Functional analysis of the competence transcription factor ComK of *Bacillus subtilis* by characterization of truncation variants

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The competence transcription factor ComK is the master regulator of competence development in *Bacillus subtilis*. In the regulatory pathway, ComK is involved in different interactions: (i) protein–DNA interactions to stimulate transcription of ComK-dependent genes and (ii) protein–protein interactions, divided into interactions with other proteins and interactions between ComK proteins involving oligomerization. The fact that ComK displays different types of interactions suggests the presence of specific, distinct domains in the protein. This paper describes a search for functional domains, by constructing ComK truncation variants, which were tested for DNA binding, oligomerization and transcription activation. Truncations at the C-terminal end of ComK demonstrated the requirement of this part for transcription activation, but not for DNA binding. The C-terminal region is probably involved in oligomerization of ComK-dimers into tetramers. Surprisingly, a ComK truncation variant lacking 9 aa from the N-terminal end (Δ N9ComK) showed higher transcription activation than wild-type ComK, when expressed in *Lactococcus lactis*. However, in *B. subtilis*, transcription activation by Δ N9ComK was twofold lower than that by wild-type ComK, resulting from a five- to sixfold lower protein level of ComK Δ N9. Thus, relatively, Δ N9ComK is more active in transcription activation than wild-type ComK. These results suggest that the presence of this N-terminal extension on ComK is a trade-off between high transcription activation and a thus far unidentified role in regulation of ComK.

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INTRODUCTION

The competence transcription factor ComK is the key regulatory protein in competence development in *Bacillus subtilis*. Genetic competence is a differentiation process, initiated at the onset of the stationary growth phase, which enables the cell to take up and incorporate exogenous DNA. Development of competence is tightly regulated via a complex regulatory system, centred around ComK (reviews: Dubnau & Lovett, 2002; Hamoen *et al.*, 2003b). During exponential growth, competence development is prevented by regulating the level of ComK in the cell via both transcriptional and post-translational control. Transcription of *comK* is repressed by binding of AbrB, CodY and Rok

to the *comK* promoter (Hamoen *et al.*, 2003a; Hoa *et al.*, 2002; Serrero & Sonenshein, 1996). Any ComK that is synthesized at this stage is bound by the adaptor protein MecA, which targets ComK for proteolytic degradation by the ClpCP protease complex (Turgay *et al.*, 1998). At the onset of stationary growth, the cell responds to environmental changes, such as nutrient deprivation and increased cell densities, by relieving transcriptional repression of *comK* by AbrB and CodY (Hahn *et al.*, 1995; Serrero & Sonenshein, 1996) and by synthesis of ComS. This small protein binds to MecA and replaces ComK, resulting in the release of ComK from the proteolytic complex (Turgay *et al.*, 1997). Once ComK is free in the cell, it activates transcription of its cognate gene, by binding to the promoter region, where it can overcome repression by Rok (Hoa *et al.*, 2002; Van Sinderen & Venema, 1994; Van Sinderen *et al.*, 1995). Via this autostimulatory loop, ComK levels increase rapidly and, subsequently, ComK activates transcription of other genes, e.g. the late competence genes, encoding the DNA-binding, -uptake and -integration machinery (Van Sinderen & Venema, 1994).

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Abbreviations: EMSA, electrophoretic mobility shift assay; MBP, maltose-binding protein.

ComK activates transcription by binding to specific sequences, so-called K-boxes, located upstream of ComK-dependent genes. Each K-box consists of two AT-boxes, separated by a spacing of two, three or four helical turns between the start positions of the repeating AT-box units. Functional ComK consists of a tetramer, composed of two dimers, which each bind to an AT-box. ComK binding is accompanied by DNA bending (Hamoen *et al.*, 1998). The major role of ComK in transcription activation is to stabilize the binding of RNA polymerase, probably by facilitating interactions with upstream DNA, through bending of the promoter area (Susanna *et al.*, 2004).

As becomes clear from the regulatory pathway, ComK is involved in different types of interactions: (i) protein–DNA binding, which is of particular interest because contacts occur through the minor groove of the DNA helix (Hamoen *et al.*, 1998), and (ii) protein–protein interactions, involving either interactions with other proteins, such as MecA, or between ComK proteins, resulting in dimerization and tetramerization. The occurrence of different interactions suggests that the ComK protein contains specific, distinct domains, responsible for one or more of the interactions. ComK is a relatively small protein of only 22·8 kDa. Amino acid sequence comparisons and structural prediction studies did not reveal the presence of any clear domains. However, an alignment with ComK-like proteins from other bacterial species shows a high degree of conservation throughout the entire amino acid sequence, with the exception of the extreme N-terminal part (Fig. 1). The C-terminal region is not conserved throughout all listed bacteria, but shows conservation when subgroups are concerned, e.g. between

Listeria species or between *Bacillus licheniformis* and *B. subtilis*.

In this study we focused on the role of the N- and C-terminal regions of ComK in oligomerization, DNA binding and transcription activation. His-tagged ComK wild-type (His-tag on the N- or C-terminus) and N- or C-terminal truncation variants (C- or N-terminal His-tag, respectively) were expressed in *Lactococcus lactis*, in which transcription activation was tested using a (*B. subtilis*) *comG*–*lacZ* reporter fusion. DNA binding was demonstrated *in vitro* using electrophoretic mobility shift assays. This approach was chosen to enable a clear dissection between the effect of truncations on DNA binding, oligomerization and transcription activation. Furthermore by using an inducible system in *L. lactis*, effects on ComK of MecA binding and interactions with other *B. subtilis* regulators could be circumvented.

