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Regulation of Potassium-Dependent Kdp-ATPase Expression in the Nitrogen-Fixing Cyanobacterium *Anabaena torulosa*

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The KdpB polypeptides in the cyanobacterium *Anabaena torulosa* were shown to be two membrane-bound proteins of about 78 kDa, expressed strictly under K^+ deficiency and repressed or degraded upon readdition of K^+. In both *Anabaena* and *Escherichia coli* strain MC4100, osmotic and ionic stresses caused no significant induction of steady-state KdpB levels during extreme potassium starvation.

Potassium is an important nutritional requirement for all bacteria. The major roles attributed to this alkali metal cation in bacterial cells relate to maintenance of turgor (13) and intracellular pH (10), enzyme activation (35), gene expression (19, 37), and regulation of stress responses (12, 27). In a heterocystous, nitrogen-fixing cyanobacterium, *Anabaena torulosa*, we earlier showed that deprivation of K^+ caused pleiotropic effects, resulting especially in the impairment of the vital metabolic processes of photosynthesis and nitrogen fixation in addition to the expected loss of turgor (2).

In bacterial cells, K^+ levels range from 0.2 to 0.6 M (15) and are maintained by multiple K^+ transport systems whose variety increases further during stress conditions (12). For example, *Escherichia coli* possesses at least two constitutive, low-affinity K^+ transport systems, the Trk and Kup systems (34), and an inducible high-affinity K^+-specific transport system, the Kdp system (3, 23). The Kdp system is an emergency or backup system expressed and used to scavenge K^+ from very low (less than 1 mM)-K^+ environs (3) when the other transport systems are unable to meet the cell's need for potassium (13).

The *E. coli* Kdp system is an ion-motive P-type ATPase comprising the KdpA, KdpB, KdpC, and KdpF proteins (3, 18) encoded by a single *kdpFABC* operon whose expression is regulated by an adjoining *kdpDE* operon. The *kdpDE* operon encodes a membrane-spanning sensor kinase, KdpD, and a cytosolic transcriptional activator, KdpE (28, 32). The KdpD protein in *E. coli* consists of (i) a cytosolic N-terminal domain of about 400 amino acids, (ii) four hydrophobic transmembrane domains (TMDs) of nearly 100 amino acid residues, and (iii) a hydrophilic cytosolic C-terminal domain (CTD) of about 400 amino acids (4). Most of the N-terminal domain is dispensable (29), except for amino acid residues 12 to 128, whose deletion deregulates the phosphatase activity of the KdpD protein (20). Modification of TMDs inactivates KdpD and desensitizes *E. coli* cells to K^+ levels (36). The CTD closely resembles the transmitter domain of other bacterial sensor kinases. In response to an appropriate signal(s), it transphosphorylates the KdpE protein (4), which in turn switches on the transcription of the *kdpFABC* operon (26). KdpD is believed to sense at least the following two types of signals: (i) a change in membrane stretch (36), possibly caused by an alteration in turgor pressure (14, 25), and (ii) the external and internal potassium levels (33, 36). The turgor model of regulation has been questioned at times (8, 17).

The *kdp* genes are widely distributed among bacteria (38). DNA sequencing has also revealed the presence of *kdp* homologs in the genomes of two filamentous heterocystous cyanobacteria, *Anabaena* sp. strain L-31 (GenBank accession no. AF213466) and *Anabaena* sp. strain PCC7120 (www.kazusa.or.jp/cyano/anabaena), and in the genome of a unicellular cyanobacterium, *Synechocystis* sp. strain PCC6803 (21). In all three genomes the *kdpD* gene is truncated and completely lacks the coding sequences for the TMDs and the CTD of *E. coli*. Also, the *kdpE* gene appears to be absent. This raises interesting questions about the expression and regulation of the Kdp system in cyanobacteria. We have earlier reported the presence of cross-reactive KdpB-like proteins of very similar molecular mass in three different *Anabaena* spp. (5). The present work examines the regulation of KdpB expression in *A. torulosa*, a brackish water, salt-tolerant (7), but relatively osmosensitive (16) cyanobacterial strain. Our results show that *Anabaena* Kdp-ATPase expression displays physiological regulation by environmental K^+ levels but is not significantly influenced by osmotic stresses.

