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A 29-mer site regulates transcription of the initiator gene as well as function of the replication origin of *Vibrio cholerae* chromosome II

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Abstract

The region responsible for replication of *Vibrio cholerae* chromosome II (chrII) resembles those of plasmids that have repeated initiator binding sites (iterons) and an autorepressed initiator gene. ChrII has additional features: Its iterons require full methylation for initiator (RctB) binding, which makes them inactive for a part of the cell cycle when they are hemi-methylated. RctB also binds to a second kind of site, called 39-mers, in a methylation independent manner. This binding is inhibitory to chrII replication. The site that RctB uses for autorepression has not been identified. Here we show that a 29-mer sequence, similar to the 39-mers, serves as that site, as we find that it binds RctB *in vitro* and suffices to repress the *rctB* promoter *in vivo*. The site is not subject to methylation and is likely to be active throughout the cell cycle. The 29-mer, like the 39-mers, could inhibit RctB-dependent mini-chrII replication in *Escherichia coli*, possibly by coupling with iterons via RctB bridges, as was seen *in vitro*. The 29-mer thus appears to play a dual role in regulating chrII replication: one independent of the cell cycle, the other dependent upon iteron methylation, hence responsive to the cell cycle.

Keywords

Vibrio cholerae; Secondary chromosome; Transcriptional autorepression; Operator site; Replication control; Hetero-handcuffing

1. Introduction

About 10% of bacteria whose DNA has been sequenced are found to have divided their genome into multiple chromosomes (Harrison *et al.*, 2010, Egan *et al.*, 2005). How the chromosomes are maintained remains largely unknown, except in the case of *Vibrio cholerae*. The genome of this bacterium is divided into two chromosomes (chrI and chrII), which are maintained by separate programs, both for replication and segregation (Heidelberg *et al.*, 2000, Egan & Waldor, 2003, Fogel & Waldor, 2006). There are also indications that the maintenance of the two chromosomes might be coordinated (Rasmussen *et al.*, 2007, Val *et al.*, 2008).

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Here we are concerned with chrII replication control. Great strides in understanding chromosome replication in *V. cholerae* were possible primarily because of the nearly identical replication systems of chrI and the *Escherichia coli* chromosome, and the similarity of the chrII replication system to that of plasmids that have iterons (Egan & Waldor, 2003, Chattoraj, 2000). Iterons bind plasmid-encoded replication initiator proteins, and the binding is essential for initiation as well as controlling the initiation rate. Whereas the replication of chrI is regulated by the universal bacterial initiator DnaA (Duigou *et al.*, 2006), replication of chrII is regulated by its own initiator RctB, which binds to the chrII iterons (Duigou *et al.*, 2006, Pal *et al.*, 2005). The chrII iterons are distinguished from plasmid iterons in having GATC sequences in them, whose adenine residue is methylated by Dam (Egan & Waldor, 2003). Methylation of both the strands of duplex DNA is required for efficient RctB binding, which is one reason why Dam is essential for *V. cholerae* survival (Demarre & Chattoraj, 2010, Julio *et al.*, 2001). The methylation requirement also makes the iterons ineffective following replication, when they become hemimethylated. The iterons stay hemimethylated for about two-thirds of the cell cycle; prevention of initiator binding thus could be a major regulatory event to prevent premature reinitiation of replication.

It was realized early on that transcription of the chrII initiator gene *rctB* is repressed by the product of the same gene (autorepression), like the transcription of many plasmid initiator genes (Egan *et al.*, 2006, Pal *et al.*, 2005). The mechanism of autorepression remained obscure since unlike the situation in plasmids, no iterons could be recognized in the vicinity of the promoter of *rctB*. Recently, RctB was found to bind a second kind of specific site, called 39-mers (Venkova-Canova & Chattoraj, 2011). These sites are key to the negative control of chrII replication. Here we report that a 29-mer site, which has the conserved features of the 39-mers, is the one used by RctB for autorepression. The 29-mer additionally could contribute to the negative regulation of chrII replication, possibly via interactions with the origin iterons, as 39-mers do. The 29-mer thus controls replication indirectly by controlling transcription of the initiator gene and possibly directly by inhibiting origin function.

2. Methods

2.1 Bacterial strains and plasmids

V. cholerae and *E. coli* strains, and plasmids used in this study are listed in Table 1. For cloning, the fragments were obtained either by PCR amplification of N16961 (CVC205) DNA using Phusion High-Fidelity polymerase (NEB, Beverly, MA) or by annealing complementary oligonucleotides (Venkova-Canova *et al.*, 2006). The *oriII* plasmids in Fig. 5 contained a second origin (R6K $\text{ori}\gamma$) that was utilized during plasmid construction, where the host was CVC23, which supplies the cognate initiator (π protein).

2.2 β -galactosidase assay

This was done from late log phase cultures in L broth (OD = 0.4–0.5) of *E. coli* strains, BR8706 (Fig. 1) and CVC72 -73 (Fig. 2), and of *V. cholerae recA* strain CVC250 (Fig. 1B), as described (Pal *et al.*, 2005). Some of the cultures were simultaneously monitored for RctB induction by Western blotting (Fig. S1).

2.3 Plasmid copy-number measurements

The copy number was measured by supplying RctB from pTVC11, where *rctB* is expressed from an arabinose-inducible promoter, P_{BAD}. *E. coli* BR8706 was used as a host, where the *araE* transporter gene is expressed constitutively from the chromosome. BR8706/pTVC11 cells were further transformed with *oriII* plasmids shown in Fig. 5. For Fig. S2, BR8706/pTVC11 cells were first transformed with a plasmid carrying the minimal *oriII* (pTVC35).

Next, the BR8706/pTVC11/pTVC35 cells were made competent for further transformation with pBR322-derived plasmids carrying the 39-mer or different derivatives of the 29-mer. Plasmid copy number measurements were done exactly as described (Venkova-Canova & Chattoraj, 2011).

