Supporting Information

Structural basis for anti-CRISPR repression mediated by bacterial operon proteins Aca1 and Aca2

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Supplementary Table 1. DNA substrates for crystallization and ITC experiments.

DNA substrates for crystallization

Name	Sequence
19-bp 5'-overhang JBD30 IR2 DNA	5′-TATAGGCACAATGTGCCTAA -3′ 3′- TATCCGTGTTACACGGATTA-5′
26-bp 5'-overhang ZF40-IR1 DNA	5′-TTGCTTGTTCGCGATTGCGAACATATA -3′ 3′- ACGAACAAGCGCTAACGCTTGTATATA-5′

DNA substrates for ITC experiments

Name	Sequence
17-bp IR2	5′-TAGGCACAATGTGCCTA-3′ 3′-ATCCGTGTTACACGGAT-5′
19-bp IR2	5′-TTAGGCACAATGTGCCTAA-3′ 3′-AATCCGTGTTACACGGATT-5′
21-bp IR2	5′-ATTAGGCACAATGTGCCTAAT-3′ 3′-TAATCCGTGTTACACGGATTA-5′
21-bp IR1	5′-AAGCGGCACACTGTGCCTATT-3′ 3′-TTCGCCGTGTGACACGGATAA-5′
23-bp IR2	5′-AATTAGGCACAATGTGCCTAATC-3′ 3′-TTAATCCGTGTTACACGGATTAG-5′
26-bp ZF40-IR1 DNA	5′-TGCTTGTTCGCGATTGCGAACATATA-3′ 3′-ACGAACAAGCGCTAACGCTTGTATAT-5′

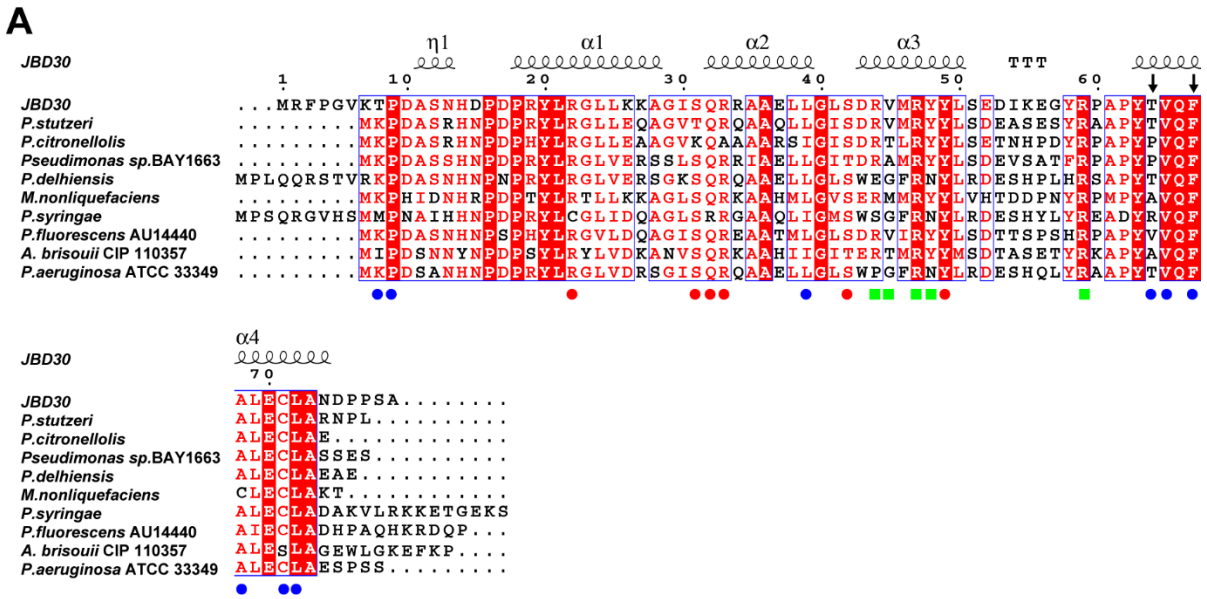
Supplementary Table 2. Thermodynamic parameters of ITC experiments.

DNA	Protein	*N*	K_d (nM)	ΔH (cal/mol)	ΔS (cal/mol/K)
21-bp IR2	Aca1 wt	1.00*	74.1	-7450	7.64
21-bp IR2	R44A		N.D.		
21-bp IR2	R47A		N.D.		
21-bp IR2	V45A	1.00	363.6	-4982	12.8
21-bp IR2	Y48A	1.00	2857.1	4383	40.1
21-bp IR2	R59A		N.D.		
21-bp IR2	R22A/Q32A		N.D.		
17-bp IR2	Aca1 wt		N.D.		
19-bp IR2	Aca1 wt	1.00	740.7	-9239	-2.93
23-bp IR2	Aca1 wt	1.00	32.2	-4346	19.7
21-bp IR1	Aca1 wt	1.00	53.2	-7290	8.85
21-bp IR2	S42A		N.D.		
21-bp IR2	S42G	1.00	59.2	-12250	-8.0
21-bp IR2	Y49A		W.B.		
26-bp ZF40IR1	Aca2 wt	1.00	2.8	-34990	-78.2
26-bp ZF40IR1	Aca2 R30A		N.D.		
26-bp ZF40IR1	Aca2 Q33A	1.00	37.2	-14930	-16.1
26-bp ZF40IR1	Aca2 Y34A		N.D.		
26-bp ZF40IR1	Aca2 R39A		N.D.		

