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Regulation of anti-CRISPR operons by structurally distinct families of Aca proteins

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CRISPR-Cas systems provide bacteria with adaptive immunity against bacteriophages and mobile genetic elements, driving an evolutionary arms race in which phages deploy anti-CRISPR (Acr) proteins. Acr proteins are often co-encoded in operons with anti-CRISPR-associated (Aca) proteins, which coordinate the regulation of *acr* gene expression. Here, we reveal the molecular basis of DNA binding that mediates transcriptional repression by two distinct Aca family members: Aca7 and Aca11. Crystal structures of Aca7 and Aca11 highlight conserved helix-turn-helix (HTH) motifs within α -helix bundles, providing a universal DNA-binding platform. Aca7 forms a symmetrical dimer to recognize a 19-bp inverted repeat (IR) within the *acrIF11-aca7* operon. Strikingly, Aca11 binds 22-bp IRs in two distinct promoters, suggesting that Aca proteins can control multiple target operons. Mutagenesis and electrophoretic mobility shift assays (EMSAs) confirm that dimerization and sequence-specific IR recognition are essential for DNA binding. Despite mechanistic similarities, these and other Aca proteins exhibit notable differences. Structural comparisons across Aca families reveal that while monomer structures are generally similar with conserved HTH motifs, the structures of their dimeric functional units vary significantly. These structural differences might be essential for Aca proteins to bind to various promoters and regulate the expression of different Acr proteins.

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR associated (Cas) systems are adaptive immune defenses found in bacteria and archaea. These systems are responsible for detecting and degrading invading nucleic acids, such as those from bacteriophages (phages) and plasmids, providing sequence-specific immunity through its multi-step mechanisms^{1–3}. CRISPR arrays store segments of foreign DNA, which are transcribed and processed into guide CRISPR RNAs (crRNAs). These crRNAs then direct Cas proteins to recognize and cleave complementary sequences in foreign genetic material.

In recent years, a growing body of research has focused on the evasion strategies employed by phages to evade CRISPR-Cas-mediated immunity^{4,5}. The most thoroughly studied of these strategies is the production of anti-CRISPR (Acr) proteins⁶. These proteins are typically small and usually inhibit CRISPR-Cas systems by targeting specific Cas proteins, neutralizing the immune response and enabling phages to propagate^{7–11}. In infected bacterial cells, Acr proteins are often expressed at high levels due to strong

phage promoters. Once produced, Acrs act rapidly to suppress the CRISPR-Cas activity through a variety of mechanisms, including interference with crRNA processing, inhibition of target DNA recognition, or blockade of DNA cleavage. By disrupting these key stages of the CRISPR pathway, Acrs ensure successful phage replication and survival. Numerous Acr proteins have been identified that inhibit diverse CRISPR-Cas systems, each with unique mechanisms. For example, AcrIIA4 binds the HNH nuclease domain of Cas9 to inhibit DNA cleavage¹², AcrIIA28 sterically blocks target DNA binding¹³, and AcrIIA17 interacts with bridge helix domain to disrupt Cas9 RNP formation¹⁴. These mechanistic differences provide insight into the dynamic evolutionary arms race between host immunity and viral countermeasures^{15–17}.

Alongside Acr proteins, Acr-associated (Aca) proteins with helix-turn-helix (HTH) motifs are encoded within the same operons as the genes encoding Acrs^{6,15,18–24}. For example, the *Pseudomonas aeruginosa* phage JBD30 contains an *acrIF1-aca1* operon, while the *Pectobacterium carotovorum* phage ZF40 contains an *acrIF8-aca2* operon^{25,26}. Aca

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proteins repress the transcription of *acr* operons and regulate the timing and level of Acr expression in the bacterial host by binding to specific inverted repeat (IR) sequences located near the promoter region^{25–27}. This regulation is critical for the proper functioning of the Acr operon, ensuring that anti-CRISPR production is optimal for phage survival^{25,27}.

Aca proteins seem to generally act as transcriptional repressors of their operons²⁸. However, the detailed molecular mechanisms of promoter binding and repressor function have only been elucidated for Aca1, Aca2, and Aca10^{25–27,29–32}, which inhibit *acr-aca* operon transcription by directly interacting with specific inverted repeats (IRs) located around the promoter, presumably preventing RNA polymerase recruitment. Interestingly, Aca2 was recently shown to additionally use its HTH motif to bind RNA and regulate *acr* translation²⁷. This finding highlights that characterizing Aca proteins might uncover further unexpected mechanisms involved in the regulation of Acr production. Therefore, we applied a structural and biochemical approach to elucidate the interactions of the Aca proteins Aca7 and Aca11 with their cognate *acr* operon promoters. Understanding the role of these Aca proteins in controlling the expression and activity of anti-CRISPR proteins will provide important insights into how phages overcome CRISPR-Cas immunity.

Results and discussion

Structure of Aca7 derived from *Halomonas caseinilytica*

Aca7 was first discovered in a screen for the type I and type V CRISPR-Cas inhibitors²⁰, sharing an operon with *acrIF11*, which encodes an ADP ribosyltransferase inhibiting the type I-F CRISPR interference complex³³. Aca7 was shown to repress transcription from the *acrIF11-aca7* promoter²⁸. However, the specific promoter sequence(s) it recognizes and the mechanism through which it exerts its Aca function remain unknown. To investigate the structural basis for Aca7-mediated anti-CRISPR repression, the full-length Aca7 protein was first purified using a two-step method, consisting of Ni-NTA affinity chromatography followed by size-exclusion chromatography (SEC). SEC revealed two distinct peaks: the first eluted at 8–10 mL, corresponding to the column's void volume and indicating the presence of high-molecular weight aggregates (apparent MW > 670 kDa) and the main peak at approximately 17–18 mL (apparent 1.35 kDa < MW < 17 kDa) (Fig. 1A). Analysis of the eluted fractions via SDS-PAGE indicated that only the fractions corresponding to the 17–18 mL peak contained highly pure Aca7 protein, whereas no Aca7 was detected in the fractions corresponding to the aggregation peak (Fig. 1B). The apparent molecular weight of Aca7 in the solution was estimated as 17 kDa based on its SEC elution volume relative to a column calibration curve, suggesting

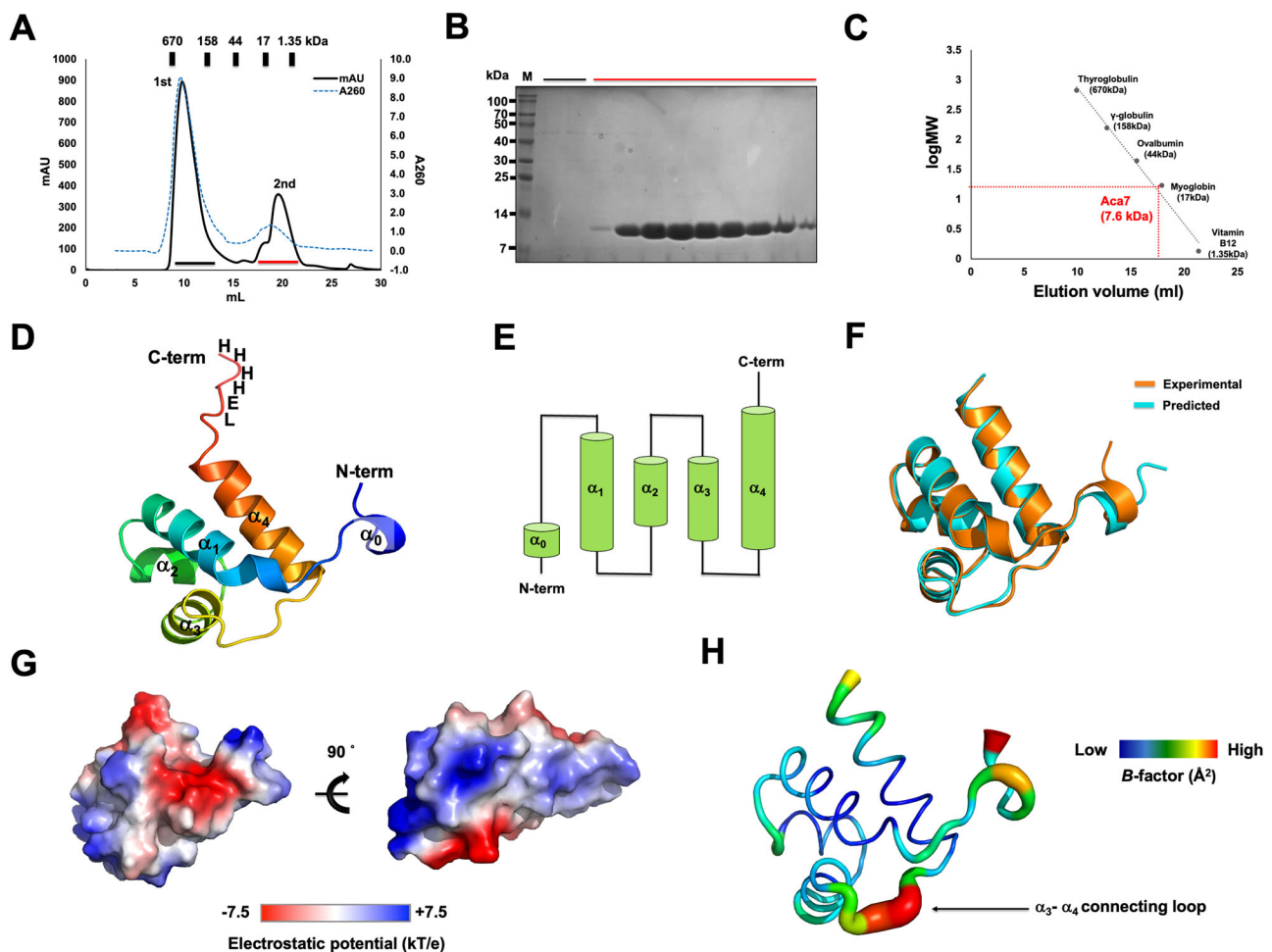


Fig. 1 | Crystal structure of Aca7 derived from *Halomonas caseinilytica*. **A** Profile of size-exclusion chromatography of Aca7. The solid black line represents the A280 absorbance profile, and the dashed blue line represents the A260 absorbance profile. **B** SDS-PAGE analysis of protein fractions corresponding to the elution peaks, indicating the purity of Aca7. **C** Calibration curve of elution volumes from SEC, showing the logarithmic relationship between molecular weight and elution volume. The experimental elution volume of Aca7 is plotted for comparison. **D** Cartoon representation of the monomeric structure of Aca7. The chain is colored in a

gradient transitioning from blue at the N-terminus to red at the C-terminus. **E** Schematic representation of Aca7's secondary structure topology, highlighting its arrangement of helices and loops. **F** Superimposition of experimentally resolved Aca7 structure with the AlphaFold2 predicted model. **G** Surface electrostatic potential of Aca7, depicted using a color gradient from negative potential (red, -7.5 kT/e) to positive potential (blue, +7.5 kT/e). **H** B-factor representation of Aca7, visualized with a putty style rendering. The structure is colored in a spectrum from red to violet to indicate regions of increasing flexibility based on B-factor values.

Table 1 | Data collection and refinement statistics of Aca7 and Aca11

Data collection	Aca7 (9LX1)	Aca11 (9LX2)
Space group	$P3_121$	$P1$
Unit cell parameters		
a, b, c (Å)	$a = 39.46, b = 39.46, c = 90.93$	$a = 24.06, b = 52.6, c = 58.29$
α, β, γ (°)	$\alpha = 90, \beta = 90, \gamma = 120$	$\alpha = 105.55, \beta = 96.9, \gamma = 100.55$
Resolution range (Å) ^a	27.32–1.25	27.63–1.94
Total reflections	426,986	62,551
Unique reflections	22,730	17,422
Multiplicity	18.8 (18.7)	3.6 (3.6)
Completeness (%) ^a	96.88 (94.59)	88.34 (90.78)
Mean $I/\sigma(I)$ ^a	52.51 (11.98)	12.48 (3.18)
R_{merge} (%) ^{a,b}	3.322 (26.51)	6.918 (50.66)
Wilson B -factor (Å ²)	12.40	26.33
Refinement	27.32–1.25	27.63–1.94
Resolution range (Å)	22,728	17,419
Reflections	17.56	19.92
R_{work} (%)	18.60	22.68
R_{free} (%)	1	4
No. of molecules in the asymmetric unit		
No. of non-hydrogen atoms	713	2196
Macromolecules	593	2069
Solvent	120	127
Average B -factor values (Å ²)	16.55	32.36
Macromolecules	14.66	32.06
Solvent	25.88	37.22
Ramachandran plot:		
favoured/allowed/outliers (%)	98.61/1.39/0.00	98.83/1.17/0.00
Rotamer outliers (%)	0.00	0.00
Clashscore	1.68	10.82
RMSD bonds (Å) / angles (°)	0.009 / 1.15	0.020 / 3.17

^aValues for the outermost resolution shell are shown in parentheses.

