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Research Letter

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Identification and characterization of a CI binding operator at a distant location in the temperate staphylococcal phage ϕ11

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One sentence summary: Identification of a new operator has extended the unique genetic circuit of phage ϕ11.

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ABSTRACT

Bacteriophage ϕ11 encodes repressors CI and Cro for executing its growth in *Staphylococcus aureus*, a human pathogen. There are three homologous operators *O1*, *O2* and *O3* between the repressor-expressing genes. While CI binds to *O1* and *O2*, Cro interacts only with *O3*. To locate additional CI binding operators in ϕ11, we searched its genome using the *O1*/*O2* sequence as a probe. The results show the presence of a putative CI binding operator (*O4*) at the 3′ end of the *cro*. *O4* differs from *O2* and *O1* by one base and five bases, respectively. A specific interaction was noticed between *O4* and rCI, a recombinant CI. However, *O4* shows no interaction with rCro, a chimeric Cro. Additionally, six guanine bases, situated in and around *O4*, have interacted with rCI. Interestingly, the rCI binding affinity of *O4* or *O1* is about 15 times higher than that of *O2*. A comparative study indicates that some bases and structural alteration, unique to *O1* and *O4*, may contribute to their enhanced rCI binding affinity. Collectively, the study has not only broadened the distinct gene regulatory circuit of ϕ11 but also suggested that it possibly employs a complex mechanism for its development in *S. aureus*.

Keywords: phage ϕ11; *Staphylococcus aureus*; repressor; operator; DNA binding activity; genetic circuit

INTRODUCTION

Bacteriophages or phages appear to be one of the predominant species in the biosphere (Clokie *et al.* 2011). Of the phages, the life cycles of the temperate phages are typically controlled by some genetic switches that are primarily composed of early promoters, operators and the repressor coding genes (Heinrich, Velleman and Schuster 1995; Oppenheim *et al.* 2005; Hatfull 2010). Most operators of the temperate phages partly overlap with their early promoters. Therefore, repressor binding to such operators inhibits the expression of their early genes.

Bacteriophage ϕ11 is a temperate phage and exploits *Staphylococcus aureus* for its lytic and lysogenic growth (Iandolo *et al.* 2002). Two repressors Cro and CI appear to regulate its development in the host (Das *et al.* 2007, 2009; Ganguly *et al.* 2009). While CI promotes the lysogenic growth, Cro is critical for the lytic growth of ϕ11. The *cI* and *cro* genes of ϕ11, encoding CI and Cro, respectively, are transcribed in opposite directions. Three homologous operators, *O1*, *O2* and *O3* are located between the two repressor genes. The 15 bp sequence of each operator has a roughly two-fold dyad symmetry. The operators partly coincide with the putative promoters of the repressor genes. The *cro* and *cI* genes are located downstream of *O1* and *O3*, respectively (Ganguly *et al.* 2009). CI carries two domains, forms dimers in the solution, interacts with the operator as a dimer and shares significant homology with other immunity repressors at the sequence level. The CI binding mode to the adjacent *O1* and *O2* is cooperative in nature (Ganguly *et al.* 2009). Conversely, Cro

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shows interaction only with *O3*. The mechanism of binding of ϕ11 repressors to the cognate operators differs completely from those of lambdoid phages and the phages infecting other gram-positive bacteria (Johansen, Brøndsted and Hammer 2003; Oppenheim *et al*. 2005; Kenny *et al*. 2006; Alsing *et al*. 2011; Koberg *et al*. 2015). Additionally, the N-terminal domain of ϕ11 CI, unlike the similar domains of other phage repressors (Jordan and Pabo 1988; Mondragón *et al*. 1989; Sevilla-Sierra, Otting and Wüthrich 1994; Pinkett *et al*. 2006; Pedersen *et al*. 2008), primarily exists as a dimer in solution and binds to *O1* and *O2* with different affinity (Biswas, Mandal and Sau 2014).

