Genetic switching into the competent state

Bacillus subtilis: a single cell approach

Madeleine Leisner

München 2008

Genetic switching into the competent state

Bacillus subtilis: a single cell approach

Madeleine Leisner

Dissertation
Fakultät für Physik
Ludwig–Maximilians–Universität München

vorgelegt von
Madeleine Leisner
aus Landshut

4. Februar 2008

Erstgutachter: Prof. Dr. Joachim O. Rädler
Zweitgutachter: Prof. Dr. Erwin Frey
Tag der mündlichen Prüfung: 5. Mai 2008

Contents

1 Zusammenfassung

Im Rahmen dieser Doktorarbeit wurde das Schaltverhalten von *Bacillus subtilis* vom nicht-kompetenten (geringe ComK Konzentration, B-state) in den kompetenten Zustand (hohe ComK Konzentration, K-state) untersucht. Die Dynamik des Überganges wurde dabei näher charakterisiert und mit einem biochemischen Model verglichen. Um die ComK Konzentration in einer Zelle zu bestimmen, wurde ein ComK-GFP Reporter Konstrukt verwendet und die relative ComK-Konzentration der einzelnen Zellen mittels Fluoreszenz-Mikroskopie gemessen. Hierbei wurde sowohl das Verhalten der Gesamtbakterienpopulation betrachtet, als auch Einzelzellzeitserien aufgenommen, um das Schaltverhalten individueller Bakterien genauer zu analysieren. Des Weiteren wurde ein Zeitfenster, in dem Schalten in den kompetenten Zustand möglich ist, postuliert und näher charakterisiert, sowie Faktoren ermittelt, die das Entkommen aus dem K-state ermöglichen.

Bakterien sind in der Lage DNA aus ihrer Umgebung aufzunehmen (= Transformation) und sie ganz oder teilweise in ihr Genom zu integrieren. Sie erlangen dadurch neue Fähigkeiten wie z.B. Resistenzen gegen Antibiotika. *Bacillus subtilis* transformiert lediglich während einer bestimmten Wachstumsphase, der sog. stationären Phase. Um DNA aufnehmen zu können, also kompetent zu werden, benötigt *Bacillus subtilis* einen aus vielen verschiedenen Proteinen bestehenden DNA Aufnahme Apparat. Für dessen Synthese ist ComK, der allgemeine Transkriptionsfaktor des Netzwerkes, von Nöten. Dieser reguliert die Expression von über 100 Kompetenzgenen und ist damit essentiell für die Entwicklung der Kompetenz (= key regulator). Die Expression von ComK setzt nur dann ein, wenn im umgebenden Medium eine ausreichende Menge des Pheromons ComX vorhanden ist (quorum sensing). Sie wird zusätzlich von vielen verschieden Mechanismen auf transkriptioneller und translationeller Ebene reguliert. Arbeiten verschiedener Gruppen postulierten, dass sich der Übergang aus dem nicht kompetenten in den kompetenten Zustand wie ein bistabiler Schalter verhält.

Untersuchungen der Gesamtpopulation auf Einzelzellniveau zeigten ein sigmoidales Anschaltverhalten, wobei ein bestimmter Prozentsatz (∼14%) der Zellen in der stationären Phase kompetent wurde. Die Kompetenzentwicklung in *Bacillus subtilis* ist daher ein bistabiler Prozess: d.h. es existieren zwei Phenotypen (kompetent und nicht-kompetent) nebeneinander. Der Prozentsatz kompetenter Zellen konnte durch künstliche Erhöhung der Pheromonkonzentration in der exponentiellen Wachstumsphase auf 36% erhöht werden. Senkte man dagegen die Pheromonkonzentration auf Null, fiel der

Anteil der kompetenten Zellen auf 8%. Gezielte transkriptionale und translationale Veränderungen des ComK-Regulationsnetzwerkes erhöhten den Anteil der kompetenten Zellen stark (85-99%) und führten zusätzlich zu einer höheren Schaltrate als im Wildtyp. Des Weiteren konnte gezeigt werden dass die Promotoraktivität des *comK* Gens (gemessen anhand der basalen *comK* Konzentration) für das Erreichen der Kompetenz wichtig ist und ein definiertes Zeitfenster für den Übergang in den kompetenten Zustand öffnet. Wildtyp Zellen schalteten in einem Zeitraum von 1.5 h. Dieses Zeitfenster konnte durch den oben bereits erwähnten künstlich herbeigeführten Anstieg des Pheromons ComX in der exponentiellen Phase auf 2.5 h verlängert werden.

Um das Schaltverhalten einzelner Zellen zu charakterisieren, wurden Einzelzellzeitserien aufgenommen. Hierbei zeigte sich eine starke Korrelation zwischen Anschaltwahrscheinlichkeit und Zellzyklus. Die Anschaltwahrscheinlichkeit war maximal, wenn sich die Zellen 15 min zuvor geteilt hatten. Die starke Regulation zwischen Zellzyklus und Kompetenz zeigte sich auch darin, dass kompetente Zellen nach Anschalten $\sim$ 1 h lang kein Wachstum aufwiesen und sich über einen Zeitraum von 2 h nicht teilten. Einmal angeschaltet benötigten die Zellen $\sim$1.5 h um ihr maximales ComK Level zu erreichen. Die experimentellen Daten wurden durch die Ergebnisse eines dynamischen, nicht-linearen theoretischen Modells bestätigt, welches in Zusammenarbeit mit J.-T. Kuhr (LS Frey, LMU) erstellt wurde. Das Modell reproduzierte den Anteil kompetenter Zellen in der stationären Phase mit $\sim$17%. Zellen, die den K-state erreichten, benötigten dafür nach dem Model 1.9h. Auch der Anstieg der basalen *comK* Expression $\sim$45 min vor Eintritt in die stationäre Phase konnte durch das Model reproduziert werden.

Des Weiteren konnte experimentell gezeigt werden, dass kompetente Zellen in der späten stationären Phase Stoffe in das umgebende Medium abgeben, die zum Abschalten des *comK* Promoters führen und damit das oben beschriebene Zeitfenster schließen. Nicht kompetente Zellen geben ebenfalls inhibitorische Stoffe ab. Diese reduzieren die *comK* Expression und verhindern dadurch möglicherweise unnötige Energieverluste durch den Aufbau eines nicht benötigten DNA-Aufnahme Apparates.

In dieser Arbeit wurden neue Informationen über den zeitlichen Verlauf der Kompetenzentwicklung in *Bacillus subtilis* erlangt. Die Existenz eines Zeitfensters in dem stochastisches Schalten in den kompetenten Zustand möglich ist, konnte gezeigt werden, sowie dessen zeitliche Begrenzung durch externe Faktoren. Die starke zeitliche Regulation der Kompetenz zeigte sich ebenfalls durch die Kopplung des Schaltens in den kompetenten Zustand mit dem Zellzyklus.

2 Summary

This PhD thesis presents studies concerning *Bacillus subtilis* switching from the non-competent (low ComK concentration, B-state) into the competent state (high ComK concentration, K-state). The dynamics of switching was analysed in detail and compared to a biochemical model of the system. To determine the ComK-concentration inside the cell, a GFP-ComK reporter construct was used and the relative ComK-concentration of single cells was obtained via fluorescence microscopy. Therefore alteration of ComK expression with time was observed for the whole bacterial population and single cell time-lapse experiments were performed to analyse the switching behaviour of individual bacteria. Furthermore, a time window, allowing switching into the K-state has been postulated and analysed, as well as factors responsible for the escape from the competent state.

Bacteria can acquire genetic diversity, including antibiotic resistance and virulent traits, by horizontal gene transfer. In particular, many bacteria are naturally competent for uptake of naked DNA from the environment in a process called transformation. *Bacillus subtilis* only transforms in a specific growth phase, the stationary phase. To take up the DNA *Bacillus subtilis* needs a huge ensemble of genes and proteins to assemble the DNA uptake machinery. For its synthesis, ComK, the main transcription factor of the network is necessary. ComK regulates the expression of over 100 competence genes and is therefore essential for the development of competence. The key regulator, ComK is only expressed, when the amount of pheromone ComX in the surrounding medium exceeds a specific concentration (quorum sensing). The expression of ComK is additionally regulated by several mechanisms on the transcriptional and translational level. Different groups postulated that the transition from the non-competent state to the competent state is switch-like.

Analysis of the whole bacterial population on the single cell level showed a sigmoidal switching behaviour, whereas a specific percentage ($\sim$14%) of the cells got competent in the stationary phase. Competence development in *Bacillus subtilis* is therefore a bistable process, that is the coexistance of two phenotypes (competent and non-competent) at the same time. The percentage of competent cells could be enhanced to 36 % by artificially increasing the ComX pheromone concentration in the exponential growth phase. Reduction of the pheromone concentration down to zero lowered the percentage to 8%. Systematic transcriptional and translational changes in the ComK regulatory network strongly increased the fraction of competent cells (85-99%) and lead additionally to an

increased switching rate as compared to the wild-type. Furthermore, it could be shown, that the promoter activity of the *comK* gene (measured as basal *comK* expression) is important for the development of competence and opens up a fine tuned time window for switching into the competent state. Wild-type cells switched into the K-state during a time period of 1.5 h. Increasing the pheromone concentration artificially as mentioned previously, lead to an elongation of the time window to 2.5 h.

To characterize the switching behaviour of single cells, time-lapse microscopy was performed. A strong correlation between switching probability and cell cycle was found. The switching probability was maximal when cells had divided 15 min prior to switching. Competent cells did not grow for about 1 h after switching and did not divide over a time period of 2 h after switching indicating a strong coupling between cell cycle and competence. After switching, cells needed $\sim$ 1.5 h to reach their maximal ComK levels. The obtained experimental data have been explained in the framework of a non-linear theoretical model that incorporated low-number stochastic effects. The model was developed in collaboration with J.-T. Kuhr (LS Frey, LMU). The model reproduced the percentage of competent cells in the stationary phase with $\sim$17%. Cells that entered the K-state needed 1.9 h to do so. The increase of the basal *comK* expression $\sim$45 min before entry into the stationary phase could also be reproduced.

In another series of experiments it could be shown, that competent cells release substances into the surrounding medium in the stationary phase, leading to shut down of the *comK* promoter, thereby terminating the switching window described above. Non competent cells also released inhibitory substances, reducing *comK* expression, possibly to prevent loss of energy by assembly of unused DNA uptake machineries.

Concentrating on the main transcription factor ComK, this work gave further insights on the kinetics of competence development in *Bacillus subtilis* and clearly showed the existence of a well defined competence window [65], in which cells can stochastically switch into the competent state. The dependence of this switching window on external factors could be shown. The strong temporal regulation of switching into the competent state could also be seen in the coupling of switching to cell cycle.

3 Introduction

Why do bacteria take up DNA? What is the purpose of DNA uptake? While most procaryots can alter their genetical properties only by spontaneous mutation, some bacteria have additionally obtained the ability to take up DNA from the surrounding environment (= competence) in a process called transformation (Fig. 3.1). While spontaneous mutation is very rare (10^{-6} per gene per generation) [1], but can take place throughout the lifetime of an organism, the uptake of DNA is only possible under specific conditions.

Transformation in *Bacillus subtilis* for example occurs only when the cells enter the stationary growth phase, a phase in which living conditions are no longer optimal, due to limited nutriments or the increase of toxic substances produced by the bacteria themselves. Under these conditions of stress bacterial cells compete with each other. The possibility to gain an advantage compared to other cells may therefore be very attractive. Transformation can offer this advantage when the DNA fragments uptaken contain for example information for a faster uptake of nutrition. In this case the advantage would manifest immediately.
Uptake of DNA fragments, that do not cause an immediate advantage, seems to be use-

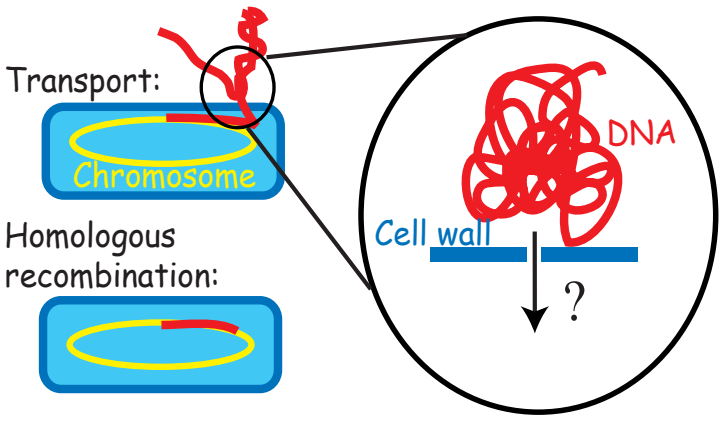

Figure 3.1: Transformation The process to take up naked DNA from the surrounding environment is called transformation. The uptaken DNA fragments are then incorporated into the genome via genomic recombination.

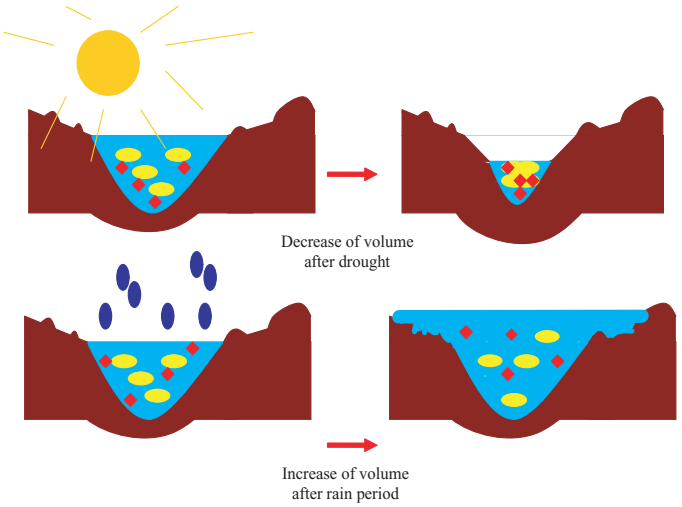

Figure 3.2: Influence of environmental changes on protein concentration Alteration of environmental conditions changes the concentration of pheromones: Draught leads to an increase of pheromone concentration by decreasing the volume of living space. Rain dilutes out the pheromone.

less at the time-point of uptake when integrated into the genome, but may help survive its owner later during its lifetime, when conditions change. For example, the information to take up the sugar fructose may be useless, when the only sugar available is glucose. As soon as environmental conditions change, glucose is used up and instead fructose is available as a nutrient, those cells will survive, that have gained the ability to use fructose as a nutrition.

But how do bacteria sense the debasement of their living conditions? What triggers bacterial cells to take up DNA from their surrounding environment? Firstly, the cells need to acquire information about the actual living situation. This happens, in case of *Bacillus subtilis*, using the so called quorum sensing mechanism (See chapter 4: DNA uptake regulation): the concentration of a pheromone in the surrounding medium is sensed by the cells and leads to the activation of the bacterial competence machinery once a specific threshold concentration is reached. Alterations of environmental conditions may cause such changes of protein (or pheromone) concentrations. Figure 3.2 sketches the influence of environmental changes on protein concentration. It is assumed that draught can lead to an increase of protein concentration by decreasing the volume of living space. Rain increases the volume, thereby thining out the protein concentration. Therefore draught may lead to increased pheromone concentrations, thereby activating a specific signaling pathway causing the assembly of the DNA uptake machinery (see chapter 4).

Figure 3.3: Decisions How to decide? When is the best time-point to make a decision?

In *Bacillus subtilis* only a specific amount of cells of a given population develops the ability to take up DNA from the surrounding environment. The population consists of genetically identical cells, that are growing under the same environmental conditions. How can it be that a part of the bacterial population is able to take up DNA, while the other is not? The individual bacteria seem to decide whether to become competent or not. But what exactly triggers decision making in bacteria and when does it happen (Fig. 3.3)?

It was shown that differences in gene expression can lead to the establishment of bistability (see chapter 4) [2], a phenomenon known as the coexistence of two different phenotypes; in case of competence developement, the presence of non-competent and competent cells at the same time. Recent studies revealed the importance of the positive feedback loop of ComK, the main transcription factor of competence development, for the establishment of bistability in *Bacillus subtilis* [2–4]. Which phenotyp is present in a single cell is dependent on the feedback loop being active or not. But why is a specific feedback loop active in some cells, but not in all? Many groups addressed this question and pinpointed the importance of noise [5,6] for the establishment of a bistable expression pattern, concerning comptence development. In a single cell little differences in transcription, mRNA decay, translation or protein degradation are known as intrinsic noise. Those little concentration differences can be amplified by regulatory feedback loops thereby determining cell fate.

We see, competence development is influenced by many factors (external and internal ones), and the underlying regulatory network is huge (see chapter 4). Progress in molecular biology, particularly in genome sequencing and high-throughput measurements enabled biological science to gain information on the underlying molecules important for competence development. While an understanding of genes and proteins continues

to be important, the focus of systems biology [7] is to understand a system's structure and dynamics. Because a system is not just an assembly of genes and proteins, its properties cannot be fully understood merely by drawing diagrams of their interconnections [8]. Such diagrams provide knowledge of how changes to one part of a system may affect other parts, but fail i.e. to reveal information about stochasticity of the described interactions. To understand the physics of a system, one must examine how the individual components dynamically interact during operation, and how the system reacts when conditions are varied. The underlying properties of such a regulatory network can be discovered in close collaboration of experimental biology and physical theory. This enables scientists to create predictive models of regulatory networks.

In this work the methods of classical biological science were combined with the physics oriented approach of systems biology [8], and revealed new insights on protein kinetics involved in competence development. To obtain quantitative data about external or interal factors being important for competence development, in combination with time-resolved information on protein kinetics, single cell time-lapse microscopy was used in this study. The dynamcis of competence development represented by the key regulator ComK were examined and factors opening up a time window for switching into the competent state have been investigated (see chapter 7). Single cell time-lapse microscopy revealed a well defined switching period as an intrinsic property of individual cells (see chapter 8). A non-linear dynamics theoretical model reproduced the obtained data qualitatively and quantitatively and is presented in chapter 8. The basic principles underlying this model will be explained in chapter 5. The strong temporal regulation of switching into the competent state can also be seen in the dependence of switching on the cell cycle as shown in chapter 9. Studies on the termination of the switching window for competence will be shown in chapter 10.

4 DNA uptake, regulation and bistability

4.1 DNA uptake regulation

The group of bacteria called *Bacilli* is mainly characterised by their ability to create endospores, a dormant that allows the bacterium to survive periods of starvation and draught. Some members of this group like *Bacillus anthracis* are pathogenic or produce extracellular hydrolytical enzymes. *Bacillus licheniformis* is responsible for the production of the antibiotic Bacitracin.

Bacillus subtilis (Fig. 4.1) is an aerobic gram positive bacterium that lives in the soil. It is able to take up naked DNA from the surrounding environment. The ability to take up DNA is called competence. By taking up DNA, bacteria can acquire genetic diversity. They integrate the uptaken DNA in their genomes via genetic recombination and therefore obtain new properties such as the resistance to antibiotics. While other bacteria like *Escherichia coli* have to be made competent using heat-shock protocols, *Bacillus subtilis* is naturally competent for the uptake of naked DNA from the surrounding environment [9], a process called transformation. *Bacillus subtilis* is only able to take up the DNA in a specific growth phase: the stationary phase [10].

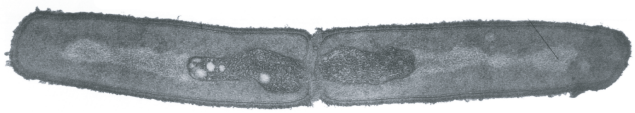

Figure 4.1: *Bacillus subtilis* is a gram positive soil bacterium [1]

Double-stranded DNA from the surrounding environment is first bound to a so called pseudopilus, that allows the DNA to bind to the receptor ComEA (Fig. 4.2). The DNA is then transported through the membrane, but only one single strand of the DNA passes the membrane complex ComEC, while the other strand is degraded outside the cell [11, 12]. The sDNA inside the cell is then bound by single strand binding proteins such as YwpH [13] that may be important firstly, for transportation of the uptaken DNA to the bacterial chromosome and secondly, for integration of the uptaken DNA into the

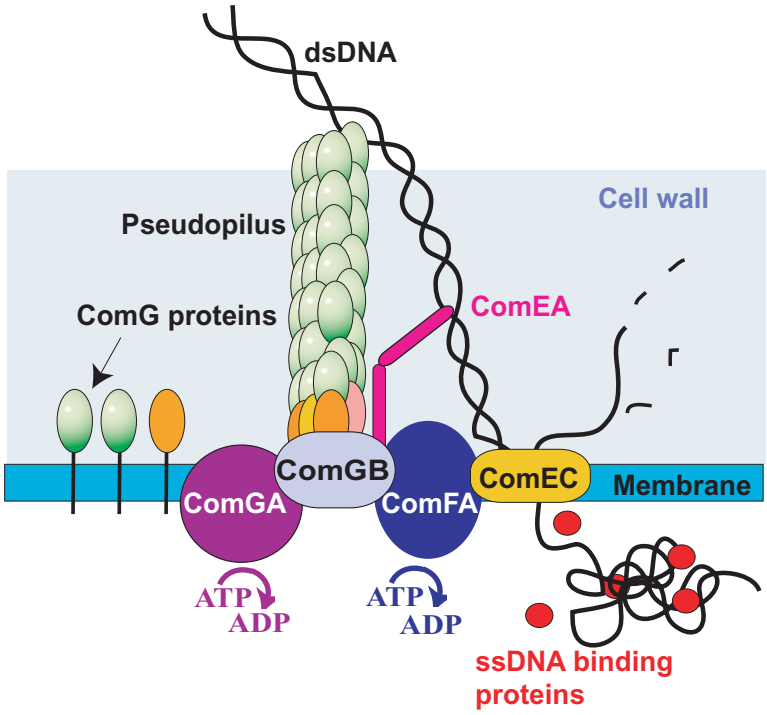

Figure 4.2: DNA uptake Molecular model of the DNA transport through the cell envelope in *Bacillus subtilis*. For detailed description see main text [11].

genome via genetic recombination.

Competence in *Bacillus subtilis* is controlled by quorum sensing, the ability of bacteria to communicate and coordinate their behaviour via signaling molecules [14]. Quorum sensing occurs at high cell density in many microorganisms and regulates specialized processes such as genetic competence (*Streptococcus pneumoniae* [9]), bioluminscence (*Vibrio harveyi* [14]), virulence and sporulation [15]. It was first observed in the bacterium *Vibrio fischeri* [14], where it is necessary for the development of luminescence regulated by the LuxR-autoinducer. While gram-negative bacteria use N-acyl-homoserine lactones (AHLs) [16] as signaling molecules, gram-positiv bacteria such as *Bacillus subtilis* utilize small peptides [17, 18]. The signaling molecule in case of *Bacillus subtilis* is the pheromone ComX [9, 19, 20]. ComX is produced inside the cell as a precursor and is then transported through the membrane. The more cells are present, the higher is the concentration of active ComX pheromone in the surrounding medium. When the ComX concentration exceeds a specific threshold it is sensed by the histidine kinase ComP [20]. This kinase then phosphorylates ComA [21], which in turn activates ComS. ComS inhibits the degradation of ComK, the main transcription factor of the competence system, by competing with ComK for binding to the MecA/ClpC/ClpP protease

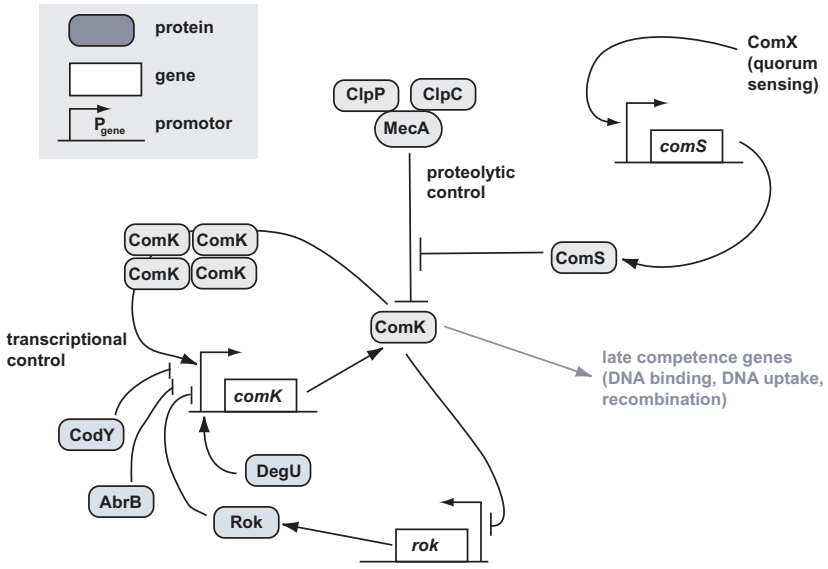

Figure 4.3: Core of the competence circuit Arrows denote up-regulation and blunt ends denote down-regulation.

complex [22]. Therefore ComK accumulates inside the cell and leads to the activation of over 100 competence genes [23].

The concentration of ComK is tightly controlled at the transcriptional and protein level. ComK positively auto-regulates its own transcription [24] by binding as a dimer of dimers to its own promoter [25]. In non-competent cells the positive auto-regulatory loop is not activated and ComK expression is low (B-state) [5, 26]. During exponential growth the level of ComK is kept at a basal concentration through the action of the MecA/ClpC/ClpP protease complex, which actively degrades the ComK protein, and by tight regulation on the transcriptional level. It has been suggested that the initiation of the autostimulatory response requires the response regulator DegU, which stabilizes binding of ComK tetramers to P_{comK} by dual interaction with the promoter region and ComK [27]. In order to prevent premature transcription of *comK* , three repressors Rok [28], AbrB [71] and CodY [29](Fig. 4.3), bind to the promoter region of *comK*. CodY represses early stationary-phase genes when intracellular GTP levels are high, linking amino acid availability to gene expression [30]. AbrB has been reported as a transition state regulator, as it is down-regulated by the transcriptional regulator SpoOA [31]. It is activated by phosphorylation via a complex phosphorelay system in response to nutrient limitation [32]. Therefore, AbrB levels decrease when SpoOA increases probably around entry into stationary phase. With the transition into the competent state, the so called K-state [33], ComK acts as an activator at its own promotor by antagonizing the action

of the two repressors Rok and CodY [28] leading to high expression levels of ComK. Depending on the activity of the ComK auto-regulatory loop cells enter the K-state or not, leading to the establishment of a bistable expression pattern (see section 4.2, this chapter).

The development of competence is also coupled to the sporulation pathway. The master regulator of sporulation, SpoOA for example, is necessary at low levels for competence development, potentially by negatively affecting the transcription of the repressor for *comK*, AbrB [34,35]. SpoOA represses *abrB* only in its phosphorylated state. A gene called *sda* is responsible for the regulation of the activity of two kinases phosphorylating SpoOA. Sda itself is inhibited by the presence of the principle initiator of replication [36], DnaA, indicating the importance of a strong separation of sporulation and replication. Burkholder *et al.* showed that via the DnaA-Sda interaction a checkpoint, that inhibits activation of SpoOA and with it sporulation, was established [37]. Such checkpoint mechanisms ensure the proper timing of cell cycle events, while preventing interfering operations. Even if one of the steps takes longer than usual, the chronology of events is preserved by stopping the cell-cycle at various checkpoints. In this way the control system does not trigger the next step in the cycle before the previous one has been completed. Most cells possess checkpoints for cell size, where the cell-cycle is halted until the cell has grown to an appropriate size. Other checkpoints, like the one for sporulation, allow the cell to check that DNA replication is complete before proceeding to sporulation initiation. Checkpoints also offer the possibility to regulate the control system by external signals [38].

4.2 Bistability

Although exposed to the same environmental conditions, populations of genetically identical bacteria are sometimes heterogeneous, with certain genes being expressed in a non-uniform manner across the population [2, 39–42]. The benefits of such non-genotype-derived heterogeneity lie in the enhanced adaptability to environmental changes of the population as a whole [2–4]. In some cases, heterogeneity is manifested by the bifurcation into distinct subpopulations, a phenomenon known as bistability [41, 43] (Fig. 4.4). Bistability arises from uni-modal noise in the expression of a master regulatory gene (in our case ComK). If a cell passes a threshold in expression of this gene, the quantitative change becomes qualitative and a new pattern of gene expression emerges: the population bifurcates into coexisting cell types. Bistability implies that a regulatory system can switch between two alternative states but cannot rest at intermediate states. Two mechanisms have been proposed to drive this kind of bifurcation [44]. The first mechanism requires that the master gene (*comK*) is positively auto-regulated and that it responds to itself non-linearly (Fig. 4.5a). Cells with an activated autoregulatory

loop will quickly exceed a specific threshold concentration of the master regulator and accumulate it at high levels inside the cell. A second mechanism (Fig. 4.5b) requires the presence of a pair of mutually repressing repressors. If R_2 is inactivated, R_1 is produced, shutting off the synthesis of R_2. Note that this is equivalent to positive autoregulation, because the increase in R_1 results in even more R_1 production.

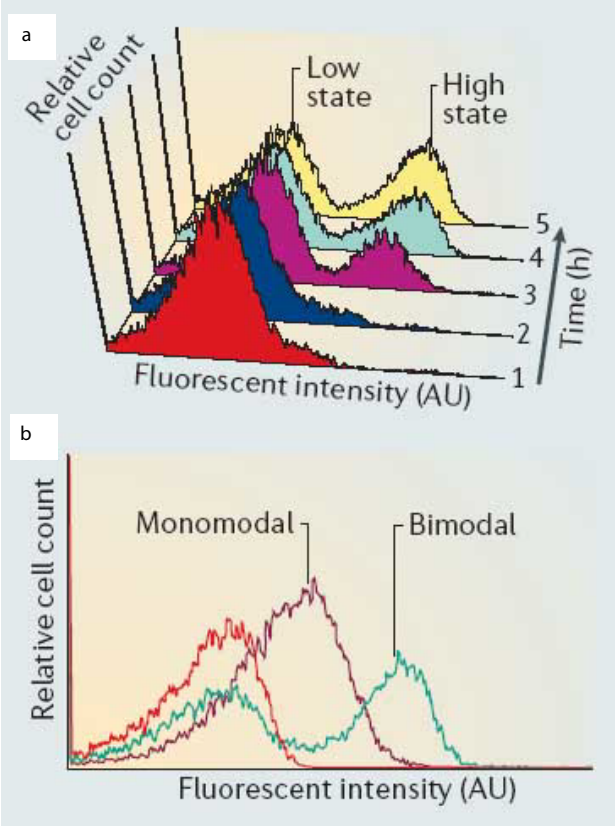

Figure 4.4: Bistable expression patterns a) with increasing time, a fraction of the population begins expressing GFP and a clear bistable expression pattern is established. b) red: background fluorescence of a non expressing culture, purple: expression pattern of a culture in which cells express at both low and high level but in a monomodal fashion, green: typical bistable expression pattern [42].

