DNA Adenine Methylation Is Required to Replicate Both Vibrio cholerae Chromosomes Once per Cell Cycle

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Abstract

DNA adenine methylation is widely used to control many DNA transactions, including replication. In Escherichia coli, methylation serves to silence newly synthesized (hemimethylated) sister origins. SeqA, a protein that binds to hemimethylated DNA, mediates the silencing, and this is necessary to restrict replication to once per cell cycle. The methylation, however, is not essential for replication initiation per se but appeared so when the origins (oriI and oriII) of the two Vibrio cholerae chromosomes were used to drive plasmid replication in E. coli. Here we show that, as in the case of E. coli, methylation is not essential for oriI when it drives chromosomal replication and is needed for once-per-cell-cycle replication in a SeqA-dependent fashion. We found that oriII also needs SeqA for once-per-cell-cycle replication and, additionally, full methylation for efficient initiator binding. The requirement for initiator binding might suffice to make methylation an essential function in V. cholerae. The structure of oriII suggests that it originated from a plasmid, but unlike plasmids, oriII makes use of methylation for once-per-cell-cycle replication, the norm for chromosomal but not plasmid replication.

Introduction

The regulatory potential of canonical DNA sequences can be greatly expanded by epigenetic modifications. Methylation is the most common modification of DNA and is widely used to control many cellular processes [1]. In bacteria, DNA methylation is restricted to adenine and cytosine residues [2], and can facilitate or interfere with DNA-protein interactions, thereby modulating various DNA transactions [3]. Such transactions include gene expression, DNA restriction, DNA mismatch repair, and chromosome replication and segregation [4,5].

Most of our knowledge regarding the role of methylation in chromosome replication comes from studies in Caulobacter crescentus and Escherichia coli. In C. crescentus, initiation of DNA replication requires the adenines of the GANTC sequences in the origin of replication to be methylated on both the top and bottom strands by the methylase CcrM. How the methylation helps the origin function is not known, although methylation lowers DNA stability [6,7] and thereby could facilitate origin-opening, an essential step in the replication initiation process. It is also possible that the methylation changes DNA structure to facilitate protein-DNA interactions at the origin [8]. Irrespective of the mechanism, methylation not only controls the timing of initiation but also restricts initiation to once per cell cycle [9]. Following initiation, the hemimethylated sister origins cannot be reused in the same cell cycle, as the CcrM methylase is not synthesized until the end of the replication cycle.

In E. coli, the methylase is called Dam and acts on the adenines of GATC sequences, which are particularly frequent in the origin of replication, oriC. In this bacterium also the methylation most likely helps in origin-opening [8,10] but plays a more definite role in restricting the initiation to once per cell cycle [11]. In E. coli, immediate reinitiation is prevented, not by delaying the synthesis of the methylase, but by preventing its action through sequestration of hemimethylated sister origins by a hemimethylation-specific DNA binding protein, SeqA [12]. Sequestration renders DNA unavailable to the methylase. The sequestration also allows initiation synchrony whereby the multiple origins that E. coli maintains during rapid growth fire nearly simultaneously. It is believed that the sequestration process continues at least until all the origins have fired. This happens in a narrow window of time giving rise to the initiation synchrony phenotype [13]. In the absence of Dam, the newly replicated origins, without their hemimethylation marks, remain indistinguishable from the unreplicated ones. The choice of origin for replication being random, once-per-cell-cycle initiation from each origin is no longer guaranteed. As a result, in dam mutants, the initiation becomes asynchronous and cells can have origins that do not fire at all or fire more than once in the same cell cycle. The consequences are the same in seqA mutants, because without sequestration, replicated origins also remain competent for reinitiation.

The lack of discrimination between replicated and unreplicated origins can lead to origin incompatibility [14]. If extra copies of oriC are introduced as plasmids into wild type (WT) E. coli, the plasmid copies do not compete with the chromosomal oriC because of sequestration of newly replicated origins. Without sequestration, in dam or seqA mutants, the plasmid copies remain available for reinitiation, and under selection they can block the growth of cells.
in which the chromosomal origins did not get a chance to fire. Sequestration-deficient strains are therefore not easily transformed with oriC plasmids [15]. Thus, although not normally required, Dam or SeqA can be essential in a competitive situation.

Vibrio cholerae has two chromosomes (chrI and chrII). The origin of chrI (oriII) shares 58% identity with the E. coli oriC, and both have similarly high densities of GATC sites. The origin of chrII (oriII) also has a high density of GATC sites but has a second feature of a major class of plasmids: repeated initiator-binding sites (iterons) [16]. The dam gene is also essential for V. cholerae, although the reason has remained unknown [17]. Our interest in the role of methylation in V. cholerae chromosomal replication stems from the fact that although the bacterium is a close relative of E. coli, plasmids with either oriI or oriII could transform WT E. coli, but not when it lacked Dam [18]. It remained unclear whether the failure to recover transformants in the case of oriI is because the origin could not function or because of competition (incompatibility) with the closely related chromosomal oriC [14,18]. Incompatibility is unlikely the case of oriII, since it has little similarity to oriC. Moreover, while oriI and oriC are regulated by the DnaA initiator protein, oriII is regulated by its own specific initiator, RctB [19]. The reason for the Dam requirement of oriII could thus be for the functioning of the origin itself.

Here we show that oriC can be replaced by oriI in the E. coli chromosome, and in this chromosomal context oriI functions without requiring Dam or SeqA. Incompatibility with the chromosomal oriC thus remains a satisfactory explanation of the earlier finding of a Dam requirement for oriI plasmids [18]. For oriII, Dam but not SeqA appears to be required as only fully methylated oriI DNA, but not hemi- or unmethylated DNA, could bind efficiently to the oriII-specific initiator RctB in vitro. Since the binding of RctB is a prerequisite for oriII function, this explains an explanation for why Dam is essential for V. cholerae, chrII being indispensable. Finally, we show that SeqA is necessary to restrict initiation to once per cell cycle for both oriI and oriII, as is the norm for chromosomal origins. Although chrII is believed to have originated from a plasmid, our findings of the methylation requirement for its initiation and cell-cycle specific regulation are unprecedented in studies of plasmids [20,21]. It appears that a plasmid origin acquired methylation to function as a chromosomal origin, thus providing a novel example of origin evolution in bacteria.

