

KdpE of *Clostridium acetobutylicum* is a Highly Specific Response Regulator Controlling only the Expression of the *kdp* Operon

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Abstract

KdpE from *Clostridium acetobutylicum* was enriched in form of its Strep-tag-derivative to allow easy immunodetection. It could be artificially phosphorylated by acetyl phosphate or carbamyl phosphate. Only phosphorylated clostridial KdpE was able to bind to a region upstream of the clostridial *kdp* structural genes. The minimal sequence requirements for binding were determined and found to share significant similarity with the *Escherichia coli* KdpE binding motif. However, the clostridial protein proved to be much more specific and did not bind in unphosphorylated form or to other similar sequences either from *C. acetobutylicum* or *E. coli*. In contrast, the enterobacterial protein recognized the clostridial binding motif. An HPt domain has been detected in KdpD from *C. acetobutylicum*, the cognate sensor kinase of KdpE. The data reported indicate that in *E. coli*, KdpE might represent a regulatory checkpoint for different phosphorelay signalling pathways, whereas in *C. acetobutylicum* KdpD might serve this function.

Introduction

K⁺ is one of the most important components in osmoadaptation of bacteria and controls turgor pressure and the activity of a number of enzymes (for a review see Csonka and Epstein, 1996). Thus, several different efflux and influx systems for this cation have been developed in cells (Stumpe *et al.*, 1996). Under potassium-limiting conditions, high-affinity uptake permeases are active such as the Kdp transporter, which occurs in a number of prokaryotes and has been well characterized (Altendorf and Epstein, 1993; Siebers and Altendorf 1993; Stumpe *et al.*, 1996; Altendorf *et al.*, 1998).

The Kdp system represents an unusual P-type ATPase, and detailed information on this protein complex is available for *Escherichia coli* (Altendorf and Epstein, 1993; Altendorf *et al.*, 1998) and *Clostridium acetobutylicum* (Treuner-Lange *et al.*, 1997). It consists of four (KdpFABC) or six (KdpZYABCX) subunits, respectively, and its

expression at the transcriptional level is controlled by a so-called two-component system consisting of the sensor kinase KdpD and the response regulator KdpE (Polarek *et al.*, 1992; Walderhaug *et al.*, 1992; Treuner-Lange and Dürre, 1996). The fact that induction was not only caused by K⁺-limitation but also upon osmotic stress, led to the hypothesis that changes in turgor pressure represent the signal that leads to induction of transcription of the ATPase structural genes (Laimins *et al.*, 1981; Malli and Epstein, 1998). However, this hypothesis is questioned by data showing e. g. that nonionic solutes changed the osmolarity of the growth medium, but did not exert an effect on *kdp* expression (Gowrishankar, 1987; Asha and Gowrishankar, 1993; Frymier *et al.*, 1997).

As a consequence much work has been devoted to elucidating the signal(s) recognized by the sensor kinase KdpD. The protein has been purified, reconstituted in proteoliposomes, mutated in a number of different residues, and shown to function as a homodimer (Voelkner *et al.*, 1993; Heermann *et al.*, 1998; Jung and Altendorf, 1998; Jung *et al.*, 1998). An analysis of its membrane topology revealed that it possesses four membrane-spanning segments in the middle of the polypeptide chain, whereas N and C terminus are both cytoplasmic (Zimmann *et al.*, 1995). KdpD is an unusual sensor kinase in that it contains a very conserved N-terminal region that is not found in other histidine kinase signal transducers (Treuner-Lange and Dürre, 1996). Interestingly, a gene encoding a homologue of this conserved N terminus has been detected in the cyanobacterium *Synechocystis* sp. PCC 6803 without an accompanying *kdpE* gene (Kaneko *et al.*, 1996).

Comparatively few data are available for the cognate response regulator KdpE. After demonstration that it is a member of two-component systems (Polarek *et al.*, 1992; Walderhaug *et al.*, 1992) its phosphorylation/dephosphorylation by KdpD (and also EnvZ) and determination of its binding site upstream of the *kdp* structural genes have been reported (Nakashima *et al.*, 1992; Sugiura *et al.*, 1992; Nakashima *et al.*, 1993; Sugiura *et al.*, 1993).

