Distinct Roles of ComK1 and ComK2 in Gene Regulation in *Bacillus cereus*

Aleksandra M. Mironczuk1, Amagoia Mañu1, Oscar P. Kuipers1,2*, Ákos T. Kovács1

1 Molecular Genetics Group, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, The Netherlands, 2 Kluyver Centre for Genomics of Industrial Fermentation, Groningen, The Netherlands

Abstract

The *B. subtilis* transcriptional factor ComK regulates a set of genes coding for DNA uptake from the environment and for its integration into the genome. In previous work we showed that *Bacillus cereus* expressing the *B. subtilis* ComK protein is able to take up DNA and integrate it into its own genome. To extend our knowledge on the effect of *B. subtilis* ComK overexpression in *B. cereus* we first determined which genes are significantly altered. Transcriptome analysis showed that only part of the competence gene cluster is significantly upregulated. Two ComK homologues can be identified in *B. cereus* that differ in their respective homologies to other ComK proteins. ComK1 is most similar, while ComK2 lacks the C-terminal region previously shown to be important for transcription activation by *B. subtilis* ComK. *comK1* and *comK2* overexpression and deletion studies using transcriptionics techniques showed that ComK1 enhances and ComK2 decreases expression of the *comG* operon, when *B. subtilis* ComK was overexpressed simultaneously.

Introduction

*Bacillus cereus* is a foodborne pathogenic bacterium and a common contaminant of food and dairy products. This gram-positive, spore-forming bacterium is an agent of two types of foodborne diseases, the emetic and the diarrheal forms. The most important virulence factors are heat-stable emetic toxins and enterotoxins. Symptoms are commonly mild and self-limiting, from diarrhea to vomiting [1]. *B. cereus* can also cause severe infections, especially in immunocompromised patients [2]. To survive in changing environments and under stress conditions, bacteria evolved adaptive networks related to e.g. biofilm formation, spore formation or competence development. Competence is defined as a physiological state of bacteria in which exogenous DNA can be incorporated leading to a genetic transformation event. 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 clear homologues for the ComGE, ComGF, ComGG proteins [3,4], although the presence of functional homologues has been suggested [5]. Interestingly, under laboratory conditions only a fraction of cells become competent, ranging between 10–20% of the population in the model organism *B. subtilis* [6]. Competence for genetic transformation in *B. subtilis* is elaborately regulated, and pivotal to this process is the level of the ComK protein. This protein is an activator of the so called ComK-regulon that also comprises all late-competence genes that are required for transformation [7,8]. ComK activity is controlled by multiple mechanisms, such as quorum sensing, proteolytic degradation by the MecA/ClpCP complex, and transcriptional control by multiple transcription factors [9–11]. Premature transcription of *comK* is prevented by three different repressors: AbrB, CodY and Rok, which all bind to the *comK* promoter region. In addition, during the exponential growth phase, the small amount of ComK that is produced, is trapped by MecA, which targets it for proteolytic degradation by the ClpCP proteasome complex [11,12]. When the competence quorum sensing mechanism is activated at the end of exponential growth, the small protein ComS is produced and liberates ComK from the MecA complex [9,13]. Subsequently, ComK activates a number of promoters including its own [14,15]. Among the known ComK targets are genes involved in the DNA uptake machinery [16].

In *B. cereus* ATCC 14579 two homologues of ComK (BC1134, hereafter referred to as ComK1; BC5250 as ComK2) could be identified [5]. Notably, *in silico* analysis demonstrated that these two putative ComK proteins have a different level of homology to *B. subtilis* ComK, i.e. ComK1 61%, whereas ComK2 shows only 44%. The ComK1 protein is similar in length to the *B. subtilis* ComK protein, while the ComK2 protein of *B. cereus* appears to be C-terminally truncated by 22 aminoacids. Taken together, this data suggests that the regulation of gene expression by ComK proteins in *B. cereus* differs significantly from that known in *B. subtilis*.

In a recent study we reported that a minimal system for functional DNA uptake exists in *B. cereus* [17]. We introduced the *B. subtilis* comK gene (hereafter referred to as comK*{	extsubscript{comK}}*) under the IPTG-inducible hyper-spank promoter (pNWcomKBsu) into the *B. cereus* ATCC 14579 strain. After induction of comK*{	extsubscript{comK}}* cells grown
in minimal medium displayed modest functional DNA uptake, as genomic DNA or plasmid DNA was shown to be taken up by the cells. This DNA uptake is less efficient compared to B. subtilis and B. licheniformis, where overexpression of their own comK is resulted in highly efficient genetic competence [10,19].

Here, we investigate the effect of the overexpression of various comK genes in B. cereus. Our study reveals that upon overexpression of comK$_{com}$ in B. cereus only part of the late competence gene cluster is upregulated. Further, we explore the roles of ComK1 and ComK2 in B. cereus. While we observe no genes to be differentially expressed connected to competence when either comk1 or comk2 are overexpressed, interestingly, ComK1 and ComK2 influence comG$\alpha$ expression under comK$_{com}$ inducing conditions.