The *cI–cro* intergenic region in the temperate phages usually carries two to three operator sites that interact with CI (Johansen, Brøndsted and Hammer 2003; Sumby and Waldor 2003; Oppenheim *et al*. 2005; Pinkett *et al*. 2006; Koberg *et al*. 2015). In the temperate lactococcal or streptococcal phages, an additional CI binding operator site is situated at the 3′ end of *cro* or another lytic gene. A loop structure, postulated to be formed using the CI proteins bound at distant operator sites, possibly ensures the tight regulation of gene expression in such temperate phages (Oppenheim *et al*. 2005; Alsing *et al*. 2011). Of the *S. aureus* phages, Sa3ms seems to carry a CI binding operator site at the 3′ end of its *cro* (Sumby and Waldor 2003). However, the distantly located Sa3ms operator or the way Sa3ms CI binds to the cognate operators has not been characterized yet at length. Herein, we report that *S. aureus* phage ϕ11 also carries a distantly situated operator (*O4*) having more than 60% sequence identity with both *O1* and *O2*. The *O4* operator shows interaction only with rCI, a recombinant ϕ11 CI. Interestingly, the order of rCI binding affinity of the operators is $O1 \approx O4 > O2$ though the closest sequence homolog of *O4* is *O2*. Some structural determinants in the *O1* and *O4* may be responsible for their higher CI binding affinity.

MATERIALS AND METHODS

Bacterial and phage strains

Staphylococcus aureus and *Escherichia coli* were routinely grown in Trypticase soy broth and in Luria-Bertani broth, respectively (Biswas, Mandal and Sau 2014). The growth media were supplemented with the appropriate antibiotic whenever required. Phage ϕ11 was grown according to a standard procedure (Iandolo *et al*. 2002).

Molecular biological methods

Plasmid DNA isolation, enzymatic treatment of DNA, agarose gel electrophoresis, polymerase chain reaction (PCR), purification of DNA fragments, labeling of DNA fragment with [γ-^{32}P]Adenosine triphosphate (ATP), protein estimation, native polyacrylamide gel electrophoresis (PAGE), Sodium dodecyl sulfate (SDS)-PAGE, urea-PAGE and polyacrylamide gel staining were carried out as described (Sambrook and Russell 2001; Biswas, Mandal and Sau 2014). Recombinant ϕ11 CI and recombinant ϕ11 Cro (rCro) were purified as described (Das *et al*. 2009; Biswas, Mandal and Sau 2014).

Preparation of DNA fragments

The gel mobility shift assays were performed using different DNA fragments such as O1, O2, O4 and cspC. The sizes of O1, O2, O4 and cspC fragments are 34, 49, 192 and 214 bp, respectively. The cspC DNA does not carry any CI binding operator, whereas O1 and O2 DNAs carry *O1* and *O2* operator, respectively (Ganguly *et al*. 2009; Biswas, Mandal and Sau 2014). Conversely, O4 DNA carries the putative CI binding operator *O4*. The O1, O2 and cspC DNA fragments were synthesized as stated before (Ganguly *et al*. 2009). The O4 DNA fragment was synthesized by PCR using the primer pairs O4–1 (5′ TGACGAA AATATTGGAGA 3′) and O4–2 (5′ AAGATCTCGAGTGTATCT 3′) and the ϕ11 genomic DNA as the template (Ganguly *et al*. 2009).

The top and bottom strands of O4 DNA were separately labeled with ^{32}P using a standard procedure (Biswas, Mandal and Sau 2014) with modifications. Briefly, the top strand-labeled O4 DNA was obtained by amplifying the ϕ11 genomic DNA with O4–2 and the ^{32}P-labeled O4–1 primers. Conversely, labeling of the bottom strand of O4 DNA was done using ϕ11 genomic DNA, O4–1 and the ^{32}P-labeled O4–2. To label the bottom strand of 94 bp O4′ DNA, it was amplified using ϕ11 genomic DNA, O4–1 and the ^{32}P-labeled O4–3 (5′ ATGATTGGTCTTGCAT TTG 3′).

Gel shift assay

To study the rCI–DNA interaction, gel shift assay was performed as described previously (Biswas, Mandal and Sau 2014). Briefly, the reaction mixtures containing different concentrations of rCI and ∼0.1 nM ^{32}P-labeled DNA were incubated followed by their analysis by a 10% native PAGE. The resulting autoradiogram was scanned using a densitometer (Bio-Rad). The extents of bound DNA, determined from the scanned data, were used to determine the half-maximal rCI binding concentration (K_D) as described (Biswas, Mandal and Sau 2014).