Results

Dam and SeqA are not essential for replication initiation at oriI in E. coli

The E. coli origin of replication, oriC, does not require dam and seqA to initiate replication. In contrast, plasmids driven by oriC are highly deficient in transformation of dam mutants [11]. This is believed to be due to irreversible sequestration of hemimethylated plasmid origins by the SeqA protein after the first round of replication [22]. Indeed, seqA and dam seqA strains can be transformed by oriC plasmids, although the efficiency is lower compared to WT due to incompatibility with the chromosomal copy of the origin [15]. The requirement of dam thus is not intrinsic to oriC function and appears so only in the plasmid context. The Dam requirement of V. cholerae oriI has so far been studied only in the plasmid context. However, in contrast to oriC plasmids, oriI plasmids only not failed to transform an E. coli dam mutant but also a seqA or a dam seqA mutant, raising the possibility that the genes could be essential for oriI [11,18]. We confirmed the plasmid results using E. coli MG1655 (BR1703) and its dam (CVC1415), seqA (BR1704) and dam seqA (CVC1424) mutant derivatives. As before, the dam, seqA and dam seqA mutants could not be transformed with an oriI plasmid, and only the dam mutant could not be transformed with the oriC plasmid (Figure 1A). We suggest below that the oriI plasmid possibly replicated in the absence of dam or seqA, which competed out replication from the chromosomal oriC and led to inviability of the transformants.

To avoid plasmid-mediated competition (incompatibility), we studied oriI by placing it in the E. coli chromosome. Using the Red recombinase system, we replaced the minimal oriC region with the corresponding oriI region (Materials and Methods). The resultant strain, MG1655damC::oriI (CVC1400; Table 1; hereafter called MG1655 damC::oriI), could be made dam minus by PI transduction, using dam-16::aph (CVC1383) as the source of the mutant dam allele [23]. We could also replace the oriC region of MG1655::seqA10 with DamC::oriI by PI transduction. The viability of dam, seqA or dam seqA mutant derivatives of MG1655 damC::oriI (CVC1401, CVC1416 and CVC1425, respectively) indicates that oriI does not require Dam and SeqA for functioning in E. coli.

To understand why oriI and oriC behave similarly in the chromosomal context but differently in the plasmid context, we repeated the transformation experiments using MG1655 damC::oriI cells as the host. The oriI plasmid could now transform the seqA and the dam seqA derivatives of MG1655 damC::oriI efficiently but not the dam derivative (Figure 1A and 1B). The failure to transform the dam derivative can be attributed to permanent sequestration. In contrast to oriI, oriC not only failed to transform the dam derivative but also the dam seqA derivative of MG1655 damC::oriI. The results can be understood assuming initiation from oriI to be more efficient than from oriC. Most likely, the weaker oriI failed to compete with oriC in the chromosome (incompatibility) that led to inviability of the transformants. It is known in E. coli that incompatibility problems can be aggravated when the incoming and recipient origins have unequal efficiencies [24].

Dam and SeqA make initiation synchronous and once-per-cell-cycle for oriI in E. coli

oriI and oriC were further analyzed using low cytometry [25]. Replication initiation and cell division were blocked by antibiotics rifampicin and cephalaxin, respectively, but sufficient time was allowed after drug addition to complete replication elongation.
(replication run-out). This method provides a measure of the fraction of the population that already initiated replication at the time of drug addition. In LB, after the replication run-out, MG1655 cells were distributed mostly into two populations, one with four and the other with eight full chromosomes (Figure 2A). This indicates that cells were born with four origins and they all fired synchronously once, giving rise to the eight chromosome peak. In the Dam and SeqA mutants, cells had a widely varying number of chromosomes indicating asynchronous initiation (Figure 2C and 2E) [22,26]. There were also cells with more than eight chromosomes indicating that initiation was no longer restricted to once per cell cycle. In the engineered strain, MG1655ΔoriC::oriI, replication initiation was synchronous (Figure 2B) but not in its dam or seqA derivatives (Figure 2D and 2F). The requirements of Dam and SeqA for synchronous and once-per-cell-cycle initiation are thus maintained when oriI replaces oriC.

Compared to the WT, replication initiation was less frequent in dam mutants but more frequent in seqA mutants in the case of both the origins. As is oriC, Dam seems to be playing a positive role and SeqA a negative role in replication initiation from oriI.

**Dam is required for initiator binding to oriIl**

It was reported earlier, and we confirmed, that oriIl plasmids cannot transform an E. coli dam mutant but can transform a seqA...
mutant [18]. The oriII mutant, indicating that irreversible sequestration cannot account for the dam requirement. The oriII function could not be tested in the chromosomal context, as was done for oriI, because attempts to replace oriC with oriII failed. In any event, incompatibility between oriC and oriII appears to be an unlikely explanation for the dam requirement, as the structure and control elements of the two origins are different [19]. We show below that the reason for the dam requirement could be for binding of oriII to its specific initiator RctB.

A distinguishing feature of oriII is that its putative RctB binding sites, called 11- and 12-mers, all contain a GATC site. This prompted us to test whether methylation of the sites might be important for RctB binding (Figure 3A). We first tested binding to the six tandem 12-mers within the minimal oriII by an electrophoretic mobility shift assay. Purified RctB bound efficiently to the 12-mer fragment, when it was fully methylated (Figure 3B).

The binding was nearly saturated because most of the DNA molecules were maximally retarded. Binding to hemimethylated DNA, where either the top or the bottom strand carried the methylation marks, and to unmethylated DNA was significantly weaker and the cloned plasmid failed. In any event, incompatibility between oriC and oriII appears to be an unlikely explanation for the dam requirement, as the structure and control elements of the two origins are different [19]. We show below that the reason for the dam requirement could be for binding of oriII to its specific initiator RctB.

To test how well the results obtained in vitro and in E. coli reproduce in the native host, the dam gene of V. cholerae was deleted in the presence of a complementing plasmid, pTS-PBAD-dam (pGD93, Table 2). The replication of this plasmid is temperature sensitive and the cloned V. cholerae dam gene is under the control of an arabinose-inducible and glucose-repressible promoter, PBAD. On LB plates, under the permissive condition (30°C and in the presence of arabinose), the Δdam/pTS-PBAD-dam strain grew as well as the WT but under the restrictive condition (42°C and in the presence of glucose), single colonies were barely visible (Figure 4A). In LB broth, under the restrictive condition, the mutant grew slower than the WT (with generation times of 27 min and 22 min, respectively), and the growth plateaued to an OD of 0.53 only one generation, while the WT continued to grow.

