

# Differential Expression of the Two *kdp* Operons in the Nitrogen-Fixing Cyanobacterium *Anabaena* sp. Strain L-31

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In several types of bacteria, the Kdp ATPase (comprising of the KdpABC complex) is an inducible, high-affinity potassium transporter that scavenges  $K^+$  from the environment. The cyanobacterium *Anabaena* sp. strain L-31 showed the presence of not one but two distinct *kdp* operons in its genome. The *kdp1* consisted of *kdpA1B1G1C1D* genes, whereas the *kdp2* contained the *kdpA2B2G2C2* genes. Among the regulatory genes, the *kdpD* open reading frame of *Anabaena* sp. strain L-31 was truncated compared to the *kdpD* of other bacteria, whereas a *kdpE*-like gene was absent in the vicinity of the two *kdp* operons. In response to  $K^+$  limitation (<0.05 mM external  $K^+$ ), only *kdp2* (and not *kdp1*) expression could be detected as a 5.3-kb transcript on Northern blots, indicating that *kdpA2B2G2C2* genes constitute a polycistronic operon. Unlike *E. coli*, addition of osmolytes like NaCl, or a change in pH of the medium did not enhance the *kdp* expression in *Anabaena* sp. strain L-31. Interestingly, the *Anabaena* sp. strain L-31 *kdp2* operon was strongly induced in response to desiccation stress. The addition of  $K^+$  to  $K^+$ -starved cultures resulted in repression and degradation of *kdp2* transcripts. Our results clearly show that *kdp2* is the major *kdp* operon expressed in *Anabaena* sp. strain L-31 and may play an important role in adaptation to  $K^+$  limitation and desiccation stress.

Potassium, the major intracellular cation in bacteria, is involved in various physiological processes such as turgor adaptation (15), activation of cellular enzymes (29), and pH homeostasis (12). In nitrogen-fixing cyanobacteria such as *Anabaena* spp.,  $K^+$  also regulates gene expression and vital metabolic processes such as photosynthesis and nitrogen fixation (1, 2). Despite very low levels of  $K^+$  available in most environments (0.1 to 10 mM), the bacteria maintain a very high concentration of  $K^+$  (0.2 to 0.6 M) within their cells and have evolved several distinct  $K^+$  uptake and efflux systems to regulate their internal  $K^+$  concentration (15). In *Escherichia coli* the constitutively expressed TrkG, TrkH, and Kup uptake systems have a low affinity for  $K^+$  but are competent to maintain the required levels  $K^+$  under normal physiological conditions (20). Under conditions of severe  $K^+$  limitation or osmotic upshift or when the low-affinity transporters are unable to meet the cell's demand for  $K^+$ , the high-affinity KdpATPase ( $K^+$ -dependent ATPase) is expressed (4). The  $K_m$  of *E. coli* KdpATPase for  $K^+$  is 2  $\mu$ M and the cells expressing Kdp can reduce the  $K^+$  concentration in the medium to as low as 50 nM (28).

Earlier studies had shown the *kdp* homologs to be widely distributed among the gram-negative bacteria (32). Genome sequencing has now shown *kdp* homologs to be present also in cyanobacteria, gram-positive bacteria, and the *Archaea*. We have previously demonstrated the presence of KdpB-like polypeptide in three different strains of the nitrogen-fixing cyanobacteria *Anabaena* spp. (6). The KdpB was induced under conditions of  $K^+$  limitation, and the KdpB protein was shown to be located in *Anabaena* membranes (3). In order to

understand the organization and the regulation of expression of *kdp* genes, we undertook the cloning and sequencing of the *kdp* operon from *Anabaena* sp. strain L-31. Degenerate primers based on evolutionarily conserved amino acid stretches within different Kdp proteins from several bacteria were used to amplify *Anabaena* sp. strain L-31 chromosomal DNA. Interestingly, sequence analysis of *kdp*-like PCR fragments showed presence of not one but two distinct *kdp* operons (*kdp1* and *kdp2*) in *Anabaena* sp. strain L-31. We examined here the expression of the two *kdp* operons under a variety of environmental stress conditions. Our data show that *Anabaena* sp. strain L-31 *kdp2* (and not *kdp1*) is the major *kdp* operon transcriptionally activated in response to  $K^+$  limitation and desiccation stress. Unlike *E. coli* and other bacteria, osmotic upshift (both ionic and nonionic) did not induce *kdp* expression in *Anabaena* sp. strain L-31.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** A filamentous, heterocystous, nitrogen-fixing freshwater cyanobacterium *Anabaena* sp. strain L-31 isolated in our laboratory (31) was used under axenic conditions. The *Anabaena* sp. strain L-31 cultures were maintained in the BG-11 medium (13) without combined nitrogen under continuous illumination (30  $\mu$ E/m<sup>2</sup>/s) and aeration (2.0 liters/min). The  $K_2HPO_4$  from BG-11 was replaced with equimolar  $Na_2HPO_4$  to obtain BG-11/K0 medium. For  $K^+$  limitation experiments, *Anabaena* sp. strain L-31 cells were inoculated into BG-11/K5 (BG-11/K0 plus 5 mM KCl) and allowed to grow (with aeration) to a chlorophyll *a* concentration of 4 to 6  $\mu$ g/ml. The cells were harvested by centrifugation, washed five times with 5 volumes of BG-11/K0 containing appropriate concentrations of KCl, and inoculated in identical medium at a concentration of ca. 4 to 6  $\mu$ g of chlorophyll *a*/ml. The BG-11N<sup>+</sup> medium was prepared by the addition of  $NaNO_3$  (17 mM) to the BG-11/K5 or BG-11/K0 media. The initial pH of all media, unless otherwise specified, was adjusted to 7.0. For RNA isolation, *Anabaena* sp. strain L-31 cells were harvested after 16 h or at the time points indicated in Results. For osmotic upshift, the BG-11/K0 or BG-11/K0.05 (BG-11/K0 plus 0.05 mM KCl) medium was supplemented with 65 mM NaCl or 110 mM sucrose or 12% polyethylene glycol (PEG). For desiccation experiments, *Anabaena* sp. strain L-31 cells grown in BG-11/K5 were thoroughly washed with and resuspended in BG-11/K0.1 medium. After 16 h the cells were filtered (0.45- $\mu$ m pore size, mixed cellulose ester; Millipore)

