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Purification and Characterization of Repressor of Temperate *S. aureus* Phage ϕ11

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To gain insight into the structure and function of repressor proteins of bacteriophages of gram-positive bacteria, repressor of temperate *Staphylococcus aureus* phage ϕ11 was undertaken as a model system here and purified as an N-terminal histidine-tagged variant (His-CI) by affinity chromatography. A ~19 kDa protein copurified with intact His-CI (~ 30 kDa) at low level was resulted most possibly due to partial cleavage at its Ala-Gly site. At ~10 nM and higher concentrations, His-CI forms significant amount of dimers in solution. There are two repressor binding sites in ϕ11 *cI-cro* intergenic region and binding to two sites occurs possibly by a cooperative manner. Two sites dissected by *Hinc*II digestion were designated operators O_L and O_R, respectively. Equilibrium binding studies indicate that His-CI binds to O_R with a little more strongly than O_L and binding species is probably dimeric in nature. Interestingly His-CI binding affinity reduces drastically at elevated temperatures (32-42°C). Both O_L and O_R harbor a nearly identical inverted repeat and studies show that ϕ11 repressor binds to each repeat efficiently. Additional analyses indicate that ϕ11 repressor, like λ repressor, harbors an N-terminal domain and a C-terminal domain which are separated by a hinge region. Secondary structure of ϕ11 CI even nearly resembles to that of λ phage repressor though they differ at sequence level. The putative N-terminal HTH (helix-turn-helix) motif of ϕ11 repressor belongs to the HTH -XRE-family of proteins and shows significant identity to the HTH motifs of some proteins of evolutionary distant organisms but not to HTH motifs of most *S. aureus* phage repressors.

Keywords: Dimer, Operator, Phi 11, Repressor (CI), Secondary structure and HTH motif

Introduction

Repressor proteins of temperate bacteriophages are generally involved in their lytic/lysogenic switching off/on function. Studies on such transcriptional regulators had not only enriched the molecular biology of phages and their hosts (Oppenheim *et al*., 2005) but also assist in generating expression vectors (Christensen, 2001) and protecting dairy industry (Martin *et al*., 2000). Since early 20th century several lytic and lysogenic phages of *Staphylococcus aureus* had been discovered but the *cis*- and *trans*-acting elements involved in lytic and lysogenic development of phages were cloned only from a handful of *S. aureus* phages and characterized to a little extent at molecular level. Accessory elements (such as *attP*, *int*, and *xis*) which are involved in lysogenic development in λ- and related phages (Ptashne, 1986) were also detected in *S. aureus* phages ϕ11 (Lee and Iandolo, 1988), L54a (Lee and Iandolo, 1986; Lee and Buranen, 1989; Ye and Lee, 1989), ϕ12 (Iandolo *et al*., 2002), ϕ13 and ϕ42 (Carroll *et al*., 1995), ϕPVL (Kaneko *et al*., 1998), ϕPV83, ϕSLT (Narita *et al*., 2001), ϕETA (Yamaguchi *et al*., 2000), ϕSa3ms (Sumby and Waldor, 2003), etc. Interestingly most of the above temperate phages including ϕ11 (Iandolo *et al*., 2002) also harbor the *cI* and *cro* genes in divergent orientation like that of lamboid phages (Ptashne, 1986). Studies showed that repressor of ϕSa3ms binds to three sites: two are located in *cI-cro* intergenic region and one within 3' end of *cro* gene. In contrast, the above intergenic region harbors a single binding site for Cro (Sumby and Waldor, 2003). Thus far, structure-function relationship and mechanism of action of the repressor protein of any temperate *S. aureus* phage had not been studied in details and also it was not investigated as to whether the molecular

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mechanism of lysogenic development of *S. aureues* phages differs from that of lamboid phages.

In this communication, we have reported the overexpression and purification of ϕ11 repressor as an N-terminal histidine tagged variant (His-CI) and demonstrated that it forms dimers in solution at nM concentration. It binds to two operator sites (O_L and O_R) which are present in its *cI-cro* intergenic region. Binding of His-CI to above operators is cooperative in nature and severely affected by elevated temperatures. A nearly common inverted repeat was detected in both O_L and O_R and our studies reveal that repressor binds to each repeat efficiently. The ϕ11 repressor which is highly similar to distantly related λ repressor at secondary structural level harbors a putative N-terminal domain and a C-terminal domain. The putative N-terminal HTH motif of ϕ11 repressor shows significant identity to the HTH motifs of some proteins of distantly related organisms and belongs to HTH-XRE family of proteins.

Materials and Methods

Antibody, chemicals, enzymes, growth media, and oligonucleotides. All fine chemicals were either purchased from Sigma or Merck. The Ni-NTA resin and anti-his antibody were purchased from Qiagen. The alkaline phosphatase-tagged goat anti-mouse antibody IgG1-AP was purchased from Santa Cruz Biotechnology. [^{32}P-γ] ATP was purchased from BARC. All the restriction and modifying enzymes, PCR kit, oligonucleotides, protein and DNA markers were purchased from Genei, Genetix Biotech Asia Pvt Ltd., and Roche. Growth media were purchased from Difco Laboratories and HiMedia.

