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Effects of the *ccd* Function of the F Plasmid on Bacterial Growth

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The *ccd* segment of the mini F plasmid containing the *ccdA* and *ccdB* genes controls the coordination between plasmid proliferation and cell physiology and fate. When the DNA replication of a thermosensitive-replication plasmid carrying the *ccd* segment of mini F is blocked, plasmid DNA molecules are progressively diluted through cell division until the copy number reaches 1 per cell. From this time on, there is little increase in the number of viable cells, although cells continue to divide, resulting in a mixed population of viable cells (mostly plasmid containing), nonviable but residually dividing cells, and nonviable nondividing cells. Results are presented suggesting that plasmid-containing cells are viable and continue to divide, whereas plasmid-free segregants are nonviable and form filaments after a few residual divisions, with DNA synthesis reduced or arrested in the filaments. Although the *ccd* functions are known to induce the SOS response when plasmid replication is blocked, the production of nonviable plasmid-free segregants is independent of the SOS cell division inhibition mechanism determined by the *sfiA* and *sfiC* genes.

Cell division in *Escherichia coli* strains harboring the F plasmid is coupled to the proliferation of this plasmid. When the copy number of the plasmid decreases to the critical level of 1 per cell, the formation of viable plasmid-free segregants is inhibited. This inhibition depends on the *ccd* (coupled cell division) segment, which lies between coordinates 42.9 and 43.6 kilobases on the map of F, outside the regions necessary for autonomous replication and partition of the plasmid (17).

The *ccd* segment specifies two functions: the *ccdB* function, responsible for this inhibition, and the *ccdA* function, which suppresses the inhibition as long as the plasmid copy number is greater than 1 per cell (17). These functions have also been called proteins H and G (2, 10), *letA* and *letD* (13, 14), and *lynA* (1).

In the present work, we show that when replication of a thermosensitive plasmid carrying the *ccd* segment of F is blocked at the nonpermissive temperature, colony formers nearly stop increasing after a lag, but cell division continues and nonviable cells are produced. To account for this phenomenon, we propose a new model of the *ccd* functions, the nonviable segregant model.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains used derive from *E. coli* K-12 SH392 (same as strain KH802 in reference 17) F⁻ *met hsdR sfiC*. It was found in this work that strain SH392 is SfiC. The *sfiA100*::Tn*5* marker was introduced by transduction with P1 *vir* phage. The genotype of strain SH2743 is F⁻ *met hsdR pyrD sfiA100*::Tn*5 sfiC*. Strain SH2746 is the same as SH2743 except that it is also *thyA deo*. The SfiC phenotype was determined as described by D'Ari and Huisman (4). The construction of plasmids pXX332 and pXX333 was described by Mori et al. (15). These plasmids are derivatives of pHSG415 (6), a derivative of pSC101 unable to replicate at 42°C. Both plasmids carry the chloramphenicol resistance gene. pXX333 carries the *ccd* segment of a mini F plasmid coding for the *ccdA* and *ccdB* functions; pXX332 is a pHSG415 derivative and lacks the *ccd* segment.

Media. Experiments were carried out in either L broth containing 0.5% glucose or ME medium (18) supplemented with 0.4% Casamino Acids, 0.4% glucose, 20 μg of uracil per ml, and, when necessary, thymine at 50 or 5 μg/ml. Antibiotics were used at the following concentrations: kanamycin, 25 μg/ml; chloramphenicol, 20 μg/ml; and rifampin, 100 μg/ml. The counting solution for the Coulter Counter contained 350 g of NaCl and 100 ml of Formalin in 20 liters of distilled water. It was filtered twice through a filter (0.45-μm pore size; Millipore Corp.). Percoll (density, 1.130 g/ml) was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. P agar medium contains 5 g of NaCl, 10 g of polypeptone, and 12 g of agar per liter. L-[^{35}S]methionine (1.110 Ci/mmol), [6-^{3}H]thymidine (20 Ci/mmol), and [*methyl*-^{3}H]thymine (20 Ci/mmol) were purchased from New England Nuclear Corp.

Kinetics of cell growth. Cultures of plasmid-carrying cells were incubated overnight at 30°C in the presence of chloramphenicol. They were then diluted 100-fold into fresh medium containing chloramphenicol and grown for another 2 h at 30°C. The cells were centrifuged, suspended in nonselective medium, and transferred to 42°C. To maintain cells in the exponential phase, the cultures were diluted at intervals with fresh prewarmed medium. Samples were taken at appropriate intervals. The following parameters were evaluated: number of particles, colony formers, and plasmid-free segregants per milliliter, cell volume distribution, and turbidity. The turbidity of cultures was measured in a Klett-Summerson colorimeter with a no. 54 filter. Colony formers were determined by plating appropriately diluted samples onto P agar plates supplemented with uracil. Colonies were scored after incubation at 30°C. To test for chloramphenicol resistance, colonies were picked with sterile toothpicks and streaked on L agar plates containing chloramphenicol; the plates were incubated at 30°C overnight.

Determination of particle number and cell volume distribution. Particles were counted and their volume distribution was determined in a Coulter Counter (industrial model B;

