# Supplementary Information

# The autoregulator Aca2 mediates anti-CRISPR repression

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### Supplementary Table S1: Bacterial strains used in this study

| Name | Genotype/Phenotype | Reference |
| --- | --- | --- |
| ***Escherichia coli*** | | |
| DH5α | $F^-$, ϕ80Δd*lacZ*M15, Δ(*lacZYA–argF*)U169, *endA1*, *recA1*, *hsd*R17 ($r_K^- m_K^+$), *deoR*, *thi-1*, *sup*E44, $\lambda^-$, *gyr*A96, *rel*A1 | (1) |
| BL21(DE3) | Str., B, F–, *ompT*, *gal*, *dcm*, *lon*, $hsdS_B(r_B^- m_B^-)$, λ(DE3, [*lacI*, *lacUV5-T7p07*, *ind1*, *sam7*, *nin5*]), [*malB*$^+$]$_{K-12}$($\lambda^S$) | (2) |
| ***Pectobacterium carotovorum*** | | |
| ZM1 | Lysogen for prophage ZF40 | (3) |
| RC5297 | Carotovoricin-resistant *P. carotovorum* derivative, lacks ZF40 prophage | (3) |
| PCF425 | Derivative of str. RC5297, markerless knockout of two restriction endonuclease genes by homologous recombination for improved transformation efficiency | Birkholz et al., unpublished |