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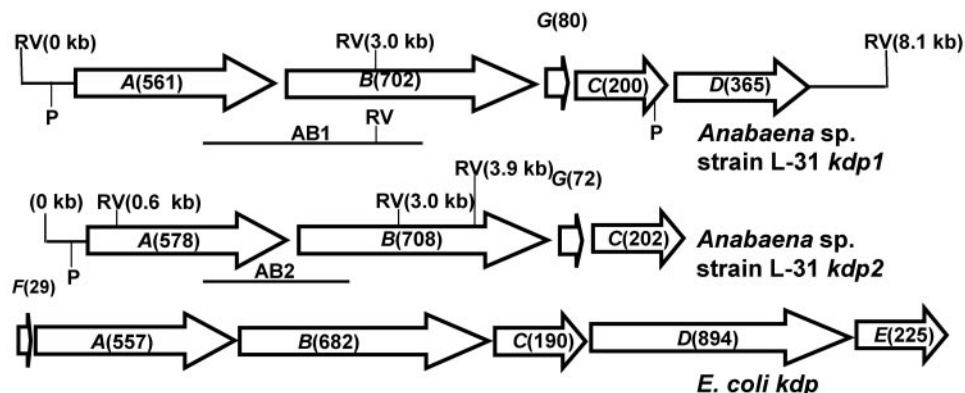


FIG. 1. Two *kdp* operons from *Anabaena* sp. strain L-31. The arrangement of the *kdp* genes within the *Anabaena* sp. strain L-31 *kdp* operons is schematically depicted. The *E. coli* *kdp* operon is also shown for comparison. ORFs are shown as arrowheads indicating the direction of transcription. The expected number of amino acids for each ORF is shown in parentheses. The positions of putative promoters (P) are indicated. The chromosomal locations of the two DNA probes (AB1 and AB2) used for Southern hybridization experiments (see Fig. 2) are shown below their respective operons. The location of the EcoRV restriction enzyme site within the *kdp* operons is indicated (RV).

using a vacuum assembly. The filter paper disks containing the *Anabaena* sp. strain L-31 cells were allowed to desiccate in air at room temperature for different time intervals under illumination (30  $\mu\text{E}/\text{m}^2/\text{s}$ ).

*E. coli* DH5 $\alpha$  [ $\Delta(\text{argF-lac})\text{U169 supE44 } \phi 80\text{d lacZ}\Delta\text{M15 recA1 endA1 gyrA96 thi-1 relA1 hsdR17}$ ] was used as host to maintain all the plasmids used in the present study.

**Cloning of *kdp* operons from *Anabaena* sp. strain L-31.** Degenerate primers based on evolutionary conserved amino acid stretches within different Kdp proteins from several bacteria such as *E. coli*, *Synechocystis* sp. strain PCC 6803, *Clostridium acetobutylicum*, and *Alicyclobacillus acidocaldarius* were used to amplify *Anabaena* sp. strain L-31 *kdp* genes from chromosomal DNA. The PCR products were cloned, sequenced, and the deduced amino acid sequence was subjected to BLAST search (5). PCR with *kdp*-specific primers generated two different *kdp*AB-like DNA products (AB1 and AB2) that showed only 70% nucleotide identity to each other and also hybridized to different restriction enzyme-digested *Anabaena* sp. strain L-31 DNA fragments on Southern blots (Fig. 1 and 2). This indicated the presence of two *kdp* operons in *Anabaena* sp. strain L-31. The *kdp*-like DNA fragments obtained from PCR were used as probes to clone the *Anabaena* sp. strain L-31 *kdp* operons by chromosomal walking (data not shown). The entire *kdp1* and *kdp2* operons cloned as described above were completely sequenced (GenBank accession numbers AF213466 and AY753299 for *kdp1* and *kdp2*, respectively).

