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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 suppressed 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 nonpermissive 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 nonpermissive 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 leakiness 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 pRR12 (31) is an Inc^- Cop^- mutant of NR1 that contains a single-base-pair substitution in the *inc*RNA gene that regulates the translation of the mRNA for the *repA1* 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 *Pst*I 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 *Pst*I site of the ColE1-like cloning vector pBR322 (3). Plasmids pRR775 and pRR790 (58) contain the 1.1-kb *Pst*I *inc* fragment from NR1 and pRR12, respectively, inserted at the *Pst*I site of pUC8, a cloning vector derived from pBR322 (51). Plasmid pRR720 (39, 49) is an NR1-derived miniplasmid that contains the wild-type

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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*::Tn*10*(Tc^r) *rnh224*	B. Kline (19)
AQ3553	*dnaA5*(Ts) *rnh*$^+$ *chr*::pKN500(Km^r)	B. Kline (19)
AQ3563	*dnaA850*::Tn*10*(Tc^r) *rnh*$^+$ *chr*::pKN500(Km^r)	B. Kline (19)

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 *lacZY* operon. Plasmid pKN500 (30) is a miniplasmid composed of *Eco*RI 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 10^6-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*::Tn*10* (null) mutant suppressed by a copy of a mini-R1 plasmid integrated into the chromosome (*chr*::pKN500), whereas recipient strain AQ3585 is a *dnaA*::Tn*10* 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*(Ts) mutant that also contains an integrated copy of mini-R1, and AQ666, which is *dnaA*$^+$ *rnh*. The NR1-derived tester plas-

TABLE 2. Transformation of *dnaA* mutant strains[a]

Plasmid(s)	Selection	Relevant genotype of recipient strain				
		AQ634 *dnaA*$^+$	AQ3553 *dnaA*(Ts) *chr*::pKN500	AQ3563 *dnaA*::Tn*10* *chr*::pKN500	AQ666 *dnaA*$^+$ *rnh*	AQ3585 *dnaA*::Tn*10* *rnh*
pRR933	Cm	+	+	−	+	+
pRR942	Cm	+	+	+	+	+
pRR714	Cm	+	+	+	+	+
pFZY1	Ap	+	+	−	+	−
pUC8	Ap	+	+[b]	+	+	+
pRR933 + pBR322	Cm	+	+	−		
	Ap	+	+	+		
pRR942 + pBR322	Cm	+	+	+		
	Ap	+	+	+		
pFZY1 + pRR714	Ap	+	+	−		
	Cm	+	+	+		
pRR775	Ap	+	+[b]	−		
pRR790	Ap	+	+[b]	+		

[a] 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 Tn*10* 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.

[b] 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	Relative plasmid copy number[a]		
		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::Tn*10* *chr*::pKN500	0.32 ± 0.06[b]	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::Tn*10* *chr*::pKN500		3.1 ± 0.1	9.3 ± 0.4
	dnaA$^+$ *rnh*	1.3 ± 0.1	6.8 ± 0.2	17 ± 2
	dnaA::Tn*10* *rnh*	0.42 ± 0.07	2.4 ± 0.9	10 ± 2

[a] 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.

[b] This strain, AQ3563(pRR933), was obtained from one of the rare transformant colonies that arose after several days of incubation.

mids were pRR933, which is composed of the wild-type NR1 minimal replicon, and pRR942, which is the equivalent construct from pRR12, a high-copy-number mutant of NR1. Various pBR322 derivatives served as DnaA-independent control plasmids, and the mini-F derivative pFZY1 served as the DnaA-dependent control plasmid. Cells were cultured at 37°C, a temperature at which the mutant *dnaA*(Ts) protein in the AQ3553 control strain is still active. All plasmids were able to transform the three control strains, AQ634, AQ3553, and AQ666, at similar frequencies, with the formation of normal-sized colonies under standard incubation conditions. This indicated that the integrated copy of pKN500 in AQ3553 and the *rnh* mutation in AQ666 did not interfere with the establishment of any of these plasmids. Although pRR933 and the integrated mini-R1 might express mutual incompatibility in AQ3553, they were apparently capable of coexisting under the conditions of antibiotic selection used here. Also as expected, the mini-F derivative pFZY1 was unable to transform AQ3563 (*dnaA*::Tn*10* *chr*::pKN500) or AQ3585 (*dnaA*::Tn*10* *rnh*), since mini-F has an absolute requirement for DnaA. In contrast, the pBR322 derivatives pRR714 and pUC8 were able to transform both AQ3563 and AQ3585 owing to their DnaA independence. Because both pRR933 and pRR942 were also able to transform the *dnaA* null mutant AQ3585 at normal frequency, it appears that NR1-derived miniplasmids have no absolute requirement for DnaA protein for replication in vivo.

An interesting difference in the abilities of pRR933 (wild-type) and pRR942 (Inc$^-$ Cop$^-$) to transform AQ3563 (*dnaA*::Tn*10* *chr*::pKN500) was observed. The failure of pRR933 to transform AQ3563 might be explained by the mutual incompatibility of pRR933 and the integrated mini-R1, upon which strain AQ3563 has an absolute dependence for survival. The Inc$^-$ mutant pRR942, however, is compatible with NR1 and R1 and therefore does not cause any interference with replication of the integrated mini-R1 plasmid.