^b $R_{\text{merge}} = \sum_i \sum_h |I(h) - \langle I(h) \rangle| / \sum_i \sum_h I(h)$, where $I(h)$ is the observed intensity of reflection h , and $\langle I(h) \rangle$ is the average intensity obtained from multiple measurements.

Aca7 is a dimer in solution (the theoretical molecular weight of monomeric His-tagged Aca7 is 7.6 kDa) (Fig. 1C). Purified Aca7 was crystallized, and high-resolution diffraction data were collected at 1.24 Å. Initial attempts to determine the structure through ab initio phasing using ARCIMBOLDO_BORGES software³⁴ were unsuccessful due to the absence of a suitable structural homolog required for molecular replacement (MR)^{35,36}. This was resolved by using a structural model generated using AlphaFold2³⁷. The final structural model of Aca7 was refined to $R_{\text{work}} = 17.6\%$ and $R_{\text{free}} = 18.7\%$ (Table 1). The crystal belonged to the space group $P3_121$, with a single molecule occupying the asymmetric unit (ASU). The complete sequence of Aca7 (residues M1 to T68) was resolved in the final model. Additionally, the XhoI linker (L69 and E70) and the 6x His-tag at the C-terminus were partially modeled (Fig. 1D).

The Aca7 structure consists of an α -helical bundle fold comprising five α -helices (α_0 – α_4), with α_2 and α_3 constituting a helix-turn-helix (HTH) motif (Fig. 1D–E). A very small helix was observed at the N-terminus, but since it was ambiguous to classify as a distinct helix, we designated it as “ α_0 ” (Fig. 1D). The remaining four helices formed a

compact structure (Fig. 1D). Structural superimposition between the experimentally resolved Aca7 model and the AlphaFold2-predicted model revealed a highly similar overall fold, with only minor deviations observed in the N-terminal α_0 region. The root-mean-square deviation (RMSD) between the two models was 0.857 Å (Fig. 1F). Surface electrostatic potential analysis of Aca7 revealed regions of both partial positive and partial negative charge (Fig. 1G). Notably, high positive potential was concentrated around the α_2 and α_3 helices, which could facilitate interactions with negatively charged molecules, such as DNA. In contrast, negative potential was distributed across other regions of the surface (Fig. 1G). Further analysis of the B -factor distribution indicated that the overall structure of Aca7 is predominantly rigid, with a low average B -factor of 13.32 Å². However, the α_3 – α_4 connecting loop exhibits a slightly elevated average B -factor of 17.97 Å², suggesting localized flexibility. While the difference is modest, this increased flexibility is indicative of subtle conformational adjustments that could facilitate functionally crucial binding interactions (Fig. 1H). In summary, Aca7 is a five α -helical bundle protein with a distinctively charged surface, featuring strong positive potential near the α_2 and α_3 helices and localized flexibility in the α_3 – α_4 loop.

Aca7 forms a dimer in solution

Initial genetic and biochemical studies of Aca family members suggested a dimeric form, which was later confirmed for Aca1, Aca2 and Aca10^{26,29,31,32}. However, it remains unclear whether all Aca proteins function as dimers, and the stoichiometry of Aca7 had not yet been determined. Given that stoichiometry likely influences the mechanism of Aca proteins, we sought to determine the oligomeric state of Aca7 in solution by calculating its absolute molecular mass using SEC-multi-angle light scattering (MALS). The molecular mass of the main elution peak of Aca7 was determined to be 18.93 kDa (5.8% fitting error) (Fig. 2A). Considering the theoretical molecular weight of monomeric Aca7 with a C-terminal histidine-tag is 7.6 kDa, this result strongly supports that Aca7 exists as a dimer in solution.

To further investigate the molecular basis of Aca7 dimerization, we examined the crystallographic unit cell and analyzed protein-protein interactions (PPIs) using the PDBePISA server³⁸. The PISA score, which ranges from 0 to 1 with higher values indicating greater relevance of an interface to complex formation, was 1.000 for the dimer formed by Molecule A (MolA) and its symmetry counterpart (SymA), suggesting that this dimer is the most likely biologically relevant assembly (Fig. 2B–C). The interface between MolA and SymA buried a surface area of 828.6 Å², accounting for 15.5% of the total molecular surface, with 20 residues (27.0% of total residues) contributing to the interface (Fig. 2B). The Aca7 (MolA/SymA) dimer is characteristic of DNA-binding proteins that recognize palindromic DNA sequences and forms a symmetric assembly stabilized primarily by interactions between helix α_0 of one molecule and helices α_1 and α_2 of the other (Fig. 2C). These interactions involve multiple hydrogen bonds and salt bridges, with key contributions from residues R5, E29 and E32 (Fig. 2D–F). Notably, sequence conservation analysis using the ConSurf server revealed that these residues are highly conserved among Aca7 orthologues, despite the overall sequence variability typical of Aca family proteins (Supplementary Fig. 1A–B). This suggests that dimer interface and its associated DNA recognition strategy are likely functionally important and evolutionarily maintained.

To experimentally validate the importance of the MolA/SymA interface in dimer formation, site-directed mutagenesis was performed to disrupt key residues involved in the dimeric interaction. Residues identified as critical for the interface were mutated to tryptophan (W), the bulkiest neutral amino acid, to disrupt the interactions stabilizing the dimer. Mutants R5W, E29W and E32W were generated, and their oligomeric states were analyzed using SEC-MALS (Fig. 2G). As expected, all three mutants disrupted dimer formation, as indicated by delayed elution corresponding to a monomeric size and measured molecular weights consistent with a monomer (Fig. 2H). In summary, Aca7 is a dimer in solution, which likely represents its biologically relevant multimerization state.

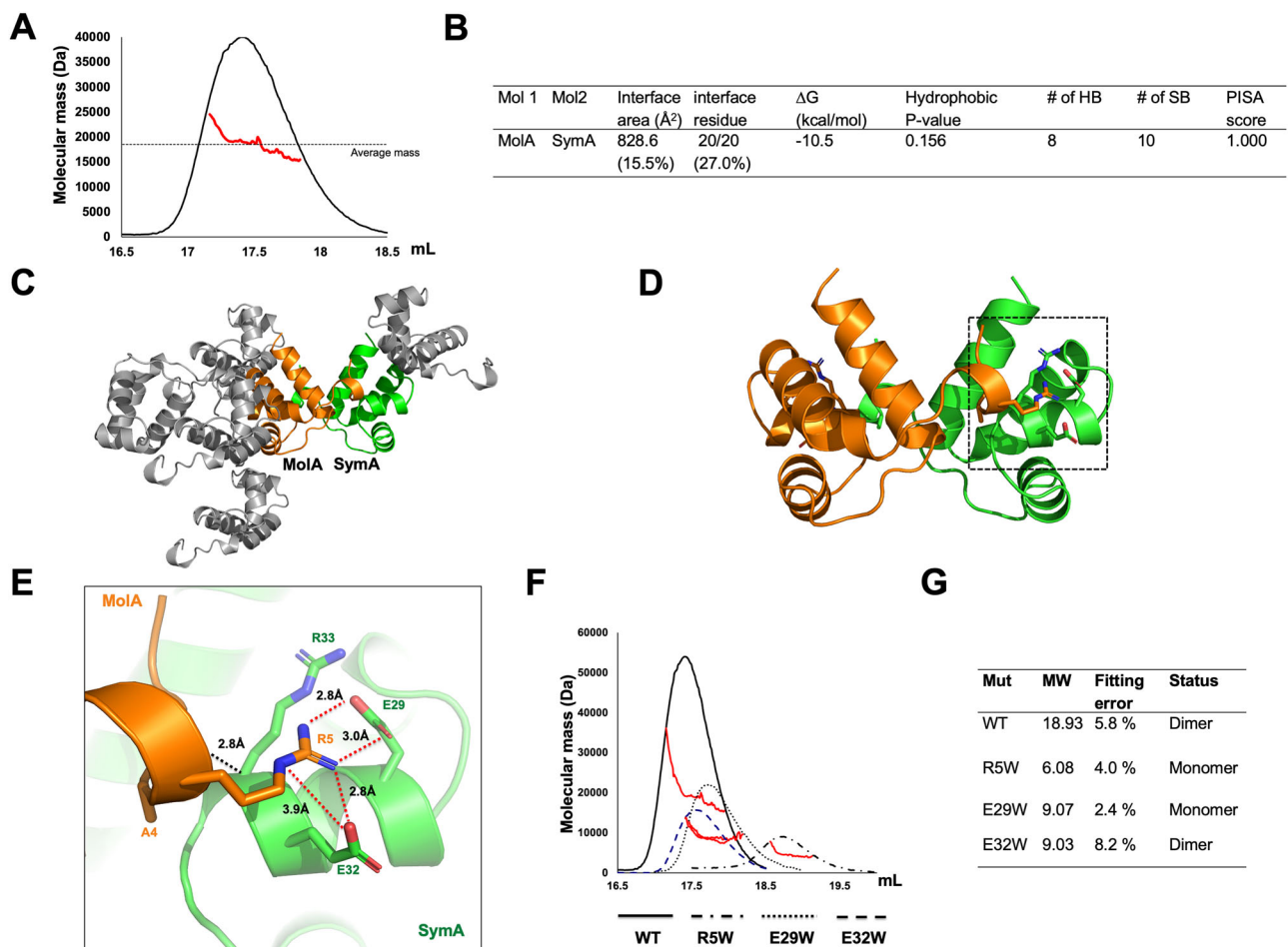


Fig. 2 | Dimeric structure of Aca7 and its interface analysis and conformation. **A** Multi-angle light scattering (MALS) profile corresponding to the main SEC peak of Aca7. The experimental molecular weight determined by MALS, is shown red, and the dashed line represents the average molecular mass within this region (~18.6 kDa). **B** Summary table of dimer interface properties analyzed using the PISA server. **C** Symmetry analysis of crystallographic packing, illustrating the MolA/SymA dimeric form identified in the asymmetric unit. **D** Regions contributing to protein-protein interactions (PPIs) within the MolA/SymA dimer are marked with

black dashed boxes. **E**, **F** Magnified view of the PPI regions from panel **D**, highlighting salt bridges (red dashed lines) and hydrogen bonds (black dashed lines). **G** Mutational analysis of PPI regions, comparing SEC-MALS profiles of wild-type Aca7 and mutants. Elution positions and molecular masses derived from MALS are indicated, with experimental molecular weights shown as red lines. **H** Table summarizing the MALS results, displaying molecular weights, fitting errors, and monomeric or dimeric status of the wild-type and mutant Aca7 proteins.

Aca7 recognizes specific IRs in the *acrIF11-aca7* promoter

Aca7 functions within the *acrIF11-aca7* operon to repress expression from the operon promoter. AcrIF11 inhibits the type I-F CRISPR-Cas system by ADP-ribosylation of the Cascade complex, preventing target DNA binding³³. Transcriptional regulators, including other Aca proteins, often bind inverted repeats (IR) within promoter regions to repress transcription by blocking RNA polymerase binding³⁹. Therefore, we hypothesized that Aca7 recognizes IRs within the promoter of the *acrIF11-aca7* operon to regulate transcription. We analyzed the 5' upstream region of the *acrIF11-aca7* operon and identified a putative promoter featuring -35 (TTGAAA) and -10 (TATACT) elements separated by 17 nucleotides (Fig. 3A). A prediction of transcription initiation rates along the operon revealed a peak of transcription initiation rate consistent with the location of these promoter elements (Supplementary Fig. 2). Within this region, we identified several IRs. Overlapping the -35 region, an 18 bp IR (IR1; TTCAGGGAATGGCTTGAA) was detected, consisting of two matching 6 bp half-sites. Further analysis revealed a 19 bp IR (IR2; ATGATAACTCAGTTATCAT) overlapping both the -35 and -10 elements, comprising two perfect 9 bp half-sites separated by one nucleotide. Finally, a smaller sequence (IR3; CTGTATTCAACAG) overlapping the -10 region was identified as a potential IR site but featured only short, partial

palindromic elements, consisting of two matching 4 bp half-sites (Fig. 3A–B).

