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Asymmetry of Dam remethylation on the leading and lagging arms of plasmid replicative intermediates

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In *Escherichia coli*, adenine methylation at the sequence GATC allows coupling of cellular processes to chromosome replication and the cell cycle. The transient presence of hemimethylated DNA after replication facilitates post-replicative mismatch repair, induces transcription of some genes and allows transposition of mobile elements. We were interested in estimating the half-life of hemimethylated DNA behind the replication fork in plasmid molecules and in determining whether Dam methyltransferase restores N6 adenine methylation simultaneously on both replicative arms. We show that remethylation takes place asynchronously on the leading and lagging daughter strands shortly after replication. On the leading arm the fully methylated adenine is restored ~2000 bp (corresponding to 2 s) behind the replication fork, while remethylation takes twice as long (at 3500–4000 bp or ~3.5–4 s) on the lagging replicative arm. This observation suggests that Dam remethylation of the lagging arm requires ligated Okazaki fragments.

Keywords: Dam methyltransferase/*Escherichia coli*/plasmid replication

Introduction

In *Escherichia coli*, methyl groups are enzymatically introduced into DNA to form 6-methyladenine or 5-methylcytosine by three proteins: the Dam, Dcm and Hsd methyltransferases (for review see Barras and Marinus, 1989). Whereas Hsd is known to be involved in a defence mechanism that protects cellular DNA from its own Hsd endonuclease, the function of Dcm remains unclear (Gomez-Eichelmann and Ramirez-Santos, 1993; Palmer and Marinus, 1994). Dam methyltransferase, on the other hand, is present at ~130 molecules per cell (Boye *et al.*, 1992) and is involved in a variety of cellular functions (Messer and Noyer-Weidner, 1998). Dam recognizes a specific sequence (5′-GATC-3′) and after DNA replication transfers methyl groups to position 6 of adenine. Nearly all GATC sites are methylated on the *E.coli* chromosome and on plasmid DNA molecules in wild-type strains (Marinus *et al.*, 1984; Russel and Zinder, 1987). The density and distribution of GATC sequences is non-random on the *E.coli* chromosome (Barras and Marinus, 1988): GATCs are found more frequently in translated regions than in non-coding and non-translated sequences, with the exception of the rRNA cluster and some tRNA genes. The spacing between GATC sites never exceeds 2 kb (Barras and Marinus, 1988). Eleven GATCs are present in the *E.coli* chromosomal origin (*oriC*) and it has been demonstrated that methylation at *oriC* facilitates initiation of *E.coli* chromosome replication *in vivo* (Messer *et al.*, 1985; Smith *et al.*, 1985; Baker and Smith, 1989; Boye and Lobner-Olesen, 1990). The origin binds more efficiently to the cellular membrane when hemimethylated, as opposite to after being fully methylated. This implies that methylation at GATC sites might play a role in segregation of the *E.coli* chromosomes (Odgen *et al.*, 1988). Apart from the fact that Dam methylation coordinates the timing of replication initiation events, a number of processes are coupled to the cell cycle by their dependence upon the half-life of hemimethylated DNA (Barras and Marinus, 1989). The mismatch repair machinery requires hemimethylated DNA in order to discriminate between the parental and nascent DNA strands (Laengle-Roulant *et al.*, 1986; Lahue *et al.*, 1987; reviewed by Modrich, 1987, 1994). The transcription of genes such as dnaA2P, mioC, trpS and trpR is induced when their promoters are transiently hemimethylated after replication (Marinus, 1985; Theisen *et al.*, 1993). The same strategy is utilized by some transposons that replicate and transpose simultaneously (Roberts *et al.*, 1985). The accurate function of cellular processes that depend upon the presence of hemimethylated GATC sites suggests that the rate of post-replicative Dam remethylation is subject to control. Strains of *E.coli* that either overexpress Dam or are deficient in Dam methyltransferase activity show a high frequency of spontaneous mutations (Bale *et al.*, 1979; Marinus *et al.*, 1984) and altered timing of chromosome replication (Lobner-Olesen *et al.*, 1994). Minichromosomes containing *oriC* are unstable in Dam$^-$ strains (Messer *et al.*, 1985), but do replicate more frequently when Dam is overproduced (Russel and Zinder, 1987).

Early estimates of the rate of post-replicative Dam remethylation in *E.coli* were based on pulse labelling experiments and chromatography measurements of 6-methyladenine in the fractions of newly synthesized chromosomal DNA. These experiments, however, represent quite controversial data. As shown by Marinus (1976), the fraction of newly synthesized DNA of a *polA lig-4* mutant strain that is enriched for Okazaki fragments contains 0.96 mol% methyladenine compared with 1.4 mol% for bulk DNA. Lyons and Schendl (1984) argue that in *E.coli* K-12 hemimethylated DNA persists at least 60 kb after replication (0.3 mol% 6-methyladenine compared with 1.76 mol% for bulk DNA). These calculations classify Dam remethylation as a distant post-

replicative event. Meanwhile, Szyf *et al.* (1982) reported that pulse-labelled newly synthesized DNA could be completely digested by *Dpn*I, which is indicative of fully methylated GATC sites. From more recent studies it became apparent that the GATC sites of *oriC*, compared with those located at other chromosomal sequences, have different kinetics of remethylation (Campbell and Kleckner, 1988, 1990). Binding of the SeqA protein to the newly replicated, hemimethylated origin delays remethylation of its GATC sites by ~20 min (Campbell and Kleckner, 1990; Lu *et al.*, 1994), whereas for several *E.coli* genes the half-life of hemimethylated DNA was calculated to be between 0.5 and 4 min (Campbell and Kleckner, 1988, 1990), which corresponds to a distance >30 kb behind the replication fork.

Considering the discrepancy in the above data, we have used an approach that permitted the rate of post-replicative Dam remethylation to be estimated with greater accuracy. We analysed the reappearance of methylated adenine in a single *Taq*I site overlapped by the Dam methyltransferase recognition sequence, on plasmid molecules replicating in *E.coli* Xl-1 cells. The inverted orientation of the *Taq*I site with respect to the ColE1 origin in two individual plasmids enabled us to follow the kinetics of remethylation on the leading and lagging daughter strands separately. Using 2D gel electrophoresis, remethylation of replicative intermediates was analysed in short time intervals (corresponding to ~0.5–1 s). The methylation state of the target *Taq*I site was estimated immediately before, during and after replication. We show that methylated adenine is restored to both newly synthesized DNA strands in a few seconds after replication and that this event occurs asynchronously on the leading and the lagging replicative arms. Our data are in agreement with the previously found asymmetry of heteroduplex mutagenesis on the two replicative arms of *E.coli* plasmids (Trinch and Sinden, 1991; Veaute and Fuchs, 1993), suggesting that not only repair mechanisms, but also the function of Dam methyltransferase might be partially hindered on the lagging strand immediately after replication.