METHODS

Bacterial strains, media and growth conditions. The strains used in this study are listed in Table 1. *Escherichia coli* strains were grown in TY medium, supplemented with 100 µg ampicillin ml^{−1} when required. To purify the maltose-binding protein (MBP)–ComK fusion protein, cells were grown in the presence of 0·2 % glucose and 0·3 mM IPTG (pMal protein-fusion and purification system, New England Biolabs). *L. lactis* strains were grown at 30 °C in twofold-diluted M17-based medium, supplemented with 0·5 % glucose (GM17) and appropriate antibiotics (chloramphenicol and/or erythromycin, both 4 µg ml^{−1}). Protein expression was induced from the nisin-inducible promoter with 1 : 10 000 dilutions of supernatant of an overnight culture of nisin-producing *L. lactis* NZ9700 in GM17 (De Ruyter *et al.*, 1996).

Fig. 1. Overview of homology between ComK proteins from different bacteria. Amino acids are conserved throughout the entire protein, except for the N- and C-terminal parts. The C-terminal region shows conservation between subgroups (grey shadings). Asterisks (*), colons (:), and periods (.) indicate identical residues in all sequences, conserved substitutions and semi-conserved substitutions, respectively. Alignments were made using CLUSTALW (www.ebi.ac.uk/clustalw).

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant features	Reference
<i>E. coli</i>		
XL1blue	<i>endA1 gyrA96 thi hsdR17 (r_k⁻ m_k⁻) supE44 relA1 lacI F' proAB lacI^q lacZΔM15 Tn10</i>	Stratagene
<i>L. lactis</i>		
NZ9000	MG1363 <i>pepN::nisRK</i>	Kuipers <i>et al.</i> (1998)
NZ9700	Nisin producing, contains Tn5276	Kuipers <i>et al.</i> (1993)
<i>B. subtilis</i>		
168	<i>trpC2</i>	Anagnostopoulos & Spizizen (1961)
BV2004	Δ <i>comK</i> , Sp ^r	Hamoen <i>et al.</i> (2002)
QB4650	Δ <i>mecA</i> , Km ^r	Msadek <i>et al.</i> (1998)
168 <i>amyE::ΔN9comK</i>	<i>amyE::ΔN9comK</i> , Cm ^r	This work
pG9	<i>amyE::ΔN9comK</i> , pG-wt, Cm ^r Ery ^r	This work
pG9ΔK	pG9 + Δ <i>comK</i> , Cm ^r Ery ^r Sp ^r	This work
pG9ΔK-ΔM	pG9ΔK + Δ <i>mecA</i> , Cm ^r Ery ^r Sp ^r Km ^r	This work
pGwt	pG-wt, Ery ^r	K. A. Susanna and others, unpublished
pGwt-ΔM	pG-wt, Δ <i>mecA</i> , Ery ^r Km ^r	This work
Plasmids		
pMal-c2X	Amp ^r , IPTG-inducible <i>malE</i> with MCS	New England Biolabs
pMal-ComK	Amp ^r , IPTG-inducible <i>malE-comK</i>	This work
pDL	Cm ^r , plasmid for integration in <i>amyE</i>	Yuan & Wong (1995)
pDL-ΔN9ComK	Cm ^r , Δ <i>N9comK</i> region flanked by <i>amyE</i>	This work
pNZ8048	Cm ^r , inducible expression vector with P _{<i>nisA</i>}	Kuipers <i>et al.</i> (1998)
pNZ-His ₆ -ComK	Cm ^r , <i>his₆-comK</i> under control of P _{<i>nis</i>}	This work
pNZ-ComK-His ₆	Cm ^r , <i>comK-his₆</i> under control of P _{<i>nis</i>}	This work
pNZ-ComKΔC25	Cm ^r , <i>his₆-comKΔC25</i> under P _{<i>nisA</i>} control	This work
pNZ-ComKΔC35	Cm ^r , <i>his₆-comKΔC35</i> under P _{<i>nisA</i>} control	This work
pNZ-ComKΔC50	Cm ^r , <i>his₆-comKΔC50</i> under P _{<i>nisA</i>} control	This work
pNZ-ΔN9ComK	Cm ^r , Δ <i>N9comK-his₆</i> under P _{<i>nisA</i>} control	This work
pILORI4	Ery ^r , pIL252 with MCS and promoterless <i>lacZ</i> of pORI13	Larsen <i>et al.</i> (2004)
pG-wt	Ery ^r , <i>comG-lacZ</i> with common K-box	K. A. Susanna and others, unpublished

DNA manipulations, materials and transformations. Standard molecular biology methods were used (Ausubel *et al.*, 1998; Sambrook *et al.*, 1989). Enzymes were obtained from Roche, New England Biolabs or Pharmacia and radiolabelled nucleotides from Amersham. For isolation of plasmids and purification of PCR products, the High Pure Plasmid Isolation kit and the High Pure PCR Product Purification kit, respectively, were used (Roche). Chromosomal DNA of *B. subtilis* was isolated as described by Venema *et al.* (1965). *E. coli* strains were transformed using CaCl₂-induced competence (Sambrook *et al.*, 1989) and *L. lactis* strains using electroporation with a gene-pulser (Bio-Rad) as described by Leenhouts & Venema (1993). *B. subtilis* strains were transformed as described by Anagnostopoulos & Spizizen (1961).

PCR amplifications and plasmid constructions. PCR reactions were performed as described by Innes & Gelfand (1990), using Pwo or Expand DNA polymerase (Roche) on chromosomal DNA of *B. subtilis* 168 as a template. Plasmids and primers are listed in Table 1 and Table 2, respectively. For purification purposes, an MBP-ComK fusion was constructed in *E. coli*. To construct this fusion, the *comK* gene was amplified with primers ComK-start and ComK-end. The

resulting PCR product was digested with *XmnI* and *HindIII* and cloned into *XmnI/HindIII*-digested pMal-c2X, yielding plasmid pMal-ComK.