Bacterial and phage strains and their growth conditions. *S. aureus* RN4220 and *E. coli* cells were routinely grown in Trypticase soy broth (Lee and Iandolo, 1986) and Luria broth (Sambrook and Russell, 2001), respectively. Growth media were supplemented with appropriate antibiotics if needed. Temperate phage ϕ11 and its growth conditions were described previously (Lee and Iandolo, 1988).

Molecular biological techniques. Molecular biological techniques including plasmid DNA isolation, digestion of DNA by restriction enzymes, ligation of DNA fragments, transformation, polymerase chain reaction were carried out according to standard procedures (Sambrook and Russell, 2001). Protein estimation, native and SDS-PAGE, and staining of polyacrylamide gel, western blotting were carried out by the standard procedures (Ausubel *et al*., 1998). DNA from ϕ11 phage particles was isolated by a standard method (Lee and Iandolo, 1988). Sequencing of all ϕ11 DNA inserts (amplified by PCR) were performed in UDSC (New Delhi, India).

Cloning, overexpression and purification of ϕ11 repressor. To clone the putative repressor protein encoding gene of temperate phage ϕ11, a polymerase chain reaction was carried out by Taq polymerase with ϕ11 genomic DNA as template and pair of primers PCI4 (5'-AAGCTTAGGCGCTATTAATCAC) and PCI5 (5'-GAAT TCAAAATGGATAAAAAAGAATTAG). The resulting 748 bp fragment was cloned into pGEMT-Easy cloning vector according to the manufacturer's protocol (Promega Biotech). One of the recombinant pGEM-T-Easy vectors carrying no mutation in the cloned *c*I gene of ϕ11 (confirmed by DNA sequence analysis) was selected for further work and designated pSAU1173. DNA insert of pSAU1173 harboring *c*I gene was then subcloned into an *E. coli* over expression vector, pET28a (Novagen) to construct pSAU1220. This cloning has included 37 extra amino acid residues including six histidine residues at the N-terminal end of repressor. After transformation of pSAU1220 to competent *E. coli* BL21(DE3) cells (Novagen, USA), a healthy transformant was selected for expression of His-CI.

To induce the expression of His-CI, IPTG (isopropyl β-D-1-thiogalactopyranoside) was added to one liter of exponentially growing *E. coli* cells (pSAU1220) to a final concentration of 0.5 mM and the growth was continued for another 3 h at 32°C. The induced cells were sequentially harvested, washed with 0.9% of NaCl and finally resuspended in 10 ml of lysis buffer A [20 mM Tris-chloride buffer (pH 8.0), 300 mM NaCl, 5% glycerol, 10 mM imidazole and 10 μg/ml of PMSF (phenylmethane sulfonylfluoride)]. After disruption of cells His-CI was purified from crude extract by Ni-NTA column chromatography according to manufacturer's protocol.

Cloning of *c*I-*cro* intergenic region from phage ϕ11. The DNA region carrying the divergent promoter elements for *c*I and *cro* genes of ϕ11 was amplified by Proof Start Polymerase (Qiagen) using ϕ11 genomic DNA as template and a pair of primers, pHC1 (5'-GGATCCTAAATCTTCTTGAG) and pHC2 (5'-GAATTCTTG GTTCTATAGTATCTG). The resulting 269 bp DNA fragment was cloned into *Pvu*II site of a plasmid to generate pSAU1201. The above ϕ11 DNA insert which was found to bear correct sequence was named O_LO_R as it carries two operator sites for ϕ11 CI (see below). Two daughter fragments, a 153 bp *Eco*RI-*Hinc*II (designated O_L) and a 116 bp *Hinc*II-*Bam*HI (designated O_R) fragments, which were prepared by digestion of O_LO_R with *Hinc*II were also used in the present study.

Gel shift assay The gel shift assay was performed by a standard method as described earlier (Ausubel *et al*., 1998; Ganguly *et al*., 2004). Briefly, desired amount of ϕ11 His-CI was mixed with ~2 ng of ^{32}P end- labeled O_LO_R or O_L or O_R in 20 μl 1X buffer B [100 μg/ml of BSA (bovine serum albumin), 200 mM NaCl, 10 mM Tris (pH 8.0), 5% glycerol, and 1 mM EDTA] followed by incubation of reaction mixture on ice for 20 min. Next, samples were successively analyzed by native polyacrylamide gel electrophoresis and densitometric scanning of gel autoradiographs.

To study the equilibrium binding of repressor to O_L or O_R operator, a 20 μl of reaction mixture containing varying amounts of His-CI, fixed (~0.1 nM) amount of [^{32}P-γ] ATP (BARC) end labeled O_L or O_R and 100 μg/ml of BSA was incubated at 0°C for 20 min. As reaction between repressor and cognate operator is very fast (our unpublished observation), it was assumed that 20 min is sufficient for reaching equilibrium between the above two species. Analysis of the reaction mixtures was then carried out by standard methods as stated above.