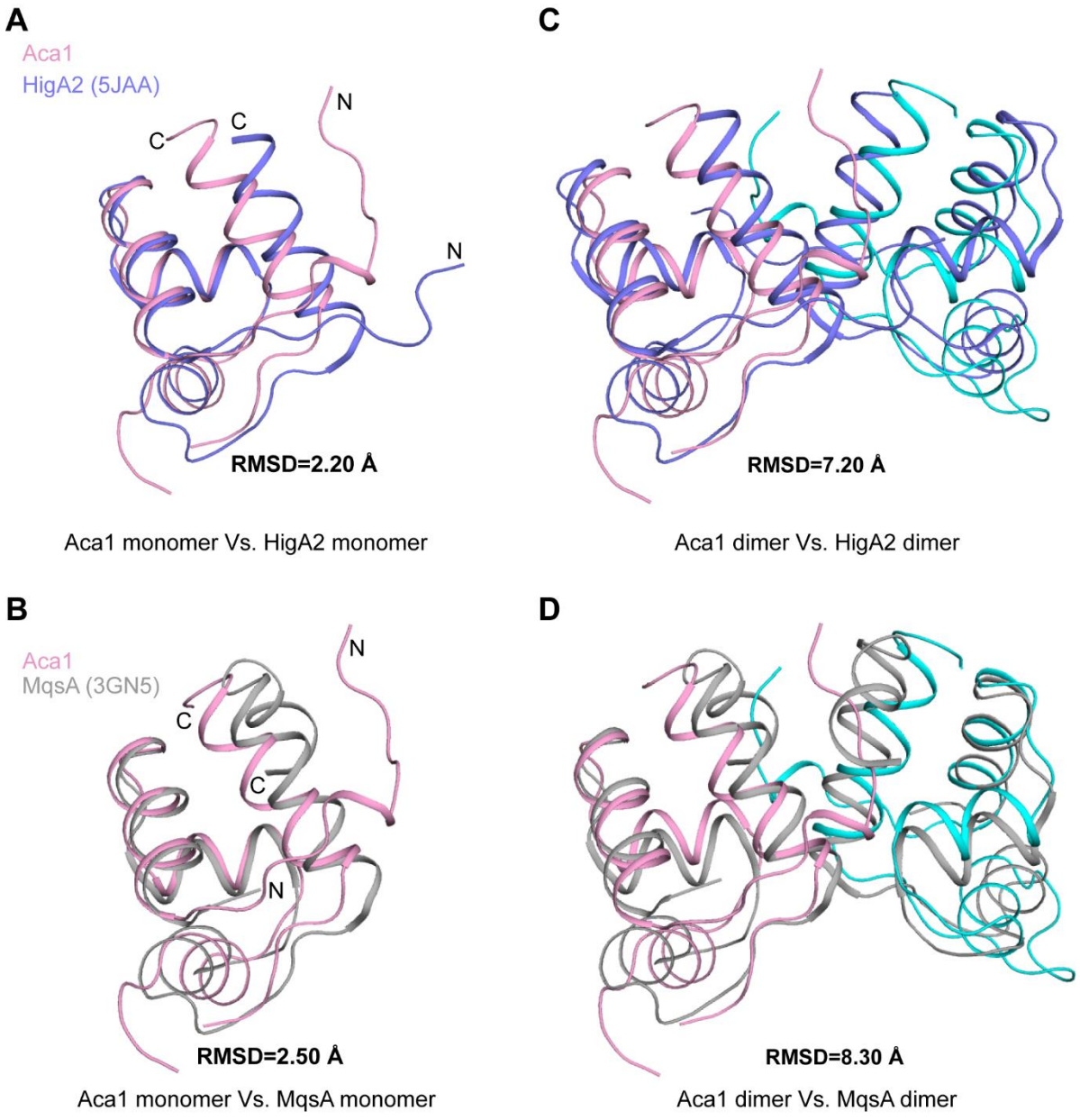
N.D.: Non-detectable. W.B.: Weak binding. Please note that all raw data of the ITC experiments are shown in **Supplementary Figures 5A-F and 6A-H**.

* As the concentrations of the Aca proteins might be intrinsically not determined accurately, or the recombinant proteins might be not 100% active, we fixed the N=1 during data fitting to get better estimation of K_d values.

