JOURNAL OF BACTERIOLOGY, Nov. 1975, p. 633-640
Vol. 124, No. 2

Printed in U.S.A.

Inhibition of Cell Division in *Escherichia coli* K-12 by the R-Factor R1 and Copy Mutants of R1

BIRGITTA ENGBERG, KARIN HJALMARSSON, AND KURT NORDSTRÖM*

Department of Microbiology, University of Umeå, S-901 87 Umeå, Sweden

Received for publication 20 June 1975

The effect of the copy number of plasmid R1*drd-19* on cell division of *Escherichia coli* K-12 was studied in populations growing as steady-state cultures at different growth rates, the growth rate being varied by use of different carbon sources. The plasmid copy number was also varied by using copy mutants of the R-factor. The mean cell size was larger in populations carrying an R-factor than in R-factorless populations, an effect that was more pronounced at low growth rates and in populations carrying R-factor copy mutants. The increased cell size was due to formation of elongated cells in a fraction of the population and to an increase in the diameter of all cells. The majority of the cells divided at a normal cell length, but the presence of an R-factor caused some cells to elongate, probably by the uncoupling of chromosome replication and cell division. This can be explained as a competition between the chromosome and plasmid replicons for some replication factor(s), presumably acting on both initiation and elongation of replication. The formation of elongated cells was a reversible process, but occasionally some of the elongated cells reached lengths 20 times that of newborn cells. If cell division did not occur at the normal cell size, the septum was not formed until the cell size was four times that of a newborn cell. When an elongated cell divided, it usually formed a polar septum, thus producing a newborn cell of normal cell length. The ability of plasmid-containing cells to omit one cell division but to retain the capacity of dividing one mass doubling later is compatible with a mechanical model for septum formation and cell division.

The cell cycle in *Escherichia coli* contains a series of events (initiation and termination of deoxyribonucleic acid [DNA] replication followed by septum formation) that ultimately lead to cell division. The first process, initiation of chromosome duplication, is generally acknowledged to be rate limiting for DNA synthesis and, hence, for growth of the bacterium. Cooper and Helmstetter (8) developed a theory that describes the cell cycle in a simple fashion and divides it into specific periods. The time periods required to complete one round of replication and to proceed from termination of DNA replication to complete cell division have been referred to as the C and D periods, respectively. Above a growth rate of one generation per hour, the C and D periods seem to be constant (about 45 and 25 min, respectively). The situation at lower growth rates is less clear, but recently collected data suggest that the C and D periods are constant under these conditions as well (23, 24). It is possible to interfere with the cell cycle. Pritchard and co-workers (28, 37, 47) have shown that, in response to decreasing the concentration of thymine, a thymine-requiring strain will increase the C period and decrease the D period without changing its growth rate. Agents that interfere with DNA synthesis generally tend to induce filament formation (42). This also applies to mutations that cause initiation or elongation factors of DNA replication to be temperature sensitive (16).

Several workers have attempted to determine when plasmids replicate during the cell cycle and to estimate the steady-state concentration of different plasmids in a population (6, 7, 10, 48). Collins and Pritchard (6) made the observation that the mean cell size of *E. coli* is increased by the presence of the plasmid F'*lac*. In studies of the growth rate control of the replication of the plasmid R1, we observed that the increased mean cell size was due to a tendency for R1-containing cells to become elongated (11, 31). Some years ago we discovered a novel type of R-factor mutant which causes an increase in the steady-state concentration of the plasmid in the cells (33). In this paper we have used such mutants to further study the

effect of R-factors on cell size and cell division. A preliminary account has been published elsewhere (31).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* K-12 strains EC1005 (*met, nal*) (15), D11 (*pro, trp, his, lac, strA, tsx*) (2), and D114 (*pro, trp, lac, strA, tsx, recA*) (3) were used. All three strains are free from plasmids, as judged by the absence of covalently closed circular plasmid DNA (15; Engberg, unpublished data). R-factor R1*drd-19* was transferred into both strains as described previously (33). R-factor R1 mediates resistance to ampicillin, chloramphenicol, kanamycin, streptomycin, and sulfonamides (29). Plasmid R1*drd-19* is a mutant plasmid of R1, derepressed with respect to transfer (30). The R-factor mutants B2 and B3 are so-called copy mutants of plasmid R1*drd-19*. This means that, by mutation in the R-factor, the steady-state concentration of the plasmid (plasmid DNA per chromosomal DNA) is increased 3.5 (mutant B2) and 2.0 (mutant B3) times, respectively (33, 44). There are no chromosomal mutations involved since, as has been described previously (33), clones carrying copy mutant R-factors were isolated by using one host strain after which the plasmids were transferred to another strain of bacteria. The copy effect always is transferred with the plasmid (33).

