Available online at www.sciencedirect.com
ScienceDirect
Archives of Biochemistry and Biophysics 474 (2008) 65–71
ABB
www.elsevier.com/locate/yabbi

Characterization of a response regulator protein that binds to *Anabaena* sp. strain L-31 *kdp*-promoter region ☆

Anand Ballal, Shree Kumar Apte *

Molecular Biology Division, Bhabha Atomic Research Centre, Trombay, Mumbai, Maharashtra 400085, India

Received 25 December 2007, and in revised form 7 February 2008
Available online 23 February 2008

Abstract

The potassium dependent adenosine triphosphatase (Kdp-ATPase), encoded by the *kdp* operon, is a potassium uptake system found in several prokaryotes. The cyanobacterium *Anabaena* sp. strain L-31 shows the presence of two *kdp* operons (*kdp1* and *kdp2*) of which the *kdp2* is predominantly induced in response to potassium limitation or desiccation stress. Two ORFs, encoding a sensor kinase and a response regulator, respectively, were identified upstream of the *kdp2* operon in *Anabaena* sp. strain L-31. The response regulator protein, tagged with 6 additional C-terminal histidine residues, was over-expressed in *Escherichia coli* and purified by affinity chromatography. Employing the protein-specific antiserum, the response regulator protein was detected in *Anabaena* L-31 cytosolic fractions. The response regulator protein bound to the *kdp2* promoter region with higher affinity than *kdp1* promoter region. Addition of acetyl phosphate increased the ability of the protein to bind to *kdp2* promoter region by several fold, suggesting a phosphorylation-dependent binding of response regulator to the promoter. These data implicate the response regulator protein in regulation of *kdp2* expression in *Anabaena* sp. strain L-31.

Keywords: *Anabaena*; *kdp* Operons; Response regulator; Acetyl phosphate; Differential regulation

The Kdp-ATPase (K^+-dependent Adenosine Triphosphatase), comprising of KdpABC complex, is a high affinity, inducible, potassium (K^+) transporting system found in several bacteria [1,2]. The Kdp complex is expressed under conditions of extreme K^+ limitation, hyperosmotic stress (especially salt stress) or when other K^+ transporters (e.g. Trk, Kup) are unable to fulfill cell's requirement of K^+ [3,4]. In *Escherichia coli* the *kdpFABC* operon, which encodes the Kdp-complex, is regulated by KdpDE proteins, which together constitute a typical two-component signal transduction system [5]. The *E. coli* KdpD is a large protein (894 amino acids, 99 kDa) comprising of two large cytosolic domains, the N-terminal domain (NTD) and the C-terminal domain (CTD), interconnected by 4 transmembrane segments [6]. In response to appropriate stimulus, the sensor kinase KdpD, is autophosphorylated at a specific histidine residue (His-673) and subsequently this phosphoryl group is transferred to a specific aspartate residue (Asp-52) of KdpE [7]. The phospho-KdpE binds to DNA sequence upstream of *kdpFABC* promoter and activates the transcription of *kdpFABC* operon in *E. coli* [8].

We have earlier demonstrated the presence of Kdp-ATPase system in three different strains of *Anabaena* [9,10] and cloned *kdp* genes from one of them, *Anabaena* sp. strain L-31 [4]. The cyanobacterium *Anabaena* sp. strain L-31 shows the presence of not one but two *kdp* operons, *kdp1* (comprising of *kdpA1B1G1C1D,* GenBank Accession No. AF213466) and *kdp2* (comprising of *kdpA2B2G2C2*, GenBank Accession No. AY753299). The *kdpD* from *Anabaena* L-31 is naturally truncated and a *kdpE*-like gene is absent in the vicinity of the *kdpD* gene [11]. The *Anabaena*

☆ Nucleotide sequence reported here has been deposited at GenBank under Accession No. EU146154.

* Corresponding author. Fax: +91 22 25505189.
E-mail address: aptesk@barc.gov.in (S.K. Apte).

0003-9861/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2008.02.017

L-31 KdpD displays homology only to KdpD-NTD of *E. coli* and other bacteria but lacks KdpD-CTD, the C-terminal histidine kinase domain [12].

We have recently shown the expression of *kdp* operons in response to K^+ limitation and desiccation stress in *Anabaena* sp. strain L-31 [13]. However, due to absence of KdpD-CTD and KdpE-like protein, the mechanism by which *kdp* operons are regulated in *Anabaena* sp. strain L-31 remained enigmatic. In this manuscript, we report on the identification, cloning and expression of a response regulator (*rr*)[1] ORF present upstream of *kdpA2*. Our data show that the response regulator (RR) protein is synthesized *in vivo* in *Anabaena* sp. strain L-31 and preferentially binds to *kdp2* promoter region with higher affinity than *kdp1* promoter region, suggesting a role for RR protein in *Anabaena* sp. strain L-31 *kdp* regulation.

Materials and methods

Strains and plasmids

The filamentous, heterocystous, nitrogen-fixing freshwater cyanobacterium, *Anabaena* sp. strain L-31 [14], was grown under axenic conditions. Stock cultures were maintained in standard BG-11 medium [15] without combined nitrogen. *E. coli* DH5α[(Δ *(argF-lac)U169 supE33φ80d lacZc M15 recA1 endA1 gyrA96 thi-1relA1 hsdR1*] was employed as carrier for plasmids described. *E. coli* BL21 [F^- *ompT* h_s $dS_B(r_B^- m_B^-)$ *gal dcm* (DE3) pLysS Cm^r] (Novagen) was used to overproduce the *Anabaena* sp. strain L-31 RR(6His).