Results

Transcriptome changes in B. cereus by overexpression of comK$_{com}$

We previously showed that B. cereus contains a minimal functional DNA uptake apparatus and is able to integrate exogenous DNA into its own chromosome [17]. Under these conditions the late competence gene comG$\alpha$ was shown to be induced upon comK$_{com}$ overexpression. We investigated the impact of comK$_{com}$ overexpression using transcriptomics. DNA microarray analysis was used to compare the transcriptional profiles of B. cereus ATCC 14579 containing plasmid pNW33N (empty vector) with those of B. cereus ATCC 14579 containing plasmid pNWcomK$_{com}$ (comK$_{com}$ overexpression) grown in MM medium. Three independent cultures were used for both the control and the target strains in this experiment. Samples were taken for transcriptome analyses at 3.5 hours after IPTG-induction. To verify the occurrence of DNA uptake in this experiment, genomic DNA was added to the cells, as described before [17]. The transformation was monitored by plating cells on TY plates containing 2.5 µg mL$^{-1}$ erythromycin. The transformation efficiency of the comK$_{com}$ overexpression strain was comparable to that found in previous experiments (5–9 samples compared to the control strain). In addition, a stimulatory effect was observed for transcripts of the cytosolic proteins Smf, YwpH and RadC. Smf is required in Streptococcus pneumoniae YwpH and RadC. Smf is required in effector was observed for transcripts of the cytosolic proteins Smf, comEA shown elevated expression levels similar to the genes of the other com operon (Table 1). The expression of comEA was followed using promoter-gfp constructs. The transcription driven from the comEA promoter was not altered in response to overexpression of comK$_{com}$ and was comparable to the wild type cells without reporter constructs (Figure S1). Upon comK$_{com}$ overexpression we also found many genes showing homology with genes unrelated to DNA uptake and recombination e.g. BC4679 (homolog of B. subtilis YcgA putative integral inner membrane protein), BC0497 (homolog of B. subtilis YihF putative nucleotide binding protein) or BC1734 (homolog of B. subtilis YilT putative ABC transporter, ATP-binding protein) (see Table S2). Genes belonging to the selected clusters were grouped into functional classes (Fig 1).

Overrepresented clusters upon overexpression of comK$_{com}$ gene contained the categories of amino acid transport and metabolism, energy production and conversion and defense mechanism.

These microarray data might explain the low efficiency of natural transformation in B. cereus ATCC 14579, because in the absence of e.g. ComEA, ComFA and NucA proteins the efficiency of natural transformation is reduced in B. subtilis, although still possible [23–26].

ComK$_{com}$ was previously shown to bind in vitro to the promoter regions of both comK homologues of B. cereus, comK1 and comK2 [17]. In our microarray experiments, comk1 showed no significant change in expression levels, while expression of comk2 was slightly elevated (i.e. less than 2 fold). Thus, overexpression of comK$_{com}$ results in elevated expression of selected genes coding for DNA uptake and recombination, but not of all genes described to be essential for efficient DNA uptake in B. subtilis.

Transcriptional profiles of comk1 and comk2 overexpression strains

Since functional DNA uptake in B. cereus ATCC 14579 could be induced by overexpressing comK$_{com}$, we addressed the question whether the ComK$_{com}$ homologues ComK1 and ComK2 can induce expression of competence-related genes in B. cereus. Therefore, the comk1 and comk2 genes were separately cloned behind the spoT promoter [27] that can be induced by isopropyl-$\beta$-D-thiogalactopyranoside (IPTG) addition. The resulting plasmids pATK31 and pATK32, containing comk1 and comk2, respectively, were introduced into B. cereus ATCC 14579 by electroporation, and an empty plasmid pLM5 was used as a control. The experiment was performed as described for ComK$_{com}$ overexpression. To test the occurrence of DNA uptake in these experiments, genomic DNA was added to the cells, but no transformants were observed. The analysis of the microarray data showed that comk1 and comk2 are responsible for activation of different sets of genes (see the 20 most up- or down-regulated genes in Table S2 and Table S3). Microarray results validated the overexpression of comk1 and comk2, as the levels of both comk1 and comk2 mRNA were about 140 times enhanced in overexpressed strains for comk1 and comk2. Unexpectedly, we did not find any genes related to DNA uptake or recombination. Upon comk1 overexpression the most differentially expressed genes belonged to the functional categories representing amino acid transport and metabolism (e.g. BC1404, BC3317) and energy production and conversion (Fig 1). Interestingly, upon comk2 overexpression we found mostly transcriptional regulators (e.g. BC4930, BC0938) and hypothetical proteins (e.g. BC5247, BC3399), but the same functional categories were also affected, e.g. amino acid transport and metabolism and energy production and conversion (Fig 1). Interestingly, we observed the upregulation of BC5251 when comk2 was overexpressed in B. cereus. BC5251 codes for a RNA polymerase sigma factor in B. cereus ATCC 14579 and located downstream of comk2 in reverse direction. The upregulation of BC5251 sigma factor in the comk2 overexpression strains was also validated using quantitative RT-PCR experiments on independent samples. The BC5251 expression level was found to be more than 1000 times enhanced in the comk2 overexpression samples compared to that of the wild type strains. Expression of BC5251 showed very weak changes when comK$_{com}$ or comK1 was overexpressed (1.9±0.1 and 2.6±0.1, respectively) compared to...
Table 1. Transcriptional changes of the functional homologues of the *B. subtilis* DNA uptake apparatus in *B. cereus* ATCC15479 upon overexpression of *B. subtilis* ComK.

| Locus tag | *B. subtilis* competence-related protein | Description in SubtilList database | Ratio | Significance (p-value)
|-----------|----------------------------------------|----------------------------------|-------|---------------------|
| BC1134    | ComK (1)                               | Competence transcription factor   | 0.96  | 10^-1              |
| BC5250    | ComK (2)                               | Competence transcription factor   | 1.3   | 10^-5              |
| BC1306    | ComC                                   | Prepilin peptidase                | NA    | NA                 |
| BC4324    | ComEA                                  | Exogenous DNA-binding protein     | 1.3   | 10^-5              |
| BC4323    | ComEB                                  | DNA binding and uptake            | 1.0   | 10^-1              |
| BC4322    | ComEC                                  | DNA binding and uptake            | 1.3   | 10^-3              |
| BC5193    | ComFA                                  | DNA binding and uptake            | 1.15  | 10^-2              |
|           | ComFB                                  | Late competence gene              | -     | -                  |
| BC5192    | ComFC                                  | Late competence gene              | 1.3   | 10^-3              |
| BC4239    | ComGA                                  | Late competence gene              | 39.7  | 10^-15             |
| BC4238    | ComGB                                  | DNA transport machinery           | 29.9  | 10^-16             |
| BC4237    | ComGC                                  | Exogenous DNA-binding             | 39.0  | 10^-13             |
| BC4236    | ComGD                                  | DNA transport machinery           | 17.5  | 10^-6              |
| BC4235    | ComGE                                  | DNA transport machinery           | NA    | NA                 |
| BC4234    | ComGF                                  | DNA transport machinery           | 31.1  | 10^-16             |
| BC4233    | ComGG                                  | DNA transport machinery           | NA    | NA                 |