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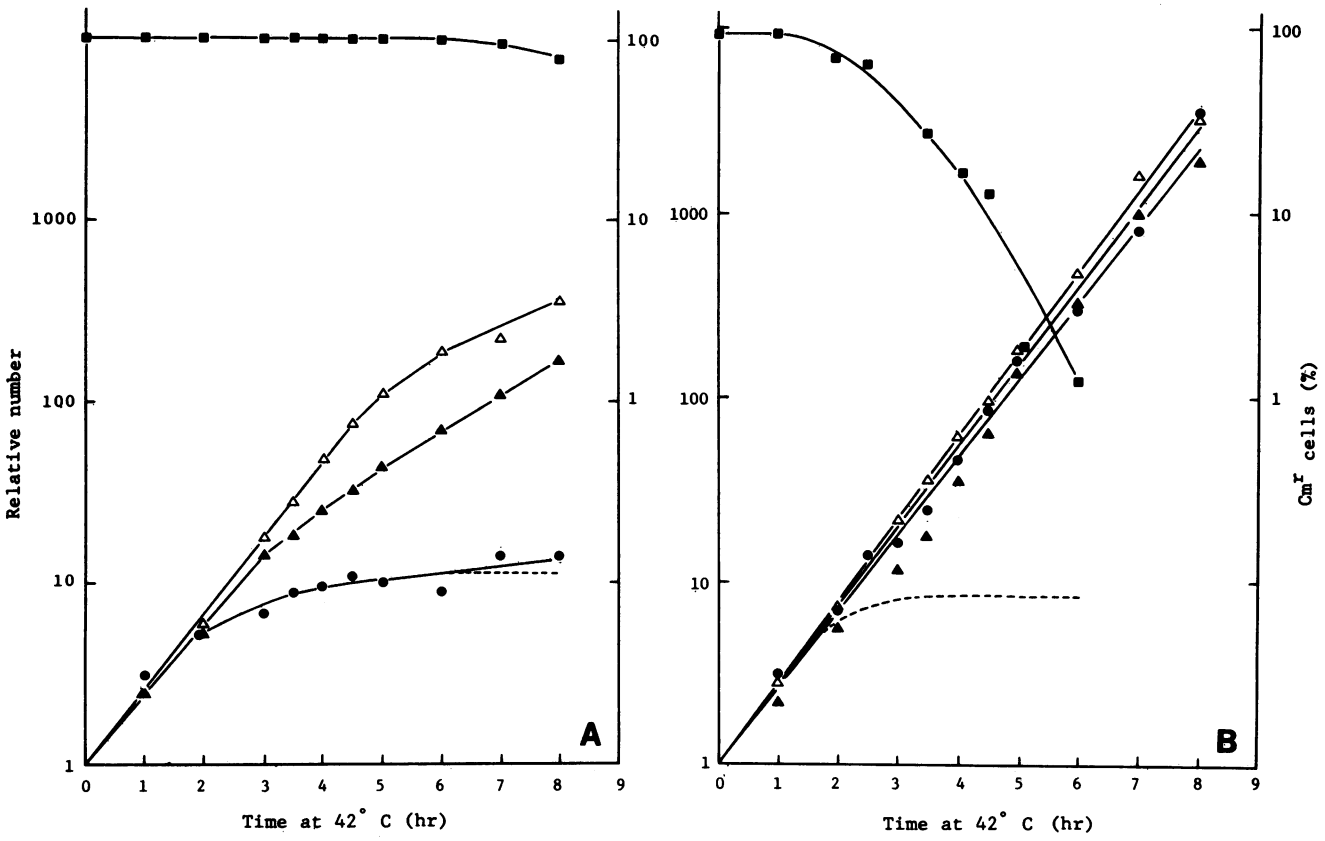

FIG. 1. Kinetics of cell growth and plasmid stability after transfer to 42°C. Cells of strains SH2743(pXX333) (A) and SH2743(pXX332) (B) growing exponentially at 30°C in enriched ME-glucose medium containing chloramphenicol were collected and suspended in nonselective medium and transferred to 42°C at time zero. Samples were analyzed as described in the text. The generation time of strain SH2743(pXX332) was 47 min at 42°C. △, turbidity; ▲, number of particles; ●, number of colony formers (both Cm^r and Cm^s); - - - - -, Cm^r colony formers; ■, clones carrying the plasmid as a percentage of the total number of colony formers.

Coulter Electronic Industrial Division) with a 30-μm orifice, 50-μl manometer, attenuation setting of 1/4, and current aperture of 1/2. Cell volume distributions were recorded with a Channelyser.

Percoll density gradient. Samples (30 ml) of cultures were centrifuged, suspended in 1 ml of saline, and deposited on the top of a 30-ml Percoll linear density gradient (15 to 80%) in polyethylene tubes. They were then centrifuged for 90 s at 2,200 rpm in a Kubota model KN-70 centrifuge with swinging buckets. After centrifugation, either 27 or 54 fractions were collected from the bottom of the tube. Fractions were numbered from the top to the bottom.

Autoradiography of continuously labeled cells. Strains were cultivated for 20 generations at 30°C in ME medium supplemented with Casamino Acids, glucose, chloramphenicol, and [^{3}H]thymine (5 μg/ml; specific activity, 2.5 Ci/mmol). Before the transfer to 42°C, cultures were diluted 1,000-fold in the same medium without chloramphenicol. Samples were withdrawn, fixed in 2% Formalin for 15 min, filtered, washed, and suspended in water. Bacterial suspensions were spread on microslides and treated as described previously (10). The slides were immersed in Sakura photographic emulsion NR-M2 (Konishiroku Photo Ind. Co., Osaka, Japan) and developed after different exposure times at 4°C. The slides were then stained with Giemsa dye. Color photographs were taken through a phase contrast microscope to analyze the number of grains per cell. In color prints, cells were green, grains were dark violet or white, and the background was light orange. Monocolor photographs, taken with a red filter, are shown in this paper.

Measurement of radioactive material in acid-insoluble fraction. Samples (200 μl) were added to 3 ml of cold 5% trichloroacetic acid and 50 μl of bovine serum albumin (5 mg/ml). Acid-insoluble fractions were collected on glass filters and washed repeatedly with 5% trichloroacetic acid and ethanol. The filters were dried and immersed in a toluene scintillator; radioactivity was counted in a scintillation counter.

Measurement of plasmid DNA. Bacterial cultures grown in enriched ME-glucose medium at 30°C were transferred to 42°C. Samples were removed at intervals, cells were collected by centrifugation, and plasmid DNA was extracted by the modified alkaline method (12). Plasmid DNA obtained from the same mass of each culture (60 Klett units, 15 ml) was analyzed by electrophoresis on a 0.8% agarose gel. The gel was stained with ethidium bromide, and a photograph was taken with a red filter under UV light. The photographic film was analyzed with a densitometer.

RESULTS

Kinetics of cell division. The growth of strain SH2743 (*sfiA sfiC*) harboring the thermosensitive plasmid pXX333 which carried the *ccd* segment (*ccdA ccdB*) was monitored at the restrictive temperature (42°C) for the replication of the

