An Atypical KdpD Homologue from the Cyanobacterium *Anabaena* sp. Strain L-31: Cloning, In Vivo Expression, and Interaction with *Escherichia coli* KdpD-CTD

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The *kdpFABC* operon of *Escherichia coli*, coding for the high-affinity K^+ transport system KdpFABC, is transcriptionally regulated by the products of the adjacently located *kdpDE* genes. The KdpD protein is a membrane-bound sensor kinase consisting of a large N-terminal domain and a C-terminal transmitter domain interconnected by four transmembrane segments (the transmembrane segments together with the C-terminal transmitter domain of KdpD are referred to as CTD), while KdpE is a cytosolic response regulator. We have cloned and sequenced the *kdp* operon from a nitrogen-fixing, filamentous cyanobacterium, *Anabaena* sp. strain L-31 (GenBank accession. number AF213466). The *kdpABC* genes are similar in size to those of *E. coli*, but the *kdpD* gene is short (coding only for 365 amino acids), showing homology only to the N-terminal domain of *E. coli* KdpD. A *kdpE*-like gene is absent in the vicinity of this operon. *Anabaena* KdpD with six C-terminal histidines was overproduced in *E. coli* and purified by Ni^{2+}-nitrilotriacetic acid affinity chromatography. With antisera raised against the purified *Anabaena* KdpD, the protein was detected in *Anabaena* sp. strain L-31 membranes. The membrane-associated or soluble form of the *Anabaena* KdpD(6His) could be photoaffinity labeled with the ATP analog 8-azido-ATP, indicating the presence of an ATP binding site. The coproduction of *Anabaena* KdpD with *E. coli* KdpD-CTD decreased *E. coli* *kdpFABC* expression in response to K^+ limitation in vivo relative to the wild-type KdpD-CTD protein. In vitro experiments revealed that the kinase activity of the *E. coli* KdpD-CTD was unaffected, but its phosphatase activity increased in the presence of *Anabaena* KdpD(6His). To our knowledge this is the first report where a heterologous N-terminal domain (*Anabaena* KdpD) is shown to affect in *trans* KdpD-CTD (*E. coli*) activity, which is just opposite to that observed for the KdpD-N-terminal domain of *E. coli*.

In bacteria, K^+ plays an important role in the maintenance of intracellular pH, cell turgor, and control of cellular enzyme activities (10) and is also known to positively regulate the expression of certain osmoresponsive genes (26). In bacteria the intracellular K^+ levels are maintained by a variety of K^+ uptake and efflux systems. The KdpFABC complex (K^+-dependent ATPase), encoded by the *kdpFABC* operon (2), is an inducible, high-affinity K^+ transporter synthesized by *Escherichia coli* during potassium limitation (11). The expression of the *kdpFABC* operon is regulated by the products of the adjacent *kdpDE* genes (27). The membrane-bound sensor kinase KdpD and the cytosolic response regulator KdpE constitute a typical prokaryotic two-component (sensor kinase/response regulator) signal transduction system (37).

The *E. coli* KdpD (894 amino acids, 99 kDa) consists of a cytosolic N-terminal domain (NTD) and a cytosolic C-terminal domain interconnected by four transmembrane segments (the transmembrane segments together with the C-terminal trans-mitter domain of KdpD are referred to as CTD) (40). In response to appropriate stimuli, KdpD becomes autophosphorylated. The phosphoryl group is transferred to KdpE, and the phosphorylated KdpE (KdpE~P) activates transcription of the *kdpFABC* operon (24). KdpD is a bifunctional enzyme, i.e., it phosphorylates KdpE and is also responsible for dephosphorylation of KdpE~P. This phosphatase activity is stimulated by ATP or its nonhydrolyzable analogs (17). The C-terminal domain resembles the transmitter domain of other kinases and carries the conserved His673, critical for phosphotransfer reactions (35) and all the other essential features of sensor histidine kinases. The NTD has been shown to be essential for maximal *kdpFABC* expression in *E. coli* (28). KdpD lacking the NTD responds poorly to loss of turgor or K^+ limitation in vivo and shows deregulated (ATP independent) phosphatase activity in vitro (17). However, the exact stimulus that is sensed by KdpD is not clear (for more details, see references 5, 16, 23, and 32).

kdp homologues were initially shown to be present among gram-negative bacteria (36). Genome sequence analyses have now revealed that gram-positive bacteria and *Archaea* also possess *kdp* homologues. Apte and Alahari (3) have earlier demonstrated the presence of the KdpB polypeptide in three strains of *Anabaena*. *Anabaena* KdpB protein has been shown

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to be present in the cytoplasmic membrane under K^+-limiting conditions (1).