His-tagged variants of wild-type and mutant ComK were constructed in *L. lactis*. To yield wild-type ComK (wtComK) with an N-terminal His-tag, the *comK* gene was amplified with primers H6Xa-comK and comK-end. The PCR-product was digested with *NcoI* and *HindIII* and cloned into *NcoI/HindIII*-digested plasmid pNZ8048, resulting in pNZ-His₆-ComK. For wtComK with a C-terminal His-tag, primers comK-Nice1 and comK-H6 were used to amplify *comK*. After digestion with *RcaI* and *HindIII*, the PCR product was cloned into *NcoI/HindIII*-digested pNZ8048, yielding plasmid pNZ-ComK-His₆. ComK truncations were constructed by amplifying the *comK* gene with primers comK-dN9-Nice and comK-H6 for an N-terminal 9 aa truncation and primers H6Xa-ComK combined with stop1 or comK-dC35 for the truncation of 25 or 35 aa from the C-terminus, respectively. The PCR products were digested with *NcoI/HindIII* and cloned into pNZ8048, also digested with *NcoI/HindIII*, yielding pNZ-ΔN9ComK, pNZ-ComKΔC25 and pNZ-ComK ΔC35, respectively.

Table 2. Oligonucleotide primers used for PCR

Restriction sites are in bold. The annealing sequences are underlined.

Primer	Sequence (5'–3')
comK-start	<u>ATGAGTCAGAAAAACAGACGC</u>
comK-end	<u>GGGGAAGCTTCGGACAAGGCACGCCGCC</u>
H6Xa-ComK	CATGCCATGGGGCACCATCACCATCACCATATCGAGGGAAGG- <u>ATGAGTCAGAAAAACAGACGC</u>
comK-Nice1	<u>GCATTTCATGAGTCAGAAAAACAGACGC</u>
comK-H6	<u>GATCAAGCTTCTAATGGTGATGGTGATGGTGCCATACCGTTCCCC-</u> GAGCTCAC
comK-dN9-Nice	<u>GATCCCATGGAATCGTATGAAGTGAACGGCGC</u>
stop1	<u>CCGCAAGCTTACTATTCTGTCTTTTCGGCACGCG</u>
comK-dC35	<u>CCGCAAGCTTCTATCTGTCTTGGAAATTTGGTTCTGAG</u>
comK-dC50	<u>CCGCAAGCTTCTAGTTCTCGAACGAATTATAAGAGATCGGC</u>
yhxC-H3	<u>GCCGAAGCTTATCAGGACGCCGAGGAA</u>
comK-start-BbsI	<u>GATCGAAGACATCATATTATGGCCTCCATCCTTTTTCTGC</u>
comK-dN9-BbsI	<u>GATCGAAGACATTATGGAATCGTATGAAGTGAACGGCGC</u>
comK + 500	<u>GATCGGATCCACTCGGCGGACTTGATGTGC</u>
comG-AT2-EcoRI	<u>GATCGAATTCAGAATTGGTTTTTCAGCATATAACATCTCAC</u>
comG-end-XbaI	<u>GATCTCTAGATTATGCCTCTTCAATCAAGTTTTTGC</u>

To produce an N-terminal 9 aa truncation variant of ComK in *B. subtilis*, a $\Delta N9comK$ copy was integrated into the *amyE* locus of *B. subtilis* 168 under control of the *comK* promoter. For this purpose, the *comK* region was amplified with primers yhxC-H3 and comK-start-BbsI and with comK-dN9-BbsI and comK + 500. Both PCR products were digested with *BbsI* and ligated to form a 2.3 kb product. Using the ligation mixture as a template, the $\Delta N9comK$ region was amplified with primers yhxC-H3 and ComK + 500. The PCR product was digested with *BamHI* and *HindIII* and ligated into *BamHI/HindIII*-digested pDL, resulting in the integration vector pDL- $\Delta N9comK$. Transformation of this plasmid into *B. subtilis* 168 resulted in strain *amyE::\Delta N9comK*. To test transcription activation by $\Delta N9comK$, the replicative plasmid pG-wt was introduced, yielding strain *B. subtilis* pG9. Finally, to have $\Delta N9comK$ as the only ComK protein in the cell, the *wtcomK* copy was deleted by transformation with chromosomal DNA of *B. subtilis* BV2004 (Hamoen *et al.*, 2002), resulting in strain pG9 ΔK . The control strain for transcription activation by wtComK consisted of *B. subtilis* 168 transformed with pG-wt, yielding strain pGwt.

To investigate potential differences in interactions of wtComK and $\Delta N9comK$ with the proteolytic system, a *mecA* deletion was introduced into *B. subtilis* pG9 ΔK and *B. subtilis* pGwt via transformation with chromosomal DNA of *B. subtilis* QB4650 (Msadek *et al.*, 1998). This transformation resulted in strains pG9 ΔK - ΔM and pGwt- ΔM , which contain $\Delta N9comK$ or wtComK, respectively, as the only ComK protein in the cell. Despite the changes necessary for the experiments, all the *B. subtilis* strains used in this study are isogenic.

Overexpression and purification of MBP-ComK. Overexpression of the MBP-ComK fusion protein was induced by adding IPTG (final concentration 0.3 mM) to an exponentially growing *E. coli* culture (OD_{600} 0.7–0.8). After 2 h of induction, cells were harvested by centrifugation (10 min, 8000 r.p.m., Beckman centrifuge). ComK was purified as described by Hamoen *et al.* (1998), yielding either native ComK (after cleavage with factor Xa) or the MBP-ComK fusion.

Expression and cell extracts of ComK in *L. lactis*. Expression of wtComK and mutants with an N- or C-terminal His-tag was induced from the nisin-inducible promoter by adding supernatant

from an overnight culture of *L. lactis* NZ9700 in 1:10 000 dilutions to *L. lactis* cultures at the end of the exponential growth phase. To obtain ComK for electrophoretic mobility shift assays, cells were harvested after 2 h of induction (10 min, 8000 r.p.m., Beckman centrifuge). Cell extracts were prepared by resuspending the cell pellet from 2 ml of culture in 200 μ l resuspension buffer (10 mM EDTA, 50 mM Tris/HCl, pH 8). Cells were disrupted by fast prep (45 s, speed 6, Bio101 analyser).

