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# Chromosomal replication incompatibility in Dam methyltransferase deficient *Escherichia coli* cells

**Anders Løbner-Olesen[1,2,3] and Ulrik von Freiesleben[4]**

[1]University of Colorado at Boulder, Dept of Molecular Cellular and Developmental Biology, Campus Box 347, Boulder, CO 80309 and [4]Tufts University Medical School, Dept of Molecular Biology and Microbiology, 136 Harrison Ave, Boston, MA 02111, USA
[2]Present address: Novo Nordisk A/S, Dept of Molecular Biology and Virology, Mørkhøj Bygade 28, DK-2860 Søborg, Denmark

[3]Corresponding author

**Dam methyltransferase deficient *Escherichia coli* cells containing minichromosomes were constructed. Free plasmid DNA could not be detected in these cells and the minichromosomes were found to be integrated in multiple copies in the origin of replication (*oriC*) region of the host chromosome. The absence of the initiation cascade in Dam⁻ cells is proposed to account for this observation of apparent incompatibility between plasmid and chromosomal copies of *oriC*. Studies using *oriC*–pBR322 chimeric plasmids and their deletion derivatives indicated that the incompatibility determinant is an intact and functional *oriC* sequence. The *seqA2* mutation was found to overcome the incompatibility phenotype by increasing the cellular *oriC* copy number 3-fold thereby allowing minichromosomes to coexist with the chromosome. The replication pattern of a wild-type strain with multiple integrated minichromosomes in the *oriC* region of the chromosome, led to the conclusion that initiation of DNA replication commences at a fixed cell mass, irrespective of the number of origins contained on the chromosome.**
*Keywords*: chromosome replication/Dam methylation/ *E.coli*/initiation cascade/minichromosomes

## Introduction

Initiation of chromosome replication in *Escherichia coli* occurs at a unique sequence, *oriC* (Marsh and Worcel, 1977; von Meyenburg *et al.*, 1978). The first step in the initation process is the binding of the initiator protein DnaA to its four recognition sequences (DnaA boxes) within *oriC* (Fuller *et al.*, 1984). This induces opening of the double helix at the three adjacent 13-mer AT-rich sequences and allows for entry of the replication machinery (Bramhill and Kornberg, 1988; Gille and Messer, 1991). It is generally believed that chromosome replication is controlled in such a way that initiation occurs when a certain cell mass per origin (the initiation mass) has accumulated (Donachie, 1968). Accumulation of DnaA protein seems to be the element controlling the initiation mass, since a good correlation between *dnaA* gene expression and time of initiation in the cell cycle has been established (Løbner-Olesen *et al.*, 1989). The initiation mass is fairly constant at different bacterial growth rates (Hansen *et al.*, 1991b; Wold *et al.*, 1994).

A complete cycle of chromosome replication and cell division takes ~60 min (Cooper and Helmstetter, 1968). Consequently, initiation in fast growing cells occurs before the previous cycle has completed. Fast growing cells, therefore, initiate DNA replication on multiple origins. A remarkable feature of the initiation process is that all origins within a single cell are initiated virtually simultaneously, and wild-type cells contain $2^n$ origins [where $n = 0, 1, 2, 3$ etc. (Skarstad *et al.*, 1986)], which is referred to as the synchrony phenotype. The initiation cascade has been proposed to account for this synchrony by initiating all cellular origins within a short time interval in a cascade like manner (Hansen *et al.*, 1991a; Løbner-Olesen *et al.*, 1994). The initiation cascade allows maintenance of a high copy number of minichromosomes, i.e. plasmids replicating exclusively from *oriC* (Messer *et al.*, 1978; von Meyenburg *et al.*, 1979; Stuitje and Meijer, 1983; Løbner-Olesen *et al.* 1987; Jensen *et al.*, 1990), and their replication occurs in synchrony with the host chromosome (Leonard and Helmstetter, 1986).

When fully methylated minichromosomes are introduced into Dam methyltransferase (*dam*) deficient host cells, the hemimethylated form of the plasmid accumulates which is inert for further initiations (Russell and Zinder, 1987). The hemimethylated minichromosomes are bound specifically to the outer membrane (Ogden *et al.*, 1988) and they stay sequestered for approximately one third of the cell cycle (Campbell and Kleckner, 1990). Attempts to identify the hemimethylated origin binding protein has resulted in the identification of the *seqA* gene product (Lu *et al.*, 1994; von Freiesleben *et al.*, 1994). Apart from this block on establishment of minichromosomes in *dam* mutant cells, other observations point toward a second, hitherto unknown, mechanism preventing the stable maintenance of minichromosomes (Messer *et al.*, 1985; Abeles *et al.*, 1993).

In this work we demonstrate that replication incompatibility exists between the chromosome and extrachromosomal copies of *oriC* in *dam* mutant cells. By introducing plasmids carrying complete and truncated *oriC* sequences into Dam⁻ cells we show that the incompatibility determinant is a complete and functional *oriC* sequence. The *seqA2* mutation originally thought to be a sequestration mutation and thus result in a replication phenotype similar to that of *dam* mutant strains was identified as a mutation increasing the copy number of *oriC*, thereby allowing minichromosomes to coexist with the host chromosome of *dam* mutant cells. Finally, we have constructed a strain containing as many as eight copies of *oriC* tandemly repeated in the chromosome. Analysis of the cell cycle of this mutant strain and other data obtained will be used in a discussion of initiation control in *E.coli* cells.

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**Table I.** Minichromosome transformation efficiencies

| Plasmid DNA | Dam methylation | Transformants per μg DNA ($\times10^{-3}$) | |
|---|---|---|---|
| | | MG1655(Dam$^+$) | ALO1213 (Dam$^-$) |
| pALO22 | + | 917 | 1 |
| pALO22 | –[a] | 1146 | 93 |
| pACYC184 | + | 1150 | 189 |
| pACYC184 | – | 851 | 131 |

[a]Unmethylated pALO22 DNA was obtained by propagating the plasmid in strain UF339.

**Table II.** Frequency of transduction of *daml6::Km* into minichromosome-containing cells

| Phage lysate | Transductants per ml phage lysate[b] | |
|---|---|---|
| | $Str^RTet^R$ | $Km^RTet^R$ |
| P1(ALO452)[a] | $2.2\times10^4$ | $8.0\times10^5$ |

[a]Relevant genotype of strain ALO452 is *daml6::Km rpsL* (Løbner-Olesen *et al.*, 1992). The titer of the P1 phage lysate used was $2.0\times10^{11}$ phages/ml.
[b]Recipient strain was ALO1208 (MG1655/pALO22).

