On the bacterial cell cycle: *Escherichia coli* mutants with altered ploidy

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We describe a scheme for isolation of new classes of mutants in the cell cycle of *Escherichia coli*. The mutants were selected as resistant to camphor vapors, which results in increased ploidy, and were subsequently screened for an increase in cell density and an increase in the gene dosage of the *lac* operon. Our mutations are located at four different places in the chromosome; we have named these loci *mbr* (moth ball resistant). *mbr*A maps to 68 min on the *E. coli* chromosome, *mbr*B to 88.5 min, *mbr*C to 89.5 min, and *mbr*D to 90 min. *mbr*D mutations may be alleles of *rpoB* (a subunit of RNA polymerase). In addition to the selected or screened phenotypes, most of the mutants fail to grow on rich media or at high temperatures. We have examined the nine mutants under nonpermissive conditions, using several techniques to determine the cause of death. We have also coupled our mutations with lesions in *dnaA*, which is required for cell-cycle-specific DNA replication, and *rnh* (the gene for RNase H), which is required for specificity in the DNA initiation reaction, and determined the effects of the double and triple mutants under permissive and nonpermissive conditions. These tests have shown that bacteria mutated at *mbr*A do not tolerate a null mutation in *rnh*, indicating that they are dependent on DNA replication initiating at *oriC*. In contrast, mutations at *mbr*B, *mbr*C, and *mbr*D exhibit their phenotypes independent of *oriC* initiation of DNA replication, suggesting that the mutations affect factors that influence the DNA/cell ratio regardless of the origin of DNA replication. Based on our results, the *mbr* mutations appear to have defects in cell-cycle timing and/or defects in chromosomal partitioning.

[Key Words: *Escherichia coli*, altered ploidy, camphor resistance]

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The cell division cycle in *Escherichia coli* is a carefully controlled series of events, beginning with cell elongation and chromosome replication, followed by chromosome separation, septum formation, and cell separation (for review, see Donachie and Robinson 1987). These events must occur in a precisely timed sequence, as well as in the correct spatial location within the cell. If any single part of the cell cycle fails to occur within these constraints, one or both of the resulting daughter cells could be inviable. In a wild-type *E. coli*, very few inviable daughter cells are produced (~1 in 10^-3 to 10^-4), indicating that the overall process of cell division is extremely accurate.

At the onset of the division cycle, DNA replication is initiated from a precise location on the chromosome (*oriC*; for review, see von Meyenburg and Hansen 1987). A major protein involved in determining the frequency of initiation is DnaA (Lobner-Olesen et al. 1989). Various other proteins, including products of many of the *dna* genes, RNase H, and RNA polymerase, are subsequently needed to complete the initiation process (for review, see von Meyenburg and Hansen 1987). Replication proceeds bidirectionally around the chromosome until it reaches the terminus (*terC*; Kuempel et al. 1977; Louarn et al. 1977). After it has replicated, the two copies of the chromosome must be physically moved toward the two poles of the cell so that the septum can be placed between them. The frequency of septum formation is dependent on the FtsZ protein (Bi and Lutkenhaus 1990). Additional proteins (products of the *fts* and *pbp* genes and others) are required for localization and synthesis of the septum and separation of the two daughter cells (for review, see Donachie et al. 1984).

Most of the currently identified cell division mutants were isolated by use of a screen to detect thermosensitive defects that cause cells to filament (Hirota et al. 1968). Whereas this screen has yielded mutations in many different genes, it has failed to uncover several classes, namely chromosomal partitioning defects and mutations altered in coupling of cell-cycle events. Potential partition mutants have been identified by use of screens to detect DNA-less cells (Hiraga et al. 1989). However, it is not clear what role these genes may play in the partition process.

In this paper we describe a scheme for identifying cell-cycle mutants that maintain more than the normal number of chromosomes. Our mutants were selected as resistant to camphor vapors, which results in increased ploidy in *E. coli* (Ogg and Zelle 1957), in yeasts (Bauch 1941), and in molds (Sansome 1946). Subsequently, they were screened for increased density and an increase in the gene dosage of the *lac* operon. We have identified

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nine mutants, which map to four different chromosomal locations. The mutants have several novel combinations of defects and may represent new classes of cell division mutants in E. coli.

Results

Rationale for the altered ploidy mutant selection

Because the coupling and spatial constraints of chromosome replication, partitioning of daughter chromosomes, and cell division are so poorly understood, we wished to identify, by mutation, genes that play a role in these processes. The selection that we have employed has the potential to yield several interesting classes of mutations. A collection of altered ploidy mutants should contain (1) mutants in the DNA replication machinery that overinitiate chromosome replication; (2) mutants that have lost the coupling between growth rate and ploidy and thus initiate DNA replication as if they are growing more rapidly than they are; (3) mutants defective in cell division timing such that they have lost the coupling between DNA replication and other cell division events; and (4) mutants defective in chromosomal partitioning. Although each of these classes is defective in a different and discrete step of the cell cycle, all of the mutants could provide useful information on how the events in cell division are choreographed.

