JOURNAL OF BACTERIOLOGY, Aug. 2010, p. 3903–3914 Vol. 192, No. 15
0021-9193/10/$12.00 doi:10.1128/JB.00311-10

Replication of *Vibrio cholerae* Chromosome I in *Escherichia coli*: Dependence on Dam Methylation[▽]

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Received 19 March 2010/Accepted 19 May 2010

We successfully substituted *Escherichia coli*'s origin of replication *oriC* with the origin region of *Vibrio cholerae* chromosome I ($oriCI_{Vc}$). Replication from $oriCI_{Vc}$ initiated at a similar or slightly reduced cell mass compared to that of normal *E. coli oriC*. With respect to sequestration-dependent synchrony of initiation and stimulation of initiation by the loss of Hda activity, replication initiation from *oriC* and $oriCI_{Vc}$ were similar. Since Hda is involved in the conversion of $DnaA^{ATP}$ (DnaA bound to ATP) to $DnaA^{ADP}$ (DnaA bound to ADP), this indicates that DnaA associated with ATP is limiting for *V. cholerae* chromosome I replication, which similar to what is observed for *E. coli*. No *hda* homologue has been identified in *V. cholerae* yet. In *V. cholerae*, *dam* is essential for viability, whereas in *E. coli*, *dam* mutants are viable. Replacement of *E. coli oriC* with $oriCI_{Vc}$ allowed us to specifically address the role of the Dam methyltransferase and SeqA in replication initiation from $oriCI_{Vc}$. We show that when *E. coli*'s origin of replication is substituted by $oriCI_{Vc}$, *dam*, but not *seqA*, becomes important for growth, arguing that Dam methylation exerts a critical function at the origin of replication itself. We propose that Dam methylation promotes DnaA-assisted successful duplex opening and replisome assembly at $oriCI_{Vc}$ in *E. coli*. In this model, methylation at $oriCI_{Vc}$ would ease DNA melting. This is supported by the fact that the requirement for *dam* can be alleviated by increasing negative supercoiling of the chromosome through oversupply of the DNA gyrase or loss of SeqA activity.

The genomes of *Vibrio cholerae* and several related *Vibrio* spp. are distributed between two circular chromosomes. Characterization of the origins of replication of *V. cholerae* chromosomes I and II ($oriCI_{Vc}$ and $oriCII_{Vc}$, respectively) has shown that $oriCI_{Vc}$ is similar to the origin of replication of the *Escherichia coli* chromosome, *oriC*, whereas $oriCII_{Vc}$ is completely different (20). Like *oriC*, $oriCI_{Vc}$ has five R-type DnaA boxes (53) as well as boxes conforming to the I and τ types (52, 61), and the DnaA protein is the rate-limiting factor in the initiation of replication in both cases (18). In *E. coli*, DnaA associates with both ATP and ADP, and the ATP-bound form is absolutely required for initiation to take place (reviewed in reference 60). When reaching a critical level, $DnaA^{ATP}$ (DnaA bound to ATP) protein is proposed to form a helical filament, anchored at one or more R-boxes (54, 69), in which origin DNA wraps around the outside of the DnaA core (21) or where the DnaA wraps around *oriC* (61). In both cases, the topology of the DnaA-*oriC* nucleoprotein complex leads to formation of compensatory negative supercoiling that facilitates unwinding of the adjacent AT-rich region resulting in initiation. In both models, $DnaA^{ATP}$ is absolutely required for initiation, and in agreement with this, $DnaA^{ATP}$ was found to be the rate-limiting factor for initiation *in vivo* (69).

The *V. cholerae* $oriCI_{Vc}$ also resembles *oriC* in having many potential sites for methylation by DNA adenine methyltransferase (Dam), although the number and position of the GATC sites differ slightly (see Fig. 1). The role of Dam in initiation of chromosome replication has been studied mainly in *E. coli*. After initiation of DNA replication has occurred on a fully methylated *oriC*, the newly replicated hemimethylated origins are sequestered from the Dam methyltransferase and from reinitiation for approximately one-third of a doubling time. During this time interval, the activity and amount of DnaA available for initiation are reduced to prevent immediate reinitiation (reviewed in references 57 and 83). The sequestration is carried out by the SeqA protein that binds hemimethylated *oriC* GATC sequences with high affinity (48). In the absence of Dam methylation or SeqA, the same origin can be reinitiated in the same cell cycle, and initiations become asynchronous (9, 48).

Genes encoding a Dam homologue and a SeqA homologue are present on *Vibrio* genomes, but there appear to be some differences between the functions of the proteins in *E. coli* and *V. cholerae*. *dam* has been found to be an essential gene in *V. cholerae* (33, 15), which is not the case in *E. coli* (48, 51). Conflicting data exist concerning the essentiality of *seqA* in *V. cholerae* (15, 72). The roles of Dam and SeqA in $oriCI_{Vc}$ replication have been studied using minichromosomes, i.e., plasmids replicating exclusively from a cloned copy of $oriCI_{Vc}$ (20). $oriCI_{Vc}$-based minichromosomes can replicate in wild-type *E. coli* cells but were unable to replicate in *dam*, *seqA*, and *seqA dam* mutants (20). The extrachromosomal existence of minichromosomes is dependent on their ability to initiate replication in synchrony with the chromosomal origin (46, 75). In *E. coli* cells mutated in *dam* or *seqA*, incompatibility exists between the *oriC* carried on minichromosomes and that of the chromosome due to origin competition (13), and when minichromosomes are maintained under selective pressure, they integrate into the origin region of the host chromosome (46, 75). Minichromosomes based on $oriCI_{Vc}$ may also compete with the *E. coli oriC* for initiations in *dam* or *seqA* mutant

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▽ Published ahead of print on 28 May 2010.

cells. However, due to limited sequence identity, they may not be able to integrate into the *E. coli* chromosome. This could provide an explanation for the failure to introduce $oriCI_{Vc}$ minichromosomes into *dam* and *seqA* mutant cells (20). Both *dam* and *seqA* genes could therefore be required for viability of *V. cholerae* for reasons not related to chromosome replication. In addition to its role in DNA replication, roles for Dam methylation in gene regulation and DNA repair have also been demonstrated in a number of bacteria (for reviews, see references 11, 45, 47, and 50). For *V. cholerae* as well as for *Salmonella* spp. and *Yersinia pseudotuberculosis*, Dam plays a role in virulence possibly through regulation of virulence gene expression (33). Less is known about the functions of *seqA* apart from its role in *E. coli* replication, but it has been suggested that SeqA functions as a nucleoid-organizing protein (for a review, see reference 83), and the *E. coli* chromosome has been demonstrated to have increased supercoiling in a *seqA* strain (85).

Here we describe the first *in vivo* evidence that Dam plays an important role in the initiation of replication by facilitating the replication initiation at $oriCI_{Vc}$ in *E. coli*. In addition, we show that SeqA does not carry an essential role in the initiation of replication.

MATERIALS AND METHODS

Growth conditions. Cells were grown in LB medium (8) or AB minimal medium (12) supplemented with 10 μg/ml thiamine and with either 0.2% glucose (Glu) or 0.2% glycerol as a carbon source. Also, 0.5% Casamino Acids (CAA) was added when fast growth conditions were required. Pictures of agarose plates were taken with a Canon digital camera (IXUS 870IS). Some changes to the brightness, contrast, and intensity of photos of agarose plates and microscopy images were made by using CorelDraw v. 9 (Corel Corp.). Antibiotics were used at the following concentrations: tetracycline (10 μg/ml), ampicillin (100 μg/ml), chloramphenicol (10 μg/ml), kanamycin (50 μg/ml), and streptomycin (50 μg/ml).

Bacterial strains and plasmids. All bacterial strains and plasmids used in this study are listed in Table 1. The pOriCImini plasmid has the 256-bp *V. cholerae* strain Bah-2 region corresponding to the *E. coli* minimal origin (see Fig. 1) cloned into pSW29TsacB. Plasmid pOriCI has the entire region between the *mioC* and *gidA* genes and a small part of the *mioC* and *gidA* genes from *V. cholerae* Bah-2, i.e., 555 bp cloned into pSW29TsacB. For construction of pOriCI and pOriCImini, the *oriCI* region equipped with NotI and SacI restriction sites were PCR amplified from *V. cholerae* Bah-2 (64) using the primers AAAGGC CAGAGCTCATTAAATATATATAAAGATCTATATAGAGATCTTTTTA TTAG and TAGGAAAAAAGCGGCCGCTGTGGATAACTATACGATTA TCCG for pOriCImini and primers AAAGGCCAGAGCTCTCATCAATCGC TTCTAAATAATGACC and TAGGAAAAAAGCGGCCGCGGCTTCGTTC CTGCGTACCG for pOriCI. The NotI/SacI-digested PCR products were cloned into NotI/SacI-digested pSW29TsacB (16), giving pOriCImini and pOriCI. Compared to the published sequence for *V. cholerae* El Tor N16961 (28), one sequenced pOriCI clone (pOriCI-1) had a C→T base shift in a GATC site 11 bp before the start of the minimal *oriCI* region as defined in Fig. 1. This mutation was not found in another pOriCI clone, pOriCI-2, or in the *V. cholerae* El Tor derivative Bah-2 (B. Koch and A. Løbner-Olesen, unpublished observations).

For replacement of *E. coli oriC* with *oriCI* from *Vibrio cholerae*, a ΩSm^r cassette was cut out from pHRP315 (62) with BamHI and cloned into the BamHI site of pOriCI-1, giving pOriCISmB, or cloned into the BamHI site of pOriCI-2, giving pOriCISmF. Recombinational replacement of *E. coli oriC* with *oriCI* from *Vibrio cholerae* ($oriCI_{Vc}$) was achieved by using a PCR-based method (14). Using pOriCISmB or pOriCISmF as a template, the ΩSm cassette in combination with $oriCI_{Vc}$ (product A) or only the ΩSm cassette (product B) was PCR amplified with primer gidA-Sm (**GATTGAAGCCCGGGCCGTGGATTCTACTCAACTTTG TCGGCTTGAGAAAG**TGATATCGAATTCCTGCAGC and primer for product A (**AAGATCCGGCAGAAGAATGGCTGGGATCGT GGGTTAATTTACTCAAA TAAATAA**TGACCTATTCCATGCAG) or primer gidA-Sm in combination with primer for product B (**TCACAATAGAACAGATCTCTAAATAAATAGATCTTCT TTTTAATACCCAGGATCCCAGGTC**GCTCTAGAACTAGTGGATC). The parts of the primers homologous to sequences in the *oriC* region in *E. coli* are shown in boldface type. The PCR products were introduced into *E. coli* BW25113 *rnhA*(pKD46) or *E. coli* BW25113(pKD46) by electroporation. Streptomycin-resistant recombinants were analyzed by PCR using primers recognizing genes flanking *oriC*, i.e., *gidA* (primer gidA-conf [CACGGCCACCGCTGTAA TTAT]) and *mioC* (primer mioC-conf [ATCCCATACTTTTCCACAGG]) in combination with primers recognizing the streptomycin resistance cassette (primer Sm-conf [GAAGAAGATCGCTTGGCCTC]) and $oriCI_{Vc}$ (primer oriCI-conf [GTGATAAAGCATGAACGACCT]). Sequence analyses of three mutants that passed the PCR test showed that in two mutants, *E. coli oriC* was replaced by $oriCI_{Vc}$ and the $oriCI_{Vc}$ sequence matched the corresponding sequence from *V. cholerae* N16961 (28). The third mutant carried two mutations compared to the sequences for *V. cholerae* N16961 (28), the C→T base shift in a GATC site 11 bp before the start of the minimal *oriCI* region originating from pOriCISmB and a T→C base shift near R1 (see Fig. 1) at a nonconserved position (87). This mutant and derivatives of this mutant will be referred to as $oriCI_{Vc}$*, while the strain carrying $oriCI_{Vc}$ without point mutations will be referred to as $oriCI_{Vc}$. $oriCI_{Vc}$::Sm^r, $oriCI_{Vc}$*::Sm^r, and ΩSm^r were transferred to *E. coli* MG1655 (24) and combined with various mutations by P1 phage-mediated transduction (55).

