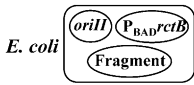


Supporting Information

Venkova-Canova and Chattoraj 10.1073/pnas.1013244108



Fragment	Relative copy # of <i>orill</i> plasmid
(vector)	1
GCGGAAGCATGTAA	0.9±0.1
GCGGAAGCATGTAAATTCATTATCAATTTACGGTCGATG	<0.1
ATGTAAATTCATTATCAATTTACGGTCGATG	1.0±0.1
ATTCATTATCAATTTACGGTCGATG	1.0±0.1
AATTTACGGTCGATG	0.9±0.2
GCGGAAGCATGTAAATTCATTATCAATTTACGGTC	0.2±0.1
GCGGAAGCATGTAAATTCATTATCAATTTAC	0.9±0.1
GTAATTCATTATCAATTTAC	0.9±0.1
<i>Ggccttcgtac</i> TAAATTCATTATCAATTTACGGTCGATG	0.9±0.2
<i>Gtaaggatgca</i> TAAATTCATTATCAATTTACGGTCGATG	0.9±0.1
GCGGAAGCATGTAAATTCATTATCAATTTA <i>gccagctac</i>	0.8±0.1
GCGGAAGCATGTAAATTCATTATCAATTTA <i>taactagca</i>	0.9±0.2
GCGGAAGCATGTAAATTCAT (N5) TATCAATTTACGGTCGATG	0.8±0.1
GCGGAAGCATGTAAATTCAT (N10) TATCAATTTACGGTCGATG	0.8±0.2
GCGGAAGCATGTAAAT-----ATCAATTTACGGTCGATG	1.0±0.2
GCGGAAGCATG-----ATCAATTTACGGTCGATG	0.5±0.1
GCGGAAGCATGTAAATTC-----CAATTTACGGTCGATG	1.1±0.2
GCGGAAGCATGTAAA-----ATTTACGGTCGATG	0.6±0.2
GCGGAACGATTAACCGACCACTAAGTTACGGTGAATG 39mer _{rctA}	0.4±0.1
TTGGAACATA-----GTGATATTACGGTAAAGT 29mer _{rctB}	0.6±0.1
TCGGAACAATGATCAAGTGCACATAAATTACGGTCAATG 39mer _{Pvca1074}	<0.1

Fig. S1. An in vivo system to define the 39-mer site. A three-plasmid system in *E. coli* (Top) was used to define the 39-mer in order for it to function as a negative regulator of copy number of an *orill* plasmid, pTVC35. The other two plasmids were pTVC11, which supplied RctB under arabinose control at near physiological level (Fig. S2), and pTVC61 (or pTVC243), which supplied the 39-mer or its various mutated derivatives. Oligonucleotide fragments were cloned in a transcription-free region of pBR322-derived plasmids pTVC61 or pTVC243. The resulting plasmids (Top to Bottom) were pTVC-132, -151, -156, -157, -190, -162, -119, -120, -182 to -185, -165, -181, -329* to -332*, -191, -400*, and -350*, respectively. The vector for plasmids with asterisks was pTVC243. Fragment 1 is the previously reported 14-mer (1). The sequences of the direct repeats flanking the 19-bp AT-rich linker are shown in bold. Substitutions are shown in lowercase letters and in italics. Insertions of 5 bp (5'-TACTG-3') and 10 bp (5'-TACTGATGAT-3') in the AT-rich linker are indicated as (N)₅ and (N)₁₀, respectively. Five- and 10-bp deletions are shown as dashes. The copy number of the *orill* plasmid in the presence of 0.002% arabinose and without the 39-mer *in trans* was taken as 1, and other copy numbers are relative to this value. The *orill* copy number of <0.1 indicates that the plasmid band was too faint to be measurable under our experimental conditions.








