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Genetics

Mini-F plasmid genes that couple host cell division to plasmid proliferation

(stable maintenance of plasmid/plasmid partition/host–plasmid interaction/*oriC* plasmid/SOS-like function)

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ABSTRACT **A mechanism for stable maintenance of plasmids, besides the replication and partition mechanisms, has been found to be specified by genes of a mini-F plasmid. An *oriC* plasmid carrying both a mini-F segment necessary for partition [coordinates 46.4–49.4 kilobase pairs (kb) on the F map] and another segment (42.9–43.6 kb), designated *ccd* (coupled cell division), is more stably maintained than are *oriC* plasmids carrying only the partition segment; the stability is comparable to that of the parental mini-F plasmid. When replication of a plasmid carrying *ccd* is prevented and the plasmid copy number decreases, to as few as one per cell, host cell division is inhibited, but not increase of turbidity or chromosome replication. Appearance of plasmid-free segregants is therefore effectively prevented under such conditions. Experimental results suggest that reduction of the copy number of plasmids carrying the *ccd* region causes an inhibition of cell division and that the *ccd* region can be dissected into two functional regions; one (*ccdB*) inhibits cell division and the other (*ccdA*) releases the inhibition. The interplay of the *ccdA* and *ccdB* genes promotes stable plasmid maintenance by coupling host cell division to plasmid proliferation.**

Plasmids that replicate by using the replication origin (*oriC*) of the *Escherichia coli* chromosome are not stably maintained through cell division under nonselective conditions (1). We have previously found that when a particular segment of mini-F plasmid is inserted into such plasmids, the resulting *oriC* plasmids become stable (1, 2). The segment that contributes to this stability has been located within the 46.4–49.4 kilobase pairs (kb) coordinates on the F map (3), which is outside of the region essential for mini-F replication (44.0–46.35 kb), and the stabilization is achieved without a detectable increase in plasmid copy number (2). Apparently, this segment specifies the partition rather than the replication control of such plasmids. It has been shown that the segment includes three functional regions necessary for stable maintenance; two (*sopA* and *sopB*) act in *trans* and one (*sopC*) acts in *cis* (2) (Fig. 1). However, even *oriC* plasmids stabilized by the partition mechanism of mini-F are not fully stable. This observation prompted us to investigate additional DNA segments for stabilization properties. In this paper, we describe the characterization of a mini-F DNA segment that seems to play an important role in stable maintenance of plasmids in host bacteria. This segment (42.9–43.6 kb), designated *ccd* (coupled cell division), is located outside of the regions essential for autonomous replication and for partition of mini-F (Fig. 1). The *ccd* segment appears to act by coupling host cell division to proliferation of plasmids. We propose a hypothesis that explains the functions of the *ccd* segment.

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MATERIALS AND METHODS

Bacterial strains used were all derivatives of *E. coli* K-12. Strains KY7231 (F⁻ *trpB9578 tna-2 rpsL recA1*) and KZ200 (F⁻ *ilv thr metE trp tyr thy rpsL recA1*) were our laboratory stocks. Strains km1213 (F⁻ *polA*^{ts} *his argG metB leu rpsL xyl lacY thy*) (5) and KH802 (F⁻ *met gal supE hsdR*) (6) have been described. Plasmid pBR322 (7) was obtained from H. W. Boyer; pSC138 (8), from C. Wada; pHSG415 (9), from T. Hashimoto-Gotoh; pKP1033, from Takeyoshi Miki. Other plasmid strains were constructed in our laboratory. Bacterial cells were grown in L broth (10), supplemented when necessary with thymine at 25 μg/ml. Plasmid DNA was prepared as described (11–13). DNA synthesis was measured by determining incorporation of [^{14}C]-thymine into trichloroacetic acid-insoluble material.

