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The ATP-binding motif in AcoK is required for regulation of acetoin catabolism in *Klebsiella pneumoniae* CG43

Jye-Lin Hsu [a], Hwei-Ling Peng [b], Hwan-You Chang [a,*]

[a] Institute of Molecular Medicine, National Tsing Hua University, 101 Kuang Fu Road 2nd Section, Hsin Chu 300, Taiwan, ROC
[b] Department of Biological Science and Technology, National Chiao Tung University, 75 Po-Ai Street, Hsin Chu, Taiwan, ROC

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ABSTRACT

Many bacterial species utilize acetoin as a carbon source. In *Klebsiella pneumoniae*, the utilization of acetoin is catalyzed by an acetoin dehydrogenase complex encoded by the *acoABCD* operon, which is positively regulated in the presence of acetoin by the transcriptional factor AcoK. AcoK contains a LuxR type DNA-binding domain at the C-terminal region and putative Walker A and B nucleotide-binding motifs in the N-terminal region. The comprehensive deletion and mutation study performed here shows that mutations in the putative Walker A motif result in a significant reduction of ATP hydrolysis and *trans*-activation by AcoK of *acoABCD* expression, presumably due to a loss of ATP-binding ability. AcoK was shown to bind specifically to nucleotides -66 to -36 of the *acoABCD* promoter, though the DNA-binding ability was not affected by the Walker A motif mutation. Thus, this study provides an additional example of how a member of the signal transduction ATPases with numerous domains family activates its target gene expression.

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Many bacterial species can utilize acetoin (3-hydroxy 2-butanone) as their sole carbon source. The utilization of acetoin is catalyzed by the acetoin dehydrogenase (AcoDHase) complex, which is encoded sequentially by the genes of *acoABCD* operon. In *Klebsiella pneumoniae*, a gene designated *acoK,* located upstream of the *acoABCD* operon, but transcribed from a divergent promoter, encodes a transcription factor that positively regulates the operon in the presence of acetoin [1]. AcoK is a polypeptide of 921 amino acid residues, which is somewhat large compared to other transcriptional regulators. Among transcription factors that have been characterized previously, AcoK most closely resembles MalT, a regulator of the *Escherichia coli* maltose regulon [2], with an overall amino acid similarity of 58%. The C-terminal region of AcoK contains a helix–turn–helix DNA-binding domain (Gly-X_2-Gly-X-Gly-Lys-Thr-Thr) of the LuxR family of transcription activators. Besides the DNA-binding property, AcoK contains nucleotide-binding motifs and exhibits a weak intrinsic ATPase activity [1].

Using sequence-based structure predictions, MalT and AcoK can be classified into a recently recognized P-loop NTPase family named signal transduction ATPases with numerous domains (STAND) [3]. The STAND NTPases control various cellular processes and are characterized by two strongly conserved sequence signatures, the Walker A and Walker B nucleotide-binding motifs, which indicate a common mechanism of NTP hydrolysis in signaling. The STAND family members, such as Apaf-1 and MalT, are normally stabilized in an inactive form by a negative effector [4]. In the presence of ATP, an inducer binds to the sensor domain and triggers protein self-association, thereby activating the signaling pathway [5,6].

Unlike MalT, which is well characterized in its domain structure and biochemical properties, little is known about the structure–function relationship of AcoK. Despite their sequence similarities, the biochemical properties of AcoK may also be different from those of MalT since they have each evolved to control different metabolic systems. In order to understand the contribution of different domains, in particular the nucleotide-binding motif, on the transcriptional activity of AcoK, several truncation and site-directed mutants of AcoK have constructed and the biochemical and *trans*-activation activities of the resulting proteins have been determined and are reported herein.

Materials and methods

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Supplementary Table 1. The DNA sequence of the *aco* operon was amplified from genomic DNA of *K. pneumoniae* CG43 [7]. Unless otherwise indicated, bacterial strains were grown in Luria–Bertani (LB) broth alone or supplemented with 100 μg ampicillin ml^{-1}, 30 μg chloramphenicol ml^{-1}, or 25 μg kanamycin ml^{-1}.

* Corresponding author. Fax: +886 3 5742910.
E-mail address: hychang@life.nthu.edu.tw (H.-Y. Chang).