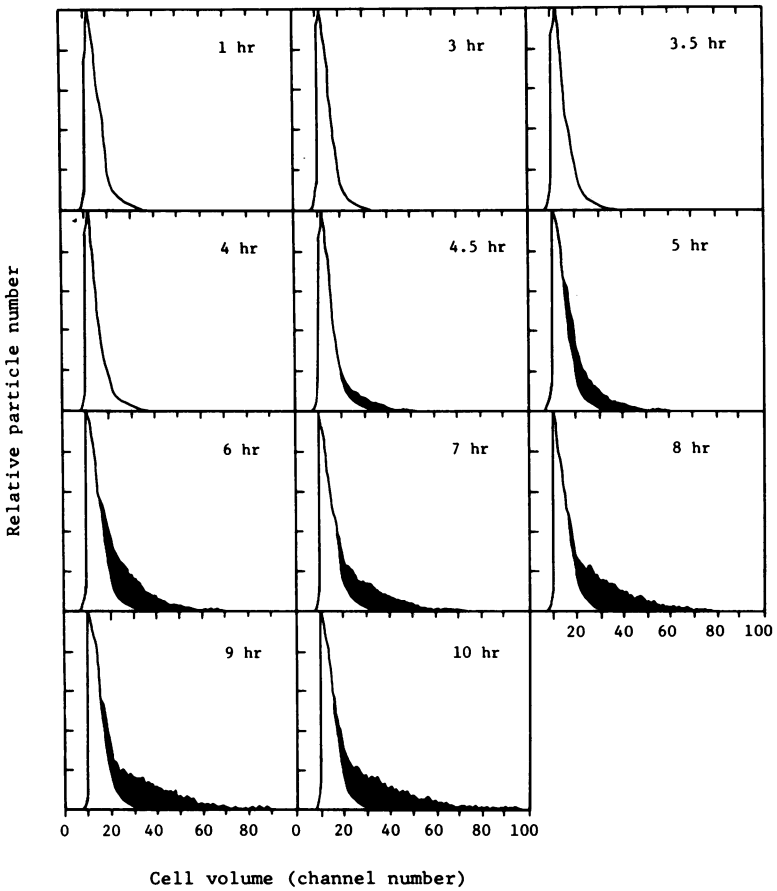

FIG. 2. Distribution of cell volume in strain SH2743(pXX333). A culture of SH2743(pXX333) was transferred to 42°C as described in the legend to Fig. 1. Samples were removed at intervals and analyzed with a Coulter Counter with a Channelyser. Solid areas indicate elongated cells.

plasmid. When a culture was transferred to 42°C in nonselective ME medium, the number of viable cells continued to increase exponentially for 2 h (2.6 generation times) and then at a much reduced rate (Fig. 1A). However, the cell mass and particle number continued to increase for at least 7 to 8 h. Plasmid-free segregants appeared at a low frequency after 5 h and constituted 20% of the colony formers after 8 h of incubation at 42°C. The continued increase in the particle number suggests that after 2 h of incubation, residual cell division occurred, giving rise to nonviable particles. After 8 h at 42°C, these nonviable particles constituted 90% of the total population. An analysis of the cell size distribution (Fig. 2) revealed that during the first 4 h at 42°C, the population comprised cells whose size was normal and uniform. After 4 h filamentous cells appeared, and after 8 h these filaments constituted about 40% of the population.

A culture of strain SH2743 harboring the plasmid pXX332 was grown under the same conditions. It showed an exponential increase in particle number, colony formers, and turbidity (Fig. 1B). There was no change in the cell size distribution throughout the incubation period at 42°C (data not shown). Plasmid-free segregants appeared at high frequency, and the time when these segregants first appeared (2 h at 42°C) coincided with the time when colony formers practically ceased to increase in the strain harboring the *ccd*$^+$ plasmid. A similar coincidence was observed when these two strains were grown in L broth medium: after 3 h at 42°C, the number of colony formers ceased to increase in the strain harboring the *ccd*$^+$ plasmid, and this was the time when plasmid-free segregants were observed in the control strain harboring the *ccd* plasmid (data not shown).

The data presented here show that, although the number of colony formers remained nearly constant after 2 h at 42°C, residual divisions occurrred and a mixed population was produced. This population comprised 90% nonviable cells, 8% viable plasmid-carrying cells, and 2% viable plasmid-free segregants after 8 h (about 10 generations) at 42°C. The *sfiA sfiC* strain SH392(pXX333) gave similar results after incubation at 42°C, except that there were more filamentous cells (data not shown).

We next looked at the kinetics with which the viable cells resumed division after being returned to 30°C. Strain SH392(pXX333) was grown in nonselective L medium at 42°C, and samples were taken after 5 h at 42°C and transferred to 30°C. Transfer to the permissive temperature resulted in an immediate increase in colony-forming ability at 30°C; the particle number continued to increase (Fig. 3), and 3% plasmid-free segregants were detected 2.5 h after the temperature downshift. Under these conditions, viable cells resumed normal division, whereas the nonviable cells were not rescued but continued their residual cell division.

To see whether the restoration of cell division after return to 30°C required protein synthesis, rifampin was added to the samples just before the shift to the permissive temperature

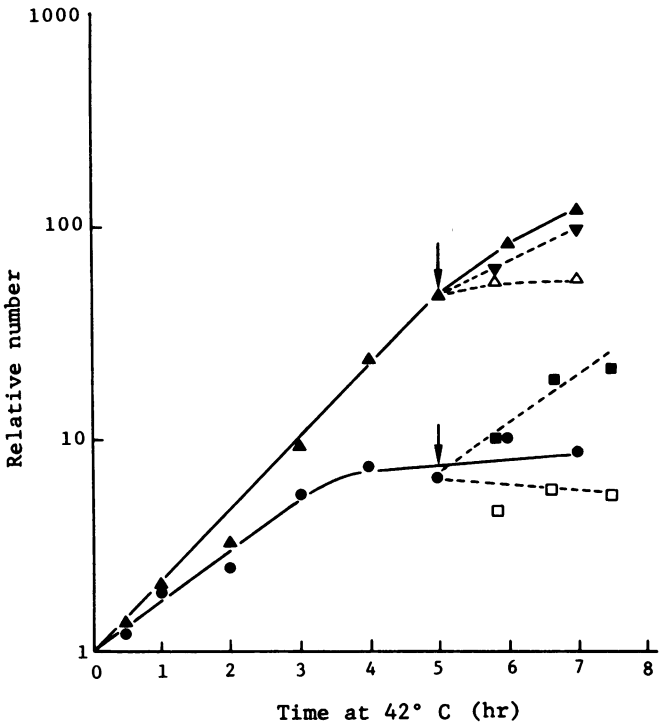

FIG. 3. Kinetics of cell growth after a shiftdown to 30°C. A culture of strain SH392(pXX333) was incubated at 42°C as described in the legend to Fig. 1. After 5 h, samples were removed (arrows) and incubated at 30°C with or without rifampin (100 μg/ml). The number of particles was determined for the culture incubated at 42°C (▲) and after the shiftdown to 30°C with (△) and without (▼) rifampin. The number of colony formers was determined for the culture incubated at 42°C (●) and after the shiftdown to 30°C with (□) and without (■) rifampin.

(Fig. 3). Under these conditions, the increase in particle number was immediately halted and the number of colony formers remained constant, showing that the ability to produce both viable and nonviable cells depended on protein synthesis.

Thermosensitivity of plasmid DNA replication. To confirm that plasmid DNA replication is inhibited after transfer to 42°C, the amount of plasmid DNA per cell mass was measured. Cultures of SH2743(pXX332) and SH2743 (pXX333) grown in enriched ME-glucose medium at 30°C were transferred to 42°C and sampled during the first 3 h. Cell mass was then adjusted before plasmid DNA was extracted. Plasmid DNA stained with ethidium bromide was analyzed by agarose gel electrophoresis. Densitometry revealed that for all samples, more than 95% of the plasmid DNA was in covalently closed circular form, the rest of the DNA was in open circular form, and linear plasmid DNA was not detected. The plasmid DNA/cell mass ratio decreased exponentially with time at a rate equal to that of the mass increase (generation time, 47 min), confirming that replication of both plasmids was blocked at 42°C (Fig. 4).