Isolation of altered ploidy mutants

To isolate a collection of altered ploidy mutants, we have taken advantage of an old observation that cells that can grow in the presence of camphor vapors are polyploid. Although the mechanism by which camphor alters the cell is not clear, when bacterial cells are treated with camphor vapors, $10^{-3}$ to $10^{-4}$ are capable of growth. Most of these cells have an epigenetic change which confers Cmr$^+$ that is rapidly lost when the camphor is removed (Ogg and Zelle 1957). Ogg and Zelle have shown that in E. coli B/r, it is possible to isolate a stable heritable mutation that confers Cmr$^+$ (Ogg and Zelle 1957).

First, we repeated the camphor selection with NT3 (E. coli K-12); the frequency of survival is $10^{-3}$ to $10^{-4}$, the same as E. coli B/r. To increase the frequency of stable Cmr$^+$ mutants, we then mutagenized NT3 with nitroguanidine. Twenty-nine stable Cmr$^+$ mutants, each from a different mutagenized culture, were identified and used to determine whether there is linkage between any of the mutations. The mutations fell into two clusters: mbr-4 and mbr-15 are close to the same Tn10. mbr-3, mbr-7, mbr-11, mbr-17, mbr-18, mbr-19, and mbr-20 are also linked to the same Tn10 but, based on additional tests, appear to map to different clusters (see below). Hfr matings were used to determine the position on the E. coli linkage map (Bachmann 1990) of the Tn10s, and P1 transductions were used to further refine the map position.

Mapping the mbr mutations

To facilitate mapping, Tn10s linked to mbr-3, mbr-4, mbr-11, and mbr-17 were identified and used to determine whether there is linkage between any of the mutations. The mutations fell into two clusters: mbr-4 and mbr-15 are linked to the same Tn10. mbr-3, mbr-7, mbr-11, mbr-17, mbr-18, mbr-19, and mbr-20 are also linked to the same Tn10 but, based on additional tests, appear to map to different clusters (see below). Hfr matings were used to determine the position on the E. coli linkage map (Bachmann 1990) of the Tn10s, and P1 transductions were used to further refine the map position.

The locus at 68 min, designated mbrA (Fig. 1A), is defined by mbr-4, and the Tn10 linked to this cluster was designated zgi4::Tn10. mbrA4 exhibits linkage to zgi::Tn10 [SK2251], zgi4::Tn10, and argG. mbr-15 also shows these linkages and may be an allele of mbrA. mbrB, as defined by mbr-20, is 60% linked to zit17::Tn10 (the Tn10 isolated near mbr-17) but not linked to zia3::Tn10 (the Tn10 near mbr-3), placing it between 88 and 89 min (Fig. 1B). mbrC, as defined by mbr-17, is close to btuB at 89.6 min (Fig. 1B). mbr-11 and mbr-18 are probably allelic to mbrC. mbrD, as defined by mbr-19, exhibits the same linkage to zit17::Tn10 and zia3::Tn10 as a Rif$^+$ mutation in rpoB and shows $<10\%$ linkage to btuB, indicating that mbrD is located at 90 min (Fig. 1B). mbr-3 and mbr-7 show the same linkages as mbrD19 and are probably in the same locus. Strains carrying mbrD19 are resistant to rifam-
Trun and Gottesman

Table 1. Initial characterization of the mbr mutations

| Mutation     | Map* | Cmr* | Density* | Lcb* | Phenotypes* |
|--------------|------|------|----------|------|-------------|
| Wild type    | -    | wt   | ±        | ±    | ±           |
| Wild-type F'lac | -   | wt   | ±        | ±    | ±           |
| mbrA4        | 68'  | + + +| heavy    | ±    | LB - Mac - |
| mbrA15       | 68'  | + +  | wt + light| +   | Ts          |
| mbrB20       | 88.5'| +    | heavy    | +    | Ts LB - Mac - |
| mbrC11       | 89.5'| + + +| heavy    | +    | Ts LB - Mac - |
| mbrC17       | 89.5'| + +  | heavy    | + +  | Ts LB - Mac - |
| mbrC18       | 89.5'| + +  | heavy    | + +  | Ts LB - Mac - |
| mbrD3        | 90'  | + + +| heavy    | +    | Ts LB - Mac - |
| mbrD7        | 90'  | + +  | heavy    | + +  | Ts LB - Mac - |
| mbrD19       | 90'  | + +  | heavy    | +    | Ts LB - Mac - Rif' |

*Map refers to the location on the E. coli chromosome [Bachmann 1990].

*Cmr is growth with camphor. (+) Slight growth; (+ +) better growth; (++) growth equal to that of NT3 in the absence of camphor.

