Supplementary Data

Replication regulation of *Vibrio cholerae* chromosome II involves initiator binding to the origin both as monomer and as dimer

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Table S1. Primers used in this study

Name	Sequence (5′-3′)	Used to construct
JJ1	CGTCCATATGAGCTCAGAAGAAAAACGA	pJJ03
JJ2	ATCTGCGGCCGCTTACTGCTTGGAAACCAGCTC	pJJ02
JJ36	CATACCTTGGTATCTCAGCTCTACTCCTAT	pJJ58
JJ52	AATTCGTAAATCGTCGCGGAAGCATGTAAATTC ATTATCAATTTACGGTCGATGTCAGGCAGAGTG	pJJ17
JJ53	AATTCACTCTGCCTGACATCGACCGTAAATTGA TAATGAATTTACATGCTTCCGCGACGATTTACG	pJJ17
JJ54	AATTCTTGTGGATAAACTGTGTGAGCACCTTGA TCATCGTTCTGAATGGCTTAAAATAATCTCTTG	pJJ16
JJ55	AATTCAAGAGATTATTTTAAGCCATTCAGAACG ATGATCAAGGTGCTCACACAGTTTATCCACAAG	pJJ16
JJ60	GTATCTCGAGTTACTGCTTGGAAACCAGCTC	pJJ03
JJ64	TAGGAGTAGAGCTGAGATACCAAGGTATGTTGTT	pJJ58
JJ70	AGTAGGATCCTTAGGCTCCAGCGGCCATCTC	pJJ56
JJ115	ATGCGAATTCATGAGCTCAGAAGAAAAACG	pJJ56
GD1	TATGGAATTCGCGGCCGCGAGCTCGGATCCGTCGACAAGCTTCAAT TGGGTACCC	pGD14
GD2	TCGAGGGTACCCAATTGAAGCTTGTCGACGGATCCGAGCTCGCGGC CGCGAATTCCA	pGD14
GD4	ATAATATTGAAAAAGGAAGAGTATGAGGGAAGCGGTGATCG	pGD12

GD5	CGATCACCGCTTCCCTCATACTCTTCCTTTTTCAATATTAT	pGD12
GD6	AAAACTGCAGTTATTTGCCGACTACCTTGGTG	pGD12
GD7	GCCTTTCATGATATATCTCCC	pGD12
GD9	CGGTATCATTGCAGCACTGG	pGD12
GD10	CCGGAATTCAAGAAGGAGATATACATGAGCTCAGAAGAAAAACGATT	pGD24
GD13	GAGGCACCTGTGGAAGGATCCGATGGCGAGATCCGCATC	pGD16
GD14	GATGCGGATCTCGCCATCGGATCCTTCCACAGGTGCCTC	pGD16
GD16	AAAACTGCAGCTGTCAGACCAAGTTTACTCAT	pGD12
GD17	TCTCGCGTCAATAGCTCTTC	pGD16 and pGD19
GD18	AGTGAGAATATGCCAGAAGGC	pGD16 and pGD19
GD23	CATACCTTGGTATCTCAGCTCTAC	pGD28 and pGD43
GD24	GTAGAGCTGAGATACCAAGGTATG	pGD28 and pGD43
GD27	CGGGGTACCTTACTGCTTGGAAACCAGCTC	pGD24
GD30	ATTAATACGACTCACTATAGG	pGD16 and pGD19
GD31	GCTAGTTATTGCTCAGCGG	pGD16 and pGD19
TVC1	GAACTATAGTGCTAGCACGGTAAGTGTGA	pJJ02
TVC141 TVC142	AATTCCAAATTTTTCTTTATTTATGATCTCTTTTTCTTTATTCTCTTGGAACTATAGTGATATTACGGTAAGTGTGATACGGATCTAACCG AATTCGGTTAGATCCGTATCACACTTACCGTAATATCACTATAGTTCCAAGAGAATAAAGAAAAAGAGATCATAAATAAAGAAAAATTTGG	pAS1
TVC143 TVC144	GGCCGCTCGATCTTGTATTGATCATGGTTTCCATCGATACATGATCATGCTTCTGAATGGCTTAAAATAATCTCTTTTAAC TCGAGTTAAAAGAGATTATTTTAAGCCATTCAGAAGCATGATCATGTATCGATGGAAACCATGATCAATACAAGATCGAGC	pTVC148
TVC145 TVC146	AATTCCAAATTTTTCTTTATTTATGATCTCTTTTTCTTTATTCTCTCGGAAGCATGTAAATTCATTATCAATTTACGGTCGATGTGATACGGATCTAACCG AATTCGGTTAGATCCGTATCACATCGACCGTAAATTGATAATGAATTTACATGCTTCCGAGAGAATAAAGAAAAAGAGATCATAAATAAAGAAAAATTTGG	pTVC500

