Original Article

Plasticity of Regulation of Mannitol Phosphotransferase System Operon by CRP-cAMP Complex in *Vibrio cholerae*[*]

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Abstract

Objective The complex of the cyclic AMP receptor protein (CRP) and cAMP is an important transcriptional regulator of numerous genes in prokaryotes. The transport of mannitol through the phosphotransferase systems (PTS) is regulated by the CRP-cAMP complex. The aim of the study is to investigate how the CRP-cAMP complex acting on the mannitol PTS operon *mtl* of the *Vibrio cholerae* El Tor biotype.

Methods The *crp* mutant strain was generated by homologous recombination to assess the need of CRP to activate the mannitol PTS operon of *V. cholerae* El Tor. Electrophoretic mobility shift assays (EMSA) and the reporter plasmid pBBRlux were used to confirm the role that the CRP-cAMP complex playing on the mannitol PTS operon *mtl.*

Results In this study, we confirmed that CRP is strictly needed for the activation of the *mtl* operon. We further experimentally identified five CRP binding sites within the promoter region upstream of the mannitol PTS operon *mtl* of the *Vibrio cholerae* El Tor biotype and found that these sites display different affinities for CRP and provide different contributions to the activation of the operon.

Conclusion The five binding sites collectively confer the strong activation of mannitol transfer by CRP in *V. cholerae*, indicating an elaborate and subtle CRP activation mechanism.

Key words: Cyclic AMP receptor protein; CRP-cAMP complex; CRP-binding sites; *Vibrio cholerae*

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INTRODUCTION

The 3',5'-cyclic adenosine monophosphate (cAMP) receptor protein (CRP) is an important regulator in the global gene control of prokaryotes. CRP was firstly identified as a transcriptional activator in *Escherichia coli* [1-2], and it has been experimentally demonstrated that CRP regulates more than 100 promoters involved in responses to carbon and energy shortages in proteobacteria[3-4]. The transcriptional regulation activity of CRP is dependent on the binding of cAMP

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and on conformational changes that allow for binding to the promoter region of a target operon via its C-terminal helix-turn-helix DNA-binding domain; this binding promotes transcriptional initiation[5]. The negative regulation of promoters by CRP has also been identified[6]. In *E. coli*, the CRP homodimer binds to its cofactor, cAMP, to form the CRP-cAMP complex. This complex binds to symmetrical 22 bp DNA sites such as AAATGTGATCT:AGATCACATTT[7] that contain a common core motif of TGTGA…TCACA, and most CRP-cAMP sites are mismatched at one or more positions of this 10 nt core motif[4].

CRP regulates gene transcription by binding to one or more specific sites upstream of promoters. In *E. coli*, many transcriptional activation mechanisms mediated by CRP have been reported and classified into classes I, II, and III based on the interaction of CRP and the recruited RNA polymerase[8-9] and the need (or lack thereof) for co-activators. When CRP works as the sole activator, it binds at positions-41.5 bp, -61.5 bp (or nearby) upon the transcriptional start site[10]. When CRP acts as a synergistic activator, it binds to high-affinity sites and bends the DNA of these sites to facilitate the access by other activators, such as MalT[11]. In other instances, the cooperativity between CRP and either MelR or CytR is needed to allow the binding of CRP to low-affinity sites[12-13]. There are also CRP-S sites (TGCGA) which need Sxy to enhance the ability of CRP to bind the DNA[4].

The PEP-dependent phosphotransferase (PTS) system is a carbohydrate transport system consists of phospho-carrier or histidine protein (HPr), protein kinase enzyme I (EI) and substrate-specific enzyme II (EII)[14]. When an more efficiently utilized carbon source is present in the growth medium, the uptake of certain carbon sources is restricted[15], this process is called carbon catabolite repression (CCR), and CRP-cAMP plays a role in it through activate or repress transcription of relevant genes. This regulation by CRP-cAMP is achieved through the activation of adenylate cyclase, which catalyzes the conversion of ATP into cAMP by the phosphorylated form of $EIIA^{Glc}$ (Glucose-permease IIA component)[16]. And then the increasing levels of cAMP form a complex with CRP and bind to the upstream sequences of numerous catabolic operons to activate (or in certain cases, repress) transcription[3-4].

