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Multipartite Regulation of *rctB*, the Replication Initiator Gene of *Vibrio cholerae* Chromosome II

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Replication initiator proteins in bacteria not only allow DNA replication but also often regulate the rate of replication initiation as well. The regulation is mediated by limiting the synthesis or availability of initiator proteins. The applicability of this principle is demonstrated here for RctB, the replication initiator for the smaller of the two chromosomes of *Vibrio cholerae*. A strong promoter for the *rctB* gene named *rctBp* was identified and found to be autoregulated in *Escherichia coli*. Promoter activity was lower in *V. cholerae* than in *E. coli*, and a part of this reduction is likely to be due to autorepression. Sequences upstream of *rctBp*, implicated earlier in replication control, enhanced the repression. The action of the upstream sequences required that they be present in *cis*, implying long-range interactions in the control of the promoter activity. A second gene specific for chromosome II replication, *rctA*, reduced *rctB* translation, most likely by antisense RNA control. Finally, optimal *rctBp* activity was found to be dependent on Dam. Increasing RctB in *trans* increased the copy number of a miniplasmid carrying $oriCII_{VC}$, implying that RctB can be rate limiting for chromosome II replication. The multiple modes of control on RctB are expected to reduce fluctuations in the initiator concentration and thereby help maintain chromosome copy number homeostasis.

Vibrio cholerae causes the potentially lethal diarrheal disease cholera and has been studied extensively from the pathogenesis perspective. Since the finding that the genome of this bacterium is divided between two chromosomes, chromosome I and chromosome II (chrI and chrII), there has been additional interest in studying chromosome maintenance in this organism (9, 10, 13). In fact, the postgenomic era has shown that multichromosome bacteria are not uncommon (9), raising the question of how the replication and segregation of multiple chromosomes are coordinated for stable maintenance of the genome. Among the multichromosome bacteria, *V. cholerae* is particularly attractive for basic research because of its similarity to *Escherichia coli*, ease of culturing in the laboratory, and amenability to genetic manipulations. Pioneering studies from the Waldor lab have identified the origins of replication of the two chromosomes and laid the foundation for studying their function (11). Of particular interest is the fact that, although the origins are disparate, the two chromosomes initiate replication synchronously in a growing culture (10). Understanding the mechanism of synchrony has the potential to reveal cell cycle controls particular to multichromosome bacteria.

The origin of *V. cholerae* chrI ($oriCI_{VC}$) is similar to that of *E. coli*, and a minichromosome carrying $oriCI_{VC}$ functions well in *E. coli* (11). The origin of chrII ($oriCII_{VC}$) has repeat sequences similar to the well-studied iteron-based plasmids (2) and is functional in *E. coli* when provided with the $oriCII_{VC}$-specific initiator RctB (11) (T. Venkova-Canova, unpublished results).

Studies on replication of the *E. coli* chromosome and bacterial plasmids have established that the regulation of replication is largely mediated through initiator proteins (6, 8). For example, in *E. coli*, the initiator DnaA is regulated by multiple mechanisms. The *dnaA* gene is transcriptionally autoregulated, and additionally, the *dnaA* promoter is made inactive by sequestration following replication initiation for about one third of the cell generation time. During the elongation phase of replication, the DnaA protein is inactivated by Hda and DnaN proteins (18) and is titrated, most notably by the *datA* locus in addition to some 300 DnaA boxes scattered around the chromosome (23). The plasmid initiators are also down regulated at the level of transcription and/or translation (7) and post-translationally by inactivation due to covalent modification (27) or simply dimerization (15, 34).

Although it is called plasmid like, $oriCII_{VC}$ deviates fundamentally from the plasmid paradigm, in that the origin is not an incompatibility determinant. Also, the origin is associated with a negative control region more elaborate than those in iteron-based plasmids (11). To understand the control of $oriCII_{VC}$ activity, we studied regulation of its specific initiator RctB. As is common among other bacterial initiators, the gene for RctB is autoregulated, and this regulation involves elements present in a stretch of about 800 bp of the promoter upstream sequences. These sequences are also involved in replication initiation and its control. Additionally, a second $oriCII_{VC}$-specific gene, *rctA*, and a global regulator, Dam, also modulate promoter activity. We further show that the copy number of a minichromosome carrying $oriCII_{VC}$ depends on the RctB concentration, which implies that controlling the initiator concentration is a mechanism to control the replication of chrII.

MATERIALS AND METHODS

Bacterial strains, phages, plasmids, and growth conditions. Relevant details of strains used in this study are described in Table 1.

CVC250, a *recA* derivative of the sequenced *V. cholerae* El Tor strain N16961 (CVC16) (Table 1) (17), was constructed as follows. First, about 3 kb of DNA (573022 to 576657) spanning the *recA* gene of N16961 was amplified by

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TABLE 1. Bacterial strains, phages, and plasmids used in this study