Minimal medium E (45) was used throughout this work. It was supplemented with thiamine (1 μg/ml). Proline, tryptophan, histidine, and methionine (25 μg each of the L-epimer per ml) were added to the medium when necessary. The growth rate was varied by using different carbon sources and other additions to the medium (see Table 1).

The bacteria were incubated at 37 C as shaken cultures. Growth was followed by measuring the optical density at 450 nm or in a Klett-Summerson colorimeter (filter W66). Cells were kept growing in densities below 0.5 (measured at 450 nm).

Analytical methods. Plasmid DNA was determined by alkaline sucrose gradient centrifugation or by ethidium bromide-cesium chloride gradient centrifugation of lysozyme-ethylenediaminetetraacetic acid lysates of bacteria labeled with [^{3}H]thymidine (33). The content of R1-DNA was determined by dividing the total counts per minute in the covalently closed circular DNA peak by the total counts per minute in the peak containing chromosomal DNA.

Protein was determined by the method of Lowry et al. (26). For determination of cell number and cell size, cell growth was inhibited by the addition of formalin (1% final concentration). Cell number was measured in a microscope equipped with a Petroff-Hauser counting chamber. Cells were spread on an agar gel slide, and cell lengths were measured by using a microscope equipped with an occular micrometer.

The nuclei in cells were stained by the method of Feulgen (9) before being counted.

Scanning electron microscopy. Cells for scanning electron microscopy were harvested at an optical density at 450 nm of 0.3 to 0.4, fixed in 3% glutaraldehyde, collected on membrane filters, dried by the critical-point drying procedure (B. Engberg, P. Hörstedt, and B. Winblad, manuscript in preparation), coated with gold, and examined in a Cambridge stereoscan S4 scanning electron microscope.

Materials. Benzylpenicillin was kindly donated by AB Astra, Södertälje, Sweden. Lysozyme (crystallized three times from egg white; 35,000 Sigma units per μg), ribonuclease A, and bovine albumin were obtained from Sigma Chemical Co., St. Louis, Mo. Vitamine-free Casamino Acids were obtained from Difco Laboratories, Detroit, Mich. [*methyl*-^{3}H]thymidine (5 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, England. Triton X-100, 2,5-diphenyloxazole and 1,4-di-(2-[5-phenyloxazolyl])-benzene were obtained from Koch-Light Laboratories, Ltd., Colnbrook, England. Toluene was obtained from Kebo AB, Stockholm, Sweden.

RESULTS

Effect of R-factor copy number on cell size. Strain EC1005 without R-factor or carrying the wild-type plasmid R1*drd-19* or its copy mutants B2 and B3 was grown in the steady-state logarithmic growth phase in different media (Table 1). The presence of an R-factor had only a slight depressing effect on the growth rate, an effect that was somewhat more pronounced for increasing copy number (cf. copy mutant B2 and the wild-type plasmid R1*drd-19*).

As reported previously for strain EC1005 carrying the wild-type plasmid R1*drd-19*, the amount of R1 DNA per chromosome equivalent decreases significantly with increasing growth rate (11, 31). The same result was obtained with strain EC1005 carrying the R-factor copy mutants R1*drd-19B2* or *B3* (Fig. 1); hence, the copy effect (3.5 and 2.0, respectively) expressed in all media tested. By using different copy mutants and different media, the number of R1 copies per genome can be varied from 0.4 to 4;