To test for direct interaction between Aca7 and the promoter DNA, we first performed electrophoretic mobility shift assays (EMSAs) using 5' FAM-labeled DNAs including all IR sites (wIR1-wIR2-wIR3). Aca7 exhibited concentration-dependent binding to this DNA fragment, producing a clear single band shift (Fig. 3C). To test binding specificity, we pre-incubated Aca7 with an excess of unlabeled target-specific DNA, which completely blocked the band shift of the FAM-labeled DNA, indicating that Aca7 binds specifically within the wIR1-wIR2-wIR3 fragment. In contrast, pre-incubation with an excess of non-specific DNA failed to prevent the band shift confirming that Aca7 does not bind non-specific sequences under these conditions (Fig. 3C). These results demonstrate that Aca7 specifically recognizes and directly binds within the *acrIF11-aca7* promoter in *H. caseinilytica*. To further assess the sequence-specificity and requirements of IR1, IR2 and IR3 for Aca7 binding, we generated four mutant DNA fragments: mIR1-wIR2-wIR3 (IR1 disruption), wIR1-mIR2-wIR3 (IR2 disruption), wIR1-wIR2-mIR3 (IR3 disruption), and mIR1-mIR2-mIR3 (IR1, IR2 and IR3 disruption) (Fig. 3D). Aca7 only bound to DNA fragments that contained the IR2 sequence, indicating that IR2 is essential for Aca7 binding, while IR1

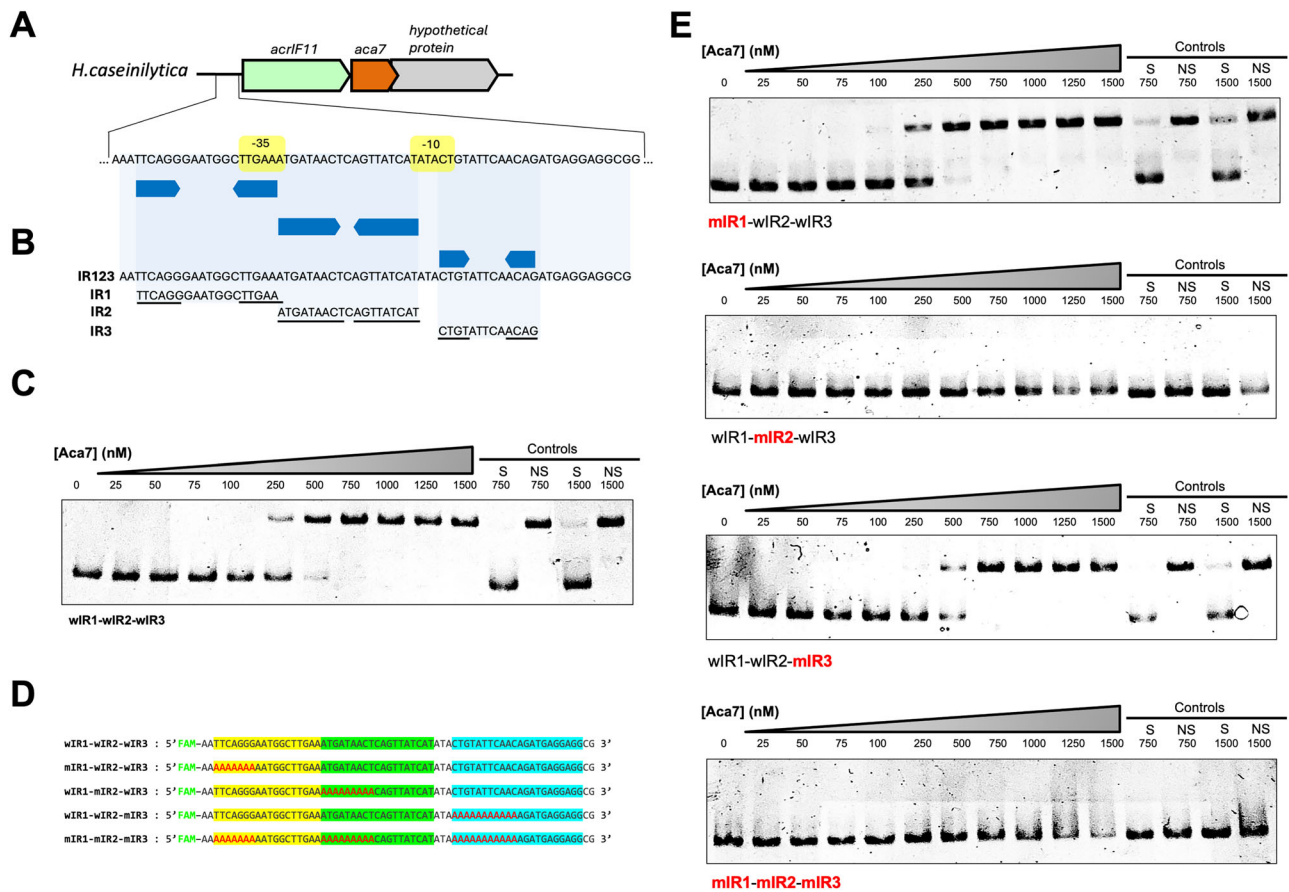


Fig. 3 | Promoter identification and IR-specific interaction by Aca7. **A** Genomic organization of the *acrIF11-aca7* operon, showing the predicted regulatory region with highlighted -35 and -10 promoter elements. Inverted repeat (IR) sequences are represented by blue arrows beneath the regulatory region. **B** Sequences of the identified IR regions, divided into IR1, IR2 and IR3 DNA probes, with each inverted repeat sequence labeled accordingly. **C** Electrophoretic mobility shift assay (EMSA) showing the binding of Aca7 to a 5'-FAM-labeled DNA probe containing wIR1-wIR2-wIR3 sequences. A black triangle indicates increasing concentrations of Aca7.

and IR3 are dispensable (Fig. 3E). In summary, Aca7 is a dimeric HTH transcriptional regulator that specifically binds a 19 bp IR overlapping the *acrIF11-aca7* promoter to regulate gene expression.

Strategy of promoter recognition by Aca7

Members of the Aca family, such as Aca1 from *P. aeruginosa* phage JBD30, Aca2 from *Pectobacterium carotovorum* phage ZF40, and Aca10 from *Pseudomonas citronellolis*, form dimers and their HTH motifs play a crucial role in binding inverted repeats (IRs) within promoter regions to repress transcription^{26,29,30,32}. We predicted that Aca7 also functions as a dimeric transcriptional repressor with an HTH motif. To further elucidate the mechanism of promoter recognition, we employed a combination of sequence-based and structure-based analyses to identify residues critical for DNA binding. The electrostatic surface potential of dimerized Aca7 revealed a highly charged surface (± 7.6 kT/e), with positive charges around α_2 and α_3 helices, suggesting their key role in promoter recognition (Fig. 4A). The positively charged residues, such as R38, K46 and K49, were prominently distributed near the α_3 helix (Fig. 4B). A model of the Aca7/dsDNA (IR2) complex, predicted by AlphaFold3, further supported the role of the HTH motif in DNA binding, showing the α_3 helix and its connecting loop positioned close to the DNA (Fig. 4C). Detailed analysis of the complex identified residues R38, Y40, K46 and K49 as potential DNA-contacting residues, with their positively charged side chains extending toward the major groove of the DNA (Fig. 4D).

Excessive unlabeled wIR-wIR-wIR and non-labeled nonspecific DNA probes were used as competitive controls to distinguish specific from nonspecific binding. **D** Mutated version of the wIR1-wIR2-wIR3 DNA probe used to disrupt individual or all IR regions. Constructs include mIR1-wIR2-wIR3 (IR1 disruption), wIR1-mIR2-wIR3 (IR2 disruption), wIR1-wIR2-mIR3 (IR3 disruption) and mIR1-mIR2-mIR3 (complete IR disruption), all 5'-FAM labeled for subsequent binding experiments. **E** EMSA results showing Aca7 binding to the mutated DNA probes. Black triangles indicate increasing concentrations of Aca7.

A schematic representation of the predicted Aca7-DNA complex further supports that multiple residues, including R38, Y40, K46 and K49, form direct polar contacts with the DNA, underscoring their critical role in promoter recognition (Supplementary Fig. 3). Although these DNA-interacting residues are not strictly conserved among Aca7 homologs, many are partially conserved or substituted with residues of similar physico-chemical properties, particularly positively charged side chains, suggesting that electrostatic DNA recognition features are preserved across homologs.

To experimentally validate the promoter recognition strategies of Aca7, site-directed mutagenesis was performed to generate Aca7 mutants (R5W, E29W, E32W, R38W, Y40W and K46W), followed by EMSAs to compare the DNA-binding affinities of wild-type (W) Aca7 and its mutants. AcrIIA13b, a protein with a highly negatively charged surface and no nucleotide-binding capability⁴⁰, was used as a negative control. EMSA results revealed significantly reduced DNA-binding affinities for both dimer-disruption mutants (R5W, E29W and E32W) and DNA-binding mutants (R38W, Y40W, K46W and K49W) (Fig. 4E). Specifically, the R5W and E29W mutants exhibited the most pronounced DNA binding disruption among those dimer disruption mutants. The E32W and Y40W mutants partially retained binding, suggesting a less critical but still supportive role in Aca7 function for DNA binding (Fig. 4E). Altogether, the structural and mutagenesis analyses demonstrate that dimerization of Aca7 is essential for its ability to recognize IR sequences, with the α_3 helix playing a central role in interacting with the target promoter to perform as transcriptional regulator.

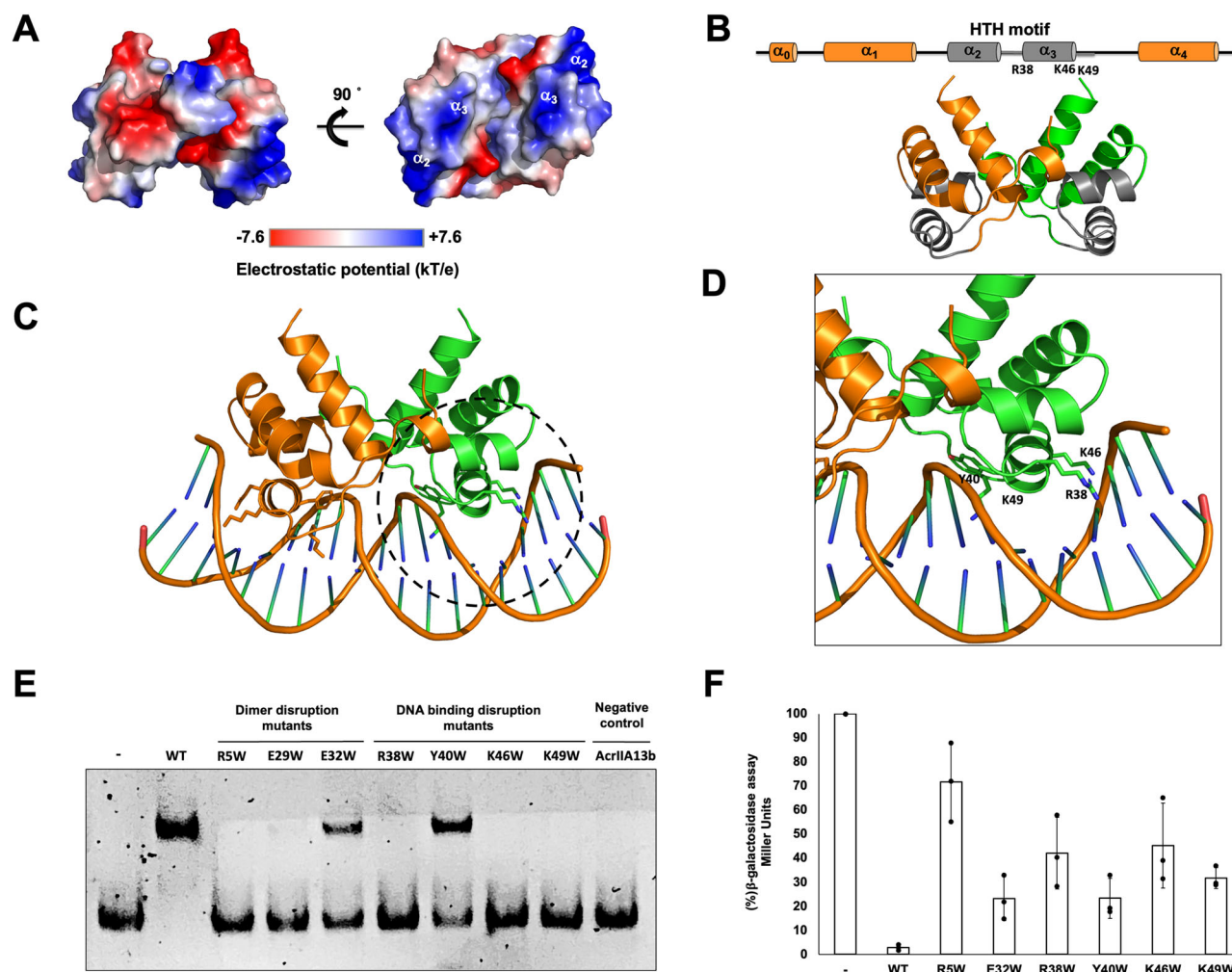


Fig. 4 | Strategy of promoter recognition by dimeric Aca7. **A** Surface electrostatic potential of dimeric Aca7. **B** Location of the predicted helix-turn-helix (HTH) motif on Aca7 structure. **C** Predicted complex model of Aca7 bound to its target IR2 DNA sequence, generated using AlphaFold3. **D** Magnified view of Aca7-DNA interaction region, highlighted with a black dashed circle in (B). Key residues involved in recognizing the palindromic sequence, R38, Y40, K46 and K49, are labeled in the structure using one-letter amino acid codes. **E** EMSA results comparing the binding ability of wild-type Aca7 and mutant variants with a 5'-FAM-labeled wIR-wIR-wIR

DNA probe. R5W, E29W and E32W are dimer disruption mutants and R38W, Y40W, K46W and K49W are DNA binding disruption mutants. AcrlIA13b, a protein not expected to bind DNA, was included as a negative control. **F** β -galactosidase reporter assay for the *acrIF11-aca7-LacZ* construct co-expressed with Aca7 wild-type or the indicated mutants. Activities were calculated as Miller units, and background-corrected by subtracting Aca only values, and normalized to the LacZ only (-) control (set to 100%). $N = 3$ biologically independent experiments.

