CRP-Cyclic AMP Dependent Inhibition of the Xylene-Responsive $\sigma^{54}$-Promoter Pu in Escherichia coli

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Abstract

The expression of $\sigma^{54}$-dependent Pseudomonas putida Pu promoter is activated by XylR activator when cells are exposed to a variety of aromatic inducers. In this study, the transcriptional activation of the P. putida Pu promoter was recreated in the heterologous host Escherichia coli. Here we show that the cAMP receptor protein (CRP), a well-known carbon utilization regulator, had an inhibitory effect on the expression of Pu promoter in a CAMP-dependent manner. The inhibitory effect was not activator specific. In vivo MnO$_4$ and DMS footprinting analysis indicated that CRP-cAMP poised the RNA polymerase at Pu promoter, inhibiting the isomerization step of the transcription initiation even in the presence of an activator. Therefore, the presence of PTS-sugar, which eliminates cAMP, could activate the poised RNA polymerase at Pu promoter to transcribe. Moreover, the activation region 1 (AR1) of CRP, which interacts directly with the $\sigma^{54}$-subunit of RNA polymerase, was found essential for the CRP-mediated inhibition at Pu promoter. A model for the above observations is discussed.

Introduction

The $\sigma^{54}$-dependent Pu promoter drives transcription of the upper operon of Pseudomonas putida mt-2 TOL plasmid pWW0 for degradation of toluene and xylenes [1–4]. This promoter region includes two upstream activating sites (UASs) for the activator protein XylR [5,6], a $-12/-24$ region recognized by Es$\sigma^{54}$ RNA polymerase, a single integration host factor (IHF) binding site located in the intervening region[5,7] and the adjacent UP-like elements for docking of the Es$\sigma^{54}$ [8]. IHF-mediated DNA bending facilitates the direct interactions between two CTDs of the RNA polymerase and two separated UP-like elements located $-78$ and $-104$ upstream of the transcriptional start site [8], recruiting Es$\sigma^{54}$ to the Pu promoter when cells reach stationary phase [9,10]. It is generally believed that this IHF-dependent closed complex formation is the rate-limiting step for the transcriptional initiation at the Pu promoter [8,11].

Recently, the architectural organization of the $\sigma^{54}$-dependent promoter was investigated and led to the conclusion that the activator must approach the Es$\sigma^{54}$ closed complexes from the unbound (activator accessible) face of the promoter DNA helix to catalyze open complex formation [12]. This conclusion is further supported by the first modeling of activator-promoter DNA-Es$\sigma^{54}$ complex [12]. Since the contact between the UAS bound activator and promoter bound Es$\sigma^{54}$ depends on the orientation of the DNA bending between UAS and $-12/-24$ region of a promoter [13,14], the optimal IHF induced DNA bending at Pu promoter is essential for the transcription initiation [8].

CRP, the cyclic AMP (cAMP) receptor protein, is one of the best studied transcriptional factor, which is responsible for the regulation of more than 100 genes mainly involved in catabolism of sugars, amino acids and nucleotides in E.coli [15,16]. The CRP-mediated regulation requires initially the binding of cAMP to form an active CRP homodimer when intracellular cAMP level is high, but in the presence of PTS (phosphoenolpyruvate-sugar phosphotransferase system)-sugars such as glucose the low cAMP level diminishes the activity of CRP [16]. At the $\sigma^{70}$-dependent promoters, the dimeric CRP protein enhances the ability of Es$\sigma^{70}$ to bind DNA and initiate transcription by interacting with Es$\sigma^{70}$ directly [17]. Two discrete surfaces of CRP, known as Activating Region 1 (AR1, consisting of residues 156–164 of CRP) and Activating Region 2 (AR2, consisting of residues His19, His21 and Lys101 of CRP), interact with the C-terminal and N-terminal domains, respectively, of the $\sigma^{70}$ subunit of RNA polymerase [15] and the structural basis of CRP-zCTD-DNA complex has been determined [18]. A third contact surface (AR3) of CRP which is thought to interact with $\sigma^{70}$ only at Class II promoters has also been identified. AR3 is defined as containing both the activating (residues 53, 54, 55 and 58 of CRP) and inhibitory (residue 52 of CRP) determinants [19].
Previous studies showed that CRP-cAMP down-regulated the σ44-dependent *dctA* and *glnP2* promoters in *Escherichia coli* [20–22]. Two mechanisms are involved for the CRP-mediated inhibition of the expression of *glnP2* promoter [23]. First, CRP affects GlnB signaling through direct activation of glnHPQ operon and in turn de-activates *glnP2*. Second, in vitro studies show that CRP can be recruited by Ert74 to a site upstream of *glnP2* through the direct interaction between σCTD of Ert74 and AR1 of CRP, preventing the activator protein from approaching the activator-accessible face of the promoter-bound Ert74 closed complex [23]. Therefore, as the major transcriptional effector of the ‘glucose effect’, CRP affects both the signal transduction pathway and the overall geometry of the transcriptional machinery of components of the nitrogen regulon. As a result, *E. coli* *glnP2*, *glnHP2* [23], *glnk* (unpublished observations) and astABCD promoters [24] together with the *Klebsiella pneumoniae* nifB, nifE, nifF, nifH, nifJ, nifL and nifU promoters [25] are all down-regulated by the CRP-cAMP complex in *E. coli*.