## Supplementary Table S2: Oligonucleotides used in this study

| Name | Sequence (5'-3') | Notes | Restriction site |
|---|---|---|---|
| PF138 | CACACTTTGCTATGCCATAG | F primer for screening of pBAD-derived plasmids | |
| PF139 | GCTACTGCCGCCAGG | R primer for screening of pBAD-derived plasmids | |
| PF209 | TCGTCTTCACCTCGAGAAATC | F primer for screening of pPF1067-derived plasmids | |
| PF210 | GTCATTACTGGATCTATCAACAGG | R primer for screening of pPF1067-derived plasmids | |
| PF2517 | TCC**AAGCTT**GACTCCTGTTGATAGAT | F primer for screening of pPF1439-derived plasmids | HindIII |
| PF2241 | CCAGCTCGACCAGGATGG | R primer for screening of pPF1439-derived plasmids | |
| *Oligonucleotides used for construction of* eyfp *reporter plasmids (pPF1439-derived)* | | | |
| PF2960 | TTTT**ACTAGT**TATTGTGGCGCTGTGTGATTTAC | F primer for *acrIF8–aca2* promoter inserts | SpeI |
| PF2961 | TTTT**ATGCAT**TGTGGAATCCTCGTTAGGAG | R primer for *acrIF8–aca2* promoter inserts | NsiI |
| PF3116 | TCGTCTTCACCTCGAGAAATC**ACTAGT**TATTGTGGCGCTGTGTGATTTACAGGAAATAAAAAGGCCACGAATGCGGCCTTAGCGATTAAAAAATATGAAATGCCTTGCTTGAGACACGAGAGTCTCATATAATTTATTCATCGGTTCGAGATGGCTCGAATCGCTCCTAACGAGGATTCCACA**ATGCAT**CCTGTTGATAGATCCAGTAATGAC | gBlock template for amplification of scIR1-wtIR2 *acrIF8–aca2* promoter insert | SpeI, NsiI |
| PF3117 | TCGTCTTCACCTCGAGAAATC**ACTAGT**TATTGTGGCGCTGTGTGATTTACAGGAAATAAAAAGGCCACGAATGCGGCCTTAGCGATTAAAAAATATGAAATGCCTTGCTTGTTCGCGATTGCGAACATATAATTTATTCATCGGAGACTACTGCAGGTCTTCGCTCCTAACGAGGATTCCACA**ATGCAT**CCTGTTGATAGATCCAGTAATGAC | gBlock template for amplification of wtIR1-scIR2 *acrIF8–aca2* promoter insert | SpeI, NsiI |
| PF3118 | TCGTCTTCACCTCGAGAAATC**ACTAGT**TATTGTGGCGCTGTGTGATTTACAGGAAATAAAAAGGCCACGAATGCGGCCTTAGCGATTAAAAAATATGAAATGCCTTGCTTGAGACACGAGAGTCTCATATAATTTATTCATCGGAGACTACTGCAGGTCTTCGCTCCTAACGAGGATTCCACA**ATGCAT**CCTGTTGATAGATCCAGTAATGAC | gBlock template for amplification of scIR1-scIR2 *acrIF8–aca2* promoter insert | SpeI, NsiI |
| PF3556 | TCGTCTTCACCTCGAGAAATC**ACTAGT**TATTGTGGCGCTGTGTGATTTACAGGAAATAAAAAGGCCACGAATGCGGCCTTAGCGATTAAAAAATATGAAATGCCTTGCTTGTTCGCGATTGCGAACATATAATTTATTCATCTCCTAACGAGGATTCCACA**ATGCAT**CCTGTTGATAGATCCAGTAATGAC | gBlock template for amplification of wtIR1-ΔIR2 *acrIF8–aca2* promoter insert | SpeI, NsiI |
| PF3557 | TCGTCTTCACCTCGAGAAATC**ACTAGT**TATTGTGGCGCTGTGTGATTTACAGGAAATAAAAAGGCCACGAATGCGGCCTTAGCGATTAAAAAATATGAAATGCCTTGCTTGAGACACGAGAGTCTCATATAATTTATTCATCTCCTAACGAGGATTCCACA**ATGCAT**CCTGTTGATAGATCCAGTAATGAC | gBlock template for amplification of scIR1-ΔIR2 *acrIF8–aca2* promoter insert | SpeI, NsiI |
| PF3560 | TCGTCTTCACCTCGAGAAATC**ACTAGT**TATTGTGGCGCTGTGTGATTTACAGGAAATAAAAAGGCCACGAATGCGGCCTTAGCGATTAAAAAATATGAAATGCCTTGCTTGTTCGCGATTGCGAACATCTAAGTTATTCATCGGTTCGAGATGGCTCGAATCGCTCCTAACGAGGATTCCACA**ATGCAT**CCTGTTGATAGATCCAGTAATGAC | gBlock template for amplification of -10 pm *acrIF8–aca2* promoter insert | SpeI, NsiI |
| *Oligonucleotides used for construction of IPTG-inducible* aca2 *expression plasmids (pPF1067-derived)* | | | |
| PF3114 | TTT**GGATCC**ATGACAAACAAAGAACTTCAG | F primer for cloning *aca2* into pPF1067 | BamHI |
| PF3115 | TTT**AAGCTT**TTAGATTAAATCCGCGTGAC | R primer for cloning *aca2* into pPF1067 | HindIII |
| PF3500 | TTTT**AAGCTT**TCGTTAGATTAAATCCGCGTGAC | R primer for overlap extension downstream fragment | HindIII |
| PF3504 | TTTT**GGATCC**ATGACAAACAAAGAACTTCAGGCA | F primer for overlap extension upstream fragment | BamHI |
| PF3505 | AACGCGGCCAATGTGTTC | R primer for overlap extension upstream fragment | |
| PF3507 | GAACACATTGGCCGCGTTTCCGCCGCGAGTTGGCAATATTGGGAGTCTG | F primer for overlap extension downstream fragment; R30A mutant | |