## Results

### *Introduction of minichromosomes into dam mutant cells*

When fully methylated pALO22 (Figure 6A) minichromosomes were transformed into an isogenic Dam$^+$/Dam$^-$ strain pair, we observed a 1000-fold lower transformation frequency when the Dam$^-$ strain was used as recipient (Table I). This poor transformation ability was most likely the result of hemimethylated minichromosomes being generated by one round of replication. Hemimethylated minichromosomes are sequestered into the cell membrane and not available for initiation of replication (Russell and Zinder, 1987; Ogden *et al.*, 1988; Campbell and Kleckner, 1990).

Unmethylated pALO22 minichromosomes were found to transform Dam$^-$ strains as efficiently as the methylation insensitive plasmid pACYC184 (Chang and Cohen, 1978; Table I). Note that the Dam$^-$ strain yielded fewer transformants per microgram of DNA of either plasmid, relative to the isogenic wild-type strain. In agreement with previous results (Russell and Zinder, 1987) these data suggest that the difficulties in establishing minichromosomes in Dam$^-$ cells arise only from passage through a hemimethylated stage. Once minichromosomes are established in their unmethylated form, they can be maintained in Dam$^-$ cells.

To test this further, we introduced the *daml6::Km* mutation (Parker and Marinus, 1988) into minichromosome-containing wild-type cells. By this procedure we expected that the dilution of the wild-type methylase over time would result in a slow decline in the methylation state of the cellular DNA. The conversion of minichromosome-containing cells to the Dam$^-$ phenotype would therefore occur without the formation of a large hemithylated origin region. Selection for the presence of the minichromosome was maintained throughout the procedure. The *dam* mutant allele was transduced into strain MG1655/pALO22 (ALO1208) with high frequency (Table II). Note that the control locus *rpsL* had a lower transduction efficiency than the *dam* locus. This may reflect that a period of phenotypic expression longer than the 2 h we used, is needed to score *rpsL* transductants by streptomycin resistance.

The above data demonstrate that Dam$^-$ cells carrying minichromosomes can be obtained either by transforming Dam$^-$ cells with unmethylated minichromosomal DNA or by introducing the *dam* mutation into wild-type cells containing minichromosomes. Both of these procedures avoids the generation of truly hemimethylated minichromosomes. It is therefore likely that sequestering of hemimethylated minichromosomes (Ogden *et al.*, 1988; Campbell and Kleckner, 1990) represents the only block to establishing minichromosomes in Dam$^-$ cells.

### *Integration of oriC plasmids into the chromosome of dam mutant strains*

Previous studies reported that transformation of minichromosomes into Dam$^-$ host cells resulted in the plasmid DNA being integrated into the host chromosome (Messer *et al.*, 1985), or that the minichromosome replicated very inefficiently (Abeles *et al.*, 1993). We therefore decided to determine the structure and copy number of minichromosome pALO22 in Dam$^-$ cells. Three independent isolates of the strains MG1655/pALO22 (ALO1208) and its derivative (by P1 transduction) MG1655 *daml6::Km*/pALO22 (ALO1214-1216) were grown exponentially, and total DNA was isolated and analyzed by Southern blot hybridization experiments. In order to readily obtain the copy number of the plasmid, some DNA samples were digested with *Eco*RI prior to electrophoresis and blotting. When hybridized to an *oriC* specific probe, the chromosomal *oriC* carrying fragment will be 9.4 kb (Burland *et al.*, 1993), and the plasmid derived *oriC* carrying fragment will be 6.2 kb (Gerdes *et al.*, 1986). Since the region of *oriC* recognized by our probe is the same for the two fragments, the relative intensity of the 6.2 kb band to the 9.4 kb band represents the plasmid copy number per chromosomal origin. In the wild-type strain, free minichromosomal DNA could be readily detected (Figure 1A, lanes 4–6). After digestion with *Eco*RI two fragments of ~6 and ~9 kb were seen (Figure 1A, lanes 10–12). The relative intensities of the 6.2 kb band to the 9.4 kb band indicated that the copy number of pALO22 was ~8–10 per chromosomal origin (not shown). This is in good agreement with previous estimates (Løbner-Olesen *et al.*, 1987). In the three isolates from the Dam$^-$ strain, no free minichromosomal DNA could be detected (Figure 1A, lanes 1–3). When digested with *Eco*RI the minichromosome specific fragment of 6.2 kb appeared, as well as two new bands corresponding to fragments of ~7 kb and 8–9 kb. The 9.4 kb fragment specific for the chromosomal origin had disappeared (Figure 1A, lanes 7–9).

The most likely explanation for the observed patterns in the Dam$^-$ strain is that the plasmid pALO22 had integrated into the *oriC* region of the chromosome in multiple copies (see Figure 1B for a schematic representation). Densitometric scanning of the autoradiogram indicated that 8–10 copies of *oriC* were present in each cell (not shown). Note that each of the new fragments of ~7 kb and 8–9 kb carries one copy of *oriC* and thus serves as an internal standard. These fragments are flanking the multiple integrated plasmids and are in good agreement