Mannitol is one of the abundant sugar sources in nature. The mannitol PTS has been confirmed in bacteria, plants and fungi[17-18] and has been shown to play important roles in osmoregulation, stress tolerance and the storage and regeneration of reducing power[19-20]. The marine bacterium species *Vibrio cholerae* efficiently utilizes mannitol derived from algae in oligotrophic water, and the function of the mannitol operon (*mtl*) has been identified in the *V. cholerae* strain O395[21]. In *V. cholerae,* the mannitol transport system consists of mannitol-specific enzyme II (*mtlA*), mannitol-1-phosphate dehydrogenase (*mtlD*) and mannitol operon repressor (*mtlR*)[21]. There were five cAMP-CRP binding sites in the promoter region of the mannitol PTS operon in *E. coli*[22]. In *V. cholerae*, the promoter sequence of the *mtl* operon is conserved[23] and very divergent from that in *E. coli*. Possible CRP binding site(s) in the *V. cholerae mtl* promoter region were not included in the CRP motif search lists at regulonDB and regtransbase databases[4] and have not been experimentally determined. In *V. cholerae*, fewer regulatory factors and binding sites of the CRP-cAMP complex have been experimentally identified. These regulatory factors include not only the transcription factors TcpP and TcpH[24] but also *ompT* (encoding the outer membrane protease)[25]. In this study, we analyzed the transcription characteristics of the *mtl* operon of the *V. cholerae* El Tor biotype and identified cAMP-CRP complex binding sites within the promoter in the *V. cholerae* O1 El Tor strain. Multiple CRP binding sites and various core motifs were revealed.

MATERIALS AND METHODS

Bacterium Strains and Culture Conditions

The strains and plasmids used in this study are listed in Table 1. *V. cholerae* and *E. coli* strains were routinely cultured in LB medium or LB agar plates and stored at LB medium containing 15% glycerol. M9 medium (48 mmol/L $Na_2HPO_4 \cdot 7H_2O$, 22 mmol/L KH_2PO_4, 8.5 mmol/L NaCl, 19 mmol/L NH_4Cl, 2 mmol/L $MgSO_4$, and 0.1 mmol/L $CaCl_2$) supplemented with 0.2% mannitol was used in mannitol utilization assays. While Mannitol fermentation medium (containing 0.5% NaCl, 0.1% tryptone, and 0.2% mannitol, adjusted to pH 8.5 with NaOH) was used to culture cells prior to luminescence measurements of the *mtl::luxCDABE* transcriptional unit. If appropriate, the culture media were supplemented with ampicillin (100 μg/mL), chloramphenicol (10 μg/mL for *E. coli* and 3 μg/mL for *V. cholerae*) and/or kanamycin (50 μg/mL).

Table 1. The Strains and Plasmids that were Used in this Study

Strains or Plasmids	Relevant Properties	Source or Reference
V. cholerae strains		
N16961	O1, El Tor, Inaba, ctxAB$^+$, tcpA$^+$	Laboratory stock
N16961Δcrp	N16961 Δcrp::cat	This study
N16961Δcya	N16961 Δcya::kan	This study
N16961ΔmtlA	N16961 ΔmtlA::cat	This study
pBADcrp-N16961Δcrp	N16961 containing plasmid pBADcrp	This study
E. coli strains		
JM109	endA1 glnV44 thi-1 relA1 gyrA96 mcrB$^+$Δ(lac-proAB)(F$^-$ traD36 proAB$^+$ laclq lacZΔ15)	TaKaRa
SM10λpir	thi recA thr leu tonA lacY supE RP4-2-Tc::Mu_::pir; Kan^r	Laboratory stock
Top10	F$^-$ mcrAΔ(mrr-hsdRMS-mcrBC) Φ80 lacZ ΔM15 ΔlacX74 recA1 araD139 Δ(ara leu)7697 galU galK rpsL(Str^R) endA1 nupG	Invitrogen
Plasmids		
pUC18	Cloning vector; ori lacZ Amp^r	Laboratory stock
pDS132	Suicide plasmid, R6k ori mob RP4 cat sacB	Laboratory stock[27]
pCVD442	Suicide vector (oriR6K mobRP4 sacB, Amp^r)	Laboratory stock[39]
pBADcrp	CRP ORF cloned in pBADHisB	[28]
pCVDΔCya-Km	pCVD442 with km gene inserted between the upstream and downstream fragments flanking cya of N16961	[29]
pBBRlux	Plasmid containing a promoterless Lux reporter, Cm^r	[33]
pmtlP4504	pBBRlux with mtl promoter region containing five CRP-binding sites	This study
pmtlDCRP1	pBBRlux with the same mtl promoter region as pmtlP4504 but with the CRP binding site CRP1 deleted	This study
pmtlDCRP2	pBBRlux with the same mtl promoter region as pmtlP4504 but with the CRP binding site CRP2 deleted	This study
pmtlDCRP3	pBBRlux with the same mtl promoter region as pmtlP4504 but with the CRP binding site CRP3 deleted	This study
pmtlDCRP4	pBBRlux with the same mtl promoter region as pmtlP4504 but with the CRP binding site CRP4 deleted	This study
pmtlDCRP5	pBBRlux with the same mtl promoter region as pmtlP4504 but with the CRP binding site CRP5 deleted	This study