Axenic cultures of the filamentous, heterocystous, nitrogen-fixing cyanobacterium *A. torulosa* (2, 6, 7) were grown in BG-11 medium (11) without combined nitrogen. The medium was modified to obtain either BG-11/K0 (wherein K_2HPO_4 was replaced by equimolar Na_2HPO_4) or BG-11/K5 (BG-11/K0 containing 5 mM KCl) medium (2). When required, media were supplemented with nitrogen by the addition of 5 mM NH_4Cl and 5 mM MOPS. The initial pH of all media was adjusted to 7.0. Cultures were grown under continuous aeration (2 liters min^{-1}) and illumination (2.5 mW cm^{-2}) from white fluorescent lamps at 25 ± 2°C. Protein content was determined by the Folin phenol method (24). Cellular extracts containing proteins (150 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto Boehringer Mannheim positively charged nylon mem-

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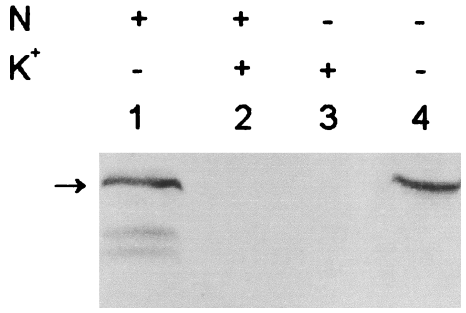

FIG. 1. Identification of *Anabaena* KdpB homolog. Proteins were extracted from *A. torulosa* cultures grown in N-supplemented (lanes 1 and 2) or N-deficient (lanes 3 and 4) conditions in BG-11/K5 (lanes 2 and 3) or BG-11/K0 (lanes 1 and 4) medium for 3 days. Equal amounts of protein (150 μg) were resolved by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (100 V for 1 h followed by 200 V for 3.5 h) and then electroblotted. The blots were allowed to cross-react with a primary anti-*E. coli* KdpB antiserum followed by a secondary anti-rabbit immunoglobulin G coupled to alkaline phosphatase.

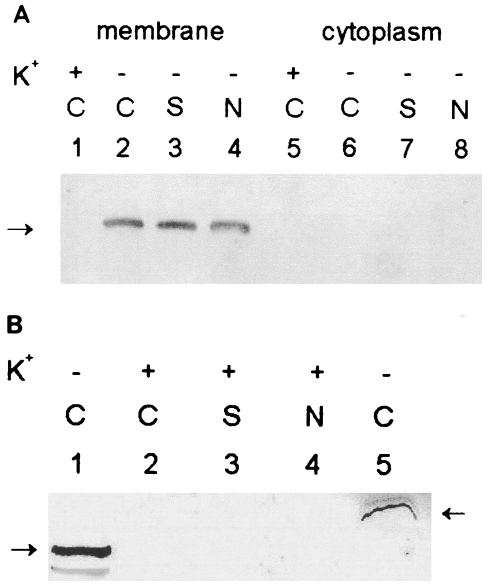

FIG. 2. Cellular location of and effect of osmotic stresses on KdpB levels in *Anabaena*. (A) Nitrogen-fixing cultures were grown in BG-11/K5 (lanes 1 and 5) or BG-11/K0 (lanes 2 and 6) medium. On day 2, parts of the BG-11/K0 culture were stressed with either 0.2 M sucrose (lanes 3 and 7) or 0.1 M NaCl (lanes 4 and 8) for 24 h. Cellular extracts were separated into membrane (lanes 1 to 4) and cytosolic (lanes 5 to 8) fractions and the proteins were resolved by electrophoresis, as described for Fig. 1. C, medium controls; S, addition of sucrose; N, addition of NaCl. (B) Nitrogen-fixing cultures grown in BG-11/K5 (lane 2) medium for 2 days were subjected to 0.2 M sucrose (lane 3) or 0.1 M NaCl (lane 4) for 24 h. Membrane fractions were isolated and electrophoretically resolved for a longer duration (100 V for 1 h followed by 200 V for 4.5 h). Protein samples from *E. coli* MC4100 grown in BG-11/K0 medium for 6 h (lane 1) and from *A. torulosa* grown in BG-11/K0 for 24 h (lane 5) were included for comparison. Other details were as given for Fig. 1.

branes, as described earlier (2, 5). The two antisera (anti-KdpB and anti-KdpABC) raised in rabbits against the corresponding purified proteins from *E. coli* were kindly provided by K. Altendorf (University of Osnabrück, Osnabrück, Germany) and were used at dilutions of 1:5,000 and 1:2,000, respectively. Immunodetection of KdpB was carried out as described earlier (2).

