Competence and Transformation in *Bacillus subtilis*

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Abstract

Transformation is the process of import and inheritable integration of DNA from the environment. As such, it is believed to be a major driving force for evolution. Competence for transformation is widespread among bacterial species. Recent findings draw a picture of a conserved molecular machine that binds DNA at the cell surface and subsequently transports it through the cell envelope. Within the cytoplasm the DNA is coated by proteins that mediate recombination or self-annealing. The regulatory mechanisms and environmental signals affecting competence are very diverse between different bacterial species. Competence in *Bacillus subtilis* has become a paradigm for stochastic determination of cell-fate. Quantitative analysis at the single cell level in conjunction with mathematical modelling allowed understanding of induction and decline of competence at the systems level. Currently, the picture is emerging of stochastic differentiation as a fitness trade-off in fluctuating environments.

Introduction

Transformation is one of three mechanisms of bacterial horizontal gene transfer. The earliest reports concerning transformation date back to the identification of DNA as the genetic material (Avery *et al.*, 1944; Griffith, 1928). Here, we define transformation as the active acquisition of extracellular DNA and the heritable incorporation of its genetic information into the host cell. The word 'active' is chosen to stress the fact that DNA import is driven by a cellular machine that utilizes energy for DNA transport. Bacteria expressing such DNA import machines are naturally competent for transformation. Natural competence is a genetically programmed physiological state which is distinct from artificial transformation involving electroporation or heat shock/chemical treatment.

Transformation has been studied mainly in five model organisms, namely the Gram-positive species *B. subtilis* and *Streptococcus pneumoniae*, and the Gram-negative species *Neisseria gonorrhoeae*, *Haemophilus influenzae* and *Helicobacter pylori*. Although the molecular mechanism of DNA uptake appears to be well conserved amongst different species (with the exception of *H. pylori*), the mechanisms of genetic regulation of competence and DNA specificity differ strongly. For example, *B. subtilis* and *S. pneumoniae* develop competence only during distinct growth phases, whereas *N. gonorrhoeae* is constitutively competent. Furthermore, the sequence specificity of DNA import differs between species. *N. gonorrhoeae* and *H. influenzae* possess a strong preference for importing species-own DNA by recognizing short specific DNA uptake sequences, which have multiple repeats on their chromosomes. Such preference for a DNA uptake sequence is absent in *B. subtilis*. So far, ~50 bacterial species have been shown to develop natural competence. However, many more bacterial species may develop competence under presently unidentified conditions (Kovacs *et al.*, 2009), in analogy to *Vibrio cholerae*, which develops competence only on chitin surfaces (Meibom *et al.*, 2005).

Transformation in *B. subtilis* has served as an important model system in molecular microbiology in at least two respects. First, the regulatory network that controls competence development has been investigated in much detail since the 1960ies. Since the molecular interactions are relatively well

described, a systems biology approach has recently enabled a modular description of the competence network and an understanding of why only a subset of all cells induces competence. Second, proteins involved in DNA import, their interactions, and their dynamics have now been well characterized. The results support the picture of a transformation machine in which DNA binding, transport, and recombination are coordinated and occur at a single specific site in the cell. Therefore this article is divided into two parts. The first part of this review presents the process of differentiation into the state of competence and the second part describes the molecular mechanism of transformation.

Development of competence for transformation

Regulatory networks

Coarse overview

The existence of a competence regime in *B. subtilis* was established in the 1950s (Spizizen, 1958),

making it genetically accessible for molecular research. Bacteria are transformable only during the stationary growth phase. Competence is controlled by the master regulator ComK (Hahn *et al.*, 1994; van Sinderen *et al.*, 1995; van Sinderen and Venema, 1994).

Fig. 13.1 shows a strongly simplified network of regulatory interactions that control the concentration of ComK. The entire regulatory network is complex (Fig. 13.2). Therefore, this paragraph presents the coarse idea of our current state-of-the-art concerning competence regulation. *comK* transcription is up-regulated through binding of four ComK proteins to their own promoter region. This non-linear autocatalytic feedback of ComK is essential for the switch-like behaviour of competence (Maamar and Dubnau, 2005; Smits *et al.*, 2005); at low ComK concentrations cells do not differentiate into the competent state, at high ComK concentrations, several cells enter the state of competence (also named K-state). In this state the expression of more than 100 genes is up-regulated (Berka *et al.*, 2002; Hamoen *et al.*, 2002; Ogura *et al.*, 2002). The late competence genes (including
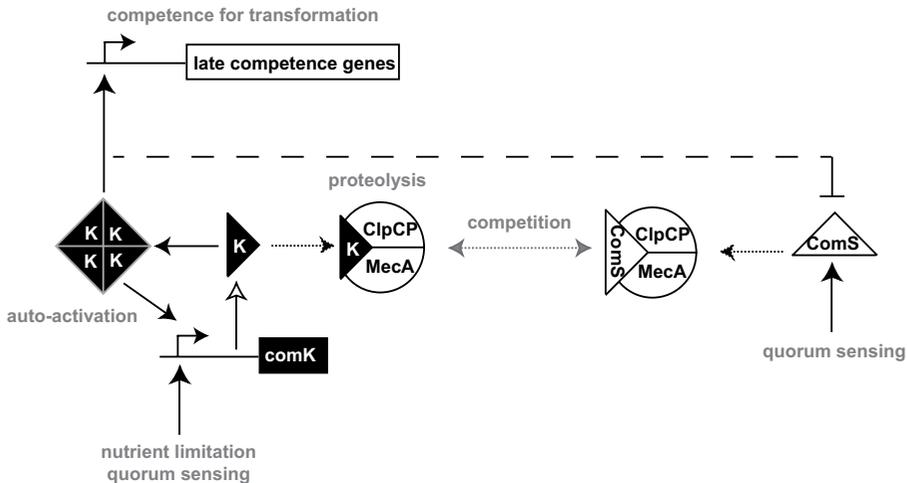

