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# Random Replication of the Stringent Plasmid R1 in *Escherichia coli* K-12

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The R-factor R1 is present in a low number of copies per genome (near unity, so-called stringent control of replication). The replication of R1 was studied in a density-shift experiment. One generation after the shift about 20% of the R1 copies had not replicated, whereas about 20% had replicated at least twice. The results are in quantitative accordance with a random replication of R1 in which the replicating molecules are taken from a cytoplasmic plasmid pool and transferred back to the pool after replication. This is analogous to the results obtained by Bazaral and Helinski (1970) and Rownd (1969) for plasmids that are present in 10 to 20 copies per genome (so-called relaxed control of replication). Hence, there seems to be no difference between stringent and relaxed plasmids with respect to selection of plasmid molecules for replication. However, we cannot tell whether all R1 copies in a cell replicate during a fraction of or throughout the cell cycle. The random selection of plasmid copies for replication has to be considered when models for control of replication are constructed.

Bacterial plasmids are replicating DNA molecules that normally are present in a defined number of copies per cell. This number can either be close to unity (so-called stringent control of replication) or 10 or higher (so-called relaxed control of replication) per genome of the host (3). Bazaral and Helinski (2) and Rownd (25) have reported that plasmids with a relaxed control are replicated at random; during one cell generation some molecules are not replicated at all, the majority replicates once, whereas others are replicated two or more times. The replicating molecules are presumably taken from a cytoplasmic pool of plasmids. The other group of plasmids is present in a low number per cell and seems to be replicated during a short interval during the cell division cycle (5, 6, 28). The question then arises as to whether all copies of a stringent plasmid in a cell are replicated once and only once during one cell generation.

In this paper we present the result of a density-shift experiment that shows that the R-factor R1 also is replicated at random in *Escherichia coli* K-12, where the number of R1 copies per genome is low, implying that there is no difference with respect to selection for replication between the two groups of plasmids mentioned. Whether there also is a randomness with respect to timing of replication cannot be concluded from our density-shift experiment.

## MATERIALS AND METHODS

**Strains, media, and growth conditions used.** *E. coli* K-12 strain EC1005 (*met*) (12) containing the R-factor R1*drd-19* (*amp, cml, kan, str, sul*) was used (18); hereafter the R-factor is denoted R1. The medium used was MOPS medium (19) with glycerol (0.4% wt/vol) as carbon source and supplemented with 100 μg of L-methionine per ml. MOPS medium with high density contained $D_2O$ (80%) instead of normal water and [$^{15}$N]ammonium sulphate (200 μg/ml) as nitrogen source. TES buffer contained 0.05 M tris(hydroxymethyl)aminomethane (pH 8.0), 0.05 M NaCl, and 0.005 M ethylenediaminetetraacetate.

The bacteria were grown at 37 C as shaken cultures in a thermostated water bath. Growth was recorded as optical density at 450 nm or by using a Klett-Summerson Colorimeter with filter W66.

**Determination of frequency of $R^+$ cells.** About 100 to 200 cells of the bacteria to be tested for the presence of the R-factor R1 were spread on LA plates (20) containing different concentrations of D-ampicillin. The colonies were counted after incubation overnight, and the resistance level as well as the frequency of $R^+$ cells was calculated (20).

**Materials.** D-Ampicillin was kindly provided by AB Astra, Södertälje, Sweden. Deuterium oxide and [$^{15}$N]ammonium sulphate were obtained from Bio-Rad Laboratories (Richmond, Calif.). [$^3$H]thymidine and [$^{14}$C]thymidine were purchased from the Radiochemical Centre (Amersham, Buckinghamshire, England). CsCl and the chemicals for the TES buffer were obtained from Merck (Darmstadt, Germany). Sigma Chemical Co. (St. Louis, Mo.) supplied lysozyme and

ribonuclease A. To destroy deoxyribonuclease activity, solutions of ribonuclease were heated at 90 C for 10 min before use.