**Southern blotting and hybridizations.** *Anabaena* sp. strain L-31 chromosomal DNA was prepared as described earlier (7). Spooled *Anabaena* sp. strain L-31 chromosomal DNA digested with desired restriction enzymes was resolved by electrophoresis on 0.8% agarose gels, transferred to a positively charged nylon membrane (Roche) by capillary blotting, and processed as instructed (DIG

*System User's Guide* [Roche]). The *kdp* genes were labeled with digoxigenin (DIG) by random priming and used as probes for Southern blots. The hybridization was performed in DIG Easy Hyb buffer (Roche) at 42°C overnight. Posthybridization, the membranes were washed twice with solution A (2 $\times$  SSC [1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate [SDS]) at room temperature and twice with solution B (0.1 $\times$  SSC, 0.1% SDS) at 68°C. The chemiluminescent detection was subsequently carried out according to the instructions provided by the manufacturer for filter hybridizations (Roche).

**Isolation of *Anabaena* sp. strain L-31 total RNA.** A total of 50 ml of *Anabaena* sp. strain L-31 cells (4 to 6  $\mu\text{g}$  of chlorophyll *a*/ml) was harvested by centrifugation at the indicated time points and immediately shock frozen in liquid nitrogen. Then, 1 ml of RNA isolation reagent (RNAwiz; Ambion) was added to the cells, and the RNA was isolated according to the recommended protocol. The RNA pellet was resuspended in DNase I reaction buffer (0.1 M sodium acetate [pH 5.0], 5 mM  $\text{MgSO}_4$ ) and subjected to DNase I (RNase free, 5 U) treatment. At the end of 45 min, 2 M LiCl (final concentration) was added to the tubes, and the tubes were kept at  $-20^\circ\text{C}$  for at least 1 h. The RNA was collected by centrifugation (15,000  $\times$  g, 20 min) at 4°C. The RNA pellet was washed with 75% ethanol, air dried, and dissolved in RNase-free water.

**Northern blotting and dot blot hybridization.** For Northern blotting experiments, 15  $\mu\text{g}$  total RNA was electrophoretically resolved on denaturing formaldehyde agarose gels using MOPS buffer (0.02 M 3-[*N*-morpholino]propanesulfonic acid, 5 mM sodium acetate [pH 7.0], 0.01 M disodium EDTA). After electrophoresis the gels were washed with diethyl pyrocarbonate-treated water, and the RNA was transferred to positively charged nylon membrane by capillary blotting with 10 $\times$  SSC. For dot blots, 4  $\mu\text{g}$  of RNA was spotted directly onto a nylon membrane with a micropipette. Prehybridization was carried out in DIG Easy Hyb buffer (Roche) for at least 1 h at 50°C. The individual *kdp* genes were amplified by PCR, labeled with DIG, and hybridized to the RNA in DIG Easy Hyb buffer (overnight at 50°C). After hybridization the membranes were washed twice with solution A (2 $\times$  SSC, 0.1% SDS) for 15 min at room temperature and twice with solution B (0.1 $\times$  SSC, 0.1% SDS) for 15 min at 66°C. The chemiluminescent detection was subsequently carried out according to the instructions provided by the manufacturer for filter hybridizations (Roche).

**RT-PCR.** Reverse primers specific for *kdpA1*, *kdpC1*, and *kdpA2* were used to reverse transcribe 0.5  $\mu\text{g}$  of total RNA using the enhanced avian reverse transcriptase (RT; Sigma). The following forward and reverse primers were used: GCCACCATGTGCGGCGCAGTC (RtkdpA1fwd) and TAAACTATGCCG GCGGTGAT (RtkdpA1rev) for *kdpA1*, ATGTTACAAGGCTGGATACAA (RtkdpA2fwd) and CCCTGCTAATGTTTCCGGCAC (RtkdpA2rev) for *kdpA2*, and ATCTCCTTGATTGAGAAGCTT (RtkdpC1fwd) and CCCATTC CTTAATCTTATCGAT (RtkdpC1rev) for *kdpC2*. The RT products were directly used as templates for amplification with the specific forward and reverse primers. Additional PCRs were performed with RNA without the RT reaction (negative control) and chromosomal DNA (positive control) using the same master mix containing all of the necessary components. The amplification prod-

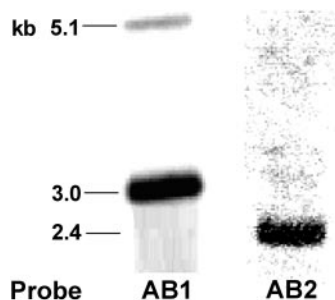


FIG. 2. Southern hybridization analysis. The DNA probes AB1 (2.1 kb) and AB2 (1.5 kb) (see Fig. 1) were generated by PCR, labeled with DIG, and hybridized separately to the EcoRV-digested *Anabaena* sp. strain L-31 chromosomal DNA (3  $\mu\text{g}$ ) on Southern blots. The hybridization signals obtained with the two probes and their respective sizes are shown.