A surprising finding was that upon induction of *kdp* structural gene expression, transcription continued through the adjacent *kdpDE* operon in *C. acetobutylicum* (Treuner-Lange *et al.*, 1997). The same seems to be true for *E. coli* (Polarek *et al.*, 1992; Voelkner *et al.*, 1993). Since it does not make much sense for a regulator to be expressed at high levels after induction has taken place, it has been hypothesized that KdpD and/or KdpE might additionally regulate other operons (Treuner-Lange *et al.*, 1997). The data reported here describe significant different characteristics of KdpE proteins from *C. acetobutylicum* and *E. coli*, support the role of enterobacterial KdpE as being a junction of different regulatory pathways, rule out such a function for its clostridial counterpart, and provide

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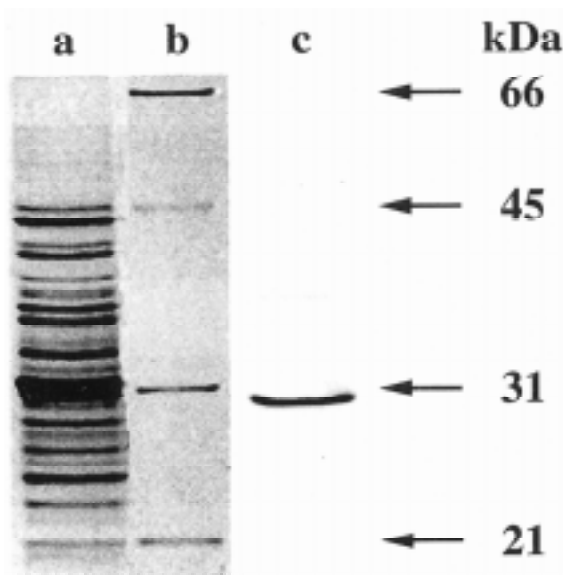


Figure 1. Immunodetection of overexpressed, recombinant clostridial KdpE-Strep-tag from *E. coli*. Lane a, crude periplasmic extract containing KdpE-Strep-tag (2.5 µg protein); lane b, molecular size marker (sizes are shown on the right). In lanes a and b, proteins were stained with silver. Lane c, immunodetection of clostridial KdpE-Strep-tag in crude periplasmic extract (2.5 µg protein).

indications that in *C. acetobutylicum*, KdpD might serve as a regulatory checkpoint controlling signal flow in different phosphorelays.

Results

Overproduction of KdpE-Strep-tag (*C. acetobutylicum*)

To study the interaction of clostridial KdpE with DNA operator regions, we constructed a recombinant plasmid (pE1) which carried the entire *kdpE* coding sequence connected downstream to the *tetA* promoter-operator, the *ompA* signal sequence, and upstream to the *Strep-tag*. *E. coli* JM109 transformed with pE1 was grown in Luria broth, with anhydrotetracycline being added in the exponential phase to induce the synthesis of KdpE-Strep-tag. The engineered *ompA* signal sequence allowed transport of the originally cytoplasmic KdpE protein into the periplasm. After permeabilization of the outer membrane of *E. coli*, the soluble components of the periplasm including the KdpE-Strep-tag protein were released, and the fused Strep-tag allowed easy confirmation of overexpression by immunodetection (Figure 1). The apparent molecular mass estimated for the KdpE-Strep-tag band (29 kDa) was in good agreement with that calculated from the amino acid sequence for the tagged protein (27.5 kDa). The periplasmic extract was used in the band shift assays.

Binding of the Phosphorylated Clostridial KdpE-Strep-tag to the Clostridial *kdp* Promoter Region

We compared the *in vitro* DNA-binding ability of phospho-KdpE-Strep-tag with that of non-phospho-KdpE-Strep-tag, by means of non-denaturing gel retardation analysis. A 238-bp PCR-generated DNA fragment (primers: Bandshift + KdpZP, see Table 1) end-labelled with IRD800, which encompassed the *kdpZYABCX* promoter region and its

upstream sequence (Treuner-Lange *et al.*, 1997), was incubated with periplasmic extract containing either non-phospho-KdpE-Strep-tag or phospho-KdpE-Strep-tag of *C. acetobutylicum*. The latter was prepared by incubation of the extract with 20 mM acetyl phosphate (or carbamyl phosphate) as described in experimental procedures. The reaction mixtures were subjected to a DNA-binding gel retardation assay and a retardation of the labelled DNA fragment could be observed (Figure 2). However, the shift only occurred when using phosphorylated KdpE-Strep-tag. The unphosphorylated form did not bind to the *kdp* promoter (Figure 2). The controls included clearly revealed that the band shift was only caused by fractions containing phospho-KdpE-Strep-tag (Figure 2), and, thus, this periplasmic preparation could be used in the following experiments. There was no difference observed between using acetyl phosphate or carbamyl phosphate as a phosphodonor.