2.4 EMSA and Ligation Assay

These were done essentially as described (Venkova-Canova *et al.*, 2006, Venkova-Canova & Chattoraj, 2011, Das & Chattoraj, 2004). The fragments used in Fig. 3 were PCR amplified from the plasmids identified in the figure legend. The fragments contained, in addition to the specific target sequences, 100 bp of non-specific sequences at both the flanks. The flanking sequences were identical in all cases. The fragments used in Fig. 4 were cut out by HpaI and EcoRV from pTVC228 and pTVC552 that created 100 bp flanks for 6×12-mers and 300 bp flanks for the 29-mer, respectively. The ends of the fragments were dephosphorylated using shrimp alkaline phosphatase, after which the fragments were gel purified. A total of 1–5 pmol ends of each fragment were labeled in 50 μ l with 8.5 pmol [γ -³²P] ATP (50 μ Ci/reaction, Perkin-Elmer, Waltham, MA) using 30 units of T4 polynucleotide kinase (NEB) for 30 min at 37°C. The enzyme was inactivated for 15 min at 65°C and the fragments were purified using Sephadex G-50 column (0.8 ml bed volume; Roche Diagnostics, Indianapolis, IN). The amount of labeled DNA was determined by TCA precipitation (Mallinckrodt Baker Inc., Phillipsburg, NJ) using salmon sperm DNA (20 μ g; Invitrogen, Carlsbad, CA) as a carrier. The binding was done exactly as described (Venkova-Canova *et al.*, 2006). In the experiments of Fig. 4, the binding buffer was supplemented with 4% PVA (polyvinyl alcohol, Sigma, St. Luis, MO), a molecular crowding agent. One half of the binding reaction was loaded onto a 5% polyacrylamide gel (National Diagnostics, Atlanta, Georgia), run at room temperature at 12 V cm^{-1} in TBE buffer (89mMTris/89mM boric acid/2.5 mM EDTA, pH 8.3). The gels were dried, and the band intensities were determined using a phosphoimager (FLA-5100, Fujifilm, Edison, NJ). The other half of the binding reaction was treated with 300 Units T4 Ligase (NEB) for 10 min at RT. The ligation reaction was stopped by equal volume of 2× stop solution (2× Bromphenol blue/Xylencyanol dye with additional 2% SDS and 20% Glycerol). The mixture was vortexed, heated at 65°C for 10 min, and spun for 5 min at 13,000 rpm. One fourth of the supernatant was loaded on a 5% polyacrylamide gel. The subsequent steps were exactly as described for EMSA gels.

3. Results

3.1 A 29-mer site suffices for autorepression of *rctB*

The promoter of *rctB*, *PrctB*, was defined earlier by mapping the start site of *rctB* message (Pal *et al.*, 2005, Egan *et al.*, 2006). These studies also showed that the promoter is autorepressed. To identify the binding site(s) of RctB relevant to autorepression, the promoter upstream region was gradually shortened (Fig. 1). The promoter activity was measured in *E. coli* as before, using *lacZ* as the reporter gene, without and with RctB induction from pTVC11, in which the initiator gene is under the control of an arabinose-inducible promoter, *P_{BAD}*. The promoter of plasmid P1 initiator, *PrepA*, present in pTVC234, served as the negative control. The DNA upstream of *PrctB* did not affect significantly the promoter activity in the absence of RctB induction. Upon RctB induction (Fig. S1), the promoter activities decreased up to about four-fold, and the level of decrease depended upon the extent of the upstream region present. The reason for variable repression is likely to be due to the presence of several RctB binding sites in the upstream sequences that could influence the repression indirectly by titrating the initiator or directly by coupling interactions with the promoter region (discussed in section 3.4). Although a clearer interpretation of these results must await further analysis, it appears that the shortest region

tested for promoter regulation (as in pAS1) showed not only optimal promoter activity but also near maximal repression (3.1 fold). This region will be referred hereafter as minimal *PrctB*.

RctB binds to multiple sites that are of two kinds, the iterons (11- and 12-mers) and the 39-mers (Fig. 1A, top map) (Venkova-Canova & Chattoraj, 2011). For efficient binding, the iterons need to be methylated at the adenine residues of GATC sequences, which are found internal to all the iterons. Inspection of the chrII region present in pAS1 revealed two GATC sites but the homology to iterons did not extend beyond those four bases. However, a 29-mer site was found within the *PrctB* that was detected earlier by searching for sequences homologous to the 39-mers (Venkova-Canova & Chattoraj, 2011). The 29-mer lacks the first 10 bp of the AT-rich linker between the two conserved GC-rich direct repeats of the 39-mers (hyphens; Fig. 3 top). The 29-mer covers the -4 to +25 bp of the template DNA for *rctB* transcription, where +1 corresponds to the start site of *rctB* mRNA (Fig. 1A, bottom). The 29-mer appears to be ideally situated to serve as an operator in that bound RctB should interfere with RNA polymerase binding and transcription initiation.

In an assay for replication driven by *oriII*, the 29-mer was shown earlier to be active in RctB titration, indicating that the site was able to bind the initiator in vivo (Venkova-Canova & Chattoraj, 2011). The fragment used had six extra bases that are naturally present, flanking the 29-mer. Without the flanks, the titration activity was less (Fig. S2; fragments 4 vs. 5). Deleting a good part of the 29-mer abrogated the titration activity entirely (Fig. S2; fragment 2). Same deletion in pSA5 did not affect the promoter activity but abolished the capacity for autorepression. It appears that a few extra flanking bases can make the 29-mer more efficient in RctB titration.

The 29-mer was effective as an operator in the native host also. Although the amount of RctB in *V. cholerae* was ~2 fold less than that present under the inducible conditions used in *E. coli* (Fig. S1), *PrctB* activity from pAS1 was significantly lower than the activity from pSA5 (Fig. 1B). In summary, it appears that the minimal promoter, present in pAS1, is necessary and sufficient for *rctB* expression and autorepression, where the 29-mer serves as the operator, in both *E. coli* and *V. cholerae*.

3.2. *rctB* autorepression does not require methylation by Dam

As stated above, the minimal promoter, in addition to the 29-mer, contains two GATC sites (Fig. 1A, bottom). These two could be part of putative iterons; although iteron bases show some homology over a stretch of 11 to 12 bp, full conservation is found only in six consecutive positions that include GATC. Since the iterons require methylation for efficient RctB binding, we hypothesized that if the putative iterons of the minimal promoter were required for autorepression, it might be compromised in *dam* mutant cells. To test the hypothesis, we used *PrctA* as positive control, which carries two natural 11-mers, one overlapping with its -35 element; RctB binding and repression of this promoter is therefore expected to be dependent on Dam. As a negative control, we used *PrepA*, which is devoid of GATC sequences. The promoter activities were measured exactly as in Fig. 1. In wild-type (*dam*⁺) cells, *PrctA* and *PrctB* were repressed upon induction of RctB, as opposed to the negative control *PrepA*, whose activity was unchanged by the induction (Fig. 2). This indicates that minimal *PrctB* region includes a binding site of RctB. In *dam* mutant cells, activity of all the three promoters tested was down, even without RctB induction. This could be due to altered physiology of the mutant cells and/or the altered DNA structure (Lobner-Olesen *et al.*, 2005, Polaczek *et al.*, 1998). Induction of RctB in the *dam* mutant not only failed to repress *PrepA* but also *PrctA*, consistent with the requirement of methylation for RctB binding to iterons (Fig. 2). In contrast, *PrctB* remained sensitive to RctB induction; the promoter activity dropped by ~50%, similar to what was found in *dam*⁺ cells. Thus, the only

identifiable RctB binding site within the minimal *PrctB* region, the 29-mer, appears to be the operator for *rctB* autorepression, and it functions without requiring Dam.