To understand the organization and regulation of expression of the *kdp* genes in *Anabaena* spp., we cloned and sequenced the *kdp* operon (GenBank accession number AF213466) from a heterocystous, filamentous cyanobacterium, *Anabaena* sp. strain L-31. In comparison to the *E. coli kdpFABC* operon, *kdpF* is missing, but a new gene, *kdpG*, coding for a small hydrophobic protein was found between *kdpB* and *kdpC*. Interestingly, only a short *kdpD* gene (coding for 365 amino acids) was observed, while a *kdpE*-like gene was absent in the vicinity of the *Anabaena* sp. strain L-31 *kdp* operon. *Anabaena* KdpD showed homology to the NTD of *E. coli* KdpD, while the transmembrane segments and the C-terminal domain (which exhibits the histidine kinase activity) were missing. Genome sequencing has revealed the presence of similar short versions of *kdpD* in other bacteria, such as *Synechocystis* sp. strain PCC 6803 (20), *Anabaena* sp. strain PCC 7120 (21), and *Deinococcus radiodurans* R1 (39), but they have not been characterized from any of them. We report here on the cloning and the in vivo expression of a naturally short KdpD homolog from *Anabaena* sp. strain L-31, and show that it affects, in *trans*, the activity of the membrane-bound *E. coli* KdpD-CTD.

MATERIALS AND METHODS

Bacterial strains and plasmids. The filamentous, heterocystous, nitrogen-fixing freshwater cyanobacterium *Anabaena* sp. strain L-31 (33) was grown under axenic conditions. Stock cultures were maintained in standard BG-11 medium (8) without combined nitrogen. *E. coli* DH5α [F⁻φ80d*lacZ*ΔM15 Δ(*lac-argF*)*U169 recA1 endA1 hsdR17 supE44 thi-1 gyrA96relA1*] was employed as the carrier for the plasmids. *E. coli* BL21(DE3)pLysS [F⁻ *ompT hsdS*$_B$(r$_B$ − m$_B$⁻) *gal dcm* (DE3) pLysS(Cm^r)] (Novagen) was used to overproduce *Anabaena* KdpD with a six-histidine tag (6His). *E. coli* TKW22692 (*kdpD6-92 trkA405 trkD1*) was used for in vivo studies of KdpD/Δ12–395 (CTD) with *Anabaena* KdpD(6His). *E. coli* TKR2000 (Δ *kdpFABCDE trkA405 trkD1 atp706*) was used for coexpression of *E. coli* KdpD-CTD and *Anabaena* KdpD(6His). pBD6-92 (17) was digested with XmaI and HindIII and the 1.2-kb DNA fragment was transferred to similarly digested pBAD33 (13) vector to obtain pBD3/Δ12-395.

Cloning of the *Anabaena* kdpD gene. *Anabaena* sp. strain L-31 chromosomal DNA was prepared as described previously (4). Degenerate primers D2 (GGN GTN GGN AAA ACC TAN CGN ATG TT) and D3 (CGY AAN GCN AGY TCY CTS AGN GC) were used to amplify the *Anabaena* sp. strain L-31 chromosomal DNA with 2.5 U Red *Taq* (Sigma). The 0.56-kb PCR product was cloned into pUC18 to yield vector pABD500 using the Sure Clone kit (Pharmacia) and sequenced. *Anabaena* sp. strain L-31 chromosomal DNA was digested with different restriction endonucleases, resolved on 0.8% agarose gel, and transferred to a nylon membrane (0.45 μm, Schleicher and Schuell). The 0.56-kb pABD500 insert was labeled with digoxigenin by the random priming method and hybridized to the restriction-digested *Anabaena* sp. strain L-31 DNA. The hybridization and posthybridization washes and chemiluminescent detection were carried out according to the manufacturer's instructions (Roche Biochemicals).

Construction of *Anabaena* KdpD(6His). *Anabaena* sp. strain L-31 chromosomal DNA was amplified with primers KdpDoefwd 5′ GCG <u>CCC ATG G</u>CT AAT TAC CAA TTA AAA 3′, KdpDoerev 5′ GCG GAT CCC TAT TAG TGA TGG TGA TGG TGA TGG <u>CGT TTG GAT GAG ACT TTT TCG CTG GC</u> 3′. The forward primer contained a site for the restriction enzyme NcoI (underlined), while the reverse primer contained six His codons followed by an in-frame stop codon with the last sense codon of the *Anabaena* sp. strain L-31 *kdpD* (the *Anabaena* sp. strain L-31 sequence is underlined). The 1.1-kb PCR product obtained was cloned into pBluescript SK⁻ vector (pBScD1) and sequenced to confirm the identity of the *Anabaena kdpD*(6His). The *Anabaena kdpD*(6His) fragment was transferred as an NcoI-BamHI or NcoI-KpnI fragment to the pET16(b) vector (pETD1) or pBAD24 vector (pBADD1). The plasmid DNA was sequenced on both strands by the dideoxy chain termination reaction (SEQLAB, Goettingen, Germany).