To see the effect of temperatures on the DNA binding activities of ϕ11 repressor, equilibrium binding of ϕ11 His-CI to O_L were

performed at 32, 37, and 42°C in different temperature-regulated water baths according to the method as demonstrated above.

Analytical gel filtration chromatography. Analytical gel filtration chromatography was performed according to standard procedure (Ausubel *et al*., 1998) in an HPLC system using a gel filtration column Protein Pak (3000 sw). Both HPLC system and column had been purchased from Waters. The column was equilibrated with 1X phosphate buffer [50 mM Na-phosphate (pH 6.0), 200 mM NaCl, 5% glycerol] before passage of His-CI protein.

Glutaraldehyde cross-linking of His-CI. Protein-protein cross-linking reactions of His-CI were performed in Buffer C [10 mM Tris-Cl (pH 8.0), 200 mM NaCl, 1 mM EDTA, 5% glycerol] in 20 µl total volume at 25°C. Repressor containing solution was incubated at 25°C for 20 min. Next, glutaraldehyde solution (0.1%) was added to repressor solution and incubated for 2 min. The reaction was stopped by adding 5 µl of 4X SDS gel loading dye. After boiling the sample for 2 min, it was analyzed by SDS-10% PAGE.

Bioinformatic analyses. Sequence similarity search was carried out by different programs of BLAST server (http://www.ncbi.nlm.nih.gov/BLAST). The putative HTH motifs in the repressors were predicted by a standard program (http://npsa-pbil.ibcp.fr). Sequences similar to putative HTH motif sequence of ϕ11 CI was detected from databases by BLAST program. Alignment of HTH motifs of different DNA-binding proteins including ϕ11 CI was performed by ClustalW program (http://www.ebi.ac.uk/clustalw). Using all the HTH motifs described here, a dendrogram was constructed by ClustalW program with neighbor-joining setting. Putative promoter elements including transcription start site was determined according to a program NNPP (http://searchlauncher.bcm.tmc.edu). Putative inverted repeats in DNA sequence was searched by a program designated EINVERTED (http://npsa-pbil.ibcp.fr). Secondary structural data of different phage repressor proteins presented here were predicted by Jpred server (http://www.compbio.dundee.ac.uk/~www-jpred/). Several other programs like PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred), Prof (http://www.aber.ac.uk/~phiwww/prof) etc. were also consulted to verify the data obtained by Jpred. Protein data bank (PDB at http://www.rcsb.org) has also been consulted for structural information of some repressor like 434 CI and λ CI.

Results and Discussion

Purification of histidine tagged ϕ11 repressor. To study the structure and function of ϕ11 repressor at length, it was cloned, overexpressed in *E. coli* (pSAU1220) cells, and purified as an N-terminal histidine tagged variant (His-CI) by single step affinity chromatography. Nearly equal amount of protein from each fraction of affinity chromatography, cell debris, and uninduced *E. coli* cell extract had been analyzed by SDS-12% PAGE (Fig. 1) and it was observed that about 25% repressor was accumulated in cell debris as inclusion bodies. As purification of any protein from inclusion bodies is cumbersome, only supernatant fraction of crude extract was considered in order to purify His-CI. The gel picture also shows that elution fraction contains two proteins of molecular masses ~30 and ~19 kDa respectively. The ~30 kDa protein might be the intact His-CI as calculated molecular weight of intact His-CI (from its primary structure) is 31.1 kDa and showed ϕ11-specific DNA binding activity *in vitro* (see below). Estimation shows that there is about 3 fold purification of repressor by affinity chromatography and the content of intact repressor in the elution fraction is around 80%. Analysis also reveals that content of intact repressor in the induced whole cell extract is nearly 24%.

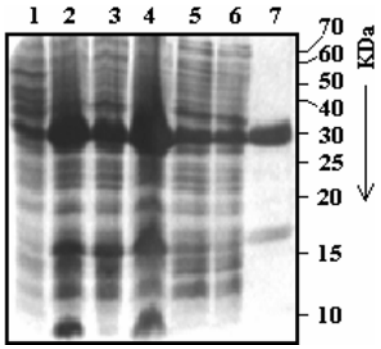

Fig. 1. Purification of histidine-tagged ϕ11 repressor (His-CI). Different protein fractions were analyzed by SDS-13.5% PAGE. Nearly 10 µg protein was loaded in each lane. Lanes: 1, cell extract from uninduced *E. coli* BL21 (DE3) (pSAU1220); 2, whole cell extract from induced *E. coli* BL21 (DE3) (pSAU1220); 3, cell debris fraction from induced whole cell extract; lane 4, induced whole cell extract without cell debris; 5, fraction collected from Ni-NTA column just after passage of induced whole cell extract minus cell debris; 6, washing fraction, and 7, elution fraction, respectively. Molecular masses (in KDa) of marker proteins were shown on the right side of gel.