RESULTS

Construction of Stable *oriC* Plasmid pXX299. Mini-F plasmids such as pSC138 (8) consist of an *Eco*RI-generated f5 fragment (Fig. 1) and a drug-resistance fragment (see also Fig. 5), and are stably maintained through cell division. Stable inheritance of mini-F plasmids should be due to controlled replication and accurate partitioning of replicated plasmid molecules into daughter cells. We have recently found that *oriC* plasmids (e.g., pXX258 and pXX199 shown in Fig. 1) carrying the "C-A2 segment" of mini-F but lacking most of the region necessary for autonomous replication are more stably maintained than *oriC* plasmids carrying only a part of the C-A2 segment (e.g., pXX206 carrying the A2 segment and pXX230 carrying the A2 segment with a deletion; see Fig. 1) even under nonselective conditions. The C-A2 segment seems to be responsible for plasmid partitioning (2).

However, even *oriC* plasmids stabilized by the mini-F partition function(s) (e.g., pXX199) are not fully stable; strains carrying such plasmids give segregants lacking the plasmid at low frequencies (Fig. 2), unlike strains carrying the parental mini-F plasmid pSC138. This suggests that mini-F has another stabilizing function in addition to the partitioning mechanism. If this were the case, such a function would be specified by a DNA segment located outside of the regions essential for autonomous replication and for partition. Accordingly, we constructed *oriC* plasmid pXX299 carrying both the partition segment and an *Xho* I segment (42.1–44.8 kb) of mini-F (Fig. 1). This plasmid was found to be extremely stable under nonselective conditions (Fig. 2). Similar results were obtained when the plasmid was in another bacterial strain, KY7231. Thus the *Xho* I seg-

Abbreviations: kb, kilobase pairs; kDa, kilodalton(s); Ap^r, ampicillin resistance.

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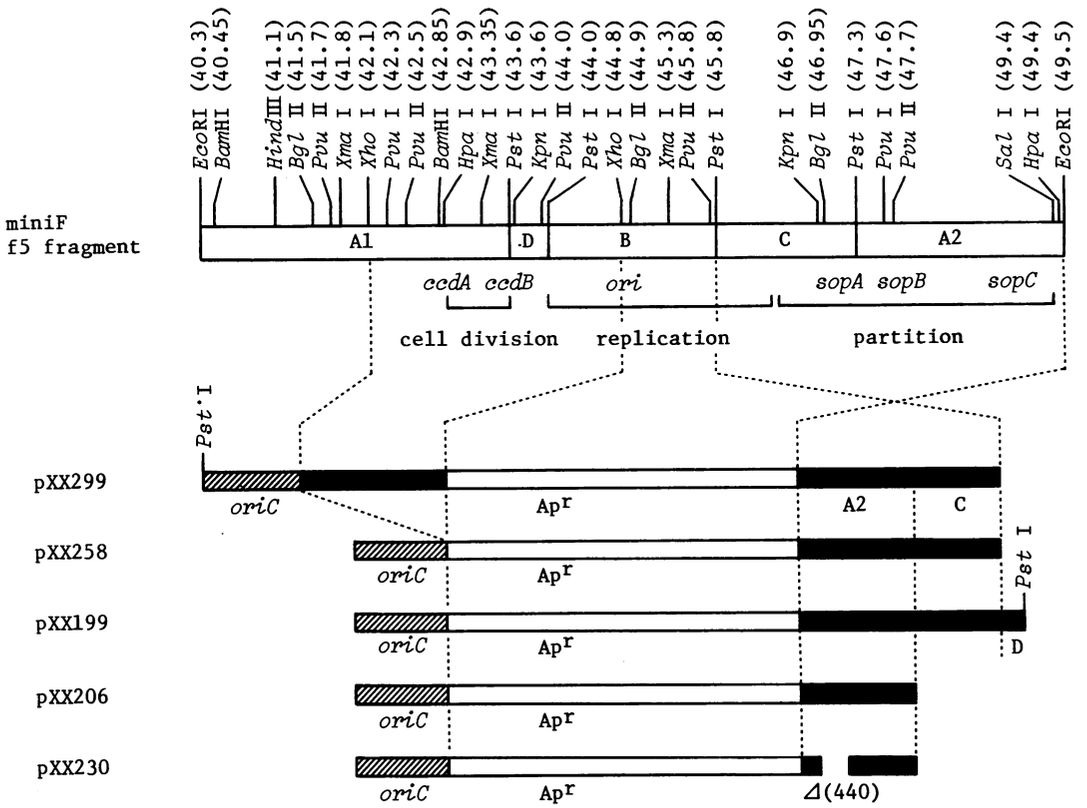