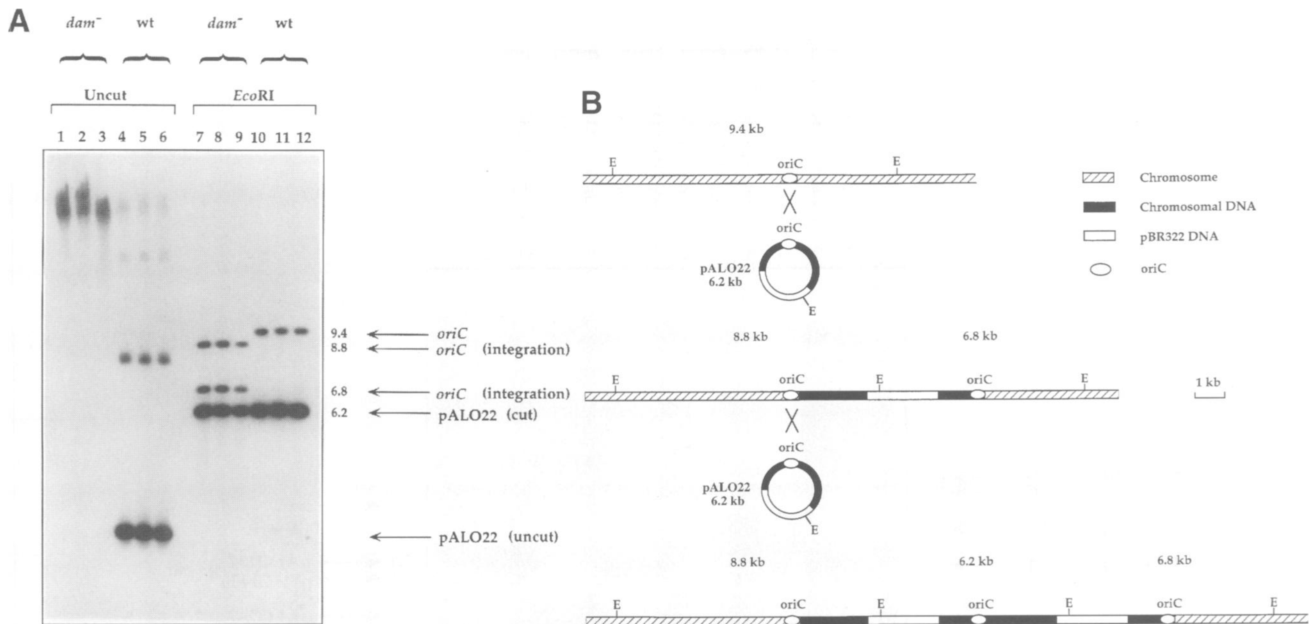


**Fig. 1.** Minichromosomes integrate into the *oriC* region of *dam* mutant cells. (**A**) Cells containing minichromosome pALO22 were grown. DNA isolated and a Southern blot hybridization performed as described in Materials and methods. The individual DNA samples were treated as indicated on the figure. The probe was the *oriC* containing 463 bp *Ava*I fragment from pGO46 (Ogden *et al.*, 1988) that had been labelled with $^{32}$P (for details see text). Sizes (in kb) of selected fragments are indicated by arrows. Lanes are: (1) ALO1214; (2) ALO1215; (3) ALO1216; (4–6) ALO1208; (7) ALO1214; (8) ALO1215; (9) ALO1216; (10–12) ALO1208. (**B**) Recombination of one copy of minichromosome pALO22 into its homologous *oriC* region on the chromosome converts the 9.4 kb *Eco*RI *oriC* fragment to two *Eco*RI fragments of 8.8 kb and 6.8 kb, respectively, each carrying one copy of *oriC*. Integration of one additional copy of minichromosome pALO22 (lower line) gives rise to an additional *oriC* carrying *Eco*RI fragment of 6.2 kb (corresponding to a complete linear pALO22 plasmid). Additional integration of pALO22 plasmids will generate additional 6.2 kb *oriC*-containing fragments, leaving the 6.8 kb and 8.8 kb flanking regions intact (not shown).

with the sizes of 8.8 kb and 6.8 kb that are expected from integration of plasmid pALO22 into the 9.4 *Eco*RI *oriC* fragment (Figure 1B). In an experiment similar to the one described above, multiple copies of plasmid pALO26 (Figure 6A) which is a MioC⁻ derivative of plasmid pALO22 was found to integrate into the *oriC* region of the Dam⁻ cells (data not shown).

When the fully methylated composite *oriC*–pBR322 plasmid pFHC271 (Figure 6B; Hansen *et al.*, 1987) was transformed into strains MG1655 and MG1655 *dam16::Km*, a somewhat different situation appeared (Figure 2). In the uncut samples of both the wild-type strain (lane 1) and the Dam⁻ strain (lane 2) free plasmid DNA could be detected. When the DNA was digested with *Ssp*I before electrophoresis a fragment of ~12 kb carrying the *oriC* (the size of the *Ssp*I *oriC* carrying fragment is 12 095 bp; Burland *et al.*, 1993) and a fragment of ~9 kb carrying the plasmid borne *oriC* (pFHC271 is ~9.1 kb; Hansen *et al.*, 1981) were seen in the wild-type strain in addition to some residual uncut plasmid pFHC271 DNA (lane 3). The relative intensity of the 9.1 kb band to the 12.1 kb band indicated a very high copy number of pFHC271, this is in agreement with previous results (Hansen *et al.*, 1987). In the Dam⁻ strain the 12.1 kb fragment carrying the chromosomal origin was no longer present, but instead a new larger fragment of 12–16 kb was found to hybridize to *oriC*, in addition to the 9.1 kb plasmid pFHC271 specific fragment and some residual uncut plasmid pFHC271 DNA (lane 4). This new fragment size is in agreement with the expected 15.7 kb arising from a copy of pFHC271 having integrated into *oriC*. The other new fragment generated by homologous recombination is 5.5 kb and is hidden by uncut plasmid pFHC271 DNA. The complete disappearance of the 12 kb *oriC* fragment shows that all cells of the Dam⁻ strain contain at least one copy of pFHC271 integrated in the chromosome.

We also transformed the fully methylated plasmids pFHC1425 and pALO237 (Figure 6B; Hansen *et al.*, 1987; this work) into both strains and analyzed them for plasmid structure (Figure 2). These plasmids are both pBR322–*oriC* composite plasmids derived from pFHC271. Plasmid pFHC1425 is made OriC⁻ by a *Bgl*II deletion that removes the leftmost 13-mer of *oriC* (Bramhill and Kornberg, 1988). Therefore the pBR322 origin of replication is the only active origin, but most of the *oriC* sequence, including the four DnaA boxes, and the *mioC* DnaA box is still present. Plasmid pALO237 has a deletion in the *mioC* promoter region which removes the *mioC* DnaA box and makes it MioC⁻, whereas the minimal *oriC* is still intact. For both plasmids pFHC1425 and pALO237, free plasmid DNA could be detected in wild-type cells (lanes 5 and 9) and Dam⁻ cells (lanes 6 and 10). After digestion with *Ssp*I only two fragments were found to hybridize to the *oriC* probe used. This was true for both wild-type cells (lanes 7 and 11) and Dam⁻ cells (lanes 8 and 12). The ~12 kb fragment carried the *oriC* and the ~8 kb pFHC1425 or ~8 kb pALO237 fragment carried the plasmid borne *oriC*. The plasmids pFHC1425 and pALO237 therefore exist only in the extrachromosomal state in both Dam⁺ and Dam⁻ cells.

We conclude that when unmethylated plasmid borne *oriC* sequences were introduced into, or generated in Dam⁻ cells, a mechanism acted to inhibit their replication.