Bistable systems have been analysed using single-cell analysis and quantification of fluorescence through fluorescence microscopy, which was also done in this study. In this work a ComK-*GFP* reporter construct was used to visualize the ComK concentration within a single cell. With increasing time, a fraction of the population begins expressing GFP and a clear bistable expression pattern is established (see Fig. 4.4 for schematic presentation or Chapter 7, Fig 7.1 for bistable expression in *Bacillus subtilis*). An ,,on"

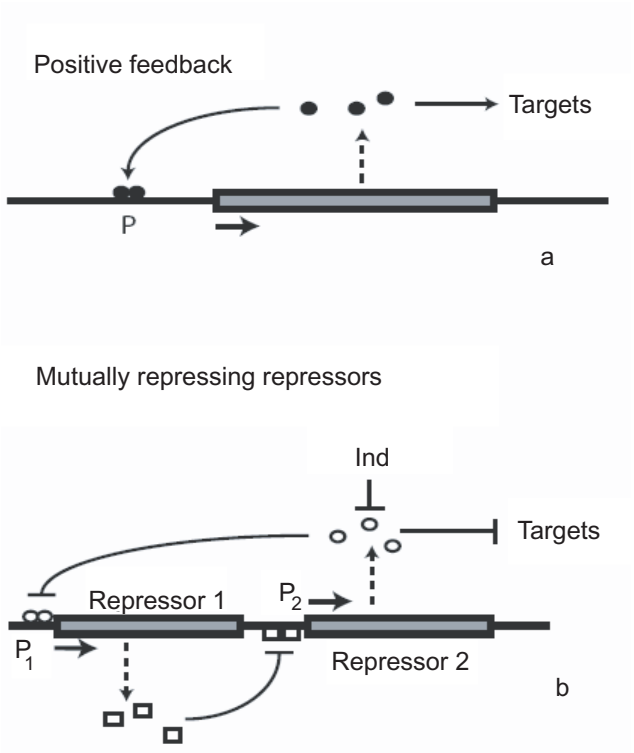

Figure 4.5: Two network configurations that lead to bistable expression a) A positive transcriptional autoregulatory loop together with cooperativity leads to bistability [45]. b) Two mutually repressing repressors induce bistability [46]. Figure after Dubnau and Losick 2006 [41].

and an ,,off" population would be observed when the low state corresponds to non-fluorescent cells. In our case the low state (B-state) corresponds to weakly fluorescent cells, whereas the high state (K-state) represents the fraction of competent cells.

5 Basic theoretical concepts

This chapter presents the basic theoretical principles necessary to characterize gene regulatory networks such as the network responsible for the development of competence in *Bacillus subtilis.*
Transcription regulation networks describe the interactions between transcription factor proteins and the genes that they regulate [47–51]. Transcription factors usually respond to biological signals and accordingly change the transcription rate of genes, allowing cells to make the proteins they need at the appropriate times and amounts. A regulation network as the one for competence development contains over 100 genes and proteins interacting with each other. To understand such complex networks, scientists started to build up similar complex models [52] in order to conceptually describe, define and analyze cellular signaling at a molecular level. Recent work shows that most transcription networks have something in common: they contain a small set of recurring regulation patterns, so called network motives [47,53,54]. A good overview of theory and experimental approaches concerning network motives is given by Uri Alon in his review of June 2007 [55]. Particularly in bistable systems motives like negative or positive autoregulation (NAR or PAR, respectively) are abundant. Those are usually characterized by the presence of a key regulator (in case of competence development this would be ComK) [42]. This key regulator is responsible for the regulation of most genes active in the studied network. Therefore, a good part of the system can be understood by analysing production and degradation of the key regulator only.

5.1 Production of the key regulator: Hill equation

The production rate of any given protein in a system is dependent on the transcription rate of the protein´s gene. In the simplest case the transcription rate is constant and leads to a linear increase of the protein concentration. This may be the case when the protein concentration stays very low over time. As soon as regulatory feedback loops (positive or negative) are present, the dynamics of protein expression becomes non-linear. In bistable systems, featured by so-called key regulators that are only active when present as a multimer [42], regulatory feedback loops are common. The development of competence also exhibits bistability. Here the main transcription factor ComK positively auto-regulates its own expression (PAR) [25], by binding as a dimer of dimers to it´s own promoter (Fig. 5.1 and Fig. 5.2). PAR leads to slowed response times

and cell-cell variation in protein levels [55]. This is due to a slow protein production at early stages. Production picks up only when the protein concentration approaches the activation threshold for its own promoter. Thus the desired steady state is reached in an S-shaped curve.

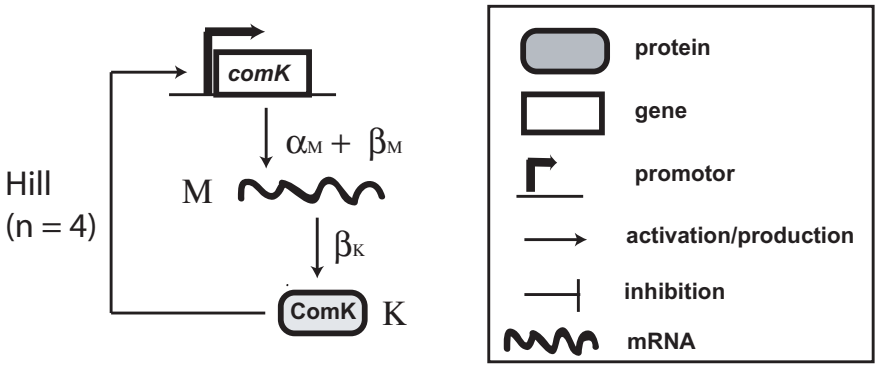

Figure 5.1: Production of the key regulator, ComK ComK activates its own expression by binding as a dimer of dimers to its own promoter (cooperativity n=4). Production of the *comK* mRNA [M]: basal *comK* mRNA production given by rate α_M, *comK* mRNA production in presence of active autocatalytic feedback loop of *comK* given by rate β_M. Translation of ComK [K] given by maximal translation rate β_K.

The multimerization of the key regulator is often related to cooperativity and occurs when the affinity of a given macromolecule for its ligand changes with the amount of ligand already bound. If the affinity is increased, one speaks of positive cooperativity versus negative cooperativity when binding of the ligand lowers the affinity for another ligand. Assigned for the production rate of our key regulator, cooperativity takes place when the key regulator itself binds to its own DNA thereby in- or decreasing its own production rate.

A quantitative method for characterizing binding cooperativity is provided by the Hill coefficient n [56]. The macromolecule (in our case the DNA = D) is assumed to bind to n ligands (in our case the key regulator itself = K) simultaneously to form the complex DK.

$$D + nK \underset{\kappa_{-1}}{\overset{\kappa_1}{\rightleftarrows}} DK \tag{5.1}$$

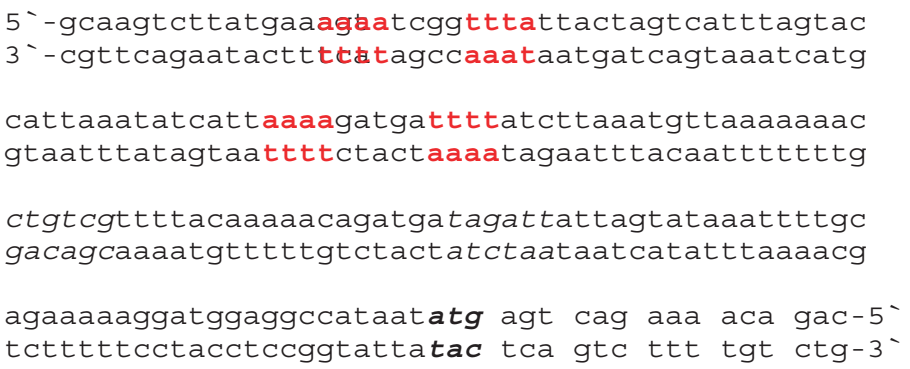

Figure 5.2: The *comK* promoter Red: the ComK-dimer recognition sequences (AT-boxes), italic/black: the -35 and the -10 promoter sequence, bold/black: the ATG start-codon.

At equilibrium the probability for the for- and backward reaction to happen are equal, therefore:

$$[D] \cdot [K]^n \cdot \kappa_1 = [DK] \cdot \kappa_{-1} \tag{5.2}$$

Hence the dissociation constant (K_d) equals to:

$$K_d = \frac{[D] \cdot [K]^n}{[DK]} = \frac{\kappa_1}{\kappa_{-1}} \tag{5.3}$$

At equilibrium, the ratio of bound to total receptors is given by the Hill equation:

$$\frac{Bound}{Total} = \frac{[DK]}{[D] + [DK]} \tag{5.4}$$

or

$$\frac{Bound}{Total} = \frac{[K]^n}{K_d + [K]^n} \tag{5.5}$$

To obtain the production rate of the key regulator ComK, rate equations have to be set up (see section 5.3, this chapter). To do so, one has to consider the production rates' dependence on transcription and translation of ComK, whereas the translation of ComK

itself is depending on the mRNA level $[M]$ available through transcription of the *comK* gene. To obtain $[M]$, the constant (ComK independent) production of ComK together with the non-linear, ComK dependent production triggered by the auto-catalytic feedback loop of ComK have to be taken into account.

5.2 Michaelis-Menten degradation

The key regulator of a given system has also a certain deactivation rate (see Fig. 5.3 for degradation of ComK), which normally can be described by a linear-type function [42]. However, as mentioned earlier (see chapter 4), ComK, the main transcription factor of the *Bacillus subtilis* competence system, is degraded by the protease activity of the MecA/ClpC/ClpP complex. In the stationary growth phase approximately 300 protease complexes are responsible for the degradation of over 10000 ComK proteins. Therefore degradation of ComK can not be described by a linear-type function. Instead, degradation of ComK is more likely an enzymatic reaction obeying Michaelis-Menten kinetics .

Figure 5.3: Degradation of the key regulator, ComK Degradation of ComK by Michaelis Menten kinetics with the rate δ_K

The Michaelis-Menten theory describes the kinetics of many enzymatic reactions. The enzyme $[E_{free}]$ binds its substrate [S], thereby building the enzyme/substrate complex [ES] and catalyses a specific reaction, for example the degradation of the substrate, or the conversion of the substrate into a product [P]. However, the Michaelis-Menten theory is valid only if the concentration of the enzyme is much less than the concentration of the substrate and if the concentration of the enzyme stays constant in time. It postulates that enzyme (catalyst) and substrate (reactant) are in fast equilibrium with their complex, which then dissociates to yield product and free enzyme. To determine the maximum rate of an enzyme mediated reaction, the substrate concentration (here ComK $= [K]$) is increased until a constant rate of product formation is achieved (Fig. 5.4). This is the maximum turnover rate V_{max}, also called maximum velocity of the enzyme. In this state enzyme active sites are saturated with substrate. With increasing substrate concentration [S], the enzyme $[E_{free}]$ is asymptotically approaching its maximum

turnover rate, V_{max}, but never actually reaching it. Therefore, the characteristic value for the enzymes' efficiency is defined by the substrate concentration at half-maximum velocity $V_{max}/2$. This K_M value is also called the Michaelis-Menten constant.

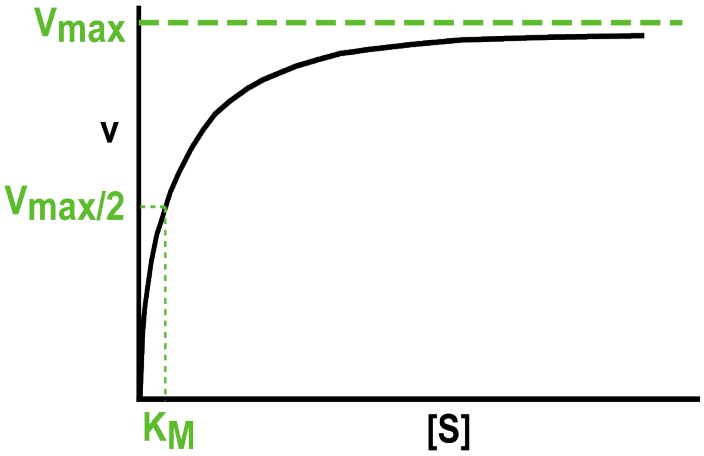

Figure 5.4: Michaelis-Menten kinetics Saturation curve for an enzyme showing the relation between the concentration of substrate $[S]$ and turnover rate $[V]$.

The enzymatic reaction is supposed to be irreversible, meaning that the product does not rebind the enzyme. Since in our case ComK is degraded, no product occurs.

$$E_{free} + K \underset{\lambda_{-1}}{\overset{\lambda_1}{\rightleftarrows}} EK \xrightarrow{\lambda_2} E_{free} + K_{degraded} \tag{5.6}$$

Using the quasi steady state approximation, the concentration of the intermediate is assumed to equilibrate much faster than the concentration of the product, i.e. its time derivative is zero:

$$\frac{d[EK]}{dt} = \lambda_1[E_{free}][K] - \lambda_{-1}[EK] - \lambda_2[EK] = 0 \tag{5.7}$$

rearranging gives

$$[EK] = \frac{\lambda_1[E_{free}][K]}{\lambda_{-1} + \lambda_2} \tag{5.8}$$

With the total concentration of the enzyme $[E_{tot}]$ being a sum of the enzyme free $[E_{free}]$ in solution and the enzyme bound to the substrate $[EK]$:

$$[E_{tot}] = [E_{free}] + [EK] \tag{5.9}$$

and the Michaelis Menten constant given by:

$$K_m = \frac{\lambda_{-1} + \lambda_2}{\lambda_1} \tag{5.10}$$

and equation (5.7) simplifies to:

$$[EK] = \frac{([E_{tot}] - [EK])[K]}{K_m} \tag{5.11}$$

or rearranged:

$$[EK] = [E_{tot}]\frac{1}{1 + \frac{K_m}{[K]}} \tag{5.12}$$

the rate of the reaction is (with $\lambda_2[\mathrm{E}_{tot}] = \mathrm{V}_{max}$):

$$\frac{d[K_{degraded}]}{dt} = V_{max}\frac{[K]}{K_m + [K]} \tag{5.13}$$

Please note that proteases normally degrade not only one substrate. Therefore the substrates compete for the enzyme to react and those competitive reactions have then also to be taken into account. In our case ComS competes with ComK for binding to the MecA/ClpC/ClpP protease complex without being degraded. This limits the amount of free protease complexes available for ComK. For further details see Chapter 8.

5.3 Rate equations

To obtain information about the concentration of a specific protein at a specific time rate equations have to be set up. To determine the rate equation for a particular system one combines the reaction rate with a mass balance for the system. For a generic reaction nA + mB $\xrightarrow{\kappa}$ C, the simple rate equation is of the form:

$$r = \kappa[A]^n[B]^m \tag{5.14}$$

for r beeing the production rate of C, with κ the rate constant and the exponents n and m as the reaction orders.
A zero-order reaction (n = m = 0) has a rate which is independent of the concentration of the reactants. Increasing the concentration of the reacting reagents will not speed up the rate of the reaction. For our key regulator ComK this is the case at low ComK concentrations when the positive feedback loop has not been activated yet.

$$r = \kappa \tag{5.15}$$

with r the reaction rate and κ the rate coefficient. Differently written as:

$$\frac{d[C]}{dt} = \kappa \tag{5.16}$$

A first-order reaction (n = 1, m = 0) depends on the concentration of only one reactant.

$$r = \kappa[A] \tag{5.17}$$

or

$$\frac{d[C]}{dt} = \kappa[A] \tag{5.18}$$

A second-order reaction depends on the concentration of one second-order reactant (n = 2, m = 0), or two first order reactants (n = 1, m = 1) .

$$r = \kappa[A]^2 \quad \text{or} \quad r = \kappa[A][B] \tag{5.19}$$

or

$$\frac{d[C]}{dt} = \kappa[A]^2 \quad \text{or} \quad \frac{d[C]}{dt} = \kappa[A][B] \tag{5.20}$$

Higher order reactions are usually very rare or composed of consecutive binary reactions and are therefore not mentioned in this chapter.

5.4 Stochastic Simulations

Cell to cell variability in protein concentration is caused by stochasticity in chemical reactions. Network motives like positive autoregulation can amplify these stochastic effects leading to broad protein level distributions represented by the distributions of fluorescence values in the experiment. As one will see in chapter 7 the concentration of our key regulator ComK does indeed vary from cell to cell and the whole distribution of protein levels is shifted to higher values with time. Theoretical predictions were necessary to accomplish stochastic simulations using the Gillespie algorithm to describe the behaviour of the system of competence development in *Bacillus subtilis*. This has been done in collaboration with Jan-Timm Kuhr (LS Frey). Single ordinary differential equations are usually not suitable to describe the behaviour of a complex system, since they only give the mean values, but not the distributions of the mean. Therefore a master equation has to be set up.

The Master Equation:
The master equation is a set of first-order differential equations describing the time-evolution of the probability of a given system to occupy each one of a discrete set of states:

$$P(S, t \mid S_0, t_0) \tag{5.21}$$

where P is the probability that the system, starting in state S_0 at time t_0 is found in state S at time t.
To give an example, consider a simple system with only two possible states. In case of competence development in *Bacillus subtilis* cells can either be in the state C (the competent state) or the state NC (the non-competent state). Then possible states and transitions can be characterized and chemical reaction equations can be set up:

$$NC \xrightarrow{\alpha} C \tag{5.22}$$

$$C \xrightarrow{\beta} NC \tag{5.23}$$

with α the reaction rate that a non-competent cell becomes competent, and β the reaction rate that a competent cell reenters the non-competent state. The ordinary differential equations will give us the mean of the probability for one cell being in the competent or non-competent state:

$$\frac{d[C]}{dt} = \alpha[NC] - \beta[C] \tag{5.24}$$

$$\frac{d[NC]}{dt} = \beta[C] - \alpha[NC] \tag{5.25}$$

Considering 100 cells, each of these cells can now be either in the competent or non-competent state. Therefore, the total system is now described by 100 different states,

0	bacteria	[C],	100	[NC]
1	bacterium	[C],	99	[NC]
	.			
	.			
100	bacteria	[C],	0	[NC]

and the system can switch between these states with specific rates.

$$1C \underset{\beta\cdot 2}{\overset{\alpha\cdot 99}{\rightleftarrows}} 2C \underset{\beta\cdot 3}{\overset{\alpha\cdot 98}{\rightleftarrows}} 3C... \underset{\beta\cdot 100}{\overset{\alpha\cdot 1}{\rightleftarrows}} 100C \tag{5.26}$$

The master equation gives us now the probability $P(C,t)$ of one single bacterium to be competent or non-competent, with $W(\mathcal{C}' \to \mathcal{C})$ the rate to reach state C starting from state C':

$$\frac{d}{dt}P(\mathcal{C},t) = \sum_{\mathcal{C}'}[W(\mathcal{C}' \to \mathcal{C})P(\mathcal{C}') - W(\mathcal{C} \to \mathcal{C}')P(\mathcal{C})] \tag{5.27}$$

Unfortunately, as soon as coherences become non-linear, it becomes very complex to solve the equation and to calculate the time evolution in this manner. To analyze competence development in *Bacillus subtilis* on a single cell level, deterministic rate equations are also not the method of choice since they rely on bulk reactions that require the interactions of huge amounts of molecules and therefore do not accurately predict cellular reactions on the single cell level. For reactions with few participating molecules stochastic effects have to be taken into account.

The Gillespie algorithm

The Gillespie algorithm was developed in 1977 [57] by Dan Gillespie to simulate chemical or biochemical systems of reactions efficiently and accurately using limited computational power. The algorithm is particularly useful for simulating reactions within cells where the number of reagents is typically in the tens of molecules or less. The Gillspie algorithm allows a discrete and stochastic simulation of a system with few reactants, because every reaction is explicitly simulated. It is possible to generate single trajectories by repeatedly picking the next reaction stochastically and executing it, starting from a well-defined, initial state. The Gillespie algorithm allows to simulate complex systems

whose Master equations are insolvable. By allowing the chosen parameters to vary one can even introduce extrinsic noise to the system.

Imagine a system that can switch between the two states NC and C:

$$NC \underset{\kappa}{\overset{\lambda}{\rightleftarrows}} C \tag{5.28}$$

The Gillespie algorithm describes which reaction will happen next and when this reaction will take place. Here it is important that we consider a well stirred system so that reaction rates do not change with time and no spatial degrees of freedom are present.

The Algorithm:
1.) Initialization:
- initial values for the reaction rates and the participating species have to be set.
- set time $t = 0$ and choose an end time t_e (or some other final condition).
2.) Calculate propensities
3.) Determine time-point and type of reaction happening next
4.) Update the system:
After the reaction of step 3 has taken place, the state of the system has changed and the initial values have to be adapted.
5.) As long as $t < t_0$ or the final condition is not met yet, return to step 2.

For our example the initial values may be $NC = 99$ and $C = 1$, with the constant rates λ and κ. Therefore the probability that NC goes over to C is:

$$P(NC \to C) = \frac{99\lambda}{99\lambda + \kappa} \tag{5.29}$$

and the probability for C to go over to NC is:

$$P(C \to NC) = \frac{\kappa}{\kappa + 99\lambda} \tag{5.30}$$

The Gillespie algorithm will then provide one possible time evolution if the system is well-stirred. To make statements on the evolution of mean, variance or the distribution itself, one has to perform an ensemble-average over many such runs.

5.5 Bistable Behaviour

An interesting property of non linear systems is the development of bistability (see chapter 4). As we will see in chapters 7 and 8 parts of the *Bacillus* population switch from the non-competent state to the competent state and this transition is mainly due to the concentration of the main transcription factor of the system: ComK. In the end the system has changed from being in one single state (non-competent) to a state were two different solutions (competent or non-competent) are possible for each single cell. This is called bifurcation. Bifurcations occur when a small smooth change made to the parameter values (the bifurcation parameters) of a system causes a sudden "qualitative"change in its long-term dynamical behaviour.

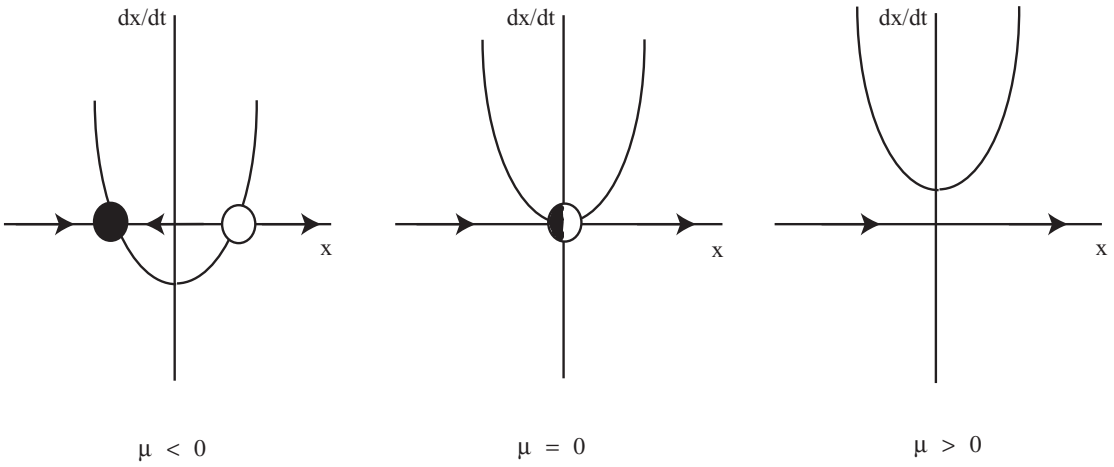

Figure 5.5: Saddle-Node Bifurcation Left: When μ is negative, two fixed points are present, one stable and one unstable. When $\mu = 0$, the fixed points coalesce into a half-stable fixed point, the bifurcation point. For $\mu > 0$ there are no fixed points at all.

There are two types of bifurcations: local and global ones. Local bifurcations can be analysed entirely through changes in the local stability properties of equilibria or other invariant sets as parameters cross through critical thresholds, while global bifurcations often occur when larger invariant sets of the system"collide" with each other or with the equilibria of the system.

One type of the local bifurcation is the saddle-node bifurcation in which two fixed points (or equilibria) of a dynamical system collide and annihilate each other. Fig. 5.5 shows the principle schematic of a saddle-node bifurcation. The prototypical example of a saddle-node bifurcation is given by the first-order system [58]:

$$\frac{dx}{dt} = \mu + x^2 \tag{5.31}$$

where μ is the parameter, which may be positive, negative or zero. When μ is negative, two fixed points are present, one stable and one unstable. When $\mu = 0$, the fixed points coalesce into a half-stable fixed point, the bifurcation point. For $\mu > 0$ there are no fixed points at all (according to Strogatz 2000 [58]).

6 Preparation techniques and experimental methods

6.1 Materials and preparation techniques

6.1.1 Materials and strains

The *Bacillus subtilis* strains used are all derivatives of strain BD 630 and are described in Table 6.1. *Bacillus* strains were grown in liquid competence medium [59] supplemented with glucose 0.5 %, L-histidine, L-leucine, L-methionine ($50\,\mu$g ml^{-1}) and chloramphenicol, kanamycin, erythromycin ($5\,\mu$g ml^{-1}) or spectinomycin ($100\,\mu$g ml^{-1}) at 37 °C (for further details on the medium see appendix A). Competent cells were prepared as described previously [59]. BM 50 was created by transformation of genomic DNA of BD 2711 into BD 3458 [11, 60]. BM 77 was created by transformation of pUB110::T7T3::ComS [60, 61] into BD 2711. BM 101 was created by transformation of genomic DNA of BD 2711 into BD 2955 [11, 60]. For detailed strain description see Table 6.1.

Strain	Genotype	Source
BD 630	*his leu met*	-
BD 3458	*his leu met, rok- (spc^a), hag- (erm^a)*	[62]
BD 2711	*his leu met, comK-gfp (CBL^b, cat^a)*	[63]
BM 50	*his leu met, rok- (spc^a), hag- (erm^a), comK-gfp (CBL^b, cat^a)*	this study
BM 101	*his leu met, comK-gfp (CBL^b, cat^a), rok- (spc^a)*	this study
BM 77	*his leu met, comK-gfp (CBL^b, cat^a), mc^c comS (kan^a)*	this study
BD 2528	*his leu met, mc^c comS (kan^a)*	[64]
BD 2955	*his leu met, rok- (spc^a)*	[62]
BD 630 sda-	*his leu met, sda-(kan^a)*	Donation^d
BK 630 comK-	*his leu met, comK-(kan^a)*	Donation^d

Table 6.1: Strains

Strains *Bacillus subtilis* strains used in this study. ^{a} kan, cat, erm and spc stand for resistance to kanamycin, chloramphenicol and spectinomycin respectively. ^{b} Inserted by Campell like integration. ^{c} multicopy. ^{d} Gift of K. Stingl.

6.1.2 Preparation of conditioned and inhibitory media

To analyse the influence of quorum sensing represented by the pheromone ComX concentration it was necessary to obtain medium with a maximal pheromone ComX concentration, so called conditioned medium. Therefore T_0 BM 50 cells (cells at the transition point from the exponential to the stationary phase) were centrifuged at 13000 r.p.m. The supernatant was collected and exchanged with the medium of T_{-2} cells (T_{-2} = timepoint two hours before T_0 .
To test the hypothesis that competent cells release inhibitory substances into the surrounding medium in the stationary phase to exit the competent state after 2 hours, the supernatant of BM 50 T_2 cells (cells two hours after the transition point T_0) or supernatant of BD 630 *comK*- (with 0% transformation rate), was exchanged for medium of $T_{-0.5}$ cells.

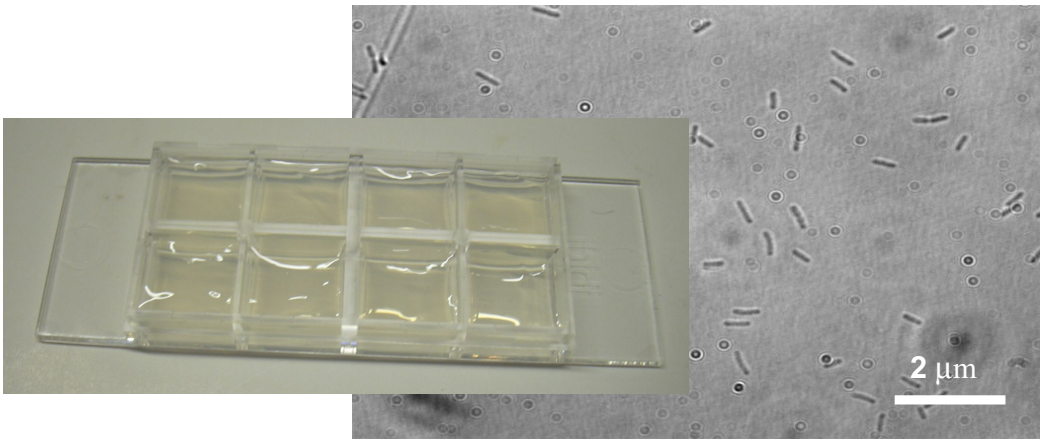

Figure 6.1: Bacteria fixation To immobilize the bacteria they were permitted to attach to an eight-well IBIDI-Chamber (ibitreat), covered with 1% agarose.

6.1.3 Preparation of polystyrene cover slides

For quantitative analysis of fluorescence in bacteria it is necessary that the single cell sits nice and flat without any movement on a specific surface. For time-lapse microscopy it is also essential to flush the bacteria with fresh medium and to supply them with enough oxygen. Therefore, the adhesion of the cells to the surface has to be very strong. On surfaces like polylysine or aminosilane *Bacillus subtilis* did only adhere as long as those surfaces were not flushed. Other silanes that enabled flushing, but contained chlorides

or other halogens inhibited competence. Surfaces like PDMS were also not useful since bacteria tended to grow in chunks. Only on a 2% polystyrene surface adhesion was strong enough (Fig. 6.2). Note that this adhesion was mainly due to the presence of the bacterial flagella since bacteria without flagella showed no adhesion at all.
To prepare 2% polystyrene surfaces cover slides were cleaned for 2h in 1% hellmanex-solution (Hellma, Mühlheim), rinsed 3 times with bidestilled water and dried with nitrogen. 100 μl of a 2% polystyrene-toluene solution were spin-coated onto cover slides at 3000 rpm for 30 sec. To remove toluene, cover slides were dried at 120°C under vacuum for 30 min.

