DnaA Protein Is Not Essential for Replication of IncFII Plasmid NR1

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By transformation of dnaA null mutant host cells that are suppressor either by an rnh mutation or by chromosomal integration of a mini-R1 plasmid, it was shown that replication of miniplasmids composed of the NR1 minimal replicon had no absolute dependence upon DnaA protein. In addition, the suppression of the dnaA null mutation by the integrated mini-R1, which is an IncFII relative of NR1, was found to be sensitive to the expression of IncFII-specific plasmid incompatibility. This suggests that the integrative suppression by mini-R1 is under the control of the normal IncFII plasmid replication circuitry. Although NR1 replication had no absolute requirement for DnaA, the copy numbers of NR1-derived miniplasmids were lower in dnaA null mutants, and the plasmids exhibited a much reduced stability of inheritance during subculture in the absence of selection. This suggests that DnaA protein may participate in IncFII plasmid replication in some auxiliary way, such as by increasing the efficiency of formation of an open initiation complex at the plasmid replication origin. Such an auxiliary role for DnaA in IncFII replication would be different from that for replication of most other plasmids examined, for which DnaA has been found to be either essential or unimportant.

Escherichia coli DnaA protein plays an essential role in the initiation of chromosomal DNA replication by binding to specific sites in the replication origin, oriC (5, 12-14, 18, 25, 26, 52). Many plasmids, including IncFII plasmids such as NR1, also contain DnaA protein-binding sites, referred to as DnaA boxes, in or near their replication origins (1, 12, 33, 37, 40, 58). However, most of these plasmids encode their own plasmid origin-specific replication initiation proteins (1, 33, 37, 40, 45, 49). Previously, the abilities of many plasmids to replicate in a dnaA(Ts) mutant host after a shift to the nonpermisive temperature (11, 15, 43, 50, 55) or to integratively suppress a dnaA(Ts) mutation (6, 7, 29, 35, 50) were taken as evidence for the DnaA independence of these plasmids. One exception was plasmid pSC101, which was incapable of replication at the nonpermisive temperature in a dnaA(Ts) host (10, 11, 17). Most recently, however, with the use of dnaA null mutants that contain no detectable DnaA protein, it has been shown that plasmids mini-F and mini-P1 have an absolute requirement for DnaA protein for their replication (16, 19, 32). The ability of the mini-F and mini-P1 plasmids to integratively suppress dnaA(Ts) mutations has therefore been ascribed to the lack of the mutant DnaA protein in dnaA(Ts) hosts (21). In contrast, the replication of ColE1-like plasmids was found to be independent of DnaA protein both in vivo (16, 19) and in vitro (38).

The survival of dnaA null mutants is dependent on one of two means of suppression: a secondary mutation in the RNase H gene, rnh, or chromosomal integration of a miniplasmid derived from IncFII plasmid R1 (16, 19, 20, 36). The integrative suppression of dnaA null mutants by mini-R1 was taken to indicate that R1 plasmid replication was DnaA independent. However, no direct tests of this hypothesis have been presented. More recently, from studies of the in vitro replication of IncFII plasmid DNA in extracts prepared from dnaA(Ts) mutant cells, it has been suggested that replication of IncFII plasmids has a strict dependence on DnaA protein in the in vitro systems (24, 38). It has also been suggested that the ability of the mini-R1 plasmid to integratively suppress a dnaA null mutant might be explained if the mini-R1 plasmid contained a second, DnaA-independent replicon (24, 38).

In this article, we present evidence that replication of the IncFII plasmid NR1 in vivo has no absolute dependence on DnaA protein and that integrative suppression of a dnaA null mutation by mini-R1 results from the normal mechanism of IncFII plasmid replication.

MATERIALS AND METHODS

Bacterial strains and plasmids. The Escherichia coli K-12 strains used in this study are listed in Table 1. The strains form a nearly isogenic set that contains various alleles of dnaA (19, 36). Two independent sets of each of the five strains were obtained from B. C. Kline and T. Kogoma. Experimental results obtained with the two sets of strains were indistinguishable.

