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Degradation of Individual Chromosomes in *recA* Mutants of *Escherichia coli*

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Rapidly growing wild-type *Escherichia coli* cells contain two, four, or eight fully replicated chromosomes after treatment with rifampin, reflecting that all replication origins are initiated simultanously. Cells with defects in the timing of the initiation of replication may contain three, five, six, or seven fully replicated chromosomes after such treatment. This phenotype, termed the asynchrony phenotype, is also seen in recombination-deficient *recA* mutants. It is shown here that for *recA* strains, the phenotype can be explained by a selective and complete degradation of individual chromosomes. The selective degradation is largely *recD* dependent and is thus carried out by the RecBCD exonuclease.

The precision with which DNA replication initiates at the several origins in a rapidly growing cell can be determined measuring the number of fully replicated chromosomes after initiation is inhibited by rifampin and ongoing rounds of replication are allowed to be completed (24). Cells with a normal coordination of initiation initiate replication simultaneously at either two or four origins (depending on the growth rate) and therefore contain either two and four or four and eight fully replicated chromosomes. Cells that contain aberrant chromosome numbers exhibit the so-called asynchrony phenotype, which in some cases reflects a perturbation of the coordination of initiation, such that initiation of replication does not occur simultanously at all origins within each cell. In the case of *dnaA* and *dam* mutants (6, 8, 24, 26), the phenotype is accompanied by the loss of a precise interinitiation time, as demonstrated in density shift experiments (3, 28), showing that the time between two initiation events at the same origin varies greatly from cell to cell and from generation to generation in these mutants.

Recombination-deficient *recA* strains also exhibit the asynchrony phenotype (23) but appear to have almost a constant interinitiation time (15, 29), indicating that initiation is essentially normal and occurs at a specific time in the cell cycle.

The origin of the asynchrony phenotype for recombination-deficient *recA* strains has been an enigma for several years. Initially, two alternative models were presented (23). The first model suggested a role for RecA at the replication fork but was not compatible with the density shift data unless it was assumed that one of the two partly replicated daughter chromosomes at a stalled fork was degraded.

The second model invoked the selective degradation of individual chromosomes before and during rifampin treatment (23). The observed asynchrony phenotype could be accommodated within the degradation measured, about 15%. This model was plausible but was not favored because it was difficult to envision how such a selective and complete degradation could occur.

Recently, it was proposed that the recombination function of the RecA protein is necessary for the proper partitioning of daughter nucleoids (29). The asynchrony phenotype could then be explained by the aberrant segregation of daughter nucleoids, but a secondary initiation defect would have to be assumed to explain the appearance of cells containing one, three, five, or seven origins (7). If initiation is synchronous and the growth rate is relatively high, all cells contain two replicating structures with four origins at the time of segregation. Correct segregation yields 2 + 2; unequal segregation yields 4 + 0. In the latter case, the cell will grow to contain eight origins in a 2 + 2 + 2 + 2 configuration. Thus, correctly timed initiation will always produce chromosomes that are replicating at the time of segragation, and the lowest number of origins to be segregated will be two. Therefore, the occurrence of cells with one, three, five, or seven origins may be explained by defective segregation plus a secondary initiation defect.

Here, we have attempted to determine which of the latter two models explains the asynchrony phenotype most adequately. We present evidence that the asynchrony phenotype in recombination-deficient *recA* mutants is caused by the complete degradation of individual chromosomes and that aberrant nucleoid segregation is not the reason for the asynchrony phenotype.

MATERIALS AND METHODS

Bacterial strains. The *Escherichia coli* K-12 strains used are listed in Table 1.

Media and growth conditions. The bacteria were grown in AB medium (10) containing thiamine (1 μg/ml), 0.5% Casamino Acids, and 0.2% glucose. Strain CR34 and its derivatives were supplemented with thymine (10 to 20 μg/ml), threonine (50 μg/ml), and leucine (50 μg/ml). Antibiotics were included in overnight cultures. The growth of the cultures was monitored by measuring the optical density at 450 nm (OD_{450}).