*The ratio of gene expression is shown. Ratio: expression in the ComK<sub>bsu</sub> overexpressed samples over that in not ComK<sub>bsu</sub> overexpressed samples.
1Bayesian p value.
NA, no data are available (no probe are available for the gene).

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comK2 overexpression. This data suggests that the primary function of ComK1 and ComK2 in *B. cereus* might not be in competence development.

**PcomK1-gfp and PcomK2-gfp expression in wild type *B. cereus* and in comK1 and comK2 mutants**

To get more insight into the effect of *B. cereus* ComK proteins on regulation of their own promoters and in the network between these two genes, we constructed fusions of comK1 and comK2 promoter regions with the gfp gene. The resulting plasmids, pLcomK1-gfp and pLcomK2-gfp, contain in an frame fusion of gfp with the first 6 codons of *B. cereus* comK1 and comK2, respectively, and were used to determine the expression from these genes in the wild type strains under various growth conditions. Using strains grown in minimal medium (MM), a low signal of PcomK1-gfp was detected compared to the wild type strain lacking the gfp construct, using flow cytometry analysis. In contrast, no comK2 expression could be detected. The microarrays data, showed that overexpression of one of the comK genes did not alter the expression level of its paralog. To validate these results, we introduced comK1 (pATK31) or comK2 (pATK32) inducible constructs into strains harboring either pLcomK1-gfp or pLcomK2-gfp. In agreement with the microarray analysis, we did not observe any difference in the gfp expression for any of the promoters in the presence of ComK1 or ComK2 (data not shown).

Subsequently, we examined the effect of comK1 and comK2 mutations on the expression of the comK promoters. We replaced either comK1 or comK2 by a chloramphenicol cassette, resulting in strains ΔcomK1 and ΔcomK2, respectively. First, we introduced pLcomK1-gfp and pLcomK2-gfp into the ΔcomK1 strain and used the wild type *B. cereus* as a control containing the corresponding reporter constructs. While mutation of comK1 did not alter the expression of PcomK1-gfp in comparison to the wild type (Fig 2A), a higher PcomK2-gfp expression was observed in the comK1 mutant background (Fig 2B). This suggests that mutation in comK1 affects only the expression of comK2, directly or indirectly, while it might not be involved in the regulation of its own transcription under the conditions examined.

We noticed that a mutation in the comK2 gene did not alter the expression of PcomK1-gfp (Fig 2C). In contrast, the comK2 mutant harboring pLcomK2-gfp showed increased gfp expression (Fig 2D), indicating that ComK2 might repress its own expression. Thus, while expression of the comK1 gene seems to be independent of the presence or absence of the ComK1 or ComK2 protein, comK2 expression depends on the presence of comK genes, but is not affected by the overexpression of comK1 or comK2 in the wild type cells.

**Expression of either comK1 or comK2 in the presence of ComK<sub>bsu</sub>**

So far, we could only detect enhanced comG expression or low levels of transformation in *B. cereus*, when comK<sub>bsu</sub> was overexpressed [17]. To examine whether ComK1 and/or ComK2 have an influence on this comG inducing effect of comK<sub>bsu</sub> overexpression, we overexpressed either comK1 or comK2 in the presence of ComK<sub>bsu</sub> and monitored the effect on PcomGA-gfp expression. First, we tested the effect of simultaneous comK1 and comK2 expression on PcomGA-gfp transcription. Strains grown in minimal medium were induced with 1 mM IPTG after reaching an OD<sub>600</sub> of 0.75 and samples were taken for flow cytometric analysis every hour after induction. Overexpression of comK1 and comK2 in minimal medium resulted in enhanced PcomGA-gfp expression (Fig 3G and Table 2). It is noteworthy that non-induced samples showed also enhanced GFP levels (Fig 3A and Table 2). Most likely, this is due to the leakiness of the used promoters that was previously also reported [17], resulting in a small amount of protein that might activate PcomGA transcription at a low level.

A similar experiment for comK2 overexpression was performed as described before for comK1. The transcription from PcomGA in
response to coexpression of comK2 and comKBsu was decreased in comparison to single induction of comKBsu (Fig 3D and Table 2), suggesting a repressing role of ComK2. Notably, we could also detect a decreased P\textsubscript{comGA-gfp} expression in noninduced samples (Fig 3B and Table 2).

ComKBsu overexpression in comK1 and comK2 mutants

The results above indicate that comK1 and comK2 might have opposite roles in the ComKBsu induced comG expression in B. cereus. To verify these data, we monitored the effect of the mutations in comK1 and comK2 genes on a P\textsubscript{comGA-gfp} expression in the presence of ComKBsu.