IncFII plasmid NR1 (8, 34, 42, 57) is a transmissible, multiple-antibiotic-resistant (R) plasmid with a size of 94.5 kilobase pairs (kb) and a low copy number of about 2 per bacterial chromosome (43, 44, 59). Plasmid pR912 (31) is an Incâ Copâ mutant of NR1 that contains a single-base-pair substitution in the incRNA gene that regulates the translation of the mRNA for the repAl gene (40, 41, 44, 53, 54), which encodes the replication initiation protein of the plasmid (37, 40, 45). Plasmids pRR933 and pRR942 (9, 27) are 4.9-kb miniplasmids derived from NR1 and pRR12, respectively, that contain the minimal replicons of the plasmids. Each is composed of three PstI restriction fragments: a 1.1-kb fragment that encodes plasmid replication control and expresses plasmid-specific incompatibility, a 1.6-kb fragment that contains the plasmid replication origin, and a 2.2-kb fragment that contains the chloramphenicol resistance gene (cat). Plasmid pRR714 (9, 27) contains this cat fragment inserted at the PstI site of the ColEl-like cloning vector pBR322 (3). Plasmids pRR775 and pRR790 (58) contain the 1.1-kb PstI inc fragment from NR1 and pRR12, respectively, inserted at the PstI site of pUC8, a cloning vector derived from pBR322 (51). Plasmid pRR720 (39, 49) is an NR1-derived miniplasmid that contains the wild-type...
NR1 minimal replicon, the cat gene, and the stb locus responsible for stable plasmid inheritance (27, 49). Plasmid pFZY1 (22) is a cloning vector derived from mini-F that confers resistance to ampicillin and contains a polylinker sequence and a promoterless lacZ operon. Plasmid pKN500 (30) is a miniplasmid composed of EcoRI fragments B and F of R1, another member of the IncFII group of R plasmids, and confers resistance to kanamycin.

**Culture media and conditions.** Strains were cultured at 37°C in LB medium (28) supplemented with thymine (20 mg/liter) or in M9 minimal medium (28) containing 0.2% glucose and supplemented with thymine (20 mg/liter) and appropriate amino acids. Minimal medium was used to culture rnh mutant strains owing to their sensitivity to growth in rich medium (20, 36). Agar media contained 15 g of Bacto agar (Difco Laboratories) per liter. When appropriate, antibiotics were also included in some cultures: chloramphenicol, 25 mg/liter; tetracycline hydrochloride, 5 mg/liter; sodium ampicillin, 20 mg/liter; and kanamycin sulfate, 50 mg/liter. Cell growth was monitored by turbidity at 650 nm with a Gilford model 260 spectrophotometer.

**Purification and manipulation of plasmid DNA.** Plasmid DNA isolation, restriction endonuclease digestion, gel electrophoresis, and transformation of E. coli cells with plasmid DNA were performed by standard methods as described before (9, 27). Plasmid identification and cellular plasmid DNA content in the transformants were monitored by the alkaline minilysate method (2).

**Plasmid copy number measurements.** The relative copy numbers of plasmids that carry the cat gene were estimated from gene dosage effects by measuring chloramphenicol acetyltransferase (CAT) specific activity in cell extracts prepared from exponential-phase cultures as described before (9, 47), with total protein content measured by the method of Bradford (4). Plasmid DNA content was also monitored by the minilysate method (2).

**Plasmid stability test.** The stability of plasmid inheritance was determined by measuring the decrease in the fraction of antibiotic-resistant cells during a period of nonselective growth (27). To begin the experiments with a uniform population of plasmid-bearing cells, the cells were initially cultured in medium containing the antibiotic to which the plasmid conferred resistance. The cells were then repeatedly subcultured by 10-fold dilution into drug-free medium, followed by overnight incubation. After each subculture, appropriate dilutions were spread on drug-free agar plates, and the antibiotic resistance of at least 50 of the resulting colonies was tested by replica plating. The plasmid DNA content of representative antibiotic-resistant and -sensitive colonies was examined by the minilysate method (2).

**RESULTS AND DISCUSSION**

**Transformation of dnaA mutant strains with plasmid DNA.** To test the requirement of NR1-derived miniplasmids for DnaA protein, several different E. coli strains were transformed with various plasmid derivatives (Table 2).Recipient strain AQ3563 is a dnaA::Tn10 (null) mutant suppressed by a copy of a mini-R1 plasmid integrated into the chromosome (chr::pKN500), whereas recipient strain AQ3585 is a dnaA::Tn10 null mutant suppressed by an rnh mutation. Recipient strain AQ634 served as the wild-type dnaA+ control. The other control strains were AQ3553, which is a dnaA+ mutant that also contains an integrated copy of mini-R1, and AQ666, which is dnaA+ rnh. The NR1-derived tester plas-