Results

General strategy

Our strategy to analyse the rate of post-replicative Dam remethylation is based on the approach described by Campbell and Kleckner (1990). We used the *Taq*I restriction enzyme, which is inhibited by adenine methylation in its recognition site (Figure 1). Since pBluescript SK+ plasmid does not contain any suitable sequences of this kind, we introduced a single *Taq*I site TCGA partially overlapping with a GATC Dam methyltransferase recognition sequence by cloning a 390 bp rat rDNA *Kpn*I–*Sal*I fragment (KS) and in reverse orientation (SK) into pBluescript KS+ and pBluescript SK+, respectively. The two plasmids were designated pDam/KS.o and pDam/NK.c. *Taq*I will not cleave when the Dam methylase recognition sequence is either methylated in the adenines of both DNA strands, as for the parental DNA, or is hemimethylated, as is the case for one of the newly synthesized daughter strands (Nelson and McClelland, 1991). The other daughter strand is accessible to the *Taq*I enzyme (see Figure 1) because methylation is absent in its recognition sequence. Placing the KS fragment in two inverted orientations with respect to the unidirectional ColE1 origin of replication allowed us to follow the remethylation of the leading replicative arm on pDam/KS.o (see Figure 1A) and of the lagging replicative arm on pDam/NK.c, respectively (see Figure 1B). Note that the unidirectional replication fork that initiates at the ColE1 origin enters and moves through the KS (or SK) fragments in both cases from right to left (see Figures 1A, 2A and 3A). We used two additional restriction enzymes, *Mbo*I and *Sau*3AI, to confirm the methylation state of the analysed sequence. Both enzymes recognize GATC but *Mbo*I does not cut when this sequence is hemimethylated or symmetrically methylated at the N6 of adenine. *Sau*3AI cleaves independently of adenine methylation. In order to introduce an internal control for partial restriction enzyme digestion, all the samples were mixed with an appropriate amount (see Materials and methods) of the yeast plasmid TRP1ARS1. This plasmid was chosen for two reasons: first, the absence of methylation in yeast allows DNA to be equally accessible to digestion by *Taq*I, *Mbo*I or *Sau*3AI restriction enzyme; secondly, the size of the TRP1ARS1 digestion products does not interfere with those resulting from the *Kpn*I–*Sal*I fragment used for our analysis (see Figures 2F and 3D).

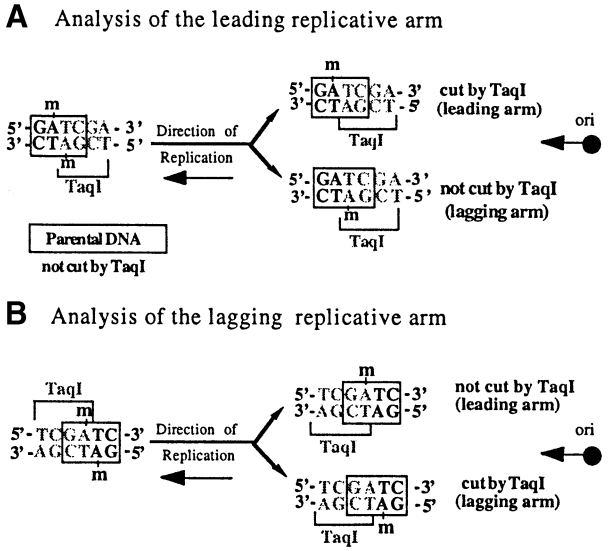


Fig. 1. *Taq*I restriction assay was used to detect the methylation state of leading and lagging replicative arms. (**A**) On plasmid pDam/KS.o the target KS fragment contains a unique *Taq*I recognition site (bracket) partially overlapped by the Dam methylase recognition sequence (square). KS is oriented in such a way with respect to the ColE1 ori that only the leading replicative arm can be cut by *Taq*I if its site is hemimethylated after replication. The parental strand and the lagging arm are not digestible by *Taq*I. (**B**) When on pDam/SK.c the same sequence is inverted with respect to the origin, *Taq*I cuts only the hemimethylated lagging replicative arm.

We performed the assay described above on KS and SK fragments derived from plasmid replicative intermediates that have been resolved by 2D gel electrophoresis and purified from agarose gels. The migration of replicating molecules in neutral/neutral 2D gels depends upon their mass (first dimension) and shape (second dimension). Cleavage with an appropriate restriction enzyme allows one to distinguish between replicative intermediates with different locations of the replication fork according to

their position in the 2D gels (Friedman and Brewer, 1995). Assuming that the speed of DNA replication is ~1000 bp/s (Marians, 1992) and that the distance between the origin of replication and the cleavage point is constant in all the cases analysed, the accessibility of replicative intermediates to *Taq*I enabled us to calculate the remethylation rate.

Since the levels of Dam enzyme mRNA and protein in plasmid (pDam/KS.o or pDam/NK.c)-transfected Xl-1 blue cells grown in the absence of isopropyl-β-D-thiogalactopyranoside (IPTG) induction are similar to that of the wild-type Xl-1 blue cells (for details see Material and methods) we conclude that these plasmids are suitable for the analysis of adenine remethylation on replicative intermediates.

Dam remethylation rate on the leading replicative arm

Pre- and post-replicative molecules can be resolved in 2D agarose gels and eluted with high precision. Plasmid pDam/KS.o (Figure 2A) containing the KS fragment in the orientation that allows the reappearance of methylated adenine to be analysed on the leading replicative arm (Figure 1A), was grown in *E.coli* and enriched for replicating molecules. After cutting with *Kpn*I, four equal samples were resolved in parallel by preparative neutral/neutral 2D gel electrophoresis. One of the 2D gels was blotted and hybridized with a KS probe while the other three were stored for elution of replicative intermediates. The single *Kpn*I recognition site is located 2400 bp from the replication initiation/termination point at the distal end (with respect to ColE1 ori) of the KS fragment, which is approximately half the size of the plasmid length. *Kpn*I digestion resulted in two classes of replicating molecules, differing in mass and shape (Figure 2B), namely bubble-containing replicative intermediates (the early stages of pDam/KS.o replication) and asymmetric double-Y forms (late replicative intermediates). As shown schematically (Figure 2C) and visualized experimentally by Southern