Plasmid-free segregants appeared after 2 h at 42°C in strain SH2743(pXX332) (Fig. 1B). By the equation described by Durkacz and Sherratt (5), these segregation kinetics show that the copy number of pXX332 per cell was 3 to 4 at time zero. Therefore, the copy number should reach 1 after about 2 h at 42°C.

Fractionation of cells by size. Strain SH2743(pXX333) grown at the nonpermissive temperature produced cells of different sizes (Fig. 2). This population comprised both viable and nonviable particles (Fig. 1A). To determine whether the viable cells were of normal size or filamentous, the cells were separated by size by Percoll gradient centrifugation. Strain SH2743(pXX332) was examined in parallel. After 8 h of incubation at 42°C, the bacteria were centrifuged on a Percoll gradient and collected in 27 fractions. Each fraction was analyzed for number of particles, viable cells, and plasmid-free segregants and the distribution of cell sizes. More than 99% of the particles were in the top five fractions (Fig. 5).

The top two fractions (fractions 1 and 2) of the strain SH2743(pXX333) culture contained mainly normal-size cells, whereas fractions 3 and 4 contained normal-size cells and filaments (Fig. 5A and 6A). In fraction 4 the majority of the cells were filamentous. The majority of viable cells were in fractions 1 and 2 (Fig. 5C). These results indicate that viable cells are normal in size and that filamentous cells have lost colony-forming ability. Nevertheless it should be noted that 90% of the cells in fraction 1 and 2 were nonviable.

In the strain SH2743(pXX332) control experiment, the ratio of viable cell to particle number was constant in all five fractions, and more than 99% of the cells were chloramphen-

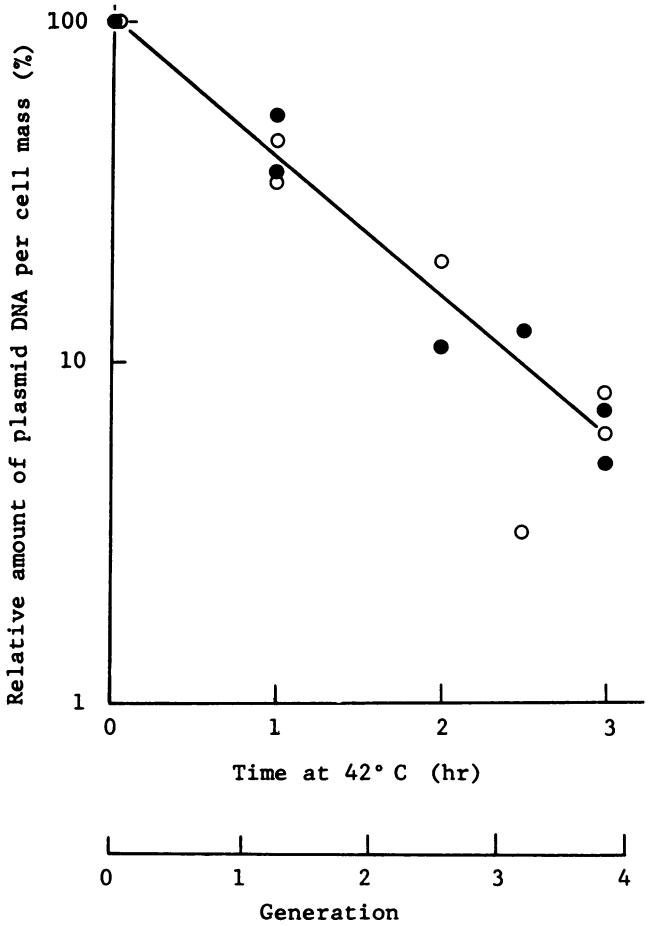

FIG. 4. Amount of plasmid DNA per cell mass after transfer to 42°C. Bacterial cultures of strains SH2743(pXX332) (●) and SH2743(pXX333) (○) grown at 30°C were transferred to 42°C. After incubation for indicated time, plasmid DNA was extracted and analyzed as described in the text.

icol-sensitive (Cm^s) segregants (Fig. 5B and D). Cell size was normal in all fractions (Fig. 6B).

DNA synthesis. The rate of DNA synthesis in the different cell populations was measured by pulse-labeling with [^{3}H]thymidine, followed by cell fractionation on a Percoll gradient. Cultures of strains SH2743(pXX333) and SH2743(pXX332) were grown at 30°C in the presence of [^{35}S]methionine for 4 h and then transferred to 42°C in the presence of [^{35}S]methionine. After 8 h of incubation, the cultures were pulse-labeled with [^{3}H]thymidine for 10 min and then centrifuged on a Percoll gradient and collected in 54 fractions. For strain SH2743(pXX333), the ^{3}H/^{35}S ratio calculated from the curves (Fig. 7B) was highest in fraction 3, which contained cells of normal size (Fig. 7A). From fractions 4 to 8, the ^{3}H/^{35}S ratio decreased progressively while the proportion of elongated cells increased, suggesting that in elongated cells DNA synthesis proceeded at a slower rate per unit mass. In fractions 1 and 2, the ^{3}H/^{35}S ratio was also lower than in fraction 3; we show below that these fractions contained small anucleate cells.