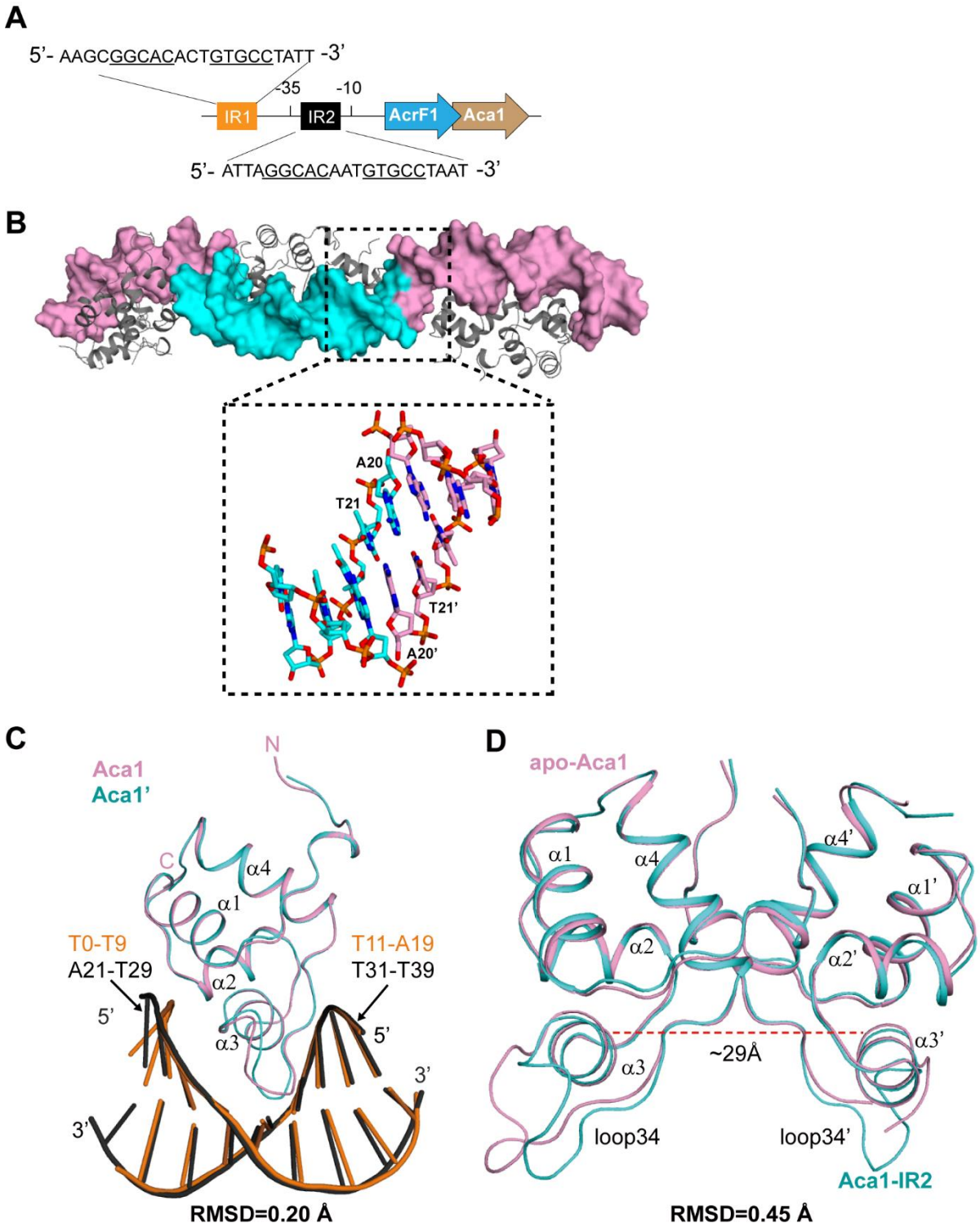
Figures and Figure legends



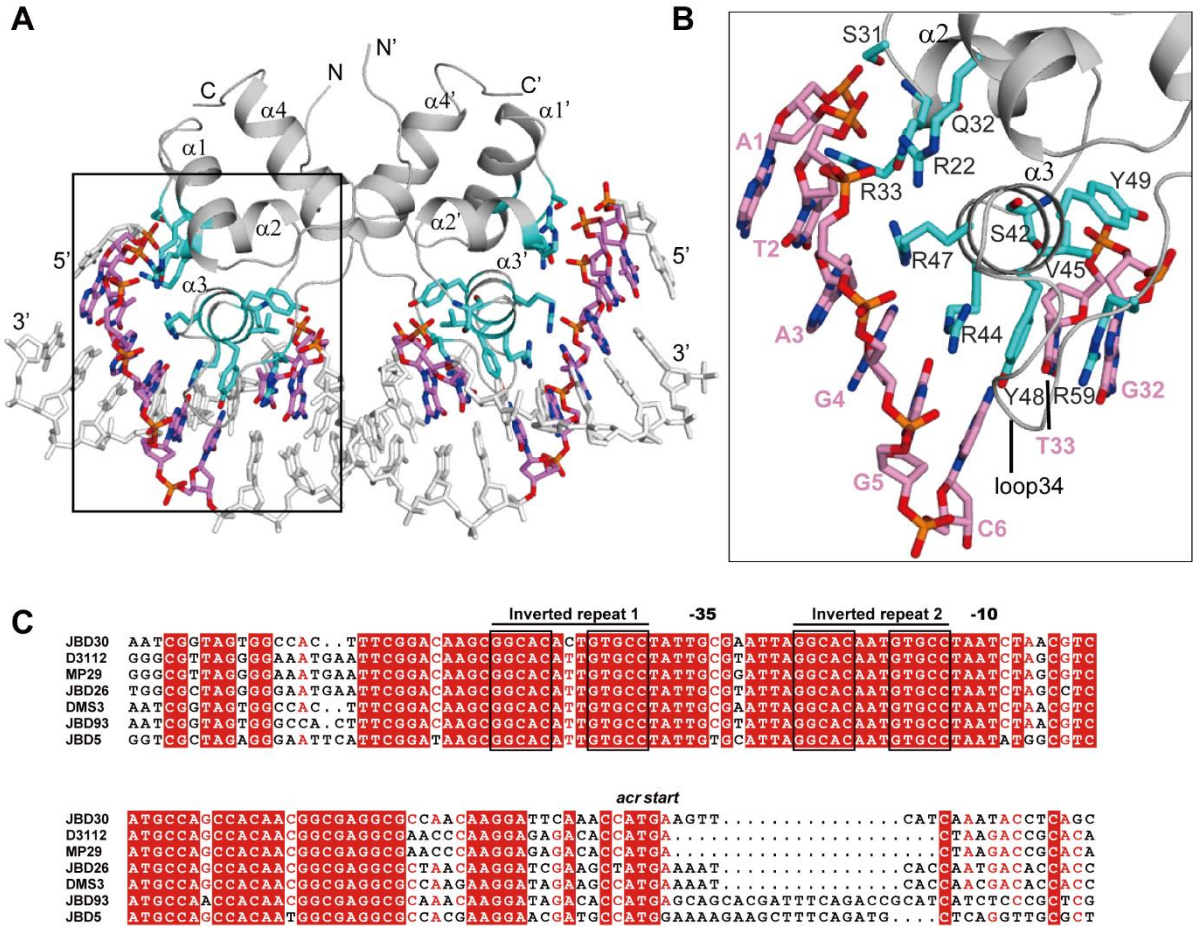
Supplementary Figure 1. Sequence alignment of JBD30 Aca1 with its homologs from different bacteria. (A) Under alignment, the residues involved in dimer formation are indicated with blue circles, while the residues interact with the DNA backbone and bases are indicated with red circles and green rectangles, respectively. Secondary structures of Aca1 from Pseudomonas phage JBD30 are shown on top of the alignment. The two residues Thr64 and Phe67 selected for the double mutant T64D/F67D in Fig. 1D are indicated with arrows.