TABLE 1. Predicted homology (% identity) of *Anabaena* sp. strain L-31 Kdp proteins with corresponding KdpA, KdpB, or KdpC proteins from other bacteria

<i>Anabaena</i> sp. strain L-31 Kdp	% Identity with:				
	<i>Anabaena</i> sp. strain PCC 7120 Kdp1	<i>Anabaena</i> sp. strain PCC 7120 Kdp2	<i>Synechocystis</i> sp. strain PCC 6803 Kdp	<i>Escherichia coli</i> Kdp	<i>Mycobacterium tuberculosis</i> Kdp
KdpA1	94	64	66	44	38
KdpA2	65	91	65	43	40
KdpB1	92	72	75	58	54
KdpB2	72	91	73	59	50
KdpC1	85	51	59	46	45
KdpC2	54	91	61	48	41

ucts were resolved by electrophoresis on agarose gels and detected by staining with ethidium bromide.

## RESULTS

**Organization of the *kdp* operons in *Anabaena* sp. strain L-31.** By using PCR with *kdp*-specific degenerate primers, we detected the presence of not one but two *kdp* operons in *Anabaena* sp. strain L-31. Both of the *kdp* operons have been cloned and sequenced by standard molecular biology techniques (GenBank accession numbers AF213466 and AY753299 for *kdp1* and *kdp2*, respectively). The arrangement of individual *kdp* genes within the two *Anabaena* sp. strain L-31 *kdp* operons is shown in Fig. 1 and closely resembles the arrangement of *kdp* operons observed in *Anabaena* sp. strain 7120 (21). Sequence analysis showed the presence of five open reading frames (ORFs; *kdpA1*, *kdpB1*, *kdpG1*, *kdpC1*, and *kdpD*) in *kdp1* (9) and four ORFs (*kdpA2*, *kdpB2*, *kdpG2*, and *kdpC2*) in *kdp2*. In all cyanobacterial *kdp* operons sequenced thus far, no genes with overlapping ORF have been observed. The position of putative promoters (based on homology search) identified upstream of *kdpA1*, *kdpA2*, and *kdpD* is depicted in Fig. 1. The BLAST search with deduced KdpABC amino acid sequences from both *Anabaena* sp. strain L-31 *kdp* operons showed very good homology to other bacterial KdpABC proteins (Table 1). Many *kdp* operons from other bacteria show the presence of small ORFs encoding for hydrophobic peptides, e.g., *kdpF* in *E. coli* (17) and *M. tuberculosis* (14) and *kdpZ* and *kdpY* in *C. acetobutylicum* (10). Although a *kdpF*-like gene was absent from the two *Anabaena* sp. strain L-31 *kdp* operons, an additional ORF, *kdpG*, was found located between *kdpB* and *kdpC*. *Anabaena* sp. strain L-31 *kdpG* appeared to encode a hydrophobic protein with two transmembrane segments. The KdpG, which is unique to cyanobacterial genomes, may play a role in stabilization or activity of the KdpATPase complex, as has been shown for KdpF and KdpY-Z in other organisms (10, 17). A naturally short *kdpD* ORF was observed downstream of *kdpC1*, while no such ORF was observed in the *kdp2* operon of *Anabaena* sp. strain L-31. The *kdpD* ORF encoded a protein of only 365 amino acids corresponding to the KdpD N-terminal domain of *E. coli*, whereas the C-terminal histidine kinase domain was missing from *Anabaena* sp. strain L-31 KdpD (9). We have evidence that *Anabaena* sp. strain L-31 KdpD is synthesized constitutively irrespective of the K<sup>+</sup> concentration in the medium (8a), which implies that *kdpD* gene is transcribed from its own pro-

moter independent of the *kdpA1* promoter. No *kdpE* like gene was found in the vicinity of the two *kdp* operons.

***kdp2* is the major *kdp* operon expressed under K<sup>+</sup> limitation.** *Anabaena* sp. strain L-31 *kdpA1B1C1* showed 60 to 75% nucleotide homology with the corresponding genes from the *kdp2* operon. Southern blotting and hybridization experiments were performed with probes AB1 and AB2 (see Fig. 1 for details) to ascertain the ability of the *Anabaena* sp. strain L-31 *kdp1* gene (DNA) probe to cross-hybridize with the corresponding *kdp2* genes and vice versa. The probe AB1 showed hybridization signals of 5.1 and 3.0 kb on Southern blots (Fig. 2) of EcoRV-digested *Anabaena* sp. strain L-31 chromosomal DNA. When probe AB2 was used, neither the 5.1- nor the 3.0-kb signal was observed, although a 2.4-kb signal was detected (Fig. 2). This demonstrated the inability of the probes to cross-hybridize with the corresponding DNA from the other *kdp* operon under the stringency conditions used (see Materials and Methods). Hence, Northern blotting (of the *Anabaena* sp. strain L-31 total RNA), followed by hybridization to *kdp1* or *kdp2* DIG-labeled DNA probes, was used to monitor the expression of one *kdp* operon vis a vis the other.