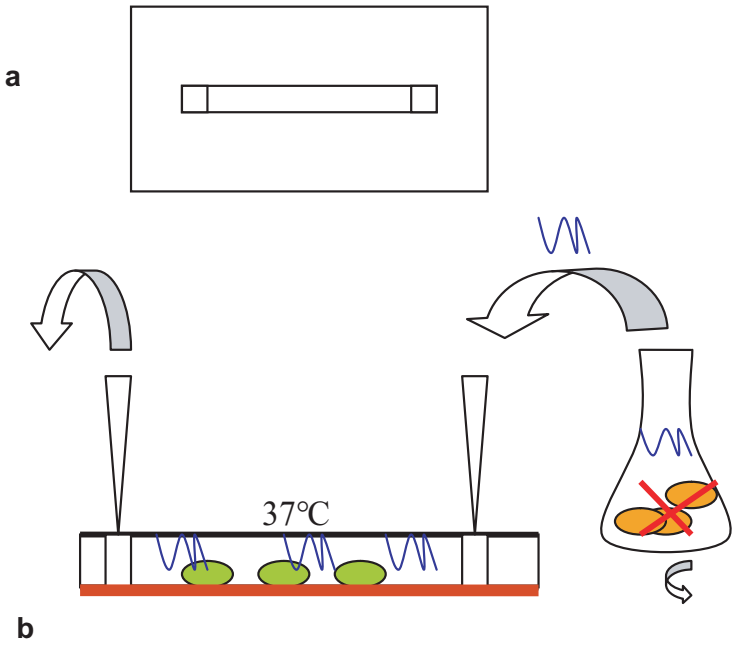

Figure 6.2: Bacteria fixation: For time-lapse microscopy bacteria were fixed on 2% polystyrene slides (red bottom line) and flushed with competence medium obtained from the supernatant of a parallel grown culture every 15 min. a) Top view of the flow chamber with fluidic channel. b) Side view of the flow chamber with cells attached to the polystyrene slide.

6.2 Experimental methods

6.2.1 Fluorescence microscopy

For snapshots of the whole bacterial population samples were taken during growth as indicated. Cells were permitted to attach to eight-well IBIDI-Chambers (ibitreat), and covered with 1% agarose (Fig. 6.1) . For single cell time-lapse microscopy cells were taken at T_0 and permitted to attach to a polystyrene-coated cover slide and mounted onto a flow chamber. An image was acquired every 15 min. To confirm that the conditions under the microscope were equal to the conditions in a shaking flask, the medium was exchanged directly after every exposure for the supernatant of a parallel culture grown in an Erlenmeyer flask (shaken at 300rpm, 37°C).
Microscopy was performed with a Zeiss Axiovert 200M microscope equipped with an Andor Digital Camera, and a Zeiss EC Plan-Neofluar 100x/1.30 Oil immersion objective. Andor software was used for image acquisition. The stability of absolute fluorescence values was verified using a microscope image intensity calibration kit (Molecular Probes, in Speck TM Green (505/515), 2.5 μm) or a microscope image intensity calibration kit (Molecular Probes, Focal Check TM fluorescence microscope test slide ♯3, F 36914). Microsopheres showed a deviation of mean gray value of less than 1% under the experimental conditions used for detection of GFP fluorescence. Homogeneity of illumination was tested using fluorescent slides and the maximum deviation was less than 5%.

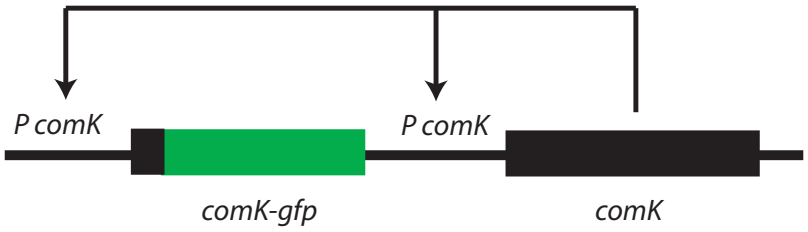

Figure 6.3: $ComK - GFP$ **promoter construct** All strains used in this study contain a functional *comK* gene standing under the *comK* promoter and carry additionally a *comK*-GFP reporter construct also standing under the control of the *comK* promoter. Therefore, the expression rates, i.e. the production rate of ComK and GFP, as well as the time-point of expression are expected to be similar. The *comK*-GFP reporter is constructed by connecting the first part of the gene sequence of *comK* (black) with the gene coding for GFP (green) [63].

6.2.2 Analysis

To analyse the ComK protein levels in single cells, strains containing a GFP-*comK* reporter construct (Fig. 6.3) standing under the control of the promoter of *comK* (P_{comK}) in addition to the original copy of the *comK* gene were used [63]. As the GFP molecule is not likely to be a substrate for MecA/ClpC/ClpP proteolysis, the concentration of GFP controlled by P_{comK} is not a direct measure of ComK. However, the expression rates, i.e. the production rate of ComK and GFP, are expected to be similar, as the complete native promoter of *comK* including all native signals for *comK* expression is present. ComK expression in single cells was analysed as the fluorescence intensity (FI) represented by the mean gray value (measured in arbitrary fluorescence units (FU)).
Images were processed using ImageJ software. The image background was corrected using a rolling ball algorithm with a radius of 50. An intensity threshold tool was used to delimit the boundaries of the cells in the bright field image (Fig. 6.4 left).The areas encircled by the boundaries were then selected as regions of interest (ROI) using the ImageJ wand tool. These ROIs were then applied to the fluorescence images and the mean gray value of each cell was obtained using the ImageJ measurement tool.

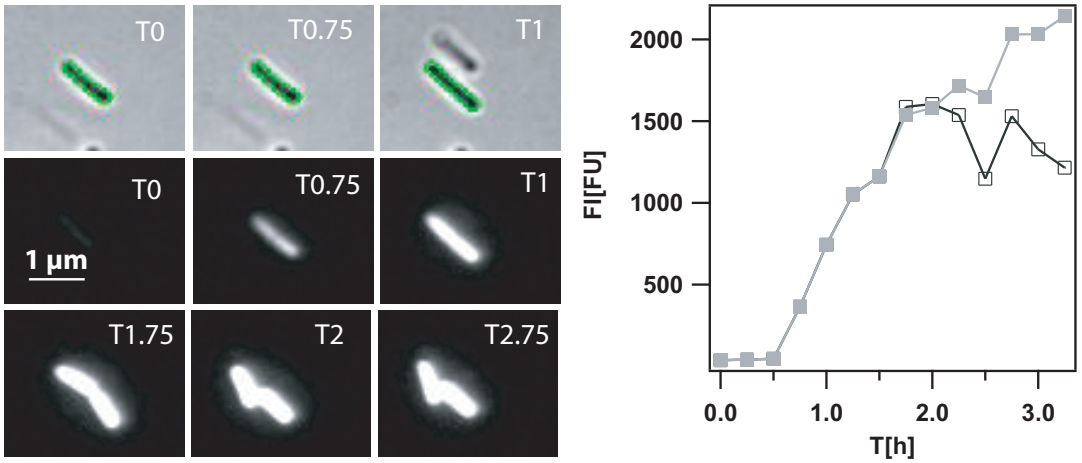

Figure 6.4: Analysis method An intensity threshold tool was used to delimit the boundaries of the cells in the bright field image. The areas encircled by the boundaries were then selected as regions of interest (ROI). These ROIs were then applied to the fluorescence images and the mean gray value of each cell was obtained. Left) Time-lapse of a switching cell. Scale bar 1μm. Green contour: outline of the cell. Right) Corresponding plot of fluorescence intensity FI of a) as a function of time.

As *Bacillus subtilis* showed a bimodal distribution with time [65], the switching threshold for analysis of whole bacterial populations was defined as the value that best separated the two distributions (100, 150 and 120 mean grey value for BD 2711, BM 50 and BM 77 respectively). Cells with lower fluorescence intensity were defined as non-competent, and cells with higher intensity were defined to be in the K-state. For single cell time-lapse experiments the switching time was defined as the point where the intensity increased sharply in the FI(t) plot. This lead to slightly lower values for the threshold but the fraction of competent cells was not affected.
The auto-fluorescence level of non-competent BD 630 cells was $FI_{autoT_{-2}} = 23 \pm 1$ FU, the width of the Gaussian fit was $\sigma_{autoT_{-2}} = 7.6 \pm 0.1$ FU [65]. To verify that auto-fluorescence level did not shift until the basal fluorescence level reached its maximum, the auto-fluorescence level of BD 2528 [64] at T_1 was tested and showed $FI_{autoT_{-2}} = 23 \pm 1$ FU, $\sigma_{autoT_{-1}} = 6.4 \pm 0.14$ FU.
For analysis of the basal expression rate, the average mean grey value of non-competent cells was calculated for each time point. The expression rate r was determined as the first derivative with respect to time of a sigmoid fit to the fluorescence data.

6.2.3 GFP maturation

To determine the time delay between expression of the GFP reporter and the onset of fluorescence, BM 77 was grown in competence medium on a platereader and erythromycin was added at 270 min.

Erythromycin inhibits the protein biosynthesis of gram-positive bacteria like *Bacillus*. Increase of fluorescence after 270 min must therefore be due to folding of already synthesized GFP (Fig. 6.5b). Assuming first-order kinetics, the data were fitted with a single exponential function and a characteristic time of 5 min was obtained. As switching into the K-state occurred on a significantly longer time scale, the time scale was not corrected for GFP folding. To determine whether GFP was degraded on the relevant time scale of 3h, the same assay was used (Fig. 6.5a) and it was found that the fluorescence intensity did not decrease within 3h. Therefore, the expression rate and switching rate were not corrected for GFP degradation.

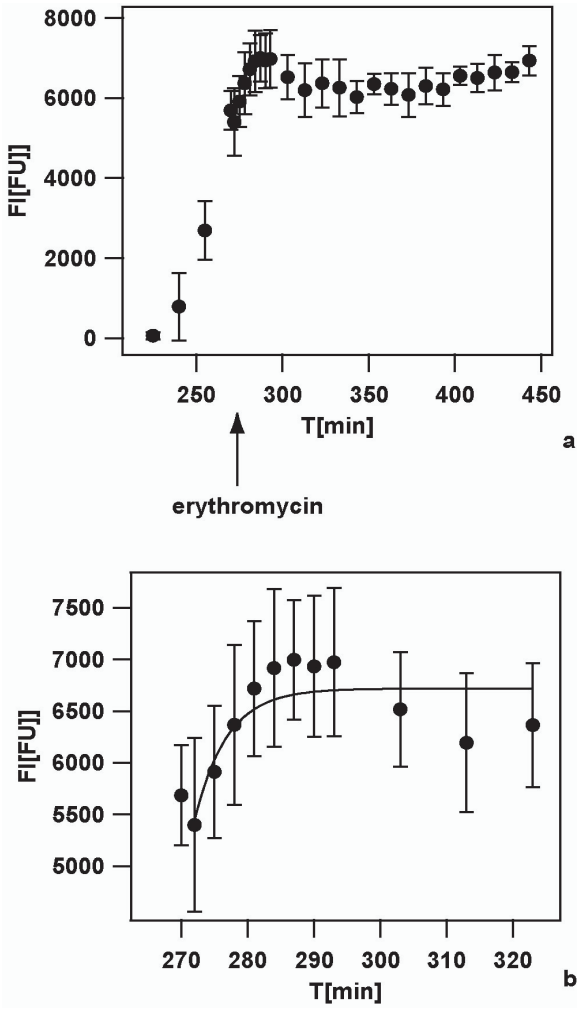

Figure 6.5: Analysis method Fluorescence intensity values of BM 77 during growth on a plate reader. Erythromycin was added at T = 270 min. a) Stability of GFP fluorescence b) Time scale of GFP maturation. Full line: exponential fit to the black circles yielding a half life time of 5 min. Note that the half life time may even be overestimated considering the time delay of inhibition of protein biosynthesis by erythromycin used in our folding assay.

6.2.4 Verification of growth conditions

Bacteria have to be fixed on specific surfaces for quantitative time-lapse microscopy. It is therefore of high importance to verify that the conditions on a microscopic slide are equal to the normal growth conditions of the bacterium. For the development of competence in *Bacillus subtilis* this was done by comparing the temporal development of the fraction of cells in the K-state of cells grown normally in a well shaken Erlenmayr flask, with the fraction of cells in the K-state of cells grown on a microscopic slide (Fig. 6.6). Using this method the conditions on the microscope could be adjusted until the same amount of cells in the K-state was obtained as when grown in an Erlenmayr flask. Adjustments have been for example, changes in surface preparation (see above: section 6.1.3), usage of additional filter sets or the improvement of oxygen supply.

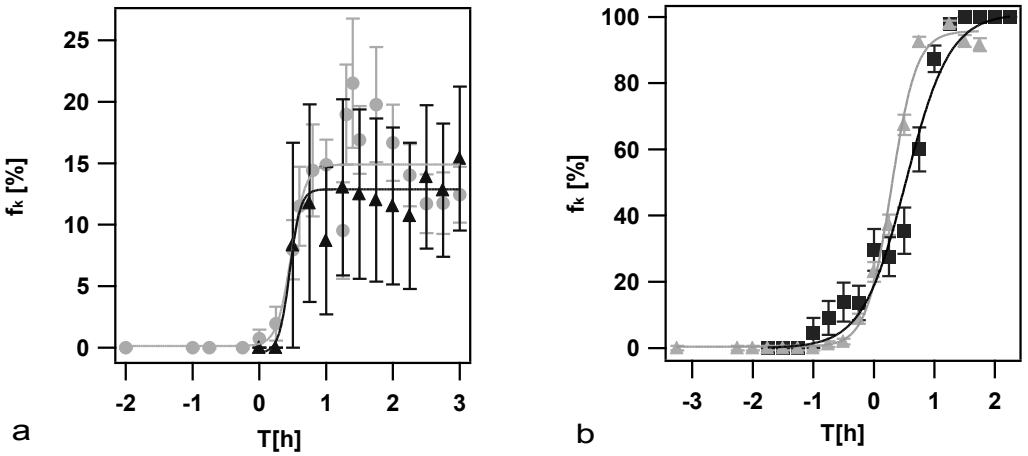

Figure 6.6: Comparison of growth conditions a) grey circles: wt grown in an Erlenmayr flask, black triangles: wt grown on a microscopic slide. b) grey triangles: *rok-* grown in an Erlenmayr flask, black squares: *rok-* grown on a microscopic slide. Full lines: best fit to a sigmoid function.

7 Basal expression rate of ComK sets a switching window into the K-state of *Bacillus subtilis*

This chapter, as well as the following ones, presents the main results of this work. The importance of the general transcription factor ComK as the main regulating protein of the competence system was shown previously [24] and it has been known that ComK positively auto-regulates its own expression. Maamar and Dubnau revealed the necessity of the positive feedback-loop of ComK for the establishment of bistability [60]. Only ∼15% of the cells of a given population in *Bacillus subtilis* become competent in the stationary phase. While Maamar *et al* [5] pointed out the importance of stochastic effects, especially intrinsic noise, the work presented in this chapter pinpoints a second factor, the existence of a well defined switching window, responsible for a fraction of the cells remaining in the non-competent state.
To address the question why not all cells switch into the K-state and the system remains bimodal in the stationary phase, a single cell assay was used: *Bacillus* cells were grown in competence medium [59] (shaking at 300 rpm, 37°C). A sample of the whole population was taken every 15 min. Please note that the cells watched differ from time-point to time-point. Cells were allowed to attach to eight-well IBIDI-Chambers (ibitreat) and covered with 1% agarose to fix the cells and enable appropriate data acquisition. For detailed experimental information see chapter 6 and appendix A.
The results presented in this chapter have been published previously in [65]. Since ComK is known to be of high importance for competence development, a ComK-gfp reporter construct was used to visualize ComK expression in individual cells (see chapter 6) [63]. The experiments showed that *comK* expression was measurable before T_0 (the transition into the stationary phase), and the expression rate of *comK* in non-competent cells, i.e. the basal expression rate, was determined. The maximum of the basal expression rate preceded the maximum of the switching probability and decreased to near zero values in the stationary phase. Together the data indicate that the fraction of cells that switch into the K-state is at least partially controlled by shut-down of *comK* expression in non-competent cells after T_0, thereby opening up a well defined switching window.

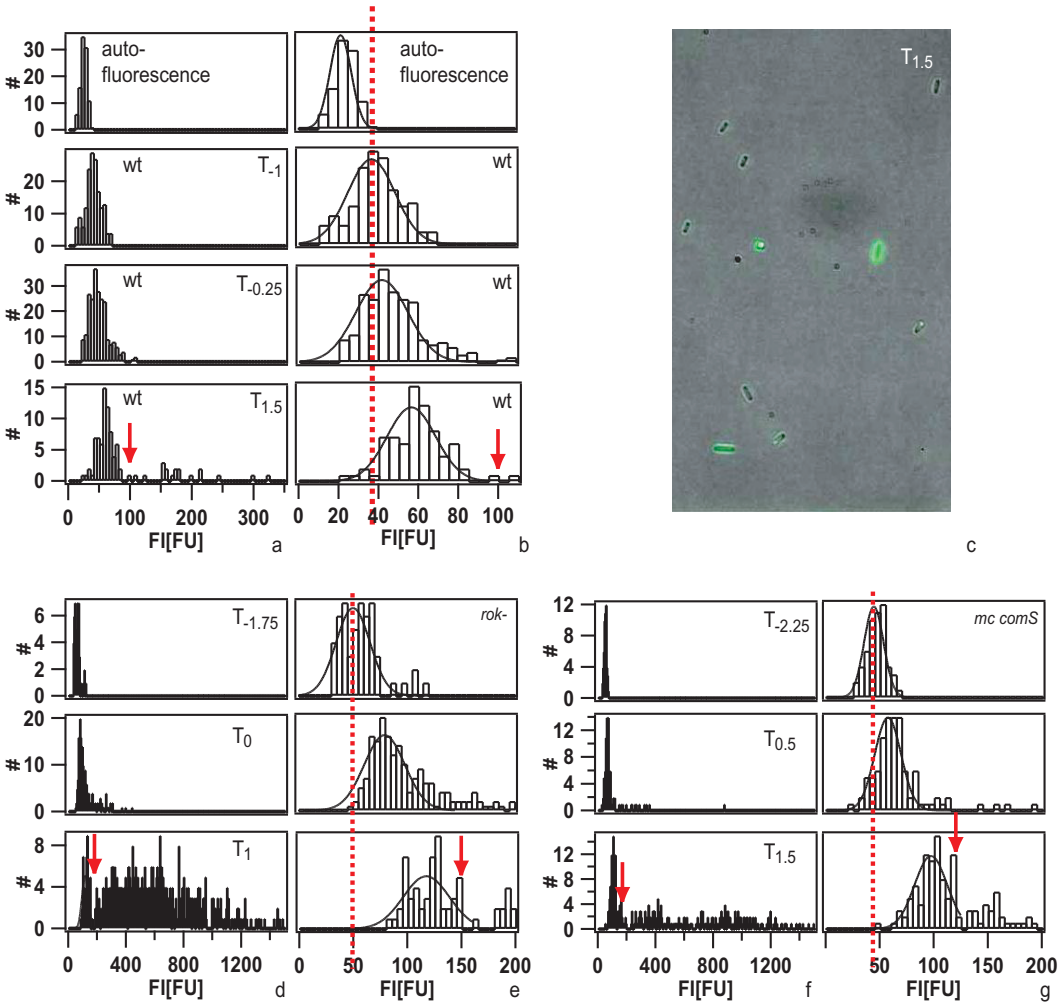

Figure 7.1: Bistability in *Bacillus subtilis* With increasing time more and more cells enter the K-state leading to a bimodal fluorescence distribution. a-c) BD 2711 (P_{comK}*-gfp)*; d and e) BM 50 (P_{comK}*-gfp), rok-* ; f and g) BM 77 (P_{comK}*-gfp), mc comS*; a, d and f) Distribution of fluorescence intensities of individual cells at various time points. The arrow depicts the threshold intensity chosen to distinguish between cells with basal *comK* expression and cells in the K-state (high *comK* expression). b, e and g) Details of the distribution of fluorescence intensities. Dotted lines: most likely FI at early time points. Full line: best fit to a Gaussian function. c) Overlay of a fluorescence micrograph (green) and a bright field image at $T_{1.5}$. a and b first line: distribution of autofluorescence of BD 630, 2-4 th line: distribution of fluorescence of BD 2711 = wild-type strain.

7.1 Switching rate into the K-state is not constant with time

The fraction of cells in the K-state was characterized as a function of time $f_K(T)$. To define whether an individual cell was in the K-state, quantitative fluorescence microscopy was used and the fluorescence intensity of GPF in individual P_{comK}-*gfp* cells was measured. Before T_0 i.e. before entry into the stationary phase, an unimodal distribution of mean gray values FI was found. Both the width and the average FI value of the distribution were clearly larger as compared with the distribution of autofluorescence (Fig. 7.1a and b). With increasing time, the distribution became bimodal, in agreement with previous studies [26, 60]. The switching threshold was defined as the value that best separated both modes of the distribution (see chapter 6). Cells with an intensity exceeding the threshold intensity were defined to be in the K-state.
Subsequently, the fraction of cells in the K-state was determined as a function of time. With the wild-type (P_{comK}-*gfp)*, a significant number of cells had switched into the K-state (Fig 7.2a) at T_0. Around $T_{1.5}$ the K-fraction saturated at $f_{Kmax} = 14 \pm 1\%$ (Fig. 7.2a). The fraction of K-cells as a function of time was well described with a sigmoid behaviour given by

$$f_K = f_{base} + \frac{f_{max}}{1 + exp[k(T_{half} - T)]} \tag{7.1}$$

with f_K the fraction of cells in the K-state, f_{max} the maximum fraction of cells in the K-state, T_{half} the half time and k the switching rate (Fig. 7.2b). (Fit parameters see Table B.1 in appendix B).
Smits *et al.* [26] had proposed that the repressor Rok was a candidate to lower the threshold for switching of basal ComK level, as Rok down-regulates the transcription of *comK* [62]. Therefore, the same experiment as above was done with a (P_{comK}-*gfp), rok*-strain (BM 50). The distribution of fluorescence intensities was unimodal at early times, became bimodal between T_{-1} and T_1 and at T_1 the lower peak disappeared (Fig 7.1d and e). Again, the fraction of cells in the K-state f_K increased with a sigmoid shape (Fig 7.2c). The maximum fraction of cells in the K-state was $f_{Kmax} = 99 \pm 3\%$ (Fig 7.2c).

The next question to answer was whether or not the concentration of ComS, another ComK regulating protein (see chapter 4), inside a single cell would influence the switching process. ComS inhibits the degradation of ComK by binding to the MecA/ClpP/ClpC protease complex (see chapter 4), thereby increasing ComK availability inside the cells. Hahn *et al.* [66] showed that when *comS* is over-expressed, ComK levels rise at earlier time points and that approximately 70 % of the cells entered the K-state [60]. With the ComS-overproducing strain, the distribution of fluorescence intensities was unimodal at

early times, and became bimodal around T_{-1} (Fig. 7.1f and g) . The K-fraction did not increase significantly after $T_{1.5}$ but remained at 83 ± 3% (Fig.7.2e).
Since all strains showed a sigmoidal switching behaviour, the switching rate p_{fK} was determined as the first derivative of the sigmoid fit to f_K with respect to time (Fig 7.2). The rate of escape from the K-state was not taken into account, as recent studies have shown that under the experimental conditions used in this study, the competent state was maintained until T_3 - T_4 [63, 67]. To determine the intrinsic switching rate p_K, the data were normalized to the fraction of non-competent cells [100%-f_K(T)] (Fig 7.2). The maximum values were $p_{Kmax} = 0.3 \pm 0.1$ h^{-1} for the wild type, $p_{Kmax} = 4 \pm 1$ h^{-1} for the *rok*- strain and $p_{Kmax} = 1 \pm 0.1$ h^{-1} for the ComS-overproducing strain. Therefore the *rok*- and the ComS-overproducing strain showed an increased switching rate as compared to the wild type strain.
In the simplest scenario, the cells would switch into the K-state at a constant rate once they reached a threshold concentration of ComK. In this case the fraction of cells in the non-competent state would decay like an exponential function. The data shown here are not in agreement with this simple assumption, as the fraction of cells in the K-state increased with a sigmoid shape. Therefore the switching rate is not constant with time.

7.2 Basal *ComK* expression sets a switching window

As Rok down-regulates the expression of *comK* it was interesting to see whether the basal *comK* expression rate was also enhanced as compared to the wild type and the *comS*-over-expressing strain. The expression rate r was measured as the first derivative with respect to time of the mean grey value of those cells that had not entered the K-state (Fig. 7.3). Unexpectedly, the basal fluorescence intesities, FI(T), saturated after T_0. FI(T) increased with a sigmoid shape similar to the fraction of cells in the K-state. However, FI(T) was shifted towards earlier times as compared with f_K(T), indicating that the basal expression rate r had a maximum and that the maximum expression rate was shifted to earlier times as compared with the maximum switching rate p_K. At the beginning of the exponential growth phase, the basal levels of *comK* expression were similar in the wild type (Fig. 7.3a and b) and the ComS-overproducing strain (Fig. 7.3e and f) but were elevated by a factor of ~ 2 in the *rok*- strain (Fig. 7.3c and d). Most likely, the *rok*- strain switched earlier than the wild type because basal expression rate was increased.
The expression rate was determined as the first derivative with respect to time of the sigmoidal fit (Fig 7.3). As expected, the maximum expression rate of the wild type and the ComS overproducing strain showed similar absolute values and the expression rate of the *rok*- strain was enhanced by a factor of 2.

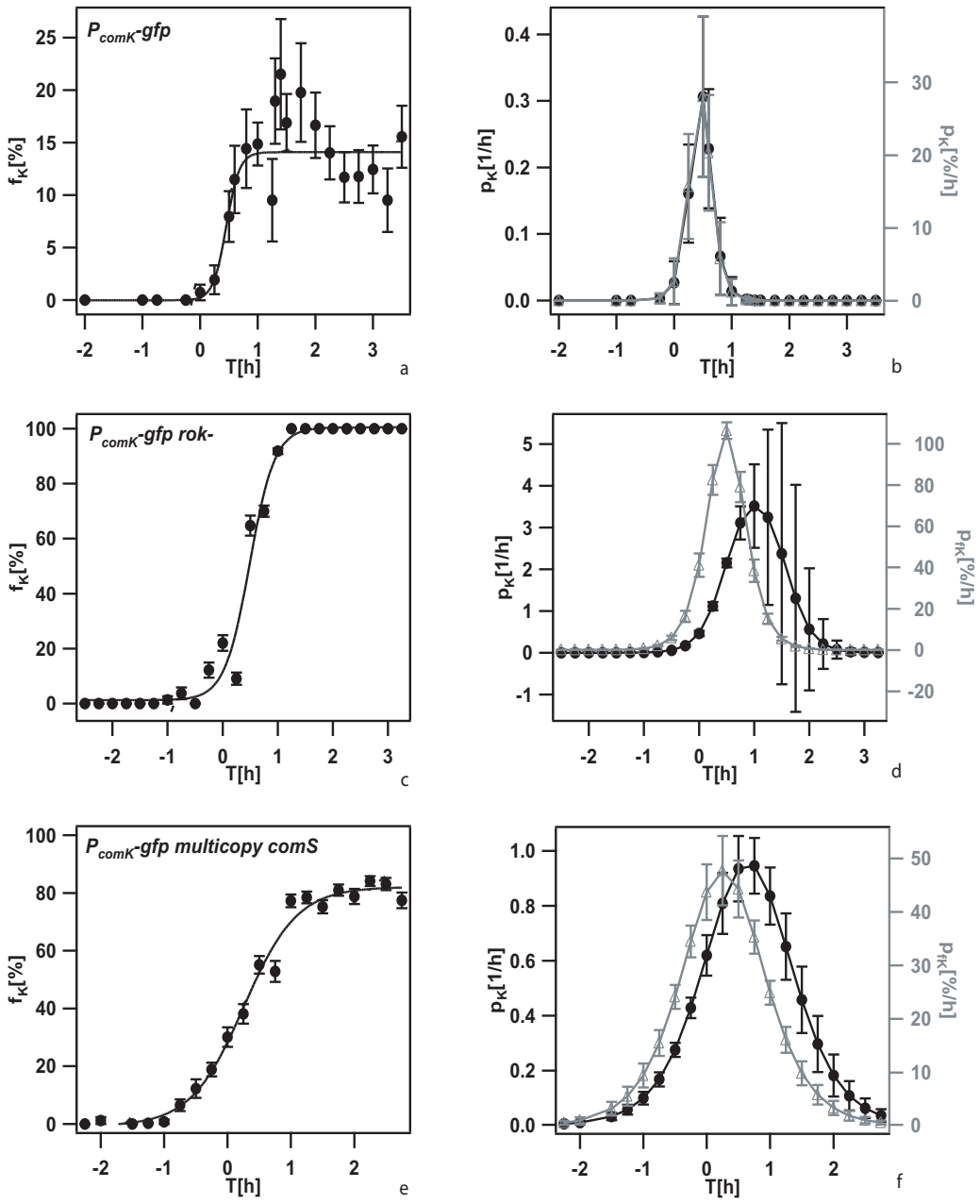

Figure 7.2: Fraction of cells in the K-state as a function of time and switching rate a, c and e). Fraction of cells in the K-state as a function of time f_K. Full line: best fit to a sigmoid function (Eqn. 7.1). b, d and f) Switching rate p_{fK} (grey triangles) and intrinsic switching rate p_K (black circles). a and b) BD 2711; c and d) BM 50; e and f) BM 77. Parameters for sigmoid fits are listed in Table B.1 in appendix B.