Generation of Mutant Strains N16961Δcrp, N16961Δcya, and N16961ΔmtlA

The primers used in this study are listed in Supplemental Table. For the deletion of the *crp* gene, the upstream and downstream flanking fragments were amplified from N16961 using the following primers: crp1, crp2, crp3, and crp4. The chloramphenicol acetyltransferase (*cat*) gene was amplified from the plasmid pCOS5[26] with primers CmF and CmR and then inserted between the flanking sequences. The amplified fragments were then sequentially cloned into pUC18 to generate pUCNcrp. The pUCNcrp fragment was amplified using primers crp5 and crp4 and cloned into suicide plasmid pDS132[27] to generate pDSNcrp, which was transferred from SM10*λpir* to N16961 by conjugation. The *crp* mutant (N16961Δcrp) was selected in LB agar containing 10% sucrose and chloramphenicol and then confirmed by PCR analysis and DNA sequencing. To complement the *crp* gene, the arabinose-induced plasmid pBADcrp[28] was transformed into N16961Δcrp by electroporation (Gene Pulser, Bio-Rad), yielding the strain pBADcrp-N16961Δcrp. The *cya* deletion mutant of N16961 (N16961Δcya) was constructed using suicide vector pCVDΔCya-Km[29]. Through a similar approach, the *mtlA* deletion mutant (N16961ΔmtlA) was constructed using primers mtlAUF, mtlAUR, mtlADF, mtlADR, CmF, and CmR.

The Purification of His-CRP

E. coli TOP10 harboring the CRP expression plasmid pBADcrp[28] were cultured at 37 °C in LB

medium. The overexpression of CRP was induced by the addition of 0.1 % (w/v) arabinose; the protein was then extracted by ultrasonication, purified using the His•Bind purification kit (Novagen) according to the instructions provided by the manufacturer and analyzed by SDS-PAGE.

Electrophoretic Mobility Shift Assays

EMSA assays were performed using previously described methods[30]. A DNA fragment encompassing the 360 bp promoter region of *mtl* was amplified by PCR assay using biotin-labeled (Invitrogen) primers ECRP1F and ECRP5R. Subsequently, the DNA fragments containing the CRP binding sites CRP1, CRP2, CRP3, CRP4, and CRP5 were amplified by PCR using biotin-labeled primers ECRP1F and ECRP1R, ECRP2F and ECRP2R, ECRP3F and ECRP3R, ECRP4F and ECRP4R, and ECRP5F and ECRP5R, respectively. The fragments covering -549.5 and -395.5 were amplified by PCR using biotin-labeled primers ECRP-2F and ECRP-2R, ECRP-1F and ECRP-1R. Binding reactions were performed by incubating each biotin-labeled DNA fragment with various quantities of purified CRP in 20 μL of binding buffer [50 mmol/L Tris-HCl (pH 8.3), 250 mmol/L KCl, 5 mmol/L $MgCl_2$, 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 1 μg Poly (dI.dC) and 0.5 μmol/L cAMP]. Following incubation at room temperature for 30 min, a one-fourth volume of loading buffer [200 mmol/L Tris-HCl (pH 6.8), 40% glycerol, and 0.2% bromphenol blue (w/v)] were added, and the mixtures were electrophoresed in a 6% non-denaturing polyacrylamide gel in 0.5× TBE buffer (45 mmol/L Tris-borate and 1 mmol/L EDTA, pH 8.3)[31]. The bands were detected with Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific).

Mapping Transcription Start Site for mtl by 5' RACE-PCR

RNeasy Mini Kit (Qiagen) was used according to the manufacturer's instructions to purify total RNA. N16961 cDNA was generated using SMARTer™ RACE cDNA Amplification Kit (Clontech). The cDNA was then amplified by PCR using UPM (universal primer) and primer mtlA-race-in. The PCR product was purified using QIAquick Gel Extraction Kit (Qiagen), and the transcription start site was confirmed by DNA sequence analysis[32].

Construction of Pmtl-luxCDABE Fusion Plasmids

To determine the activation of the *mtl* promoter by CRP, upper primer sac4504F and forward primer BamH4880 were used to amplify the *mtl* promoter fragment, which was cloned into the reporter plasmid pBBRlux[33] to generate the fusion plasmid pmtlP4504 with *luxCDABE*. This plasmid was transferred into N16961 and mutants N16961Δcrp/ N16961Δcya for the measurement of luminescence activities. After the CRP binding sites in the upstream region of the *mtl* gene cluster were determined, a series of mutations involving the deletion of one of these CRP binding sites (CRP1, CRP2, CRP3, CRP4, or CRP5) from the *mtl* promoter fragments was created. This series of CRP-binding-site deletion mutations were firstly generated with the following partially overlapping primer pairs: CRP1R and CRP1F, CRP2R and CRP2F, CRP3R and CRP3F, CRP4R and CRP4F, CRP5R and CRP5F. These primer pairs were matched with 4126F or 5258R, respectively. Subsequently, PCR was used to construct *Pmtl-luxCDABE* fusion plasmids by amplifying the above overlapping fragments with primers sac4504F and BamH4880. And then the resulting plasmids were transformed into *E. coli* SM10λpir, named pmtlDCRP1, pmtlDCRP2, pmtlDCRP3, pmtlDCRP4, and pmtlDCRP5, respectively. Finally, these plasmids were transferred into N16961 by conjugation.