*Density refers to relative position in a Percoll gradient. Heavy is more dense than wild type (wt); light is less dense.

*Lcb refers to growth on lactobionate minimal agar.

(Ts) Thermosensitive, (LB-) inability to grow on LB agar; (Mac-) inability to grow on MacConkey agar; (Rif') rifampicin resistant.

Reversion analysis of the mutants

Using the linked Tn10s, the nine mbr mutations were transduced back into NT3 [NT596–NT620]. Without exception, all of the phenotypes of the mutations moved together, suggesting that they are the result of a single lesion. As a further test, the mutants were reverted to either growth on LB or growth at 42°C. Many of the revertants had changed only the characteristic that was selected. However, with all nine of the mutants, at least one revertant had restored all of the phenotypes to wild type, indicating that a single mutation is responsible for the pleiotropic phenotypes observed in the mutant strains. All subsequent experiments were performed using the strains with the mbr mutations transduced into an unmutagenized NT3 background [NT596–NT620].

Reaction of the mutants to nonpermissive conditions

To investigate the nature of the mbr defects, we wished to determine the reasons that they fail to grow under nonpermissive conditions. The mutants were grown in glucose minimal media to mid-log and subcultured into prewarmed LB at 37°C [mbrA4, mbrB20, mbrC11, mbrC17, mbrD3, mbrD7, mbrD19] or prewarmed glucose minimal media at 42°C [mbrA15, mbrC18]. At 1-hr intervals a sample was removed to determine the number of viable cells and the OD_600. At 2-hr intervals, samples were stained with the DNA-specific fluorescent dye, 4,6-diamidino-2-phenylindole (DAPI), and viewed in a phase-contrast microscope by use of both UV and visible light sources. The data for mbrA4, mbrB20, mbrC17, and mbrD19 are shown in Figures 2 and 3. mbrA15 behaves essentially like mbrA4, mbrC11 and mbrC18 behave similarly to mbrC17, and mbrD3 and
mbrD7 behave identically to mbrD19. As can be seen in Figure 2A, the OD_{600} measurements continued to increase under nonpermissive conditions, while the viable cell counts varied widely (Fig. 2B). mbrC17 actually lost viability for the first few hours and subsequently began to grow. mbrA4, mbrB20, and mbrD19 did not change in cell number for the first few hours but eventually resumed growth. mbrA4, mbrC17, and mbrD19 began to elongate shortly after they were exposed to LB. As the cells filament, the DNA stains as long ribbons that criss-cross the cell (Fig. 3, column 2). At the same time the cells exhibited this staining pattern, the viable cell counts indicated that the mutants either lost viability (mbrC17) or did not increase in number (mbrA4, mbrB20, mbrD19).

Between 4 and 6 hr after introduction to the nonpermissive conditions, the mutants that did not grow in LB exhibited unusual behavior. The DNA within the filamentous cells began to be divided into visually separate nucleoids (Fig. 3, column 3). After the DNA separated, septation initiated between virtually every nucleoid and the filaments divided en masse, resulting in a large increase in the number of viable cells. Very few of the cells that resulted from division of the filaments were devoid of DNA. If the mutants exposed to LB for 6 hr are re-inoculated into LB at low densities, they again exhibit filamentation for the first 4–6 hr, followed by division of the filaments en masse. We do not believe that revertants are overtaking the culture because when the cells that have grown in LB for the viable cell measurements and DAPI stains are streaked on LB agar, they fail to grow. Additionally, if less than ~10^6 mutant cells are inoculated into LB media, they fail to grow, suggesting that they are not capable of the maximal growth rates required for extended growth in LB.

Effects of the mbr mutations with dnaA204(Ts)

dnaA204(Ts) is a nonreversible temperature-sensitive mutation in dnaA that blocks initiation of replication at the nonpermissive temperature [above 37°C; Hansen et al. 1984]. We combined this lesion in dnaA with the mbr mutations for several reasons. First, mutations that suppress dnaA(Ts) lesions have been identified [sdr (Kogoma and von Meyenburg 1983) and das (Atlung 1981)]. Both sdr and das are alleles of rnh [see below]. In these mutants DNA replication is no longer coupled to the cell division cycle. We wished to determine whether any of the mbr mutants are similar to these suppressors of dnaA(Ts). Second, we wished to know the effects of blocking chromosome replication from oriC on the mbr mutants. If the mbr mutants require the normal, cell-cycle-controlled replication from oriC for Cmr^ or filamentation at the nonpermissive conditions, then blocking this replication should prevent the visualization of these defects. If, however, the mbr lesions bypass this replication or exert their effects indirectly, blocking replication from oriC should have no effect.
The most striking effect of dnaA204(Ts) is that it blocks resistance to camphor in all of the mbr mutants. This indicates that the extra DNA replication that is responsible for the increased ploidy is DnaA-dependent, most likely originating at oriC. Under nonpermissive conditions, all of the double mutants exhibited the growth defects of both the mbr mutation and dnaA204(Ts) (Table 2). Thus, the mbr mutations do not suppress dnaA204(Ts) and vice versa. These results indicate that none of the mbr mutations are equivalent to sdi or das.