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Promoter activity assay. Plasmid pUCD1752 (kindly provided by S.-T. Liu, Department of Microbiology and Immunology, Chang-Gung University, Taiwan), a derivative of *Vibro fischeri luxAB* genes containing pUCD607, was used for the reporter gene assay. The recombinant plasmids containing $P_{acoABCD}$-*luxAB* (pHPA129) were transformed into *E. coli* BL21 (DE3) and the activity of LuxAB was determined. Overnight bacterial cultures were inoculated at 1:50 ratio into fresh LB broth supplemented with either 0.01% (w/v) acetoin or 20 mM glucose. After further incubation at 37 °C at different time intervals, the cells were kept at room temperature for 1 h and the cell density was measured as the optical density at 600 nm (OD_{600}) using a UV–Vis spectrophotometer (UV1201, Shimadzu Co.). *N*-decyl aldehyde (Sigma) was prepared in 20 mg fatty acid free BSA ml^{-1} (Sigma) to a final concentration of 0.1% (w/w). For each reaction, 500 μL of the *N*-decyl aldehyde solution was added immediately to 500 μL of the cultures in a polypropylene test tube designed for the luminometer (TD-20/20 Luminometer, Turner Designs). The light emitted was then recorded over an integration time of 1 min. The readings from the luminometer were normalized to bacterial density into relative light units (RLU). The data presented are the mean values from triplicates of at least two independent experiments.

Sequence comparison of AcoK and proteins catalyzing ATP-triggered reaction. The nucleotide and putative amino acid sequences of *acoK* (U10553) [1] were used to search for homologous data files in GenBank. A sequence motif search was performed in the PROSITE profile at GenomeNet WWW Server (http://www.genome.ad.jp).

Construction of AcoK mutants. Site-directed mutagenesis was performed using the megaprimer PCR method. An *acoK* expressing plasmid, pHPA31 [1], was used as the template in the PCR and the sequence of the primers are listed in Supplementary Table 2. Deletion mutants of *acoK* were constructed primarily by utilizing convenient restriction endonuclease cutting sites present in the gene (Supplementary Table 1). The fragments of different lengths were then religated and transformed into *E. coli* NovaBlue (DE3). The deletion length and reading frame of the mutants were determined by nucleotide sequencing.

Purification of the recombinant proteins. The bacterial strains harboring an AcoK expressing plasmid were grown in LB broth until the OD_{600} reached 0.6. Synthesis of the recombinant protein was induced by incubating the bacteria in LB containing 0.1 mM isopropyl-β-D-thiogalactopyranoside at 16 °C for 24 h. The bacterial cells were collected by centrifugation, resuspended in lysis buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 10% (v/v) glycerol, 20 mM imidazole, and 1 mM PMSF) and disrupted on ice by ultrasonication. After centrifugation at 24,000*g* for 20 min at 4 °C to remove the debris, the clarified cell lysate was loaded onto a nickel-charged affinity column (GE-Amersham Biosciences), which was washed sequentially with buffer W1 (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 0.1 mM ATP, 5 mM $MgCl_2$, 10% (v/v) glycerol, and 20 mM imidazole), and buffer W2 (W1 with 40 mM imidazole instead of 20 mM), and the His_6-tagged proteins were eluted using buffer E (W1 with 1 mM ATP and 500 mM imidazole instead). The purity of the recombinant proteins was determined by SDS–PAGE and the results indicated that these proteins were nearly homogeneous (Supplementary Fig. 1). To eliminate contaminating ATPase, the proteins used for the ATPase assay were purified with additional wash steps, sequentially with buffer A (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 20 mM imidazole, 5 mM ATP, and 5 mM $MgCl_2$), buffer B (20 mM Tris–HCl, pH 8.0, 250 mM NaCl, 0.1 μg hexokinase ml^{-1}, 1 mM glucose, and 5 mM $MgCl_2$), and buffer C (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 40 mM imidazole) prior to samples elution [8]. The purified proteins were dialyzed against storage buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 20 mM tri-sodium citrate, 1 mM dithiothreitol, and 10% (v/v) glycerol) and their concentrations determined by the Bradford method using a Bio-Rad kit.

ATPase assay. ATP hydrolysis activity of the AcoK mutants was assayed in 20 μL reaction buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 20 mM tri-sodium citrate, 1 mM dithiothreitol, and 5 mM $MgCl_2$) containing 0.5 μg of a testing protein. Reactions were carried out at 25 °C and were initiated by addition of 0.5 μCi (1.85×10^4 Bq) of [γ-^{32}P]ATP. Aliquots (3 μL) were sampled at the indicated times, immediately mixed with 3 μL of stop buffer (25 mM EDTA/5 mM ADP), analyzed by thin layer chromatography [8] and the amount of P_i released was determined by using a phosphorimager (InstantImage, PACKARD Instrument Co.).

