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

Kim A. Susanna, Fabrizia Fusetti, Andy-Mark W. H. Thunnissen, Leendert W. Hamoen and Oscar P. Kuipers

1Department of Genetics, University of Groningen, NL-9751 NN Haren, The Netherlands
2Laboratory of Biophysical Chemistry, Department of Chemistry, University of Groningen, NL-9747 AG Groningen, The Netherlands

The competence transcription factor ComK is the master regulator of competence development in *B. 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 (∆N9ComK) showed higher transcription activation than wild-type ComK, when expressed in *Lactococcus lactis*. However, in *B. subtilis*, transcription activation by ∆N9ComK was twofold lower than that by wild-type ComK, resulting from a five- to sixfold lower protein level of ComK∆N9. Thus, relatively, ∆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.

INTRODUCTION

The competence transcription factor ComK is the key regulatory protein in competence development in *B. 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; Serror & 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; Serror & 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).
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 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).
Functional analysis of ComK

Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant features</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL1blue</td>
<td>endA1 gyrA96 thi hsdR17 (rK– mC–) supE44 relA1 lacZ F' proAB lacY1 lacZΔM15 Tn10</td>
<td>Stratagene</td>
</tr>
<tr>
<td><strong>L. lactis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZ90000</td>
<td>MG1363 pepN::nisRK</td>
<td>Kuipers et al. (1998)</td>
</tr>
<tr>
<td>NZ97000</td>
<td>Nisin producing, contains Tn5276</td>
<td>Kuipers et al. (1993)</td>
</tr>
<tr>
<td><strong>B. subtilis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>168</td>
<td>trpC2</td>
<td>Anagnostopoulos &amp; Spizizen (1961)</td>
</tr>
<tr>
<td>QB4650</td>
<td>ΔmecA, Km'</td>
<td>Msaed et al. (1998)</td>
</tr>
<tr>
<td>168 amyE::ΔN9comK</td>
<td>amyE::ΔN9comK, Cm'</td>
<td>This work</td>
</tr>
<tr>
<td>pG9</td>
<td>amyE::ΔN9comK, pG-wt, Cm', Ery' Sp'</td>
<td>This work</td>
</tr>
<tr>
<td>pG9AK</td>
<td>pG9 + ΔcomK, Cm', Ery' Sp'</td>
<td>This work</td>
</tr>
<tr>
<td>pG9AK-ΔM</td>
<td>pG9AK + ΔmecA, Cm', Ery' Sp' Km'</td>
<td>This work</td>
</tr>
<tr>
<td>pGwt</td>
<td>pG-wt, Ery'</td>
<td>K. A. Susanna and others, unpublished</td>
</tr>
<tr>
<td>pGwt-ΔM</td>
<td>pG-wt, ΔmecA, Ery' Km'</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMal-c2X</td>
<td>Amp', IPTG-inducible malE with MCS</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>pMal-ComK</td>
<td>Amp', IPTG-inducible malE-comK</td>
<td>This work</td>
</tr>
<tr>
<td>pDL-ΔN9ComK</td>
<td>Cm', ΔN9comK region flanked by amyE</td>
<td>This work</td>
</tr>
<tr>
<td>pNZ8048</td>
<td>Cm', inductible expression vector with PnisA</td>
<td>Kuipers et al. (1998)</td>
</tr>
<tr>
<td>pNZ-Hisr-ComK</td>
<td>Cm', hisr-comK under control of Pnis</td>
<td>This work</td>
</tr>
<tr>
<td>pNZ-ComK-Hisr</td>
<td>Cm', comK-hisr, under control of Pnis</td>
<td>This work</td>
</tr>
<tr>
<td>pNZ-ComKAC25</td>
<td>Cm', hisr-comKAC25 under Pnis control</td>
<td>This work</td>
</tr>
<tr>
<td>pNZ-ComKAC35</td>
<td>Cm', hisr-comKAC35 under Pnis control</td>
<td>This work</td>
</tr>
<tr>
<td>pNZ-ΔN9ComK</td>
<td>Cm', ΔN9comK-hisr under Pnis control</td>
<td>This work</td>
</tr>
<tr>
<td>pLOR14</td>
<td>Ery', pl232 with MCS and promoterless lacZ of pOR113</td>
<td>Larsen et al. (2004)</td>
</tr>
<tr>
<td>pG-wt</td>
<td>Ery', comG-lacZ with common K-box</td>
<td>K. A. Susanna and others, unpublished</td>
</tr>
</tbody>
</table>