Overproduction, detection, and purification of *Anabaena* KdpD(6His). *E. coli* BL21(DE3)/pLysS/pETDI was grown in complex KML medium (1% tryptone, 0.5% yeast extract, 1% KCl) with appropriate antibiotics to an optical density at 600 nm (OD$_{600}$) of ~1.0 and induced with 1 mM isopropylthiogalactopyranoside (IPTG) for 3 h. The cells were shock frozen in liquid nitrogen, thawed on ice, and resuspended in lysis buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, pH 8.0) and sonicated (10-sec bursts at 200 to 300 W with a 10-sec cooling period between each burst). The cytosolic and membrane fractions were prepared as described before (19) and aliquots of each were electrophoretically resolved on 11% sodium dodecyl sulfate (SDS)-polyacrylamide gels. *Anabaena* KdpD(6His) was detected by staining with Coomassie brilliant blue or on Western blots using the monoclonal penta-His antibody (QIAGEN). For purification purposes, the *E. coli* BL21(DE3)/pLysS/pETD1 membrane containing *Anabaena* KdpD(6His) was solubilized with the detergent Aminoxid WS 35 (1% final concentration) for 30 min at 4°C with gentle stirring. The solubilized mixture was centrifuged at 240,000 × *g* for 45 min. The membrane-solubilized or cytosolic *Anabaena* KdpD(6His) was allowed to bind to Ni^{2+}-nitrilotriacetic acid (NTA)-agarose with gentle shaking at 4°C for 1 h. Binding to the Ni^{2+}-NTA resin slurry, washing, and subsequent elution steps were performed as described (QIAexpressionist). To raise antisera against *Anabaena* KdpD, *Anabaena* KdpD(6His) purified from *E. coli* membranes was employed for immunization. The primary and booster immunizations (subcutaneous injections in the back of a rabbit) and collection of the antisera were performed as described (29).

Photoaffinity labeling of *Anabaena* KdpD(6His) with 8-azido-[α-^{32}P]ATP. Everted vesicles from *E. coli* BL21(DE3)/pLysS containing *Anabaena* KdpD(6 His) were washed once with buffer of low ionic strength (1 mM Tris, pH 7.5, 3 mM EDTA). Vesicles with or without the His-tagged *Anabaena* KdpD or the purified *Anabaena* KdpD(6His) were incubated with 5 μCi of 8-azido-[α-^{32}P]ATP (20 Ci/mmol) in lysis buffer (total reaction volume, 25 μl) containing 10 mM MgCl$_2$, in the presence or absence of 2 mM ATP, for 5 min at room temperature. Cross-linking was achieved by irradiating with UV light at 302 nm for 0.5 min. All samples were immediately subjected to electrophoresis on 11% SDS-polyacrylamide gels. The gels were dried and the radiolabeled proteins detected by a PhosphorImager (Molecular Dynamics). Gels were also stained with Coomassie blue to confirm equal protein loading.

Coexpression, coelution, and phosphorylation of *Anabena* KdpD(6His) and *E. coli* KdpD-CTD. For in vivo coexpression experiments *E. coli* TKW22692 transformed with pBluescript or pBScD1 was grown in K115 medium (12) to an OD$_{600}$ of 1.0. Cells were collected by centrifugation and, after washing, immediately resuspended in the same volume of medium containing 0.1 mM K⁺. After 30 min the cells were centrifuged, lysed in SDS sample buffer, and semi-dry blotted onto a nitrocellulose membrane. The expression of the *kdpFABC* operon was monitored using antisera raised against purified *E. coli* KdpC (30).

The in vitro studies were performed with *E. coli* TKR2000 cells cotransformed with pBADDI and pBD3/Δ12-395. The cells were grown in KML medium containing 0.2% arabinose to an OD$_{600}$ of ~1.0 and harvested by centrifugation, and everted membranes were prepared as described before (19). The kinase assay was performed as described previously (17). The dephosphorylation of KdpE~^{32}P by *E. coli* KdpD-CTD in the presence or absence of *Anabaena* KdpD(6His) with ATP-γ-S was performed as described before (15). For coelution experiments *E. coli* TKR2000/pBADD1/pBD3/Δ12-395 cells coexpressing *Anabaena* KdpD(6His) and *E. coli* KdpD-CTD were lysed by sonication, and the lysate was solubilized with 1% Aminoxid. The solubilized proteins (5 mg/ml total protein) were allowed to bind to Ni^{2+}-NTA resin at 4°C for 1 h. The Ni^{2+}-NTA resin was thoroughly washed with buffer B [50 mM Tris/HCl, pH 7.5, 10% (vol/vol) glycerol, 0.3 M NaCl, 0.04% Aminoxid, 10 mM imidazole], and the bound proteins were eluted by increasing the imidazole concentration to 200 mM.

Analytical procedures. Protein concentrations were determined by a modified Lowry method (25) with bovine serum albumin as the standard. Proteins were separated on SDS-polyacrylamide gel electrophoresis (PAGE) using 11% or 12.5% polyacrylamide gels (22). For microsequencing the Coomassie-stained protein bands were cut from polyacrylamide gels, transferred to a polyvinylidene difluoride membrane (Millipore) by electroblotting, and sequenced on an Applied Biosystems A473a protein sequencer. A dendrogram depicting evolutionary relationships between 20 different KdpD protein sequences was constructed with the ClustalW software program, available at www.clustalw.genome .ad.jp.

Nucleotide sequence accession number. The nucleotide sequence of *Anabaena kdpD* has been submitted to GenBank (accession number AF213466).
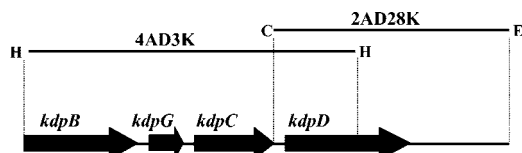
FIG. 1. Location of *Anabaena* sp. strain L-31 *kdp* genes in p4AD3K and p2AD28K. The *kdpB*, *kdpG*, *kdpC*, and *kdpD* ORFs are shown by thick arrows. The p4AD3K insert contains the 3′ end of *Anabaena kdpB*, complete *kdpG*, *kdpC*, and 840 bp of the *Anabaena kdpD* gene. The p2AD28K insert shows a 974-bp overlap with the p4AD3K insert and contains the entire *kdpD* gene. Restriction sites: H, HindIII; C, ClaI; and E, EcoRV.