Based on the striking inhibitory effect of CRP-cAMP on the expression of σ44-dependent promoters as mentioned above, this study was undertaken to investigate the influence of CRP-cAMP on the Pu promoter expression in *E. coli*. Our data show that CRP had an inhibitory effect on the expression of Pu promoter in a cAMP-dependent manner and the inhibitory effect was not activator specific. In vivo KmnO₄ and DMS footprinting analysis indicated that CRP-cAMP poised the RNA polymerase at Pu promoter, inhibiting the isomerization step of the transcription initiation even in the presence of an activator. This transcription program leads to the maximal production of toluene and xylenes degradation enzymes only in the absence of cAMP signal. Moreover, AR1 of CRP, which interacts directly with the σCTD of RNA polymerase, was found essential for this CRP-mediated inhibition at Pu promoter. A model for the above observations is discussed.

**Materials and Methods**

**Bacterial strains and plasmids**

Bacterial strains and plasmids used in this study are listed in Table 1.

**Growth media and enzyme assays**

M63 modified medium was prepared as previously described [26]. Cells were grown at 30°C. β-galactosidase assays were performed according to Miller [27].

**Genetic manipulations**

Preparation of plasmid DNA, restriction enzyme digestions, ligation and horizontal agarose gel electrophoresis in Tris-borate-EDTA buffer were performed according to the standard methods [28]. DNA sequence analysis was either performed at TaKaRa Corporation, Japan or using a GenomyxLR™-OPTIMIZED sequencing kit for DMS and KmnO₄ footprinting experiment.

**Plasmid construction**

The complete nucleotide sequence of *xyl* upper operon of TOL plasmid pWW0 was sequenced previously (Harayama et al., unpublished). In order to amplify the Pu promoter, two primers were synthesized: 5′-CCCAACGTGATGCAGATACGTTTTTATC-3′ (p1, *HindIII*) and 5′-CGGGATCCGATGGAAATATACACAT-3′ (p2, *BamHI*). Restriction sites present in oligonucleotide primers used for cloning are underlined. Polymerase Chain Reactions (PCRs) were carried out, and the entire Pu region and the first 7 codons of the *xylU* open reading frame (ORF) (from –200 to +50 of *xylU*) together with artificially introduced restriction sites was amplified, using TOL plasmid pWW0 from *P. putida* strain mt-2 as template and p1, p2 as primers. The DNA fragment was restricted with *HindIII* and *BamHI*, and cloned into pBlue-script-SK. This resulted in pBS/Pu and its DNA sequence was verified. The 250 bp *HindIII*-*BamHI* fragment of pBS/Pu was subsequently inserted into pgD926 to produce an in frame *Puc::lacZ* fusion, plasmid pKU700. In order to amplify the *P. putida* crp gene, PCR was carried out by using *P. putida* strain mt-2 genome as template and oligonucleotides p3 (5′-CGCGGATCCGCTACCGGCGAGTTACG, *BamHI*) and p4 (5′-AGCGCTGACCTAGCGGGTACCG, *SalI*) as primers. The *BamHI*-SalI fragment was verified subsequently and inserted into pLG339 to gain the plasmid designated as pLG339PpCRP.