Table S2. Transformation efficiency of *E. coli* WT and Δ*dnaKJ* cells by a low-copy *oriII* plasmid

E. coli / pRctB (Sp^R)	Number of transformants[a] with	
	pACYC177 (positive control, Ap^R)	Low-copy p*oriII* (pTVC22, Ap^R)
WT / pGD16	~500	~140
Δ*dnaKJ* / pGD16	~500	0
Δ*dnaKJ* / plasmid library[b]	~2000	32

[a]Transformants were selected on plates containing Ap (100 μg/ml) and Sp (40 μg/ml).

[b]The library also contained pGD16 except that its *rctB* gene was mutagenized randomly.

Table S3. Description of RctB mutants isolated in this study

Mutant name	Amino acid substitutions	Initiator activity[a] with multiple amino acid substitutions	Initiator activity[a] with single amino acid substitution
JK1[b]	T149M F378L A567E	Yes	NT[c] Yes NT
JK2	P190T V201L F233I	Yes	No No Yes
JK3	S122A E496V K502ochre	Yes	No No Yes
JK4	F378S R525I	Yes	Yes No
CD26[d]	E335D F378Y L420I R651H K580N	No	NT Yes NT NT NT

[a]The initiator activity was checked by the ability to replicate pTVC22 in Δ*dnaKJ* cells.

[b] JK1 and JK4 were found once each, and JK3 and JK4 were found four times each.

[c]NT stands for not tested.

[d]This mutant was isolated in a different screen, not detailed in this study, but is included here because one of the amino-acid substitutions affected the position F378.

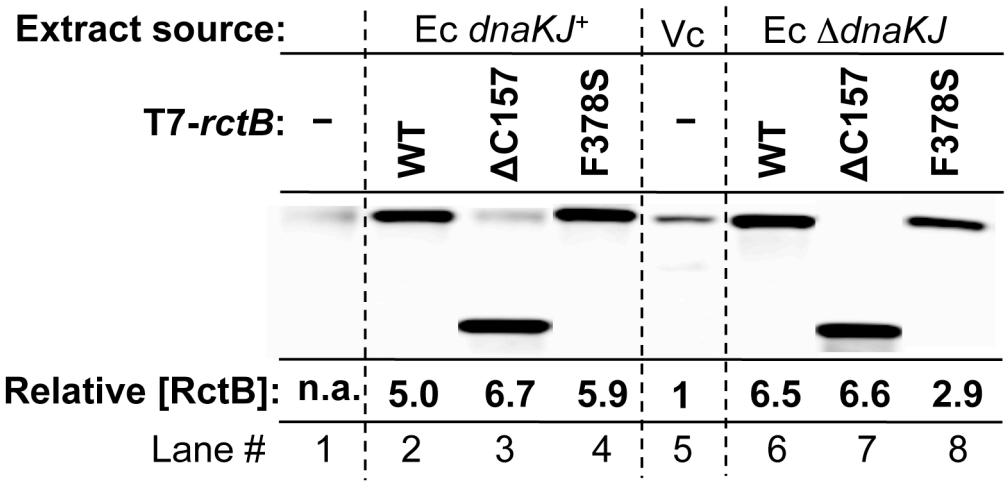


Figure S1. Western blotting analysis of the *dnaJK*$^{+}$ (WT) and Δ*dnaJK* cells expressing RctB. The numbers below the blots show RctB amounts relative to that of *V. cholerae* cells (N16961). The average intensity of the cross reacting bands seen in lanes 1 and 3 was subtracted from the intensities of the bands seen in lanes 2 and 4 while calculating relative amounts of RctB.