PCR verification of the *E. coli* MG1655 *dam16*::Km^r P1 transductants were carried with the primer pair dam-forward (CGCTTTTTTGAAGTGGGCAG) and dam-reverse (TTTCGCGGGTGAAACGAC) and primer pair dam-forward/kan1 (AATTGCAGTTTCATTTGATGCTC) and dam-reverse/kan2 (GA GCAAGACGTTTCCCGTTG). PCR verification of the *hda*::*cat* mutation was done as described previously (69).

Genomic blot. To determine the copy numbers of pOriCI-1, pOriCI-2, and pOriCImini, cellular DNAs were isolated from cells growing exponentially at 37°C in AB minimal medium supplemented with glucose, Casamino Acids, and kanamycin. The DNA was double digested with BamHI and XhoI. A ^{33}P-labeled probe recognizing the chromosomal *terC* region located on a 1.6-kb fragment was made essentially as described previously (56). Similarly, a probe specific for the Tn*5*-derived kanamycin resistance gene on the minichromosome was made using the primers k-m1 (GCGATACCGTAAAGCACGAG) and k-m2 (GGCTATTC GGCTATGACTGG). Hybridization was carried out as previously described (46).

Dam depletion. The cells were grown at 42°C for at least 6 generations in AB minimal medium supplemented with glucose and Casamino Acids containing tetracycline to select for plasmid pKG339 and in AB minimal medium supplemented with glucose and Casamino Acids containing ampicillin to select for plasmid pALO160, before the cultures were diluted in the same medium supplemented with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) but without ampicillin. To maintain the cells in exponential growth, the cultures were diluted in fresh prewarmed medium, and samples were taken at appropriate time intervals for flow cytometry.

Changing the intracellular gyrase activity. In order to study the effects of variations in the cellular gyrase level, we used derivatives of *E. coli* strain PJ4240, in which gyrase expression is IPTG dependent (31). To obtain a streptomycin-sensitive derivative of strain PJ4240, *rpsL*$^+$ was P1 transduced from *E. coli* strain CAG18456 *zhe-3084*::Tn*10* to PJ4240, resulting in strain ALO3470. A *seqA*Δ10 deletion derivative of strain ALO3470 (strain ALO3509) was obtained by the method described in reference 77, and the presence of the *seqA*Δ10 deletion was confirmed by PCR using the primer pair ybf (TTTACCAGATCGCGAGCCAG)/pgm (CCCTGCTTCTGGTTTCAGTAC). $oriCI_{Vc}$, $oriCI_{Vc}$*, and ΩSm derivatives of strains ALO3470 and ALO3509 were obtained by P1 transduction.

Sequence analysis. The WEB-THERMODYN sequence analysis software for profiling DNA helical stability (30) was used to analyze the helical stability of the *E. coli oriC* region and the *V. cholerae* $oriCI_{Vc}$ region.

Flow cytometry. Flow cytometry was performed as described previously (44) using an Apogee A10 flow cytometer.

RESULTS

Structure of *V. cholerae oriCI*. A 447-bp DNA fragment situated between the *mioC* and *gidA* genes on *Vibrio cholerae* chromosome I can function as an autonomous replicating sequence (ARS) in both *V. cholerae* and surrogate host *E. coli* (20). By sequence alignment, a 256-bp region corresponding to the 257-bp minimal *E. coli* origin (5) can be identified within the 447-bp DNA fragment in *V. cholerae* (Fig. 1). To examine whether this 256-bp region could also replicate in *E. coli*, it was

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or characteristic(s)[a]	Reference or source
Vibrio cholerae Bah-2	Derivative of El Tor strain E7946	64
E. coli strains		
ALO863	LJ24 *damX*::mini-Tn*10*; Tc^r	42
ALO1639	MG1655 *seqA*Δ10	78
ALO1917	MG1655 *hda*::Cm^r	69
ALO2031	MG1655 *dam16*::Km^r	63
ALO3150	MG1655 *oriC* replaced by *oriCI*$_{Vc}$*; Sm^r	This study
ALO3152	MG1655 *seqA*Δ10 *oriC* replaced by *oriCI*$_{Vc}$*; Sm^r	This study
ALO3189	MG1655 *oriC* replaced by *oriCI*$_{Vc}$*; *hda*::Cm^r; Sm^r	This study
ALO3199	MG1655 *damX*::mini-Tn*10*; Tc^r	This study
ALO3203	MG1655 *oriC* replaced by *oriCI*$_{Vc}$*; *damX*::mini-Tn*10*; Tc^r Sm^r	This study
ALO3270	MG1655 I2 G3→T I3 G3→T	68
ALO3470	MC1000 P$_{A1lacO1}$ *gyrA gyrB zhe-3084*::Tn*10*; Tc^r	This study
ALO3481	MC1000 P$_{A1lacO1}$ *gyrA gyrB zhe-3084*::Tn*10*; *oriC* replaced by *oriCI*$_{Vc}$*	This study
ALO3509	MC1000 P$_{A1lacO1}$ *gyrA gyrB zhe-3084*::Tn*10 seqA*Δ10	This study
ALO3521	MC1000 P$_{A1lacO1}$ *gyrA gyrB zhe-3084*::Tn*10*; *seqA*Δ10 *oriC* replaced by *oriCI*$_{Vc}$*	This study
ALO3689	MG1655 *dam13*::Tn*9 argE86*::Tn*10*; Tc^r Cm^r	This study
ALO3699	MG1655 ΩSm cassette inserted between *gidA* and *oriC*; Sm^r	This study
ALO3702	MG1655 *oriC* replaced by *oriCI*$_{Vc}$; Sm^r	This study
ALO3705	MC1000 P$_{lacO1}$ *gyrA gyrB zhe-3084*::Tn*10*ΩSm; Sm^r Tc^r	This study
ALO3733	MC1000 P$_{lacO1}$ *gyrA gyrB zhe-3084*::Tn*10 seqA*Δ10ΩSm; Sm^r Tc^r	This study
ALO3736	MG1655 *oriC* replaced by *oriCI*$_{Vc}$*; *damX*::mini-Tn*10*; Tc^r Sm^r	This study
ALO3745	MG1655 *seqA*Δ10 *oriC* replaced by *oriCI*$_{Vc}$; Sm^r	This study
ALO3749	MG1655 ΩSm cassette inserted between *gidA* and *oriC*; *hda*::Cm^r; Sm^r	This study
ALO3751	MG1655 *oriC* replaced by *oriCI*$_{Vc}$; *hda*::Cm^r; Sm^r	This study
ALO3753	MG1655 *seqA*Δ10ΩSm cassette inserted between *gidA* and *oriC*; Sm^r	This study
ALO3756	MC1000 P$_{lacO1}$ *gyrA gyrB zhe-3084*::Tn*10*; *oriC* replaced by *oriCI*$_{Vc}$	This study
BW25113	Δ*araBAD hsdR*	14
CAG18456	MG1655 *zhe-3084*::Tn*10*; Tc^r	Martin Martinus
CAG 12185	MG1655 *argE86*::Tn*10*; Tc^r	Martin Martinus
GM2927	*dam13*::Tn*9*; Cm^r	49
DH5αλpir	Pir$^+$	Rasmus Bugge Jensen
EH3827	Δ*dnaA zia*::pKN500	25
PJ4240	MC1000 P$_{A1lacO1}$ *gyrA gyrB*	31
MG1655	Wild type	24
SS211	MG1655 pBIP- *seqA*Δ10 cointegrant	Kirsten Skarstad
WM2482	MG1655	84
WM2759	MG1655 *oriC160 asnA*::*km*; Km^r	84
WM2762	MG1655 *oriC13 asnA*::*km*; Km^r	84
WM2764	MG1655 *oriC15 asnA*::*km*; Km^r	84
WM2844	MG1655 *oriC17 asnA*::*km*; Km^r	84
WM28 45	MG1655 *oriC162 asnA*::*km*; Km^r	84
XL1-Blue	Tc^r	Stratagene
Plasmids		
pFHC539	DnaA (wild type); Ap^r	27
pRUC672	DnaAK178T; Ap^r	76
pLR40	pBR322 derivative with *E. coli dnaA* exclusively under *lac* promoter control	69
pHRP315	pUCBM20 with ΩSm^r/Sp^r cassette; Ap^r Sm^r	62
pSW29T	R6K replicon-based cloning vector; Km^r	16
pSW29TsacB	pSW29T with *sacB* from *Bacillus subtilis* cloned in BglII site; Km^r	Ole Skovgaard
pKD46	P$_{araB}$ γ β *exo*; Ap^r	14
pOriCImini	pSW29TsacB with a 256-bp o*riCI* region from *V. cholerae* Bah-2	This study
pOriCI-1	pSW29TsacB with a 555-bp *oriCI* region from *V. cholerae* Bah-2 with C→T point mutation	This study
pOriCI-2	pSW29TsacB with a 555-bp *oriCI* region from *V. cholerae* Bah-2	This study
pOriCISmB	pOriCI-1 with ΩSm^r/Sp^r cassette from pHRP315; Km^r Sm^r	This study
pOriCISmF	poriCI-2 with ΩSm^r/Sp^r cassette from pHRP315; Km^r Sm^r	This study
pKG339	P$_{lac}$::*copA*; Tc^r Ap^r	32
pALO160	Rep$_A$, contains a BglII fragment carrying the *E. coli dam* operon; Ap^r	42
pRUC1443	pBR322 derivative with *V. cholerae dnaA* under P$_{A10403}$ promoter control	Ole Skovgaard

[a] Km^r, kanamycin resistance; Ap^r, ampicillin resistance; Sm^r, streptomycin resistance; Tc^r, tetracyclin resistance; Cm^r, chlorampenicol resistance.

cloned into the *oriV*$_{R6Kγ}$-based plasmid pSW29TsacB, giving pOriCImini. Replication from *oriV*$_{R6Kγ}$ depends on the *pir*-encoded protein. pOriCImini was able to replicate as a minichromosome in a Pir-deficient *E. coli* strain (strain XL1-Blue), demonstrating the ARS activity of the cloned fragment. We also cloned a longer 555-bp region covering the intergenic region between the *gidA* and *mioC* genes into pSW29TsacB, giving pOriCI. Compared to the published sequence for *V.*

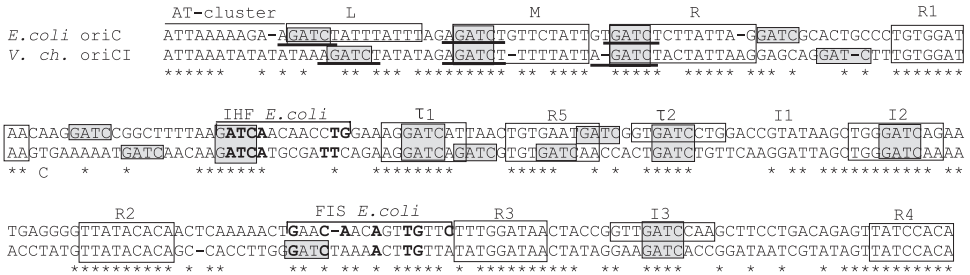

FIG. 1. Alignment of the *E. coli* minimal *oriC* with the corresponding region from *V. cholerae* chromosome I. The AT-rich sequence and the three 13-mer repeats L, M, and R found in *E. coli* (5) are indicated above the alignment. The 6-mer (A/T)GATCT boxes (80) are underlined. Other DnaA binding sites, i.e., R-boxes (53), I-boxes (52), and τ-boxes (61), are shown as boxed regions. Dam methylation sites (GATC) are shaded gray. The experimentally defined binding sites for integration host factor (IHF) (22) and factor for inversion stimulation (FIS) (65) in *E. coli* are indicated, and bases that match the consensus sequence are in boldface type. The single base difference between $oriCI_{Vc}$ and $oriCI_{Vc}$* (see Materials and Methods) in the minimal origin region is shown below the two sequences. A gap introduced to maximize alignment of the two sequences is indicated by a dash in the sequence. Nucleotides that are identical in the two sequences are indicated by an asterisk below the two sequences.

cholerae El Tor N16961 (28) and to the *V. cholerae* Bah-2 chromosomal sequence, one sequenced pOriCI clone (pOriCI-1) had a C→T base shift in a GATC site 11 bp before the start of the minimal *oriCI* region as defined in Fig. 1 in a nonconserved region (71). This mutation was not found in another pOriCI clone pOriCI-2 or in *V. cholerae* El Tor derivative Bah-2 (64). A genomic blot was performed to compare the minichromosome copy numbers for pOriCI-1 (4.4 kb), pOriCI-2 (4.4 kb), and pOriCImini (4.1 kb) (Fig. 2) (see Materials and Methods). The genomic blot was hybridized with two probes, one recognizing the *terC* region on the chromosome (1.6 kb) and one recognizing the kanamycin resistance gene on the linearized minichromosomes. No major difference in copy number for the three minichromosomes was observed (Fig. 2).

