Functional Analysis of the ComK Protein of Bacillus coagulans

Ákos T. Kovács1, Tom H. Eckhardt1, Richard van Kranenburg2, Oscar P. Kuipers1,3

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

Abstract

The genes for DNA uptake and recombination in Bacilli are commonly regulated by the transcriptional factor ComK. We have identified a ComK homologue in Bacillus coagulans, an industrial relevant organism that is recalcitrant for transformation. Introduction of B. coagulans comK gene under its own promoter region into Bacillus subtilis comK strain results in low transcriptional induction of the late competence gene comGA, but lacking bistable expression. The promoter regions of B. coagulans comK and the comGA genes are recognized in B. subtilis and expression from these promoters is activated by S. subtilis ComK. Purified ComK protein of B. coagulans showed DNA-binding ability in gel retardation assays with S. subtilis- and B. coagulans-derived probes. These experiments suggest that the function of B. coagulans ComK is similar to that of ComK of B. subtilis. When its own comK is overexpressed in B. coagulans the comGA gene expression increases 40-fold, while the expression of another late competence gene, comC is not elevated and no reproducible DNA-uptake could be observed under these conditions. Our results demonstrate that B. coagulans ComK can recognize several S. subtilis comK-responsive elements, and vice versa, but indicate that the activation of the transcription of complete sets of genes coding for a putative DNA uptake apparatus in B. coagulans might differ from that of B. subtilis.

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E-mail: o.p.kuipers@rug.nl

Current address: Terrestrial Biofilms, Institute of Microbiology, Friedrich Schiller University of Jena, Jena, Germany

Introduction

The ability to take-up DNA from the environment is widely spread among eubacteria, including Gram-positive and Gram-negative species [1]. It allows the exchange of genetic material, possibly contributing to the survival of bacteria under harsh growth conditions [2–4]. Cells that activate the expression of genes coding for a DNA uptake and recombination apparatus can benefit from foreign DNA after it recombines into the genome. Due to the need for homologous sequences for recombination it is proposed that DNA is utilized more efficiently from closely related species [5]. The induction of the competence genes has been studied in various bacteria [2,6]. In Gram-positives, a global transcription factor or sigma factor coordinate the expression of genes required for efficient DNA uptake and recombination, the so-called late competence genes. In Streptococci, the conserved ComX sigma factor activates the late competence genes [5], while the global transcription factor ComK has been identified in various Bacilli to activate gene expression of genes related to DNA uptake [7]. As the induction of functional DNA uptake can be a useful tool for molecular biotechnological applications, numerous studies aim to better characterize the regulators involved in competence and try to achieve highly transformable strains [8–14].

The genes coding for DNA uptake and recombination are conserved among Bacilli [7]. The functional uptake of exogenously provided genomic DNA has been shown in various strains of B. subtilis [8,12,15], and also in other Bacilli, like B. licheniformis [10,16], B. amyloliquefaciens [17], and B. cereus [11]. The regulation and function of late competence genes have been mainly studied in B. subtilis [18]. The 7 genes containing comG operon encodes a type IV pilus that facilitates DNA to pass the cell wall and reach the cell membrane [19]. The maturation of the pilin like proteins is facilitated by the ComG prepilin protease [20]. DNA is bound and transported across the membrane in a single stranded form by the ComEA protein and ComEC permease, respectively, with the aid of ComFA and NucA proteins [18]. The single-stranded DNA is then integrated via recombination by a protein complex containing among others RecA, SsbB, DprA and YjbF [21].

The late competence genes are scattered around the B. subtilis chromosome. To coordinate the expression of these genes and operons, B. subtilis utilizes the global transcription factor ComK. If the protein level of ComK increases in the cells, ComK directly or indirectly activates more than 100 genes [6,22–24]. ComK binds to the so-called K-boxes, that contains two AT-boxes (AAAA-N5-TTTT) separated by a spacer of a discrete number of helical turns [25–27]. To ensure that competence develops only under particular conditions, the expression of the comK gene and the
protein level of ComK are tightly regulated. Transcription of comK is repressed by AbrB, CodY, and Rok and activated by the DegU protein [6], while the ComK protein is trapped by the adaptor protein MecA, and targeted to proteolysis by ClpCP [29]. At high cell densities, the expression of the comG gene, embedded in the comA operon, is activated in a quorum sensing dependent manner [29]. ComS protein hijacks the MecA protein and prevents ComK degradation [29]. The increase of ComK amounts in the cells leads to a positive feedback loop and the protein level further increases. However, the enhanced level of ComK is only developed in a subpopulation of cells [30,31]. The occurrence of two subpopulations of cells with a distinct expression state is called bistability [32] and has not been only described for competence, but also for other phenotypes of B. subtilis, like sporulation, motility, biofilm formation, and protease production [33–37].