Due to conflict in the applied antibiotic resistance markers of our constructs (i.e. both comK mutants and comKBsu overexpression construct were constructed using cat resistance genes), we constructed a new comKBsu inducible construct (pNW-Km), where the kanamycin cassette was inserted into the chloramphenicol resistance gene. This comKBsu overexpression construct (pNW-Km) showed moderately increased comG expression compared to the original comKBsu construct (pNWcomKBsu) when cultures were not induced with IPTG. Changing the antibiotic resistance gene on the vector could cause differences in copy number or in the transcription activation on the plasmid, resulting in enhanced basal expression from the hyperspank promoter. After obtaining a comK1 mutant strain containing pNW-Km, the P\textsubscript{comGA-gfp} promoter fusion construct (pILcomGA-gfp) was subsequently introduced by electroporation. Strains were grown under the same condition as described before and the cells were induced with...
IPTG. In comK1 mutant strain a lower PcomGA-gfp expression was detected in the comKBsu overexpression samples (Fig 4C and Table 2). This indicates in agreement with the overexpression constructs that ComK1 positively effects the expression of comG operon when comKBsu is overexpressed in B. cereus.

Data on comK2 overexpression suggested a negative effect of ComK2 on the expression of the comG gene in comKBsu overexpressing B. cereus. As previously, the comK2 mutant strain was subsequently transformed with pNWK-Km and pILcomGA-gfp constructs. In the comK2 mutant background comG1 transcrip-

Figure 2. Flow cytometric analysis of PcomK1-gfp (A,C) and PcomK2-gfp (B,D) in liquid minimal medium. Promoter fusions in wild type (black) and in comK1(A,B) or comK2(C,D) mutant (gray). Analyses were performed 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 on a logarithmic scale. For each experiment at least 20,000 cells were analysed. The graphs are the representative of at least three independent experiments.
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Figure 3. Flow cytometric analyses of PcomGA-gfp after overexpression of comKBsu in the wild type containing pNWcomKBsu (black) and in the co-expressed comKBsu and comK7 (pATK31(A,C)) or comK2 (pATK32(B,C)) (gray). The samples were analyzed three hours after IPTG induction; panels A,B- strains without induction, panels C,D- strains after induction with IPTG. 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 each experiment at least 20,000 cells were analysed. The graphs are the representative of at least three independent experiments.
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three independent experiments. Induction, panels C,D- strains after induction with IPTG. The numbers of cells are indicated on the axis on a logarithmic scale. For each experiment at least 20,000 cells were analysed. The graphs are the representative of at least three independent experiments. Values are the geometric mean value of the whole population from the flow cytometric experiments (Figure 3–4) and given in arbitrary units with extracted background auto-fluorescence. Standard deviations are indicated.

| Strains         | PcomGA-gfp | PcomK1-gfp | PcomK2-gfp |
|-----------------|------------|------------|------------|
| ATCC 14579 (WT) | 1.1±0.3    | 3.4±0.7    | 0.9±0.1    |
| ATCC 14579 pNWcomK
| comKBsu | 26.6±1.9 | ND         | ND         |
| ATCC 14579 pNWcomK
| comKBsu, pATK31 | 42.0±3.0 | ND         | ND         |
| ATCC 14579 pNWcomK
| comKBsu, pATH32 | 16.8±2.7 | ND         | ND         |
| ATCC 14579 Δ comK1 | 1.1±0.5   | 2.7±0.8    | 5.0±0.6    |
| ATCC 14579 Δ comK1
| pNWcomK
| comKBsu | 8.2±0.7 | ND         | ND         |
| ATCC 14579 Δ comK2 | 1.1±0.2   | 2.4±0.2    | 6.6±2.7    |
| ATCC 14579 Δ comK2
| pNWcomK
| comKBsu | 33.7±3.4 | ND         | ND         |

Values are the geometric mean value of the whole population from the flow cytometric experiments (Figure 3–4) and given in arbitrary units with extracted background auto-fluorescence. Standard deviations are indicated. doi:10.1371/journal.pone.0021859.t002

Table 2. Expression of the reporter gene (gfp) under different promoters.

Figure 4. Single cell analyses of PcomGA-gfp and in liquid minimal medium. The effect of ComK
| Strains         | PcomGA-gfp | PcomK1-gfp | PcomK2-gfp |
|-----------------|------------|------------|------------|
| ATCC 14579 (WT) | 1.1±0.3    | 3.4±0.7    | 0.9±0.1    |
| ATCC 14579 pNWcomK
| comKBsu | 26.6±1.9 | ND         | ND         |
| ATCC 14579 pNWcomK
| comKBsu, pATK31 | 42.0±3.0 | ND         | ND         |
| ATCC 14579 pNWcomK
| comKBsu, pATH32 | 16.8±2.7 | ND         | ND         |
| ATCC 14579 Δ comK1 | 1.1±0.5   | 2.7±0.8    | 5.0±0.6    |
| ATCC 14579 Δ comK1
| pNWcomK
| comKBsu | 8.2±0.7 | ND         | ND         |
| ATCC 14579 Δ comK2 | 1.1±0.2   | 2.4±0.2    | 6.6±2.7    |
| ATCC 14579 Δ comK2
| pNWcomK
| comKBsu | 33.7±3.4 | ND         | ND         |

Values are the geometric mean value of the whole population from the flow cytometric experiments (Figure 3–4) and given in arbitrary units with extracted background auto-fluorescence. Standard deviations are indicated. doi:10.1371/journal.pone.0021859.t002

Table 2. Expression of the reporter gene (gfp) under different promoters.