Figure 13.1 Simplified network of competence regulation. The ComK protein binds to the *comK* promoter as a dimer of dimers and activates *comK* transcription. This autocatalytic feedback is the basis for switch-like behaviour of competence. The adapter protein MecA mediates binding of ComK to the protease ClpCP. ComS competes for binding to the protease. When the cell density is high, multiple external signals are integrated leading to increased ComS concentration, which allow for increased ComK levels. The intracellular energy state regulates *comK* at the transcriptional level. As ComK switches to the high expression state, late competence genes are activated through binding of ComK to their promoters. ComK indirectly down-regulates *comS*, thereby enabling escape from competence. Filled arrows indicate positive regulation, dotted arrows biochemical interaction, dashed arrows indirect regulation. Kinked arrows illustrate promoter regions, open arrows expression.
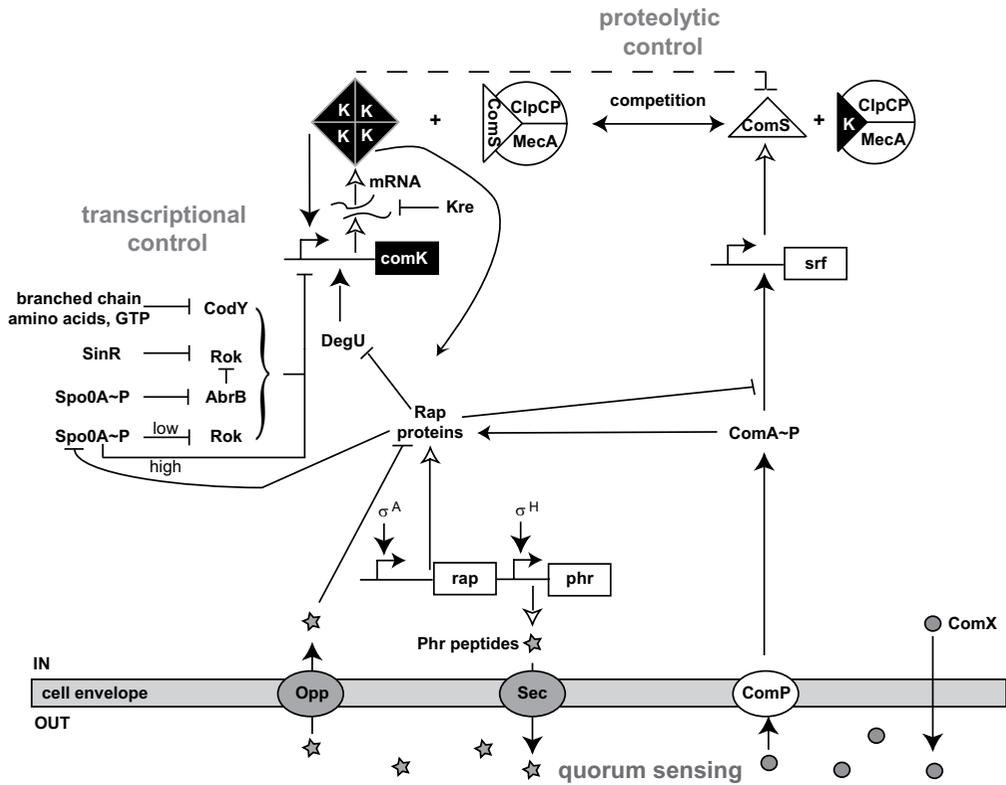
Figure 13.2 Core of the regulatory network for competence in *B. subtilis.* Filled arrows and T-bars indicate positive and negative regulation, respectively. Kinked arrows illustrate promoter regions, open arrows expression.

genes encoding for proteins essential for DNA import) are among the most strongly up-regulated genes. The concentration of ComK is regulated at different levels. At the level of transcription, signals from various pleiotropic regulators are integrated, resulting in the regulated synthesis of ComK. At the post-translational level, the ComK concentration is regulated through proteolysis by the ClpCP protease. *B. subtilis* detects the density of the population through quorum sensing, which down-regulates ComK proteolysis through up-regulation of ComS. The latter competes with ComK for binding to the protease. Furthermore, quorum sensing and nutrient limitation up-regulate *comK* transcription. During the competent state, *comS* is down-regulated enabling escape from competence (Suel *et al.*, 2006).

Transcriptional regulation of *comK*

The regulation of ComK concentration relies on a complex regulatory network (Fig. 13.2). Signals from various pleiotropic regulators are integrated, resulting in the regulated synthesis of ComK. Footprinting experiments showed that ComK functions as a tetramer composed of two dimers each recognizing the motif AAAA-$[N]_5$-TTTT (K-box) (Hamoen *et al.*, 1998). Among the competence genes three classes of K-boxes have been characterized; type I (including *addAB, recA, nucA*), type II (including the late competence gene *comC*, and the operons *comE, comF, comG*), and type III (represented by *comK*), in which the AT-boxes are separated by 1, 2, or 3 helical turns respectively (Hamoen *et al.*, 2002; Hamoen *et al.*, 1998). The mechanism of transcriptional activation

was investigated for the promoter of the late competence gene *comG*. Susanna *et al.* showed that ComK stabilizes the binding of RNA polymerase to the *comG* promoter, probably through interactions with the upstream DNA (Susanna *et al.*, 2004).

ComK acts as an activator at its own promoter by antagonizing the action of two repressors, Rok and CodY (Smits *et al.*, 2007b) (Fig. 13.2). Anti-repression occurs without preventing binding of the repressing proteins, suggesting that ComK and the repressors might bind at distinct surfaces of the DNA helix. Rok (repressor of ComK) is a potent repressor of *comK* transcription (Hoa *et al.*, 2002) and of various other A+T-rich genes, many of which appear to have been acquired by horizontal gene transfer (Smits and Grossman, 2010). SinR and AbrB act negatively on *rok* transcription (Hamoen *et al.*, 2003), and it has been suggested that the dependence of *comK* expression on SinR and AbrB may be a result of their repression of *rok* transcription. It has also been shown *in vivo*, that Rok and ComK can individually repress *rok* transcription, introducing a positive feedback for *comK* transcription. GTP functions as a nutritional signal for competence development, through the regulatory role of CodY, a GTP binding protein (Inaoka and Ochi, 2002; Serror and Sonenshein, 1996). Moreover CodY senses the pool of branched chain amino acids (Shivers and Sonenshein, 2004). CodY directly binds to the *srfA* and *comK* promoter region. The transition state regulator AbrB is responsible for repression of stationary phase proteins during exponential growth including the *comK* promoter. AbrB levels are down-regulated by Spo0A~P (Fujita *et al.*, 2005; Hahn *et al.*, 1995b), promoting transcription of *comK*. Please refer to Graumann (2017) for the regulation of phosphorylated Spo0A~P. In the promoter of *comK* the spacer between the two ComK-boxes is rather long, leading to a relatively low binding affinity for ComK (Susanna *et al.*, 2007). This probably explains the need for another pleiotropic response regulator, DegU, as priming protein for ComK binding to its promoter at low ComK concentrations (Hamoen *et al.*, 2000).

Regulation of *comK* mRNA stability

The search for unknown regulators governing differentiation into the K-state has led to the discovery of ComK control at the level of *comK* mRNA stability (Gamba *et al.*, 2015). The Kre protein moderately reduces the stability of *comK* mRNA; conversely, *kre* expression itself is down-regulated by ComK, establishing a double negative feedback situation. Since small variations in the basal ComK levels strongly affect the probability of competence development, the reduction of ComK levels via the effect of Kre on mRNA stability contributes significantly to bimodal gene expression.

Proteolysis of ComK

Post-translationally, ComK is sequestered by MecA and thereby targeted for degradation by the ClpCP protease (Turgay *et al.*, 1998; Turgay *et al.*, 1997). A 17 residue peptide of ComK is sufficient for binding to MecA (Prepiak and Dubnau, 2007). A small protein, encoded by the *sfr* operon, ComS, competes for binding to MecA, preventing ComK degradation (D'Souza *et al.*, 1994; Turgay *et al.*, 1997). The expression of *sfr* is regulated by quorum sensing through the ComPA two-component system (Griffith and Grossman, 2008), leading to expression of *comS*. Regulatory processes involving the ClpCP proteases are described in more detail in Graumann, 2017.

Quorum sensing

Differentiation into the state of competence is most likely to occur during early stationary phase. Quorum sensing and nutrient limitation have been identified as important determinants of competence development, both of which induce stationary phase growth. Bacterial quorum sensing has been mostly studied in well-mixed populations residing within closed systems that do not exchange mass with their surroundings (Magnuson *et al.*, 1994; Solomon *et al.*, 1996). Recently, density-dependent differentiation was studied in micro-structured environments that allowed for mass transfer by coupling microscopic growth reservoirs to fluid flow (Ribbe and Maier, 2016). This system mimics natural environments of *B. subtilis* such as the rhizosphere. Spatial heterogeneity promotes density-dependent differentiation in open systems, albeit at a strongly increased cell density compared to closed systems.