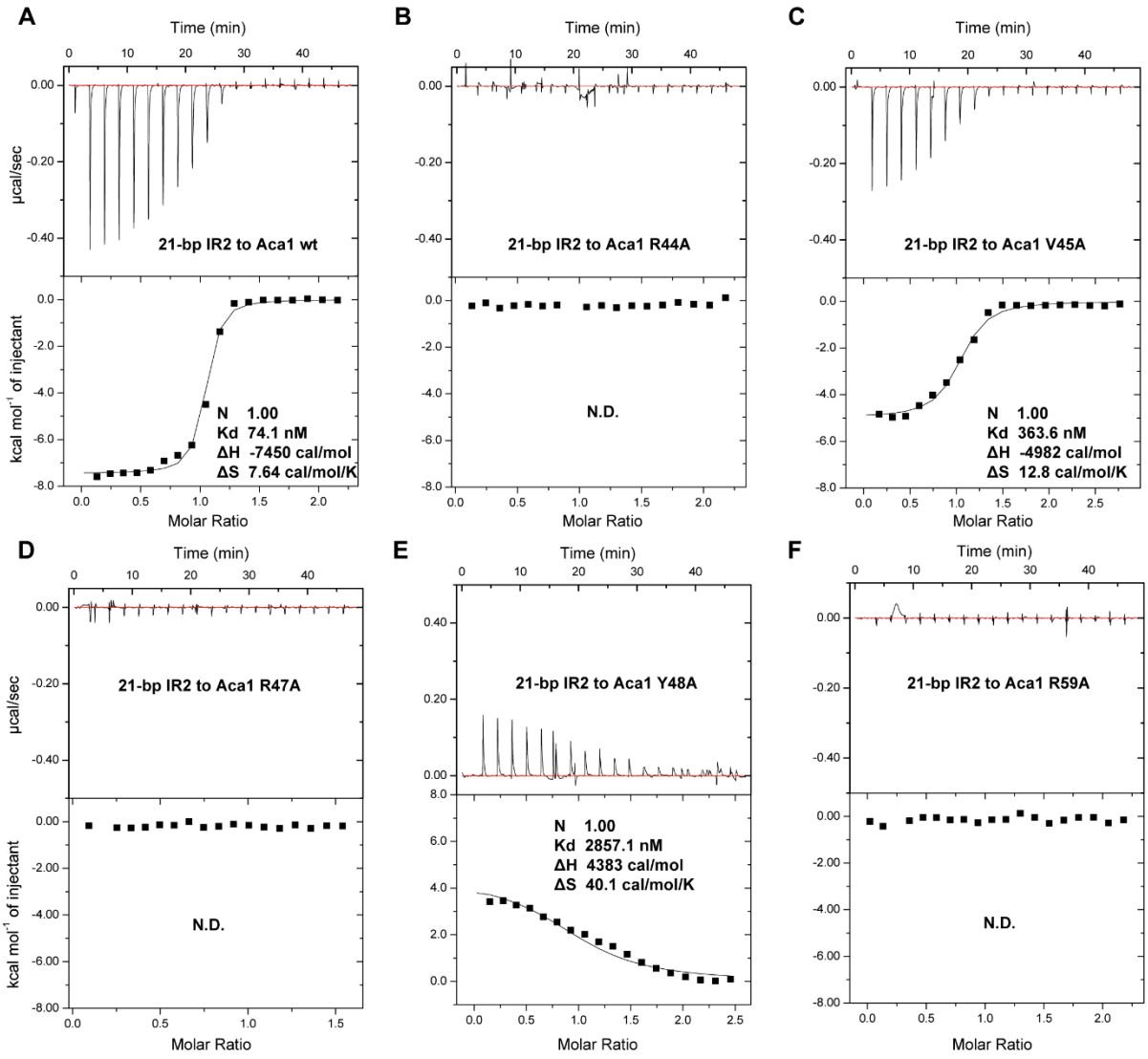
Supplementary Figure 2. Structural comparisons of Aca1 with the *Vibrio cholerae* antitoxin and autorepressor HigA2 and the *Escherichia coli* antitoxin and transcription repressor MqsA. (A) Superposition of Aca1 monomer with HigA2 monomer (PDB ID: 5JAA; Z-score 7.5; 2.20 Å RMSD for 61 Cα atoms; Sequence identity 11%). (B) Superposition of Aca1 monomer with MqsA monomer (PDB ID: 3GN5; Z-score 6.9; 2.50 Å RMSD for 63 Cα atoms; Sequence identity 17%). (C) Superposition of Aca1 dimer with HigA2 dimer (RMSD 7.20 Å). (D) Superposition of Aca1 dimer with MqsA dimer (RMSD 8.30 Å).