**Analyses of DNA by density-shift cultivation.** The experiments for analyses of deoxyribonucleic acid (DNA) were essentially as described by Barazaral and Helinski (2). The bacteria were pregrown in heavy medium containing [$^{14}$C]thymidine (10 $\mu$Ci, specific activity 59 Ci/mol). At a cell density of about 0.5, 30 ml of the culture was chilled, harvested by centrifugation, and resuspended in 30 ml of normal-density medium (37 C) containing [$^{3}$H]thymidine (400 $\mu$Ci, specific activity 19 Ci/mol). Incubation was continued, and 10-ml samples were taken at intervals, chilled, centrifuged, washed twice by ice-cold TES buffer, and lysed as described before (21). The total material was then diluted to 5 ml. The sample (4.8 ml), 2 ml of propidium iodide (700 $\mu$g/ml), and 6.55 g of CsCl were mixed. A 7-ml portion of the mixture was added to centrifuge tubes and centrifuged at 24,000 rpm for 40 h in a SW40 rotor in a Beckman L2-65B centrifuge. About 40 fractions were collected, and the radioactivity was measured in 10 $\mu$l of each fraction. The fractions containing chromosomal DNA and covalently closed circular (CCC) plasmid DNA, respectively, were pooled (about 5 to 7 fractions in each case), and the propidium iodide was removed by extraction with isobutanol.

The pooled samples were dialyzed against TES buffer at 4 C, and 1 ml of the dialyzed plasmid samples was purified by neutral sucrose gradient centrifugation (5 to 20%) as described previously (21). About 35 fractions were collected, and the radioactivity was measured in 10 $\mu$l of each fraction. The fractions containing CCC RI DNA were pooled and dialyzed against TES buffer at 4 C.

The chromosomal DNA and the purified R1 DNA were diluted to 3.75 ml, 4.75 g CsCl being added, and the solution so obtained was centrifuged for 70 h at 38,000 rpm in a type 65 rotor of a Beckman L2-65B centrifuge. About 40 fractions were collected, and the radioactivity was measured in 100 $\mu$l of each fraction. The concentration of CsCl was estimated refractometrically.

## RESULTS

Strain EC1005 carrying the R-factor R1 has been analyzed before (7). In the steady state in different media the amount of CCC R-factor DNA (measured per chromosomal DNA or per cell) decreases with increasing growth rate. In glycerol-minimal medium the amount of R1 DNA is 3 to 4% of the amount of chromosomal DNA (see Fig. 1) or on the average four copies per cell (7).

The kinetics of R1 DNA replication was studied in a density-shift experiment (see above). Strain EC1005 R1 was grown logarithmically for at least 10 doublings at 37 C in heavy glycerol-minimal medium ($D_2O$, $^{15}$N) containing [$^{14}$C]thymidine. At an optical density at 450 nm of about 0.5, the culture was harvested and resuspended in prewarmed (37 C) normal-density medium containing [$^{3}$H]thymidine. The generation time was 95 min before the shift and 75 min after the shift. At 0, 1, and 1.5 doubling times after the shift (measured as increase in optical density at 450 nm), samples were taken for analysis of the DNA and for the presence of the R-factor in the population. At least 99% of the cells still contained the R-factor and showed the normal level of ampicillin resistance even 1.5 doublings after the density shift.

The R-factor DNA amounts to just a few percent of the total DNA. It was therefore necessary to obtain the plasmid DNA free from contamination by chromosomal DNA; this was accomplished by two purification steps. The samples for DNA analysis were lysed and subjected to propidium iodide-CsCl centrifugation (Fig. 1). This first step separates CCC R1 DNA from the rest of the DNA. To further purify the CCC R1 DNA it was run on a neutral sucrose gradient (21) (Fig. 2). No extensive breakdown of CCC DNA to open circular DNA or linear fragments was observed. There was virtually no background of chromosomal DNA. The CCC DNA as well as the chromosomal DNA from the dye-bouyant density centrifugation were then analyzed by CsCl gradient centrifugation (Fig. 3). In this final step, the molecular form of the DNA is irrelevant.