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**TABLE 1. E. coli strains**

| Strain | Relevant genotype and properties | Source and reference |
|--------|---------------------------------|----------------------|
| AQ634  | dnaA+ rnh+                       | B. Kline (19)         |
| AQ666  | dnaA+ rnh-224                    | B. Kline (19)         |
| AQ3585 | dnaA850::Tn10(Tc) rnh224         | B. Kline (19)         |
| AQ3553 | dnaA5(Ts) rnh+ chr::pKN500(km')  | B. Kline (19)         |
| AQ3563 | dnaA850::Tn10(Tc) rnh+ chr::pKN500(km') | B. Kline (19) |

**TABLE 2. Transformation of dnaA mutant strains**

| Plasmid(s) | Selection | Relevant genotype of recipient strain |
|------------|-----------|-------------------------------------|
| pRR933     | Cm        | AQ634      | AQ3553      | AQ3563      | AQ666       | AQ3585      |
|            | +         | +          | +           | +           | +           | +           |
| pRR942     | Cm        | AQ3553     | AQ3563      | AQ666       | +           | +           |
| pRR714     | Cm        | AQ3563     | AQ666       | +           | +           | +           |
| pFZY1      | Ap        | AQ3553     | AQ3563      | +           | +           | +           |
| pUC8       | Ap        | AQ3553     | AQ3563      | +           | +           | +           |
| pRR933 + pBR322 | Cm | + | + | + | + | + |
| pRR942 + pBR322 | Cm | + | + | + | + | + |
| pFZY1 + pRR714 | Ap | + | + | + | + | + |
| pRR775     | Ap        | +          | +           | +           | +           | +           |
| pRR790     | Ap        | +          | +           | +           | +           | +           |

* Experiments with AQ3553 and AQ3563 were carried out on rich medium, whereas experiments with AQ666 and AQ3585 were carried out on minimal medium. The wild-type control strain AQ634 was plated on both rich and minimal media, with identical results. Plasmid transformants were selected with either chloramphenicol (Cm) or ampicillin (Ap). Host strains with integrated pKN500 were also selected with kanamycin, and those with Tn10 were selected with tetracycline. Symbols: +, in multiple experiments, colonies of normal size and frequency, i.e., several hundred to several thousand colonies per plate, were present after 18 h (rich medium) or 40 h (minimal medium) of incubation at 37°C; -, normal-sized colonies were not present after the standard period of incubation. For the negative plates from AQ3563 with pRR933 and pFZY1, a few colonies were often observed after several days of incubation (see text). For the negative plates from AQ3585 with pFZY1, incubation for additional time usually failed to give even a few colonies.

* For the negative plates from AQ3585 with pFZY1, incubation for additional time usually failed to give even a few colonies.

+ The AQ3553 transformants that contained pUC8 and pRR790 also formed colonies during incubation at 30 and 42°C, whereas those that contained pRR775 formed colonies at 30 and 37°C but not at 42°C.
TABLE 3. Plasmid copy number measurements for dnaA mutant strains

| Medium | Host genotype | pRR933 | pRR942 | pRR714 |
|--------|--------------|--------|--------|--------|
| LB     | dnaA         | 1.0    | 5.0 ± 0.6 | 3.2 ± 0.2 |
|        | dnaA(Ts) chr::pKN500 | 0.69 ± 0.04 | 4.6 ± 0.1 | 4.7 ± 0.2 |
|        | dnaA::Tn10 chr::pKN500 | 0.32 ± 0.06 | 1.5 ± 0.0 | 4.9 ± 0.8 |
| Minimal | dnaA+ rnh   | 1.0    | 4.6 ± 0.4 | 7.8 ± 1.4 |
|        | dnaA(Ts) chr::pKN500 | 0.66 ± 0.03 | 5.0 ± 0.2 | 8.3 ± 0.1 |
|        | dnaA::Tn10 chr::pKN500 | 3.1 ± 0.1 | 9.3 ± 0.4 |
|        | dnaA+ rnh   | 1.3 ± 0.1 | 6.8 ± 0.2 | 17 ± 2   |
|        | dnaA::Tn10 rnh | 0.42 ± 0.07 | 2.4 ± 0.9 | 10 ± 2   |

*Measured by CAT enzyme activity in comparison with that of plasmid pRR933 in the wild-type host, AQ634. The copy numbers for strains cultured in rich medium were compared with those of pRR933 in AQ634 cultured in rich medium, whereas the copy numbers for strains cultured in minimal medium were compared with those of pRR933 in AQ634 cultured in minimal medium. The results are presented as the averages of two or more determinations.