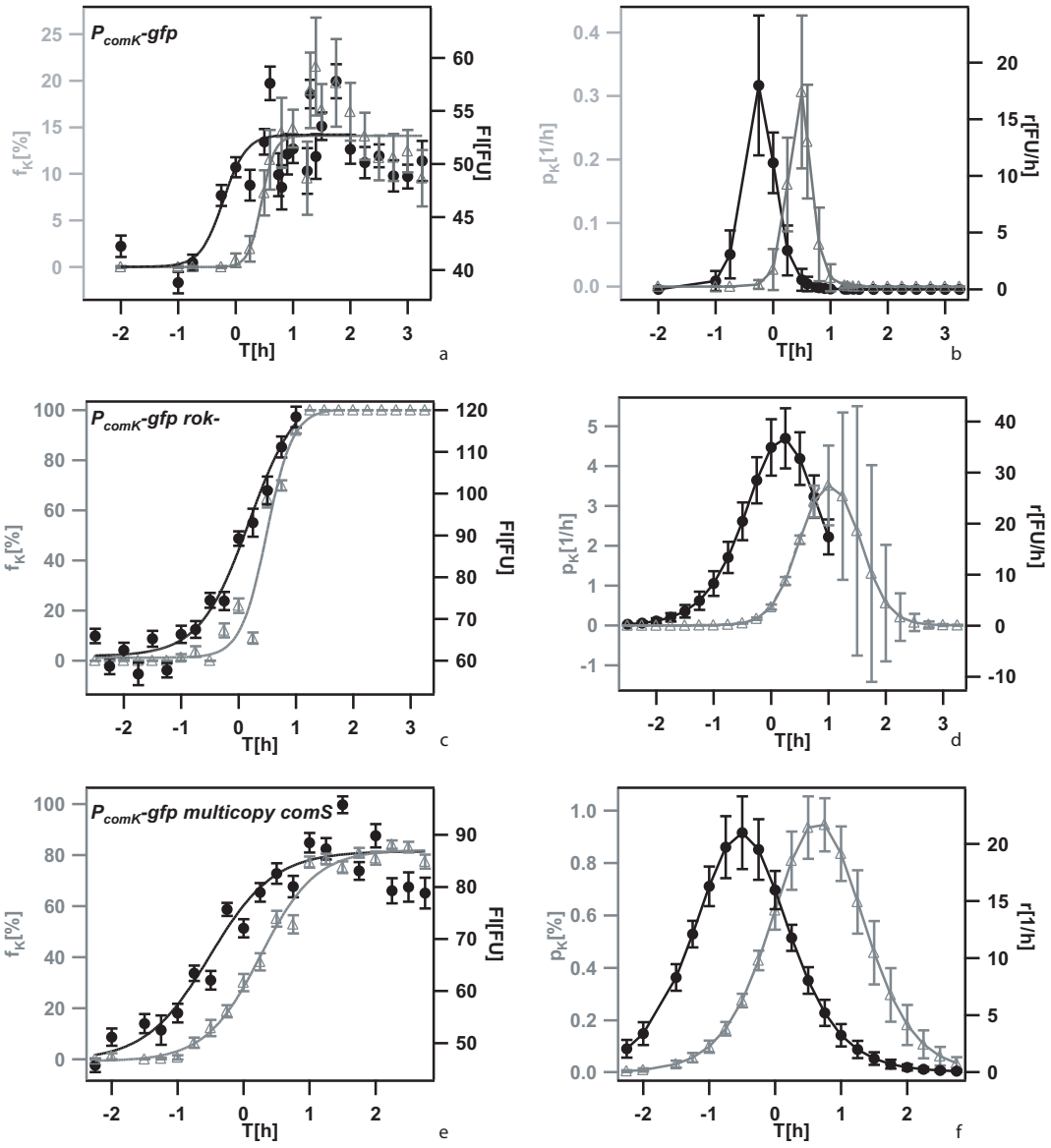

Figure 7.3: Basal *comK* expression rate: a, c and e) Black circles: basal fluorescence intensity FI. Grey triangles: fraction of cells in the K-state f_K. Full lines: best fit to a sigmoid function (Eqn. 7.1). b, d and f) Black circles: basal expression rate r determined from the first derivative with respect to time of the sigmoid fit. Grey triangles: intrinsic switching probability P_K determined from the first derivative with respect to time of the sigmoid fit and normalized by the fraction of non-competent cells (100-f_K). a and b) BD 2711; c and d) BM 50; e and f) BM 77. Fit parameters are listed in Table B.2 in appendix B.

The maximum expression rate and the maximum intrinsic switching rate showed a significant time delay for all three strains. However the delay was only 0.3 ± 0.1 h with the *rok*- strain as compared with 0.7 ± 0.1 h with the wild-type and 0.8 ± 0.1 h with the ComS-overproducing strain, indicating a faster activation of the positive ComK feedback loop (Fig. 7.6).
Since the basal *comK* expression rate showed its maximum prior to switching the *comK* promoter activity seems to open a time window in which cells can switch into the K-state. The time point when the K-fraction saturated coincided with the time point when the basal *comK* expression rate decreased to non-measurable values. Thus, some event turns off the expression of *comK* shortly after cells enter the stationary phase. This event seems to be unrelated to competence since turn-off was measured in cells that had not switched into the K-state. Shut-down of basal expression rate after entry into the stationary phase might be controlled by an additional unknown repressor interacting with P_{comK}.

7.3 Elongation of switching window enables more cells to enter the K-state

It had been reported that bacteria enter the competent state at earlier time points if the medium was exchanged for conditioned medium (medium with a maximal pheromone ComX concentration) during the exponential growth phase [19]. To assess whether basal *comK* expression was influenced by exchange of medium, the competence medium was exchanged for conditioned medium at T_{-2} with the wild-type strain (Fig 7.4a and b). Basal *comK* expression rate increased at earlier times, but again the shut-down of *comK* expression occurred shortly after entry into the stationary phase. Similar behaviour was observed with the *rok*- and ComS-overproducing strain (Fig. 7.5). The maximum fraction of wild-type cells in the K-state raised to 36 ± 3% (Fig 7.4a). When instead the medium was exchanged for fresh medium at T_{-2} basal *comK* expression occurred at later times and the maximum fraction of cells in the K-state decreased to 8 ± 1% (Fig 7.4c). This observation is in agreement with the assumption that the basal activity increased with an internally determined rate and decreased in the stationary phase. As a consequence, the fraction of cells that switches into the K-state depends on the window in which the *comK* promoter is active.

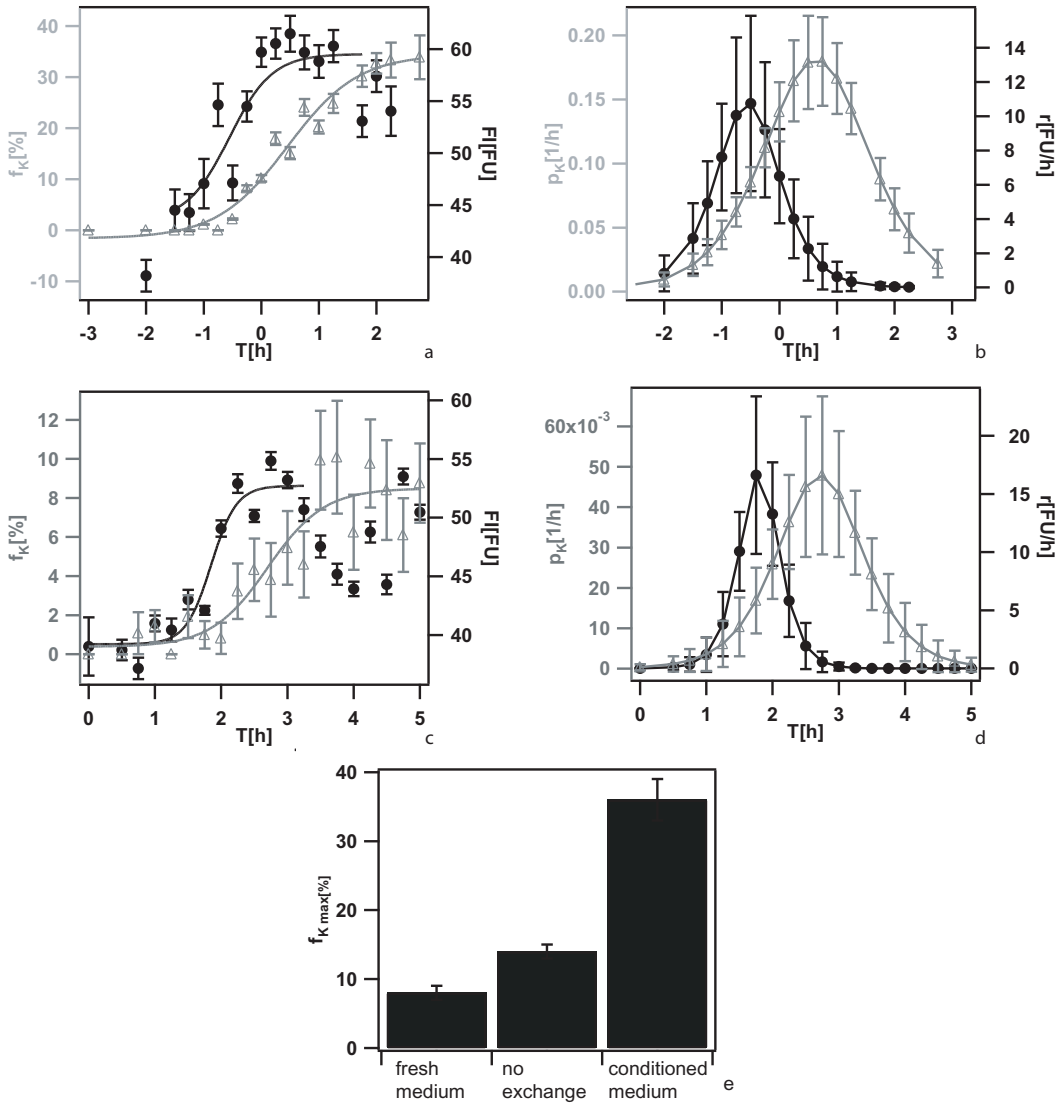

Figure 7.4: Basal *comK* expression rate of BD 2711 P_{comK}-gfp grown in conditioned or fresh medium a and b) Exchange for conditioned medium at T_{-2}. c and d) Exchange for fresh medium at T_{-2}. a and c) Black circles: basal fluorescence intensity FI. Grey triangles: fraction of cells in the K-state f_K. Full lines: best fit to a sigmoid function (Eqn. 7.1). b and d) Black circles: basal expression rate r determined from the first derivative with respect to time of the sigmoid fit. Grey triangles: intrinsic switching probability p_K determined from the first derivative with respect to time of the sigmoid fit and normalized by the fraction of non-competent cells (100-f_K). Fit parameters are listed in Tables B.1 and 2 in appendix B. e) Maximum fraction of cells in the K-state.

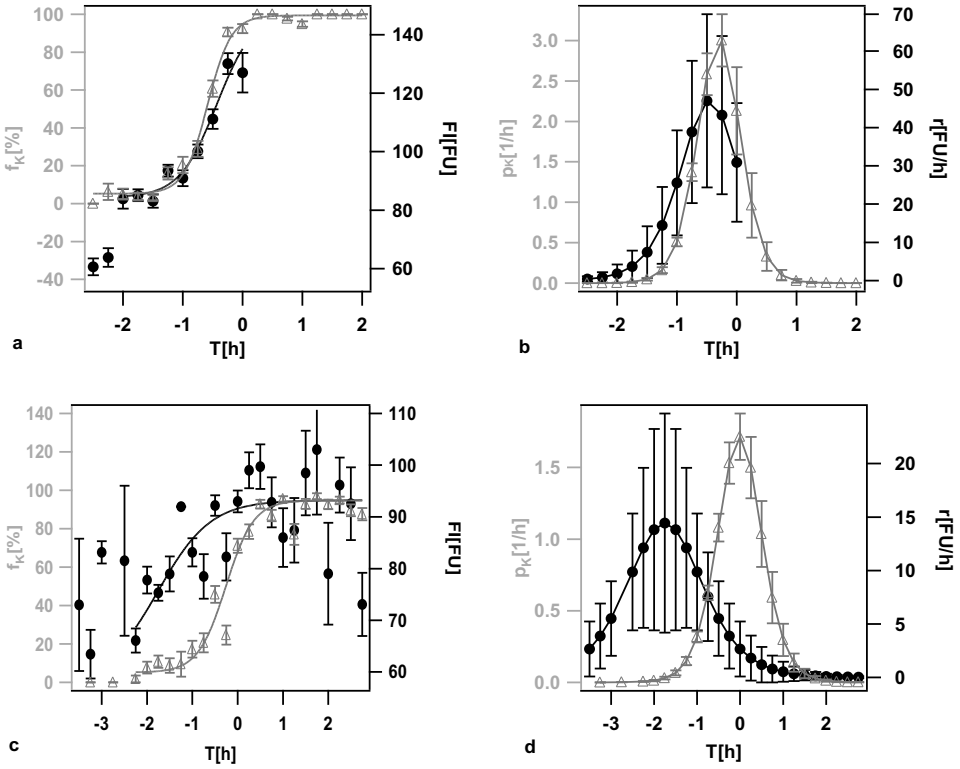

Figure 7.5: Basal *comK* expression rate of strains BM 50 and BM 77 grown in conditioned medium: Exchange for conditioned medium at T_{-2}. a and c) Black circles: basal fluorescence intensity FI. Grey triangles: fraction of cells in the K-state f_K. Full lines: best fit to a sigmoid function (eqn. 7.1). b and d) Black circles: basal expression rate r determined from the first derivative with respect to time of the sigmoid fit. Grey triangles: intrinsic switching probability p_K determined from the first derivative with respect to time of the sigmoid fit and normalized by the fraction of non-competent cells (100-f_K).

Artificially increasing the pheromone concentration lead to an elongation of the switching window so that more cells had time to enter the K-state. When the medium was exchanged at earlier time points than T_{-2}, for example at $T_{-2.5}$ the same results could be obtained, meaning that basal *comK* expression rate still increased at $T_{-1.5}$. This indicates that cells in the early exponential growth phase are not able to react to an enhanced pheromone concentration, possibly due to the absence of the competence machinery in the early exponential growth phase. Nevertheless, the effect of artificially in- or decreasing the pheromone concentration by medium exchange clearly shows the importance of external signaling like quorum sensing and the strong connection of cells with their environment (see chapter 3).

7.4 Conclusion

Using a single-cell assay, one factor that controls the K-fraction in the bimodal population of *Bacillus subtilis* in the stationary phase was defined. It was found that basal *comK* expression rate was shut down after T_0 and that this shut-down was followed by a drop in switching rate with a time lag that depended quantitatively on expression rate and ComK proteolysis rate. Furthermore the basal expression level of *comK* showed strong cell-to-cell variation. The obtained data support the hypothesis that the average basal level of ComK rises during late exponential phase and that due to noise in basal *comK* expression only those cells that are on the high end of *comK* expression reach the threshold of ComK concentration during a specific time window set by the basal *comK* expression rate.

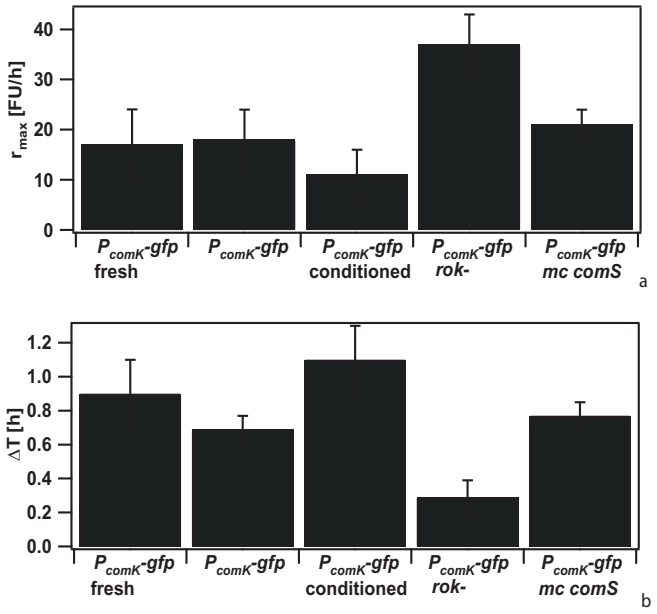

Figure 7.6: Comparison of important values obtained for different strains a) Maximum expression rate r_{max}. b) Shift of half life period $\triangle$ T between basal fluorescence level and switching distribution. Values were determined from fits in Fig. 7.2 and 7.3. BD 2711 P_{comK}*-gfp*, BM 50 P_{comK}*-gfp, rok-*, BM 77 P_{comK}*-gfp, mc comS*, conditioned: exchange for conditioned medium, fresh: exchange for fresh medium.

8 Switching into the K-state: real-time measurements with single cells

Now knowing the switching behaviour of the whole *Bacillus* population, an experimental approach following the switching behaviour of individual cells was performed. Therefore, cells were taken at $T_0 \pm$ 1h and permitted to attach to a polystyrene-coated cover slide and mounted onto a flow chamber. To stimulate growth phase dependent development of cells on the microscope and to supply the cells with oxygen, the medium in the flow chamber was exchanged with medium of a cell culture growing under standard competence conditions [59] every 15 min (see chapter 6 details on the method).
As seen in chapter 7, *Bacillus subtilis* cells can only switch into the K-state during a specific switching window. While intrinsic noise was known to be important for the onset of switching [5], the real-time experiments indicated the responsibility of extrinsic noise for the development of a broad distribution of ComK values in the K-state. Time-lapse microscopy did not reveal additional factors important for switching into the K-state, but showed the existence of an intrinsically set switching period (time interval between onset and saturation of ComK level) of approximately 1.5h.

8.1 The basal $comK$ expression rate increases in the entire cell population

The expression of the master regulator *comK* in individual cells was quantified by measuring the fluorescence intensity of GFP fused to the promoter of *comK* . (For details on the method see chapter 6). The expression of the *comK* promoter was quantified by measuring the average fluorescence intensity *FI* of the single cells (chapter 6). In agreement with the experiments performed for the whole bacterial population (see chapter 7), 15 % of the cells switched into the K-state with the real-time approach (see Fig. 6.5, chapter 6). Again, the individual cells showed a sigmoidal switching behaviour (Fig 8.1b). Before entry into the stationary phase at T_0, basal expression of *comK* increased significantly in all cells between $T_{-0.75}$ and $T_{-0.5}$ (Fig 8.1a and b). The development and magnitude of increase was similar in cells that remained in the B-state and cells that switched into the K-state. This result indicates that the sharp rise in the expression rate of the *comK* promoter upon switching is not a result of pre-determination in the

B-state. The switching probability of individual cells was independent of their mean basal ComK concentration prior to switching. This observation is in agreement with the finding that noise in transcription from the *comK* promoter was mainly intrinsic, i.e. that the transcription probability from two copies of P_{comK} is uncorrelated [5]. Therefore quorum sensing (see chapter 4) seems to set the cells into an "on" state before the stationary phase enabling all cells to switch, but the determination if a cell switches or not is then dependent on other factors such as the intrinsic noise.

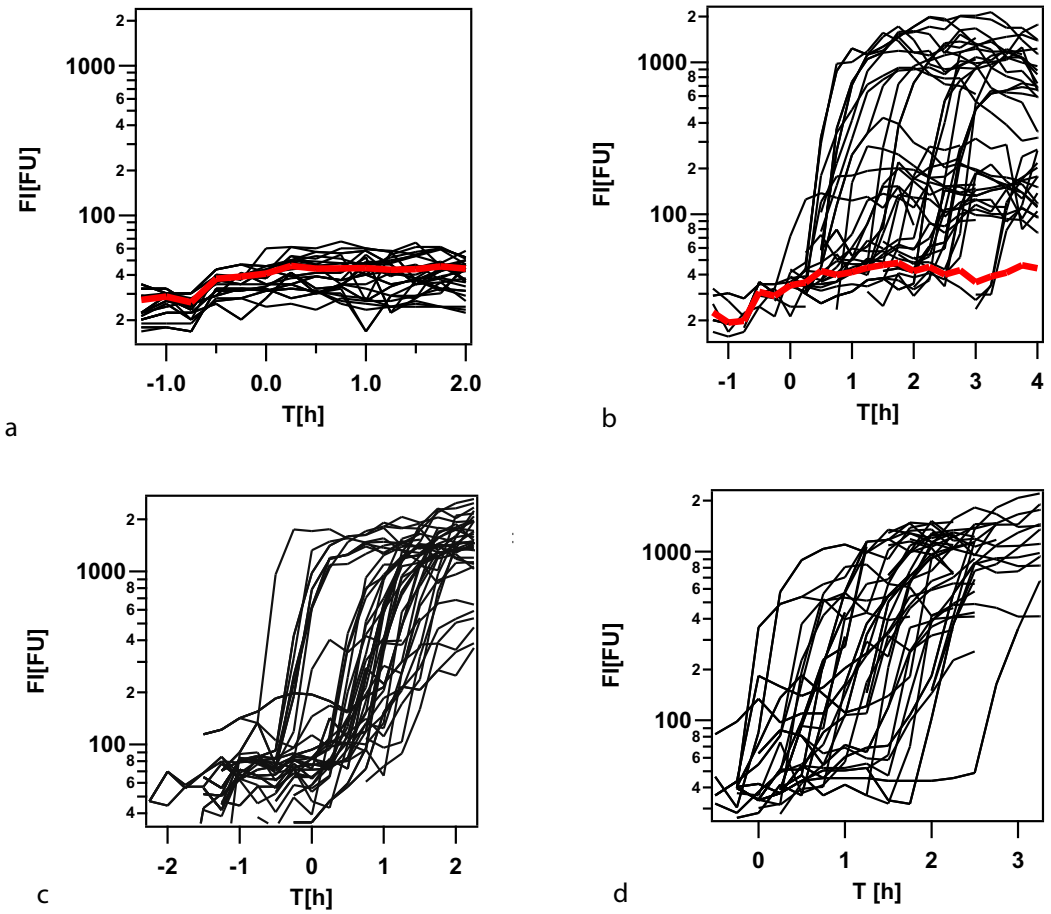

Figure 8.1: Time course of P_{comK}-gfp expression in individual cells a) Time course of fluorescence intensity of BD 2711 cells remaining in the B-state. Red line indicates the average fluorescence intensity of cells remaining in the B-state. b) Time course of fluorescence intensity of BD 2711 cells switching into the K-state. Red line indicates the average fluorescence intensity of cells of cells still in the B-state prior to switching. c) Time course of fluorescence intensity of BM 101 cells switching into the K-state. d) Time course of fluorescence intensity of BM 77 cells switching into the K-state.

8.2 The switching period of individual cells is well defined

The individual switching curves, i.e. the fluorescence intensity as a function of time showed a steep slope indicating that the autocatalytic feedback loop of ComK was active (Fig. 8.1b). The increase saturated after a specific time interval which was defined as the switching period.

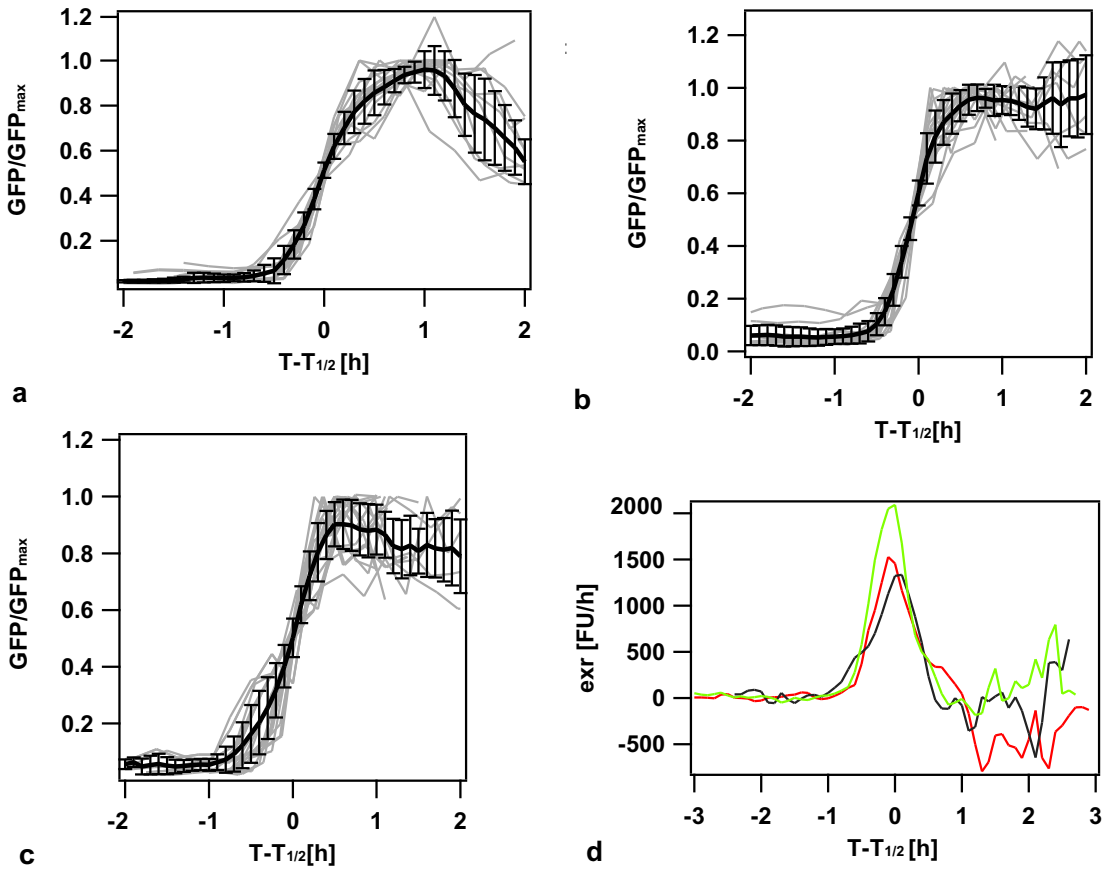

Figure 8.2: Switching kinetics of individual cells Black line: average fluorescence intensity, grey lines: individual cells. a, b and c) The fluorescence intensity of individual cells were normalized to the cumulative expression (maximum fluorescence intensity). The time axis was shifted to $T_{1/2}$, where cells had half maximum fluorescence intensity. a) BD 2711, b) BM 101, c) BM 77. d) Expression rates of wt (red), *rok* (green) and ComS (black) overproducing strain. Expression rates were obtained by multiplication of the normalized values with the average maximum value of 28-50 cells.

To address the question whether the switching period was well-defined in individual cells, the individual switching curves were normalized to their saturation level and shifted the T-axis to overlay all curves at half maximum expression level (Fig 8.2). To ensure that only cells in the K-state were observed, only cells whose accumulated *gfp* expression exceeded 300 FU were analysed. The switching period in individual cells did not show strong variation and the average period was approximately 1.5h, independent of growth phase (Fig 8.1). The expression rate was defined as the first derivative with respect to time of the fluorescence intensity FI (Fig. 8.2). Please note that the cells did not grow during approximately 1h after the onset of switching (see chapter 9) and therefore the expression rate of *comK* was not corrected for cell length. However the negative expression rate at $T\text{-}T_{1/2} > 1$ is attributed to cell growth, indicating cells exiting the competent state. Deletion of *rok*, a repressor of *comK* enhanced the switching rate by a factor of 1.5 and over-expression of *comS* leading to increased ComK stability (see chapter 4), did not affect the switching rate (Fig 8.1 and 8.2). For all strains the switching period was approximately 1.5 h, indicating that this period may be an intrinsic property of the individual cells. The expression of *comK* seems to be switched off after this period of time. One explanation could be that dependent on growth phase an external signal accumulated in the surrounding medium. But an overlay of the switching curves between T_0 and T_4 revealed no large variation, indicating that the expression rate and switching period did not depend on growth phase. Probably the positive feedback of ComK is turned off by an internal signal. A possible mechanism may be that with increasing ComK the expression of *comS* was repressed [68]. Another candidate for this task would be RapH [69]. RapH indirectly suppresses the expression of *comS* . In both cases, the overproduction of ComS should increase the switching period in individual cells. However, it was observed, that the temporal behaviour of the expression rate was not significantly different in the wild-type and in a *comS* over-expressing strain.

8.3 Maximum ComK concentration differs from cell to cell

Since the switching period in individual cells seemed to be well defined, the next question to address was if individual cells would also reach well defined ComK values while entering the competent state. The wild-type strain showed clearly that ComK values differ from cell to cell, and that there is not one distinct ComK concentration reachable by the cells. Instead two clearly distinguishable FU distributions were found. One distributed around 350 FU and one around 1050 FU (Fig. 8.3). The theoretical model described in the following showed that the variations in ComK levels could be explained by extrinsic noise, that was implemented to the model by variations in parameter sets. But still the two different distributions could not be explained. No correlations to the

basal *comK* expression rate or the time-point of switching could be found. BM 77, the ComS overproducing strain, showed a similar ComK distribution as the wild-type strain (Fig. 8.3c). However with the *rok* knockout strain the lower ComK distribution vanished and the higher ComK distribution broadened so that even higher ComK values could be reached by the individual cells (Fig. 8.3b). This could be explained considering posttranskriptional regulation. The *rok* knockout strain showed a two fold higher promoter activity as the wild-type and the comS over-producing strain, mainly due to a faster activation of the positive feedback loop of comK (see chapter 4). Therefore, posttranskriptional regulation via RNA interference for example could possibly be not fast enough in the *rok*- strain to stop the further increase of ComK levels, leading to the second ComK concentration distribution at 1050 FU.

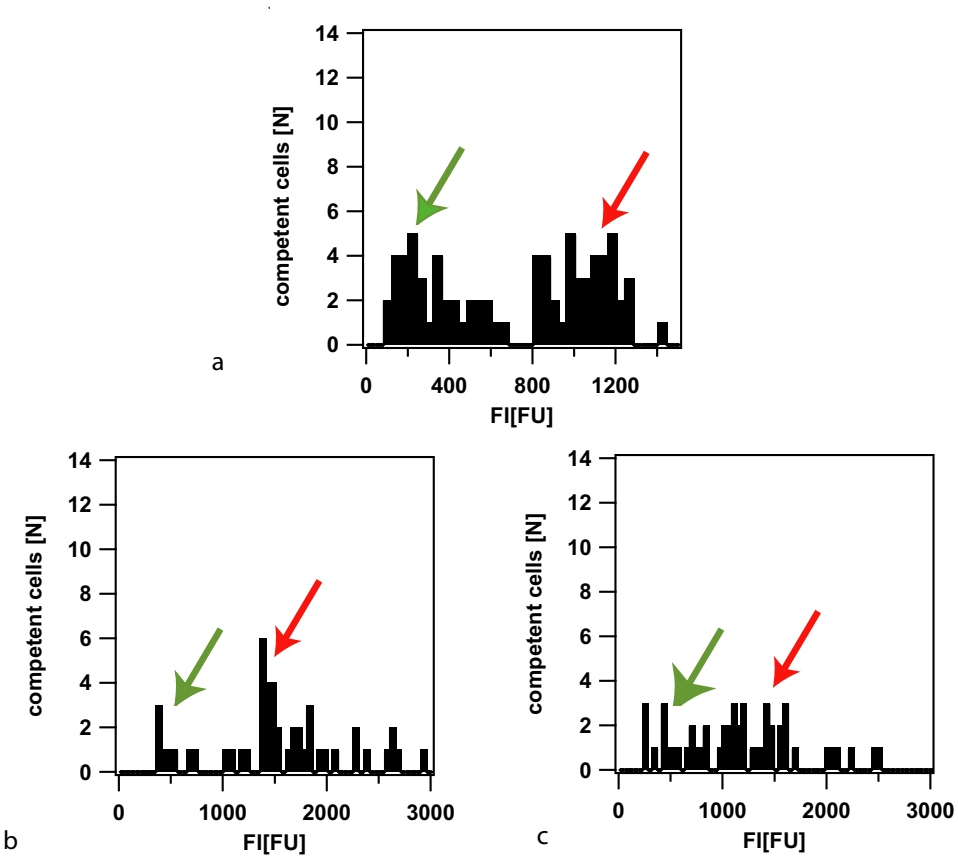

Figure 8.3: Histograms: comparison of maximum ComK values a) BD 2711, b) BM 101, c) BM 77. Green arrows indicate ComK value distribution around 350 FU. Red arrows indicate ComK value distribution around 1050 FU.