Lux Activity Measurement

V. cholerae strains containing *Pmtl-luxCDABE* plasmids were cultured in mannitol fermentation medium. Samples were collected at the fourth hour after culture, and luminescence was measured using 96-well white flat-bottomed plates (Corning) and a microplate reader (Infinite M200 Pro, Tecan). Lux activity was reported as relative light units per OD600 after the relative light units and OD600 were blank corrected, respectively. In this assay, the OD600 was shown as the values from the measurements of 200 μL culture medium in flat-bottomed 96-well microplates (Corning) with a microplate reader at 600 nm.

RESULTS AND DISCUSSION

CRP is Required for Activation of Mannitol PTS Operon of V. cholerae El Tor

To assess the crucial or synergistic activation of the *mtl* operon in *V. cholerae* by the CRP-cAMP complex, we compared the regulation of CRP in the wild-type and CRP gene mutant strains. M9 medium

supplemented with mannitol was used to measure the growth curve of the wild-type and mutant strains. A flat growth curve compared with the wild-type strain was observed in N16961ΔmtlA, in which mannitol transportation is blocked by the defect in mannitol PTS, and the *crp* and *cya* mutants demonstrated similar defective growth patterns (Figure 1a). The strain pBADcrp-N16961Δcrp restored normal growth through the trans-complementation of intact *crp* induced by arabinose, while N16961 could not use the arabinose as a carbon source (Figure 1a)[34]. We also used reporter plasmids to determine the activation of CRP. The parental strain N16961 and the mutants N16961Δcrp and N16961Δcya, which harbored the *mtl* promoter-*luxCDABE* fusion plasmid pmtlP4504, were used to measure the activities of luminescence in the mannitol fermentation medium. The activity of wild-type strain N16961 was 4 388 043±308 490 at 2 h, 3 203 317±311 460 at 4 h, while the activity of N16961Δcrp and N16961Δcya were only 1050±211, 3980±758 at 2 h, 1158±256, 1654±141 at 4 h, respectively. The activity of wild-type strain N16961 was significantly higher (P<0.0001) than the activity of N16961Δcrp and N16961Δcya at both 2 h and 4 h of the test, and no obvious luminescence activity was observed in the *crp* or *cya* mutants (Figure 1b), suggesting the absolute need for CRP-cAMP for the activation of the *mtl* operon in the *V. cholerae* El Tor strain.

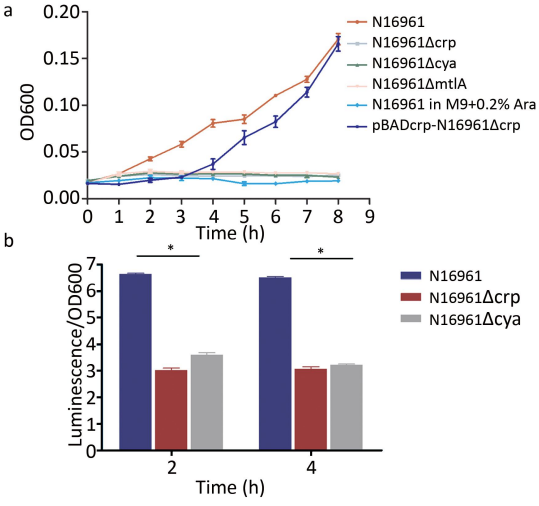

Figure 1. Mannitol utilization assays. (a) The growth curve of N16961 WT strain, *crp* mutant (N16961Δcrp), *cya* mutant (N16961Δcya) and *mtlA* mutant (N16961ΔmtlA) in M9 medium supplemented with 0.2% mannitol; pBADcrp-N16961Δcrp in M9 medium supplemented with 0.2% mannitol, 0.1% arabinose (Ara) and ampicillin; and N16961 in M9 medium supplemented with 0.2% Ara were measured by spectrophotometry at different time points. The assay was performed in triplicate, and the data are presented as the means with the standard deviation from three experiments. (b) Comparisons of *mtl* promoter activities in N16961 and its mutants which were cultured in mannitol fermentation medium. The asterisks signify that statistically significant differences were found (P<0.0001, two-tailed t test) in comparisons of N16961Δcrp and N16961Δcya with N16961. The data are presented in terms of the averages and standard deviations from three experiments. The activity of wild-type strain N16961 was significantly higher (P<0.0001) than the activity of N16961Δcrp and N16916Δcya at both 2 h and 4 h of the test, and no obvious luminescence activity was observed in the *crp* or *cra* mutants.