Multiple extracellular factors mediate competence development in response to cellular crowding (Solomon *et al.*, 1995). The pheromone ComX is required for competence development (Magnuson

et al., 1994) (Fig. 13.2). The ComX peptide is post-translationally modified by coupling of a geranyl group (Okada *et al.*, 2005). At sufficient extracellular concentration, ComX activates the ComPA two-component system (Weinrauch *et al.*, 1990). ComA is a transcriptional regulator whose DNA-binding activity is stimulated through phosphorylation (Ogura *et al.*, 2001; Roggiani and Dubnau, 1993). In particular, four recognition elements on the promoter P*sfrA* are targeted by ComA (Griffith and Grossman, 2008; Wolf *et al.*, 2016), activating the expression of *comS*.

The expression of *comS* is fine-tuned by multiple Rap proteins, which are usually counteracted by their cognate Phr peptides. The structural basis of Phr inhibition of Rap proteins has recently been investigated. Phr binding induces a pronounced conformational change in Rap proteins, locking them into the inactive state (Gallego del Sol and Marina, 2013; Parashar *et al.*, 2013). Most Phr peptides are expressed in stationary phase under σ^H control. For example, PhrC, PhrF, and PhrH counteract the activity of their respective Rap protein. The latter independently inhibit ComA binding to DNA (Bongiorni *et al.*, 2005; Core and Perego, 2003; Smits *et al.*, 2007a). An analogous regulatory mechanism on ComA-dependent genes was attributed to PhrK/RapK (Auchtung *et al.*, 2006), and to RapD, which has no cognate Phr peptide (Ogura and Fujita, 2007). Likewise, the PhrG/RapG system modulates DegU binding to target DNA (Ogura *et al.*, 2003). Since *rapC* and *rapF* are members of the ComA regulon and *rapH* is antirepressed by ComK, additional regulatory circuits are present for competence development as well as for escape from the competence phase (Bongiorni *et al.*, 2005).

PhrC is exported through the Sec system and its cleavage product CSF (competence stimulating factor) is imported through the oligopeptide permease encoded by *spo0K* (Lazazzera *et al.*, 1997; Ogura *et al.*, 2003; Solomon *et al.*, 1996). Interestingly, CSF reveals a dose-dependent response that triggers different differentiation pathways depending on its concentration. Low concentrations of CSF stimulate competence by activation of *srfA* (and thereby *comS*), whereas high concentrations of CSF stimulate sporulation by phosphorylating Spo0A (Solomon *et al.*, 1996). Spo0A is the master regulator of sporulation, another stationary phase differentiation process (see Graumann, 2017). Phosphorylation of the protein leads to dimerization and is required for its function as a transcription factor (Lewis *et al.*, 2002). The phosphor group is transferred from membrane-located and soluble kinases via a series of cytoplasmic kinases to Spo0A (phosphorelay) (Perego, 1997). Among the phosphatases that dephosphorylate Spo0A-P are members of the Rap family. Differentiation into endospores is described in detail in Graumann, 2017.

Growth arrest and decline of competence

Competence in *B. subtilis* is a transient state. Fig. 13.3 illustrates rise and decline of the ComK concentration by monitoring a reporter strain that carries a *gfp* gene under the control of the *comK* promoter. Competent cells are growth-inhibited (Haijema *et al.*, 2001; Nester and Stocker, 1963). Growth arrest can be seen in Fig. 13.3 as an
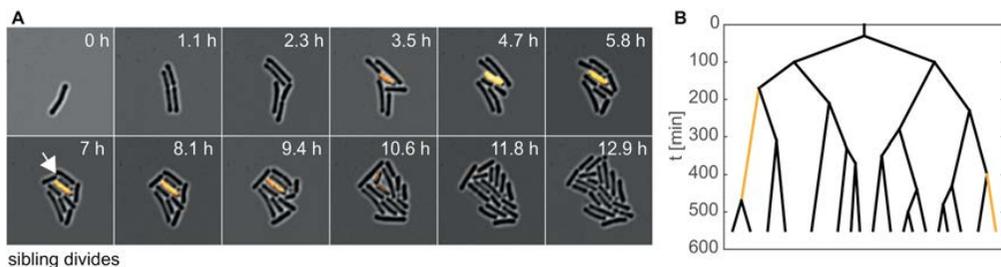

Figure 13.3 The competent state is transient and associated with growth arrest. (A) Time lapse of *B. subtilis* carrying a competent state reporter (PcomK gfp) in the stationary growth phase. At t = 3.5 h a single cell differentiates into the competent state (orange). This cell is growth-arrested while its sibling elongates and divides around t = 7 h. At t = 9.4 h the cells escapes from the competent state and resumes elongation and division. (B) Typical genealogy in the stationary phase. Black: non-competent cells, orange: competent cells.

increased time between two cell division events. Competent cells up-regulate multiple factors that are required for growth arrest. The late competence protein ComGA is involved in growth arrest by various mechanisms. It sequesters the actin-like protein MreB, most likely inhibiting cell elongation (Mirouze *et al.*, 2015). Moreover, ComGA inhibits replisome formation (Haijema *et al.*, 2001). Finally, it directly interacts with RelA to increase the (p) ppGpp-pool in the cell (Fig. 15.1), inhibiting replication elongation and rRNA synthesis (Hahn *et al.*, 2015b). Downstream of ComGA, Maf is involved in arresting cell division at a step following Z-ring formation (Briley *et al.*, 2011b; Hahn *et al.*, 2015b). The adaptor protein MecA that directs ComK to proteolysis is required for relieving the growth arrest, indicating that proteolysis of ComK is important for escape from the competent state (Hahn *et al.*, 1995a).

Phenotypic variability

Although an isogenic population of *B. subtilis* cells enters the stationary growth phase, only a subpopulation of cells (up to 20%) resides in the competent state at a given point of time (Nester and Stocker, 1963). The fraction of competent cells has been measured by single cell techniques including fluorescence microscopy and flow cytometry. When a fluorescent reporter (green fluorescent protein) for competence was introduced through a translational fusion to ComK, only 10–20% of the cells in a competent culture are fluorescent, indicating

that ComK synthesis is an all-or-nothing event (Haijema *et al.*, 2001) (Fig. 13.4). Importantly, the autocatalytic feedback of *comK* expression (Fig. 13.1) is necessary and can be sufficient for phenotypic variability (Maamar and Dubnau, 2005; Smits *et al.*, 2005). Phenotypic heterogeneity of gene expression has been reviewed in various recent articles (Ackermann, 2015; Leisner *et al.*, 2008; Martins and Locke, 2015; Smits *et al.*, 2006; Veening *et al.*, 2008).