Many proteins including lexA and λ repressors undergo RecA- as well as alkaline pH-mediated autocleavage at their Ala-Gly (Daniels *et al*., 1983; Little, 1984), Cys-Gly (Nohmi *et al*., 1988), and Leu-Gly (Koudelka *et al*., 2004) dipeptide positions. The ϕ11 CI also carries Ala and Gly residues at its 130th and 131st positions, respectively and the molecular weight of its N-terminal 1 -130 amino acid residues was estimated to be ~17 kDa. Because of histidine tag at N-terminal end, the molecular weight of above N-terminal fragment may be increased to ~19 kDa. Western blot analysis with anti-his antibody indeed revealed that ~19 kDa protein which was co-purified with the above His-CI belongs to the N-terminal end of intact repressor (data not shown). Thus ~19 kDa fragment present at low level in elution fraction might have been arisen by the partial degradation of intact His-CI in recA positive *E. coli* BL21(DE3) cells and/or due to use of buffers with alkaline pH (8.0) during purification.

His-CI forms dimer in solution. To identify the oligomeric status of ϕ11 repressor in solution analytical gel filtration

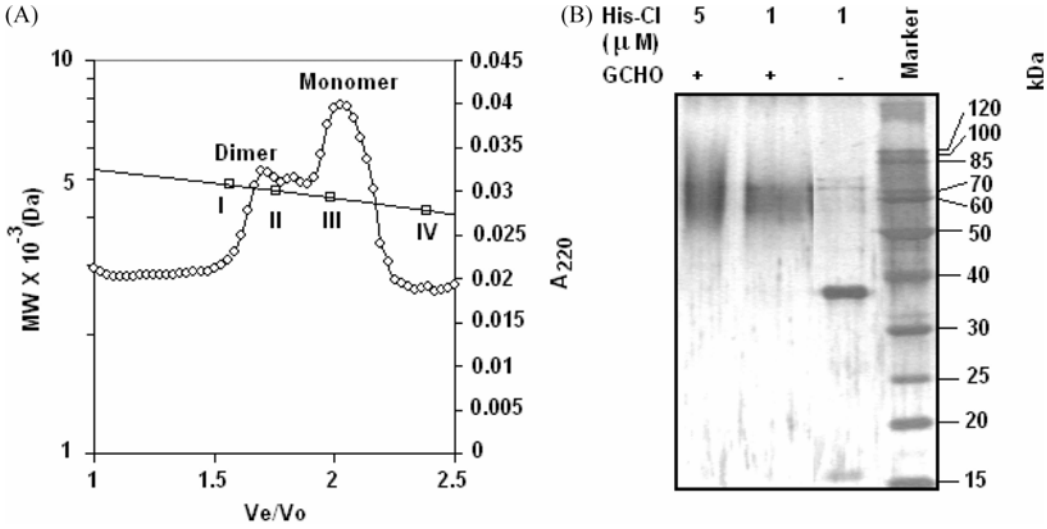

Fig. 2. Oligomerization of ϕ11 repressor in solution. (A) Nearly 5 μM His-CI was loaded onto HPLC gel filtration column and absorbance of eluted fractions were determined at 220 nm. Column was calibrated with BSA (66 kDa), ovalbumin (46 kDa), carbonic anhydrase (29 kDa), and lysozyme (14.4 kDa). Molecular weights were plotted against V_e/V_o, where V_e and V_o denote elution volume and void volume respectively. Void volume of column was determined from elution of blue dextran (2,000 kDa). Abbreviations, I-IV denote BSA, ovalbumin, carbonic anhydrase, and lysozyme, respectively. (B) Glutaraldehyde (GCHO) cross-linking of His-CI. Indicated amounts of protein were cross-linked with 0.1% glutaraldehyde separately and samples were analyzed by SDS- 12% PAGE. Protein bands were visualized by silver staining.

chromatography was carried out with His-CI according to standard techniques. As shown in Fig. 2A, passage of 5 μM His-CI through gel filtration column had produced two major peaks. In comparison with the elution profiles of some standard proteins (also shown in Fig. 2A), two peaks correspond to ~31.62 kDa and ~56.23 kDa, respectively. Both the peaks had also appeared when 1 and 15 μM solutions of His-CI were chromatographed (data not shown). The data together indicate that repressor forms significant amount of dimers in solution at ~10 nM repressor concentration and its formation is concentration-dependent.

Protein- protein cross-linking experiment also shows (Fig. 2B) that at 0.5 μM or higher concentrations His-CI forms mostly dimeric (~60 kDa) repressor molecules. The ~19 kDa N-terminal end of repressor did not dimerize by itself.