TABLE 1. *Growth rate of strain EC1005 in different media*

Addition to medium E (%, wt/vol)	Growth rate (doublings/h)		
	EC1005	EC1005-R1*drd-19*	EC1005-R1*drd-19B2*
Sodium acetate (0.4)	0.45	0.43	0.40
Sodium succinate (0.2)	0.78	0.74	0.68
Glycerol (0.2)	0.88	0.80	0.80
Glucose (0.2)	1.15	1.10	1.05
Casamino Acids (2.0)	1.45	1.40	1.38
Casamino Acids (1.5) + glucose (0.2)	2.03	1.82	1.70

an R1 DNA content of 2.6% of the chromosomal DNA corresponds to one R1 copy per chromosome equivalent (2.5×10^9 daltons [8]) (Table 2). The data for R1 DNA in Fig. 1 and Table 2 were obtained by determination of covalently closed circles of R1 DNA using alkaline sucrose gradient centrifugations. However, control experiments using ethidium bromide-cesium chloride gradient centrifugations gave essentially the same results (cf. reference 33).

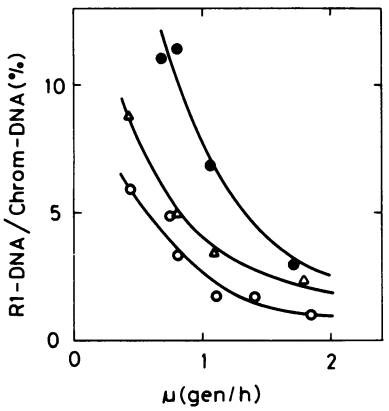

FIG. 1. *Relative content of R1 DNA in strain EC1005 carrying R-factor R1drd-19* (○) *or its copy mutants B2* (●) *or B3* (△) *grown in the steady state at different growth rates* (μ). *The bacteria were grown for at least 10 doublings in the logarithmic growth phase before harvesting. The growth rate was varied by using different carbon sources and other additions to the medium; the growth rates are given in Table 1. The values given are the mean values obtained in at least three different experiments using alkaline sucrose gradient centrifugation to determine the content of covalently closed circles of plasmid R1.*

The mean cell size was determined as protein per bacterium in cultures grown in the steady state in different media. The results (Fig. 2) show that the presence of the R-factor caused an increase in the mean cell size. This effect was more pronounced at a higher copy number and overshadowed the normal reduction of cell size at lower growth rates. This is also apparent from Fig. 3, which shows that the mean cell size increased with increasing copy number when bacteria containing R-factor R1*drd-19* or copy mutants B2 and B3 were grown in minimal-succinate medium. In Casamino Acids-glucose medium, the R-factor did not affect cell size.

Size distribution in steady-state populations. Examination of steady-state cultures by phase-contrast microscopy and by scanning electron microscopy showed that the increased mean cell size was due to the formation of elongated cells and to an increased cell diameter at higher plasmid copy number (Table 2). Only a fraction of the cells were elongated, but some of the cells had lengths many times longer than that of normal bacteria. Electron microscopy

TABLE 2. *Analysis of strain E. coli K-12 EC1005 carrying R-factor R1drd-19 and copy mutants of the R-factor*

Plasmid	Medium	R1 copies per chromosome equivalent[a]	Length of newborn cells[b] (n)(μm)	Avg cell length[c] (μm)	Diameter of cells[d] (μm)	Frequency of elongated cells[e] (%)	Septa in elongated cells[f] (%)
None	Minimal-succinate	0.0	1.3 ± 0.1 (20)	2.0	0.57 ± 0.01 (17)	3	32
R1*drd-19*	Minimal-succinate	1.3	1.6 ± 0.1 (20)	2.1	0.57 ± 0.01 (27)	6	27
R1*drd-19B3*	Minimal-succinate	2.2	1.6 ± 0.1 (20)	3.1	0.76 ± 0.01 (25)	20	19
R1*drd-19B2*	Minimal-succinate	4.3	1.6 ± 0.1 (20)	2.8	0.71 ± 0.01 (18)	19	15
None	Casamino Acids + glucose	0.0	1.3 ± 0.1 (20)	2.1	0.79 ± 0.01 (20)	5	
R1*drd-19*	Casamino Acids + glucose	0.4	1.3 ± 0.1 (20)	1.9	0.79 ± 0.01 (17)	3	
R1*drd-19B3*	Casamino Acids + glucose	0.9	1.2 ± 0.1 (20)	2.0	0.77 ± 0.01 (18)	4	
R1*drd-19B2*	Casamino Acids + glucose	1.3	1.4 ± 0.1 (20)	2.2	0.75 ± 0.01 (29)	4	

[a] Calculated from data of Fig. 1.