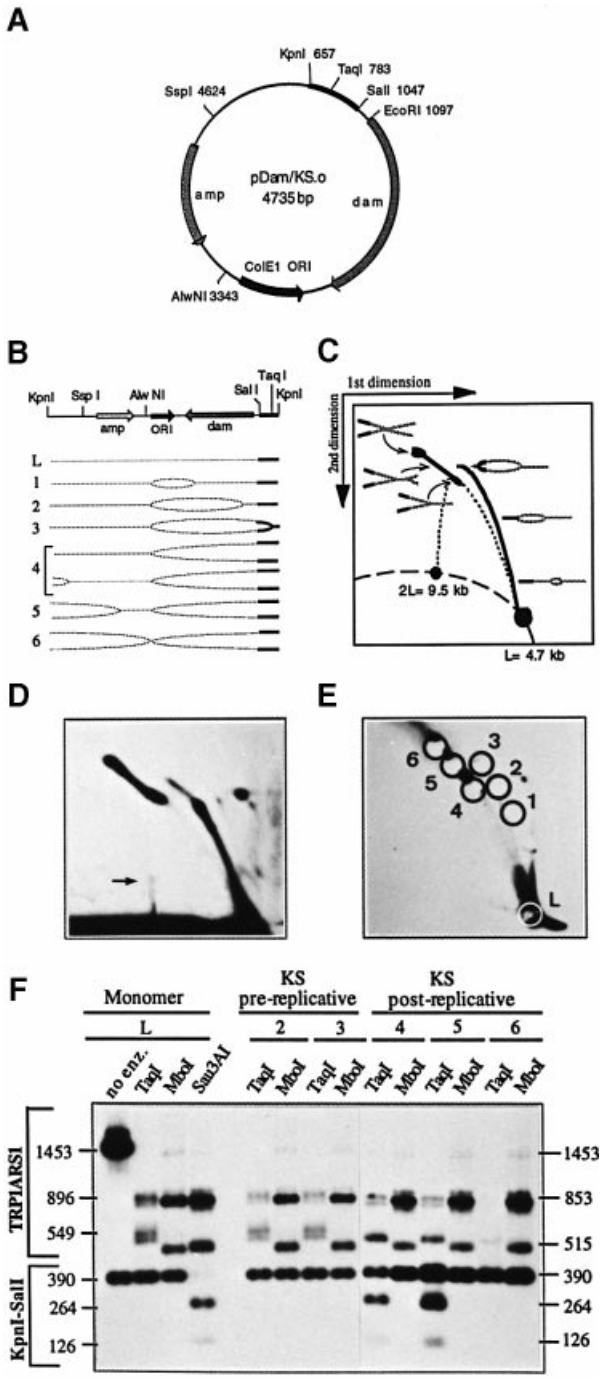


Fig. 2. Analysis of Dam remethylation on the leading replicative arm. (**A**) Restriction map of plasmid pDam/KS.o. The inserted *Kpn*I–*Sal*I fragment, replication origin and the two genes β-lactamase (*amp*) and *Dam* methylase are indicated. (**B**) Schematic representation of *Kpn*I-linearized pDam/KS.o and pDam/KS.o replicative intermediates. Note that the target KS fragment (black bar) remains at the very end of the linearized molecules (L). 1, 2 and 3 are the molecules containing the replication bubble in advancing stages of progress through the plasmid sequence and the KS fragment in pre-replicative state. After the replication fork passes the *Kpn*I recognition site, the digested replicating molecules have asymmetric double-Y shape (see 4, 5 and 6) and there the KS sequence has already been replicated. (**C**) Graphical representation of the arcs containing bubble and asymmetrical double-Y replicating molecules in 2D gel electrophoresis. Note that the arcs representing the two classes of replicating molecules (1, 2, 3 and 4, 5, 6 in B) do not overlap at any point. Linear non-replicating monomers (L) are represented by the spot of 4.7 kb. When Y-shaped intermediates are nearly fully replicated (Figure 4C) they migrate at position 9.5 kb (2L). The dotted-Y arc corresponds to simple-Y replicative intermediates that arise from circular plasmid dimers (see also Figure 4). (**D**) 2D gel of pDam/KS.o replicative intermediates. Both the bubble and the asymmetric double-Y arcs are clearly visible. The arrow indicates that the simple-Y arcs arise from the dimers (see also Figure 4). (**E**) Seven agarose pieces were excised along the arcs from preparative gels: L from linear molecules; 1, 2 and 3 from the bubble arc; 4, 5 and 6 from asymmetrical double-Y. They were blotted and hybridized with KS probe. The empty circles represent the position of excised agarose regions containing replicating molecules that were assayed with *Taq*I for Dam methylation. The very faint intensity of the residual bubble arc is due to inefficient transfer to membranes from the low melting agarose gels. (**F**) All recovered samples of pDam/KS.o replicative intermediates were redigested with *Sal*I, mixed with 500 ng of *Eco*RI-linearized yeast plasmid TRP1ARS1, split into two aliquots and cut with *Taq*I or *Mbo*I. Linear molecules were divided into four aliquots and cut with *Taq*I, *Mbo*I or *Sau*3A. The very first lane is undigested KS fragment from linear plasmid. The sizes of *Taq*I-derived DNA fragments are shown at the left side of the panel, *Mbo*I digestion products on the right side. The upper part of the panel shows that TRP1ARS1 were digested to completion. KS fragments digested with *Taq*I (246 and 126 bp) appear immediately after replication (4) and disappear rapidly at a distance of 1.4–2 kb behind the replication fork. Note that the inefficient transfer of the 126 bp fragment is responsible for underrepresentation. In the line corresponding to *Taq*I digestion of sample 6, the added TRP1ARS1aliquot was underestimated. However, the barely detectable 515 bp band suggests that the digestion was complete.