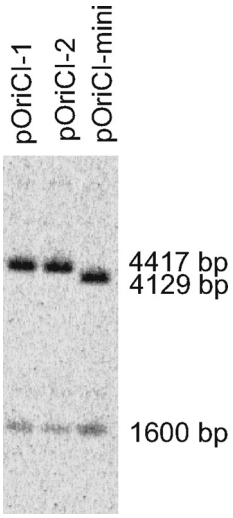

FIG. 2. Copy number of *oriCI*-based minichromosomes. Total DNA was isolated from *E. coli* strain XL1-Blue containing the indicated minichromosomes. Cells were grown exponentially at 37°C in AB minimal medium supplemented with glucose and Casamino Acids and containing 50 μg/ml of kanamycin. Individual DNA samples were digested with BamHI plus XhoI before Southern blot hybridization was performed. Two probes were used simultaneously; the two probes were homologous to sequences specific for *terC* on the chromosome and the kanamycin resistance gene carried by the minichromosomes.

The *E. coli* replication origin can be replaced with $oriCI_{Vc}$. The *oriC* region of the *E. coli* chromosome was replaced by $oriCI_{Vc}$ by lambda *red*-mediated homologous recombination (14) as described in Materials and Methods. Data from *E. coli* indicate that origin activity is stimulated by transcription from the *gidA* promoter located immediately adjacent to *oriC* and transcribing away from *oriC* (5, 59), probably due to increased negative supercoiling behind transcribing RNA polymerases (40).The constructed strain has *aadA* inserted between $oriCI_{Vc}$ and the *gidA* promoter (Fig. 3C), i.e., the native *E. coli gidA* promoter is moved about 2 kb away from the origin. We were uncertain whether the promoter would still be able to exert its stimulatory effect on $oriCI_{Vc}$ at this new location, since it was previously shown that the presence of a GC-rich element between the stimulatory D-loop and the origin can be alleviated if a GC-rich element is present in the intervening sequence (73). Therefore, we decided to include the native *V. cholerae gidA* promoter on the replacement cassette. The *aadA* promoter directs transcription away from $oriCI_{Vc}$ and is situated more than 500 bp away from the *V. cholerae gidA* ($gidA_{Vc}$) promoter (66). Whereas the downstream *aadA* gene may very

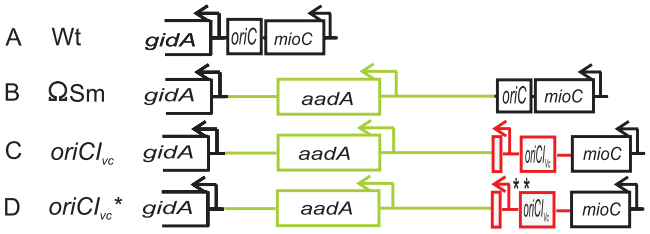

FIG. 3. Structure of genes in the origin regions of the strains used in this study. (A) *E. coli* wild type (Wt). (B) The *aadA* gene (GenBank accession no. AAC33912) encoding streptomycin adenylyltransferase flanked by transcription and translation termination signals (the ΩSm cassette [66]) is inserted upstream of the *E. coli gidA* promoter as defined in reference 37. (C) The *E. coli oriC* was replaced by the $oriCI_{Vc}$ region comprising the intergenic region between the *V. cholerae mioC* and *gidA* gene homologues and the sequence encoding the first 20 amino acids of the *V. cholerae gidA* gene homologue in combination with the ΩSm cassette. (D) Same as panel C but with two point mutations. The precise locations of the point mutations are described in the text.

TABLE 2. Growth rate, cell mass, and origin content of wild-type and mutant strains

Medium supplement[a]	Strain[b]	Doubling time (min)	No. of origins/cell[c]	Cell mass (LS)[d]	Mass/origin ratio[e]
Glycerol	wt	84	2.3	1.0	1.0
	ΩSm	84	2.4	1.0	1.0
	*oriCI**	84	2.5	1.1	1.0
	oriCI	95	2.2	0.7	0.7
Glucose	wt	57	3.2	1.7	1.2
	ΩSm	57	3.3	1.8	1.2
	*oriCI**	57	3.2	1.8	1.3
	oriCI	70	2.6	1.2	1.1
Glucose + Casamino Acids	wt	36	4.7	2.3	1.1
	ΩSm	36	4.7	2.4	1.2
	*oriCI**	36	4.7	2.3	1.1
	oriCI	40	5.1	2.3	1.0

[a] The cells were grown in minimal medium supplemented with glycerol, glucose, or glucose plus Casamino Acids.
[b] Four strains, i.e., *E. coli* MG1655 (wild type [wt]), MG1655 $oriCI_{Vc}$ ($oriCI_{Vc}$), MG1655 $oriCI_{Vc}$* ($oriCI_{Vc}$*), and MG1655 ΩSm (ΩSm) strains, were analyzed.
[c] Based on flow cytometric analysis of rifampin- and cephalexin-treated cells.
[d] Based on flow cytometric analysis of exponentially growing cells. The cell mass of wt cells growing in medium supplemented with glycerol is set at 1.0. LS, light scatter.
[e] The ratio of cell mass to the number of origins per cell for wt cells growing in minimal medium supplemented with glycerol is set at 1.0.

well be affected by transcription from the $gidA_{Vc}$ promoter (4), the opposite should not be the case. To look for possible effects of the insertion of the resistance cassette, we constructed a control strain that retained the *E. coli oriC* but had the ΩSm cassette inserted (Fig. 3B). Following replacement, $oriCI_{Vc}$ was amplified from the *E. coli* chromosome by PCR, and the nucleotide sequence was determined. One mutant strain had a $oriCI_{Vc}$ sequence identical to the *V. cholerae* Bah-2 chromosomal sequence and to the published sequence for *V. cholerae* El Tor N16961 (28). A second mutant strain carried two mutations, the described C→T base shift in a GATC site

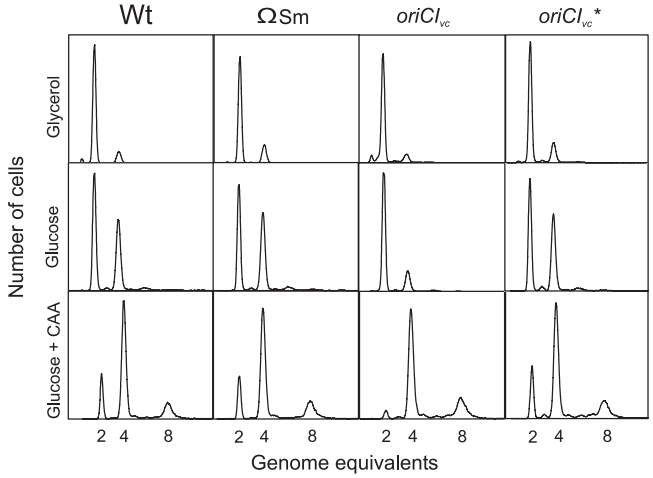

FIG. 4. Cell cycle parameters for wild-type (Wt) (*E. coli* MG1655), ΩSm, $oriCI_{Vc}$, and $oriCI_{Vc}$* strains. Wt, ΩSm, $oriCI_{Vc}$, and $oriCI_{Vc}$* cells were grown at 37°C in minimal medium supplemented with glycerol, glucose, or glucose plus Casamino Acids (CAA). Cells were treated with rifampin and cephalexin (Materials and Methods) prior to flow cytometric analysis.

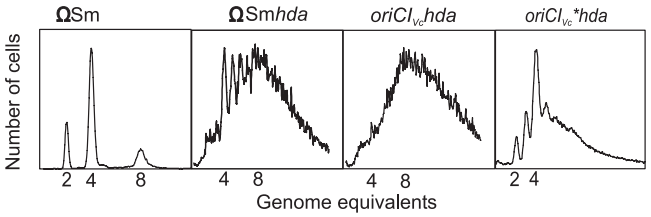

FIG. 5. Loss of Hda activity stimulates replication initiation from $oriCI_{Vc}$. ΩSm cells and *hda*::*cat* derivatives of ΩSm, $oriCI_{Vc}$, and $oriCI_{Vc}$* cells were grown at 37°C in minimal medium supplemented with glucose (Glu) plus Casamino Acids (CAA). The cells were treated with rifampin and cephalexin (Materials and Methods) prior to flow cytometric analysis.

11 bp before the start of the minimal *oriCI* region and a T→C base shift at a nonconserved position (87) immediately to the right of DnaA box R1 (Fig. 1). The $oriCI_{Vc}$ region with these two mutations will be referred to as $oriCI_{Vc}$*, while the $oriCI_{Vc}$ region without mutations will be referred to as $oriCI_{Vc}$ (Fig. 3). The $oriCI_{Vc}$, $oriCI_{Vc}$*, and the ΩSm cassette were transduced into a wild-type *E. coli* strain (MG1655) with equally high frequency (not shown), demonstrating that the *V. cholerae oriCI* is capable of directing replication of the *E. coli* chromosome. Most analyses were carried with all four strains, i.e., MG1655 (referred to as the wild type), MG1655 $oriCI_{Vc}$ (referred to as $oriCI_{Vc}$), MG1655 $oriCI_{Vc}$* (referred to as $oriCI_{Vc}$*), and MG1655 ΩSm (referred to as ΩSm) strains (Fig. 3).

When the wild-type, ΩSm, and $oriCI_{Vc}$* strains were grown in minimal medium supplemented with either glycerol, glucose, or glucose and Casamino Acids, the cells grew with similar doubling times (Table 2). In contrast, the doubling time of $oriCI_{Vc}$ cells were 10 to 20% longer in all three media (Table 2). When analyzed by flow cytometry, all four strains contained mainly two and four or four and eight origins of replication, depending on the culture doubling time, indicating that the origins were initiated in synchrony (Fig. 4) (74).

