

Induction of natural competence in *Bacillus cereus* ATCC14579

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Summary

Natural competence is the ability of certain microbes to take up exogenous DNA from the environment and integrate it in their genome. Competence development has been described for a variety of bacteria, but has so far not been shown to occur in *Bacillus cereus*. However, orthologues of most proteins involved in natural DNA uptake in *Bacillus subtilis* could be identified in *B. cereus*. Here, we report that *B. cereus* ATCC14579 can become naturally competent. When expressing the *B. subtilis* ComK protein using an IPTG-inducible system in *B. cereus* ATCC14579, cells grown in minimal medium displayed natural competence, as either genomic DNA or plasmid DNA was shown to be taken up by the cells and integrated into the genome or stably maintained respectively. This work proves that a sufficient structural system for DNA uptake exists in *B. cereus*. *Bacillus cereus* can be employed as a model system to investigate the mechanism of DNA uptake in related bacteria such as *Bacillus anthracis* and *Bacillus thuringiensis*. Moreover, natural competence provides an important tool for biotechnology, as it will allow more efficient transformation of *B. cereus* and related organisms, e.g. to knockout genes in a high-throughput way.

Introduction

Genetic competence is the natural ability to take up exogenous DNA from the environment and integrate it into the own genome (Dubnau, 1999), a phenomenon that is widespread among eubacteria (Lorenz and Wackernagel, 1994). One of the best-studied naturally competent

bacteria is *Bacillus subtilis*. To enable incorporation of DNA from the environment, *B. subtilis* cells synthesize a specific DNA-binding and uptake system. Several operons have been identified that are required for functional DNA uptake including *comC*, *comE*, *comF*, *comG* and *nucA*. A *comG* operon-coded type IV pilus helps the DNA to pass the cell wall and reach the cell membrane (Dubnau, 1997; Chen *et al.*, 2006), where it is bound by ComEA and taken up by the ComEC permease with the involvement of ComFA and NucA (Chen and Dubnau, 2004). DNA is then integrated via recombination by the action of a protein complex consisting at least of RecA, SsbB, DprA and YjbF (Kramer *et al.*, 2007).

Competence development is controlled by a complex signal transduction network in *B. subtilis*, and is expressed under strict conditions (Hamoen *et al.*, 2003). ComK plays an important role in the regulation of expression of late-competence genes under these conditions. The induction of ComK is strictly controlled at the level of *comK* transcription as well as post-translationally. The transcription of *comK* is repressed by at least three different transcription factors: AbrB, Rok and CodY, but is also positively regulated by the DegSU two-component system and by ComK itself (Hamoen *et al.*, 2003). ComK protein is trapped by the adaptor protein MecA, and targeted to the protease complex ClpCP by which it is degraded (Turgay *et al.*, 1998). In response to high cell density, quorum-sensing pathways induce the expression of the small anti-adaptor protein ComS, which destabilizes the protein complex and liberates ComK (D'Souza *et al.*, 1994; Hamoen *et al.*, 1995; Solomon *et al.*, 1995; Prepiak and Dubnau 2007). ComK then binds to its own promoter region and derepresses transcription resulting in high level of ComK in the cells and development of competence state. However, the enhanced level of ComK and the concomitant development of competence is only achieved in part of the cells (Nester and Stocker 1963; Maamar and Dubnau, 2005; Smits *et al.*, 2005). ComK is directly or indirectly responsible for the activation of more than 100 genes (Berka *et al.*, 2002; Hamoen *et al.*, 2002; Ogura *et al.*, 2002). Activation of late-competence genes is achieved by binding of ComK to specific sequences, the so-called K-boxes, that consist of two AT-boxes with the consensus sequence AAAA-N₅-TTTT and separated by a spacer of a discrete number of helical turns (Hamoen *et al.*, 2002; Susanna *et al.*, 2007).

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Within the *Bacillus* family natural DNA uptake has been described for *B. subtilis*, *Bacillus licheniformis* and *Bacillus amyloliquefaciens* (Spizizen, 1958; Thorne and Stull 1966; Koumoutsis *et al.*, 2004), but the phenomenon has been never demonstrated to occur in the *Bacillus cereus sensu lato* group.

Bacillus cereus is a rod-shaped, Gram-positive spore-forming bacterium and an important food-borne human pathogen. *Bacillus cereus* occurs ubiquitously in soil and in a diversity of foods as milk, rice, dairy products and vegetables. As a member of the *B. cereus sensu lato* group, *B. cereus* is closely related to *Bacillus anthracis* and *Bacillus thuringiensis* (Helgason *et al.*, 2000; Rasko *et al.*, 2005). The former one is the ethiological agent of anthrax, and the latter is widely used as an insecticidal biopesticide (Helgason *et al.*, 2000).

The appearance of whole genome sequences of the *Bacillus* group showed that the presence of competence genes is not restricted to *B. subtilis* and closely related species, but is apparent throughout the *Bacillus* genus. Homologues of most structural proteins required for transformation in *B. subtilis* have been found in *B. cereus*, with the exception of the ComGE, ComGF, ComGG and ComFB homologues, suggesting that this strain has a potential for developing natural competence (Rasko *et al.*, 2004). Interestingly, two homologues of ComK could be identified in *B. cereus* ATCC14579, suggesting that the regulation of competence gene expression might differ from that in *B. subtilis*.