Figure 4, column 2, shows the DNA-staining phenotypes of some of the double mutants at the nonpermissive conditions. The double mutants have DNA-staining patterns similar to dnaA204(Ts), indicating that DNA replication is required for the formation of the characteristic long ribbons of DNA seen in the elongated mbr mutant cells.

Effects of the mbr mutations with rnh339 :: cat

RNase H, the product of the rnh gene, is required for the cell to initiate DNA replication specifically at oriC. In the absence of RNase H, initiation of DNA replication occurs at oriC, as well as alternate origins, collectively called oriK. Replication from oriK is not cell cycle regulated, occurs in the absence of protein synthesis, and does not require DnaA (Ogawa et al. 1984). In an rnh− strain, dnaA is no longer an essential gene (Kogoma and von Meyenburg 1983). To determine the effects of altering the origin of replication on the mbr mutations, we first transduced rnh339 :: cat into strains carrying the mutations and checked the growth defect phenotypes, Cmr− (Table 2), and DAPI staining patterns at the permissive and nonpermissive conditions (Fig. 4, column 3). When the null mutation rnh339 :: cat was combined with the alleles of mbrB, mbrC, and mbrD, the strains still exhibited essentially all of the phenotypes of the mbr mutations (Table 2). The DAPI staining of the double mutants indicated that the cell size is somewhat increased under all conditions (Fig. 4, column 3). Thus, allowing initiation of replication from alternate origins does not significantly affect these mutants, indicating that mbrB, mbrC, and mbrD are not specific for oriC-dependent replication. When mbrA15 is combined with rnh339 :: cat, the double mutant exhibits the phenotypes of mbrA15. However, when rnh339 :: cat is transduced into an mbrA4-containing strain, the cells exhibit several novel phenotypes. The most obvious defect is that the double mutant fails to form single colonies on
Table 2. Phenotypes of mbr mutations with dnaA204(Ts) and rnh339 :: cat

| Mutation | C* | M* | L* | T* | dnaA204(Ts)* | rnh339 :: cat* | dnaA204(Ts) | rnh339 :: cat* |
|----------|----|----|----|----|--------------|---------------|-------------|---------------|
| mbrA4    | R  | -  | -  | R  | S            | S             | S           | S             |
| mbrA15   | R  | +  | +  | S  | S            | R             | R           | R             |
| mbrB20   | R  | -  | -  | S  | -            | S             | S           | S             |
| mbrC11   | R  | -  | -  | S  | -            | R             | R           | R             |
| mbrC17   | R  | +  | +  | S  | S            | R             | R           | R             |
| mbrC18   | R  | +  | +  | S  | S            | R             | R           | R             |
| mbrD3    | R  | -  | -  | S  | -            | R             | R           | R             |
| mbrD7    | R  | -  | -  | S  | -            | R             | R           | R             |
| mbrD19   | R  | -  | -  | S  | -            | R             | R           | R             |
| NT3      | S  | +  | +  | R  | S            | S             | S           | S             |

(C) Sensitivity or resistance to the presence of camphor vapors at 32 and 37°C; (M) growth on MacConkey agar at 32 and 37°C; (L) growth on LB agar at 32 and 37°C; (T) sensitivity or resistance to growth at 42°C.

(R) Resistance; (S) sensitivity; (-) no growth; (+) growth.

Effects of mbr mutations with dnaA204(Ts) and rnh339 :: cat

To determine the effects of completely blocking oriC-dependent replication and demanding that the cells use only the alternate origins, we have combined the mbr mutations with both dnaA204(Ts) and rnh339 :: cat. If the polyploidy of the mbr mutants does not depend on oriC-initiated replication, the triple mutants should be Cmr+ because dnaA204(Ts) is silent in the rnh339 :: cat background. If, however, the polyploidy of the mutants requires initiation of DNA replication from oriC, eliminating it should mask the mbr defects and the triple mutants should be camphor sensitive.

Addition of the rnh null mutation to strains carrying mbrB, mbrC, and mbrD restores Cmr+, suggesting that the triple mutants are again polyploid. Thus, these mutants affect factors that influence the DNA/cell ratio regardless of the origin of DNA replication. These triple mutants retain the growth defects characteristic of the mbr mutations, and in some cases, notably mbrC17 and mbrC18, additional growth defects are seen in the presence of rnh339 :: cat (Table 2). mbrA4 again exhibits severe growth defects in the presence of the rnh mutation, and for both mbrA4 and mbrA15, rnh339 :: cat does not restore Cmr+. These results suggest that the mutations at mbrA require DNA replication to initiate at oriC to exhibit Cmr+ and are unable to tolerate cell cycle independent replication initiation.

