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Published in:
Microbial Biotechnology

DOI:
10.1111/j.1751-7915.2008.00023.x

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2008

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Mironczuk, A. M., Kovács, Á. T., & Kuipers, O. P. (2008). Induction of natural competence in Bacillus cereus ATCC14579. Microbial Biotechnology, 1(3), 226-235. https://doi.org/10.1111/j.1751-7915.2008.00023.x

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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; Maamor 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).
Within the *Bacillus* family natural DNA uptake has been described for *B. subtilis*, *Bacillus licheniformis* and *Bacillus amyloliquefaciens* (Spizizen, 1958; Thorne and Stull 1966; Koumoutsi 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-born 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*

Competition 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 pNWcomKBsu 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 ComKBsu-specific antibodies (see Experimental procedures). The arrow indicates ComKBsu-specific signal.](image)

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 *comKBsu*) was cloned behind the Phyper-sparck promoter that can be induced by isopropyl-β-D-thiogalactoside (IPTG) addition. The resulting plasmid pNWcomKBsu (or the empty plasmid pNW33N used as a control) was introduced by electroporation into *B. cereus* ATCC14579. To verify the overexpression of ComKBsu following IPTG addition, Western blot analysis was performed using ComKBsu-specific antibodies (Fig. 1). The most favourable condition was selected, where ComK protein production was most effectively induced. Cells

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collected at two different time points, 2 or 3 h after induction, showed high expression of ComK<sub>BSu</sub> in the induced <i>B. cereus</i> strain containing pNWcomK<sub>BSu</sub> 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<sub>BSu</sub> can be achieved in <i>B. cereus</i>.

The <i>B. subtilis</i> ComK protein can induce transcription from PcomGA of <i>B. cereus</i>

As comG<sub>4</sub> has been shown to be a suitable competence-specific reporter (Smits <i>et al.</i>, 2005) and transcription of this gene is under strict control of ComK in <i>B. subtilis</i>, we constructed a fusion between the promoter region of <i>B. cereus</i> comGA (PcomGA<sub>Bce</sub>) 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 <i>B. cereus</i> comG<sub>4</sub> was used to determine the effect of ComK<sub>BSu</sub> 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 <i>et al.</i>, 2005). This construct was introduced into <i>B. cereus</i> by electroporation. Also the pIL253 control plasmid was introduced into <i>B. cereus</i>. Both fluorescence microscopy and flow cytometry were used to visualize the expression of GFP from the PcomGA<sub>Bce</sub> in individual cells.

Experiments were performed with strains harbouring either pNWcomK<sub>BSu</sub> or the control pNW33N plasmid, both carrying the pLcomGA–gfp vector to determine whether the expression system used could result in the high ComK<sub>BSu</sub> level that is probably required for activation of the <i>B. cereus</i> 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<sub>Bce</sub> in IPTG induced <i>B. cereus</i> samples carrying the pNWcomK<sub>BSu</sub> 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 cytometry 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 <i>B. cereus</i> 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<sub>BSu</sub> either induced or not induced, the GFP signal was enhanced (3.4 ± 0.6 and 12.3 ± 1.0 AU in
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 pNWcomKBsu strain, is most likely originating from the low basal expression of comKBsu gene from the Phyperspank 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 ± 2.5% of the culture).

Our results show that high levels of ComKBsu 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.

ComKBsu 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 comGAbsu promoter is solely dependent on ComK (Susanna et al., 2004). Here, electrophoretic mobility shift assays (EMSAs) were performed to determine whether purified ComKBsu protein can bind to the comGA and comE promoter regions of B. cereus (hereafter referred to as PcomGABce and PcomEABce). The results from the gel shift analyses showed that ComKBsu can bind in vitro to both promoter fragments, leading to reduced mobility of the probes (Fig. 4A). These data confirm that ComKBsu may activate transcription at the PcomGABce promoter region by
directly binding to an operator region. Reaction mixtures contained poly(dI-dC) that is known to eliminate non-specific DNA binding of ComK<sub>Bsu</sub> (Hamoen <i>et al.</i>, 1998).

Analysis of the P<sub>comGABce</sub> sequence showed the presence of a putative ComK binding site upstream of the putative –35 and –10 promoter elements of comG<sub>ABce</sub> (Fig. S1).