RESULTS

Cloning and sequence analysis of *kdpD* from *Anabaena* sp strain L-31. Two degenerate PCR primers used to amplify the *Anabaena* sp. strain L-31 *kdpD* from chromosomal DNA were based on the conserved amino acid sequences GVGKT(W/Y)(A/R)ML and ALR(E/K)LAL present in KdpD-NTD. The deduced amino acid sequence of the 0.56-kb product showed high similarity to the KdpD-NTD of different bacteria (e.g., 68% with *Synechocystis* sp. strain 6803, 37% with *E. coli*). This *Anabaena kdpD* gene fragment, when used as a probe on Southern blots with restriction-digested *Anabaena* sp. strain L-31 DNA, showed single bands at 3.1 kb (HindIII), 5.5 kb (EcoRV), and 2.8 kb (EcoRV and ClaI) (data not shown). The 3.1-kb region of the HindIII or the 2.8-kb region of the EcoRV and ClaI restriction-digested *Anabaena* sp. strain L-31 chromosomal DNA was eluted from the gel and ligated to the HindIII-digested pUC19 or EcoRV- and ClaI-digested pBluescript SK⁻, respectively.

The colonies obtained upon transformation were hybridized to the digoxigenin-labeled pABD500 insert to identify the larger genomic *Anabaena kdpD* fragment. Two clones, 4AD3K (3.1-kb HindIII fragment in pUC19) and 2AD28K (2.8-kb ClaI-EcoRV fragment in pBluescript SK⁻) were identified by colony hybridizations with the *Anabaena* sp. strain L-31 *kdpD* probe and sequenced. The two plasmids showed an overlap of 974 bp, with p2AD28K containing the entire *kdpD* gene (Fig. 1).

The *Anabaena* sp. strain L-31 *kdpD* gene was located immediately downstream of *kdpC* in the *kdp* operon, while no "*kdpE*-like" gene was found downstream of *kdpD* (Fig. 1). The *Anabaena* sp. strain L-31 *kdpD* open reading frame (ORF) was found to be 1,098 bp in length and encoded a KdpD protein of only 365 amino acids, compared to 894 amino acids in *E. coli* KdpD. The *Anabaena* KdpD protein corresponded only to the NTD of *E. coli* KdpD, while the four transmembrane segments and the C-terminal transmitter domain found in other full-length KdpDs were missing. All the conserved amino acids present in KdpD-NTD, especially the slightly modified Walker A and Walker B motifs (14, 38), were also present in *Anabaena* KdpD.

Overproduction and purification of *Anabaena* KdpD(6His) from *E. coli*. To facilitate its biochemical characterization and purification, *Anabaena* KdpD with six additional histidine codons in-frame was cloned into the pET16b vector (pETD1) and transformed into *E. coli* BL21(DE3)/pLysS. Upon induction with IPTG, the *E. coli* BL21(DE3)/pLysS/pETD1 cells
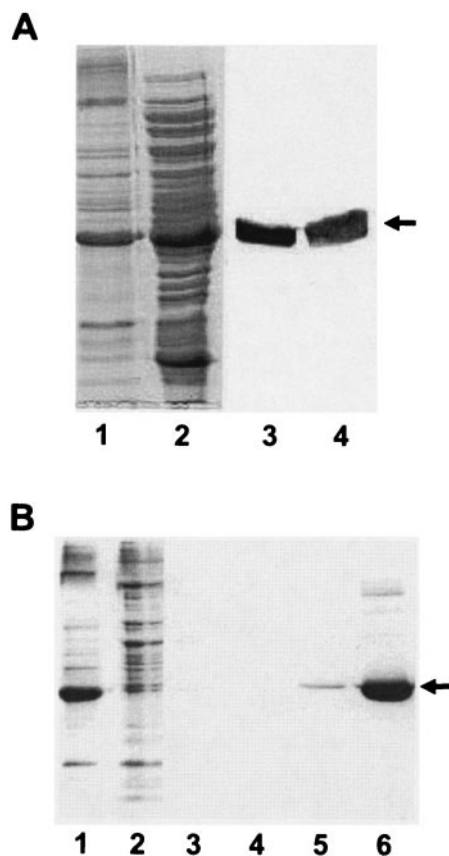