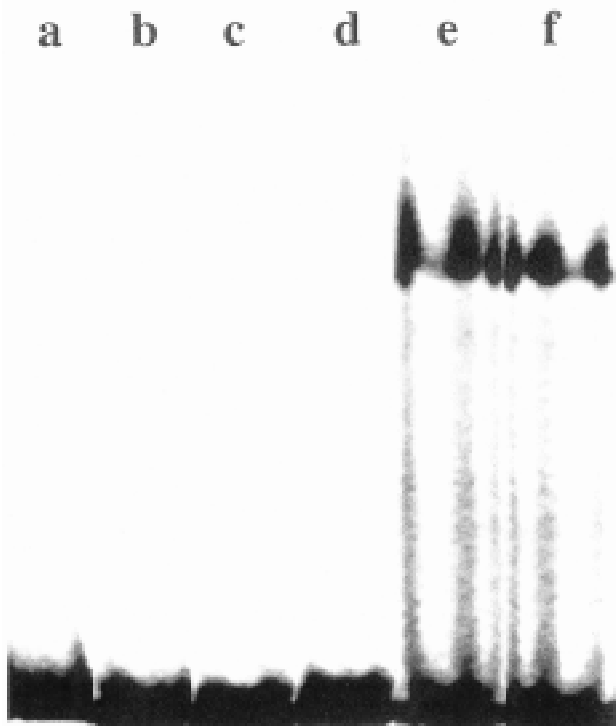


Figure 2. DNA-binding analysis of clostridial phospho-KdpE-Strep-tag. A 238-bp PCR-generated DNA fragment (primer: Bandshift + KdpZP, see Table 1) end-labelled with IRD800 and comprising the *kdpZYABCX* promoter region and its upstream sequence, was incubated with either unphosphorylated KdpE-Strep-tag or phospho-KdpE-Strep-tag. The latter was prepared by incubation with 20 mM acetyl phosphate (or carbamyl phosphate) as described in experimental procedures. Lane a, DNA fragment only; lane b, DNA fragment plus periplasmic protein fraction of a culture containing only vector (pASK-IBA1); lane c, DNA fragment plus phosphorylated periplasmic protein fraction of a culture containing only vector; lane d, DNA fragment plus unphosphorylated periplasmic protein fraction of a KdpE-Strep-tag-overexpressing culture; lane e, DNA fragment plus acetyl phosphate-phosphorylated periplasmic protein fraction of a KdpE-Strep-tag-overexpressing culture; lane f, DNA fragment plus carbamyl phosphate-phosphorylated periplasmic protein fraction of a KdpE-Strep-tag-overexpressing culture.

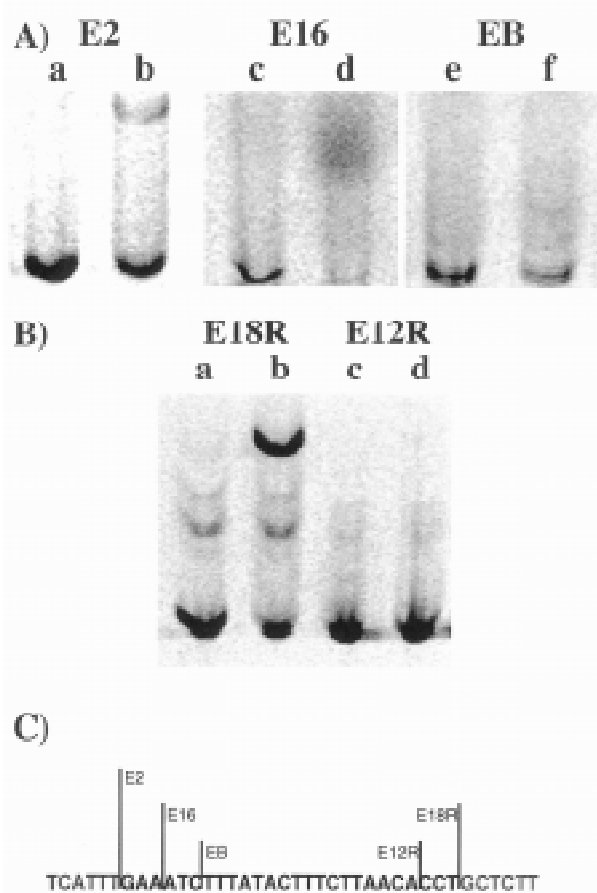


Figure 3. Determination of the minimal sequence requirements of the clostridial KdpE binding site.

Several PCR-generated DNA fragments containing different parts of the *kdpZYABCX* promoter sequence were used to determine the KdpE binding site by gel retardation analysis.