For Western blotting, *E. coli* and *V. cholerae* cells of total OD = 1 or 2, were harvested at late log phase (OD 0.4-0.5) by centrifugation for 5 min at 13,000 rpm (Biofuge fresco) at room temperature and the pellet was frozen. Upon thawing, the pellet was dissolved in 100 μl 1x SDS Red Loading Sample Buffer with 1x DTT (NEB), vortexed, boiled for 7 min, vortexed again and spun for 5 min 13,000 rpm at room temperature. An aliquot of the supernatant was loaded on 7.5% Tris-HCl Pre-cast Criterion gel (Bio-Rad). The gel was run in 1x Tris-HCl SDS buffer (Bio-Rad) at constant 200V for ~45-50 min (5 min after the front dye ran out of the gel). The proteins were transferred onto a membrane using iBlot Fast Transfer apparatus, following the manufacturer's protocol (Invitrogen). The membrane was blocked for 1 hour in 5% blocking reagent (GE Healthcare) in PBS-T [1x PBS (KD Medical, Columbia, MD) + 0.1% Tween 20 (Affymetrix, Santa Clara, CA)], and was incubated for 1 hour with purified RctB antibody (1:3500 dilution in 5% blocking reagent in PBS-T). The membrane was washed with PBS-T three times, 20 min each, and incubated for 1 hour in 5% blocking reagent in PBS-T containing 1:5000 diluted anti-rabbit horseradish peroxidase-conjugated secondary antibody from donkey (GE Healthcare). The membrane was finally washed three times, 20 min each, with PBS-T and kept in fresh PBS-T before staining and imaging. Four ml of each detection solution of SuperSignal* West Pico Chemiluminescent ECL Substrate (ThermoScientific, Waltham, MA) was mixed and incubated with the membrane for 1 min. The images were visualized using Fuji LAS-3000 imaging system.