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Table 1. Bacterial strains.

| Strains       | Description/relevant characteristics                                           | Reference or source |
|---------------|--------------------------------------------------------------------------------|---------------------|
| BR1703 (=MG1655) | Wild type                                                                         | [29]                |
| BR1704        |                                                                                  | [29]                |
| BR2699 (=DH5α) | supE44 ΔlacU169 (Δ80lacZ’ΔM15) ΔargF hsdR17 recA1 endA1 gyrA96 thi-1 relA1 | [53]                |
| CVC209        | Ni6961 Str                                                                        | [46]                |
| CVC769        | CVC209 with parS-Kn at ~90 kb in chrI                                             | R. K. Ghosh         |
| CVC827        | CVC209 with parS-Kn at ~40 kb in chrII                                            | R. K. Ghosh         |
| CVC1060 (=GM48) | (F−) thr leu lty iacY galT galT ara fhuA tss dcm supE44                          | D. Mazel            |
| CVC1061 (=I110) | CVC1060 ΔthyA::(erm–pir116)                                                    | [54]                |
| CVC1121       | Ni6961 hapR° Δdns                                                                | M. Blokesch         |
| CVC1363 (=I13813) | B462 ΔthyA::(erm–pir116)                                                   | [54]                |
| CVC1364 (=I3914) | MG1655 ΔdapA::(erm–pir) RP4-2-Tc::Mu gyrA462 zei-298:Tn10                  | [48]                |
| CVC1383 (=GM3819) | dam16::aph                                                                       | [23]                |
| CVC1394 (=NM1100) | MG1655 mini−Δ Tet                                                                | [45]                |
| CVC1400       | MG1655 ΔoriC::iri-zeo                                                              | This study          |
| CVC1401       | CVC1400 dam16::aph                                                                 | This study          |
| CVC1410       | CVC209 ΔseqA−::zeo                                                                | This study          |
| CVC1415       | MG1655 dam16::aph                                                                  | This study          |
| CVC1416       | CVC1400 ΔseqA10                                                                    | This study          |
| CVC1424       | MG1655 dam16::aph ΔseqA10                                                          | This study          |
| CVC1425       | CVC1400 dam16::aph ΔseqA10                                                         | This study          |
| CVC1455       | CVC1410 with parS-Kn at ~40 kb in chrII                                           | This study          |
| CVC1457       | CVC1410 with parS-Kn at ~90 kb in chrII                                           | This study          |
| CVC2003       | CVC1121 ΔseqA−::zeo                                                                | This study          |
| CVC2023       | CVC209 Δdam::zeo/pGD93                                                             | This study          |
Hemimethylation period is prolonged at oriI and oriII

The hemimethylation period, the time to remethylate a GATC site after passage of the replication fork, is particularly prolonged at oriC because of the presence of high density of GATC sites within the origin [12]. The prevalence of high density of GATC sites in both oriI and oriII (Figure 5A) prompted us to examine their hemimethylation period, as was done using asynchronous exponential cultures [27,28].

We examined the hemimethylation period of a GATC site within the origin and, for comparison, another site external to the origin (about 300 kb away) for each of the chromosomes. In oriI, the GATC site chosen is between DnaA boxes R3 and R4, and in oriII, it is between the fourth and the fifth 12-mers (arrows, Figure 5A). Total genomic DNA was extracted and digested with restriction enzymes whose recognition sequences overlap a GATC site and whose cleavage is inhibited when the site is fully methylated but not in one of the two hemimethylated sister sites, generated by passage of the replication fork (Figure 5B). The fraction of hemimethylated (cut) DNA at each of the origin sites was significantly higher than at the external markers (Figure 5C). The values were 11 ± 3% and 56 ± 8% for oriI and oriII, respectively, while at the external markers they were 4 ± 0.8% and 8 ± 3%, respectively (Figure 5D). The results indicate that as in E. coli, the hemimethylation period is prolonged at the two V. cholerae origins but the duration of the period can be significantly different for the two.

From the E. coli paradigm, we expected that SeqA would be required to prolong the hemimethylation periods at both the origins [22]. To test for the requirement, a partial in-frame deletion of seqA was made where the deleted region was substituted with a zeocin drug-resistance cassette, maintaining the seqA reading frame (Figure S2A). The resulting gene was called ΔseqA, and the strain CVC1410. Replication run-out experiments indicated that initiation of one or both the chromosomes has become asynchronous (Figure S2B), and in this respect, V. cholerae appears to be similar to E. coli (Figure 2A and 2E) [22].

For the GATC site tested in oriI, the fraction of hemimethylated DNA increased from 11% in WT to 68% in ΔseqA (Figure 6A and 6C). Providing Dam or SeqA from a plasmid in the ΔseqA background decreased the fraction of hemimethylated DNA. The decrease by providing excess of Dam was expected because it converts hemimethylated DNA to fully methylated DNA. The increase in the absence of SeqA and decrease in its presence were unexpected, if SeqA were responsible for prolonging the period. The seqA plasmid did not change the period significantly in the WT background (Figure S3). The results indicate that it is the absence of SeqA that causes the increase of hemimethylated oriI DNA, a result opposite to that found for oriC [29]. The behavior of oriII was similar to that of oriC. The fraction of hemimethylated DNA decreased from 75% in WT to 17% in ΔseqA (Figure 6B and 6C). Thus seqA effects can be opposite in different origins at specific GATC sites. It remains to be seen whether the results are site-specific or true for the entire origins.

The opposite response of the GATC sites tested in oriI and oriII was also seen in a V. cholerae mutant where seqA was completely deleted (ΔseqA1, CVC2003; Figure S4). oriI also responded opposite to oriC in E. coli (Figure 7). While the percent of hemimethylated DNA at oriC dropped from 13% in MG1655 to 9% in MG1655 ΔoriC::oriI, the values at oriI increased from 9% in MG1655 ΔoriC::oriI to 25% in its ΔseqA1 derivative. These results suggest that the opposite behavior of oriI and oriII upon seqA deletion is intrinsic to the sequence context of the GATC sites tested in the two origins rather than the sequestration machinery of the two bacteria. Thus depending upon the context, SeqA can both shorten and prolong the hemimethylation period of a GATC site.