3.3 The 29-mer is a binding site of RctB in vitro

To confirm RctB binding to the 29-mer in vitro, EMSA was performed with pure protein. As controls, the 39-mers from *rctA* and from the middle of *incII* with its various deletion derivatives were used (Fig. 3, top). The alterations were made in the linker region of the 39-mer by deleting either 10 bp to mimic the 29-mer, or five bp, which served as a control. The 29-mer bound RctB with affinity similar to that of the 39-mer. The binding was also comparably efficient when the 39-mer was shortened by 10 bp at two different places of the linker (Fig. 3, bottom). The binding was reduced when five bp was deleted. These results suggest that the 29-mer is a natural example of a 39-mer deleted for 10 bp, and the deletion does not affect the binding affinity of the sites.

The better binders were also more effective in reducing the copy number of a plasmid driven by *oriII* in vivo (Venkova-Canova & Chatteraj, 2011). In this assay, the intact 39-mer was most potent in reducing the copy number, followed by the 39-mer from *rctA* and the three 29-mers, the natural and the two artificially made ones, which were ~50% as effective. Thus, although the 29-mers and the 39-mer bound equally well in vitro, their activities in vivo were not the same. Since in spite of similar binding the control activity of the deleted sites were reduced by 50%, the results suggest that either EMSA overestimated the binding affinity of the 29-mers or the structure of RctB bound to 29-mer and 39-mer differs enough to affect the function of the complexes.

3.4 The 29-mer, like the 39-mer, participates in handcuffing with iterons

It is known that RctB, like the plasmid initiators, not only binds its target sites but also couples them, a reaction commonly referred to as handcuffing (Venkova-Canova & Chatteraj, 2011). RctB can couple iterons, iterons with 39-mers (hetero-handcuffing) but not the 39-mers. The 39-mers, however, improved handcuffing of iterons with themselves. Most likely, RctB binding to 39-mers changes the conformation of the initiator such that it becomes more proficient in handcuffing of iterons. The handcuffing is believed to engage the iterons of the origin in a way that impairs origin function. Because of the sequence similarity between the 29-mer and the 39-mers, we tested whether the 29-mer could also participate in handcuffing, as do the 39-mers. EMSA, followed by ligation assay were performed exactly as for the 39-mer (Venkova-Canova & Chatteraj, 2011). First, binding was tested by EMSA (Fig. 4, top). The fragments carrying the 29-mer and the origin 6×12-mers bound RctB to a comparable extent, whether the fragments were tested separately (lanes 4 and 5) or together (lane 6). After treatment of the binding reaction with ligase, the 29-mer showed little difference in ligated products in the absence or the presence of RctB (lanes 2 and 5, Fig. 4 Ligation assay). This result is consistent with our earlier result showing that the 39-mers do not handcuff with themselves (Venkova-Canova & Chatteraj, 2011). In contrast, the 6×12-mers clearly showed more ligated products upon addition of RctB, confirming the earlier finding that RctB can handcuff iterons (lanes 1 and 4, Fig. 4 Ligation assay). When the two fragments were together, the band representing ligation of the 29-mer fragment with the 6×12-mer fragment was enhanced in the presence of RctB, indicating that the 29-mer can hetero-handcuff with the iterons (Fig. 4, arrows, and the black bars in graph below). An increase in the ligated products among the iteron fragments themselves in presence of the 29-mer was also evident (Fig. 4, lane 6 in the graph below). In these experiments, the handcuffing of iterons needs to be kept low since homo-handcuffing of iterons dominates hetero-handcuffing, making the latter reaction harder to detect. In conclusion, it appears that the handcuffing properties of the 29-mer are quite similar to those of the 39-mers.

3.5 The 29-mer reduces replication of orill-based plasmids in *E. coli*

The similarity of the handcuffing properties of the 29-mer with those of the 39-mers in vitro suggests that the 29-mer could be controlling *chrII* replication in vivo, as do the 39-mers. This was tested by measuring the copy number of *oriII* plasmids with and without the 29-mer *in cis*. A two-plasmid system was used in *E. coli*, where one had *oriII* and the other provided RctB *in trans* from pTVC11. The use of a surrogate host has the advantage that the issue of incompatibility between the plasmid and chromosomal origins does not arise (Venkova-Canova & Chatteraj, 2011, Egan & Waldor, 2003, Yamaichi *et al.*, 2011). In these experiments, *oriII* plasmids were electroporated into an *E. coli* that already harbored the RctB plasmid. No colonies were seen when pTVC25 was used for transformation, most probably because the 29-mer acted as a negative regulator on top of the strong negative regulator, the 39-mer. Transformants were obtained with an isogenic plasmid pTVC523 lacking the 29-mer. The presence of the plasmid in the transformants was confirmed by measuring its copy number (Fig. 5). The results were similar with the plasmid pair pTVC31 and pTVC524, with and without the 29-mer, respectively. Both these plasmids replicated with high copy numbers because they lack the negative control locus, *incII*, entirely. Even in this high-copy-number plasmid set, the one without the 29-mer had the higher copy number. This could be due to several reasons: (i) lack of titration of RctB by the 29-mer, which will increase initiator availability for replication initiation; (ii) lack of interactions between 29-mer and the origin iterons (hetero-handcuffing) which will improve the origin function; and (iii) activation of the origin by increased activity of *PrctB* in the absence of the operator site (transcriptional activation (Baker & Kornberg, 1988)). We conclude that the 29-mer is capable of inhibiting replication even in the presence of other regulators of *incII*. This regulatory role is in addition to the role of the 29-mer in autorepression, since in the present experiment RctB was supplied from a foreign promoter (P_{BAD}) *in trans* that is not under RctB control.

4. Discussion

Autorepression is an efficient mechanism to maintain gene products within narrow limits and to dampen out the effect of changes in gene-dosage (Becksei & Serrano, 2000, Simpson *et al.*, 2003). Many bacterial and plasmid initiators are autorepressed at the transcriptional level (Das *et al.*, 2005, Atlung *et al.*, 1985). This is easily rationalized since initiators are essential for replication and often the rate-limiting component of replication; therefore maintaining their concentration within narrow limits becomes necessary to maintain the rate of replication.