Footprinting assay

DNase I footprinting assay was performed by a standard method with minor modifications (Ganguly *et al*. 2009). Briefly, the ^{32}P-labeled O4/O4′ DNA (∼5000 cpm) in the presence or absence of rCI was incubated on ice for 20 min. The reaction mixtures were made 1 mM with $MgCl_2$ and treated with 0.15 units of DNase I for 5 min at room temperature. After terminating the reactions with a stop solution, the digested DNA fragments were purified as described (Sambrook and Russell 2001). The adenine plus guanine (A + G) and guanine (G) sequencing ladders were produced from the same labeled DNA fragments as described (Ganguly *et al*. 2009). Finally, the DNA fragments, separated by the 8% urea-PAGE, were analyzed as demonstrated (Biswas, Mandal and Sau 2014).

Dimethyl sulfate (DMS) protection assay was performed as described earlier (Biswas, Mandal and Sau 2014). Briefly, the reaction mixtures containing the ^{32}P-labeled O4 DNA (∼5000 cpm) and varying concentrations of rCI were incubated followed by their treatment with 0.2% DMS for 2 min at room temperature. The O4 DNAs, purified from the stopped reaction mixtures, were used to produce the G-specific DNA fragments as stated before (Ganguly *et al*. 2009). The DNA fragments, resolved along with the A + G ladders, were analyzed as described above.

RESULTS AND DISCUSSION

Identification of a new operator

Phage ϕ11, like other temperate phages (Johansen, Brøndsted and Hammer 2003; Oppenheim *et al*. 2005; Koberg *et al*. 2015), may carry a CI binding operator at location away from the operators in its *cI–cro* intergenic region (Ganguly *et al*. 2009). To identify such operator, we manually searched the genome sequence of ϕ11 using the sequence ‘TACACG’ as a probe. The 5′

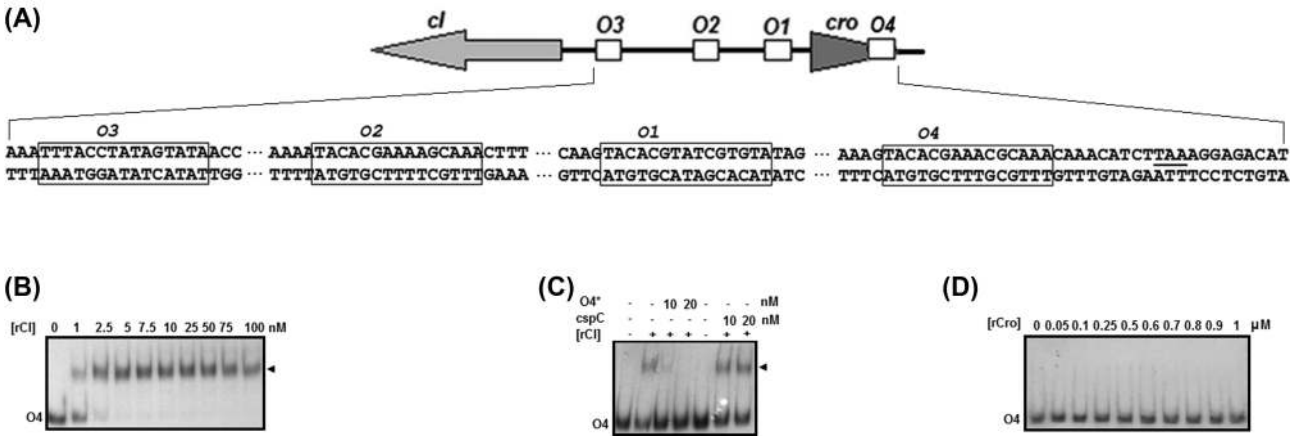

Figure 1. Identification of operator *O4*. **(A)** A schematic representation of the ϕ11 gene regulatory region. The sequences of four homologous operators (*O1*, *O2*, *O3* and *O4*) are covered by the rectangular boxes. The *cI* and *cro* genes are shown by two arrows pointing in opposite directions. The stop codon of Cro has been underlined. The gene regulatory region has not been drawn to scale. The autoradiogram of gel shift assay shows the interaction between the labeled O4 DNA fragment and the indicated concentrations of rCI **(B** or **C)** or rCro **(D)**. The binding of rCI (1 nM) to the labeled O4 DNA was also tested in the presence of indicated amount of cold O4 DNA (O4∗) or cspC DNA **(C)**. The '+' and '−' denote presence and absence of DNA or protein. Arrowhead indicates rCI–O4 DNA complex.

ends of both *O1* and *O2* carry the above sequence (Ganguly *et al.* 2009), prompting us to consider it in our investigation. The analysis shows the presence of a putative CI binding operator (designated as *O4*) at the 9 bp upstream of the stop codon of *cro* (Fig. 1A). The tentative 15 bp *O4* that shares ∼93% sequence identity with *O2* differs from the latter by a single base (C/G versus A/T) at position 6′ (Fig. 1A). Conversely, *O4* shows more than 65% sequence identity with *O1* that varies from the former by five bases (Fig. 1A). Interestingly, the base at the position 6′ (C/G) in *O1* and *O4* are identical.