Purification of His₆-ComK expressed in *L. lactis*. Expression of His₆-ComK was induced in *L. lactis* as indicated above. Purification was performed in a batch procedure under denaturing conditions. Cells from a 1 l culture were resuspended in 6 ml buffer A (0.1 M NaH₂PO₄, 10 mM Tris/HCl, pH 8.0, 2% Triton X-100) with lysozyme (1 μ g μ l⁻¹). After 20 min at 37 °C, cells were disrupted by French press. A clear cell extract was obtained by ultracentrifugation (25 min, 77 000 g, 4 °C, Beckman ultracentrifuge, SW-41 rotor). To this extract, buffer B (buffer A + 8 M urea) was added to a volume of 50 ml. The extract was mixed with 2.5 ml Talon column material (Clontech). After 1 h at room temperature, the column material was centrifuged (10 min, 9000 g, Eppendorf centrifuge), then washed sequentially with buffer B and buffer C (8 M urea, 0.1 M NaH₂PO₄, 10 mM Tris/HCl, pH 6.3). His₆-ComK was eluted in 1 ml buffer E (8 M urea, 0.1 M NaH₂PO₄, 10 mM Tris/HCl, pH 4.5) and dialysed against dialysis buffer (20 mM Tris/HCl, pH 8, 1 mM EDTA, 0.5 mM DTT, 0.1 M Na₂SO₄) with gradually decreasing urea concentrations. Upon reaching 1 M urea, the sample was dialysed overnight at 4 °C against dialysis buffer without urea. The purified protein was divided into aliquots and stored at –80 °C.

Transcription activation test system in *L. lactis*. Transcription activation by wtComK and ComK variants was first tested in *L. lactis*. ComK expression was induced at the end of the exponential growth phase from the nisin-inducible promoter on the pNZ plasmids (De Ruyter *et al.*, 1996). Transcription activation was tested using the *lacZ* reporter under control of the *B. subtilis* *comG* promoter on plasmid pG-wt (K. A. Susanna and others, unpublished). Samples were taken at the moment of induction and at 30 min intervals for 2 h. β -Galactosidase activity was determined as described by Israelsen *et al.*

(1995). Control samples were analysed for protein expression by SDS-PAGE (Laemmli, 1970) and Western blotting (Towbin *et al.*, 1979). His-tagged ComK proteins were detected with a His₆-specific first antibody and an anti-mouse horseradish peroxidase secondary antibody (both from Amersham) and visualized by chemiluminescent detection using the ECL Western blotting analysis system (Amersham).

Transcription activation test system in *B. subtilis*. Transcription activation by Δ N9ComK was compared with that by wtComK in *B. subtilis* using the *lacZ* gene on plasmid pG-wt as reporter. Samples for β -galactosidase assays were taken from the transition point until 4 h into the stationary growth phase, at 1 h intervals. Expression of wtComK and Δ N9ComK was checked by Western blot analysis, using a ComK-specific first antibody (Van Sinderen & Venema, 1994) and an anti-rabbit horseradish peroxidase secondary antibody (Amersham). To indicate the relative amounts of wtComK and Δ N9ComK in *B. subtilis*, the intensity of similar-sized areas covering the signal of wtComK or Δ N9ComK was determined using the software package of Quantity One. The intensity of wtComK in *B. subtilis* strain pGwt was set to 100% and the intensity of wtComK and Δ N9ComK in the other strains was represented as the percentage of this wtComK level.

Electrophoretic mobility shift assays (EMSAs). These were performed essentially as described previously (Hamoen *et al.*, 1998). A *comG*-promoter fragment was used as a probe, amplified by PCR with primers comG-AT2-EcoRI and comG-end-XbaI. The product was end-labelled with T4 polynucleotide kinase using [γ -³²P]ATP. EMSAs were performed with cell extracts and compared with shifts from pure ComK (purified from MBP-ComK fusions). Proteins and probes were premixed on ice in 20 μ l binding buffer (20 mM Tris/HCl (pH 8.0), 5 mM MgCl₂, 100 mM KCl, 0.5 mM dithiothreitol, 0.05 mg poly[d(I-C)] ml⁻¹, 0.05 mg BSA ml⁻¹ and 8.7%, v/v, glycerol). Complexes were allowed to form for 15 min at 37°C, followed by running 15 μ l of each sample on a non-denaturing 6%

polyacrylamide gel. Gels were run in TAE buffer (40 mM Tris/acetate, pH 8.2, and 2 mM EDTA) at 100 V, dried, and autoradiographed using a phosphor-screen (Packard). Read-outs of the screens were made using the Perkin Elmer-Packard Cyclone Storage phosphor system.

RESULTS

B. subtilis ComK is active in transcription in *L. lactis*

In order to determine the characteristics of the ComK truncation variants, a test system was developed to discriminate between effects on transcription activation, DNA binding and oligomerization. This was achieved by using an *in vitro* system to investigate DNA binding and oligomerization (discussed below) and an *in vivo* system in *L. lactis* to determine transcription activation. For this purpose, wild-type and mutant *B. subtilis* ComK were produced in *L. lactis* under control of the nisin-inducible promoter. As reporter for transcription activation, a (*B. subtilis*) *comG*-promoter-*lacZ* fusion on plasmid pG-wt was used. By using an inducible system in *L. lactis*, possible effects of the autostimulatory loop and of other *B. subtilis* regulators, such as MecA, could be circumvented, enabling a clean investigation of the transcription activation abilities of the ComK mutants. Before the test system could be used for the truncation variants, it was established for wild-type ComK (wtComK), which was expressed with a His-tag fusion to the N-terminus (His₆-ComK) or C-terminus (ComK-His₆). β -Galactosidase studies demonstrated that transcription activation at the *comG* promoter was stimulated by both