Treatment with rifampin and furazlocillin. At an OD_{450} of 0.1 to 0.2, rifampin was added to the cultures at a final concentration of 150 μg/ml to inhibit the initiation of replication, and in some experiments, 4 μg of furazlocillin (a kind gift from R. D'Ari) (22) per ml was added to inhibit cell division (23). After drug addition, incubation was continued to allow the completion of ongoing rounds of replication.

For the experiment shown in Fig. 1, strain N1332 was grown at 32°C (doubling time, 75 min), and at an OD_{450} of 0.15, furazlocillin was added to 4 μg/ml. After 10 min, the

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TABLE 1. *E. coli* strains

Strain	Relevant genotype	Source, reference, or construction details
N1331	*recA*$^+$	E. Seeberg (17)
N1332	N1331 *recA200*	E. Seeberg (17)
MG1655	*rec*$^+$	J. Zyskind (14)
DBP271	MG1655 *recD1903*::mini-*tet*	J. Zyskind (14)
ALS972	MG1655 *recA938*::*cat*	J. Zyskind (29)
ALS973	MG1655 *recA938*::*cat recD1903*::mini-*tet*	J. Zyskind (29)
CR34	*rec*$^{+a}$	K. Rasmussen and T. Atlung (2)
KS932	CR34 *recD1903*::mini-*tet*	This work; CR34×P1.DPB271
KS933	CR34 *recA938*::*cat*	This work; CR34×P1.ALS972
KS934	CR34 *recA938*::*cat recD1903*::mini-*tet*	This work; KS932×P1.ALS972

[a] Remaining genotype: *thyA deoC thr leu thi.*

culture was split in two; one half was kept at 32°C, and the other half was shifted to 42°C to inactivate the temperature-sensitive RecA protein. At 5 min after the temperature shift, rifampin was added (to 150 μg/ml) to both cultures, and ongoing rounds of replication were allowed to run to completion (5 h).

Flow cytometry. Cell preparation and flow cytometry with an Argus flow cytometer (Skatron, Tranby, Norway) were performed as described previously (25).

DNA degradation. The level of DNA breakdown during drug treatment was measured by labeling the DNA with [^{3}H]thymidine (Amersham, Buckinghamshire, England) for two generations, removing the label by filtration, and reincubating the cells in warm medium supplemented with unlabeled thymidine (20 μg/ml) for about one generation. Rifampin (150 μg/ml) was added, samples were withdrawn after 0, 30, 120, and 240 min into 5% trichloroacetic acid, filtered through glass fiber filters, washed with 10 mM HCl–96% ethanol, and dried, and the ^{3}H activity was measured by scintillation counting.

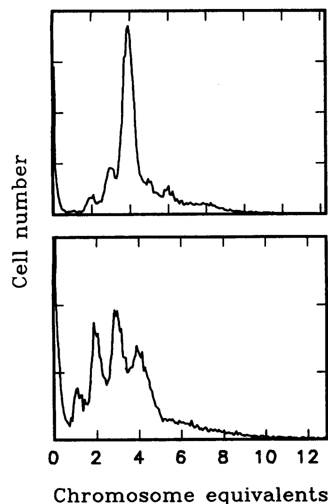

FIG. 1. Specific degradation of individual chromosomes occurs when RecA is inactivated. Shown are DNA histograms of N1332 cells grown at 32°C and treated with furazlocillin to inhibit cell division. After 10 min, half of the culture was shifted to 42°C (lower panel), and the other half was kept at 32°C (upper panel). After 5 min, rifampin was added to inhibit the initiation of replication. After 5 h, samples for flow cytometry were collected and stained as described previously (25). The fractions of non-DNA-containing and one-, two-, three-, and four-chromosome cells were 7, 1, 4, 10, and 53% in the upper panel and 11, 8, 18, 24, and 20% in the lower panel.

