A dnaC Mutation in Escherichia coli That Affects Copy Number of ColE1-Like Plasmids and the PriA-PriB (but Not Rep-PriC) Pathway of Chromosomal Replication Restart

R. Harinarayanan* and J. Gowrishankar†

*Centre for Cellular and Molecular Biology, Hyderabad 500 007, India and †Laboratory of Bacterial Genetics, Centre for DNA Fingerprinting and Diagnostics, Hyderabad 500 076, India

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ABSTRACT

Escherichia coli wusG and rho mutants, which are defective in transcription termination, are killed following transformation with several ColE1-like plasmids that lack the plasmid-encoded copy-number regulator gene ron because of uncontrolled plasmid replication within the cells. In this study, a mutation [dnaC1331(A84T)] in the dnaC gene encoding the replicative helicase-loading protein was characterized as a suppressor of this plasmid-mediated lethality phenotype. The mutation also reduced the copy number of the plasmids in otherwise wild-type strains. In comparison with the isogenic dnaC+ strain, the dnaC mutant was largely unaffected for (i) growth on rich or minimal medium, (ii) tolerance to UV irradiation, or (iii) survival in the absence of the PriA, RecA, or RecB proteins. However, it was moderately SOS-induced and absolutely dependent on both the Rep helicase and the PriC protein for its viability. A dnaC1331(A84T) dam mutant, but not its mutH derivative, exhibited sensitivity to growth on rich medium, suggestive of a reduced capacity in the dnaC1331(A84T) strains to survive chromosomal double-strand breaks. We propose that DnaC-A84T is proficient in the assembly of replication forks for both initiation of chromosome replication (at oriC) and replication restart via the Rep-PriC pathway, but that it is specifically defective for replication restart via the PriA-PriB pathway (and consequently also for replication of the Rom+ ColE1-like plasmids).

The replication fork represents a complex of enzymes that act in concert to achieve unidirectionally processive semiconservative replication of the template DNA, notwithstanding the opposing polarity of its two strands. In Escherichia coli, the replication fork that is involved in replication of the chromosome as well as of various plasmids and coliphages is composed of the 5'→3' replicative helicase DnaB, the primase DnaG, and two catalytic polymerase subassemblies of the DNA polymerase III holoenzyme (one each for the leading and lagging template DNA strands; reviewed in Kornberg and Baker 1992; MARIANS 1996; MESSER and WIEGEL 1996).

Initiation of replication fork assembly on the chromosome involves the delivery by DnaC protein of the DnaB helicase to a specific region oriC at which the initiator protein DnaA is bound. This assembly is also known as the ABC primosome (MARIANS 1996). DnaB then recruits DnaG (TOUGU and MARIANS 1996) and DNA polymerase III (Kim et al. 1996) to constitute the replication fork. Two replication forks so constituted at oriC then travel in opposite directions to achieve replication of the entire chromosome.

In the past few years, it has been recognized that replication forks initiated at oriC often do not progress to completion around the chromosome but instead collapse or disintegrate spontaneously at, for example, sites of template DNA damage. In such cases, the fork may be reassembled at the site of its collapse in a process called replication restart, which in turn is intimately connected to the process of RecA-dependent recombinational repair of the damaged DNA (reviewed in KOGOMA 1997; KUZMINOV 1999; COX et al. 2000; MICHEL 2000; SANDLER and MARIANS 2000; COX 2001; McGLYNN and LLOYD 2002).

Although the mechanism of replication restart is not understood in all its detail, it is DnaA independent and shares many features with phage φX174 replication. Biochemical studies with purified proteins in vitro have suggested that during replication restart, a restart primosome (also called the φX-type primosome) is constituted at a three-way-junction DNA structure, the D-loop, which is an intermediate in recombinational repair (McGLYNN et al. 1997; LIU and MARIANS 1999; LIU et al. 1999; XU and MARIANS 2003). The restart primosome is formed by the sequential action of three proteins, PriA, PriB, and DnaT, at the D-loop. PriA is a bifunctional protein that both initiates φX-type primosome assembly and acts as a 3'→5' DNA helicase, but studies in vitro (LIU et al. 1999) and in vivo (SANDLER et al. 1996, 2001; JAKTAI and LLOYD 2003) have suggested that the