It is known that in the *B. cereus sensu lato* group, pathogens have adapted to various environments, because of insertion of DNA through homologous recombination, a process called horizontal gene transfer (Read *et al.*, 2003). However, uptake of genomic DNA has never been shown to occur in the *B. cereus* group. Here, we prove that a minimal system for functional DNA uptake exists and can be induced in *B. cereus*, which enables us to elucidate how natural transformation proceeds in this organism.

Results

Overexpression of *B. subtilis* ComK in *B. cereus*

Competence development in *B. subtilis* depends on the expression of a set of late-competence genes that are transcribed only in the presence of the competence transcription factor ComK. Previously, it has been shown that induction of *comK* combined with positive auto-stimulation of native *comK* results in an increased percentage of competence cells in the population (Maamar and Dubnau 2005; Smits *et al.*, 2005). Interestingly *B. cereus* ATCC14579 contains two homologues of the *B. subtilis* *comK* gene, i.e. *comK1* (BC1134) and *comK2* (BC5250).

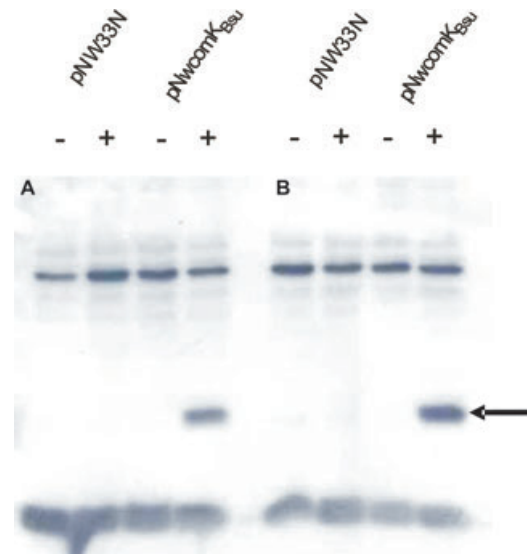


Fig. 1. Detection of *B. subtilis* ComK protein. Equal amounts of proteins were loaded in each lane. Strains carrying an empty plasmid pNW33N or pNWcomK_{Bsu} were grown in MM, supplemented with chloramphenicol (5 µg ml⁻¹). Samples were taken 2 h (A) and 3 h (B) after IPTG induction (+) (final concentration 1 mM) or without IPTG induction (-). Cells were separated from the growth medium by centrifugation, lysed and analysed by Western blotting using ComK_{Bsu}-specific antibodies (see *Experimental procedures*). The arrow indicates ComK_{Bsu}-specific signal.

The first putative ComK protein (ComK1) shows 62% homology to *B. subtilis* ComK, while ComK2 has only 48% homology and is 32 amino acids shorter at the C-terminal than the *B. subtilis* ComK protein.

Natural transformation is an efficient tool for genetic manipulation, which has been used successfully in *B. subtilis* for many years. Therefore, we set out to determine whether *B. cereus* ATCC14579 is naturally transformable. Driven by the fact that many late-competence genes and recombination genes are present in the genome of this strain (Table S1), the first step to enhance expression of late-competence genes in *B. cereus* was to increase ComK levels in the cells. The regulation and function of the two *comK* genes are unknown in this bacterium. Here, we employed the *comK* gene of *B. subtilis* and investigated whether functional DNA uptake exists in *B. cereus*. For this purpose the *B. subtilis* *comK* gene (hereafter referred to as *comK*_{Bsu}) was cloned behind the Phyper-spark promoter that can be induced by isopropyl-β-D-thiogalactoside (IPTG) addition. The resulting plasmid pNWcomK_{Bsu} (or the empty plasmid pNW33N used as a control) was introduced by electroporation into *B. cereus* ATCC14579. To verify the overexpression of ComK_{Bsu} following IPTG addition, Western blot analysis was performed using ComK_{Bsu}-specific antibodies (Fig. 1). The most favourable condition was selected, where ComK protein production was most effectively induced. Cells