FIG. 1. Physical and functional map of *Eco*RI-generated f5 fragment of F plasmid and structures of *oriC* plasmids. The cleavage sites for restriction endonucleases are indicated. The numbers in parentheses denote kb coordinates on the F genome (3). The *Pst* I segments are called A1, D, B, C, and A2 according to Murotsu *et al.* (4). The region essential for autonomous replication is taken from Murotsu *et al.* (4). *ori* denotes the replication origin of the mini-F plasmid. The region essential for plasmid partitioning contains three *sop* genes (2). The *ccd* region is described in this paper. Filled region on maps of *oriC* plasmids, DNA derived from mini-F plasmid; hatched region, DNA from the *E. coli* chromosome; open region, the ampicillin-resistance (Ap^r) segment; *oriC*, replication origin of the *E. coli* chromosome. Δ denotes a spontaneous deletion, and the number in parentheses indicates the size of the deletion (base pairs).

ment together with the C-A2 segment seems to confer complete stabilization on *oriC* plasmids.

Inhibition of Host Cell Division by Plasmid Carrying a Specific Mini-F Segment. How does the *Xho* I segment (42.1–44.8 kb) stabilize the plasmid? We assumed that the segment might be responsible for coupling host cell division to plasmid proliferation (see *Discussion*). To examine the possible effect of the *Xho* I segment on cell division when plasmid replication is blocked, we constructed several pBR322 derivatives carrying various regions of mini-F, and we tested colony-forming ability of *polA*^{ts} cells (strain km1213) carrying them at 42°C (see column I in Fig. 3). Because replication of pBR322, but not mini-F, depends on DNA polymerase I, which is encoded by the *polA* gene, these plasmids cannot replicate in *polA*^{ts} cells at 42°C unless they contain a mini-F segment essential for replication. We found that on nonselective L agar plates at 42°C, cells carrying pXX306, pXX312, pXX315, or pXX334 form minute colonies and form fewer colonies than cells lacking plasmid. These plasmids all carry the *Hpa* I/*Pst* I (42.9–43.6 kb) segment, but not the mini-F origin. Microscopical observation revealed that *polA*^{ts} cells carrying these plasmids become much elongated upon incubation at 42°C, indicating that cell division is inhibited. By contrast, cells carrying pKP1033 or pXX304, which contains the *Hpa* I/*Pst* I segment and can replicate at 42°C by using the replication origin of mini-F, form normal-size colonies as well as cells carrying the parental pBR322. Similarly, cells carrying pXX167, which has neither the *Hpa* I/*Pst* I segment nor the mini-F origin, also form normal-size colonies at 42°C. It is therefore concluded that when replication of a plasmid carrying the *Hpa* I/*Pst* I (42.9–43.6 kb) segment of mini-

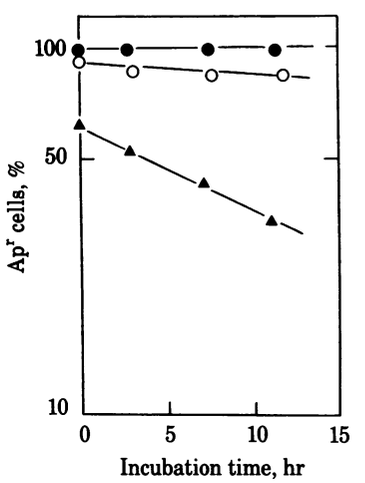

FIG. 2. Stability of *oriC* plasmids. Cells of *E. coli* KZ200 carrying an *oriC* plasmid were examined for plasmid stability in a nonselective medium. Bacteria were grown overnight at 37°C in a selective medium containing ampicillin, diluted 1:500 to 1:1,000 with a nonselective medium (L broth), and incubated at 37°C. The culture was diluted at appropriate intervals with a prewarmed fresh nonselective medium to maintain exponential growing conditions throughout the experiment. Samples were taken and diluted, and plates were incubated overnight at 37°C. At least 200 colonies from each sample were scored for the presence of the Ap^r plasmid by transferring them to selective agar plates containing ampicillin by toothpicks. Cells carrying each plasmid showed no detectable differences in growth rate as compared with plasmid-free cells. The doubling time as determined by turbidity was 50–52 min in all strains tested. ●, pXX299; ○, pXX199; ▲, pXX230.