The radioactivity pattern shown in Fig. 3 was then used to estimate the relative frequency of heavy, hybrid, and light DNA molecules (Fig. 4). After one generation the internal control (the chromosomal DNA) was present only as hybrid

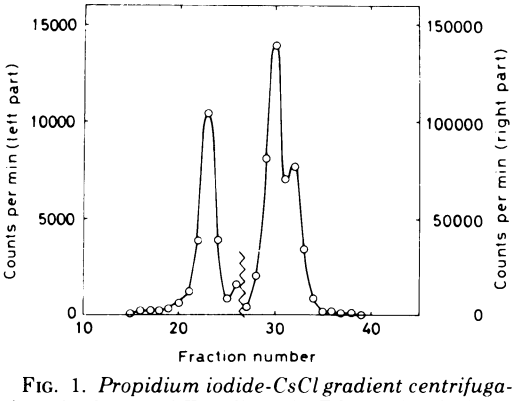


FIG. 1. *Propidium iodide-CsCl gradient centrifugation of a lysate of E. coli strain EC1005 R1 during a density-shift experiment. The sample was taken at 1.5 cell doublings after the shift from high-density to low-density medium (see Materials and Methods). Note the change in scale in the middle of the gradient. Fractions 18 to 26 (CCC R1 DNA) and 27 to 36 (chromosomal DNA) were pooled and analyzed as described in the legends to Fig. 2 and 3.*

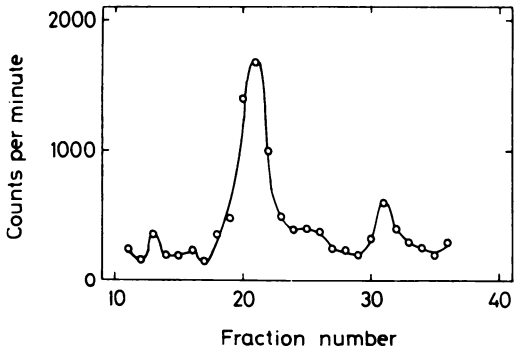


FIG. 2. *Neutral sucrose gradient centrifugation of the CCC R1 DNA from Fig. 1. Fractions 16 to 25 containing the CCC DNA were pooled and analyzed further as described in the legend to Fig. 3.*

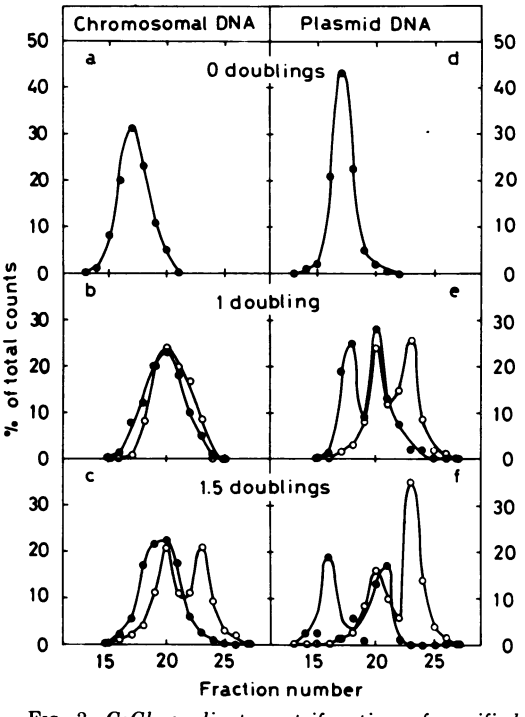


FIG. 3. *CsCl gradient centrifugation of purified chromosomal DNA and CCC plasmid DNA from a density-shift experiment. The samples were taken at 0, 1, and 1.5 cell doublings after the shift from high-density to low-density medium. The scale given is relative. Total counts for each graph were ($^{14}C$ and $^{3}H$, respectively): (a) 3,934, 0; (b) 36,449, 896,299; (c) 6,605, 278,136; (d) 275, 0; (e) 705, 21,093; (f) 93, 4,603.*

DNA, which shows that the steady state growth was unaffected by the density shift. On the other hand, a considerable fraction of the plasmid was present as unreplicated heavy molecules even after 1.5 cell generations. After one generation, the frequency of nonreplicated R1 molecules equalled that of those that had replicated at least twice. The experimental data have been compared to the theoretical values deduced by Rownd (25) (Table 1 and Fig. 4).