Interaction between rCI and O4

To test whether ϕ11 CI interacts with the putative operator *O4*, a gel shift assay was carried out using a ^{32}P-ATP labeled O4 DNA fragment (Fig. 1B) and the varying concentrations of rCI. The resulting autoradiogram reveals the gradual formation of a shifted complex upon increasing the concentration of rCI, indicating that rCI binds to *O4*.

To determine whether the binding of rCI to the O4 DNA is specific, an additional gel shift assay was carried out with rCI in the presence of cold O4 DNA or cspC DNA. As seen in Fig. 1C, the labeled *O4* DNA competed with the cold O4 DNA but not with cspC DNA for the binding of rCI, suggesting a specific interaction between rCI and *O4*.

The *O4* operator shows ∼47% identity with *O3* at the sequence level (data not shown), indicating that ϕ11 Cro may interact with O4 DNA. To verify it, we performed a gel shift assay using the labeled O4 DNA and increasing concentrations of rCro. However, no detectable shifted band was observed, even at μM rCro, indicating that it does not bind to O4 DNA under the conditions tested (Fig. 1D). Together, the data suggest that rCI and not rCro binds to the *O4* operator.

Localization of O4 operator

To localize the CI binding site in the *O4* DNA, a DNase I footprinting experiment was carried out using two different concentrations of rCI and the labeled O4 or O4′ DNA (Fig. 2A). The footprints, generated using the labeled top and bottom strands of DNA, revealed that a single region of DNA was protected from DNase I digestion in the presence of rCI. Notably, rCI shielded the +212 to +239 region of the top strand and the +207 to +231 region of the bottom strand, respectively (Fig. 2B). The 15 bp *O4* operator is located at the center of the protected site. Thus, the data confirmed the gel shift assay data (Fig. 1B) and also demonstrated the presence of a single rCI binding site in the O4 DNA.

Interaction between CI and 15 bp O4

To verify whether *O4* interacts with ϕ11 CI, a DMS protection assay was performed using two different concentrations of rCI and the labeled *O4* DNA. The resulting autoradiograms show that intensity of two G bases in the top strand and four G bases in the bottom strand is decreased in the presence of rCI (Fig. 2C). A densitometric analysis indicates that there is ∼50%–70% reduction of the intensity of the bottom strand-specific G bases in the presence of rCI. Under similar conditions, the intensity of the G bases in the top strand was decreased nearly 80%–90%. While the protected G bases in the top strand are located at positions +214 and +225, those protected at the bottom strand are situated at positions +217, +219, +226 and +230 (Fig. 2C). All of the protected G bases appear to be present in and around the 15 bp *O4* and may be responsible for interaction with rCI (Fig. 2C). The intensity of the +224G base in the bottom strand was increased notably (Fig. 2C) indicating that it was more exposed as a result of the rCI-induced conformational change of *O4*.

Comparative binding affinity of rCI

To compare the rCI binding affinity of *O4* with those of *O1* and *O2*, gel shift assays were separately performed with increasing concentrations of rCI and ^{32}P-labeled O4 (Fig. 3A), O1 (Fig. 3B) and O2 (Fig. 3C) DNAs. As expected, gradual formation of one shifted complex by each set of DNA and rCI was observed. The amount of the operator DNA bound, determined from the resulting autoradiograms, was plotted against the concentrations of rCI (Fig. 3D). The yielded K_D values for the interactions between rCI–*O1*, rCI–*O2* and rCI–*O4* DNAs are 1.55 ± 0.17, 27.55 ± 3.67 and 1.30 ± 0.06 nM, respectively. Further analysis revealed that rCI bound to *O1* and *O4* sites with a nearly similar affinity ($P = 0.29$). As noticed before with *O1* (Ganguly *et al.* 2009), the rCI binding affinity of *O4* is significantly higher than that of *O2* ($P = 0.03$).