Strain, phage, or plasmid	Relevant description[a]	Source or reference
Strains		
BR3239	BR8706(λDKC347)	This study
BR8706	Stbl2Δ(*araFGH*)Δ*araEp* P_{CP18}-*araE* (*araE* under constitutive CP18 promoter)	12
CVC16 (N16961)	*Vibrio cholerae* El Tor	R. Taylor
CVC23 [DH5α(λ*pir*)]	Source of R6K pu protein	M. Waldor
CVC72 (GM7589)	MG1655 *rph*-1 *lacU169 mutL459*::Kan *dam-13*::Tn*9*	M. Marinus
CVC73 (GM7576)	MG1655 *rph*-1 *lacU169 recA56 srl300*::Tn*10* Tet^r	M. Marinus
CVC201 (SM10λ*pir*)	Donor strain for conjugation	10
CVC205	N16961 *lacZ*::*res*::*tet*::*res*	M. Waldor
CVC250	CVC205 *recA*::Kan	This study
CVC432 [BR8706(λDKC372)]	*lacZ* fusion from pDP310 transferred to λDKC331	This study
CVC444 [BR8706(λDKC373)]	*lacZ* fusion from pDP336 transferred to λDKC331	This study
CVC446 [BR8706(λDKC375)]	*lacZ* fusion from pDP351 transferred to λDKC331	This study
CVC447 [BR8706(λDKC376)]	*lacZ* fusion from pDP352 transferred to λDKC331	This study
DH5Δ*lac* (BR2846)	K-12 *recA1* Δ(*argF-lac*)*U169*	32
Stbl2	*recA1* Δ(*lac-proAB*)	Invitrogen
Phages		
λDKC331 (λRS45)	Vector for transferring *lacZ* fusions to chromosome	31
λDKC347 (λDKC331 + *repA′*)	*repA′-′lacZ* protein fusion from pRJM358	32
λDKC372 (λDKC331 + *rctB′*)	*rctB′-′lacZ* protein fusion from pDP310	This study
Plasmids		
pALA162	pBR322 + *repA*	3
pALA169	pST52 + *repA*	3
pALA326	pMLB1034 + *repA′*	3
pDP309	Coordinates 256 to 1157 of N16961 in pMLB1109 between EcoRI and BamHI sites	This study
pDP310	Coordinates 727 to 1157, otherwise same as pDP309	This study
pDP311	Same as pDP309, except the vector is pMLB1034	This study
pDP312	Same as pDP310, except the vector is pMLB1034	This study
pDP332	Coordinates 547 to 1157, otherwise same as pDP309	This study
pDP333	Coordinates 648 to 1157, otherwise same as pDP309	This study
pDP334	pDP310 mutated at −35 box of *rctBp*	This study
pDP335	pDP310 mutated at −10 box of *rctBp*	This study
pDP336	Coordinates 925 to 1157, otherwise same as pDP309	This study
pDP337	Coordinates 365 to 1058, otherwise same as pDP309	This study
pDP338	Coordinates 365 to 631, otherwise same as pDP309	This study
pDP339	Coordinates 365 to 539, otherwise same as pDP309	This study
pDP340	Coordinates 727 to 1058, otherwise same as pDP309	This study
pDP348	Coordinates 547 to 751 of N16961 in pACYC184 between BamHI and XbaI sites	This study
pDP349	Coordinates 649 to 751, otherwise same as pDP348	This study
pDP350	Coordinates 256 to 559, otherwise same as pDP348	This study
pDP351	Same as pDP336, except the vector is pMLB1034	This study
pDP352	Coordinates 787 to 1157, otherwise same as pDP351	This study
pDP354	Coordinates 1072274 to 1072845 of N16961 in pRFG110 between NotI and NheI sites	This study
pDS132	R6K*ori*γ*sacB* RP4Mob, Cm^r	24
pET28a(+)	Vector with T7 promoter, Kn^r	Novagen
pGP704	R6K*ori*γ, Ap^r	10
pMLB1109	pBR322*ori*, promoter-cloning vector; Ap^r	M. L. Berman
pMLB1034	pBR322*ori*, gene fusion vector; Ap^r	30
pNEB193	pUC19-derived vector, Ap^r	NEB
pPP112	pBR322*ori*, P1*prepA* fused to *lacZ*, Ap^r	23
pPS25	Coordinates 573022 to 576657 carrying *recA* of N16961 in pDS132 between SphI and SacI sites	This study
pPS26	Same as pPS25, except *recA* replaced with Kn^r cassette	This study
pRFB110	Source of Kn^r cassette	12
pRFG123	pSC101*ori* with *lacI-yfp* and λ*cI-cfp* fusion genes under P_{BAD}, Sp^r	12
pRFG110	pRFG123 without the λ*cI-cfp* fusion gene	R. Fekete
pSP-*luc*+	Vector for transcriptional fusion to luciferase gene, Ap^r	Promega
pST52	Multicopy pBR322-compatible vector, Cm^r	3
pTVC11	*rctB* (coordinates 1118–3115) with its own SD sequence cloned under P_{BAD} in pRFG110. The *luc* gene from pSP-*luc*$^+$ was fused to *rctB* with a 21-bp spacer between the TAA codon of *rctB* and SD of *luc*	This study
pTVC12	*luc* gene with its own SD from pSP-*luc*$^+$ cloned under P_{BAD} in pRFG110	This study
pTVC13	*rctB* (coordinates 1134–3111) in pET28a+	This study
pTVC31	pGP704 carrying coordinates 775–1133 of *ig2* (coordinates 247–1133)	This study

[a] Unless otherwise mentioned, all coordinates are from *V. cholerae* chrII.

PCR using primers PS39 (5′-ACATGCATGCGTGACACAATGAAACAGAAG CGAGAGGC) and PS40 (5′-TGCAGAGCTCCATCCCTGCGTTGGTAAACA ACAG) containing sites for restriction enzymes SphI and SacI, respectively, at their 5′ end. The PCR product after digestion with these enzymes was cloned into a suicide vector, pDS132 (25), yielding pPS25. Next, the *recA* gene was deleted and substituted with a Kan cassette as follows. pPS25 was digested with MfeI and NotI, and between the two sites a Kan cassette (about 1.2 kb, obtained from pRFB110 after digestion with HindIII) was ligated after blunting of all four ends, yielding pPS26 (pPSΔ*recA*::Kan). The plasmid was transferred to *V. cholerae* strain CVC205 by conjugation using SM10λ*pir*/pPS26 as donor. The transconjugants were selected and purified on plates containing 25 μg/ml chloramphenicol (Cm), 40 μg/ml kanamycin (Kan), and 20 μg/ml tetracycline (Tet). Transconjugants were screened by colony PCR to confirm integration of pPS26 at the *recA* locus using screening primers PS41 (5′-GCAAAGTAGCCCGAGCGTCCTACGCTGGC) and PS42 (5′-CGTTGAGCGGTCGTGGCGCGAGTGTAGGCGCGC), which flanked the 3-kb *recA* locus, and primers PS37 (5′-CAGTCATAGCCGAATAGCCT) and PS38 (5′-CGGTGCCCTGAATGAACTGC)within the Kan gene. Out of 24 colonies tested, only 3 gave the expected PCR products. A Cm-sensitive but Kan- and Tet-resistant derivative was obtained from one of the colonies by sucrose selection (25). The *recA* locus was again examined by PCR, and the loss of *recA* function of the strain was confirmed by its sensitivity to UV light compared to the parent strain. The resultant strain was named CVC250.

Fragments containing the *rctB* control region were obtained from N16961 by PCR amplification. The fragments were fused to the *lacZ* gene present in promoter and protein fusion vectors pMLB1109 (21) and pMLB1034 (31), respectively, and the resultant plasmids are described in Table 1. The identity of all cloned fragments was verified by DNA sequencing. They matched with published sequences in all cases.

Both *E. coli* and *V. cholerae* strains were grown in L broth at 37°C, and log-phase cultures (optical density at 600 nm [OD_{600}] ≈ 0.4) were used to measure promoter activities.

Primer extension analysis. Primer extension analysis was done using α-^{32}P-labeled primer (Univ RF) and an E3030 kit (Promega, Madison, WI). Total RNA was isolated from DH5Δ*lac* (33) carrying pDP309 grown in L broth to an OD_{600} of ~0.4 using an RNeasy Mini kit (QIAGEN). Typically, approximately 10 μg of total RNA was mixed with 1 pmol primer in a buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 mM spermidine, and 5 mM dithiothreitol in an 11-μl reaction volume. The mixture was incubated at 58°C for 20 min to anneal the primer to RNA before the reaction was cooled at room temperature for 10 min in a Perkin-Elmer PCR system 2400. A mixture of 1.4 μl of 40 mM sodium pyrophosphate, 5 μl of 2× avian myeloblastosis virus primer extension buffer, 0.1 U of avian myeloblastosis virus reverse transcriptase (0.05 U μl^{-1}), and 0.6 μl water was added to the annealed reaction products and incubated at 42°C for 30 min. An equal volume (20 μl) of loading dye (98% formamide, 10 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue) was added to terminate the reactions. A 2-μl aliquot of the reaction was loaded onto a 6% sequencing gel together with a sequencing ladder generated using the primer and plasmid identical to those in the primer extension reaction. The ladder was generated using an fmol DNA sequencing kit (Promega, Madison, WI). The bands were visualized using a Phosphoimager (Fujix BAS 2000).