A) DNA-binding assay of phospho-KdpE-Strep-tag with fragments starting with the upstream end of the KdpE binding site. Primer pair E2 + KdpZP: lane a, phosphorylated periplasmic protein fraction of a culture containing only vector (pASK-IBA1); lane b, acetyl phosphate-phosphorylated periplasmic protein fraction of a KdpE-Strep-tag-overexpressing culture; primer pair E16 + KdpZP: lane c, phosphorylated periplasmic protein fraction of a culture containing only vector; lane d, acetyl phosphate-phosphorylated periplasmic protein fraction of a KdpE-Strep-tag-overexpressing culture; primer pair EB + KdpZP: lane e, phosphorylated periplasmic protein fraction of a culture containing only vector; lane f, acetyl phosphate-phosphorylated periplasmic protein fraction of a KdpE-Strep-tag-overexpressing culture.

B) DNA-binding assay of phospho-KdpE-Strep-tag with fragments starting with the downstream end of the KdpE binding site. Primer pair E18R + E5IRD: lane a, phosphorylated periplasmic protein fraction of a culture containing only vector (pASK-IBA1) (additional bands were caused by contaminating non-specific PCR products); lane b, acetyl phosphate-phosphorylated periplasmic protein fraction of a KdpE-Strep-tag-overexpressing culture; primer pair E12R + E5IRD: lane c, phosphorylated periplasmic protein fraction of a culture containing only vector; lane d, acetyl phosphate-phosphorylated periplasmic protein fraction of a KdpE-Strep-tag-overexpressing culture.

C) Schematic representation of the results of the gel retardation assays. The 5' end of the primers E2, E16 and EB (sense strand) as well as the 5' end of the primers E12R and E18R (antisense strand) are indicated by lines. The minimal sequence requirement for binding of KdpE from *C. acetobutylicum* is indicated by bold letters.

Determination of the Minimal Sequence Requirements for KdpE Binding

The minimal sequence requirements for binding of KdpE to the *kdpZYABCX* promoter region were determined by use of different PCR-generated DNA fragments. The following primers (Table 1) were used: upstream end of the KdpE binding site: E2 + KdpZP (yielding a DNA fragment of 145 bp), E16 + KdpZP (142 bp), EB + KdpZP (139 bp); downstream end of the KdpE binding site: E18R + E5IRD (136 bp), E12R + E5IRD (133 bp). These probes were analyzed with periplasmic extract containing clostridial phospho-KdpE-Strep-tag in gel retardation assays (Figure 3). Band shifts were only obtained if the fragments included sequences encompassing the E2 or E18R positions shown in Figure 3C. A gradual decrease of the binding ability could be observed on the upstream side when 3 or 6 bp were missing (E16 and EB). On the other hand, a drastic decrease of band shift formation occurred if the downstream side was shortened by only 3 bp (E18R

versus E12R). The DNA binding site of clostridial KdpE includes a sequence of 26 bp and is thus only slightly longer than the one from *E. coli*, with which it shares significant sequence similarity (Figure 4).

Only one KdpE Binding Site is Present in the *C. acetobutylicum* Genome

Recently, raw sequence data on the *C. acetobutylicum* genome became available (National center for biotechnology information, <http://www.ncbi.nlm.nih.gov/>). Thus, it was possible to search the chromosome for regions with homology or identity to the experimentally determined KdpE binding site. This investigation was of interest because upon induction of the *kdp* system, the amount of *kdpDE* transcripts increased as well, raising the speculation that KdpE might regulate additional operons (Treuner-Lange *et al.*, 1997).

The search for regions with homology to the KdpE binding site in the *C. acetobutylicum* genome revealed three



Figure 4. Comparison of KdpE binding motifs of *E. coli* and *C. acetobutylicum*.

The binding sites are indicated by shaded boxes. E stands for *E. coli* and C for *C. acetobutylicum*. The numbers refer to the distance from the transcription startpoints of *kdpFABC* and *kdpZYABCX*, respectively.

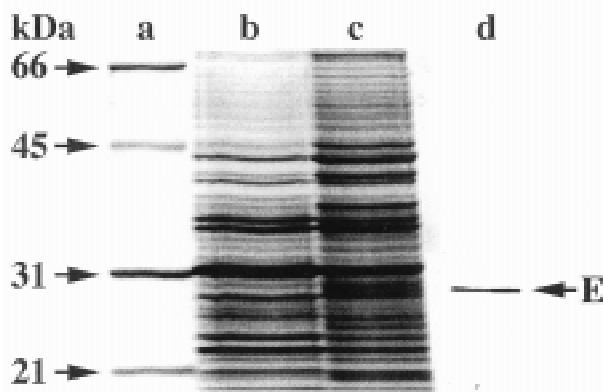


Figure 7. Overproduction of KdpE-Strep-tag from *E. coli*. Lane a, molecular size marker (sizes are shown on the left); lane b, crude periplasmic extract of a culture containing only vector (pASK-IBA1) (2.5 μ g protein); lane c, crude periplasmic extract containing enterobacterial KdpE-Strep-tag (2.5 μ g protein). In lanes a-c, proteins were stained with silver. Lane d, immunodetection of enterobacterial KdpE-Strep-tag (E, indicated by arrow) in crude periplasmic extract (2.5 μ g protein).