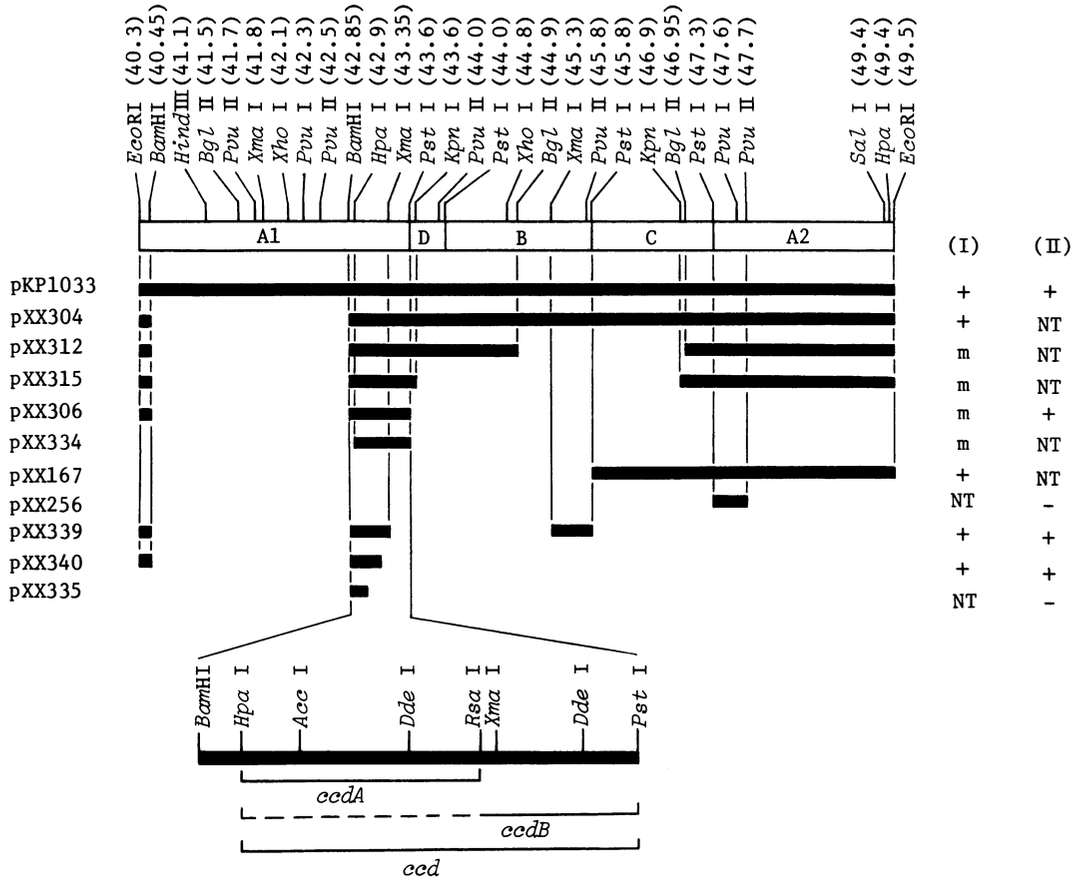

FIG. 3. Structures of mini-F segments carried by pBR322 derivatives and properties of the derivatives. Column I, colony-forming ability of km1213 cells (*polA*^{ts}) carrying the indicated plasmids on thymine-supplemented nonselective L agar plates at 42°C. +, Normal-size colonies; m, minute colonies, in reduced numbers. Column II, suppression effect of the indicated plasmid on the inhibition of cell division that is caused at 42°C by a thermosensitive plasmid pXX333 carrying the *ccd* region (see text). KH802 cells (*polA*$^{+}$) harboring both pXX333 and the indicated plasmid were transferred to 42°C, and samples were removed and tested for viable cells as described in the legend to Fig. 4. +, The inhibition of cell division by pXX333 was suppressed in the presence of the indicated plasmid; −, cell division was inhibited by pXX333 even in the presence of the indicated plasmid. NT, not tested.