However, it has been reported that plasmid DNA is present as CCC DNA and as the so-called relaxation complex (13). In our procedure the latter is lost in the first step (16). For the interpretation of our results, CCC DNA must be representative of the total plasmid DNA. We regard this as likely since during one doubling in cell mass and in chromosomal DNA the CCC plasmid DNA has on the average replicated once; the fraction of CCC DNA which has not replicated during one generation equals that which has replicated twice or more (Fig. 4).

## DISCUSSION

The data presented in this paper clearly show that after one doubling in cell mass the R-factor R1 exists as unreplicated (old), once-replicated (hybrid), as well as at least twice-replicated (new) molecules, whereas the internal control,

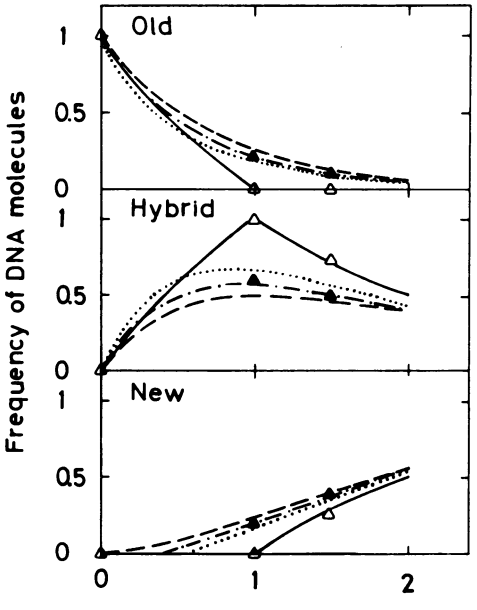


FIG. 4. *Distribution of old, hybrid, and new molecules of R1-DNA (▲) and chromosomal DNA (△) in a density-shift experiment. The experimental data were taken from Fig. 2. The theoretical curves corresponding to random replication assuming n to be 2 (·····), 4 (-·-·), or ∞ (- - -) R1 copies in the pool of plasmids (see Rownd [25] and Table 1) and the theoretical curve corresponding to nonrandom replication (i.e., all DNA copies replicate once and only once during one cell generation) are also included in the figure (——).*

TABLE 1. *Distribution between old, hybrid, and new plasmid molecules in a density-shift experiment if replication is random (25)*

| Plasmid molecule | Relative frequency[a] |
|---|---|
| Old | $\frac{n(n-1)}{(n+R)(n+R-1)}$ |
| Hybrid | $\frac{2nR}{(n+R)(n+R-1)}$ |
| New | $\frac{R(R-1)}{(n+R)(n+R-1)}$ |

[a] $n$, Number of plasmid copies per cell; $R$, number of replications after the shift (after 1 generation $R = n$, and after 1.5 generations $R = n\sqrt{2}$).

the bacterial chromosome, is composed solely of hybrid DNA. Several possibilities are open to explain this result.

(i) Randomness in the distribution of plasmid molecules between the daughter cells is possible. Since the average number of R1 molecules per newborn cell was about 3, the poisson distribution should give plasmid-free cells in a frequency of about 5% per cell generation. This value is much higher than the rate found even if transfer is taken into account. Hence, this possibility is very unlikely.

(ii) All plasmid copies in a cell (about 3) replicate simultaneously at a certain stage of the cell cycle, but there is a statistical spread in the time at which this event takes place. As a result, during one cell doubling, the plasmids in some cells do not replicate at all, whereas in other cells plasmid replication occurs twice. However, an extremely great spread in timing of plasmid replication is required to give the frequency of hybrid molecules found experimentally (Fig. 5 and 6). This spread is much greater than that of the interdivision times measured in synchronous cultures (17) or by microscope studies of individual cells in microdrops (8, 23, 26). In both cases the standard deviation was about 15 to 20% of the generation time.