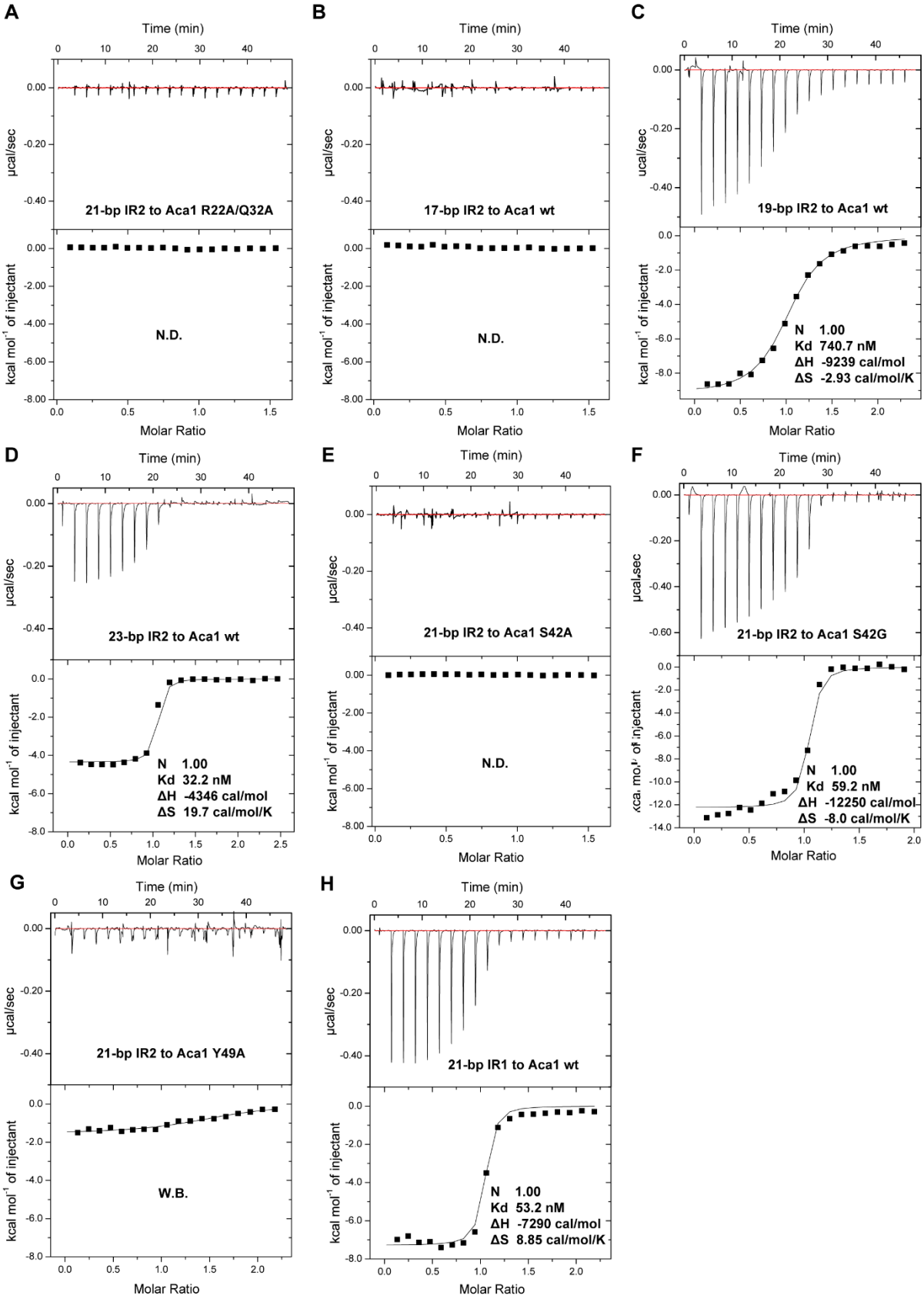
Supplementary Figure 3. Aca1 forms a rigid homodimer. (A) Schematic representation of the *acrIF1-aca1* operon. (B) A cartoon view highlighted that the 5′ overhangs facilitate crystal packing in the symmetric mates. (C) Superimposition of the two symmetric halves from the Aca1–IR2 complex structure, revealing little differences. (D) Superimposition of the structures of apo Aca1 and Aca1–IR2 DNA complex, highlighted little differences with small adjustments in the loops 34 and 34'.



Supplementary Figure 4. Interactions of Aca1 with IR2 DNA. (A-B) A 3D view of the overall interactions between Aca1 and IR2 DNA. Panel B is the zoom in view on the DNA binding interface of one Aca1 protomer. (C) DNA sequence alignment of the promoter regions of the *acr-aca1* operons from phages JBD30 and the other phages. Black line on top of the alignment indicted the inverted repeats (IR2 and IR1), and the core motif involved in specific recognitions are indicated with black boxes.