F, designated the *ccd* region, is prevented, host cell division is inhibited and the cells cannot form normal-size colonies on nonselective plates.

Kinetics of Inhibition of Host Cell Division. To examine further the relationship between inhibition of plasmid replication and that of host cell division, the kinetics of increase of turbidity, number of viable cells, proportion of plasmid-carrying cells, and host chromosomal DNA synthesis were followed by using *polA*^{ts} cells harboring pBR322 or pXX306 carrying the *ccd* segment. In a strain carrying pXX306, colony-formers (viable cells) continue to increase exponentially for 3–3.5 hr (3.5–4 generations) after transfer to 42°C and then stop increasing (Fig. 4), whereas turbidity increases for at least 6–7 hr. Plasmid-free segregants appeared only infrequently (about 10% of total colony formers) after 7 hr of incubation at 42°C (Fig. 4). In contrast, the inhibition of cell division was not observed in a strain carrying pBR322, and segregants appeared at high frequency under the same conditions (about 90% of total colony-formers). It should be noted that the time when colony-formers stop increasing in the pXX306-carrying strain nearly coincides with the time when plasmid-free segregants begin to appear in pBR322-carrying cells. DNA synthesis of the cells carrying pXX306 was not inhibited significantly as compared with that of the cells carrying pBR322 (data not shown). These results indicate that the primary effect on host cell growth caused by the replication inhibition of plasmids carrying the *ccd* region is inhibition of cell division. It seems unlikely that the inhibition of plasmid replication *per se* inhibits cell division, because the strain carrying pXX306 grows normally and number of colony formers increases for 3–3.5 hr after transfer to 42°C (Fig. 4), even though replication of ColE1-type plasmids in the strain km1213 (*polA*^{ts}) is inhibited immediately after transfer to high temperature (5). The inhibition of cell division may be caused by reduction of plasmid copy number.

Effect of Thermosensitive pSC101 Derivative Carrying the *ccd* Segment on Host Cell Division. To confirm the effect of plasmids carrying the *ccd* region on cell division, we recloned the mini-F segment carried by the pXX306 [*Eco*RI/*Bam*HI (40.3–40.45 kb) fragment and the *Bam*HI/*Pst* I (42.85–43.6 kb) fragment containing the *ccd* region; see Fig. 3] onto a thermosensitive pSC101 derivative vector, pHSG415 (9), which cannot replicate at 42°C. The resulting thermosensitive plasmid pXX333 (structure not shown), which has a chloramphenicol resistance gene, was introduced into a *polA*$^{+}$ strain (KH802) (6), and cell division was examined after transfer to 42°C. The increase of colony-formers began to be markedly prevented at 2–2.5 hr (about 3 generations), whereas turbidity continued to increase for at least 4.5 hr. Plasmid-free segregants (chloramphenicol-sensitive cells) appeared only at low frequencies (<5%) at 4.5 hr after transfer. By contrast, no detectable inhibition of cell division was observed in a parallel culture of KH802 carrying the parental pHSG415, and segregants started to appear at 2–2.5 hr, the proportion of segregants being more than 80% at 4.5 hr (data not shown). These results are consistent with those