8.4 Theoretical model reproduces the experimental findings

To better understand the obtained data a mathematical model describing the switching process by non-linear dynamics coupled to low-number stochastic effects and accounting for extrinsic noise was set up. The main parameters incorporated in this model are presented in Fig. 8.4. The model was developed in cooperation with Jan-Timm Kuhr (LS Frey, LMU). The basic theoretical principles underlying this model are presented in chapter 5. The model showed that in the stationary phase 17.7% of the cells overcame the switching threshold, reaching the K-state with a broad ComK level distribution within 1.9 h ± 0.7 in remarkable agreement with the experimental findings.

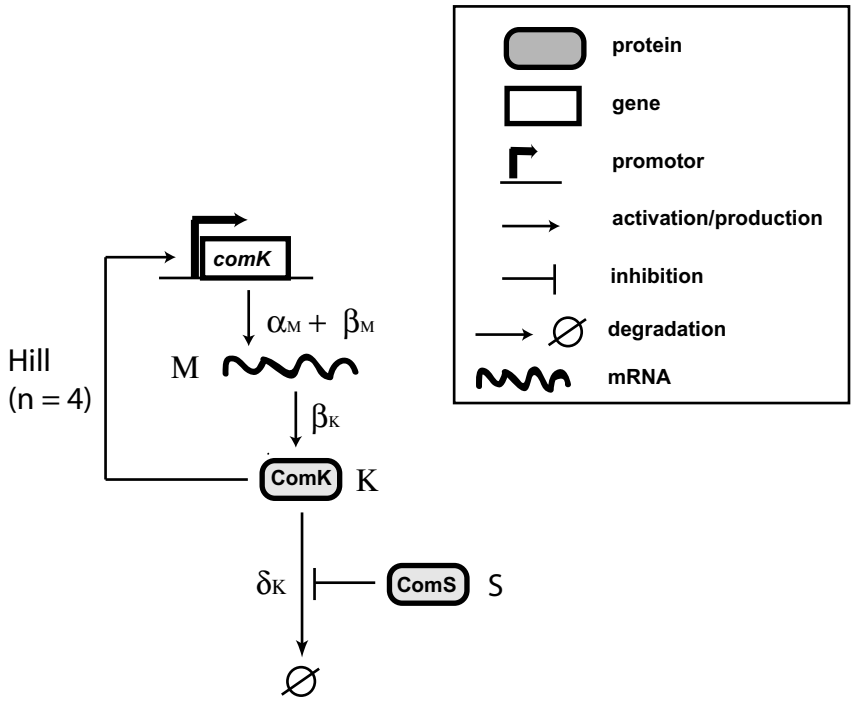

Figure 8.4: Reactions incorporated in this model Production of the *comK* mRNA [M]: basal *comK* mRNA production given by rate α_M, *comK* mRNA production in presence of active autocatalytic feedback loop of *comK* given by rate β_M. Translation of ComK [K] given by maximal translation rate β_K. Cooperativity of ComK: ComK activates its own promoter by binding as a dimer of dimers (n=4). Degradation of ComK by Michaelis Menten kinetics with rate δ_K. Degradation of ComK is inhibited by ComS [S].

8.4.1 Mathematical rate equation model

It is known that non-linear dynamics together with fluctuations can lead to bimodal protein distributions [70]. The following descriptive rate-equation model was set up for the number of ComK proteins (K) and the corresponding mRNA molecules (M):

$$\frac{\partial K}{\partial t} = \beta_K M - \frac{\delta_K K}{q_K + S + K} \tag{8.1}$$

$$\frac{\partial M}{\partial t} = \alpha_M + \frac{\beta_M}{1 + (p_K / K_f(K,S))^{\gamma_K}} - \delta_M M \tag{8.2}$$

The first term in eqn. (8.1) describes translation of K from M, which happens with rate β_K for each M. The second term describes Michaelis-Menten-like degradation of K (degradation at a constant rate). Here K and S refer to the total ComK and ComS numbers, respectively. For very high K degradation happens at a maximal rate δ_K, and half-maximal degradation is found when K = S + q_K. In eqn. (8.2) α_M is the basal transcription rate in absence of K. The next term describes the autocatalytic feedback of ComK: Two ComK dimers bind cooperatively to the *comK* promoter [27, 71] thereby strongly enhancing transcription (production of M). If dimer formation and dissociation as well as promoter binding and unbinding are fast enough to equilibrate, autocatalytic transcription can be modelled by a Hill function, with maximal (for large K) transcription rate β_M, half-maximal concentration p_K and cooperativity γ_K. K_f, appearing in the denominator, is the number of free ComK, i.e. not bound to the MecA/ClpC/ClpP protease complex and able to bind to the promoter. Finally degradation of mRNA is proportional to M and the rate δ_M.

8.4.2 Rise of ComS decreases degradation of ComK

Degradation of ComK follows Michaelis-Menten kinetics [24](see chapter 5), catalysed by the MecA/ClpC/ClpP protease complex (D). The reaction scheme is:

$$K_f + D_f \underset{\lambda_{-1}}{\overset{\lambda_1}{\rightleftharpoons}} [KD] \xrightarrow{\lambda_2} D_f \tag{8.3}$$

where K_f, D_f, and [KD] refer to free ComK, free protease complex and ComK bound to the protease complex, respectively. Reactions λ_1 and λ_{-1} are fast in comparison with the actual degradation λ_2. Cells respond to high density by increasing ComS concentration that couples into the competence decision network [71](Fig. 8.5). ComS is a small peptide that is also degraded by the MecA/ClpC/ClpP protease complex [22]. Protease complexes are thus partially occupied by ComS and degradation of ComK is slowed

down.

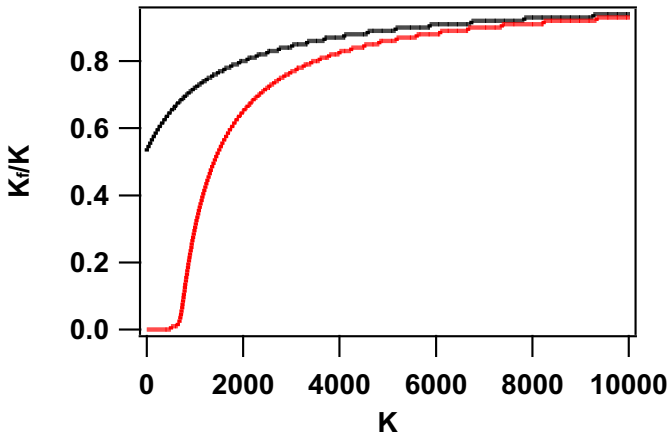

Figure 8.5: Influence of ComS on the fraction of free ComK ComS binds competitively to the MecA/ClpC/ClpP protease complex, thereby increasing the amount of free ComK (K_f), see eqn. 8.6. Red line: S = 0, black line: S = 1500. Near the lower stable and unstable fixed points (K ≈ 200 and K ≈ 600, respectively) this has an important effect on free ComK concentration.

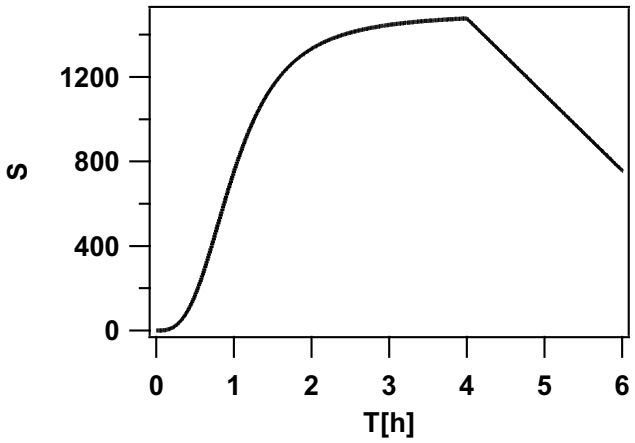

Figure 8.6: ComS concentration over time Empirical development of the number of ComS molecules S(t) used for the simulations. At time zero the bacterial population started growing. Therefore, the transition into stationary phase (T0) takes place between time-point 2 and 3 for the individual simulations.

Furthermore, K_f is increased in the presence of S and thus more K is available for dimer formation and binding to the *comK* promoter, which has a prominent effect near the lower fixed point and threshold (Fig. 8.7). Since degradation of ComS and ComK happens through the same processes [22] identical rates are assumed:

$$S_f + D_f \underset{\lambda_{-1}}{\overset{\lambda_1}{\rightleftharpoons}} [SD] \xrightarrow{\lambda_2} D_f \tag{8.4}$$

By the law of mass action one finds:

$$K_f(K,s) = \frac{1}{2}\frac{K}{K+S}\left(K+S-D-\frac{\lambda_1}{\lambda_{-1}}+\sqrt{\left(K+S-D-\frac{\lambda_1}{\lambda_{-1}}\right)^2+4\frac{\lambda_1}{\lambda_{-1}}(K+S)}\right) \tag{8.5}$$

which appears in eqn. 8.2. To obtain K_f particle conservation, i.e. $K = K_f + [KD]$, $S = S_f + [SD]$, and $D = D_f + [KD] + [SD]$, was used. S as a function of time (Fig. 8.6) was set empirically as an external control parameter:

$$S(t) = \begin{cases} \frac{1500}{1+\left(\frac{1h}{t}\right)^3} & ; t \leq 4h \\ 1500 - 360(t-4h) & ; 4h < t < 6h \end{cases} \tag{8.6}$$

8.4.3 Parameters

The model includes ten parameters (see Table 8.1) and the external control function S(t) (Fig. 8.6). All parameters were chosen consistently with results of earlier studies. δ_K, D and β_K/δ_M, could be obtained directly from literature. Further constraints were $M \approx 1$ in the vegetative state in stationary phase [5], a switching threshold of a few hundred ComK proteins and a saturation value of $10^4 - 10^5$ proteins in the K-state [24]. The rates of the degradation reaction λ_1, λ_{-1} and λ_2 have not been addressed experimentally. However, this is not a drawback to the model, since λ_2 is incorporated in δ_K (Michaelis-Menten theory). λ_1, λ_{-1} appeared explicitly in K_f(K,S) and since both rates are fast, only their ratio is of interest. Varying λ_1/λ_{-1} has only minor influence on the model dynamics.

Parameter	Significance	Mean value	Motivation/Explanation
β_K	Translation rate per mRNA	$1.0s^{-1}$	burstfactor $\beta_K/\delta_M \approx 50$ [5]
δ_K	Maximal protein degradation rate	$11.5s^{-1}$	$\approx 11.5s^{-1}$, [24], Figure 1A therein
q_K	Half-maximal degradation	400	< 9000; [24] Figure 1A therein; sets position of fixed points
a_M	Minimal transcription rate	$0.023s^{-1}$	$M \approx 1$ before switching; [5]
β_M	Maximal additional transcription rate by feedback	$0.19s^{-1}$	position of upper fixed points/ saturation
p_K	Half-maximal feedback	600	position of switching threshold
γ_K	Cooperativity/Hill Coefficient	4	two dimers bind to promoter; cooperativity
δ_M	Degradation rate per mRNA	$0.022s^{-1}$	burstfactor $\beta_K/\delta_M \approx 50$ and $M \approx 1$ before switching; ([5])
D	Number of protease complexes	700	[24]
$\frac{\lambda_1}{\lambda_{-1}}$	Equilibrium constant of degradation reaction	1	variation of this parameter are of minor influence only

Table 8.1: Parameters of the model.

8.4.4 Stochastic simulations and results

If molecular fluctuations are neglected, initial conditions determined the dynamics of the model and each cell developed towards one of the fixed points of the model. Since numbers of M and K are low initially, a deterministic numerical simulation would end up with 0 % cells in the K-state. To explain for the heterogeneity of a cell culture molecular fluctuations are thus of prime importance. To evaluate the model including stochastic fluctuation we implemented Gillespie's stochastic algorithm [57]. To render the model more realistic, parameter values were allowed to vary thereby incorporating cell-to-cell variability (size, variable number of the cellular machinery, etc.) i.e. extrinsic noise was introduced [72]. For each realization of the simulation every parameter was chosen out of a Gaussian distribution about its mean with a standard deviation of 5% of its mean, and then held constant for that run (γ_K was not varied, since its value is set by the topology of the model).

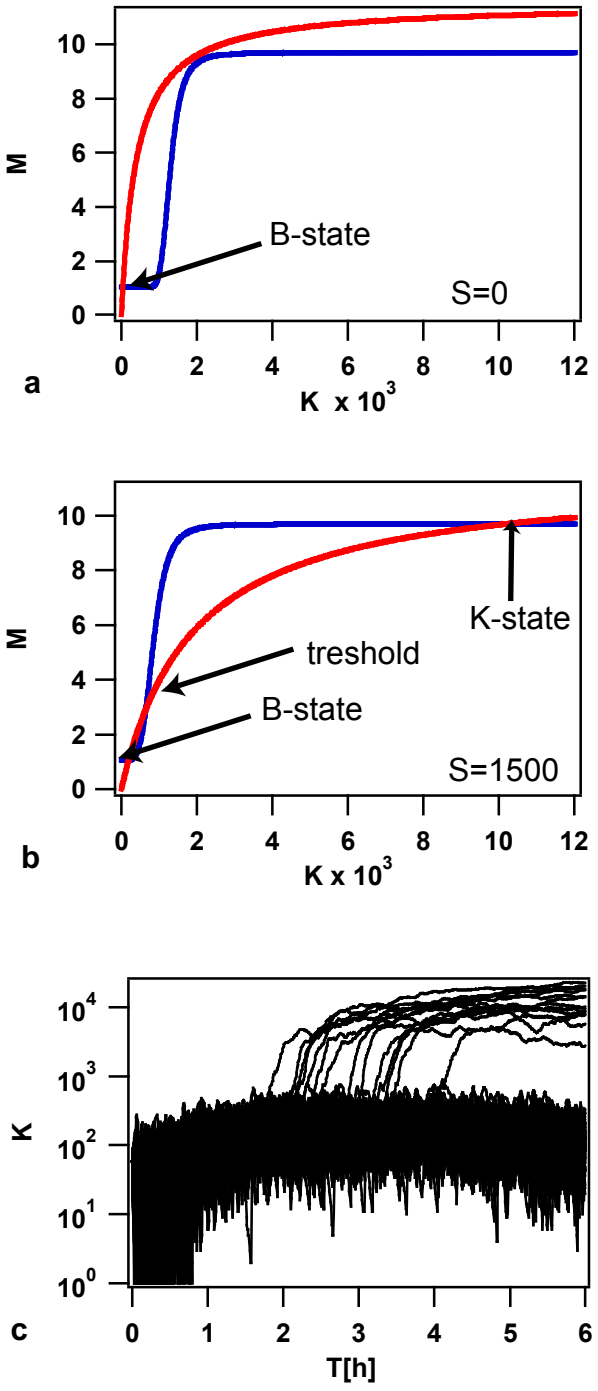

Figure 8.7: Analysis of the non-linear dynamics model and stochastic simulations. a) and b) Bifurcation analysis. Nullclines of eqn. (8.1) and (8.2). For a) S = 0. b) S = 1500 are plotted (blue: dM/dt = 0; red: dK/dt = 0). c) 100 realizations of the stochastic simulation of the model are shown.

In each run the evolution of an individual cell was simulated for 6 hours. The influence of the cell population as a whole (quorum sensing) was incorporated via S(t) (described above) (Fig. 8.6). For low numbers of ComS, S, only one fixed point corresponding to the vegetative state (K $\approx$ 100, M $\approx$ 1) was found (Fig. 8.6). As S rises, a saddle-node bifurcation appears, which, in principle, would allow cells to stochastically switch to a state with higher K and M. However, due to the high activation threshold this is highly unlikely. At S $\sim$ 1500 three fixed points emerge. The lower stable fixed point at K $\approx$ 200, M $\approx$ 1 corresponds to the B-state. The intermediate unstable fixed point at K $\approx$ 600, M $\approx$ 3 is very close to the B-state (Fig. 8.7). The lower stable and the unstable fixed point almost meet in another bifurcation, rendering the vegetative state unstable to stochastic fluctuations. Under theses conditions intrinsic noise is strong enough to carry some cells across the switching threshold. Cells that cross the threshold evolve towards the upper stable fixed point (K-state = K $>$ 900, K $\approx$ 10000, M $\approx$ 10), i.e. bacteria switch into the K-state. Back-transitions from the K-state into the B-state were highly improbable, because in the K-state fluctuations are too small to revert switching.

Quantitative data, obtained through evaluation of 1000 stochastic simulations [57], corroborated the predictive value of the model. Before entry into the stationary phase, i.e. at values S $<$ 1500, cells showed fluctuations about the vegetative state, but no escape was possible. Similar to the experiment, the simulations showed an increase of basal ComK concentration before individual cells started to switch. 17.7 % of all cells entered the K-state, showing saturation in ComK level within 1.9 $\pm$ 0.7h, in good accordance with the experimental findings of 15 % competent cells and a switching period of 1.5h.
The spread in saturation levels (Fig. 8.7c and 8.3) can be explained by cell-to-cell variations in the chemical rates, known as extrinsic noise [72], which is present in any cell population. In the model extrinsic noise is represented as variations in the model parameters. These have a huge effect on the dynamics since the nullclines in Fig. 8.7 are close to parallel at the upper fixed point. As a consequence the exact ComK number in the K-state is very sensitive to variability in the model parameters. To account for this variance, model parameters were allowed to vary slightly for each realization of the stochastic simulation. Results were consistent with the experiment (Fig. 8.1) indicating that extrinsic noise in addition to intrinsic noise is an important determinant of ComK levels. Furthermore, the two-variable model explains the saturation of ComK by the emergence of a second stable fixed point.

8.5 Conclusion and Outlook

Using time-lapse microscopy the switching behaviour of single cells could be determined. Switching curves of individual cells confirmed the switching behaviour seen for the whole bacterial population (chapter 7). It could be shown that the switching process is restricted to an intrinsically defined period in individual cells. Simulations of the non-linear dynamics model employing two variables reflected the major features of the switching process in remarkable agreement with the experiments. While escape from the B-state is governed by low number fluctuations (intrinsic noise), the spread in the level of ComK saturation is set by cell-to-cell variations (extrinsic noise).

The model described above is based on the assumption that at T_4 the ComS level decreases (Fig. 8.6) due to degradation and additionally to the possible action of a negative feedback loop of ComK inhibiting the promoter of ComS at high ComK levels. When instead an S(T)function was used, that saturated in the stationary phase (no degradation anymore), the experimentally obtained data of 14% cells being competent in the stationary phase could only be explained by lowering the basal *comK* expression α_M at T_4 in the model. This indicates that at the time period watched in the experiment, a negative feedback loop of ComK inhibiting ComS expression, leading to increased ComK degradation and thereby lowering ComK expression, has not to be taken into account. Instead, shut down of the *comK* promoter seems to be due to additional still unknown repressing factors in the late stationary phase, enabling the exit from competence.

9 Cell cycle controls entry into the competent state

Cell cycle dependent gene expression has been reported for eukaryotes as well as procaryotes and is often coupled to checkpoint mechanisms. In particular, genetic switching between alternative states of gene expression is coupled to the cell cycle in many organisms. Developmental checkpoints have been identified to regulate sporulation in *Bacillus subtilis* in response to DNA replication or damage [36,37,73]. Other checkpoint mechanisms have been found to block cell cycle progression [63, 74]. Therefore, it was interesting to see whether the development of competence is dependent on cell cycle as well. So far it has been demonstrated that cell growth is inhibited after initiation of competence. ComGA, a late competence protein, involved in the DNA-uptake complex, was pinpointed as a checkpoint protein, responsible for growth arrest [63]. To address the question whether the entry into the competent state is coupled to cell cycle, real-time experiments have been performed at the level of individual cells (see chapter 6 for details on the method) and the switching probability between the vegetative and the competent state with respect to cell cycle progression was analysed.

9.1 Growth is inhibited during the switching period

It was previously reported that cell growth and division is inhibited after initiation of competence. Therefore, the temporal development of cell growth was monitored by measuring cell-length with switching into the K-state in individual cells (Fig 9.1). The initiation of switching was defined as an abrupt increase in fluorescence intensity *FI* of the single cells. The concentration of the GFP, representing ComK concentration (for further details on the method see chapter 6) increased strongly during a switching period of appox. 90 min (see chapter 8). Cell division was defined as the time-point when a septum was visible in the bright field image. Cell length was constant while the ComK levels increased and the cells resumed growth in length, once the ComK amplification was shut off. On average, growth in cell-length was inhibited during 50 min after initiation of competence (Fig 9.1) Non-competent cells in the stationary growth phase were not inhibited. After 60 min, the competent cells resumed growth in cell length at their normal rate (Fig 9.1). The data indicate that the inhibition of growth in cell-length is coupled to the interval in which the ComK concentration is amplified, i.e. to the

switching interval.

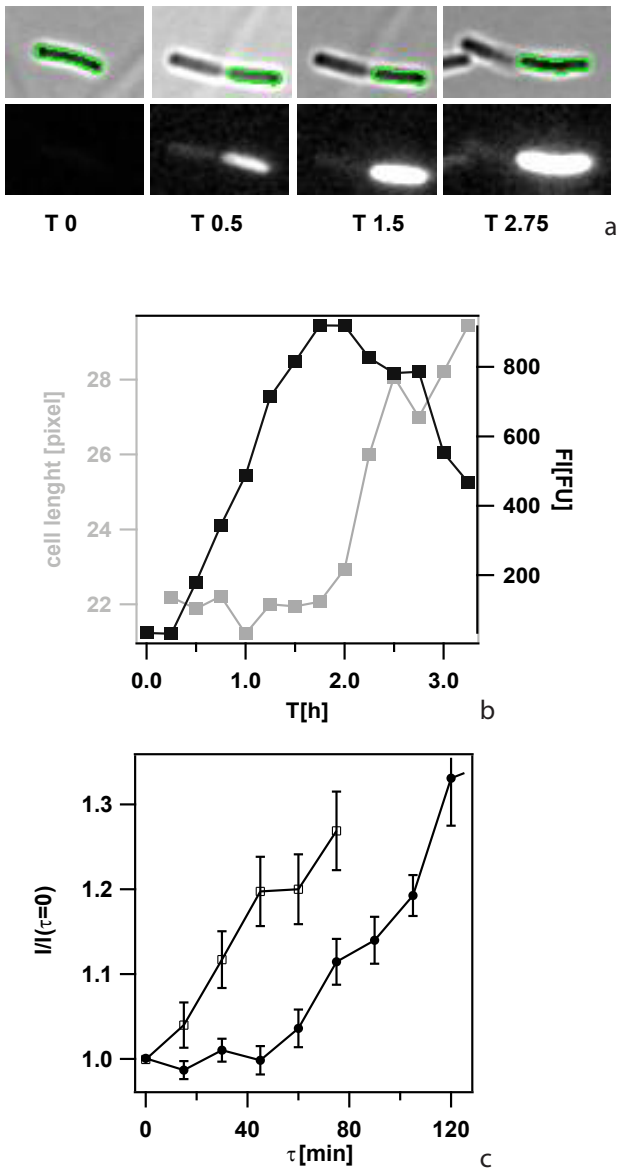

Figure 9.1: Growth in cell-length is inhibited during the switching interval a) Time-lapse of a cell expression P_{comK}-GFP. Top row: Brightfield image. Green contour: outline of the cell. Bottom row: fluorescence intensity of wild type cell expressing P_{comK}-GFP. b) Fluorescence intensity of a switching cell and corresponding cell length during switching. T = 0 refers to the time of entry into stationary phase. c) Cell length of competent (black, closed symbols) and non-competent cells (grey, open symbols) during switching. $\tau = 0$ refers to the time of cell division.

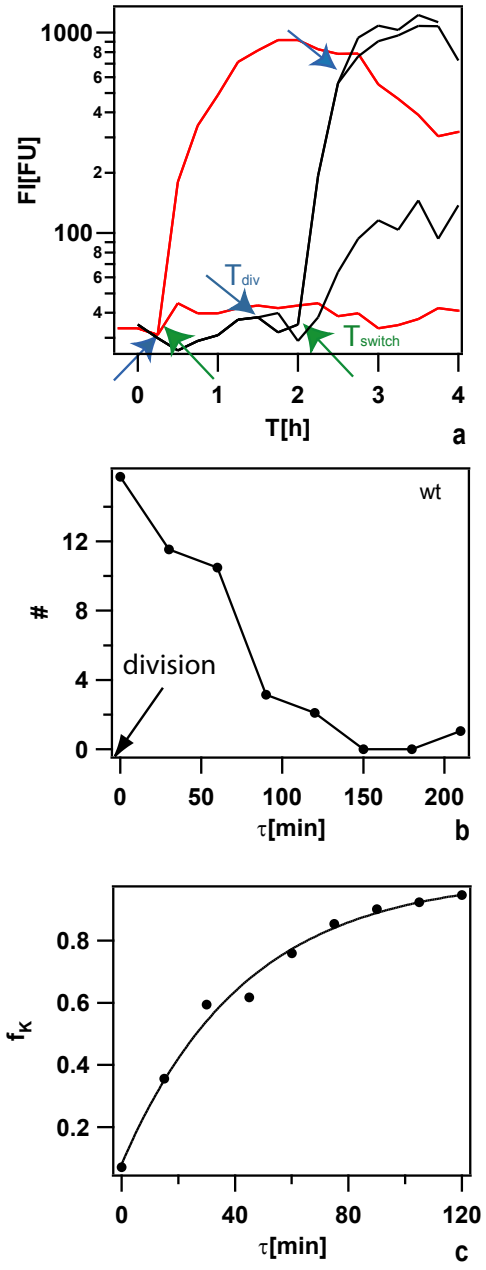

Figure 9.2: Cells switch preferentially after cell division a) Two examples of time-course of fluorescence intensity *FI* in dividing cells. Daughter cells are plotted in equal colors. T = 0 refers to the time of entry into stationary phase. Blue arrows depict time-points of cell division. Green arrows depict time-points of switching. b) Number of cells that initiate switching at time delay $\tau = 0$. c) Fraction of cells in the K-state at delay τ after cell division. Full line: exponential fit with rate k = 0.023 ± 0.003 min^1.

9.2 Genetic switching is initiated after cell division

To assess whether the probability to initiate competence development was correlated with the cell cycle, the temporal delay τ between cell division, as detected in bright field microscopy (Fig. 9.1), and the onset of switching (Fig. 9.2) was measured. The number of cells that initiated switching was maximal after cell division and decayed exponentially with increasing temporal delay τ between cell division and initiation of switching (Fig. 9.2). The exponential distribution underlines the coupling between cell cycle and switching probability. As a measure of coupling strength between cell division and probability for competence initiation (or switching) an exponential fit was applied to the cumulative histogram and a half life of $\tau_h = 27 \pm 7$ min was found. The half life is a measure for the coupling strength. In case the probability for switching was constant throughout the cell cycle, the cumulative histogram would have the shape of a straight horizontal line.
To verify that the distribution of cell-cycle dependent switching probabilities was independent of growth phase and of the time window of data acquisition, the switching probability after cell division for all cells analysed was compared with the switching probability for cells that switched after T_1 (Fig. 9.3). No significant difference was found, indicating that the maximum of switching probability directly after cell division was not an artefact of time-lapse microscopy.

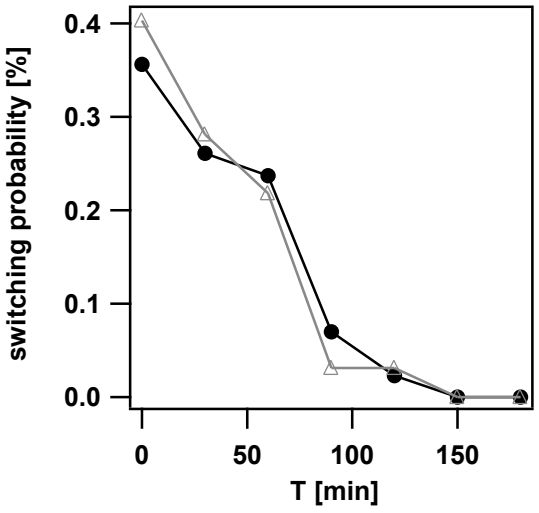

Figure 9.3: The coupling between cell cycle and switching probability p is independent of growth phase: Black: switching probability p of cells switching after T_{-1}. Grey: switching probability p of cells switching after T_1 (1 hour after transition into stationary phase).

9.3 Variances in ComK availability do not decouple cell-cycle and switching

It has been proposed that switching into the K-state was initiated when the basal level of ComK proteins exceeded a threshold for the initiation of the positive feedback loop for *comK* transcription by stochastic fluctuations [26]. Therefore in this set of experiments the switching probability was modified, either by reducing ComK degradation (see chapter 4) or by enhancing *comK* transcription. If coupling between cell cycle and switching probability was caused by unequal partitioning of mRNA or ComK molecules, one would expect that increasing the ComK level or reducing the ComK threshold for switching, would decrease the coupling between cell division and switching, leading to a flattening of the exponential decay of switching probability after cell division.

First the switching delay τ in *rok*- cells in which the Rok repressor control for the *comK* promoter is lifted, tantamount to decreasing the switching threshold, was quantified. In this strain 100 % of the cells switched into the K-state. The distribution of delay times between cell division and switching initiation τ was not significantly different from the wild-type strain with a half life of $\tau_h = 32 \pm 7$ min (Fig. 9.4). Therefore increasing the promoter activity did not influence coupling of competence initiation to cell division.

To assess the effect of enhancing the basal level of ComK, the switching probability after cell division was measured in a strain with reduced proteolysis of ComK by overproduction of the protease inhibitor ComS (see Chapter 4). Unexpectedly, the probability of switching in the *comS* over-expresser ($comS++$) strain was even higher after cell division than in the wild-type or the *rok*- strain, with 76 % of the cells switching within 30 min after cell division (Fig. 9.4). The half time was $\tau_h = 4 \pm 7$min (Fig. 9.4). To assess whether the increase in coupling was due to modified duration of cell cell-cycle, the duration of the *comS++* strain and the wild-type strain was measured. The average durations were 108 ± 7 min in the wild-type strain and 52 ± 7min in the *comS++* strain (Fig. 9.5). Thus the increased coupling between switching rate and cell division is correlated with a decrease of cell cycle duration in the *comS* over-expression strain. For the *rok*- strain the average division time could not be measured for non-competent cells within the time range of data acquisition, since here all cells switched with exponentially decaying probability after the first cell division.
These observations indicate that the probability for initiation of switching is not purely stochastic and demonstrate the existence of a developmental checkpoint for the initiation of switching that is coupled to cell division.