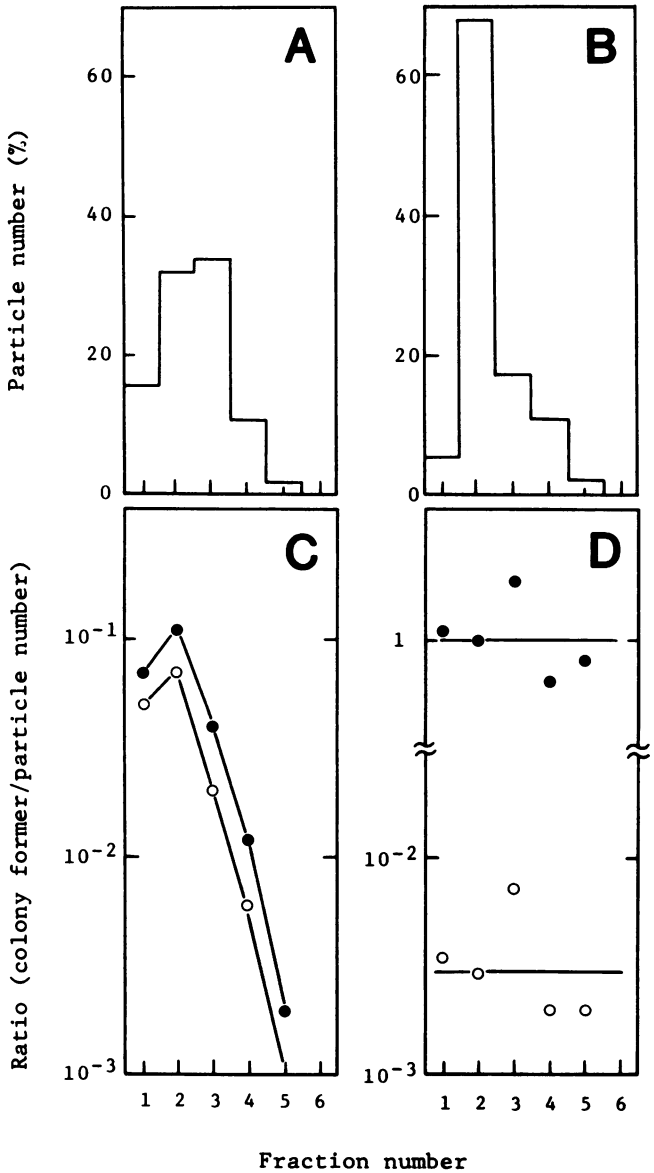

FIG. 5. Fractionation of cells by Percoll gradient centrifugation. Cells of strains SH2743(pXX333) (A and C) and SH2743(pXX332) (B and D) were incubated at 42°C for 8 h as described in the legend to Fig. 1, collected, and analyzed by Percoll gradient centrifugation. Twenty-seven fractions were collected and numbered from top to bottom. Symbols: ●, Cm^r and Cm^s colony formers per particle; ○, Cm^r colony formers per particle.

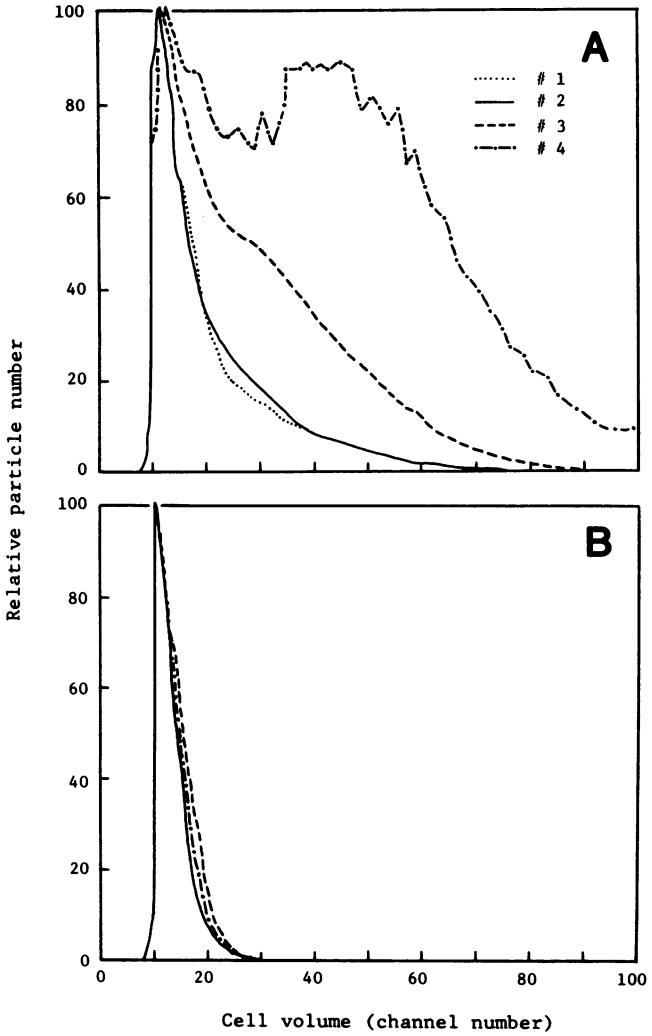

FIG. 6. Cell volume distribution. Fractions separated by Percoll gradient centrifugation were analyzed with a Coulter Counter. Fraction numbers correspond to the numbers shown in Fig. 5. (A) strain SH2743(pXX333); (B) strain SH2743(pXX332).

In contrast, with the control strain SH2743(pXX332), the ^{3}H/^{35}S ratio was the same for all fractions (data not shown). The ratio was essentially the same as that of fraction 3 of SH2743(pXX333). Therefore, it seems likely that for the strain carrying pXX333, essentially all cells in fraction 3 were synthesizing DNA at the normal rate, although 90% of the cells in this fraction were nonviable.

Autoradiography of cells. To determine the rate of DNA synthesis in individual cells, a culture of strain SH2746 (*sfiA sfiC thy deo*)(pXX333) was pulse-labeled with [^{3}H]thymidine for 3 min after 8 h of incubation at 42°C, and autoradiography was then carried out (Fig. 8). The distribution of grains was analyzed after 14 days of exposure (Table 1 and Fig. 9).