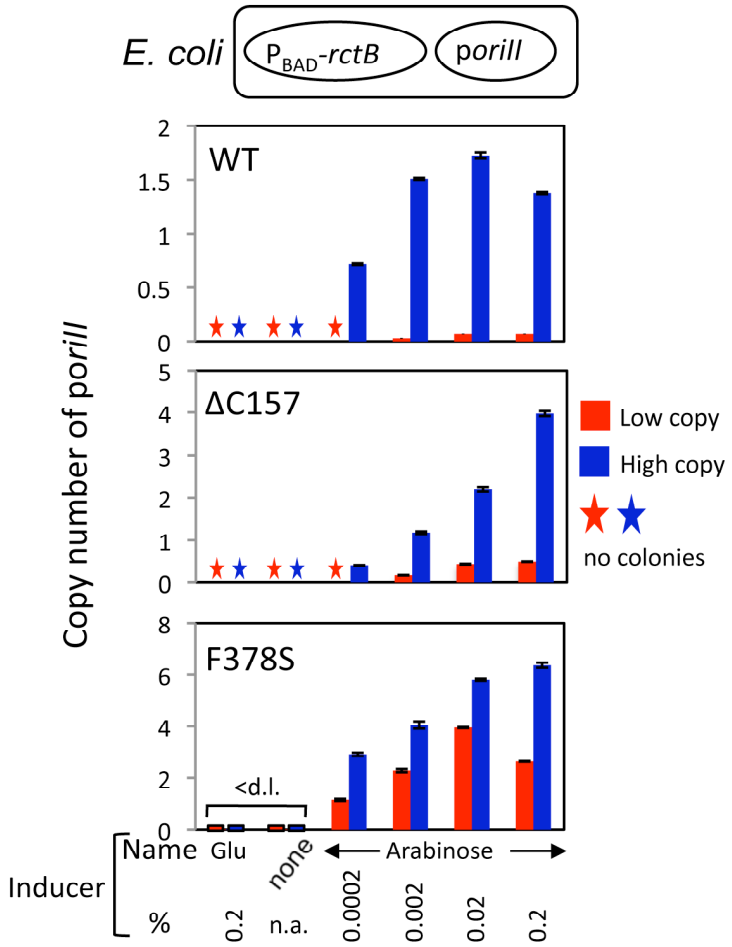


Figure S2A. Replication of low- and high-copy *oriII*-plasmids in WT *E. coli* where *rctB* supply was gradually increased from an inducible promoter. The cartoon at the top of the figure shows the two-plasmid system used to measure the *oriII* plasmid copy number. The system is same as in Figure 1 except that RctB was supplied from a low-copy vector, pSC101, instead of pBR322, and the *rctB* genes were under the control of a glucose (Glu) inhibited and arabinose (Ara) inducible promoter, P_{BAD}, and the host was BR8706, where the arabinose transporter is made constitutively to avoid all or none induction (44). The plasmids supplying the WT, ΔC157 and F378S RctB proteins were pTVC11, pJJ02 and pJJ58, respectively. Red and blue bars represent replication of low-copy (pTVC251) and high-copy (pTVC336) plasmids, respectively, as in Figure 1. The stars represent cases where the copy number could not be measured because no transformants were seen. In some cases transformants were seen but the intensity of the plasmid band was below the detection limit (<d.l.).

44. Khlebnikov, A., Datsenko, K.A., Skaug, T., Wanner, B.L. and Keasling, J.D. (2001) Homogeneous expression of the P_{BAD} promoter in *Escherichia coli* by constitutive expression of the low-affinity high-capacity AraE transporter. *Microbiology*, **147**, 3241-3247.

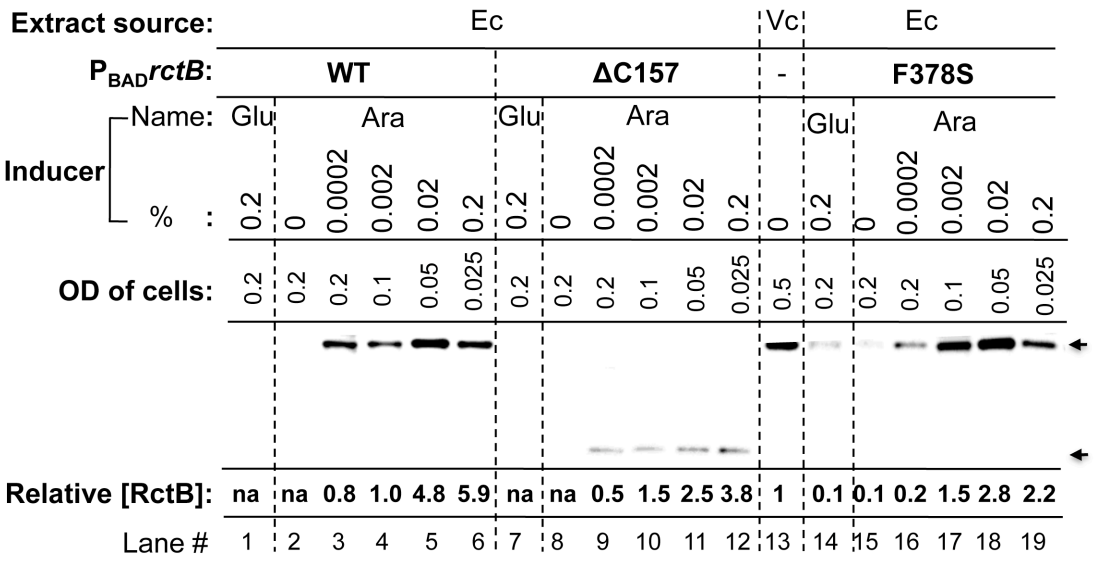
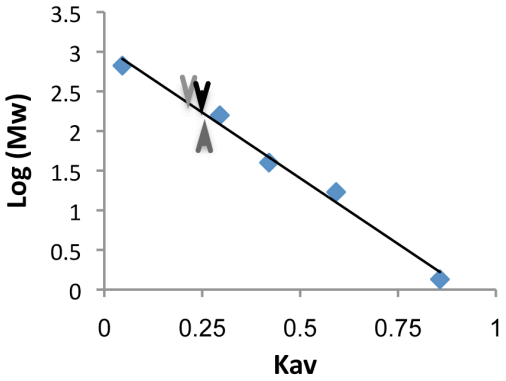


Figure S2B. Western blotting to confirm RctB induction from P_{BAD}. The samples are same as in Figure S2A and in Figure 2, and the details of Western blotting are same as in Figure S1. The RctB concentrations shown are normalized with respect to OD of cells and to the intensity of the *V. cholerae* band.