The sizes and origin content for ΩSm and $oriCI_{Vc}$* strains were similar to wild-type cells in all three media. On the other hand, $oriCI_{Vc}$ cells were smaller than wild-type cells in the two slow growing cultures (Table 2). The $oriCI_{Vc}$ cells also had a reduced origin content at slow growth, but not to the same extent, and the average cell mass/origin was lower for $oriCI_{Vc}$ cells relative to wild-type cells. This indicates that the initiation mass (17) was reduced during slow growth (Table 2) and that $oriCI_{Vc}$ is slightly more efficient than *oriC.* One or both of the point mutations in $oriCI_{Vc}$* influence it in such a way that it becomes similar to *oriC* with respect to growth and initiation parameters (Table 2). Insertion of an ΩSm cassette between *gidA* and *oriC* (Fig. 3) did not influence growth or initiation of replication (Table 2).

Initiation from $oriCI_{Vc}$ is stimulated by the loss of Hda activity. *V. cholerae* does not contain an *hda* homologue (18). Since Hda is essential for RIDA (regulatory inactivation of DnaA), a process described for *E. coli* where active DnaA protein associated with ATP is converted to the inactive ADP-bound form (34), it was unclear whether replication initiation from $oriCI_{Vc}$ is affected by the nucleotide-bound form of DnaA. Deletion of *hda* (69) from $oriCI_{Vc}$ cells led to asyn-

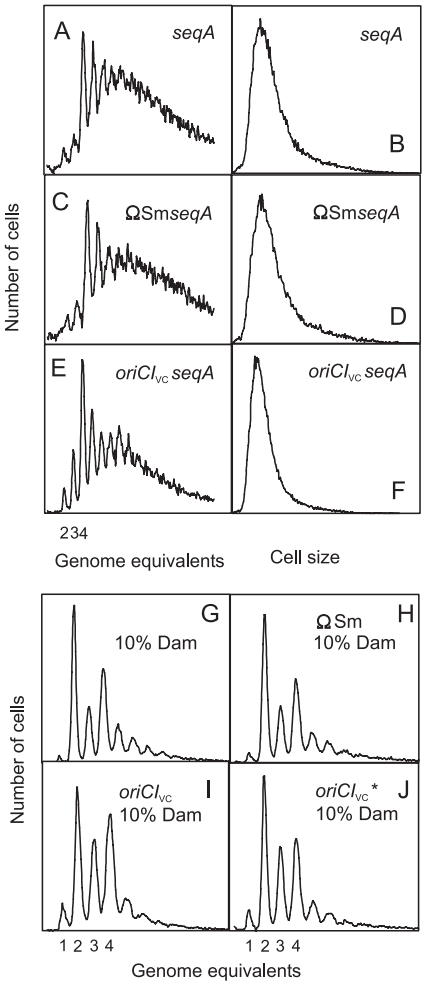

FIG. 6. Synchronous replication initiation from $oriCI_{Vc}$ is dependent on *seqA* and *dam* gene products. Cells were grown at 37°C in minimal medium supplemented with Glu plus CAA. Panels A, C, E, G, H, I, and J show cells treated with rifampin and cephalexin prior to flow cytometric analysis, whereas the cells in panels B, D, and F were in the exponential growth phase. *seqA* (A and B), ΩSm *seqA* (C and D), $oriCI_{Vc}$ *seqA* (E and F), *damX*::mini-Tn*10* (G), ΩSm *damX*::mini-Tn*10* (H), $oriCI_{Vc}$*damX*::mini-Tn*10* (I), and $oriCI_{Vc}$* *damX*::mini-Tn*10* (J) strains were studied. Insertion of mini-Tn*10* in *damX* reduces the transcription of the *dam* gene to approximately 10% of the level in wild-type cells (42).

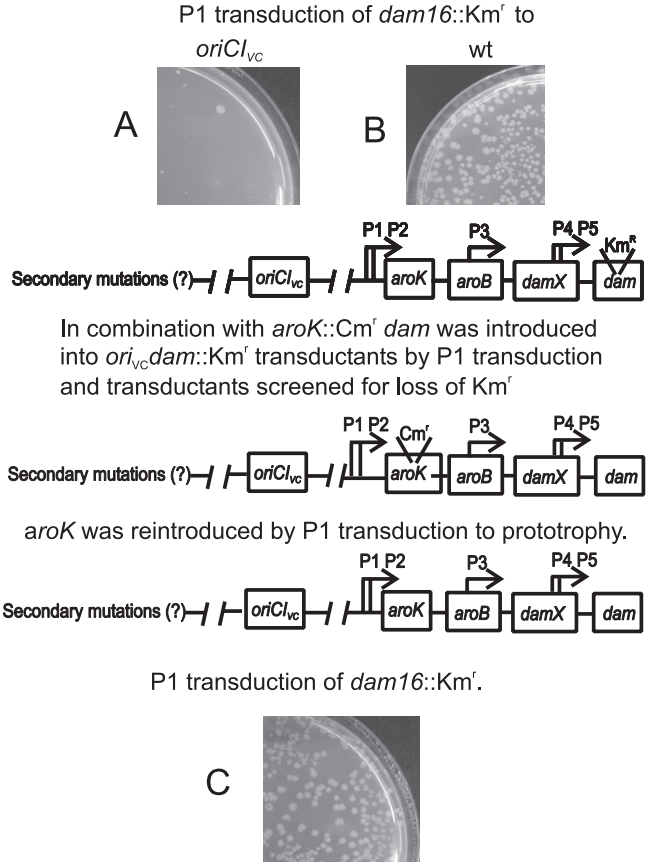

FIG. 7. Dam-deficient $oriCI_{Vc}$* cells contain suppressor mutations. The *dam16*::Km^r allele was P1 transduced into $oriCI_{Vc}$* (A) and wild-type (wt) (B) cells. Four independent $oriCI_{Vc}$* *dam* cells (from panel A) were transduced back to Dam$^+$ by a two-step procedure (details in Materials and Methods) (46). By this procedure, putative *dam* suppressor mutations (*dsm* mutations) will be present in an otherwise Dam$^+$ background. The *dam16*::Km^r allele was subsequently transduced back into the $oriCI_{Vc}$* (*hsm?*) cells. This transduction resulted in a high number of transductants, indicating that all four $oriCI_{Vc}$* *dam* clones tested contained secondary mutations to compensate for loss of Dam activity. The same *dam16*::Km^r P1 lysate was used for all strains, and the same number of cells was plated on selective media and incubated for 24 h at 37°C prior to inspection.

chrony of initiation and an elevated number of origins per cell (Fig. 5). Hda deficiency in $oriCI_{Vc}$, $oriCI_{Vc}$*, and ΩSm strains led to different degrees of overinitiation, which is not surprising, since such cells are known to accumulate secondary mutations (69). Because loss of Hda and thereby RIDA activity was previously shown to increase the $DnaA^{ATP}/DnaA^{ADP}$ ratio ($DnaA^{ADP}$ is DnaA bound to ADP) (35), these data suggest that $DnaA^{ATP}$ is also the active form of the initiator protein for $oriCI_{Vc}$-dependent initiation. In agreement with this, we observed that $oriCI_{Vc}$ minichromosomes could be introduced into a Δ*dnaA E. coli* strain EH3827 (25) when it contained the *dnaA* plasmid pFH539 (27) but not when it contained the *dnaAK178T* plasmid pRUC672 (76). The latter directs synthesis of the DnaAK178T protein that is mutated in the ATP binding site (data not shown).

SeqA is required for synchronous replication from $oriCI_{Vc}$. The ΩSm, $oriCI_{Vc}$*, and $oriCI_{Vc}$ genetic elements (Fig. 3) were transferred into *seqA*Δ10 mutant cells (78) by P1 transduction. Transductants were obtained at similar frequencies, indicating that the SeqA protein is dispensable for function of $oriCI_{Vc}$. Flow cytometric analysis revealed initiation asynchrony and an increased number of origins for *seqA* cells initiating from both *oriC* and $oriCI_{Vc}$ cells relative to SeqA$^+$ cells (Fig. 6). This is in agreement with previous data for *E. coli* (48). Both cell mass and number of origins per cell of *seqA* $oriCI_{Vc}$ cells were somewhat lower than for their *oriC* counterparts (compare Fig. 6E and F with Fig. 6A, B, C, and D), giving similar origin/mass ratios (not shown).

***E. coli* cells carrying $oriCI_{Vc}$ requires Dam methylation for viability.** The $oriCI_{Vc}$* region could not be transduced into *dam16*::Km^r (63) cells. In contrast, the *dam16*::Km^r mutation could be transduced into cells replicating from $oriCI_{Vc}$ and

TABLE 3. Relative P1 transduction of *argE*::Tn*10* and *dam13*::Tn*9* into strains with mutated *oriC* regions[a]

Recipient	Mnemonic	Minichromosome phenotype[b]	Cm^r/Tc^r ratio[c]
WM2482	Wild-type	++	0.9
WM2759 (*oriC160*)	Deletion of 77 bp to the right of R4	+	1.0
WM2762 (*oriC13*)	Scrambled R2	+	0.9
WM2764 (*oriC15*)	Scrambled R4	−	9.0×10^{-4}
WM2844 (*oriC17*)	Scrambled R5	−	3.3×10^{-4}
WM2845 (*oriC162*)	Addition of 14 bp between R3 and R4	−	2.6×10^{-4}
ALO3270 (*oriC-I3,I6*)	G3T in I2 and G3→T in I3	+++	1.4
ALO3699 (ΩSm)	ΩSm inserted next to *oriC*	ND	1.2
ALO3150 (*oriCI*$_{Vc}$*)	*oriC* replaced by *oriCI*$_{Vc}$*	ND	2.2×10^{-4}
ALO3702 (*oriCI*$_{Vc}$)	*oriC* replaced by *oriCI*$_{Vc}$		
Incubated at 37°C[d]		ND	1.0×10^{-3}
Incubated at 42°C[d]		ND	1.3×10^{-4}

[a] Plates were inspected after overnight incubation at 37°C. Strains with *oriC* mutations *oriC14*, *oriC21*, *oriC131*, *oriC132*, and *oriC136* (84) behaved similar to wild-type (wt), *oriC160*, and *oriC13* strains.

[b] Data from reference 84, except for the *oriC-I3,I6* mutant (data from reference 23). Symbols: +++, increased *oriC* function; ++, normal *oriC* function; +, reduced *oriC* function; −, inactive *oriC*. ND, not determined.

[c] Transduction efficiency of *argE*::Tn*9* into the recipient was 1×10^{-6} to 2×10^{-6} in all cases except for *oriC15* and *oriC162* strains, where it was approximately 5-fold lower.

[d] There were about as many microcolonies as seen on the plates containing tetracycline. These microcolonies could not really restreak and did not appear when the plates were incubated at 42°C.

oriCI$_{Vc}$* with low frequency and resulting in transductants that were heterogeneous in colony size and generally appeared slow growing (Fig. 7A). Approximately 25% of the transductants contained both a mutated *dam* gene and a wild-type *dam* gene (not shown). Such cells were never observed for transductants with the *dam16*::Km^r allele introduced into wild-type cells (not shown). The low number of *dam16*::Km^r transductants obtained in *oriCI*$_{Vc}$ and *oriCI*$_{Vc}$* cells and the fact that different phenotypes were observed for the transductants indicate that the attempt to combine these origins with a null mutation in *dam* led to the selection of strains with secondary *dam* suppressor mutations (*dsm* mutations). To test this possibility, we constructed four independent isolates of *oriCI*$_{Vc}$* *dam16*::Km^r cells and transduced these back to Dam$^+$ by a two-step procedure as described previously (Fig. 7) (46). The resultant cells were Dam$^+$ but contained putative *hsm* mutations. When these cells were subsequently transduced to *dam16*::Km^r, this occurred with the same frequency as the frequency observed for wild-type cells (Fig. 7C). This demonstrates that all four initial *oriCI*$_{Vc}$* *dam* clones tested carried secondary mutations. The nature of the *dsm* mutations is not known at present, although transductant data indicate that neither of the *dsm* mutations were linked to *oriCI*$_{Vc}$.