SeqA is required for once-per-cell-cycle initiation from both oriI and oriII

Although a role of SeqA in restraining replication initiation in V. cholerae was suggested by the flow cytometry results (Figure S2B), they did not allow us to distinguish whether one or both the
Chromosomes were affected. We used fluorescence microscopy to follow replication initiation of the two chromosomes individually. The numbers and positions of oriI and oriII were determined in WT and ΔseqA strains of V. cholerae by the GFP-P1ParB/parS system [30,31]. For oriI in WT, 94% of the cells had two to four foci and the rest one or three foci, indicating synchronous and once-per-cell-cycle initiation (Figure 8A and 8E). In contrast, only 45% of ΔseqA cells showed this pattern (Figure 8D and 8E). The remaining cells showed three to six foci. The significant increase in the number of cells with odd numbers of foci and more than four foci indicates that initiation is no longer synchronous and no longer limited to once per cell cycle in the absence of SeqA.

The regulation of chrII initiation was also affected. While 100% of the cells in the presence of SeqA showed one to two foci (Figure 8C and 8E), this was true for 83% of the ΔseqA cells (Figure 8D and 8E). The remaining cells showed three to six foci. SeqA thus contributes to synchronous and once-per-cell-cycle initiation of both the chromosomes.

Discussion

Here we have addressed the role of DNA adenine methylation in replication of the two V. cholerae chromosomes. In bacterial replication, adenine methylation can contribute by regulating gene

Figure 3. RctB binding to variously methylated oriII DNA. (A) A schematic showing the features of the oriII region. Two open reading frames, rctA and rctB (white arrows), border the region. The 11- and 12-mers (white or hatched arrowheads, respectively) are the putative RctB binding sites with GATC sequences (black dots). The origin also has a conserved sequence, 14-mer, and putative binding sites for DnaA (DnaA box) and IHF. (B) Electrophoretic mobility shift assay with fullymethylated DNA (Full), unmethylated DNA (None), the same DNA methylated in vitro by Dam (None + in vitro), hemimethylated DNA (Hemi) and the same DNA methylated in vitro by Dam (Hemi + in vitro). The Hemi DNA was methylated on the top strand. RctB amount was 0, 50 or 100 ng per 20 μl binding reaction. The free DNA band (black arrow head) refers to fragments not bound by RctB. The fraction of bound DNA was deduced from the loss of intensity of the free DNA band (Bound DNA = 1-free DNA). (C) Chromatin immunoprecipitation analysis of RctB binding in vivo. The precipitation was done with RctB antibody and the cells were either MG1655 (black bars) or its dam derivative (CVC1415, white bars), and each carried either the six-12mers (pGD61) or the empty vector (pRLM167). The histogram shows the average of three experiments. doi:10.1371/journal.pgen.1000939.g003
expression, by helping origin opening, and by regulating initiation so that it occurs only once per cell cycle. From the regulatory point of view, the major contribution of methylation is the marking of promoters/origins so that unreplicated DNA can be distinguished from the replicated ones. Newly replicated DNA is uniquely marked with hemimethylated sites that lend themselves to regulation in various ways. In E. coli, the newly replicated initiator (dnaE) promoter and the origin (oriC) are silenced (sequestered) by the SeqA protein, which prevents their reuse for a significant period of the cell cycle. In C. crescentus, the hemimethylated origin and the initiator promoter are also less active but the mechanisms remain unclear. In V. cholerae, we show that full methylation of oriII promotes initiator binding, providing a new role of the marks in replication initiation, and that SeqA is required for once-per-cell-cycle replication from both the origins (oriI and oriII), as in the case of oriC. By contributing to both initiation and its regulation, methylation thus serves two fundamental requirements for genome maintenance in V. cholerae. A comparison of oriII to plasmid origins also allowed us to address how a plasmid origin could have evolved to drive a chromosome in a cell-cycle specific fashion. We elaborate on these issues below.

Methylation and oriI

Our work started by questioning the essentiality of Dam and SeqA for functioning of oriI since a similar origin, oriC, can do without them [18]. We find that the requirements are not real for oriI but were imposed due to the use of plasmids to check the origin function. When we replaced oriC in the E. coli chromosome with oriI, making it the only origin in the cell, both the dam and seqA genes could be deleted (Figure 1). Thus for the functioning of oriI and oriC, methylation is not essential but it improves chromosomal replication initiation and its control (Figure 2A–2D), including the ability to tolerate extra copies of the origin in trans (Figure 1). In bacteria such as Bacillus subtilis that are naturally devoid of the methylation system, ori plasmids can exert an inhibitory effect (incompatibility) on chromosomal replication [32]. Methylation thus can help bacterial survival in a competitive situation.

Methylation and oriII

Dam plays a previously unrecognized role for oriII. It significantly promotes binding of the chrII-specific initiator, RctB, to the origin, thus possibly serving an essential function (Figure 3). Origin methylation is known to be essential for replication of C. crescentus chromosome, and of plasmids P1 and ColV-K30 in E. coli [33,34,35]. The reason is not clear in these cases, but unlikely to be for initiator binding. The initiator binding sites in these systems lack the sequences required for methylation. In contrast, RctB binding sites have an internal Dam recognition site, and methylation of the sites is required for initiator binding (Figure 3 and Figure S1). Thus, for oriII, the mechanism whereby methylation could be essential for its function and, therefore, for the bacterial survival is clear.