observed (Figure 6), indicating that the *C. acetobutylicum* protein does not recognize the *E. coli* DNA despite the high similarity between the two DNA sequences (Figure 4).

Does the KdpE-Strep-tag Protein of *E. coli* Recognize the KdpE Binding Site of *C. acetobutylicum*?

An analogous experiment was performed using the KdpE-Strep-tag protein of *E. coli* and DNA containing the clostridial KdpE binding sequence. Overproduction of KdpE-Strep-tag from *E. coli* was performed as described above. The expression of *E. coli* KdpE-Strep-tag was analyzed by Western blot analysis (Figure 7). For gel retardation DNA fragments containing the KdpE binding motifs of *C. acetobutylicum* and *E. coli* were used. The positive control (*E. coli* DNA) with the above described 251-bp PCR fragment (primers: EE1IRD + EE2) showed a significant bandshift. Using a 173-bp *C. acetobutylicum* DNA fragment (primers: E51RD + E7R) a weaker, but still significant bandshift could be observed as well (Figure 8). Thus, the *E. coli* KdpE protein proved to have a less specific binding ability than the regulator from *C. acetobutylicum*.

Discussion

The data presented provide evidence that the response regulator KdpE from *C. acetobutylicum* binds in its phosphorylated form to a specific sequence motif upstream of the promoter for the structural genes (*kdpZYABCX*) of the potassium-transporting P-type ATPase Kdp. Thus, it allows induction of transcription that has been observed under potassium limitation (Treuner-Lange *et al.*, 1997). Like KdpE from *E. coli* (cited as unpublished in Bouché *et al.*, 1998) and other response regulators (Feng *et al.*, 1992; Lukat *et al.*, 1992), it could be phosphorylated by acetyl phosphate or carbamyl phosphate. In these respects, KdpE in *C. acetobutylicum* seems similar to KdpE in *E. coli*. However, several significant differences exist that might have important consequences for the regulatory network.

In *C. acetobutylicum*, the genes for the sensor kinase KdpD and the response regulator KdpE are organized in a common operon and transcribed from its separate promoter under conditions of potassium excess (Treuner-Lange and Dürre, 1996). At low potassium concentration this promoter is shut down. Instead, transcription is initiated at a promoter upstream of the contiguous, upstream operon *kdpZYABCX* which runs through to the end of *kdpE* (Treuner-Lange *et al.*, 1997). Since it does not make much sense that transcription of the regulator genes, whose products control expression of the transport ATPase system, is increased after the induction already took place, it has been hypothesized that KdpD and/or KdpE regulate in addition other systems (Treuner-Lange *et al.*, 1997). The data reported here, however, argue against such a function of KdpE. Although there are some similar binding motifs in the genome of *C. acetobutylicum*, no specific interaction of the respective DNA fragments and the phosphorylated response regulator could be detected. The theoretical possibility that an additional accessory protein may be required for binding seems very unlikely, since all regulators of this type investigated so far act on their own. The high degree of specificity is a significant difference of clostridial KdpE to the analogous KdpE protein of *E. coli*. As shown here, the enterobacterial regulator recognizes similar binding motifs, such as the clostridial sequence. It has also been suggested that DNA curvature induced by stretches