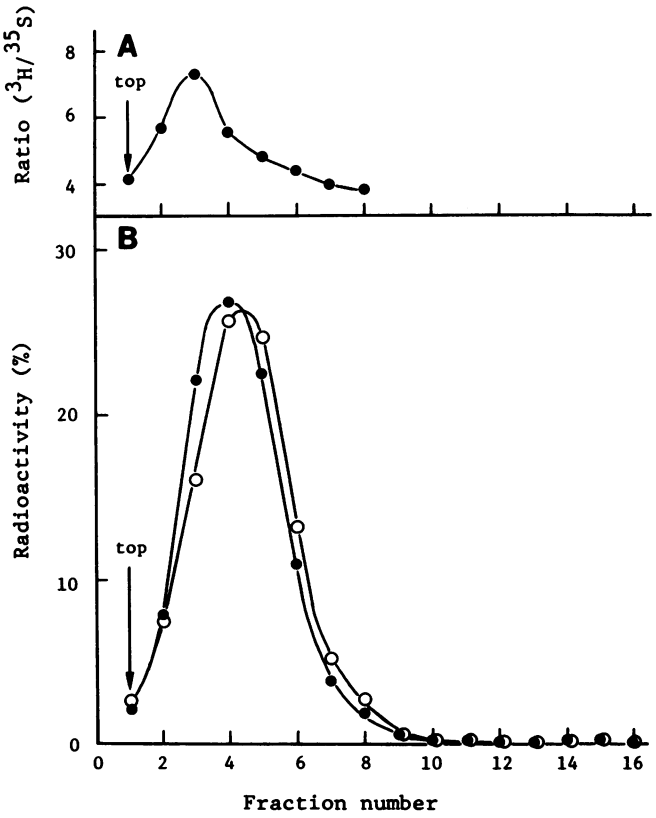

FIG. 7. Fractionation of cells labeled with [^{35}S]methionine and [^{3}H]thymine. Bacterial cells of strain SH2743(pXX333) were incubated in enriched ME-glucose medium containing [^{35}S]methionine (22 μCi/ml) for 4 h at 30°C and then transferred to 42°C and incubated for 8 h in the presence of [^{35}S]methionine. After incubation, the culture was pulse-labeled with [^{3}H]thymidine (40 μCi/ml) for 10 min at 42°C, and labeling was stopped by the addition of 0.2% sodium azide. Cells were collected and analyzed by Percoll gradient centrifugation. Fifty-four fractions were collected, and 0.2 ml of each fraction was analyzed for acid-insoluble radioactivity. (B) Symbols: ●, ^{3}H; ○, ^{35}S.

Although only 10% of the cells were viable, 71% were still synthesizing DNA, and half of these were filaments. Significant fractions of both normal-size and filamentous cells were not actively synthesizing DNA.

To see whether these cells contained DNA, the *thyA deo* strain SH2746(pXX333) was labeled with [^{3}H]thymine for 20 generations at 30°C and then divided into two portions. These cultures were incubated at 42 or 30°C for 8 h in the presence of [^{3}H]thymine. Samples were fixed and prepared for autoradiography. After 4 days of exposure, nuclear bodies were clearly visible in most cells, and anucleate cells were easily detected under the microscope. The 30°C control culture contained only 1.6% anucleate cells. The average number of grains per cell was about 8 (data not shown).

In contrast, in the culture incubated for 8 h at 42°C, 22% of the population was anucleate (Table 1). The anucleate cells were all small; no filaments without grains were detected. These anucleate cells presumably corresponded to the nonincorporating normal-size cells detected by pulse-labeling. Most filaments displayed one localized mass of DNA, although some revealed DNA distributed throughout the cell. After continuous labeling and transfer to 42°C, filamentous cells contained about 2.4 times more DNA per cell than did DNA-containing cells of normal size (Table 1). After 6 to 10 h at 42°C, a culture of strain SH2743(pXX333) was examined directly under a phase contrast microscope. Filamentous cells were observed with constrictions near one or both ends at distances similar to the length of small or normal cells. It seems likely that the anucleate cells were produced by aberrant division of the filaments.

From the results described above, four types of cells can be distinguished: normal-size cells that contained and synthesized DNA, normal-size anucleate cells, filaments that contained DNA but no longer synthesized DNA, and filaments that contained and synthesized DNA. The rate of DNA synthesis per cell in these filaments was similar to that in normal-size cells, although their DNA content and volume were greater. Thus, the rate of DNA synthesis per chromosome is reduced in filamentous cells and ultimately stops in some.

Origin of plasmid-free segregants. Viable plasmid-free segregants were detected after 5 h at 42°C in the culture of strain SH2743(pXX333) (Fig. 1A). These viable segregants could be either preexisting or formed during the incubation period at high temperature. Since the plasmid confers chloramphenicol resistance, the growth of preexisting segregants can be inhibited by adding chloramphenicol to the culture medium. Assuming that inhibition is complete, the absolute number of segregants can only increase through segregation at 42°C. We tested the colony-forming ability of strain SH2743 harboring the *ccd*$^+$ plasmid pXX333 after growth in liquid medium at 30 or 42°C for 10 h in the presence of chloramphenicol. As described above, the cultures were diluted at intervals in fresh chloramphenicol-containing medium to maintain a low bacterial concentration, avoiding inactivation of the drug in liquid culture. The cultures were spread onto selective and nonselective plates and incubated overnight at 30 or 42°C. The number of particles in the cultures was measured with a Coulter Counter, and the proportion of viable plasmid-free segregants was determined by replica plating at 30°C.

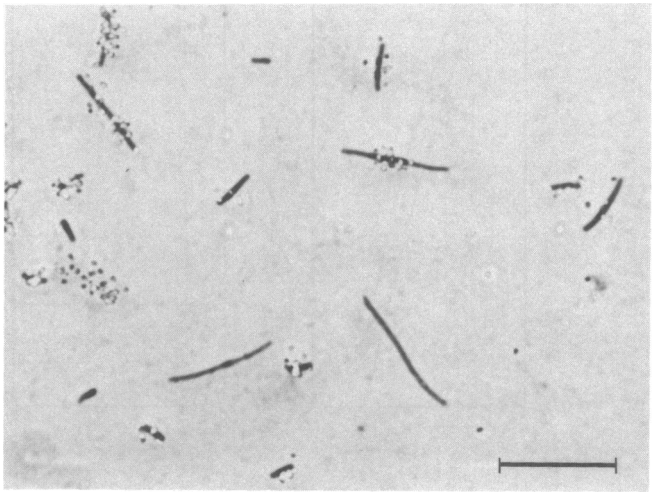

FIG. 8. Autoradiograph of cells pulse-labeled with [^{3}H]thymidine. Strain SH2746(pXX333) was incubated at 42°C for 8 h in enriched ME-glucose medium containing thymine (5.3 μg/ml). A 2-ml sample (25 Klett units) was removed, added to 200 μl of [^{3}H]thymidine (20 Ci/mmol), and labeled for 3 min at 42°C. Labeling was stopped by the addition of 40 μl of 2% sodium azide. Autoradiography was performed as described in the text. Bar, 10 μm.

TABLE 1. Analysis of autoradiographs for SH2746(pXX333)

Cell size or type	% of total cells labeled (avg. no. of grains/cell)			
	Pulse labeling (n = 287)		Continuous labeling (n = 711)	
	Anucleate	Nucleate	Anucleate	Nucleate
Normal and small	19	38 (7.3)	22	37 (6.9)
Filamentous	10	33 (9.6)	<0.1	41 (16.6)

In the cultures grown at 30°C, the plating efficiency on nonselective plates was ca. 100% at 30 and 42°C (Table 2). One hundred colonies grown at 30°C were tested by replica plating; all were resistant to chloramphenicol. This suggests that the cultures grown at 30°C contained fewer than 1% plasmid-free segregants.

The colonies grown at 42°C on nonselective plates were smaller than those grown at 30°C and consisted primarily of chloramphenicol-sensitive viable cells. This suggests that every cell from the cultures grown at 30°C can form a colony at 42°C and that these colonies consist of the progeny of viable plasmid-free segregants which escaped the inhibitory action of the *ccdB* gene product.