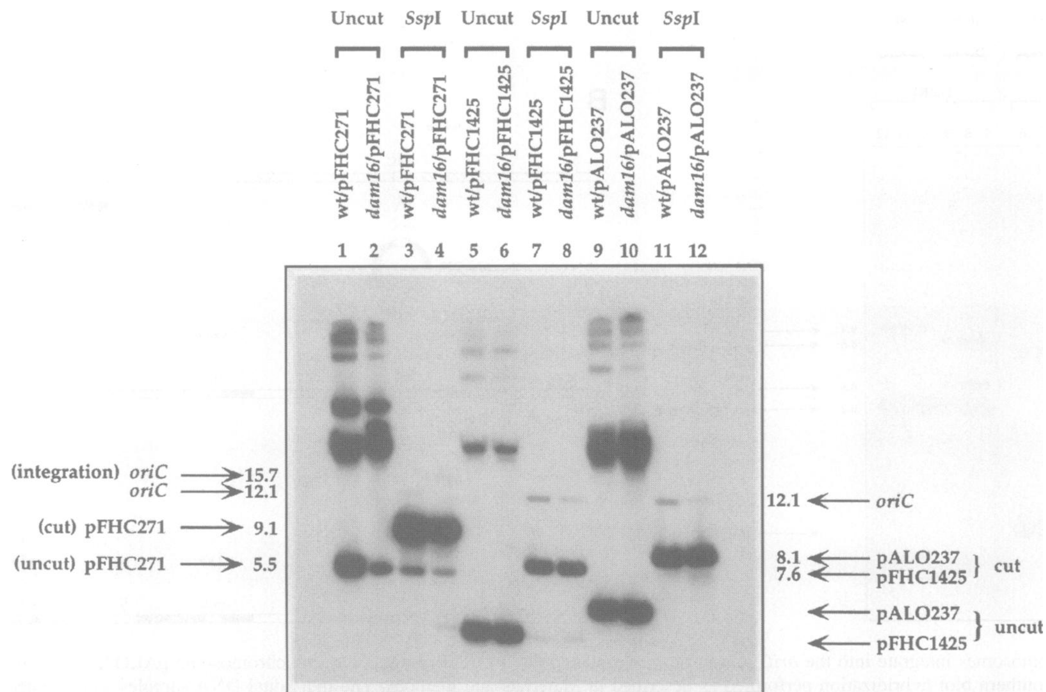


**Fig. 2.** The structure of *oriC*-pBR322 composite plasmids in wild-type and *dam* mutant cells. Cells containing the indicated plasmids were grown, DNA isolated and a Southern blot hybridization performed as described in Materials and methods. The individual DNA samples were treated as indicated on the figure. The probe was the *oriC* containing 463 bp *Ava*I fragment from pGO46 (Ogden *et al.*, 1988) that had been labelled with $^{32}P$ (for details see text). Sizes (in kb) of selected fragments are indicated by arrows. Lanes are: (1) ALO1244; (2) ALO1246; (3) ALO1244; (4) ALO1246; (5) ALO1261; (6) ALO1263; (7) ALO1261); (8) ALO1263; (9) ALO1285; (10) ALO1287; (11)ALO1285; (12) ALO1287.

If the plasmid replicated only from *oriC*, all copies of the plasmid were integrated into the host chromosome, and no free plasmid DNA was detected. For *oriC*–pBR composite replicons the situation was somewhat different; free plasmid DNA could be detected, yet cells always contained integrated copies of the plasmid, provided that a complete *oriC* sequence (*mioC* included) was present. It is difficult to estimate the fraction of plasmids integrated into the *oriC* region of the chromosome relative to those present as free plasmids. When the *oriC* region of composite replicons was truncated by deleting the leftmost 13-mer of the origin or by removing the *mioC* gene promoter region, integration was no longer observed despite extensive sequence homology. Therefore, integration of complete extrachromosomal *oriC* sequences into the chromosome of Dam$^-$ cells, is likely to be the result of an active extrachromosomal origin competing with the chromosomal origin for replication factors, i.e. incompatibility.

### *Strains with multiple replication origins*

The stable presence of minichromosome pALO22 in multiple copies in the genome of Dam$^-$ strains ALO1214-1216 prompted us to investigate if this was also a stable situation in Dam$^+$ cells. Strains ALO1214 and ALO1215 (MG1655 *dam16::Km*/pALO22::int) were transduced back to Dam$^+$ (for selection procedure see Materials and methods). We analyzed the total DNA content of the two resulting transductants (ALO1250-1251) for minichromosome structure (Figure 3A). Surprisingly, we found that even though the resultant transductants had become phenotypically Dam$^+$, as seen by the ability of *Dpn*I (cuts methylated DNA only) to cut chromosomal DNA prepared from these strains (Figure 3B), all plasmid pALO22 DNA was still contained within the chromosomal DNA (Figure 3A, lanes 5 and 6 and 11 and 12). After digestion with *Eco*RI the band pattern of the Dam$^+$ transductants was indistinguishable from that of the Dam$^-$ parent (compare lanes 9 and 10 with 11 and 12), indicating that these cells still contained multiple copies of pALO22 integrated in their chromosomes.

Introduction of minichromosome pALO22 had no detectable effect on either size or DNA content of the wild-type strain MG1655 (Table III), while the presence of both the *dam16::Km* mutation and the integrated copies of plasmid pALO22 resulted in increased size and DNA content. The DNA concentration in the latter was slightly lowered. For the Dam$^+$ strain containing multiple integrated copies of pALO22 we found the cell size to be similar to that of the wild-type. The DNA content and therefore the DNA concentration was reduced by ~15%. The wild-type strain MG1655 contained two, four or eight replication origins after replication runout in the presence of rifampicin, an indication of initiation synchrony (Figure 4A; Skarstad *et al.*, 1986). Introduction of the minichromosome pALO22 did not change the distribution much although the frequency of cells containing five, six and seven replication origins was increased (Figure 4B). Cells of strain MG1655 *dam16::Km* with multiple integrated pALO22 plasmids frequently contained three, five, six or seven origins (the asynchrony phenotype) a phenotype found in Dam$^-$ cells (Figure 4C; Boye *et al.*, 1988; Boye and Løbner-Olesen, 1990). Cells of the Dam$^+$ strain with multiple origins (integrated pALO22 plasmids) contained mainly two, four or eight replication origins (Figure 4D). However, when compared with the wild-type cells, or with wild-type cells containing non-integrated plasmid