The calculations presented so far were based on the assumption that consecutive replications were randomly distributed within the curves shown in Fig. 5. However, Kubitschek (15) and Schaechter et al. (26) have shown that there is a strong negative correlation between the generation times of the mother cell and its daughters. We have made the analogous assumption with respect to the timing of R1 replication, i.e., if R1 replication in a cell is, for example, delayed 5 min from the average value, the next replication will take place 5 min earlier than in the average cell. Also, in this case a broad distribution curve is required to give the experimentally found distribution between old, hybrid, and new R1 molecules (Fig. 6). The high frequency of unreplicated R1 molecules even after 1.5 generations (11%) is also highly against the model discussed in this paragraph.

(iii) Random selection for replication of plasmid copies in a cell (2, 25) is also possible. All molecules of R1 have the same probability of being replicated, and when replicated the two daughter plasmids have the same probability as the other R1 copies in the cell to be replicated. This can be explained by assuming that all R1 copies in a cell are located in one cytoplasmic pool of plasmids. Such a pool is compatible with the fact that the plasmids seem to be membrane bound only when they replicate (9). The distribution between old, hybrid, and new R1 molecules after a shift from high- to low-density medium fits nicely into the formulas deduced by Rownd (25).

The R-factor R1 is a so-called stringent plasmid, i.e., the number of plasmid copies per chromosome is low (about 1 to 2). Abe (1) has recently reported that phage P1 is stringently controlled in a P1 lysogen. He performed a density-shift experiment at the nonpermissive temperature of a mutant of *E. coli* that was thermosensitive with respect to initiation of chromosome replication; i.e., the culture was not in the steady state. However, he found replication of prophage P1 to be random. Hence,

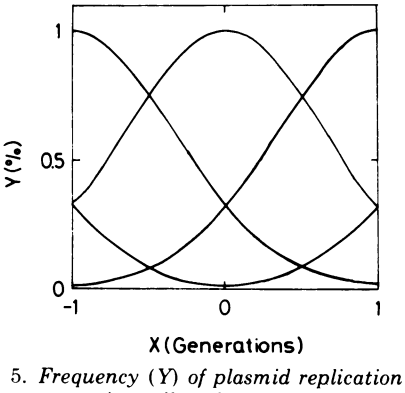


FIG. 5. *Frequency (Y) of plasmid replication during two consecutive cell cycles assuming a gaussian distribution. The figure shows the distribution at a standard deviation ($\sigma$) or 2/3 of the mean generation time. $Y = (1/\sigma\sqrt{2\pi}) \times e^{x^2/2\sigma^2}$, where x is the time at which the plasmid replicates minus the time at which the average plasmid replicates. In this case four different replication cycles may occur during one cell cycle.*

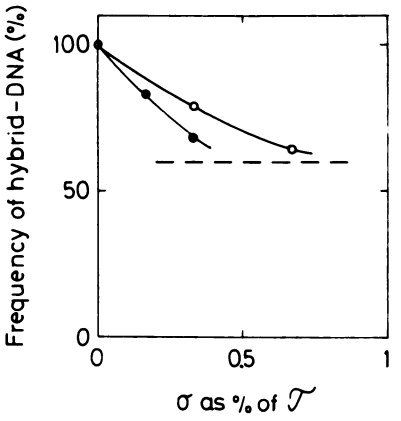


FIG. 6. *Frequency of hybrid plasmid DNA one generation after a density-shift. If all plasmids in a cell replicated simultaneously but the inter-replication times showed a gaussian spread (see Fig. 5), replication occurs twice in some cells and not at all in others during one doubling in mass. The inter-replication times for two consecutive replications were assumed either to be independent of each other* (○) *or strictly negatively correlated* (●). *In the latter case, a delay in one replication event determines that the next replication takes place earlier than in the average cell. The dotted line shows the frequency of hybrid DNA when replication is completely random* (25).

there seems to be no difference between the mode of replication of the stringent plasmids R1 and P1 and the relaxed plasmids ColE1 (2) and NR1 (25); all these plasmids are replicated randomly.