The role of noise in initiation and decline of competence

One mechanism of generating phenotypic variability is noise in the concentration of master regulators (Ben-Jacob *et al.*, 2014; Leisner *et al.*, 2008; Mirouze and Dubnau, 2013; Tsimring, 2014). In general, the relative amplitude of noise increases with decreasing concentration of molecules and it has been demonstrated that this rule is also valid for gene expression (Elowitz *et al.*, 2002). Using single molecule FISH, Maamar *et al.* revealed that at the entry into stationary phase the average number of mRNA molecules encoding for ComK is ~1 mRNA per cell (Maamar *et al.*, 2007). In this regime small number fluctuations are, relative to the mean, of paramount importance. This is especially noteworthy since the reaction kinetics of ComK is highly non-linear (Fig. 13.1).

The positive cooperative feedback of *comK* transcription implies a threshold with the following significance: The autocatalytic feedback (Fig.
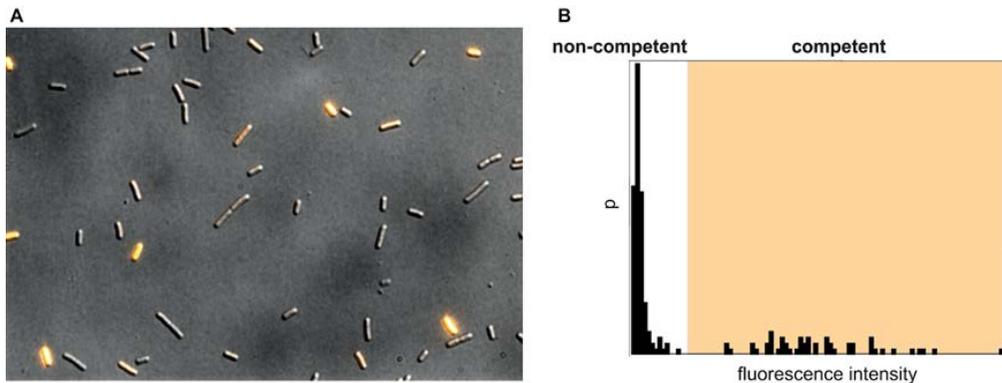

Figure 13.4 Phenotypic heterogeneity in terms of competence development. (A) Stationary phase *B. subtilis* cells. Brightfield and fluorescence image of a *comK* reporter strain (*P_comK gfp*) are merged, revealing that only a subpopulation develops competence. (B) Typical distribution of fluorescence intensities obtained from images shown in (A) demonstrates bimodal fluorescence.

13.1) is triggered whenever the concentration of ComK exceeds a threshold concentration; since the feedback is positive (i.e. self-amplifying) the ComK concentration raises rapidly (Fig. 13.5a and b). At equal average concentrations of ComK the amplitude of fluctuations (noise) would then determine the fraction of competent cells as illustrated in the simulation in (Fig. 13.5a and b). Please note that the distribution of ComK concentrations is slightly broader in Fig. 13.5a than in Fig. 13.5b, leading to a larger number of cells that develop competence. It has been shown that the major source of noise in gene expression generally results from transcription and is unaffected by translation (Elowitz *et al.*, 2002). Accordingly, Maamar *et al.* showed that by decreasing translation rate and increasing the transcription rate noise in gene expression of *comK* was reduced. Reduction of noise resulted in a dramatic reduction of competence initiation frequency (Maamar *et al.*, 2007). The importance of global

noise on the transition into the competent state was demonstrated by Süel *et al.* (Suel *et al.*, 2007). They reduced noise in ComK concentration by using elongated filamentous cells while maintaining the mean concentrations of cellular components. When they reduced the noise ~2-fold through a ~7-fold increase in cell length, the switching probability dropped ~10-fold.

The period of competence is highly variable between cells. To explain the high variance, Catagay *et al.* generated alternative network architectures that match the competence initiation probability but show reduced variance of competence duration (Cagatay *et al.*, 2009). In the natural architecture, *comS* is repressed in the state of competence, suggesting that small number effects may give rise to noise. By systematically varying the noise in ComS concentration, they provided evidence that low number noise of ComS governs the probability of escaping from the competent state. Moreover, noise
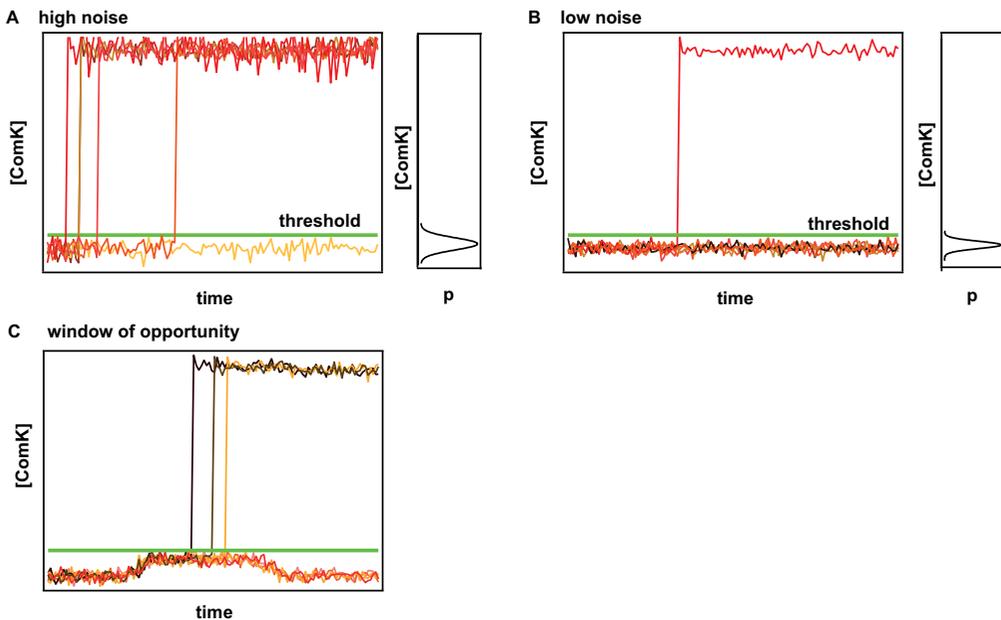

Figure 13.5 The amplitude of noise in ComK concentration governs the probability of differentiation into the competent state. Computer simulations of noise-triggered initiation of competence development. Each curve (red shades) simulates the ComK concentration in a single cell as a function of time. The average ComK concentration is slightly lower than the threshold (green) for triggering the autocatalytic feedback of *comK* transcription. Random fluctuations drive the system over the threshold. Although the average ComK concentrations in (A) and (B) are equal, the distribution *p* is broader in A) than in B), leading to a higher fraction of competent cells. (C) Transient up-regulation of basal *comK* transcription during entry into stationary phase brings the average ComK concentration close to the threshold. As a consequence, the probability of differentiation is highest during early stationary phase.

expands the range of stress levels to which the cells are responsive (Mugler *et al.*, 2016), suggesting that the network architecture may have evolved to cope with variable environmental conditions.

Apart from differentiating into the competent state, *B. subtilis* can elicit spore formation in the stationary phase. The level of phosphorylated Spo0A~P controls the probabilities of competence initiation and sporulation. To address the question how the differentiation programmes interact, Kuchina *et al.* simultaneously characterized competence and sporulation transcriptional activities in individual cells (Kuchina *et al.*, 2011). They found that the probability of competence development remained constant during the approach to spore formation. The fate was determined by the relative timing between the two programs, supporting a 'molecular race' between the differentiation programs. This finding nicely agrees with stochastic choice of cell fate.