Electrophoretic mobility shift assay (EMSA). Oligonucleotide probe 1 (31 bp), probe 2 (30 bp), and probe 3 (36 bp) used in the EMSA were commercially synthesized and annealed at room temperature prior to labeling. Probe 4 (115 bp) was prepared by PCR amplification with primer set JL007F/JL006R (Supplementary Table 2). These probes were end-labeled with [γ-^{32}P]ATP as described previously [9] and 2 nM of them were used in the studies. The purified wild-type or mutant AcoK proteins, in amounts ranging from 125 ng to 1.0 μg, were mixed with the DNA probes individually in a 20 μL reaction mixture containing 50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 20 mM tri-sodium citrate, 1 mM dithiothreitol, 5 mM $MgCl_2$, and 5% (v/v) glycerol. The mixtures were incubated at room temperature for 15 min, and then loaded onto a 5% nondenaturing polyacrylamide gel containing 4% (v/v) glycerol in 0.5× TBE buffer. Gels were electrophoresed at 4 °C, dried under vacuum, and the results were detected via autoradiography using a phosphorimager (InstantImage, PACKARD Instrument Co.). The percentage of the probe that formed a complex with the recombinant AcoK was determined using the baseline subtracting function provided by the instrument software.

Results

Sequence comparison of AcoK and proteins catalyzing ATP-triggered reaction

There are two putative ATP-binding domains observed in AcoK. One (Motif I) is located at amino acid positions 64–71 (AGAGFGKT) and 153–157 (YLIID) as Walker A and B boxes, respectively; the other (Motif II) is located at 776–783 (ALWNSGKT) and 851–855 (SGSPD) (Fig. 1A). Based on Yoshida and Amano's hypothesis [10], a Glu or Asp at 24 ± 2 residues from the Lys of motif A is essential for the ATPase activity of its carboxyl group. Comparison of the amino acid sequences around the Walker A boxes of AcoK and those of ATP-binding proteins of known structures revealed the presence of potentially catalytic carboxylate residues located at positions 95 and 118, in the proximity of ATP-binding domains I and II, respectively, of AcoK (Fig. 1A). The region identified as a β-strand-loop, which consists of several hydrophobic residues following a Gly cluster was identified upstream of the Walker A motif. The α_C helix region, consisting of several amphiphilic characteristic residues, was also revealed around ATP-binding domain I (Fig. 1A). On the other hand, a Walker B box signature, four hydrophobic residues preceding a conserved Asp, was observed only in ATP-binding domain I, but not in domain II. This finding suggests that the ATP-binding activity of AcoK is most likely contributed by ATP-binding domain I.

Construction of site-directed and deletion mutants of AcoK

To verify the significance of the two putative ATP-binding sites on AcoK activity, the Lys residues in these ATP-binding domains were mutated to a Ser residue individually. The plasmids carrying