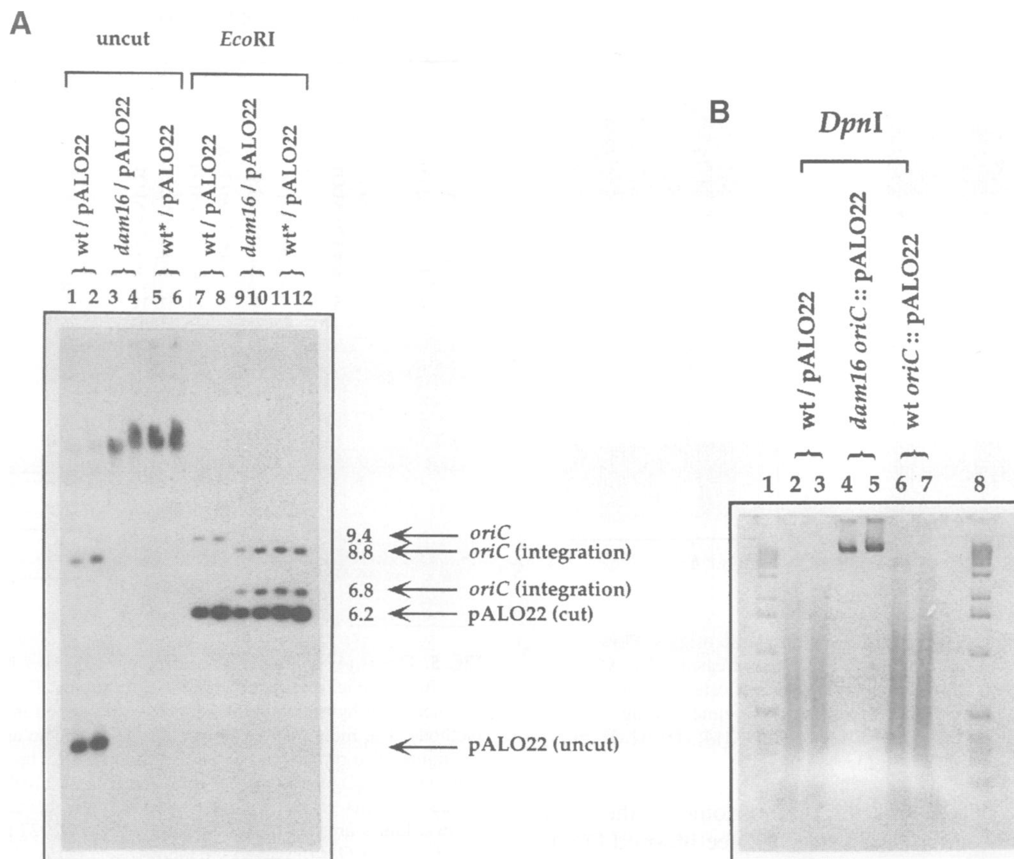


**Fig. 3.** Stable presence of multiple integrated minichromosomes in an otherwise wild-type strain. Cells containing minichromosome pALO22 were grown and DNA isolated as described in Materials and methods. (**A**) A Southern blot hybridization performed as described in Materials and methods. The individual DNA samples were treated as indicated on the figure. The probe was the *oriC* containing 463 bp *Ava*I fragment from pGO46 (Ogden *et al.*, 1988) that had been labelled with $^{32}P$ (for details see text). (*) These strains were transduced from *dam16::Km* back to $Dam^+$ as described in Materials and methods. Sizes (in kb) of selected fragments are indicated by arrows. Lanes are: (1–2) ALO1208; (3) ALO1214; (4) ALO1215; (5) ALO1250; (6) ALO1251; (7–8) ALO1208; (9) ALO1214; (10) ALO1215; (11) ALO1250; (12) ALO1251. (**B**) Methylation status of total DNA isolated as described above. DNA was cut with restriction enzyme *Dpn*I which only cuts fully methylated DNA. Lanes are: (1 and 8) 1 kb ladder molecular weight marker; (2 and 3) ALO1208); (4) ALO1214; (5) ALO1215; (6) ALO1250; (7) ALO1251.

pALO22, a significantly larger fraction of the cells contained irregular numbers of origins such as three, five, six and seven. This is indicative of a slightly disturbed timing of initiation of replication at multiple origins.

A wild-type cell, therefore, seems capable of stably maintaining 8–10 copies of *oriC* extrachromosomally or in its normal origin position on the chromosome without significant change in cell cycle parameters such as cell mass, DNA content and DNA concentration. Synchrony of multiple initiations in the division cycle, seemed to be slightly perturbed, when cells contained multiple integrated minichromosomes.

### *Replication incompatibility in dam mutant strains is overcome by the seqA2 mutation*

Recently mutations were isolated in the *seqA* gene which allowed for an efficient transformation of fully methylated minichromosomes into *dam* mutant cells (Lu *et al.*, 1994; von Freiesleben *et al.*, 1994).

We decided to investigate whether the *seqA* mutation would allow *oriC* plasmids to exist in Dam⁻ cells without integrating into the chromosome. Independent isolates of strains wt, *dam13::Cm*, *seqA2* and *dam13::Cm seqA2* (AB1157, GM2927, UF340 and UF339 respectively) transformed with methylated pALO1 minichromosomes (Figure 6A), were grown exponentially, and total DNA was isolated and used for Southern blot hybridization. The DNA samples analyzed were either uncut or digested with *Eco*RI and *Sal*I. This enzyme combination will give rise to a 9.4 kb chromosomal DNA fragment carrying *oriC* and a pALO1 derived fragment of 7.0 kb. If at least one copy of the minichromosome pALO1 has integrated into the origin region on the chromosome one should expect to see additional fragments of 6.1 kb and 10.3 kb.

**Table III.** Cell size and DNA content of strains with multiple origins

| *dam* allele | Plasmid | Cell mass | DNA content | DNA/ mass |
|---|---|---|---|---|
| $Dam^+$ | none | 1.00 | 1.00 | 1.00 |
| $Dam^+$ | pALO22 | 1.00 | 0.98 | 0.98 |
| *dam16::Km* | pALO22::int | 1.36 | 1.13 | 0.83 |
| $Dam^+$ | pALO22::int | 0.98 | 0.85 | 0.87 |

The *seqA2* mutation increased the cellular copy number of minichromosome pALO1 ~3-fold relative to wild-type, (Figure 5, compare lane 1 with lanes 5 and 6). Less than 5% plasmid integration was seen in the *seqA2* mutant (lanes 11 and 12) and none in the wild-type (lane 7). In contrast, all detectable plasmid pALO1 DNA was integra-

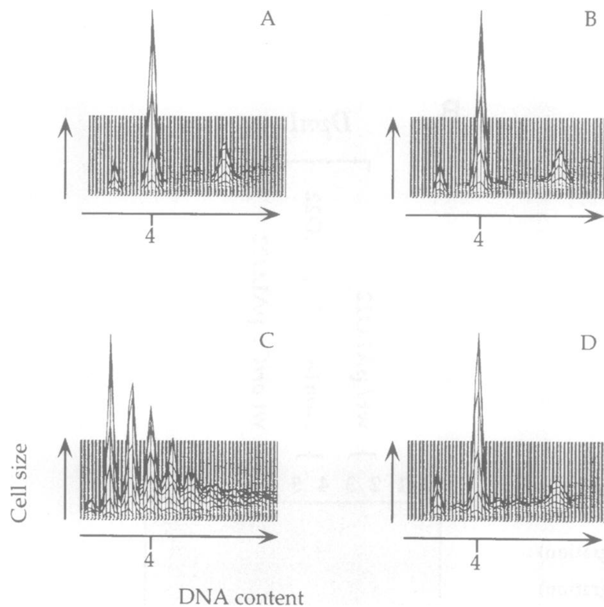


**Fig. 4.** Initiation synchrony of strains with multiple origins. Flow cytometric analysis of rifampicin and cephalexin treated cells. After run-out of replication, cells end up with fully replicated chromosomes equivalent to the number of origins present at the time of drug addition. (**A**) MG1655; (**B**) ALO1208; (**C**) ALO1214; (**D**) ALO1250.