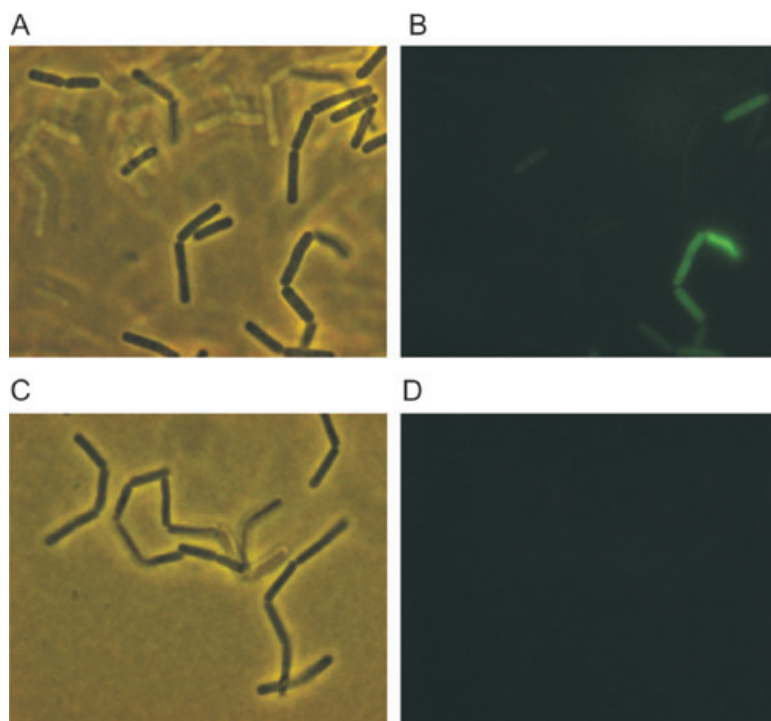


Fig. 2. Visualization of PComGA_{Bce}–GFP protein production in *B. cereus* by fluorescence microscopy. A and B. *Bacillus cereus* pNWcomK_{Bsu} carrying pLcomGA–gfp. C and D. *Bacillus cereus* carrying pLcomGA–gfp. (A) and (C) are phase contrast pictures of cells from (B) and (D) respectively. Strains were grown in MM medium, and samples were taken 3.5 h after IPTG induction. Cells producing GFP from the *comGA*_{Bce} promoter were visualized on (B) and (D). Production of GFP, whose expression was driven by activity of *comGA*_{Bce} promoter, was visualized by fluorescence microscopy, as described in *Experimental procedures*.

collected at two different time points, 2 or 3 h after induction, showed high expression of ComK_{Bsu} in the induced *B. cereus* strain containing pNWcomK_{Bsu} plasmid. Slightly higher expression was observed with the samples taken after 3 h of induction. In contrast, no ComK could be detected either in the non-induced strains or when the induced strain contained the empty plasmid pNW33N. The experiments above demonstrate that a reasonably high yield of IPTG-dependent overexpression of ComK_{Bsu} can be achieved in *B. cereus*.

The B. subtilis ComK protein can induce transcription from PcomGA of B. cereus

As *comG_A* has been shown to be a suitable competence-specific reporter (Smits *et al.*, 2005) and transcription of this gene is under strict control of ComK in *B. subtilis*, we constructed a fusion between the promoter region of *B. cereus comGA* (*PcomGA*_{Bce}) and the green fluorescence protein (*gfp*) gene. The resulting plasmid (pLcomGA–gfp) containing an in-frame fusion of *gfp* with the first six codons of *B. cereus comG_A* was used to determine the effect of ComK_{Bsu} overexpression under various growth conditions. As the fusion contains only a few N-terminal amino acid residues of ComGA protein, it should not specifically localize in the cells, similar to previous experiments (Maamar and Dubnau, 2005; Smits *et al.*, 2005). This construct was introduced into *B. cereus* by electroporation. Also the pIL253 control plasmid was introduced into *B. cereus*. Both fluorescence microscopy and flow

cytometry were used to visualize the expression of GFP from the *PcomGA*_{Bce} in individual cells.

Experiments were performed with strains harbouring either pNWcomK_{Bsu} or the control pNW33N plasmid, both carrying the pLcomGA–gfp vector to determine whether the expression system used could result in the high ComK_{Bsu} level that is probably required for activation of the *B. cereus* competence cascade. The strains were grown in presumed competence stimulating medium in the presence of glucose and induced at different time points and with different concentrations of IPTG (varying from 0.1 to 1.0 mM). Subsequently, cultures were sampled every hour after induction and analysed. Fluorescence microscopy showed the presence of GFP signal from *PcomGA*_{Bce} in IPTG induced *B. cereus* samples carrying the pNWcomK_{Bsu} vector (Fig. 2). Interestingly, only a few percent of cells in the culture showed the expression of GFP. In agreement with the microscopy experiments, flow cytometric analysis also proved that only part of the population expressed high levels of GFP (Fig. 3). Flow cytometry experiments showed that highest *PcomGA*–*gfp* expression occurred after 3 h of induction with a 1 mM IPTG concentration. We did not notice GFP expression in *B. cereus* carrying the control plasmid pNW33N neither under non-inducing or inducing conditions [1.6 ± 0.5 and 1.6 ± 0.6 arbitrary units (AU) in non-induced and induced cells respectively]. In the strain harbouring pNWcomK_{Bsu}, either induced or not induced, the GFP signal was enhanced (3.4 ± 0.6 and 12.3 ± 1.0 AU in