Extract source: <i>V. cholerae</i>	<i>E. coli</i> / P _{BAD}		<i>E. coli</i> / <i>porII</i> / P _{BAD} <i>rctB</i>				
% Arabinose	0	0.002	0.2	+ Frag. 3		+ Frag. 11	
OD ₆₀₀ of cells	0.5	0.2	0.2	0.2	0.05	0.2	0.05
							
[RctB]	1	n.a.	n.a.	1.6	11	0.8	13
Lane #	1	2	3	4	5	6	7

Fig. S2. Western blotting to confirm RctB induction by arabinose. The method used was as described (2). The amount of RctB expressed in wild-type *V. cholerae* (CVC250 in our strain collection) is used as a standard (arrow, lane 1), and RctB concentrations in other samples are relative to this value. *E. coli* (BR8706) with P_{BAD} vector (pTVC12) was used as the negative control (lanes 2 and 3). The experimental cultures are from Fig. 2, and they had *E. coli* (BR8706) with three plasmids: *orill* plasmid (pTVC35), P_{BAD}*rctB* plasmid (pTVC11), and the plasmid with the *inclI* fragment 3 (pTVC94) or fragment 11 (pTVC175; lanes 4–7). RctB concentrations are shown for the two arabinose (inducer) concentrations used in Fig. 2 to supply low and high RctB, respectively. The RctB band (arrow) was recognized using polyclonal antibodies against RctB. The antibodies also recognized another *E. coli* protein (seen above the RctB band and in negative controls), the intensity of which was used to normalize loading amounts. Total OD₆₀₀ of cells loaded were 0.2 in lanes 2–7. The samples in lanes 5 and 7 were diluted fourfold with the extract from lane 3. Values of [RctB] shown are for the undiluted cultures in all cases. The results show that the low [RctB] used approximated the physiological level and the high [RctB] was about 10-fold higher.

	Locus				
<i>V. cho</i>	(<i>incII</i>)	450	CGGAAGCATG ----	TAAATTCATTATCA ATTACGGTCG -ATG	487
	(<i>rctA</i>)	177	CGGAAGCATT ----	AAACCGAGCCACT AGTTACGGTGA -ATG	214
	(<i>PrctB</i>)	1090	TGGA ACTATA -----	GTGAT ATTACGGTAA -GTG	1117
<i>V. vul</i>	(<i>incII</i>)	1027996	CGGAACAATG ----	ATCAAGTGCACATA AAATTACGGTCA -ATG	1028033
	(<i>rctA</i>)	1272270	CGGAAGCATG ----	AAAAAATATCACT AGTTACGGTCAAATG	1272308
	(<i>PrctB</i>)	1271794	CGGAAGCATT ----	GACATTGGTCACT AAACTACGGTCA -ATG	1271831
<i>V. par</i>	(<i>incII</i>)	1272906	TGGA ACTATA -----	GCGCT TTTACGGTCA -ATG	1272933
	(<i>PrctB</i>)	23218	CGGAAGCATT ACC	ACTGCGGCAGTACAG AACGATCGGCTGGCTC	23262
	(<i>rctA</i>)	458	CGGAAGCATG ----	CTTTT AATGCGCTATTACGGCTGAGTG	496
<i>V. fis</i>	(<i>incII</i>)	143	CGGAAGCATT ----	ATATAAGTGGGCT AAATTACGGATT -ATG	180
	(<i>PrctB</i>)	1107	CGGAACCATA -----	GCAC ACTACGGCTAGTGT	1135
	(<i>rctA</i>)	448	CGGAAGCATG ----	TATCTTCTTTCT AAATTACAGAGG -ATG	485
<i>V. sal</i>	(<i>incII</i>)	217	CGGAAGCGTG ----	AAAAATAT TGGCTAAATTACGGTAA -GTG	254
	(<i>PrctB</i>)	1015	CGAAAGAATG -----	ATT AAATACAAATTA -ATG	1042
	(<i>rctA</i>)	465	CGGAAGCATG ----	ATAAGCAAAGGATA AAATACGGAGA -GTG	502
<i>V. spl</i>	(<i>incII</i>)	215	CGGAAGCGTG ----	ATAATAGTCACT AAACTACGGAGGTGT	253
	(<i>PrctB</i>)	1046	CGAAACAATG -----	ATG AAATACAAATGA -ATG	1073
	(<i>rctA</i>)	1675350	CGGAAGCATG ----	TAATTTAAGAGCT AGTTACGGCAAATG	1675312
	(<i>rctA</i>)	157	CGGAAGCATT ----	GAAACACAACCAT AGATTACGGTGTG -GTG	115
	(<i>PrctB</i>)	1674705	AGGA ACTATA-----	GTG AAATTACGGTTA -GTG	1674678