Different conditions support bimodality of gene expression and identify noise as a necessary ingredient, indicating that noise-induced bimodality is a robust property of competence development that occurs in different environments (Leisner *et al.*, 2009; Maamar *et al.*, 2007; Suel *et al.*, 2007; Veening *et al.*, 2006). When the competence decision network of *B. subtilis* is taken together, noise was identified as a necessary ingredient, supporting the idea of 'functional noise' (Ackermann, 2015; Eldar and Elowitz, 2010).

Stochastic competence initiation governed by growth phase

The complex regulatory network (Fig. 13.2) can fine-tune basal ComK levels in non-competent cells. In other words, it modulates the concentration of ComK relative to the threshold (Fig. 13.5). Natural 'inducers' of competence include cell density and nutrient limitation. As a consequence, the probability that stochastic fluctuations can carry the system over the threshold is high in the stationary phase but low in the exponential growth phase. Temporal regulation of the basal levels of *comK* expression in non-competent cells was identified as an important factor that can tune the fraction of competent cells (Leisner *et al.*, 2008; Leisner *et al.*, 2007; Maamar *et al.*, 2007). Basal expression increased before entry into the stationary growth phase, reached a maximum and decreased towards zero in the stationary

phase, setting a 'window of opportunity' or 'switching window' for competence initiation (Fig. 13.5c).

Phosphorylated Spo0A is an important player in setting this window (Mirouze *et al.*, 2012). The concentration of Spo0A~P increases with cell density (Fig. 13.2). At the entry into stationary phase Spo0A~P activates *comK* by antagonizing the repressor of ComK, Rok (Mirouze *et al.*, 2012). When the Spo0A~P concentration increases to even higher levels, it directly binds to the *comK* promoter repressing *comK* expression. Another important player is ComS that up-regulates the ComK concentration by competing for binding to the proteolytic complex (Fig. 13.2). With increasing cell density the levels of ComS increase. During exponential growth, when ComS levels are low and ComK degradation is high, differentiation into the competent state is very unlikely. Only during entry into stationary phase do ComK levels increase due to reduced degradation and reside close to the transition threshold. In support of this picture, up-regulation of *comS* expression causes the basal ComK levels to rise before the cells enter the stationary phase, enabling competence differentiation at earlier time points (Leisner *et al.*, 2007).

Taken together, the complex regulatory network shown in Fig. 13.2 deterministically regulates the average ComK levels and the proximity to the threshold determines the probability of stochastic differentiation.

Synthetic control of differentiation dynamics

Different dynamic phenotypes can generate phenotypic hetereogeneity (Martins and Locke, 2015). Using quantitative fluorescence time-lapse microscopy under nutrient-poor conditions, Süel *et al.* investigated the promoter activities of *comG* (encoding for a late competence proteins) and *comS* and found that they were anti-correlated (Suel *et al.*, 2006). This behaviour led to an 'excitable' model consisting of positive autocatalytic feedback for *comK* transcription and a negative feedback in which ComK inhibits *comS* transcription (Schultz *et al.*, 2007; Suel *et al.*, 2006; Suel *et al.*, 2007).

Espinar *et al.* have used a synthetic approach for defining different dynamic regimes (Espinar *et al.*, 2013). In particular, they controlled the expression of *comK* by adding an additional copy of the gene

under the control of an inducible promoter. Additionally, they introduced multiple copies of the *comS* gene under its native promoter (thus keeping the indirect negative feedback from ComK to *comS* intact). In this system, the levels of ComS and ComK were varied independently. They found that at low concentrations of both ComK and ComS the system showed excitable behaviour, defined by low initiation probability and high exit probability (Fig. 13.6). A subpopulation formed spores under these conditions. At high ComS levels, cells are unlikely to exit from the competent state (Suel *et al.*, 2006). In this regime both the initiation and the exit probability were low generating bistability (Espinar *et al.*, 2013). At low ComS levels but high ComK levels, the system showed oscillatory behaviour (Fig. 13.6). Finally, when both protein levels are high, then the system is monostable with all cells in the competent state. Alternatively, the monostable state can be reached by increasing the basal *comK* expression rate through deletion of the repressor of *comK*, *rok* (Leisner *et al.*, 2009).

In summary, by independently varying the transcription rates of *comK* and *comS*, one can screen through the different dynamical behaviours of competence differentiation including excitable, bistable, oscillatory, and monostable patterns.

Costs and benefits of competence

Evolutionary costs and benefits of transformation are still a matter of debate (Chen and Dubnau, 2004; Redfield, 2001). Three major hypotheses for the benefit of transformation are currently discussed (de Visser and Elena, 2007; Fisher, 1930). First, transformation has the potential to reduce maladaptation due to the accumulation of deleterious mutations. When clones are selected for because of a strongly beneficial mutation, additional weakly deleterious mutations are likely to accumulate. Recombination with DNA from a different subpopulation can cure these deleterious mutations. Second, transformation can reduce clonal interference by recombining different beneficial sequences from clones that would otherwise compete. Third, transformation supports acquisition from adaptive alleles from other species (e.g. resistance genes). On the other hand, competence for transformation is likely to induce costs including the physiological cost of generating machines for DNA exchange, and the genetic costs of acquiring deleterious alleles and reducing fitness in the presence of strong epistasis (Engelmoer and Rozen, 2011; Moradigaravand and Engelstadter, 2013).

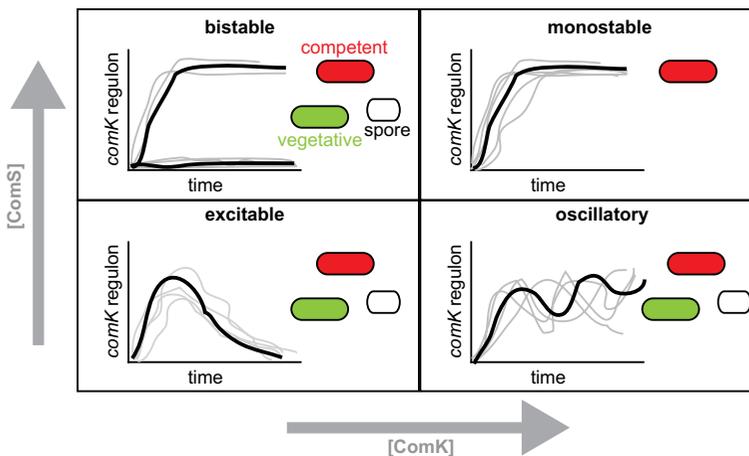In addition to the general costs of transformation, competent *B. subtilis* are growth-arrested.

Figure 13.6 The concentrations of ComS and ComK govern the differentiation dynamics. Systematic variation of *comS* and *comK* expression levels revealed different dynamic regimes that generate phenotypic heterogeneity (Espinar *et al*., 2013).

It is not entirely clear how the ability to develop competence is stably maintained, despite its cost. Importantly, the state of competence confers a benefit under penicillin treatment (Nester and Stocker, 1963). Johnson *et al.* proposed that competence may be selected for because growth arrest increases antibiotic tolerance under episodic treatment with penicillin (Johnsen *et al.*, 2009). Computer simulations predicted a benefit, and this benefit was verified experimentally using time-kill kinetics and head-to-head competition experiments between a laboratory strain of *B. subtilis* with a fraction of ~ 15% competent cells and a non-competent strain (Johnsen *et al.*, 2009). Recent time-kill and competition experiments using bactericidal and bacteriostatic antibiotics with different targets revealed that persistence is a general benefit of the competent state and independent of transformation (Hahn *et al.*, 2015b; Yüksel *et al.*, 2016).