**In vivo KmnO₄ footprinting experiments**

To detect binding of Eσ44 RNA polymerase to Pu promoter, *E. coli* strain carrying the indicated plasmids was pre-grown aerobically at 30°C to late-logarithmic phase in the LB medium, diluted into 10 mM of the same medium with an addition of 0.2 mmol/L m-methylbenzyl alcohol (mMBA) as the inducer at an initial OD₆₀₀ of 0.05 and then grown out aerobically. At 0.9 OD₆₀₀, each sample was treated with 40 μL of 50 mg mL⁻¹ rifampicin (dissolved in methanol) for 5 min. The cells were immediately spun down, followed by resuspension in 5 mL of 0.09 mol/L KmnO₄ for 2 min. The reaction was stopped by adding 100 μL of β-mercaptoethanol. The cells were spun down, and the plasmid DNA was isolated using SV DNA purification kit (Promega Corporation). 7 μL of 100 μL eluted plasmid DNA was analyzed by PCR amplified primer extension. 7 μL of DNA solution, 2 μL of 5× Taq polymerase buffer, 1.5 μL dNTP (2.5 mmol/L each), 0.25 μL of 5′-32P-labeled p3 (0.4 pmol μL⁻¹, 7.4×10⁶ Bq μL⁻¹) (p3: 5′-GGGATTCGGTACCGGCGAGTTACG, *BamHI*) and p4 (5′-AGCGCTGACCTAGCGGGTACCG, *SalI*) as primers. The *BamHI*-SalI fragment was verified subsequently and inserted into pLG339 to gain the plasmid designated as pLG339PpCRP.
mixed well and then heated for 2 min at 90°C, followed plunged into ice. Then 8 μL of dNTP (2.5 mmol each), 10 μL of 10×EcoPol buffer, 41 μL of ddH₂O, 0.2 μL of Klenow DNA polymerase (5 U μL⁻¹, New England Biolabs, Inc) was added and extended at 37°C for 45 min. The samples were analyzed on a 6% (w/v) polyacrylamide sequencing gel, calibrated with the corresponding sequencing reactions.

Results

Differential induction of the Pu promoter in E. coli cya mutant TP2006 and its isogenic wild type TP2101

In this study, XylR-mediated activation of the P. putida Pu promoter was recreated in the heterologous host E. coli. A translatable Pu::lacZ fusion was constructed in the 28 kb low-copy reporter plasmid pGD926 [29], a derivative of the RK2, the best-studied IncP-1 plasmid whose replication and, hence, its copy number are tightly regulated [30]. This resulting construct was named pKU700. Plasmid pKU700 was co-transformed with pTS174 into E. coli cya mutant TP2006 (unable to produce cAMP) and its isogenic wild type TP2101 separately. β-galactosidase activities were measured in the presence of different concentrations of mMBA, an effective aromatic inducer for XyR [32]. Activator XyR-mediated and inducer mMBA-dependent activation of the Pu promoter was observed in both E. coli strains (Figure 1). However, under the same mMBA concentration, XyR-activated expression of Pu was much higher in the cya mutant TP2006 than that in its isogenic wild type strain TP2101 (Figure 1). In addition, control experiment by using empty vector of pTS174 showed little Pu activation in both E. coli strains (data not shown). These results suggest that the presence of cAMP affects Pu expression. Hence, it was thought necessary to examine whether the CRP-cAMP complex influenced Pu expression.