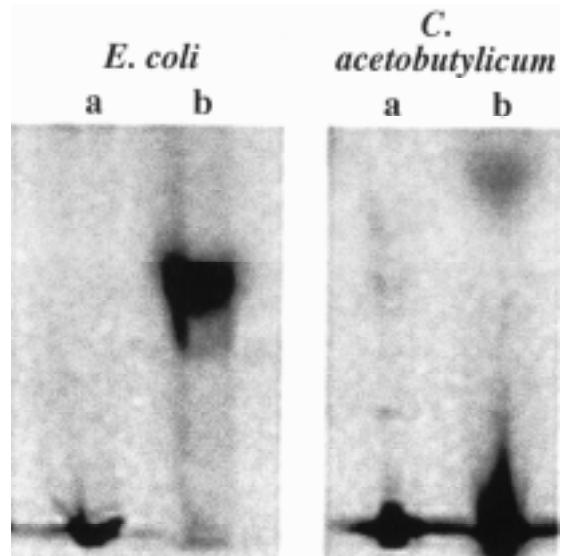


Figure 8. Specificity of binding of KdpE-Strep-tag from *E. coli*. *E. coli*: A DNA fragment containing the *kdpFABC* promoter sequence of *E. coli* generated by PCR with the primer pair EE1IRD + EE2 was used. Lane a, DNA fragment plus phosphorylated periplasmic protein fraction of a culture containing only vector (pASK-IBA1); lane b, DNA fragment plus acetyl phosphate-phosphorylated periplasmic protein fraction of an enterobacterial KdpE-Strep-tag-overexpressing culture. *C. acetobutylicum*: A DNA fragment containing the *kdpZYABCX* promoter sequence of *C. acetobutylicum* generated by PCR with the primer pair E51RD + E7R was used. Lane a, DNA fragment plus phosphorylated periplasmic protein fraction of a culture containing only vector (pASK-IBA1) (additional bands were caused by contaminating unspecific PCR products); lane b, DNA fragment plus acetyl phosphate-phosphorylated periplasmic protein fraction of an enterobacterial KdpE-Strep-tag-overexpressing culture.

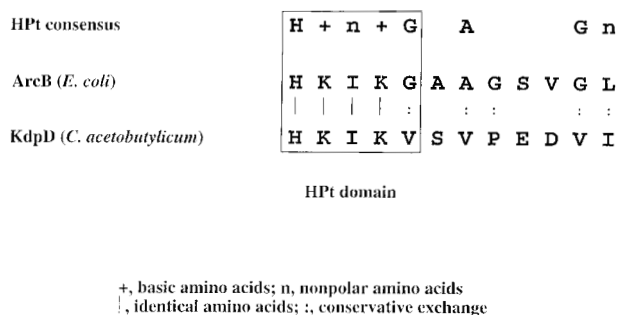


Figure 9. Alignment of HPt domains in ArcB (*E. coli*) and KdpD (*C. acetobutylicum*) compared to the consensus according to Ishige *et al.* (1994) and Mizuno (1998).

The proper domain is boxed, downstream consensus residues are indicated by capitals or small letters. The single letter code is used for amino acids. Identical residues are joined by a line, conservative exchanges are indicated by a colon. +, basic amino acid residue; n, nonpolar amino acid residue.

of T's around the binding site is important for enterobacterial KdpE-DNA interaction (Sugiura *et al.*, 1993). This is not the case with clostridial KdpE, since removal of either upstream or downstream T-stretches did not result in a loss of band shift formation ability. Although the minimal binding site of *C. acetobutylicum* showed some differences to that determined for *E. coli* (Sugiura *et al.*, 1992), stretches of T's are also found in its vicinity. This, however, is not surprising in an organism with an A+T content of the DNA of 72% (Cummins and Johnson, 1971). Thus, the high specificity of the clostridial protein prevents erroneous binding to related sequences.

Another difference between the two regulators is the finding that enterobacterial KdpE is able to bind its target DNA even in unphosphorylated form (Sugiura *et al.*, 1992; Nakashima *et al.*, 1993). At first glance, this is a surprising feature for a response regulator supposed to exert its control by its phosphorylation status. However, it should be kept in mind that binding of unphosphorylated KdpE was observed only with high protein concentrations (Sugiura *et al.*, 1992; Nakashima *et al.*, 1993). Readthrough into the *kdpDE* operon upon induction of *kdpFABC* has also been found in *E. coli* as verified by *kdpD-lac* fusions and expression of KdpD protein (Polarek *et al.*, 1992; Voelkner *et al.*, 1993). Thus, one could speculate that also in *E. coli* KdpD and/or KdpE serve additional regulatory functions. In this organism, the higher degree of binding unspecificity would render KdpE a suitable candidate. Support for this hypothesis comes from the observation that KdpE interacts with phosphorylated EnvZ, a protein kinase different from its cognate sensor KdpD (Nakashima *et al.*, 1992). This could be an example of not only "cross talk" but "cross regulation" allowing the cell to link different regulatory networks (Wanner, 1995).