Luciferase assay. The luciferase assay was performed using the luciferase assay system (Promega, Madison, WI). To 50 μl of culture, 40 μl of a BR8706 culture (OD_{600} = 0.4) (carrier cells) and 10 μl of 1 M K_2HPO_4 (pH 7.8)–20 mM EDTA solution were added. The mixture was placed on dry ice for 5 min and allowed to equilibrate to room temperature for 15 min. The cells were then lysed with 300 μl of a lysis mix, and 10 μl of the lysed extract was added to 100 μl of the substrate in a flat-bottom 96-well microtiter plate (Costar 3596, Corning, NY). Light emission was measured for 1 s at intervals of 10 s for a total of 10 readings using a Wallace 1420-Victor Multilabel counter (Perkin-Elmer, Shelton, CT). The mean value of 10 readings was determined and used in the figures.

Western blot analysis. Two OD units of log-phase cells were resuspended in 100 μl of 1× sodium dodecyl sulfate sample buffer (NEB, Beverly, MA), boiled for 5 min, and centrifuged. Twenty-five microliters of the supernatant was loaded on a 7.5% Tris-HCl Criterion gel (Bio-Rad, Hercules, CA). After electrophoretic separation, the bands were blotted onto a nitrocellulose filter (Schleicher & Schuell, Keene, NH) using 1× CAPS [3-(cyclohexylamino)-1-propanesulfonic acid] transfer buffer (pH 11, USB) containing 10% methanol. The membrane was then blocked by incubation with 5% membrane-blocking agent (Amersham Biosciences, Piscataway, NJ) and treated with 1:2,000 diluted anti-RctB antiserum (Medicore, Gaithersburg, MD) and finally with 1:5,000 diluted anti-rabbit horseradish peroxidase-conjugated secondary antibody from donkey (Amersham Biosciences, Piscataway, NJ). The anti-RctB antiserum was raised against untagged RctB purified using an IMPACT system (NEB, Beverly, MA). The blot was developed in ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ), and the light intensities were captured by a LAS1000 system (Fujifilm, Stamford, CT).

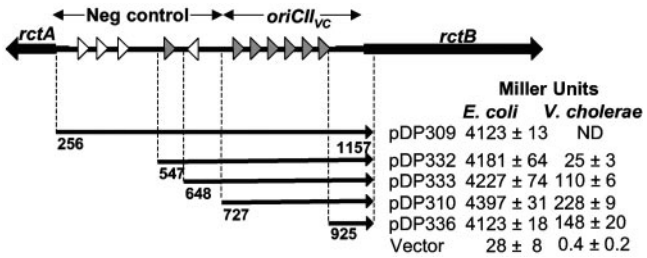

FIG. 1. Identification by deletion mapping of a region containing a promoter for the initiator gene *rctB*. The top line shows the region involved in chromosome II replication. It consists of *rctB*, the origin (*oriCII*$_{VC}$), and elements of negative control of replication. The region has two kinds of repeats, 11-mers (open triangles) and 12-mers (filled triangles), and these were used as guides for choosing deletion end points (next five lines). The coordinates are as published previously (17). All fragments ended at coordinate 1157 and included the first eight codons of the *rctB* ORF. They were cloned in front of the *lacZ* reporter in a promoter-cloning vector (pMLB1109), and their promoter activities were measured after the resultant plasmids were transferred to *E. coli* (DH5Δ*lac*) and *V. cholerae* (CVC250). The arrows indicate the orientation in front of the *lacZ* gene. The reporter (β-galactosidase) activities were measured and presented in Miller units as described previously (20). Values presented are averages from at least two independent transformants, each assayed in duplicate.

Plasmid copy number. The plasmid copy number was measured as has been described previously (4).

RESULTS

Mapping of a promoter for *rctB*. A minichromosome carrying the two initiator genes *rctA* and *rctB* and the intergenic region (*ig2*) of chrII of the sequenced *Vibrio* strain N16961 replicates in *E. coli*, suggesting that the promoters for the two genes must be functional in the surrogate host (11). To identify the sequences responsible for *rctB* transcription, the first eight codons of the *rctB* open reading frame (ORF) and various sequences upstream were fused to *lacZ* in a pBR322-derived promoter-cloning vector, pMLB1109 (21) (Fig. 1). The sequences included the minimal origin and some of the elements identified earlier to be involved in the negative regulation of replication initiated from *oriCII*$_{VC}$. In *E. coli*, a strong promoter activity was detected in all of the fragments tested. The shortest one containing 209 of bp upstream sequences from the putative start codon of the *rctB* ORF (pDP336) (Fig. 1).

In order to locate the start of the *rctB* message, a primer extension analysis was performed with total RNA isolated from an *E. coli* strain, DH5Δ*lac*, carrying pDP309 (Fig. 2A). A clear signal was detected corresponding to a start site at an A at coordinate 1092. Suitable matches to consensus −10 and −35 boxes were detected at the expected positions. To confirm the promoter, some of the bases were mutated in the putative −10 and −35 boxes (Fig. 2B). The activity of the promoter was reduced in both cases, confirming that the sequences are essential elements of the *rctB* promoter, hereafter called *rctBp* (Fig. 2C). The results were qualitatively similar in a *recA* mutant derivative of N16961, CVC250, indicating that *rctBp* is also functional in the native host (Fig. 1 and 2C). The promoter

