Supplementary data for:

TUNING THE RELATIVE AFFINITIES FOR ACTIVATING AND REPRESSING OPERATORS OF A TEMPORALLY-REGULATED RESTRICTION-MODIFICATION SYSTEM

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SUPPLEMENTAL TABLES AND FIGURES

All used methods were the same as those described in the text of the paper.

Table S1. Plasmids used in this study

Name	Relevant feature(s)	Reference
pACYC177	cloning vector (AmpR, KanR)	(1)
pACYC-kan	as pACYC177, but *bla* gene deleted (AmpS, KanR)	This study
pBAD24	arabinose inducible *araBAD* promoter, *araC*, AmpR, ColE1 ori	(2)
pBadMTwt	WT *pvuIIM* under control of *araBAD* promoter in pBAD24, ColE1 ori, AmpR	This study
pBadMT2	as pBadMTwt, but M.PvuII two substitutions: N4S (AAU-AGU) and S10R (AGC-CGC)	This study
pBadMT3	as pBadMT2, but additional third M.PvuII substitution M8L (AUG-UUG)	This study
pBadMTwt-kan	WT *pvuIIM* under control of *araBAD* promoter in pBAD24, p15A ori, KanR	This study
pBadMT2-kan	as pBadMT2, but p15A ori, KanR	This study
pBadMT3-kan	as pBadMT3, but p15A ori, KanR	This study
pBend2	cloning vector for DNA bending analysis	(3)
pBR322	cloning vector (AmpR, TetR)	(4)
pDK178 (WWWW)	transcriptional fusion of *pvuIIC* promoter including C boxes with the symmetrical core - WWWW (GATCcat AGCTtgtaGACTcaaAGCT) to the *cat* gene in pKK232-8	(5)
pDK435	transcriptional fusion of *pvuIIC* promoter including WT C boxes (positions -93 to +88) in front of promotorless *lacZ* reporter gene in pKK232-8, TetR, p15A ori	(5)
pIM1	*pvuIIC* under control of *araBAD* promoter in pBAD24, AmpR, ColE1 ori	(5)
pIM2	Kan cassette inserted to break *bla* gene in pIM1, KanR, ColE1 ori	(5)
pIM4	as pIM2 but p15A ori	(5)
pIM6	nonrepression mutant, as pPvuRM3.4, but C box (GACTcatAGTCtgtaGACTcaaGATC)	(5)
pIM8	as pDK435, but symmetrized C-box, as for pDK178	(5)
pIM9	as pPvuRM3.4, but symmetrized C-box, as nonrepression mutant pIM6	(5)
pIM10	as pIM8, but mutation in spacer of O_R (GACTcatAGTCtgtaGACTcatGATC)	This study
pIM13	as pIM8, but mutation in spacer of O_L (GACTcaaAGTCtgtaGACTcaaGATC)	This study
pIM14	as pIM8, but mutation in both spacers of O_L and O_R (GACTcaaAGTCtgtaGACTcatGATC)	This study
pIM17	as pPvuRM3.4 but mutation in spacer of O_R (as pIM10)	This study
pIM18	as pPvuRM3.4 but mutation in spacer of O_L (as pIM13)	This study