In *E. coli* and several other bacteria the *kdp* operon is induced by K<sup>+</sup> limitation and repressed by high levels of K<sup>+</sup> (20). Earlier studies in our laboratory showed that high K<sup>+</sup> (5 mM) repressed KdpB expression in *Anabaena* spp. (3, 6). Based on this observation the *Anabaena* sp. strain L-31 cells were grown in BG-11/K5 (to completely suppress *kdp* expression) and transferred to BG-11/K0 medium after thorough washing in the same medium. When hybridized with the *kdpA1* probe, no signal was detected in the BG-11/K5 or BG-11/K0 total RNA (Fig. 3A). However, when the same RNA was probed with *kdpA2* or *kdpC2*, a strong signal of 5.3 kb was observed in the K<sup>+</sup> starved samples while none was observed in the BG-11/K5 samples (Fig. 3A). This indicated that *kdp2* and not *kdp1* was the *kdp* operon induced under K<sup>+</sup> limiting conditions and that the *kdpA2B2G2C2* did form a polycistronic operon.

A more sensitive RT-PCR technique was used to verify whether *kdp1* was expressed at all. Reverse primers (see Materials and Methods) specific for *kdpA1*, *kdpC1*, and *kdpA2* were utilized to reverse transcribe RNA isolated from *Anabaena* sp. strain L-31 cells grown in BG-11/K5 or BG-11/K0. On PCR amplification with the specific forward primers, *kdpA1* and *kdpC1* amplification products were detected in BG-11/K0 RNA. However, the amount of *kdpA1/C1* PCR products

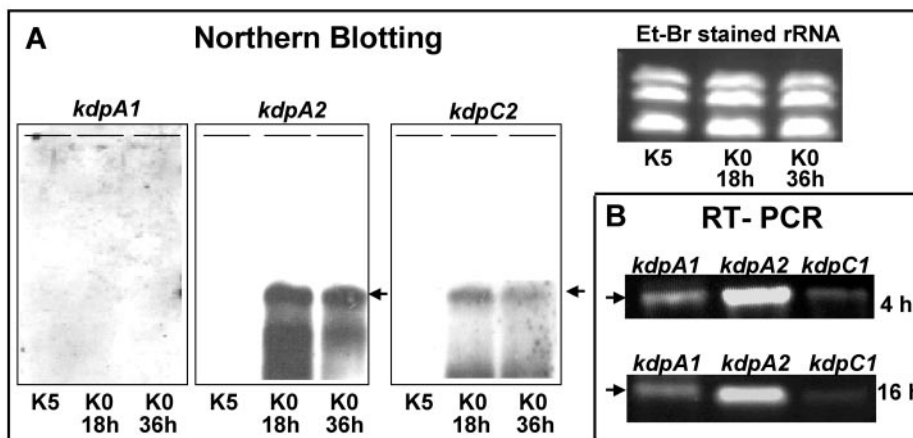


FIG. 3. Differential expression of the *kdp* operons in *Anabaena* sp. strain L-31. (A) Northern blotting. Total RNA was isolated from *Anabaena* sp. strain L-31 cells grown in BG-11/K5 or BG-11/K0 for 18 and 36 h and electrophoretically resolved (15  $\mu$ g per lane) on formaldehyde agarose gels. First, 600 bp of *kdpA1* ORF, 1 to 400 bp of *kdpA2* ORF, and 200 to 600 bp of *kdpC2* ORF were amplified by PCR, labeled with DIG, and used as *kdpA1*, *kdpA2*, or *kdpC2* probes, respectively. The RNA was transferred to nylon membrane and subsequently hybridized to the individual probes. The 5.3-kb transcript is indicated by an arrow. Equal loading of RNA was confirmed by ethidium bromide staining of rRNA from various samples. (B) RT-PCR. Total RNA was isolated from cells grown in BG-11/K0 for 4 or 16 h after inoculation and subjected to RT reaction with primers specific for *Anabaena* sp. strain L-31 *kdpA1*, *kdpA2*, or *kdpC1* genes. The RT reaction was subsequently subjected to PCR, and the amplification products were resolved by electrophoresis and stained with ethidium bromide.

was severalfold lower than that obtained with *kdpA2*-specific primers (Fig. 3B), clearly showing that *kdp2* is the major *kdp* operon expressed under conditions of  $K^+$  starvation in *Anabaena* sp. strain L-31. No PCR product was observed when RNA isolated from *Anabaena* sp. strain L-31 cells grown in BG-11/K5 was subjected to RT-PCR with the above-mentioned primers (data not included). PCRs without the RT reaction (negative control) yielded no amplification products (data not shown).