Fig. S3. Conservation of 39-mer-like sequences in the *Vibrio* family. Several 39-mer-like sequences were found in *chrII* of completely sequenced *Vibrio* genomes recorded in GenBank. Their accession numbers are as follows: NC_002506 for *V. cholerae* (*V. cho*), NC_004460 for *V. vulnificus* (*V. vul*), NC_004605 for *V. parahaemoliticus* (*V. par*), NC_006841 for *V. fisheri* (*V. fis*), NC_011313 for *Aliiv. salmonicida* (*V. sa*), and NC_011744 for *V. splendidus* (*V. spl*). The locus names and end coordinates of the 39-mer-like sequences are shown. The conserved bases are highlighted in bold and the direct repeats are boxed.

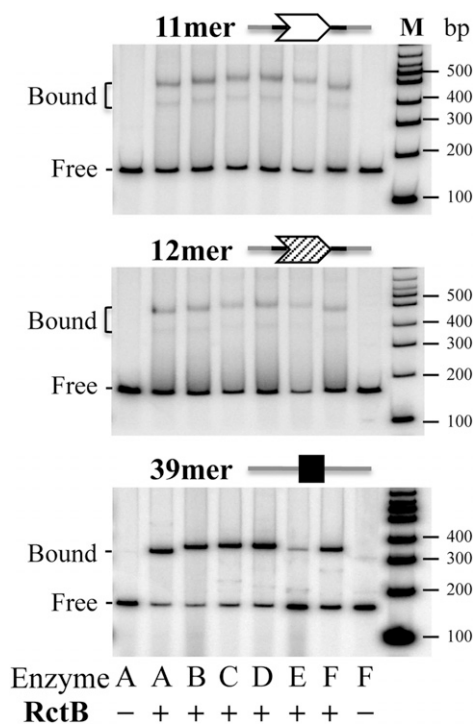


Fig. S4. RctB binds and bends 11-, 12-, and 39-mers in vitro. A 5% PAGE showing binding of purified RctB protein to circularly permuted DNA fragments carrying single 11-, 12-, or 39-mer. The fragments were obtained from plasmids pTVC174, pTVC194, and pTVC195, all derivatives of pBEND-2 (Table S1), after digestion by the following enzymes: MluI (A), NheI (B), XhoI (C), EcoRV (D), NruI (E), and BamHI (F). RctB (100 ng) was used in all binding reactions, and the protein was activated with chaperones before use (3). Lane M contains the 2-Log Ladder (NEB), which was dephosphorylated by SAP and end-labeled by γ -ATP using T4 kinase. Note that the iterons (11- and 12-mers) show two retarded species, the basis of which remains to be understood.