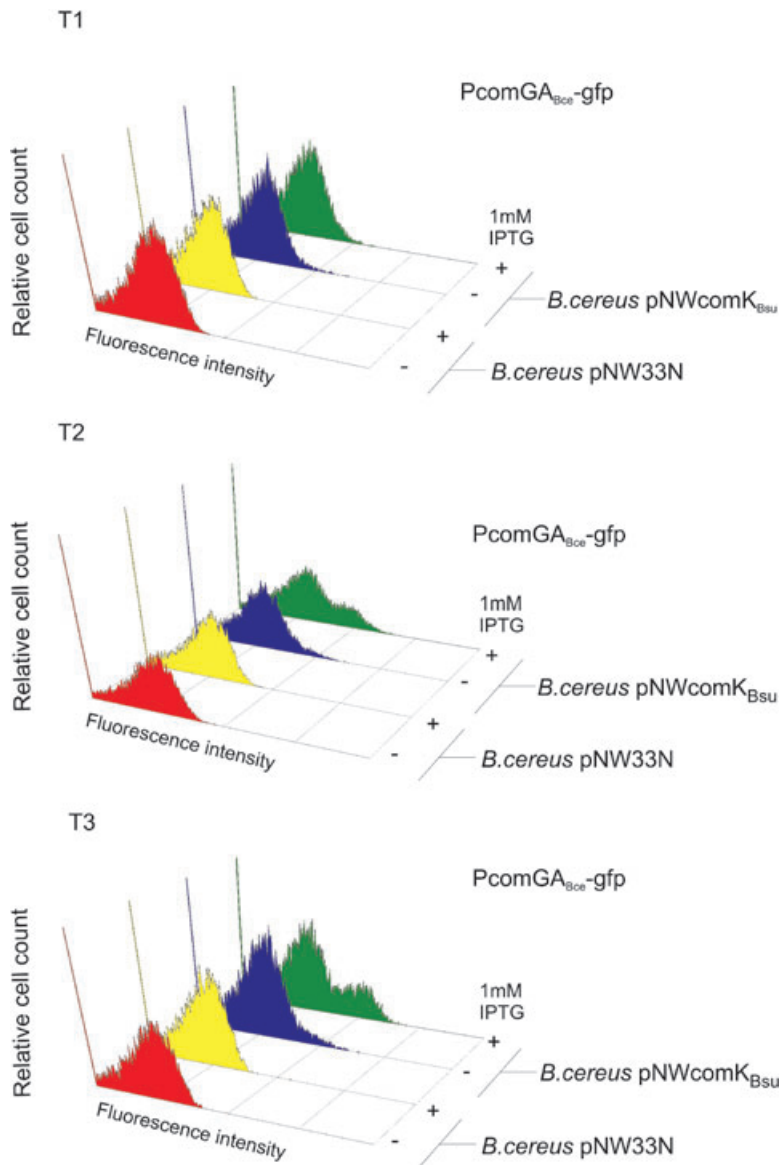


Fig. 3. Flow cytometric analysis of $P_{comGA_{Bce}}-gfp$ in liquid MM medium. Analyses were performed as described in *Experimental procedures*. The panels present the different time points in hours relative to the induction point at OD_{600} of 0.75 (1 h after IPTG induction is T1, etc.). Strains carrying $P_{comGA_{Bce}}-gfp$ fusion were grown in liquid MM, supplemented with chloramphenicol ($2.5 \mu\text{l ml}^{-1}$) and erythromycin ($2.5 \mu\text{l ml}^{-1}$). *Bacillus cereus* strains contain the pNW33N control plasmid or the $ComK_{Bsu}$ -overexpressing plasmid, pNWcomK_{Bsu}. The plus and minus symbols denote samples incubated in the presence or absence of IPTG respectively. The numbers of cells are indicated on the y-axis, and their relative fluorescence levels are indicated on the x-axis on a logarithmic scale. For detailed description of the visualization, see also the WinMDI manual (<http://facs.scripps.edu/software.html>).

non-induced and induced cells respectively). However, in the induced strain, the GFP signal was stronger. The low level fluorescence, which occurs in the non-induced *B. cereus* pNWcomK_{Bsu} strain, is most likely originating from the low basal expression of *comK_{Bsu}* gene from the P_{hyperspank} promoter. In the *ComK*-expressing samples, two subpopulation of cells could be separated in flow experiments, one that possesses GFP activity (2.3 ± 0.3 AU) similar to the control strain (*B. cereus* ATCC14579 with pNW33N), while in the other part of the cells high GFP activity could be detected (117 ± 11 AU in $23 \pm 2.5\%$ of the culture).

Our results show that high levels of *ComK_{Bsu}* in the cells can activate transcription of the *comGA* gene either directly or indirectly and probably also increases expression of the other late-competence genes.

ComK_{Bsu} binds to the promoter regions of potential competence related genes in *B. cereus*

Previously, it has been shown that in *B. subtilis* *ComK* can bind directly to a region upstream of late-competence genes (Hamoen *et al.*, 1998). Expression activation at the *comGA_{Bsu}* promoter is solely dependent on *ComK* (Susanna *et al.*, 2004). Here, electrophoretic mobility shift assays (EMSA) were performed to determine whether purified *ComK_{Bsu}* protein can bind to the *comGA* and *comE* promoter regions of *B. cereus* (hereafter referred to as $P_{comGA_{Bce}}$ and $P_{comEA_{Bce}}$). The results from the gel shift analyses showed that *ComK_{Bsu}* can bind *in vitro* to both promoter fragments, leading to reduced mobility of the probes (Fig. 4A). These data confirm that *ComK_{Bsu}* may activate transcription at the $P_{comGA_{Bce}}$ promoter region by