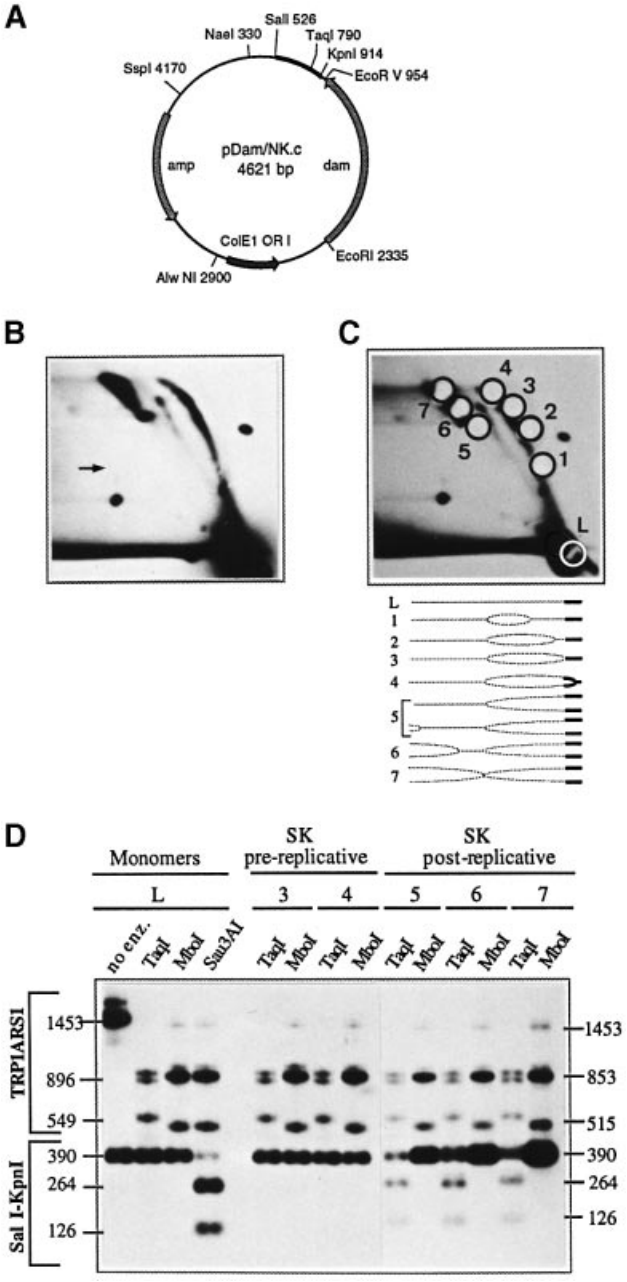


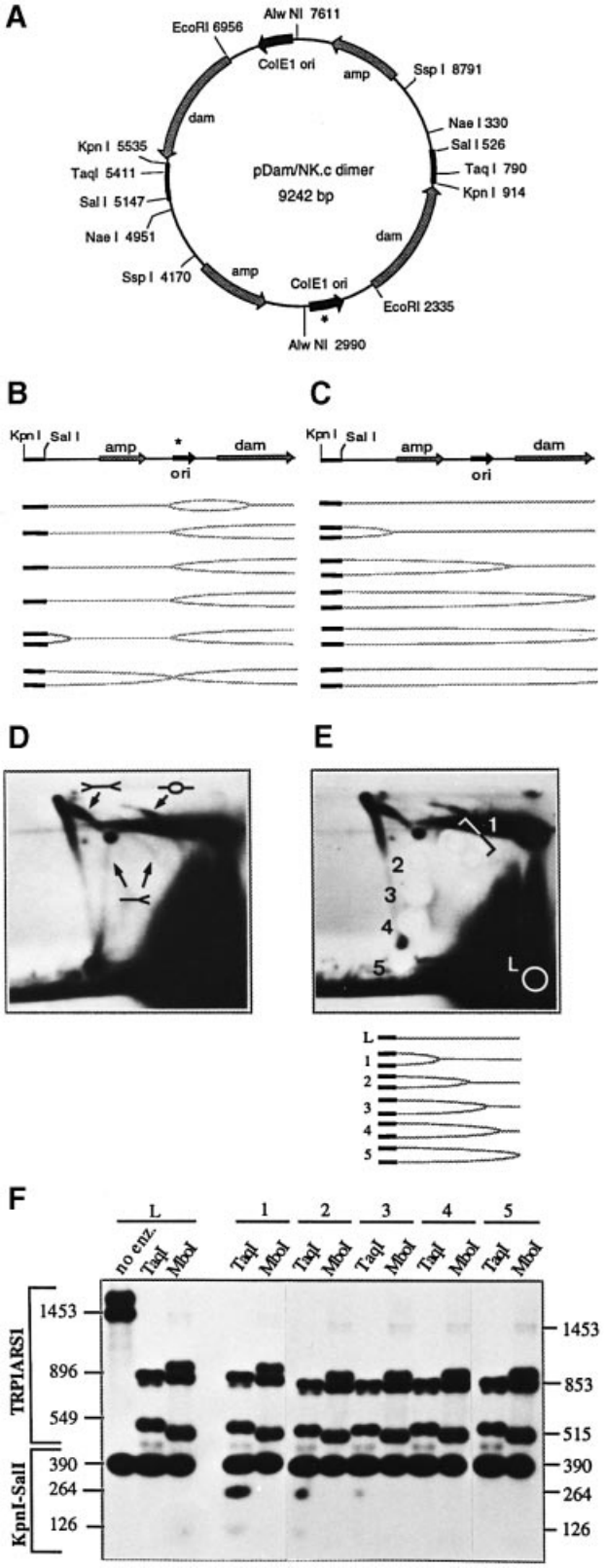
Fig. 3. Dam remethylation of the lagging replicative arm. (**A**) A circular map of plasmid pDam/NK.c. (**B**) 2D gel electrophoresis of pDam/NK.c replicative intermediates linearized with *Nae*I. *Nae*I, similar to *Kpn*I for pDam/KS.o, produces a gel pattern that consists of two arcs, a bubble arc where the SK fragment is not yet replicated and an asymmetrical double-Y arc carrying recently replicated SK. (**C**) Example excision of samples containing pDam/NK.c linear molecules (L) and replicative intermediates (1–7). The drawing below indicates that samples 1, 2, 3 and 4 represent bubble-shaped replicating molecules, 5, 6 and 7 those having an asymmetrical double-Y fork. (**D**) Purified linear plasmid molecules and replicative forms were digested with *Sal*I and *Kpn*I to obtain SK fragments, divided into two aliquots and analysed for Dam methylation by *Taq*I and *Mbo*I restriction enzymes in the presense of TRP1ARS1. Eluted linear molecules were divided into four aliquots and were further digested with *Taq*I, *Mbo*I and in addition with *Sau*3A. The fragments in the upper part of the panel reveal complete digestion of the control plasmid TRP1ARA1. In the lower part, progressively decreasing *Taq*I digestion of the SK fragment can be seen in all three samples 5, 6 and 7 where the replication fork is moving away from the analysed sequence. Therefore, the 2.4 kb length of the newly replicated plasmid is too short to detect the restored fully methylated *Taq*I site.

blot hybridization (Figures 2D and 3B), the bubble-containing molecules produced by *Kpn*I cleavage migrated in 2D gel electrophoresis as a shortened bubble arc. The asymmetric double-Y forms were represented by an incomplete diagonal, which started from a point immediately below the end of the bubble arc and proceeded up to the termination point (Figure 2C and D). The bubble arc contained plasmid molecules where the KS fragment had not yet been replicated (pre-replicative KS) or those where the fragment was in a process of replication (at the very end of the bubble arc). The asymmetric double-Y forms were those where the replication fork was moving away from the KS sequence (post-replicative KS). Note that a simple-Y arc was also visible, especially after long exposures of the autoradiograph, due to the presence of plasmid dimers (dotted arc in Figure 2C and arrow in 2D). The different migration of replicative intermediates (Friedman and Brewer, 1995) carrying KS in the pre- and post-replicative states enabled their separate purification from the agarose gels and analysis for Dam remethylation.

The autoradiograph of the hybridized 2D gel was superimposed on the three stored gels. Seven agarose slabs of equal size were excised and the DNA was quantitatively recovered from agarose (see Figure 2E and Materials and methods for details). Sample L contained DNA derived from the gel area corresponding to the position of the non-replicating plasmid monomers. Samples 1, 2 and 3 were excised along the bubble arc and contained KS pre-replicative molecules where the replication fork was located ~800, 1600 and 2300 bp from the ColE1 ori. DNA in the last three samples 4, 5 and 6 (~3100, 3900 and 4700 bp from ColE1 ori) was purified from areas along the asymmetric double-Y diagonal that contained replicative intermediates with post-replicative KS sequences (Figure 2E). Sample 3, from the very edge of the bubble arc most probably contained plasmid molecules where the KS sequence was in the process of replication. All eluted samples were cleaved with *Sal*I, mixed with 50 ng *Eco*RI-linearized yeast TRP1ARS1 plasmid and divided into two aliquots each. The two sets of aliquots were digested with *Taq*I or *Mbo*I, respectively. Since enough DNA was available from agarose-purified non-replicating monomers (L) this sample was divided into four equal aliquots. One was left as undigested control and the other three were digested with *Taq*I, *Mbo*I and *Sau*3AI. All the digests were electrophoresed in 1.8% agarose gels, blotted and hybridized with the KS–TRP1ARS1 hybrid probe. The hybridization signals were detected by autoradiography or by PhosphorImager for quantitative analysis.

In sample L (Figure 2F), as expected, ~100% of 390 bp KS fragments were resistant to *Taq*I and *Mbo*I, and almost completely (93 ± 2%) cleaved by *Sau*3AI (into 246 and 126 bp), indicating the fully methylated state of the non-replicating parental strand also maintained in samples 1 (not shown), 2 and 3. The absence of any *Taq*I and *Mbo*I digestion products in samples 2 and 3 indicates that the symmetrically methylated parental state has not been changed before replication (Figure 2F, 2 and 3). A clear change in the digestion pattern can be observed in sample 4, corresponding to plasmid molecules where the KS sequence has very recently been replicated (~800 bp from KS and ~2300–3100 bp from ori). Of the order of 45%

of the fragments were cleaved by *Taq*I, but not by *Mbo*I (Figure 2F, 4). This is consistent with the presence of hemimethylated DNA in the *Taq*I recognition site on the leading replicative arm immediately after replication. The lack of *Mbo*I digestion suggests that both daughter strands are hemimethylated at this time point and do not undergo sudden, complete demethylation. In the next sample, 5, where the replication fork is moving up to 1600 bp away from the KS fragment (~3100–3900 bp from ori), the methylation of the *Taq*I recognition sequence on the leading strand starts to be restored (Figure 2F, 5) and ~70% of the KS fragments (Figure 5, dotted curve) are already resistant to *Taq*I. Symmetrically methylated *Taq*I sites are present in the last sample 6, where the replication fork is located between ~1600–2300 bp from the analysed sequence (and ~3900–4300 bp from ori), since no *Taq*I cleavage can be detected (data summarized in Figure 5). Simultaneous digestion of the internal control plasmid TRP1ARS1 (the upper part of Figure 2F) reveals the virtual absence of partially digested DNA in any of the reactions. This indicates that the inability of *Taq*I to cleave in its recognition site is determined by the methylation state within the analysed KS sequence. From the above experiment, we conclude that Dam remethylation takes place ~2000 bp behind the replication fork on the leading replicative arm (see also Figure 5 for the data summary). Since the average speed of replication fork progression in prokaryotes *in vivo* is ~1000 bp/s (Marians, 1992), consistent with the doubling time of plasmid-transfected Xl-1 cells used in our assay (see Materials and methods), the distance of 2000 bp corresponds to ~2 s after replication.