Role of ComK Proteins in B. cereus

Discussion

Regulation of DNA uptake and recombination is achieved in various ways in bacteria. Within the Bacillus genus natural competence has been shown to be activated by the transcription factor ComK [5]. So far, only a limited number of Bacillus sp. has been shown to have the ability of reaching high efficient natural competence, such as B. subtilis, B. licheniformis and B. amyloliquefaciens [28–30]. However, only a limited number of strains within a species show this phenotype under laboratory conditions, while in other cases to achieve high transformability the protein level of the ComK transcription factor should be increased by overexpression of its own comk gene [18,19] or by disrupting the degradation of the ComK protein [31]. We have used similar methods previously to show the presence of functional DNA uptake in B. cereus ATCC 14579 [17]. However, we achieved a low-efficient DNA uptake induction only by overexpression of the heterologous comKBsu. In this study we used various molecular methods to follow the effect of different comk species overexpression in B. cereus. We followed the effect of comKBsu, comK1 and comK2 overexpression in B. cereus using microarray techniques and showed that competence-related genes are induced only when the comKBsu was overexpressed. However, only part of the competence-related genes is activated, while comK1 expression was not changed and comK2 showed a slightly increased level of expression in the B. cereus strain containing overexpressed comKBsu. As shown before, ComKBsu binds to the promoter regions of several late competence genes and comK genes of B. cereus [17]. In agreement with flow cytometric analysis the comG operon in the comKBsu overexpression strain was highly up-regulated, while we did not notice significantly enhanced expression of the comE or comF operons. This implies that ComKBsu might not activate transcription of these genes in vivo although ComKBsu was shown to bind to the comE, comK1 and comK2 promoter fragments in vitro [17].
vivo and in vitro observation might originate from the position of the ComK binding site relative to the promoter -35 and -10 sites. Transcription activation by ComK in B. subtilis is helix face dependent as a 6-bp insertion between the ComK box and -35 hexamer of the B. subtilis comG promoter abolished activation of transcription [32]. On the other hand, we could not identify the so-called K-box (ComK binding site) in any of the B. cereus genes coding for the homology of the DNA uptake apparatus. Interestingly, other competence-related genes involved in DNA binding or recombination, like ycdC and yapH, showed an enhanced level of expression when comK was overexpressed. The lack of high induction of the complete set of competence-related genes in the comK overexpressing B. cereus strain might explain the observed low transformability of B. cereus under the conditions used. Previous studies on B. subtilis showed that when not all the genes for the competence machinery are functional, transformation is still possible, though the efficiency is much lower (e.g. ΔcomE [23]).

In contrast to comK overexpression, increasing the level of comK or comK2 alone, activated different sets of genes unrelated to DNA uptake. We noticed altered expression of more than 100 genes by comK overexpression, while more than 300 genes have altered gene expression in the comK2 overexpressing B. cereus, and several of these genes were located in operons. The lack of altered gene expression related to DNA uptake could be the result of overexpression of comK1 and comK2 separately or because the target genes of these regulators are different under the conditions used. It is also possible that ComK1 and ComK2 have other primary functions in B. cereus than modulating the expression of genes related to DNA uptake and recombination. This is also supported by the increased transcription of a σ-factor (BC5251) located adjacent to comK when comK2 was overexpressed in B. cereus. Interestingly, upon overexpression of comK in B. cereus, similar functional categories (e.g. amino acid transport and metabolism, energy production and conversion) were overrepresented (Fig 1), although the list of genes was not overlapping. The connection between regulation of competence related genes and amino acid metabolism is not unprecedented, e.g. B. subtilis CodY which, next to its major function as branched-chain amino acid metabolism regulator, modulates competence development in B. subtilis [33]. Strikingly, although similar functional categories are overrepresented in the microarray experiments, we did not find common genes upregulated upon comK1 and comK2 overexpression, suggesting highly different regulation for these two comK homologues. The relatively high level of mRNA increase in the induced overexpression strains suggest a very low or almost absent expression of comK genes in wild type cells.

The target genes of ComK1 and ComK2 are different in the overexpression strains which shows the divergence of the two ComK proteins in B. cereus. Although both ComK1 and ComK2 show conserved regions homologous to ComK in B. subtilis, the ComK2 protein lacks the 22 amino acids long C-terminal region [5]. Interestingly, deletion of the 25 amino acids C-terminal part disrupted the ability of ComK in B. subtilis to activate transcription on the comG promoter in vivo, but preserved its DNA binding ability [34]. One could hypothesize that ComK2 represses genes, like comG, in its short form, but once was able to activate genes, like comG in an ancient longer form (without deletion at the C-terminus) of the protein, which lost these amino acids during evolution. It will be interesting to investigate the target genes and promoters found in the comK1 and comK2 overexpression studies and perform EMSA experiments to see if the genes identified by transcriptomics are directly or indirectly regulated by the different ComK proteins and to define a DNA binding site for both ComK proteins of B. cereus. However, in this study we concentrated on the ability of ComK1 and ComK2 to modulate the expression of competence-related genes and other genes. Interestingly, we show that overexpression of comK1 or comK2 in the presence of comK resulted in changed activation of comGA::gfp. The simultaneous overexpression of comK1 and comK in B. cereus resulted in enhanced expression from comGA::gfp compared to single induction of comK, while deletion of comK1 reduced the effect of comK on the comGA expression.

In contrast to comK1, the overexpression of comK2 or deletion of comK2 in the presence of comK resulted in reduced or increased comGA expression in B. cereus, respectively.

The overexpression or deletion of comK1 and comK2 genes modulate the ComK transcription, but has no effect on comG expression in the absence of the comK protein. It is therefore possible that B. cereus ATCC 14579, if it is a naturally competent bacterium under specific conditions, has a different regulatory mechanism than that of the model organism B. subtilis. This view is also supported by the observation that the upstream regulatory pathway is less conserved in Bacilli [5].

Taken together, we propose that ComK1 and ComK2 take an opposite role on the modulation of the ComK effect in B. cereus. Future studies should reveal the functions of ComK1 and ComK2, and whether any protein-protein interaction exists between the ComK proteins, how ComK1 and ComK2 proteins activate or repress transcription in vivo and in vitro, and if they compete for DNA binding sites at target promoters.