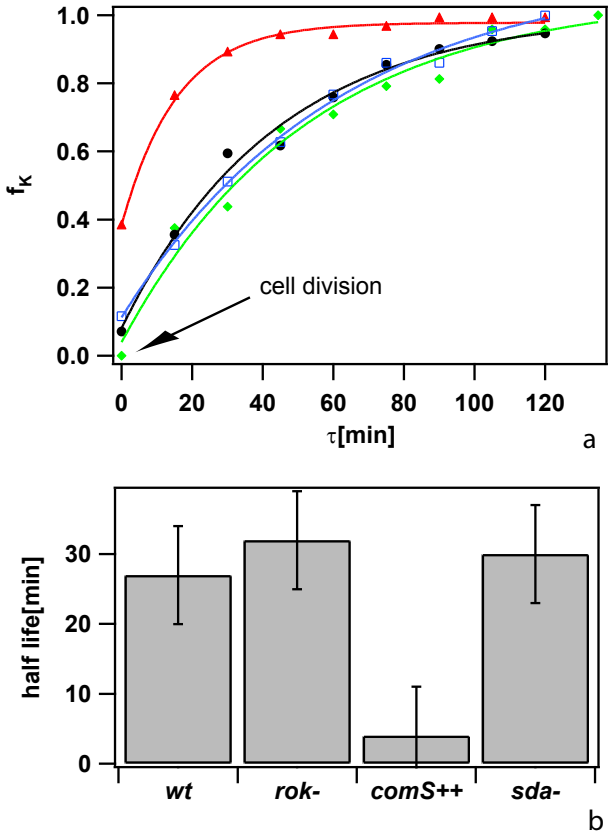

Figure 9.4: Coupling of cell division and switching initiation in various strains a) Fraction of cells in the K-state at delay $\tau = 0$ in wt (black circles), *sda-* (blue squares), full blue line: exponential fit with rate k = 0.016 ± 0.002 min^{-1}, *rok-* (green diamonds), full green line: exponential fit with rate k = 0.019 ± 0.003 min^{-1}, *comS++* (red triangles), full red line: exponential fit with rate k = 0.064 ± 0.004 min^{-1}. b) Half life times τ_h of different strains indicating the time after cell division at which 50 % of the cells initiated competence.

9.4 Influence of the checkpoint protein for sporulation on cell cycle

During cell-cycle progression DNA-replication is crucial and was shown to serve as a checkpoint for initiation of sporulation in *Bacillus subtilis*. DnaA was identified as a crucial protein for replication initiation [37, 75]. In particular, the expression of the master regulator for sporulation SpoOA is coupled to replication initiation via DnaA-

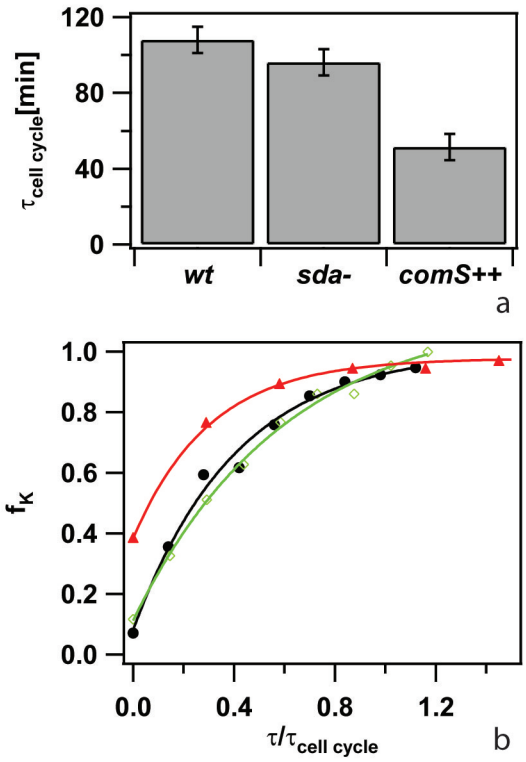

Figure 9.5: Cell division frequency enhances coupling between cell cycle and switching probability a) Average cell division frequency of vegetative cells in the stationary phase. b) Fraction of cells in the K-state at delay after cell division τ normalized to cell division period τ_c in wt (black circles), *sda-* (green diamonds), *comS++* (red squares).

regulated *sda* . Knock-out of *sda* results in increased phosphorylation of SpoOA which in turn represses *abrB*, itself coding for a repressor of *comK* transcription. Therefore, the switching behaviour in a *sda-* strain was analysed and found that the fraction of competent cells increased from 13 % in the wild-type to 34 % in the *sda-* strain (Fig. 9.6), consistent with the proposed effect on SpoOA phosphorylation and repression of AbrB. However, the correlation between switching initiation and cell division was not significantly different from the wild-type (Fig. 9.4). The fraction of cells that had initiated switching increased exponentially as a function of time after cell division τ, showing that the switching probability was still coupled to cell cycle. Thus, the control circuit that prevents cells from initiating competence during cell division is different from the one that regulates sporulation.
Interestingly, the half time of the time delay between cell division and switching was similar with $\tau_h = 27 \pm 7$min in the wild type strain (or $\tau_h = 32 \pm 2$min in the *rok-*

strain) and $\tau_h = 30 \pm 7$min in the *sda-* strain (Fig 9.4). In addition, the average division time for the *sda-* strain was similar to that of the wild-type strain (Fig. 9.5). Therefore, increasing the fraction of competent cells (2-3 fold as compared to the wild-type) did not lead to enhanced coupling of the cell-cycle to switching. This corroborates the conclusion that the observed higher coupling of the *comS++* strain is not an artefact, potentially caused by an unknown additional effect of ComS on competence development.

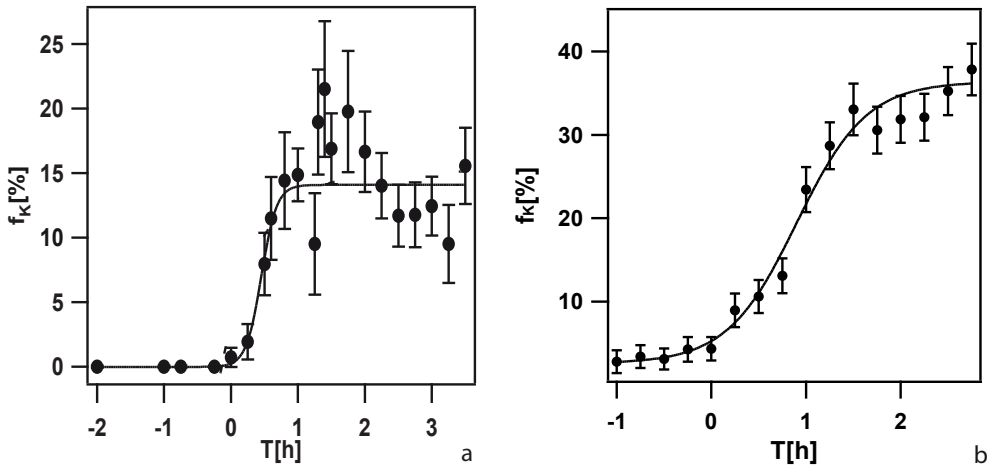

Figure 9.6: Fraction of cells in the competent state a) Wild-type b) *sda* knockout strain.

9.5 Discussion

For a cell it would be potentially detrimental to acquire external DNA during replication of its genome due to interference of recombination with replication. Haijema *et al* found a checkpoint that limits growth and DNA replication once cells have entered the competent state [63]. The real-time experiments described above showed that growth in cell-length was suspended for 50 min once genetic switching was initiated, supporting Haijemas findings. Therefore, the inhibition of growth after entry into the K-state seems to be well understood. In order to avoid parallel processes like replication and incorporation of DNA, not only growth has to be inhibited in competent cells, but vice versa initiation of competence would be risky during ongoing replication. The data described above clearly demonstrate, that a second checkpoint is present to ensure that competence does not interfere with cell division.

9.5.1 A cell division related checkpoint controls the initiation of competence

The probability for switching into the K-state is highest shortly after cell division. It would be quite interesting if this observation could be understood by considering purely stochastic concentration fluctuations of the master regulator ComK or its mRNA. The current model for competence initiation is that the system is bistable. Vegetative cells express ComK at a low concentration and the expression is noisy, generating a broad distribution of concentrations in individual cells (Fig. 9.7). By random fluctuations the concentration of ComK may exceed a threshold concentration and due to the nonlinear amplification of *comK* transcription the cells switch into the K-state in which the expression of ComK is high. Fig. 9.7 shows that indeed the results presented in the cumulative histogram of the *comS++* strain (Fig. 9.4), in which the basal level of ComK is increased as compared to the wild-type can be modeled stochastically:
Assuming that the distribution of ComK levels in vegetative cells between $N(\tau)$ individual cells is Gaussian with an average of [ComK] = 200 molecules and a width (standard variation) of w = 200 molecules. The switching threshold is s = 600 molecules. These values are taken from the stochastic model described in chapter 8. The $M(\tau)$ cells that exceed the threshold at time τ switch and at time $\tau + 1$ a new random distribution with N(τ +1) = N(τ) - M(τ) is generated. Thus after several runs the amplitude of the Gaussian distribution decreases and less cells switch within a given period of time. In the ComS overproducing strain the concentration of ComK should be higher as compared to the wild-type strain, therefore the value was set to [ComK] = 300 molecules and thus reproduced the behaviour of the ComS over-producing strain.
If this observed correlation between cell-cycle and switching probability was really caused by stochastic effects, then increasing the basal transcription rate should lower the switching threshold and weaken the coupling between cell cycle and competence initiation. To lower the threshold the strain *rok-* was used, but the correlation between switching probability and cell division did not weaken. Furthermore, enhancing basal ComK levels should approach the system towards the threshold and weaken the correlation. However, the *ComS++* strain instead showed increased correlation. Both observations exclude purely stochastic correlations.
The correlation between switching and cell cycle could also be due to a general up-regulation of gene expression in the daughter cells. To address this question the average basal fluorescence intensity of 25 cells in the B-state before division was measured and compared to the intensity 15-30 min after cell division. The basal fluorescence intensity increased from 33 ± 2 FU to 37 ± 1 FU. If this subtle effect of up-regulation of *comK* promoter activity was the major cause for cell cycle dependence of switching probability then cell-cycle correlation must be depleted by knocking out *rok*, coding for a strong repressor of *comK* transcription. Since no variation in cell-cycle correlation in the *rok-* strain could be detected, one can conclude, that this increase of *comK* expression after cell division is not a leading cause for cell cycle correlation. Thus the data strongly

indicate a checkpoint before initiation of competence to ensure that competence development is initiated after a cell-cycle round is completed in order to avoid ongoing replication interfering with incorporation of external DNA (Fig. 9.8)

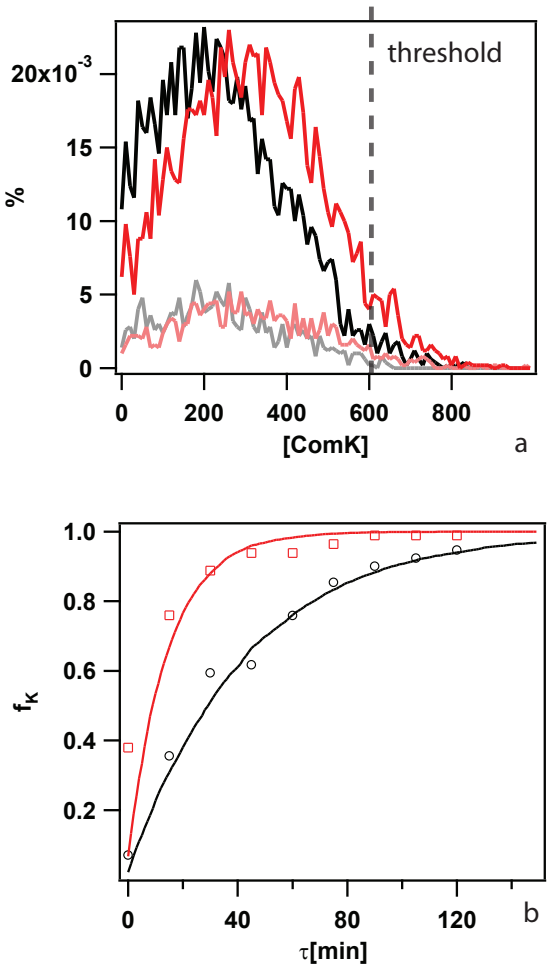

Figure 9.7: Model for stochastic switching after release of cell-division related inhibition of competence development. Details on the model see main text. a) wt: [ComK]= 200 (black), *comS++* [ComK]= 300 (red). Light curves: Gaussian distribution of cells at later time points when most cells have already switched. b) The number of cells in the vegetative state decreases with an exponential rate with N(τ)=1-A exp(kτ), where k is the decay rate. Fraction of cells that have switched at time τ after cell division. Black circles: wt, red diamonds: *comS++*. Black full line: simulation with average [ComK] = 200, w = 200 molecules, s = 600 molecules. Red full line: simulation with average [ComK] = 300, w = 200 molecules, s = 600 molecules.

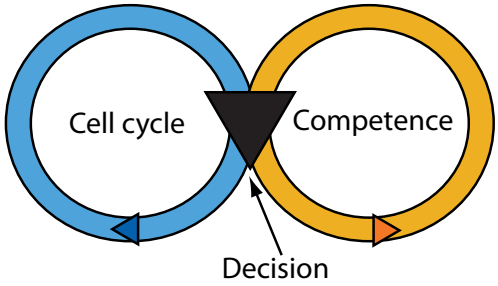

Figure 9.8: Cycles of competence development and cell division are mutually exclusive. When cell division is completed, non-competent cells decide whether they initiate competence or progress along the cell cycle.

9.5.2 Cell cycle frequency tunes the coupling strength between cell cycle and competence initiation

In the current model of competence initiation, switching into the K-state is triggered when the basal levels of ComK exceed a threshold concentration. Here it was observed that the switching probability decayed exponentially after cell division. This could be explained by stochastic switching as described above. If the cells would switch stochastically once inhibition is lifted, then an exponential decay of switching probability as observed in the experiment would be the result. But some experimental results, such as the fact that the switching rate saturates when 13 % of the wild-type cells have switched, cannot be explained by the stochastic model.
Other explanations for the exponential decay of the switching rate would be the accumulation of inhibitor proteins. For initiation of sporulation rapid proteolysis of the replication checkpoint protein Sda was shown to be necessary and the half life was 4 min [75]. A similar mechanism may trigger the initiation of competence development after cell division, i.e. the checkpoint protein would be rapidly degraded allowing the cells to initiate competence. The concentration of the inhibitor may then build up gradually until the next round of division is initiated. Alternatively, an activator concentration could increase rapidly after cell division and be gradually degraded as cell cycle progresses. This hypothesis can explain the observation that the duration of the cell cycle tunes the temporal development of the switching probability. To investigate whether the half life time of the switching probability depended linearly on the duration of the cell cycle, the data were normalized to the duration of one cell cycle τ_c (Fig. 9.5). It was found that coupling in the *comS* over-expressing strain did not match with the wild-type and the *rok*- strain. This observation indicates that the process of cell-cycle checkpoint is not simply linear, i.e. that the cell-cycle/competence coupling regulator is produced/degraded in a non-linear fashion during cell-cycle.

When looking for the checkpoint mechanism firstly similarities to the sporulation pathway could be assumed. In *Bacillus subtilis* sporulation is inhibited upon inhibition of DNA replication [36] and control of *sda* by the principal initiator of replication, DnaA, establishes a checkpoint that inhibits activation of SpoOA [37]. Phosphorylation of SpoOA represses *abrB* , itself coding of a repressor for *comK* . Therefore, it appeared likely that DnaA controlled the initiation of competence via the same pathway. However, although the deletion of *sda* had a strong effect on the fraction of competent cells, *sda* knockout showed no effect on cell cycle correlation of switching probability. Competence development seems to employ a distinct checkpoint pathway to prevent cells from initiating competence during an ongoing cell-cycle round (Fig. 9.8). DnaA regulates the initiation of replication by cell-cycle dependent binding to DnaA motifs (DnaA boxes) on the chromosome. DnaA boxes were mapped not only at the origin of replication and before *sda*, but also in other regions of the *Bacillus subtilis* genome, indicating that additional genes might be coupled to initiation of replication [76].

10 Termination of switching window

While the entry into the K-state is now pretty well understood, still little is known why switching into the K-state stops after a certain time. Different scenarios try to explain the shut-down of the auto-catalytic feedback of *comK* expression. While Suel *et al* [68] suggested repression of ComS production by ComK, Smits *et al* [69] showed that a member of the Rap family of proteins, RapH can indirectly suppress the expression of ComS with increasing concentration of ComK [69]. Furthermore, accumulation of pheromones such as CSF may switch off the feedback in individual cells dependent on growth phase [35]. Chapter 7 and 8 clearly demonstrate the presence of a well defined switching window during which cells are able to stochastically switch into the K-state. While chapter 7 pinpoints the importance of quorum sensing for the beginning of the switching window, this chapter presents experiments pointing out the importance of extracellular inhibitory substances for the termination of the switching window of competence development. It will be shown that both highly competent and non-competent cells release inhibitory substances into the surrounding medium in the stationary growth phase, terminating or preventing the development of competence, respectively.

10.1 Inhibitory substances derived from highly competent cells decrease the switching rate

To assess whether an external factor could be important for termination of the switching window for competence, the competence medium of cells at $T_{-0.5}$ (half an hour prior to the transition from exponential to stationary phase) was exchanged for the supernatant of highly competent BM 50 cells at T_2. At T_2 it was shown, that cells start to exit the competent state [63, 65]. Therefore, any inhibitory elements should have maximal concentration at this time-point. The fraction of K-cells as a function of time was still well described with a sigmoid behaviour as described in chapter 7, eqn. 7.1. As expected the fraction of K-cells decreased after addition of T_2 BM 50 supernatant to $7 \pm 0.7\%$ as compared to $14 \pm 1\%$ for the wild-type under normal growth conditions (Fig. 10.1c and Fig 10.5). The onset of switching did not alter, but duration of switching was doubled. The switching rate p_K was again (see chapter 7) determined as the first derivative of the sigmoid fit to f_K with respect to time [65]. To determine the intrinsic switching rate p_K, the data were normalized to the fraction of non-competent cells [100%-f_K] (Fig 10.1d).

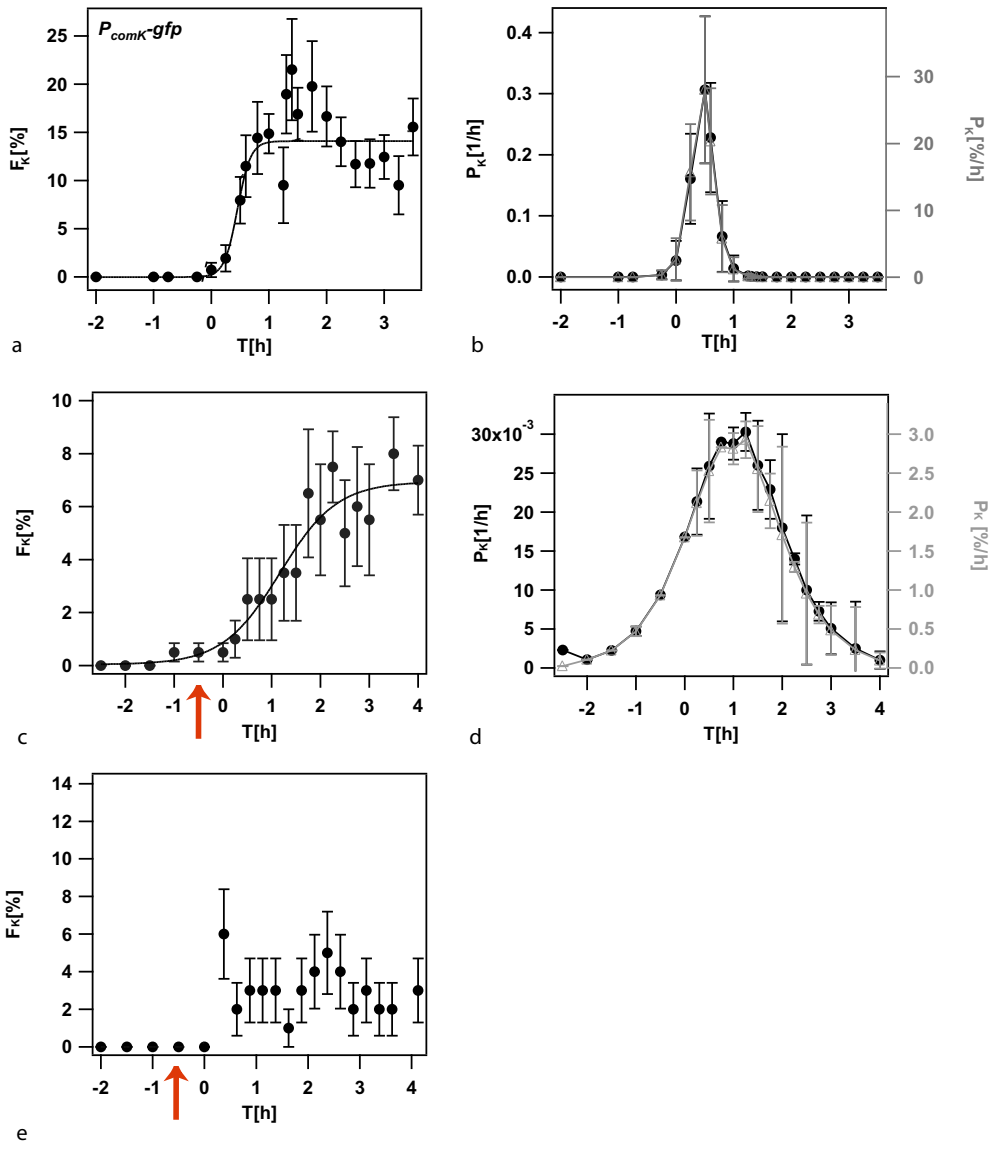

Figure 10.1: Fraction of BD 2711 cells in the K-state as a function of time and switching rate a, c and e) Fraction of cells in the K-state as a function of time f_K. Full line: best fit to a sigmoid function (Eqn. 7.1). b and d) switching rate p_{fK} (grey) and intrinsic switching rate p_K (black). a and b) BD 2711 under normal conditions. c and d) BD 2711 after exchange for supernatant obtained from highly competent (T_2) BM 50 cells at $T_{-0.5}$. e) BD 2711 after exchange for supernatant obtained from non-competent (T_2) BD 630 *comK*- cells at $T_{-0.5}$. Arrows depict timepoints of medium exchange. Parameters for sigmoid fits are listed in Table B.3 in appendix B.

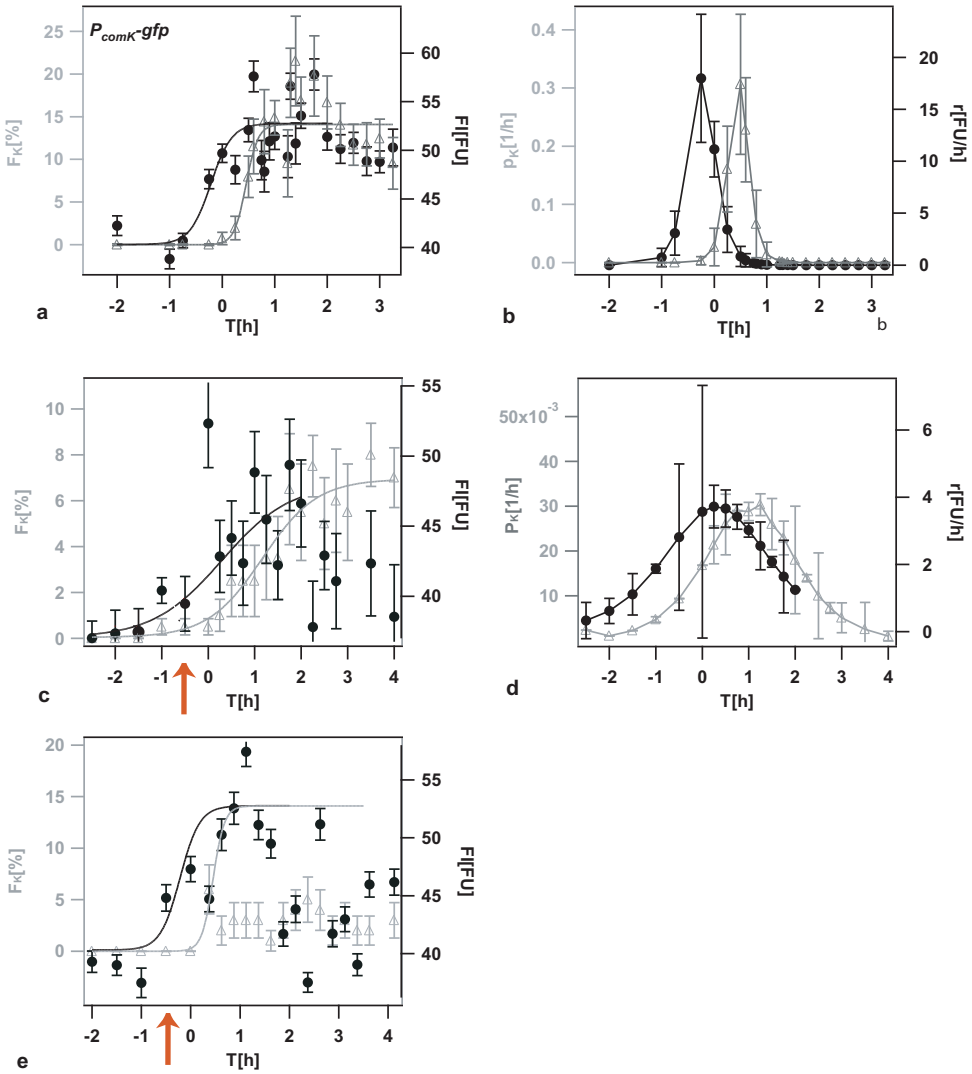

Figure 10.2: Basal *comK* expression rate of BD 2711 a, c, and e) Black: basal fluorescence intensity FI. Grey: fraction of cells in the K-state f_K. Full lines: best fit to a sigmoid function (eqn 7.1). b and d) Black: basal expression rate r determined from the first derivative with respect to time of the sigmoid fit. Grey: intrinsic switching probability p_K determined from the first derivative with respect to time of the sigmoid fit and normalized by the fraction of non-competent cells (100-f_K). a and b) BD 2711 under normal growth conditions. c and d) BD 2711 after exchange for supernatant obtained from highly competent (T_2) BM 50 cells at $T_{-0.5}$. e) BD 2711 after exchange for supernatant obtained from non-competent (T_2) BD 630 *comK*-cells at $T_{-0.5}$. Red arrows indicate time-points of medium exchange. Parameters for sigmoid fits are listed in Table B.4 in appendix B.

The maximum values were $p_{Kmax} = 0.3 \pm 0.1$ h_{-1} for the wild-type under normal growth conditions and $p_{Kmax} = 0.035 \pm 0.05 h_{-1}$ for the wild-type after medium exchange.

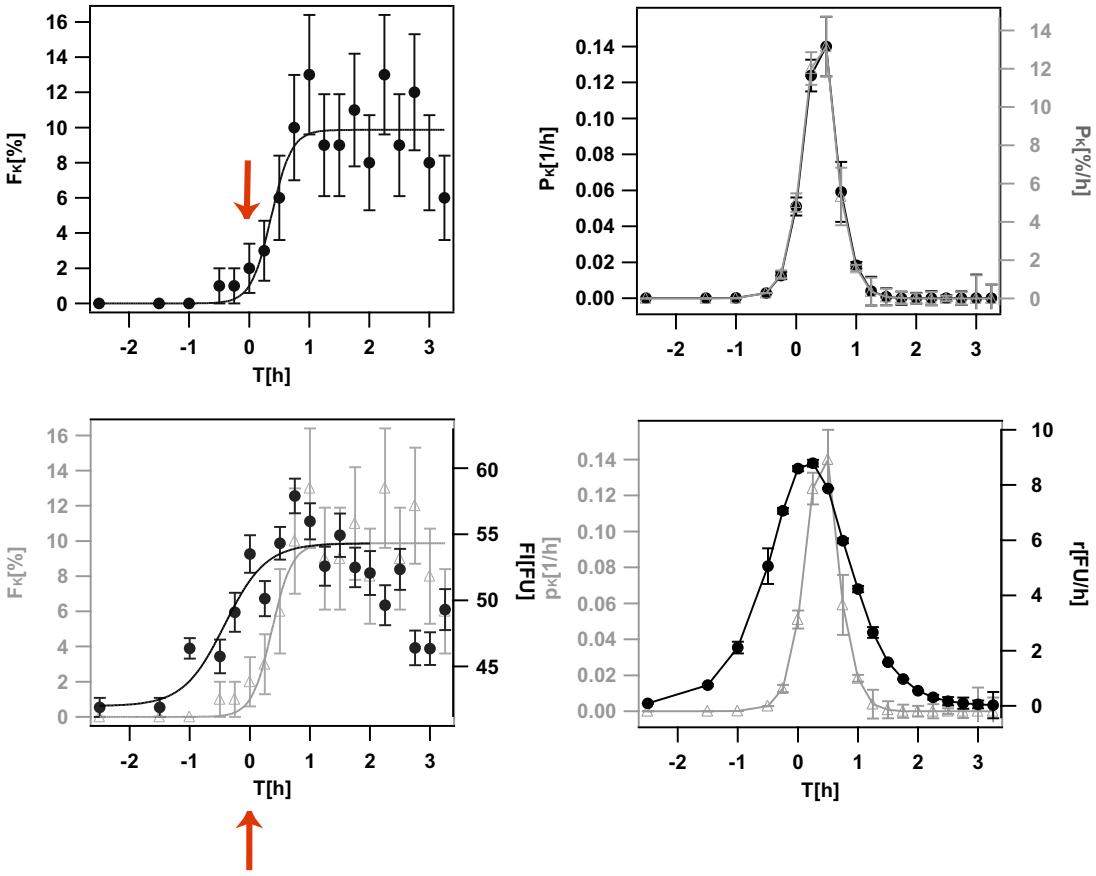

Figure 10.3: Fraction of BD 2711 cells in the K-state as a function of time and switching rate a) Fraction of BD 2711 cells in the K-state as a function of time f_K after exchange for supernatant obtained from highly competent (T_2) BM 50 cells at T_0. Full line: best fit to a sigmoid function. b) Switching rate p_{fK} (grey) and intrinsic switching rate p_K (black). c) Black: basal fluorescence intensity FI. Grey: fraction of cells in the K-state f_K. Full lines: best fit to sigmoid function. d) Black: basal expression rate r determined from the first derivative with respect to time of the sigmoid fit. Grey: intrinsic switching probability p_K determined from the first derivative with respect to time of the sigmoid fit and normalized by the fraction of non-competent cells (100-f_K). Red arrows indicate time-points of medium exchange. Parameters of the fits are listed in Table B.3 (see appendix B).