CRP-cAMP Binds Directly to mtl Promoter of V. Cholerae Strain N16961 in vitro

EMSA was performed using purified *V. cholerae* CRP protein and 360 bp *mtl* promoter fragment of N16961 to verify the binding of the CRP-cAMP complex to the promoter. The quantity of CRP-promoter DNA complex increased with the amount of CRP in the reaction mixture and the binding is absent if cAMP or CRP was cleared from the reaction mixture (Figure 2a), demonstrating that cAMP-CRP per se directly binds to the *mtl* promoter region in *V. cholerae*.

Mapping of Transcriptional Start Site of mtl Operon

The determination of the initiation site of *mtl* transcription may assist in determining the positions of the possible CRP binding sites. With 5'RACE, 5'GUACUA was identified as the initiation sequence of the *mtl* mRNA. This information placed the transcription start site at a location 75 nucleotides upstream of the *mtl* open reading frame (as illustrated in Figure 3a); this result is consistent with a previous report[35]. Based on this site, sequences that share similarities with the *E. coli* consensus -10 (TATAAT) and -35 (TTGACA) hexamers were also identified; these sequences are located proximally downstream of the predicted CRP binding site CRP5 (Figure 3a).

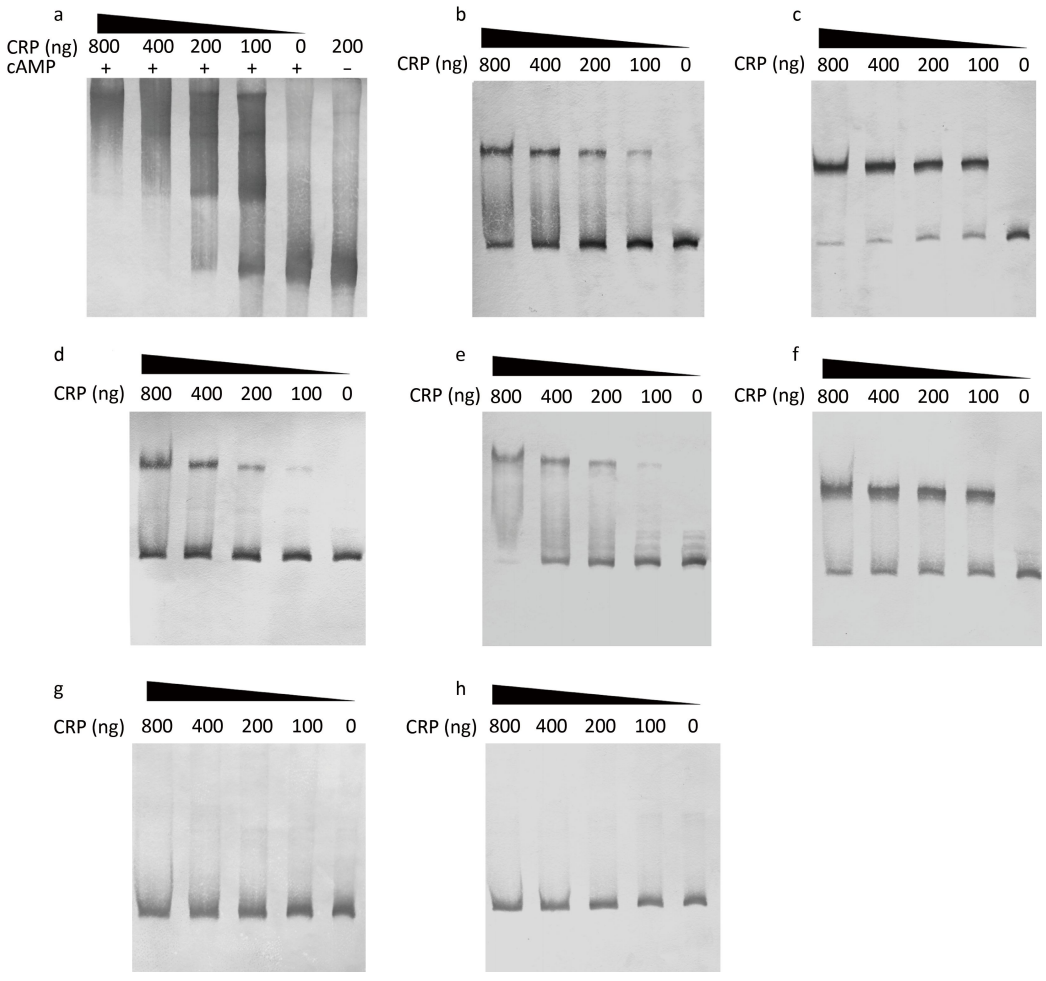

Figure 2. EMSA assays for binding of CRP. (a) Biotin-labeled promoter fragments (containing all five CRP binding sites) were titrated with decreasing amounts of purified CRP (final concentrations of 800, 400, 200, 100, and 0 ng). All of the reaction mixtures except for lane 6 included 10 nmol cAMP. (b) to (h) Biotin-labeled DNA fragments of *mtl* were titrated with decreasing quantities of purified CRP (final concentrations of 800, 400, 200, 100, and 0 ng). All of these reaction mixtures included 0.5 μmol/L cAMP. (b) to (f) The mobility of the *mtl* promoter probes were retarded in a CRP-concentration-ependent manner. Panels B, C, D, E, and F represent the reactions between CRP-cAMP complex and DNA fragment containing CRP binding site CRP1, CRP2, CRP3, CRP4, or CRP5, respectively. (g) and (h) The mobility of the *mtl* promoter probes were not retarded by CRP-cAMP complex. Panels G and H represent the reaction between CRP-cAMP complex and DNA fragment covering -549.5 bp and -395.5 bp, respectively.

CRP-cAMP Binds to Five Individual Sites in mtl Promoter Region with Different Affinity