To further assess the importance of these Aca7 residues in vivo, we performed β -galactosidase reporter assay using an *acrIF11-aca7* promoter-LacZ construct. Wild-type Aca7 markedly reduced β -galactosidase activity relative to LacZ alone, indicating the Aca7 represses promoter driven LacZ expression through direct promoter recognition. Notably, among the mutants, R5W showed the most significant loss-of-function phenotype in vivo. Despite some differences in the extent of the effects, all mutants displayed in vivo outcomes that were consistent with their impaired DNA-binding abilities observed in vitro, supporting the direct link between promoter recognition and transcriptional repression (Fig. 4F).

Structural characterization of Aca11 from *Streptococcus* sp

Aca11 was identified through a guilt-by-association approach but little is known about how it might exert its predicted regulatory effect⁴¹. The role of Aca11 was examined by purifying the full-length protein encoded in a *Streptococcus* prophage and determining its structure. Two purification strategies were employed, involving Ni-NTA affinity chromatography followed by SEC. SEC analysis revealed two elution peaks: one near the column void volume (8–10 mL) and another at 18–19 mL (Fig. 5A). SDS-PAGE

analysis confirmed that the 18–19 mL fractions contained highly purified Aca11 (Fig. 5B). The molecular weight of Aca11 in solution, estimated from its SEC profile, was approximately 17 kDa, consistent with a dimeric state, given the theoretical molecular weight of 8.1 kDa for monomeric Aca11 with its vector-derived histidine tag.

High quality crystals were obtained, and diffraction data were collected at 1.94 Å resolution. A structural model predicted by AlphaFold2 was used as a search model for MR phasing. The final structure was refined to $R_{\text{work}} = 19.9\%$ and $R_{\text{free}} = 22.6\%$ (Table 1). Aca11 crystallized in the P1 space group, with four molecules occupying the asymmetric unit (ASU) (Fig. 5C). The complete Aca11 sequence (M1 to P63) was successfully included in the final model (Fig. 5C).

Aca11 comprises a short β -sheet at its N-terminus and four α -helices (α_1 – α_4), of which α_2 and α_3 form a HTH motif (Fig. 5C–E). Structural superimposition of the four molecules in the asymmetric unit revealed a highly conserved conformation, with only slight variations in the N- and C-terminal regions (Fig. 5F). Surface electrostatic potential analysis of monomeric Aca11 revealed a predominantly positively charged surface. The strongest positive potential was observed around the α_3 helix and its

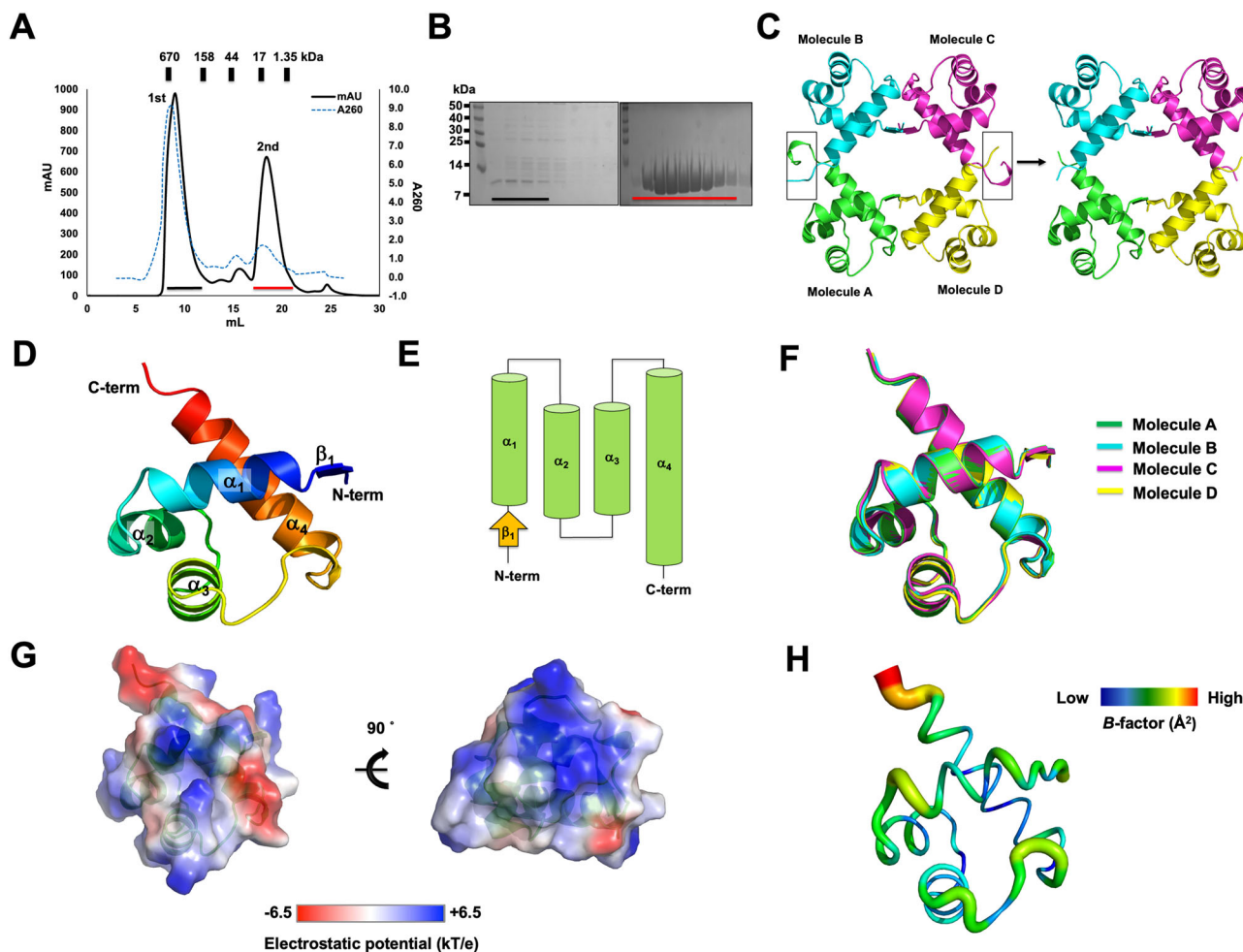


Fig. 5 | Crystal structure of Aca11 derived from *Streptococcus* sp. **A** Size-exclusion chromatography profile of Aca11. The solid black line represents the A280 absorbance profile, and the dashed blue line represents the A260 absorbance profile. **B** SDS-PAGE gel analysis of protein fractions corresponding to the main SEC peak, confirming the purity of Aca11. **C** Cartoon representation of the four Aca11 molecules present in the crystallographic asymmetric unit, with each molecule colored uniquely. **D** Cartoon representation of the monomeric structure of Aca11. The chain is colored in a gradient transitioning from blue at their N-terminus to red

at the C-terminus. **E** Schematic topology of Aca11's secondary structure, illustrating the arrangement of its helices and β -strand. **F** Structural alignment of the four Aca11 molecules identified in one asymmetric unit, with colors corresponding to the monomers shown in panel (C). **G** Surface electrostatic potential of monomeric Aca11, depicted with a color gradient ranging from negative potential (red, -6.5 kT/e) to positive potential (blue, $+6.5$ kT/e). **H** B -factor distribution in the structure of Aca11, visualized with a putty-style rendering. Rainbow colors from red to violet indicate regions of increasing flexibility, based on B -factor values.

connecting loop with α_4 , suggesting these regions as potential key sites for interactions with negatively charged molecules, such as nucleic acids (Fig. 5G). B -factor analysis indicated that Aca11 is mostly rigid, with an average B -factor of $27.12 \text{ \AA}^2$, reflecting structural stability (Fig. 5H). However, the α_3 - α_4 connecting loop exhibited localized flexibility, potentially reflecting their roles in facilitating dynamic interactions or structural adaptations. Additionally, the N- and C-terminal regions showed slightly elevated B -factors, consistent with their less well-defined electron density in the crystal structure. In summary, Aca11 adopts a four α -helical bundle fold with an additional N-terminal β -sheet, having a distinct positively charged surface. The strong positive potential around the α_3 - α_4 region, along with localized flexibility in specific structural elements, suggests a potential role in nucleotide binding and regulatory interactions.

Aca11 forms a dimer in solution

To test whether Aca11 is dimeric, we employed SEC-MALS to determine the molecular weight of Aca11. The primary elution peak corresponded to a molecular weight of 18.63 kDa (7.7% fitting error), which is consistent with a dimeric assembly, given that the theoretical molecular weight of the monomer is 8.12 kDa (Fig. 6A). To further elucidate the molecular basis of Aca11 dimerization, we analyzed the crystallographic unit cell using the

PDBEPIA server to identify the relevant protein-protein interactions (PPIs). The analysis revealed a PISA score of 1.000 for the dimer formed by Molecule A (MolA) and Molecule D (MolD), indicating a highly stable and biologically significant assembly (Fig. 6B). The interaction interface buried a total surface area of $596.2 \text{ \AA}^2$, representing 13.7% of the total molecular surface, with 15 residues (23.4% of total residues) contributing to the interaction. This dimer symmetry, characteristic of DNA-binding proteins that recognize palindromic sequences, is stabilized by 13 hydrogen bonds, primarily between the N-terminal regions that form an antiparallel β -sheet and the α_3 - α_4 connecting loop, extending into the α_4 helix (Fig. 6C). In contrast, the interactions between MolA and Molecule B (MolB) scored a significantly lower PISA score of 0.109 , with only eight hydrogen bonds, predominantly located at the C-terminal region (Fig. 6D). These findings suggest that the MolA/MolD dimer is the most biologically relevant configuration for Aca11 in solution, warranting further investigation into its dimerization mode and key interacting residues.

Two main interaction regions were identified as crucial for the stability of the MolA/MolD dimer: the antiparallel β -sheet at the N-terminus and the α_4 helix along with its connecting loop (Fig. 6E-F). The antiparallel β -sheets from both molecules were positioned in close proximity, approximately $2.8 \text{ \AA}$ apart, forming multiple hydrogen bonds involving residues V3 and T5

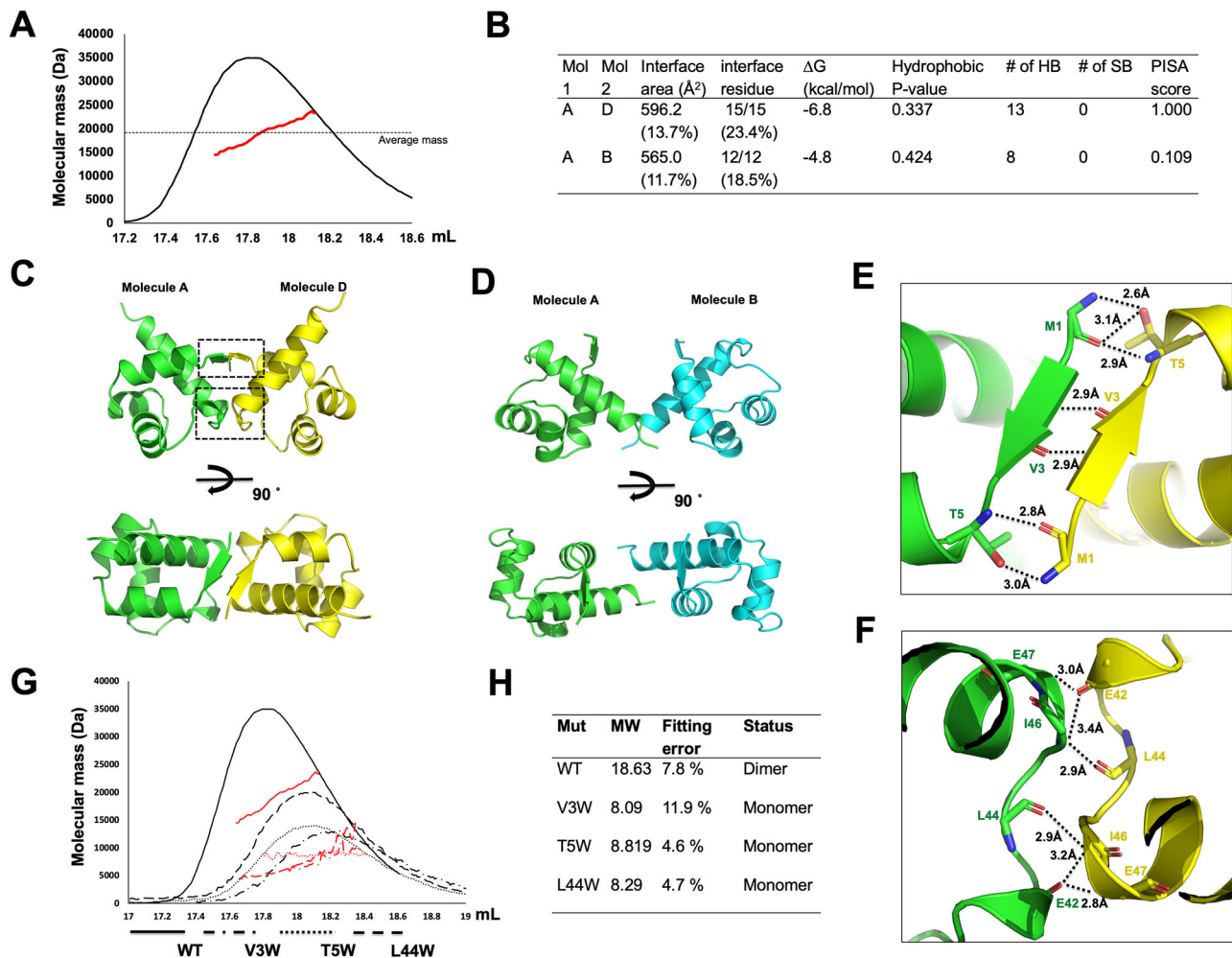


Fig. 6 | Dimeric structure of Aca11 and its interface analysis and conformation. **A** Multi-angle light scattering (MALS) profile corresponding to the main SEC peak of Aca11, with the experimental molecular weight determined by MALS shown as a red line, and the average molecular mass indicated by a dashed line (~19.1 kDa). **B** Summary table of PPI details for the two possible dimeric form of Aca11, analyzed using the PISA server. Symmetry analysis of crystallographic packing, illustrating two potential dimeric configurations: MolA/MolD dimer (**C**) and MolA/MolB dimer (**D**) identified in the asymmetric unit. Black dashed boxes highlight the PPI