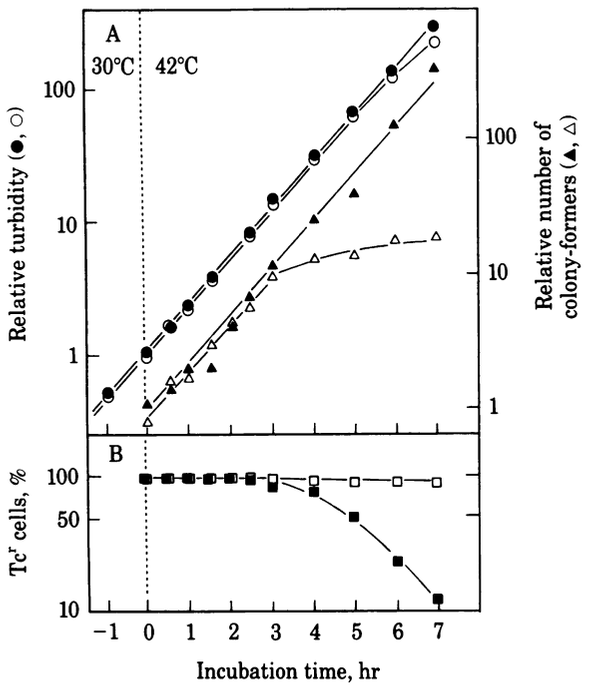

FIG. 4. Kinetics of cell growth and plasmid stability after transfer to 42°C. Cells of km1213 (*polA*^{ts}) carrying pXX306 (Fig. 3) or pBR322 were grown at 30°C to a midlogarithmic phase in a nonselective medium (L broth supplemented with thymine) and transferred to 42°C. The culture was diluted at intervals with a prewarmed fresh nonselective medium to maintain exponential growing conditions. (*A*) Turbidity of cultures was measured in a Klett–Summerson colorimeter with a no. 54 filter. Number of colony formers was determined by plating samples with appropriate dilutions of cells onto L agar supplemented with thymine. Colonies were scored after incubation overnight at 30°C. Generation time of both strains was 50 min. (*B*) Tetracycline-resistant clones carrying the plasmid as a percentage of total colony-formers was determined as described in the legend to Fig. 2, except that selective agar plates contained tetracycline and they were incubated at 30°C. Open symbols, pXX306; closed symbols, pBR322.

described above for pBR322-derived plasmids in *polA*^{ts} cells.

When a *recA1 polA*$^{+}$ strain (KZ200) carrying pXX333 was transferred to 42°C, colony-formers stopped increasing at about the same time that plasmid-free segregants began to appear in a parallel control culture of KZ200 cells carrying pHSG415 after transfer to 42°C. This indicates that the *ccd* function blocking host cell division is independent of *recA*$^{+}$ activity.

Suppression of Growth Inhibition by a Coexisting Replication-Proficient Plasmid Carrying the *ccd* Region. If we assume that the reduction of copy number of a *ccd*-carrying plasmid induces inhibition of cell division, it might be expected that the inhibition is suppressed by the presence of another plasmid that also has the *ccd* segment and can replicate at 42°C in the *polA*^{ts} cells. Accordingly, we introduced mini-F plasmid pSC138 (see Fig. 5) into the *polA*^{ts} strain carrying pXX306 (see Fig. 3) and examined its cell division after transfer to 42°C. The cell division of the transformant carrying both pXX306 and pSC138 was no longer inhibited after transfer to high temperature, supporting our expectation (data not shown). Similar suppression of the inhibition was observed in a *polA*$^{+}$ strain (KH802) carrying thermosensitive pXX333 in the presence of pKP1033 or pXX306, which replicates at 42°C (see column II in Fig. 3). In contrast, inhibition of cell division by pXX333 was not suppressed by coexisting pBR322 derivatives lacking the *ccd* region (e.g., pXX256; Fig. 3). Therefore the inhibition of cell division caused by a nonreplicative plasmid carrying *ccd* is suppressed by the simultaneous presence of a replicative plasmid that also carries *ccd*.

Further Dissection of the *ccd* Region. We have constructed a pBR322 derivative, pXX339, carrying a *Bam*HI/*Xma* I (42.85–43.35 kb) segment, which is part of the *ccd* region, and examined its properties. Although cell division of *polA*^{ts} cells carrying this plasmid is not inhibited at 42°C (see column I in Fig. 3), the plasmid suppresses the inhibition of cell division of *polA*$^{+}$ cells exerted by pXX333 at 42°C (see column II in Fig. 3). The inhibition of cell division by pXX333 is suppressed also by coexisting pXX340 but not pXX335, which carries the *Bam*HI/*Acc* I (42.85–43.0 kb) segment (see column II in Fig. 3). These results suggest that the *ccd* region contains two distinct functional regions; one (*ccdA*) specifies the suppression function and the other (*ccdB*) specifies the inhibitory function for cell division (see *Discussion*). The *ccdA* gene seems to be located within the *Hpa* I/*Rsa* I (42.9–43.35 kb) segment (Fig. 3). *ccdB* may be located at the right side of *ccdA* in Fig. 3, because the *Xma* I/*Pst* I (43.35–43.6 kb) segment, which is deleted in pXX339, seems to be essential for the inhibitory function (Fig. 3; see also *Discussion*).