FIG. 2. (A) Localization of *Anabaena* KdpD(6His) in *E. coli* cell fractions. Membranes and cytosolic fractions of BL21(DE3)/pLysS/pETDI were prepared as described in Materials and Methods. For comparison, the membrane pellet obtained after the ultracentrifugation step was resuspended (in lysis buffer) in a volume equal to that of the supernatant (cytosol). Lane 1, membrane vesicles (20 μl); lane 2, cytosol (20 μl). The samples were also electroblotted onto a nitrocellulose membrane and probed with the monoclonal anti-His antibody. Detection was carried out by a secondary anti-mouse immunoglobulin G coupled to alkaline phosphatase (lanes 3 and 4). *Anabaena* KdpD(His6) is indicated by an arrow. (B) Purification of *Anabaena* KdpD(6His) from *E. coli* membranes. *Anabaena* KdpD(6His) was solubilized with 1% Aminoxid WS35 from *E. coli* BL21(DE3)/pLysS/pETDI membranes and purified by affinity chromatography on an Ni-NTA matrix. The samples were resolved on an SDS-polyacrylamide (11%) gel and stained with Coomassie brilliant blue. Lane 1, solubilized membranes (10 μg); lane 2, unbound fraction (10 μg); lane 3, 10 mM imidazole wash; lane 4, 20 mM imidazole wash; lane 5, 40 mM imidazole wash; lane 6, 200 mM imidazole elution (5 μg). The position of *Anabaena* KdpD(His6) is indicated by an arrow.

synthesized a 42-kDa protein that could clearly be seen on SDS gels. The identity of the protein was confirmed by Western blot analysis using monoclonal penta-His antibodies (data not shown).

Membrane and cytosolic fractions were prepared from *E. coli* BL21(DE3)/pLysS/pETD1 cells containing the overproduced *Anabaena* KdpD(6His). Electrophoretic resolution of these fractions followed by Western blotting and immunodetection (with anti-penta-His) revealed that the *Anabaena* KdpD(6His) protein was present in both the cytosolic and membrane fractions (Fig. 2A). *Anabaena* KdpD(6His) was purified to near homogeneity from *E. coli* cytosolic and solubi-
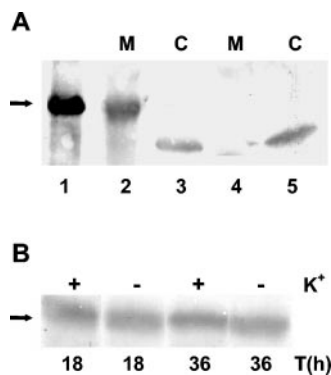
FIG. 3. (A) Localization of KdpD in *Anabaena* sp. strain L-31. The antiserum raised against purified KdpD(6His) was used to detect the KdpD protein from *Anabaena* sp. strain L-31. *Anabaena* sp. strain L-31 membrane (M) and cytosolic (C) fractions from cells grown in BG11K5 medium were prepared as described (1). About 200 μg of protein was loaded in lanes 2 to 5, while 150 ng of purified *Anabaena* KdpD(6His) was loaded in lane 1. Lanes 1 to 3 were probed with the *Anabaena* KdpD(6His) antiserum, while lanes 4 and 5 were probed with the control preimmune serum. (B) Effect of K$^+$ limitation on *kdpD* expression in *Anabaena* sp. strain L-31. *Anabaena* sp. strain L-31 cells were grown in BG11K5 medium and then transferred to BG11 medium containing 0 mM or 5 mM K$^+$. At the times indicated, the cells were harvested, membranes were isolated, 200 μg total membrane protein was electrophoresed, and *Anabaena* KdpD (shown by the arrow) was detected as before.

FIG. 4. Detection of ATP binding by *Anabaena* KdpD(6His) by photoaffinity labeling with 8-azido-[α-^{32}P]ATP. *E. coli* BL21(DE3)/pLysS everted membranes containing *Anabaena* KdpD(6His) (lanes 1 and 2) and purified cytosolic *Anabaena* KdpD(6His) (lanes 3 and 4) were labeled with 8-azido-[α-^{32}P]ATP in the presence or absence of 2 mM ATP as indicated. After electrophoresis on 11% SDS-polyacrylamide gels, the radiolabeled proteins were detected by a PhosphorImager system (Molecular Dynamics). *Anabaena* KdpD(6His) is shown by an arrow.

lized membrane fractions by affinity chromatography using Ni^{2+}-NTA-agarose. In Fig. 2B the purification steps are only shown for the membrane fraction. The identity of *Anabaena* KdpD(6His) was confirmed by immunoblotting using the monoclonal penta-His antibodies and by N-terminal amino acid microsequencing (data not shown).

In vivo synthesis of the KdpD protein in *Anabaena* sp. strain L-31. Polyclonal antisera were raised against *Anabaena* KdpD(6His) (purified from *E. coli* membranes) and used to detect the KdpD protein in *Anabaena* sp. strain L-31. The 42-kDa *Anabaena* KdpD protein could be clearly detected in the *Anabaena* sp. strain L-31 membrane fraction, while no protein was observed in the cytosol (Fig. 3A). The 39-kDa band seen in the cytosol appears to be a nonspecific signal, as it was also observed with the preimmune serum (control).

E. coli KdpD has been shown to be located in the cell membrane, and the amount of this protein increases with K$^+$ limitation (35). To test whether this also holds true for *Anabaena* KdpD, cells were grown in the presence or absence of 5 mM K$^+$ and the membranes were isolated. When probed with *Anabaena* KdpD antiserum, an almost equal amount of KdpD was seen under all conditions (Fig. 3B). Thus, the *Anabaena kdpD* gene appeared to be constitutively expressed, and K$^+$ limitation did not enhance its expression level.