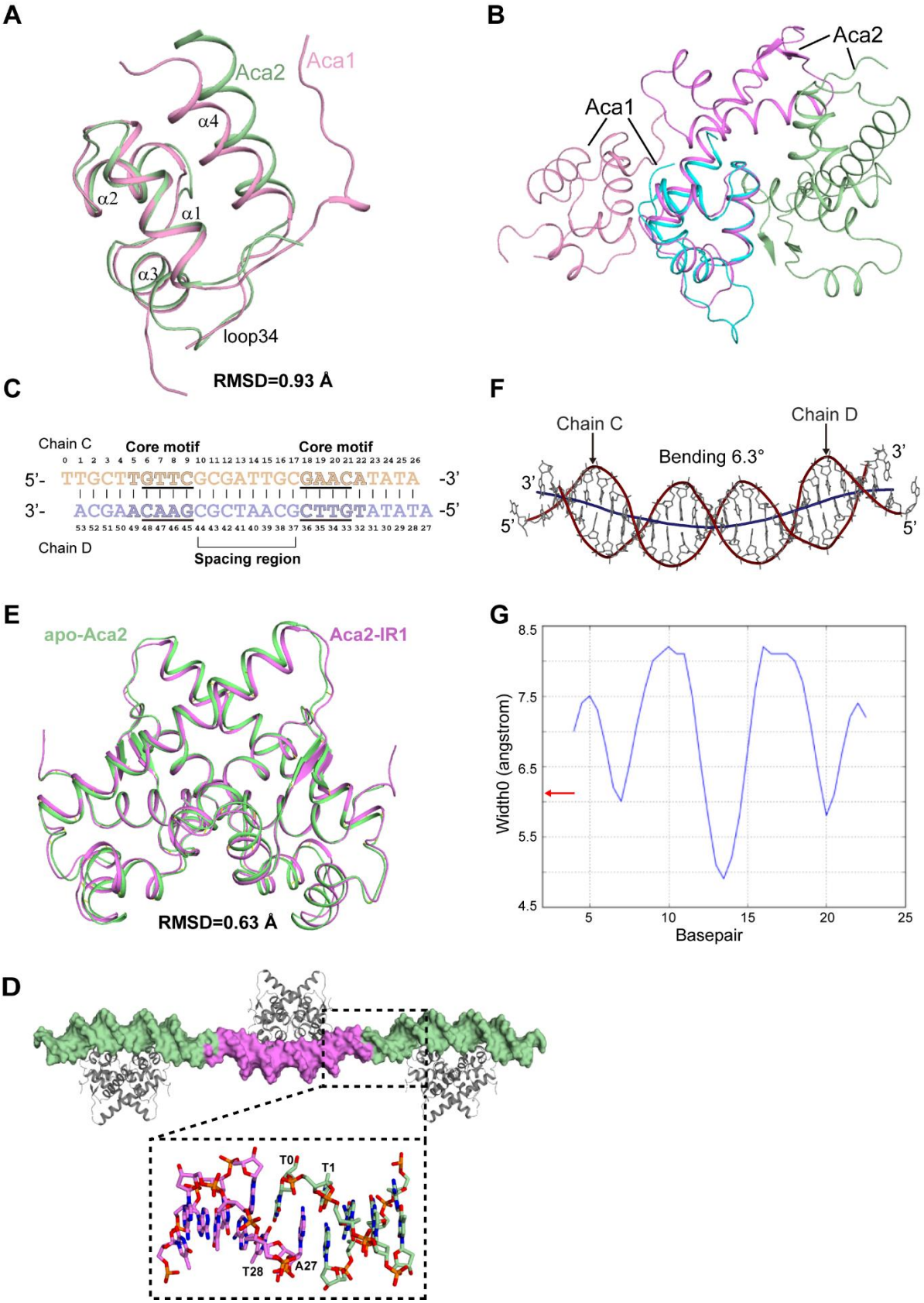


Supplementary Figure 5. ITC results corresponding to the main Fig. 3. (A-F) ITC results of wild-type Aca1 and mutants with the 21-bp blunt-end IR2 DNA. The upper of each panel is the raw data of heat pulses resulting from each injection, whereas the lower of each panel is the integrated heat pulses which are normalized at per mole of injectant and as a function of molar ratio. N.D., not detected.



