are subsequent processes (Figure 1). The fact that amongst the total population of spores it was possible to identify those transformed earlier in growth indicates that the two developmental pathways are not mutually exclusive (Table 2). Competence bistability can therefore be classified as a reversible bistable process that does not exclude cells from entering the sporulation pathway. Through the introduction of the comK mutation in the IIA-gfp strain, it was established that abolishment of competence does not significantly affect the final bistable sporulation outcome (Table 3). This indicates that
there is no direct relation between \textit{comK} expression and the bistable expression pattern of sporulation genes. Taken together, these results form a strong argument to view both bistable processes as independently regulated under the conditions tested. The origin of this strong independency in regulation remains an intriguing question, since both processes respond to similar triggers (Hoch, 1991; Dubnau, 1991a). In addition, the key-regulator of sporulation, Spo0A, is also essential for competence development. This was verified by single cell analysis of our reporter strains in which a \textit{spo0A} mutation was introduced (comG-gfp/spo0A and IIA/spo0A). For both strains, the bistable expression patterns were abolished, and no fluorescence was observed (data not shown). It is important to realize that the fact that no bias is observed in sporulation of cells that have or have not been competent, does not exclude the existence of a regulatory mechanism that temporally separates the two developmental pathways.

A bistable expression pattern was also observed under biofilm-promoting conditions. Interestingly, development of spores seems to occur preferentially at the liquid air interface of pellicle-like biofilms on liquid. These results are consistent with the results of Branda and co-workers, in which it was shown that sporulation is preferably initiated within aerial structures of a biofilm (Branda \textit{et al}., 2001). Formation of biofilms was recently reported to involve a master regulator, SinR (Kearns \textit{et al}., 2005). Interestingly, SinR was initially identified as a sporulation inhibitor (Bai \textit{et al}., 1993; Mandic-Mulec \textit{et al}., 1995), indicating the close relationship between both processes. Competence in biofilms in CDM seems to be poorly developed (Figures 2 and 3), while in colonies grown on solid MM agar, competence is well initiated (Table 4). This difference might be attributed to the fact that for the flow cytometry analyses of colonies from CDM, cells were harvested after prolonged incubation, up to 72 hours and for the noise measurements after 24 hours. Since competence is a reversible process, cells may have reverted to a non-expressing state or have lysed. Interestingly, the cells that did express \textit{comG}, showed very high levels of expression, and little or no intermediate levels of expression (Figures 2 and 3). Conceivably, in biofilms, micro-environments differ significantly between areas, and lead to strong induction of a specific developmental pathway.

Finally, the quantitative analysis of the expression of competence and sporulation reporters under atypical conditions indeed seems to suggest a role for noise in both processes (Table 4). Although unlikely, it cannot be excluded that some of the observed fluorescent cells demonstrate expression due to a secondary mutation. This could be tested by sorting out the ‘noisy’ cells and perform competence and/or sporulation efficiency assays. However, the results are in excellent agreement with previous reports that suggest that noise in gene expression is key to both competence (Maamar and Dubnau, 2005) and sporulation, and that the decision to enter either of these pathways is stochastic (Maughan and Nicholson, 2004). From an industrial or medical point of view, noise in gene expression has major implications. 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. On a laboratory scale, these may be negligible, but in industrial fermentations or food-industrial processes for instance, the occurrence of highly resistant spores in the system can have big consequences. Also, increased resistance to a wide-range of antibiotics, which can be linked to genetic transformation (Dobay \textit{et al}., 2004), is more and more becoming a serious epidemic threat for the community. Therefore, future research must be aimed at localizing the noisiest regulatory components in developmental pathways and neutralizing these targets.
Materials and methods