Fig. 2. ComK of *B. subtilis* is active when expressed in *L. lactis*. (a) β -Galactosidase assays were used to monitor transcription activation by His₆-ComK and ComK-His₆. ComK expression was induced in *L. lactis* at $t=3$ by adding nisin. A *comG*-promoter-*lacZ* fusion was used as a reporter for regulation by ComK. The mean transcription level and the standard deviation over three experiments is shown. Transcription is observed in cells expressing His₆-ComK ($\blacktriangle$) or ComK-His₆ ($\blacksquare$), but not in cells with empty plasmid pNZ8048 ($\blacklozenge$). (b) DNA binding of purified His₆-ComK shown using EMSAs. The *comG* promoter (with common K-box) was used as a probe. Binding of His₆-ComK purified from *L. lactis* (L) was compared with ComK purified from the MBP-fusion system in *E. coli* (E). Both proteins show a similar shift relative to unbound DNA (-). (c) Binding of His-tagged ComK was tested directly from extracts of induced cells with pNZ8048 (NZ), pNZ-His₆-ComK (H₆-K) or pNZ-ComK-His₆ (K-H₆).

His-tag ComK variants, indicating that ComK of *B. subtilis* is active in *L. lactis* (Fig. 2a). However, transcription activation by His₆-ComK was three to four times higher than that by ComK-His₆, despite a smaller difference (at most twofold) in protein expression, as was determined by Western blotting with an anti-His antibody (results not shown). This suggests that the position of the His-tag influences the ability of ComK to activate transcription. Furthermore, transcription is about 10-fold lower than normally observed for wtComK without a tag at the same promoter in *B. subtilis*.

ComK isolated from *L. lactis* shows normal binding

Extensive research on ComK revealed the binding characteristics of purified ComK from *B. subtilis* using EMSAs (Hamoen *et al.*, 1998). In the present study, ComK was expressed as a His-tag fusion, which might influence DNA binding or oligomerization of the protein. In order to determine the effect of the His-tag, His₆-ComK was isolated from *L. lactis* using a Talon column. However, all the His₆-ComK ended up in the unbound fraction, indicating that the His-tag is inaccessible for column binding. To solve this problem, purification was performed under denaturing conditions, using buffers with 8 M urea. Under these conditions, His₆-ComK did bind to the column and could be purified. The purified protein could successfully be renatured to active DNA-binding His₆-ComK, as was demonstrated by EMSAs (Fig. 2b). The binding characteristics of His₆-ComK were comparable to binding of ComK purified from the MBP-ComK system in *E. coli*, as described by Hamoen *et al.* (1998). Since His₆-ComK could only be

purified under denaturing conditions, an alternative system was tested, using cell extracts. Although the observed band is weaker than for purified proteins, DNA binding with typical ComK characteristics could be demonstrated using cell extracts of *L. lactis* producing either His₆-ComK or ComK-His₆ (Fig. 2c). This possibility provided a quick tool to test binding of ComK truncation variants directly from cell extracts. Furthermore, this approach prevents wrong interpretations of binding characteristics of mutants, due to potential unsuccessful renaturing of the truncation variants. Therefore, all *in vitro* binding experiments in this study were performed with cell extracts instead of purified ComK variants.

The C-terminal region of ComK is required for transcription activation

As can be seen in Fig. 1, ComK proteins from different bacterial species show clear sequence conservation throughout the entire protein, with the exception of the N-terminal end. The C-terminal part is not conserved for all ComK proteins, but shows conservation when subgroups are considered, e.g. *B. subtilis* and *B. licheniformis*, for which most of the last 35 aa are identical or similar. Since the C-terminal region is partially conserved, a specific role for this part of ComK is expected. To investigate this role, truncations lacking 25 or 35 aa from the C-terminus were constructed and expressed as N-terminal His-tag fusions in *L. lactis*. β -Galactosidase assays were used to determine transcription at the (*B. subtilis*) *comG*-promoter, demonstrating that transcription activation by both His₆-ComK Δ C25 and His₆-ComK Δ C35 is completely abolished (Fig. 3a), despite a normal protein production level as

Fig. 3. The C-terminal region of ComK is required for transcription activation. (a) β -Galactosidase assays were performed, comparing His₆-ComK (■) with His₆-ComK Δ C25 (×) and His₆-ComK Δ C35 (▲) in *L. lactis*. As a negative control *L. lactis* pNZ8048 (◆) was used. Protein overexpression was induced at $t = 3$. The mean transcription level and the standard deviation over three experiments is shown. β -Galactosidase activity in cells overexpressing truncation variants is comparable with the negative control, demonstrating the requirement of the C-terminal part of ComK for transcription activation. (b) EMSAs were performed with cell extracts demonstrating binding of wtComK and the C-terminal truncation variants, although with a smaller shift for His₆-ComK Δ C25 and His₆-ComK Δ C35. (c) Western blots were performed using a His₆-specific first antibody to compare expression levels of the different ComK variants in *L. lactis*. Lanes in (b) and (c): -, blank; H₆-K, His₆-ComK; C25, His₆-ComK Δ C25; C35, His₆-ComK Δ C35.

shown by SDS-PAGE and Western blot experiments using an anti-His antibody (Fig. 3c). The loss of transcription activation might be due to a loss of DNA binding or to a defect in the transcription activation mechanism. To discriminate between these possibilities, EMSAs were performed with cell extracts containing His₆-ComK, His₆-ComKΔC25 or His₆-ComKΔC35; these demonstrated that both ComK mutants could still bind DNA (Fig. 3b). However, the observed shift by binding of the mutant proteins was smaller than the wtComK shift and, for His₆-ComKΔC35, the band is much weaker.