pIM19	as pPvuRM3.4 but mutation in both spacers of O_L and O_R (as pIM14)	This study
pIM20	as pPvuRM3.4, but symmetrized C-box, as pDK178	This study
pIM22	as pDK178, but symmetrized C-box with mutation in spacer of O_L (as pIM13)	This study
pIM57	as pDK435, symmetrized C-box, intra-operator variant (GACTTATAGTCtgtaGACTtatGATC)	This study
pIM58	as pDK435, symmetrized C-box, intra-operator variant (GACTTATAGTCtgtaGACTcgcGATC)	This study
pIM67	as pDK435, symmetrized C-box, intra-operator variant (GACTcgcAGTCtgtaGACTtatGATC)	This study
pIM68	as pDK435, symmetrized C-box, intra-operator variant (GACTcgcAGTCtgtaGACTcgcGATC)	This study
pWWWR	as pDK178, but symmetrized C-box with mutation in 2A box of O_R (as pIM6), WT spacers, non-repressing	(5)
pKK232-8	promotorless *cat* reporter gene	(6)
pLex3B	vector for testing gene translational fusion to LacZ	(7)
pLex-met1	as pLexB but WT *pvuIIM* 5' fragments fused in the same open reading frame to LacZ (starting with codons for M_1M_2TLNLQTM_3SSNDM_4L)	This study
pLex-met2	as pLex-met1 but M_3 (ATG) codon mutated to L (TTG) (codons M_1M_2TLNLQTLSSNDM_4L)	This study
pLex-met3	as pLex-met1 but M_4 (ATG) codon mutated to L (TTG) (codons M_1M_2TLNLQTM_3SSNDLL)	This study
pLex-met4	as pLex-met1 but M_1 (ATG) codon mutated to L (TTG) (codons LM_2TLNLQTM_3SSNDM_4L)	This study
pLex-met5	as pLex-met1 but M_2 (ATG) codon mutated to L (TTG) (codons M_1LTLNLQTM_3SSNDM_4L)	This study
pPvuRM3.4	WT PvuII R-M system in pBR322 backbone	(8)
pPvuM1.9-ACYC	as pPvuM1.9 (M+R-C-), but p15A ori, Cm^R	(8)
pUC×7PvuII	pUC19 with 7 PvuII sites	This study