CRP-cAMP-mediated conditional inhibition of the Pu promoter in E. coli

To explore the role of CRP-cAMP in controlling Pu output in E. coli, promoter activity was monitored in the E. coli wild-type strain TP2101, the cya mutant TP2006 and the cya csp double mutant TP2339-1 respectively. All strains harbor plasmids pTS174 and pKU700. In addition, low copy pLG339-derived plasmids [33], harboring either the wild-type E. coli csp gene (pLG339CRP), P. putida (pLG339PcCRP), or a deleted csp gene (pLG339ARS, empty vector), were introduced into TP2339-1. Expression of Pu was monitored as the absence or presence of cAMP. In the absence of cAMP, the Pu promoter was expressed at high levels in TP2006 and TP2339-1 (i.e., in hosts lacking cAMP) (Table 2). In contrast, it was inhibited in the wild-type strain TP2101 (Table 2). When exogenous cAMP was added in the growth medium, expression of Pu was comparatively lower than that in the absence of cAMP in TP2006 and TP2339-1 containing pLG339CRP (carrying the E.coli csp gene) or pLG339PcCRP (carrying the P. putida csp gene), but it remained constant at a high level in TP2339-1 containing pLG339ARS (Table 2). These results suggested that CRP proteins (both from E. coli and P. putida) had a cAMP-dependent inhibitory effect on Pu expression.

Table 1. Bacterial strains and plasmids used in this work.

| Strain/Plasmid  | Relevant characteristics | Source/Reference |
|-----------------|--------------------------|------------------|
| **E.coli strains** |                          |                  |
| TP2101          | F-, xyl, lacX74, argH1    | A. Danchin       |
| TP2006          | F-, xyl, cya, lacX74, argH1, glpD³ | [57] |
| TP2339          | F-, xyl, cya, csp-39, lacX74, argH1 | [22] |
| TP2339-1        | F-, xyl, cya, csp-39, lacX74, argH1, glpD³ | [22] |
| TH1             | ΔlacU669, endA1, thi-1, hsdR17, supE44, ΔproN2518 |                  |
| **Plasmids**    |                          |                  |
| pWW0            | TOL*, IncP9 incompatibility group plasmid | Juan L. Ramos |
| pBluescript-SK  | ColE1, lacZ, ApB           | Stratagene       |
| pBS/Pu          | pBluescript-SK-Pp Pu, ApB  | This work        |
| pGD926          | lacZYA translational fusion vector, TcB | [29] |
| pKU101          | gliA1p2mCRP::lacZYA fusion in pGD926, TcB | [22] |
| pKU700          | Puc::lacZYA fusion in pGD926, TcB | This work        |
| pTS174          | pACYC184 derivative, expresses xylR, CmR | [31] |
| pVTRA          | pVTR-A derivative, expresses xylRA, CmR | V. De Lorenzo   |
| pLG339CRP      | pLG339 carrying E.coli csp under the control of the csp promoter, KmR | This work        |
| pLG339PcCRP    | pLG339 carrying P.putida csp under the control of constitutive Tc promoter, KmR | This work        |
| pLG339ARS      | pLG339 with EcoRl/SaiI internal deletion, KmR | S. Busby         |
| pLG339CpCRP    | pLG339CRP derivative, KmR, CRP with defective AR1 | S. Busby         |
| pLG339CpK101E  | pLG339CRP derivative, KmR, CRP with defective AR2 | S. Busby         |
| pLG339CpK52N   | pLG339CRP derivative, KmR, CRP with improved AR3 | S. Busby         |
| pLG339CpE8K    | pLG339CRP derivative, KmR, CRP with defective AR3 | S. Busby         |

Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline; *, resistance; Pp, Pseudomonas putida; Δ, deletion; s, novel joint; lac operon; glpD³ is mutation near the argH gene that allows growth of this strain on glycerol [57].

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CRP-cAMP-mediated inhibition on *Pu* is at the transcriptional level *in vivo*

In order to examine whether the inhibitory effect of CRP-cAMP on *Pu* operates at the transcriptional level, we detected the influence of CRP-cAMP on the open complex formation at *Pu*. The presence of open complexes may be probed by preventing transcription elongation with rifampicin to trap open complexes and then footprinting with potassium permanganate (KMnO₄), an agent that primarily oxidizes T and C residues in single-stranded DNA of melted promoters [34]. In the presence or absence of exogenous cAMP with and without rifampicin, the *E. coli cya* mutant TP2006 containing plasmid pKU700 and pTS174 was chemically treated with KMnO₄. The footprints obtained with the bottom strand of plasmid DNA pKU700 from intact cells are shown in Figure 2. It can be seen that in the absence of exogenous cAMP, the residues between position −9 and −5 upstream the transcriptional start site are strongly hypersensitive to attack of KMnO₄, indicating that open complex formed at *Pu* (Figure 2, lane 1). In contrast, in the presence of exogenous cAMP, no DNA open complexes are observed (Figure 2, lane 3), indicating that CRP-cAMP prevented XylR-dependent open complex formation at *Pu*. As negative controls, without rifampicin treating, no DNA open complexes were observed regardless of the presence or absence of exogenous cAMP (Figure 2, lanes 3 and 4). We conclude therefore, that the CRP-cAMP-mediated inhibition on *Pu* expression is at the transcriptional level.