Effect of C-terminal truncations on tetramerization of ComK

Since the loss of transcription activation by the C-terminal truncation variants of ComK is not due to a loss of DNA binding, it is expected that the C-terminal region is involved in another aspect of ComK interactions. An interesting hypothesis is that the C-terminal part is required for tetramerization of ComK, which would explain the smaller shift observed for the mutants as binding of a single ComK dimer, due to the loss of cooperative binding of two dimers forming a tetramer. To separate tetramerization from dimer binding, EMSAs were performed using a probe of the wild-type *addAB* promoter and one in which 5 bp were introduced in between the two AT-boxes (*addAB* + 5). Introduction of 5 bp, corresponding to half a helical turn, positions both AT-boxes (and thus the two bound ComK dimers) on opposite sides of the DNA helix, thereby eliminating the possibility of forming a tetramer (Hamoen *et al.*, 1998). Using this approach, we demonstrated that small differences in protein size, due to the truncations, only resulted in small differences in shift when the AT-boxes were located on opposite sides of the DNA helix so that only dimer binding could occur (Fig. 4, lower panel). This can by no means explain the large differences in shifts observed when both AT-boxes were located on the same side of the DNA helix (Fig. 4, upper panel). Furthermore, the shifts by His₆-ComKΔC25 and His₆-ComKΔC35 are similar for both probes, indicating that in both situations, these mutants bind as dimers instead of tetramers. The wtComK shift on the other hand increases from the *addAB* + 5 to the *addABwt* K-box, corresponding to oligomerization from dimers into tetramers. For wtComK binding, tetramerization is accompanied by DNA bending. A loss of tetramerization for the C-terminal truncation variants and thus a decrease of induced bending would explain the loss of transcription activation, since previous studies demonstrated that the main effect of ComK in transcription activation at the *comG* promoter is to stabilize binding of RNA polymerase, by facilitating interactions with the upstream DNA through bending of the promoter region (Susanna *et al.*, 2004).

Hyperactive ComK by truncation of 9 aa from the N-terminus

As noted, the N-terminal part of ComK is not strictly conserved (Fig. 1). This observation raises curiosity about

Fig. 4. ComK C-terminal truncations bind as dimers instead of tetramers. Binding of His₆-ComK, His₆-ComKΔC25 and His₆-ComKΔC35 was compared on a K-box with both AT-boxes on the same side of the DNA-helix (*addABwt*) and a K-box with the AT-boxes on opposite sides of the helix (*addAB* + 5). Increasing amounts of cell extract were used (0.5, 1 and 2 μl) for each protein overexpression, indicated by the concentration bar (–, no cell extract added). Arrows indicate the positions of ComK-dimer (d) and ComK-tetramer (t) binding. Both gels were run under the same conditions and for equal times.

the function of this region. As a first attempt to elucidate its role in different interactions, a truncation of 9 aa from the N-terminus was constructed and expressed in *L. lactis* (His-tag fusion to C-terminus). The ability of this mutant, ΔN9ComK-His₆, to activate transcription was tested as described for the C-terminal truncations. Surprisingly, transcription activation by ΔN9ComK-His₆ was increased about two- to threefold compared to wtComK-His₆ (Fig. 5a). As for the other mutants, DNA binding was determined using EMSAs, demonstrating that binding by ΔN9ComK-His₆ was also increased compared to ComK-His₆ (Fig. 5b), while Western blots indicated similar expression levels for both proteins (results not shown).

ΔN9ComK is relatively more active than wtComK in *B. subtilis*

The transcription test system in *L. lactis* provides a quick method to investigate the characteristics of the ComK truncation mutants. However, compared with *B. subtilis*, the test system in *L. lactis* is incomplete, considering all known other regulatory inputs, like the autostimulatory loop involved in *comK* expression and the proteolytic degradation system consisting of MecA/ClpCP. The finding of a hyperactive ComK variant raises the question whether this characteristic is specific for the truncation of the extreme N-terminal region or whether it results from a lack of regulatory input in *L. lactis*. In order to answer this question, ΔN9ComK (without His-tag) was expressed in *B. subtilis*. The constructed test strain, *B. subtilis* pG9ΔK, contained the

Fig. 5. An N-terminal truncation results in a hyperactive ComK protein. (a) β -Galactosidase assays were performed to determine transcription activation at the *comG* promoter-*lacZ* fusion. ComK overexpression was induced in *L. lactis* at $t=3$. As a negative control, *L. lactis* pNZ8048 ($\blacklozenge$) was used. The mean transcription level and the standard deviation over three experiments is shown. Induction of Δ N9ComK-His₆ (▲) results in a two- to threefold higher transcription activation than overexpression of ComK-His₆ (■). (b). EMSAs were performed to investigate DNA binding to the K-box of the *comG* promoter. DNA binding from an extract of cells overexpressing Δ N9ComK-His₆ was compared with *L. lactis* overexpressing ComK-His₆. -, Blank; K-H₆, ComK-His₆; N9, Δ N9ComK-His₆.

comK region with Δ N9ComK under control of the *wtcomK* promoter integrated into the *amyE* locus, plasmid pG-wt to determine transcription activation and a deletion of the wild-type copy of *comK*. As a control *B. subtilis* pGwt was

Fig. 6. Transcription activation by Δ N9ComK is lower than that by wtComK. β -Galactosidase assays were performed to determine transcription activation by wtComK and Δ N9ComK in *B. subtilis*, using a *comG*-promoter-*lacZ* fusion on plasmid pG-wt as a reporter. Both wtComK and Δ N9ComK were transcribed under control of the *comK* promoter, located upstream of their respective genes. As a negative control, *B. subtilis* Δ *comK* was used. The mean transcription level and the standard deviation over two experiments is shown. ■, *B. subtilis* pGwt; ▲, *B. subtilis* pG9 Δ K; ◆, *B. subtilis* Δ *comK*.

constructed, containing wtComK and plasmid pG-wt. β -Galactosidase assays with these two strains demonstrated that the level of transcription activation by Δ N9ComK was about twofold lower than for wtComK (Figs 6 and 7). However, Western blot analysis showed that the expression level for Δ N9ComK was about five- to sixfold lower than for wtComK (Fig. 7b), indicating that, also in *B. subtilis*, Δ N9ComK is relatively more active in transcription activation than wtComK.