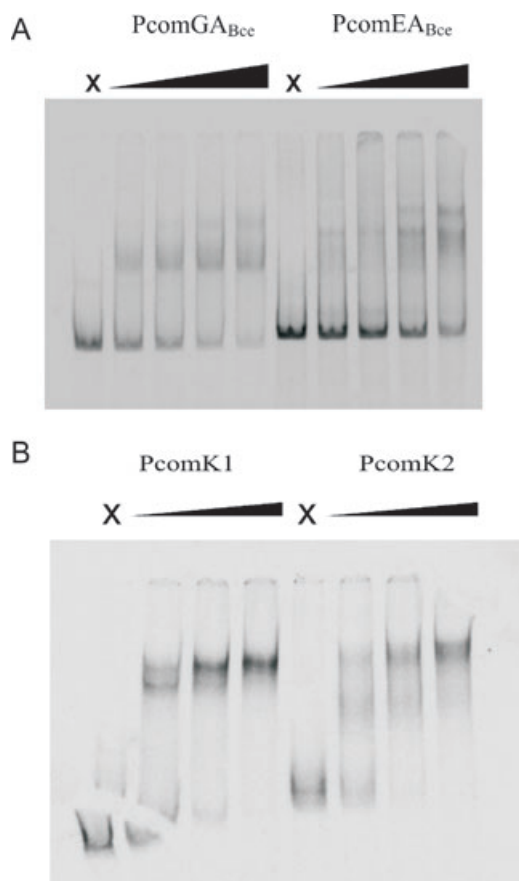


Fig. 4. *Bacillus subtilis* ComK binds to both *B. cereus* late-competence genes promoters *PcomGA_{Bce}* and *PcomEA_{Bce}* (A), and the promoters of *comK1* and *comK2* genes (B). Assessment of binding of ComK_{Bsu} was performed as described in *Experimental procedures*. DNA fragments from the indicated promoter regions were prepared by PCR and end-labelled with ³³P. In the panel, the lanes marked with an X correspond to probe alone. The remaining lanes correspond to probe samples incubated with ComK_{Bsu}, at concentration ranging from 0.2 μM to 1.6 μM. Each lane, from left to right, corresponds to a doubling in the concentration of ComK_{Bsu}.

directly binding to an operator region. Reaction mixtures contained poly(dI-dC) that is known to eliminate non-specific DNA binding of ComK_{Bsu} (Hamoen *et al.*, 1998).

Analysis of the *PcomGA_{Bce}* sequence showed the presence of a putative ComK binding site upstream of the

putative –35 and –10 promoter elements of *comGA_{Bce}* (Fig. S1).

Subsequently, we performed EMSA analysis with the *comK1* and *comK2* promoter regions to determine whether ComK_{Bsu} could also affect the expression of these genes. In both cases, DNA binding was observed by ComK_{Bsu} (Fig. 4B), showing it might also directly affect *comK1* or *comK2* expression, thereby passing by their putative natural regulation.

Occurrence of the competence state in B. cereus ATCC14579

The previous experiments showed that high expression of ComK_{Bsu} can activate *PcomGA-gfp* expression in *B. cereus* ATCC14579. Having constructed an inducible ComK_{Bsu} system, which efficiently activated transcription level of *comGA*, we next investigated whether the presumed competence machinery of *B. cereus* could be fully functional. For this, we tested the presence of natural transformation using *B. cereus* pNWcomK_{Bsu} and, as a control, a *B. cereus* strain carrying the empty plasmid pNW33N. Both *B. cereus* strains were grown in *B. subtilis*-like competence minimal medium (see *Experimental procedures*). When they reached an optical density at 600 nm (OD₆₀₀) of 0.7, they were induced with 1 mM IPTG. Either genomic DNA of *B. cereus* FM1400 (van Schaik *et al.*, 2004) containing a *sigB* null mutation carrying an erythromycin cassette on the chromosome or the replicative plasmid pNG8048E (Kuipers *et al.*, 1998) was used as donor DNA during the transformation experiments. In these experiments, genomic or plasmid DNA was added to the cells, which were incubated for 30 min at 30°C with shaking. Subsequently, TY medium was added and the incubation was continued for an additional 1 h (see *Experimental procedures*). The transformation was monitored by plating cells on TY plates containing 2.5 μg ml⁻¹ erythromycin. Colony counts are presented in Table 1. Although the transformation efficiency was relatively low, only the *B. cereus* strain containing the inducible *comK_{Bsu}* could act as a recipient of plasmid or genomic DNA, being stably maintained or integrated respectively. In all transformants the presence of

Table 1. Natural transformation experiments.

Strain	Induction with 1 mM IPTG	cfu					
		pNG8048e		FM1400			
<i>Bacillus cereus</i> pNWcomK _{Bsu}	+	3	1	3	9	1	3
<i>Bacillus cereus</i> pNWcomK _{Bsu}	–	0	0	0	0	0	0
<i>Bacillus cereus</i> pNW33N	+	0	0	0	0	0	0
<i>Bacillus cereus</i> pNW33N	–	0	0	0	0	0	0

Numbers present colonies obtained after transformation with 1.5 μg of genomic DNA or plasmid. cfu, colony-forming units.

erythromycin- and chloramphenicol-resistant markers was confirmed by PCR (data not shown), proving the uptake of the foreign DNA by the cells containing the pNWcomK_{BSU}. Deletion in the *sigB* gene was also proven in specific PCR reactions (data not shown). Strains transformed with the genomic DNA of FM1400 were checked for their ability to survive a 60 min exposure to 50°C after pre-treatment at 42°C for 30 min. The donor *sigB* mutant strain and the transformed strains showed the similar 10³ times reduction in viable counts upon these treatments. Plasmid pNG8048E could be isolated and retransformed into *Lactococcus lactis* proving that it is stably maintained. These results confirm that ComK_{BSU} is able to induce natural competence in *B. cereus* and above all, that *B. cereus* contains the minimal structural machinery required to bind and take up DNA.