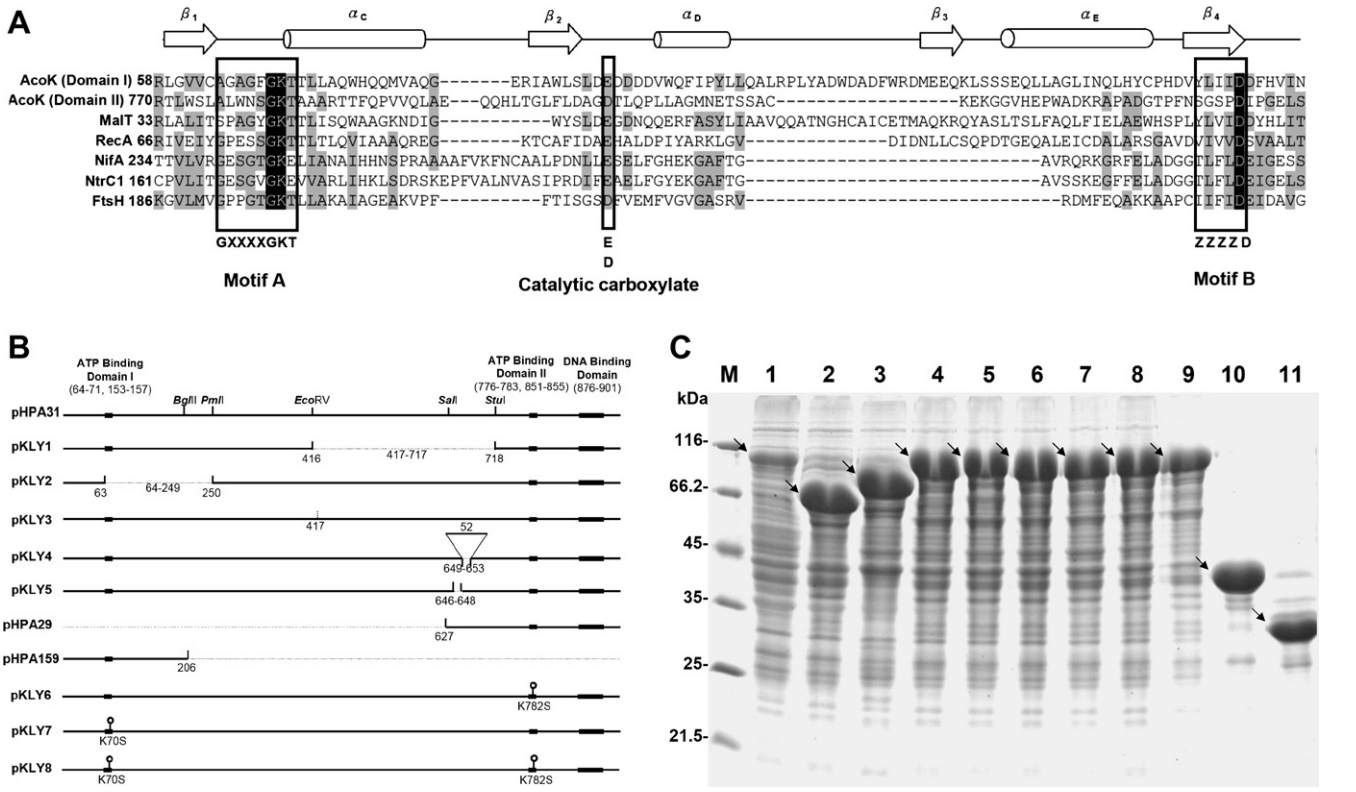

Fig. 1. (A) Alignment of the nucleotide-binding domains of representative P-loop NTPases. The 100% consensus sequence was boxed with black in the alignment. Related residues are shaded in gray. Secondary structure elements of *E. coli* RecA are shown above the sequences, and conserved motifs are indicated below. Walker A, GX_4GK/T (X, any amino acid); catalytic carboxylate, either E or D; and Walker B, Z_4D (Z, hydrophobic amino acid). The GenBank accession numbers for each of the protein sequences are as follows: *E. coli* MalT, P06993; *E. coli* RecA, YP853918; *K. pneumoniae* NifA, P03027; *Aquifex aeolicus* NtrC1, 1NY5 A; *E. coli* FtsH, P0AAI3. (B) Relative positions of truncation mutations for AcoK. The numbers stand for the location of amino acid residues. The black boxes represent the ATP and DNA-binding domains. Dotted lines indicate the deleted part of AcoK. (C) Overexpression of *acoK* mutants in *E. coli* NovaBlue (DE3). The proteins were induced with 0.01 mM IPTG for 6 h at 37 °C. Total cellular proteins are resolved on a 12.5% SDS–polyacrylamide gel and visualized by means of Coomassie Brilliant Blue R250 staining. The name of the plasmid for producing the recombinant AcoK and the expected size of the gene product in each lane are: 1, pHPA31 (108 kDa); 2, pKLY1 (75 kDa); 3, pKLY2 (85 kDa); 4, pKLY3 (108 kDa); 5, pKLY4 (113 kDa); 6, pKLY5 (108 kDa); 7, pKLY6 (108 kDa); 8, pKLY7 (108 kDa); 9, pKLY8 (108 kDa); 10, pHPA29 (36 kDa); 11, pHPA159 (27 kDa). Recombinant AcoKs are marked by an arrow.

genes encoding AcoKK70S, AcoKK782S, and AcoKK70S-K782S were designated pKLY7, pKLY6, and pKLY8, respectively. Several truncated versions of AcoK were also constructed (Fig 1B) to dissect the functional domain of this large transcription regulator. Plasmid pKLY3 encodes an AcoK with only one amino acid (Ile-417) deletion. The AcoK encoded by pKLY4 suffered a truncation from amino acid residues 649 to 653, which was substituted by a 52-amino acid fragment of a kanamycin resistance gene with a sequence of VAVFLRRLHSIPVCNCPFNSDRVFRLAQAQSRMNNGLVDASDFDDERN GWPV. All the mutant constructs could express recombinant proteins of expected sizes as demonstrated by SDS–PAGE analysis (Fig. 1C).

ATP-binding domain I in AcoK is essential for activation of acoABCD transcription

We have previously shown that AcoK was required for acetoin-induced AcoDHase synthesis [1]. To determine whether *acoABCD* promoter is regulated directly by AcoK, a 430-bp DNA fragment containing the putative *acoABCD* promoter was cloned in front of the promoterless *luxAB* genes in pHPA138 (Supplementary Table 1). The resulting plasmid pHPA129 was then transformed into *E. coli* BL21 (DE3) with the AcoK-expressing vector pHPA31, and monitored in the presence or absence of acetoin at different time points (Supplementary Fig. 2). The luciferase activity was considerably induced by acetoin and reached maximum at about 150 min post-inoculation. In contrast, the luciferase activity in regular LB or LB supplemented with glucose did not show significant enhancement. Subsequent experiments of expression of $P_{acoABCD}$ in *E. coli* were then performed about 150 and 180 min after refreshing the overnight cultures.