In the case of plasmids of the iteron-family, transcriptional autorepression is the main mechanism that limits initiator synthesis (Paulsson & Chatteraj, 2006). An exception has been found in plasmid RK2, where the initiator gene is regulated by the products of three other plasmid genes, *korA*, *korB* and *kilB1* (Kolatka *et al.*, 2010). There is a post-translational mechanism of autorepression as well, which operates by initiator dimerization (Paulsson & Chatteraj, 2006). In iteron-based plasmids, initiator dimers are inactive since only monomers bind iterons. The role of dimerization in dampening initiator monomer fluctuation has been deduced from sound theoretical considerations, and its contribution to maintaining monomers within narrow limits is predicted to be similar to that of transcriptional autorepression. In either of the mechanisms, the autorepression is not known to depend on the cell cycle.

The *chrII* iterons require full methylation for binding to the initiator, whereas plasmid iterons do not (Demarre & Chatteraj, 2010). *ChrII* also replicates at a particular time of the cell cycle whereas initiation of plasmid replication is distributed throughout the cell cycle. The involvement of methylation allows controlling the iteron activity in the cell cycle, since

they become inactive following replication, and stay inactive for about two-thirds of the cell cycle. In this context, use of a 29-mer as the operator should help maintain the initiator in a cell-cycle independent fashion, since the site lacks the sequences for methylation. Finally, the methylation-independent regulation of the initiator expression should allow restricting the amount of the newly-synthesized RctB during the hemi-methylated period, which would prevent over-loading of cells with RctB when *oriII* becomes methylated again. This should be beneficial for the once-per-cell-cycle replication initiation.

The *chrII* replication initiation rate increases in response to increases in RctB concentration; in contrast, the copy number of iteron-carrying plasmids hardly changes when initiator concentration is increased (Das *et al.*, 2005, Pal *et al.*, 2005, Duigou *et al.*, 2006, Pal & Chattoraj, 1988, McEachern *et al.*, 1989). This suggests that there might be a greater demand to maintain RctB concentration within narrower limits than what might suffice for the plasmid initiators. In *chrII*, the mechanism of transcription repression itself might be more complex, involving sequences other than the 29-mer, as suggested by the results of Fig. 1, where sequences upstream of *PrctB* could influence the magnitude of autorepression. The influence could also vary during the cell cycle, since the iterons remain active only a part of the cell cycle (Demarre & Chattoraj, 2010). There could also be mechanisms other than transcription autorepression to make the regulation of RctB concentration more stringent.

The 29-mer, like the 39-mers, was able to inhibit *oriII*-based plasmid replication (Fig. 5). The site thus has a dual role in *chrII* replication. The role as transcription operator is perhaps more important since it is required to control the rate-limiting component of replication – the RctB concentration. The role as a replication inhibitor should be relatively minor since the 29-mer is one of several other regulatory sites involved in controlling *chrII* replication. Also, compared to the 39-mers, the 29-mer is less potent in inhibiting *oriII*-based plasmid replication (Venkova-Canova & Chattoraj, 2011). It is not clear whether there is any significance of having a 29-mer as the operator and 39-mers as purely replication inhibitors. To this end, we note that autorepression could also be achieved equally efficiently when the 29-mer was replaced with the 39-mer (data not shown). In any event, the ability of RctB to recognize 39-mer like sites when the linker sequences were changed from normally 19 to either nine or 29 bp shows the inherent adaptability of the RctB structure. Since not all binding proficient sites were functional *in vivo*, this suggests that the sites not merely titrate initiators but play more active role in replication, such as handcuffing. Here we have shown that the 29-mer, like the 39-mer, was capable of promoting self-ligation of iteron fragments *in trans* most likely by remodeling RctB. Further understanding of these results calls for more structural information of the complexes.

Highlights

- The replication initiator of *V. cholerae* chromosome II is limiting for replication.
- The autorepression site for the initiator gene expression is identified.
- The same site additionally serves as a negative regulator of replication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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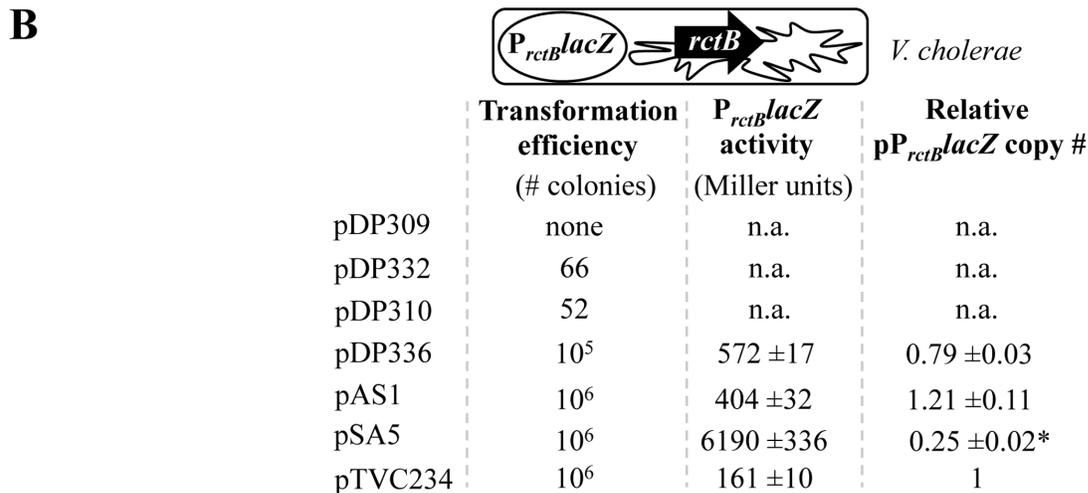
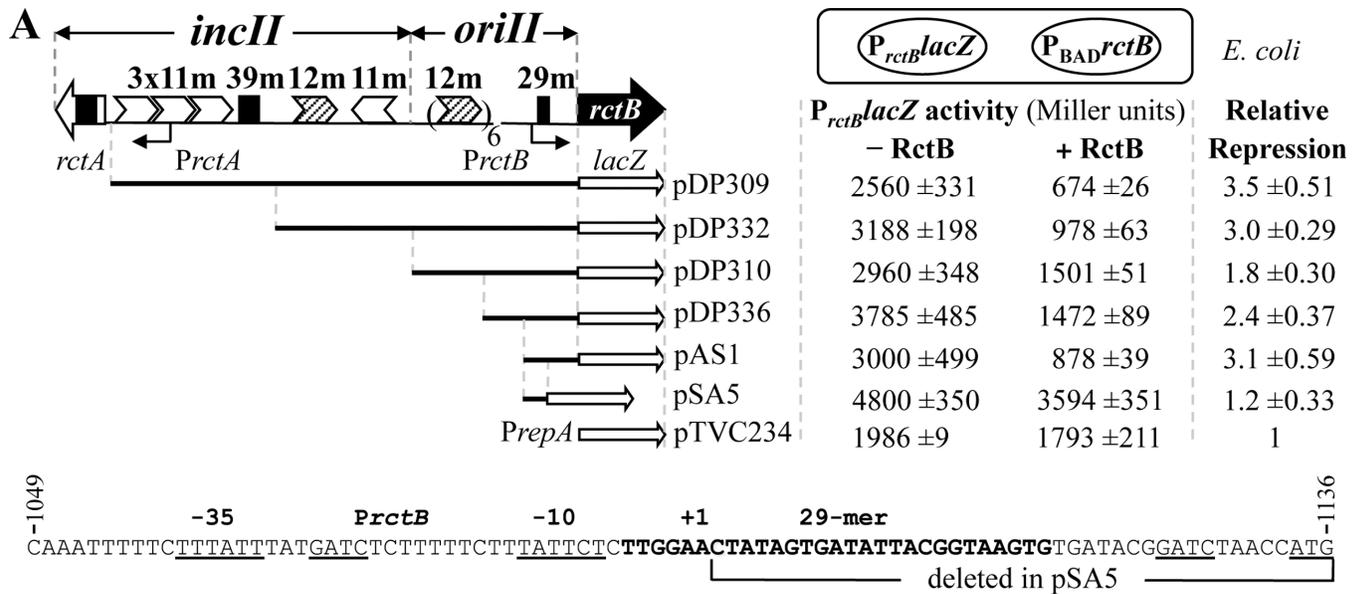