	Kav	Mw (kDa)
WT	0.257	163
F378S	0.249	173
ΔC157	0.280	136

Figure S3. Molecular weight determination of WT and mutant RctB proteins by size exclusion chromatography. The chromatography was performed using Superdex 200 10/300 GL column (GE Healthcare) equilibrated with the RctB buffer {25 mM Tris-HCl (pH 8.0), 300 mM NaCl, 5% glycerol (v/v) and 1 mM EDTA}, and connected to ACTA FPLC (GE Healthcare). The column void volume was determined with Blue Dextran 2000. The column was calibrated with Biorad gel filtration standard #151-1901 and Blue Dexran. The molecular weight of RctB proteins (Table) was calculated from the plot of the gel-phase distribution coefficient (*K*av) vs. log Mw of protein standards. *K*av=(*V*e-*V*o)/(*V*c-*V*o), where *V*e = elution volume, *V*o = column void volume, and *V*c = geometric column volume. *K*av values are shown by arrows and the corresponding molecular mass in kDa in the Table.

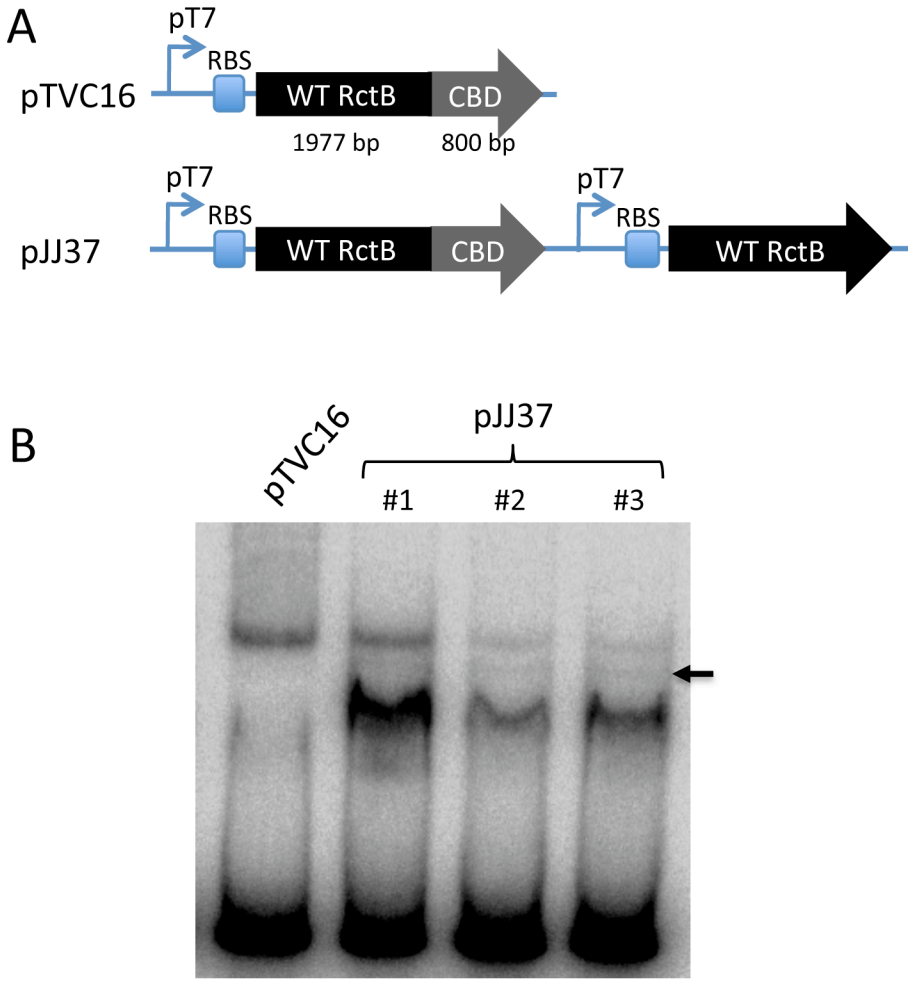