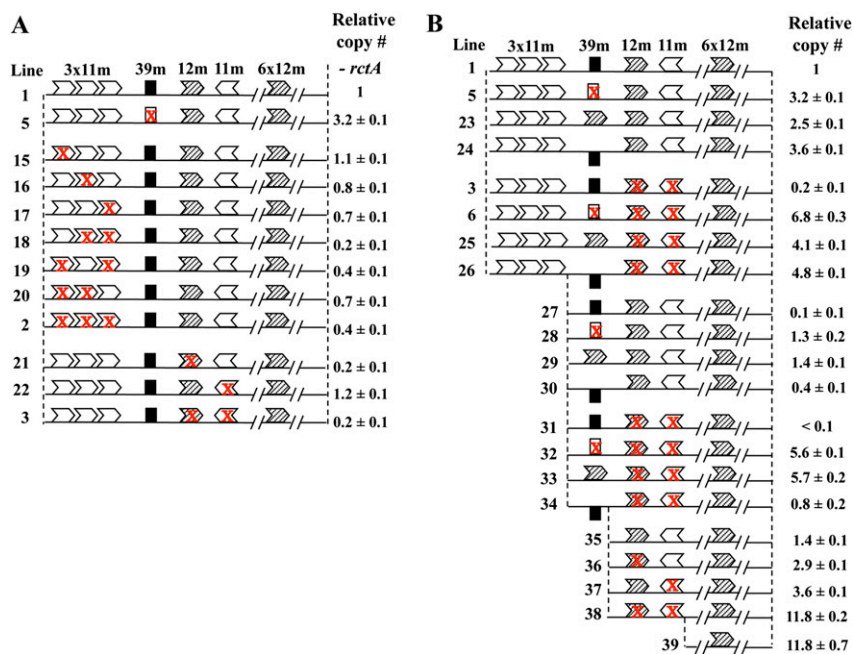


Fig. 55. (A and B) Regulatory activity *in cis* from individual elements of *inclI*. The details of these experiments are identical to those of Fig. 3A except that the *inclI* elements have been mutated one or two at a time. Plasmids in lines 1, 2, 3, 5, and 6 are the same as the ones used in Fig. 3A. Plasmids 15–22 carrying *rctA in cis* are pTVC-391, -392, -410, -412, -411, -399, -279, and -307. Plasmids 15–39 lacking *rctA* are pTVC-395, -396, -423, -424, -425, -426, -295, -317, -319, -394, -333, -427, -251, -278, -322, -398, -321, -336, -334, -428, -253, -281, -323, -324, and -337. (A) These experiments show that individual iterons can dampen the negative regulatory action of the 39-mer but their location is important. Mutating the two extreme iterons had little effect on copy number (line 1 vs. 15 and 22), whereas the iterons proximal to the 39-mer had the most profound effect (line 1 vs. 17 and 21). (B) The *inclI* 12-mer is not as effective compared with the 39-mer as a negative regulator. The replacement of the 39-mer by a 12-mer had essentially the same effect on copy number as its inactivation or inversion. The magnitude of the loss of the 39-mer activity upon inversion was dependent on iterons. For example, inversion of the 39-mer essentially inactivated the site in lines 24 and 26 but retained considerable activity in lines 30 and 34. The last set of plasmids (lines 35–39) show that unlike the earlier situation where the 12-mer neutralized the 39-mer much more effectively than the rightmost 11-mer, the two iterons can contribute equivalently to the copy number control in the absence of the 39-mer. Thus, the role of iterons also changes depending upon the context. The basic behavior of the 39-mer and its neutralization by iterons, however, was retained in these simplified contexts (e.g., lines 27, 28, and 31).

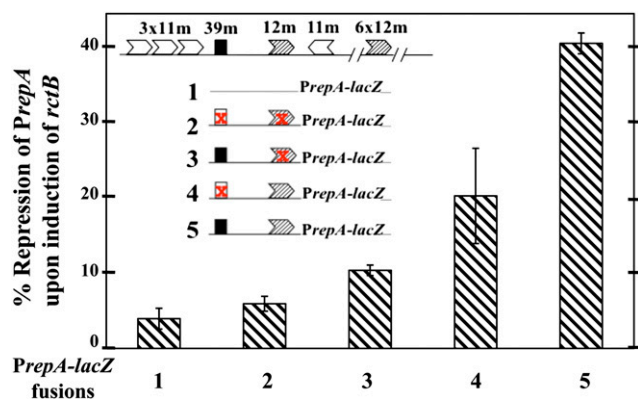


Fig. 56. Interactions between the 39-mer and the *incll* 12-mer in vivo. Because the 39-mer was more active *in cis* than when present in a multicopy plasmid *in trans* (Fig. 2, fragment 7 vs. Fig. 3A, line 4), a likely mechanism for the *cis* preference could be DNA looping. To test this, an in vivo assay for DNA looping was devised with the premise that the looping could interfere with transcription initiation if it improved binding of a repressor that otherwise would bind weakly (4). To test whether the 39-mer could improve binding of a 12-mer by directly interacting with it, the *incll* region containing the 39-mer and the 12-mer was tested (*inset*). The region with the sites variously mutated was cloned in front of a foreign promoter, *PrepA*, fused to *lacZ* (*PrepA-lacZ*), and β -galactosidase activity was used as a reporter for RctB binding. The resultant plasmids were pTVC234 (called 1), pTVC481 (called 2), pTVC479 (called 3), pTVC480 (called 4), and pTVC470 (called 5). The *incll* region was cloned such that the 12-mer is placed 23 nt upstream of the -35 box of *PrepA*. The RctB was supplied from $P_{BAD}rctB$ -containing plasmid, pTVC11, as in Figs. 2 and 3. β -Galactosidase activity was measured with and without induction of $P_{BAD}rctB$ (0% and 0.2% arabinose, respectively) for each plasmid, as described (5). Percent repression was calculated separately for each plasmid and is the difference between the uninduced and induced β -galactosidase activities expressed as a percent of the uninduced activity. The 12-mer alone interfered with transcription moderately. Addition of the 39-mer *in cis* improved repression, thus supporting the notion of DNA looping. Together with the in vitro evidence of heterologous interactions between the 39-mer and the iterons *in trans* (Fig. 5), it appears that the two kinds of RctB binding sites can interact with each other directly.