Compared with the full-length AcoK, activation of $P_{acoABCD}$-luxAB was eliminated in AcoKΔ64-249 (pKLY2), AcoKK70S (pKLY7), and AcoKK70S-K782S (pKLY8), which have either deletions of or point mutations in ATP-binding domain I (Fig. 2). In contrast, AcoKK782S (pKLY6), which has a mutation in the putative ATP-binding domain II showed only a slight decrease on its transcriptional activity (Fig. 2). These results strongly suggest that ATP-binding domain I, but not the domain II, is essential for AcoK transcriptional activity.

While most of the *acoK* deletion mutants were inactive, AcoKΔ417-717 (pKLY1) and Δ646-648 (pKLY5) were partially functional (Fig. 2). The acetoin induction effect was less evident in AcoKΔ417-717 (pKLY1), which seemed to be constitutively active at 120 (data not shown) and 150 min (Fig. 2A). This finding is consistent with the hypothesis that the central portion of AcoK is required for recognition of inducer molecules.

ATP-binding domain I in AcoK is required for its ATPase activity

Since all mutations involving ATP-binding domain I completely abolished the AcoK-mediated transcriptional activation, it is reasonable to assume that the ATPase activity is also lost in these mutant AcoKs. To verify this hypothesis, the mutant proteins including AcoKK70S (pKLY7), AcoKK782S (pKLY6), and AcoKK70S-K782S (pKLY8) were purified and their ATPase activity

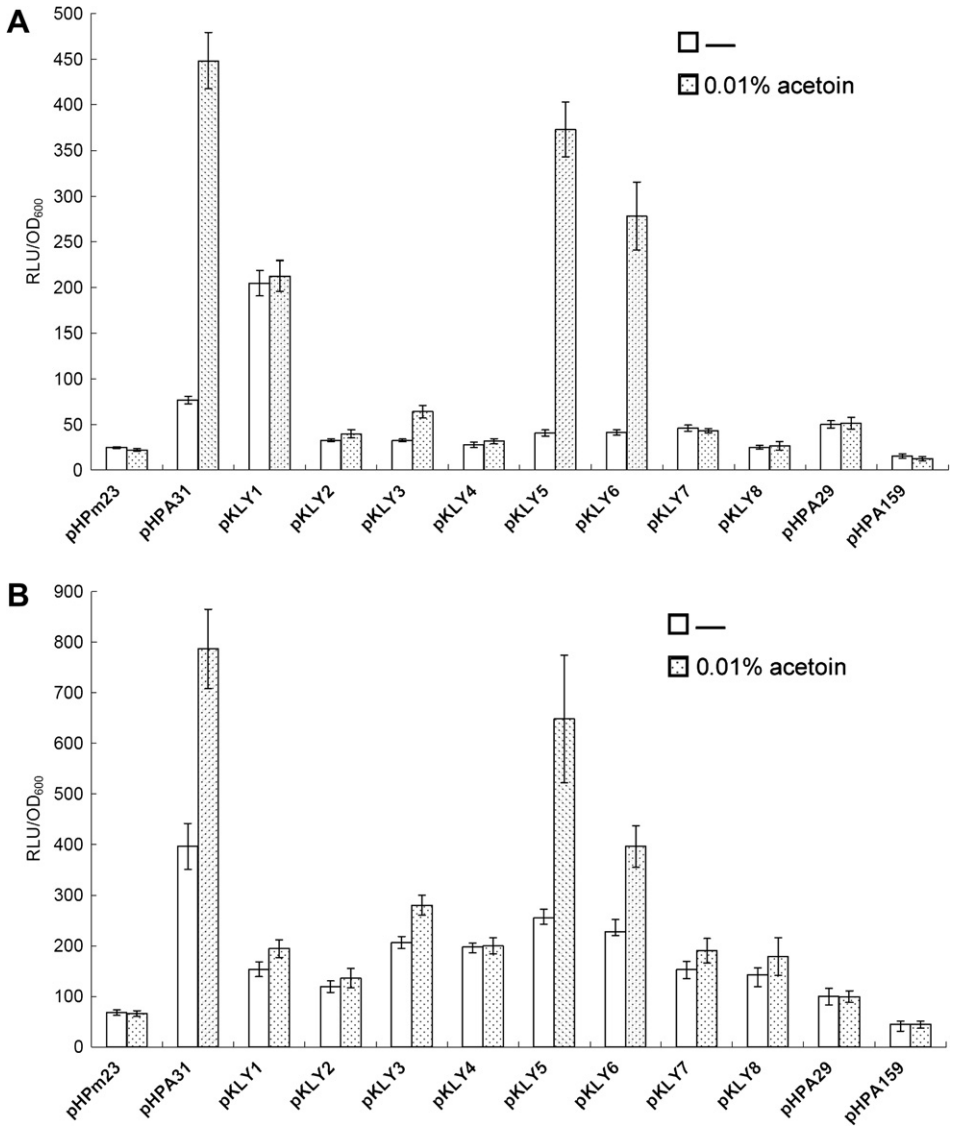

Fig. 2. *Trans*-activation activity of AcoK mutants on the *acoABCD* promoter. Transcriptional activities were assayed in *E. coli* BL21 (DE3) expressing a wild-type or mutant AcoK protein and a $P_{acoABCD}$-*luxAB* fusion construct. Experiments were performed at (A) 150 min and (B) 180 min after refreshing the cultures.