Initially, Virtual Footprint[36] was used to search for possible CRP binding sites on the 650 bp *mtl* promoter region of *V. cholerae* El Tor strain N16961, using the consensus CRP binding motif of *E. coli* K12. Seven probable CRP binding sites were selected to determine the possible binding of CRP-cAMP complex by EMSA. All seven predicted CRP binding sites were located at the upstream of -35 region of *mtl* and located at -549.5 bp, -395.5 bp, -267.5 bp, -217.5 bp, -173.5 bp, -100.5 bp, and -57.5 bp, with respect to the *mtl* transcription start site. Seven DNA fragments, each of which contained one of the predicted CRP binding sites were amplified by PCR using biotin-labeled primers. Retarded DNA fragments were observed with five fragments covering -267.5 bp, to -57.5 bp in the presence of increasing amounts of CRP with cAMP (Figure 2b-f), except the fragments covering -549.5 bp and -395.5 bp (Figure 2g, h). We then named the binding motifs covering -267.5 bp to -57.5 bp as CRP1 to CRP5, sequentially. Differential affinities of these binding sites may presumably exist. Based on the lanes that contained 100 ng CRP, CRP-cAMP may have a greater binding

affinity to CRP2 and CRP5 than to CRP1, CRP3, and CRP4 according to the observed densities of retarded DNA fragments (Figure 2b-f).

The roles of these five binding sites were further investigated using site deletion and *luxCDABE* reporter fusion plasmids. The reporter plasmids pmtlDCRP1, pmtlDCRP2, pmtlDCRP3, pmtlDCRP4), and pmtlDCRP5 contain the *mtl* promoter region with the deletion of sites CRP1, CRP2, CRP3, CRP4, and CRP5, respectively. Compared with the plasmid pmtlP4504, which contained the intact *mtl* promoter, approximately 2.7, 42.7, 10.0, 35.8, and 21.6-fold decreases in the luminescence activity were found for pmtlDCRP1, pmtlDCRP2, pmtlDCRP3, pmtlDCRP4, and pmtlDCRP5, respectively (Figure 4). This experiment demonstrated the roles of these five CRP-cAMP

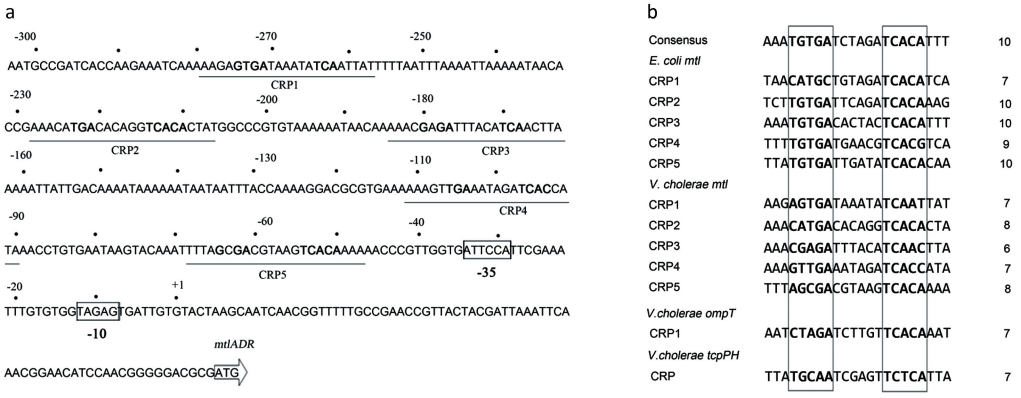

Figure 3. (a) The nucleotide sequence of *mtlADR* from *V. cholerae* strain N16961. The start codon (ATG) is shown at the 3' terminus. The location of the transcription start site (+1) established by 5'RACE-PCR and the inferred -35 and -10 hexamers are boxed. The CRP binding regions are underlined and labeled. The CRP binding sites are compared with the CRP consensus sequence and are shown in boldface type. (b) A comparison of the CRP binding motif sequences in the *mtl* promoter of *E. coli* and in the *mtl*, *ompT* and *tcpPH* promoters of *V. cholerae*. The consensus motif of TGTGA…TCACA is shown in the top sequence. The CRP binding motif sequences are boxed and highlighted in bold, and the numbers of nucleotides in each conserved motif are indicated on the right.