Methods

Bacterial strains and media

The strains and plasmids used in this study are listed in Table 3. B. cereus strains were grown in TY (10 g L⁻¹ trypton, 5 g L⁻¹ yeast extract, 5 g L⁻¹ NaCl, 0.1 mM MnCl₂) or in minimal medium MM (62 mM KH₂PO₄, 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 growth factors [35]). Growth factors were made by adding tyrosine, tryptophan, methionine, histidine, adenine, uracil (final concentration 20 μg/mL), nicotinic acid and riboflavin (final concentration 0.5 μg/mL) to water. For cloning, Escherichia coli MC1061 and Lactococcus lactis MG1363 were grown in TY and GM17 (37.5 g L⁻¹ M17 broth (Difco), 0.5% glucose) broth medium, respectively. Bacterial strains were grown at 30°C or 37°C, supplemented with appropriate antibiotics, erythromycin (5 μg mL⁻¹), chloramphenicol (3–5 μg mL⁻¹) or kanamycin (30 μg mL⁻¹).

RNA isolation, preparation of labeled cDNA, and hybridization

Cells were grown overnight in 10 mL of TY medium supplemented with chloramphenicol (5 μg mL⁻¹) or kanamycin (30 μg mL⁻¹) for comK or comK1/comK2 overexpression, respectively. Next, the cultures were diluted to an OD₆₀₀ of 0.15 in 25 mL of minimal medium containing appropriate antibiotics. Samples for transcriptome analyses were induced at the exponential-growth phase (OD₆₀₀=0.75) with isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cells were harvested 3 h after induction. Three independent cultures of each strain were used. RNA was isolated from 15 mL of culture, as described previously [36]. RNA was eluted with 60 μL of elution buffer. A total amount of 20 μg RNA was used for the reverse transcription reaction with SuperscriptIII (Gibco BRL). DNA-microarrays containing ampiclons of 5200 annotated genes in the genome of B. cereus ATCC 14579 were designed and produced as described.
previously [37]. Slide spotting, slide treatment after spotting, and slide quality control were performed as described elsewhere. Data were analyzed essentially as described before [38]. Each ORF is represented by duplicate spots on the array. After hybridization, fluorescent signals were quantified with the ArrayPro analyzer, and processed with Micro-Prep [37]. Statistical analysis was performed using CyberT [39]. Genes with a Bayes P-value below 1.0 \times 10^{-2} with at least twofold differential expression were considered to be significantly affected. Microarrays data is MIAME compliant and that the raw data has been deposited in Table 3.

| Strains and plasmids | Relevant characteristics/plasmids | Reference |
|----------------------|-----------------------------------|-----------|
| **E. coli**           |                                   |           |
| HB101                | pRK24; strain for conjugation      | [41]      |
| MC1061               | F- araD139 (ara-leu) 7696 galE15 galK16 (Str)L hasR2 (Km, Mck) mcrA mcrB1 | Laboratory stock |
| **L. lactis**         |                                   |           |
| MG1363               | Lac’ Prt’; plasmid-free derivative of NCDO712 | [45]      |
| **B. cereus**        |                                   |           |
| ATCC 14579           | type strain                       | BGSC      |
| ATCC 14579           | pATK31, pNWcomKmec plLcomGA-gfp    | This study |
| ATCC 14579           | pATK32, pNWcomKmec plLcomGA-gfp    | This study |
| ATCC 14579           | pNWcomKmec plLcomGA-gfp           | This study |
| ATCC 14579 \( \Delta \text{comK1} \) | comK1:: cm                        | This study |
| ATCC 14579 \( \Delta \text{comK1} \) | plLcomK1-gfp                     | This study |
| ATCC 14579 \( \Delta \text{comK1} \) | plLcomK2-gfp                     | This study |
| ATCC 14579 \( \Delta \text{comK1} \) | pNWK-km, plLcomGA-gfp            | This study |
| ATCC 14579 \( \Delta \text{comK1} \) | pNWK-km, plLcomEA-gfp            | This study |
| ATCC 14579 \( \Delta \text{comK2} \) | comK2:: cm                        | This study |
| ATCC 14579 \( \Delta \text{comK2} \) | plLcomK1-gfp                     | This study |
| ATCC 14579 \( \Delta \text{comK2} \) | plLcomK2-gfp                     | This study |
| ATCC 14579 \( \Delta \text{comK2} \) | pNWK-Km, plLcomGA-gfp            | This study |
| ATCC 14579 \( \Delta \text{comK2} \) | pNWK-Km, plLcomEA-gfp            | This study |
| plasmids             |                                   |           |
| pNWcomKmec           | pNW33N containing B. subtilis comK | [17]      |
| pNW33N               | Geobacillus-Bacillus-E. coli shuttle vector, Cm
| pil253               | ermAM, ery'                       | [46]      |
| pSG1151              | ampr, cat                         | [43]      |
| pcomK1-gfp           | pSG1151 containing PcomK1 fused to gfp | This study |
| pcomK2-gfp           | pSG1151 containing PcomK2 fused to gfp | This study |
| pcomEA-gfp           | pSG1151 containing PcomEA fused to gfp | This study |
| pilLcomK1-gfp        | pil253 containing PcomK1-gfp      | This study |
| pilLcomK2-gfp        | pil253 containing PcomK2-gfp      | This study |
| pilLcomEA-gfp        | pil253 containing PcomEA-gfp      | This study |
| pilLcomGA-gfp        | pil253 containing PcomGA-gfp      | [17]      |
| pNWK-km              | pNWcomKmec containing km\( ^n \) cassette | This study |
| pLM5                 | Vector containing spac promoter and lac repressor, km\( ^n \) | [27]      |
| pATK31               | pLM5 containing B. cereus comK1   | This study |
| pATK32               | pLM5 containing B. cereus comK2   | This study |
| pUC19C               | pUC19 vector containing cat\( ^n \) | Laboratory stock |
| pBlueScript SK       | ampr\( ^n \)                       | Stratagene |
| pATAS28              | tra-, conjugative suicide vector for B. cereus group, Spc\( ^n \) | [41]      |
| pBtcomK1             | pBtSK, containing comK1 region with cat\( ^n \) cassette | This study |
| pAT\( \Delta \text{comK1} \) | pATAS28, containing comK1 region with cat\( ^n \) cassette | This study |
| pBtcomK2             | pBtSK, containing comK2 region with cat\( ^n \) cassette | This study |
| pAT\( \Delta \text{comK2} \) | pATAS28, containing comK2 region with cat\( ^n \) cassette | This study |