Fig. 1. A minimal promoter region that suffices for autorepression of *rctB* contains a 29-mer sequence

A. Repression of *PrctB* by an inducible source of RctB in *E. coli*. A map of the replication region of *V. cholerae* chrII is shown at the top left. The region is divided into three segments: *incII* that regulates replication, *oriII* that is required *in cis* to initiate replication, and *rctB* (black arrow), the gene that codes for the initiator, RctB. RctB binds to two kinds of site: iterons, which are 11- or 12-mers (white or hatched arrowheads, respectively), and the 39-mers (black rectangles). The region has two promoters, *PrctA* and *PrctB*, the latter for transcribing *rctB*. A 29-mer, which has the conserved GC-rich direct repeats of 39-mers (boxed bases shown in Fig. 3) serves as the operator regulating *PrctB*, as shown below.

To identify the minimal region required for autorepression of *rctB*, the promoter with decreasing upstream sequences was fused to *lacZ* (white arrows) in a pBR322-derived vector. An identical construct (pTVC234) except where *PrctB* was replaced with a foreign promoter, *PrepA*, was used as the negative control. The autorepression was checked by supplying RctB from an inducible source, pTVC11, where *rctB* was under P_{BAD} control

(P_{BAD}*rctB*, cartoon on top right). 0.2% arabinose was used as the inducer. β -galactosidase activities were normalized with respect to the copy number of the relevant *lacZ*-plasmids (see Material and Methods). The values are mean \pm one standard deviation from three independent measurements.

The sequence of the *PrctB* region present in pSA1 is shown at the bottom. The sequence coordinates are from the annotated chrII sequence at NCBI (NC_002506). -35 and -10 elements of *PrctB* are underlined. Also underlined are the two Dam methylation sites (GATC) and the starting ATG codon of *rctB*. +1 corresponds to the start position *rctB* mRNA. The 29-mer site is shown in bold. The region of pAS1 deleted in pSA5 (coordinates 1095–1136) is shown by a bracket.

B. Repression of *PrctB* by the natural source of RctB in *V. cholerae*. The same plasmids as in A were electroporated into CVC250 (*V. cholerae recA* mutant strain) and where colonies with normal size were obtained, β -galactosidase activity and copy number of the *lacZ*-carrying plasmids were measured. In the case of plasmids pDP309, -332 and -310, either transformants were not seen or they were bigger than normal size colonies (most likely these were revertants where the *incII* got deleted). The star indicates a slow-growing culture. The values are mean \pm one standard deviation from three cultures inoculated with independent colonies.