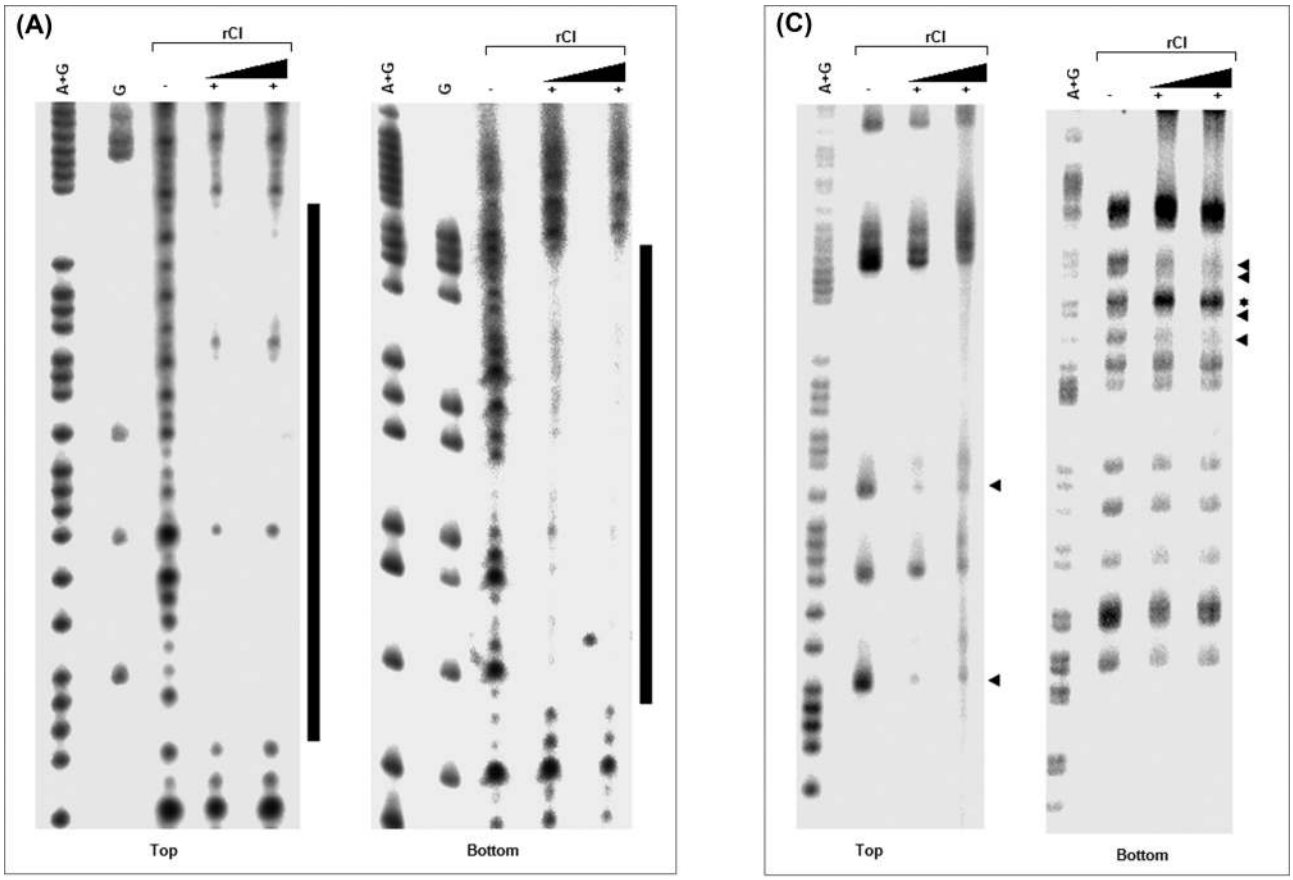

Figure 2. Footprinting assay. **(A)** Autoradiograms of DNase I footprinting assays produced using the top (Top) strand labeled O4 DNA and the bottom (Bottom) strand labeled O4′ DNA. The labeled DNAs were incubated with 250 and 500 nM rCI (+) or without (–) rCI prior to DNase I digestion as described (Ganguly *et al.* 2009). The resulting DNA fragments were separated using the G and the A + G markers. The location of the 15 bp *O4* site within the shielded region is presented by a solid bar. **(B)** Summary of different footprinting assay. The lines above and below the *O4* carrying DNA sequence denote the DNase I-protected sites. The 15 bp *O4* is surrounded by a rectangular box. The protected and the hypermethylated G bases are indicated by arrowhead and star, respectively. Considering the putative initiation site of *cro* as +1, the entire O4 DNA sequence is numbered. The stop codon of Cro is underlined. **(C)** Autoradiograms of DMS footprinting assay. The top (Top) and bottom (Bottom) strand labeled O4 DNA were incubated with 250 and 500 nM rCI (+)/without (–) rCI and exposed to DMS as described (Ganguly *et al.* 2009). The resulting DNA fragments were analyzed using the A + G marker. The shielded and the hypermethylated G bases are indicated by arrowhead and star, respectively.

In the present study, the identification of *O4* has not only extended the CI-regulated genetic circuit of ϕ11 (Fig. 4A) but also indicated that its gene regulation is more complex than what has been presumed earlier (Ganguly *et al.* 2009). The gene regulatory region in ϕ11 is nearly identical to that in the streptococcal phage TP-J34 (Koberg *et al.