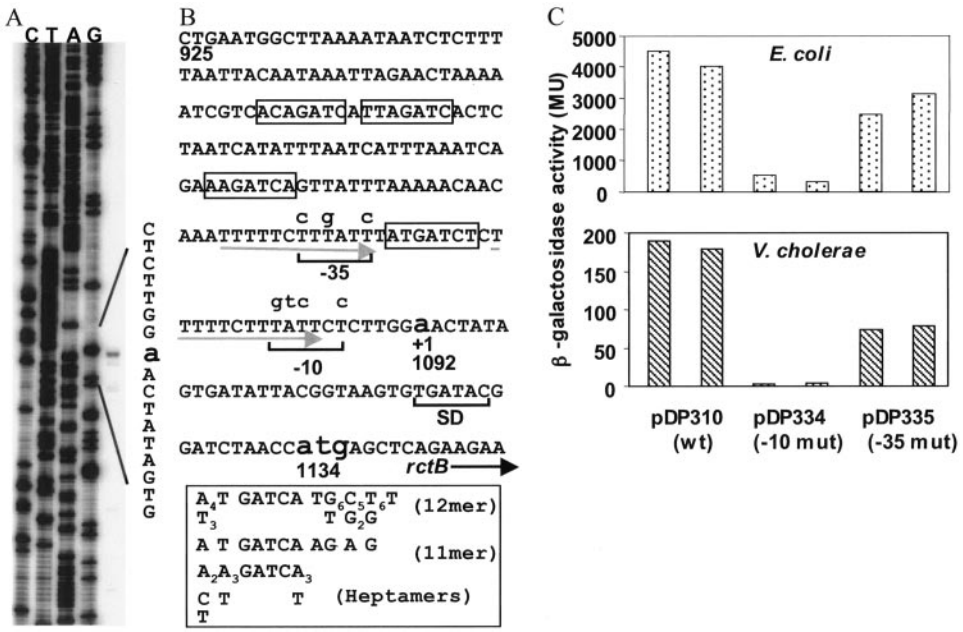

FIG. 2. Precise location of *rctBp*. (A) Primer extension results showing the sequence around the start site of the *rctB* message. The starting base is shown in bold. (B) The predicted −10 and −35 boxes, assuming that +1 is at coordinate 1092, are bracketed. Also shown are the predicted start codon (in bold at 1134) and a putative SD sequence (underlined) of the *rctB* ORF. The promoter region has four heptameric sequences (boxes) containing GATC that are partially homologous to 11- and 12-mer repeats. Their sequence relationships are shown in the box at the bottom. Also shown are two potential ToxT binding sites (gray arrows) overlapping the −35 and −10 boxes. (C) Promoter activities when the putative −10 and −35 boxes of *rctBp* were mutated. The mutated sequences are shown above the boxes in panel B.

activities in general were considerably reduced in *V. cholerae* compared to *E. coli*, suggesting that host-specific genes could negatively regulate *rctBp* in *trans*.

Regulation of *rctBp*. Since initiator genes are often autoregulated, we determined the effect of RctB in *trans* on the activity of *rctBp*. The protein was supplied from an arabinose-inducible promoter, P_{BAD}, from plasmid pTVC11 (Fig. 3; Table 1) (16). The initiator expression was varied by varying the inducer (arabinose) concentration, and the host used (BR8706) carried a constitutively expressing arabinose transport system to avoid all-or-none induction at suboptimal inducer concentration (12, 19). pTVC11 also contained the firefly luciferase gene downstream of *rctB*, so that both genes were transcribed from P_{BAD} and luciferase activity could serve as a reporter for the relative levels of *rctB* expression. When *rctB* expression was induced with arabinose, the activity of *rctBp* present in pDP309 initially decreased but then reached a plateau, with significant activity still remaining (Fig. 3A, pDP309/pTVC11). Measurement of luciferase activity confirmed that *rctB* transcription increased in the plateau region in proportion to the inducer concentration (Fig. 3C) and suggests that the incomplete repression of *rctBp* was unlikely to be due to insufficient RctB supply. This was further confirmed by Western analysis (Fig. 3D, lanes 7 to 13). Increasing the arabinose concentration from 0.002 to 0.02% did increase RctB concentration about 2.1-fold.

To confirm the specificity of RctB in promoter repression, another well-characterized autoregulated promoter, *repAp* of plasmid P1, present in plasmid pALA326, was used (3). Supplying RctB from pTVC11 repressed the activity of *rctBp* as before but not that of *repAp* (Fig. 3B). Under identical conditions, *repAp* was efficiently repressed when the cognate repressor RepA was supplied in *trans* from pALA169. The repression activity of RctB, therefore, appears specific to its own promoter.

A second level of regulation of *rctBp* was evident when the repressed levels of transcription in plasmids pDP309 and pDP310 were compared (Fig. 3A, pDP309/pTVC11 and pDP310/pTVC11). The repressed level was lower in pDP309. An effect of upstream sequences was also evident in *V. cholerae* strain CVC250 (Fig. 1). The level of repression depended in a complex way on the extent of upstream sequences present, pDP310 being the least repressed of all. (pDP309, which contains $oriCII_{VC}$ as well as the negative control locus, could not be established in the *V. cholerae* strain, most likely because of incompatibility with the chromosomal origin as suggested previously [11].) Thus, upstream sequences seem to regulate the promoter both in *E. coli* and in *V. cholerae*. When a few fragments containing the additional upstream sequences present in pDP309 compared to pDP310 were provided in *trans* from the pACYC184 vector (yielding pDP348, pDP349, and pDP350 [Table 1]), they were ineffective in exerting any repressive effect (data not shown). The *cis* action of upstream sequences suggests that they aid in repression, possibly by DNA looping or altering the structure of the promoter DNA (14).

Additional promoters of *rctB*. To understand the basis of why the repression in pDP309 was higher than that in pDP310, we searched for sequences upstream of *rctBp* for additional promoters not regulated by RctB. Weak promoter activities were detected when fragments without the *rctBp* sequences were analyzed by fusion to *lacZ*, but the levels of their activities (≤800 Miller units [MU]) were not high enough to fully account for the plateau level seen from plasmid pDP310 (Fig. 4).