Discussion

Competence development has been described for a variety of species belonging to both Gram-negative and Gram-positive bacteria. Natural DNA uptake presents a valuable tool for genetic studies. This beneficial property made *B. subtilis* the model organism of the *Bacillus* family. Since the description of natural transformability of *B. subtilis* (Spizizen, 1958), the phenomenon has been described only in closely related species [*B. licheniformis* and *B. amyloliquefaciens* (Thorne and Stull, 1966; Koumoutsi *et al.*, 2004)] within *Bacillaceae*. In contrast, competence development has not been described for *B. cereus* or any of its close relatives, such as *B. anthracis*. The growing number of available genome sequences indicates the presence of several genes capable of coding for such a DNA uptake apparatus in these species. The presence of these orthologous genes raises the question whether these species can become competent.

To address this question, we tested natural DNA uptake in *B. cereus* ATCC14579 harbouring an inducible *comK*_{BSU} gene, using either genomic or plasmid DNA containing an erythromycin-resistant marker. We showed the occurrence of a low level of transformation (one to six colonies per microgram of DNA) under the given condition. Considering the low efficiency, it is still needed to find the best environmental conditions for obtaining enhanced rates of DNA uptake. The presence of this low transformation frequency can be explained in several ways. Although many homologues for competence-related genes are found in *B. cereus* ATCC14579, some genes are lacking or could be non-functional. Two striking examples are the absence of the *comF_B* gene in the *comF* operon and the missing homology between the ComG_{EF_G} proteins of *B. cereus* and *B. subtilis*, expressed from the *comG* locus (Table S1). However, the *comF_B* gene is also missing in *Streptococcus pneumoniae*, another example of a natural

competent Gram-positive bacterium (Berge *et al.*, 2002). Interestingly, there are three non-homologous genes present in the *comG* gene cluster of *B. cereus* at the analogous position as *comGE*, *comGF* and *comGG* in *B. subtilis* that could be the functional complements of these *B. subtilis* genes. Another possible explanation for the relatively low transformation efficiency might be related to a decreased recombination efficiency or the result of a low level of induction of recombination genes.

In spite of the presumably homogeneous overexpression of ComK_{BSU}, the transcription initiation from the P*comGA_{Bce}* region occurs in a low, bistable manner (Fig. 2 and 3). Bistable expression of genes in bacteria depends on a positive or double negative regulatory cascade (Smits *et al.*, 2006; Dubnau and Losick, 2006). In *B. subtilis* only 10–20% of the cells of a given culture express the late-competence genes (Nester *et al.*, 1963; Hadden and Nester, 1968). This bistable outcome requires positive autoregulation at the P*comK*, and the basal level of ComK_{BSU} determines the fraction of cells that develop competence (Maamar and Dubnau, 2005; Smits *et al.*, 2005). The fraction of *B. subtilis* cells that develop competence is increased if the level of ComK_{BSU} is enhanced (e.g. by inducing *comK*_{BSU} expression from a heterologous promoter or by inactivating MecA involved in proteolytic degradation of ComK_{BSU}) (Smits *et al.*, 2005). A bistable outcome of P*comGA_{Bce}* upon monomodal overexpression of ComK_{BSU} suggests that one or more regulators of *B. cereus* are also involved in the expression of *comGA_{Bce}*. It is interesting to note that expression from P*comGA_{Bce}* was not enhanced by overexpressed ComK_{BSU} in rich medium (Fig. S2), suggesting that medium-dependent factors are also affecting this heterogeneous regulation of competence genes. In *B. cereus* ATCC14579 two *comK* genes could be identified based on the homology to ComK_{BSU} sequence. The roles of these proteins are unknown in *B. cereus*, although this is not the only example of doubled regulators in the *B. cereus* group. *Bacillus cereus* G9241 was shown to contain two copies of the *atxA* gene, the regulator shown to be involved in pathogenicity of *B. cereus* (Hoffmaster *et al.*, 2004).

Electrophoretic mobility shift assay experiments demonstrated binding of ComK_{BSU} to the promoter regions of two late-competence genes (*comGA_{Bce}* and *comEA_{Bce}*), but also to P*comK1* and P*comK2*. The overexpressed ComK_{BSU} might bind and modulate the expression of *comK1* and *comK2* genes in *B. cereus*, thereby promoting the occurrence of a bistable expression of competence genes. Thus the ComK1 and/or ComK2 proteins might play a role in this regulation. As ComK_{BSU} binds to P*comGA_{Bce}* and P*comEA_{Bce}* regions, the effect of ComK_{BSU} might be independent of the *B. cereus* *comK* genes. The investigation of the ComK1 and ComK2 functions in the transcriptional regulation of late-

Table 2. Bacterial strains and plasmids.

Strains/plasmids	Relevant characteristics	Reference
Strains		
ATCC14579	Type strain	Bacillus Genetic Stock Center
FM1400	<i>sigB::ery</i>	van Schaik <i>et al.</i> (2004)
MG1363	<i>Lac⁻ Prt⁻</i> ; plasmid-free derivative of NCDO712	Gasson (1983)
MC1061	<i>F- araD139 Δ(ara-leu) 7696 galE15 galK16 Δ(lac)X74 rpsL (Strr) hsdR2 (rK₋ mK₊) mcrA mcrB1</i>	Wertman <i>et al.</i> (1986)
Plasmids		
pSG1151	<i>amp^r, cat</i>	Lewis and Marston (1999)
pL253	<i>ermAM, ery^r</i>	Simon and Chopin (1988)
pNW33N		Genetic Stock Center
pNWcomK _{Bsu}	pNW33N containing <i>comK_{Bsu}</i>	This study
pSC1151-comGA	pSG1151 containing <i>PcomGA</i> fused to <i>gfp</i>	This study
pLcomGA-gfp	pL253 containing <i>PcomGA-gfp</i>	This study

competence genes is the subject of future studies in our laboratory. The intent of our current study was to show the presence of a functional DNA uptake machinery in *B. cereus* ATCC14579.