To quantify the transfer of a *dam* mutation into cells with various mutations in *oriC*, we used a strain carrying both the *dam13*::Tn*9* and *argE*::Tn*10* mutations (strain ALO3689) as a donor. The efficiency of *dam13*::Tn*9* transduction could thus be determined relative to the unlinked *argE*::Tn*10* mutation carried in the same P1 lysate (Table 3). The *dam13*::Tn*9* and *argE*::Tn*10* mutations were transduced into wild-type and ΩSm cells with the same frequency, whereas the efficiency of *dam13*::Tn*9* transduction into *oriCI*$_{Vc}$ or *oriCI*$_{Vc}$* cells was reduced approximately 10,000-fold (Table 3). Transduction of *dam13*::Tn*9* into *oriCI*$_{Vc}$ at 37°C resulted in microcolonies that could not be restreaked, while incubation of plates at 42°C resulted in very few heterogeneous colonies similar to what we observed for *oriCI*$_{Vc}$*. Because replication initiation is facilitated by negative supercoiling, which in turn decreases with temperature (3), these observations suggest that the events leading to duplex opening and productive initiation at *oriCI*$_{Vc}$ are severely compromised in the absence of Dam methylation.

Next, we wanted to determine whether *oriCI*$_{Vc}$ and *oriCI*$_{Vc}$* cells were viable when the amount of Dam was significantly reduced. We used the *damX*::mini-Tn*10* mutation resulting in *dam* gene transcription from promoters P4 and P5 only (out of a total of five) at approximately 10% of the wild-type level (42). The *damX*::mini-Tn*10* mutation was transduced into wild-type, ΩSm, *oriCI*$_{Vc}$, and *oriCI*$_{Vc}$* cells with similar frequencies, showing that only small amounts of Dam are needed for viability of *oriCI*$_{Vc}$ and *oriCI*$_{Vc}$* cells. The reduction in *dam* gene expression led to initiation asynchrony in all cells (Fig. 6G to J).

Only severely compromised *E. coli* origins require Dam methylation for viability. In order to examine whether changes in initiation efficiency from the *E. coli* replication origin itself could result in a requirement for Dam methylation, we transduced *dam13*::Tn*9* into a previously characterized set of *oriC* mutants (69, 84), again using the *argE*::Tn*10* mutation as an internal control (Table 3).

For *oriC* mutants (*oriC13*, *oriC14*, *oriC21*, *oriC131*, *oriC132*, *oriC136*, and *oriC160* mutants) that all show reduced *oriC* function when carried on a minichromosome (84), the two markers were transduced with the same frequency, indicating that Dam methylation is dispensable for the function of these origins (Table 3). For an *oriC* mutation (MG1655 *oriC-I3,I6*) that increases origin function when present on the chromosome (68), Dam methylation was also dispensable (Table 3). We found methylation to be essential only for the viability of the *oriC15*, *oriC17*, and *oriC162* mutants that carry severely truncated origins that cannot sustain minichromosome replication (i.e., with an average replication rate well below that of *oriC*) and that result in severe asynchrony when carried on the chromosome (84).

Together, these data indicate that *dam* is dispensable for replication initiation from mutant *oriC*s, provided that origin function is increased or only somewhat reduced relative to wild type. A severe reduction in *oriC* function, on the other hand, results in a requirement for Dam methylation. In contrast,

oriCI$_{Vc}$ does not seem to be so severely defective as to warrant a Dam requirement, suggesting a different role for Dam in initiation of replication from *oriCI*$_{Vc}$ compared to *oriC*.

Overexpression of DnaA does not restore the viability of Dam-deficient *oriCI*$_{Vc}$ cells. *E. coli* cells deficient in Dam methylation have decreased expression of *dnaA* (10, 36). A possibility for the inviability of Dam-deficient *oriCI*$_{Vc}$ cells could be that their reduced DnaA content was insufficient for initiation of replication. We therefore transduced the *dam16*::Km^r mutation into *oriCI*$_{Vc}$ and *oriCI*$_{Vc}$* cells carrying either plasmid pRUC1443, which carries the *V. cholerae dnaA* gene under the control of the strong *lac* P$_{A10403}$ promoter (O. Skovgaard, unpublished observation), or plasmid pLR40, which carries the *E. coli dnaA* gene under the control of the indigenous *lac* promoter (69). Plasmid pRUC1443 complemented an *E. coli dnaA46* mutant at low IPTG levels, whereas full induction was deleterious to cells, suggesting that this plasmid directs high-level *V. cholerae* DnaA production. Plasmid pLR40 was previously shown to overproduce *E. coli* DnaA about 3-fold when fully induced (69).

We did not obtain an increased transduction frequency at any IPTG concentration for either plasmid (not shown). Therefore, additional DnaA protein cannot compensate for loss of Dam methylation in the process of *oriCI*$_{Vc}$-specific initiation.

Dam methylation is required for initiation from *oriCI*$_{Vc}$. To further study the effect of Dam on replication initiation from *oriCI*$_{Vc}$, we used a conditional replication system based on pKG339 that carries *copA* under *lac* promoter control (18, 32) for Dam depletion. This system relies on the replication control system of plasmid R1, where the copy number is negatively regulated by the CopA antisense RNA. Hence, if CopA is overproduced from a coresident plasmid, R1 replication is blocked within a few minutes (39). We introduced the *dam16*::Km^r mutation into wt, ΩSm, *oriCI*$_{Vc}$, and *oriCI*$_{Vc}$* cells containing pALO160, a R1-based plasmid carrying the entire *dam* operon under the control of its indigenous promoters (42). A further introduction of plasmid pKG339 that carries *copA* under *lac* promoter control (39) allowed Dam depletion in both strains after the addition of IPTG (Materials and Methods).

Dam depletion in wild-type cells gradually led to decreased initiation synchrony, and 7 h after the addition of IPTG, the cells were completely asynchronous (Fig. 8A). The cells appeared relatively homogeneous with a good correlation between cell size and DNA content, although a small subpopulation of cells with variable size containing one fully replicated chromosome was observed after 7 h (Fig. 8B). For *oriCI*$_{Vc}$ and *oriCI*$_{Vc}$* cells, the situation was aggravated. Approximately 7 h after the addition of IPTG, cells containing one origin of replication started to dominate the population (Fig. 8A). For these cells, there was poor correlation between size and DNA content and cells appeared "trapped" with only one fully replicated chromosome (Fig. 8B). The accumulation of large cells containing only one fully replicated *oriCI*$_{Vc}$ chromosome suggests that these cells were able to grow but were unable to initiate replication from *oriCI*$_{Vc}$. Therefore, Dam methylation was required for one or more processes leading to initiation from *oriCI*$_{Vc}$. After longer times of incubation, *oriCI*$_{Vc}$ and

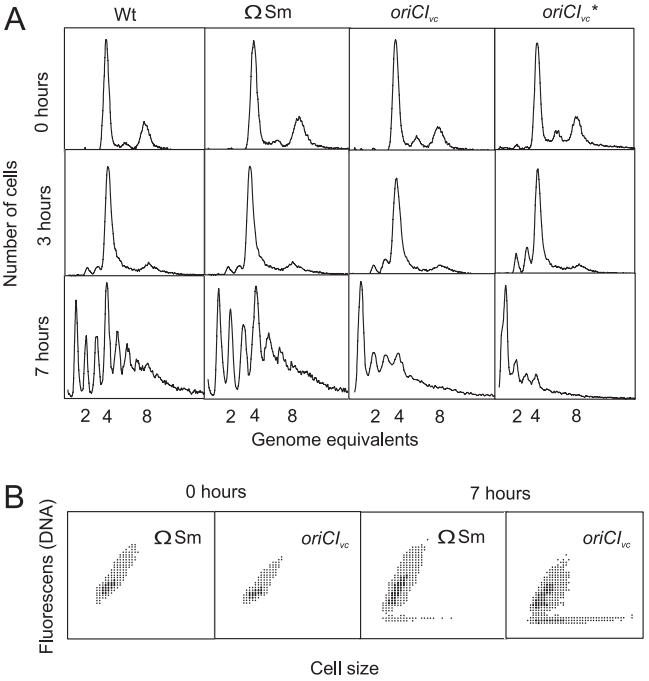

FIG. 8. Depletion of Dam methylase leads to *oriCI*$_{Vc}$ initiation arrest. Dam methylase was depleted from wt, ΩSm, *oriCI*$_{Vc}$, and *oriCI*$_{Vc}$* cells as described in Materials and Methods. Samples were incubated with rifampin and cephalexin prior to flow cytometric analysis. (A) DNA histograms of *oriC* and *oriCI*$_{Vc}$ after incubation in the presence of IPTG for the indicated time period. Cells were treated with rifampin and cephalexin prior to flow cytometric analysis. (B) Two-parameter histograms showing DNA content versus cell size after incubation with IPTG for 0 and 7 h.

oriCI$_{Vc}$* cells regained the ability to initiate replication, most likely due to the accumulation of secondary mutations.

Increased negative supercoiling restores viability of Dam-deficient *E. coli* cells carrying *oriCI*$_{Vc}$. The *dam16*::Km^r mutation was transduced into ΩSm *seqA* and *oriCI*$_{Vc}$ *seqA* cells with the same frequency although colonies of the latter were smaller (Fig. 9A). The absence of SeqA therefore rendered *oriCI*$_{Vc}$ *dam* mutants viable although somewhat growth compromised. Because loss of SeqA activity leads to increased negative supercoiling of the chromosome (85), that in turn facilitates duplex opening, we speculated that this was a way of reactivating an otherwise inactive *oriCI*$_{Vc}$ in *dam* mutant cells. This could provide the explanation for the ability of a *seqA* mutation to suppress the initiation defect of *oriCI*$_{Vc}$ *dam* cells.

In order to test this hypothesis further, we used strain ALO3470, a derivative of PJ4240 where the level of supercoiling could be exogenously controlled (31). In ALO3470, DNA gyrase (*gyrAB*) is under the control of the IPTG-inducible P$_{A1lacO1}$ promoter (48a). Since DNA gyrase is an essential gene, the ALO3470 strain grows only in the presence of IPTG. Approximately 70 μM IPTG has been determined to give a wild-type expression level of DNA gyrase, whereas higher IPTG levels result in more negatively supercoiled DNA (31). The *dam16*::Km^r mutation was transduced into *oriCI*$_{Vc}$, ΩSm, ΩSm *seqA*, and *oriCI*$_{Vc}$ *seqA* mutant derivatives of ALO3470, and transductants were plated in the presence of various IPTG

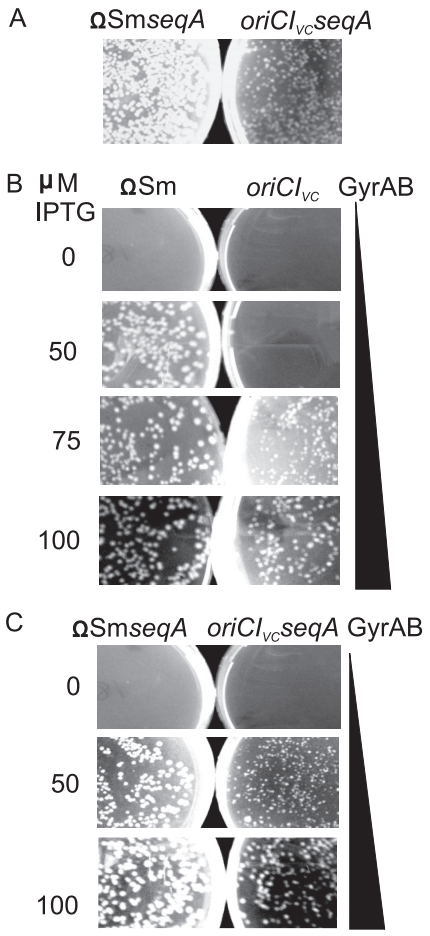

FIG. 9. Dam methylation is dispensable for $oriCI_{Vc}$ function in cells with increased negative supercoiling. (A) The *dam16*::Km^r allele was transduced into ΩSm *seqA* and $oriCI_{Vc}$ *seqA* cells derived from *E. coli* MG1655. (B) The *dam16*::Km^r allele was transduced into ΩSm and $oriCI_{Vc}$ cells carrying the *gyrAB* genes under the control of the IPTG-inducible $P_{A1lacO1}$ promoter. (C) The *dam16*::Km^r allele was transduced into ΩSm *seqA* and $oriCI_{Vc}$ *seqA* cells carrying the *gyrAB* genes under the control of the IPTG-inducible $P_{A1lacO1}$ promoter. Transductants were plated on LB agar plates containing kanamycin (A) or kanamycin, tetracycline, and the indicated IPTG concentration (B and C) and were inspected after 22 h of incubation at 42°C. The same *dam16*::Km^r P1 lysate was used for all strains, and the same number of cells was plated.