Discussion

Growth in the presence of camphor vapors: the mbr mutants

Beginning with slowly growing cells, we have isolated nine mutants with altered ploidy by selecting for growth in the presence of camphor vapors. Very few of the genes known to play a role in DNA replication and cell division were identified by the Cmr+ selection, implying that overinitiating replication or slowing septation is not enough to allow the cells to survive this selection. Although the mechanism of Cmr+ is unknown, it is clear that it selects for a novel class of mutants with cell cycle defects, termed mbr. The Cmr+ mutants map to four chromosomal locations: mbrA at 68 min; mbrB at 88.5 min; mbrC at 89.5 min; and mbrD at 90 min. mbrA, mbrB, and mbrC define either new genes or genes not previously implicated in the cell cycle. The mutations at 68 min (mbrA) may define a gene analogous to the E. coli B lar gene, which also maps to 68 min and has the phenotypes of resistance to camphor and increased cell size and DNA content (Ogg and Zelle 1957; Kvetkas et al. 1970). mbrD appears to be allelic to rpoB.

Increased ploidy in the mbr mutants

The Cmr+ of the mbr mutants is reversed by introducing a dnaA(Ts) mutation and restored in most of the mutants (mbrB, mbrC, and mbrD) when a mutation defective in RNase H is added to the mbr dnaA(Ts) cells. The loss of Cmr+ in all of the mbr dnaA(Ts) strains supports the idea that Cmr+ reflects increased ploidy. When replication initiation is compromised as it is in the dnaA(Ts) even at permissive temperatures (von Meyenburg and Hansen 1987), the extra DNA and, therefore, the Cmr+ of the mbr strains, is lost. This also implies that the increased ploidy of mbr is normally dnaA-dependent, initiated at oriC.

Bypassing oriC and allowing secondary origins to ini-
Trun and Gottesman

Figure 4. Photomicrographs of single, double, and triple mutant combinations of mbr, dnaA204(Ts), and rnh339::cat incubated at the nonpermissive conditions. (Column 1) mbr single mutants [dnaA+ rnh+] exposed to LB broth at 37°C for 4 hr; (column 2) mbr dnaA204(Ts) exposed to LB broth at 42°C for 2 hr [DAPI-stained cells from the 2- and 4-hr time points appeared identical]; (column 3) mbr rnh339::cat exposed to LB broth at 37°C for 4 hr; (column 4) triple mutants [mbr dnaA204(Ts) rnh339::cat] exposed to LB broth at 37°C for 4 hr. (Row 1) mbr+; (row 2) mbrA4; (row 3) mbrB20; (row 4) mbrC17; (row 5) mbrD19.

tiate is not sufficient to increase ploidy and make cells Cmr+, since neither an rnh single mutant nor an rnh dnaA double mutant is Cmr+. Finally, the rnh mutant allows the strains carrying mbrB, mbrC, and mbrD in combination with dnaA(Ts) to initiate replication at origins other than oriC and simultaneously restores Cmr+. Therefore, the defect that increases ploidy in these mutants requires DNA replication but can function independently of oriC.

E. coli cell cycle

Under nonpermissive conditions, the mbr mutants are capable of undergoing a complete cell cycle but with abnormal timing of the cycle relative to wild-type strains. Instead of doubling at 20- to 30-min intervals in LB, the mutants divide every 4-6 hr. When the filaments produced at the nonpermissive conditions do divide, very few of the resulting cells are devoid of DNA. Thus, in the mbr mutants, division is delayed until every future cell has been partitioned a chromosome. These two striking characteristics of the mutants, in combination with the results from the dnaA(Ts) and rnh- experiments, allow us to make a preliminary placement of the mbr defects within the context of the normal cell cycle.

Figure 5 summarizes the interrelationships of many of the key events in the timing of the E. coli cell cycle. The genes known to be required to complete most of these stages, as well as those genes involved in determining the location of each process, have not been listed (for reviews, see Donachie et al. 1984; von Meyenburg and Hansen 1987). Bernander and Nordstrom (1990) have proposed that the chromosome replication and cell division cycles run in parallel but independently of one another. To ensure productive cell duplication, both cycles must be connected at specific points. The cell division cycle [Fig. 5, outer circle] begins with newly separated daughter cells. The newborn cells elongate until they have reached twice their initial length, whereupon they initiate formation of the septum in the middle of the long axis of the cell. After the septum has formed, the two daughter cells are separated and the cycle begins again (for review, see Donachie and Robinson 1987).