In this study we characterized the function of the Bacillus coagulans ComK homologue in B. coagulans and in B. subtilis. B. coagulans is a spore forming, microaerophilic, lactic acid producing species of the Bacillus genus. It is frequently isolated as food spoilage organism [38], while its propitious features are used in biotechnological procedures and various molecular tools have been developed recently [40–42]. The genome sequences of several strains have been determined that facilitate genomics studies in this group of organisms [43–45]. As there is no study published on DNA uptake in B. coagulans, our aim was to better characterize the ComK homologue from B. coagulans DSM 1. First, we assayed the B. coagulans comK gene (denoted as comKBco) and promoter regions of comKBco and comGABco in the heterologous host, B. subtilis. In vitro studies further supported the conserved role of ComKABco as a DNA binding protein. Finally, we assayed the effect of comKBco overexpression in B. coagulans.

**Results**

**Identification of comK Homologue in B. coagulans**

Genomic inspection of various B. coagulans strains, including DSM 1 (unpublished data), 36D1 and 2–6 showed that several genes and operons can be identified with high sequence similarity to Bacillus genes that code for the late competence genes in B. subtilis and their homologues in other Bacillus species (Fig. 1A). BLAST analyses revealed the presence of many orthologous genes putatively involved in DNA uptake and recombination, which we visualized with Genesis software. As in the case of many Bacillus species [7], the comFB gene is missing in the comF operon. While the putative ComGEGF proteins lack high similarity to the corresponding proteins of B. subtilis, the number of genes in the comG operon is conserved and the coded proteins show higher similarity to the corresponding proteins of B. cereus, where functional DNA uptake has been shown [11]. Interestingly, the genomes of all B. coagulans species also lack the muck-nah operon that is required for the DNA cleavage during transformation in B. subtilis [46]. The absence of these genes reduces transformation efficiency to 8–13% of the wild type in B. subtilis. Still, the genomic analysis of competence genes shows that homologues of the majority of genes coding for the B. subtilis DNA-uptake and recombination machinery are present and conserved in all B. coagulans strains.

Homologues of the comK gene are present in all B. coagulans species. Although the comK homologue is not annotated in the complete genome of B. coagulans 2–6, a gene that codes for a putative ComK homologue can be identified between nucleotides 860449 and 860961 of the B. coagulans 2–6 chromosome (NCBI reference sequence NC_015634.1). The B. coagulans ComK homologues are somewhat shorter than the ComK protein of B. subtilis (13 amino acids shorter compared to ComK of B. subtilis), but most regions are conserved (Fig. 1B). The C-terminal region of B. coagulans ComK proteins is truncated by 11 amino acids. Previous studies have shown that a 25–35 amino acid C-terminal truncation is incapable of transcriptional induction of comK operon [47]. The ComK proteins of B. coagulans strains have half of this C-terminal part. Interestingly, as shown in many Bacillus species, the region recognized by the adaptor protein MecA is not conserved in any of the B. coagulans species suggesting that the interaction site is different or that the ComK level is not controlled by a MecA homologue in B. coagulans species, while putative MecA homologues are present in all B. coagulans strains (Fig. 1A).

Examination of the presence of the early regulatory competence genes suggest that pleiotropic regulators (DegU, CodY, AbrB, and Spo0A) that directly or indirectly control comK transcription in B. subtilis are present in B. coagulans, while SinR and the Rap-Phr signaling systems seem to be less conserved or absent in B. coagulans (Fig. 1A). Interestingly, rok can be identified in B. coagulans, while it was previously described to be present only in the B. subtilis/amyloliquefaciens/pumilus/bicheniformis group [7].

**Introduction of comKBco into B. subtilis Results in Activation of Gene Expression from PcomGABsu**

On the basis of its protein sequence analysis, the comKBco gene of B. coagulans DSM1 appears to code for another member of the ComK family. Therefore, we wanted to test if the product of the comK gene can activate transcription. For this we first introduced the comKsu gene (cloned in pATK1) into B. subtilis harboring a PcomGABsu::gfp reporter that enables us to monitor the activation of gene expression. The expression of comKsu is driven by its own promoter region. Subsequently, we deleted the endogenous comKsu gene in this strain so we can solely monitor the effect of comKBco. In this synthetic background, the reporter activity observed depends on the presence of ComKsu. As depicted in Fig. 2, we observed reporter activity from the comGABsu promoter when the comKsu gene was introduced, but not when the empty plasmid was present. The gene expression was detected using both flow cytometry (Fig. 2A) and fluorescence microscopy (Fig. 2B). The expression from PcomGABsu was low compared to the strain in which wild type comKsu was present, and expression of comGABsu was not bistable in contrast to the expression observed in the wild type B. subtilis strain. However, the lack of bistable gene expression of the reporter gene could also originate from a low expression level from the comKsu promoter in B. subtilis. These experiments suggest that comKBco is able to affect gene expression in Bacilli. Introduction of comKBco into B. subtilis resulted in low comGABsu expression, which suggests the lack of complete functional complementation of comKsu deletion under the tested conditions. Accordingly, no natural transformation was observed in the complemented B. subtilis strain (data not shown).