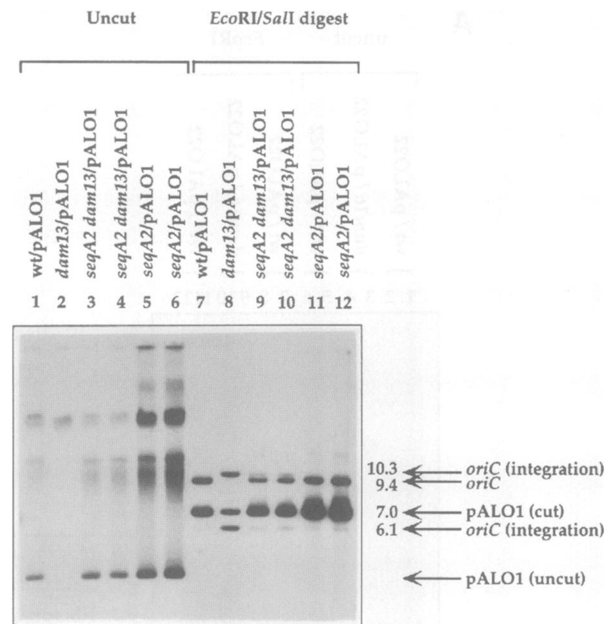


**Fig. 5.** The *seqA2* mutation as an *oriC* copy number mutation. Cells containing minichromosome pALO1 were grown, DNA isolated and a Southern blot hybridization performed as described in Materials and methods. The individual DNA samples were treated as indicated on the figure. The probe was the *oriC* containing 463 bp *AvaI* fragment from pGO46 (Ogden *et al.*, 1988) that had been labelled with $^{32}P$ (for details see text). Sizes (in kb) of selected fragments are indicated by arrows. Lanes are: (1) AB1157/pALO1; (2) GM2927/pALO1; (3 and 4) UF339/pALO1; (5 and 6) UF340/pALO1; (7) AB1157/pALO1; (8) GM2927/pALO1; (9 and 10) UF339/pALO1; (11 and 12) UF340/pALO1.

ted as multiple copies into the chromosome of the *dam* mutant cells as seen by the absence of free plasmid DNA (lane 2) and the appearance of three bands of ~10.5 kb, 7 kb and 6 kb after enzyme digestion (lane 8). When the *dam13::Cm* mutation was introduced into the *seqA2* strain containing pALO1, the copy number was reduced to that of the wild-type (Figure 5, compare lane 1 with lanes 3 and 4) and most plasmid DNA was still found in the extrachromosomal state. Integration was observed in the *seqA2 dam13::Cm* double mutant cells but was considerably reduced compared with what was seen in the *dam13::Cm* single mutant cells (Figure 5; compare lane 8 with 9 and 10). The *seqA2* mutation can thus overcome the incompatibility phenotype observed between the chromosomal *oriC* and minichromosomal *oriC* copies in the *dam* mutant strain. Therefore, we suggest that the *seqA2* mutation, in addition to being a copy number mutation, is also an incompatibility mutation.

## Discussion

We have demonstrated that introduction of minichromosomes into *dam* mutant strains is accompanied by integration of the entire plasmid into homologous regions of the chromosome itself. All Dam$^-$ host cells were shown to contain integrated plasmid DNA, in multiple copies, indicative of replicational incompatibility between copies of *oriC*. Only when *oriC* sequences were carried on a plasmid also containing a separate origin of replication, could free plasmid DNA be detected. The integration phenotype could be overcome by introducing a mutation in the *seqA* gene, which has previously been identified as a mutation that increases *oriC* copy number. Finally, we have shown that integrated copies of minichromosomes are stably maintained in the chromosome when the strain is converted back to Dam$^+$ phenotype.

### *Minichromosomes in wild-type cells*

The absence of replication incompatibility between the chromosome and minichromosomes in Dam$^+$ cells is best explained by the presence of the initiation cascade (see below; Hansen *et al.*, 1991a; Løbner-Olesen *et al.*, 1994); the essential aspects of which are as follows. In wild-type cells, accumulation of DnaA protein to a critical level triggers initiation on one of the origins within the cell. As the origin is initiated the DnaA protein is released. Since the two newly formed origins are hemimethylated and membrane bound (Ogden *et al.*, 1988) and therefore inaccessible to the DnaA protein (Landoulsi *et al.*, 1990), the ratio of free DnaA protein to the accessible origins will be momentarily increased. Consequently, initations on 'old' origins will follow in a cascade-like manner. In a flow cytometry analysis this leads to the synchrony phenotype (Figure 4A). The initiation cascade ensures initiation of all origins in the cell, chromosomal or extrachromosomal, within a short time window in the cell cycle. The number of origins initiated by the cascade can be high, as demonstrated by the finding that minichromosomes, having a copy number of 8–10 per chromosomal origin (Figure 1A; Løbner-Olesen *et al.*, 1987), replicate in synchrony with the chromosome (Leonard and Helmstetter, 1986). Introduction of additional origins of replication was expected to increase the initiation time window slightly, with a resultant slight disturbance of the single cell initiation synchrony. Surprisingly this was not observed (Figure 4B), suggesting that the chromosomal borne origins are chosen for replication before the extrachromosomal origins. The reason for this is not known but may