Stochastic differentiation has been proposed as a 'bet-hedging' strategy in varying environments; under benign conditions the majority of the population remains undifferentiated and retains rapid growth (Hahn *et al.*, 2015; Veening *et al.*, 2008). Under stress, a small subpopulation of competent cells is more likely to persist and can ensure the survival of the population as a whole. Recent pair-wise competition experiments under benign conditions and transient antibiotic stress support this hypothesis (Yüksel *et al.*, 2016). They indicate that the generation of phenotypic heterogeneity by means of the K-state is a useful strategy for exploiting the persister phenotype of the K-state when under stress, while minimizing the cost under benign conditions. Persistence is usually associated with transitory growth arrest that renders bacteria more tolerant to antibiotic exposure (Balaban *et al.*, 2013). It is also interesting to note that this strategy of dealing with fluctuating levels of antibiotics is very different for *Streptococcus pneumoniae*. *S. pneumoniae* induces competence in response to sub-MIC levels of antibiotics (Prudhomme *et al.*, 2006; Slager *et al.*, 2014), whereas *B. subtilis* does not (Yüksel *et al.*, 2016).

We conclude that under transient antibiotic exposure the competent state is beneficial in two ways. First, it increases the probability of surviving transient antibiotic exposure. Second, this tolerance increases the window in which the bacteria can sample their environment for genetic information that confers genetic resistance by transformation.

Molecular mechanism of transformation

The process of transformation can be divided into different steps, including DNA binding to the extracellular side, transport through the cell envelope, protection from nuclease attack, and recombination into the chromosome or annealing into self-replicating plasmids (Burton and Dubnau, 2010; Chen and Dubnau, 2004; Dubnau, 1999). Our current knowledge is consistent with the formation of a DNA import machine that delivers the DNA to the recombination machinery for integration into the chromosome. In the following, the individual steps of transformation will be described and the idea of a DNA import machine will be promoted.

The DNA import machine

The so-called 'late competence proteins' are up-regulated at high ComK concentration and are required for transformation. These proteins include proteins located in the membrane or at the extracellular side including ComGB, ComGC, ComEC, and ComEA (Fig. 13.7A). Furthermore, proteins that associate with the membrane at the intracellular side are required, including ComFA and ComGA. Finally, the cytosolic proteins RecA, SsbB (YwpH), DprA (Smf), and YjbF (CoiA) modulate the transformation efficiency. These proteins are involved in different steps of transformation and have been discussed by other authors (Graumann, 2017).

Representatives of membrane bound proteins and cytoplasmic proteins accumulate at the cell poles (Hahn *et al.*, 2005; Kidane and Graumann, 2005) (Fig. 13.7B). As competence develops proteins accumulate and as transformability wanes proteins delocalize. Dynamic fluorescence data are consistent with a diffusion-capture mechanism for polar accumulation, although the identity of the anchoring protein remains elusive (Hahn *et al.*, 2009). Most likely, subsequent delocalization from the poles is caused by degradation of the anchor protein by the McsA-McsB-Clp(C or E)P complex. In agreement with polar localization of
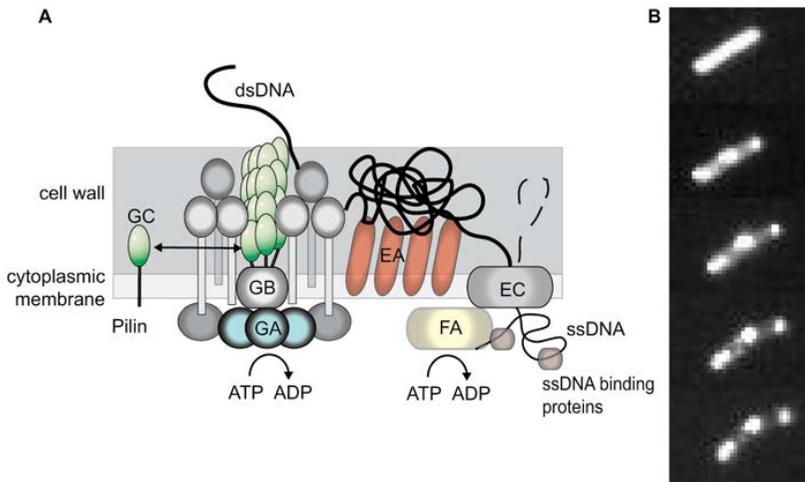
Figure 13.7 (A) Current model of the DNA import machine. A pseudopilus is required for binding of DNA at the extracellular side. Multiple components are essential for its biogenesis including the major pilin subunit ComGC, the integral membrane protein ComGB, and the cytoplasmic ATPase ComGA. ComEA masses DNA in a DNase-resistant state. ComEC forms an integral membrane complex essential for DNA import into the cytoplasm. ComFA supports transport of a single strand of DNA while the second strand is degraded. In the cytoplasm, the DNA is decorated with single-strand binding proteins. (B) Example of dynamics of focus formation by ComGA-GFP recorded over several minutes in the stationary phase.

the DNA import proteins, DNA is taken up preferentially at the poles, as revealed by single molecule experiments (Hahn *et al.*, 2005). These experiments suggest that a whole machine assembles at the pole for efficient DNA transport. To further support the picture of a multi-component DNA transport machine, Kramer *et al.* (2007) used a combination of co-localization, FRET, and protein stability analysis to investigate interactions among DNA import proteins. They found that interactions occurred between membrane-associated proteins and cytosolic proteins, confirming the existence of a complex machine for binding, transport, and integration of transforming DNA. FRAP experiments showed remarkably low exchange of late competence proteins at the cell pole at a time scale of minutes, with the exception of the DNA receptor ComEA, which was mobile within the cell membrane (Kaufenstein *et al.*, 2011). The DNA import complex was stable even in round spheroblasts, indicating that polar localization is not required for maintenance of the import complex. Together, these experiments strongly support the picture of a stable DNA import machine that transiently assembles at a defined position in competent cells.

DNA binding

The first step towards transformation is binding of exogenous double-stranded DNA (dsDNA) to the bacterial cell surface. In Gram-positive bacteria this is complicated by the presence of a peptidoglycan-containing cell wall. A group of proteins essential for transformation is related to type 4 pili and type 2 secretion (Chen and Dubnau, 2004). These may either act as a receptor, or through modification of the cell wall. The pseudopilins ComGC to ComGG are P-shaped with a hydrophobic α-helix traversing the membrane. After removal of several amino acids by the prepilin protease ComC (Chung and Dubnau, 1995) and formation of an intramolecular disulphate bond through the oxidoreductase pair BdbD and BdbC, they assemble into a helical polymeric structure, called the pseudopilus (Chen *et al.*, 2006) (Fig. 13.7A). A major pseudopilin (ComGC) and three minor pseudopilins (ComGD, GE, GG) are required to form the pseudopilus. Furthermore, the traffic ATPase ComGA and the polytopic membrane protein ComGB are essential for the assembly of the pseudopilus. The latter is polydisperse with an average length that allows protrusion from the cell wall (Chen *et al.*,

2006; Kaufenstein *et al.*, 2011). Interestingly, the pseudopilus is dispensable for transformation in the absence of a cell wall (Provvedi 1999). Type 4 pilus proteins are necessary for transformation in all naturally competent species characterized so far, with the exception of *H. pylori*. Although very low DNA binding activity has been reported in with pili of *Neisseria meningiditis* (Lang *et al.*, 2009), it remains unclear whether the primary role of the pilus is DNA binding, or modification of the cell wall.