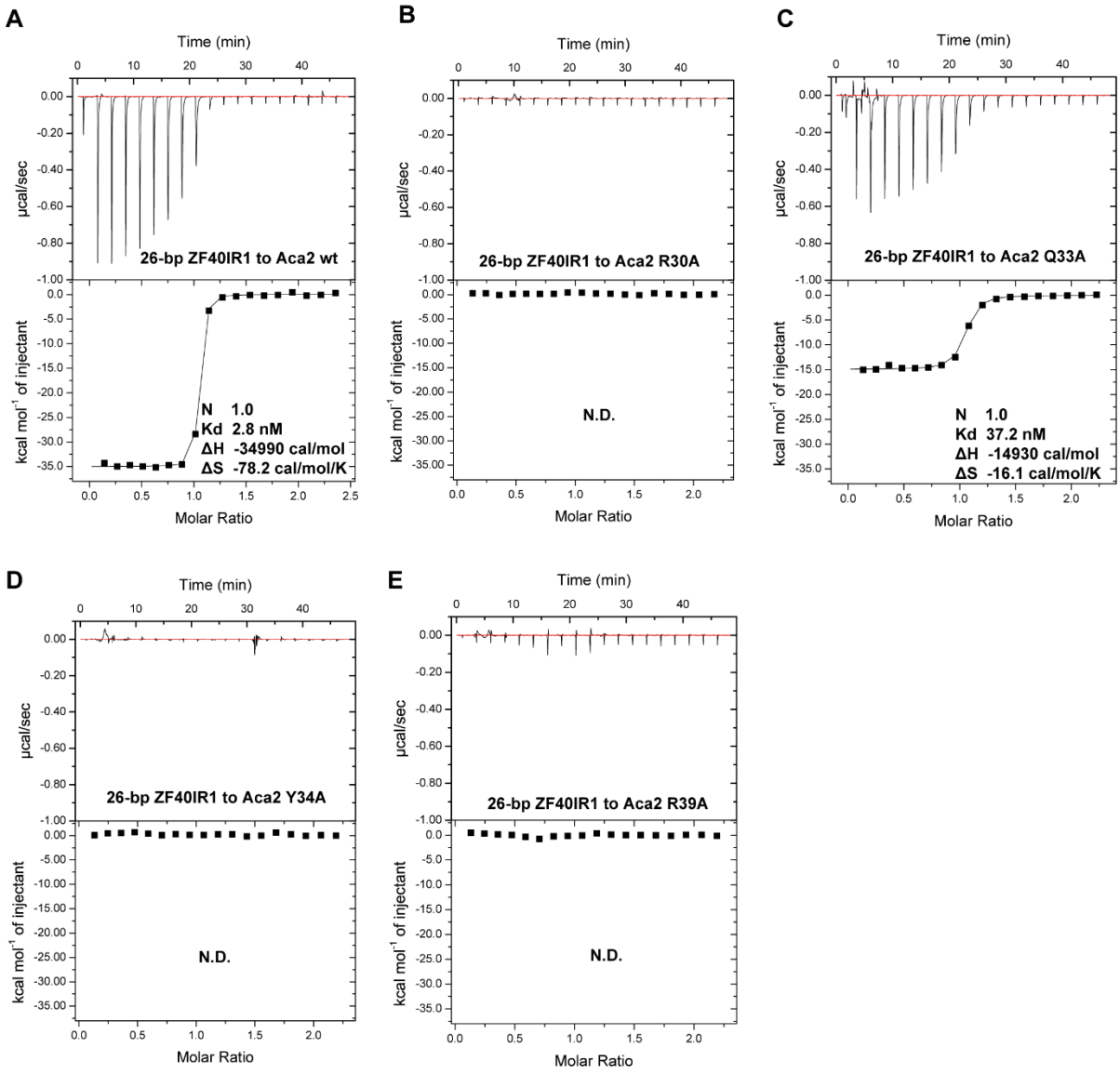
Supplementary Figure 6. ITC results corresponding to the main Fig. 3. (A-H) The upper of each panel is the raw data of heat pulses resulting from each injection, whereas the lower of each panel is the integrated heat pulses which are normalized at per mole of injectant and as a function of molar ratio. Panel A, ITC results of the Aca1 R22A/Q32A mutant with the 21-bp IR2 DNA; Panels B-D,

ITC results of the IR2 DNA with different length of flanking regions to wild-type Aca1; Panels E-G, ITC results of Aca1 mutants S42A, S42G and Y49A with the 21-bp IR2 DNA; Panels H, ITC results of the 21-bp IR1 DNA to wild-type Aca1. N.D., not detected; W.B., week binding.