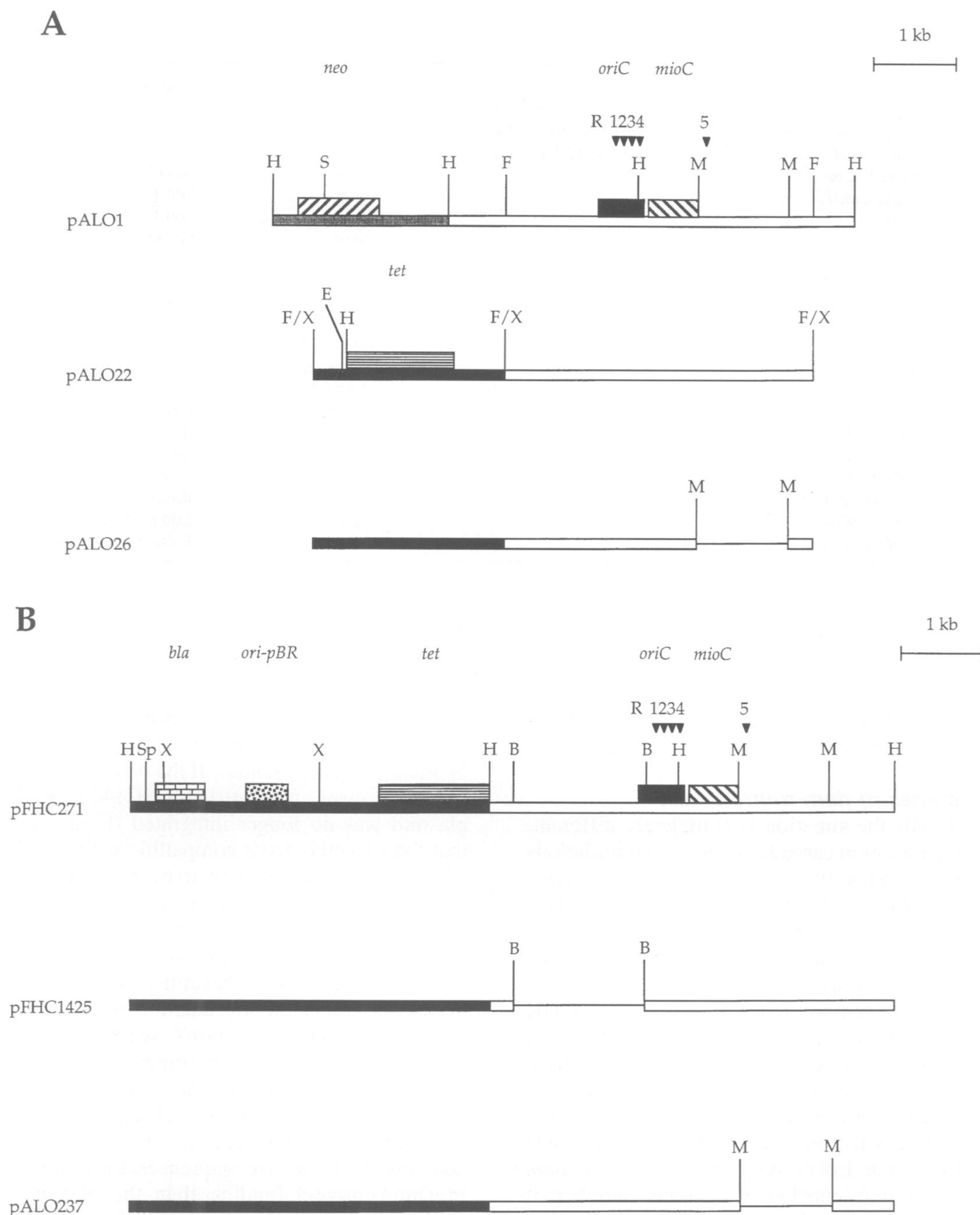


**Fig. 6.** Plasmids used. Structure of minichromosomes (**A**) and chimeric *oriC* and ori–pBR322 plasmids (**B**). The location of relevant genetic elements are indicated on the drawing. The symbols for restriction enzyme recognition are: (H) *Hin*dIII; (F) *Fsp*I; (M) *Mlu*I; (X) *Xmn*I; B; *Bgl*II; (S) *Sal*I; (E) *Eco*RI; (Sp) *Ssp*I. DnaA protein binding sites are indicated as R1–5 and symbolized by arrowheads.

be the result of small differences in superhelical densities that can lead to slightly different binding affinities for the DnaA protein. When multiple integrated origins were present in a wild-type cell a slight asynchrony was observed (Figure 4D), demonstrating that the integrated copies of the minichromosome were active and that the duration of the initiation window was increased. The presence of multiple origins in Dam$^+$ cells had otherwise little effect on the cell cycle, since cell cycle parameters such as cell mass, DNA content and DNA concentration were similar to those of the wild-type (Table III). It is, therefore, unlikely that the cell has means of sensing or 'counting' the number of origins it contains. The term initiation mass, defined as cell mass/origin at the time of initiation (Donachie, 1968), therefore does not make any sense in the classical definition. Rather, it seems that the cell senses that it has reached a certain mass per general starting area of chromosome replication. If the starting area contains multiple origins, and replication commences on several of these, the end result will be the same as if only one origin was present; one replication fork leaving the area in each direction. The remaining started replication forks will meet each other and terminate after a short distance of replication. Note that, following an initiation event, the transient hemimethylation of the area will prevent initiations on newly synthesized DNA even on

**Table IV.** Bacterial strains

| Strain | Relevant genotype | Plasmid | Source/reference |
|---|---|---|---|
| AB1157 | *thr-1 ara-14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 supE44 galK2* $\lambda^-$ Rac$^-$ *hisG4(Oc) rfbD1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1* | none | Howard-Flanders *et al.* (1964) |
| GM2927 | *dam13::Cm*[a] | none | M.G.Marinus |
| UF339 | *seqA2 dam13::Cm*[a] | none | von Freiesleben *et al.* (1994) |
| UF340 | *seqA2*[a] | none | von Freiesleben *et al.* (1994) |
| MG1655 | $\lambda^-$ $F^-$ | none | Guyer *et al.* (1980) |
| ALO1208 | wt[b] | pALO22 | this work |
| ALO1213 | *dam16::Km*[b] | none | this work |
| ALO1214-1216 | *dam16::Km*[b] | pALO22::int[c] | this work |
| ALO1244-1245 | [b] | pFHC271 | this work |
| ALO1246-1247 | *dam16::Km*[b] | pFHC271 | this work |
| ALO1248 | *aroK17::Cm*[b] | pALO22::int[c] | this work |
| ALO1250-1252 | [b] | pALO22::int[c] | this work |
| ALO1261-1262 | [b] | pFHC1425 | this work |
| ALO1263-1264 | *dam16::Km*[b] | pFHC1425 | this work |
| ALO1285-1286 | [b] | pALO237 | this work |
| ALO1287-1288 | *dam16::Km*[b] | pALO237 | this work |
| LJ24 | *supE44 rpsL rfbD1 thi-1, Δ(lacIZ) tonA21* | none | Rasmussen *et al.* (1991) |
| ALO452 | *dam16::Km*[d] | none | Løbner-Olesen *et al.* (1992) |
| ALO803 | *aroK17::Cm* | none | Løbner-Olesen and Marinus (1992) |

[a]Genotype as AB1157.
[b]Genotype as MG1655.
[c]The plasmid is integrated in multiple copies into the *oriC* region of the host chromosome.
[d]Genotype as LJ24.

adjacent origins that have not yet been utilized for an initiation event, thereby preventing over-replication.

### *Minichromosomes in dam mutant cells*

In *dam* mutant cells the situation is completely different. In these cells the initiation cascade is absent and initiations occur sequentially rather than synchronously on origins picked randomly from the pool within each cell (Løbner-Olesen *et al.*, 1994). When the amount of DnaA protein builds up to the initiation level, initiation occurs on one origin only. The two newly synthesized origins will be unmethylated and not sequestered into the cell membrane (Campbell and Kleckner, 1990). Consequently the level of free DnaA protein to accessible origin will be lowered, and a new period of accumulation is necessary before the next initiation can occur on a randomly picked origin. This leads to the asynchrony phenotype of Dam$^-$ cells (Figure 4C; Boye and Løbner-Olesen, 1990). The *dam* mutant cell is thus only capable of a limited number of initiations per cell cycle, which is similar to the number of origins present in the cell (Løbner-Olesen *et al.*, 1994). This number is set by the availability of DnaA protein. Minichromosomes in Dam$^-$ cells will, therefore, compete with the chromosome for the DnaA protein available, i.e. incompatibility. Initiation of replication on a minichromosomal *oriC* will provide a sink for DnaA protein and lead to under-replication of the host chromosome unless the minichromosomes are integrated into the chromosome and become a functional part of it. In this case each initiation event, chromosomal or minichromosomal, will lead to replication of the entire chromosome. In agreement with this we found all minichromosomal DNA integrated into the *oriC* region of Dam$^-$ cells (Figures 1, 3 and 5).