concentrations. The *dam16*::Km^r mutation could be efficiently transferred into ΩSm cells at IPTG concentrations of 50 μM or higher (Fig. 9B). On the other hand, the *dam16*::Km^r mutation could be efficiently transduced into $oriCI_{Vc}$ cells only when IPTG concentrations were 75 μM or above, and growth appeared to be best at the highest concentration tested (Fig. 9) (similar data were observed for $oriCI_{Vc}$* cells). The absence of SeqA reduced the IPTG requirement for growth of *dam16*::Km^r transductants into $oriCI_{Vc}$ cells to approximately 50 mM (Fig. 9C), but better growth was observed at a higher IPTG concentration (Fig. 9C).

These data strongly suggest that unmethylated $oriCI_{Vc}$ is initiated poorly, but the origin can gain activity by loss of SeqA activity or if the level of negative superhelicity is increased by increased expression of the DNA gyrase.

DISCUSSION

Studies of *in vivo* replication initiation in *V. cholerae* are complicated by the presence of two chromosomes in this bacterial species; it is often difficult to attribute certain replication defects or phenotypes to a specific chromosome (19, 67). In order to study replication initiation from $oriCI_{Vc}$, the origin of the larger chromosome, chromosome I, we replaced the *E. coli oriC* region with the corresponding $oriCI_{Vc}$ region. When the cells were growing slowly, replication initiation at $oriCI_{Vc}$ took place at a similar or slightly reduced initiation mass relative to initiation from *oriC*. Otherwise, replication initiation at $oriCI_{Vc}$ resembles *oriC*-dependent initiation with respect to cell cycle parameters, such as initiation frequency, SeqA-dependent initiation synchrony, and stimulation of initiation by loss of Hda activity, but differ with respect to the requirement for Dam methylation.

The minimal $oriCI_{Vc}$ region. The minimal replication origins from the *E. coli* chromosome and chromosome I of *V. cholerae* are quite similar. The left-hand side of *E. coli oriC* contains an AT-rich cluster followed by three 13-mer repeats (L, M, and R) each starting with (A/T)GATCT, a 6-mer sequence that binds $DnaA^{ATP}$ in both single- and double-stranded DNA (Fig. 1) (80). The corresponding region in $oriCI_{Vc}$ contains two 13-mer regions corresponding to M and R and three 6-mer sequences, and the rightmost two 6-mer sequences are found within M and R 13-mer regions (Fig. 1). The DnaA R boxes R1, R2, and R4 are completely conserved between *E. coli* and *V. cholerae*, while R3, R5, I2, and τ1 are highly conserved (52, 53, 61) (Fig. 1).

We found no significant difference in copy number of $oriCI_{Vc}$ minichromosomes carrying the entire region between the *mioC* and *gidA* genes and of those carrying a minimal origin only (Fig. 2). This is different from the situation in *E. coli* where the presence of the *gidA* and *mioC* promoters stimulates replication initiation (5, 41, 81).

Replication initiation from *oriC* and $oriCI_{Vc}$. The DnaA protein serves as the initiator protein for both *E. coli oriC* and $oriCI_{Vc}$ (18). The facts that $oriCI_{Vc}$ minichromosomes can replicate in *E. coli* and that $oriCI_{Vc}$ can replace *oriC* show that *E. coli* DnaA can functionally replace *V. cholerae* DnaA. Similarly, the DnaA proteins from *Vibrio harveyi* and *V. cholerae* can replace the *E. coli* DnaA protein (7). In *V. cholerae*, a homologue of the *hda* gene has not been identified (18). Because Hda is essential for RIDA (regulatory inactivation of DnaA), this process may be absent or operate differently in this organism. The construction of the $oriCI_{Vc}$ strain allowed us to address the roles of the different nucleotide-bound forms of DnaA (i.e., $DnaA^{ATP}$ and $DnaA^{ADP}$) in regulation of *V. cholerae* chromosome I replication. Deletion of *hda* from the $oriCI_{Vc}$ strain, which presumably raised the $DnaA^{ATP}$/$DnaA^{ADP}$ ratio, stimulated replication initiation (Fig. 5) and $oriCI_{Vc}$ minichromosomes could not be introduced into *E. coli* cells having only a DnaA protein mutated in the ATP binding site. We believe that initiation from $oriCI_{Vc}$ is similar to initiation from *oriC* with an absolute requirement for $DnaA^{ATP}$.

In wild-type *E. coli* cells, regulation of the initiation frequency at *oriC* depends primarily on sequences outside the origin (13). This is because formation of the prereplication complex (pre-RC) is a low-affinity process that takes place at a

critical level of $DnaA^{ATP}$ that is reached only when all DnaA binding sites outside the origin are filled (6, 26, 29). Therefore, differences in initiation efficiency between *oriC* and $oriCI_{Vc}$ that can be observed by the analyses done here are expected to be small. In agreement with this, we observed only a 10% reduction in growth rate and no difference in cell cycle parameters, such as the number of origins per cell, origin concentration, or single cell synchrony between *E. coli* cells initiating from their normal *oriC* sequence and those initiating from $oriCI_{Vc}$ when the cells were grown in rich medium. At lower growth rates, cells replicating from $oriCI_{Vc}$ initiated replication in synchrony with a reduced cell mass, suggesting that the pre-RC complex forms more efficiently at this origin than at *oriC*. Therefore, $oriCI_{Vc}$ seems more efficient than *oriC* in initiation of replication in *E. coli*. Despite this, $oriCI_{Vc}$-based minichromosomes did not compete with the chromosomal origin when present in *E. coli* and were maintained as extrachromosomal plasmids. Therefore, the cascade of initiations triggered by firing of the first origin, whether the origin is located on the chromosome or minichromosome, has the potential to initiate all remaining origins in the cell, suggesting that the differences between *oriC* and $oriCI_{Vc}$ are small (75).

Roles of *dam* and *seqA* genes in replication from $oriCI_{Vc}$. In *V. cholerae*, the *dam* gene is essential, while there is conflicting data on whether *seqA* is essential in *V. cholerae* (15, 72). We found that the loss of SeqA function led to overinitiation from $oriCI_{Vc}$ and asynchrony, but the cells remained viable. A reduction in the level of Dam methylase also led to initiation asynchrony. It is therefore likely that sequestration of hemimethylated $oriCI_{Vc}$ is involved in prevention of immediate reinitiation, similar to the case for *oriC*-dependent replication. In addition to this postinitiation role of Dam methylation, our data also show that *dam* is required for efficient initiation from $oriCI_{Vc}$ in *E. coli*. This requirement was not a result of limited DnaA availability or quality, because overproduction of neither the *E. coli* or *V. cholerae* DnaA protein could compensate for loss of Dam activity. Our data are in agreement with the observation that overexpression of SeqA in *V. cholerae* led to loss of viability and replication arrest (72); because Dam and SeqA proteins compete for the same GATC sites on DNA, overexpression of SeqA leads to the phenotype associated with the lack of the Dam protein (43).

Dam methylation may be required for duplex opening at $oriCI_{Vc}$. On the basis of our data, Dam methylation seems to play dual roles for replication from *V. cholerae oriCI* in *E. coli*. One of these is the well-characterized role in SeqA-dependent sequestration of newly initiated and hemimethylated origins that is required to prevent rereplication within the same cell cycle. The second role of Dam methylation is the requirement for methylation of $oriCI_{Vc}$ prior to initiation to allow for efficient duplex opening either directly or indirectly. Similar roles for Dam methylation have previously been reported for replication of plasmid P1 (1, 2). The first step in initiation of chromosome replication in *E. coli* is unwinding of the origin in the region containing the AT-rich cluster and 13-mer repeats in a process assisted by DnaA as described above. This part of the *E. coli* origin exhibits helical instability and can unwind even in the absence of DnaA when present on a negatively supercoiled plasmid (38). Therefore, it is not surprising that mutations in gyrase and topoisomerase I, key players in controlling DNA supercoiling in *E. coli* (79), influence initiation of replication in *E. coli*, leading to asynchrony and changes in origin concentration (82). Thermal melting determinations of a *oriC*-carrying plasmid by differential scanning calorimetry indicated that Dam methylation can lower the *oriC* melting point and thereby facilitate strand separation in the *oriC* region (86). The inability to delete *dam* from cells with *oriC15*, *oriC17*, and *oriC162* mutations suggest that DnaA fails to promote strand separation at these truncated origins when unmethylated. The lower intrinsic melting point of the fully methylated mutant origins may, however, augment DnaA in the duplex opening process to allow for initiation, albeit at reduced frequency compared to that of wild-type *oriC*. A similar explanation for the inability to initiate replication from unmethylated $oriCI_{Vc}$ is therefore that an increase in thermodynamic stability of DNA, associated with the absence of N^6 methylation, renders the AT-rich region of $oriCI_{Vc}$ unable to unwind in response to DnaA binding. In agreement with this hypothesis, conditions that decrease helical stability, such as increased expression of *gyrAB* genes (31) or deletion of *seqA* (85), can compensate for the loss of Dam activity. It is not clear why methylation is required for initiation from $oriCI_{Vc}$ but not *oriC*. The 256-bp $oriCI_{Vc}$ minimal origin region has a lower GC content than the corresponding 257-bp *oriC* region (87 and 102 bp, respectively). The helical stability of the *oriC* and $oriCI_{Vc}$ minimal origin regions was analyzed using WEB-THERMODYNE sequence analysis software (30). As expected, the lowest helical stability for both minimal origins was found in the left part of the sequence where the initial unwinding of the *E. coli* origin occurs (38). The helical stability in this region as well as the overall helical stability is lower for the $oriCI_{Vc}$ minimal origin than for the corresponding *oriC* regions, indicating that the increased negative supercoiling needed to suppress the Dam requirement of $oriCI_{Vc}$ does not result from differences in the DNA sequence between the minimal origin regions. Another possible explanation is that the formation of the orisome is perturbed on unmethylated origins. The Dam/SeqA system normally ensures a highly organized orisome assembly at the origin (58). In its absence, one might imagine that less active complexes are formed and productive initiations from these complexes could be augmented by increased negative superhelicity. The difference between *oriC* and $oriCI_{Vc}$ could then be due to the slightly different positions of the GATC sites and/or the extra GATC sites in the minimal $oriCI_{Vc}$ region compared to the minimal *oriC* region (Fig. 1). $oriCI_{Vc}$ contains two extra GATC sequences relative to *oriC*. One is located between τ1 and R5 and could, in the absence of methylation, promote untimely DnaA binding to these sites and hence interfere with orisome formation. The other is located within the region corresponding to the *E. coli* FIS (factor for inversion stimulation) binding site (Fig. 1). Methylation of this site could influence FIS binding and thereby pre-RC assembly (70). We have not yet tested this possibility.