[b] The distance between the septum and the nearest pole was measured with the aid of a microscope and is given as the mean ± the standard deviation (number of cells tested).

[c] Calculated from the data in Fig. 4 and analogous experiments.

[d] Calculated from scanning electron micrographs and given as the mean ± the standard deviation (number of cells measured).

[e] The frequency of cells longer than $2n$ was calculated from the data of Fig. 4 and analogous experiments.

[f] Cells longer than $3n$ were scored for septa.

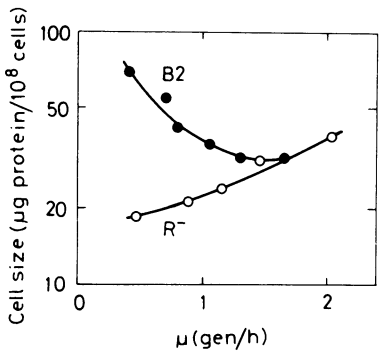

FIG. 2. *Cell size of strain EC1005 with (●) or without (○) plasmid R1drd-19B2 at different growth rates. Experimental conditions were as given in the legend to Fig. 1.*

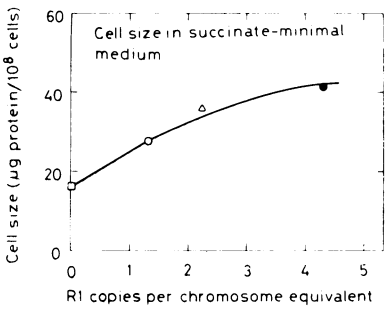

FIG. 3. *Cell size in succinate-minimal medium as a function of the R-factor content. The bacteria were grown in the steady state. The copy effects 0, 1, 2, and 3.5 correspond to strain EC1005 carrying no R-factor (□), R1drd-19 (○), R1drd-19B3 (△), and R1drd-19B2 (●), respectively.*

showed that the elongated cells were filaments since no septa could be seen.

The size distribution of strain EC1005 without any plasmid, as well as carrying R-factors R1*drd-19*, R1*drd-19B2*, and R1*drd-19B3*, was determined in minimal-succinate medium by measuring, with the aid of a microscope, the cell lengths (Fig. 4). In all cases, the majority of the cells showed almost identical length distributions. This indicates that cell division normally took place at the same cell length in the R-factorless, as well as in the R-factor-containing, populations and that the length of the newborn cells was unaffected by the plasmids. The latter finding was verified by direct measurements of the location of septa in dividing cells (Table 2). The mean length of the newborn cells will hereafter be denoted *n* (see Table 2).

Figure 4 also demonstrates that in the presence of an R-factor a significant fraction of the population was longer than 2*n*; this was particularly evident for cells carrying R-factor copy mutants. The length distribution formed a plateau in the range 2*n* to 4*n*. A minor fraction of the cells were even longer; cells with a cell length of at least 20*n* were found in populations carrying the R-factor copy mutants. This is more evident from the histograms shown in Fig. 5.

The mean cell length was calculated (Table 2). The higher the copy number, the longer was the average cell. However, the effect of the R-factor on cell length was less than that on cell size (protein content per cell). This is explained by the fact that R-factor-containing cells had a larger diameter than R-factorless cells (Table 2).