Dam remethylation rate on the lagging replicative arm

This experiment employed plasmid pDam/NK.c (Figure 3A), which contains the KS fragment in opposite orientation with respect to ColE1 ori (SK). Here, only the lagging replicative arm is accessible to *Taq*I when hemimethylated after replication (Figure 1B). The leading arm and parental unreplicated strand are not digestible by *Taq*I and *Mbo*I. Replicative intermediates of pDam/NK.c were isolated as described above, linearized with *Nae*I and resolved in four 2D neutral/neutral gels. *Nae*I restriction

Fig. 4. Extended analysis of Dam remethylation on the lagging replicative arm. (**A**) A circular map of pDam/NK.c plasmid dimer. The potentially active origin is indicated with an asterisk. (**B** and **C**) Schematic representations of replicative intermediates derived of *Kpn*I-linearized pDam/NK.c plasmid dimers. Left drawing (B) shows the half of the plasmid that contains the actively initiating ColE1 ori, the right drawing (C), the passively replicated part. Note that in (B), the SK fragment is located at the very beginning of the molecule and the replication fork is moving away from the SK sequences. Replicative intermediates have a similar shape to those shown in Figure 3. In (C), the whole second half of the plasmid is replicated from a replication fork initiated in (B) and therefore containing only simple-Y replicative forms. The SK fragment is located at the very beginning of the molecule but the replication fork enters it immediately after passing the *Kpn*I site. (**D**) Overexposed autoradiograph of pDam/NK.c digested with *Kpn*I. The actively replicated half of the plasmid displays both the bubble and asymmetric double-Y arcs, which overlap with those from the plasmid monomer and therefore show higher intensity after hybridization. Here, compared with the *Kpn*I digestion shown in Figure 3, the bubble arc is 584 bp shorter and the asymmetric double-Y arc is 584 bp longer. The passively replicated half of the dimer appears as a faint simple-Y arc which meets the asymmetric double-Y arc at the point corresponding to 2.3 kb as expected. The positions of the three arcs are indicated with arrows and drawings. (**E**) Four agarose slices were excised along the simple-Y arc; 1 comprises the broader area shown with a bracket. In 3 and 4, the replication fork had moved further than in samples 5, 6 and 7 of Figure 3. (**F**) Replicative intermediates were recovered from the agarose slices and analysed by *Taq*I and *Mbo*I digestion as described previously.

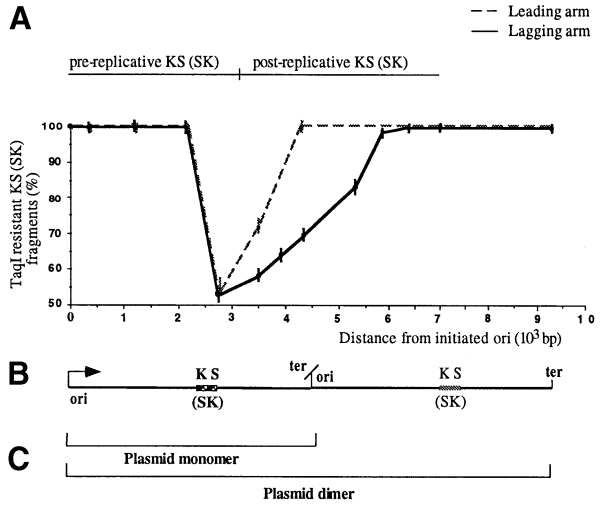


Fig. 5. Post-replicative Dam remethylation rates. (**A**) The amount of post-replicative KS (SK) fragments uncut by *Taq*I (in %) from the experiments shown in Figures 2, 3 and 4 were plotted against the distance from the ColE1 origin of replication. The amount of *Taq*I-resistant fragments is the average of the data obtained from three individual gels for each replicative arm. The bars indicate the standard deviation. (**B** and **C**) A graphical alignment of plasmid sequences corresponding to the distance from ori indicated in (A). The position of KS (SK) fragments that were analysed for Dam remethylation on the leading (plasmid monomer only) and on the lagging replicative arm (monomer and dimer) are indicated.

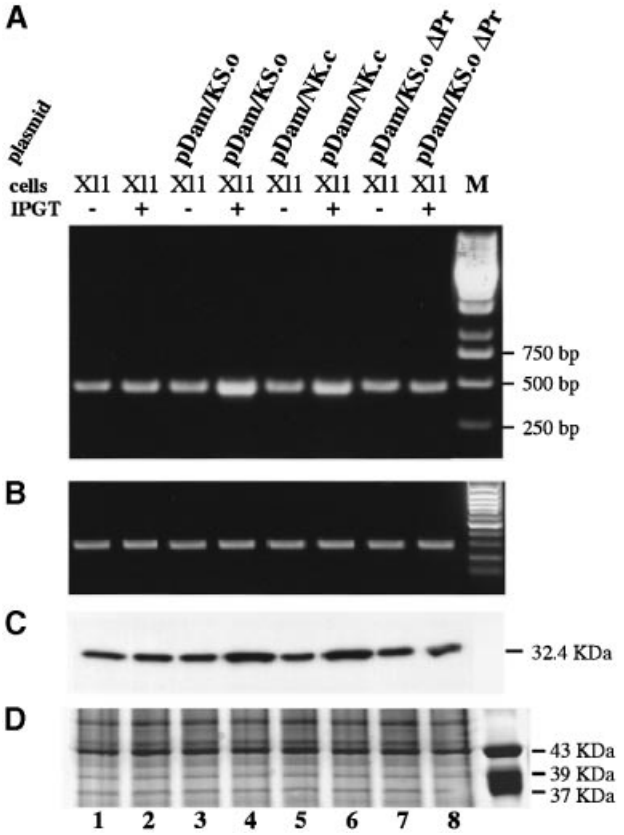


Fig. 6. Dam gene expression in wild-type and plasmid-transfected Xl-1 cells. (**A**) Total RNA was purified from Xl-1 blue untransfected and plasmid-transfected cells grown with or without IPTG (1 mM final concentration). Quantitative RT–PCR of Dam methyltransferase transcripts revealed that Dam is not overexpressed in the absence of IPTG. M is a 1 kb ladder DNA marker (Fermentas). The sizes of the bands are indicated. (**B**) Control RT–PCR for ribosomal protein L32 mRNA derived from the same cells as in (A). M is a 100 bp ladder DNA marker (Fermentas). (**C**) Immunological detection of Dam protein in wild-type and plasmid-transfected Xl-1 blue cells grown without or with IPTG (as in A and B). (**D**) A fragment from a 10% SDS–polyacrylamide gel similar to the one used in (C), indicating equal loading of protein extracts. The order of loading on the gel is the same as in A, B and C. Lane M contains a protein size marker.