**ComKBsu Activates Transcription from the Promoter Regions of comKBsu and comGABsu**

ComKBsu can activate gene expression in the heterologous host B. subtilis. The transcription activation by ComK proteins depends on the promoter sequences they bind and their relative amount, and they either activate gene expression (e.g. comGABsu promoter [26]) or relieve transcription repression (e.g. comKBsu promoter [48]). To test whether the elements of the comGABsu and comKBsu promoters are functionally conserved, we assayed the effect of the ComKBsu protein on these promoter fragments in the heterologous host, B. subtilis. For this we introduced the promoter-gfp constructs.
Figure 1. Survey on the presence of competence genes and the alignment of ComK protein sequences from various Bacillus strains. (A) Results of BLAST searches were visualized with Genesis 1.6 software: white is absent (with E-value of E–0), dark blue is present (E-value < E–20). BLAST analysis was performed with B. subtilis protein sequences against translated protein database of a given genome. Protein names are indicated on the right. Bsu, B. subtilis; Bli, B. licheniformis; Bam, B. amyloquefaciens; Bce, B. cereus; Bco, B. coagulans. Question marks denote small ORFs where identification is uncertain using the available bioinformatic tools that can miss homologues. (B) Multiple alignment of ComK homologues. Black background represents conserved amino acids and grey background represents similar amino acids. Alignment was performed using ClustalW [59], and presented using Boxshade 3.21 program. The N- and C-terminal deletions analyzed by Susanna et al [47] are marked (DN9 and DC25, respectively). Boxed amino acid residues indicate the residues involved in interaction with MecA [60]. Alpha-helices and beta-sheets of B. subtilis ComK protein are indicated with rectangles and arrows under the alignment, respectively.

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pATK5 and pATK6 into B. subtilis and subsequently also assayed the effect of the comKBsu mutation on the expression from these promoters. Expression of a reporter gene from both the comKBco and comGABco promoters was observed in B. subtilis (Fig. 3). This expression was dependent on the presence of the ComK Bsu protein. The activation of gene expression from the introduced promoters showed a bimodal expression pattern that could originate from the bimodal level of ComK Bsu protein in B. subtilis or due to use of plasmid based system to monitor gene expression. However, we can conclude that the comGABco and comKBco promoters are recognized in B. subtilis in a comKBsu-dependent manner. Introduction of comKBsu (pATK4) into the ΔcomKBsu strain containing the pATK6 plasmid showed that ComK Bsu can activate gene expression from the promoter of comGABsu (Fig. 3C, pATK6, ΔcomKBsu with pATK4) similarly to that observed for the comGABsu promoter (Fig. 2A, PcomGABsu-gfp, ΔcomKBsu with pATK4).

ComK Bco is a DNA Binding Protein

Experiments presented above show that ComK Bco affects expression from the comGABsu promoter and that the comGABco and comKBco promoters are also recognized by ComK Bco. To test this in more details, we examined the in vitro DNA binding ability

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**Figure 2. Single cell analysis of PcomGABsu-gfp in the presence of comKBsu in B. subtilis.** Samples were taken 2 hours after the transition point between the exponential and stationary growth phase. (A) Flow cytometric analyses of comGABsu expression in wild type (red line), ΔcomK mutant (blue line), ΔcomK strain with the comKBsu containing plasmid pATK4 (green line), and ΔcomK strain with the empty plasmid (black line). The relative 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 analyzed. The graph is the representative of at least three independent experiments. (B) Light-microscopic phase-contrast picture (top row) and fluorescence image (bottom row) of cells. Strains used from left to right are wild type, ΔcomK mutant, ΔcomK with pATK4, and ΔcomK with pEM53, respectively.
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Figure 3. Expression from the comKBco and comGAco promoters in B. subtilis. Single cell analysis of B. subtilis strains containing plasmids with promoter-less gfp (A), with PcomKBco-gfp fusion (B), and the PcomGAco-gfp reporter (C). Samples were taken at the indicated time points given in hours relative to the transition point between the exponential and stationary growth phase (T0). The single cell expression pattern in the wild type strain is indicated with light grey, the ΔcomK mutant is designated with dark grey, and the ΔcomK strain with the comKBco containing plasmid pATK4 is shown in white. The relative 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 analyzed. The graph is the representative of at least three independent experiments. doi:10.1371/journal.pone.0053471.g003
of ComK<sub>Bco</sub>. We overexpressed a maltE-comK<sub>Bco</sub> fusion construct in <i>Escherichia coli</i> and purified the ComK<sub>Bco</sub> protein with the aid of the maltose binding protein (MBP) tag (Fig. 4A). MBP fusion tag is generally used to purify DNA binding proteins and assay the <i>in vitro</i> DNA binding ability of target proteins. The MBP tag did not alter the binding ability of ComK<sub>Bco</sub> protein in previous studies [25]. As a control, we also obtained the MBP-ComK<sub>Bco</sub> protein using the same purification procedure. The MBP-ComK<sub>Bco</sub> protein was overexpressed in <i>Escherichia coli</i> and purified as described in the Methods section (Fig. 4A). The integrity of the purified MBP-ComK<sub>Bco</sub> protein was also verified using antibodies developed against the ComK<sub>Bco</sub> protein (Fig. 4B). A smaller protein band was copurified and recognized by the ComK<sub>Bco</sub> antibody. The purified MBP-ComK<sub>Bco</sub> clearly bound to the DNA fragment containing the comK<sub>Bco</sub> and comGA<sub>Bco</sub> promoter regions in gel retardation assay (Fig. 4C and D). We also observed a weaker DNA binding of MBP-ComK<sub>Bco</sub> to the comG<sub>Bco</sub> and comFA<sub>Bco</sub> promoters (Fig. 4E and F). The MBP-ComK<sub>Bco</sub> showed no binding towards the <i>B. coagulans</i> tspD promoter fragment that is used as a non-specific control in our experiments (Fig. 4G).