regions for the MolA/MolD dimer. **E–F** Magnified view of the PPI regions within the MolA/MolD dimer. Hydrogen bonds stabilizing the interface are represented by black dashed lines. **G** Mutational analysis of PPI regions, comparing SEC-MALS profiles of wild-type Aca11 and its mutant variants. Elution positions and molecular weights derived from MALS are shown, with experimental molecular weights indicated by red lines. **H** Table summarizing the result of MALS, listing molecular weights, fitting errors, and the monomeric or dimeric status of wild-type and mutant Aca11 proteins.

(Fig. 6E). Meanwhile, the α_4 helix and its connecting loop contributed to dimer stabilization through interactions mediated by L44, which was closely associated with E47 and I46 of the opposing molecule (Fig. 6F). Consistent with their structural importance, sequence conservation analysis revealed that V3, T5 and L44 are partially conserved among Aca11 orthologues, suggesting that the dimer interface is evolutionarily conserved (Supplementary Fig. 4A–B).

Building on the structural insights from the MolA/MolD interface analysis, we performed site-directed mutagenesis to experimentally validate its importance in dimer formation. Mutations were introduced at V3, T5 and L44, generating V3W, T5W and L44W variants. The oligomeric states of these mutants were analyzed using SEC-MALS, revealing that all three mutations abolished dimer formation, as indicated by delayed elution peaks corresponding to monomeric forms (Fig. 6G). The measured molecular weights of V3W, T5W and L44W were 8.09 kDa (11.9% fitting error), 8.82 kDa (4.6% fitting error) and 8.29 kDa (4.9% fitting error), respectively, closely matching the theoretical molecular weight of monomeric Aca11 (Fig. 6H). These results confirm that disrupting key residues at the MolA/MolD interface effectively prevents dimerization, highlighting their essential role in dimer stability. The integration of structural analysis, crystal packing

evaluations, and mutagenesis studies provides strong evidence that Aca11 exists as a symmetric MolA/MolD dimer in solution. The integrity of this interface is essential for maintaining dimeric assembly with its hypothetical role in anti-CRISPR regulation.

Aca11 recognizes specific IRs upstream of two distinct operons

Aca11 is encoded 82 bp downstream of *acrIIA25.1* and 149 bp upstream of an apparent operon consisting of *acrIIA3* and a hypothetical protein with a predicted AP2 domain (Fig. 7A). A prediction of transcription initiation rates across this locus using the De Novo DNA Server⁴² suggested two distinct regions with high initiation rates: one upstream of *acrIIA25.1* and one upstream of *acrIIA3* (Supplementary Fig. 5A), suggesting that *aca11* is part of an operon with *acrIIA25.1*. Interestingly, the predicted promoter regions of the two operons share extensive sequence identity (Fig. 7B and Supplementary Fig. 5B). Both promoters comprised identical -35 (TTGACA) and -10 (TATACT) elements separated by 17 nucleotides (Fig. 7A–B). Overlapping the *acrIIA25.1-aca11* promoter we discovered a distinct IR sequence consisting of two perfect 11 bp half-sites separated by two nucleotides (IR_{acrIIA25}: AGTATATAACAAATGTTATATACT); a near-identical IR with one mismatch between the half sites was found

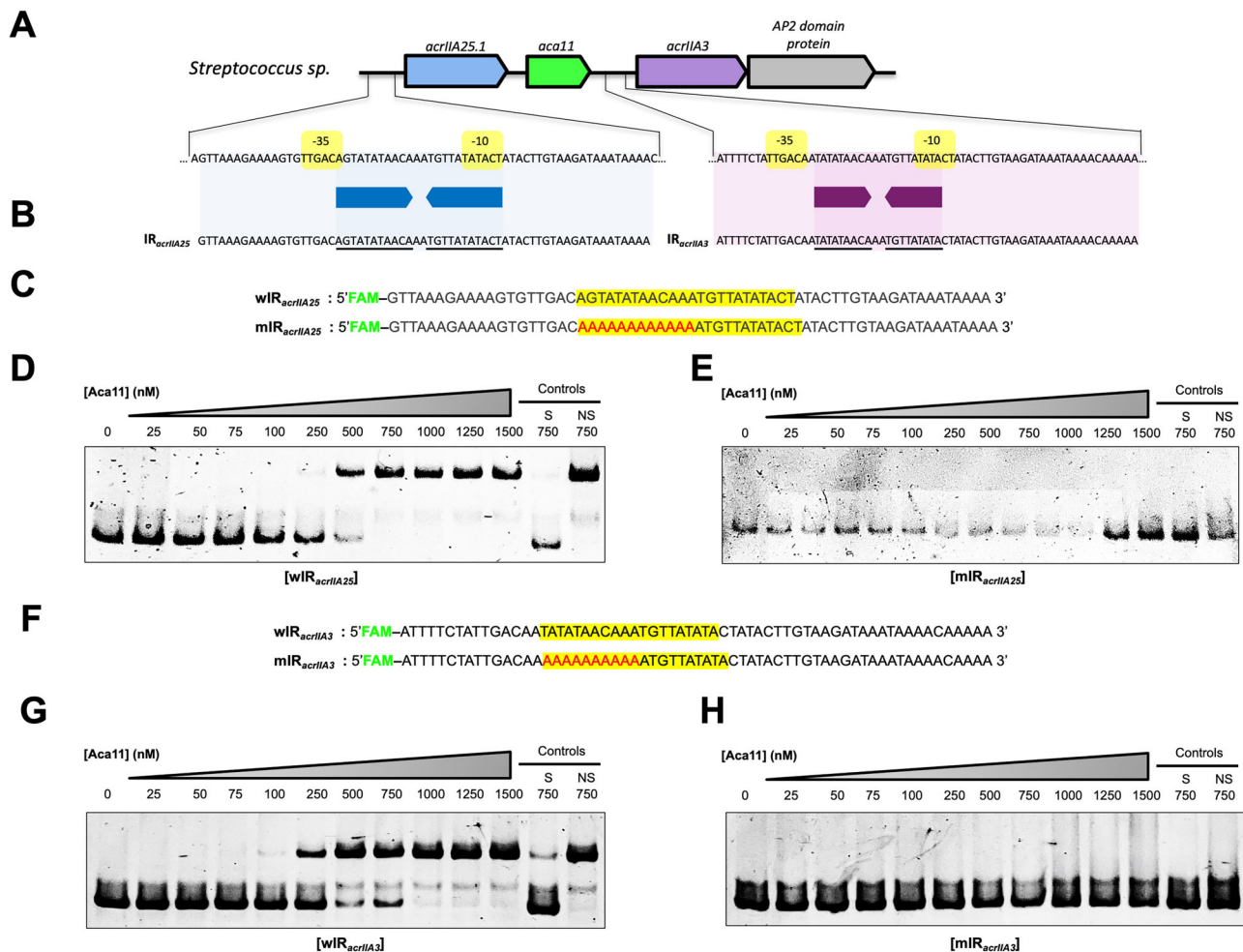


Fig. 7 | Promoter identification and specific interactions with Aca11. **A** Genomic organization of the *acrIIA25.1-aca11* and its neighboring operon with highlighted -35 and -10 promoter elements. IR sequences are represented by blue and purple arrows beneath the regulatory region. **B** DNA probe sequences used for analysis, including the putative *acrIIA25* promoter (IR_{acrIIA25}) and the putative *acrIIA3* promoter (IR_{acrIIA3}). **C** Sequences of *acrIIA25.1* promoter DNA probes tested for Aca11 binding, including the wild-type IR (wIR_{acrIIA25}) and the mutated IR (mIR_{acrIIA25}) with the disrupted IR region highlighted. **D** EMSA showing the binding of Aca11 to a 5'-FAM-labeled wIR_{acrIIA25} DNA probe. A black triangle indicates increasing concentration of Aca11. Excessive unlabeled wIR_{acrIIA25} and nonspecific

DNA probes were used as competitive controls to distinguish specific from non-specific binding. **E** EMSA results of Aca11 binding to the mIR_{acrIIA25} DNA probe. A black triangle indicates increasing concentration of Aca11. **F** Sequences of *acrIIA3* promoter DNA probes tested for Aca11 binding, including the wild-type IR (wIR_{acrIIA3}) and the mutated IR (mIR_{acrIIA3}) with the disrupted IR region highlighted. **G** EMSA showing the binding of Aca11 to a 5'-FAM-labeled wIR_{acrIIA3} DNA probe. Excessive unlabeled wIR_{acrIIA3} and nonspecific DNA probes were used as competitive controls to distinguish specific from nonspecific binding. **H** EMSA results of Aca11 binding to the mIR_{acrIIA3} DNA probe.

upstream of *acrIIA3* (IR_{acrIIA3}: AATATATAACAATGTTATATACT). The positions of these IRs suggest that Aca11 binding to the palindromic sequence could sterically hinder RNA polymerase binding, thereby repressing transcription initiation of the downstream operons.

Due to the perfect complementarity of the IR half sites in the promoter upstream of *acrIIA25.1*, we first tested a direct interaction between Aca11 and 5' FAM-labeled DNA containing the sequence of the *acrIIA25* promoter (Fig. 7C). Purified Aca11 exhibited concentration-dependent binding to this DNA, producing a clear band shift on the gel (Fig. 7D). Pre-incubation with an excess of unlabeled target-specific DNA completely abolished the shift of FAM-wIR_{acrIIA25}, confirming the specificity of Aca11 binding. In contrast, excess non-specific DNA had no effect on the FAM-wIR_{acrIIA25} band shift, demonstrating that Aca11 selectively recognizes its target sequence over non-specific DNA (Fig. 7D). Further testing of sequence-specificity was carried out using a mutant DNA fragment (mIR_{acrIIA25}) in which the inverted repeat sequence was disrupted (Fig. 7C). EMSAs with this DNA showed no detectable band shift, indicating that the inverted repeat sequence is indispensable for Aca11 binding (Fig. 7E).

We next tested whether Aca11 could also bind the promoter region upstream of the operon encoding *AcrlIA3* and the AP2-domain protein. Intriguingly, an EMSA using this DNA probe (wIR_{acrIIA3}) also led to a distinct shift upon addition of Aca11 that was abrogated by mutations in the inverted repeat sequence (mIR_{acrIIA3}), suggesting that Aca11 regulates not only its own but also a separate, neighboring operon (Fig. 7F–G). Since this operon also encodes at least one Acr protein, we hypothesized that dual regulation by the same Aca could aid in coordinating the Acr response. Considering the high similarity of the promoter sequences, this may have been enabled through a duplication event.

Together, these results establish that Aca11 specifically binds two IRs overlapping the promoters of the *acrIIA25.1-aca11* and *acrIIA3-AP2* operons. As a dimeric HTH transcriptional regulator, Aca11 likely regulates gene expression by directly interacting with these IR sequences.