enzyme (analogous to *Kpn*I in the case of pDam/KS.o) cleaves the plasmid at a distance 2370 bp from the ColE1 ori. The *Nae*I cleavage results in similar 2D gel patterns to these described above for pDam/KS.o with a bubble and asymmetrical double-Y arc (Figure 3B). Replicative intermediates were recovered from seven gel slabs (Figure 3B). Four of them (along the bubble arc) contained pre-replicative SK sequences and the other three (along the asymmetric double-Y arc) contained the recently replicated SK fragment (Figure 3C). Non-replicating plasmid monomers (L) were also purified from the gels as control for the initial methylation state (Figure 3C, L). All replicative intermediates recovered were cleaved with *Sal*I and *Kpn*I. Two of the SK pre-replicative samples (Figure 3C, 3 and 4) and the three post-replicative samples (Figure 3C, 5, 6 and 7) were split into two aliquots, mixed with 50 ng of *Eco*RI-linearized TRP1ARS1 and further digested with *Taq*I or *Mbo*I. Four aliquots of purified pDam/NK.c monomers (L) were processed as described for pDam/KS.o. For the non-replicating monomers (Figure 3D, L) and the SK pre-replicative samples (Figure 3D, 3 and 4), there was no detectable cleavage by *Taq*I of either the parental strand or of replicative intermediates where the replication fork was ~1600 and ~2300 bp away from the ColE1 ori. However, when SK is replicated (~2300–3100 bp from ori), nearly half of the SK fragments were accessible to *Taq*I, indicating the presence of hemimethylated DNA on the lagging replicative arm behind the replication fork (Figure 3D, 5). The proportion of *Taq*I-digested SK fragments decreased slightly in the next two samples (Figure 3D, 6 and 7) where the replication fork had moved ~1600 bp (~3900 bp from ori) and ~2300 bp (~4700 bp from ori) away from the SK sequence (data summarized in Figure 5; note that on the abscissa, the numbers correspond to the proportion of *Taq*I-resistant SK fragments). Surprisingly, in comparison with the leading replicative arm, even in the last sample, 7 (Figure 3D), >30% of the SK fragments were still accessible to *Taq*I when the replication fork was approaching the ori/termination point of pDam/NK.c (Figure 3D, 7). This indicates that the post-replicative Dam remethylation on the lagging arm was incomplete when the symmetrically methylated adenines of the GATC sites were already restored on the leading replicative arm (compare with Figure 2F and see Figure 5).

Extended analysis of Dam remethylation on the lagging replicative arm

To follow further the remethylation on the lagging replicative arm, we analysed the plasmid dimers present in all preparations of plasmid replicative intermediates. Dimerization is a naturally occurring event (Summers and Sherrat, 1984). In *E.coli* a recombination mechanism exists that resolves plasmid dimers to monomers (Baker, 1991). As demonstrated by Martin-Parras *et al.* (1991) on dimerized plasmid molecules, replication can initiate

efficiently from only one of the two ColE1 origins: the second origin causes temporal pausing of the traversing replication fork. After cleavage with a restriction enzyme, the replicating plasmid monomers that have the passive origin appear as a simple-Y arc in 2D neutral/neutral gels (easily visible in overexposed autoradiographs Figures 2D and 3B, and not shown). The presence of these dimers in plasmid preparations (~10% of the total replicative intermediates) permitted us to analyse Dam remethylation at >2300 bp from the target SK sequence (Figure 4A, B and C). We digested isolated pDam/NK.c replicative intermediates with *Kpn*I. The cleavage again resulted in bubble and asymmetric double-Y arcs in 2D gels and, in addition, in a simple-Y arc (Figure 4D). *Kpn*I digestion places the SK sequence behind the replication fork on the half of the plasmid dimer that contains the active origin (Figure 4B). On the second half which has the passive ori, the SK fragment is located at the entry point of the replication fork (Figure 4C), enabling Dam remethylation to be followed over the remaining 4621 bp of the plasmid dimer (see also Figure 4A). We excised five areas along the simple-Y arc from preparative 2D gels. Sample 1 (Figure 4E) contained replicative intermediates where the replication fork was moving between ~1800 and ~2100 bp away from SK. Samples 2, 3, 4 and 5 contained late replicative intermediates where the replication fork was moving through the second half of the plasmid between ~4900 and ~7000 bp from the active ori (Figure 4E). Control non-replicating monomers (L) were excised and processed as described above. The DNA was recovered from the agarose, digested with *Sal*I, mixed with TRP1ARS1 and additionally cut, either with *Taq*I or *Mbo*I. The digests were analysed in 1.8% agarose gels followed by Southern blot transfer and hybridization (Figure 4F). The *Taq*I digestion pattern reveals that from sample 1 (which corresponds to a point between 6 and 7 of Figure 3D with SK located from ~1800 to ~2100 bp behind the replication fork) to samples 2 and 3 (Figure 4F), the proportion of *Taq*I-resistant SKs progressively increases as the replication fork proceeds further away from the SK sequence (Figure 4F, 2 and 3). SK fragments completely (100%) inaccessible to *Taq*I were present only in samples 4 and 5, ~3700 to ~4300 bp away, repectively, from the analysed sequence (Figure 4F, 4 and 5). We conclude that the time needed to restore symmetrically methylated adenines in the analysed GATC site on the lagging arm is about twice as long as on the leading strand, respectively 3.5–4 s (corresponding to 3500–4000 bp) compared with ~2 s (see also Figure 5).

Discussion

The rate of remethylation of the *E.coli* genome by Dam has been a disputed issue. The methyladenine content in radioactively pulse-labelled (from 30 s to 30 min) fractions of newly synthesized DNA measured in different experiments varied from complete absence immediately after replication (Lyons and Schendl, 1984) to its definite presence already in the replication fork (Szyf *et al.*, 1982). However, even the shortest pulse of 30 s allows the replication fork to travel over 30 kb. In addition, neither of the above-mentioned studies excluded the possible contamination of the labelled fractions by sequences that derive from DNA excision repair. More accurate studies to estimate the remethylation rate of various *E.coli* chromosomal sequences utilized a synchronously replicating *E.coli* strain carrying a *dnaC*^{ts} allele (Campbell and Kleckner, 1990). According to the accessibility of seven gene-specific fragments to methylation-sensitive restriction enzymes in defined time intervals post-replication, the half-life of hemimethylated DNA was calculated to be between 0.5 and 4.5 min. Assuming that the migration speed of DNA polymerase *in vivo* is, on average, 1000 bp/s (Marians, 1992), this corresponds to a distance of 30–270 kb behind the replication fork. Perhaps both the replication fork movement and remethylation rate may exhibit certain locus and sequence specificity and therefore can be non-uniform over the entire *E.coli* chromosome.

We have also used a methylation-sensitive restriction enzyme assay similar to that previously described by Campbell and Kleckner (1990), to estimate the rate of Dam remethylation immediately behind the replication fork. To avoid sequence bias, a single GATC site was analysed in plasmid replicative intermediates isolated from 2D gels. 2D gel electrophoresis enabled us to distinguish between pre-replicative and post-replicative forms of the target sequence KS (SK) and these forms were analysed separately for their methylation status. Such an approach is of high resolution and provides individual information for the two newly synthesized daughter strands. It also excludes any contamination by sequences that derive from DNA repair. Our analysis of the remethylation rate indicates that methyl-adenine in a single GATC site can be restored as fast as 3.5–4 s after replication on both daughter strands. Independent analysis of the leading and the lagging replicative arms shows that in fact they become remethylated asynchronously. This corresponds to ~2 s for the leading arm and ~3.5–4 s for the lagging replicative arm (Figure 5), assuming that the speed of the fork movement is constant and is ~1000 bp/s (Marians, 1992) and as calculated for the Xl-1 blue cells used in our assay (~800 bp/s).