Stability of Mini-F Plasmids. To investigate whether the *ccd* functions are also involved in stable maintenance of the parental mini-F plasmid as observed in *oriC* plasmids, we constructed several deletion derivatives of pSC138 (Fig. 5) and examined their stabilities. pXX9 and pXX325 were stably maintained like pSC138, whereas pXX318, which lacks the partition mechanism (*sop*), was unstable (Fig. 6*A*). pXX327, which lacks both the partition mechanism and the *ccd* mechanism, was apparently more unstable than pXX318 (Fig. 6*A*). Similar results were obtained in bacterial strains KZ200 and KH802. These results suggest that the *ccd* functions are involved in the maintenance of parental mini-F as well as *oriC* plasmids.

If the stabilizing effect by the *ccd* segment is exerted by coupling host cell division to plasmid proliferation as suggested in the case of nonreplicative plasmids carrying *ccd*, we can also expect that pXX318 is made unstable by the simultaneous presence of another plasmid carrying *ccd*. We have therefore examined the stability of pXX318 in the presence of pXX306, a pBR322 derivative carrying *ccd* (see Fig. 3). As shown in Fig. 6*B*, pXX306 markedly disturbs maintenance of pXX318, and segregants lacking pXX318 appear frequently, as much as in a strain carrying pXX327 (Fig. 6*A*), whereas a pBR322 derivative pXX256 lacking *ccd* (see Fig. 3) exhibits no detectable effects on stability of pXX318 (Fig. 6*B*). Thus the *ccd* functions responsible for stable plasmid maintenance seem to be also suppressed by the presence of another replication-proficient plasmid carrying *ccd*.

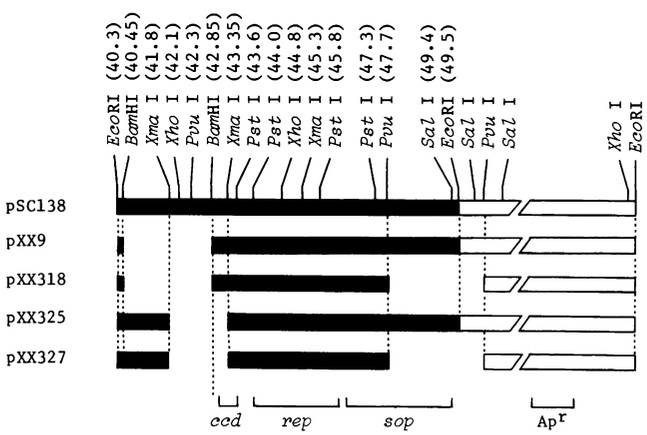

FIG. 5. Structures of deletion mini-F plasmids. Filled region and open region indicate DNA derived from mini-F and the Ap^{r} segment, respectively. *rep*, Minimal region essential for mini-F replication (4).

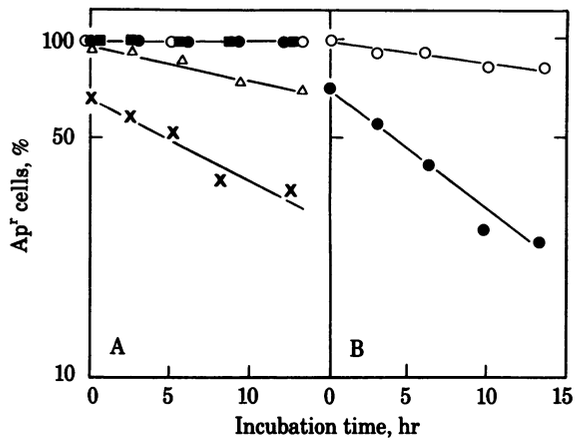

FIG. 6. Stability of mini-F plasmids. (*A*) Exponentially growing cells of *E. coli* KY7231 carrying a mini-F plasmid were examined for stability as described in the legend to Fig. 2. ●, pSC138; ○, pXX9; △, pXX318; ■, pXX325; ×, pXX327. (*B*) Stability of pXX318 in KY7231 was examined in the simultaneous presence of pXX306 (●) or pXX256 (○). The doubling time as measured by turbidity was 40–42 min in all strains tested in both experiments A and B, showing no detectable differences in growth rate as compared with plasmid-free cells.