**Table 2. Plasmids.**

| Plasmids       | Description/Relevant characteristics                              | Reference or source |
|----------------|------------------------------------------------------------------|---------------------|
| pBAD24         | Cloning vector                                                   | [55]                |
| pDS132         | Suicide plasmid for allele exchange                               | [56]                |
| pEM7/Zeo       | Cloning vector                                                   | Invitrogen          |
| pET22b (+)     | Cloning vector                                                   | Novagen             |
| pGD55          | pBAD24 Flag-dam<sup>16mers</sup> = pdam                          | This study          |
| pGD57          | pRLM167::<sup>12mutant</sup>+11 mers                            | This study          |
| pGD58          | pRLM167::<sup>12+11mutant</sup> mers                             | This study          |
| pGD59          | pRLM167::<sup>12mutant</sup>+<sup>11mutant</sup> mers            | This study          |
| pGD61          | pRLM167::6 x 12mers (coordinates 788-934)                        | This study          |
| pGD63          | pBAD24 Flag-seqA<sup>16mers</sup> = pseqA                        | This study          |
| pGD69          | poriC (coordinates 4639498-1497)-bla                             | This study          |
| pGD70          | pSW4426T::SeqA<sup>16</sup>:zeo                                 | This study          |
| pGD79          | pSW23-oriI-zeo                                                   | This study          |
| pGD93          | pTS-P<sub>64</sub>dam (from pKOBEGA)                             | This study          |
| pGD114         | pEM7-1 seqA<sub>16</sub>:zeo                                    | This study          |
| pGD118         | pEM7-<sup>1</sup>dam:zeo                                         | This study          |
| pGD121         | pDS132-<sup>1</sup>:dam::zeo                                     | This study          |
| pGP704         | Cloning vector                                                   | [57]                |
| pKOBEGA        | Cloning vector; rep(ts)                                          | [58]                |
| pRKG256        | pGP704:oriI (coordinates 2955711-1848) = poriI                   | R. K. Ghosh         |
| pRLM167        | Vector for cloning into a transcription-free zone                 | R. McMacken         |
| pSW23         | Suicide vector                                                   | [54]                |
| pSW4426T       | pSW23T::aadA7-araC-P<sub>64</sub>ccdB                           | [48]                |
| pTVC11         | pSC101::ccB                                                     | [59]                |
| pTVC86         | pRLM167::3 x 11mers (coordinates 291-445)                        | T. Venkova-Canova   |
| pTVC88         | pRLM167::12 x 11mers (coordinates 549-718)                       | T. Venkova-Canova   |

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A. Growth condition

Permissive (P) vs. Restrictive (R) conditions for V. cholerae WT and Δdam strains.

B. OD$_{600}$nm vs. time (hours)

- Vc WT, P
- Vc Δdam, P
- Vc WT, R
- Vc Δdam, R

C. Cell viability and viable cells with plasmid

| Growth condition | Cell viability | Viable cells with plasmid |
|------------------|---------------|---------------------------|
|                  | P  | R  | P  | R  |
| Vc WT            | 1  | 0.60 | 0.28 | 3.2x10$^{-3}$ |
| Vc Δdam          | 0.80 | 2.0x10$^{-4}$ | 0.58 | 0.57 |

D. Ratio vs. growth condition

- orl/orll
- orl/terl
- orll/terll
- terl/terll
SeqA and once-per-cell-cycle replication of oriI and oriII

We show that seqA is not an essential gene in V. cholerae by obtaining viable seqA deletion mutants of V. cholerae. Although earlier studies suggested the gene to be essential, the finding that both oriI and oriII could function without SeqA in E. coli encouraged us to attempt isolation of the deletion mutants [18,20]. In a deletion mutant, the number of both oriI and oriII per cell was found to be greater than in the WT (Figure 6). The overreplication indicates a breakdown of once-per-cell-cycle replication and reveals that SeqA is a negative regulator of overreplication [18,28]. In a deletion mutant, the number of both oriI and oriII per cell was found to be greater than in the WT (Figure 6). The overreplication indicates a breakdown of once-per-cell-cycle replication and reveals that SeqA is a negative regulator of overreplication. The latter was also concluded when the role of SeqA was studied by SeqA overproduction [28]. There was also an increase in the number of cells with odd number of origins for both the chromosomes, indicating loss of initiation synchrony. Thus, SeqA appears to contribute to both once-per-cell-cycle replication and initiation synchrony.

Hemimethylation periods of oriIC, oriI, and oriII

An unexpected finding of this study is that the hemimethylation period of oriI and oriC changed in opposite ways upon seqA deletion: for oriC it decreased whereas for oriI it increased (Figure 6 and Figure 7). The decrease in the case of oriC is expected since SeqA is believed to be the key factor that prolongs the period [22]. A significant increase of the period without requiring SeqA shows that there are other ways to prolong the period, and that SeqA can play an opposite role of shortening the period. The opposite roles of SeqA were seen in isogenic strains of both V. cholerae and E. coli, suggesting that the reason cannot be due to species-specific factors (Figure 6 and Figure 7). The period also changed in opposite ways for oriI and oriII in the same seqA mutants of V. cholerae. SeqA thus has the capacity to both increase and decrease the duration of the period.

SeqA binding to DNA is favored in GATC-dense areas [36,37]. The density of GATC sites around the diagnostic GATC site has the capacity to both increase and decrease the duration of the period. Proteins other than SeqA that interact with origins can also explain the differences in the hemimethylated periods of the origins. DNAa is known to compete with SeqA for binding to some of the sites in oriC [38], and can significantly prolong the period even without SeqA [37]. Thus, DNAa is a likely candidate for prolonging the period for oriI in the absence of SeqA.

Upon seqA deletion, although the hemimethylation period changed oppositely for oriI and oriII, both the chromosomes over-replicated (Figure 8). The prolongation of the period thus may not always be diagnostic of the role of SeqA in the negative regulation of replication. As stated above, competition with DNAa for oriI binding could be another way for SeqA to exert its negative regulatory role [38]. The correlation of the prolongation of the period and the strength of negative regulation was also poor in the case of oriII. Although, the period reduced drastically in a seqA mutant, the corresponding relaxation of replication was modest (Figure 8). In oriII, the negative control is mediated primarily by limiting RctB, which apparently makes the contribution of sequestration to regulation less significant [19].

Plasmid versus chromosome replication

ChrII has many plasmid-like features including the organization of its origin. Plasmids generally initiate their replication randomly in the cell cycle and control it independently of the chromosome [16,20,31,39]. Plasmid copy number can vary among individual cells due to replication error and unequal segregation. To maintain the mean copy number, plasmids adjust for fluctuations in copy number by replicating more in cells that receive fewer copies than the mean, and replicating less in cells with more copies than the mean. Thus, once-per-cell-cycle replication is not suited for the maintenance of plasmid copy number. We show here that unlike plasmids, chrII replicates once per cell cycle, like other bacterial chromosomes. The high density of GATC sites of oriII is not typical for plasmid origins but is a conserved feature of all sequenced strains of the family Vibrionaceae [18]. It appears that the involvement of methylation has rendered functioning of a plasmid-like origin similar to that of a chromosomal origin.