When *oriC* sequences were introduced into Dam$^-$ cells as *oriC*–pBR322 composite replicons (i.e. pFHC271) free plasmid DNA could be detected. However, plasmid copies were always found integrated into the *oriC* region of the host cells (Figure 2). Again, this suggests that the probability of replicating the chromosome was increased by integrating *oriC* copies. If the *oriC* sequence on plasmid pFHC271 was truncated (pFHC1425, pALO237) the plasmid was no longer integrated (Figure 2), suggesting that the plasmids were compatible with the chromosome. This compatability is likely to be the result of the plasmids replicating from their pBR322 origins of replication; plasmid pFHC1425 does not contain a functional *oriC*, due to a deletion of the leftmost 13-mer sequence (Bramhill and Kornberg, 1988). Plasmid pALO237 carries a functional but truncated (by deletion of the *mioC* promoter and the *mioC* DnaA box) *oriC* sequence. Removal of this sequence from minichromosomes is known to result in a lowered copy number (Løbner-Olesen *et al.*, 1987), probably resulting from a less efficient binding of DnaA protein to *oriC* (Hansen *et al.*, 1987). Since the host cell contains complete *oriC* sequences, these are more efficient in DnaA protein binding than the plasmid borne, and are consequently always chosen for initiation over the truncated plasmid copies of *oriC*, leaving plasmid pFHC237 to be replicated from its pBR322 origin of replication.

Plasmid pBR322 replicates randomly in the cell cycle (Leonard and Helmstetter, 1986). The presence of plasmids pFHC1425 and pALO237 in Dam$^-$ cells, is therefore not expected to result in a sudden sink in DnaA protein concentration at the precise time of chromosome initiation at a given *oriC*. This explains why both of these plasmids are fully compatible with the host chromosome, despite carrying additional DnaA boxes. The incompatibility determinant in Dam$^-$ cells is therefore a complete origin of replication.

### *seqA2 as an incompatibility mutation*

The *seqA* gene was recently identified as a factor inhibiting initiation of replication of hemimethylated chromosomal

origins (Lu *et al.*, 1994; von Freiesleben *et al.*, 1994), by binding hemimethylated *oriC* sequences (Slater *et al.*, 1995). The initiation cascade was therefore expected to be absent in *seqA* mutant cells. This was in turn expected to result in initiation incompatibility between minichromosomes and the chromosome, similar to what was observed for the Dam⁻ cells. This was not the case (Figure 5) for *seqA*. Rather, the *seqA2* mutant was found to increase the copy number of *oriC* ~3-fold. In the *seqA2*, *dam13::Cm* double mutant the copy number of *oriC* was close to wild-type level and most of the minichromosomes existed in the extrachromosomal state. This finding indicates a methylation-independent role of SeqA. The SeqA protein could inhibit the utilization of DnaA protein of the origin as previously suggested (von Freiesleben *et al.*, 1994) in addition to a possible role in sequestration. Loss of SeqA protein function therefore resembles the situation of DnaA protein overproduction, which also leads to an elevated number of origins per cell (Atlung *et al.*, 1987; Løbner-Olesen *et al.*, 1989; Atlung and Hansen, 1993).

## Materials and methods

### Bacterial strains and growth conditions

All bacteria used were *E.coli* K-12, and are listed in Table IV. Cells were grown in M9 minimal Medium (Miller, 1972) supplemented with 0.2% glucose and 0.5% casamino acids. Antibiotics were used at the following concentrations; 8 μg/ml of tetracycline, 25 μg/ml of kanamycin, 100 μg/ml of ampicillin and 100 μg/ml of streptomycin.

### Plasmids

The plasmids used are shown in Figure 6. Detailed descriptions of the plasmids can be found elsewhere; pALO1 (Løbner-Olesen *et al.*, 1987), pALO22 (Gerdes *et al.*, 1986), pFHC271 (Hansen *et al.*, 1987), pFHC1425 (this plasmid is similar to pFH352; Hansen *et al.*, 1981).

Plasmids pALO26 and pALO237 were made as *Mlu*I cutbacks of plasmids pALO22 and pFHC271, respectively.

### DNA manipulations

Restriction enzyme digests and ligations were done as recommended by the manufacturer (New England Biolabs Inc.).

### P1 transduction

Transduction (Miller, 1972) of *dam13::Cm* or *dam16::Km* cells to the Dam⁺ phenotype was accomplished in two steps. First, the Dam⁺ allele was introduced by cotransduction with the *aroK17::Cm* mutation of strain ALO803 (Løbner-Olesen and Marinus, 1992). Second, the *aroK17::Cm* cells were transduced back to the Aro⁺ phenotype using a P1 lysate grown on LJ24 (Rasmussen *et al.*, 1991).

### Southern blot analysis

For preparation of total cellular DNA, 25 ml of exponentially growing cells at an $OD_{450}$ = 0.4 were collected into 8 g of crushed ice to stop further cell growth immediately. Total DNA (chromosomal and plasmid) was prepared by treatment of cells with 300 μg/ml lysozyme in 50 mM Tris–HCl, 50 mM EDTA, pH 8.0, for 15 min at 0°C prior to incubation with 1% SDS for 10 min at room temperature, followed by two phenol and one chloroform extractions and ethanol precipitation. After digestion with appropriate restriction enzymes (New England Biolabs), the fragments were separated on 0.7% agarose gels, and transferred by capillary transfer to GeneScreen membranes (New England Nuclear Corp., Boston, MA) and probed with the *oriC* containing 463 bp *Ava*I fragment from pGO46 (Ogden *et al.*, 1988) that had been labelled with $^{32}$P (New England Nuclear Corp.) using the Random Primer system ('Prime-a-gene'; Promega Biotech).

### Flow cytometry

Flow cytometry was performed using an Argus 100 flow cytometer as previously described (Skarstad *et al.*, 1985).

For determination of numbers of origins per cell, samples were treated with 300 μg/ml of rifampicin (Sigma) to stop further initiations, and 36 μg/ml of cephalexin (Sigma) to stop further cell division prior to flow cytometry (Løbner-Olesen *et al.*, 1989). Cell size, cellular DNA content and DNA concentration were determined as described previously (Løbner-Olesen *et al.*, 1989).

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