This still leaves the question why readthrough of the *kdpDE* operon occurs in *C. acetobutylicum*. If there is also "cross regulation" it is unlikely to be executed by KdpE as shown here. This leaves only KdpD as a potential candidate. In the last few years several bacterial two-component signal transduction systems have been identified in which the sensor kinase contains additional receptor and transmitter domains (Iuchi, 1993; Ishige *et al.*, 1994; Tsuzuki *et al.*, 1995; Appleby *et al.*, 1996;

Georgellis *et al.*, 1997; Kato *et al.*, 1997; Mizuno, 1997; Matsushika and Mizuno, 1998; Mizuno, 1998). Thus, phosphotransfer can occur from either one of two different histidines providing the opportunity for interactions with different response regulators. The respective sensors contain in their transmitter domain an additional receiver and a histidine-containing phosphotransfer (HPt) domain (Mizuno, 1998). A typical example of such a sensor is ArcB from *E. coli* (Ishige *et al.*, 1994). Upon inspection of the clostridial KdpD sequence we found a typical HPt domain (AA 773-784, Treuner-Lange and Dürre, 1996) which is almost identical to the *E. coli* ArcB sequence (Figure 9). The enterobacterial KdpD does not contain such a region. Thus, in *C. acetobutylicum* readthrough of *kdpDE* might provide additional KdpD as a junction point for communication with other signaling pathways, whereas in *E. coli* KdpE might serve this function by being less specific and interacting with other sensor kinases. Future experiments will try to evaluate this hypothesis.

Experimental Procedures

Materials

All chemicals were of analytical grade and obtained from Sigma Chemie GmbH, Deisenhofen, Germany. Restriction endonucleases were purchased from MBI Fermentas. Deep Vent™ DNA polymerase was from New England Biolabs GmbH, Schwalbach, Germany and T4 DNA ligase was purchased from Boehringer Mannheim, Mannheim, Germany. Nylon membranes were obtained from Amersham Buchler GmbH, Braunschweig, Germany.

Bacterial Strains and Growth Conditions

E. coli strain JM109 (*recA1*, *endA1*, *gyrA96*, *thi*, *relA1*, *hsdR17*, *supE44*, λ , Δ (*lac-proAB*), *F'*, *traD36*, *proAB*, *laqPZ* Δ M15) was used as host for the plasmids described (Yanisch-Perron *et al.*, 1985). Bacteria were grown in Luria broth (Sambrook *et al.*, 1989) or on plates containing 1.5% agar (Difco Laboratories, Detroit, USA). Plasmid-carrying strains were grown with the appropriate antibiotic, ampicillin (100 μ g/ml).

Preparation of Genomic DNA

The preparation of genomic DNA was carried out according to a conventional laboratory manual (Ausubel *et al.*, 1994).

Recombinant DNA Techniques

The conditions used for DNA manipulating enzymes, such as restriction endonucleases and T4 ligase, were those recommended by the supplier. Other recombinant DNA techniques were carried out according to a conventional laboratory manual (Ausubel *et al.*, 1994).

Plasmids

The following plasmids were used: pASK-IBA1 (ampicillin resistance), a high-level expression vector (IBA-GmbH, Göttingen, Germany) carrying the *tetA* promoter-operator, the *ompA* signal sequence (*ompA* can be used for secretion of KdpE-Strep-tag into the periplasmic space of *E. coli*), and the C-terminal fusion of the Strep-tag (sequence: Ser-Ala-Trp-Arg-His-Pro-Gln-Phe-Gly-Gly) allowing easy immunodetection with streptavidin alkaline phosphatase conjugate (Biometra biomedizinische Analytik GmbH, Göttingen, Germany); pT10 carrying the *kdpDE* genes of *C. acetobutylicum* (ampicillin resistance) (Treuner-Lange and Dürre, 1996); and pT111 carrying the *kdpZYABCX* genes of *C. acetobutylicum* (ampicillin resistance) (Treuner-Lange and Dürre, 1996).

Construction of kdpE-Strep-tag (*C. acetobutylicum*) and of kdpE-Strep-tag (*E. coli*)

The conditions for the construction of *kdpE-Strep-tag* were those recommended by the supplier (Biometra biomedizinische Analytik GmbH, Göttingen, Germany). To facilitate mutant construction, two *BsaI* restriction sites, one upstream of the ATG start codon and one after the stop codon, were created by polymerase chain reaction using plasmid pT10 (sense primer: 5'-AGA GTT ATA GTG GGT CTC TGG CCA TGG ATA ATA AAC CTT ATA-3', antisense primer: 5'-CAA AAT CCT TAT GGT CTC CGC GCT TTC ATC AAT CAA TCT ATA G-3'). The polymerase chain reaction product was purified by phenol/chloroform extraction, digested with *BsaI*, and ligated into similarly treated pASK-IBA1, resulting in pE1 (*kdpE-Strep-tag C. acetobutylicum*). The conditions for the construction of *kdpE-Strep-tag (E.*

coli) were the same as described above for the *kdpE-Strep-tag* (*C. acetobutylicum*). The polymerase chain reaction was performed with genomic *E. coli* DNA and the following primers: sense primer: 5'-CTT GAA GAA TTT GGT CTC GGG CCG TGA CAAACG TTC TGA TTG-3'; antisense primer: 5'-AGG CTG TAT TAA GGT CTC TGC AAG CAT AAA CCG ATA GCC A-3'. The resulting plasmid was pEE1.