At a specific cell mass, termed the initiation mass [M1], DNA replication is initiated from oriC (Donachie...
Additional evidence suggests that chromosomal partitioning occurs concomitantly with DNA replication (Kleckner 1990). Ogden et al. (1988) have shown that the presence of oriC, and the inability of the dnaA mbrA4, rnh~ double mutant to grow at any appreciable rate. We show that the mbrA mutants indicate that it be intimately involved in chromosomal partitioning. DnaA is responsible for sensing M₀ and, accompanied by protein and RNA synthesis, initiating replication (Fig. 5, inner solid line; Lobner-Olesen et al. 1989). It has been shown that replication of the entire chromosome requires at least 40 min (Cooper and Helmstetter 1968). When a cell is dividing every 20 min, it must initiate multiple rounds of DNA replication within one cell division cycle. Thus, the level of initiation of DNA replication must be coupled to the growth rate of the cells.

The phenotypes of the mbrB mutant indicate that it may be defective in this coupling of growth rate and DNA replication. At the permissive conditions, mbrB is increased in ploidy, is uniform in cell size, and has a uniformly high density. At the nonpermissive conditions, the mutant exhibits a wide range of cell sizes that vary in their DNA content. This suggests that in minimal media, the mbrB mutant is growing as if it were in rich media, and upon the shift to LB, the mutant cannot keep the cell cycle coordinated with the growth rate. If the increased ploidy at the permissive conditions were simply due to extra initiations per cell, the phenotypes of mbrB should be dependent on the presence of dnaA, and the activation of oriK. It is clear that initiation of DNA replication in a dnaA+ strain is tied to the cell cycle through M₀. If this were the only connection, an mbrB+ mutant that bypasses M₀ should have completely uncoupled DNA replication from the cell cycle, and the prediction is that an mbrB+ mutant should produce a large number of DNA-less cells, as well as cells with widely varying DNA contents. Because mbrB- cells appear to be uniform in cell size and DNA content (Fig. 4), this suggests that DNA replication is still coupled to the cell division cycle by a second level of control. If the mbrA mutants are defective in this second level of control, their only coordination between DNA replication and cell division would be through dnaA and M₀, eliminating dnaA would completely uncouple DNA replication and cell division. This would explain the dependence of mbrA on the presence of dnaA and the inability of the mbrA4, mbrB- double mutant to grow at any appreciable rate. We show this new coupling tentatively in Figure 5, located beyond initiation of DNA replication but before termination of chromosome replication.

When oriC has been replicated, the newly synthesized strand remains unmethylated for a longer period of time than other newly replicated DNA (Campbell and Kleckner 1990). Ogden et al. (1988) have shown that hemimethylated oriC binds specifically to two membrane proteins and have proposed that this binding may be intimately involved in chromosomal partitioning. Additional evidence suggests that chromosomal partitioning occurs concomitantly with DNA replication.
[Woldringh 1976]. Very little is known about partitioning of oriK-initiated DNA replication.

DNA replication proceeds bidirectionally from oriC (or oriK) around the chromosome until the forks reach terC, where replication stops and the chromosomes separate completely from one another [Kuempel et al. 1977; Louarn et al. 1977]. terC is dispensable. In its absence, chromosome segregation still occurs [Henson et al. 1984]. The mechanism by which segregation takes place in the terC deletion and in wild-type cells remains to be elucidated.

The phenotypes of the mbrC mutations suggest that they may be defective in chromosomal partitioning or in the coupling of partitioning to the cell division cycle. At the permissive conditions, the mutants are increased in cell length and ploidy. The increase in ploidy does not depend on dnaA. At the nonpermissive conditions, the mutants form very long filaments with the DNA in a novel ribbon-like structure that crossovers the cell. Before the filaments begin to divide, the DNA ribbons is separated into discrete nucleoids and segregated into distinct sections of the filaments. These results suggest that the mbrC mutations are slowed in chromosomal segregation. However, given enough time, the mutant cells can move the chromosomes apart.

The phenotypes of the mbrD mutations are similar to those of mbrC. At the permissive conditions, the mutants are uniform in cell length and increased in ploidy in a dnaA-independent manner. At the nonpermissive conditions, the mutants form long filaments of DNA that must be divided into discrete nucleoids before the filaments can septate. mbrD is probably allelic to rpoB; increased ploidy mutants have been identified previously in rpoB [Rasmussen et al. 1983; Tanaka et al. 1983]. It is known that RNA synthesis is required for initiation of DNA replication [Lark 1972] and protein synthesis is required for initiation of DNA replication and between termination of DNA replication and initiation of septation [Donachie and Begg 1989b]. Because mbrC and mbrD mutations are similar in appearance, they might be defective in similar steps. It is also possible that mbrD mutations are defective in expression of a specific gene or genes. Our alleles of rpoB fail to grow on rich media, MacConkey media, and at high temperatures, which makes them different from any previously isolated rpoB mutants. The mbrD alleles may provide a way to examine the role of RNA polymerase in different parts of the cell cycle.