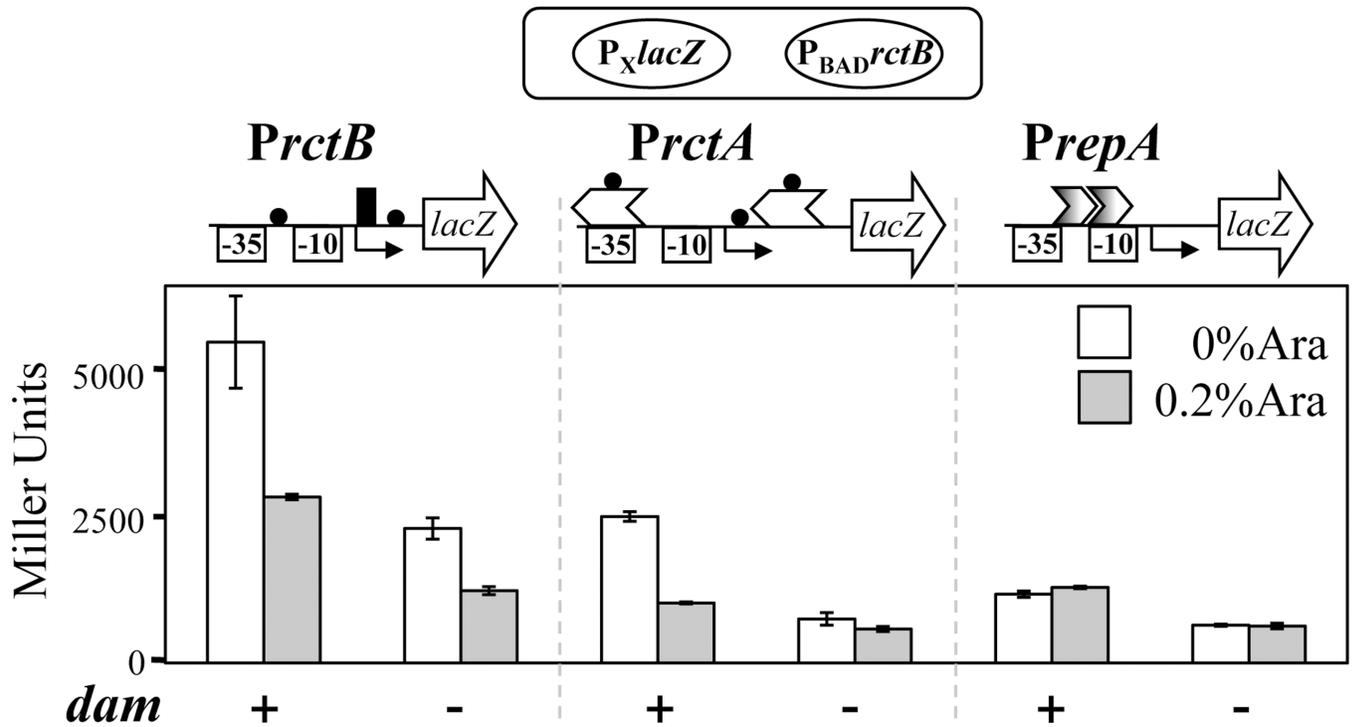


Fig. 2. Autorepression of *rctB* does not require methylation by Dam

Repression by RctB was determined, as in Fig. 1, for three promoters, *PrctB*, *PrctA* and *PrepA*, present in plasmids pAS1, pTVC126 and pTV234, respectively. All promoter fragments contained the untranslated leader region and ended with their respective ATG start codon, which was fused to *lacZ*. The symbols are as in Fig. 1, except that GATC methylation sites are represented as black circles, and the -35 and -10 elements of the promoter are boxed. The bars show β galactosidase activity in Miller units, measured before (white bars) and after (grey bars) induction with 0.2% arabinose. The host cells either had the *dam* gene intact (+, CVC73) or inactivated (-, CVC72). The data are from three independent colonies for each of the fusions. The error bars here and elsewhere represent one standard deviation.

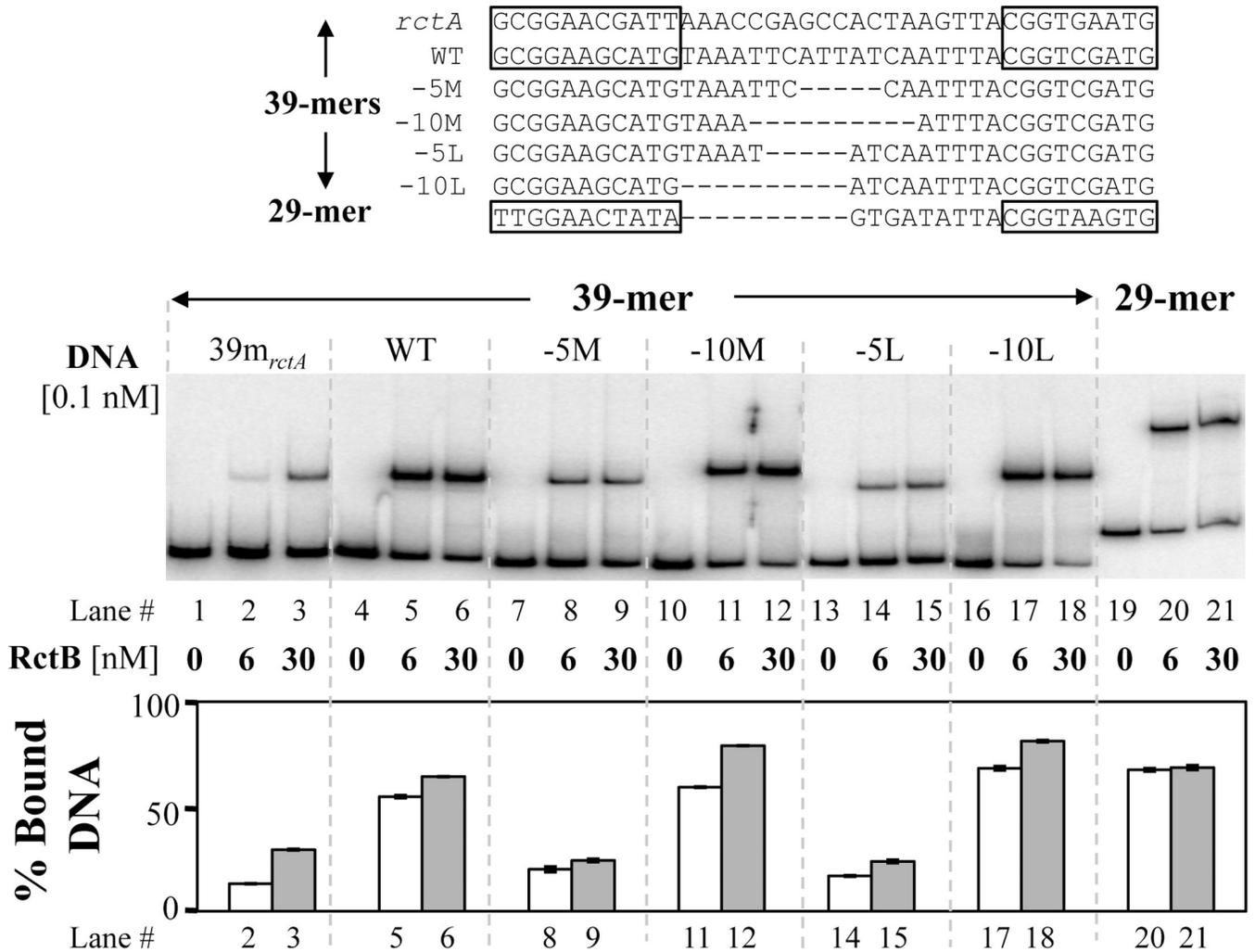


Fig. 3. The 29-mer, like the 39-mer, binds RctB in vitro

The sequences of the 39-mers in *rctA* and in the middle of *incII*, and of several deletion derivatives of the latter are shown in the top, along with the sequence of the 29-mer. The 29-mer carrying fragment was bigger than the other fragments used here, as it contained more natural sequences flanking the site, exactly as present in pAS1 and shown on Fig. 1A (bottom sequence). To determine binding proficiency of these sequences to RctB, they were amplified by PCR (i.e. they were unmethylated) from the following plasmids: pTVC191, -222, -331, -332, -329, -330 and -327. The binding was monitored by EMSA (middle panel) at two different RctB concentrations. Percent binding ($[\text{intensity of the retarded band} / \text{combined intensities of free and retarded bands}] \times 100$) at 6 nM and 30 nM are shown by white and grey bars, respectively. The error bars are from three independent measurements of band intensities from the same gel, which reflect the reproducibility of our measurements.