DISCUSSION

We have found that a function specified by the segment of mini-F at 42.9–43.6 kb is necessary for complete stabilization of *oriC* plasmids or mini-F plasmids, in addition to the segment responsible for the partition function. This functional region, designated *ccd,* seems to operate by coupling host cell division to plasmid proliferation, because host cell division is inhibited when the copy number of a plasmid carrying the *ccd* segment decreases (to as few as one copy per cell). Moreover, there seem to be two functional regions within the *ccd* segment; one (*ccdB*) inhibits host cell division and the other (*ccdA*) suppresses the inhibitory function of the *ccdB* gene.

Replication of F and *oriC* plasmids has been shown not to be coupled to the cell division cycle; i.e., it takes place throughout the division cycle (14, 15). Although it is not known if the mini-F and *oriC* plasmids used here also replicate throughout the division cycle, if each plasmid molecule of these plasmids replicates at an arbitrary stage of the division cycle, a small fraction of cell population might fail to replicate plasmid during one division cycle. These cells would have only one copy of plasmid at the time of cell division and should produce daughter cells that have lost the plasmid after cell division. The *ccd* functions may serve to prevent appearance of plasmid-free segregants at appreciable frequencies by temporarily inhibiting host cell division.

We hypothesize that the *ccd* region acts for stable maintenance of plasmid by the following mechanism: the *ccdA* gene usually suppresses the inhibition of cell division caused by the *ccdB* gene. When the plasmid copy number decreases to as few as only one per cell, the inhibitory function of the *ccdB* gene can be no longer suppressed, and cell division is stopped temporarily.

Takeyoshi Miki, Katsuji Yoshioka, and Tadao Horiuchi (personal communication) have isolated a type of amber mutant (*letA*) of F′ plasmid that inhibits cell division when transferred into recipient cells lacking suppressor functions. They have determined the nucleotide sequence of the *Bam*HI/*Pst* I (42.85–43.6 kb) region of mini-F that contains *letA* and have found two possible open reading frames coding for proteins with molecular masses of 8 and 11 kilodaltons (kDa), respectively. The *letA* mutations were suggested to be located in the gene encoding the 8-kDa protein. Moreover, they found that, when the segment containing the gene for 11-kDa protein (but not the gene for 8-kDa protein) is cloned onto pBR322, the resulting plasmid inhibits the cell division of the host bacteria unless the 8-kDa protein gene is supplied in *trans*. On the basis of these results, they hypothesized that the 11- and 8-kDa proteins act for coupling cell division to F DNA replication; the 11-kDa protein acts for inhibition of cell division and the 8-kDa protein suppresses this inhibitory function and induces cell division when DNA replication is completed. In accordance with their hypothesis, these workers found that a temperature-sensitive replication mutant of mini-F that contains the *Bam*HI/*Pst* I (42.85–43.6 kb) segment inhibits cell division at high temperature, whereas the same mini-F mutant lacking the segment does not. In line with their expectation, the presumptive *ccdA* and *ccdB* genes seem to correspond to the genes for 8- and 11-kDa proteins, respectively.

In addition to the inhibition of cell division, the *ccd* function also causes induction of phage λ in lysogenic bacteria (our unpublished data; Takeyoshi Miki, personal communication), suggesting that this function might be related to SOS functions (16). It should be noted, however, that the inhibition of cell division by the *ccd* function is independent of the *recA*$^+$ activity.

Several thermosensitive mutants of F′ plasmids for replication have been isolated (e.g., see refs. 17 and 18). The mutant plasmids fail to inhibit cell division, and plasmid-free segregants appear at high frequencies after transfer to high temperatures. These observations appear to contradict our present findings and those by Miki *et al.* described above. We assume that this type of mutant has double mutations at both the gene essential for replication and the *ccdB* gene, because the mutants were isolated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, which tends to induce closely linked mutations. Most of natural stringent plasmids might also have function(s) similar to the *ccd* functions as observed in the mini-F plasmid for their stable inheritance.

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