Detection of ATP binding to *Anabaena* KdpD(6His) by photoaffinity labeling with 8-azido-[α-^{32}P]ATP. *Anabaena* KdpD has a region similar to the Walker A (GMAPGVGKT) and Walker B (LVLVD) motifs (38). These sites have been proposed to play an important role in modulating the activity of *E. coli* KdpD (17). The NTD of *E. coli* KdpD has been shown to bind 8-azido[α-^{32}P]ATP (14). Everted *E. coli* BL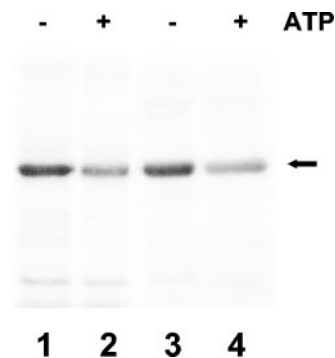-21 membrane vesicles or purified *Anabaena* KdpD(6His) were labeled with 8-azido[α-^{32}P]ATP in the presence or absence of 2 mM unlabeled ATP. Both membrane-bound and purified *Anabaena* KdpD could be labeled with 8-azido[α-^{32}P]ATP, and binding was significantly reduced in the presence of 2 mM ATP (Fig. 4).

In vivo interaction of *Anabaena* KdpD with *E. coli* KdpD-CTD. *E. coli* strain TKW22692 carries the *kdpABCE* genes, but the *kdpD* gene is truncated (KdpD-NTD deletion; *kdpD*/Δ12–395). This truncated KdpD does not affect the ability of this strain to grow normally at very low K$^+$ concentrations (0.1 mM), but the expression of the *kdpFABC* operon is significantly reduced (28). However, when transformed with pBScD1, which constitutively produces the *Anabaena* KdpD(6His) (data not shown), strain TKW22692 grew poorly at 0.1 mM K$^+$, while at 115 mM K$^+$ the growth of TKW22692/pBScD1 and TKW22692/pBluescript was identical.

Based on this observation, *kdp* expression in *E. coli* TKW22692 with and without *Anabaena* KdpD(6His) was tested by monitoring the levels of KdpC in response to K$^+$ limitation. A decrease in KdpC production was observed when *Anabaena* KdpD(6His) was coproduced with *E. coli* KdpD-CTD (Fig. 5A). This indicated the ability of *Anabaena* KdpD(6His) to interact with *E. coli* KdpD-CTD in vivo. In this case the interaction decreased the ability of KdpD-CTD to bring about *kdp* expression.

In vitro interaction of *Anabaena* KdpD(6His) with *E. coli* KdpD-CTD. The kinase and phosphatase activities of *E. coli* KdpD-CTD in the presence and absence of *Anabaena* KdpD(6His) were tested. Since *E. coli kdpD*/Δ12–395 (cloned in the pBAD18 vector) was not compatible with pBADD1 (as they have similar origins of replication), *E. coli kdpD*/Δ12-395 was transferred to pBAD33 (compatible with pBAD24) (13), and the resulting pBD3/Δ12-395 was used for in vitro coexpression. Everted vesicles of strain TKR2000 cotransformed with pBD3/Δ12-395 and pBADD1 were prepared, and the coproduction of *Anabaena* KdpD(6His) and *E. coli* KdpD-CTD was checked on Western blots using penta-His and *E. coli* KdpD antisera, respectively (data not shown).

FIG. 6. Organization of the *kdp* genes in different bacteria. The arrowhead indicates the direction of transcription. SK, sensor kinase; RR, response regulator.

The kinase activity of KdpD-CTD remained unaltered in the presence of *Anabaena* KdpD(6His), but the phosphatase activity of KdpD-CTD was about 34% higher in the presence of *Anabaena* KdpD(6His) (Fig. 5B). In other control experiments *E. coli* TKR2000/pBADD1 vesicles expressing *Anabaena* KdpD(6His) could not dephosphorylate KdpE~P at all (data not shown). These data demonstrated the ability of *Anabaena* KdpD(6His) to modulate *E. coli* KdpD-CTD activity and provided indirect evidence for an interaction between the two proteins.

Coelution of *E. coli* KdpD-CTD with *Anabaena* KdpD(6His). To obtain direct evidence for the interaction between the two proteins, coelution experiments with *Anabaena* KdpD(6His) and *E. coli* KdpD-CTD were performed. The *E. coli* TKR2000/pBADD1/pBD3/Δ12-395 cell lysate was solubilized with the detergent Aminoxid, and the solubilized proteins were allowed to bind to the Ni^{2+}-NTA slurry. After removal of unbound proteins, the bound proteins were eluted with 200 mM imidazole. The protein in the eluted fraction were resolved on SDS-PAGE and probed with *E. coli* KdpD or *Anabaena* KdpD(6His) antiserum separately. *E. coli* KdpD-CTD could be clearly detected in the eluted fraction along with *Anabaena* KdpD(6His) (Fig. 5C). This confirmed the ability of *Anabaena* KdpD(6His) to interact directly with *E. coli* KdpD-CTD.