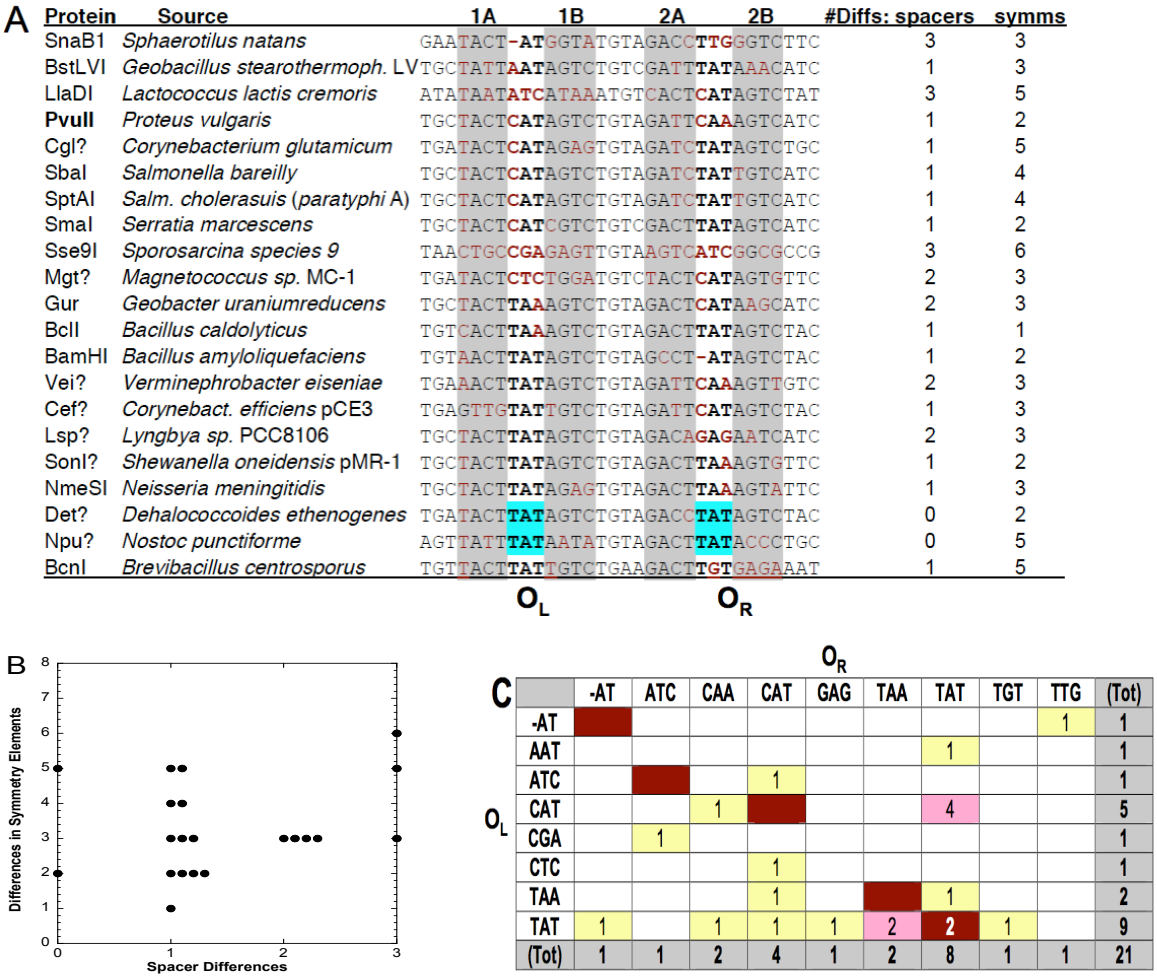

Figure S1. Control region for the PvuII regulation R-M system.

(A) Comparison of upstream regions from known and some putative C protein–coding genes of the C.PvuII–like subfamily, which have predicted recognition helices containing HRTY. The shaded nucleotides indicate the C-box symmetrical cores, where 1A and 1B form operator left (O_L) and 2A and 2B form operator right (O_R). The proposed consensus sequence for this region is GATC-TAT-AGTC-TGTA-GATC-TAT-AGTC, as explained in Figure 1 of the main text. Any difference from this consensus within the symmetrical boxes or intra-operator spacers is indicated by red font, and the number of differences is shown in columns to the right. Two exact matches to consensus for the intra-operator spacers are highlighted in blue. A question mark in the name indicates hypothetical C proteins.

(B) Using data from Figure S1A, the relationship is shown between O_L/ O_R variation in GATCnnnAGTC *vs*. that in the intra-operator spacers.

(C) The distribution of specific pairs of intra-operator spacers is shown. Dark red boxes indicate occurrences of identical spacers; only two cases are known (blue shading in Figure S1A).

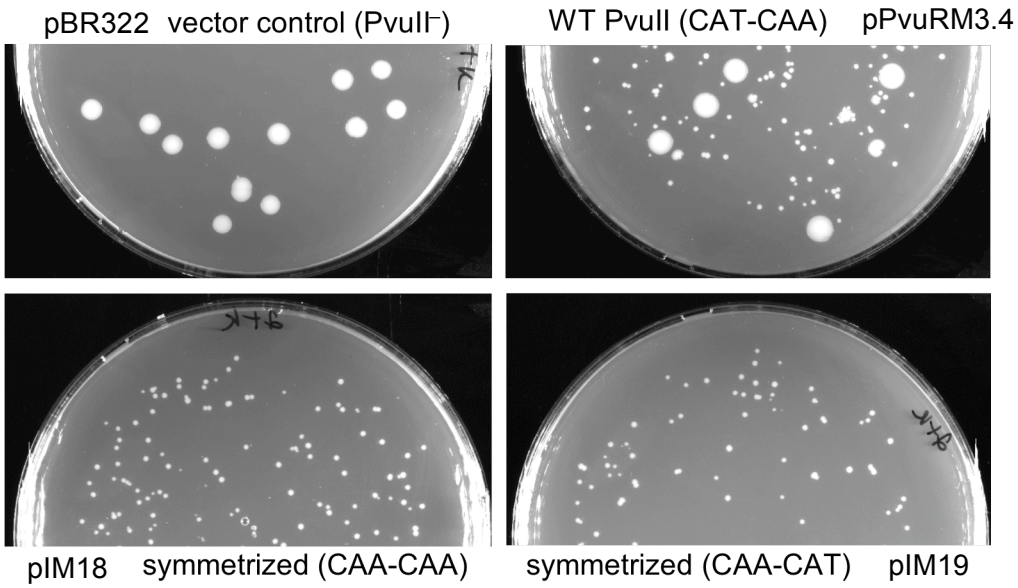

Figure S2. The effect of altered spacers in O_R and O_L of C-boxes on PvuII R-M system maintenance.

Plasmid DNAs carrying the intact PvuII R-M system and its C-box spacer variants were used. These plasmids were introduced into competent *E. coli* TOP10 [pPvuM1.9-ACYC; Cm^R], cells that already carry a plasmid specifying PvuII MTase (M^+). These cells were again transformed with pACYC-kan (M^-) and grown without antibiotics for 2 h, to allow plasmid segregation.