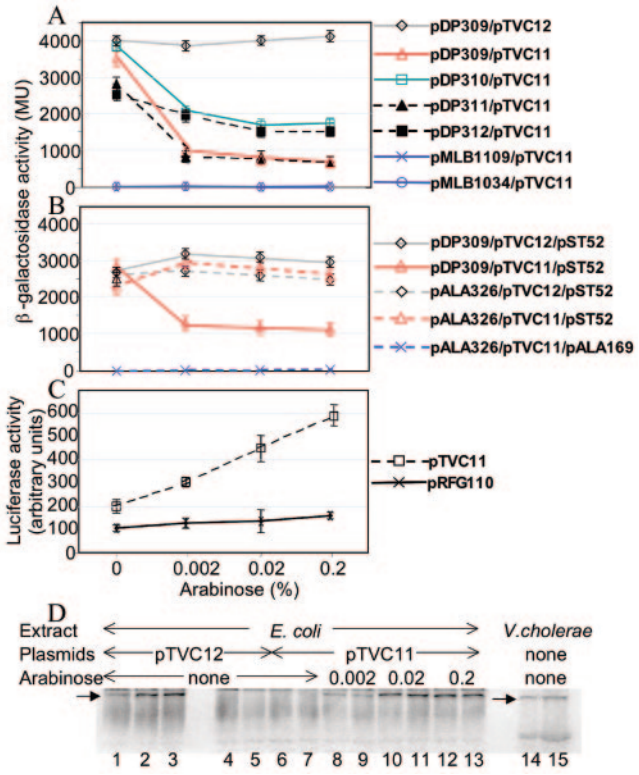

FIG. 3. Transcriptional autorepression of *rctBp*. (A) The promoter activities from two different fragments were determined: when they were present in transcriptional fusion vector pMLB1109, the resultant plasmids were called pDP309 and pDP310 (Fig. 1), and when in translational fusion vector pMLB1034, the resultant plasmids were called pDP311 and pDP312. To study autorepression, various levels of RctB protein were supplied from pTVC11 (pTVC12+*rctB*) where the protein was under arabinose-inducible promoter P_{BAD} present in vector pTVC12. The host was BR8706 in these experiments, with a constitutive source of arabinose transporter. (B) To show specificity of promoter repression by RctB, activity of another fusion, *repAp-lacZ* (present in pALA326), was tested. RepA was supplied from pALA169, where *repA* is constitutively made. pST52 is the vector used to clone *repA* that generated pALA169. (C) The arabinose induction of P_{BAD} was confirmed by measuring the activity of the luciferase gene present downstream of *rctB* in pTVC11. pTVC12 is identical to pRFG110 except that *rctB* and the downstream luciferase gene replaced the *lacI-yfp* fusion gene. (D) RctB production after P_{BAD} induction was measured by immunoblotting with antibody against RctB. Extracts from equal ODs of cells were added to all lanes, except that the ODs of *V. cholerae* cells were two times higher (lanes 14 and 15). Purified RctB (5, 10, and 20 ng) (arrow) were added to extracts from vector (pTVC12)-carrying cells for reference purposes (lanes 1 to 3). The band inferred as RctB (arrow, lanes 14 and 15) comigrated with purified RctB when it was mixed with the *V. cholerae* extract (data not shown), confirming the identity of the protein. Samples were loaded in duplicates (lanes 4 to 15).

For pDP310, the plateau level was around 1,500 MU, but the maximal promoter activity obtained from sequences upstream of *rctBp* was around 300 MU in the presence of RctB. It appears that additional promoters are not adequate to explain the difference in *rctBp* activity between pDP309 and pDP310 in the presence of RctB.

Translational control of *rctB*. The fragments containing *rctBp* present in plasmids pDP309 and pDP310 were fused to *lacZ* in the protein fusion vector pMLB1034 so that the *rctB*

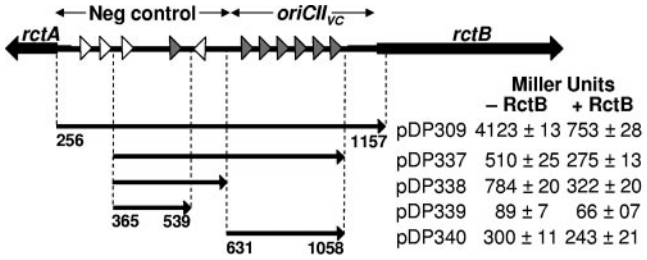

FIG. 4. Search for additional promoters of *rctB*. The fragments shown were fused to *lacZ* of the promoter-cloning vector pMLB1109, as in Fig. 1. The region containing *rctBp* is deleted in all but pDP309. In the +RctB column, the protein was supplied from pTVC11 after adding 0.2% arabinose to the culture; otherwise, the experiments were done as described in the legend to Fig. 3. In the −RctB column, the vector pTVC12 replaced pTVC11.

ORF continued into the reporter gene (31). The repression profiles from the resultant plasmids, pDP311 and pDP312, in the presence of various levels of RctB were similar to those seen from the transcriptional fusion plasmids pDP309 and pDP310 (Fig. 3A, dashed lines). These results indicate that RctB is autoregulated at the level of transcription only. However, we show below that some translational regulation is possible in the presence of *rctA*.

Since replication initiation from *oriCII*$_{VC}$ also requires a second initiator, *rctA*, we examined whether it could be responsible for the higher level of repression of *rctBp* seen in *V. cholerae*. A putative promoter for *rctA* was identified by gene fusion studies (D. Pal, unpublished results), and a fragment excluding this promoter but containing the *rctA* ORF, including the putative Shine-Dalgarno (SD) sequence and considerable sequences downstream to the ORF (coordinates 1072274 to 1072845), was chosen. The fragment was placed under P_{BAD} control in plasmid pDP354. To test the effect of *rctA* in conjunction with RctB, *rctB* was expressed constitutively from pTVC13, a derivative of pET28a. Although the gene was under the control of a phage T7 promoter, the basal level of expression was sufficient, even though no T7 RNA polymerase was present in these experiments. The transcription initiated from unidentified vector promoters apparently produced enough RctB to support replication of an *oriCII*$_{VC}$ plasmid in *E. coli* (T. Venkova-Canova, unpublished results). To the extent that it was analyzed, no significant effect of *rctA* expression alone or in conjunction with RctB could be seen on *rctBp* (Fig. 5A). In these experiments, the *rctBp-lacZ* transcriptional fusion was transferred to a λ vector (λRS45 [32]), and the resultant phage, λDKC372, was integrated at the *att*$_{\lambda}$ site in the chromosome. In other words, no effect of *rctA* could be seen on *rctBp* even when the promoter was present in a single copy. The results were similar when a shorter fusion present in pDP336 was tested (data not shown).

We next determined the effect of *rctA* on an *rctB′-′lacZ* translational fusion after it was transferred to the chromosome as described above (CVC446 [Table 1]). In *rctA*-carrying cells, β-galactosidase activities were slightly but reproducibly lower, indicating that the gene could repress, albeit to a small extent, *rctB* translation (Fig. 5B). This effect was also seen when the cells had a source of RctB (pTVC13), but the protein did not seem to alter the level of repression by *rctA*. The repression

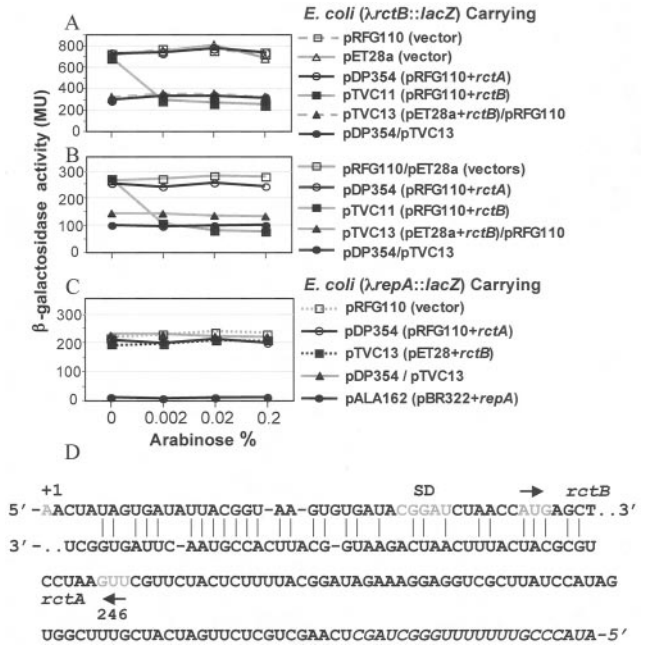

FIG. 5. Effect of *rctA* on *rctB* expression. The *rctA* effect was determined either alone or in conjunction with RctB. (A) A transcriptional *rctBp-lacZ* fusion as in pDP336 (Fig. 1) was used, but the fusion was present in an integrated λ prophage in CVC444 (Table 1). RctB was supplied either from an inducible source, pTVC11, or constitutively from pTVC13 (a pET28a derivative, but the cells did not have a source of T7 polymerase) when *rctA* was also present in the same cell. When present, *rctA* was under P_{BAD} control as in pDP354. (B) Same as panel A except the fusion was translational as in pDP351. (C) Same as panel B except *rctBp* was replaced with *repAp* and the resultant strain called BR3239. pALA162 was used to supply RepA to confirm the repressibility of *repAp*. (D) The extent of complementarity between the *rctA* message and the leader of *rctB* message is shown by vertical lines. The first 48 nucleotides of the leader of the *rctB* mRNA are shown in the top line. The predicted sequence of the 5′ end of *rctA* message as made from the P_{BAD} promoter present in pDP354 is shown below. The predicted start codon of *rctA* ORF is shown in gray (coordinate 246), and the vector-derived 5′ end sequences are in italics. The chromosomal sequences covering *rctA* present in pDP354 span the coordinates 845 to 192.