DISCUSSION

The full-length KdpD protein of *E. coli* and other bacteria is the only known bacterial sensor kinase with a large cytosolic NTD, comprising almost 400 amino acids (17). The *Anabaena* sp. strain L-31 *kdpD* ORF codes for a short KdpD protein of 365 amino acids showing homology only to the KdpD-NTD of *E. coli* and other bacteria. The *kdpDE* genes in other bacteria are located either downstream of *kdpABC* (*E. coli* and *Clostridium acetobutylicum* [34, 37]) or upstream of *kdpABC* (*Mycobacterium tuberculosis* [9]). Although a short version of *kdpD* is present downstream of *kdpC* in *Anabaena* sp. strain L-31, no *kdpE*-like gene has been found 1.5 kb downstream or upstream of *kdpD* or *kdpA*, respectively. Two other cyanobacteria, *Anabaena* sp. strain PCC 7120 and *Synechocystis* sp. strain PCC 6803, also have short *kdpD* genes at comparable positions in their *kdp* operons (20, 21; www.kazusa.or.jp) (Fig. 6). The presence of short versions of *kdpD* is not restricted to cyanobacteria. Other bacteria, such as *Deinococcus radiodurans* R1, *Bacillus anthracis*, *Leptospirillum ferrooxidans*, and *Myxococcus xanthus*, also display truncated *kdpD* genes.

Prior to this study, information was lacking on the regulation of KdpD expression in *Anabaena* spp. or any other bacteria
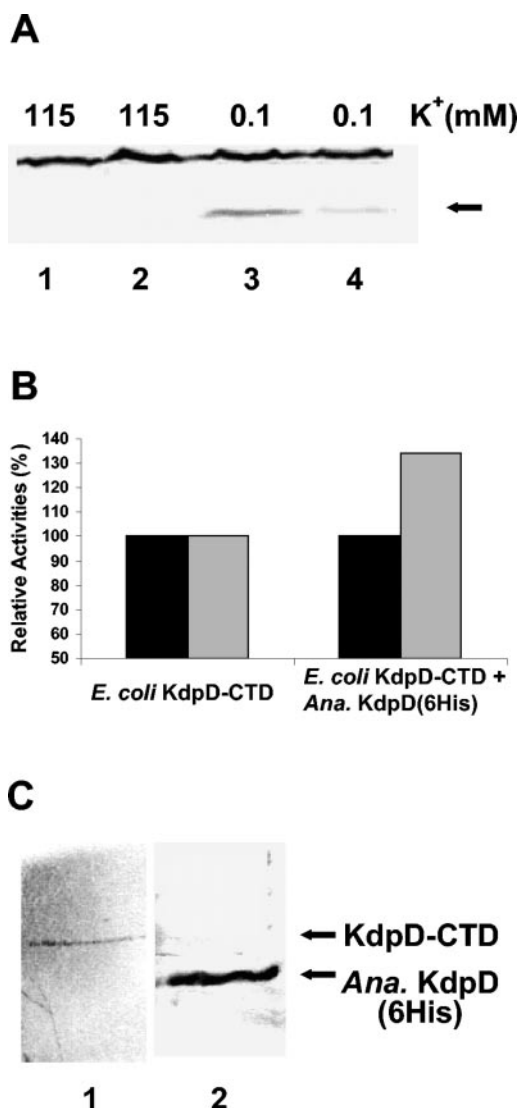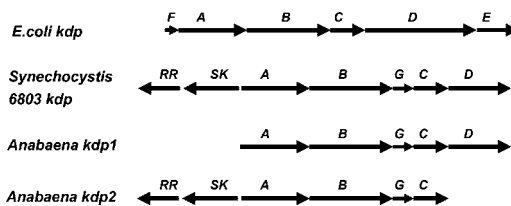

FIG. 5. (A) In vivo interaction of *Anabaena* KdpD(6His) with *E. coli* KdpD-CTD. *E. coli* TKW22692 transformed with pBScD1 or the empty vector was grown in K115 medium and then subjected to K^+ limitation as indicated. Thirty minutes after K^+ downshock, total protein was extracted from the cells and KdpC (arrow) was detected with the *E. coli* KdpC antiserum. Lanes 1 and 3, TKW22692/pBluescript; lanes 2 and 4, TKW22692/pBScD1. (B) In vitro interaction of *Anabaena* KdpD(6His) with *E. coli* KdpD-CTD. The kinase activity (black bars) and the phosphatase activity (grey bars) of *E. coli* KdpD-CTD in the presence and in the absence of *Anabaena* KdpD(6His) were determined. The data are presented as percentages of the initial rates relative to *E. coli* KdpD-CTD. The kinase reaction was initiated by addition of $[\gamma\text{-}^{32}P]$ATP to everted membrane vesicles containing *E. coli* KdpD-CTD with or without *Anabaena* KdpD(6His). For phosphatase measurements, purified KdpE~^{32}P was added to the vesicles in the presence of 1 mM ATP-γ-S. For both assays, aliquots were removed at definite time points and added to SDS sample buffer to stop the reaction. The proteins were separated by SDS-PAGE, and the radiolabeled proteins were quantified with a PhosphorImager (Molecular Dynamics). For KdpD-CTD (100% values), the phosphorylation activity was 2.5 pmol KdpD~P min^{-1} mg $protein^{-1}$ and the phosphatase activity was 0.01 pmol of P_i released min^{-1} mg $protein^{-1}$. (C) Coelution of *Anabaena* KdpD (6His) and *E. coli* KdpD-CTD. The *E. coli* TKR2000/pBADD1/pBD3/Δ12-395 cell lysate was used for coelution experiments as described in Materials and Methods. The elution fraction was resolved on SDS-PAGE and probed separately with *E. coli* KdpD (lane 1) and *Anabaena* KdpD(6His) (lane 2) antisera.