Experiments performed in <i>B. subtilis</i> suggest that the ComK proteins can activate gene expression on the heterologous comGA promoters (see above). To examine if this effect of ComK proteins is achieved by direct binding and transcription activation, we examined the <i>in vitro</i> binding of various ComK proteins on the <i>B. coagulans</i> and <i>B. subtilis</i> promoters of the comGA and comK genes. Results depicted in Fig. 5 show that the ComK proteins bind to the heterologous promoters, although the affinity of the ComK proteins was different in the case of different promoters. As our gel retardation experiments were not controlled in competition experiments with a cold probe, we can only judge the presence of DNA binding, but no indisputable conclusion can be drawn on the affinity differences. However, the binding of ComK proteins of <i>B. coagulans</i> and <i>B. subtilis</i> to the heterologous promoter fragments is in agreement with the <i>in vivo</i> experiments done in <i>B. subtilis</i>. Taken together the <i>in vivo</i> and <i>in vitro</i> experiments all suggest that <i>B. coagulans</i> possesses a functional ComK homologue that is presumably able to activate the transcription of several late competence genes in <i>B. coagulans</i> (see also below).

ComK<sub>Bco</sub> activates transcription by binding K-boxes that are composed of two AT-boxes with a consensus sequence AAAAA-N<sub>31</sub>-TTTT. The boxes are separated of a discrete number of helical turns (8-, 18- or 31-bp between the two AT-boxes), which places them on the same side of the DNA-helix [25–27]. The analysis of the promoter region of putative competence related genes in <i>B. coagulans</i> showed the presence of several AT-boxes (allowing maximum 3 mismatches to the consensus AT-box), however, K-boxes could only be found in the promoter regions of comK<sub>Bco</sub> and comGA<sub>Bco</sub> (Figure S1). Interestingly, the promoters regions of comG<sub>Bco</sub>, comEA<sub>Bco</sub> and comFA<sub>Bco</sub> contain an overrepresented GCC-N<sub>31</sub>-TGC motif (identified 1, 2, and 3 times, respectively). This motif is not found within the promoter regions of the comG<sub>Bco</sub> and comK<sub>Bco</sub> genes. However, due to the low number of analyzed promoters, we cannot conclude whether the K-boxes or this latter overrepresented motif are functional in <i>B. coagulans</i> and their role requires additional functional characterization.

Overexpression of comK<sub>Bco</sub> in <i>B. coagulans</i> Results in Elevated comGA<sub>Bco</sub> Expression

In our final experiments, we assayed the effect of comK<sub>Bco</sub> overexpression in <i>B. coagulans</i> DSM 1. For this, we cloned the comK<sub>Bco</sub> gene under control of the IPTG (isopropyl-β-d-thiogalactopyranoside) inducible <i>sph</i> promoter, resulting in plasmid pATK10. We introduced this construct into <i>B. coagulans</i> DSM 1 by electroporation and assayed whether the level of ComK protein is enhanced in <i>B. coagulans</i> containing pATK10 upon induction. An increased level of ComK protein was detected in Western blot analysis using antibodies against ComK<sub>Bco</sub> (Figure S2). Next, we monitored the expression of late competence genes using quantitative RT-PCR. As expected, the expression level of comG<sub>Bco</sub> gene was increased (ratio of 30.5±3.7) in the strain where comK<sub>Bco</sub> expression was induced with 1 mM IPTG compared to the wild type strain that lacks the plasmid. However, the expression level of another late competence gene (i.e. comG<sub>Bco</sub>) showed no significant change (ratio of 0.8±0.3) in the comK<sub>Bco</sub>-overexpression strain compared to the plasmid-free strain under this given condition. Other late competence genes (comEA<sub>Bco</sub> and comFA<sub>Bco</sub>) also lacked the increased expression in the comK<sub>Bco</sub>-overexpression strain (data not shown). Overexpression of the comK<sub>Bco</sub> gene using the previously published pNWcomK<sub>Bco</sub> plasmid [11] resulted in slightly increased comGA<sub>Bco</sub> expression (ratio of 3.2±1.2) and unaltered comG<sub>Bco</sub> transcription (ratio of 1.3±0.5) compared to the plasmid free wild type strain. These experiments demonstrate that ComK<sub>Bco</sub> can activate gene expression in <i>B. coagulans</i> in line with previous observations presented above.