Why does initiation need to be cell-cycle specific for the chromosome? Completion of cell division demands that the septum forming area be cleared of DNA [40]. Plasmids are generally small and have correspondingly short replication elongation periods. Incompletely replicated plasmids are unlikely to cause steric hindrance to cell division for a significant period, unlike incompletely replicated chromosomes [41]. If chrII were to initiate replication randomly in the cell cycle like the plasmids, late-initiating chrII would likely delay cell division and create heterogeneity in cell generation times. V. cholerae SeqA cells did form elongated cells, indicative of a cell division defect (our unpublished results). One reason for this could be steric hindrance to cell division from late-initiating chrII. We suggest that a chromosome replicating from an origin with a plasmid provenance is subject to selection pressure to make the initiation cell-cycle specific, and the acquisition of methylation sites could allow that.

Methylation and bacteria with multiple chromosomes

Understanding the role of methylation can also be important for another reason. It has been suggested that one of the common conspicuous features of the two origins being the high density of GATC sites, their methylation could be a mechanism to coordinate the replication between the two chromosomes [18]. Methylation is essential for the viability of bacteria with multiple chromosomes such as Rhizobium meliloti [42], Brucella abortus [43] and Agrobacterium tumefaciens [44] in addition to V. cholerae [17]. Although there is no evidence yet for direct communication among the chromosomes for replication initiation in any system, it is possible that in these bacteria methylation could be coordinating the replication to the cell cycle, as is does for V. cholerae and possibly other members of the family of Vibrionaceae.
Materials and Methods

Bacterial strains, plasmids, and media

Bacterial strains and plasmids used in this study are listed in Table 1 and Table 2, respectively. Primers are listed in Text S1. E. coli and V. cholerae were grown in LB (10 g tryptone +5 g yeast extract +5 g NaCl per liter, pH adjusted with NaOH to ~7) or M63 medium (KH2PO4 3 g + K2HPO4 5 g + K3HPO4 7 g + (NH4)2SO4 2 g + FeSO4 0.5 mg + MgSO4·7H2O 0.25 g, pH adjusted with KOH to ~7) supplemented with 2 mM MgSO4, 0.1 mM CaCl2, 0.01% thiamine and 0.2% glucose, and additionally 0.1% casamino acids when desired. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml for E. coli; 5 μg/ml for V. cholerae; erythromycin, 20 μg/ml; kanamycin, 25 μg/ml; spectinomycin, 50μg/ml; tetracycline, 15 μg/ml; and zeocin, 25 μg/ml. Diaminopimelic acid (DAP) was used at 0.8 mM, L-arabinose at 2 or 0.2 mg/ml, IPTG at 100 μM and thymidine at 0.3 mM.

Recombinering in E. coli

To replace oriC (coordinates 3923756–3924202) with oriI (coordinates 2961130–364), the latter was amplified from DNA of CVC209 by PCR using primers GD113 and GD114. The PCR product was digested with EcoRI and BamHI, and ligated to similarly digested pEM7-Zeo. The resulting plasmid, pGD63, was digested with SacI and BamHI, and the fragment containing the oriI-zeo region was ligated to a similarly digested vector, pSW23, generating pGD79. The oriI-zeo region of pGD79 was amplified with primers GD124 and GD125, and the product used to replace oriC of CVC1394 by the mini-Δ Red recombinering method [45]. The mini-Δ prophage was eliminated from the strain by a 30°C to 42°C temperature shift. The resultant strain was called MG1655ΔoriC::oriI-zeo (CVC1400), and the replacement was confirmed by sequencing of the origin region. The genomic DNA of the dam mutant derivative (CVC1401) was confirmed for the absence of adenine methylation by its resistance to DpnI but not to MboI and BstCI restriction enzymes (data not shown).

Flow cytometry

Cultures of E. coli were grown in LB to OD600=0.2 and processed for flow cytometry after replication run-out in the presence of rifampicin (150 μg/ml) and cephalexin (10 μg/ml) for three hours as described [46]. The peak fluorescence intensity of an overnight grown E. coli culture in M63 + 0.2% glucose medium (without casamino acids) was taken to represent one genome equivalent.

Electrophoretic mobility shift assay

A fragment with six 12-mers was obtained from pGD61 by digestion with Xhol and NotI. Fragments with three 11-mers and a pair of 12- and 11-mers were obtained from pTVC86 and pTVC88, respectively, by digestion with Xhol and BamHI. For methylated and unmethylated fragments, the plasmids were from a dam“ (BR2699) and a dam+ (CVC1060) strain, respectively. The fragments were gel-purified, dephosphorylated with shrimp alkaline phosphatase (USB Corporation), and end-labeled with 50 μCi [γ-32P]ATP (PerkinElmer) by using 30 units of T4 polynucleotide kinase (New England Biolabs) and purified through ProbeQuant G-50 micro columns (GE Healthcare). To obtain hemimethylated DNA, oligonucleotide primers, TVC64 and TVC138 (Sigma-Genosys), were end-labeled and purified as above. The labeled primers were then used for PCR one at a time with methylated DNA as template for one cycle to obtain two populations of hemimethylated DNA, one with methylation on the top strand and the other on the bottom strand. The binding reactions were essentially as described [47].

seqA deletion

A partial deletion of seqA was made by deleting codons 51 to 140 and substituting the deleted region with a zeocin cassette maintaining the seqA reading frame as follows. The seqA gene was amplified from CVC209 by PCR with primers GD87 and GD88. The product was digested with EcoRI and cloned in similarly digested vector, pSW426T. The resultant plasmid was used as template for PCR with primers GD91 and GD92 to amplify the 5’ end of seqA, the plasmid backbone and the 3’end of seqA. After digestion with MfeI, a site of which was present within GD91 and GD92 primers, the PCR product was ligated to the zeocin cassette. The cassette was obtained from pEM7-Zeo by PCR, using primers GD89 and GD90 and digested with EcoRI before ligation to the MfeI fragment. The resulting plasmid, pGD70, containing the ΔseqA::zeo allele was used to replace seqA of CVC209 by the allele-exchange method [48]. The resulting ΔseqA::zeo mutant (CVC1410) grew slower than the WT. In LB at 37°C, the doubling times of the mutant was 32±2 min as opposed to 19±2 min for the WT. The ΔseqA::zeo allele is called hereafter ΔseqA.