For K^+ starvation, 3-day-old BG-11/K5-grown *Anabaena* cells were harvested by centrifugation (5,000 × *g* for 5 min), washed three times with 5 volumes of BG-11/K0 medium each, inoculated in BG-11/K0 or BG-11/K5 medium at a density of approximately 1 μg of chlorophyll *a* ml^{-1}, and grown. Using an anti-KdpB antiserum, the KdpB was immunodetected in *A. torulosa* as a protein of about 78 kDa (Fig. 1 and 2A) which resolved into two protein bands (Fig. 2B; see Fig. 4) if a longer duration of electrophoresis was employed. K^+ deprivation caused KdpB expression in cells grown in both N-supplemented and N-deficient media. KdpB was never detected in cells grown in K^+-supplemented media. Identical results were obtained when an anti-KdpABC antiserum was used, i.e., no bands corresponding to KdpA or KdpC were observed (data not shown), as has been the case in *Alicyclobacillus acidocaldarius* and *Rhodobacter sphaeroides* (1, 9), in which only the most conserved KdpB was detected.

Under all conditions tested, in *A. torulosa* KdpB was seen exclusively in the crude membrane fraction and none was detected in the cytosol (Fig. 2A). When osmotic stress (0.1 M NaCl or 0.2 M sucrose) adequate to induce an osmotic stress response in this strain (6, 16) was imposed on K^+-deficient *A. torulosa* cultures, the steady-state levels of KdpB did not change significantly (Fig. 2A, lanes 3 and 4). Similarly, exposure of cells grown in 5 mM K^+ to such osmotic stresses also failed to cause any detectable KdpB expression (Fig. 2B). Clearly, therefore, the turgor perturbations caused by osmotic stresses did not significantly enhance the KdpB expression in *Anabaena*, irrespective of the K^+ status of cells.

In *E. coli* strains carrying mutations in either the *kdp*, *trk*, or *kup* genes and grown in media containing 1 to 5 mM K^+, the addition of NaCl enhances the steady-state levels of β-galactosidase expressed from the *kdpFABC* promoter (8, 20, 23, 26, 33, 36). This contrasts with the situation in *Anabaena*. However, the effects of osmotic stresses over and above that of severe K^+ deficiency (BG-11/K0) on the actual synthesis of Kdp polypeptides were not reported for wild-type *E. coli* strains. To examine whether the results for *Anabaena* (Fig. 2A) were really at variance with the situation in *E. coli*, an experiment was performed with *E. coli* strain MC4100, which possesses all the K^+ transport systems (Fig. 3). Cells grown in

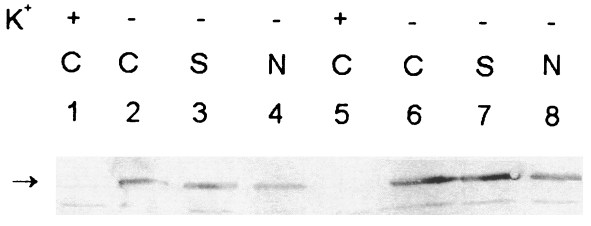

FIG. 3. Effect of osmotic and salinity stresses on KdpB levels in *E. coli* strain MC4100. Cells were grown either in K115 (lanes 1 and 5) or in BG-11/K0 (lanes 2 and 6) medium. Part of the cells grown in BG-11/K0 medium were stressed with either 0.4 M sucrose (lanes 3 and 7) or 0.25 M NaCl (lanes 4 and 8). Samples were collected at 6 (lanes 1 to 4) or 24 (lanes 5 to 8) h after the initiation of K^+ deprivation. Other details were as given for Fig. 2B.

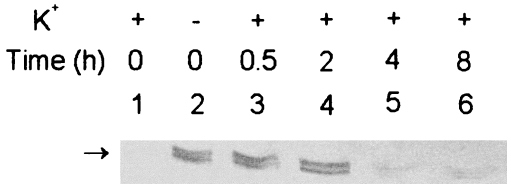

FIG. 4. The negative effect of exogenous K^+ addition on KdpB levels in *Anabaena*. Cultures were grown in BG-11/K5 (lane 1) or BG-11/K0 (lane 2) medium for 2 days. On day 2, 5 mM KCl was added to part of the BG-11/K0 culture at time zero. Samples were removed 0.5 (lane 3), 2 (lane 4), 4 (lane 5), or 8 (lane 6) h after K^+ addition, electrophoretically resolved for a longer duration, and processed for KdpB immunodetection as for Fig. 2B.