The induction of competence in *B. cereus* resulted in uptake and homologous recombination of genomic DNA. The place of recombination was verified both with molecular biology techniques (e.g. PCR) and by phenotypic characterization (e.g. the effect of *sigB* mutation on heat resistance). This indicates that the recombination apparatus was also functional under these conditions. Thus, our natural transformation method should help us to introduce mutations into the genome or plasmids of *B. cereus* ATCC14579 using linear PCR fragments containing homologous fragments and resistance genes, i.e. the method described as allelic replacement mutagenesis using long-flanking-homology PCR (Wach, 1996). This biotechnologically important and convenient method would facilitate a rapid mutagenesis of target genes and by-pass the time-consuming steps of current methods using selection of single and subsequent double-recombination events and electroporation.

In addition to conjugation and phage transduction, the observed natural transformation can also provide an explanation for the naturally occurring genome plasticity observed in *B. cereus* and related species such as *B. anthracis* and *B. thuringiensis*. Recent studies indicate that these pathogens have adapted to various environments by foreign insertion of DNA through homologous recombination (Ivanova *et al.*, 2003; Read *et al.*, 2003; Rasko *et al.*, 2004).

In this report, we have shown that a functional DNA uptake apparatus exists in *B. cereus* ATCC14579, which will further enhance *B. cereus* research. Our work presents a useful tool for up to now non-transformable *Bacilli*. Establishing a competence regime for a wider range of *Bacillus* species will greatly enhance the genetic accessibility, and thus research speed, for medically and industrially relevant *Bacilli*.

Experimental procedures

Bacterial strains and media

The strains and plasmids used in this study are listed in Table 2. *Bacillus cereus* strains were grown in TY (1% Bacto-Tryptone, 0.5% Bacto-yeast extract and 1% NaCl) or in MM [62 mM K₂HPO₄, 44 mM KH₂PO₄, 15 mM (NH₄)₂SO₄, 5.6 mM sodium citrate, 0.8 mM MgSO₄, 0.02% of casamino acids, 27.8 mM glucose and 0.1 mM L-tryptophan (Leskela *et al.*, 1996)] at 30°C, supplemented with appropriate antibiotics, erythromycin (5 µg ml⁻¹) or chloramphenicol (3–5 µg ml⁻¹). *Escherichia coli* strains were grown at 37°C in TY, supplemented with chloramphenicol (15 µg ml⁻¹) or erythromycin (150 µg ml⁻¹). *Lactococcus lactis* strains were grown in twofold-diluted M17 broth (Terzaghi and Sandine, 1975) supplemented with 0.5% glucose (GM17) at 30°C and erythromycin (5 µg ml⁻¹).

Construction of pLcomGA-gfp vector

The *comGA* promoter region including the ribosome bind site was amplified by PCR using primers comGA-EcoRI-fw (primer sequences are available in Table S2) and comGA-ApaI-rw. The PCR product was digested with EcoRI and ApaI ligated into the corresponding sites of pSG1151 (Terzaghi and Sandine, 1975; Lewis and Marston, 1999), resulting pSG1151-PcomGA. This plasmid was used as a template to amplify out PcomGA-gfp by PCR using primers oAM17 and oAM18. The resulting 1391 bp PCR fragment was digested with XbaI and Eco47III, and inserted to into XbaI-SmaI cleaved pL253 (Simon and Chopin, 1988).

The resulting plasmid, pLcomGA-gfp, was introduced into *L. lactis* MG1363 by electroporation (Holo and Nes, 1995). Correct construct was confirmed by sequencing. Subsequently, plasmid pLcomGA-gfp was introduced by electroporation (Bone and Ellar, 1989) into *B. cereus* and *B. cereus* pNWcomK_{Bsu}.

Construction of pNWcomK_{Bsu}

After amplification of *B. subtilis* *comK* gene with pDRK-F and pDRK-R, the 746 bp PCR fragment was digested with HindIII and SphI and cloned into the corresponding sites of pDR111

(kind gift of David Rudner), yielding pDRKwt. The sequence of the insert was verified with digestion and sequencing. The 2506 bp BamHI and PstI fragment of pDRKwt was inserted to the adequate sites of pNW33N (GenBank Accession No. AY237122), resulting pNWcomK_{Bsu}. The verified construct along with pNW33N as control was electroporated into *B. cereus* ATCC14579.