To test if the increased expression of comG<sub>Bco</sub> by comK<sub>Bco</sub> overexpression is sufficient to observe a functional DNA uptake in <i>B. coagulans</i>, we tested the uptake of genomic DNA (e.g. chromosomal DNA of DSM1 ΔsigF::Cmr described in [40]) or plasmid DNA (e.g. pNW93N). The expression of comK<sub>Bco</sub> was induced at mid-exponential phase and DNA was supplied at different time points (1–4 hours) after induction. Cells were plated on medium containing chloramphenicol. We could not observe reproducible DNA uptake under the above presented comK<sub>Bco</sub> overexpression conditions in <i>B. coagulans</i>, suggesting the lack of a fully functional DNA uptake machinery under these specific conditions. Similarly, DNA uptake was not detected in <i>B. coagulans</i> when comK<sub>Bco</sub> was overexpressed, in contrast to the experiments with <i>B. cereus</i> [11]. Since an increased level of ComK<sub>Bco</sub> is detected by Western blot analysis when comK<sub>Bco</sub> is overexpressed in <i>B. coagulans</i> (Figure S2) and the comG<sub>Bco</sub> gene expression was induced roughly 30 times, it may be that the resulting level of ComK<sub>Bco</sub> is not high enough to activate the whole DNA-uptake and recombination apparatus.

**Discussion**

Genetic engineering of microorganisms allows to improve them or introduce alternative biochemical reactions and thereby to develop improved or novel strains or products. However, genetic engineering can be time consuming for recalcitrant bacteria. The use of competence for DNA uptake and recombination improves the engineering process by allowing or enhancing genetic accessibility. Competence has been described for many laboratory type strains of <i>Bacillus</i> [15–17] [12]. The genes coding for functional DNA uptake and recombination are widely conserved in <i>Bacillus</i> suggesting that natural competence exists in more species than described before [7]. However, highly efficient DNA uptake is not identified under laboratory conditions in many species. Different strains of the same species might also differ in their degrees of competence. Natural isolates of <i>B. subtilis</i> show a low DNA uptake efficiency that can be improved by induction of the late competence genes through overexpression of the comK gene [12].

In this study, we present the genomic conservation of genes coding for putative homologues for DNA uptake and the recombination apparatus in <i>B. coagulans</i>. Further characterization of the comK homologue in <i>B. coagulans</i> DSM 1 indicates that comK<sub>Bco</sub>...
Figure 4. Purification of ComK<sub>bco</sub> protein and its DNA binding ability. (A) SDS-PAGE analysis of overexpression and purification of ComK<sub>bco</sub> protein from E. coli. Non-induced and 0.1 mmol l<sup>-1</sup> IPTG induced cell extracts are loaded on the first and second lanes, respectively, while purified MBP-ComK<sub>bco</sub> is run in the third lane. (B) Immunoblot analysis of the purified MBP-ComK<sub>bco</sub> protein using α-ComK<sub>bsu</sub> antibodies. Marker sizes are...
codes for a DNA binding transcriptional activator. Introduction of comK<sub>Bsu</sub> into a synthetic <i>B. subtilis</i> background that lacks its own comK<sub>Bsu</sub> gene results in gene expression activation from the promoter regions of comG operons of <i>B. subtilis</i> and <i>B. coagulans</i>. These experiments clearly suggest a conserved role of ComK homologues in Bacilli, although the set of target genes might vary. This is also supported by the induction of functional DNA uptake in <i>B. cereus</i> by the ComK<sub>Bsu</sub> protein [11]. However, overexpression of either or both comK genes of <i>B. cereus</i> into <i>B. subtilis</i> does not result in a similar induction of comG expression (unpublished observation, AM Mironczuk and ÁT Kovács). Previous studies on the binding site of ComK<sub>Bsu</sub> described K-boxes, where the distance between the two AT-boxes is important for its function [25,26]. While AT-boxes can be identified in several promoter regions of late competence genes in various Bacilli, properly spaced K-boxes are found only in the promoter regions of comK<sub>Bsu</sub> and comC<sub>Bsu</sub> genes. In contrast with these in silico observations, purified ComK<sub>Bsu</sub> protein binds in vitro to the promoter regions of late competence genes of <i>B. coagulans</i> (Fig. 5) and <i>B. cereus</i> [11] and overexpression of comK<sub>Bsu</sub> results in enhanced comG expression.