* 2015). However, the ϕ11 CI counterpart of phage TP-J34 showed binding to all three operators located between its two repressor genes. The lactococcal phages, unlike the above phages, carry two operators between their repressor and antirepressor genes and a third operator at the 3′ end of their antirepressor or another lytic gene (Kenny *et al.* 2006; Alsing *et al.* 2011). The lactococcal phage repressor binds to the distant operator and one of the operators in the immunity region with nearly similar affinity (Alsing *et al.* 2011). Conversely, the interaction between the lactococcal phage repressor and another operator in the immunity region is exceedingly weak. The function of antirepressor in the lactococcal phages does not match with the function of Cro or equivalent protein of either ϕ11 or phage JP-34 (Alsing *et al.* 2011). Collectively, the mechanism of action of ϕ11 repressors (Fig. 4A) may not be identical to those of other phage repressors.

Various DNase I footprinting assays have indicated that the 15 bp operators and their flanking regions are protected by rCI (Fig. 2; Ganguly *et al.* 2009). Conversely, different DMS footprint-

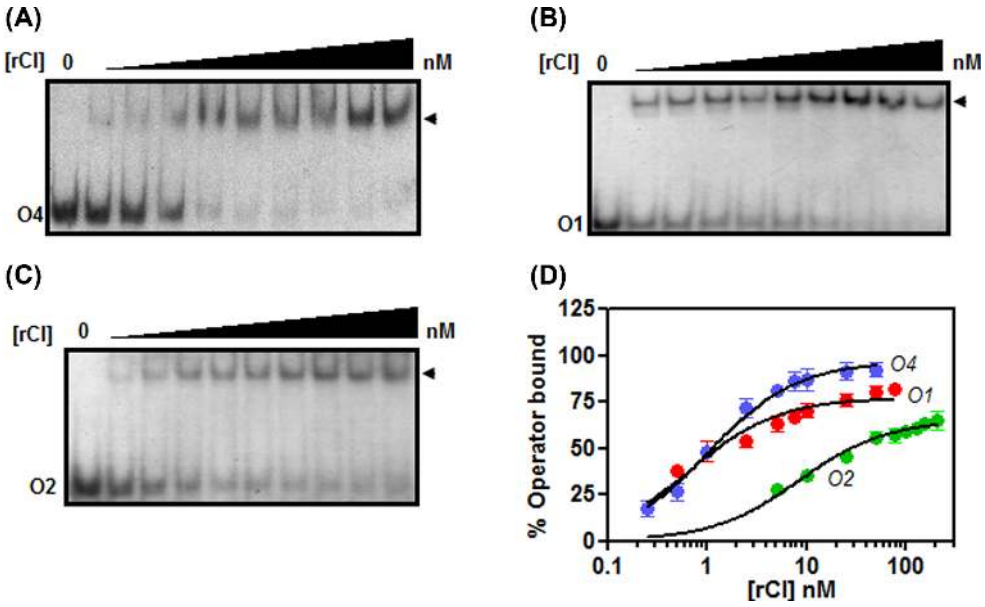

Figure 3. Interaction between rCI and different operators. Autoradiograms of gel shift assay **(A–C)** demonstrate the interaction between the labeled DNA fragments and the varying concentrations of rCI. In panels A, B and C, the concentrations of rCI are 0.25–50 nM, 0.5–75 nM and 5–200 nM, respectively. The shifted rCI-DNA complexes are denoted by an arrowhead. **(D)** The extents of operator bound (in %), determined using the data from the autoradiograms (panels A–C), are plotted against the varying concentrations of rCI. The error bars represent standard deviations of three separate experiments.