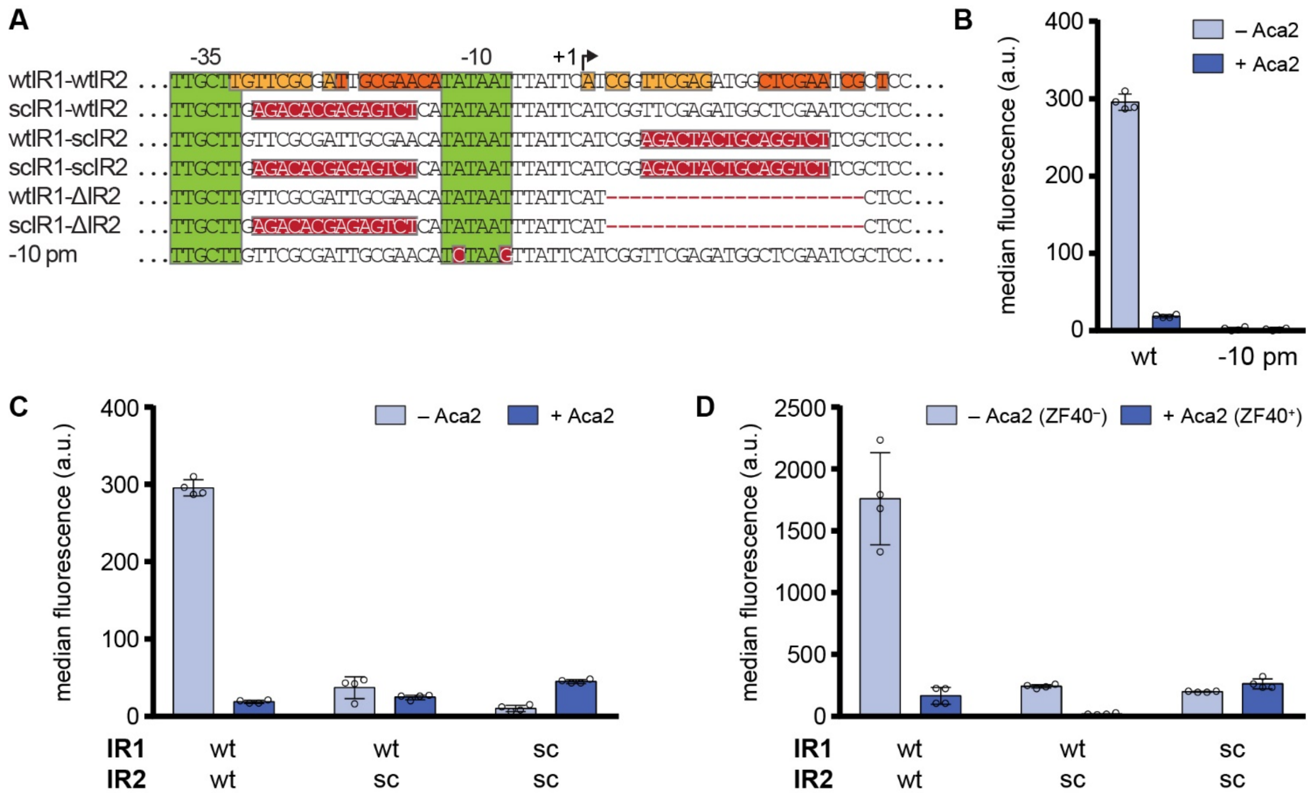
## Supplementary Table S2 continued: Oligonucleotides used in this study