Identification of two repressor binding sites in ϕ11 *c*I-*cro* intergenic region. To look for operator site(s) if any in O_LO_R (i.e., *c*I-*cro* intergenic region of ϕ11 phage; Fig. 3A) region, gel shift assay was carried out by a standard method as described above. Fig. 3B shows that two shifted complexes (designated I and II) are gradually formed when increasing amounts of His-CI were mixed with ^{32}P-labeled O_LO_R. Complexes I and II are seen in presence of ~10 nM and ~25 nM His-CI, respectively. At 45 nM His-CI, complex I is the most predominant form formed, whereas, there are formation of nearly equal amount of both the complexes when concentration of His-CI was raised to 55 nM. Complex I nearly disappears at ~75 nM His-CI. The data suggest that the above intergenic region carries at least two binding sites for repressor and binding of repressor to two sites is possibly cooperative in nature as there was dramatic increase of complex II content when repressor concentration was increased from 45 to 55 nM.

Our gel shift assays performed using two DNA fragments, O_L and O_R, in fact reveled that above *c*I-*cro* intergenic region carries two repressor binding sites (see below). To see as to whether repressor binding to O_L or O_R is specific, gel shift assay was also performed in presence of excess cold operator and non-operator DNA. As shown in Fig. 3C, ^{32}P-labeled O_L DNA competes with excess cold O_L DNA for binding to His-CI but it did not do so with non-specific DNA. Identical result was obtained when O_R was used instead of O_L (data not shown). The data suggest that binding of repressor to operator sites which are located in each side of *Hinc*II site of above *c*I-*cro* intergenic regions (Fig. 3A) is indeed specific. Binding ability of N-terminal fragment of His-CI is not clear at this moment.

Equilibrium binding of repressor to two operators. Figs. 3D and 3E show the typical equilibrium binding of His-CI to O_L and O_R, respectively. At His-CI concentrations which give 50% saturation of O_L and O_R, the apparent equilibrium dissociation constant are ~39 nM and ~30 nM, respectively indicating a little weaker affinity of repressor to former than latter operator. It also suggests that complex I (Fig. 3A) was formed most possibly due to the binding of repressor to O_R alone whereas complex II was formed when both O_L and O_R (O_LO_R) were bound by excess repressor.