investigated. Both wild-type AcoK and AcoKK782S displayed a weak but reproducible ATPase activity. In contrast, no ATPase activity could be detected in AcoKK70S and AcoKK70S-K782S (Fig. 3). These results strongly suggest that the ATP-binding domain I is essential for the ATPase activity of AcoK and that this activity is crucial for the transcriptional function of the protein. Although the sequence of the putative ATP-binding domain II displayed some similarity with Walker A box, mutation of this region did not affect the ATPase activity of AcoK. This result, together with the promoter-reporter assay, clearly indicates that ATP-binding domain II is not involved in AcoK-mediated transcriptional activation.

ATP-binding activity is not essential for DNA-binding activity of AcoK

We have previously shown that AcoK interacts with a 0.73-kb region of the *acoABCD* promoter [1]. An EMSA study was performed in this study to investigate whether the ATPase activity of AcoK is required for binding of the protein to the *acoABCD* promoter. The EMSA included four overlapping probes comprising the active 0.46-kb promoter region (Fig. 4) to further define the AcoK-binding site. As shown in Fig. 4B, both wild-type AcoK and AcoKK70S retarded the probe 1 DNA, indicating that the ATPase activity is not essential for AcoK binding to the promoter of *acoABCD*. On the other hand, AcoK could not interact with probes 2, 3, and 4 (Fig. 4C), indicating that the protein specifically recognizes the −36 to −66 sequence of the *acoABCD* promoter. A potential −35 and a −10 consensus sequence, and a putative NtrC recognition site [CAC-(N_{11}–N_{18})-GTG] were found in the proximity of the AcoK-binding site (Fig. 4A).

Discussion

AcoK possesses several typical characteristics of the STAND NTPase family, including the STAND-type nucleotide-binding domain, a LuxR-type DNA-binding effector domain and a sensor domain consisting of the tetratricopeptide repeats of the TPR_4 family (PF07721) for possible intra and intermolecular interactions [3,11]. This study provides additional biochemical evidence demonstrating that AcoK is capable of binding the *acoABCD* promoter specifically and that its ATP-binding domain is essential for ATP hydrolysis and the transcription activation function of the protein. These results further support the notion that, like MalT, AfsR, and GutR, AcoK is a member of STAND NTPases [12–14].