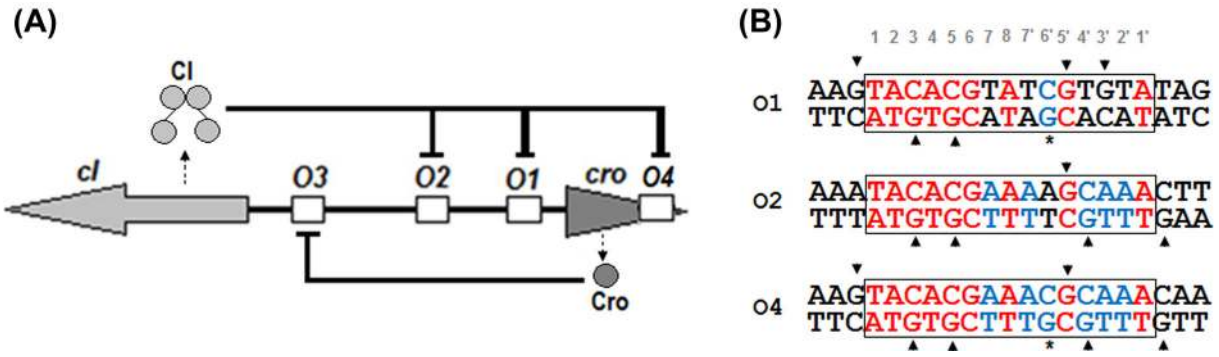

Figure 4. Gene regulatory elements of ϕ11. **(A)** A cartoon picture demonstrating the mechanisms utilized by two different ϕ11 repressors to inhibit gene expression by binding to distinct operators. **(B)** Sequences of CI binding operators (*O1*, *O2* and *O4*) and the neighboring regions. The indicated 15 bp operator sites are boxed. The operator sequences are numbered as described in Fig. 1. The red colored bases are present in all of the 15 bp operators. The blue colored bases are present in any pair of operators. The protected and the hypermethylated G bases are indicated by arrowheads and stars, respectively.

ing assays have suggested that *O4*, unlike *O1* or *O2* (Ganguly *et al.* 2009), employs one extra G base for interaction with rCI (Fig. 2). However, a comparative analysis opines that the identical sets of conserved G bases at the top and bottom strands of the *O1* or *O2* and *O4* may have interacted with rCI (Fig. 4B). The common G bases in *O2* and *O4* and their immediate flanking regions may interact with rCI, as shown by footprinting experiment. Furthermore, the G base located immediately upstream of the 15 bp *O1* or *O4* seems to possess a rCI binding ability. Such symmetry in the interaction suggests that the mode of binding of rCI to the operators is nearly similar.

Six base pairs in the left and three base pairs in the right halves of *O1* and *O4* are identical (Fig. 4B). Conversely, a total of 14 base pairs in the left and right halves of *O2* and *O4* are identical. Despite such homology, the rCI binding affinity of *O2* is significantly less than that of *O4* or *O1* (Fig. 3D). The 15 bp *O2* differs from the *O4* or *O1* at position 6′ (A/T versus C/G). The G base at the position 6′ of either *O1* (Ganguly *et al.* 2009) or *O4* does not interact with rCI (Fig. 2). However, there is a conformational alteration at position 6′ of *O1* (Ganguly *et al.* 2009) or *O4* (Fig. 2) in the presence of rCI. The possible CI-mediated conformational change of *O2* at position 6′ may not be identical as it carries a different base at this position. Such a difference in the structural alteration, in turn, may be responsible for more stable binding of rCI by *O1* or *O4*. The rCI binding G base, located immediately upstream of both *O4* and *O1*, is missing in *O2* (Fig. 4B). This unique G base at the upstream of *O1* and *O4* may also have contributed to their significantly higher CI binding affinity. Currently, the biological role of the higher CI binding affinity of *O4* is not known with certainty.

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Conflicts of interest. None declared.

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