![Figure 5. Gel retardation assay with ComK<sub>Bsu</sub> and ComK<sub>Bco</sub>](image-url)

The binding of MBP-ComK<sub>Bsu</sub> (A–D) and MBP-ComK<sub>Bco</sub> (E–F) was assayed at a doubling concentration of the proteins from 125 nmol l<sup>−1</sup> to 1 μmol l<sup>−1</sup> (lanes 2 to 5, respectively). Lane 1 of each picture lacks any added protein. DNA binding was detected on promoters of comK<sub>Bco</sub> (A), comGA<sub>Bco</sub> (B), comK<sub>Bsu</sub> (C and E), and comGA<sub>Bsu</sub> (D and F) genes. Free probes (P), shifted bands (S), and signal specific to the wells of the gel (W) are indicated.

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in vivo (RT-qPCR results and [11]). This suggests that the recognition and transcriptional activation by ComK proteins might not be so stringent in the heterologous hosts. Alternatively, ComK proteins of B. coagulans and B. cereus could act on deviating binding sites or their effect is indirect on the comK promoter.

Overexpression of various comK genes in different Bacilli results in increased transcription from the comG promoter. In the present and previous studies, we used the fusion between the comG promoter and the reporter gene gfp for general use of comG promoter as a reporter of activation of competence in B. subtilis [30,31,49]. However, microarray analysis and RT-qPCR experiments in B. cereus showed that while expression of comK genes increases comG transcription, the transcript levels of other late competence genes are not induced equally [11]. In B. coagulans, when the ComK\textsubscript{Bco} protein level is increased to a certain level that results in roughly 30 times induction of comG promoter, the expression of comG\textsubscript{Bco} is not changed. We can hypothesize that the produced ComK protein level is not high enough to activate gene expression from these promoters or one or more additional regulatory mechanisms act on the late competence genes. In vitro transcription assays using these promoter regions and purified ComK protein could show whether this is the case.

While overexpression of comK genes in B. coagulans results in increased comG expression similar to the experiments in B. cereus [11], we did not detect functional uptake of DNA under these conditions. Our survey on the presence of late competence genes in B. coagulans also points to the absence of genes that are required for high efficiency DNA uptake in B. subtilis (e.g. nucA-nin genes). However, our study clearly shows that ComK\textsubscript{Bco} is a DNA-binding protein that is capable of activating gene expression. Therefore, it presents an important element of future research for better understanding of late competence gene induction in B. coagulans.

**Methods**

**Bacterial Strains, Growth Conditions and Transformation**

The strains and plasmids used in this study are listed in Table 1. B. coagulans strains were grown in BC medium at 50°C, 120 rpm [40]. BC medium contains per liter: 10 g yeast-extract (Difco), 2 g (NH\textsubscript{4})\textsubscript{2}HPO\textsubscript{4}, 3.5 g (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 10 g Bis-Tris (bis[2-hydroxyethyl]iminomtitris[hydroxymethyl]-methane), 5 mg MgCl\textsubscript{2}·6 H\textsubscript{2}O, 3 mg CaCl\textsubscript{2}·2 H\textsubscript{2}O, 1 ml of filter sterilized trace elements (containing per liter 0.05 g ZnCl\textsubscript{2}, 0.03 g MnCl\textsubscript{2}·4 H\textsubscript{2}O, 0.5 g H\textsubscript{2}BO\textsubscript{3}, 0.2 g CoCl\textsubscript{2}·6 H\textsubscript{2}O, 0.01 g CuCl\textsubscript{2}·2 H\textsubscript{2}O, 0.02 g NiSO\textsubscript{4}·6 H\textsubscript{2}O, and 0.03 g Na\textsubscript{3}MoO\textsubscript{4}·2 H\textsubscript{2}O), pH 6.7. B. subtilis strains were grown in minimal medium [15]. For cloning, Escherichia coli DH\textsubscript{5}\textalpha and Lactococcus lactis MG1363 were grown in YT and GM17 (37.5 g M17 broth (Difco) per liter supplemented with 0.5% glucose) medium, respectively, grown at 30°C or 37°C. Antibiotics were used at a concentration of 5 μg ml\textsuperscript{-1} for chloramphenicol, 6 μg ml\textsuperscript{-1} for tetracycline, and 100 μg ml\textsuperscript{-1} for ampicillin. Transformation of L. lactis and B. coagulans was performed by electroporation as previously described [40,50]. Transformation of E. coli was performed by heat-shock [51]. DNA was introduced into B. subtilis strains using natural competence [52].