The shape of curve for equilibrium binding of repressor to

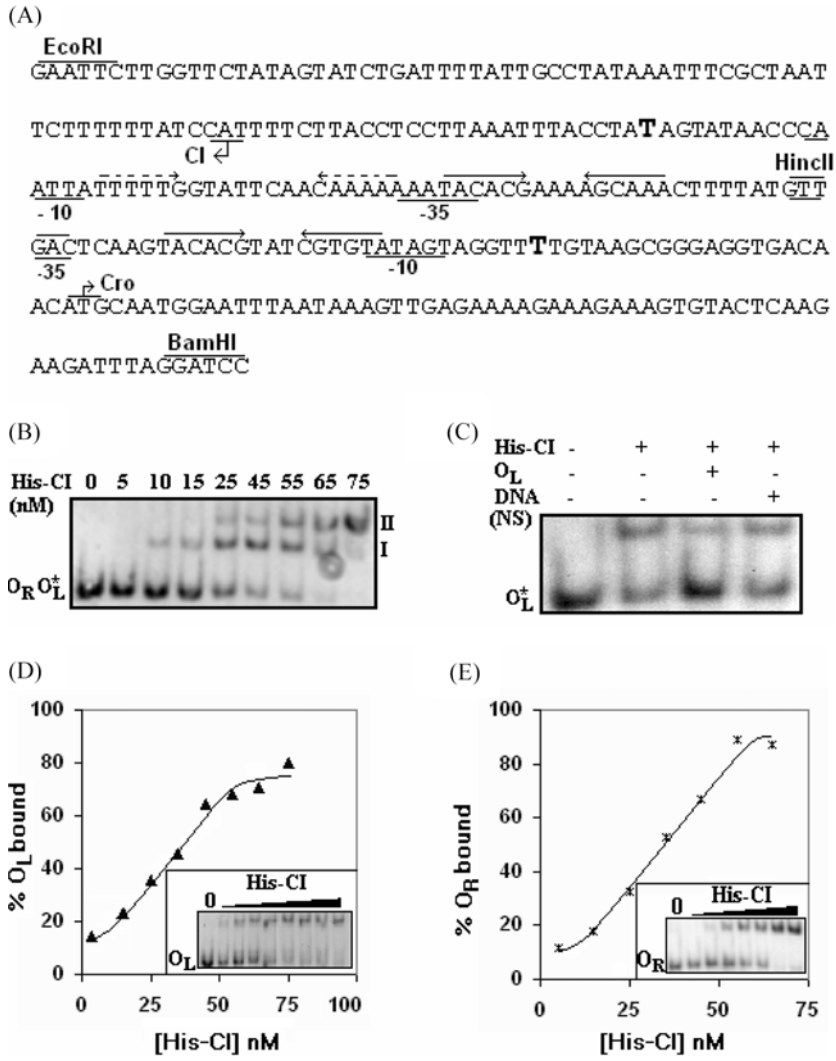

Fig. 3. Gel shift assay. (A) Sequence of *c*I-*cro* intergenic region (designated O_LO_R) of ϕ11. The O_LO_R DNA was digested by *Hinc*II and the resulting 117 bp *Hinc*II-*Bam*HI (designated O_R) and 153 bp *Hinc*II-*Eco*RI (designated O_L) DNA fragments were purified. All three fragments had been utilized in gel shift assay separately. The putative start codons of Cro and CI, and putative transcription initiation sites (shown by bold and larger font), -10, and -35 elements (both underlined) of putative promoters of *c*I and *cro* genes are indicated. Inverted repeats (IR) are represented by converging arrows on the nucleotide sequence. IRs indicated by solid arrows are the possible binding sites for CI. (B) Binding of His-CI to two sites in O_LO_R. Indicated amounts of His-CI were incubated with ~1 nM ^{32}P-labeled O_LO_R (*) separately followed by analysis of samples by the method as described in Materials and Methods. I and II denotes two shifted complexes. (C) Autoradiograph showing the specific binding of O_L to His-CI. To see the binding specificity of CI nearly 100 molar excess of either cold O_L or a non-specific DNA (135 bp *Eco*RV-*Sa*lI fragment carrying truncated *xylE* gene) was added to the reaction mixture which also contained ~1 nM of ^{32}P labeled O_L (*) and 45 nM His-CI. After 15-20 min incubation all the reaction mixtures had been analyzed by native 6% PAGE. (D) Equilibrium binding of O_L to His-CI. The gel shift assay (inset picture) was performed at 0ºC according to standard procedure using ~0.1 nM ^{32}P labeled O_L and varying amounts of His-CI ranging from 5-75 nM. Points used to construct curve were obtained from scanning gel picture. See text for details. (E) Equilibrium binding of O_R to His-CI. It was performed by similar manner as described in panel D.

O_R/O_L is nearly sigmoid which indicates that curve results possibly from two equilibria: monomer - dimer equilibrium and dimer - operator equilibrium. As no repressor oligomer except dimer was detected in solution (Fig. 2) and there was less than 20% binding with 5-15 nM repressor solutions (which carry very low amount of dimer), dimer might be the active operator binding species. This is in fact partly supported by the observation that Hill plots determined from the above equilibrium binding data of repressor yielded best fit straight lines with slopes very close to ~1.3-1.5 (data not shown).

Effect of temperature on the operator DNA binding activity of ϕ11 repressor. Temperature was shown to affect

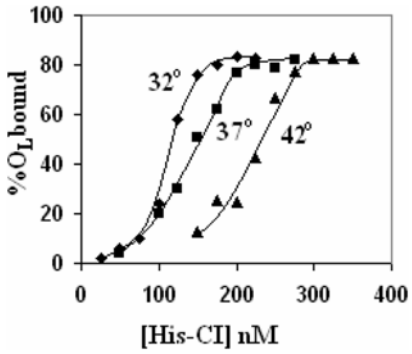

Fig. 4. Effect of temperature on the equilibrium binding of His-CI to O_L. Binding reactions were carried out at 32°, 37°, and 42°C separately. All other steps were identical to those described in Fig. 3D. See text for details.

severely the binding affinity of various repressor proteins to their cognate operator DNAs (Mandal and Leib, 1976; Ballivet and Eisen, 1978; Whitson *et al*., 1986; Vershon *et al*., 1987; Koblan and Ackers, 1991; Shaner and Gaissarian, 1996; Ganguly *et al.*, 2004). To see as to whether temperature affects the operator DNA binding activity of ϕ11 repressor also, equilibrium binding of His-CI to O_L operator was studied at temperatures 32, 37, and 42°C by standard gel shift assays and the corresponding % O_L bound versus repressor concentration plots were represented by Fig. 4. At CI concentration that gives 50% saturation of input O_L, the apparent equilibrium dissociation constant is nearly 245 nM at 42°C. This indicates that there is about 6.5 fold decrease of CI affinity to O_L when binding reaction temperature was increased from 0 to 42°C. The reason why the CI repressor had started losing its DNA-binding activity at temperatures above 32°C is not known with certainty. One explanation is that ϕ11 repressor is a true thermolabile protein and loses its conformation above 32°C. Alternatively, elevated temperatures disturb repressor - interaction by some unknown mechanism.

Inverted repeat that binds to ϕ11 repressor. To look for common binding site(s) of ϕ11 repressor in two sides of *Hinc*II site of *cI-cro* intergenic region (Fig. 3A), its nucleotide sequence was analyzed by a standard software program. A 15 bp inverted repeat (5'TACACGTATCGTGTA) identified in O_R was found 60% identical to another inverted repeat (5'TACACGAAAAGCAAA) detected in O_L (Fig. 3A). While former is a perfect one the latter is an imperfect inverted repeat. No other common repeats were detected on both sides of *Hinc*II site. Interestingly above inverted repeats were found to overlap with the putative promoters of both *cI* and *cro* genes (Fig. 3A). Our gel shift assay with 30 bp DNA fragments carrying each of above inverted repeats showed that His-CI binds to each of them efficiently (data not shown). It was noticed that the center to center distance between two inverted repeats is about 32 bp (approximately three full helical turns) which indicates that they are located on the same face of the helix. This in turn suggests that repressors bound at two inverted repeats most likely contact each other and facilitate the formation of complexes I and II (Fig. 3A) in a cooperative manner.