| Name | Sequence (5'-3') | Notes | Restriction site |
|---|---|---|---|
| *Oligonucleotides used for construction of arabinose-inducible* aca2 *expression plasmids (pBAD30-derived)* | | | |
| PF2965 | TTTT**GAGCTC**AGGAGGACACGGATGACAAACAAAGAACTTCAG | F primer for cloning wt *aca2* into pBAD30 and for overlap extension upstream fragment | SacI |
| PF3505 | AACGCGGCCAATGTGTTC | R primer for overlap extension upstream fragment | |
| PF3507 | GAACACATTGGCCGCGTTTCCGCCGCGAGTTGGCAATATTGGGAGTCTG | F primer for overlap extension downstream fragment; R30A mutant | |
| PF3508 | GAACACATTGGCCGCGTTTCCGCCCGGAGTTGGGCATATTGGGAGTCTG | F primer for overlap extension downstream fragment; Q33A mutant | |
| PF3509 | GAACACATTGGCCGCGTTTCCGCCGCGAGTTGGGCATATTGGGAGTCTG | F primer for overlap extension downstream fragment; R30A,Q33A mutant | |
| PF2966 | TTTT**GCATGC**TTAGATTAAATCCGCGTGAC | R primer for cloning wt *aca2* into pBAD30 and for overlap extension downstream fragment | SphI |
| *Oligonucleotides used for DNA probes for EMSAs and bending assays* | | | |
| PF1222 | CACGATTATGATATTCCGACC | F primer for amplification of non-specific EMSA control (template pPF1067, used in combination with PF210) | |
| PF210 | GTCATTACTGGATCTATCAACAGG | R for amplification of non-specific EMSA control (template pPF1067) | |
| PF3151 | /5IRD700/CACGAATGCGGCCTTAGCG | IRDye-700-labelled F primer for amplification EMSA probes, F primer for bending probe 1 | |
| PF3152 | CACGAATGCGGCCTTAGCG | unlabelled F primer for amplification of specific EMSA control | |
| PF3153 | GCATTGTGGAATCCTCGTTAGGAG | R primer for amplification of EMSA probes | |
| PF2081 | /5IRD700/GAACTGAGCCTGAAATTCAGGATC | IRDye-700-labelled F primer for bending probe 2 | |
| PF3088 | /5IRD700/CTTGGCGAGATTTTCAGGAGC | IRDye-700-labelled F primer for bending probe 3 | |
| PF3339 | /5IRD700/CGCCCTTGCTCAGGGATC | IRDye-700-labelled F primer for bending probe 4 | |
| PF3513 | /5IRD700/CCTTGCTTGTTCGCGATTGC | IRDye-700-labelled F primer for bending probe 5 | |
| PF3514 | TAGGAGCGATTCGAGCCATCTC | R primer for bending probe 1 (IR1+IR2) | |
| PF3515 | AATTATATGTTCGCAATCGCGAACAAG | R primer for bending probe 1 (IR1 only) | |
| PF2693 | GTGCAGGCAGCTTCCACAG | R primer for bending probe 2 | |
| PF2865 | GACCATGATTACGCCAAGCTG | R primer for bending probe 3 | |
| PF2241 | CCAGCTCGACCAGGATGG | R primer for bending probe 4 | |
| PF2024 | CGCCCATCTGCTCACCAAC | R primer for bending probe 5 | |
| PF3511 | TCGTCTTCACCTCGAGAAATCGAATTCCACGAATGCGGCCTTAGCGGTCCGAACTGAGCCTGAAATTCAGGATCTACTCACGTTAAGGGATTTTGGTCTGCTTGGCGAGATTTTCAGGAGCACGTGGATTCACCACTCCAAGAATTGGATCGCCCTTGCTCAGGGATCGAATGCCTTGCTTGTTCGCGATTGCGAACATATAATTTATTCATCGGTTCGAGATGGCTCGAATCGCTCCTAACGACTGTGGAAGCTGCCTGCACATCTCGAAAATCTTGATCACTCCGATAAACAGCTTGGCGTAATCATGGTCGCCGATAACCAACTCTGGCTAAGACATTGATACCATCCTGGTCGAGCTGGCGTCGTTGGTGAGCAGATGGGCGAAGCTTCCTGTTGATAGATCCAGTAATGAC | gBlock template for amplification of IR1+IR2 bending probes | |
| PF3512 | TCGTCTTCACCTCGAGAAATCGAATTCCACGAATGCGGCCTTAGCGGTCCGAACTGAGCCTGAAATTCAGGATCTACTCACGTTAAGGGATTTTGGTCTGCTTGGCGAGATTTTCAGGAGCACGTGGATTCACCACTCCAAGAATTGGATCGCCCTTGCTCAGGGATCGAATGCCTTGCTTGTTCGCGATTGCGAACATATAATTTATTCTGTGGAAGCTGCCTGCACATCTCGAAAATCTTGATCACTCCGATAAACAGCTTGGCGTAATCATGGTCGCCGATAACCAACTCTGGCTAAGACATTGATACCATCCTGGTCGAGCTGGCGTCGTTGGTGAGCAGATGGGCGAAGCTTCCTGTTGATAGATCCAGTAATGAC | gBlock template for amplification of IR1-only bending probes | |

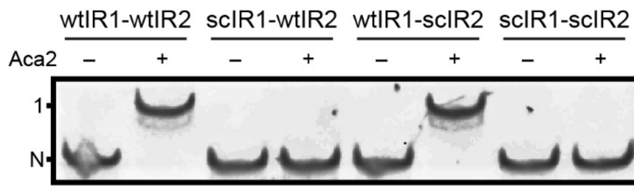
**Note:** In the oligonucleotide sequences, only the relevant restriction sites are highlighted bold (as indicated in the fourth column). Oligonucleotides are sorted by experiment. As such, they may be listed more than once with different functions.