Strategy of promoter recognition by Aca11

Based on the results so far, it is likely that Aca11, like other Aca family members, is a dimeric transcriptional regulator with an HTH motif. The HTH motif is a common feature of Aca proteins and is critical for binding

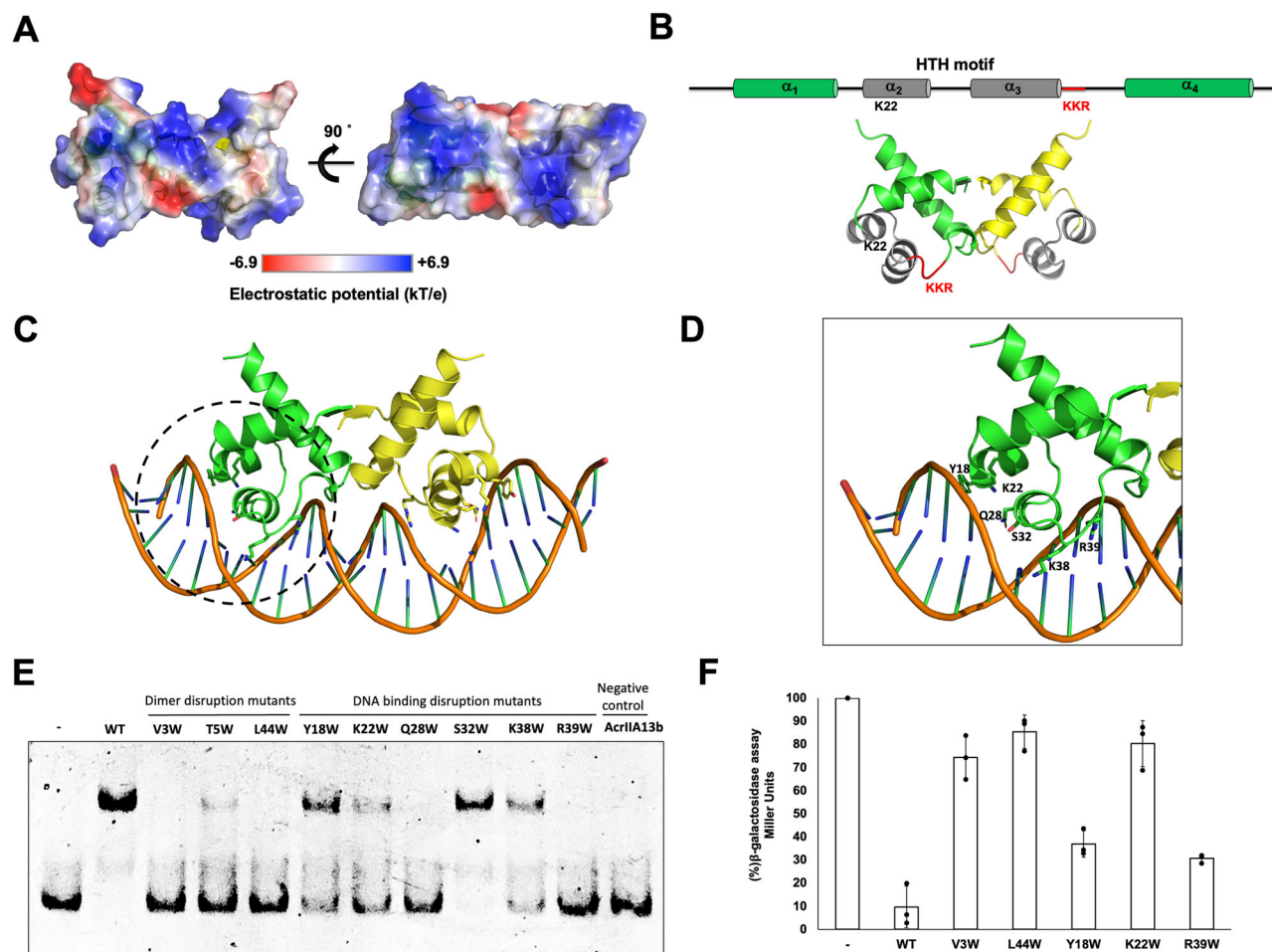


Fig. 8 | Strategy of promoter recognition by dimeric Aca11. A Surface electrostatic potential of dimeric Aca11. **B** Location of the tentative HTH motif and the adjacent KKR motif within the Aca11 structure. **C** Predicted complex model of Aca11 bound to its target IR DNA sequence, generated using AlphaFold3. **D** Magnified view of Aca11-DNA interacting region, highlighted with a black dashed circle in (B). Residues predicted to interact with the palindromic DNA sequence, Y18, K22, Q28, S32, K38 and R39, are labeled in the structure using one-letter amino acid codes. **E** EMSA results comparing binding ability of wild-type Aca11 and mutant variants

to a 5'-FAM-labeled wIR_{Long} DNA probe. V3W, T5W and L44W are dimer disruption mutants and Y18W, K22W, Q28W, S32W, K38W and R39W are DNA binding disruption mutants. AcrIIA13b, which is not expected to bind DNA, was included as a negative control. **F** β-galactosidase activity of the *acrIIA25.1-acai11*-LacZ reporter with Aca11 wild-type or its mutants. Activities were calculated as Miller units, and background-corrected by subtracting Aca only values, and normalized to the LacZ only (-) control (set to 100%). *N* = 3 biologically independent experiments.

IRs within promoter regions to repress transcription. While these general characteristics align with those observed for Aca7, we aimed to explore the specific molecular mechanism of Aca11 in recognizing its target promoter, focusing on its unique sequence and structural features.

The Aca11 dimer has a highly charged surface (± 6.9 kT/e), with positively charged regions prominently localized around the α₃ helices and the α₃-α₄ connecting loops of both monomers. These positively charged regions align with their role in interacting with the negatively charged DNA backbone and were identified as key regions for promoter recognition (Fig. 8A). Dimeric Aca11 showed symmetrically arranged HTH motifs formed by the α₂ and α₃ helices of each monomer, along with a positively charged KKR motif immediately following the α₃ helix, contributing additional electrostatic complementarity for DNA binding (Fig. 8B). A model of the Aca11/dsDNA (IR_{acrIIA25}) complex, predicted using AlphaFold3, further supported the importance of the HTH motif in DNA recognition. The model highlighted residues from α₂, α₃, and their connecting loops extending toward the DNA, suggesting specific interactions with the IR sequence (Fig. 8C). Detailed analysis of the complex identified residues Y18, K22, Q28, S32, K38 and R39 as potential DNA-contacting residues, with positively charged side chains positioned to interact with the DNA backbone (Fig. 8D). The schematic representation of the predicted Aca11-DNA complex illustrates that these key residues establish direct polar contacts with the DNA,

supporting their critical role in promoter recognition (Supplementary Fig. 6). Most of these residues are also fully conserved among Aca11 homologs, as revealed by sequence conservation analysis (Supplementary Fig. 4B).

Experimental validation of promoter recognition by Aca11 was conducted through site-directed mutagenesis, generating variants (V3W, T5W, L44W, Y18W, K22W, Q28W, S32W, K38W and R39W). Using the wIR_{acrIIA25} DNA probe, these mutants were analyzed through EMSAs to compare the DNA-binding affinities with wild-type Aca11 and its mutants. EMSAs revealed significantly reduced DNA-binding affinities for mutants that disrupted dimerization (V3W, T5W and L44W). Since these mutations were previously shown to destabilize the dimeric structure of Aca11, the corresponding loss of DNA-binding activity underscores the essential role of dimerization in its function. For DNA-binding disruption mutants (Y18W, K22W, Q28W, S32W, K38W and R39W), varied impacts on binding affinity were observed. Q28W and R39W exhibited the most severe reductions, suggesting that these residues are critical for recognizing the IR sequence. Other mutants showed milder effects, with most displaying reduced binding compared to wild type. However, S32W retained binding affinity comparable to the wild-type protein, indicating its limited involvement in direct promoter interaction (Fig. 8E). Overall, Aca11 relies on dimerization to recognize IR sequences. The α₂ and α₃ helices, along with the

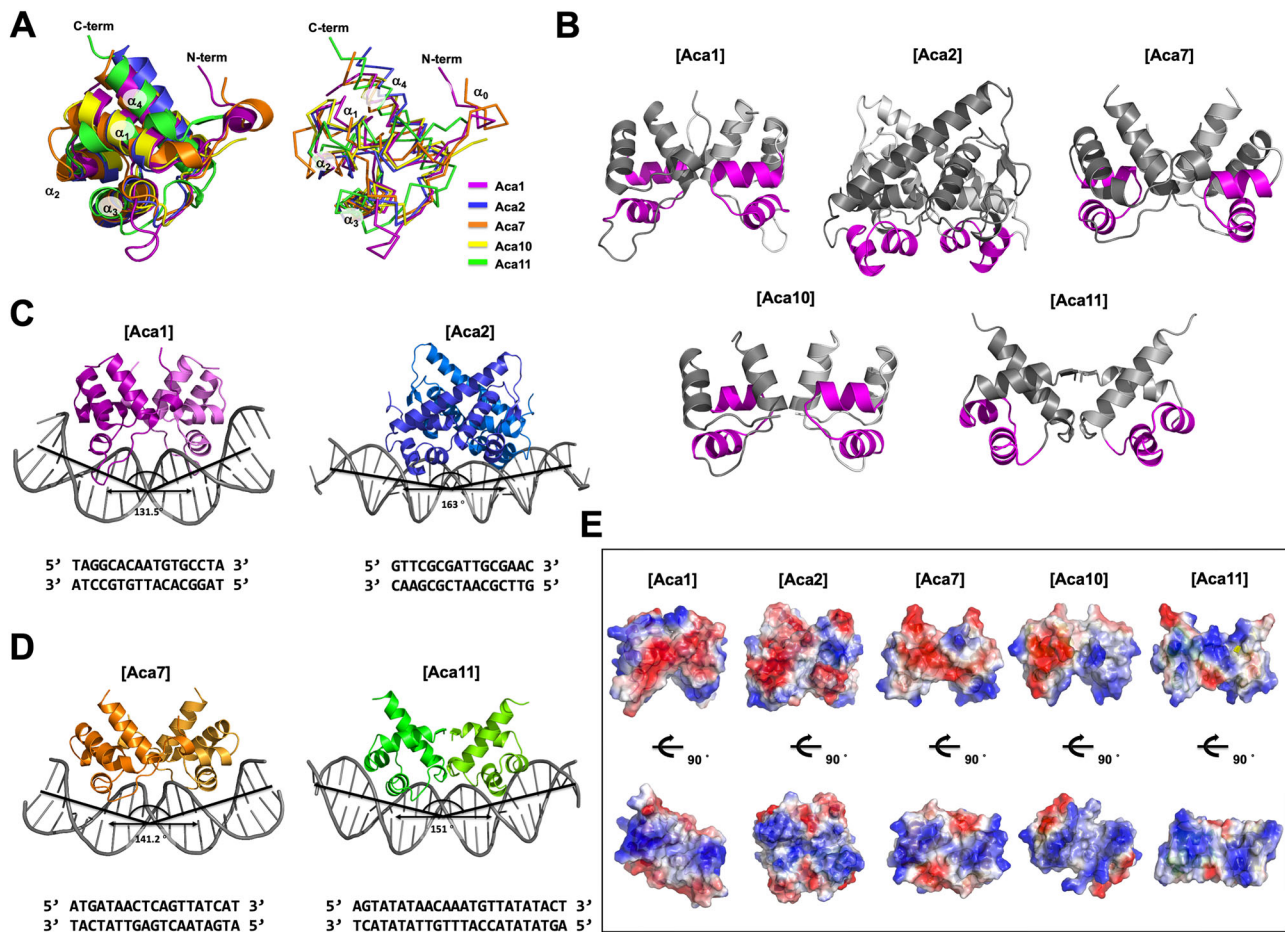


Fig. 9 | Structural comparison of Aca family members. **A** Ribbon representation showing the superimposed monomeric structure of the Aca family proteins, highlighting the conserved α -helix bundles and the variations in the positioning across members. **B** Dimeric structures of Aca1, Aca2, Aca7, Aca10, and Aca11, with the HTH motifs involved in promoter recognition highlighted in magenta.

C Experimentally determined complex structures of Aca1-DNA (PDBID: 7VJM) and Aca2-DNA (PDBID: 7VJQ)³². DNA-bending angles and IR sequences that bound to Aca are shown. **D** Modeled complex structures of Aca7-DNA and Aca11-DNA. DNA-bending angles and IR sequences that bound to Aca are shown. **E** Surface electrostatic potential of dimeric Aca protein family.

downstream KKR motif, serve as key structural elements that enable Aca11 to bind its target promoter.

To determine whether Aca11 regulates transcription *in vivo*, we performed a β -galactosidase reporter assay with the *acrIIA25.1-aca11* promoter fused to LacZ. Expression of wild-type Aca11 led to a clear decrease in LacZ activity, consistent with promoter-specific repression. In contrast, the dimer disruption mutants (V3W and L44W) largely failed to suppress LacZ expression, highlighting the importance of stable dimer formation for regulatory function *in vivo*. DNA binding mutants also reduced regulatory activity compared to wild-type, with K22W showing the strongest loss of repression. Interestingly, R39W maintained relatively strong repression *in vivo*, despite impaired binding *in vitro* (Fig. 8F). This discrepancy suggests that the residues most critical for *in vivo* repression may not fully overlap with those identified by EMSA. Nonetheless, the overall trend observed *in vitro* was also validated under *in vivo* conditions. Taken together, the *in vitro* and *in vivo* assays demonstrate that Aca11 requires dimerization and the α_2 - α_3 helices for promoter recognition and transcriptional repression.