**Cloning of comK\textsubscript{Bco} Gene**

To facilitate the purification of MBP-ComK\textsubscript{Bco} the comK\textsubscript{Bco} gene was PCR amplified from the genome of B. coagulans DSM 1 using oligos oATK26 and oATK14 (for the sequences of oligos, see Table 2) containing BamHI and SalI sites, respectively. The construct pMALcomK was created by ligating the BamHI and SalI digested PCR into the corresponding site of the pMAL-c2 (New England Biolabs). The comK\textsubscript{Bco} gene harboring its own promoter was PCR amplified with oligos oATK1 and oATK2, and cloned into the Scal site of pEM53 vector, resulting pATK4. The cloned fragment contains the comK\textsubscript{Bco} gene and the 732 bp upstream region. Vector pEM53 is derived from pNZ124 by replacing the chloramphenicol resistance gene cat by the tetracycline resistance gene tetr amplified from pGhost8::ISS/ [40]. To overexpress comK\textsubscript{Bco} in B. coagulans, the comK\textsubscript{Bco} gene was cloned after the Pspac promoter. For this, the comK\textsubscript{Bco}, containing PCR fragment was obtained with oATK13 and oATK14 oligonucleotides (Table 2), digested with HindIII-Sall enzymes, and ligated together with the Pspac containing EcoRI-HindIII fragment from pDG148 and the EcoRI-XhoI digested vector, resulting in pATK8. Subsequently, the lacI gene was introduced from pDG148 (1294 bp BamHI-Swal fragment) into the BamHI-Scal digested pATK8 vector, resulting pATK10. The resulting vectors were validated using restriction analysis and inserts were verified by sequencing.

**Construction of Promoter-gfp Reporter Plasmids**

The gfp gene was first obtained from pSG1151 using KpnI-XbaI restriction enzymes and ligated into the corresponding sites of the broad host range pNW35N vector, resulting pATK2. pATK2 was digested with KpnI and ApaI and used to ligate the promoter fragments of comK\textsubscript{Bco} and comG\textsubscript{Bco} obtained with PCR reaction using oligonucleotides oATK5 and oATK6 (for comK\textsubscript{Bco}) and oATK7 and oATK8 (for comG\textsubscript{Bco}) and digested with the same restriction enzyme pairs. The integrity of cloned fragments was verified by sequencing.

**Protein Overexpression and Purification**

1 liter culture of cells containing the pMALcomK\textsubscript{Bco} or pMALcomK [53] was grown for 2 hours at 37°C and induced with 0.1 mmol l\textsuperscript{-1} of IPTG at 0.8 of OD\textsubscript{600}. Cells were harvested by centrifugation (10 min, 4°C, 6500×g). Pellets were washed with a buffer containing 1.17% NaCl, 25 mmol l\textsuperscript{-1} EDTA, 10 mmol l\textsuperscript{-1} Na\textsubscript{2}HPO\textsubscript{4}, 0.15% DTT, 50 mmol l\textsuperscript{-1} Tris-HCl pH 7.4. Cells were lysed by sonication (15×10 s at 10 kHz with 30 s intervals), and the sonicated fractions were centrifuged (20 min, 4°C, 9000×g) to obtain a supernatant that contains the MBP-ComK. The fraction with the MBP-ComK has been loaded on an amylose column which had been equilibrated with a buffer containing 0.5 mM DTT, 20 mM Tris-HCl pH 8.0. Elution was performed with the same buffer, now containing 10 mM maltose. The fractions were stored immediately at 4°C (after analysis the fractions were pooled and stored at −80°C). The purity of MBP-ComK\textsubscript{Bco} and MBP-comG\textsubscript{Bco} was verified on SDS-PAGE and the purified proteins were also validated using Western hybridization with antibody raised against ComK\textsubscript{Bco} as described in [53]. Sample preparation and Western hybridization on the B. coagulans samples were performed as described previously for B. cereus [11]. B. coagulans wild-type and comK\textsubscript{Bco} overexpression strains were grown in BC medium until 0.8 of OD\textsubscript{600} and induced with 0.1 mmol l\textsuperscript{-1} IPTG. Three hours after induction, samples were harvested by centrifugation (10.397×g, 1 min, 4°C), disrupted using lysozyme treatment. The ComK\textsubscript{Bco} protein level was detected after SDS-PAGE using Western hybridization with ComK\textsubscript{Bco}–specific antibody [53].