The most well-studied stringent plasmid is F. The replication of F resembles that of R1 in many respects; the replication of both plasmids uses the same elongation factors (including DNA polymerase III) (10, 21a, 27) and the initiation factor, the *dnaC* product (11), but is independent of the *dnaA* product (11); the number of plasmid copies per chromosome equivalent decreases with increasing growth rate (4, 7). Kline (14) performed a density-shift experiment with the F-factor in which the bacteria were pregrown in the presence of 5-bromouracil and then shifted to the same medium lacking 5-bromouracil but containing [$^3$H]thymine. He observed that new F DNA appeared much earlier than new chromosomal DNA. Since 5-bromouracil is known to induce the formation of extra forks (22), and by assuming that replication of the plasmid F is coupled to termination of chromosome replication, Kline (14) concluded that the observed pattern of hybrid and new F DNA could be explained by the extra chromosomal forks inducing extra rounds of replication of the plasmid. However, Pritchard (24) has later shown that there is no such coupling between the replication of the plasmid F and of the chromosome. Therefore the results of Kline (14) may be reinterpreted to conclude that replication of the plasmid F is random. Hence, the replication of the plasmids R1 and F seems to be random.

Since the R-factor R1 is replicated by DNA polymerase III (21a) it should take 1 to 2 min to complete one round of replication. The daughter plasmids should then be released from the replication site to the cytoplasm before the next round of replication. The other plasmid copies in the cell have the same probability as the newly formed ones of being selected for the next replication. It is not known what time is required from termination of replication to the release of the daughter replicons into the plasmid pool. The extreme case is that this time is zero; this means that the only time to be considered is the 2 min it takes to complete one round of replication. Under these circumstances even a rather narrow distribution in timing of replication (standard deviation 5 min; cf. Fig. 7) gives almost the distribution among the DNA molecules found experimentally. However, it is not possible to measure or even estimate the time required for transfer back to the pool. Hence, analysis of results obtained one generation or more after a density shift cannot reveal whether all plasmids in a cell replicate at the same stage or throughout the cell cycle. However, it may be appropriate to mention that the experiment reported in the present paper was also performed with a copy mutant of R1 (R1*drd-19B2*) which is present in 3.5 times as many copies per chromosome equivalent as the wild-type R-factor (21); also this mutant R-factor was selected randomly for replication.

Several reports claim that the timing of replication with respect to cell division of the F-factor is accurate (5, 6, 28). Those studies were performed with the plasmid F'*lac* and *E. coli* B/r. Remarkably enough the various groups have used the very same system, but have come to quite different conclusions. At present very little definite knowledge has been gained about the mechanisms which control the steady state level of plasmids in a culture.

We want to stress that any model for the control of replication has to consider also the randomness in replication discussed here. None of the simple positive or negative control models proposed seems to be able to explain why plasmid replication takes place during a

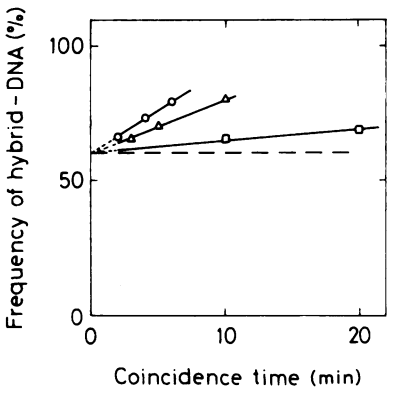


FIG. 7. *Frequency of hybrid plasmid DNA one generation after a density-shift if replication of individual plasmids was independent of that of the other plasmids in a cell and if the timing of replication followed a gaussian curve. The time during which a plasmid cannot be selected for a new round of replication has been referred to as coincidence time. The standard deviation in the gaussian distribution was assumed to be 3 (○), 5 (△), or 20 (□) min. The dotted line shows the frequency of hybrid DNA when replication is completely random (25).*

short interval of the cell cycle and why the replication is random.

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