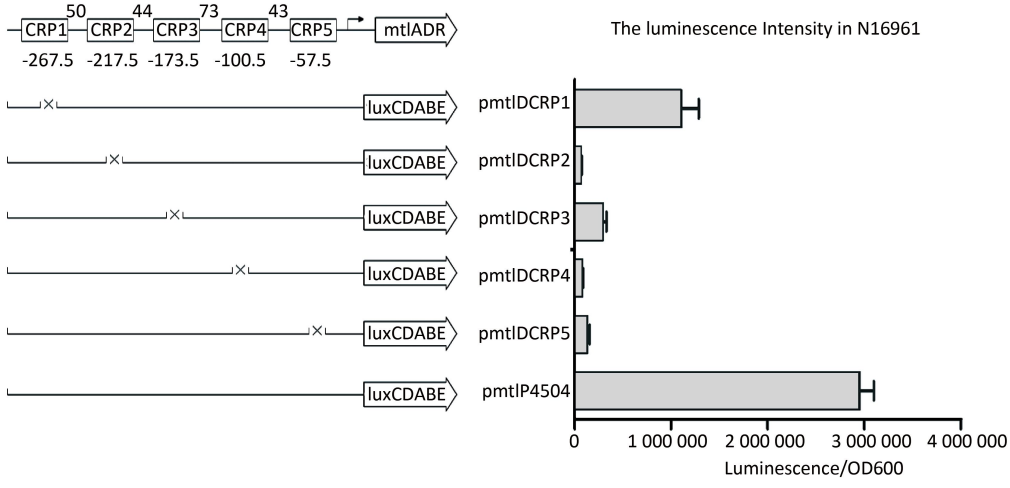

Figure 4. Expression assays of *mtl* with different promoter fragments. A schematic presentation of the promoter region of the mannitol operon *mtl* of *V. cholerae* is shown on the top (not to scale). The numbers are presented with respect to the transcriptional start site (+1). Five CRP binding sites (CRP1 to CRP5) are indicated by rectangular boxes. The numbers under the rectangular boxes indicate the center positions of the CRP binding sites (unit: bp). The numbers between the rectangular boxes indicate the center-to-center distances between the CRP binding sites (unit: bp). The Six different *mtl* promoter fragments linked with the *luxCDABE* (*lux*) gene are illustrated, representing (from top to bottom) the *mtl* promoter fragments with deletions of CRP1, CRP2, CRP3, CRP4, or CRP5 and the unbroken promoter fragment. The lux intensities of these constructed plasmids in N16961 were shown on the right side using (relative light units-lank)/(OD600-lank); these values represent the averages and standard deviations from three experiments.

binding sites in the activation of *mtl*. Tandem DNA binding sites bound individually by activators are typically considered to contribute to cumulative enhancement of transcription. The co-existence of these five sites showed the most effective activation of the *mtl* operon by the CRP-cAMP complex in our study. It also seems that CRP1 is relatively unimportant for CRP activation among these five binding sites.

In *E. coli*, the core motifs of the five binding sites within *mtl* promoter are identical or similar to the consensus sequence for CRP binding sites, TGTGA-N6-TCACA, whereas the *V. cholerae* CRP binding site sequences are of divergence from the canonical (Figure 3b). Across its 201 amino acid residues, the CRP of *V. cholerae* shares 96% identity with the CRP of *E. coli* K12[37] and can functionally complement the *E. coli crp* mutant[38]. We compared the sequences and residues of a helix-turn-helix DNA-binding motif that was identified in the C-terminal region of the *V. cholerae* CRP protein (^{140}V to ^{202}K) with the corresponding aspects of the *E. coli* CRP protein and discovered only one different residue. This comparison revealed the flexible nature of the recognition of CRP binding sites by CRP.