On the basis of minichromosome data, it has been reported that in *E. coli*, origin activity is stimulated by transcription from the *mioC* promoter traversing *oriC* and from the *gidA* promoter located immediately adjacent to *oriC* and transcribing away from *oriC* (5, 59). In the case of *gidA*, the stimulation is likely to result from increased negative supercoiling behind transcribing RNA polymerases (40) that have initiated at

P*gidA*. It seemed reasonable that the same role could apply to the *V. cholerae* P*gidA* and $oriCI_{Vc}$. However, our minichromosome data (Fig. 2) indicate that initiation from $oriCI_{Vc}$ is not stimulated significantly by the presence of the *V. cholerae gidA* promoter, which would could contribute to the apparent methylation requirement.

The data reported here are in conflict with a recent study (15) where an essential role for *dam* was not observed when *oriC* was replaced with $oriCI_{Vc}$. Although in both studies $oriCI_{Vc}$ was found to be more active than the *E. coli* origin, differences do exist. In this work, the $oriCI_{Vc}$ strain contained an intact *E. coli mioC* gene followed by the *V. cholerae* region between the *mioC* and *gidA* genes and the 20 N-terminal codons of the *V. cholerae gidA* gene. The *aadA* (Sm^r) cassette was located 1 kb further downstream of the *gidA* promoter (Fig. 3). Demarre and Chattoraj (15) used a minimal $oriCI_{Vc}$ region with an attached zeocin resistance gene to replace the minimal *E. coli oriC* sequence. In the final construct, the *zeo* gene was inserted between the *E. coli mioC* gene and $oriCI_{Vc}$ in such a way that transcription from the *zeo* promoter was directed toward $oriCI_{Vc}$. The *E. coli gidA* and the *mioC* genes remained intact (G. Demarre and D. K. Chattoraj, personal communication). This *oriCI* strain seems somewhat more active than our $oriCI_{Vc}$ strain. It is not clear to us why the Dam requirement for these two $oriCI_{Vc}$ strains should differ. The observed differences may, however, stress the contribution of transcriptional events around the origin to the initiation process and indicate that Dam methylation could modulate (i.e., lower) the requirement for transcriptional activation of $oriCI_{Vc}$. The two different sets of data further stress that extreme care should be taken when extrapolating data obtained in *E. coli* to the situation in *V. cholerae*. The precise role for *dam* in initiation of replication from $oriCI_{Vc}$ in *V. cholerae* under different conditions will require studies carried out in the native host.

ACKNOWLEDGMENTS

This work was supported by the Danish Natural Sciences Research Council and the Novo Nordisk Foundation.

We thank Ole Skovgaard for providing plasmids pSW29TsacB, and pRUC1443, Kenn Gerdes for providing plasmid pKG339, Martin Martinus for providing strain CAG18456, Kirsten Skarstad for providing strain SS211, Rasmus Bugge Jensen for providing strain DH5α*λpir*, and Peter Rudahl Jensen for providing strain PJ4240. Christa P. Nielsen is thanked for excellent technical assistance. We thank Godefroid Charbon for critically reading the manuscript.

REFERENCES

1. **Abeles, A., T. Brendler, and S. Austin.** 1993. Evidence of two levels of control of P1 *oriR* and host *oriC* replication origins by DNA adenine methylation. J. Bacteriol. **175:**7801–7807.
2. **Abeles, A. L., and S. J. Austin.** 1987. P1 plasmid replication requires methylated DNA. EMBO J. **6:**3185–3189.
3. **Adamcik, J., V. Viglasky, F. Valle, M. Antalik, D. Podhradsky, and G. Dietler.** 2002. Effect of bacteria growth temperature on the distribution of supercoiled DNA and its thermal stability. Electrophoresis **23:**3300–3309.
4. **Adhya, S., and M. Gottesman.** 1982. Promoter occlusion: transcription through a promoter may inhibit its activity. Cell **29:**939–944.
5. **Asai, T., M. Takanami, and M. Imai.** 1990. The AT richness and gid transcription determine the left border of the replication origin of the *E. coli* chromosome. EMBO J. **9:**4065–4072.
6. **Atlas, J. C., E. V. Nikolaev, S. T. Browning, and M. L. Shuler.** 2008. Incorporating genome-wide DNA sequence information into a dynamic whole-cell model of *Escherichia coli*: application to DNA replication. IET Syst. Biol. **2:**369–382.
7. **Berenstein, D., K. Olesen, C. Speck, and O. Skovgaard.** 2002. Genetic organization of the *Vibrio harveyi* DnaA gene region and analysis of the function of the *V. harveyi* DnaA protein in *Escherichia coli*. J. Bacteriol. **184:**2533–2538.
8. **Bertani, G.** 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J. Bacteriol. **62:**293–300.
9. **Boye, E., and A. Løbner-Olesen.** 1990. The role of dam methyltransferase in the control of DNA replication in *E. coli*. Cell **62:**981–989.
10. **Braun, R. E., and A. Wright.** 1986. DNA methylation differentially enhances the expression of one of the two *E. coli dnaA* promoters in vivo and in vitro. Mol. Gen. Genet. **202:**246–250.
11. **Casadesus, J., and D. Low.** 2006. Epigenetic gene regulation in the bacterial world. Microbiol. Mol. Biol. Rev. **70:**830–856.
12. **Clark, D. J., and O. Maaløe.** 1967. DNA replication and the division cycle in *Escherichia coli*. J. Mol. Biol. **23:**99–112.
13. **Dasgupta, S., and A. Løbner-Olesen.** 2004. Host controlled plasmid replication: *Escherichia coli* minichromosomes. Plasmid **52:**151–168.
14. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A. **97:**6640–6645.
15. **Demarre, G., and D. K. Chattoraj.** 2010. DNA adenine methylation is required to replicate both Vibrio cholerae chromosomes once per cell cycle. PLoS Genet. **6:**e1000939.
16. **Demarre, G., A. M. Guerout, C. Matsumoto-Mashimo, D. A. Rowe-Magnus, P. Marliere, and D. Mazel.** 2005. A new family of mobilizable suicide plasmids based on broad host range R388 plasmid (IncW) and RP4 plasmid (IncPalpha) conjugative machineries and their cognate *Escherichia coli* host strains. Res. Microbiol. **156:**245–255.
17. **Donachie, W. D.** 1968. Relationship between cell size and time of initiation of DNA replication. Nature **219:**1077–1079.
18. **Duigou, S., K. G. Knudsen, O. Skovgaard, E. S. Egan, A. Løbner-Olesen, and M. K. Waldor.** 2006. Independent control of replication initiation of the two *Vibrio cholerae* chromosomes by DnaA and RctB. J. Bacteriol. **188:**6419–6424.
19. **Egan, E. S., A. Løbner-Olesen, and M. K. Waldor.** 2004. Synchronous replication initiation of the two *Vibrio cholerae* chromosomes. Curr. Biol. **14:**R501–R502.
20. **Egan, E. S., and M. K. Waldor.** 2003. Distinct replication requirements for the two *Vibrio cholerae* chromosomes. Cell **114:**521–530.
21. **Erzberger, J. P., M. L. Mott, and J. M. Berger.** 2006. Structural basis for ATP-dependent DnaA assembly and replication-origin remodeling. Nat. Struct. Mol. Biol. **13:**676–683.
22. **Gille, H., J. B. Egan, A. Roth, and W. Messer.** 1991. The FIS protein binds and bends the origin of chromosomal DNA replication, *oriC*, of *Escherichia coli*. Nucleic Acids Res. **19:**4167–4172.
23. **Grimwade, J. E., J. J. Torgue, K. C. McGarry, T. Rozgaja, S. T. Enloe, and A. C. Leonard.** 2007. Mutational analysis reveals *Escherichia coli oriC* interacts with both DnaA-ATP and DnaA-ADP during pre-RC assembly. Mol. Microbiol. **66:**428–439.
24. **Guyer, M. S., R. R. Reed, J. A. Steitz, and K. B. Low.** 1981. Identification of a sex-factor-affinity site in *E. coli* as gamma delta. Cold Spring Harb. Symp. Quant. Biol. **45:**135–140.
25. **Hansen, E. B., and M. B. Yarmolinsky.** 1986. Host participation in plasmid maintenance: dependence upon *dnaA* of replicons derived from P1 and F. Proc. Natl. Acad. Sci. U. S. A. **83:**4423–4427.
26. **Hansen, F. G., B. B. Christensen, and T. Atlung.** 1991. The initiator titration model: computer simulation of chromosome and minichromosome control. Res. Microbiol. **142:**161–167.
27. **Hansen, F. G., S. Koefoed, and T. Atlung.** 1992. Cloning and nucleotide sequence determination of twelve mutant *dnaA* genes of *Escherichia coli*. Mol. Gen. Genet. **234:**14–21.
28. **Heidelberg, J. F., J. A. Eisen, W. C. Nelson, R. A. Clayton, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, L. Umayam, S. R. Gill, K. E. Nelson, T. D. Read, H. Tettelin, D. Richardson, M. D. Ermolaeva, J. Vamathevan, S. Bass, H. Qin, I. Dragoi, P. Sellers, L. McDonald, T. Utterback, R. D. Fleishmann, W. C. Nierman, O. White, S. L. Salzberg, H. O. Smith, R. R. Colwell, J. J. Mekalanos, J. C. Venter, and C. M. Fraser.** 2000. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature **406:**477–483.
29. **Herrick, J., M. Kohiyama, T. Atlung, and F. G. Hansen.** 1996. The initiation mess? Mol. Microbiol. **19:**659–666.
30. **Huang, Y., and D. Kowalski.** 2003. WEB-THERMODYN: sequence analysis software for profiling DNA helical stability. Nucleic Acids Res. **31:**3819–3821.
31. **Jensen, P. R., C. C. van der Weijden, L. B. Jensen, H. V. Westerhoff, and J. L. Snoep.** 1999. Extensive regulation compromises the extent to which DNA gyrase controls DNA supercoiling and growth rate of *Escherichia coli*. Eur. J. Biochem. **266:**865–877.
32. **Jensen, R. B., E. Grohmann, H. Schwab, R. Diaz-Orejas, and K. Gerdes.** 1995. Comparison of *ccd* of F, *parDE* of RP4, and *parD* of R1 using a novel conditional replication control system of plasmid R1. Mol. Microbiol. **17:**211–220.
33. **Julio, S. M., D. M. Heithoff, D. Provenzano, K. E. Klose, R. L. Sinsheimer, D. A. Low, and M. J. Mahan.** 2001. DNA adenine methylase is essential for viability and plays a role in the pathogenesis of *Yersinia pseudotuberculosis* and *Vibrio cholerae*. Infect. Immun. **69:**7610–7615.