**Response of *Anabaena* sp. strain L-31 *kdp2* operon to  $K^+$  starvation.** To determine the threshold of external  $K^+$  concentration that allowed *kdp2* expression, *Anabaena* sp. strain L-31 cells that had been grown in BG-11/K5 were inoculated into

BG-11 medium with different concentrations of  $K^+$  as indicated (Fig. 4A). The total RNA was isolated from all the cultures after 16 h and hybridized to the *kdpA2* probe. The highest external  $K^+$  concentration that allowed *kdpA2* expression was 0.05 mM  $K^+$ , whereas maximal expression was observed at  $<0.025$  mM  $K^+$  (Fig. 4A).

The kinetics of *Anabaena* sp. strain L-31 *kdp2* expression in response to  $K^+$  starvation was also monitored (Fig. 4B). The *kdp2* expression was clearly observed after 1 h of  $K^+$  limitation, and maximal expression occurred by 3 h of  $K^+$  starvation. The ability of external  $K^+$  to repress the previously induced *kdp2* operon was also examined. Upon addition of 5 mM  $K^+$  to a  $K^+$ -starved culture, the *kdp2* expression was drastically reduced, and 30 min after  $K^+$  addition hardly any expression could be detected (Fig. 4B). The *kdpA1* expression could not be detected on dot blots even after prolonged (24 h)  $K^+$  starvation.

**Effect of osmotic stress on *Anabaena* sp. strain L-31 *kdp2* expression.** Osmotic stress, particularly ionic upshock, is known to induce *kdp* expression in the presence of moderate external  $K^+$  in *E. coli* (8, 25, 30). To check whether the same held true for *Anabaena*, the *kdp* expression in response to NaCl and other nonionic osmolytes was examined. Initially, the *kdp* expression was monitored in BG-11/K0.05, i.e., the highest  $K^+$  concentration at which *Anabaena* sp. strain L-31 *kdp2* expression could be detected (Fig. 5A). Sucrose and PEG did not enhance *kdp2* expression, and NaCl, in fact, decreased *kdp2* expression (Fig. 5A).

Whether osmotic stress increased *kdp2* expression over and above that seen with extreme  $K^+$  deprivation (K0) was also determined. Sucrose and PEG did not enhance *kdp2* expression (data not shown), but with NaCl a significant reduction in the *kdp2* expression was again observed. The addition of NaCl at the beginning of  $K^+$  starvation or at a later point during  $K^+$  starvation had the same effect of reducing *kdp2* expression

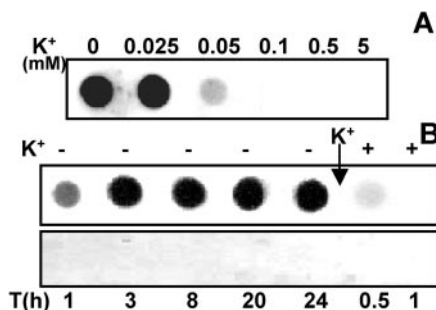


FIG. 4. Effect of  $K^+$  limitation on *Anabaena* sp. strain L-31 *kdp2* expression. (A) *Anabaena* sp. strain L-31 cells grown in BG-11/K5 were inoculated into BG-11 with different concentrations of external  $K^+$  and grown for 16 h. Total RNA (4  $\mu$ g) was spotted onto nylon membrane and hybridized to the *kdpA2* probe. (B) *Anabaena* sp. strain L-31 cells grown in BG-11/K5 were inoculated into BG-11/K0 medium, and the total RNA isolated at the indicated time points. At the end of 24 h, KCl (5 mM) was added to the medium (shown by an arrow), and the total RNA was isolated 0.5 and 1.0 h after  $K^+$  repletion. All of the RNA samples (4  $\mu$ g each) were hybridized to the *kdpA2* probe (upper panel) or the *kdpA1* probe (lower panel).

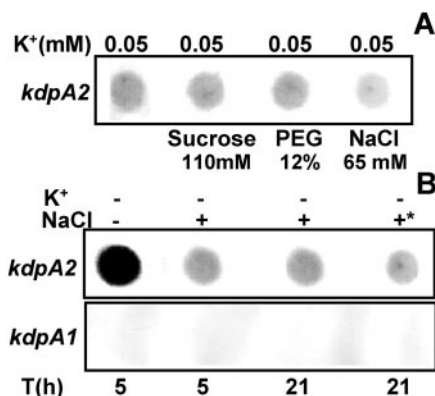


FIG. 5. Effect of osmotic stress on *Anabaena* sp. strain L-31 *kdp* expression. (A) *Anabaena* sp. strain L-31 cells were inoculated into BG-11/K0.05 medium containing different osmolytes as indicated. Total RNA (4  $\mu$ g each) was hybridized to the *kdpA2* probe. (B) Combined effect of extreme  $K^+$  limitation and NaCl stress on *kdp2* expression. *Anabaena* sp. strain L-31 cells grown in BG-11/K5 were inoculated into BG-11/K0 with or without NaCl (65 mM) for the indicated time intervals. In another experiment, the cells grown in BG-11/K0 medium for 5 h were supplemented with NaCl (65 mM), and the culture was incubated for a further 16 h with NaCl (indicated by an asterisk). Total RNA was isolated and hybridized to the *kdpA2* (upper panel) or *kdpA1* (lower panel) probe.