DNA import through the cell envelope

After binding of dsDNA to the cell surface, one strand is degraded and the other strand is imported into the cytoplasm. Good experimental evidence suggests that the polytopic membrane protein ComEC forms the pore for DNA uptake (Draskovic and Dubnau, 2005) (Fig. 13.7A). In *B. subtilis*, deletion of ComEC also prevents degradation of the non-transforming DNA-strand.

In one of the previous paragraphs, the idea of a DNA-import machine has been promoted. It was therefore important to characterize the kinetics and force generation by this machine. To this end, Maier *et al.* investigated DNA import at the level of single DNA molecules using laser tweezers (Maier *et al.*, 2004) (Fig. 13.8). In this approach, DNA was attached to a micrometre-sized bead at one end and to the bacterium at the other end. The length of imported DNA was monitored in real time and at a resolution of 10 nm. These experiments revealed a highly processive molecular motor that transports DNA at a velocity of 80 bases/s, generating a force

of at least 50 pN. Import is irreversible even at large counteracting force. Thus, a remarkably strong, efficient, and irreversible machine supports DNA import (Maier *et al.*, 2004).

From a biophysical point of view, the DNA import machine can be regarded as a translocation motor that uses chemical energy for performing mechanical work. Recent experiments have shed some light onto the biophysical mechanism, but many questions remain open. We propose that DNA uptake from the environment to the cytoplasm is driven by two separate molecular machines. First, DNA is taken up reversibly into the periplasmic space or cell wall driven by a translocation ratchet mechanism with ComEA as a chaperone. Second, the stronger and irreversible cytoplasmic motor (potentially ComFA) drives import from the periplasm to the cytoplasm. In the following we will discuss experiments that are in agreement with this hypothesis. We note, however, that presently other mechanisms are possible.

In *B. subtilis*, ComEA strongly enhances DNA-uptake into a Dnase-resistant state, but is not important for initial DNA binding (Briley *et al.*, 2011a). ComEA is a membrane-bound protein containing a DNA-binding motif (Inamine and Dubnau, 1995). Interestingly, DNase-resistance of externally added DNA in *B. subtilis* does not require transport into the cytoplasm (Briley *et al.*, 2011a). Briley *et al.* showed that the larger part of DNase-resistant DNA remained in the supernatant after protoplast formation. ComEA was not essential for protoplast transformation but increased its probability by several orders of magnitude (Takeno
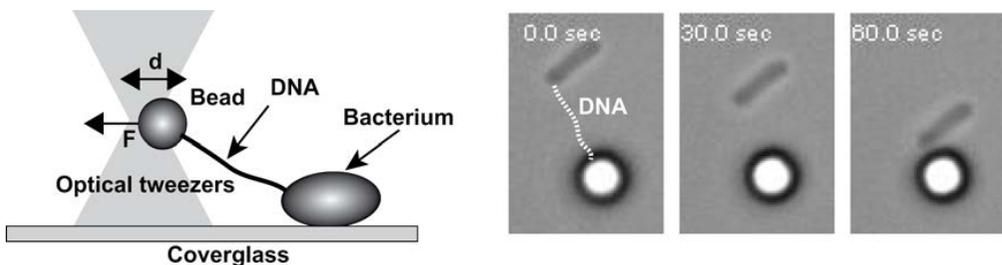

Figure 13.8 Setup for characterizing DNA import at the single molecule level. *B. subtilis* is immobilized at a microscope cover slide. Micrometre-sized beads are coated with DNA and brought into contact with the bacterium. Upon binding, the DNA (drawn as dashed line in panel at $t = 0.0$ s) is stretched and as the bacterium starts importing DNA, the distance between the bacterium and the bead decreases. Thus the distance between the latter is a measure for the length of imported DNA as a function of time.

et al., 2012). Furthermore, there is evidence that the speed of DNA-translocation is reduced in a *comEA* deletion strain (Takeno *et al.*, 2012). These experiments suggest that ComEA may be involved in masking DNA within the cell wall or the Gram-positive periplasm prior to transport through the cytoplasmic membrane. This hypothesis nicely agrees with recent experiments in Gram-negative systems including *Neisseria gonorrhoeae* and *Vibrio cholerae.* They have adapted the type 4 pilus system for transformation and the essential DNA uptake proteins are largely conserved (Chen and Dubnau, 2004). DNA uptake through the outer membrane can proceed independently from transport through the inner membrane (Facius and Meyer, 1993). *N. gonorrhoeae* and *V. cholerae* have ComEA homologues (ComE for *N. gonorrhoeae*) lacking the membrane binding domain (Chen and Gotschlich, 2001). Thus ComE(A) is homogeneously distributed in the periplasm and relocates to transforming DNA as it enters the periplasm (Gangel *et al.*, 2014; Seitz *et al.*, 2014). Gonococcal ComE governs the carrying capacity of the periplasm for transforming DNA in a gene-dosage dependent fashion (Gangel *et al.*, 2014). The force-dependent speed of DNA uptake obtained from single molecule experiments as shown in Fig. 13.8, shows that DNA uptake is reversible at a force of 20 pN (Hepp and Maier, 2016), i.e. the amount of force generated during outer membrane transport is considerably lower than by cytoplasmic transport (Maier *et al.*, 2004). The force-dependent velocity is affected by the concentration of ComE and is in remarkable agreement with the mechanism of a translocation ratchet (Hepp and Maier, 2016). The translocation ratchet model has been proposed by Peskin *et al.* (Peskin *et al.*, 1993). In this concept, movement of DNA within the membrane pore is generated by Brownian motion. DNA-binding proteins in the periplasm hinder diffusion of the DNA into the environment inducing net movement of the DNA from the environment into the periplasm. In analogy to *N. gonorrhoeae*, we propose that *B. subtilis* ComEA binds the DNA and accumulates it within the Gram-positive periplasm or cell wall.

Because DNA import observed by single molecule experiments with *B. subtilis* was dependent on ComEC (Maier *et al.*, 2004), we conclude that transport through the cytoplasmic membrane and not binding to ComEA was monitored in these experiments. ComFA is an interesting candidate for driving transport through the cytoplasmic membrane. In *B. subtilis* ComFA is important but dispensable for DNA transport (Londono-Vallejo and Dubnau, 1994a), since DNA import proceeds at 1000-fold reduced rate in a deletion strain. ComFA is a membrane-bound ATPase that resembles DEAD box helicases. Titration experiments using ComFA with mutations in the putative ATP-binding site showed a dominant negative effect suggesting that more than one copy of ComFA is included in an active DNA translocation complex (Londono-Vallejo and Dubnau, 1994b). Furthermore the fact that overexpression of ComFA has a detrimental effect on DNA uptake indicates that the correct stoichiometry within the DNA import machine is important for proper function (Londono-Vallejo and Dubnau, 1994b). Thus, ComFA may work like a cyclic molecular motor that drives DNA import by successive binding and conformational changes.