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Quantitative RT-PCR

Samples obtained as described above for the microarray experiments were treated with RNase-free DNase I (Fermentas, St. Leon-Rot, Germany) for 60 min at 37 °C in DNaseI buffer (10 mmol·L⁻¹ Tris-HCl (pH 7.5), 2.5 mmol·L⁻¹ MgCl₂, 0.1 mmol·L⁻¹ CaCl₂). Samples were purified with the Roche RNA isolation Kit. Reverse transcription was performed with 50 pmol random nonamers on 4 µg of total RNA using RevertAid™H Minus M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany). Quantification of cDNA was performed on an CFX96 Real-Time PCR System (BioRad, Hercules, CA) using Maxima SYBR Green qPCR Master Mix (Fermentas, St. Leon-Rot, Germany). The following primers were used: for BC4239, qBCE9 and qBCE10, for BC5251, qBCE19 and qBCE20 and for rpoA gene of B. cereus, qBCE3 and qBCE4 (primer sequences are listed in Table 4). The amount of BC07535 cDNA was normalized to the level of rpoA1 cDNA using the 2⁻ΔΔCT method [40].

Construction of pATK31 and pATK32

To overexpress ComK homologues the comK1 and comK2 genes were amplified with oAM19 and oAM20, oAM21 and oAM22, respectively. PCR products were cloned into the Eco47III site of pLM5 vector [27], resulting in pATK31 and pATK32, respectively. Plasmids were introduced into the pLM5 vector HB101/pRK24 and the resulting strains were used in conjugation experiments with B. cereus. Conjugation was performed as described by Trieu-Cuot [42]. Transconjugants were selected for chloramphenicol resistance and spectinomycin sensitivity. PCR and Southern analysis confirmed that the strain harbored the deleted allele of comK1 and comK2 and that the chloramphenicol resistance cassette had recombined into the chromosome through a double-crossover event (data not shown).

Table 4. Oligonucleotides used in this study.

| Oligonucleotides | Sequence (5'-3') |
|------------------|-----------------|
| oAM9             | TTTCCTCAATGATACGCTCCTTT |
| oAM11            | CGGAAGCTTCGTGTTTGGGAGACTAAGAC |
| oAM12            | CAACATTATGGAGGCTGACGGTGTA |
| oAM17-b          | CGGCCCCGGGCACATAGGAAGGAAAAG |
| oAM18            | CTGCTAGACATAGTTGGGAGAGGAG |
| oAM20            | CATGGCCATGCGGGTCTTACTGCCCACAAA |
| oAM21            | CGCAGACCTTTAGGGGAGACCATGAAAA |
| oAM22            | CATCAGCTGATCCGAGCAACACTCGAGAAA |
| comK1-Apaf-F     | CAGGGGCGGTGTTAGCAGAAGTACG |
| comK1-EcoRI-R    | CGGGATCTCCACTTACTTCCTACAT |
| pEA-Apaf-F       | GCAGGGCTCCGTGTTATGAGGAGTACAT |
| pEA-EcoRI-R      | GCCGGGATCTCCACTTACTTCCTACAT |
| comK2-Apaf-F     | CGGCCCCGTCGCGGGTCTTACTGCAAATG |
| comK2-EcoRI-R    | CGGAGCTTCTCATTCACTCATGATGTT |
| K1-F             | CGCCGAGCATCAGCTCTCTCTCTCTTT |
| K1-R             | CGCCGAGCATCAGCTCTCTCTCTCTCTTT |
| K2-S-F           | CGCCGAGCATCAGCTCTCTCTCTCTCTTT |
| qBCE3            | CTGCTAGACATAGTTGGGAGAGGAG |
| qBCE4            | TTTCCTCAATGATACGCTCCTTT |
| qBCE9            | ATCGAAGAAGTCTCCTGATTGGCC |
| qBCE10           | GTGCAATGACCAAGAAGTACG |
| qBCE19           | CATTGGTGTATATTGGTGTGTT |
| qBCE20           | TTTCCTCAATGATACGCTCCTTT |

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Role of ComK Proteins in B. cereus

Construction of a comK1 and comK2 null mutant

First, the comK1 region was amplified from genomic DNA of B. cereus ATCC15479 with primers oAM9 and oAM12. The PCR product was cloned into pBlSK digested with HinII and ScaI, resulting in pBSKcomK1. Subsequently, the comK1 gene was cut out with XbaI and EcoRV and replaced by a chloramphenicol cassette from pUC19C. Finally, the insert containing comk1 upstream and downstream flanking regions with chloramphenicol cassette was amplified from the vector with primers K1-F and K1-R. Subsequently, this fragment was cloned into pATAS28, resulting in pATcomK1.

To knockout the comk2 gene, first the comK2 region was amplified with primers K2-S-F and K2-E-R. The resulting fragment was cloned into the pBlSK vector digested with ApaI and XbaI and blunted by Klenow polymerase. Next, comK2 was cut out with XhoI and SfuI and replaced by a chloramphenicol cassette from pUC19C. The resulting plasmid pcomK2 was digested with PvuII and the insert containing comk2 upstream and downstream flanking regions with a chloramphenicol cassette was cloned into pATAS28 [41], resulting in pATcomK2. The orientation of the inserts in the vectors was checked by restriction analysis.