Figure S4. EMSA of RctB binding to 12-mer as dimer. A) Shows the relevant region of plasmid pTVC16 expressing WT RctB fused in frame to chitin binding domain (RctB-CBD), and plasmid pJJ37expressing WT RctB + RctB-CBD simultaneously. The proteins were produced in BL21(DE3) cells using 0.2 mM IPTG at 20°C. B) EMSA using an end-labeled 12-mer fragment and 10 µg of whole cell lysate of cells expressing RctB as described above. The samples were run in a 5% polyacrlyamide gel. Binding is shown for extracts starting from three independent colonies.

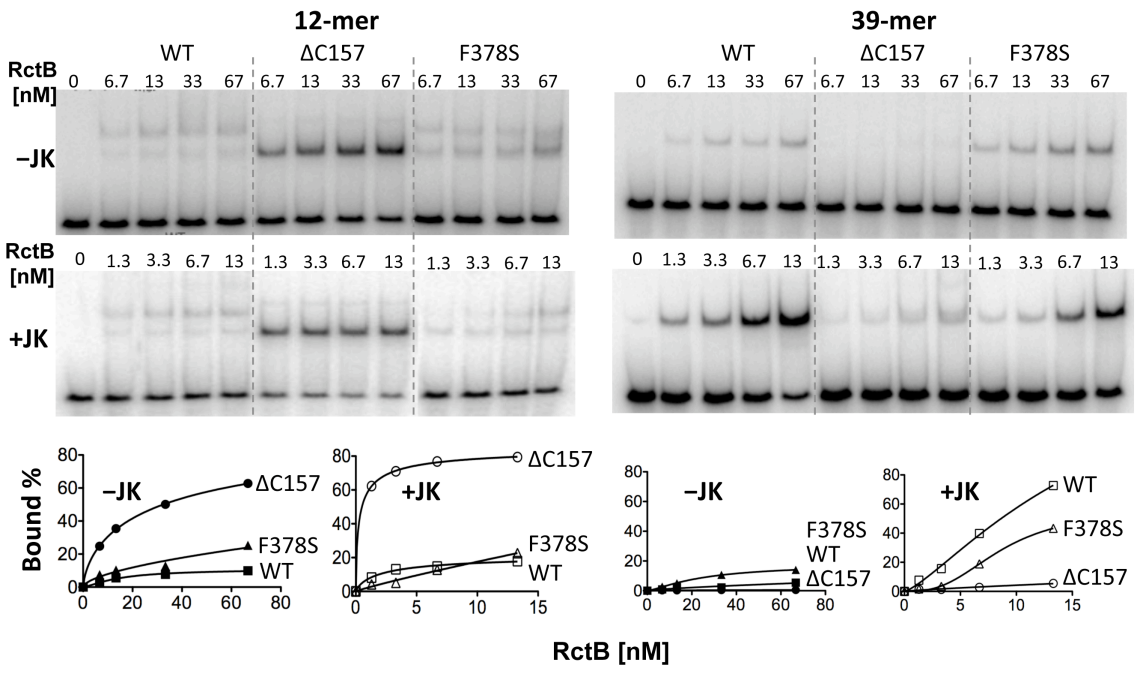


Figure S5. EMSA of RctB binding to a 12- or a 39-mer fragment in the presence or in the absence of chaperones. EMSA was performed with end-labeled (^{32}P) DNA fragments carrying a single copy of either a 12-mer or a 39-mer, same as in Figure 3. The upper panels represent binding in the absence of chaperones DnaJ and DnaK (− JK) and the bottom panels in the presence of chaperones (+JK). RctB concentrations ranged from 1.3-13 nM in +JK conditions and 6.7-67nM in − JK conditions. The binding was analyzed using a 5% polyacrlyamide gel. Binding profiles were obtained by fitting the data using the equation shown the legend to Figure 3.