Taken together, we propose a 'two force-generating' mechanisms during DNA-uptake. First, weak and reversible binding to ComEA causes DNA accumulation within the cell wall or periplasm. Subsequently, a strong and irreversible motor drives the translocation through the cytoplasmic membrane.

Recombination and annealing

The final steps of the transformation process are either recombination with the chromosome or strand annealing to form self-replicating plasmids. The recombinase RecA is the key protein that mediates incorporation of incoming ssDNA into the chromosome via homologous recombination. RecA itself forms filamentous structures termed threads that move away from the cell pole upon addition of external DNA (Kidane and Graumann, 2005). These findings are consistent with the hypothesis that RecA threads are involved in the transport of ssDNA from the pole to the chromosome, where homology is sought throughout the chromosome. The extended nucleoprotein filaments can integrate into the DNA double helix and displace one DNA strand (which is later degraded), if sufficient homology exists (see Graumann, 2017). However, by itself, RecA has rather low affinity to ssDNA, much lower than many single strand binding (SSB) proteins. Therefore,

SSB proteins cover the ssDNA immediately after import.

SsbB (YwpH) is a single strand-binding protein required for optimal transformation (Lindner *et al.*, 2004). SsbB is part of the *comK* regulon (Berka *et al.*, 2002) and localizes to the cell pole (Hahn *et al.*, 2005). *In vitro* studies indicate that SsbB rapidly binds to ssDNA at the ComEC pore, protecting the incoming DNA (Yadav *et al.*, 2012). SsbA co-assembles onto SsbB-coated ssDNA and the two proteins inhibit binding of RecA. During chromosomal transformation the RecA mediators RecO and DprA provide access for RecA to the DNA (Yadav *et al.*, 2013) (Fig. 13.9a). At the cellular level, fluorescent DprA changed its localization when DNA is added, i.e. the polar DprA assembly dissipated in a RecA dependent manner when monitored by real-time microscopy, in agreement with the *in vitro* studies (Tadesse and Graumann, 2007). Once RecA is nucleated, filament elongation dislodges SsbA and SsbB and enables RecA-mediated DNA strand exchange (Yadav *et al.*, 2013; Yadav *et al.*, 2012).

Transformation with plasmid DNA has different requirements than chromosomal transformation. While import of plasmid DNA occurs through the uptake machinery, it does not require RecA protein, but RecO and RecU proteins. Imported ssDNA (both, Watson and Crick strands) must first anneal to form dsDNA, and this DNA must intramolecularly recombine to yield circular plasmid DNA that can be replicated autonomously. This explains why only multimeric plasmid DNA can lead to transformation of *B. subtilis* cells (de Vos *et al.*, 1981). RecO can efficiently anneal ssDNA and promote intramolecular recombination (Kidane *et al.*, 2009), while RecU can modulate RecA activity (Carrasco *et al.*, 2005). Interestingly, transformation with chromosomal or plasmid DNA follows visibly different pathways (Kidane *et al.*, 2009). Uptake of plasmid DNA, but not of chromosomal DNA, leads to the accumulation of RecO at the competence pole, where RecU accumulates dependent on RecA, most likely for annealing and replacement of RecA, respectively (Fig. 13.9a). *In vitro* experiments indicate that during plasmid transformation, RecO can catalyse annealing of SsbA- and SsbB- coated complementary ssDNA strands to from duplex DNA (Fig. 13.9b) (Yadav *et al.*, 2012).

These experiments show that proteins that determine the fate of incoming ssDNA are directly coupled to the import machinery and operate
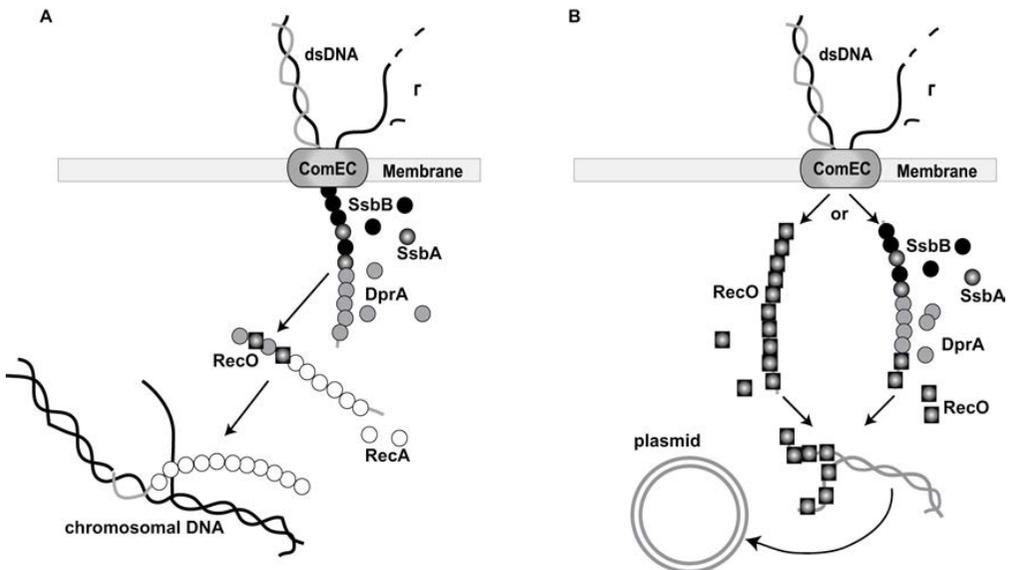

Figure 13.9 Model for intracellular processing of imported DNA. (a) Import and homologous recombination of chromosomal DNA (adapted from Burton and Dubnau, 2010). (b) Import and annealing of plasmids.

from a single position within *B. subtilis* cells. For an extended description of recombination and self-annealing the reader is referred to (Kidane *et al.*, 2012).

Conclusions

A consistent picture of a coordinated DNA uptake and transformation machine is emerging that enables bacteria to bind external DNA, to transport it through the cell envelope, to protect it from degradation in the cytoplasm, and to guide it to the chromosome for homologous recombination. While the components of the transformation machine are well conserved between different bacterial species, the mechanisms of competence regulation are very different. Even for different isolates of *B. subtilis* the probabilities of competence differentiation vary. For example, natural plasmids can encode for factors that alter the ComK levels and thereby reduce competence levels in a plasmid-containing population (Konkol *et al.*, 2013; Singh *et al.*, 2012). Phenotypic heterogeneity in terms of competence is likely to act as a bet-hedging strategy (Hahn *et al.*, 2015; Veening *et al.*, 2008; Wylie *et al.*, 2010), which enables the major fraction of the population to grow quickly under benign conditions. Under conditions of stress, however, the small subpopulation of growth-arrested K-state cells may be at an advantage and ensure the survival of the population. Recent work supports the idea that tuning the differentiation probability may be an efficient strategy for rapidly adapting to different environments (Yüksel *et al.*, 2016).

Theoretical efforts attempt to model the full differentiation strategy of *B. subtilis* including competence, sporulation, and cannibalism by subdividing the decision network into corresponding modules (Schultz *et al.*, 2009). It will be exciting to understand the entire *B. subtilis* regulatory network including all intracellular, developmental and multicellular processes in its entity (Mirouze and Dubnau, 2013).

Acknowledgements

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