Only about 5% of the cells grown at 42°C for 10 h in the absence or presence of chloramphenicol were able to form a colony on nonselective plates at either temperature. In the 42°C culture grown in the absence of chloramphenicol, 49% of the colony formers were plasmid-free segregants. Even in the 42°C culture grown in the presence of chloramphenicol, 18% of the colony formers were plasmid-free segregants (Table 2). These viable plasmid-free segregants were presumably produced during the 10-h incubation at 42°C, escaping the inhibitory function of the *ccdB* gene.

In all these experiments (Table 2), the colonies formed on nonselective plates at 42°C were small and consisted of plasmid-free viable cells. On the other hand, no culture gave rise to colonies on selective plates at 42°C. This inhibition of colony formation at 42°C was reversible; when the plates incubated at 42°C were transferred to 30°C and further incubated overnight, colonies did appear. The number of colonies was similar to that obtained on selective plates incubated directly at 30°C. These colonies contained a majority of chloramphenicol-sensitive cells, which presumably descended from plasmid-free segregants escaping the inhibitory action of *ccdB*.

The kinetic experiments (e.g., Fig. 1A) showed that viable plasmid-free segregants must generally appear late after transfer to 42°C. This delay explains the small colony size on nonselective plates at 42°C. The results (Table 2) thus demonstrate that plasmid-free viable cells were produced during the long incubation at 42°C, escaping the *ccdB* inhibitory function.

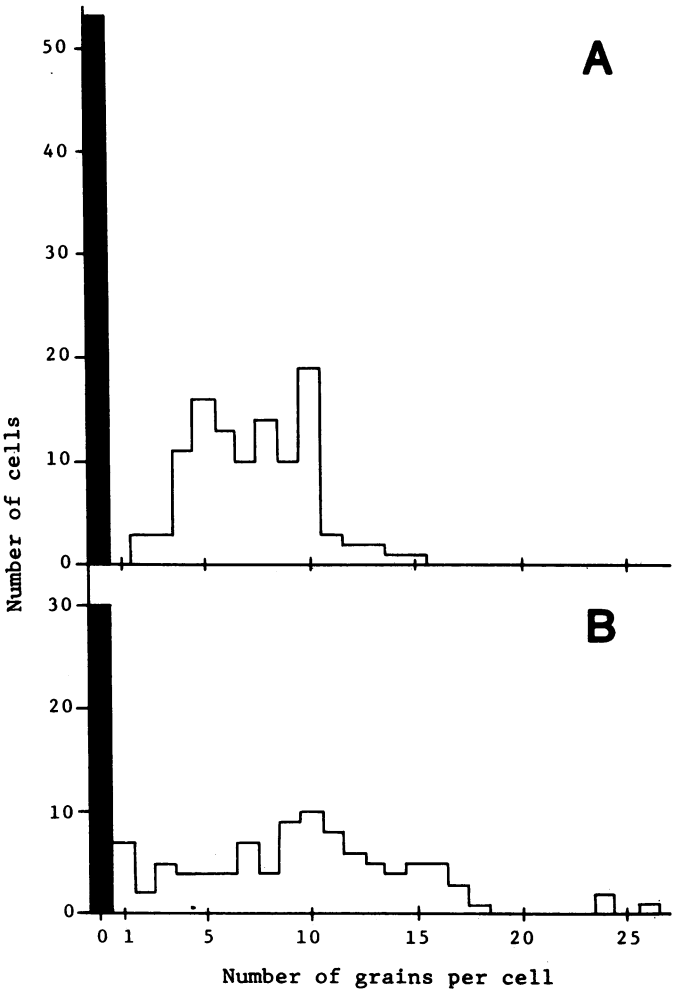

FIG 9. Distribution of grains in strain SH2746(pXX333) pulse-labeled with [^{3}H]thymidine for 3 min after 8 h of incubation at 42°C. (A) Cells of normal size. (B) Filamentous cells. Solid bar, Cells having no grains. The number of cells analyzed was 340.

We obtained similar results with strain SH392(pXX333) (data not shown).

DISCUSSION

A model to explain the functions of the *ccdA* and *ccdB* genes when *ccd*$^+$ plasmid replication is blocked must take

TABLE 2. Colony-forming ability of strain SH2743(pXX333)

Expt no.	Growth conditions[a]		No. of particles (10^8 per ml)	No. of colony formers (10^8 per ml)				% Cm^r colonies[d]
	CM	Temp (°C)		30°C	30°C, CM	42°C[b]	42°C, CM[c]	
1	−	30	4.3	4.9	3.3	5.1	0 (3.0)	100
2	−	42	5.3	0.27	0.16	0.2	0 (0.12)	51
3	+	30	3.8	3.2	1.8	3.4	0 (2.5)	100
4	+	42	5.2	0.2	0.12	0.17	0 (0.16)	82

[a] Liquid cultures were incubated in ME medium with or without chloramphenicol (CM) for 10 h at the indicated temperature.
[b] Small colonies formed on plates incubated at 42°C without chloramphenicol.
[c] Numbers in parentheses are the number of colonies formed when the selective plates which had been incubated at 42°C were further incubated at 30°C overnight.
[d] One hundred colonies from nonselective plates incubated at 30°C were analyzed.