Subsequently, we performed EMSA analysis with the comK<sub>1</sub> and comK<sub>2</sub> promoter regions to determine whether ComK<sub>Bsu</sub> could also affect the expression of these genes. In both cases, DNA binding was observed by ComK<sub>Bsu</sub> (Fig. 4B), showing it might also directly affect comK<sub>1</sub> or comK<sub>2</sub> 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<sub>Bsu</sub> can activate PcomGA–gfp expression in B. cereus ATCC14579. Having constructed an inducible ComK<sub>Bsu</sub> 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<sub>Bsu</sub> 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 (OD<sub>600</sub>) of 0.7, they were induced with 1 mM IPTG. Either genomic DNA of B. cereus FM1400 (van Schaik <i>et al.</i>, 2004) containing a sigB null mutation carrying an erythromycin cassette on the chromosome or the replicative plasmid pNG8048E (Kuipers <i>et al.</i>, 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<sup>–1</sup> erythromycin. Colony counts are presented in Table 1. Although the transformation efficiency was relatively low, only the B. cereus strain containing the inducible comK<sub>Bsu</sub> could act as a recipient of plasmid or genomic DNA, being stably maintained or integrated respectively. In all transformants the presence of

![Fig. 4. Bacillus subtilis ComK binds to both B. cereus late-competence genes promoters PcomGA<sub>ABce</sub> and PcomE<sub>ABce</sub> (A), and the promoters of comK<sub>1</sub> and comK<sub>2</sub> genes (B). Assessment of binding of ComK<sub>Bsu</sub> was performed as described in Experimental procedures. DNA fragments from the indicated promoter regions were prepared by PCR and end-labelled with <sup>32</sup>P. In the panel, the lanes marked with an X correspond to probe alone. The remaining lanes correspond to probe samples incubated with ComK<sub>Bsu</sub> 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<sub>Bsu</sub>.](image)

| Strain                  | Induction with 1 mM IPTG | pNG8048e | FM1400 |
|------------------------|-------------------------|----------|--------|
| Bacillus cereus pNWcomK<sub>Bsu</sub> + | 3 | 1 | 3 | 9 | 1 | 3 |
| Bacillus cereus pNWcomK<sub>Bsu</sub> – | 0 | 0 | 0 | 0 | 0 | 0 |
| Bacillus cereus pNW33N + | 0 | 0 | 0 | 0 | 0 | 0 |
| Bacillus cereus 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 plasmid. 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; Kourmoutsi 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_{G} gene in the comF operon and the missing homology between the ComGEFG proteins of B. cereus and B. subtilis, expressed from the comG locus (Table S1). However, the comF_{G} 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 PcomG_{Bsu} 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 PcomK, 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 PcomGA_{Bsu} upon monomodal overexpression of ComK_{Bsu} suggests that one or more regulators of B. cereus are also involved in the expression of comGA_{Bsu}. It is interesting to note that expression from PcomGA_{Bsu} 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_{Bsu} and comEA_{Bsu}), but also to PcomK1 and PcomK2. 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 PcomGA_{Bsu} and PcomEA_{Bsu} 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-
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 \textit{B. cereus} ATCC14579.

The induction of competence in \textit{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 \textit{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 \textit{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 \textit{B. cereus} and related species such as \textit{B. anthracis} and \textit{B. thuringiensis}. Recent studies indicate that these pathogens have adapted to various environments by foreign insertion of DNA through homologous recombination (Ivanova \textit{et al.}, 2003; Read \textit{et al.}, 2003; Rasko \textit{et al.}, 2004).

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


table

| Strains/plasmids | Relevant characteristics | Reference |
|------------------|--------------------------|-----------|
| Strains          |                          |           |
| ATCC14579        | Type strain              | Bacillus Genetic Stock Center |
| FM1400           | sigB::ery                | van Schaik \textit{et al.} (2004) |
| MG1363           | Lac:: Prt; plasmid-free derivative of NCDO712 | Gasson (1983) |
| MC1061           | F- araD139 Δ(ara-leu) 7696 galE15 galK16 Δ(lac)X74 rpsL (Strr) | Wertman \textit{et al.} (1986) |
| Plasmids         |                          |           |
| pSG1151          | amp’, cat                | Lewis and Marston (1999) |
| pil23            | ermAM, ery’             | Simon and Chopin (1988) |
| pNW33N           | pNW33N containing comK\textsubscript{Bsu} | Genetic Stock Center |
| pSC1151-comGA    | pSG1151 containing PcomGA fused to \textit{gfp} | This study |
| pilcomGA-gfp     | pilIL253 containing PcomGA-gfp | This study |