RESULTS

Degradation of individual chromosomes. In an attempt to experimentally distinguish between the selective degradation of individual chromosomes and the aberrant partitioning of nucleoids, we used strain N1332 carrying a temperature-sensitive *recA* allele (Table 1). Cell division was inhibited by the addition of furazlocillin (22) before the RecA protein was inactivated by shifting of the cells to the nonpermissive temperature. After the temperature shift, the cells were treated with rifampin, and ongoing rounds of replication were allowed to run to completion. A defect in nucleoid partitioning should have had no effect on the cellular DNA content in this experiment, since cell division was stopped before RecA was inactivated. Control experiments demonstrated that less than 10% of the cells divided after the furazlocillin treatment (data not shown).

Figure 1 shows the DNA histograms of strain N1332 after the described treatment (lower panel) and a control culture given the same treatment without the temperature shift (upper panel). There were substantially more non-DNA-containing and one-, two-, and three-chromosome cells in the culture that was shifted to 42°C than in the culture that was kept at 32°C. Strain N1331 given the same treatment showed a negligible amount of non-DNA-containing and one-, two-, and three-chromosome cells at both 32 and 42°C (data not shown). These results can be explained by the selective degradation of individual chromosomes in cells in which the RecA protein is inactive but cannot be explained by the aberrant segregation of daughter chromosomes.

Selective degradation is *recD* dependent. A substantial amount of the degradation occurring in *recA* mutants is due to the exonuclease activity of the RecBCD enzyme (9, 21). Cells that lack the RecD subunit of this enzyme lack RecBCD exonuclease activity (4, 21). The phenotype of a *recA recD* double mutant was therefore investigated. Strains MG1655 (*rec*$^+$), DPB271 (*recD*), ALS972 (*recA*), and ALS973 (*recA recD*) (Table 1) had doubling times of 39, 39, 47, and 52 min, respectively. At an OD_{450} of about 0.15, rifampin was added, and ongoing rounds of replication were allowed to run to completion. No aberrant phenotype could be detected for the *recD* single mutant (Fig. 2C). The *recA* single mutant exhibited the asynchrony phenotype, as shown before, with a decrease in the amount of four-chromosome cells and an increase in the amounts of one-, two-, and three-chromosome cells (Fig. 2E). The *recA recD*

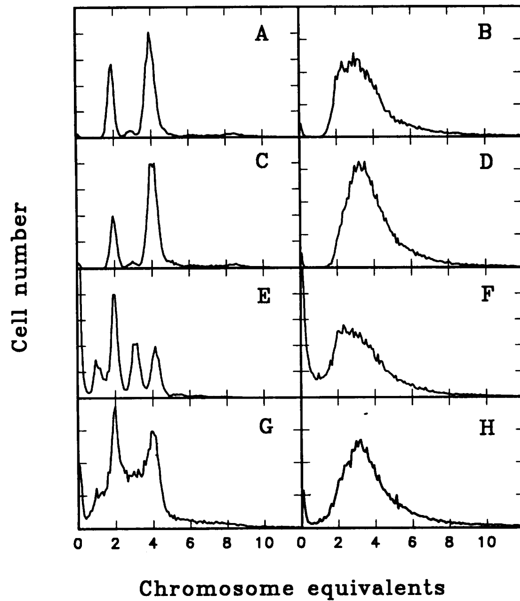

FIG. 2. Selective and complete degradation of individual chromosomes does not occur in *recA recD* cells. Shown are DNA histograms of exponentially growing (B, D, F, and H) and rifampin-treated (A, C, E, and G) strains MG1655 (wild type; A and B), DPB271 (*recD*; C and D), ALS972 (*recA*; E and F), and ALS973 (*recA recD*; G and H).