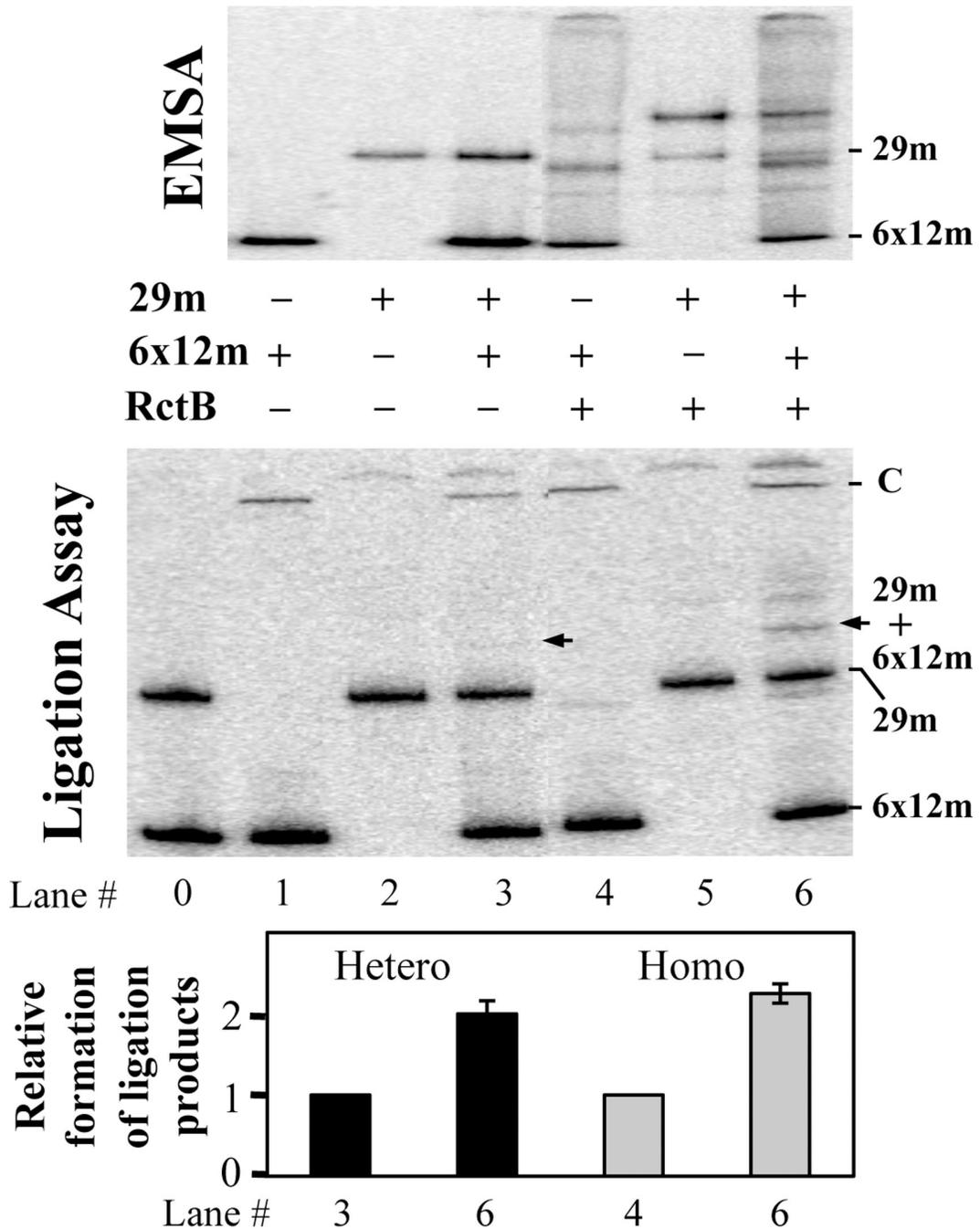


Fig. 4. The 29-mer, like the 39-mer, participates in handcuffing with iterons

Two fragments (both at 0.02 nM), carrying the 29-mer in one and the 6×12-mers in the other, were analyzed by EMSA with 7 nM RctB, and subsequently, after addition of ligase to the binding reaction (Ligation Assay). The fragments were obtained by enzyme digestion from pTVC552 and pTVC228, respectively (see Material and Methods). Half of the binding reaction was loaded onto a 5% polyacrylamide gel (EMSA, top panel). The remaining half was treated with ligase to record coupling rate of the fragments. The products of ligation, after deproteinization, were analyzed also in a 5% polyacrylamide gel (Ligation Assay, middle panel). The sample in lane 0 did not contain ligase. C stands for circular products of ligation, which migrate poorly in polyacrylamide gels. The DNA species representing a

heterodimer of 29-mer and a 6×12-mer fragments is indicated by arrows. The intensities of this band before (lane 3) and after (lane 6) addition of RctB, are shown by black bars on the graph below. The intensities of the self-ligated products of the 6×12-mer fragments (Homo), are shown by grey bars after excluding the intensity of the heterodimer band and the 29-mer bands from lane 6. Note that self-ligation of the 29-mer fragment is not enhanced by RctB. Lanes 0 and 1 were used for background correction. The error bars are from measurement of three independent gels.

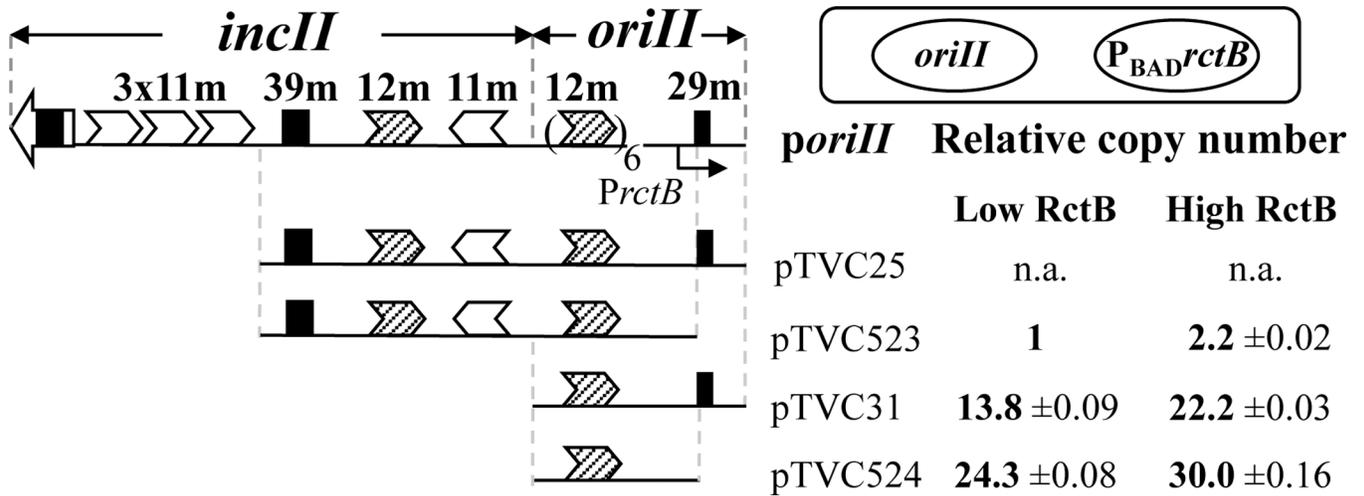


Fig. 5. The 29-mer, like the 39-mer, reduces replication of *oriII*-based plasmids in *E. coli*
 Four *oriII*-plasmids were tested for replication in the presence of an inducible source of RctB, as in Fig. 1 (cartoon on the top). Plasmids pTVC25 and pTVC523 are isogenic except for the presence of the 29-mer. They both have a part of the *incII* control region. Plasmids pTVC31 and pTVC524 are also isogenic except for the 29-mer but they lack *incII* totally. The *oriII* plasmid copy number was measured upon induction with 0.002% (Low RctB) and 0.2% (High RctB) arabinose, as described in Material and Methods. There was seven-fold more RctB at the higher inducer concentration, as was found earlier (Fig. S2 in Venkova-Canova & Chattoraj, 2011). The copy numbers shown are mean values from three cultures, each inoculated with separate single colonies. No transformants were obtained using pTVC25, hence its copy number is not shown (n.a.).