*This strain, AQ3563p(pRR933), was obtained from one of the rare transformant colonies that arose after several days of incubation.
FIG. 1. Analysis of plasmid DNA content in minilysates of dnaA mutant strains. Minilysate DNA was prepared from strains AQ634 (dnaA+</small>) (lanes 7 to 9), AQ3553 [dnaATs chr::pKN500] (lanes 4 to 6), and AQ3563 [dnaA::Tn10 chr::pKN500] (lanes 1 to 3) harboring either pRR933, pRR942, or pRR714, as indicated above each lane. The minilysate DNA was then electrophoresed in 1% agarose and stained with ethidium bromide. Marker DNA was electrophoresed in the same lane. The positions of the supercoiled forms of plasmids pRR714 (6.5 kb) and pRR933 and pRR942 (4.9 kb) are indicated on the right.

that the copy numbers of the NR1-derived plasmids pRR933 and pRR942 were lower in the dnaA null mutant strains. Although the relative copy number of pBR322 derivative pRR714 was higher in minimal medium than in LB, the absence of DnaA was inconsequential. This suggests that the replication of pRR933 and pRR942 is less efficient in the absence of DnaA protein. The copy number of pRR933, but not of pRR942 or pRR714, was also reduced in strain AQ3553 [dnaATs chr::pKN500]. This most likely reflects the incompatibility interaction between pRR933 and the integrated mini-R1 in this strain, in which the total number of copies of pRR933 plus mini-R1 may have been approximately equivalent to that of pRR933 alone in the wild-type strain. In a test of one of the pRR933 transformant colonies of strain AQ3563 (dnaA::Tn10 chr::pKN500) that had appeared after prolonged incubation, as described above, the apparent copy number of pRR933 was also quite low (Table 3). Although this might be consistent with the combined effects of plasmid incompatibility and the absence of DnaA, an alternative explanation could be chromosomal integration of the plasmid, as discussed below.

The plasmid DNA contents of strains AQ634 (wild type), AQ3553 [dnaATs chr::pKN500], and AQ3563 [dnaA::Tn10 chr::pKN500], visualized by the minilysate method (Fig. 1), were consistent with the CAT assay data in Table 3. That is, the copy numbers of pRR942 and pRR714 were high in both AQ634 and AQ3553, whereas the copy number of pRR933 was low in both strains. The copy number of pRR714 was also high in AQ3563 (lane 3). However, the copy number of pRR942 (lane 1) was lower in this dnaA null mutant strain. In one of the dnaA null AQ3563 transformants that had appeared after prolonged incubation, no pRR933 plasmid DNA was detected at all (lane 2). One possibility is that this represents a case of integration of pRR933 into the chromosome. pRR933 shows homology with the integrated copy of mini-R1 in this strain and also has some homology from segments of ISJ on either side of the cat gene with ISJ elements located in the E. coli chromosome. A similar explanation might apply to the rare colonies that arose after transformation and prolonged incubation of the dnaA null mutants with the pFZY1 plasmid, which shares lac homology with the chromosome (22).

Tests of plasmid stability in dnaA mutant strains. Miniplasmids pRR933 and pRR942 contain the complete minimal replicon necessary for proper control of replication, but they lack the stb locus required for proper partitioning (27). Therefore, even in a wild-type host strain, pRR933 was not inherited stably in the absence of continuous antibiotic selection (Table 4). However, owing to its elevated copy number, the inheritance of pRR942 was quite stable under the same conditions (Table 4). Whereas pRR942 was stable in the dnaA+ and dnaATs strains, it was very unstable in the dnaA (null) strains. The stability of pRR933 was even lower than normal in AQ3585 (dnaA::Tn10 rnh). These results are consistent with a reduced efficiency of plasmid replication for NR1 derivatives in the absence of DnaA protein, as reflected in a reduction of plasmid copy number, which would cause a decrease in stable inheritance. The very low stability of pRR933 in strain AQ3553 [dnaATs chr::pKN500] compared with the stable inheritance of pRR942 in the same strain can be explained by the incompatibility between pRR933 and the integrated mini-R1.

pRR720 is a miniplasmid that contains the stb locus responsible for stable inheritance, i.e., for plasmid partitioning at cell division (27, 49), in addition to the wild-type NR1 minimal replicon. The results of transformation and copy number measurements of the various tester strains with pRR720 DNA were similar to those with pRR933 (data not shown). Whereas pRR720 was inherited stably in the wild-type host, AQ634, and in the rnh mutant host, AQ666, it was not inherited stably in the dnaA null mutant AQ3585 (Table 4). Although pRR720 was unstable in the dnaA null mutant, it was much more stable than pRR933 stb in the same host strain (Table 4, and data not shown). This suggested that the stb locus was able to function in a dnaA null mutant and that the instability of pRR720 in this host was caused by its lower-than-normal copy number resulting from the absence of DnaA protein.