The lower protein level of Δ N9ComK is probably not due to altered MecA interactions

As described above, expression of Δ N9ComK in *B. subtilis* is four to five times lower than for wtComK. This wtComK level in strain pGwt is set to 100% in Fig. 7 and is in this text referred to as the standard wtComK-level. A possible explanation for the lower expression of Δ N9ComK could be that the truncation of 9 aa from the N-terminus alters the interaction between ComK and MecA, which targets ComK for proteolysis. To investigate this possibility, a *mecA* deletion was introduced into *B. subtilis* pG9 Δ K, resulting in strain pG9 Δ K- Δ M. As shown in Fig. 7, the levels of Δ N9ComK expression and β -galactosidase activity increased upon introduction of Δ *mecA*, resulting in protein expression comparable to the standard wtComK-level; so effectively, in this pG9 Δ K- Δ M strain, four to five times as much Δ N9ComK is produced as when a functional *mecA* gene is present. Also transcription activation is increased to about four- to fivefold of the pGwt level. Δ N9ComK expression and transcription activation could not be compared with wtComK under the same conditions, since *B. subtilis* strain pGwt- Δ M turned out to be unstable. However, previous research demonstrated a dramatic overexpression of wtComK in a Δ *mecA* background to levels several times the wild-type ComK level (Kong & Dubnau, 1994). So although the level of Δ N9ComK is increased upon introduction of a *mecA* deletion, the level is still lower than what would be expected based on previous research for wtComK, suggesting that altered interactions of Δ N9ComK with MecA are not the main reason for the low protein expression level.

DISCUSSION

In competence regulation, ComK is involved in protein-DNA as well as protein-protein interactions (with MecA and in ComK oligomerization). DNA binding and MecA interactions could be exclusive, but at least DNA binding and oligomerization occur simultaneously. An interesting question is whether the small ComK protein (22.8 kDa) contains specific functional domains responsible for one or more of these interactions. In this study, an attempt was made to elucidate the roles of the N- and C-terminal regions of ComK. A test system with a maximal dissection of ComK functions was developed successfully. We demonstrated that *B. subtilis* ComK could be expressed with an N- or C-terminal His-tag fusion in *L. lactis*, while retaining

Fig. 7. Overview of transcription activation and protein expression for ΔN9ComK and wtComK in different genetic backgrounds. (a) For each strain, the schematic drawing indicates whether the cell contains wtComK or ΔN9ComK (expressed under control of its own promoter in the *amyE* locus) and *mecA*. Deletions are indicated by crosses. *comG* transcription activation and ComK expression are shown as percentage of the level observed for *B. subtilis* pGwt. (b) Western blots were performed with a ComK-specific first antibody to demonstrate the level of ComK expression in the different strains. wt, pGwt; 9ΔK, pG9ΔK; 9ΔM, pG9ΔK-ΔM. X indicates a non-specific band which serves as a control for the total protein level of the samples.

transcription activation activity and normal DNA-binding characteristics (Fig. 2). However, the transcription activation observed from a *comG-lacZ* fusion in *L. lactis* is about 10-fold lower than that from the same promoter in *B. subtilis*, although nisin-induced ComK expression in *L. lactis* should be sufficient to stimulate *comG* transcription to high levels. It might be that the presence of the His-tag on ComK decreases transcription activation. At least the position of this tag influences DNA binding and transcription activation by ComK (Fig. 2). Alternatively, the lower transcription in *L. lactis* could result from differences between the RNA polymerases of *L. lactis* and *B. subtilis*. Nevertheless, the fact that *B. subtilis* ComK can activate transcription from the *comG* promoter in *L. lactis* provides a suitable test-system for ComK-truncation mutants.

The C-terminal region of ComK was shown to be required for transcription activation, which is completely abolished upon removal of 25 aa (Fig. 3). Western blot assays showed that protein production levels were comparable to the level of His₆-ComK, indicating that the loss of transcription activation does not result from reduced protein production. Furthermore, we have shown that increasing the ComK level in *L. lactis* by a higher induction does not lead to a higher transcription level, indicating that the ComK concentration is not limiting for transcription activation and that an increase in ComK levels cannot compensate for the observed transcription activation deficiency of the mutants

(unpublished data). The DNA-binding affinity of His₆-ComKΔC25 is not affected, but the extent of the shift is smaller, suggesting binding of a single ComK dimer instead of a tetramer. Previous research showed that oligomerization of ComK dimers into a tetramer can only occur when both AT-boxes are located on the same side of the DNA helix (Hamoen *et al.*, 1998). When the two boxes are positioned on opposite sides of the helix, ComK is forced to bind as a dimer. By inverting the orientation of the AT-boxes, a similar shift for His₆-ComKΔC25 or His₆-ComKΔC35 was demonstrated for both the probe with inverted AT-boxes and the one with normal AT-boxes, indicating dimer binding in both cases, whereas the shift for wtComK differs from dimer to tetramer binding between the two situations. Binding of ComK tetramers is accompanied by DNA bending, which will be affected by the loss of tetramerization in the C-terminal truncation variants. This explains the loss of transcription activation, since previous research showed that the major effect of ComK on transcription activation is on stabilizing RNAP binding, probably via facilitating contacts between RNA polymerase and upstream DNA, enabled by DNA bending (Susanna *et al.*, 2004).