When the SK fragments are released by *Sal*I and *Kpn*I cleavage from replicative intermediates of pDam/NK.c with post-replicated SK sequence, 50% of the SK fragments arise from the leading strand. As mentioned in the rationale of the assay (Figure 1), these SK fragments are resistant to *Taq*I digestion. The remaining 50% of the *Kpn*I–*Sal*I cleavage-released SK fragments derive from the lagging strand. Therefore, when analysing replicative intermediates with moving forks between 3600 and 4300 bp away from the origin we find that ~65% of the SK fragments are resistant to *Taq*I digestion (Figure 4, lanes 1 and Figure 5), 50% of them belong to the leading arm sequences and only 15% represent the SK fragments that have been remethylated on the lagging arm. Since the KS insert is localized 2400 bp from the origin and since the length of the Okazaki fragments in *E.coli* is between 1000 and 2000 bp (Baker and Wickner, 1992; Kornberg and Baker, 1992), it can be deduced that the SK sequences are in the Okazaki fragments only until the elongation point has moved ~2000 bp away from the SK sequence, or 4400 bp from the origin. We conclude that Dam remethylation on the lagging replicative arm occurs preferentially after ligation of the Okazaki fragments. Surprisingly, a similar estimation has been made recently

for cytosine methylation of DNA behind the replication fork in eukaryotes (Araujo *et al.*, 1998).

Further support for our observations comes from the finding of differences in the heteroduplex mutation frequency on the two replicative daughter arms (Trinh and Sinden, 1991; Veaute and Fuchs, 1993). Secondary structure mispairs, corrected by the mismatch repair pathway (Trinh and Singen, 1991) and covalent adducts removed by excision repair (Veaute and Fuchs, 1993) are both ~20-fold less efficiently corrected on the lagging replicative arm than on the leading strands of *E.coli* plasmids. It seems not only that the replication complex itself possesses an internal asymmetry, but also that the processes occurring immediately after replication tend to be asynchronous. The efficacy of Dam methyltransferase and repair machinery on the lagging replicative arm might be hindered by the large protein complexes (RNase H, DNA polymerase, DNA ligase, etc.) that are involved in the processing of the Okazaki fragments (Baker and Wickner, 1992; Kornberg and Baker, 1992). In eukaryotic cells, DNA cytosine-methyltransferase (Dnmt1) interacts directly with DNA polymerase processivity factor PCNA (Chuang *et al.*, 1997). As elegantly demonstrated by Shibahara and Stillman (1999), PCNA remains bound to the newly replicated SV40 DNA after a round of replication is completed and is involved in chromatin assembly and presumably post-replicative remethylation of the daughter strands. It would be of great interest to find out whether similar mechanisms are also utilized by prokaryotes. In conclusion, our data demonstrate that on plasmid molecules, the Dam enzyme completes its function 2000–4000 bp behind the replication fork and, as such, remethylation can be considered as a rapid but not immediate post-replicative event. Additional experiments are required to determine whether the calculated remethylation rates are representative for more individual GATC sites and whether they apply to the sequences of the *E.coli* chromosome.

Materials and methods

Cells and plasmids

Xl-1 blue *E.coli* strain [F′ Tn*10*proA+B+*lac*^{Iq} (*lacZ*) M15/*recA1 endA1 gyrA96*(*Nalr*) *thi hsdR17*(*rk-mk+*) *supE44 relA1 lac*] was used for analysis of post-replicative Dam remethylation after transfection with plasmids based on the high copy number Bluescript II series (Bluescript II SK+, Stratagene). The 390 bp *Kpn*I–*Sal*I (KS) target sequence was subcloned from the non-transcribed spacer of rat rRNA genes (Yavachev *et al.*, 1986) into pBluescript II SK+. The KS fragment has a unique *Taq*I site overlapped by the Dam methylase recognition sequence (see Figure 1, *Taq*I assay rationale). In pDam/KS.o, the KS fragment was cloned in the orientation 5′*Kpn*I–3′*Sal*I with respect to the direction of replication from the ColE1 origin. pDam/NK.c carries the same KS fragment inserted in the opposite orientation (5′*Sal*I–3′*Kpn*I), indicated as SK. To generate pDam/NK.c, the 390 bp KS sequence was first subcloned in pUC 19, then excised by *Pvu*II and *Kpn*I and introduced in the *Nae*I and *Kpn*I sites of pBluescript SK+. An additional 1.38 kb fragment containing the Dam methylase gene derived from plasmid pTP166 (Marinus *et al.*, 1984) was inserted into both plasmids pDam/KS.o and pDam/NK.c between *Eco*RI and *Eco*RV sites as a *Eco*RI–*Pvu*II fragment. Plasmid pDam/KS.o ΔPr carries *Eco*RI–*Xba*I deletion of the p_{taq} promoter. The doubling time for both plasmid-transfected Xl-1 blue strains used to estimate the Dam remethylation rates was ~50 min, which recalculated into the speed of replication fork progression gives ~800 bp/s. Yeast plasmid TRP1ARS1 was purified from yeast JM12 strain as described by Thoma *et al.* (1984).

Levels of Dam expression in untransfected and plasmid transfected XL-1 blue cells

We introduced into both plasmids pDam/KS.o and pDam/NK.c a copy of the *Dam* gene under the control of the IPTG-inducible p_{taq} promoter (Brosius and Holy, 1984), since initially we intended to overexpress the *Dam* gene. To exclude the possibility that in the absence of IPTG induction, plasmid-transfected cells contained higher than wild-type levels of Dam methyltransferase, total RNA was isolated from untransfected Xl-1 blue cells and cells transfected with pDam/KS.o and pDam/NK.c. Total RNA was also prepared from the same three strains grown in the presence of IPTG. Additional controls included IPTG-induced and uninduced Xl-1 cells transfected with pDam/KS.o ΔPr, where the p_{taq} promoter had been deleted from the original pDam/KS.o plasmid. The abundance of *Dam* transcripts in all strains was estimated by quantitative RT–PCR (Figure 6A) in comparison with ribosomal protein L32 (Figure 6B). Untransfected Xl-1 cells and those carrying pDam/KS.o and pDam/NK.c, but grown without IPTG, contained a similar amount of *Dam* mRNA (compare lanes 1, 3 and 5 in Figure 6A). A similar amount of *Dam* RNA was present in Xl-1 cells transfected with pDam/KS.o ΔPr (Figure 6A, lane 7 and 8). Xl-1 cells transfected with pDam/KS.o and pDam/NK.c and grown upon IPTG induction contained slightly more *Dam* transcripts than the corresponding uninduced transfected strains and the wild-type Xl-1 blue cells (Figure 6A, compare lanes 3 with 4 and 5 with 6). RT–PCR was confirmed by immunoblot analysis (see below) of protein extracts prepared from uninduced and IPTG-induced bacterial strains using Dam methyltransferase antibodies (Figure 6C).

Quantification of Dam methylase mRNA by RT–PCR

A single colony of *E.coli* Xl-1 Blue cells, untransfected or transfected with pDam/SK.o, pDam /NK.c or pDam/KS.o ΔPr, was grown in Luria–Bertani (LB) media to OD 0.1. The cultures were divided to two halves. One was grown as uninduced (IPTG–) control and to the second IPTG was added to a final concentration of 1 mM. The cells were allowed to grow to OD_{600} 0.3–0.4. The cultures were pelleted by centrifugation at 4000 r.p.m. (Joan CR 422) and total RNA was isolated using the Purescript RNA isolation kit (Gentra) according to the manufacturer's protocol. RNA concentration was measured by spectrophotometry ($A_{260/280}$). RNA samples were treated with 5 U RNase-free DNase (Promega) for 15 min at 37°C and all the samples were checked by electrophoresis in denaturing 1% agarose gels.