DNA manipulations, materials and transformations. Standard molecular biology methods were used (Ausubel et al., 1989; 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 Products Purification kit, respectively, were used (Roche). Chromosomal DNA of B. subtilis was isolated as described by Venema et al. (1967). E. coli strains were transformed using CaCl2-induced competence (Sambrook et al., 1989) and L. lactis strains using electroporation with a gene-pulsed (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.

Histagged 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 Ncol and HindIII and cloned into Ncol/HindIII-digested plasmid pNZ8048, resulting in pNZ-ComK-Hisr-ComK. For wtComK with a C-terminal His-tag, primers comK-Nice and comK-H6 were used to amplify comK. After digestion with RecI and HindIII, the PCR product was cloned into Ncol/HindIII-digested pNZ8048, yielding plasmid pNZ-ComK-Hisr-ComK. 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 with stop1 or comK-H6 for the truncation of 25 or 35 aa from the C-terminus, respectively. The PCR products were digested with Ncol/HindIII and cloned into pNZ8048, also digested with Ncol/HindIII, yielding pNZ-ΔN9ComK, pNZ-ComKAC25 and pNZ-ComK ΔC35, respectively.
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 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 μl 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 11 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 μg ml-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 (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.
Functional analysis of ComK

Transcription activation test system in *B. subtilis*. Transcription activation by ΔN9ComK 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 ΔN9ComK 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 ΔN9ComK in *B. subtilis*, the intensity of similar-sized areas covering the signal of wtComK or ΔN9ComK 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 ΔN9ComK 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 μl binding buffer (20 mM Tris/HCl (pH 8.0), 5 mM MgCl2, 100 mM KCl, 0.5 mM dithiothreitol, 0.05 mg poly[d[1-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 μl 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 clear 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 (His8-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 His8-ComK and ComK-His8. 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 His8-ComK (▲) or ComK-His8 (●), but not in cells with empty plasmid pNZ8048 (○). (b) DNA binding of purified His8-ComK shown using EMSAs. The comG promoter (with common K-box) was used as a probe. Binding of His8-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).](http://mic.sgmjournals.org)
His-tag ComK variants, indicating that ComK of *B. subtilis* is active in *L. lactis* (Fig. 2a). However, transcription activation by His<sub>6</sub>-ComK was three to four times higher than that by ComK-His<sub>6</sub>, 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, His<sub>6</sub>-ComK was isolated from *L. lactis* using a Talon column. However, all the His<sub>6</sub>-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, His<sub>6</sub>-ComK did bind to the column and could be purified. The purified protein could successfully be renatured to active DNA-binding His<sub>6</sub>-ComK, as was demonstrated by EMSAs (Fig. 2b). The binding characteristics of His<sub>6</sub>-ComK were comparable to binding of ComK purified from the MBP–ComK system in *E. coli*, as described by Hamoen *et al.* (1998). Since His<sub>6</sub>-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 His<sub>6</sub>-ComK or ComK-His<sub>6</sub> (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 His<sub>6</sub>-ComKΔC25 and His<sub>6</sub>-ComKΔC35 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 His<sub>6</sub>-ComK (■) with His<sub>6</sub>-ComKΔC25 (×) and His<sub>6</sub>-ComKΔC35 (▲) 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 His<sub>6</sub>-ComKΔC25 and His<sub>6</sub>-ComKΔC35. (c) Western blots were performed using a His<sub>6</sub>-specific first antibody to compare expression levels of the different ComK variants in *L. lactis*. Lanes in (b) and (c): −, blank; H<sub>6</sub>-K, His<sub>6</sub>-ComK; C25, His<sub>6</sub>-ComKΔC25; C35, His<sub>6</sub>-ComKΔC35.
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-ComKAC25 or His6-ComKAC35; 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-ComKAC35, 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-ComKAC25 and His6-ComKAC35 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 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 MeC/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
negative control, L. lactis ComK overexpression was induced in L. lactis D binding from an extract of cells overexpressing L. lactis. Transcription activation by Fig. 6. was compared with L. lactis mine transcription activation at the comG b protein. (a) An N-terminal truncation results in a hyperactive ComK K. A. Susanna and others Dments is shown. Induction of ComK-His6 (▲) results in a two- to threefold higher transcription activation than overexpres- sion 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 ΔN9ComK-His6 was compared with L. lactis overexpressing ComK-His6 —, Blank; K-H6, ComK-His6; N9, ΔN9ComK-His6.