Media and growth conditions

Chemicals used were of analytical grade and, unless indicated otherwise, obtained from Merck, or Baker Chemical Co. Liquid B. subtilis cultures were grown at 37 °C and continuously shaken at 230 rpm, unless indicated otherwise. To study the development of competence and sporulation in colonies, cells were spread onto plates consisting of the indicated media type, supplemented with 1.5% agar and incubated overnight at 37°C. Biofilms with a liquid-air interface were obtained by inoculating B. subtilis directly from a frozen stock (-80°C) to 10 ml of chemically defined medium as indicated, and subsequent incubation at 37°C for 72 hours without shaking. TY medium (tryptone/yeast extract) contained Bacto-Tryptone (1%), Bacto-yeast extract (0.5%) and NaCl (1%). Sporulation medium (SM) was prepared as described before (Hageman et al., 1984) and contained KHPO₄ (62 mM), KH₂PO₄ (44 mM), (NH₄)₂SO₄ (15 mM), sodium citrate (6.5 mM), MgSO₄ (0.8 mM), 0.02% of casamino acids, glucose (27.8 mM) and L-tryptophan (0.1 mM). The pH was set to 7 using a KOH solution. Chemically defined competition medium (CDCM) was prepared as described before (Vasantha and Freese, 1980) and contained MgCl₂ (5.0 mM), L-glutamic acid (8.0 mM), L-tryptophan (0.1 mM), D-glucose (20 mM) and 1x MT mix. A 50x MT mix was prepared as described before (Leskela et al., 1996) and contained K₂HPO₄ (62 mM), KH₂PO₄ (44 mM), MnCl₂ (0.05 mM), (NH₄)₂SO₄ (15 mM), sodium citrate (6.5 mM), MgSO₄ (0.8 mM), glucose (27.8 mM), L-glutamic acid (5.35 mM) and L-tryptophan (0.1 mM). The pH was set to 7 using a KOH solution. Chemically defined sporulation medium (CDSM) was prepared as described before (Vasantha and Freese, 1980) and contained MgCl₂ (0.2 M), CaCl₂ (70 mM), MnCl₂ (5 mM), ZnCl₂ (0.1 mM), thiamin-hydrochloride (0.2 mM), HCl (2 mM) and FeCl₃ (0.5 mM). To obtain a chemically defined medium in which both competence and sporulation are initiated, CDCM and CDSM were mixed in a 1:1 ratio (50/50). When appropriate, media for B. subtilis were supplemented with chloramphenicol (Cm; 5 µg/ml), spectinomycin (Sp; 100 µg/ml) or kanamycin (Km; 10 µg/ml).

Strains and plasmids

Table 1 lists the plasmids and bacterial strains used. All strains used in this study are isogenic derivatives of B. subtilis 168 (Kunst et al., 1997). B. subtilis was transformed as described before (Leskela et al., 1996).

B. subtilis strain comG-gfp was obtained by a Campbell-type integration (single crossover) of plasmid pSGComG (Smits et al., 2005b) into the chromosomal comGA promoter region of B. subtilis 168. Transformants were selected on TY agar plates containing Cm, after overnight incubation at 37°C. Correct integration was verified by PCR (data not shown). B. subtilis strain IIA-gfp/ΔcomK (P_comA::gfp, ΔcomK) was obtained by transformation of strain IIA-gfp (Veening et al., 2005) with chromosomal DNA of strain BV2004 (Hamoen et al., 2002). Transformants were selected on TY agar plates containing Km, after overnight incubation at 37°C. Correct integration was verified by PCR (data not shown).
agar plates containing Cm and Sp, after overnight incubation at 37°C. *B. subtilis* strain IIA-gfp/Δspo0A (PṣpIIA-gfp, Δspo0A) was obtained by transformation of strain IIA-gfp (Veening et al., 2005) with chromosomal DNA of strain SWV215 (Xu and Strauch, 1996). Transformants were selected on TY agar plates containing Cm and Km, after overnight incubation at 37°C. *B. subtilis* strain G-gfp/Δspo0A (PcomG-gfp, Δspo0A) was obtained by transformation of strain comG-gfp with chromosomal DNA of strain SWV215 (Xu and Strauch, 1996). Transformants were selected on TY agar plates containing Cm and Km, after overnight incubation at 37°C. *B. subtilis* strain G-gfp/ΔcomK (PcomG-gfp, ΔcomK) was obtained by transformation of strain comG-gfp with chromosomal DNA of strain BV2004 (Hamoen et al., 2002). Transformants were selected on TY agar plates containing Cm and Sp, after overnight incubation at 37°C.

**Flow cytometric analyses**

Cells were analysed by flow cytometry essentially as described (Smits et al., 2005b; Veening et al., 2005). For each sample, at least 20,000 cells were analyzed. To determine levels of ‘noise’ (see Table 4), at least 100,000 cells were analyzed. To analyze biofilms, samples were first resuspended in 1 ml of MM and subsequently homogenized for 1 min using a mini bead-beater (Biospec Products, Bartlesville, OK, USA) before flow cytometric analysis. The gate of the forward and side scatter of the flow cytometer (which indicates the particle size) were set such that only single cells were counted for fluorescence analysis.

**Competence and sporulation assays**

Strain comG-gfp was grown in 3 ml of the chemically defined 50/50 medium and initiation of competence was determined by flow cytometric analysis at hourly intervals. Plasmid DNA (pWK-Sp; unpublished cloning vector containing a Sp marker between the flanking regions of the amyE gene of *B. subtilis*) was added to the culture when the presence of comG-gfp expressing cells first appeared. Incubation was continued for another 24 hours, and the presence of phase-bright spores was verified by phase-contrast microscopy. To determine transformation frequencies, aliquots of the culture were diluted and plated onto TY agar plates without (viable count) or with (transformation count) Sp. Furthermore, to determine the total viable spore population and the transformed spores formed, samples were treated with a 1/10 volume of chloroform for 10 min (killing vegetative cells, while the chloroform-resistant spores survive). Chloroform treated samples were plated on TY (spore count) and on TY plates containing Sp (transformed spore count).

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