Total RNA (1 μg) from each sample was used for cDNA synthesis by Superscript II reverse transcriptase (Gibco, Life Technologies) according to supplier's protocol. All cDNA samples were diluted 20-fold and RT–PCR amplification was performed with Red Hot *Taq* polymerase (BioGene) for Dam transcripts and ribosomal protein L32 as a control. Dam primers were: reverse TTCTCGTTACATCCTTGCCG, forward TCGCAATAGACGACGGATGC. L32 primers were: reverse ATGCGAAGGTGACGGTAACG, forward GCTTCTTCAGGCAGTTCACC. A typical 100 μl RT–PCR reaction included 2 μl of a 20× dilution of each cDNA (or 1 μl cDNA derived from IPTG-induced cells transfected with pDam/KS.o and pDam/NK.c), 50 pmol of each primer, 50 μM $MgCl_2$ and 1 × *Taq* buffer. Amplification cycles were: 5 min denaturing step followed by 30 s at 95°C, 1 min at 51°C and 30 s at 72°C. Last extension step was 5 min at 72°C. Dam transcripts were amplified in 35 PCR cycles, L32 in 30 cycles. After amplification the PCRs were ethanol precipitated, resuspended in 15 μl dH_2O and loaded on 1.6% agarose gels (0.5 μg/ml ethidium bromide). For quantification of PCR products, the gels were scanned directly using FLA 2000 Fluorescent Image Analyzer (Fujifilm), fluorescent reading at 473 nm excitation and the 0580 filter. RT–PCR products were quantified by Aida 2.0 (Advanced Imager Analyzer) software. Both sets of primers were tested for linear amplification range using 5×, 10× and 20× (or 10×, 20×, 50× in the case of the IPTG-induced Dam-overexpressing strains) dilutions of cDNA for each sample.

Immunodetection of Dam in protein extracts

Xl-1 cells, untransfected and transfected with plasmids pDam/KS.o, pDam/NK.c and pDam/KS.o ΔPr were grown in 10 ml of LB media to OD_{600} = 0.1 and induced by IPTG as described above. At OD_{600} = 0.3–0.4, the cells were collected by centrifugation at 4000 r.p.m., 4°C in a table-top centrifuge (Joan CR 422) and homogenized in 500 μl 1 × Laemmli buffer. Aliquots of 15 μl were run in 10% SDS–polyacrylamide gels and electrotransferred to polyvinylidenefluoride membranes (Micron Separations Inc.). The filters were blocked for 1 hour at room temperature in MAB (100 mM maleic acid, 150 mM NaCl pH 7.5) containing 2% blocking reagent (Boehringer) and incubated overnight at 4°C with 1:1000 dilution of Dam methyltransferase antibody in MAB, 2% blocking reagent, 10% fetal calf serum. Immunoblots were washed three times for 30 min each with phosphate-buffered saline (PBS), 1% Tween and incubated for 1 h with a secondary, anti-mouse HPRT-conjugated

antibody (dilution 1:1000), followed by three washes for 30 min each with PBS, 1% Tween. The signal was detected using enhanced chemiluminescence reagents (Amersham).

Isolation and BND–cellulose enrichment of replicative intermediates

Xl-1 blue cells were transfected with pDam/KS.c or pDam/NK.o and grown in 1 l LB media to low optical density, which was optimized for each plasmid (OD_{600} = 0.45 for pDam/KS.c and OD_{600} = 0.3 for pDam/NK.o). Plasmid replicative intermediates (RIs) were isolated according to the procedure described by Martin-Parras *et al.* (1991), resuspended in 500 µl TE (10 mM Tris–HCl, 1 mM EDTA, pH 8) and stored at 4°C. In some cases, before 2D gel electrophoresis, the restricted linearized plasmids were enriched for RIs on 0.4 ml bed volume BND (benzoylated naphthoylated DEAE, Sigma) cellulose columns (Bio-Rad Polyprep column) as described by Dijkwel *et al.* (1991). After BND chromatography, the two plasmid DNA-containing fractions (salt and caffeine wash) were precipitated with 1/10 vol sodium acetate and absolute ethanol overnight at –20°C, centrifuged at 4500 r.p.m. in a tabletop centrifuge (Joan CR 422), washed twice with 70% ethanol and resuspended in an appropriate volume of TE (usually 50 µl for the caffeine wash and 150 µl for the salt wash). The caffeine-eluted fraction enriched for replicating molecules was used for preparative 2D gel electrophoresis.

Neutral/neutral 2D gel electrophoresis

Neutral/neutral 2D gel electrophoresis was performed essentially as described by Brewer and Fangman (1987). The plasmid RIs were linearized with appropriate restriction enzymes and were first resolved by molecular mass in 25/25 cm 0.4% agarose gels (Sigma, Type II-medium EEO), 1 × TBE buffer (89 mM Tris, 89 mM borate, 2 mM EDTA) at 35 V for 24 h (first dimension). The lanes containing plasmid and plasmid replicative forms were excised and placed horizontally on the electrophoretic tray. The second dimension was run in 1% low gelling temperature agarose, 1 × TBE gels containing 0.5 µg/ml ethidiun bromide at 140 V for 16 h at 4°C. Four samples were resolved simultaneously in one electrophoretic tray. After electrophoresis, three of them were stored at 4°C and one was blotted and hybridized with the *Kpn*I–*Sal*I 390 bp (KS) rDNA probe labelled with [α-^{32}P]dCTP (Random Priming Kit, Pharmacia). Hybridization was performed according to the standard Z-membrane commercial protocol using Biodyne B membranes (Pall).

Purification of replicative intermediates from 2D agarose gels and TaqI assay

After hybridization of the reference filters, blotted from one out of the four preparative 2D agarose gels (see above) and exposure overnight at room temperature, the autoradiograph was superposed on each of the three stored 2D gels. The spot corresponding to the plasmid monomer was used as an adjustment point and the stripe of agarose from the first dimension, visible in the preparative ethidium bromide gels of the second dimension. Areas containing molecules corresponding to the pre-replicative and post-replicative state of the KS sequence and the plasmid monomers were excised from the gels as described by Lucchini and Sogo (1995). The plasmid DNA was recovered using the Gelase^{TM} high activity protocol (Epicentre Technologies). DNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and once by chloroform:isoamyl alcohol, ethanol precipitated and each sample was dissolved in 50 µl TE. Replicative intermediates were digested either with *Sal*I (pDam/NK.o) or with *Kpn*I and *Sal*I (pDam/SK.c) to obtain the KS fragments. All recovered samples except non-replicating monomers (L) were divided into two aliquots. The first set was further digested with *Taq*I and the second with *Mbo*I. Sample L in cases shown in Figures 2 and 3 was divided into four equal aliquots of which one was kept as undigested control and the other three were further digested with *Taq*I, *Mbo*I or *Sau*3A. Restriction digestion was done according to the manufacturer's protocol. The digestion was stopped by adding EDTA to 10 mM final concentration and all the plasmid DNA samples were loaded on a 1.8% agarose gel. After the electrophoresis (5.5 h, 65 V), the gel was blotted and hybridized with a 1:1 mixture of [α-^{32}P]dCTP labelled (as described above) KS probe and yeast plasmid TRIP1ARS1.

Quantification of TaqI-sensitive KS fragments

The hybridized filters following conventional autoradiography were scanned by PhosphorImager after 1–3 days' exposure. The amounts of *Taq*I-resistant fragments and *Taq*I-derived digestion product (260 bp) were calculated for each lane after correcting for loading error. The loading error was estimated by dividing the values of total radioactivity per lane for the control by the *Taq*I-digested lanes. The data were plotted using Microsoft Excel. All experiments shown in Figures 2, 3 and 4 were repeated at least three times. The standard deviation for each sample is indicated by bars in Figure 5.

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