As they are mutually incompatible, either pPvuM1.9-ACYC (M^+) or pACYC-kan (M^-) should be lost. Transformants were plated with selection for the R-M system plasmid (Ap^R) and pACYC-kan (M^-) (Km^R). Lack of Ap^R Km^R segregants indicate inability to maintain the R-M system variant without additional MTase expression. Plates with transformants, after overnight incubation, showed colonies for the following C-box spacer variants: pPvuRM3.4 (WT), pIM20 (CAT/CAA, Sym) and pBR322 control (no R-M system). No colonies were seen for IM18 (CAA/CAA, Sym) and pIM19 (CAA/CAT) (Table 2 of main text; not shown). However, when the same plates were incubated an additional 2 days, the growth of smaller colonies was detected (as shown here). These tiny colonies were tested and determined to possess all three plasmids as indicated by antibiotic resistance for Ap^R, Km^R and Cm^R, unlike the larger colonies that have only two antibiotic resistance markers Ap^R and Km^R.

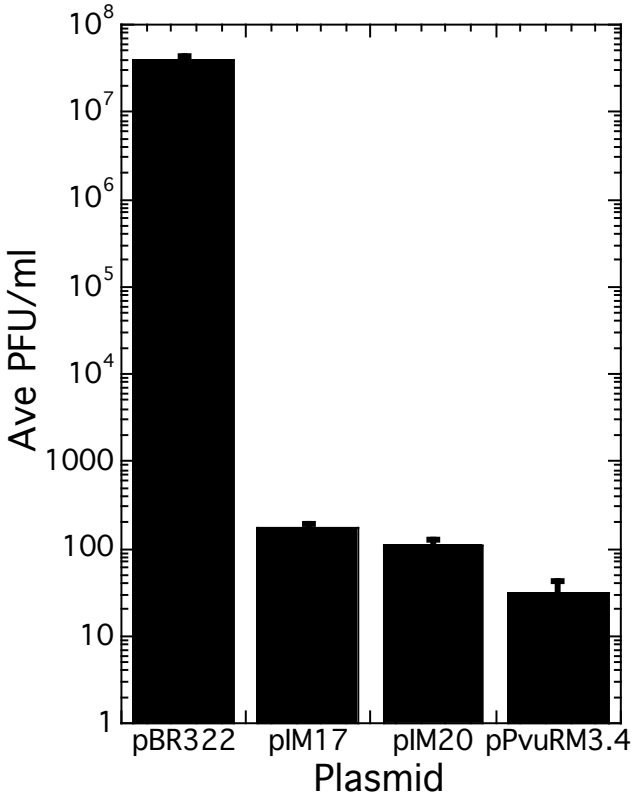

Figure S3. Restriction assay.

The restriction activity of *E. coli* cells carrying PvuII R-M system and its variants was measured through the plaquing efficiency of phage λ*vir*. The average PFU (plaque-forming units) is shown. The average comes from four replicates at each of two phage dilutions differing by 10-fold. Error bars indicate standard error of the mean. As the same phage stock was used in all cases, lower bar heights reflect increased restriction. pBR322 is a nonrestricting vector control, pPvuRM3.4 is the WT PvuII R-M system (positive control), and the two pIM plasmids are variants that are described in the main text. These data (and more) were used to calculate the relative efficiency of λ*vir* plaquing (EOP) shown in Table 2 of the main text