DNA Sequencing

Constructions were verified by sequencing the complete segment generated by the polymerase chain reaction and the ligation junctions in double-stranded plasmid DNA using the dideoxynucleotide termination method (Sanger *et al.*, 1977) and synthetic sequencing primers.

Enrichment of *C. acetobutylicum* KdpE-Strep-tag and *E. coli* KdpE-Strep-tag

Strain JM109 carrying plasmid pE1 was grown aerobically at 28°C in 100 ml Luria broth supplemented with ampicillin. When the culture reached an optical density of 0.5 at 550 nm, 10 µl of an anhydrotetracycline solution (2 mg/ml) were added to induce the *tetA* promoter while the concentration of the antibiotic is not high enough to affect growth. After 3 hours, cells were harvested by centrifugation (4°C, 4500 g, 10 min). The cells were suspended in 1 ml of precooled buffer P (100 mM Tris/HCl pH 8.0, 500 mM sucrose, 1 mM EDTA, 0.02 % (w/v) Na₃N) and incubated on ice for 30 min. Under these conditions, the outer membrane of *E. coli* is sufficiently permeabilized to release the soluble components of the periplasm. To remove the spheroplasts, the suspension was centrifuged (4°C, 13000 g, 10 min). This periplasmic extract was used for gel retardation experiments. The enrichment of the *E. coli* KdpE-Strep-tag in the periplasmic space was performed as described above using pEE1.

Immunoblotting

Before electrophoretic transfer of the proteins from SDS gels to nitrocellulose (Towbin *et al.*, 1979), gel and membrane were preincubated for 30 min in transfer buffer (125 mM Tris, 195 mM glycine, adjusted to pH 8.6 with 6 M HCl, 20 % methanol). The transfer was performed for 30 min at 4°C with 5 mA/cm² membrane (Nova-Blot Unit, Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany). The membrane was incubated in PBS buffer (4 mM KH₂PO₄, 16 mM Na₂HPO₄, 115 mM NaCl), 3 % (w/v) BSA, and 0.5 % (v/v) Tween 20 for 1 h at room temperature to block non-specific interactions. Subsequently, it was washed three times for 5 min at RT with PBS-Tween 20 (PBS with 0.1 % (v/v) Tween 20). The detection of KdpE-Strep-tag via the Strep-tag on its C-terminus was performed by incubation of the membrane in 10 ml PBS-Tween 20 and 2.5 µl of streptavidin alkaline phosphatase conjugate (Amersham Buchler GmbH, Braunschweig, Germany) for 1 h (RT). Then, the membrane was washed three times with PBS-Tween 20 and twice with PBS (1 min per washing step). The phosphatase reaction was performed with a ready to use substrate reaction mixture (BCIP/NBT tablets, Sigma Fast, Sigma Chemie GmbH, Deisenhofen, Germany).

DNA-Binding Assay

The DNA for these experiments was generated by polymerase chain reaction using IRD800-labelled oligonucleotides (MWG Biotech GmbH, Ebersberg, Germany) (Table 1). For KdpE-Strep-tag autophosphorylation, the periplasmic extract was incubated in a buffer containing 50 mM Tris/HCl (pH 7.5), 0.5 M NaCl, 10 mM MgCl₂, 2 mM DTT, 10 % (v/v) glycerol, and 20 mM acetyl phosphate (or carbamyl phosphate) at 28°C for 1 h. Then, the DNA and 2 µg poly(dI-C) (competitor DNA) per lane were added and incubated for 15 min at 22°C in the dark. The samples (80 ng protein and 23 ng DNA per lane) were immediately analyzed on a 5 % (w/v) non-denaturing polyacrylamide gel running on a LI-COR model 4000L DNA sequencer (MWG Biotech GmbH, Ebersberg, Germany) as indicated by the manufacturer.

Analytical Methods

Protein samples were analyzed by 12 % (w/v) SDS/polyacrylamide gel electrophoresis according to Laemmli (1970). Staining of gels was performed with silver as described by Blum *et al.* (1987). Protein concentration was determined by the method of Bradford (1976). The conditions for the assay were those recommended by the supplier of the reagent (Bio-Rad Laboratories GmbH, München, Germany).

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