The entire seqA ORF was also deleted and substituted with the zeocin cassette as follows. First, a kilobase region located downstream the stop codon of seqA was amplified by PCR with primers GD228 and GD230, the product digested with EcoRI and BamHI and cloned in a derivative of pEM7-Zeo (pGD111), previously digested with the same enzymes, generating pGD113. pGD111 is essentially same as pEM7-Zeo except that the multi-cloning site upstream of zeo is modified to include KpnI and NdeI restriction sites. Next, a kilobase region located upstream of the start codon of seqA was amplified by PCR with primers GD230 and GD231, the product digested with KpnI and NdeI and cloned in pGD113, previously digested with the same enzymes, generating pGD114. The flanking regions of seqA, now flanking
the zeocin cassette, was amplified by PCR with primers GD257 and GD258 and the linear product was introduced by natural transformation in a \( \text{hapR}^+ \text{D} \text{dns} \) derivative of N16961 (CVC1121) essentially as described [49,50]. The transformants were selected for zeocin resistance and checked for the replacement of the seqA gene by the zeocin cassette by PCR and DNA sequencing. The resulting \( \text{DseqA}_{T}::\text{zeo} \) mutant (CVC2003) grew as slow as the \( \text{DseqA}_{P}::\text{zeo} \) mutant with a doubling time of 32±2 min. The \( \text{DseqA}_{T}::\text{zeo} \) allele is called hereafter \( \text{DseqA}_{T} \).

dam depletion

A complete deletion of the \( \text{dam} \) ORF and its substitution with a zeocin cassette was obtained by the allele-exchange method in the presence of a complementing plasmid, pGD93. The replication of the plasmid was thermo-sensitive and it carried the \( V. \text{cholerae} \) dam under the PBAD promoter. pGD93 was made as follows: the dam gene was amplified by PCR with primers GD72 and GD73, and the product after digestion with EcoRI and KpnI was cloned in pBAD24, previously digested with the same enzymes, generating pGD55. Next, the NdeI-HindIII fragment from pGD55 containing the dam gene was cloned in pKOBEGA, previously digested by NdeI and HindIII, generating the pGD93. For allele-exchange, a kilobase region located downstream of the stop codon of dam was amplified by PCR with primers GD261 and GD262, and the product after digestion with EcoRI and BamHI was cloned in a derivative of pEM7-zeo, previously digested with the same

Figure 6. Quantification of hemimethylated GATC sites in WT and \( \Delta \text{seqA}_P \) strains of \( V. \text{cholerae} \). Autoradiographs of Southern blots of chromosomes I (A) and II (B). (C) Quantification of band intensities from (A) and (B). The analysis was done in LB in WT (CVC209/pBAD24; black bars), and in a \( \Delta \text{seqA}_P \) mutant (CVC1410) either with pBAD24 (Vec, dark gray bars) or with a plasmid overexpressing \( V. \text{cholerae} \) dam (pdam = pGD65, light gray bars) or \( V. \text{cholerae} \) seqA (pseqA = pGD63, white bars). Other details are same as in Figure 5.

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enzymes, generating pGD117. A 700 bp region located upstream of the start codon of dam was amplified by PCR with primers GD263 and GD264, and the product after digestion with KpnI and NdeI was cloned in pGD117, previously digested with the same enzymes, generating pGD118. The zeocin cassette with the flanking regions of dam was amplified by PCR with primers GD268 and GD269, and the product cloned as a blunt end fragment in pSW23, previously digested with SmaI, generating the pGD120. The plasmid was digested with SacI and SalI, and the fragment with the zeocin cassette was cloned into pDS132, previously digested also with the same enzymes. The resulting plasmid, pGD121, was used to replace dam of CVC209/pGD93. The resulting strain, CVC2023, was confirmed for the replacement of dam by the zeocin cassette by PCR and by DNA sequencing. To deplete Dam, single colonies grown in the presence of ampicillin (to select pGD93) and arabinose (to express dam) were used to inoculate LB without any drug but containing glucose (to repress dam expression) and the cultures were grown at 42°C (to stop plasmid replication).

**Southern blotting**

Genomic DNA was isolated from cells of log phase cultures (OD600=0.3), using the Genelute Bacterial Genomic DNA kit (Sigma). For analyzing chrI and E. coli DNA, 1 μg of DNA was digested 2 hours with 7.5 or 15 units of HphI (New England Biolabs) at 37°C, and the products resolved in a 1.5% agarose gel. For chrII, the conditions were similar except that TaqI was used at 65°C. The origin probes were prepared by PCR using primers GD36 and GD37 for oriC, GD40 and GD41 for oriI, GD67 and GD68 for oriC, and GD150 and GD151 for DoriC::oriI. The primers for external markers on the three chromosomes were GD38 and GD39, GD42 and GD43, and GD128 and GD129.

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**Figure 7. Quantification of hemimethylated GATC sites in oriC and oriI in dam and seqA mutants of E. coli.** (A) Schematic maps of origin regions of E. coli chromosome and V. cholerae chromosome I. The regions are very similar except that the V. cholerae origin (oriI) has an extra DnaA box (R6). Other details are described in Figure 5A. The tau and I sites of the E. coli origin (oriC) that also bind DnaA are yet to be described in oriI. The two GATC sites studied here for their methylation states are shown by vertical arrows. (B) The WT either had oriC (MG1655) or oriI (MG1655::DoriC::oriI). The GATC sites probed were located either within the origins (oriC or oriI) or external to the origin (ext) at about 300 kb away. The same ext marker was used for both the strains. The numbers below the figure show the percent of hemimethylated DNA at the origins. N.A. stands for ‘not applicable’. In these lanes the DNA being from a dam mutant is unmethylated, and is all cleaved both at the origin and the external marker. An uncharacterized cross-reacting band appears in the dam mutants only (arrow head). doi:10.1371/journal.pgen.1000939.g007
Figure 1: Dam Methylation and Replication in V. cholerae

A and B: Vc WT and Vc ΔseqAp

C and D: orill

E: Number of cells (%)

Cell length (μm)

Distance from proximal pole (μm)

- Distal pole
- 1 Focus
- 2 Foci
- 3 Foci
- 4 Foci
- 5 Foci
- 6 Foci
- 7 Foci
- 8 Foci
- 9 Foci

oril WT  oril ΔseqAp  orill WT  orill ΔseqAp
respectively. The probes for ori and the external markers were made radioactive using the RediPrimeII random primer labeling kit (GE Healthcare) and [α-32P] dCTP (PerkingElmer) and mixed separately for the two chromosomes. The band intensities were recorded and quantified as described earlier [46].