Structural conservation and diversity among Aca family members

Including Aca7 and Aca11 presented in this paper, the structures of a total of five Aca proteins (Aca1, Aca2, Aca7, Aca10 and Aca11) have been elucidated by our groups and others^{26,29,31,32}. Given the similar functions of these Aca proteins in Acr regulation, we compared and analyzed the structures of

Aca proteins to understand their DNA-binding modes and mechanisms of action. Sequence alignment using PROMALS3D showed low sequence identity across Aca proteins (Supplementary Fig. 7); however, structure-based alignment revealed a highly preserved α -helix bundle architecture with a conserved HTH motif (Fig. 9A and Supplementary Fig. 8). Structure superposition demonstrates that although the sequence identity among Aca proteins is low, the structures of the Aca monomers are highly similar (Fig. 9A).

However, when comparing the functional dimer units, significant structural differences were observed (Fig. 9B). For example, Aca11 displays wide spacing between the recognition helices of each monomer, likely reflecting its adaptation to its larger IR sequence target. When comparing Aca2 and Aca11, it becomes evident that HTH motifs are oriented in significantly different positions (Fig. 9B). Although the dimeric structure of Aca11 is the most distinct within the Aca family, it is not unusual, as there are other dimeric proteins with HTH motifs oriented similarly to that of Aca11⁴³. Recent studies have reported that Aca2 not only binds to DNA but also interacts with RNA to regulate translation²⁷. The distinct structural feature of Aca2 may enable such a function, suggesting that further research is needed to investigate whether other Aca proteins also possess this capability.

Despite this diversity in DNA-binding modes, the positioning of inverted repeat (IR) sequences within the promoter regions is largely conserved across Aca family members. IR sequences targeted by Aca proteins are typically located overlapping with the core promoter elements, spanning

the −35 and −10 regions, facilitating precise repression of RNA polymerase activity. Another interesting similarity among the Aca family members in recognizing their promoters is their ability to either induce DNA bending or bind to bent DNA. In the experimentally determined Aca1-DNA and Aca2-DNA complex structures, the bound DNA is bent to varying degrees (Fig. 9C). Additionally, modeling of Aca7 and Aca11 with their corresponding IR sequences, the bound DNA consistently adopts a bent conformation (Fig. 9D) whereas docking studies with unbent DNA show poor binding efficiency (Supplementary Fig. 9A–B), suggesting that DNA bending is an essential phenomenon for Aca family members to bind effectively to their promoters. Induction of DNA bending may be facilitated by the similar electrostatic surface properties shared across Aca families, with a highly basic surface in the DNA-binding region that may have evolved for strong DNA binding (Fig. 9E).

The structural variation of Aca dimers could contribute to the specificity of IR recognition by different Aca proteins. These findings suggest that while the conserved HTH motif serves as a universal DNA-binding scaffold within Aca proteins, the observed structural variations may enable each member to adapt to distinct regulatory functions and target specific DNA sequences. The IR-specific binding capabilities demonstrated by Aca7 and Aca11 underscore the critical role of this conserved core in mediating promoter recognition.

Methods

Protein expression and purification

All the sequences of nucleotides used in this study are provided in Table 2.

The *aca7* gene from *Halomonas caseinilytica* (accession code: WP_064702654.1) and *aca11* gene from *Streptococcus* sp. (accession code: WP_009730540.1) were synthesized by Bionics (Daejeon, Republic of Korea) and individually subcloned into the pET21a vector (Novagen, Wisconsin, USA), which was designed to include a C-terminal hexa-histidine tag to facilitate affinity chromatography. Cloning was performed using the *Nde*I and *Xho*I restriction sites. Each recombinant plasmid encoding full-length Aca7 (residue 1–68) and Aca11 (residue 1–63), was independently transformed into competent *Escherichia coli* BL21(DE3) cells.

For each protein, a single transformed colony was selected and cultured overnight at 37°C in 5 mL lysogeny broth (LB) containing 50 µg/mL ampicillin. A 1 mL aliquot of the overnight culture was then used to inoculate 1 L of LB medium containing the same antibiotic. The cultures were grown at 37°C until the optical density measured at 600 nm (OD₆₀₀) reached 0.7–0.8. After this stage, the incubation temperature was lowered to 20°C, and 0.25 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce expression of *aca7* or *aca11*. Each induced culture was further incubated for 18 h at 20°C in a 200 rpm shaking incubation.

Following incubation, cultured cells expressing *aca7* or *aca11* were harvested separately by centrifugation at 3500 g for 15 min at 20°C. The pelleted cells were individually resuspended in a lysis buffer (20 mM Tris-HCl (pH 8.0) and 500 mM NaCl) and lysed via ultrasonication at 4°C. The cell lysates and the supernatants were separated by centrifugation at 14,000 g for 30 min at 4°C. For each protein, the supernatant was collected and incubated independently with Ni-nitrilotriacetic acid (NTA) affinity resins at 4°C for 2 h. The mixtures were then loaded onto gravity-flow columns (Bio-Rad, Hercules, CA, USA), and the resin was washed with 50 mL lysis buffer to remove non-specifically bound impurities.

Elution of Aca7 and Aca11 was performed using a buffer containing 20 mM Tris-HCl (pH 8.0) 500 mM NaCl, 250 mM Imidazole. The eluted proteins were further purified individually by size-exclusion chromatography (SEC) on a Superdex 200 Increase 10/300 GL column (GE Healthcare, Waukesha, WI, USA), pre-equilibrated with SEC buffer (20 mM Tris-HCl (pH 8.0) and 150 mM NaCl). Each target protein was eluted from SEC and collected, pooled, and concentrated to a final concentration of 8.9 mg/mL for Aca7 and 8.6 mg/mL for Aca11 for use in crystallization. The purity of each protein was visually assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Crystallization and data collection

The crystallization of Aca7 was performed using the hanging-drop vapor diffusion method at 20°C. Initial crystals were grown by equilibrating a mixture of 1 µL protein solution (8.9 mg/mL protein in SEC buffer) and 1 µL of a reservoir solution (30% (w/v) PEG 4000, 0.1 M Tris pH 8.5, 0.2 M Lithium sulfate) against 0.3 mL of reservoir solution. Crystals appeared after 72 h. Optimization of the crystallization conditions resulted in improved crystals using a reservoir solution containing 32% (w/v) PEG 4000, 0.1 M Tris pH 8.5, 0.2 M Lithium sulfate, equilibrated against 0.5 mL of reservoir solution.

Similarly, the crystallization of Aca11 was performed using a hanging-drop vapor diffusion method under analogous conditions. A 1 µL protein solution (8.6 mg/mL protein in SEC buffer) was mixed with 1 µL of a reservoir solution containing 20% (w/v) PEG 3000, 0.1 M Sodium citrate pH 5.2. The drops were equilibrated against 0.3 mL of reservoir solution, and crystals of Aca11 obtained within 24 h at 20°C.

For cryo-protection, individual crystals of Aca7 and Aca11 were carefully transferred to their respective reservoir solutions supplemented with 30% (v/v) glycerol. X-ray diffraction data were collected at −178°C on the beamline BL-5C beamline (Aca7) or the BL-11C beamline (Aca11), both at the Pohang Accelerator Laboratory (Pohang, Korea). Data processing including indexing, integration, and scaling, was carried out using HKL3000⁴⁴.

Structure determination and analysis

The structures of Aca7 and Aca11 were determined through the molecular replacement phasing method, implemented using PHASER within the PHENIX package³⁵. Predicted structural models for each protein, generated by AlphaFold2, served as search models for the molecular replacement process³⁷. The initial models were constructed automatically using AutoBuild from the PHENIX package, followed by iterative cycles of manual model building and refinement performed in Coot⁴⁵ and phenix.refine⁴⁶. Structure refinement included reciprocal-space refinement (XYZ), real-space refinement, rigid-body refinement, and both individual and group B-factor refinement. X-ray/stereochemistry and X-ray/ADP weights were automatically optimized throughout. Secondary structure restraints and experimental phase restraints were applied, and torsion-angle non-crystallographic symmetry (NCS) restraints were used when applicable. Hydrogen atoms were automatically added, and solvent molecules were updated during refinement.

Manual rebuilding in Coot was aided by the application of torsion restraints, planar peptide restraints, and trans-peptide restraints, with careful validation using Ramachandran and rotamer outlier checks, as well as assessment of real-space density fit.

Structural quality and stereochemical accuracy were assessed using MolProbity⁴⁷, which provided comprehensive validation metrics. All visualizations and structural representations were prepared using PyMOL⁴⁸.

SEC-multi-angle light scattering (MALS) analysis

The absolute molecular masses of Aca7 and Aca11 in solution were individually measured using SEC-coupled multi-angle light scattering (SEC-MALS). For each protein, purified Aca protein solutions were separately injected onto a Superdex 200 Increase 10/300 GL column (24 mL, GE Healthcare) pre-equilibrated with SEC buffer. Chromatographic separation was carried out at a constant flow rate of 0.5 mL/min, and SEC-MALS was conducted at 20°C. Each SEC run was integrated with a DAWN-Treos MALS detector (Wyatt Technology, Santa Barbara, USA), connected to an ÄKTA explorer system (GE Healthcare). Bovine serum albumin (BSA) was used as the molecular weight standard for calibration. The molecular mass of each protein was analyzed using the ASTRA software (Wyatt Technology), providing accurate measurements of the absolute molecular masses in solution.

Mutagenesis

Site-directed mutagenesis was performed using the Quick-Change kit (Stratagene) following the manufacturer's protocol. The introduced

Table 2 | Oligonucleotides used in this study

Name	Sequence (5' → 3')
Aca7	ATGATCGATGCGGTAAACACTACGATCCGAAACCTGGCGCCGGAACCTGGTTGCTGCGCTGGCGGTTACCGCACCCAGAAA- GAACCTGGCGGAACGTCGTGATGTTCTCGTACCTACCTTCAGCTGCTGGGTAAGGTCAGAAATCTATGTCTTATGCGGTTTCAGGT- TATGCTGGAACACAGGTTATCCAGGATGGTGAACCC
Aca7 R5W(Mut)- sense	ATATACATATGATCGATGCGTGGAAACACTACGATCCGAAACCT
Aca7 R5W(Mut)- antisense	AGGTTCCGATCGTAGTGTTCACGCGATCGATCATATGTATAT
Aca7 E29W(Mut)- sense	ACCGGCACCCAGAAATGGCTGGCGGAACGTCCTG
Aca7 E29W(Mut)- antisense	ACCGGCACCCAGAAATGGCTGGCGGAACGTCCTG
Aca7 E32W(Mut)- sense	CCCAGAAAGAACTGGCGTGGCGTCTGGATGTTTCTCG
Aca7 E32W(Mut)- antisense	CGAGAAACATCCAGACGCCACGCCAGTTCTTTCTGGG
Aca7 R38W(Mut)- sense	GAACGTCGTGATGTTTCTGGACCTACCTTCAGCTGCTG
Aca7 R38W(Mut)- antisense	CAGCAGCTGAAGGTAGGTCCAGAAACATCCAGACGTTT
Aca7 Y40W(Mut)- sense	GGATGTTTCTCGTACCTGGCTTCAGCTGCTGGGTAA
Aca7 Y40W(Mut)- antisense	TTACCCAGCAGCTGAAGCCAGGTACGAGAAACATCC
Aca7 K46W(Mut)- sense	CCTACCTTCAGCTGCTGGTGGGTCAGAAATCTATGCTTA
Aca7 K46W(Mut)- antisense	TAAGACATAGATTTCTGACCCCAACCCAGCAGCTGAAGGTAGG
Aca7 K49W(Mut)- sense	GCTGCTGGGTAAAGGTCAAGTGTCTATGTCTTATGCGGTTTC
Aca7 K49W(Mut)- antisense	GAACCCGACATAGACATAGACCCACTGACCTTTACCCAGCAGC
wlR1-wlR2-wlR3(Aca7)- sense	FAM_AATTCAGGGAATGGCTTGAATGATAACTCAGTTATCATATAGTGTATTCAACAGATGAGGAGCGG
wlR1-wlR2-wlR3(Aca7)-antisense	CGCCTCCTCATCTGTTGAATACAGTATATGATAAAGTATCATTTCAAGCCATTCCTGAAATT
mlR1-wlR2-wlR3(Aca7)- sense	FAM_AAAAAAAAATGGCTTGAATGATAACTCAGTTATCATATAGTATTCAACAGATGAGGAGCGG
mlR1-wlR2-wlR3(Aca7)-antisense	CGCCTCCTCATCTGTTGAATACAGTATATGATAAAGTATCATTTCAAGCCATTTCTTTTTT
wlR1-wlR2-wlR3(Aca7)- sense	FAM_AATTCAGGGAATGGCTTGAACAAAAAACAGTTATCATATAGTGTATTCAACAGATGAGGAGCGG
wlR1-wlR2-wlR3(Aca7)-antisense	CGCCTCCTCATCTGTTGAATACAGTATATGATAAAGTATCATTTTTCAGCCATTCCTGAAATT
wlR1-wlR2-miR3(Aca7)- sense	FAM_AATTCAGGGAATGGCTTGAATGATAACTCAGTTATCATATAAAAAAAAAGATGAGGAGCGG
wlR1-wlR2-miR3(Aca7)-antisense	CGCCTCCTCATCTTTTTTTTTTATATGATAACTGAGTTATCATTTCAAGCCATTCCTGAAATT
mlR1-miR2-miR3(Aca7)- sense	FAM_AAAAAAAAATGGCTTGAACAAAAAACAGTTATCATATAAAAAAAAAGATGAGGAGCGG
mlR1-miR2-miR3(Aca7)-antisense	CGCCTCCTCATCTTTTTTTTTTATATGATAACTGTTTTTTTTTCAAGCCATTTTTTTTTT
pQF_acrIF11-aca7-promoter_lacZ- sense	GGGGGCTAGCGGTAAAAAATTCAGGGAATGGCTTGAATGATAACTCAGTTATCATATAGTGTATTCAACAGATGAGGAGCGG
pQF_acrIF11-aca7-promoter_lacZ- antisense	CCCGGTACCCCATCTGTTGAATACAGTATATGATAAAGTATCATTTCAAGCCATTCCTGAATTTTTACGGGTAGCCCCC
Aca11	ATGAAAGTTGATACCAACAGATTGAATGGCTGCTGAAAAATGCAAGCGGTTATCAGATTAGCAAAATGAGCGGTTGTCACAGCCGAC- CATTAGGCCCTGATTAAATAAAAAACGTAGCATTGAAATCTGACCAATTGAAACCGGTCATAAAGTACCCGAATCGAGATGCAGAAACCCCGG
Aca11 V3W(Mut)- sense	AGAAGGAGATATACATATGATGAATGGGATACCAACAGATTGAATGGCTGC
Aca11 V3W(Mut)- antisense	GCAGCCATTCAATCTGTTGGTATCCCATTTTCATCATATGTATATCTCTTCT
Aca11 T5W(Mut)- sense	GGAGATATACATATGATGAAGTTGATTGGAACAGATTGAATGGCTGCTGAAAAAT
Aca11 T5W(Mut)- antisense	ATTTTCAGCAGCCATTCAATCTGTTTCCCAATCAACTTCATCATATGTATATCTCC
Aca11 Y18W(Mut)- sense	CTGAAAAATGCAAGCGGTGGCAGATTAGCAAAATGAGCG
Aca11 Y18W(Mut)- antisense	CGCTCATTTTGTAAATCTGCCAACCGCTTGCATTTTTCAG
Aca11 K22W(Mut)- sense	AAGCGGTTATCAGATTAGCTGGATGAGCGGTTGTTGCACAGC
Aca11 K22W(Mut)- antisense	GCTGTGCAACACCGCTCATCCAGCTAATCTGATAACCGCTT