The completion of chromosome segregation is thought to be tied to the cell division cycle in at least two manners. The presence of DNA [unseparated chromosomes] in the center of the cell, where the septum initiates, has been proposed to prevent the septum from forming [Mulder and Woldringh 1989]. Additionally, both chromosome separation and initiation of septum formation appear to occur at a minimum cell length that is twice that of the newborn cell at any given growth rate [Donachie and Begg 1989a]. During the period characterized by termination of DNA replication, completion of chromosome segregation, and initiation of septum formation, protein synthesis is required [Donachie and Begg 1989b]. Following initiation of septum formation, the cell division cycle continues until the daughter cells separate and again reach a specific Min. The Cmr selection has not identified any genes that appear to be defective in these later stages of the cell cycle.

Conclusions

Using several novel approaches to select gain-of-function mutants, we have identified nine E. coli mutants with increased ploidy. We believe that the mutants have increased ploidy because (1) they are resistant to camphor vapors, (2) they have an increased density in Percoll gradients, (3) they have at least a slight increase in the gene dosage of the lac operon, (4) there are no longer Cmr in the presence of a defective dnaA gene; and (5) for mbrB, mbrC, and mbrD, they are again Cmr in the presence of a defective dnaA gene and an rnh mutation. Our mutants define several new classes of cell-cycle mutants in that they appear to act after DnaA-dependent initiation of DNA replication but before initiation of septum formation. Thus, the mbr mutants are likely contained within that part of the cell cycle where many different events must occur in a precisely timed fashion and very little information is known about how and where these events take place.

Materials and methods

Bacteria and bacteriophages

All of the strains used in this study are isogenic derivatives of E. coli K-12 and are listed in Table 3. The dnaA204(Ts) mbr double mutants were constructed by P1 transduction from NT452 into NTS96–NTS20, selecting Kan'. Marker rescue experiments were performed to confirm the presence of dnaA204(Ts). The rnh339 :: cat mbr double mutants were constructed by P1 transduction from MIC1020 into NTS96–NTS20, selecting Cam'. The triple mutants were constructed by P1 transduction from MIC1020 into the dnaA204(Ts) mbr double mutants. Plvir [lab stock] and T4gt7 [from K. Tilly] were used for generalized transductions. ANK561 and ANK1105 have been described [Way et al. 1984], as has the procedure for their use [Silhavy et al. 1984]. Isogenic strains containing mbrA15, mbrC11, mbrC18, mbrD3, and mbrD7 and the various combinations of dnaA204(Ts) and rnh339 :: cat were constructed, and all of the experiments described were performed using them as well. The data are not shown; in all cases, they behave similarly to the alleles presented.

Media and reagents

Liquid and solid media were as described [Silhavy et al. 1984], except for M56 minimal salts [Monod et al. 1951]. Lactobionate plates contain M56 or M63 complete salts, 2.5% lactobionate [free acid, ~pH 7 [Fluka Biochemica, Ronkonkoma, NY], the hemicalcium salt does not permit growth of E. coli] and 5 mM isopropyl-thio-β-D-galactoside (IPTG). N,N-methylnitosoguanidine (NTG), 4,6-diamidino-2-phenylindole (DAPI), and poly-L-lysine [m.w. 150,000–300,000] were purchased from Sigma [St. Louis, MO]. Camphor (± mixture) was purchased from Fluka Biochemica.
Table 3.  Bacterial strains

| Strain   | Description                                      | Source/construction  |
|----------|--------------------------------------------------|----------------------|
| BR4433   | dnaA204(Ts)                                      | D. Chattoraj         |
| HS4004   | F- [lac-pro]XIII strA relA metA rif thi          | H. Shuman            |
| MC4100   | F- arad139Δ[argF-lac]U169 rpsL150 relA1 flbB301 deoC1 ptsF25 rbsR thi-1 | Casadaban (1976)    |
| MCI1020  | AB1157 mbl339 :: cat                             | Kanaya and Crouch    |
|           |                                                  | (1984)               |
| MG1655   | wild type                                        | B. Bachmann          |
| NS1558   | dnaG(Ts) str^ leu thyA                           | N. Sternberg         |
| NT1015   | MC4100 ΔamlBl5                                   | lab stock            |
| SFN135   | MC4100 argC :: Tn10                              | Kiino and Silhavy (1984) |
| SK2251   | F- gal-lacY1 malA1 xyl-7 his-1 leuB6 metB1 rpsL104 sup-59 zgi :: Tn10 T6' | Kiino and Silhavy (1984) |
| RK4936   | araD139Δ[argF-lac]205 flbB5301 gyrA219 non-9 relA1 rpsL150 metE70 btaB :: Tn10 thi-1 deoC1 | B. Bachmann          |
| NT3      | MC4100 lac^                                      | T4gt7(MG1655) into MC4100 |
| NT441    | NT3 proAB :: Tn10 F- lac-pro                     | F' mated in selecting Pro^ |
| NT452    | BR4433 ziL2 :: Δkan                              | Δkan[ANK1105] near dnaA204 |
| NT596    | NT3 zgi4 :: Tn10                                 | Tn10[ANK561] near mbrA4 |
| NT597    | NT3 zji17 :: Tn10                                | Tn10[ANK561] near mbrC17 |
| NT603    | NT3 mbrD3 zji17 :: Tn10                          | -*                   |
| NT604    | NT3 mbrA4 zgi4 :: Tn10                           | -*                   |
| NT607    | NT3 mbrD7 zji17 :: Tn10                          | -*                   |
| NT611    | NT3 mbrC11 zji17 :: Tn10                         | -*                   |
| NT615    | NT3 mbrA15 zgi4 :: Tn10                          | -*                   |
| NT617    | NT3 mbrC17 zji17 :: Tn10                         | -*                   |
| NT618    | NT3 mbrC18 zji17 :: Tn10                         | -*                   |
| NT619    | NT3 mbrD19 zji17 :: Tn10                         | -*                   |
| NT620    | NT3 mbrB20 zji17 :: Tn10                         | -*                   |