Table 1

Bacterial strains and plasmids used in this study 500

Strains	Relevant characteristics	Source/Fig.
BR2846	K-12 <i>recA1</i> Δ (<i>argF-lac</i>) <i>U169</i> ; Strain for cloning in pMLB1109	(Simons <i>et al.</i> , 1987)
BR8706	= <i>Stb12</i> Δ (<i>lac-proAB</i>) Δ (<i>araFGH</i>) Δ <i>araEp</i> P _{CP18} - <i>araE</i> ; <i>araE</i> under constitutive CP18 promoter	(Fekete & Chattoraj, 2005, Pal <i>et al.</i> , 2005)
CVC23	DH5(λ <i>pir</i>); Strain for maintaining R6 <i>Kori</i> γ plasmids.	M. Waldor
CVC72	GM7589=MG1655 <i>rph-1 lacU169 mutL459::Kan dam-13::Tn9</i> ; Dam- strain	M. Marinus
CVC73	GM7576= MG1655 <i>rph-1 lacU169 recA56 sr1300::Tn10</i> ; Dam+ strain	M. Marinus
CVC205	<i>V. cholerae</i> El Tor N16961 <i>lacZ::res::tet::res</i>	(Pal <i>et al.</i> , 2005)
CVC250	CVC205 <i>recA::Kan</i>	(Pal <i>et al.</i> , 2005)
DH5 α	Strain for cloning	Invitrogen
Plasmids		
pAS1	<i>PrctB</i> (nt 1049–1133) in pMLB1109; Ap ^R	Figs. 1, 2
pDP309	Same as pAS1, but <i>PrctB</i> (nt 256–1157)	(Pal <i>et al.</i> , 2005)
pDP310	Same as pAS1, but <i>PrctB</i> (nt 727–1157)	(Pal <i>et al.</i> , 2005)
pDP332	Same as pAS1, but <i>PrctB</i> (nt 547–1157)	(Pal <i>et al.</i> , 2005)
pDP336	Same as pAS1, but <i>PrctB</i> (nt 925–1157)	(Pal <i>et al.</i> , 2005)
pMLB1109	Source of promoter-less <i>lacZ</i> gene; pBR322 <i>ori</i> ; Ap ^R	M. Berman
pSA5	<i>PrctB</i> (nt 1049–1094) in pMLB1109; Ap ^R	Fig. 1
pTVC11	<i>rctB</i> (nt 1118–3115) under P _{BAD} control in pSC101 <i>ori</i> ; Sp ^R	(Pal <i>et al.</i> , 2005)
pTVC12	P _{BAD} in pSC101 <i>ori</i> ; Sp ^R	(Pal <i>et al.</i> , 2005)
pTVC25	<i>orIII</i> (nt 441–1133) in R6 <i>Kori</i> γ plasmid; Ap ^R	(Venkova-Canova <i>et al.</i> , 2006)
pTVC31	Same as pTVC25, but <i>orIII</i> (nt 775–1133)	(Pal <i>et al.</i> , 2005)
pTVC35	pTVC31 lacking R6 <i>Kori</i> γ (maintained in BR8706 + pTVC11)	(Venkova-Canova <i>et al.</i> , 2006)
pTVC61	pBR322 <i>ori</i> . Digestion with EcoRV and HpaI leaves 300 bp flanks on either side of MCS; Cm ^R	(Venkova-Canova <i>et al.</i> , 2006)
pTVC126	<i>PrctA</i> (nt 377-249) in pMLB1109; Ap ^R	Fig. 2
pTVC191	pTVC61+ 39-mer from <i>rctA</i> (nt 176 – 214)	(Venkova-Canova & Chattoraj, 2011)
pTVC222	pTVC243 + 39-mer (nt 449–487)	Fig. 3
pTVC228	6 \times 12-mers (nt 788–934) in pTVC243; Cm ^R	(Venkova-Canova & Chattoraj, 2011)
pTVC234	<i>PrepA</i> from P1 (nt 556–600) in pMLB1109	(Venkova-Canova & Chattoraj, 2011)
pTVC243	pTVC61 shorter by 400 bp. Digestion with EcoRV and HpaI leaves 100 bp flanks on either side of MCS; Cm ^R	(Venkova-Canova & Chattoraj, 2011)
pTVC327	pTVC243 + <i>PrctB</i> (same as in pAS1)	Fig. 3 and S2
pTVC328	pTVC243 + <i>orIII</i> (nt 1049–1094)	Fig. S2
pTVC329	pTVC222 with 5 bp deleted from the left side of the 39-mer linker region	(Venkova-Canova & Chattoraj, 2011)
pTVC330	Same as pTVC329, but the deletion is of 10 bp	(Venkova-Canova & Chattoraj, 2011)
pTVC331	pTVC222 with 5 bp deleted from the middle of the 39-mer linker region	(Venkova-Canova & Chattoraj, 2011)
pTVC332	Same as pTVC331, but the deletion is of 10 bp	(Venkova-Canova & Chattoraj, 2011)

Strains	Relevant characteristics	Source/Fig.
pTVC338	pTVC243 + <i>orill</i> (nt 1089–1118)	Fig. S2
pTVC339	pTVC243 + <i>orill</i> (nt 1089–1132)	Fig. S2
pTVC400	pTVC243 + <i>orill</i> (nt 1083–1124)	(Venkova-Canova & Chatteraj, 2011)
pTVC522	pTVC61 + same <i>orill</i> as in pTVC400	Fig. 4
pTVC523	Same as pTVC25, but with deleted 29-mer (<i>orill</i> = nt 441–1094)	Fig. 5
pTVC524	Same as pTVC31, but with deleted 29-mer (<i>orill</i> = nt 775–1094)	Fig. 5