HTH motif of ϕ11CI belongs to HTH-XRE-family. DNA binding proteins including repressors interact with cognate DNAs through their helix-turn-helix (HTH) motifs. To look for such region in ϕ11 repressor, if any, its primary sequence was analyzed by several computer programs. It was noticed that 23-44 amino acid residues located at the N-terminal end of ϕ11 repressor harbors a putative helix-turn-helix (HTH) motif. By searching different databases it was found that HTH motif of ϕ11 repressor is 100% identical to those of *S. aureus* phages L54a, 42e, 53, 69, and 92 (Kwan *et al*., 2005). The *cI-cro* intergenic region of ϕ11 was also found 100% identical to those of above five phages at nucleotide level (data not shown). In contrast, the N-terminal HTH motif sequences of repressors of ϕSa3ms (Sumby and Waldor, 2003) and many other *S. aureus* phages do not bear significant homology with that of ϕ11 repressor and as expected, sequences of *cI-cro* intergenic regions of these 2nd group phages differ significantly (data not shown). Interestingly, C-terminal ends of the most 2nd group phages are 100% identical to C-terminal end of ϕ11 repressor.

Analyzing putative HTH motif sequences available in different databases, we identified seventeen HTH motifs (each carrying 22 amino acid residues) which show 45-65% identity with the HTH motif sequence of ϕ11 repressor. All the above seventeen HTH motifs in fact belong to helix-turn-helix XRE-family like proteins of different living and viral systems (Fig. 5A). The λ phage CI and 434 phage CI also belong to helix-turn-helix XRE-family like proteins and their HTH motifs are well characterized from diverse experimental evidences including three dimensional structural data (Aggarwal *et al*., 1988; Beamer *et al*., 1992). It was observed that HTH motif residues of 434 phage repressor bears about 32% identity with that of ϕ11 repressor. Taking together we suggest that the region of ϕ11 repressor harboring 23-44 amino acid residues must be carrying an operator DNA binding HTH motif. Next, to know how the putative HTH motifs of ϕ11 repressor and 17 other proteins are related to each other and to those of λ CI and 434 CI, a phylogenetic tree (Fig. 5B) was constructed by standard procedure. It is conspicuous from the dendrogram that HTH motif of ϕ11 repressor is evolutionarily very close to those of proteins of *L. reuteri, L. innocua, B. clausii, G. kaustophilus,* phage ϕLC3, phage Tuc2009 etc. On the contrary the HTH motifs of λ CI and 434 CI are distantly related to HTH motif of ϕ11 CI though they belong to same family of proteins.

Secondary structure of ϕ11 repressor is nearly similar with that of distantly related λ repressor. Amino acid sequence of ϕ11 repressor had shown nearly 65-100% identity over the entire lengths of repressor proteins of *S. aureus*