Overproduction of the activator alleviates the CRP-cAMP-mediated inhibitory effect on *Pu*

XylR belongs to the prokaryotic enhancer binding protein family of transcriptional regulators [35,36]. Direct interactions between activator from this family and σ₅₄ or the Eσ₅₄ holoenzyme have been studied [37–41]. We considered the possibility that CRP-cAMP may compete out XylR-Eσ₅₄ inter-

Table 2. The cAMP receptor protein (CRP) is the factor that mediates the inhibition of the *Pu* promoter in *E. coli*.

| Strain         | Plasmid                   | β-Gal activity (Miller units)* | Exogenous cAMP† |
|----------------|---------------------------|-------------------------------|-----------------|
| TP2101 (WT)    | pKU700 + pTS174           | 223±35                        | –               |
| TP2006 (cya)   | pKU700 + pTS174           | 8837±352                      | +               |
| TP2339-1(cya cpr) | pKU700 + pTS174 + pLG339ARS | 7953±307                      | +               |
| TP2339-1(cya cpr) | pKU700 + pTS174 + pLG339CPR | 7900±298                      | +               |
| TP2339-1(cya cpr) | pKU700 + pTS174 + pLG339ppCPR | 5693±269                      | +               |

*The β-galactosidase activity in LB medium with an addition of 0.2 mmol/L mMBA as the inducer was assayed after growing cells at 30°C. Mean values and standard deviations from three independent experiments are shown.

†Exogenous cAMP was at a final concentration of 2 mmol/L when added.

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Figure 2. Probing open complexes at the Pu promoter with KMnO4. Under activated situation, KMnO4 footprints were conducted on plasmid pKU700 in E. coli cya mutant TP2006. Lanes: 1, plus rifampicin; 2, no rifampicin; 3, plus rifampicin and 2 mmol/L cAMP; 4, plus 2 mmol/L cAMP; A, G, C and T refer to sequencing lanes with the same primer. The −5 to −9 region is marked with a bracket for the open complex (lane 1). Note that the open complex formation at the Pu promoter was blocked by the presence of the CRP-cAMP complex (compare lane 3 with lane 1).

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actions to prevent open complex formation at Pu. To examine this possibility, we took advantage of the constitutive activity of a truncated XylR derivative named XylRAA. In XylRAA, the N-terminal signal reception domain had been entirely deleted, but its central activation domain and the DNA binding segment remained [42]. XylRAA is able to constitutively activate Pu regardless of the presence of any inducer. Plasmid pVTRAA expresses XylRAA from a tac promoter, which can be suppressed by lacI encoded on the same plasmid, and induced by the presence of IPTG [43]. Consistent with previous studies, when XylRAA (pVTRAA) substituted for XylR, activation of the Pu promoter was observed (Figure 3). Moreover, as the expression of activator XylRAA was highly induced by increasing concentrations of IPTG present in the medium, the extent of inhibition mediated by CRP-cAMP was gradually diminished (Figure 3). It thus seems that CRP-cAMP-mediated inhibition on Pu can be alleviated by increasing the intracellular concentration of XylRAA (Figure 3). This observation suggests that the effects of CRP-cAMP are directly targeted to the transcription machinery by interfering with XylR-Eσ54 interactions.