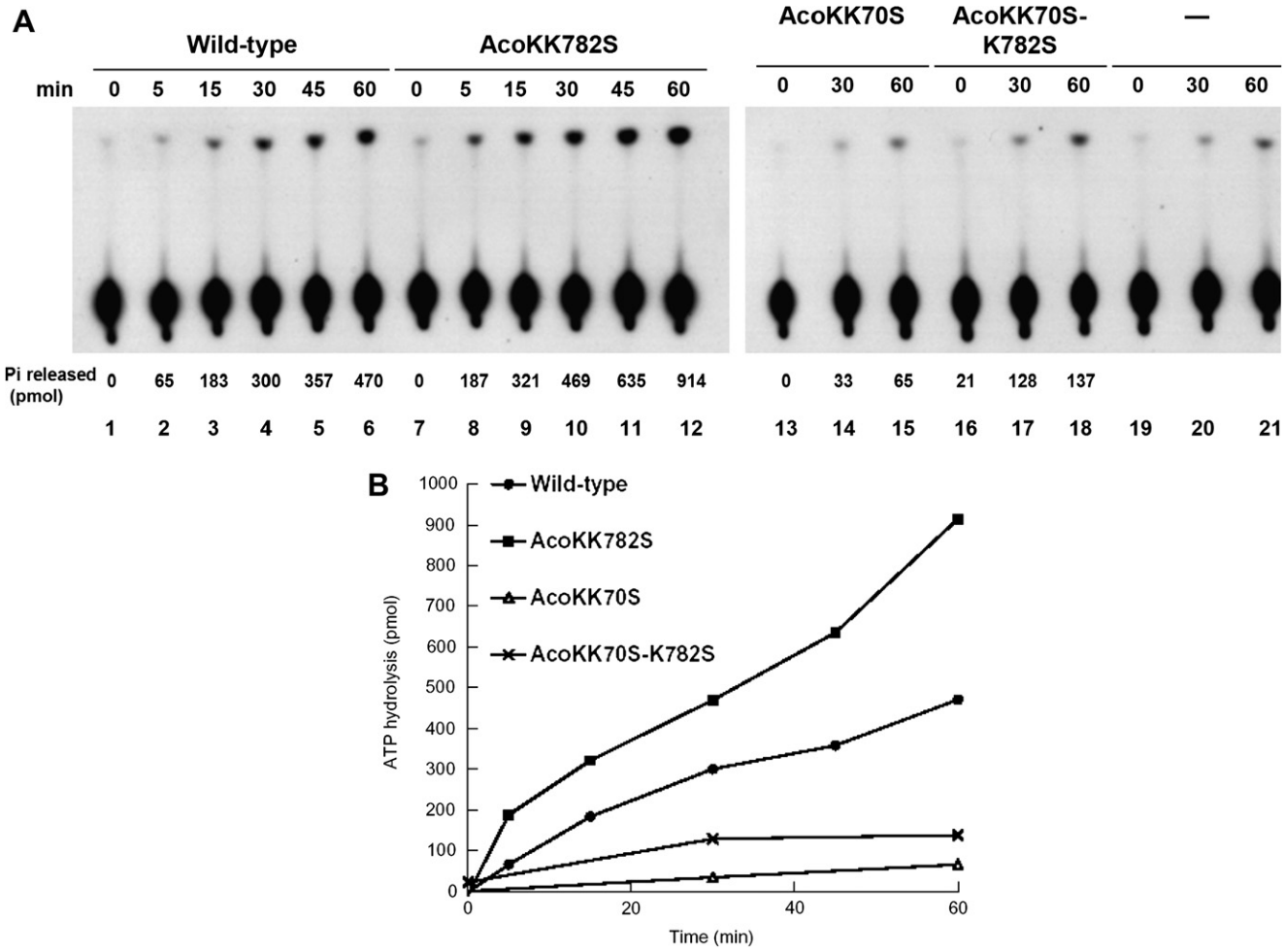

Fig. 3. ATPase activity of wild-type and mutant AcoK proteins. The reactions were performed in the presence or absence (marked by-) of 0.5 μg of a purified protein at the indicated incubation times followed by thin layer chromatography (A). The numbers at the bottom of the autoradiogram represent the amounts of $^{32}P_i$ released. Time course analysis of ATP hydrolysis by wild-type and mutant AcoK proteins relative to negative control samples were performed and plotted in (B).

It has been hypothesized that the long central portion of MalT, AfsR, GutR, and AcoK is responsible for inducer recognition. Based on sequence comparison with MalT, the region of AcoK corresponding to the sensor domain is located between residues 382 and 838. Consistent with this finding, AcoK with a deletion at Ile-417, which is located in this proposed region, showed a significant reduction in the transcriptional activation property of the protein. Moreover, a constitutive phenotype was observed in AcoKΔ417-717 (pKLY1), a clone with a deletion in this putative inducer binding region. These results support the notion that the region between residues 382 and 838 is the sensor domain of AcoK and that this domain plays a negative role in keeping the protein in an inactive state in the absence of inducer, a feature which is observed in some STAND NTPases, such as Apaf-1 and GutR [14–16]. It has been proposed that many STAND NTPases are part of complex regulatory networks that integrate many different signals [11]. Three negative effectors, MalY, Aes, and MalK, can independently stabilize the inactive form of MalT [4,17]. Similarly, cytochrome *c* and ATP are two effectors that promote Apaf-1 binding to procaspase-9 [6]. Future efforts will be focused on verifying whether the 382–838 regions is indeed the sensor domain of AcoK and also to identify effector molecules that interact with AcoK.

It should be mentioned here that, at present, it is not clear whether acetoin itself is the real effector in AcoK-mediated *acoABCD* operon activation. In the case of MalT, maltotriose, instead of maltose, has been found to be the authentic inducer in activating *mal* regulon expression in enteric bacteria [2,11]. The findings from the ATPase and DNA-binding assays with AcoK as well as the construction of several mutant proteins in this study will be useful for future investigation of whether acetoin is indeed the inducer of the *aco* operon.

A major difference between AcoK and MalT is that the latter binds its target DNA only when both ATP and maltotriose are present [18], whereas a functional ATP-binding motif is not essential for DNA-binding property of AcoK. Thus, AcoK seems to be more closely related to another STAND regulator AfsR, which binds to its recognition sequence on DNA prior to interacting with ATP or an inducer, and is characterized by its ATPase activity not being essential for its DNA-binding activity [13]. Nevertheless, how effector molecules binding and ATP hydrolysis trigger subsequent *acoABCD* gene expression requires further investigation.

In summary, this study has revealed that an integral ATP-binding domain I of AcoK is essential for the transcriptional activation property of the protein. The ATPase activity is conferred by the ATP-binding domain I of AcoK, which is essential for its *in vivo* transcriptional activity, but is not required for modulating the DNA-binding ability. However, the detailed molecular mechanism of how ATP hydrolysis relates to transcription activation remains to be investigated.