Septum formation in elongated cells. Careful examination with a phase-contrast microscope revealed that some of the elongated cells

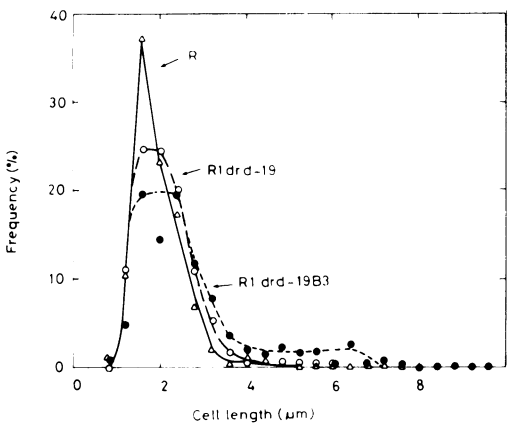

FIG. 4. *Length distribution of steady-state cultures grown in succinate-minimal medium. The lengths of about 300 bacteria in each culture were measured with the aid of a microscope.*

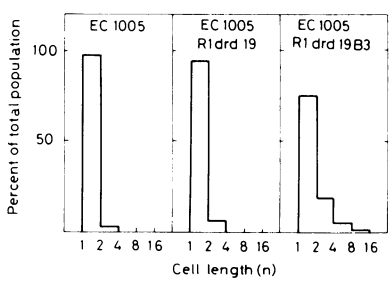

FIG. 5. *Length distribution of strain EC1005 carrying R-factor R1drd-19 or its copy mutant B3. The data were taken from Fig. 4 and replotted to give a histogram of the distribution of cells with respect to cell age (n is the length of a newborn cell); a length of 2n to 4n comprises bacteria in which one cell division has been omitted; a length of 4n to 8n comprises bacteria in which two consecutive divisions have been omitted, etc.*

contained septa (Table 2). The probability of forming a septum in an elongated cell decreased with increasing copy number of plasmid R1 (Table 2). Normally, only one septum was formed in an elongated cell. In most cases, these septa were located near one of the poles of the elongated cells (Fig. 6), about the length of one newborn cell distant from the pole of the cell (Fig. 7). However, some elongated cells formed central septa. Microslide cultures of elongated cells showed that septum formation could go to completion. Hence, the formation of elongated cells was a reversible process. If a cell had been formed, there was a great probability that a new septum would be formed in the same end of the elongated cell as the previous septum.

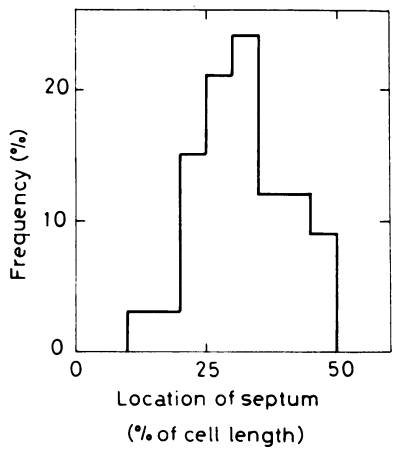

FIG. 6. *Location of septa in elongated cells of strain EC1005-R1drd-19B3. The distance from the septum to the nearest pole of the cell was measured with the aid of a microscope.*

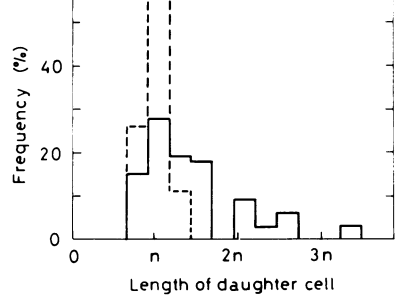

FIG. 7. *Location of cell division sites in elongated cells of strain EC1005-R1drd-19B3 (full lines). The distance between a septum and the nearest pole of the cell was measured with the aid of a microscope. Cells of strain EC1005-R1drd-19B3, at least three times longer than normal newborn cells (3n), were measured (full line). The arrows indicate the position of the cell length, n, 2n, and 3n, respectively. As a control, the corresponding measurement was performed with normal cells of R-factorless strain EC1005 (dotted line).*

There was no detectable change in the frequency of elongated cells when the population left the exponential growth phase and entered the stationary phase.

Feulgen staining revealed that the number of nuclei per unit length was about the same in normal cells as in elongated cells at least up to a cell length eight times that of newborn cells. The very long cells sometimes appeared only faintly, evenly stained by the Feulgen reagent.

Other properties of the system. Filaments of various species that result from the expression of various mutations or nutritional conditions divide after the addition of many different agents, e.g., sodium chloride (42). The addition of 1% (wt/vol) sodium chloride to minimal-glycerol medium did not cause any detectable change in the frequency of elongated cells of strain EC1005-R1*drd-19B2*.