10.2 Inhibitory substances derived from highly competent cells lower basal *comK* expression

Since the intrinsic switching rate was decreased ten fold after exchange of conditioned medium for supernatant obtained from highly competent T_2 BM50 cells, the next question to address was whether the basal *comK* expression rate was also decreased as compared to the wild-type under normal growth conditions. When the medium at $T_{-0.5}$ was exchanged for medium of BM 50 T_2 cells, the fluorescence intensity of non-competent cells increased only very little and could be well described by eqn. 7.1. The expression rate r was measured as the first derivative with respect to time of the mean grey value of those cells that had not entered the K-state as described in chapter 7. The maximum expression rate was 4 ± 0.6 FU/h as compared to 18 ± 7 FU/h for the wild-type under normal growth conditions (Fig. 10.2). The maximum expression rate and the maximum intrinsic switching rate showed a significant time delay of approximately 1 h for both experimental conditions (Fig. 10.2). To exclude these effects being due to shortage of nutrition in the late stationary phase, the experiment was repeated, but this time the medium was exchanged for supernatant of highly competent T_2 BM50 cells at T_0. At this time-point the maximal promoter activity of *comK* is already passed and therefore both the maximum fraction of cells in the K-state and the maximum *comK* expression rate should be less affected, if not other limiting factors have to be taken into account. Indeed the fraction of competent cells in the K-state did only decrease down to $10\ \% \pm 1$, while the intrinsic switching rate was lowered by a factor of 2 as well as the *comK* expression rate (Fig. 10.3). These results show clearly that the effects described here were rather due to inhibitory substances in the surrounding medium than limitation of nutrition.

10.3 Inhibitory substances derived from non-competent cells decrease basal *comK* expression dramatically

To assess the question whether or not the release of inhibitory substances obtained from supernatant of highly competent BM 50 T_2 cells could be competence induced, the experiment was repeated with supernatant obtained from non-competent T_2 BD 630 *comK*- cells. If the release of those inhibitory substances is competence induced one would expect a less intense decrease of cells in the competent state. Instead it was found that the fraction of cells in the K-state decreased down to $\approx 3\%$ as compared to the wild-type under normal growth conditions (Fig. 10.1e and Fig. 10.5). Interestingly the fraction of cells in the K-state seemed to develop as usual until $T_{0.5}$ and than dropped down to 3%, but it is unclear wheather this is an artefact of the measurement method applied (high error), or if indeed a shut down of the *comK* promoter takes place. In

the later case the fact that the fraction of cells drops down to 3% instead of staying at 7% could be explained by increased cell growth and/or division. To obtain detailed information on this subject, it will be important to improve time resolution, so that more time-points can be taken.
The basal *comK* expression rate in the *comK* knockout strain (Fig. 10.2e) increased similarly as the wild-type strain under normal growth conditions, but decreased again after T_1 to the fluorescence values obtained in the exponential growth phase. Since the values of basal fluorescence intensity decreased immediately after T_1 the promoter activity for the wild-type strain after medium exchange for the supernatent of the *comK* knockout strain was not determined.

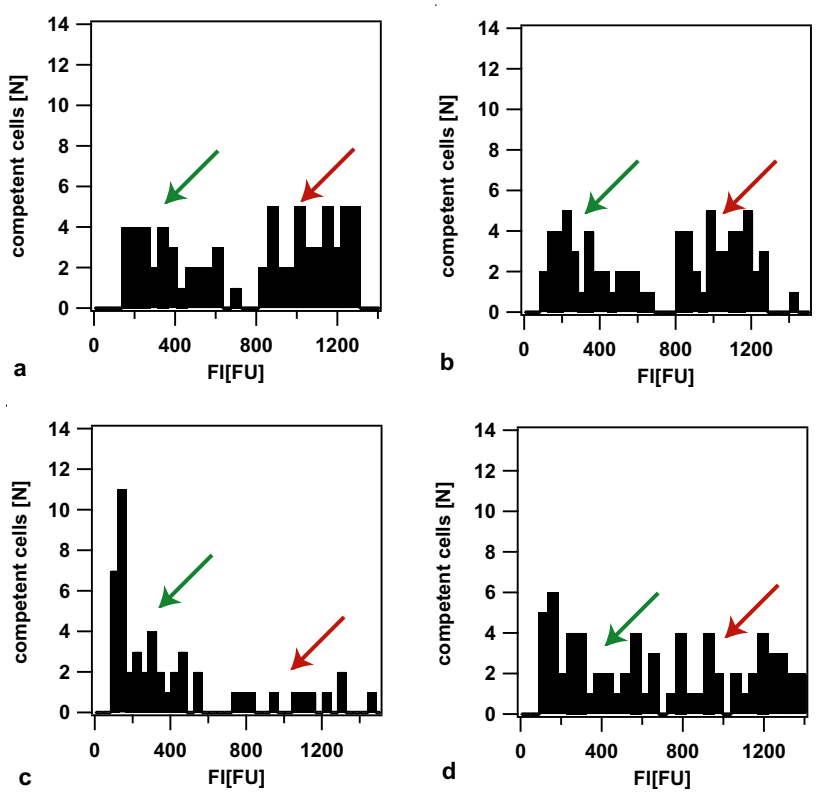

Figure 10.4: Histograms: Comparison of maximum ComK values: a) BD 2711 grown under normal conditions in an Erlenmayr flask. b) BD 2711 grown under normal conditions on a microscopic slide. c) BD 2711 after exchange for supernatant obtained from highly competent T_2 BM 50 cells at $T_{-0.5}$. d) BD 2711 after exchange for supernatant obtained from highly competent T_2 BM 50 cells at T_0.

10.4 Addition of inhibitors influences the max. fluorescence intensities of single cells

Since the exchange of medium at $T_{-0.5}$ for medium taken from BM 50 cells at T_2 decreased the basal *comK* expression, it was interesting to see whether this exchange did also influence the maximal ComK values expressed in individual cells. Therefore the fluorescence intensity distributions of ComK at T_2 and T_3 for wild-type and wild-type after medium exchange, respectively, where it was shown, that most of the cells had entered the K-state [65], were compared to each other. To ensure that indeed all the cells had reached their maximum fluorescence values, the FI values of wild-type cells taken at T_2 grown in bulk were compared to the FI values of wild-type cells grown on a microscopic slide. They showed similar FI distributions (Fig 10.4a and b). The wild-type showed a ComK distribution with two distinct peaks, one at 350 FU ± 50 and one at 1050 ± 25 FU. The maximum values of the wild-type cells after medium exchange at $T_{-0.5}$ were shifted to lower values (Fig 10.4c). Exactly, the distribution of FI values around 1050 decreased and a new peak at 150 developed. The FI peak around 350 was not altered. When instead the supernatant was exchanged at T_0, again the same FI distribution as for the wild-type under normal growth conditions could be observed. This indicated that inhibitory substances derived from highly competent cells shut down *comK* expression, but where only able to cause an effect on *comK* expression when *comK* transcription was fully active.
The maximum fluorescence values for exchange with supernatant of non-competent cells could not be obtained, since here it is not clear that variations in the FI value distribution are due to cells already having exited the competent state, or cells still being not in the competent state.

10.5 Discussion

10.5.1 Release of competence induced inhibitors terminates the switching window of competence.

To assess the question whether or not the termination of the switching window may be due to the presence of inhibitory substances in the surrounding medium, given into the medium by the competent cells itself, the fraction of cells in the K-state was measured for cells grown under normal conditions and cells where the medium was exchanged at $T_{-0.5}$ for the supernatant of highly competent T_2 BM 50 cells. While under normal growth conditions 14 ± 1% of the cells switch into the K-state, only 7 ± 0.7% cells entered the K-state when the medium was exchanged. This decrease was even higher as compared to experiments shown earlier (see chapter 7, Fig. 7.4) where the medium was exchanged for fresh medium at T_0. Therefore the higher decrease may be due to

inhibitory substances in the supernatant of highly competent BM 50 T_2 cells such as CSF [35]. This decrease can not be due to the fact of medium exchanging itself, since it was shown earlier (see chapter 7, Fig. 7.4) that exchange for conditioned medium increased the fraction of competent cells to 36%. The intrinsic switching rate was decreased 10 fold for cells where the medium was exchanged for the supernatant of highly competent BM 50 T_2 cells at $T_{-0.5}$. The basal *comK* expression rate was also repressed and showed a maximum of only 4 ± 0.6 FU/h as compared to 18 ± 7 FU/h for the wild-type grown normally. Since the medium exchange took place shortly before the time-point of maximal activity of the *comK* promoter (as seen in wild-type experiments under normal growth conditions (Fig. 10.2b)), but still had an effect on the basal expression rate of *comK* , inhibitory substances in the surrounding medium of competent cells must have an effect acting on *comK* expression. This effect is thought to be indirect since the promoter was not shut down totally but only lowered in its activity. Therefore, competence induced extra cellular inhibitors might act over the ComS and ComK pathway indirectly on the *comK* promoter (Fig. 10.6). When the medium exchange took place shortly after the time-point of maximal *comK* promoter activity, the fraction of cells in the K-state was altered very little (10 % instead 14 %) and only small differences in increase of basal *comK* expression rate could be detected. Therefore, inhibitory substances obtained from highly competent cells can only affect the basal *comK* expression rate when added prior to the time-point of maximal *comK* promoter activity, indicating an indirect action on the *comK* promoter. Therefore it is more likely that competence induced inhibitors act over the ComS pathway rather than via direct regulators of the *comK* promoter such as AbrB. Lazazzera *et al* showed that competence development is partly controlled by a pentapeptide called CSF [35]. CFS was shown to be only functional when present inside the cell after transport through the membrane by the oligopeptide permease *spoOK*. Inside the cell CSF was found to have at least three different targets corresponding to three different activities. At low concentrations (1-10nM) CSF inhibits the phosphatase RapC. Since RapC inhibits the transcription factor ComA (in its phosphorylated form), ComA can now act as an inducer of ComS expression, thereby increasing ComK stability (Fig. 10.6 pathway 1a) and stimulating competence gene expression. At high concentrations (< 20nM) CSF stimulates sporulation, but also inhibits competence gene expression [35]. It was suggested that inhibition of competence by CSF could be due to inhibition of the ComP histidin-kinase through CSF, thereby terminating ComS expression and therefore allowing degradation of ComK to take place [15]. Inhibition of ComS expression through the action of CSF might also possibly be due to activation of RapH [69] through CSF (Fig. 10.6 pathway 1b). The inhibitory effects seen in the experiments presented here might be due to the action of CSF. High ComK levels inside the cell could lead to increased CSF expression in the late stationary phase. High concentrations of CSF could than lead to the termination of the time window of stochastic switching into the K-state as described above (Fig. 10.6).

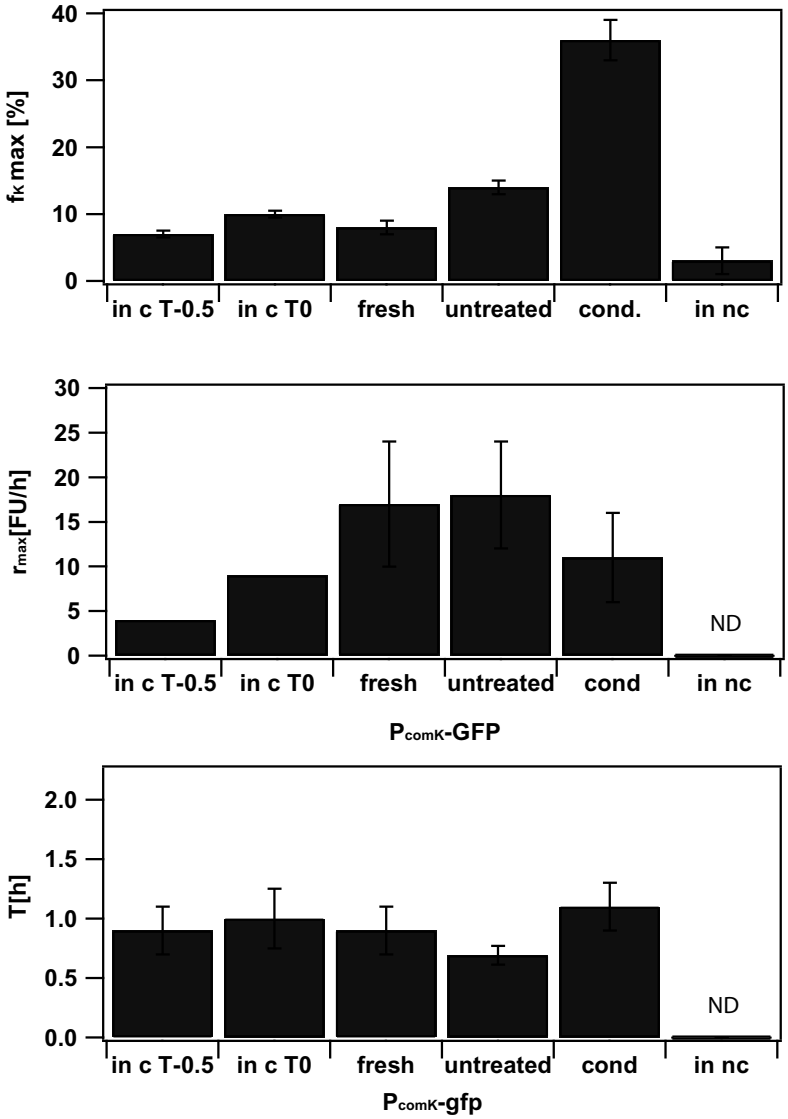

Figure 10.5: Comparison of important values for the different growth conditions of BD 2711 a) Maximum fraction of cells in the K-state f_{Kmax}. b) Maximum expression rate r_{max}. c) Shift of half life period T between basal fluorescence level and switching distribution. in = inhibition, c = competent, nc = non-competent, fresh = exchange for fresh medium, cond. = exchange for conditioned medium, ND = not definded.

10.5.2 Release of inhibitors by non-competent cells prevents switching into the competent state.

To obtain information about these effects being competence induced or not, the experiment was repeated with supernatant of non-competent T_2 BD 630 *comK*- cells. If the effect described above was only due to the development of competence, no effect should be seen with the supernatant of non-competent cells. Unexpectedly, it was found, that the fraction of cells in the K-state decreased dramatically down to 3% instead of further increasing (as seen in Fig. 10.1e), indicating that non-competent cells also release inhibitory substances into the surrounding medium in the stationary phase. The fact that the fraction of cells in the K-state decreased to 3% may be due to increased cell division of remaining non-competent cells. Inhibitory substances derived from non-competent cells seem to act differently than inhibitors originated from competent cells, since the reaction of the wild-type population is increased and takes place immediately. These inhibitory substances of non-competent cells do not seem to act primarily on the *comK* promoter since the basal *comK* expression rate was not altered until T_1 as compared to normal growth conditions. The basal fluorescence values decreased after T_1 down to fluorescence values obtained at exponential growth, indicating that inhibitory substances derived from non-competent cells may induce cell growth and division thereby enabling the cells to exit the competent state. Alternatively, inhibitory substances derived from the BD 630 *comK* knock-out strain could also increase degradation of ComK after T_1. Inhibitory substances derived from the *comK* knock-out strain seem to prevent further transitions into the K-state, in a way different from the action of inhibitors derived from competent cells. By ensuring that no competence machinery is built when cells fail to become competent, non-competent cells can prevent loss of energy, thereby enabling them to outgrow competent cells.

10.5.3 Competence induced inhibitory substances lower the K-state.

As seen in chapter 8, wild-type cells grown normally develop two distinct ComK concentration distributions: one at 350 FU and one at 1050 FU. Inhibitory substances from highly competent cells released into the surrounding medium in the stationary phase shifted the ComK values reached in single cells to lower values. While in experiments with exchange for supernatant of highly competent BM 50 T_2 cells at $T_{-0.5}$ the peak of FU values around 1050 FU was lowered, supernatant added at T_0 could no longer affect the FU values of single cells. This indicates that the regulation cannot take place once the maximum *comK* promoter activity has been passed. Therefore regulation of *comK* values in single cells might be due to increased ComK degradation by action of the CSF-ComS pathway as described above (Fig. 10.6).

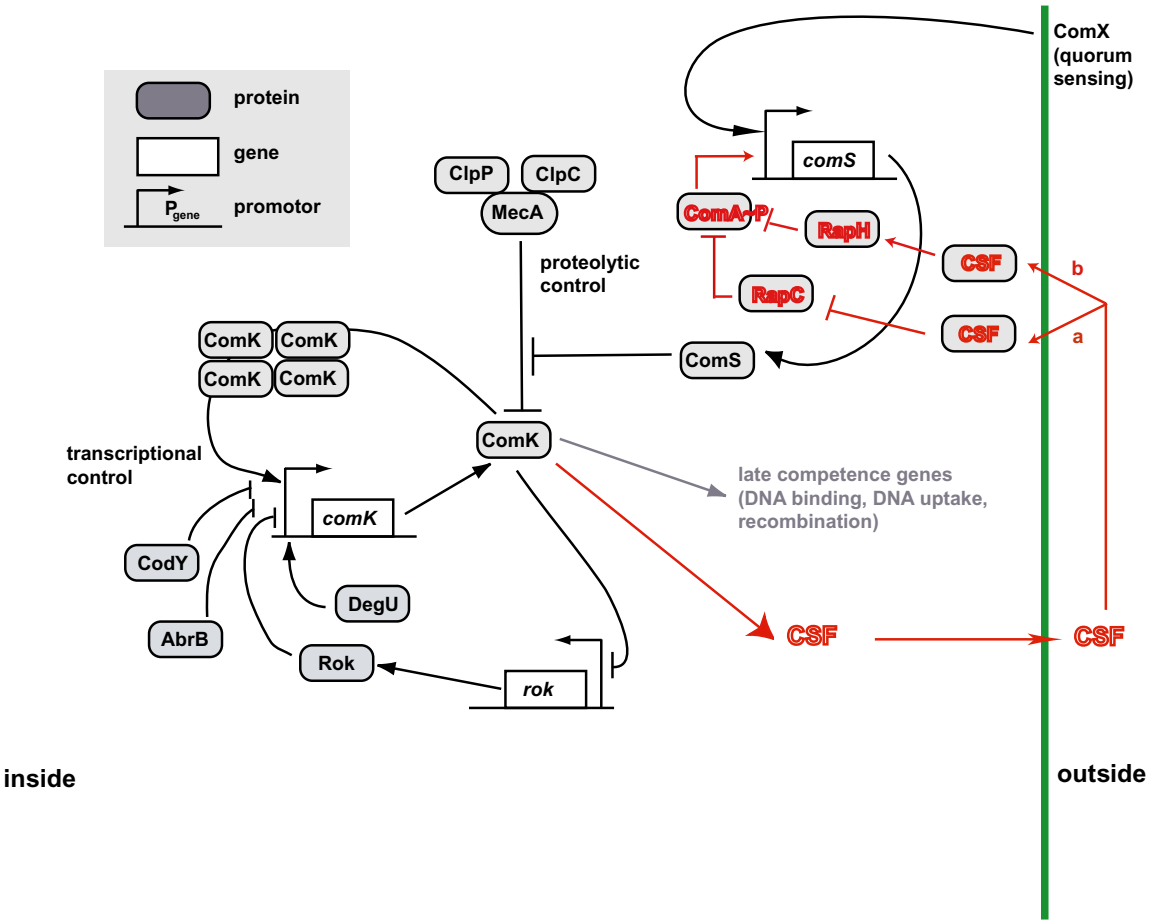

Figure 10.6: Core of the competence circuit Arrows denote up-regulation and blunt ends denote down-regulation. Green line indicates the cell wall. a): possible pathway of competence induced activation of ComK availability inside the cell during exponential growth phase at low CFS concentrations. Low ComK levels inside the cells may lead to minor expression of inhibitory factors such as CSF, which are then transported through the membrane. After reimport into the cell, those inhibitory factors lead to the activation of *comS* expression through action of RapC, thereby increasing ComK stability. b): possible pathway of competence induced repression of ComK availability inside the cell at late stationary phase at high CFS concentrations. High ComK levels inside the cells may lead to high expression of inhibitory factors such as CSF, which are then transported through the membrane. After reimport into the cell, those inhibitory factors lead to the repression of *comS* expression through action of RapH, thereby increasing ComK degradation.

10.6 Conclusion

Using a single cell assay, it was shown that inhibitory substances in the surrounding medium released by highly competent cells in the stationary phase, lead to indirect shut-down of *comK* expression probably due to the action of the pentapeptide CSF. Non-competent cells also give inhibitory substances into the surrounding medium in the late stationary phase thereby decreasing *comK* expression. Independent of the origin of the inhibitor, its presence decreased the number of cells entering the competent state.
Here we suggest a model (see Fig. 10.6) in which competent cells release inhibitors into the surrounding medium in the stationary phase, thereby terminating the switching window for competence (Fig. 10.6). High ComK levels inside the cells may lead to expression of inhibitory factors such as CSF, which are then transported through the membrane. After reimport of those inhibitory factors, they lead to the repression of *comS* expression, thereby increasing ComK degradation. Additionally, non-competent cells also release inhibitory substances into the surrounding medium with entry into the stationary phase, ensuring that no competence machinery is built without usage.

11 Future prospects

Competence in *Bacillus subtilis* has been studied for decades, but still new questions arise and new insights are delivered. The discovery that *Bacillus* is able to take up DNA from the surrounding medium fascinated scientists since it opened up a new way of evolution. First studies on competence started with the design of proper growth conditions that allow cells to become competent [59]. The discovery of proteins involved in DNA uptake and their interactions followed, leading to the model of the DNA uptake machinery as seen in Fig. 4.2 (chapter 4). Using optical tweezers, time, velocity and the force necessary for DNA uptake could be revealed [11]. In the last years more and more interest was applied on regulation mechanisms leading to the bistable expression pattern of competence in *Bacillus subtilis* [41]. Recent work focusses on kinetic aspects concerning competence development [5,6,68] or tries to explain how competent cells can exit the K-state after a certain time [35, 69].
This work contributed to the understanding of competence development. It revealed a strong coupling between cell cycle and switching into the K-state and demonstrated the importance of checkpoint mechanisms for competence development. Previously, a DNA-dependent checkpoint mechanism has been found for the sporulation pathway [37], and Sda could be shown to be the protein responsible for the mechanism. Since competence and sporulation are connected with each other via the protein AbrB, it was natural to ask if Sda could also be the protein important for the checkpoint mechanism responsible for competence induction. Unfortunately, this was not the case, and future experiments have to be performed to discover the real checkpoint protein. Sda has been shown to be regulated itself by DnaA, a protein that regulates the initiation of replication by cell-cycle dependent binding to DnaA motifs (DnaA boxes) on the chromosome. DnaA boxes were also mapped in other regions of the *Bacillus subtilis* genome, indicating that additional genes might be coupled to initiation of replication, possibly responsible for the checkpoint in competence induction [76]. Nevertheless, Sda had an effect on competence development since 32% instead of 14 % of the cells entered the K-state, indicating another still unknown coupling between sporulation and competence that has to be discovered in the future.
The checkpoint for sporulation was found to be DNA-dependent and further experiments are required to show the dependence of competence checkpoint mechansims on either DNA replication or recombination. The effect of DNA replication can be examined using replication inhibitors such as NAL (= Nalicixid acid, acting on the enzyme Gyrase) or HPura (= 6-hydroxy-phenylazo-uracil, acting on DNA Polymerase III) added prior to entry into the stationary phase. Coupling to recombination could be determined using

mutants carriing a ComK-GFP reporter and additionaly a YwpH-YFP reporter, since YwpH is ment to be important for the recombination of uptaken DNA.

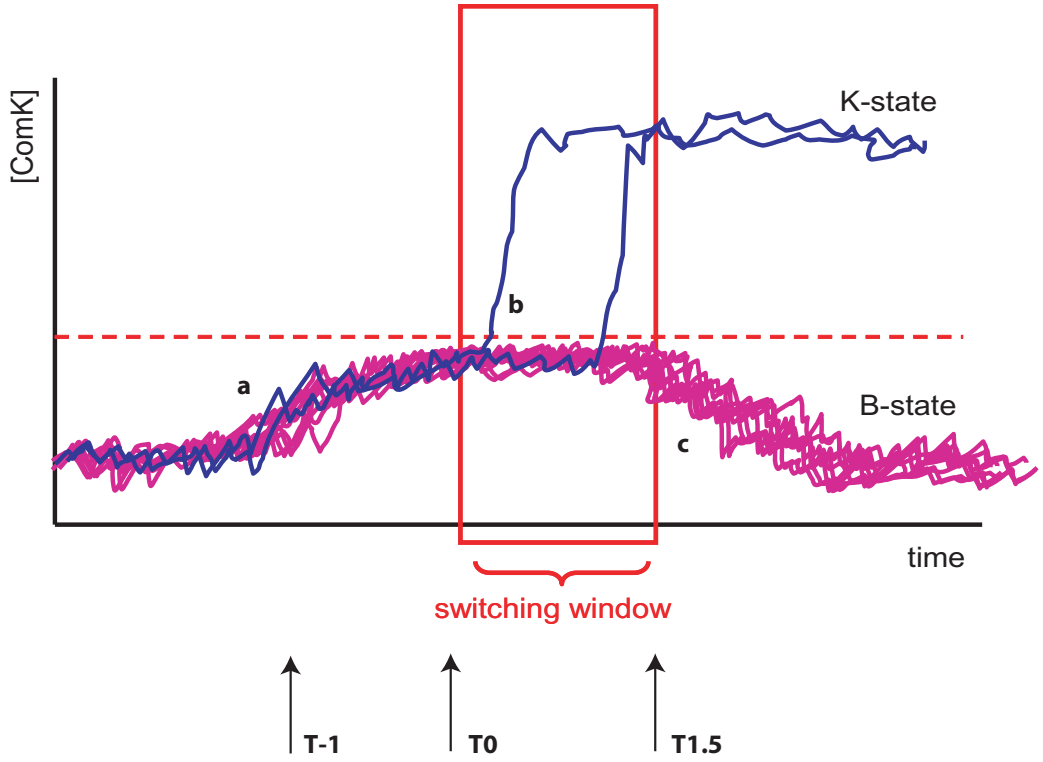

Figure 11.1: The window of competence Quorum sensing sensed over the ComS pathway increased the basal *comK* expression rate in every single cell and enables them to decide whether to switch into the K-state or not (a). Stochastic effects govern the probability for competence initiation (b). Switching is thereby only possible during a specific time window. Accumulation of competence induced inhibitory substances allow cells to slowly exit the competent state (c).

Concentrating on the main transcription factor ComK, this work gave further insights on the kinetics of competence development in *Bacillus subtilis* and clearly showed the existence of a well defined competence window [65], in which cells can enter the competent state. The obtained data lead to the model in Fig. 11.1.

In the late exponential growth phase the pheromone ComX accumulates in the surrounding medium. At about T_{-1} the ComX concentration is high enough to be sensed by the ComP histidine kinase that activates a quorum sensing pathway via ComS, thereby enhancing the basal *comK* expression rate in every single cell. This generally enables the cells to decide whether to switch into the K-state or not. Now in principle pre-activated stochastic switching into the K-state can be induced and takes place over a time period of about 1.5 h. With the end of the switching window, inhibitory substances released into the surrounding medium by the competent cells itself accumulate possibly enabling competent cells to slowly exit the competent state.
In addition this work showed the presence of inhibitory substances in the surrounding medium closing the switching window of competence. This mechanism is still not well understood. Firstly, the proteins or peptides responsible for these inhibitory effects have to be discovered. Secondly, their interactions with proteins in the cell membrane have to be examined and the pathway and the targets inside the cell have to be revealed. CSF, the pentapeptide thought to be responsible for inhibitory effects in the late stationary phase, could be synthesized artificially and added to a growing wild-type culture as done in chapter 10. This could reveal if CSF indeed causes the obtained effects. Knock-out mutants of RapH or RapC could give detailed information about interaction of CSF or other inhibitors, with either RapH or RapC dependent on growth phase, concentration of the inhibitor and its origin (competence induced or not). Knowing this, the tight regulation mechanism of competence development and competence duration can be understood in the future.
Another interesting feature seen in this work is the fact that individual cells are able to reach different ComK levels. The theoretical model described in chapter 8 showed that extrinsic noise can explain a broad distribution of ComK levels but not the existence of two clearly distinguishable ComK level distributions. It would be interesting to see whether cells with higher ComK levels are more competent than cells with low ComK levels. This could, for example, be examined using the laser tweezer technique coupled with fluorescence microscopy. With this method one could obtain the information if cells with high ComK values take up DNA from the surrounding medium faster than cells with low ComK values, or if they are able to take up longer DNA fragments as compared to their counterparts. Another question would be if those cells can incorporate the DNA more efficiently into their genomes, and if specific preferations to gene classes (genes for sugar or aminoacid usage compared to genes for enzymes) are present.