(Fig. 5B). These RNA samples were also hybridized to the *kdpA1* DNA probe, but no detectable *kdp1* expression was observed (Fig. 5B).

**Expression of *Anabaena* sp. strain L-31 *kdp2* in response to other environmental stresses.** A decrease in pH of the medium is known to induce *kdp* expression in *E. coli* (8). To test the effect of pH on *Anabaena* sp. strain L-31 *kdp* expression, the cells were inoculated in BG-11/K0 or BG-11/0.05 medium at pH 6.0 or 7.0, and the total RNA was isolated at the end of 16 h. When the RNA was probed with the *kdpA2* probe, no increase in *kdp2* due to the pH shift (7.0 to 6.0) over that at pH 7.0 was observed (Fig. 6A). Expression of *kdp1* was not detected, even at pH 6.0 (data not shown). The presence of a combined nitrogen source in the medium is known to regulate the expression of several genes, e.g., *nif*, *het* (18, 34), and several osmoreponsive proteins (19) in *Anabaena* spp. However, the addition of 17 mM  $NaNO_3$  during growth did not affect expression of *kdp1* or *kdp2* operons in *Anabaena* sp. strain L-31 (data not shown). Heat shock is known to alter the expression of several stress responsive genes in bacteria. *Anabaena* sp. strain L-31 cells grown in BG-11/K0 were exposed to 42°C for 30 min, and the RNA was isolated. Heat shock decreased the *kdpA2* expression under  $K^+$  limitation, whereas no expression was seen in cells grown with 5 mM  $K^+$  (Fig. 6B). Heat shock did not influence the *kdp1* expression (data not shown).

Cyanobacteria are periodically exposed to desiccation in the natural environment. Recently, *kdp2* operon from *Anabaena* sp. strain 7120 was reported to be induced in response to desiccation stress (24). The response of *Anabaena* sp. strain L-31 *kdp* operons to desiccation stress was also analyzed. *Anabaena* sp. strain L-31 cells grown in BG-11/K5 were inoculated in BG-11/K0.1 medium, wherein no *kdp* expression was observed (Fig. 4A). After 16 h of incubation in this medium,

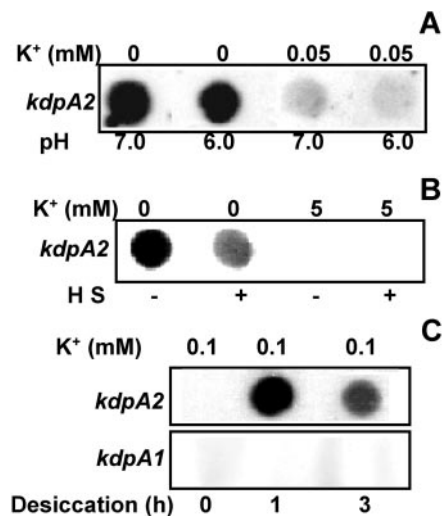


FIG. 6. Effect of different environmental stresses on *Anabaena* sp. strain L-31 *kdp2* expression. (A) *Anabaena* sp. strain L-31 cells were inoculated in BG-11 medium with  $K^+$  concentrations and pH as indicated. (B) *Anabaena* sp. strain L-31 cells were subjected to heat shock (HS) at 42°C for 30 min in the presence or absence of 5 mM  $K^+$ . (C) *Anabaena* sp. strain L-31 cells grown in BG-11/K0.1 were filtered onto filter paper disks and desiccated under illumination for the specified time intervals. The total RNA (4  $\mu$ g) from each of the above samples (A, B, and C) was hybridized to the *kdpA2* or *kdpA1* probe as denoted.

the cells were filtered (0.45- $\mu$ m pore size; Millipore) and allowed to desiccate under light for 1 and 3 h. The total RNA was isolated and hybridized to the *kdpA2* probe. No expression was observed in the BG-11/K0.1 control, whereas *kdp2* expression was clearly observed in the RNA obtained from desiccated *Anabaena* sp. strain L-31 (Fig. 6C). No expression was detected when the *kdpA1* probe was hybridized to the RNA isolated from either desiccated or nondesiccated *Anabaena* sp. strain L-31 cells (Fig. 6C).

## DISCUSSION

We earlier demonstrated the presence KdpB polypeptides in three strains of *Anabaena* (including L-31) and their regulation by  $K^+$  with the *E. coli* KdpB antiserum (3, 6). Here we report on the *Anabaena* sp. strain L-31 *kdp* operon's organization and its transcriptional activation in response to various environmental conditions. Nucleotide sequencing and Southern hybridization analysis demonstrated the presence of two distinct *kdp* operons in *Anabaena* sp. strain L-31. The genome of another filamentous cyanobacterium, *Anabaena* sp. strain PCC 7120 (21), also possesses two *kdp* operons very similar to those of strain L-31, while two unicellular cyanobacteria, *Synechocystis* sp. strain PCC 6803 (22) and *Gloeobacter violaceus* PCC 7421, possess only one *kdp* operon ([www.kazusa.or.jp/cyano/gloeobacter](http://www.kazusa.or.jp/cyano/gloeobacter)). Recently, the importance of Kdp for  $K^+$  uptake in absence of Ktr system has been suggested for *Synechocystis* sp. strain PCC 6803 (11).