In addition to a function for the C-terminal region of ComK, this study also investigated the role of the N-terminal part of ComK in the competence regulatory pathway. To our surprise, removing 9 aa from the N-terminus of ComK yielded a variant hyperactive in transcription activation in *L.*

lactis (Fig. 5). The DNA-binding affinity of this variant is higher as well. However, using *L. lactis* as a host might mask effects of the N-terminal truncation in relation to other regulatory inputs, which are present in *B. subtilis*, but not in *L. lactis*. Compared with the complex competence regulatory pathway in *B. subtilis*, the system in *L. lactis* is incomplete, since it lacks for example MecA. Furthermore, by using an inducible system, potential effects on the autostimulatory loop involved in *comK* transcription are missed. In order to determine the biological relevance of the N-terminal region of ComK, a Δ N9ComK variant was introduced into the *amyE* locus of *B. subtilis* under control of the *comK* promoter. Although the absolute level of transcription of *comG-lacZ* was twofold lower, Δ N9ComK was relatively more active in transcription activation in *B. subtilis* as well, since the protein expression level was five- to sixfold lower than wtComK expression (Figs 6 and 7). A possible explanation for the decreased expression of Δ N9ComK could be an altered interaction with MecA/ClpCP, resulting in a faster breakdown of ComK or a slower release of ComK from the complex as a response to interactions of ComS with MecA at the onset of the stationary growth phase. To investigate the interactions with MecA, a *mecA* deletion was introduced, resulting in an increase in protein expression to wild-type level accompanied by a four- to fivefold enhancement of transcription activation by Δ N9ComK. However, the proper control experiment could not be performed, since a stable *B. subtilis* Δ *mecA* strain could not be obtained, despite several attempts (Fig. 7). Previous research described the construction of such a mutant, although high revertance was reported, which might cause the instability of the strain. However, the reports described a level of wtComK overexpression that was much higher than the level achieved in this study for Δ N9ComK (Kong & Dubnau, 1994), suggesting that, despite the increase of Δ N9ComK expression in a Δ *mecA* background, an altered interaction between Δ N9ComK and MecA is not the main reason for the low expression level. As an alternative, a *clpC* deletion was introduced, since ClpC acts on the same proteolytic control system as MecA. Δ *clpC* strains could be obtained in both a *B. subtilis* pGwt and a pG9 Δ K background, although again pGwt was more affected by the lack of ClpC than pG9 Δ K, as became clear from the slower growth rate of *B. subtilis* pGwt. In the Δ *clpC* strain pGwt- Δ C, transcription activation by wtComK and its protein expression level increased three- to fourfold compared to *B. subtilis* pGwt. In contrast, however, transcription activation by Δ N9ComK increased only slightly to 50–75 % of the level of pGwt, which means little or no increase compared to the original Δ N9ComK strain, pG9- Δ K (results not shown). Taken together, these results suggest that the low expression of Δ N9ComK is not due to altered interactions with the MecA/ClpCP proteolytic degradation complex. Other possible explanations for the decreased protein expression of Δ N9ComK could be a reduced transcription activation at the *comK* promoter or, alternatively, a decreased intrinsic stability of Δ N9ComK. The latter possibility is not the most likely, since the protein could be normally expressed in *L. lactis*, without displaying

instability. Since Δ N9ComK was shown to be more active in transcription activation, it might not seem very likely that the reduced protein production is due to a lowered transcription activation of *comK*. However, in this study, transcription activation was tested at the *comG* promoter, which differs significantly from the *comK* promoter and might be easier to activate. For example, the *comG* promoter does not need any other activators than ComK, while at the *comK* promoter DegU is required to stimulate ComK-activated transcription when ComK concentrations are low. Furthermore, in the K-box of the *comK* promoter the spacing between the start positions of the two AT-boxes spans four helical turns, compared with three turns in the *comG* promoter. It might be that this longer spacing requires other abilities of the ComK protein. If the N-terminal region of ComK were required for one of these mechanisms, a truncation of this part would result in lower transcription activation at the *comK* promoter and therefore in a reduced ComK production. More research is required to determine the exact role of the N-terminal region of ComK in the function of the protein. However, the fact that truncation of the N-terminal region of ComK results in higher transcription activation, but lower protein expression, suggests that the presence of this region is a trade-off between optimal transcription activation and a thus far unidentified role in regulation of transcription, activity or stability of ComK.

This study presents a general overview of the location of two domains affecting ComK behaviour. Further research is required to determine the exact role of the N-terminal region of ComK in the activity and/or stability of the protein. Furthermore, despite the elucidation of the C-terminal region as being required for tetramerization, the exact mechanism and the amino acid residues involved remain unknown. However, it is tempting to speculate on possible mechanisms underlying tetramerization. For example, the C-terminal part of ComK contains many charged residues, suggesting that the distribution of negative and positive charges enables electrostatic interactions of this region with the C-terminal part of other ComK proteins or, alternatively, interactions with other charged regions in the protein. The same mechanism could apply for ComK proteins in other species, since all ComK proteins have a relatively high proportion of charged residues in their C-terminal region.

Functional domains for the other indicated ComK interactions, like dimerization, DNA binding and interactions with MecA, have not been identified yet. However, the significantly lower DNA-binding affinity of ComK Δ C35, as compared to wtComK and ComK Δ C25, may indicate that the C-terminal region of ComK also forms part of the DNA-binding domain. This interpretation is supported by the observation that ComK displays limited sequence homology with the DNA-binding domain of human hSR γ , which interestingly is a minor groove binder as well (Werner *et al.*, 1995). The region showing the highest homology is slightly touched in ComK Δ C35, while most of the homologous region is located further towards the middle segment of

ComK and is not affected by any of the truncations. It has been suggested before that the DNA-binding mechanism of ComK could be similar to that of hSRY (Hamoen *et al.*, 1998), which would be an interesting starting point for further research. In addition, further research is required to elucidate the domains involved in dimerization and interactions with MecA.

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