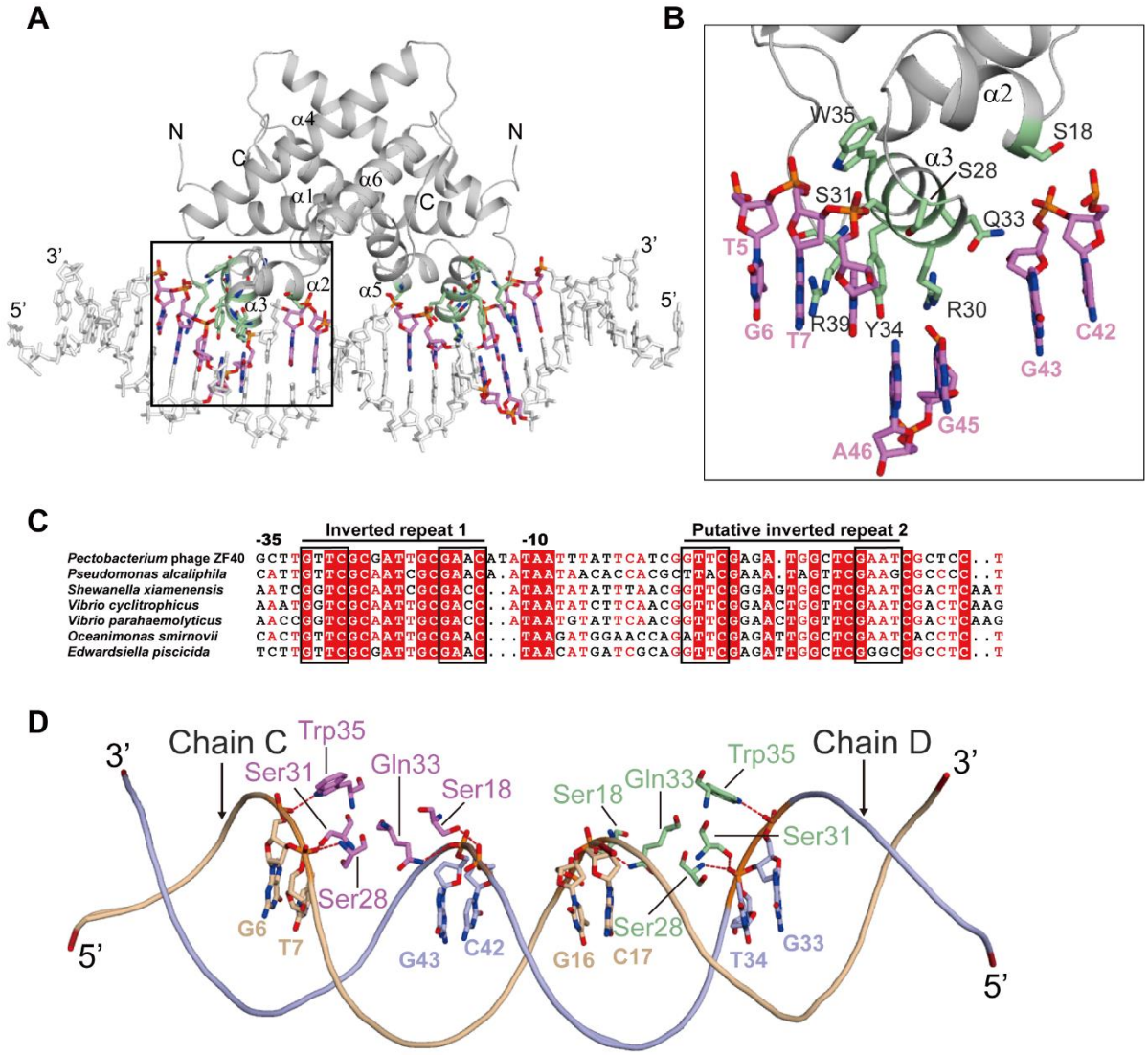


Supplementary Figure 7. Aca2 bind to the DNA target by the same element with Aca1. (A) Superposition of the HTH domains of Aca1 and Aca2. The Aca1 and Aca2 are colored in pink and pale green, respectively. (B) Comparison of Aca2 with Aca1 shows mirrored symmetry. (C) DNA

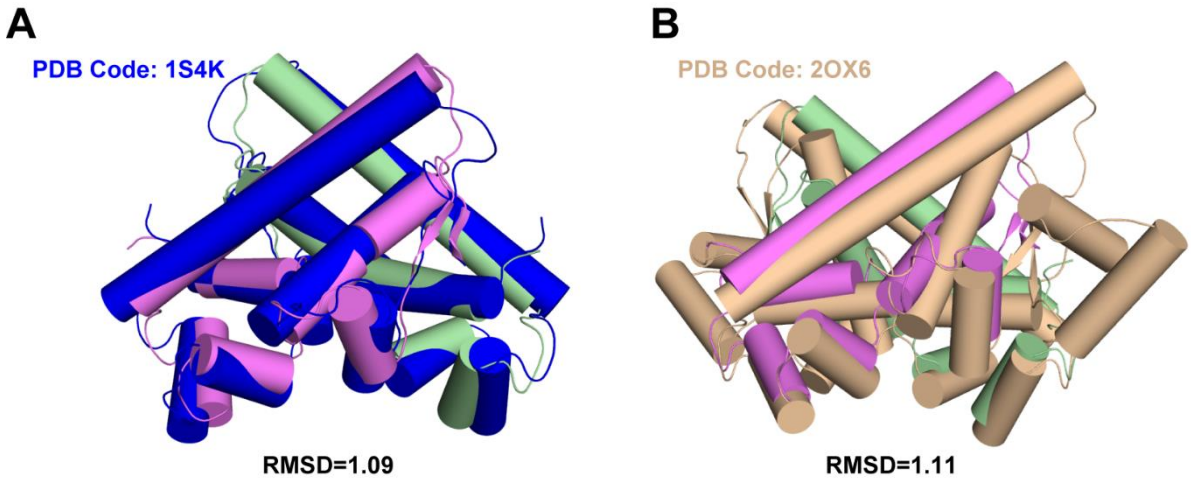
sequences used for co-crystallization with Aca2 and ITC experiments. (D) A cartoon view highlighted that the 5′ overhangs facilitate crystal packing in the symmetry mates. (E) Superimposition of the structures of apo Aca2 and Aca2–IR1 DNA complex with small RMSD of 0.63 Å. (F) Bent conformation of the IR1 DNA. The helical axis of curvature is highlighted with a dark blue line through the center of DNA. (G) The blue curve represents variation of the minor groove widths (Å) along the Aca2 bound IR1 DNA. The red arrow denotes the widths of minor groove in a canonical B-form DNA. Both panels E and F are generated using the program Curves+.



Supplementary Figure 8. ITC results of Aca2 and its mutants with target DNA. (A-E) The raw data of ITC results corresponding to the main Fig. 6I. N.D., not detected.



Supplementary Figure 9. Interactions of Aca2 with IR1 DNA. (A-B) A 3D view of the overall interactions between Aca2 and IR1 DNA. Panel B is the zoom in view on the DNA binding interface of one Aca2 protomer. (C) DNA sequence alignment of the promoter regions of different *acr-aca2* operons. Black line on top of the alignment indicted the inverted repeat1 (IR1) and the putative inverted repeat (IR2). The core motifs involved in specific recognitions are indicated with black boxes. **(**D) Some backbone phosphate groups in core motifs and spacing region of IR1 DNA are also recognized by the Aca2 dimer. Hydrogen bonds are shown in red dash lines, the residues and nucleotides involved in interactions are shown in sticks representation.



Supplementary Figure 10. Quaternary structure comparison of Aca2 with YdiL and SO_3848. (A) Superposition of Aca2 with YdiL (PDB ID: 1S4K). (B) Superposition of Aca2 with SO_3848 (PDB ID: 2OX6).