Natural competence in *B. cereus* ATCC14579

Bacillus cereus ATCC14579 strains carrying either pNW33N or pNWcomK_{Bsu} were grown overnight in TY supplemented with chloramphenicol (5 µg ml⁻¹). For IPTG-mediated induction of ComK_{Bsu} expression a fresh overnight culture of strains was inoculated into fresh minimal medium, supplemented with chloramphenicol (3 µg ml⁻¹) with an OD₆₀₀ of 0.15. When cultures achieved an OD₆₀₀ of 0.75, they were induced by 1 mM IPTG. Cultures were grown further for 3.5 h before 1.5 µg of genomic or plasmid DNA was added. Genomic DNA was extracted from *B. cereus* FM1400 with the Sigma-Aldrich GenElute™ Bacterial Genomic DNA Kit and contains no detectable spores. Plasmid DNA was isolated from *L. lactis* with the Roche High Pure Plasmid Isolation Kit. It contains mono-, di- or multimer forms of plasmid DNA as it is active in transformation of *B. subtilis* natural competence cells that requires di- or multimeric forms of plasmid. Cells were incubated at 30°C with shaking for 30 min before TY medium was added, and incubation was continued for a subsequent hour. Cells were plated out onto TY agar plate (1.5% agar) supplemented with erythromycin (2.5 µg ml⁻¹). The presence of chloramphenicol resistance gene in transformants was proved by PCR using CmForw and CmRev; the presence of erythromycin was shown in the FM4100-transformed strain using Ery2-F and Ery2-R; in pNG4048E-transformed strain using Ery-F and Ery-R; the deletion in *sigB* was confirmed by PCR using primers SigB-down-R and SigB-up-F.

Flow cytometric analyses

Cultures were grown similar to the conditions described above except that erythromycin was added (5 µg ml⁻¹ in overnight cultures and 2.5 µg ml⁻¹ in minimal medium). After induction with 1 mM IPTG at OD₆₀₀ of 0.75, samples were taken every hour.

Cells were diluted in minimal salts and analysed on Coulter Epics XL-MCL flow cytometer (Beckman Coulter Mijdrecht, NL) operating an argon laser at 488 nm. GFP signals were collected through an FITC filter with the photomultiplier voltage set between 700 and 800 V. Data were obtained using EXPO32 software (Beckman Coulter) and further analysed using WinMDI 2.8 (<http://facs.scripps.edu/software.html>). Figures were prepared using WinMDI 2.8 and Corel Graphics Suite 12.

Fluorescence microscopy

The fluorescence of the GFP reporter protein was visualized with a Zeiss Axiophot microscope, using appropriate filters. Imaging of pLcomGA-gfp in individual cells using fluores-

cence microscopy was performed as described by Smits and colleagues (2005).

Western blotting and protein detection

SDS-PAGE and Western blotting of cell lysates was performed as described (Bolhuis *et al.*, 1999). Chemiluminescent detection of bound ComK-specific antibodies (Kong and Dubnau, 1994) was performed with horseradish peroxidase-conjugated anti-rabbit IgG and the ECL Western blotting analysis system (Amersham).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays were carried out essentially as described by Susanna and colleagues (2004). The promoter regions of *B. cereus* competence genes, *comK1*, *comK2*, *comEA* and *comGA*, were obtained by PCR, using primers comK1-F and comK1-R for *PcomK1*; primers comK2-F and comK2-R for *PcomK2*; primers comGA-F and comGA-R for *PcomGA*; primers comEA-F and comEA-R for *PcomEA*. As a template chromosomal DNA of *B. cereus* ATCC14579 was used. The resulting fragments were end-labelled with T4 polynucleotide kinase and [γ -³³P]-ATP. Purified *B. subtilis* ComK protein and probe were premixed on ice in binding buffer (80 mM Tris HCl pH 8, 4 mM EDTA, 20 mM MgCl₂, 2 mM DTT, 400 mM KCl, 40% glycerol) containing 0.5 µg µl⁻¹ poly(dI-dC) as non-specific DNA and 2 µg µl⁻¹ BSA. Samples were incubated at 30°C, and were loaded on 6% polyacrylamide gel after 20 min of incubation. Gels were run in 1× TBE buffer (0,089 mM Tris, 0,089 mM Boric Acid, 0,022 mM EDTA) at 90 V for 60 min, dried in a vacuum dryer and autoradiographed using phosphoscreens and a Cyclone PhosphorImager (Packard Instruments, Meriden, CT).

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Sequence of the upstream region of *B. cereus comGA*. The start codon of the BC3940 (located upstream of *comGA*) and *comGA* (BC4239) genes are highlighted in bold. The protein sequence of ComGA is given above the DNA sequence. The location of GFP fusion in pLcomGA-gfp is indicated in the figure. Putative –35 and –10 sequences are underlined, while putative AT-boxes with 18 bp spacing are boxed.

Fig. S2. Single cell analysis of PcomGA_{Bce}-gfp in liquid rich medium. Samples were taken 3 h after the induction point at OD₆₀₀ of 0.75. Strains carrying PcomGA_{Bce}-gfp fusion were grown in liquid TY, supplemented with chloramphenicol (2.5 µl ml⁻¹) and erythromycin (2.5 µl ml⁻¹). Samples were prepared for flow cytometric analysis as described in *Experimental procedures*. The numbers of cells are indicated on the y-axis, and their relative fluorescence levels are indicated on the x-axis in log scale.

Table S1. *Bacillus cereus* homologues of *B. subtilis* proteins involved in DNA uptake and recombination during natural competence. E-values represent values resulted in BLAST analysis using the *B. subtilis* proteins against the protein database of *B. cereus* ATCC14579.

Table S2. Oligonucleotide primers used in this study.

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