Many reports claim that there is a correlation between the induction of filament formation and the induction of prophage λ (4, 5, 21, 46). We could not find any such correlation between plasmid-induced filamentation and phage induction; the frequency of plaque-forming units per bacterium was the same (about 10^{-4}) when strain D11 (λ), with or without the plasmid R1*drd-19B2*, was grown in the steady state in minimal-succinate medium.

Inouye (19) has reported that *recA* mutants have lost the ability to form filaments when DNA synthesis is blocked. No such effect was observed for the plasmid-induced filaments; strains D11 (*rec*$^+$) and D114 (*recA*) carrying the copy mutant R1*drd-19B3* and grown in glycerol-minimal medium had the same frequency of elongated cells (about 20%).

DISCUSSION

The results presented in this paper can be summarized as follows. (i) The presence of R-factor R1 causes an increase in the mean size of the cells, this effect being more pronounced the higher the ratio of R1 DNA to chromosomal DNA. (ii) The increased mean cell size is due to an increased cell diameter and to the formation of elongated cells in a fraction of the population; i.e. the presence of the plasmid increases the probability of not forming a septum when the cell has reached the size when it normally should divide. (iii) The majority of the cells divide at the same cell length as R-factorless cells divide. (iv) If cell division does not occur at the normal cell length, the cells continue to

grow until they reach a length corresponding to four times that of a newborn cell ($4n$); at that time, there is a certain probability of septum formation. Only one septum is formed per elongated cell. This cell division, in the majority of cases, takes place about the length of one newborn cell (n) from the pole of the elongated cell. (v) Cell divisions may be omitted during successive doublings in mass, which results in a small fraction of cells being very long (at least up to $20n$).

An effect of plasmids on the mean cell size has also been reported for *E. coli* carrying the plasmid F'*lac* (6). Also in this case, a fraction of the population was elongated cells while the majority of the cells showed the normal cell length (unpublished data).

The bacterial cell cycle seems to be regulated by the frequency of initiations of replication of the bacterial chromosome. Once started, elongation of replication proceeds until replication has been completed. This process is then followed by septum formation and cell division (38, 42). Two different models have been proposed for the coupling between chromosome replication and cell division. One assumes that there is a diffusible factor formed which triggers septum formation and which is consumed during septum formation (20, 43, 49). The other control model, which is purely mechanical, was proposed by Zaritsky and Pritchard (47) and has been discussed extensively by Pritchard (38) and Normark and Wolf-Watz (34). This model assumes that some cell components grow exponentially whereas others grow linearly, the linear growth rate being doubled once every generation, perhaps coinciding with termination of chromosome replication. This causes a cyclic variation in the ratio between the quantities of the two types of components. Septum formation should then be the result of the linearly growing component being synthesized faster than the exponentially growing one at a certain stage during the cell cycle. If chromosome initiation in a cell is delayed slightly, the ratio between the components discussed above will never reach the critical value that enforces constriction and cell division. In such a case, cell division would be omitted, causing the formation of filaments.

Both the omission of a cell division and the division observed in elongated cells are compatible with the mechanical model. It is hard to see how the presence of the plasmid could affect the production of a triggering substance such that cell division is omitted rather than just being delayed slightly.

The filament formation caused by plasmid R1 does not involve the *recA*$^+$ (19) gene product and is not related to the induction of prophage λ (4, 5, 21, 46) and, therefore, seems to be distinct from other described cases of filamentation.

It is very likely that the presence of plasmid R1 causes a delay in chromosome replication since these two replicons use the same elongation factors (14) as well as initiation factor C (13). As a matter of fact, we have recently reported (32) that there is a competition for DNA polymerase III between plasmid R1 and the bacterial chromosome under some circumstances. There is considerable variation in the timing of events in the cell cycle (12, 27, 36, 41). The effect of the presence of the plasmid could then be explained by the gaussean curves in Fig. 8. An alternative possibility is that the repressor of initiation of R1 replication cross-reacts with that of the chromosome (37, 39). The effect of the presence of the plasmid could be two-fold, either on initiation of replication or on the rate of elongation. It is very likely that both mechanisms are operating. The increased diameter imposed by plasmid R1, especially by the copy mutants, indicates a decrease in the rate of elongation of chromosome replication; Zaritsky and Pritchard (47) have reported that thymine-requiring *E. coli* becomes thicker when grown at suboptimal thymine concentrations and that the C period was considerably increased. A similar correlation between increased length of the C period on the cell diameter was reported by Lane and Denhardt (25) for *rep* mutants of *E. coli*.