K115 medium (23) were washed with and inoculated in either K115 medium or BG-11/K0 medium, wherein K_2HPO_4 and KH_2PO_4 were replaced with equimolar concentrations of corresponding sodium salts. Figure 3 shows that in BG-11/K0 medium, the KdpB levels were not noticeably enhanced by short (6 h) or prolonged (24 h) exposure to osmotic stress (0.25 M NaCl or 0.4 M sucrose) in this *E. coli* strain.

A characteristic feature of Kdp-ATPase is its negative regulation by high levels of external K^+. To test this, *Anabaena* cells were grown in BG-11/K0 medium for 2 days to express KdpB optimally and then supplemented with 5 mM KCl. Extended electrophoretic resolution of protein extracts from such cultures clearly revealed the presence of two KdpB bands of nearly equal intensity in K^+-starved cells (Fig. 4). In the K^+-replete cells, the presynthesized KdpB bands were not affected for at least 2 h but were rapidly degraded thereafter and were barely detectable after 4 h (Fig. 4). Thus, restoration of K^+ not only represses further Kdp synthesis as in *E. coli* (3, 13, 14) but also triggers its degradation in *Anabaena*.

DNA sequencing has revealed the existence of two independent *kdpABC* operons each in *Anabaena* sp. strain PCC7120 and *Anabaena* sp. strain L-31, while only one such operon is found in *Synechocystis* sp. strain PCC6803 (www.kazusa.or.jp/cyano/; our unpublished results). The two KdpB bands observed in *A. torulosa* (Fig. 4) may well be the products of two *kdpB* genes, apparently typical of *Anabaena* genomes. The KdpB polypeptides of *A. torulosa* have been clearly and unambiguously shown to be (i) absent in the presence of 5 mM K^+ in the medium; (ii) present solely under conditions of K^+ limitation; (iii) insensitive to osmoinduction; (iv) exclusively localized in the membranes; (v) of a molecular mass of about 78 kDa, in agreement with the expected molecular mass of the *Anabaena* KdpB protein (74.6 kDa), which is larger than the *E. coli* KdpB protein (72 kDa); (vi) strongly immunologically cross-reactive to both the anti-KdpB and anti-KdpABC antisera; and (vii) repressed and degraded upon the readdition of K^+.

The apparent osmoinsensitivity of *kdp* expression (Fig. 2) appears to be a regulatory feature unique to *Anabaena*. In *E. coli* strains carrying most K^+ transporters, the osmoinduction is maximal at 1 to 5 mM K^+ (20) and decreases both at higher (26, 36) and even at lower (33) K^+ concentrations. Similar results have been reported for *Salmonella enterica* serovar Typhimurium in which under severe K^+ deficiency (Kdp^- Trk^- strain grown at 1 mM K^+), NaCl represses Kdp expression (see Table 2 in reference 17). Our results (Fig. 2A and 3) clearly show that under extreme K^+ starvation (BG-11/K0), the Kdp expression becomes independent of the osmotic signal both in *Anabaena* and in *E. coli*. Thus, among the two signals sensed by KdpD, the K^+ signal is a dominating one compared to turgor perturbations or membrane stretch caused by osmotic upshock. Indeed, the osmoinduced β-galactosidase activities (from *kdpFABC*::*lacZ*) in any strain never exceed those observed at very low K^+ levels (0.1 to 1 mM).

A novel irreversible inactivation of presynthesized Kdp by 20 mM K^+ was recently shown in *E. coli* (33). It was suggested that when a high K^+ level is encountered the Kdp complex may be dissociated and degraded, although a previous study of *E. coli* showed that Kdp proteins were stable for at least two generations (2 h) following the readdition of excess K^+ (22). The Kdp-ATPase thus appears to be a liability when cells have enough K^+ available to them. In *Anabaena*, the Kdp system appears to be tightly regulated in that it is (i) neither expressed nor osmoinduced at 5 mM K^+ and (ii) repressed and rapidly degraded (presynthesized Kdp) when exposed to 5 mM K^+ (Fig. 4).

As stated earlier, the cyanobacterial KdpD has no membrane-spanning domains and it is not clear if it can anchor in the cytoplasmic membrane and/or sense membrane stretch or turgor drop. This, along with the apparent absence of KdpE, had raised doubts about how *kdp* expression is regulated in these microbes. The osmoinsensitivity of *kdp* expression in *Anabaena* reported here may well be related to its unique truncated KdpD, but that remains to be established. The present study clearly shows that expression of the membrane-bound Kdp-ATPase in *Anabaena* is primarily regulated by K^+ levels in the environment. This study also provides a molecular basis for earlier reports on the presence of high-affinity turgor-responsive K^+ transport systems in *Anabaena* (30, 31).

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