CRP-cAMP-mediated inhibition on the Pu promoter is not specific for XylR

CRP-cAMP may interact directly with XylR or with Eσ54 to compete out XylR-Eσ54 interactions. A simple possibility is that the quantity or activity of the regulatory protein XylR might be somehow modulated by the CRP-cAMP complex. To explore this and to see whether the inhibitory effect was activator-specific or not, alternative activator NtrC was used to activate Pu under nitrogen-deficient conditions. Under certain conditions, NtrC can activate Pu (as well as most other σ54-dependent promoters) from solution, without a need of binding to the UAS. Plasmid pKU700 was introduced into the cya mutant TP2006, and β-galactosidase activities were measured in the presence or absence of exogenous cAMP under nitrogen-deficient growth conditions. Activation of the Pu promoter was observed in the absence of CRP-cAMP, while inhibition of Pu was observed in the presence of CRP-cAMP. In addition, along with the reduced nitrogen supplied in the growth medium (which means increasing amount of active NtrC in the cells), CRP-cAMP-mediated inhibition on Pu was strongly reduced (from 50-fold down to 2-fold, Figure 4). It suggests that the increasing quantity of activator NtrC-phosphate can also lead to partial alleviation of CRP-cAMP-mediated inhibition on Pu, a result similar to that obtained with constitutively active XylRAA (Figure 5). This result indicates that CRP-cAMP-mediated inhibition on Pu is not limited to a specific activator. Therefore, it seems improbable that CRP-cAMP specifically interferes with XylR-mediated transcriptional activation, for example by somehow interfering with the activity of XylR, e.g. by inhibiting the induced activation of XylR by mMBA. Preferably, CRP-cAMP may target to the Eσ54 complex at Pu [21,22].

The repression of Pu promoter by CRP-cAMP may occur at the step of closed complex formation

The formation of Eσ54-dependent closed-complex is a rate-limiting step in the process of transcription initiation at the Pu promoter [11]. CRP-cAMP may inhibit transcription of the Pu promoter by interfering in the stable binding of Eσ54 to Pu. It was demonstrated previously that the closed-complex at the gbdh2 promoter can be detected by protection from dimethylsulphate (DMS) attack of critical guanines within the −12 and −24 regions of the promoter [44]. We therefore performed in vivo footprinting to analyze protection of the guanine residues at −14, −23 and −26 of Pu from DMS modification by bound Eσ54. In the absence or presence of 2 mmol/L exogenous cAMP, DMS footprints were conducted on plasmid pKU700 in the cya mutant TP2006. As controls, the E. coli cya mutant TH1 (unable to produce σ54) containing plasmid pKU700 was also treated with DMS. The footprints obtained with the bottom strand of plasmid DNA pKU700 from intact cells are shown in Figure 5A. It can be seen that, first, in absence of Eσ54, the bands at both the −12 and −24 regions of Pu are approximately equal in intensity to the −18 reference band (Figure 5A, Lane 1). Secondly, in the presence of Eσ54 and absence of CRP-cAMP, these two bands are both lower in intensity than the −18 band because the guanine residues within the −12 and −24 regions are protected by bound Eσ54 from methylation of DMS (Figure 5A, Lane 2). However, when CRP-cAMP is present the intensity of the band at the −12 regions was enhanced (Figure 5A, Lane 3). To obtain a more reliable reference for the influence of CRP-cAMP, the bands (Figure 5A, lanes 2 and 3) were scanned and plotted as shown in Figure 5B. Enhancement of intensity of the band at the −12 regions might reflect a conformational change of the closed complex at Pu promoter due to the presence of CRP-cAMP. In contrast, similar footprinting patterns were observed in the presence or absence of CRP-cAMP respectively when another σ54-dependent promoter gbdh2, which was also down-regulated by CRP-cAMP [22,23], was used as controls (Figure 5). Therefore, the stable closed-complex formation at the gbdh2 promoter was hardly affected by CRP-cAMP. Taken together, the above results suggest that the mechanism by which CRP-cAMP mediated inhibition on Pu is different from that observed on gbdh2.
AR1, but not AR2 or AR3, of CRP is essential for the inhibitory effect of CRP-cAMP on Pu

To explore the role of the AR1, AR2 and AR3 surface determinants of CRP on Pu, each of these CRP mutants were tested for their ability to mediate inhibitory effect on Pu in vivo.