*mbr alleles were moved into NT3 from the original isolates by P1, using the linked Tn10.

Genetic manipulations

P1 transductions and isolation of Tn10 insertions near a gene have been described previously [Silhavy et al. 1984]. Briefly, for the mbr mutations, individual random pools of Tn10 transpositions were isolated in each of the mutant strains using λNK561. The pool sizes were ~100,000 Tn10 insertions. Subsequently, P1 was grown on each of the pools and used to transduce NT3 to Tet'^ Cmr'.

NTG mutagenesis and camphor resistance

NTG mutagenesis was carried out as described previously [Silhavy et al. 1984]. Eight cultures were mutagenized, immediately split to 10 tubes each, and incubated in fresh media for 3—4 hr. The cells were serially diluted, plated at either 32°C or 37°C, and incubated overnight. After colonies had formed, they were replica plated and incubated in the presence of camphor vapors. Only whole colonies that grew in the presence of camphor were tested, and no more than two colonies from any one culture were chosen. Of 129 mutants originally isolated, 29 remained stably Cmr'. The levels of mutagenesis in the cultures were monitored by the appearance of Lac^- colonies on lactose tetrazolium agar and Gal^- colonies on galactose tetrazolium agar. In all cultures, the frequency of either Lac^- or Gal^- colonies was 1—3%.

To expose cells to camphor vapors, ~7—10 grams of solid camphor was crushed in the lid of a petri plate. The cells were spread on the agar surface in the bottom of the plate and inverted over the camphor crystals. The plates were subsequently placed in a lidded plastic container and incubated at either 32 or 37°C. At 32°C, only a small amount of camphor is vaporized so that camphor-sensitive strains grow to form small colonies. At 37°C, enough camphor is vaporized so that only Cmr' cells can grow. At temperatures above 39°C, so much vaporization of the camphor takes place that no cells survive. The frequency of survival of LB-grown cells when exposed to camphor is 10^-1 to 10^-2 and that of glucose-grown cells is 10^-3 to 10^-4.

PercoU gradients

PercoU [Sigma, St. Louis, MO] was diluted to 72% in sterile water. Sodium chloride was added to a final concentration of 30 mM. Preformed PercoU gradients were prepared by adding 10.5 ml of PercoU solution to ultracentrifuge tubes and centrifuging in a type 40 rotor at 15,000 rpm for 30 min at 4°C. Preformed gradients were stored at 4°C for up to 1 week. To run the gradients, 1 ml of a balanced growth culture was layered on top of the gradients and centrifuged in a Sorvall RC2B at 7000 rpm for 20 min at 4°C. The gradients were calibrated using color-coded density beads [Pharmacia LKB Biotechnology, Piscataway, NJ], and the position of the cell bands was measured in centimeters from the top of the tubes immediately upon removal of the gradients from the centrifuge.
**DAPI staining and microscopy of cells**

Aliquots (100 μl) of actively growing cells were mixed with 3 μl of toluene and incubated at 37°C for 15 min. Subsequently, 3 μl of a 10 μg/ml DAPI solution (in water) was added, and the cells were incubated at room temperature for 30 min. Slides were first washed with ethanol and wiped dry. Poly-L-lysine (1% stock solution in saline, 20 μl) was placed on the cleaned slide and incubated at room temperature for 10 min. Excess poly-L-lysine was removed, and the slides were washed in distilled water. A drop of stained cell suspension was placed in the poly-L-lysine and allowed to settle for 5–10 min. A drop of 1 μl/ml para-phenylenediamine (free base, Sigma) in 90% glycerol was placed on the cells, and a coverslip was placed over the suspension. After removing excess liquid and sealing the coverslip, slides were viewed under a 100× Neofluar objective on a Zeiss microscope using both UV and visible light. Photographs were taken with a 35-mm camera attached to the microscope, using Kodak Tnp-X Pan black-and-white film. The film was developed using Diafine (Chicago, IL).

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N J Trun and S Gottesman

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