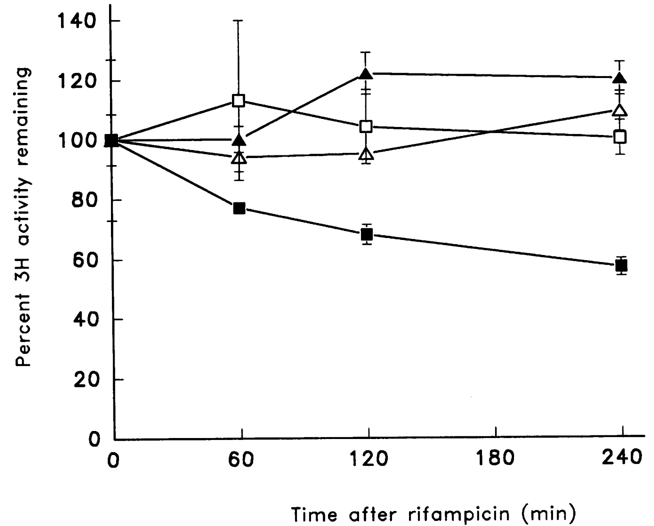

FIG. 3. DNA degradation during rifampin treatment. Cultures of CR34 (rec^+) (□), KS932 (*recD*) (△), KS933 (*recA*) (■), and KS934 (*recA recD*) (▲) prelabeled with [^{3}H]thymidine were treated with rifampin. Samples were withdrawn at the indicated times, and the amounts of acid-insoluble ^{3}H were determined.

double mutant exhibited distinct peaks only at two and four chromosomes (Fig. 2G). There were fewer four-chromosome cells than in the wild type, but this result may be explained by the somewhat longer doubling time of the mutant. Cells with one chromosome and three chromosomes were also apparent but were not separated into distinct peaks, suggesting either that some forks were unable to run to completion or that a small amount of degradation took place in the *recA recD* mutant as well.

Before treatment with rifampin, few wild-type cells and few *recD* and *recA recD* mutant cells contained less than one chromosome (Fig. 2B, D, and H), while about 10% of *recA* mutant cells contained less than one chromosome equivalent of DNA (Fig. 2F). These results also indicate a significant level of DNA degradation in the *recA* strain during exponential, unperturbed growth.

DNA degradation was measured directly in these strains (data not shown) and in the thymine-requiring strains CR34 (rec^+), KS932 (*recD*), KS933 (*recA*), and KS934 (*recA recD*) after prelabeling of cultures with [^{3}H]thymidine and comparison of amounts of acid-insoluble label in samples withdrawn at different times after rifampin treatment (Fig. 3). There was little degradation in the rec^+, *recD*, and *recA recD* strains and about 40% degradation in the *recA* strain during 4 h of rifampin treatment.

Fully replicated chromosomes are less prone to degradation. The nature of the DNA degradation during rifampin treatment was further investigated by use of the temperature-sensitive *recA* allele. Strain N1332 was grown at the permissive temperature (30°C). The culture was split into six portions, and the initiation of replication and cell division were inhibited by the addition of rifampin and furazlocillin. Cultures 1, 2, 3, 4, and 5 were kept at the permissive temperature for 10, 30, 60, 120, and 180 min, respectively, and then shifted to the nonpermissive temperature for 3 h. Culture 6 was kept at the permissive temperature for 6 h. In culture 5, most of the replication forks had reached the termini at the time of the temperature increase and RecA inactivation, while in culture 1, most of the forks were still proceeding.

The control culture contained mostly cells with four chromosomes but also some two- and three-chromosome cells (Fig. 4, panel 6, and Table 2). In comparison, all the cultures that were shifted to the nonpermissive temperature contained fewer four-chromosome cells and a substantial number of cells with zero, one, two, or three chromosomes. Cultures 1 and 2 contained more non-DNA-containing and one-chromosome cells than cultures 4 and 5 (Fig. 4 and Table 2). Thus, it seems that more degradation of the chromosomes occurred (during the 3 h at 42°C) when the chromosomes were still undergoing replication than when fully replicated. An alternative explanation is that the activity of the degrading enzymes was significantly reduced by a few hours of rifampin treatment.

DISCUSSION

The data presented show that the lack of a functional RecA protein causes the complete degradation of single chromosomes and that this degradation causes the appearance of the asynchrony phenotype. The selective degradation seems to be largely RecD dependent and is thus carried out by the RecBCD enzyme (exonuclease V).