In *E. coli*, the *kdpFABC* operon is transcriptionally controlled by the products of the adjacent *kdpDE* operon (33). In response to appropriate stimuli, the KdpD (a transmembrane sensor kinase) phosphorylates the cytosolic response regulator KdpE, and the KdpE~P binds to the *kdpFABC* promoter and

activates its transcription (20). The corresponding cyanobacterial genes appear to be distinctly different. *Anabaena* sp. strain L-31 *kdp1*, *Anabaena* sp. strain 7120 *kdp1*, and the *Synechocystis* sp. strain PCC 6803 *kdp* operon all show the presence of a naturally short *kdpD* ORF downstream of the *kdpC* and lack *kdpE*. Interestingly, the *kdp2* operon, which is the major *kdp* operon induced in *Anabaena* sp. strain L-31, has neither a *kdpD*-like nor a *kdpE*-like ORF downstream of the *kdpC* gene. The cyanobacterial KdpD resembles only the KdpD N-terminal domain of the full-length KdpD from other bacteria. What role the naturally short KdpD plays in cyanobacterial *kdp* regulation is not clear and remains to be investigated. We have earlier shown that *Anabaena* sp. strain L-31 KdpD, when fused with the *E. coli* KdpD C-terminal domain, results in a functional protein that can induce *kdpFABC* expression in vivo in *E. coli* and phosphorylate the purified *E. coli* KdpE in vitro (9).

*kdpABC* expression is tightly regulated and is induced at the transcriptional level in all bacterial strains where it has been monitored (15). *Anabaena* sp. strain L-31 *kdp* operons are no exception. Of the two *kdp* operons, the *kdp2* is the major operon expressed in response to K<sup>+</sup> limitation. Minor *kdpA1* or *kdpC1* expression could be detected only by a very sensitive RT-PCR technique (Fig. 3). Comparison of the DNA sequences upstream of the *kdpA1* and *kdpA2* (presumed promoter regions) revealed no significant homology (data not shown). This suggests that the regulatory DNA (*cis*) elements present upstream of the two *kdp* operons may be different from each other.

The *kdp2* operon is rapidly induced by transcriptional activation in response to K<sup>+</sup> limitation and immediately repressed by addition of exogenous K<sup>+</sup> (5 mM). Experiments with the transcription inhibitor rifampin showed *kdp2* mRNA to have a half-life of 30 min in *Anabaena* sp. strain L-31 (data not shown). However, on addition of K<sup>+</sup> very little *kdp2* RNA was observed after 30 min (Fig. 4B). This suggests that not only is the *kdp2* repressed by externally added K<sup>+</sup> but also that the *kdp2* mRNA is unstable in the presence of K<sup>+</sup>. We have earlier shown that the KdpB protein is degraded upon K<sup>+</sup> repletion of K<sup>+</sup>-starved *Anabaena* cells (3). This correlates well with the need of *Anabaena* cells to express/repress and degrade the *kdp2* operon products in response to changing environmental stimuli.

In *E. coli* and *Salmonella enterica* serovar Typhimurium the *kdp* operon has been shown to be induced by osmotic stress (particularly NaCl) (25, 16) and low pH (8). However, the *Anabaena* sp. strain L-31 *kdp2* operon did not respond to increase in osmolarity mediated by PEG or sucrose, and exposure to NaCl in fact repressed it. Global-scale gene expression analysis of *Synechocystis* sp. strain PCC 6803 has also shown that the *kdp* is not induced in response to osmotic shock (23, 26). A shift in pH, heat shock, or the presence or absence of combined nitrogen in the medium also did not influence *Anabaena* sp. strain L-31 *kdp* expression.

Desiccation is known to markedly affect gene expression and gene regulation in bacteria (27). The *kdp2* operon from *Anabaena* sp. strain L-31 was distinctly induced in response to desiccation. Katoh et al., using gene array technology, reported the manyfold induction of *kdpA* and *kdpB* genes of *kdp2* operon (but not *kdp1*) from *Anabaena* sp. strain 7120 in re-

sponse to desiccation stress (24). Desiccation stress increases the cellular requirement of K<sup>+</sup> and the expression of KdpAT-Pase encoded by the *Anabaena* sp. strain L-31 *kdp2* operon may fulfill such requirement of K<sup>+</sup>.

The data presented here show that, of the two *kdp* operons present in *Anabaena* sp. strain L-31, only the *kdp2* operon is predominantly expressed under K<sup>+</sup> deficiency or during desiccation and is regulated at transcriptional level in a need-based manner. Unlike the enterobacterial *kdp* operons, *Anabaena* sp. strain L-31 *kdp* is unlikely to play a role in saline environments. It is more likely to contribute to survival during K<sup>+</sup> starvation and the well-known desiccation tolerance of cyanobacteria.

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