**Experimental procedures**

**Bacterial strains and media**

The strains and plasmids used in this study are listed in Table 2. \textit{Bacillus cereus} strains were grown in TY (1% Bacto-Tryptone, 0.5% Bacto-yeast extract and 1% NaCl) or in MM [82 mM K$_2$HPO$_4$, 44 mM KH$_2$PO$_4$, 15 mM (NH$_4$)$_2$SO$_4$, 5.6 mM sodium citrate, 0.8 mM MgSO$_4$, 0.02% of casamino acids, 27.8 mM glucose and 0.1 mM L-trptophan (Leskela \textit{et al.}, 1996)] at 30°C, supplemented with appropriate antibiotics, erythromycin (5 \mu g ml$^{-1}$) or chloramphenicol (3–5 \mu g ml$^{-1}$). \textit{Escherichia coli} strains were grown at 37°C in TY, supplemented with chloramphenicol (15 \mu g ml$^{-1}$) or erythromycin (150 \mu g ml$^{-1}$). \textit{Lactococcus lactis} strains were grown in two-fold-diluted M17 broth (Terzaghi and Sandine, 1975) supplemented with 0.5% glucose (GM17) at 30°C and erythromycin (5 \mu g ml$^{-1}$).

**Construction of pilcomGA-gfp vector**

The \textit{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-Apal-rw. The PCR product was digested with EcoRI and Apal 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 Xbal and Eco47III, and inserted into XbaI–SmaI cleaved pIL253 (Simon and Chopin, 1988).

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

**Construction of pNWcomK\textsubscript{Bsu}**

After amplification of \textit{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.
Natural competence in B. cereus ATCC14579

Bacillus cereus ATCC14579 strains carrying either pNW33N or pNWcomK\textsubscript{\textit{Bsu}} were grown overnight in TY supplemented with chloramphenicol (5 \(\mu\)g ml\(^{-1}\)). For IPTG-mediated induction of ComK\textsubscript{\textit{Bsu}} expression a fresh overnight culture of strains was inoculated into fresh minimal medium, supplemented with chloramphenicol (3 \(\mu\)g ml\(^{-1}\)) with an OD\textsubscript{600} of 0.15. When cultures achieved an OD\textsubscript{600} of 0.75, they were induced by 1 mM IPTG. Cultures were grown further for 3.5 h before 1.5 mM IPTG was added. Genomic DNA was extracted from B. cereus \textit{ATCC14579} with the Sigma-Aldrich GenElute\textsuperscript{TM} Bacterial Genomic DNA Kit and contains no detectable spores. Plasmid DNA was isolated from \textit{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 \textit{B. subtilis} natural competence cells that requires di- or multimeric forms of plasmids. Cells were inoculated 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 \(\mu\)g ml\(^{-1}\)). The presence of chloramphenicol resistance gene in transformants was proved by PCR using primers SigB-down-R and SigB-up-F. The deletion in \textit{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 \(\mu\)g ml\(^{-1}\)) in overnight cultures and 2.5 \(\mu\)g ml\(^{-1}\) in minimal medium. After induction with 1 mM IPTG at OD\textsubscript{600} 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.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays were carried out essentially as described by Susanna and colleagues (2004). The promoter regions of \textit{B. cereus} competence genes, \textit{comK1}, \textit{comK2}, \textit{comEA} and \textit{comGA}, were obtained by PCR, using primers \textit{comK1}-\textit{F} and \textit{comK1}-\textit{R} for \textit{PcomK1}; primers \textit{comK2}-\textit{F} and \textit{comK2}-\textit{R} for \textit{PcomK2}; primers \textit{comGA}-\textit{F} and \textit{comGA}-\textit{R} for \textit{PcomGA}; primers \textit{comEA}-\textit{F} and \textit{comEA}-\textit{R} for \textit{PcomEA}. As a template chromosomal DNA of \textit{B. cereus} ATCC14579 was used. The resulting fragments were gel-purified with T4 polynucleotide kinase and \(\gamma\)\textsuperscript{32}P-\textit{ATP}. Purified \textit{B. subtilis} ComK protein and probe were premixed on ice in binding buffer (80 mM Tris HCl pH 8, 4 mM EDTA, 20 mM MgCl\(_2\), 2 mM DTT, 400 mM KCl, 40% glycerol) containing 0.5 \(\mu\)g ml\(^{-1}\) poly(dI-dC) as non-specific DNA and 2 \(\mu\)g ml\(^{-1}\) BSA. Samples were incubated at 30°C, and were loaded on 6% polyacrylamide gel after 20 min of incubation. The strips were visualized using a PhosphorImager (Packard Instruments, Meriden, CT).

Acknowledgements

We thank Wiep Klaas Smits for constructing the pDRKwt plasmid, Tjakko Abee for the gift of \textit{B. cereus} FM1400, David Rudner for plasmid pDR111 and Kim Susanna for purified ComK protein.

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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 pILcomGA–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 PcomGABce–gfp in liquid rich medium. Samples were taken 3 h after the induction point at OD600 of 0.75. Strains carrying PcomGABce–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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