that encode a short *kdpD* gene. To specifically address this question, polyclonal antisera were raised against purified *Anabaena* KdpD(6His). *Anabaena* KdpD, as detected by the specific antiserum, was only observed in the membrane fraction of *Anabaena* sp. strain L-31 and not in the cytosol. Similarly, *E. coli* KdpD-NTD has been shown to be attached to the *E. coli* cell membrane (14).

In *E. coli* the amount of KdpD protein synthesized depends on the K^+ concentration of the medium. The level of *E. coli* KdpD in K^+-rich medium is very low but increases severalfold under K^+ limitation (35) due to the readthrough of the *kdpFABC* operon into the *kdpDE* operon during transcription. *Anabaena* sp. strain L-31 *kdpD* was observed to be constitutively expressed but, unlike *E. coli kdpD*, its level did not increase with K^+ limitation. This suggests that a readthrough of the *kdpABGC* operon into *kdpD* is probably absent in *Anabaena* sp. strain L-31 or that expression of the operon is not regulated at the transcriptional level.

Anabaena KdpD contains a slightly modified Walker A (ATP binding) motif. A similar binding site in *E. coli* KdpD-NTD has been shown to play a role in regulating the phosphatase activity of the KdpD protein (17). Membrane-bound *E. coli* KdpD-NTD has been shown to bind 8-azido-ATP. *Anabaena* KdpD(6His) in its membrane-bound form or in its soluble form also binds 8-azido-ATP, indicating the presence of a functional ATP binding site. We have earlier constructed a chimeric KdpD (AnacoliKdpD) from *Anabaena* KdpD and *E. coli* KdpD-CTD and studied some of its properties in vivo and in vitro (6). The hybrid protein, like *E. coli* KdpD, showed very rapid ATP-dependent phosphatase activity in vitro. Therefore, the ATP binding site in *Anabaena* KdpD may play a regulatory role in *kdpFABC* expression mediated by AnacoliKdpD in *E. coli*.

The NTD of KdpD has been shown to regulate the *E. coli* KdpD phosphatase activity (17), which in turn has been implicated in the regulation of *kdpFABC* expression. KdpD mutants that show low or negligible phosphatase activity exhibit constitutive *kdpFABC* expression (7). Conversely, KdpD mutants that show high phosphatase activity exhibit reduced *kdpFABC* expression in vivo (18). *E. coli* KdpD-NTD has been shown to complement in *trans* the membrane-anchored KdpD-CTD, resulting in restoration of signal transduction in vivo (14). However, our experiments show that coexpression of *Anabaena* KdpD(6His) with *E. coli* KdpD-CTD decreased *kdpFABC* expression in vivo and increased the phosphatase activity of the *E. coli* KdpD-CTD studied in vitro.

Coelution data shown here clearly indicated the ability of *Anabaena* KdpD(6His) to interact with *E. coli* KdpD-CTD. The results show that a heterologous NTD (*Anabaena* KdpD) can affect the KdpD-CTD (*E. coli*) activity, however, this modulation is different from that observed with the KdpD-NTD from *E. coli*. Therefore, this observation lends support to the notion that the NTDs from various organisms might exert different effects on the in vivo and in vitro activities of KdpD-CTD proteins. Furthermore, it is conceivable that, in contrast to the intact KdpD protein, the separation of domains (NTD and CTD) allows more flexibility with respect to interaction not only with CTD domains, but also with additional proteins. Evidence in this direction stems from the recent observation

that the KdpD-NTD of *Mycobacterium tuberculosis* interacts with the lipoproteins LprJ and LprF (31).

How the short KdpD functions in vivo in *Anabaena* sp. strain L-31 is not clear. The genome of *Anabaena* sp. strain L-31 (our unpublished results) and that of the cyanobacterium *Anabaena* sp. strain PCC 7120 (21) show the presence of a second (*kdp2*) operon encompassing the *kdp2ABGC* genes but lacking *kdpD*. Interestingly, in both *Anabaena* strains, upstream of the *kdpA2* gene two ORFs exist, which code for a putative sensor kinase and a response regulator (Fig. 6). The sensor kinase is a membrane-anchored polypeptide consisting of N-terminal transmembrane segments followed by a cytosolic histidine kinase domain (reminiscent of *E. coli* KdpD-CTD), while the response regulator shows homology to the general class of bacterial response regulators.

Our results indicate that *Anabaena* KdpD can interact with a membrane-anchored histidine kinase (*E. coli* KdpD-CTD) and modulate its activity. Therefore, it is conceivable that in *Anabaena* sp. strain L-31, KdpD may interact in vivo with a membrane-bound histidine kinase and modulate its activity. The search for partners for *Anabaena* KdpD is currently being pursued in our laboratory.

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