Low copy pLG339-derived plasmids, harboring different mutant crp genes were introduced into E. coli cya crp double mutant TP2339-1 together with pKU700 and pTS174 respectively, using pLG339RS and pLG339CRP as controls. Expression of Pu during growth was monitored in the presence or absence of cAMP. As shown in Figure 6, the results indicate that CRP mutants
containing substitutions in AR1 lose their ability to cause inhibition on Pu (H159L). The same result was obtained with no crp gene on the plasmid (pLG339ARS, Figure 6). In contrast, the result obtained with the mutants in AR2 (K101E) or AR3, including both the inhibitory determinant (K52N), and activating determinant (E58K), had no significant difference from that obtained with the wild type CRP (Figure 6). These results indicated that surface AR1, but not AR2 or AR3, of CRP is indispensable for the inhibitory effect on Pu.

Discussion

The P. putida Pu expression system has been recreated in E. coli. Physiological, genetic and biochemical data demonstrate that CRP inhibits Pu expression in a cAMP-dependent manner (Table 2). KMnO₄ footprinting analysis indicates that the down-regulation is at the transcriptional level in vivo (Figure 2). The inhibitory effect of activated CRP on Pu remains when its cognate activator XylR is replaced by another member in the NtrC family (such as NtrC-Pu, Figure 6). In contrast, the result obtained with the mutants in AR2 (K101E) or AR3, including both the inhibitory determinant (K52N), and activating determinant (E58K), had no significant difference from that obtained with the wild type CRP (Figure 6). These results indicated that surface AR1, but not AR2 or AR3, of CRP is indispensable for the inhibitory effect on Pu.
the glnAp2 promoter, the CRP can be recruited by Eσ54 to a site upstream of glnAp2 through the direct interaction between αCTD of Eσ54 and AR1 of CRP, preventing the activator protein from approaching the activator-accessible face of the promoter-bound Eσ54 closed complex [23]. In all cases, the Eσ54 in the closed complex is poised by CRP-cAMP through direct interaction between the AR1 of CRP and the αCTD of Eσ54.

Interestingly, when either the quantity (for the constitutive activator XylRΔA) or the activity (NtrC-phosphate) of the regulatory proteins was increased, CRP-cAMP-mediated inhibition on Pu was strongly reduced (from 12- and 50-fold down to 2-fold, Figure 3 and Figure 4 respectively). Since it is well known that high concentration of activator could contact the closed complex from solution without binding to UAS, the decrease of the inhibitory fold in the presence of high concentration of activator suggests that CRP-cAMP might inhibit the direct contact between the UAS bound activator and the promoter bound RNA polymerase. Many mechanisms might be involved: 1) CRP inhibits the binding of activator to UAS at Pu promoter (as the case of dctA, see [21]). 2) The recruitment of CRP by RNA polymerase could affect the orientation of the DNA bending between UAS and the core promoter region (as the case of glnAp2, see [23]). 3) The recruitment of CRP by RNA polymerase inhibits the IHF binding to its binding site.

To date, the product of P. putida KT2440 crp gene (GenBank: AE015451.1, [53,54]) was found to have identical ARI region with CRP from E. coli [57]. Our data also showed that the CRP protein from P. putida (PpCRP) could function as a cyclic AMP receptor. When PpCRP were expressed in an E. coli cya crp minus strain and experiments concerning its role on the expression of lac promoter with exogenous cAMP were done, similar activation results were obtained from PpCRP and CRP from E. coli [55]. Furthermore, PpCRP was recently proved to be involved in the utilization of aromatic amino acid in cyclic AMP-dependent [56]. These observations confirmed that PpCRP was able to function as a cyclic AMP receptor in a cAMP-dependent manner. Moreover, our results showed that PpCRP could also inhibit Pu promoter in E. coli in the presence of exogenous cAMP (Table 2). Therefore, it is interesting to investigate how the activity of the equivalent crp cya system is controlled in its host P. putida.

Taken together, our data implicated that CRP can function on Pu promoter in E. coli, and maybe similarly in P. putida. Our results also provided proof that, despite the existence of nonspecific activator, the expression of Pu promoter could be inhibited by CRP in cAMP-dependent manner in E. coli.
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Author Contributions

Conceived and designed the experiments: YZ ZT YW. Performed the experiments: YZ FJ YH. Analyzed the data: YZ FJ YH YW. Contributed reagents/materials/analysis tools: FJ YH YS. Wrote the paper: FJ ZT YH YW.

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