Table 2 (continued) | Oligonucleotides used in this study

Name	Sequence (5' → 3')
Aca11 Q28W(Mut)- sense	GAGCGGTGTTGCATGGCCGACCATTAGCGC
Aca11 Q28W(Mut)- antisense	GCGCTAATGGTCGGCCATGCAACACCGCTC
Aca11 S32W(Mut)- sense	TGTTGCACAGCCGACCATTTGGGCCCTGATTAATAAAAAAC
Aca11 S32W(Mut)- antisense	GTITTTTATTAAATCAGGCCCAATGTGCGCTGTGCAACA
Aca11 K38W(Mut)- sense	CCATTAGCGCCCTGATTAAATAAATGGCGTAGCATTGAAAAATCTGACCAT
Aca11 K38W(Mut)- antisense	ATGGTCAGATTTTCAATGCTACGCCATTATTAAATCAGGGCGCTAATGG
Aca11 R39W(Mut)- sense	ATTAGCGCCTGATTAAATAAAAAATGGAGCATTGAAAAATCTGACCATTGAA
Aca11 R39W(Mut)- antisense	TTC AATGGTCAGATTTTCAATGCTCCATTTTATTATATCAGGGCGCTAAT
Aca11 L44W(Mut)- sense	TAATAAAAAACGTAGCATTTGAAATTTGGACCATTTGAAACCGGTCATAAACTG
Aca11 L44W(Mut)- antisense	CAGTTTATGACCGGTTTCAATGGTCCAATTTTCAATGCTACGTTTTTTATTTA
wIR _{acrlIA25} (Aca11)- sense	FAM_GTTAAAGAAAAAGTTTGACAGTATATAACAAATGTTTATATACTATACCTTGTAAGATAAAATAAAAA
wIR _{acrlIA25} (Aca11)- antisense	TTTTATTATCTTACAAGTAGTATATAACATTTTGTATATACTGTCAACACATTTTCTTTAAC
mIR _{acrlIA25} (Aca11)- sense	FAM_GTTAAAGAAAAAGTTTGACAAAAAATAAAAAATGTTATATACTATACTGTGAAGATAAAATAAAAA
mIR _{acrlIA25} (Aca11)- antisense	TTTTATTATCTTACAAGTAGTATATAACATTTTTTTTTTTTGTCAACACATTTTCTTTAAC
wIR _{acrlIA3} (Aca11)- sense	FAM_ATTTTCTATTGACAATATATAACAAATGTTTATATACTATACCTTGTAAGATAAAATAAAAA
wIR _{acrlIA3} (Aca11)- antisense	TTTTTGTTTTTATTATCTTACAAGTAGTATATAACATTTTGTATATATTGTCAATAGAAAAAT
mIR _{acrlIA3} (Aca11)- sense	FAM_ATTTTCTATTGACAAAAAATAAAAAATGTTTATATACTATACTGTGAAGATAAAATAAAAA
mIR _{acrlIA3} (Aca11)- antisense	TTTTTGTTTTTATTATCTTACAAGTAGTATATAACATTTTTTTTTTTTGTCAATAGAAAAAT
pQF_acrlIA25.1-aca11-promoter_lacZ- sense	GGGGGTAGCTTTTAAAAAATTAAAAAAGTTAAAGAAAAAGTTTGACAGTATATAACAAATGTTTATATACTATACTGTGAAGATAAAATAAAAAACGGTACCGGGG
pQF_acrlIA25.1-aca11-promoter_lacZ- antisense	CCCCGGTACCGTTTTTTTGTTTTTATTATCTTACAAGTAGTATATAACATTTTGTATATACTGTCAACACATTTTCTTTAAACATTTTTTAAATTTTAAAGCTAGCCCCC

mutations were verified by DNA sequencing. Mutant proteins were expressed and purified as described above for the wild-type proteins.

Identification of promoters and potential binding sites for Aca7 and Aca11

The 5' regulatory region upstream of the *acrIF11-aca7* operon from *H. caseinilytica* and the *acrIIA25.1-aca11-acrIIA3* operon from *Streptococcus* sp. were analyzed to predict potential promoter sequences. Promoter identification was initially conducted using BPROM (<http://www.softberry.com>), followed by manual curation to refine the predictions. Alignment of promoter sequences was performed using Clustal Omega⁴⁹. Additionally, the De Novo DNA Server (Salis Lab⁴²) was used to predict transcription initiation rates across the two loci, from 200 bp upstream of the start codon of the first gene in the operon to 30 bp downstream from the stop codon of the last gene of the operon. To assess the presence of potential binding sites for Aca7 and Aca11, the Repeat Finder plugin in Geneious Prime version 2022.1.1 (<https://www.geneious.com>) was employed. Identified repeat sequences were further curated manually to ensure accuracy and relevance to the study.

Electrophoretic mobility shift assays (EMSA)

DNA oligonucleotides containing the identified inverted repeat sequences were synthesized by Bionics. For the fluorescent labeling, sense oligonucleotides were synthesized with a 5' Amino Modifier C6. The 5' FAM labeling was achieved by reacting amino-modified oligonucleotides with 6-FAM NHS ester in a molar ratio of 10:1 (6-FAM to oligonucleotide) at 20 °C for 4 h. The labeled oligonucleotides were purified by ethanol precipitation, annealed with complementary non-labeled antisense oligonucleotides in a 1:1 molar ratio by incubating the mixture at 100 °C for 3 min, followed by gradual cooling to room temperature to form dsDNA suitable for EMSA.

For binding studies with Aca7, 230 nM of 5'-6 FAM labeled dsDNA was incubated with varying concentrations of purified Aca7 protein (0–1500 nM) at 4 °C for 1 h. To assess DNA-binding specificity, an excess (5000 nM) of unlabeled specific or non-specific dsDNA was pre-incubated with Aca7 at 4 °C for 30 min, followed by the addition of the labeled target specific DNA and an additional 1 h incubation at 4 °C.

Similarly, for Aca11, 200 nM of 5'-6FAM labeled dsDNA was incubated with increasing concentrations of purified Aca11 protein (0–1500 nM) at 4 °C for 1 h. The specificity of Aca11 binding was also examined using the same pre-incubation method with unlabeled dsDNA.

To identify the specific DNA region for Aca binding, additional EMSAs were performed using dsDNA with disrupted inverted repeat sequences. The experimental procedures were identical to those for the wild-type dsDNA, except for the sequence variations in the mutated construct.

To analyze the binding mode of Aca proteins to their promoter regions, 1000 nM of purified wild-type or mutant Aca proteins were incubated with 5'-6FAM labeled target dsDNA (230 nM) in SEC buffer at 4 °C for 1 h. All the prepared samples were then separated by gel electrophoresis at 100 V on a 10% native 0.5 X TBE (Tris-borate EDTA) polyacrylamide gel for 1 h. Afterward, gels were stained with SYBR Gold (Invitrogen, Waltham, MA, USA) and visualized according to the manufacturer's instructions.

In vivo β -galactosidase reporter assay

The β -galactosidase reporter constructs containing either the *acrIF11-aca7* promoter or the *acrIIA25.1-aca11* promoter were generated by cloning each promoter upstream of the *lacZ* gene in the pQF vector (Addgene #48094) using a Gibson Assembly Kit (NEB). For the assay, BL21(DE3) cells were transformed with one of the following plasmid combinations: (i) *LacZ* plasmid alone, (ii) *LacZ* plasmid co-expressed with *Aca7* or *Aca11*, or their variants, or (iii) *Aca7* or *Aca11* plasmid alone to measure background activity. Single colonies were selected and grown overnight at 37 °C in LB medium supplemented with 1% (w/v) glucose. Overnight cultures (0.2 mL) were inoculated into 20 mL of fresh LB medium containing 1% (w/v) glucose, followed by incubation at 37 °C with shaking at 220 rpm until reaching

an OD₆₀₀ of 0.3. Protein expression was induced by adding 0.1 mM IPTG, and the cultures were further incubated for 1 h at 37 °C. After induction, 1 mL of each culture was harvested by centrifugation at 14,000 rpm for 10 min at 4 °C. Cell pellets were resuspended in Z-buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 1 mM MgSO₄, 10 mM KCl, and 50 mM β -mercaptoethanol), followed by permeabilization with 30 μ L of 0.1% SDS and 60 μ L chloroform. Samples were vortexed for 10 s and incubated at 30 °C for 5 min. Subsequently, 4 mg/mL o-Nitrophenyl- β -D-galactopyranosid was added to each reaction, vortexed for 10 sec, and incubated for 5 min at 30 °C.

The reactions were terminated by adding 0.3 mL of 1 M Na₂CO₃, and the mixtures were vortexed for 10 s. Samples were centrifuged at 14,000 rpm for 5 min, and the absorbance values at OD₄₂₀, OD₅₅₀, and OD₆₀₀ were measured using a NanoDrop spectrophotometer with 1 mL quartz cuvette. β -galactosidase activity was calculated as Miller units. For each condition, activity from cells carrying the Aca plasmid alone was subtracted as background. The background corrected values were normalized to the LacZ only control (set to 100%). The raw data are provided in Supplementary Data 1.

Sequence alignment

Amino acid sequences of Aca7 and Aca11 homologs from various species were aligned using Clustal Omega⁴⁹.

Modeling of Aca7-DNA, Aca11-DNA complex structure

The Aca7/DNA and Aca11/DNA complexes were modeled using an AlphaFold3⁵⁰. To predict the complex structures, the amino acid sequences of Aca7 and Aca11 were individually submitted to AlphaFold3 along with their respective IR sequences (- 5'-ATGATAACTCAGTTATCAT-3' for Aca7 and 5'-AGTATATAACAAATGTTATATACT-3' for Aca11).

Statistics and reproducibility

All reporter assays were performed independently at least three times ($n = 3$) with similar results. Data are presented as mean $\pm$ standard deviation (SD).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Atomic coordinates and structure factors for the reported crystal structures have been deposited with the Protein Data Bank under accession codes 9LX1 (Aca7) and 9LX2 (Aca11). Uncropped original gels are provided at the Supplementary Fig. 10. The raw data for In vivo β -galactosidase reporter assay are provided in Supplementary Data 1.

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Author contributions

H.H.P. designed the research and supervised the project. S.Y.L. expressed and purified the proteins and performed crystallization and structure determination. S.Y.L. conducted biochemical and EMSA experiments as well as in vivo report assays. N.B. and P.C.F. performed the promoter analysis. J.H.L. assisted with structural determination. H.H.P. and S.Y.L. interpreted the data and wrote the manuscript with contributions from all authors. All authors reviewed and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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