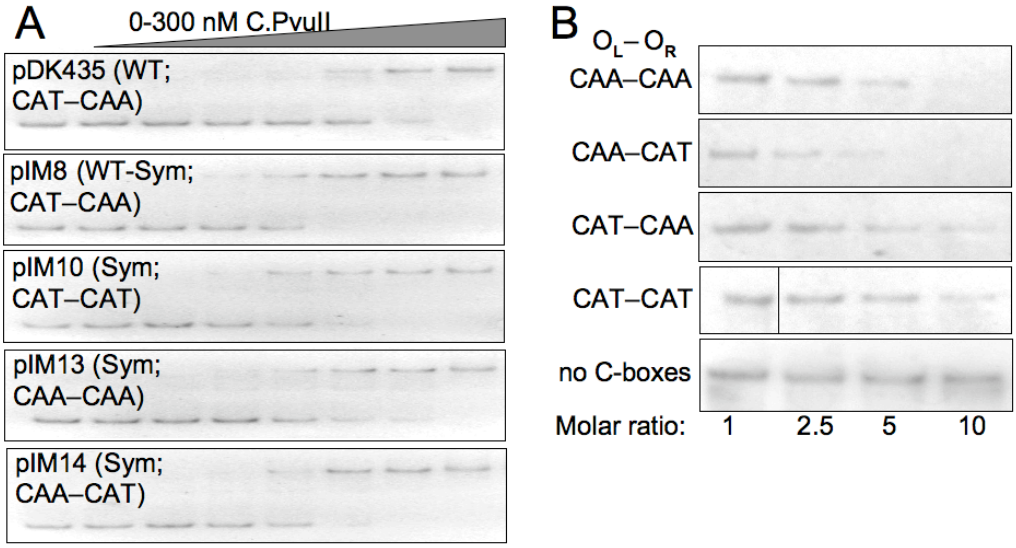

Figure S4. ***In vitro*** **interaction of C.PvuII protein with wild-type and altered C-box regions.**

(A) EMSA (electrophoretic mobility shift assay) reactions were processed as outlined in the main text. Quantitation of these results are shown in Figure 3A. "WT" refers to the native PvuII C-boxes and spacers; the others all have symmetrized spacers with varied intra-operator spacers as indicated.
(B) EMSA competition assays were performed using 200 nM of C.PvuII and 20 nM of biotin-labeled WT C-box 126-mer (as described in Methods). Competition reactions contained increasing amounts of unlabeled 126-mer DNA fragments (from 1- to 10-fold molar excess). The competitor DNAs contained the indicated intra-operator spacers, but were otherwise identical. Following EMSA and electroblotting, the shifted bands for each reaction were visualized and quantified via chemiluminescent detection of the biotinylated DNA as described in Methods.