Different transcriptional activation mechanisms of CRP in *E. coli* have been reported[4,8-9,11-13]; in particular, these mechanisms are distinguished by binding site position and by the need for other activators. We found crucial activation of the transcription of the *mtl* operon by CRP. In addition, five CRP binding sites were found, and these five sites collectively enhanced the transcription of *mtl* to a high degree. The positions of these five sites covered the -267.5 bp to -57.5 bp of *mtl* (Figure 4); by contrast, other gene promoters harbor only one or two binding sites. Interestingly, the distance between the CRP binding sites are 50 bp (between CRP1 and 2), 44 bp (between CRP2 and 3), 73 bp (between CRP3 and 4), 43 bp (between CRP4 and 5) in *V. cholerae*, while the distance between the CRP binding sites are 43 bp (between CRP1 and 2), 44 bp (between CRP2 and 3), 73 bp (between CRP3 and 4), 44 bp (between CRP4 and 5) in *E. coli*[22]. The distances of these sites in *V. cholerae* and *E. coli* are quite similar except the distance between the CRP1 and 2, although the difference in promoter sequences exists. Thus, the hypothetic conformational model, that the five binding sites make the *mtl* promoter region forming a loop[22], should be the same in *V. cholerae* and *E. coli*. We suggest that the mechanism of CRP-cAMP regulation of *mtl* in *V. cholerae* (and in *E. coli*) may be unusual with respect to its binding sites at promoter region. This mechanism produces strong activation upon CRP binding.

In conclusion, five CRP binding sites are identified within the promoter region upstream of the *mtl* operon in the *V. cholerae* El Tor biotype; these five sites display different affinities to CRP and thereby modulate CRP activation. These results may facilitate the understanding of the elaborate and subtle regulation of mannitol utilization in *V. cholerae*.

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We have no conflict of interest to declare.

Supplement Table. Primers Used in this Study

Primers	Nucleotide Sequence (5' to 3')*
crp1	CGGAATTCGACTACCTCAAATTCATCACC
crp2	GGGGTACCGTGACAATGTGAAAGAAACC
crp3	ACGGGATCCGAACCTGATCTCTGCCCAC
crp4	ACATGCATGCT TGCGCCTAAGGTTTC
crp5	CTAGTCTAGAGA CTACCTCAAATTCATCACC
CmF	GGGGTACCCGTA GCACAGGCGTTTAAG
CmR	ACGGGATCCGATCGGCACATGAGAGGTTC
mtlAUF	CCGGAATTCTTTGTGTGGTAGAGTGAT
mtlAUR	ACGCGT CGACTGGCACATAGCATAC
mtlADF	ACGCGTCGACCCAAGAATGATGA ACATA
mtlADR	ACATGCATGCACCATATTCTCACAGG
pUA-FP	TGCACTGCAG TTTGTGTGGTAGAGTGAT
pUA-RP	ACATGCATGCACCATATTCTCACAGG
ECRP1F	ACTATGTGTAGGTCTTCCTACTTACG
ECRP1R	ATGTTTCGGTGTTATTTTTAATTT
ECRP2F	TTTTTAATTTAAAATTAAAAATAACACC
ECRP2R	GTTTTTGTTATTTTTTACAC
ECRP3F	ACTATGGCCCGTGTAAAAAATAAC
ECRP3R	CACGCGTCCTTTTGGTAAATTATTAT
ECRP4F	TACATCAACTTAAAAATTATTGAC
ECRP4R	GCTAAAATTTGTACTTATTCACAG
ECRP5F	CCATAAACCTGTGAATAAGTACAAAT
ECRP5R	CGCGTCCCCCGTTGGATGTTATAGTAGTACCCCCCCCCAC

Continued

Primers	Nucleotide Sequence (5' to 3')*
ECRP-2F	GAAGGTTGTTTTTAACTTAAATGACTCGGATAGAGGAAA CAGAAACAATCAT
ECRP-2R	TATCCATCTCCCCTTCCATTTTTATT
ECRP-1F	CGGTTTGGCGAGCTGTCCCCTTAC
ECRP-1R	TTCGAGCTCGAATGCCGATCACCAAGAAAT
mtlA-race-in	CGCGGATCCGCGCGCGTCCCCCGTTGGATGTT
sac4504F	ATCAAAAAGATTTTAATTTAAAATTAAAAATAACACC
BamH4880	TAAATTAAAATCTTTTTGATTTCTTGGTGAT
CRP1F	CACCGAAACAACTATGGCCCGTGTAAAAAATAAC
CRP1R	GGCCATAGTTGTTTCGGTGTTATTTTTAATTTT
CRP2F	AACAAAAACGACTTAAAAATTATTGACAAAATAAAAAAT
CRP2R	ATTTTTAAGTCGTTTTTGTTATTTTTTACAC
CRP3F	TGAAAAAAGTCCATAAACCTGTGAATAAGTACAAAT
CRP3R	AGGTTTATGGACTTTTTTCACGCGTCCTTTTG
CRP4F	CAAATTTAGGAAAAAACCCGTTGGTGATTCCATTC
CRP4R	CGGGTTTTTTCTAAAATTTGTACTTATTCACAGGTTTAT
CRP5F	AGTTTGGCAAAATCAACTTGG
CRP5R	TTGTTGACCAACATTTCAAAG
4126F	AGTTTGGCAAAATCAACTTGG
5258R	TTGTTGACCAACATTTCAAAG

Note. *The underlined nucleotides in the primers indicate introduced restriction endonuclease recognition sites. The small letters in the primers indicate overlapping regions.

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