The vectors were then transformed into E.coli HB101/pRK24 and the resulting strains were used in conjugation experiments with B. cereus. Conjugation was performed as described by Trieu-Cuot [42]. Transconjugants were selected for chloramphenicol resistance and spectinomycin sensitivity. PCR and Southern analysis confirmed that the strain harbored the deleted allele of comk1 and comk2 and that the chloramphenicol resistance cassette had recombined into the chromosome through a double-crossover event (data not shown).

Construction of the plcomK1-gfp, plcomK2-gfp and plcomEA-gfp vectors

The comK1, comK2 and comEA promoters regions, including the ribosome binding site, were amplified by PCR using primers comK1-Apaf-F and comK1-EcoRI-R for pcomK1, comK2-Apaf-F and comK2-EcoRI-R for pcomK2 and pEA-Apaf-F and pEA-EcoRI-R for pcomEA, respectively. After digesting with EcoRI and ApaI the PCR products were ligated into the corresponding sites of pSG1151 [43], resulting in pcomK1-gfp, pcomK2-gfp and pcomEA-gfp vectors, respectively. These plasmids were used as a template to amplify PcomK1-gfp, PcomK2-gfp and PcomEA-gfp by PCR using primers oAM17-b and oAM18. The resulting PCR fragments were digested with XbaI and EcoRI and inserted into XbaI-Smal cleaved pHIL1 and inserted into L. lactis MG1363 by electroporation [44]. The correct cloned DNA sequence was confirmed by sequencing. Subsequently, plasmids pLcomK1-gfp, pLcomK2-gfp and pLcomEA-gfp were introduced into the wild type, B. cereus ΔcomK1, B. cereus ΔcomK2 by electroporation.

Analysis of reporter gene expression

For flow cytometric analyses B. cereus ATCC 15479 and B. cereus ΔcomK1 and ΔcomK2 strains carrying either pLcomGA-gfp, pLcomK1-gfp, pLcomK2-gfp or pLcomEA-gfp were grown ON in TY supplemented with erythromycin (5 µg ml⁻¹) and chloramphenicol (5 µg ml⁻¹) for the mutants strains. For the flow cytometric analyses, cultures were inoculated into fresh minimal medium with erythromycin (2.5 µg ml⁻¹ in MM) and chloramphenicol (5 µg ml⁻¹) for comK mutants After transition point, samples were taken every hour.
Cells were diluted in minimal salts and analyzed on a Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Mijdrelt, NL) operating an argon laser at 488 nm. Green fluorescent protein (GFP) signals were collected through an FITC filter with the photomultiplier voltage set between 700 and 800 V. Date were obtained using EXPO32 software (Beckman Coulter) and further analyzed using WinMDI 2.8 (The Scripps Research Institute). Figures were prepared using WinMDI 2.8 and CorelDraw X3 (Corel Corporation).

For the plate reader experiments, B. cereus cells containing either plComGA-gfp or pILcomEA-gfp were grown as described above. The OD and fluorescence were measured every 15 minutes using a TECAN F200 Microplate Reader (TECAN Group Ltd, Männedorf, Switzerland). Obtained fluorescence data from at least 3 independent experiment was normalized to OD and given in arbitrary units.

**Supporting Information**

Figure S1 Level of green fluorescent protein (gfp) in B. cereus cells carrying the pNWK-Km comK<sub>Bsu</sub> overexpression plasmid and pILComGA-gfp (triangle) or pILcomEA-gfp (circle in wild type, square in comK1 deletion and rhombus in comK2 deletion strains) reporter plasmids. (-) Strains without induction of comK<sub>Bsu</sub> overexpression are indicated with open symbol, while (+) strains with comK<sub>Bsu</sub> induction are denoted with filled symbols. Fluorescence of wild type cells without any reporter constructs (cross). OD and fluorescence was measured every 15 minutes using a TECAN F200 Microplate Reader. Obtained fluorescence data from 3 independent experiments were normalized to OD and given in arbitrary units. Time is indicated on the y axis in seconds and fluorescence in arbitrary units is given on the x axis. (PDF)

**Table S1** Summary of transcriptional changes in B. cereus ATCC15479 upon overexpression of B. subtilis comK unrelated to DNA uptake. The top 20 genes significantly up- or down-regulated are shown in the table. The complete list of transcriptional changes is available at the Gene Expression Omnibus database under the accession number GSE27267. *a* The ratio of gene expression is shown. Ratio: expression in the comK<sub>Bsu</sub> overexpressed samples over control samples. *b* Bayesian p value. (PDF)

**Table S2** Summary of transcriptional changes in B. cereus ATCC15479 upon overexpression of comK1. The top 20 genes significantly up- or down-regulated are shown in the table. The complete list of transcriptional changes is available at the Gene Expression Omnibus database under the accession number GSE27267. *a* The ratio of gene expression is shown. Ratio: expression in the comK1 overexpressed samples over control samples. *b* Bayesian p value. (PDF)

**Table S3** Summary of transcriptional changes in B. cereus ATCC15479 upon overexpression of comK2. The top 20 genes significantly up- or down-regulated are shown in the table. The complete list of transcriptional changes is available at the Gene Expression Omnibus database under the accession number GSE27267. *a* The ratio of gene expression is shown. Ratio: expression in the comK2 overexpressed samples over control samples. *b* Bayesian p value. (PDF)

**Author Contributions**

Conceived and designed the experiments: AMM ATK OPK. Performed the experiments: AMM ATK AM. Analyzed the data: AMM ATK. Contributed reagents/materials/analysis tools: AMM ATK OPK. Wrote the paper: AMM ATK OPK.

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