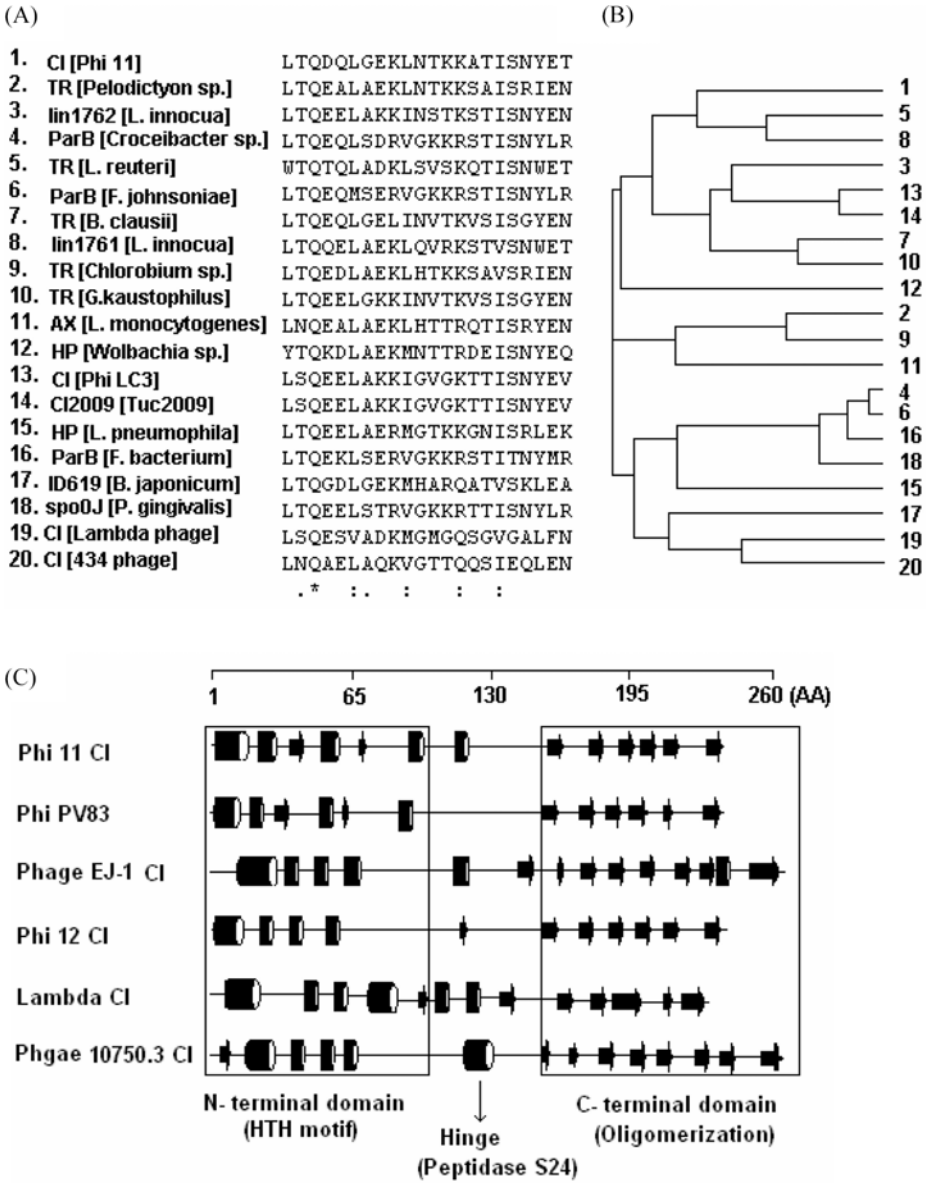

Fig. 5. Bioinformatic analysis of ϕ11 repressor. (A) Detection and evolution of helix-turn-helix (HTH) motif in ϕ11 repressor. HTH motifs homologous to that of ϕ11 repressor have been aligned with ClustalW (See Materials and Methods for details). The identical residues, highly conserved residues, and weakly conserved residues are shown by '*', ' : ', and ' . ', respectively. On the left side of each sequence carrying HTH motif, the protein name and its source organism (within first bracket) are given. Putative HTH motifs are also numbered by 1-20. Abbreviations used: TR- transcription regulator, HP -hypothetical protein. (B). Relationship among HTH motifs. A dendrogram was constructed using the 20 HTH motif carrying sequences (numbered 1-20) as shown in panel A. (C) Determination of putative secondary structures in some phage repressor proteins including ϕ11 repressor and λ CI. Extents of secondary structures were determined in all the indicated repressor proteins except λ CI by Jpred server. Secondary structural information of λ CI was collected from three dimensional structural data of its different regions (deposited in PDB as 1LRP and 1F39). See Material and Method for details. The arrows and tubes represent β-strands and α-helices, respectively. N- and C- terminal conserved regions are boxed and putative HTH motifs, hinge regions, and regions for oligomerization are shown. A scale has been given above the structures of repressor proteins to show the size of each protein.

phages such as L54a, 42e, ϕPVL, ϕ13, ϕ53, 3A, 77, ϕSa3ms, 69, and 92 (Kaneko *et al*., 1998; Iandolo *et al*., 2002; Sumby and Waldor, 2003; Kwan *et al*., 2005). It suggests that the higher ordered structures and function of above *S. aureus* phage repressors might be highly identical to each other. On the contrary, ϕ11 repressor also shows 30-40% identity with the repressor proteins of some other phages of *S. aureus*, *S. pyogenes,* and *S. pneumoniae* (data not shown). To see as to whether this second group of repressors has any similarity at structural level, secondary structures of four representative

repressors were determined and compared with those of ϕ11 and λ repressors. The data presented in Fig. 5C reveal that N-terminal ends (carrying ~1-100 amino acid residues) of all six repressors are constituted primarily with α-helix whereas C-terminal ends (initiates at ~155th amino acid residue) are made mostly with β-sheet. It is surprising to note that overall secondary structures of repressors of *S. aureus* phages ϕ11, ϕPV83, and ϕ12, and *Streptococcus* phages EJ-1 and 10750.3 are nearly similar to that of λ phage repressor though the formers have extremely weak homology with the latter at primary sequence level. In addition the secondary structure of the C-terminal end is more conserved than that of the N-terminal end.

The Ala-Gly dipeptide site responsible for autocleavage in λ phage repressor (Little, 1984) and most possibly in ϕ11 repressor are also present within ~130-160 amino acid residues of four other repressors. Bioinformatic analysis shows that all the above six proteins belong to proteins of serine protease S24 family. The 'hinge' region of λ CI harboring its Ala-Gly site holds its N-terminal and C-terminal domains (Ptashne, 1986). Based on the above discussion we suggest that the regions encompassed through ~130-160 amino acid residues of other five repressors form their respective 'hinge' regions which hold their corresponding N-terminal and C-terminal domains. While N-terminal domain is involved in binding operator DNA, C-terminal domain is engaged possibly in oligomerization especially, in dimerization of ϕ11 repressor.

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