A Competence Medium

To grow *Bacillus subtilis* to competence, a specific medium, the so called competence medium is necessary [59]. Since competence is repressed in the presence of detergents all solutions have to be made in flasks that have not been washed with a detergent! Therefore all flasks need to be washed 10 times with distilled water before usage. To obtain the competence medium firstly, a stock solution of spizizen salts 10x has to be prepared:

chemical	amount
KH_2PO_4	6 g
K_2HPO_4	14 g
$(NH_4)_2SO_4$	2 g
Na-citrate x $2H_2O$	1 g

Table A.1: Spizizen Salts
Spizizen Salts (1x SS) for 1 Liter, pH = 7.0

The ingredients of the competence medium underneath have to be autoclaved separately, therefore stock solutions as indicated have to be prepared. Please note that $MgCl_2$ has to be added to the medium directly before usage, otherwise the medium will precipitate.

chemical	amount
10 % yeast extract	0.5 ml
2 % casein hydorlyzate	0.5 ml
50 % glucose	0.5 ml
histidine (10 mg/ml)	0.25 ml
leucine (10 mg/ml)	0.25 ml
methionine (10 mg/ml)	0.25 ml
$MgCl_2$ (1 M)	0.125 ml
$MgSO_4$x $7H_2O$	0.05 ml

Table A.2: Competence Medium
Competence Medium To 50 ml 1x SS ad amounts mentioned in this table

B Fit Parameters

	k[1/h]	T_{half}[h]	f_{max}[%]	f_{base}[%]
BD 2711 P_{comK} gfp	8 ± 3	0.45 ± 0.06	14.1 ± 0.8	0.01 ± 0.05
BD 2711_{cm} P_{comK} gfp	1.6 ± 0.3	0.5 ± 0.1	36 ± 3	-1.6 ± 1.5
BD 2711_{fm} P_{comK} gfp	2.3 ± 0.9	2.7 ± 0.2	8 ± 1	0.4 ± 0.6
BM 50 P_{comK} gfp, *rok-*	4.3 ± 0.1	0.49 ± 0.04	99 ± 3	-0.5 ± 0.5
BM 77 P_{comK} gfp, *mc comS*	2.3 ± 0.3	0.26 ± 0.05	83 ± 3	-0.7 ± 2

Table B.1: Fit Parameters

Fit Parameters For the fraction of cells in the K-state f_K as a function of time T. Parameters determined from the best fit to a sigmoid function $f_K = f_{base} + \frac{f_{max}}{(1+exp[k(T_{half}-T)])}$, with f_{base} baseline ,f_{max} maximum fraction of cell in the K-state, T_{half} half time, k switching rate. cm: Exchange for conditioned medium at T_{-2}. fm: Exchange for fresh medium at T_{-2}.

	k[1/h]	T_{half}[h]	FI_{max}[FU]	FI_{base}[FU]
BD 2711 P_{comK} gfp	6 ± 2	-0.24 ± 0.06	12 ± 1	41 ± 1
BD 2711_{cm} P_{comK} gfp	3 ± 1	-0.55 ± 0.2	16 ± 4	44 ± 3
BD 2711_{fm} P_{comK} gfp	5 ± 2	1.8 ± 0.1	14 ± 2	39 ± 1
BM 50 P_{comK} gfp, *rok-*	2.3 ± 0.3	0.2 ± 0.1	64 ± 6	68 ± 2
BM 77 P_{comK} gfp, *mc comS*	2.1 ± 0.3	-0.51 ± 0.06	40 ± 2	46 ± 1

Table B.2: Fit Parameters

Fit Parameters For the basal fluorescence level FI as a function of time T. Parameters determined from the best fit to a sigmoid function $FI = FI_{base} + \frac{FI_{max}}{(1+exp[k(T_{half}-T)])}$, with FI_{base} baseline, FI_{max} maximum grey level, T_{half} half time, k switching rate. cm: Exchange for conditioned medium at T_{-2}. fm: Exchange for fresh medium at T_{-2}.

	k[1/h]	T_{half}[h]	f_{max}[%]	f_{base}[%]
BD 2711 P_{comK} gfp	8 ± 3	0.45 ± 0.06	14.1 ± 0.8	0.01 ± 0.05
BD 2711$_{cm}$ P_{comK} $gfp_{inhibition1}$	1.7 ± 1	1.2 ± 0.2	6.9 ± 1.2	0.04 ± 0.4
BD 2711$_{fm}$ P_{comK} $gfp_{inhibition2}$	5.9 ± 11	0.4 ± 0.1	10 ± 0.6	0 ± 0

Table B.3: Fit Parameters

Fit Parameters For the fraction of cells in the K-state f_K as a function of time T. Parameters determined from the best fit to a sigmoid function $f_K = f_{base} + \frac{f_{max}}{(1+exp[k(T_{half}-T)])}$, with f_{base} baseline, f_{max} maximum fraction of cell in the K-state, T_{half} half time, k switching rate. Inhibition 1: Exchange for supernatant of highly competent T_2 BM50 cells at $T_{-0.5}$. Inhibition 2: Exchange for supernatant of highly competent T_2 BM50 cells at T_0.

	k[1/h]	T_{half}[h]	FI_{max}[FU]	FI_{base}[FU]
BD 2711 P_{comK} gfp	6 ± 2	-0.24 ± 0.06	12 ± 1	41 ± 1
BD 2711$_{cm}$ P_{comK} $gfp_{inhibition1}$	1.35 ± 1.2	0.29 ± 0.7	11 ± 6.7	37 ± 2.6
BD 2711$_{fm}$ P_{comK} $gfp_{inhibition2}$	3.3 ± 5.4	-0.37 ± 0.2	12 ± 2.2	42 ± 2

Table B.4: Fit Parameters

Fit Parameters: For the basal fluorescence level FI as a function of time T. Parameters determined from the best fit to a sigmoid function $FI = FI_{base} + \frac{FI_{max}}{(1+exp[k(T_{half}-T)])}$, with FI_{base} baseline, FI_{max} maximum grey level, T_{half} half time, k switching rate. Inhibition 1: Exchange for supernatant of highly competent T_2 BM50 cells at $T_{-0.5}$. Inhibition 2: Exchange for supernatant of highly competent T_2 BM50 cells at T_0.

C Abbreviations

BM	*Bacillus* strains created by Madeleine Leisner
com	Competence
comA	Competence gene A
D = DNA	Desoxyribonucleic acid
E	Enzyme
FI	Fluorescence intensity
FU	Fluorescence unit
GFP	Green fluorescing protein
h	Hour
hrs	Hours
K	ComK
K_M	Michaelis Menten constant
L	Liter
LMU	Ludwig Maximilians Universität
LS	Lehrstuhl/chair
mg	Miligramm
ml	Mililiter
M = mRNA	Messenger RNA
NAR	Negative autoregulation
PAR	Positive autoregulation
R	Repressor
ROI	Region of interest
rpm	Rounds per minute
S	Substrate
sDNA	Single stranded DNA
T_0	Transition point into the stationary phase
V	velocity
V_{max}	Maximum velocity
w	Width
wt	Wild-type

Bibliography

[1] Brock: *Brock Microbiolgy.* Spektrum, 2000

[2] Avery, S. V.: Cell individuality: the bistability of competence development. In: *Trends in Microbiology* 13 (2005), Nr. 10, S. 459–462. – 974QM Times Cited:1 Cited References Count:22

[3] Sumner, E. R. ; Avery, S. V.: Phenotypic heterogeneity: differential stress resistance among individual cells of the yeast Saccharomyces cerevisiae. In: *Microbiology-Sgm* 148 (2002), S. 345–351. – Part 2 519WM Times Cited:13 Cited References Count:39

[4] Thattai, M. ; van Oudenaarden, A.: Stochastic gene expression in fluctuating environments. In: *Genetics* 167 (2004), Nr. 1, S. 523–530. – 826TF Times Cited:29 Cited References Count:30

[5] Maamar, H. ; Raj, A. ; Dubnau, D.: Noise in gene expression determines cell fate in Bacillus subtilis. In: *Science* 317 (2007), Nr. 5837, S. 526–529. – 194JD Times Cited:1 Cited References Count:30

[6] Suel, G. M. ; Kulkarni, R. P. ; Dworkin, J. ; Garcia-Ojalvo, J. ; Elowitz, M. B.: Tunability and noise dependence in differentiation dynamics. In: *Science* 315 (2007), Nr. 5819, S. 1716–1719. – 148WA Times Cited:2 Cited References Count:23

[7] Kitano, H.: *Foundations of Systems Biology.* Cambridge : MIT Press, 2001

[8] Kitano, H.: Systems biology: A brief overview. In: *Science* 295 (2002), Nr. 5560, S. 1662–1664. – 527XH Times Cited:471 Cited References Count:25

[9] Solomon, J. M. ; Grossman, A. D.: Who's competent and when: Regulation of natural genetic competence in bacteria. In: *Trends in Genetics* 12 (1996), Nr. 4, S. 150–155. – Uc599 Times Cited:75 Cited References Count:59

[10] Grossman, A. D.: Genetic networks controlling the initiation of sporulation and the development of genetic competence in Bacillus subtilis. In: *Annual Review of Genetics* 29 (1995), S. 477–508

[11] Maier, B. ; Chen, I. ; Dubnau, D. ; Sheetz, M. P.: DNA transport into Bacillus subtilis requires proton motive force to generate large molecular forces. In: *Nature Structural Molecular Biology* 11 (2004), Nr. 7, S. 643–649. – 832NL Times Cited:16 Cited References Count:29

[12] Dubnau, D.: DNA uptake in bacteria. In: *Annual Review of Microbiology* 53 (1999), S. 217–244. – 247EW Times Cited:204 Cited References Count:142

[13] Lindner, C. ; Nijland, R. ; van Hartskamp, M. ; Bron, S. ; Hamoen, L. W. ; Kuipers, O. P.: Differential expression of two paralogous genes of Bacillus subtilis encoding single-stranded DNA binding protein. In: *Journal of Bacteriology* 186 (2004), Nr. 4, S. 1097–1105. – 776LQ Times Cited:12 Cited References Count:30

[14] Fuqua, C. ; Winans, S. C. ; Greenberg, E. P.: Census and consensus in bacterial ecosystems: The LuxR-LuxI family of quorum-sensing transcriptional regulators. In: *Annual Review of Microbiology* 50 (1996), S. 727–751. – Vl560 Times Cited:321 Cited References Count:110

[15] Lazazzera, B. A.: Quorum sensing and starvation: signals for entry into stationary phase. In: *Current Opinion in Microbiology* 3 (2000), Nr. 2, S. 177–182. – 299QD Times Cited:64 Cited References Count:45

[16] Fuqua, C. ; Greenberg, E. P.: Self perception in bacteria: quorum sensing with acylated homoserine lactones. In: *Current Opinion in Microbiology* 1 (1998), Nr. 2, S. 183–189. – 117DJ Times Cited:131 Cited References Count:59

[17] Dunny, G. M. ; Leonard, B. A. B.: Cell-cell communication in gram-positive bacteria. In: *Annual Review of Microbiology* 51 (1997), S. 527–564. – Ya467 Times Cited:192 Cited References Count:130

[18] Kleerebezem, M. ; Quadri, L. E. N. ; Kuipers, O. P. ; deVos, W. M.: Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. In: *Molecular Microbiology* 24 (1997), Nr. 5, S. 895–904. – Xg801 Times Cited:278 Cited References Count:38

[19] Magnuson, R. ; Solomon, J. ; Grossman, A. D.: Biochemical and Genetic-Characterization of a Competence Pheromone from Bacillus-Subtilis. In: *Cell* 77 (1994), Nr. 2, S. 207–216. – Nh634 Times Cited:179 Cited References Count:73

[20] Solomon, J. M. ; Magnuson, R. ; Srivastava, A. ; Grossman, A. D.: Convergent Sensing Pathways Mediate Response to 2 Extracellular Competence Factors in Bacillus-Subtilis. In: *Genes Development* 9 (1995), Nr. 5, S. 547–558. – Qn633 Times Cited:103 Cited References Count:70

[21] Weinrauch, Y. ; Penchev, R. ; Dubnau, E. ; Smith, I. ; Dubnau, D.: A Bacillus-Subtilis Regulatory Gene-Product for Genetic Competence and Sporulation Resembles Sensor Protein Members of the Bacterial 2-Component Signal-Transduction Systems. In: *Genes Development* 4 (1990), Nr. 5, S. 860–872. – De563 Times Cited:117 Cited References Count:46

[22] Prepiak, P. ; Dubnau, D.: A peptide signal for adapter protein-mediated degradation by the AAA(+) protease ClpCP. In: *Molecular Cell* 26 (2007), Nr. 5, S. 639–647. – 180PM Times Cited:1 Cited References Count:36

[23] Tortosa, P. ; Dubnau, D.: Competence for transformation: a matter of taste. In: *Current Opinion in Microbiology* 2 (1999), Nr. 6, S. 588–592. – 261FW Times Cited:37 Cited References Count:41

[24] Turgay, K. ; Hahn, J. ; Burghoorn, J. ; Dubnau, D.: Competence in Bacillus subtilis is controlled by regulated proteolysis of a transcription factor. In: *Embo Journal* 17 (1998), Nr. 22, S. 6730–6738. – 145WC Times Cited:121 Cited References Count:55

[25] Hamoen, L. W. ; Van Werkhoven, A. F. ; Bijlsma, J. J. E. ; Dubnau, D. ; Venema, G.: The competence transcription factor of Bacillus subtilis recognizes short A/T-rich sequences arranged in a unique, flexible pattern along the DNA helix. In: *Genes Development* 12 (1998), Nr. 10, S. 1539–1550. – Zp838 Times Cited:42 Cited References Count:44

[26] Smits, W. K. ; Eschevins, C. C. ; Susanna, K. A. ; Bron, S. ; Kuipers, O. P. ; Hamoen, L. W.: Stripping Bacillus: ComK auto-stimulation is responsible for the bistable response in competence development. In: *Molecular Microbiology* 56 (2005), Nr. 3, S. 604–614. – 913UM Times Cited:24 Cited References Count:45

[27] Hamoen, L. W. ; Van Werkhoven, A. F. ; Venema, G. ; Dubnau, D.: The pleiotropic response regulator DegU functions as a priming protein in competence development in Bacillus subtilis. In: *Proceedings of the National Academy of Sciences of the United States of America* 97 (2000), Nr. 16, S. 9246–9251. – 341UL Times Cited:24 Cited References Count:30

[28] Smits, W. K. ; Bongiorni C., J.W. Hamoen L.W. Kuipers O. ; Perego, M.: Temporal separation of distinct differentiation pathways by a dual specificity Rap-Phr system in Bacillus subtilis. In: *Molecular Microbiology* 65 (2007), S. 103–120

[29] Serror, P. ; Sonenshein, A. L.: Interaction of CodY, a novel Bacillus subtilis DNA-binding protein, with the dpp promoter region. In: *Molecular Microbiology* 20 (1996), Nr. 4, S. 843–852. – Un988 Times Cited:39 Cited References Count:41

[30] Ratnayake-Lecamwasam, M. ; Serror, P. ; Wong, K. W. ; Sonenshein, A. L.: Bacillus subtilis CodY represses early-stationary-phase genes by sensing GTP levels. In: *Genes Development* 15 (2001), Nr. 9, S. 1093–1103. – 429ER Times Cited:63 Cited References Count:52

[31] Hahn, J. ; Roggiani, M. ; Dubnau, D.: The Major Role of Spooa in Genetic Competence Is to down-Regulate Abrb, an Essential Competence Gene. In: *Journal of Bacteriology* 177 (1995), Nr. 12, S. 3601–3605. – Rc461 Times Cited:36 Cited References Count:37

[32] Fujita, M. ; Gonzalez-Pastor, J. E. ; Losick, R.: High- and low-threshold genes in the Spo0A regulon of Bacillus subtilis. In: *Journal of Bacteriology* 187 (2005), Nr. 4, S. 1357–1368. – 898YQ Times Cited:24 Cited References Count:39

[33] Berka, R. M. ; Hahn, J. ; Albano, M. ; Draskovic, I. ; Persuh, M. ; Cui, X. J. ; Sloma, A. ; Widner, W. ; Dubnau, D.: Microarray analysis of the Bacillus subtilis K-state: genome-wide expression changes dependent on ComK. In: *Molecular Microbiology* 43 (2002), Nr. 5, S. 1331–1345. – 537RV Times Cited:53 Cited References Count:65

[34] Hahn, J. ; Dubnau, D.: Growth Stage Signal Transduction and the Requirements for Srfa Induction in Development of Competence. In: *Journal of Bacteriology* 173 (1991), Nr. 22, S. 7275–7282. – Gp820 Times Cited:49 Cited References Count:53

[35] Lazazzera, B. A. ; Solomon, J. M. ; Grossman, A. D.: An exported peptide functions intracellularly to contribute to cell density signaling in B-subtilis. In: *Cell* 89 (1997), Nr. 6, S. 917–925. – Xe357 Times Cited:88 Cited References Count:59

[36] Ireton, K. ; Grossman, A. D.: A Developmental Checkpoint Couples the Initiation of Sporulation to DNA-Replication in Bacillus-Subtilis. In: *Embo Journal* 13 (1994), Nr. 7, S. 1566–1573. – Nf928 Times Cited:47 Cited References Count:70

[37] Burkholder, W. F. ; Kurtser, I. ; Grossman, A. D.: Replication initiation proteins regulate a developmental checkpoint in Bacillus subtilis. In: *Cell* 104 (2001), Nr. 2, S. 269–279. – 400PB Times Cited:25 Cited References Count:54

[38] Alberts, B ; Bray, D ; Johnson, A ; Lewis, J ; Raff, M ; Roberts, K ; Water, P: *Essential Cell Biology. An Introduction to the molecular biology of the cell.* New York, London : Garland Publishing, Inc., 1998

[39] Raser, J. M. ; O'Shea, E. K.: Control of stochasticity in eukaryotic gene expression. In: *Science* 304 (2004), Nr. 5678, S. 1811–1814. – 829YU Times Cited:145 Cited References Count:23

[40] Kaern, M. ; Elston, T. C. ; Blake, W. J. ; Collins, J. J.: Stochasticity in gene expression: From theories to phenotypes. In: *Nature Reviews Genetics* 6 (2005), Nr. 6, S. 451–464. – 931MT Times Cited:138 Cited References Count:141

[41] Dubnau, D. ; Losick, R.: Bistability in bacteria. In: *Molecular Microbiology* 61 (2006), Nr. 3, S. 564–572. – 060YK Times Cited:13 Cited References Count:40

[42] Smits, W. K. ; Kuipers, O. P. ; Veening, J. W.: Phenotypic variation in bacteria: the role of feedback regulation. In: *Nature Reviews Microbiology* 4 (2006), Nr. 4, S. 259–271. – 022FJ Times Cited:16 Cited References Count:127

[43] Graumann, P. L.: Different genetic programmes within identical bacteria under identical conditions: the phenomenon of bistability greatly modifies our view on bacterial populations. In: *Molecular Microbiology* 61 (2006), Nr. 3, S. 560–563. – 060YK Times Cited:1 Cited References Count:21

[44] Ferrell, J. E.: Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability. In: *Current Opinion in Cell Biology* 14 (2002), Nr. 2, S. 140–148. – 527NZ Times Cited:189 Cited References Count:62

[45] Becskei, A. ; Seraphin, B. ; Serrano, L.: Positive feedback in eukaryotic gene networks: cell differentiation by graded to binary response conversion. In: *Embo Journal* 20 (2001), Nr. 10, S. 2528–2535. – 436PC Times Cited:151 Cited References Count:33

[46] Gardner, T. S. ; Cantor, C. R. ; Collins, J. J.: Construction of a genetic toggle switch in Escherichia coli. In: *Nature* 403 (2000), Nr. 6767, S. 339–342. – 276VU Times Cited:400 Cited References Count:26

[47] Alon, U.: *Introduction to Systems Biology: Design Principles of Biological Circuits*. Boka Raton : CRC, 2006

[48] Davidson, E.H.: *The Regulatory Genome: Gene Regulatory Networks in Development and Evolution*. Burlington : Academic, 2006

[49] Ptashne, M ; Gann, A: *Genes and Signals*. Cold Spring Harbor : Cold Spring Harbor Laboratory Press, 2002

[50] Levine, M. ; Davidson, E. H.: Gene regulatory networks for development. In: *Proceedings of the National Academy of Sciences of the United States of America* 102 (2005), Nr. 14, S. 4936–4942. – 914AC Times Cited:87 Cited References Count:66

[51] Thieffry, D. ; Huerta, A. M. ; Perez-Rueda, E. ; Collado-Vides, J.: From specific gene regulation to genomic networks: a global analysis of transcriptional

regulation in Escherichia coli. In: *Bioessays* 20 (1998), Nr. 5, S. 433–440. – 107LC Times Cited:128 Cited References Count:42

[52] Bhalla, U. S.: Understanding complex signaling networks through models and metaphors. In: *Progress in Biophysics Molecular Biology* 81 (2003), Nr. 1, S. 45–65. – 638NN Times Cited:20 Cited References Count:58

[53] Shen-Orr, S. S. ; Milo, R. ; Mangan, S. ; Alon, U.: Network motifs in the transcriptional regulation network of Escherichia coli. In: *Nature Genetics* 31 (2002), Nr. 1, S. 64–68. – 547ZF Times Cited:410 Cited References Count:21

[54] Milo, R. ; Shen-Orr, S. ; Itzkovitz, S. ; Kashtan, N. ; Chklovskii, D. ; Alon, U.: Network motifs: Simple building blocks of complex networks. In: *Science* 298 (2002), Nr. 5594, S. 824–827. – 607KR Times Cited:521 Cited References Count:24

[55] Alon, U.: Network motifs: theory and experimental approaches. In: *Nature Reviews Genetics* 8 (2007), Nr. 6, S. 450–461. – 169PF Times Cited:2 Cited References Count:100

[56] Weiss, J. N.: The Hill equation revisited: uses and misuses. In: *Faseb Journal* 11 (1997), Nr. 11, S. 835–841. – Xt790 Times Cited:87 Cited References Count:9

[57] Gillespie, D. T.: Exact Stochastic Simulation of Coupled Chemical-Reactions. In: *Journal of Physical Chemistry* 81 (1977), Nr. 25, S. 2340–2361. – Ee498 Times Cited:814 Cited References Count:32

[58] Strogatz: *Nonlinear dynamics and Chaos.* Cambrigde, Mass. : Westview Press, 2000

[59] Albano, M. ; Hahn, J. ; Dubnau, D.: Expression of Competence Genes in Bacillus-Subtilis. In: *Journal of Bacteriology* 169 (1987), Nr. 7, S. 3110–3117. – H9989 Times Cited:126 Cited References Count:30

[60] Maamar, H. ; Dubnau, D.: Bistability in the Bacillus subtilis K-state (competence) system requires a positive feedback loop. In: *Molecular Microbiology* 56 (2005), Nr. 3, S. 615–624. – 913UM Times Cited:24 Cited References Count:34

[61] Mckenzie, T. ; Hoshino, T. ; Tanaka, T. ; Sueoka, N.: The Nucleotide-Sequence of Pub110 - Some Salient Features in Relation to Replication and Its Regulation. In: *Plasmid* 15 (1986), Nr. 2, S. 93–103. – A8147 Times Cited:179 Cited References Count:33

[62] Hoa, T. T. ; Tortosa, P. ; Albano, M. ; Dubnau, D.: Rok (YkuW) regulates genetic competence in Bacillus subtilis by directly repressing comK. In: *Molecular*

Microbiology 43 (2002), Nr. 1, S. 15–26. – 523MZ Times Cited:18 Cited References Count:31

[63] Haijema, B. J. ; Hahn, J. ; Haynes, J. ; Dubnau, D.: A ComGA-dependent checkpoint limits growth during the escape from competence. In: *Molecular Microbiology* 40 (2001), Nr. 1, S. 52–64. – 422NQ Times Cited:24 Cited References Count:42

[64] Liu, L. ; Nakano, M. M. ; Lee, O. H. ; Zuber, P.: Plasmid-amplified comS enhances genetic competence and suppresses sinR in Bacillus subtilis. In: *Journal of Bacteriology* 178 (1996), Nr. 17, S. 5144–5152. – Ve371 Times Cited:21 Cited References Count:65

[65] Leisner, M. ; Stingl, K. ; Radler, J. O. ; Maier, B.: Basal expression rate of comK sets a 'switching-window' into the K-state of Bacillus subtilis. In: *Molecular Microbiology* 63 (2007), Nr. 6, S. 1806–1816. – 145SY Times Cited:1 Cited References Count:31

[66] Hahn, J. ; Luttinger, A. ; Dubnau, D.: Regulatory inputs for the synthesis of ComK, the competence transcription factor of Bacillus subtilis. In: *Molecular Microbiology* 21 (1996), Nr. 4, S. 763–775. – Ve872 Times Cited:53 Cited References Count:40

[67] Hahn, J. ; Maier, B. ; Haijema, B. J. ; Sheetz, M. ; Dubnau, D.: Transformation proteins and DNA uptake localize to the cell poles in Bacillus subtilis. In: *Cell* 122 (2005), Nr. 1, S. 59–71. – 950EC Times Cited:12 Cited References Count:42

[68] Suel, G. M. ; Garcia-Ojalvo, J. ; Liberman, L. M. ; Elowitz, M. B.: An excitable gene regulatory circuit induces transient cellular differentiation. In: *Nature* 440 (2006), Nr. 7083, S. 545–550. – 024DV Times Cited:25 Cited References Count:24

[69] Smits, W. K. ; Hoa, T. T. ; Hamoen, L. W. ; Kuipers, O. P. ; Dubnau, D.: Antirepression as a second mechanism of transcriptional activation by a minor groove binding protein. In: *Molecular Microbiology* 64 (2007), Nr. 2, S. 368–381. – 158SG Times Cited:0 Cited References Count:48

[70] Thattai, M. ; van Oudenaarden, A.: Intrinsic noise in gene regulatory networks. In: *Proceedings of the National Academy of Sciences of the United States of America* 98 (2001), Nr. 15, S. 8614–8619. – 454HK Times Cited:161 Cited References Count:29

[71] Hamoen, L. W. ; Kausche, D. ; Marahiel, M. A. ; van Sinderen, D. ; Venema, G. ; Serror, P.: The Bacillus subtilis transition state regulator AbrB binds

to the-35 promoter region of comK. In: *Fems Microbiology Letters* 218 (2003), Nr. 2, S. 299–304. – 647TQ Times Cited:11 Cited References Count:25

[72] Elowitz, M. B. ; Levine, A. J. ; Siggia, E. D. ; Swain, P. S.: Stochastic gene expression in a single cell. In: *Science* 297 (2002), Nr. 5584, S. 1183–1186. – 584BP Times Cited:433 Cited References Count:24

[73] Bejerano-Sagie, M. ; Oppenheimer-Shaanan, Y. ; Berlatzky, I. ; Rouvinski, A. ; Meyerovich, M. ; Ben-Yehuda, S.: A checkpoint protein that scans the chromosome for damage at the start of sporulation in Bacillus subtilis. In: *Cell* 125 (2006), Nr. 4, S. 679–690. – 047NM Times Cited:3 Cited References Count:41

[74] Baschal, E. E. ; Chen, K. J. ; Elliott, L. G. ; Herring, M. J. ; Verde, S. C. ; Wolkow, T. D.: The fission yeast DNA structure checkpoint protein Rad26(ATRIP/LCD1/UVSD) accumulates in the cytoplasm following microtubule destabilization. In: *Bmc Cell Biology* 7 (2006), S. –. – 092CC Times Cited:0 Cited References Count:90

[75] Ruvolo, M. V. ; Mach, K. E. ; Burkholder, W. F.: Proteolysis of the replication checkpoint protein Sda is necessary for the efficient initiation of sporulation after transient replication stress in Bacillus subtilis. In: *Molecular Microbiology* 60 (2006), Nr. 6, S. 1490–1508. – 046LG Times Cited:3 Cited References Count:74

[76] Ishikawa, S ; Ogura, Y ; Yoshimura, M ; Okumura, H ; Cho, E ; Kawai, Y ; Kurokawa, K ; Oshima, T ; Ogasawara, N: Distribution of Stable DnaA-Binding Sites on the Bacillus Subtilis Genome Detected using a Modified ChIP-chip Method. In: *DNA Research* 14 (2007), S. 155–168

List of Figures

List of Tables

Vielen Dank an ...

Prof. Joachim Rädler für die Möglichkeit, diese Arbeit an seinem Institut durchzu - führen. Die vielen Gelegenheiten, die sich mir an seinem Lehrstuhl boten, mich auch auf anderen Gebieten als der der Wissenschaft fortzubilden, haben mir bei meiner persönlichen Entwicklung sehr geholfen.

Prof. Berenike Maier für die unglaublich gute Zusammenarbeit auch über große Entfernungen. Eine bessere Betreuung hätte ich mir für mich nicht vorstellen können.

Prof. Erwin Frey für sein offenes Ohr, seine Objektivität und seine ehrliche Meinung. Unsere Gespräche haben mich auf vielen Gebieten ein gutes Stück weitergebracht.

Tobias Reichenbach und Jan-Timm Kuhr für die spannende und erfolgreiche Zusammenarbeit. Die für mich anfangs ungewohnten Ideen ließen meine Kreativität wachsen und lehrten mich gewohntes Terrain zu verlassen, oder Bekanntes unter einem neuen Blickwinkel zu betrachten.

Prof. Reinhard Wirth für die vielen motivierenden Gespräche, auch über die Diplomarbeit hinaus.

den LS Rädlers für ein unvergleichlich gutes Arbeitsklima. Aufmunternde Worte, oder ehrliche Kritik, was immer gerade von Nöten war. Besonders bedanken möchte ich mich bei Judith für die unzählbaren Latteladengänge, bei Hanna, die es geschaft hat mich wieder zum Klavierspielen und üben zu annimieren, bei Kirstin und Alicia meinen beiden Zimmergenossinen, bei Simon für das Retten der einen oder anderen Festplatte, bei Susi für viele Bladenights und ihre unermüdliche Hilfe im Labor, bei Gerlinde, die mir die Tipps und Tricks des Klonierens vermittelte und auch bei allen anderen Fragen des Laboralltages eine große Hilfe war.

der AG Maier die obwohl so fern mir immer das Gefühl gab, doch irgendwie dazuzugehöhren. Auf die Besuche in Münster habe ich mich immer sehr gefreut. Mein besonderer Dank gilt Martin, der mir mit unendlicher Geduld das Lasertweezen näher brachte. Gerda und Kerstin, die wie ich die guten und schlechten Tage der Bacillen erlebten. Andrea, Alex und Dirk für die netten geselligen Abende.

meinen Freunden den Freunden der Arbeit, des Reitens, der Schule, des Studiums, diverser Wohnheime and so on. Ich bin froh dass es euch gibt.

meiner Familie
Meinem Vater, der mich immer angespornt hat Dinge zu tun, die ich mir selbst nicht zugetraut hätte. **Meiner Mutter**, die mir ihre Neugier und Kreativität vererbt hat und mir so den Weg in die Interdisziplinarität ebnete. **Meiner Schwester**, für ihren Glauben und ihre Liebe. **Meinen Nichten/Neffe** für die rotzfrechen geradeheraus Worte, und die liebevolle Zuwendung wenn es mal nicht so lief, wie sie eben nur Kinder zu geben fähig sind.

Lebenslauf

Madeleine Luise Leisner

Geburtstag	05.05.1980
Geburtsort	Landshut
Familienstand	ledig
1999	Abitur, Note: 1.8
11/1999 - 09/2004	Studium der Biologie an der Universität Regensburg
08/2002 - 06/2003	Independent studies an der University of Colorado at Boulder, USA
01/2004 - 09/2004	Diplomarbeit bei Professor Dr. R. Wirth
09/2004	Diplom der Biologie, Note: 1.2
12/2004 -	Promotion an der Ludwigs-Maximilians-Universität München bei Professor Dr. J.O. Rädler Titel: "Genetic switching into the competent state. *Bacillus subtilis* : a single cell approach"

Publications

LEISNER, M.; STINGL, K.; RÄDLER, J.O. and B. MAIER.: Basal expression rate of *comK* sets a "switching window" into the K-state of *Bacillus subtilis. Molecular Microbiology* 63 (2007), 1806-1816.

LEISNER, M.; KUHR, J-T.; RÄDLER, J.O.; FREY, E. and B. MAIER.: Kinetics of genetic switching into the state of bacterial competence. In preparation.

LEISNER, M.; STINGL, K. and B. MAIER.: Cell cycle controls entry into the competent state. In preparation.

LEISNER, M.; STINGL, K.; RÄDLER, J.O. and B. MAIER.: Release of inhibitors terminates the "switching window" of competence of *Bacillus subtilis*. In preparation.