Evidence is presented that the asynchrony phenotype is not caused by the aberrant segregation of daughter nucleoids, as has been suggested by Zyskind et al. (29). It is still possible that RecA has a role in the partitioning of daughter chromosomes, but because of the extensive DNA degradation in *recA* cells and the possibility of a low percentage of degradation in *recA recD* cells, this possibility could not easily be addressed in the present experiments.

The origin of and initial substrate for the highly specific degradation of single chromosomes by the RecBCD enzyme are not clear. The RecBCD enzyme is important in homol-

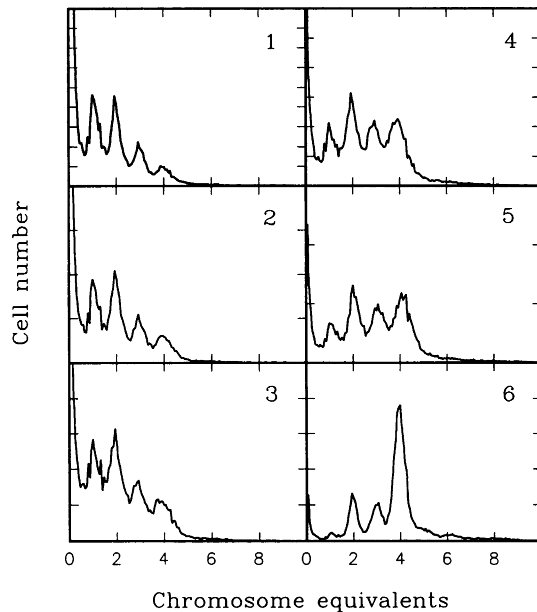

FIG. 4. Selective degradation during rifampin treatment. Strain N1332 was grown at 30°C, rifampin and furazlocillin were added, and the cultures were incubated for 10 min (1), 30 min (2), 1 h (3), 2 h (4), or 3 h (5) before a shift to 42°C for 3 h. Culture 6 was kept at 30°C for 6 h. The DNA content per individual cell was analyzed by flow cytometry. Culture numbers are given in the upper-right-hand corners of the panels.

ogous recombination and the maintenance of cell viability (27). The enzyme unwinds linear duplex DNA and has a potent double-strand nuclease activity that acts on linear but not closed circular DNA. Recombination by the RecBCD pathway has been proposed to occur when the RecBCD enzyme binds to a duplex end and unwinds the DNA. When this enzyme encounters a correctly oriented Chi sequence, it nicks the Chi sequence-containing strand and continues unwinding the DNA, spooling out a 3′-terminated strand that is proposed to invade homologous DNA with the aid of RecA and single-strand-binding protein (13, 27).

RecBCD requires a double-strand break to enter the DNA duplex and to start unwinding or degrading. Such duplex DNA ends seem to be protected from RecBCD degradation by RecA (12). Analogously, in our experiments, without protection by RecA, RecBCD could degrade one of the two duplex arms of a replicating chromosome.

Termination and segregation events may cause gaps with free ends and single-stranded regions, structures that are effective substrates for degradation (1, 18). Chromosomal

TABLE 2. Quantitation of the number of cells within each peak in the histograms of Fig. 4

Culture	% of cells with the following no. of chromosomes:					
	0	1	2	3	4	More than 4
1	38	23	20	11	6	2
2	29	21	24	14	10	2
3	24	22	25	14	12	3
4	16	14	24	17	23	6
5	12	12	22	20	27	7
6	4	2	15	13	53	13

dimers are formed by RecA and resolved by site-specific recombination at the end of the replication cycle (5, 11, 16). Recombination by RecA therefore has been proposed to be an essential part of the termination and segregation of daughter chromosomes (29). Further evidence for a function of RecA in termination involves the hyperrecombination that occurs in the terminus area (19) and the possible role of RecA as part of a "salvage pathway" for replication past *ter* sites when replication is unidirectional (12a, 20). Among these processes may be the ones creating intermediates that when unused and unprotected by RecA lead to degradation by RecBCD.

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