also did not increase when *rctA* expression was induced with arabinose and the induction was confirmed by the luciferase reporter activity. A partial complementarity between the *rctA* RNA and leader region of *rctB* mRNA could be detected by sequence comparison (Fig. 5D). Thus, there could be a weak translational regulation of *rctB* in the presence of *rctA*. We note that even in the presence of *rctA* and RctB, considerable *rctBp* activity remained (about 100 MU) (Fig. 5B). *rctA* and RctB also did not have any significant effect on the heterologous promoter, *repAp*, which could be efficiently repressed by RepA from pALA162 (Fig. 5C).

Additional control of *rctBp* in *V. cholerae*. Before invoking additional factors in the repression of *rctBp*, we wanted to determine whether the promoter activity is intrinsically lower in *V. cholerae* than in *E. coli*. To account for possible differences in promoter activity in the two strains, the P1 plasmid *repAp*, present in pPP112 (24), was again used for normalization purposes in the two strains (Table 2). Furthermore, plasmid copy numbers were measured to normalize for possible gene dosage differences between the two strains. The activity of *repAp* was lower in *V. cholerae* (946 MU versus 2,159 MU in *E. coli*); this could be due to the lower copy number of pPP112 in that strain (8.3 versus 25 in *E. coli*). However, the copy number was not sufficient to explain the difference in *rctBp* activity in the two strains. The copy number of pDP310 was roughly sixfold lower (4.4 versus 27), whereas the promoter activity was about 16-fold lower (279 versus 4,500 MU). Thus, the involvement of factors other than *rctA* and RctB cannot be ruled out in the regulation of *rctBp* in *V. cholerae*. There are two direct repeats overlapping the putative −10 and −35 boxes that are largely homologous to ToxT transcriptional factor binding sites (35), suggesting that ToxT could be an additional transcriptional repressor of *rctBp*.

Stimulation of *rctBp* activity by *E. coli* DNA adenine methylase (Dam). The minimal region tested for autoregulation spanned the coordinates 925 to 1157 (as cloned in pDP336). RctB apparently binds within this region for autoregulation. However, the region is devoid of the 11- and 12-mer repeat sequences, the two likely candidates for RctB binding (11) (Fig. 1, open and shaded triangles). However, we noted that a hexamer that is also present in the 11- and 12-mer repeats, AT GATC, is present between the −10 and −35 boxes (Fig. 2B). Three other similar sequences are also present in the minimal region tested for autorepression (Fig. 2B, boxes). These short sequences appear to be the best candidate for RctB binding for autorepression.

Since the short sequences contain an adenine methylation site (GATC), we tested the role of the *dam* gene in the regulation of *rctBp* in *E. coli*. The *dam* gene is also essential for $oriCII_{VC}$ activity (11). *rctBp* activity was reduced in a *dam* mutant strain, particularly evident in pDP336 (Table 3). The degree of autorepression could also be somewhat stronger in the mutant strain, suggesting that RctB could be competing better with RNA polymerase for binding to the promoter region. Although small, the Dam effect was not due to plasmid copy number changes (data not shown). The effect on autorepression was also unlikely to be due to a change in RctB expression, since the unrepressed promoter activity from pDP309 and pDP336 was affected differently in the *dam* mutant strain.

RctB level and the copy number of an $oriCII_{VC}$-based replicon. The regulation of initiator genes is most easily justified when they are found to be rate limiting for replication, as in plasmids R1 (22) and pT181 (26). Even when not rate limiting, regulation of initiators is required to guarantee their supply without being excessive, which could be detrimental to repli-

TABLE 2. Relative promoter activities in *E. coli* and *V. cholerae*

lacZ fusion plasmid	Copy number in:		β-Galactosidase activity			
			Miller units		Normalized[a]	
	E. coli	*V. cholerae*	*E. coli*	*V. cholerae*	*E. coli*	*V. cholerae*
pDP310 (*rctBp-lacZ*)	27	4.4	4,500	279	167	63
pPP112 (*repAp-lacZ*)	25	8.3	2,159	946	86	114

[a] Values were obtained by dividing Miller units by copy number of the corresponding source plasmids.

TABLE 3. Regulation of *rctBp* activity by Dam

lacZ fusion	Arabinose[a]	β-Galactosidase activity (MU) in		Relative promoter activity (Dam$^-$/Dam$^+$)
		CVC73 (Dam$^+$)	CVC72 (Dam$^-$)	
pDP309 (*rctBp-lacZ*)	−	5,153 ± 478 (1)	4,055 ± 652 (1)	0.79
	+	2,840 ± 157 (0.55)[b]	1,634 ± 300 (0.40)	0.58
pDP336 (*rctBp-lacZ*)	−	6,525 ± 268 (1)	3,575 ± 141 (1)	0.55
	+	4,517 ± 112 (0.69)	1,653 ± 285 (0.46)	0.37
pPP112 (*repAp-lacZ*)	−	2,300 ± 36 (1)	2,094 ± 16 (1)	0.91
	+	2,283 ± 11 (0.99)	1,978 ± 33 (0.94)	0.87

[a] To induce RctB from pBAD*rctB* present in pTVC11).
[b] Relative promoter activity after RctB induction.

cation or cell growth (5). To the extent that it was overproduced, RctB was not found to be detrimental to *E. coli* growth (Fig. 3D). To test the first possibility, we determined the copy number of an *oriCII*$_{VC}$-based minichromosome (pTVC31) when RctB was supplied in *trans* from pTVC13, where the protein was under P_{BAD} control. The copy number did increase with increasing concentration of RctB, suggesting that the protein can be rate limiting for replication (Fig. 6B). Thus, RctB is not only essential for replication initiation but can also be its regulator.

DISCUSSION

Here we report that the replication initiator gene *rctB* for *oriCII*$_{VC}$ is under multiple negative and positive controls. A strong promoter of the gene was identified and named *rctBp*. The gene is autorepressed at the level of transcription. The autorepression was enhanced by sequences further upstream when they were present in *cis*. The gene was also weakly negatively regulated at the level of translation by *rctA*, the second *oriCII*$_{VC}$-specific gene of *V. cholerae*. Additionally, the host factor Dam was required for optimal *rctBp* activity. Dam is essential for chrII replication, and it remains to be seen whether the adenine methylation is required directly for replication initiation or for optimal *rctB* expression (11). The sequences immediately upstream of the −35 box are quite AT rich and could serve as a promoter UP element (28). In contrast, the same region has two direct repeats that are candidates for binding ToxT, a transcriptional activator of a majority of *V. cholerae* virulence genes (35). Since the repeats overlap the −10 and −35 boxes, they could aid in repression rather than activation of *rctBp.* It should be noted, however, that ToxT is expressed only under special conditions and the gene is present only in a subset of *V. cholerae* strains, whereas all *Vibrio* species appear to have *rctB* (10) (M. Waldor, personal communication). In any event, establishing a connection between DNA replication and virulence expression will be of great general interest.