The fact that septum formation may be omitted during one cell cycle demonstrates that there is no obligate coupling between chromo-

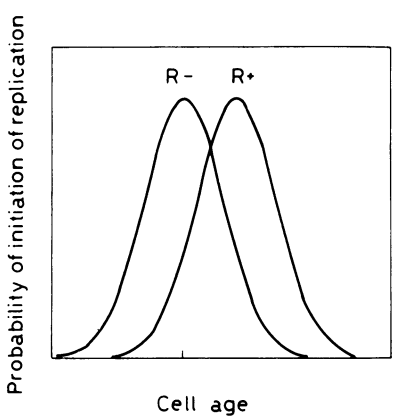

FIG. 8. *Model curves showing the distribution of initiation of chromosome replication with respect to cell age in plasmid-free cells (R^-) and in cells carrying an R-factor (R^+).*

some replication and cell division. This has been shown before (1, 16, 17, 18, 40, 42), but it should be stressed that in our case this uncoupling occurs in a steady-state culture and is not caused by the addition of toxic substances, by the use of inhibitory treatments like ultraviolet light, or by growing temperature-sensitive mutants at a nonpermissive temperature.

The elongated cell formed by the omission of one cell division could not divide until one generation later, but at that time there was the possibility of forming one septum. According to the mechanical model, the formation of a septum in a tetrameric cell implies that the next initiation of chromosome replication occurs early enough to compensate for the previous delay. Kubitschek (23) and Schaechter et al. (41) have reported that there is a strong negative correlation between the generation times of the mother cell and its daughters. The mechanical model is compatible with the fact that, in a tetrameric cell, division occurs preferentially towards the pole of the cell, i.e., in a division site that is formed later than that omitted previously. The model is also compatible with the fact that division only occurs at one of the two potential polar division sites; the two polar sites are independent of each other, and their timing of replication may be governed as explained by the curves in Fig. 8. It is unlikely that both initiations in a cell should be early enough to compensate for the previous delay. Increasing copy number of plasmid R1 should cause an increasing delay in initiation of chromosome replication; it is consistent with this view that the probability of forming septa in elongated cells decreased with increasing plasmid copy number (Table 2). The model postulates further that the formation of one septum consumes the mechanical forces and consequently inhibits the formation of a second one. Polar division has also been demonstrated in mutants of *E. coli* (49) and *Agmenellum quadruplicatum* (35) that are allowed to form filaments at 41 C and then are transferred to 30 C and treated with chloramphenicol and other inhibitory agents.

Normark and Wolf-Watz (34) have discussed the division patterns of cells carrying the *envA* mutation, which causes the formation of chains of cells. The effect of the *envA* mutation may be interpreted as being the opposite of filament formation. As a matter of fact, the introduction of R1 and F'*lac* into an *envA* strain significantly reduces the tendency for chain formation (K. Hjalmarsson, L. Norlander, S. Normark and K. Nordström, unpublished data).

The results presented in this paper indicate that the presence of a plasmid in a cell affects the cell division pattern. The situation is stable and is maintained as a steady state. Therefore, plasmid-containing cells may be useful in studies of the normal cell division cycle analogous to minicell-producing strains or to some cell division mutants such as *envA*, *envB*, or *envD*. Studies of this type may also give information about the control of chromosome and plasmid replication and the timing of initiation of the replication of these two replicons. Finally, the results may be of relevance when applying the Cooper-Helmstetter model (8) to cells containing plasmids; we believe that the effect of plasmids on cell division has to be considered in these cases.

ACKNOWLEDGMENTS

This work was supported by the Swedish Medical Research Council (project no. 4236).

The skillful technical assistance of Ann-Sofie Pettersson and Gerd Mauree is gratefully acknowledged.

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