Together, the above results indicate that replication of plasmid NR1 does not have an absolute dependence upon DnaA protein in vivo. In this respect, replication of NR1 is

| Host genotype | pRR933 | pRR942 | pRR720 |
|---------------|--------|--------|--------|
|               | 0 days | 6 days | 0 days | 6 days | 0 days | 6 days |
| dnaA+ rnh+    | 96     | 84     | 100    | 100    | 100    | 100    |
| dnaA+ rnh     | 98     | 32     | 100    | 100    | 100    | 100    |
| dnaA::Tn10 rnh| 40     | 0      | 76     | 0      | 88     | 4      |
| dnaATs chr::pKN500 | 44 | 0      | 100    | 100    | 90     | 0      |
| dnaA::Tn10 chr::pKN500 | 44 | 0      | 100    | 100    | 90     | 0      |

* pRR933 is Inc+ Cop+ Stb-, pRR942 is Inc+ Cop+ Stb- and pRR720 is Inc+ Cop+ Stb-. Each day of subculture is equivalent to 20 generations of growth. These data are representative of those obtained from multiple replicates.

TABLE 4. Plasmid stability test for dnaA mutant strains cultured in drug-free minimal medium.

NR1 DOES NOT REQUIRE DnaA
similar to that of the ColEl1-like plasmids, such as pBR322.
In contrast, the replication of mini-F and mini-P1 appears to have a strict dependence upon DnaA protein (16, 19, 32). It is interesting that the plasmids that require DnaA are those whose replication is regulated by repeated DNA sequences present in their origins, whereas the plasmids that do not require DnaA are those whose replication is regulated by the interaction of two complementary RNA transcripts (46).

Although replication of NR1 does not have an absolute requirement for DnaA protein, the copy number and stability of NR1 derivatives were lower in the dnaA null mutant strains. This suggests that DnaA protein participates in an auxiliary role in initiation of NR1 replication. However, it could be argued that such a role is "essential" to the overall survival of NR1, because any reduction in copy number of this already low-copy-number plasmid could seriously reduce its stable maintenance, as observed (Table 4). Other plasmids that have been tested for DnaA dependence had either an absolute requirement for DnaA, such as mini-F, or were indifferent, such as CoEl1. The auxiliary role played by DnaA in IncFI replication therefore may be different from that in the replication of other plasmids.

Other studies have shown that NR1 miniplasmids can survive and replicate in vivo after deletion of the single DnaA box that lies next to the replication origin (37; unpublished data), whereas deletions that proceeded further into the origin region caused inactivation of origin function (37). However, pR942 derivatives that lacked the DnaA box had a lower copy number (unpublished data). Therefore, this is also consistent with a nonessential auxiliary role for DnaA protein in NR1 replication. However, those studies were carried out with miniplasmids derived from the high-copy-number mutant pRR12. Therefore, the importance of the DnaA box for the stable replication of low-copy-number NR1-derived miniplasmids has not been tested.

Based on the replication of IncFI plasmid DNA in vitro in cellular extracts prepared from dnaA(Ts) mutant E. coli strains, it was suggested that replication of these plasmids has an absolute requirement for DnaA protein (24, 38). However, in vitro replication reactions have not been tested in extracts prepared from dnaA null mutant cells, nor have plasmids from which the DnaA box has been deleted been tested for replication in vitro in the dnaA(Ts) extracts. Therefore, an alternative interpretation might be that the mutant DnaA protein present in the extracts prepared from the dnaA(Ts) mutant cells was inhibitory to IncFI plasmid replication. This would be more consistent with the results in this paper that suggest that DnaA protein plays an auxiliary but nonessential role in the replication of NR1 in vivo. From the results of our transformation experiments, it can also be concluded that the integrative suppression of the dnaA null mutant by mini-R1 was under the control of the normal (i.e., incompatibility sensitive) IncFI plasmid replication mechanism of pKN500.

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