34. **Katayama, T., T. Kubota, K. Kurokawa, E. Crooke, and K. Sekimizu.** 1998. The initiator function of DnaA protein is negatively regulated by the sliding clamp of the *E. coli* chromosomal replicase. Cell **94:**61–71.
35. **Kato, J., and T. Katayama.** 2001. Hda, a novel DnaA-related protein, regulates the replication cycle in *Escherichia coli*. EMBO J. **20:**4253–4262.
36. **Kedar, G. C., F. Ozcan, E. C. Guzman, D. W. Smith, V. G. Newman, and J. W. Zyskind.** 2000. Role of DNA methylation at GATC sites in the *dnaA promoter*, *dnaAp2*. J. Mol. Microbiol. Biotechnol. **2:**301–310.
37. **Kolling, R., A. Gielow, W. Seufert, C. Kucherer, and W. Messer.** 1988. AsnC, a multifunctional regulator of genes located around the replication origin of *Escherichia coli*, *oriC*. Mol. Gen. Genet. **212:**99–104.
38. **Kowalski, D., and M. J. Eddy.** 1989. The DNA unwinding element: a novel, cis-acting component that facilitates opening of the *Escherichia coli* replication origin. EMBO J. **8:**4335–4344.
39. **Kruse, T., J. Bork-Jensen, and K. Gerdes.** 2005. The morphogenetic MreBCD proteins of *Escherichia coli* form an essential membrane-bound complex. Mol. Microbiol. **55:**78–89.
40. **Liu, L. F., and J. C. Wang.** 1987. Supercoiling of the DNA template during transcription. Proc. Natl. Acad. Sci. U. S. A. **84:**7024–7027.
41. **Løbner-Olesen, A., T. Atlung, and K. V. Rasmussen.** 1987. Stability and replication control of *Escherichia coli* minichromosomes. J. Bacteriol. **169:** 2835–2842.
42. **Løbner-Olesen, A., E. Boye, and M. G. Marinus.** 1992. Expression of the *Escherichia coli dam* gene. Mol. Microbiol. **6:**1841–1851.
43. **Løbner-Olesen, A., M. G. Marinus, and F. G. Hansen.** 2003. Role of SeqA and Dam in *Escherichia coli* gene expression: a global/microarray analysis. Proc. Natl. Acad. Sci. U. S. A. **100:**4672–4677.
44. **Løbner-Olesen, A., K. Skarstad, F. G. Hansen, K. von Meyenburg, and E. Boye.** 1989. The DnaA protein determines the initiation mass of *Escherichia coli* K-12. Cell **57:**881–889.
45. **Løbner-Olesen, A., O. Skovgaard, and M. G. Marinus.** 2005. Dam methylation: coordinating cellular processes. Curr. Opin. Microbiol. **8:**154–160.
46. **Løbner-Olesen, A., and U. von Freiesleben.** 1996. Chromosomal replication incompatibility in Dam methyltransferase deficient *Escherichia coli* cells. EMBO J. **15:**5999–6008.
47. **Low, D. A., and J. Casadesus.** 2008. Clocks and switches: bacterial gene regulation by DNA adenine methylation. Curr. Opin. Microbiol. **11:**106–112.
48. **Lu, M., J. L. Campbell, E. Boye, and N. Kleckner.** 1994. SeqA: a negative modulator of replication initiation in *E. coli*. Cell **77:**413–426.

48a. **Lutz, R., and H. Bujard.** 1997. Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I_1-I_2 regulatory elements. Nucleic Acids Res. **25:**1203–1210.

49. **Marinus, M. G., M. Carraway, A. Z. Frey, L. Brown, and J. A. Arraj.** 1983. Insertion mutations in the *dam* gene of *Escherichia coli* K-12. Mol. Gen. Genet. **192:**288–289.
50. **Marinus, M. G., and J. Casadesus.** 2009. Roles of DNA adenine methylation in host-pathogen interactions: mismatch repair, transcriptional regulation, and more. FEMS Microbiol. Rev. **33:**488–503.
51. **Marinus, M. G., and N. R. Morris.** 1973. Isolation of deoxyribonucleic acid methylase mutants of *Escherichia coli* K-12. J. Bacteriol. **114:**1143–1150.
52. **McGarry, K. C., V. T. Ryan, J. E. Grimwade, and A. C. Leonard.** 2004. Two discriminatory binding sites in the *Escherichia coli* replication origin are required for DNA strand opening by initiator DnaA-ATP. Proc. Natl. Acad. Sci. U. S. A. **101:**2811–2816.
53. **Messer, W.** 2002. The bacterial replication initiator DnaA. DnaA and *oriC*, the bacterial mode to initiate DNA replication. FEMS Microbiol. Rev. **26:**355–374.
54. **Miller, D. T., J. E. Grimwade, T. Betteridge, T. Rozgaja, J. J. Torgue, and A. C. Leonard.** 2009. Bacterial origin recognition complexes: direct assembly of higher-order DnaA oligomeric structures. Proc. Natl. Acad. Sci. U. S. A. **106:**18479–18484.
55. **Miller, J. S.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
56. **Morigen, E. Boye, K. Skarstad, and A. Løbner-Olesen.** 2001. Regulation of chromosomal replication by DnaA protein availability in *Escherichia coli*: effects of the *datA* region. Biochim. Biophys. Acta **1521:**73–80.
57. **Nielsen, O., and A. Løbner-Olesen.** 2008. Once in a lifetime: strategies for preventing re-replication in prokaryotic and eukaryotic cells. EMBO Rep. **9:**151–156.
58. **Nievera, C., J. J. Torgue, J. E. Grimwade, and A. C. Leonard.** 2006. SeqA blocking of DnaA-*oriC* interactions ensures staged assembly of the *E. coli* pre-RC. Mol. Cell **24:**581–592.
59. **Ogawa, T., and T. Okazaki.** 1991. Concurrent transcription from the *gid* and *mioC* promoters activates replication of an *Escherichia coli* minichromosome. Mol. Gen. Genet. **230:**193–200.
60. **Ozaki, S., and T. Katayama.** 2009. DnaA structure, function, and dynamics in the initiation at the chromosomal origin. Plasmid **62:**71–82.
61. **Ozaki, S., H. Kawakami, K. Nakamura, N. Fujikawa, W. Kagawa, S. Y. Park, S. Yokoyama, H. Kurumizaka, and T. Katayama.** 2008. A common mechanism for the ATP-DnaA-dependent formation of open complexes at the replication origin. J. Biol. Chem. **283:**8351–8362.
62. **Parales, R. E., and C. S. Harwood.** 1993. Construction and use of a new broad-host-range *lacZ* transcriptional fusion vector, pHRP309, for gram$^-$ bacteria. Gene **133:**23–30.
63. **Parker, B., and M. G. Marinus.** 1988. A simple and rapid method to obtain substitution mutations in *Escherichia coli*: isolation of a *dam* deletion/insertion mutation. Gene **73:**531–535.
64. **Pearson, G. D., A. Woods, S. L. Chiang, and J. J. Mekalanos.** 1993. CTX genetic element encodes a site-specific recombination system and an intestinal colonization factor. Proc. Natl. Acad. Sci. U. S. A. **90:**3750–3754.
65. **Polaczek, P., K. Kwan, D. A. Liberies, and J. L. Campbell.** 1997. Role of architectural elements in combinatorial regulation of initiation of DNA replication in *Escherichia coli*. Mol. Microbiol. **26:**261–275.
66. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
67. **Rasmussen, T., R. B. Jensen, and O. Skovgaard.** 2007. The two chromosomes of *Vibrio cholerae* are initiated at different time points in the cell cycle. EMBO J. **26:**3124–3131.
68. **Riber, L., K. Fujimitsu, T. Katayama, and A. Løbner-Olesen.** 2009. Loss of Hda activity stimulates replication initiation from I-box, but not R4 mutant origins in *Escherichia coli*. Mol. Microbiol. **71:**107–122.
69. **Riber, L., J. A. Olsson, R. B. Jensen, O. Skovgaard, S. Dasgupta, M. G. Marinus, and A. Løbner-Olesen.** 2006. Hda-mediated inactivation of the DnaA protein and *dnaA* gene autoregulation act in concert to ensure homeostatic maintenance of the *Escherichia coli* chromosome. Genes Dev. **20:**2121–2134.
70. **Ryan, V. T., J. E. Grimwade, J. E. Camara, E. Crooke, and A. C. Leonard.** 2004. *Escherichia coli* prereplication complex assembly is regulated by dynamic interplay among Fis, IHF and DnaA. Mol. Microbiol. **51:**1347–1359.
71. **Saha, A., S. Haralalka, and R. K. Bhadra.** 2004. A naturally occurring point mutation in the 13-mer R repeat affects the *oriC* function of the large chromosome of *Vibrio cholerae* O1 classical biotype. Arch. Microbiol. **182:**421–427.
72. **Saint-Dic, D., J. Kehrl, B. Frushour, and L. S. Kahng.** 2008. Excess SeqA leads to replication arrest and a cell division defect in *Vibrio cholerae*. J. Bacteriol. **190:**5870–5878.
73. **Skarstad, K., T. A. Baker, and A. Kornberg.** 1990. Strand separation required for initiation of replication at the chromosomal origin of *E. coli* is facilitated by a distant RNA–DNA hybrid. EMBO J. **9:**2341–2348.
74. **Skarstad, K., E. Boye, and H. B. Steen.** 1986. Timing of initiation of chromosome replication in individual *Escherichia coli* cells. EMBO J. **5:**1711–1717.
75. **Skarstad, K., and A. Løbner-Olesen.** 2003. Stable co-existence of separate replicons in *Escherichia coli* is dependent on once-per-cell-cycle initiation. EMBO J. **22:**140–150.
76. **Skovgaard, O., K. Olesen, and A. Wright.** 1998. The central lysine in the P-loop motif of the *Escherichia coli* DnaA protein is essential for initiating DNA replication from the chromosomal origin, *oriC*, and the F factor origin, *oriS*, but is dispensable for initiation from the P1 plasmid origin, *oriR*. Plasmid **40:**91–99.
77. **Slater, S., and R. Maurer.** 1993. Simple phagemid-based system for generating allele replacements in *Escherichia coli*. J. Bacteriol. **175:**4260–4262.
78. **Slater, S., S. Wold, M. Lu, E. Boye, K. Skarstad, and N. Kleckner.** 1995. *E. coli* SeqA protein binds *oriC* in two different methyl-modulated reactions appropriate to its roles in DNA replication initiation and origin sequestration. Cell **82:**927–936.
79. **Snoep, J. L., C. C. van der Weijden, H. W. Andersen, H. V. Westerhoff, and P. R. Jensen.** 2002. DNA supercoiling in *Escherichia coli* is under tight and subtle homeostatic control, involving gene expression and metabolic regulation of both topoisomerase I and DNA gyrase. Eur. J. Biochem. **269:**1662–1669.
80. **Speck, C., and W. Messer.** 2001. Mechanism of origin unwinding: sequential binding of DnaA to double- and single-stranded DNA. EMBO J. **20:**1469–1476.
81. **Stuitje, A. R., N. de Wind, J. C. van der Spek, T. H. Pors, and M. Meijer.** 1986. Dissection of promoter sequences involved in transcriptional activation of the *Escherichia coli* replication origin. Nucleic Acids Res. **14:**2333–2344.
82. **von Freiesleben, U., and K. V. Rasmussen.** 1992. The level of supercoiling affects the regulation of DNA replication in *Escherichia coli*. Res. Microbiol. **143:**655–663.
83. **Waldminghaus, T., and K. Skarstad.** 2009. The *Escherichia coli* SeqA protein. Plasmid **61:**141–150.
84. **Weigel, C., W. Messer, S. Preiss, M. Welzeck, Morigen, and E. Boye.** 2001. The sequence requirements for a functional *Escherichia coli* replication origin are different for the chromosome and a minichromosome. Mol. Microbiol. **40:**498–507.
85. **Weitao, T., K. Nordstrom, and S. Dasgupta.** 2000. *Escherichia coli* cell cycle control genes affect chromosome superhelicity. EMBO Rep. **1:**494–499.
86. **Yamaki, H., E. Ohtsubo, K. Nagai, and Y. Maeda.** 1988. The *oriC* unwinding by *dam* methylation in *Escherichia coli*. Nucleic Acids Res. **16:**5067–5073.
87. **Zyskind, J. W., J. M. Cleary, W. S. Brusilow, N. E. Harding, and D. W. Smith.** 1983. Chromosomal replication origin from the marine bacterium *Vibrio harveyi* functions in *Escherichia coli*: *oriC* consensus sequence. Proc. Natl. Acad. Sci. U. S. A. **80:**1164–1168.