Most of the regulators when tested individually affected the promoter activity by twofold or even less. Since the *rctBp* activity was found to be significantly lower in *V. cholerae* than in *E. coli*, either there are additional regulators, such as ToxT, in the native host or the conditions used in *E. coli* were not optimal. Even if weak, the multiple regulators could reduce noise in gene expression; the effect of individual regulators may fluctuate, but together the fluctuations may average out to produce more uniform initiator levels in the population of cells.

The repression profile of *rctBp* in the presence of an increasing concentration of RctB in *trans* showed two characteristics. First, after an initial drop in activity, the promoter could not be repressed any further by increasing the RctB concentration (Fig. 3A). The repressed level stayed high even when the copy number of *rctBp* was reduced by changing the vector from a multicopy plasmid to an integrated lambda prophage. This suggests that saturation of binding of RctB is not sufficient for full repression. There are two partially homologous repeat sequences in *oriCII*$_{VC}$, the 11- and 12-mers, which most likely bind RctB. Although the autorepression was seen without the 11- and 12-mers, heptameric sequences partially homologous to these repeats were found to overlap the promoter region

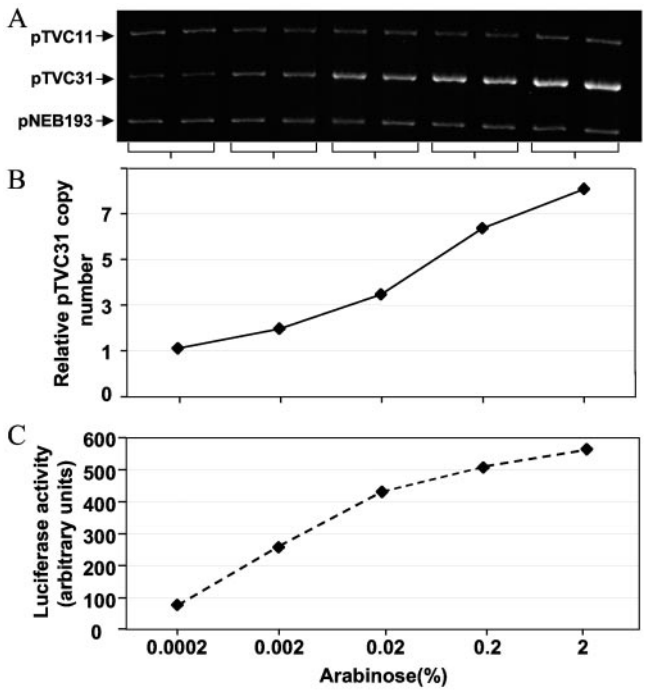

FIG. 6. Relationship of RctB concentration and the copy number of a minichromosome carrying *oriCII*$_{VC}$. The origin region was present in pTVC31, and various levels of RctB were supplied from pTVC11 by changing the arabinose concentration. A constant volume of a culture of pNEB193 was added to each sample before the start of plasmid DNA isolation to normalize for any loss of DNA during the isolation procedure. The DNAs were linearized using SalI before running on the gel to recover each plasmid in one band for easier comparison of DNA concentration.

(Fig. 2). RctB binding to a fragment without the 11- and 12-mers but containing these heptamers has been shown by electrophoretic mobility shift assay (11). The heptamers could thus be responsible for the autorepression. These putative sites are apparently weak and may account for the high basal level of *rctBp* activity in the presence of excess RctB.

The second characteristic of *rctBp* repression was that the repressed level depended on the extent of upstream sequences present in *cis*. The obligatory *cis* action of the upstream sequences suggests involvement of DNA looping in repression. Action at a distance is well known for transcriptional repression/activation (1, 29, 30). The repression of lambda p_{RM} and *galP1* requires interactions between repressors bound to distant operator sites and looping of the intervening DNA (15, 29). In these cases, the looping efficiency determines the repression efficiency. Apparently, the efficiency of a higher-order interaction, such as DNA looping, also sets the repressed level of *rctBp* transcriptional activity.

It is intriguing that although upstream sequences helped in repression, an apparent exception was found when the repressed levels in pDP310 and pDP336 were compared in *V. cholerae* (Fig. 1). Although pDP310 had six 12-mer repeats, it was repressed less than pDP336. We can think of two reasons. The $oriCII_{VC}$ sequences present in pDP310 suffice for origin function in the presence of RctB in *E. coli* (T. Venkova-Canova, unpublished results). The origin activity could have interfered with repression of *rctBp*. Alternatively, RctB binding to the 12-mer repeats of the origin could have interfered with the protein binding to the promoter region and thereby caused a decrease in repression. Since the repeats are distributed over the entire IG2 ($oriCII_{VC}$ plus negative control) region (Fig. 1), multiple higher-order structures are possible, and their effects on *rctBp* are likely to be different.

An important finding of this paper is the demonstration that RctB can be rate limiting for replication (Fig. 6). Other than plasmids like R1 and pT181, which are regulated by antisense RNA, such clear regulation of copy number by initiator concentration has not been seen in replicons with repeating initiator-binding sites, including the *E. coli oriC* replicon (5). A reason for this could be the presence of multiple modes of control. We have argued recently that such systems tend to be insensitive to changes in any one of the control modes. The control by initiator limitation alone therefore may not appear dramatic. By testing the response of the minimal origin to RctB, we may have eliminated other negative control modes of the system. Including the entire *ig2* and *rctA* in the test plasmid would indicate the contribution of RctB limitation in controlling the copy number of $oriCII_{VC}$ replicon. In any event, initiator limitation by autoregulation alone cannot control replication: the basal level of initiator increases following replication due to the increase in the number of initiator genes (5). There ought to be other mechanisms to reduce initiator synthesis or availability or origin availability following replication to prevent runaway replication. However, if initiators indeed control replication initiation, the direct relationship of RctB level and the $oriCII_{VC}$ copy number makes it all the more necessary that the fluctuations in the initiator level be reduced to avoid fluctuations in the chromosomal copy number.

We note that autoregulation of *rctB* has also been found independently (E. Egan, S. Duigou, and M. Waldor, unpublished data).