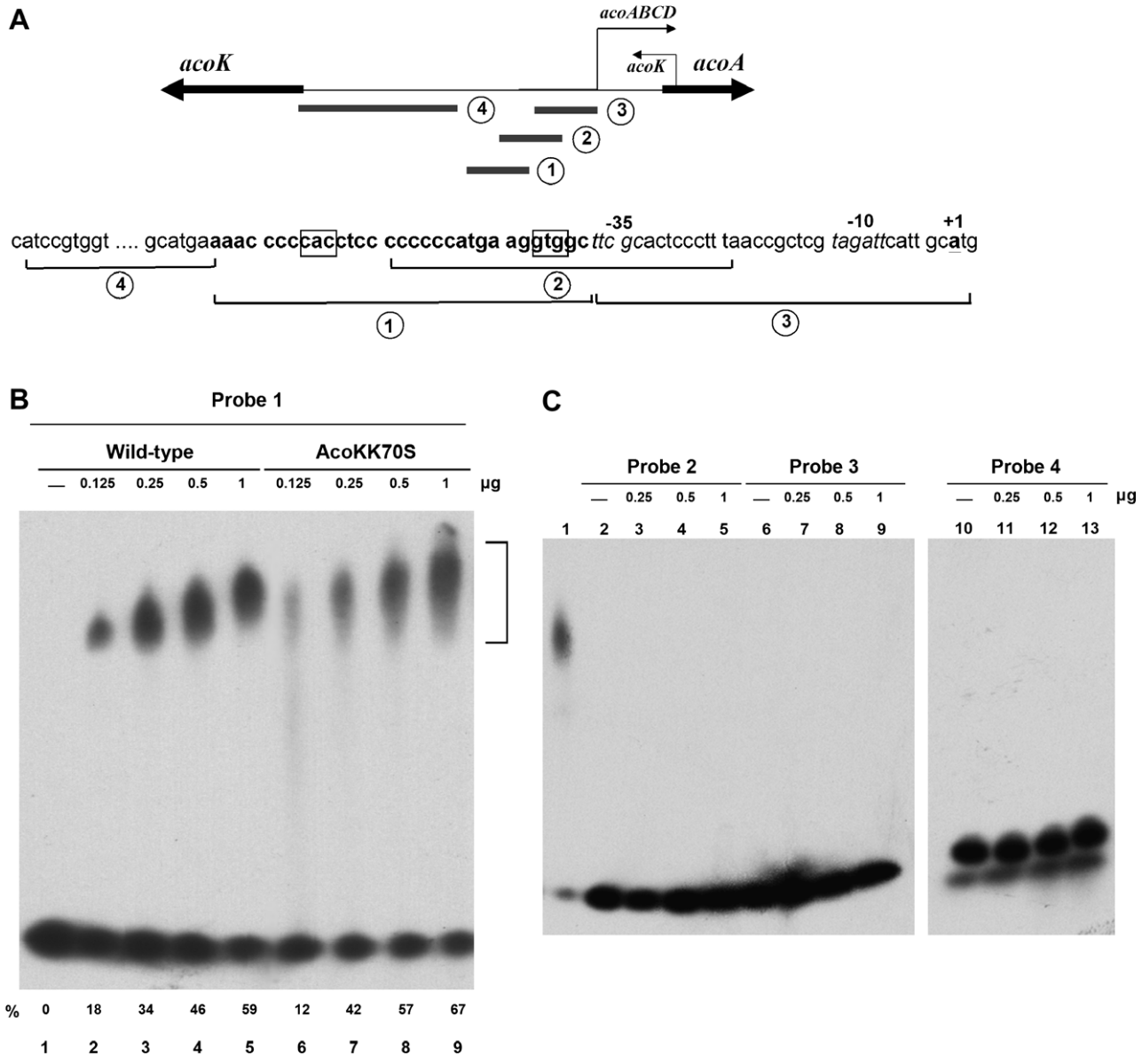

Fig. 4. Electrophoretic mobility shift assay of wild-type and mutant AcoK proteins. (A) Diagrammatic representation of the putative *acoABCD* promoter region and the location of the putative NtrC-binding site (circled). Nucleotides in italic represent −10 and −35. The underlined nucleotide is the transcriptional initiation site of the *aco* operon. Probe1 region is indicated by bold-faced nucleotides. The relative positions of the probes used in the EMSA are, respectively, indicated. (B) DNA-binding ability of wild-type and ATP-bind domain I mutant AcoK. The amounts of purified wild-type AcoK and AcoKK70S (pKLY7) used in the EMSA are indicated on top of the autoradiogram. The percentages of the probe that formed a complex with the recombinant AcoK are indicated below. (C) Localization of AcoK-binding sequence in *acoABCD* promoter. The amounts of AcoK used in the EMSA are indicated on top of the gel. Lane 1 is 1 µg wild-type AcoK mixed with DNA probe 1 as the positive control.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.08.103.

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