**Gel Retardation Assay**

Gel retardation assays were carried out essentially as described by Susanna et al. [26]. The promoter regions of B. coagulans putative competence genes comK, comG, comC and comF\textsubscript{Bco} were obtained by PCR using oligos oATK5-oATK6, oATK7-oATK8,
Table 1. Strains, plasmids used in this study.

| Strain                  | Properties                                      | Reference                      |
|-------------------------|-------------------------------------------------|--------------------------------|
| B. coagulans DSM 1      | wild type strain                                | DSMZ collection                |
| B. subtilis 168         | wild type strain                                | laboratory strain              |
| B. subtilis ΔcomK       | comK::Kn′ mutant                                 | [30]                           |
| B. subtilis PcomG-gfp   | PcomG-gfp fusion in B. subtilis 168 strain (Cm′) | [30]                           |
| L. lactis MG1363        | lac′prt′; plasmid-free derivative of NCDO712    | [61]                           |
| E. coli DH5s            | endA1 hisD17 supE44 thi-1 recA1 gyrA96 relA1    | Bethesda Research Laboratories |
|                         | ΔlacU169 (p80dlocZΔM15)                          |                                |

Plasmids

| Plasmid     | Properties                                      | Reference                      |
|-------------|-------------------------------------------------|--------------------------------|
| pNW33N      | 4.2 kb, Cm′, Geobacillus-E. coli shuttle vector | Bacillus Genetic Stock Centre  |
| pEM53       | 5.6 kb, Tc′, pNZ124-based cloning vector         | [40]                           |
| pDG148      | 8.3 kb, Amp′, Km′, pspac, lacI integration vector | [62]                          |
| pMALc2X     | 6.6 kb, Amp′, overexpression vector for MalE fusion | New England Biobabs |
| pSG1151     | 4.6 kb, Amp′, Cm′, gfpmut1 harboring plasmid     | [63]                           |
| pATK2       | 5.0 kb, Cm′, gfpmut1 cloned into pNW33N          | This study                     |
| pATK4       | 4.9 kb, Tc′, comK Δ′ gene and promoter region in pEM53 | This study |
| pATK8       | 4.5 kb, Tc′, pspac-comK Δ′ in pEM53              | This study                     |
| pATK10      | 5.8 kb, Tc′, pspac-comK Δ′ overexpression construct and lacI in pEM53 | This study |
| pATK5       | 5.6 kb, Cm′, PcomG Δ′-gfp fusion                 | This study                     |
| pATK6       | 5.5 kb, Cm′, PcomK Δ′-gfp fusion                 | This study                     |
| pMALcomK Δ′  | 7.3 kb, Amp′, MAL-ComK overproduction vector     | This study                     |

Cm′, chloramphenicol resistant; Tc′, tetracycline resistant; Km′, kanamycin resistant; Amp′, ampicillin resistant.
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Quantitative RT-PCR

B. coagulans wild type and comK overexpression strains were grown in BC medium until 0.8 of OD600 and induced with 0.1 mmol l−1 IPTG. Two hours after induction, samples were harvested by centrifugation (10,397 g, 1 min, 4°C). A total of three independent biological replicates were included. RNA preparation of quantitative PCR was performed as described before [55,56]. The pellets were immediately frozen in liquid nitrogen and stored at −80°C. RNA extraction was performed with the Macaloid/Roche protocol [57]. Samples 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), and re-purified with the Roche RNA isolation Kit. RNA concentration and purity was assessed using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed with 50 pmol random nonamers on 4 μg of total RNA using RevertAidTM 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 oligos were used: for comG4, oATK87 and oATK88, for comC, oATK83 and oATK84 (oligo sequences are listed in Table 2). The amount of comG4 and comC cDNA levels was normalized to the level of rpiA cDNA using the 2−ΔΔCt method [58].

Flow Cytometric Analyses and Microscopy

B. subtilis wild type and ΔcomK strains carrying either pATK5 or pATK6 were grown ON in minimal medium supplemented with chloramphenicol (5 μg ml−1). For the flow cytometric analyses, cultures were inoculated into fresh minimal medium. Samples were taken after transition to stationer phase every hour. Cells were diluted 10 fold in minimal salts and analyzed on a Coulter Epics XL-MCL flow cytometer (Beckman Coulter Mijdrecht, 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 Adobe CS4 Illustrator.

The fluorescence of the GFP reporter protein was visualized with a Zeiss Axiophot microscope, using filter set 09 (excitation, 450 to 490; emission, 520 nm). Imaging of ComGABsu-gfp in individual cells using fluorescence microscopy was performed as described by Smits et al. [30] using AxioVs20 software (Zeiss) for image capturing and figures were prepared for publication using Adobe CS4 Illustrator.

Nucleotide Sequence Accession Numbers

Sequences used in this study have been deposited in GenBank under accession numbers JX518619 (comKBsu), JX518620 (comGABsu), JX518621 (comGABsu), JX518622 (comFABsu), JX518623 (rpsDBsu), JX518624 (rpiABsu).

Supporting Information

Figure S1 A. Schematic presentation of the promoter region of putative competence related genes. Filled boxes indicate putative AT-boxes (maximum 3 mismatches to the consensus AAAAA-TTTT), open boxes indicate upstream open reading frames and com genes, numbers denote spacing between AT-boxes resulting in a so called K-box (8 bp and 31 bp in the case of comKBsu and comGABsu respectively).

B. Sequences of B. coagulans DSM1 promoter regions related to competence. Bold letters indicate putative AT-boxes. The putative open reading frames, com genes are indicated below the sequence.

Author Contributions

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

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