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REFERENCES

1. **Buck, M., M. T. Gallegos, D. J. Studholme, Y. Guo, and J. D. Gralla.** 2000. The bacterial enhancer-dependent σ^{54} (σ^{N}) transcription factor. J. Bacteriol. **182:**4129–4136.
2. **Chattoraj, D. K.** 2000. Control of plasmid DNA replication by iterons: no longer paradoxical. Mol. Microbiol. **37:**467–476.
3. **Chattoraj, D. K., K. M. Snyder, and A. L. Abeles.** 1985. P1 plasmid replication: multiple functions of RepA protein at the origin. Proc. Natl. Acad. Sci. USA **82:**2588–2592.
4. **Das, N., and D. K. Chattoraj.** 2004. Origin pairing ('handcuffing') and unpairing in the control of P1 plasmid replication. Mol. Microbiol. **54:** 836–849.
5. **Das, N., M. Valjavec-Gratian, A. N. Basuray, R. A. Fekete, P. P. Papp, J. Paulsson, and D. K. Chattoraj.** 2005. Multiple homeostatic mechanisms in the control of P1 plasmid replication. Proc. Natl. Acad. Sci. USA **102:** 2856–2861.
6. **Dasgupta, S., and A. Lobner-Olesen.** 2004. Host controlled plasmid replication: *Escherichia coli* minichromosomes. Plasmid **52:**151–168.
7. **del Solar, G., and M. Espinosa.** 2000. Plasmid copy number control: an ever-growing story. Mol. Microbiol. **37:**492–500.
8. **del Solar, G., R. Giraldo, M. J. Ruiz-Echevarria, M. Espinosa, and R. Diaz-Orejas.** 1998. Replication and control of circular bacterial plasmids. Microbiol. Mol. Biol. Rev. **62:**434–464.
9. **Egan, E. S., M. A. Fogel, and M. K. Waldor.** 2005. Divided genomes: negotiating the cell cycle in prokaryotes with multiple chromosomes. Mol. Microbiol. **56:**1129–1138.
10. **Egan, E. S., A. Lobner-Olesen, and M. K. Waldor.** 2004. Synchronous replication initiation of the two *Vibrio cholerae* chromosomes. Curr. Biol. **14:** R501–R502.
11. **Egan, E. S., and M. K. Waldor.** 2003. Distinct replication requirements for the two *Vibrio cholerae* chromosomes. Cell **114:**521–530.
12. **Fekete, R. A., and D. K. Chattoraj.** 2005. A *cis*-acting sequence involved in chromosome segregation in *Escherichia coli*. Mol. Microbiol. **55:**175–183.
13. **Fogel, M. A., and M. K. Waldor.** 2005. Distinct segregation dynamics of the two *Vibrio cholerae* chromosomes. Mol. Microbiol. **55:**125–136.
14. **Geiselmann, J.** 1997. The role of DNA conformation in transcriptional initiation and activation in *Escherichia coli*. Biol. Chem. **378:**599–607.
15. **Giraldo, R., C. Fernandez-Tornero, P. R. Evans, R. Diaz-Orejas, and A. Romero.** 2003. A conformational switch between transcriptional repression and replication initiation in the RepA dimerization domain. Nat. Struct. Biol. **10:**565–571.
16. **Guzman, L.-M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. **177:**4121–4130.
17. **Heidelberg, J. F., J. A. Eisen, W. C. Nelson, R. A. Clayton, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, L. Umayam, S. R. Gill, K. E. Nelson, T. D. Read, H. Tettelin, D. Richardson, M. D. Ermolaeva, J. Vamathevan, S. Bass, H. Qin, I. Dragoi, P. Sellers, L. McDonald, T. Utterback, R. D. Fleishmann, W. C. Nierman, O. White, S. L. Salzberg, H. O. Smith, R. R. Colwell, J. J. Mekalanos, J. C. Venter, and C. M. Fraser.** 2000. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature **406:**477–483.
18. **Kato, J., and T. Katayama.** 2001. Hda, a novel DnaA-related protein, regulates the replication cycle in *Escherichia coli*. EMBO J. **20:**4253–4262.
19. **Khlebnikov, A., K. A. Datsenko, T. Skaug, B. L. Wanner, and J. D. Keasling.** 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.
20. **Miller, J. H.** 1992. A short course in bacterial genetics. A laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
21. **Mukhopadhyay, S., and D. K. Chattoraj.** 2000. Replication-induced transcription of an autorepressed gene: the replication initiator gene of plasmid P1. Proc. Natl. Acad. Sci. USA **97:**7142–7147.
22. **Nordstrom, K., S. Molin, and J. Light.** 1984. Control of replication of bacterial plasmids: genetics, molecular biology, and physiology of the plasmid R1 system. Plasmid **12:**71–90.
23. **Ogawa, T., Y. Yamada, T. Kuroda, T. Kishi, and S. Moriya.** 2002. The *datA* locus predominantly contributes to the initiator titration mechanism in

the control of replication initiation in *Escherichia coli*. Mol. Microbiol. **44:** 1367–1375.
24. **Papp, P. P., G. Mukhopadhyay, and D. K. Chattoraj.** 1994. Negative control of plasmid DNA replication by iterons. Correlation with initiator binding affinity. J. Biol. Chem. **269:**23563–23568.
25. **Philippe, N., J. P. Alcaraz, E. Coursange, J. Geiselmann, and D. Schneider.** 2004. Improvement of pCVD442, a suicide plasmid for gene allele exchange in bacteria. Plasmid **51:**246–255.
26. **Rasooly, A., and R. P. Novick.** 1993. Replication-specific inactivation of the pT181 plasmid initiator protein. Science **262:**1048–1050.
27. **Rasooly, A., P. Z. Wang, and R. P. Novick.** 1994. Replication-specific conversion of the *Staphylococcus aureus* pT181 initiator protein from an active homodimer to an inactive heterodimer. EMBO J. **13:**5245–5251.
28. **Ross, W., S. E. Aiyar, J. Salomon, and R. L. Gourse.** 1998. *Escherichia coli* promoters with UP elements of different strengths: modular structure of bacterial promoters. J. Bacteriol. **180:**5375–5383.
29. **Schleif, R.** 2003. AraC protein: a love-hate relationship. Bioessays **25:**274–282.
30. **Semsey, S., M. Y. Tolstorukov, K. Virnik, V. B. Zhurkin, and S. Adhya.** 2004. DNA trajectory in the Gal repressosome. Genes Dev. **18:**1898–1907.
31. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Simons, R. W., F. Housman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:**85–96.
33. **Sozhamannan, S., and D. K. Chattoraj.** 1993. Heat shock proteins DnaJ, DnaK, and GrpE stimulate P1 plasmid replication by promoting initiator binding to the origin. J. Bacteriol. **175:**3546–3555.
34. **Wickner, S., J. Hoskins, and K. McKenney.** 1991. Monomerization of RepA dimers by heat shock proteins activates binding to DNA replication origin. Proc. Natl. Acad. Sci. USA **88:**7903–7907.
35. **Withey, J. H., and V. J. DiRita.** 2005. Activation of both *acfA* and *acfD* transcription by *Vibrio cholerae* ToxT requires binding to two centrally located DNA sites in an inverted repeat conformation. Mol. Microbiol. **56:** 1062–1077.