comK region with ΔN9ComK 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 constructed, containing wtComK and plasmid pG-wt. β-Galactosidase assays with these two strains demonstrated that the level of transcription activation by ΔN9ComK was about twofold lower than for wtComK (Figs 6 and 7). However, Western blot analysis showed that the expression level for ΔN9ComK was about five- to sixfold lower than for wtComK (Fig. 7b), indicating that, also in B. subtilis, ΔN9ComK is relatively more active in transcription activation than wtComK.

The lower protein level of ΔN9ComK is probably not due to altered MecA interactions

As described above, expression of ΔN9ComK 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 ΔN9ComK 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 pG9AK, resulting in strain pG9AK-ΔM. As shown in Fig. 7, the levels of ΔN9ComK expression and β-galactosidase activity increased upon introduction of ΔmecA, resulting in protein expression comparable to the standard wtComK-level; so effectively, in this pG9AK-ΔM strain, four to five times as much ΔN9ComK is produced as when a functional mecA gene is present. Also transcription activation is increased to about four- to fivefold of the pGwt level. ΔN9ComK expression and transcription activation could not be compared with wtComK under the same conditions, since B. subtilis strain pGwt-ΔM turned out to be unstable. However, previous research demonstrated a dramatic overexpression of wtComK in a ΔmecA background to levels several times the wild-type ComK level (Kong & Dubnau, 1994). So although the level of ΔN9ComK 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 ΔN9ComK 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 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
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 investigate the biological relevance of the N-terminal region of ComK, a ΔN9ComK 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, ΔN9ComK 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 ΔN9ComK 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 ΔN9ComK. However, the proper control experiment could not be performed, since a stable B. subtilis ΔmecA 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 ΔN9ComK (Kong & Dubnau, 1994), suggesting that, despite the increase of ΔN9ComK expression in a ΔmecA background, an altered interaction between ΔN9ComK 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. ΔclpC strains could be obtained in both a B. subtilis pGwt and a pG9ΔK background, although again pGwt was more affected by the lack of ClpC than pG9ΔK, as became clear from the slower growth rate of B. subtilis pGwt. In the ΔclpC strain pGwt–ΔC, transcription activation by wtComK and its protein expression level increased three- to fourfold compared to B. subtilis pGwt. In contrast, however, transcription activation by ΔN9ComK increased only slightly to 50–75% of the level of pGwt, which means little or no increase compared to the original ΔN9ComK strain, pG9–ΔK (results not shown). Taken together, these results suggest that the low expression of ΔN9ComK is not due to altered interactions with the MecA/ClpCP proteolytic degradation complex. Other possible explanations for the decreased protein expression of ΔN9ComK could be a reduced transcription activation at the comK promoter or, alternatively, a decreased intrinsic stability of ΔN9ComK. The latter possibility is not the most likely, since the protein could be normally expressed in L. lactis, without displaying instability. Since ΔN9ComK 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 ComKAC35, as compared to wtComK and ComKAC25, 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 hSRY, which interestingly is a minor groove binder as well (Werner et al., 1995). The region showing the highest homology is slightly touched in ComKAC35, 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.

ACKNOWLEDGEMENTS

We would like to thank Wiel Klaas Smits and Jan-Willem Veening for helpful discussions. Furthermore, we would like to thank Anton Steen and Rasmus Larsen for help with the first work in L. lactis.

REFERENCES