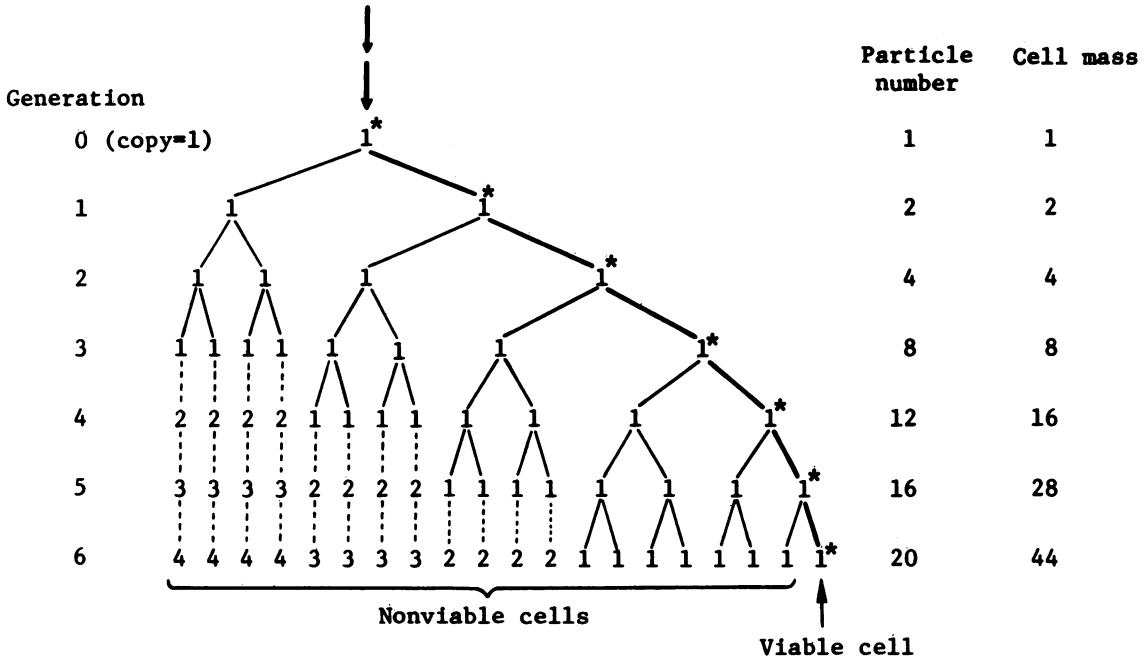

FIG. 10. Nonviable segregant model of *ccd* function. This figure shows three residual cell divisions (see the text). Numbers indicate the average cell volume in each generation. Asterisks indicate cells carrying plasmid DNA. Broken lines indicate filamentation.

the following observations into account: (i) the number of colony-forming cells nearly stops increasing when the copy number of the plasmid reaches 1 per cell; (ii) cell mass and number increase throughout incubation at the nonpermissive temperature; (iii) plasmid-free colony formers are only rarely formed; (iv) the cell population at late times is heterogeneous, comprising both viable and nonviable cells of normal size and nonviable filaments; and (v) filaments appear 2 to 3 generations after the number of colony-forming cells stops increasing.

The above observations imply that the final number of viable cells, essentially all of which carry a plasmid, is equal to the number of plasmid copies initially present. Since plasmid replication is blocked at 42°C, the vast majority of nonviable cells must be plasmid free.

During the first 2 h at the nonpermissive temperature, the copy number of the ccd^+ plasmid fell from 3 to 4 per cell to the critical level of 1 per cell. In our model, an asymmetrical division then takes place, resulting in one viable cell harboring the plasmid and one nonviable plasmid-free cell. The plasmid-free segregants, although unable to form a colony, can still undergo a few residual divisions; these cells then form filaments. The particle number and cell mass predictions of this model are shown in Fig. 10. Assuming two to three residual divisions per plasmid-free cell, our experimental results are consistent with these predictions. Slight differences may be attributed to the production of anucleate cells by filaments.

All aspects of the abnormal behavior observed depend on the presence of ccd^+ genes. The segment carrying the *ccdA* and *ccdB* genes codes for the H_1/H_2 (LetA) and G_1/G_2 (LetD) polypeptides (2, 10, 13). It has been proposed that the action of the *ccdB* gene product is under negative control by the *ccdA* gene product. In perturbed conditions, when the copy number of the ccd^+ plasmid decreases to 1 per cell (13, 15, 17) or when the *ccdA* gene is mutated (10, 13), the CcdB protein has been reported to cause inhibition of host cell division. In the work presented in this paper, cell division was not immediately inhibited: bacteria harboring 1 copy of the ccd^+ plasmid divided asymmetrically to produce a viable cell carrying 1 copy of the plasmid and a nonviable plasmid-free cell. Residual division could still take place in these plasmid-free segregants before finally being inhibited. Thus, cell division is inhibited in nonviable plasmid-free segregants but not in viable plasmid-containing cells.

In previous work with thermosensitive mutants of ColVB *trp* plasmids which induce host cell filamentation and death, Koyama and Yura (11) postulated that a plasmid-encoded mechanism is responsible for these effects. Ogura and Hiraga (17) found that the host cell formed filaments when replication of pBR322 or pSC101 plasmids carrying the mini F *ccd* region was blocked. They proposed that when the copy number of mini F falls to 1 per cell, the CcdB protein blocks cell division, preventing plasmid loss. Miki et al. (13) speculated that a temperature-sensitive mini F plasmid was unable to complete a round of replication at a nonpermissive temperature and that this inability was responsible for inhibition of cell division in the host bacteria together with a defect of chromosome partitioning.

Our present results do not support these hypotheses. Under similar experimental conditions, we observed that division continued and nonviable normal-size cells accumulated before plasmid-free filamentous cells appeared.

Limiting plasmid proliferation has been shown to induce the SOS response (15), which is known to be induced by DNA damage. Brandenburger et al. (3) proposed that impairment of the normal coregulation between partition and replication of the mini F plasmid affects the *ccd* functions, leading to the production of an SOS-inducing signal. We show here, however, that the production of nonviable plasmid-free segregants and the inhibition of cell division are independent of the SOS division inhibition mechanisms determined by the *sfiA* and *sfiC* gene functions (4, 7, 8). This is consistent with previous data showing that cell division

inhibition by the *ccd* functions, but not phage λ induction, occurs in a *recA* mutant (15, 17).

A mini F plasmid carrying an amber mutation in the *ccdA* gene was shown to induce filamentation and death in the host cell, induction of resident prophage λ, and induction of the *sfiA* gene in the absence of the CcdA protein (10, 13). These observations suggest that the inhibitory action of the CcdB protein could result from dilution of the CcdA protein until it can no longer counteract CcdB inhibitory action. They confirm, as suggested previously by Ogura and Hiraga (17), that blockage of the replication of *ccd* plasmids is not in itself the triggering event.

In the present work we show that bacteria harboring 1 copy of the plasmid carrying the $ccdA^+$ and $ccdB^+$ genes are normal with respect to size, DNA synthesis, and the ability to produce normal progeny when plasmid replication is restored. The event leading to the loss of viability is irreversible, and its effect is transmitted to essentially all plasmid-free daughter cells. Although the molecular basis of CcdB action is still unknown, the facts that DNA synthesis was perturbed and anucleate cells were generated suggest that CcdB may interfere with chromosome replication, chromosome partitioning, or both.

As described previously by Ogura and Hiraga (17), the *ccd* mechanism promotes stable maintenance of the mini F plasmid. A high proportion of filamentous cells were observed in a culture of strain SH392 harboring a ccd^+ Δ*sop* mini F plasmid grown in nonselective conditions (S. Hiraga, unpublished data). Since the ccd^+ Δ*sop* plasmid is presumably defective in equipartitioning plasmid DNA molecules into daughter cells (16), nonviable plasmid-free segregants may appear at high frequency in a culture and form filaments. This phenomenon was not observed with ccd^+ sop^+, Δ*ccd* Δ*sop*, or Δ*ccd* sop^+ mini F plasmids, as was expected. The *ccd* mechanism guarantees that plasmid-carrying cells grow preferentially in a population by killing plasmid-free segregants.

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