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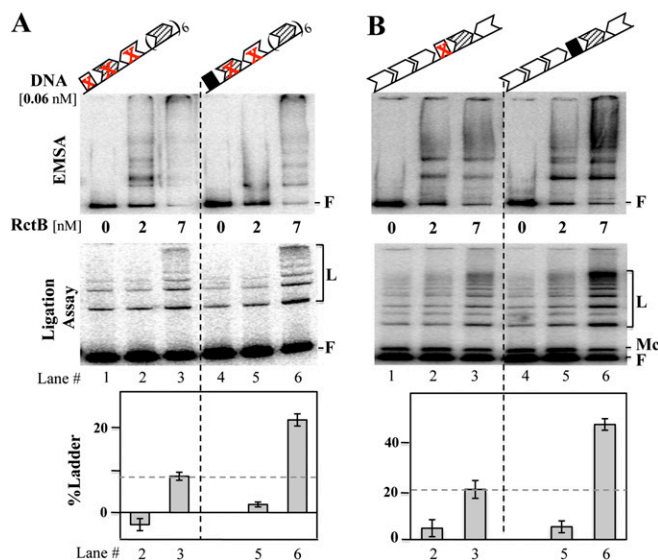


Fig. 57. Increase in ladder formation of origin 12-mers (A) and of control 11- and 12-mers (B) in the presence of the 39-mer *in cis*. (Top) Fragments used. The symbols are the same as in Fig. 3A. (A) The fragments with mutated and intact 39-mer were obtained from plasmids pTVC467 and pTVC353, respectively. The fragments have the natural sequence between 6×12 -mers and the 39-mer except that the intervening 12- and 11-mers were mutated. A 5% PAGE was used for both EMSA and the ligation assay. Circular ligated products did not enter the gel. Percent ladder was estimated as in Fig. 4. (B) The fragments with mutated and intact 39-mer were obtained from plasmids pTVC298 and pTVC301, respectively, and the ligation products were run on 1% agarose gel, where monomer circles (Mc) could enter. The other details are same as in A. At the higher RctB concentration (7 nM), the total binding as measured by the loss of free (F) DNA was essentially identical (92.5% vs. 91.4% with and without the 39-mer, respectively), but ladders were significantly higher (62% vs. 38%, respectively). The error bars are from three experiments in A and from three different exposures of the same gel in B.

Table S1. Bacterial strains and plasmids

	Relevant characteristics	Source
Strains		
BR8706	= Stbl2 $\Delta(lac-proAB)$ $\Delta(araFGH)$ $\Delta araEp$ $P_{CP18-araE}$; <i>araE</i> under constitutive CP18 promoter	(1)
CVC205	<i>V. cholerae</i> El Tor N16961 <i>lacZ::res::tet::res</i>	(2)
CVC250	CVC205 <i>recA::Kan</i>	(2)
CVC553	DH5 $\Delta lac(\lambda pir)$, where $\lambda pir = \lambda DKC370$; strain for maintaining R6Kori γ plasmids	This study
DH5 α	Strain for cloning	Invitrogen
Plasmids		
pACYC184	P15Aori plasmid with unique EcoRV site; Cm ^R	NEB
pBEND-2	Vector for bending assay with unique XbaI, Sall sites; Ap ^R	(3)
pMLB1109	Source of promoter-less <i>lacZ</i> gene; Ap ^R	(4)
pTVC11	<i>rctB</i> under P _{BAD} control in pSC101ori plasmid; Sp ^R	(2)
pTVC12	P _{BAD} in pSC101ori plasmid; Sp ^R	(2)
pTVC20	<i>orill</i> (nt 109–1,133) in R6Kori γ plasmid; Ap ^R	(4)
pTVC35	<i>orill</i> (nt 775–1,133); Requires RctB for replication; Ap ^R	(4)
pTVC61	pBR322ori plasmid with transcription-free MCS. Digestion with EcoRV and HpaI leaves 300-bp flanks on either side of MCS; Cm ^R	(4)