## Supplementary Table S3: Plasmids used in this study

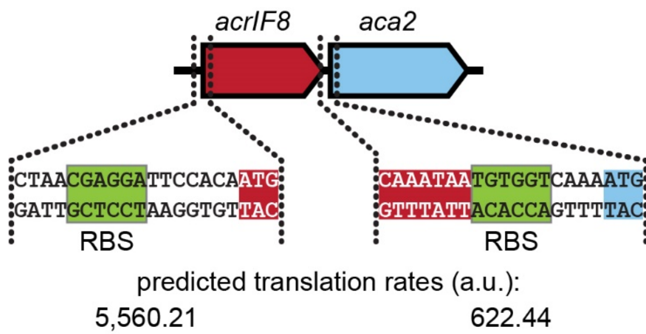
| Name | Genotype/Phenotype | Reference |
|---|---|---|
| pBAD30 | *E. coli* arabinose inducible vector, ApR, pACYC184/p15A replicon | (4) |
| pPF1532 | pBAD30-derivative for arabinose-inducible expression of Aca2 | this study |
| pPF1861 | pBAD30-derivative for arabinose-inducible expression of Aca2$^{R30A}$ | this study |
| pPF1862 | pBAD30-derivative for arabinose-inducible expression of Aca2$^{Q33A}$ | this study |
| pPF1863 | pBAD30-derivative for arabinose-inducible expression of Aca2$^{R30A,Q33A}$ | this study |
| pPF1067 | empty vector for insertion of *aca2* and tagging with a TEV and 6x His-tag; template for amplification of non-specific EMSA probe using PF336 and PF1710 | (5) |
| pPF1575 | IPTG-inducible *aca2* expression plasmid (wild-type Aca2) (TEV-cleavable 6x His-tag); pPF1067-derivative | this study |
| pPF1439 | *eyfp* reporter plasmid for insertion of *acrIF8–aca2* promoter; IPTG-inducible *mCherry* reporter, CmR, pBR322 replicon | Smith et al., unpublished |
| pPF1530 | *eyfp* combined with wild-type *acrIF8–aca2* promoter; IPTG-inducible *mCherry*; template for wtIR1-wtIR2 EMSA probe; pPF1439-derivative | this study |
| pPF1580 | *eyfp* combined with scIR1-wtIR2 *acrIF8–aca2* promoter; IPTG-inducible *mCherry*; template for scIR1-wtIR2 EMSA probe; pPF1439-derivative | this study |
| pPF1581 | *eyfp* combined with wtIR1-scIR2 *acrIF8–aca2* promoter; IPTG-inducible *mCherry*; template for wtIR1-scIR2 EMSA probe; pPF1439-derivative | this study |
| pPF1582 | *eyfp* combined with scIR1-scIR2 *acrIF8–aca2* promoter; IPTG-inducible *mCherry*; template for scIR1-scIR2 EMSA probe; pPF1439-derivative | this study |
| pPF1831 | *eyfp* combined with wtIR1-ΔIR2 *acrIF8–aca2* promoter; IPTG-inducible *mCherry*; pPF1439-derivative | this study |
| pPF1832 | *eyfp* combined with scIR1-ΔIR2 *acrIF8–aca2* promoter; IPTG-inducible *mCherry*; pPF1439-derivative | this study |
| pPF1835 | *eyfp* combined with -10 pm *acrIF8–aca2* promoter; IPTG-inducible *mCherry*; pPF1439-derivative | this study |
| pPF1857 | IPTG-inducible *aca2* expression plasmid (Aca2$^{R30A}$) (TEV-cleavable 6x His-tag): pPF1067-derivative | this study |