Marker frequency determination
Marker frequency was determined by qPCR using a PTC-200 Peltier Thermal Cycler (MJ Research) and a LightCycler 480 SYBR Green I Master [Roche]. Genomic DNA was prepared from log phase cultures in LB with Genelute Bacterial Genomic DNA kit (Sigma), and 313 pg was used in each reaction as template. The primers were used at 0.3 μM each. They were proximal to either ori (GD156 and GD137) or oriII (GD156 and GD157) or terI (GD142 and GD143) or terII (GD140 and GD141) region of the two chromosomes, and were identical to those described [39]. The primer pairs were such that they produced ~100 to 150 bp fragments in all cases. Cp (crossing point) values were determined and used for calculating the ori/oriII, oriI/terI, oriII/terII and terI/terII ratios. The ratios were normalized to those of a culture grown to stationary phase in supplemented M63 medium (without casamino acids). Mean ratios were obtained from DNA prepared from three cultures, each grown from independent colonies, and each DNA was analyzed in triplicate.

Chromatin immunoprecipitation
The method was modified from the one described by Lin and Grossman [51]. Briefly, cultures at OD600nm = 0.3 were treated with 1% formaldehyde at room temperature for 30 min. After cell lysis and sonication, RctB complexes were precipitated with antibody against RctB (IP DNA) and Dynabeads-Protein G magnetic beads (Invitrogen), followed by stringent washings (see Text S1 for the detailed ChiP protocol). After reversal of the cross-links by incubation at 65°C overnight, the samples were treated by protease K (Sigma) and then purified with a PCR purification Kit (Qiagen). To quantify the enrichment of RctB binding sites in the template. The primers were used at 0.3 μM each. They were proximal to either ori (GD156 and GD137) or oriII (GD156 and GD157) or terI (GD142 and GD143) or terII (GD140 and GD141) region of the two chromosomes, and were identical to those described [39]. The primer pairs were such that they produced ~100 to 150 bp fragments in all cases. Cp (crossing point) values were determined and used for calculating the ori/oriII, oriI/terI, oriII/terII and terI/terII ratios. The ratios were normalized to those of a culture grown to stationary phase in supplemented M63 medium (without casamino acids). Mean ratios were obtained from DNA prepared from three cultures, each grown from independent colonies, and each DNA was analyzed in triplicate.

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Supporting Information

Figure S1 Requirement of adenine methylation for RctB binding to oriII. (A) The negative control locus of oriII showing the 11- and 12-mers (hatched or white arrowheads, respectively), which are the putative RctB binding sites with GATC sequences (black dots). (B, C) Autoradiographs of EMSA showing RctB binding to methylated or unmethylated DNA. In (B), the DNA fragments contained either the 12+11-mers or the 3x11-mers. In (C), a 170 bp fragment was used containing the 12+11-mer pair but no GATC sequences outside of these two sites. The fragment was also tested when either one or both of its two GATC sites were mutated to GATG. Note that when both the GATC sites were mutated, no retarded band could be seen whether or not the DNA was extracted from dam0 or dam+ strain. These results are consistent with methylation being important for efficient DNA binding of RctB in vitro.

Figure S2 (A) Western blot analysis of extracts from E. coli (Ec) and V. cholerae (Vc) cells with either an intact or deleted seqA gene. The blots were reacted with anti-SeqA Ec and anti-RctB antibodies. The latter antibody showed a cross reacting band (~70 kDa) in all cases that was used as a loading control. The cells used were MG1655 (Ec WT) and its isogenic SeqA10 derivative (BR1704), CV129 (Vc WT) and its isogenic SeqR derivative CV1410, and CV71121 (Vc WT) and its isogenic SeqA10 derivative (CV2003). The molecular weights in kDa of protein markers are shown on the left of the autoradiograph. The proteins interacting with the antibodies are named on the right. Note that in the SeqR strain, although the SeqA band is missing, a protein of higher molecular weight interacted with the antibody. This is a SeqA-Zeo fusion protein since we deleted the seqA gene partially, and the deleted region was substituted with a ZeocinR cassette in-frame. (B) Flow cytometric analysis of DNA contents in E. coli and V. cholerae. The cells used were as identified in (A) and analyzed when grown to log phase or after replication run out in the presence of drugs that inhibit replication initiation (rifampicin at 150 μg/ml for E. coli) or chloramphenicol at 200 μg/ml for V. cholerae and cell division (cephalexin at 10 μg/ml for both bacteria) (Srivastava et al, 2006. J Bacterial 188: 1060). The fluorescence intensity at the first E. coli peak after replication run-out was taken to represent four genome equivalents (Figure 2), and this value was used as a reference to scale the abscissa in all other cases, after accounting for the size difference between the two bacterial genomes. 100,000 cells were analyzed in each experiment.

Figure S3 Effect of Dam and SeqA overproduction on the fraction of hemimethylated DNA in V. cholerae. Hemimethylation states of GATC sites were probed both in chromosome I (A) and chromosome II (B), located either within the origin (oriI or oriII) or external to the origin (extI or extII) at about 300 kb away. Autoradiographs of Southern blots show sets of three lanes representing repeat experiments from independent cultures. (C) Quantification of band intensities from (A, B). The values represent the mean and standard deviations from the set of three lanes. From the E. coli paradigm, overexpression of dam was expected to decrease the percent of hemimethylated DNA, and it did for oriI (from 18 to 4%). The decrease was less for oriII (from 54 and 43%). The results of seqA overexpression were expected to be opposite to those of dam, but the increase in hemimethylated DNA was significant only at oriII (from 54 to 70%). At the external markers, the hemimethylated DNA remained low upon overexpression. Dam and SeqA thus seem to be involved in prolonging the origin hemimethylation period but they affect the two origins differently. For oriI, Dam appears to be limiting, not SeqA, and the

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results are opposite for oriII. The longer hemimethylation period and the relative insensitivity to Dam overproduction suggest that oriII is more efficiently quenched than oriR.

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**Figure S4** Comparison of the effects of a partial and a complete deletion of seqA (ΔseqA) and ΔseqAΔr, respectively) on the hemimethylation periods of specific GATC sites of the two V. cholerae chromosomes. The WT and ΔseqA strains were identical to those used in Figure S2A. Other details are as in Figure S3. In both the deletion strains, the hemimethylation period increased in the case of oriR and decreased in the case of oriII.

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**Text S1** Primers and ChIP protocol.

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