Construction of pETRR1

The response regulator (*rr*) ORF was amplified from *Anabaena* sp. strain L-31 chromosomal DNA with primers RR_Fwd and RRHis_Rev (Table 1). The forward primer contained a site for the restriction enzyme NcoI (underlined) while the reverse primer contained 6 His codons (shown in bold) followed by a stop codon in frame with the last sense codon of the *Anabaena* sp strain L-31 *rr* ORF and a BamHI restriction site (underlined). The 729 bp PCR product obtained was cloned into pUC19 vector (designated pUC19RR1) and sequenced to confirm the identity of the *Anabaena* L-31 *rr(6His)*. The *Anabaena* L-31 *rr(6His)* fragment was transferred as NcoI–BamHI fragment to pET16b vector to obtain pETRR1.

Overproduction and purification of Anabaena sp. strain L-31 RR(6His)

Escherichia coli BL21(DE3)/pLysS cells carrying pETRR1 were grown at 20 °C in Luria Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl) with appropriate antibiotics to an OD_{600} of ~1.0. At this point the culture flask was shifted to 20 °C and after 30 min the cells were induced with IPTG (1 mM) for ~12–14 h. The cells were harvested, shock frozen in liquid nitrogen, thawed on ice, resuspended in lysis buffer (25 mM Tris, 50 mM NaCl, pH 8.0) and sonicated (10 s bursts at 200–300 W with a 10 s cooling period between each burst). After removing the cell debris by centrifugation (10000*g* × 10 min), the clarified lysate was allowed to bind to Ni^{2+}-NTA (nitrilotriacetic acid) agarose with gentle shaking at 4 °C for 1 h. The slurry was thoroughly washed with lysis buffer containing increasing amounts of imidazole (20, 30 and 40 mM, respectively). The His-tagged protein was eluted by raising the concentration of imidazole in lysis buffer to 200 mM. The *Anabaena* sp. strain L-31 RR(6His) protein purified from *E. coli* was also 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 [16] at a commercial facility (Bangalore Genei, India).

Table 1
List of primers used in the study

1	RR_Fwd	TTGGATCCATGGCTTCTAACCCTGTAAAAAT TCTG
2	RRHis_Rev	GGGGATCCTGCAGTTA**GTGATGGTGATGGT GATG**CTCTTCTAAGACTGCATTATTTTTAA
3	skstinv	GGATCCGCATTTAAGTCTGCTCAAGT
4	kdpA2inv	GGATCCAGGGGTAATTGCTACTACA
5	kdpA2upF	ATCTGCTGTATCATCCATCA
6	kdpA2upR	TGATGGATGATACAGCAGAT
7	HalfA2prmF	CACCATATATAAAAGATTAAC
8	HalfA2prmR	GTTAATCTT TTATATATGGTG
9	kdpA1F	CTTTGGTGAGCGACTACAAT
10	kdpA1R	TGCTATGACAATACACAACGT

Electrophoretic mobility shift assays (EMSA)

The various primers used for PCR amplification of *Anabaena* sp. strain L-31 *kdp* promoter regions are shown in Table 1. The different DNA fragments were obtained with following primer pairs: fragment A (skstinv and kdpA2upR), fragment B (kdpA2upF and HalfA2prmR), fragment C (HalfA2prmF and kdpA2inv), fragment D (skstinv and kdpA2inv) and fragment E (kdpA1F and kdpA1R). The end labelling of DNA fragments with digoxygenin (DIG) was performed as described by the manufacturer (Roche). About 100 pg of labelled DNA was typically employed for each reaction in a total vol. 20 µl. Labeled DNA and purified RR(6His) protein were incubated in binding buffer [20 mM Hepes pH 7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 0.2% Tween20 (v/v) and 30 mM KCl] along with 1 µg of non-specific competitor poly [d(I-C)]. After 20 min, 5 µl loading buffer [2.5× TBE buffer, 60%; glycerol, 40%, bromphenolblue, 0.2% (w/v)] was added to the tubes and the samples were electrophoretically resolved on 8% native polyacrylamide gels at 4 °C (with 0.5× TBE as running buffer). After electrophoresis the gel was electro-blotted onto nylon membrane (Roche) and cross-linked with a trans-illuminator. The subsequent chemilumenescent detection was performed as described in the DIG gel shift kit second generation hand book (Roche). In some experiments, the RR protein (500 ng) was phosphorylated *in vitro* by incubating it in phosphorylation buffer (20 mM Tris, pH 7.5, 1 mM $MgCl_2$, 50 mM KCl, 50 mM acetyl phosphate) for 1 h, before employing it for EMSA. Each EMSA was repeated at least three times and the results obtained were very consistent.

Analytical procedures

Protein concentrations were determined by a modified Lowry method [17] with bovine serum albumin as standard. Proteins were separated by SDS–PAGE using 12% polyacrylamide gels [18]. When required, the proteins were electroblotted on to nitrocellulose membrane and probed with the desired antiserum as described earlier [10]. The monoclonal anti-penta His (QIAGEN) was employed to detect RR(6His) protein.

Results

Cloning and characteristics of rr ORF from Anabaena sp. strain L-31

Approximately 3.0 kb DNA upstream of the *kdpA2* gene was cloned by standard molecular biology techniques (chromosomal walking and PCR). Nucleotide

[1] *Abbreviations used:* rr, response regulator; ORF, open reading frame.