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3. Kim J, Zwieb C, Wu C, Adhya S (1989) Bending of DNA by gene-regulatory proteins: Construction and use of a DNA bending vector. *Gene* 85:15–23.
4. Venkova-Canova T, Srivastava P, Chattoraj DK (2006) Transcriptional inactivation of a regulatory site for replication of *Vibrio cholerae* chromosome II. *Proc Natl Acad Sci USA* 103: 12051–12056.

Table S2. Plasmids in Figs. 2, 3A, 4, and 5 and Figs. S1, S4, S5, S6, and S7

Plasmid	Relevant characteristics	Used in
pTVC151	pTVC61 + <i>orill</i> (nt 449–487); Cm ^R	Fig. 5 and Fig. S1
pTVC158	pACYC184 with transcription-free Eco01019 fragment from pTVC61; Cm ^R	Fig. 2, line 1
pTVC174	pBEND-2 + <i>orill</i> (nt 449–487); Ap ^R	Fig. S4
pTVC194	pBEND-2 + <i>orill</i> (nt 344–380); Ap ^R	Fig. S4
pTVC195	pBEND-2 + <i>orill</i> (nt 565–602); Ap ^R	Fig. S4
pTVC210	pTVC20 + <i>lacZ</i> from pMLB1109; <i>PrctB-lacZ</i> ; contains <i>rctA</i> ; Ap ^R	Fig. 3A, line 1
pTVC211	pTVC210; <i>orill</i> spans nt 253–1133 (without <i>rctA</i>); Ap ^R	Fig. 3A, line 1
pTVC228	pTVC243 + <i>orill</i> (nt 788–934); Cm ^R	Fig. 5
pTVC234	pMLB1109 + <i>PrepA</i> from P1 (nt 556–600) between EcoRI and BamHI sites; Cm ^R	Fig. S6
pTVC243	pTVC61 deleted for 200 bp from each side of MCS leaving 100-bp flanks when cut by EcoRV and HpaI; Cm ^R	Fig. S1
pTVC248	pTVC243 + <i>orill</i> (nt 291–445) + 49-bp oligonucleotide linker to match the size of the <i>orill</i> in pTVC255; Cm ^R	Fig. 4
pTVC251	pTVC210 but <i>orill</i> spans nt 441–1133; Ap ^R	Fig. S5, line 27
pTVC253	pTVC210 but <i>orill</i> spans nt 468–1133; Ap ^R	Fig. S5, line 35
pTVC255	pTVC243 + <i>orill</i> (nt 289–487); Cm ^R	Fig. 4
pTVC256	pTVC255 with 5-bp insertion at chrII coordinate 444; Cm ^R	Fig. 4
pTVC257	pTVC255 with 10-bp insertion at chrII coordinate 444; Cm ^R	Fig. 4
pTVC298	pTVC243 + <i>orill</i> (nt 289–717); Cm ^R	Fig. S7B
pTVC301	pTVC298 with mutated 39-mer; Cm ^R	Fig. S7B
pTVC337	pTVC210 but <i>orill</i> spans nt 722–1133; Ap ^R	Fig. S5, line 39
pTVC351	pTVC243+ <i>orill</i> (nt 444–934); Cm ^R	Fig. S7A
pTVC353	pTVC351 with mutated 12-mer and 11-mer; Cm ^R	Fig. S7A
pTVC467	pTVC353 with mutated 39-mer; Cm ^R	Fig. S7A
pTVC470	pTVC234 + <i>orill</i> (nt 439–600), which places the 12-mer 23 bp upstream of –35 box of <i>PrepA</i> ; Ap ^R	Fig. S6
pTVC479	pTVC470 with mutated 12-mer; Ap ^R	Fig. S6
pTVC480	pTVC470 with mutated 39-mer; Ap ^R	Fig. S6
pTVC481	pTVC470 with mutated 12-mer and 39-mer; Ap ^R	Fig. S6