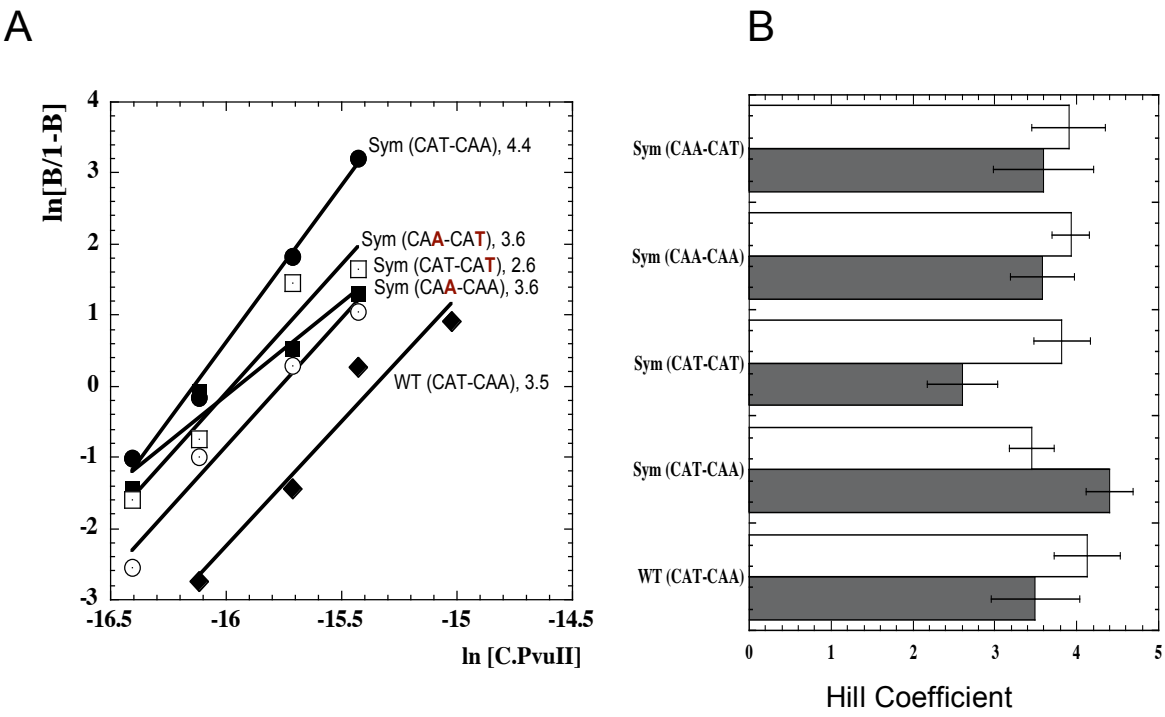

Figure S5. Hill plot showing cooperativity for C.PvuII binding to WT and altered C-boxes.

A. The natural logarithm of [bound / (1-bound)] is plotted vs. that of C.PvuII concentration. "Bound" refers to low mobility complex for C-box variants from Figure S3A. The intra-operator spacer variants (O_L - O_R) are indicated with their calculated Hill coefficient (9).

B. Two sets of repeated data are shown. The standard error bar for Hill coefficient was calculated from the correlation coefficient from the residuals using the "summary(lm(y~x))" function from the R statistical package.

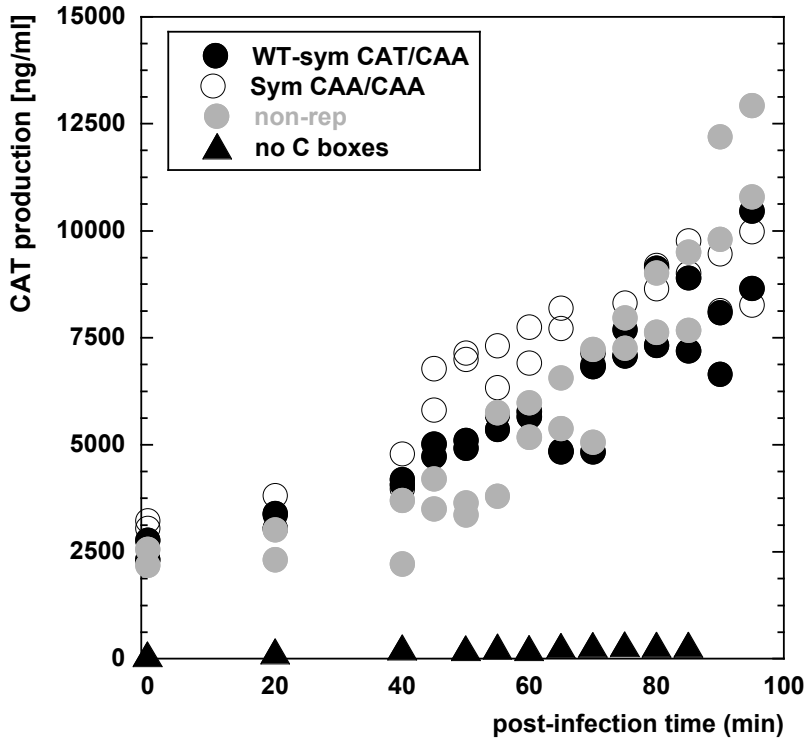

Figure S6. ***In vivo*** **effects of C-box O_L spacer variants on temporal expression of C.PvuII**

Four *E. coli* TOP10' cultures, carrying plasmids with different O_L spacers fused to a *cat* (chloramphenicol acetyltransferase) promotorless reporter gene, were infected with recombinant M13pvuIIwt phage at MOI=15. We used three spacer variants tested previously in this study: WT-Sym (CAT/CAA; pDK178; closed circles), CAA/CAA (pIM22; open circles) and a WT-Sym, but non-repressing regulatory region (box 2B reversed, pWWWR; grey circles) as a control. The first two variants have identical, symmetrized C-boxes, and differ only at a single nt in the O_R spacer. A plasmid with no C-box sequence (pKK-238; triangles) was also tested and gave only background levels of *cat* expression. Growth was monitored at OD_{600nm} before and after phage addition (see Figure 6B), but did not significantly differ between cultures. CAT production in two independently-performed experiments was measured by an ELISA sandwich method (Materials and Methods). These data were normalized for presentation in Figure 6C of the main text.

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