**Supplementary Figure S1: Point mutations in the -10 region and scrambling of IR2 severely attenuate the *acrIF8–aca2* promoter. (A)** Promoter variants used in reporter assays for this study. In the wtIR1-wtIR2 promoter, shades of orange indicate the inverted repeats (see **Figure 1A** for comparison). Red boxes indicate scrambled (sc) sequences or point mutations (pm), and dashes indicate deleted (Δ) sequences. Green boxes indicate the putative -35 (incomplete) and -10 regions and the arrow indicates the putative transcription start site (+1). **(B)** Activity of the promoter with point mutations in the putative -10 region compared to the wild-type promoter, in the presence and absence of Aca2 expression from a plasmid, determined as the median eYFP fluorescence, in the *Pca* ZF40⁻ strain (RC5297) **(C)** Activity of the indicated *acrIF8–aca2* promoter variants in the presence and absence of Aca2 expression from a plasmid, determined as the median eYFP fluorescence, in the *Pca* ZF40⁻ strain (RC5297). **(D)** Activity of the indicated promoter variants in the strain *Pca* ZF40⁺ (ZM1) and the control ZF40⁻ strain (RC5297).




**Supplementary Figure S2: IR2 is not bound by Aca2 at high protein concentrations.** Mobility of DNA probes encoding the indicated promoter variants in the absence (–) or presence (+) of 128 nM Aca2. N and 1 indicate non-shifted and single-shifted bands, respectively.




**Supplementary Figure S3: *aca2* has a suboptimal ribosome-binding site compared to *acrIF8*.** Sequences directly upstream of the *acrIF8* and *aca2* genes of phage ZF40, with coding regions highlighted in the respective gene colours and predicted ribosome-binding sites (RBS) highlighted in green. Translation rates were calculated using the De Novo DNA RBS Calculator (Salis Lab, https://salislab.net/software/).

## SUPPLEMENTARY METHODS

### Construction of $P_{acrIF8aca2}$-*eyfp* reporter plasmids

Inserts for pPF1439-derived *eyfp* expression plasmids were PCR-amplified using primers PF2960 and PF2961. For the wtIR1-wtIR2 insert (pPF1530), *Pca* ZM1 were used as template. For other promoter variants (pPF1580, pPF1581, pPF1582, pPF1831, pPF1832 and pPF1835), the gBlocks PF3116 (scIR1-wtIR2), PF3117 (wtIR1-scIR2), PF3118 (scIR1-scIR2), PF3556 (wtIR1-ΔIR2), PF3557 (scIR1-ΔIR2) or PF3560 (-10 pm) were used as templates. PCR products were digested with the enzymes SpeI and NsiI and ligated with pPF1439 digested with the same enzymes.

### Construction of the IPTG-inducible *aca2* expression plasmid

The plasmid pPF1575 for expression of $His_6$-TEV-Aca2 was constructed by PCR-amplifying *aca2* from *Pca* ZM1 using PF3114 and PF3115. The resulting fragment was digested with the enzymes BamHI and HindIII and ligated with pPF1067 digested with the same enzymes. For the construction of the plasmid pPF1837 for expression of $Aca2^{R30A}$, an upstream-fragment of *aca2* was generated using primers PF3504 and PF3505, and a downstream fragment was generated using PF3507 and PF3500. Upstream and downstream fragments were then connected by overlap extension PCR and the resulting fragments ligated with pPF1067, as described above.

### Construction of arabinose-inducible *aca2* expression plasmids

For the construction of pPF1532 for in vivo native Aca2 expression experiments, *aca2* was PCR-amplified from *Pca* ZM1 using the primers PF2965 and PF2966. The resulting fragment was digested with the enzymes SacI and SphI and ligated with pBAD30 digested with the same enzymes. For the construction of *aca2* expression plasmids with point mutations (pPF1861-pPF1863), an upstream-fragment of *aca2* was generated using primers PF2965 and PF3505, and downstream fragments were generated using the forward primers PF3507 (pPF1861, R30A mutation), PF3508 (pPF1862, Q33A mutation) or PF3509 (pPF1863, R30A,Q33A mutation) in combination with the reverse primer PF2966. Upstream and downstream fragments were then connected by overlap extension PCR and the resulting fragments ligated with pBAD30 as described above.

**REFERENCES**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