When the strains were transformed by various mixtures of plasmid DNAs, results consistent with those obtained with pure plasmid preparations were observed (Table 2). When both plasmids in the mixture were capable of transforming a given strain, some cotransformation was also detected (data not shown). When the plates that gave negative results for pRR933 and pFZY1 with recipient AQ3563 (*dnaA*::Tn*10* *chr*::pKN500) were incubated for 2 to 3 additional days, formation of a few small colonies was sometimes observed. This was not observed for the plasmid-free transformation controls (data not shown). As will be discussed in more detail below, the formation of these rare transformant colonies after prolonged incubation could result from integration of the plasmids into the chromosome.

To test the hypothesis that the failure of the wild-type miniplasmid pRR933 to transform AQ3563 (*dnaA*::Tn*10* *chr*::pKN500) was a result of the mutual incompatibility between pRR933 and the suppressing integrated mini-R1, pUC8 derivatives that contain the cloned *inc*$^+$ region of NR1 (pRR775) or the *inc* region of pRR12 (pRR790) were transformed into each of these strains. It is well established that the incompatibility expressed by the cloned NR1 plasmid DNA in these derivatives is the result of specific inhibition of IncFII plasmid replication and that the Inc$^-$ Cop$^-$ phenotypes of the pRR12 mutant result from a single-base-pair substitution that changes both the strength and specificity of this inhibition (9, 23, 27, 44, 54, 56). All three strains tested were transformed by both pRR775 and pRR790 except the *dnaA* null mutant AQ3563, which was not transformed by the *inc*$^+$ pRR775 (Table 2). This was consistent with the idea that the IncFII-specific plasmid incompatibility expressed by pRR775 was interfering with the replication of the chromosome in AQ3563, which is absolutely dependent on initiation of replication from the integrated mini-R1 plasmid.

Furthermore, when the AQ3553 (*dnaA*(Ts) *chr*::pKN500) transformants were plated at 42°C as well as at 37°C, those containing pRR775 failed to form colonies at 42°C. Transformants that contained pRR790 or pUC8 formed colonies at both temperatures. This indicated that chromosomal replication from the integrated mini-R1 in AQ3553 was being "switched off" (29) by the *inc*$^+$ pRR775 at 42°C in this temperature-sensitive *dnaA* mutant. Together, these results indicated that integrative suppression of both the *dnaA* null mutation in AQ3563 and the *dnaA*(Ts) mutation in AQ3553 by the integrated copy of mini-R1 was under control of the normal IncFII replication circuitry. We cannot rule out the possibility that replication of the IncFII miniplasmids in the *dnaA* null mutants occurs through some secondary mechanism. However, because the miniplasmids contain only the minimal IncFII replicon, and because their replication in the *dnaA* null mutants is specifically sensitive to the normal IncFII replication control elements, we consider this unlikely.

Measurements of plasmid copy number in *dnaA* mutant strains. The copy number of NR1 and its Cop$^+$ miniplasmid derivatives is approximately 2 per chromosome or approximately 4 per average *E. coli* cell (44, 56). The relative copy numbers of plasmids pRR933 (wild type), pRR942 (Inc$^-$ Cop$^-$), and pRR714 (pBR322 *cat*) in the various tester strains were determined (Table 3). These results indicate

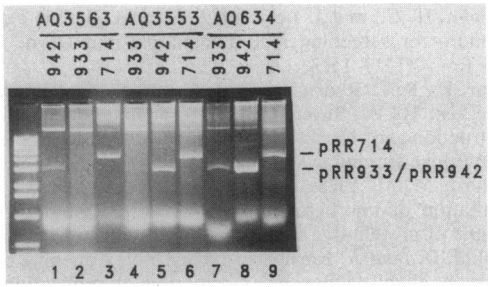

FIG. 1. Analysis of plasmid DNA content in minilysates of *dnaA* mutant strains. Minilysate DNA was prepared from strains AQ634 (*dnaA*$^+$) (lanes 7 to 9), AQ3553 [*dnaA*(Ts) *chr*::pKN500] (lanes 4 to 6), and AQ3563 (*dnaA*::Tn*10* *chr*::pKN500) (lanes 1 to 3) harboring either pRR933, pRR942, or pRR714, 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 leftmost 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 (*dnaA*(Ts) *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*::Tn*10* *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 [*dnaA*(Ts) *chr*::pKN500], and AQ3563 (*dnaA*::Tn*10* *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 IS*1* on either side of the *cat* gene with IS*1* 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 *dnaA*(Ts) strains, it was very unstable in the *dnaA* (null) strains. The stability of pRR933 was even lower than normal in AQ3585 (*dnaA*::Tn*10* *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 [*dnaA*(Ts) *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

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

Host genotype	% of cells retaining indicated plasmid after subculture for:					
	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::Tn*10* *rnh*	40	0	76	0	88	4
dnaA(Ts) *chr*::pKN500	44	0	100	100		
dnaA::Tn*10* *chr*::pKN500			90	0		

[a] 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.

similar to that of the ColE1-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 ColE1. The auxiliary role played by DnaA in IncFII 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, pRR942 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 IncFII 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 IncFII 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) IncFII plasmid replication mechanism of pKN500.

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