*Corresponding author: Laboratory of Bacterial Genetics, Centre for DNA Fingerprinting and Diagnostics, ECIL Rd., Nacharam, Hyderabad 500 076, India. E-mail: Shankar@cdfd.org.in

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latter function is dispensable for replication restart at D-loops; PriB is a structural component of the prionosome whose precise role in replication restart is not established, while DnaT apparently promotes the transfer of DnaB from the DnaB-DnaC complex in solution. PriB pathway but not for the Rep-PriC pathway of replication. Some whose precise role in replication restart is not well characterized, acting with either PriA (PriA-PriC pathway) or another 3'-5' DNA helicase Rep (Rep-PriC pathway); however, biochemical data in support of either pathway are not available.

Finally, replication fork assembly is also important for plasmid replication. In the case of the ColE1-like plasmids (see Cesareni et al. 1991; Del Solar et al. 1998 for reviews), an RNA primer generated by the processing of the plasmid-specified transcript RNA-II is extended by DNA polymerase I to form a D-loop that is then believed to serve for replication fork assembly in much the same way as that described above for replication restart on the chromosome. RNA-II processing to generate the primer is modulated by an antisense inhibitor RNA-I, and the interaction between RNA-II and its inhibitor is stabilized by the Rom protein, encoded by the plasmid-borne rom gene. Null mutants in priA do not support ColE1 plasmid replication, and point mutations in priA as well as a triple substitution mutant allele of priB that confer a reduced plasmid-copy-number phenotype have been described (Lee and Kornberg 1991; Nurse et al. 1991; Sandler et al. 1996, 2001; Berges et al. 1997; Jaktaji and Lloyd 2003).

Although it is apparent from the description above that DnaC-mediated delivery of DnaB is the first common step in all mechanisms of replication fork assembly, it is not clear whether the same domain of DnaC is involved in interacting with the different structures (such as DnaA-bound oriC or the restart primosome assembled at a D-loop). DnaC mutants that are temperature sensitive (Ts) for growth have been characterized (Wechsler 1975; Maisnier-Patin et al. 2001), consistent with the protein’s essential role in replication fork assembly at oriC (and perhaps also in replication restart). Other dnaC mutations have been recovered as suppressors of sickness or inviability associated with priA disruption (Kogoma et al. 1996; Sandler et al. 1996, 1999, 2001; Gregg et al. 2002; Rangarajan et al. 2002; Jaktaji and Lloyd 2003). In this article, we report the identification and characterization of a novel dnaC mutant [dnaCl331(A84T)] that exhibits both a reduced-copy-number phenotype with Rom ColE1-like plasmids and synthetic lethality with certain genes, such as rep and priC, implicated in replication restart. Our results suggest that DnaC-A84T is affected for the PriA-PriB pathway but not for the Rep-PriC pathway of replication restart or for replication fork assembly at oriC.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids: Genotypes of E. coli K-12 strains are listed in Table 1. The routinely employed rich and minimal growth media were, respectively, Luria broth (LB) and glucose-minimal A (Miller 1992). Antibiotics were used at the following final concentrations (micrograms per milliliter): ampicillin (Amp), 100; rifampicin (Rif), 100; chloramphenicol (Cm), 25; kanamycin (Kan), 50; spectinomycin (Spec), 50; and tetracycline (Tet), 15. Unless otherwise indicated, the growth temperature was 30°C.

Plasmids previously described include (salient features in parentheses): (i) p15A-derived: pACYC184 (CmR TetR; Chang and Cohen 1978); (ii) pMB9-derived: pBR322 (AmpR TetR; Bolivar et al. 1977), pUC19 (AmpR; Yanisch-Perron et al. 1985), pBR329 (AmpR CmR TetR; Covarrubias and Bolivar 1982), and pHYD762 (AmpR; Harinarayanan and Gowrishankar 2003); and (iii) pSC101-derived: pLG339 (KanR; Williams et al. 1996), and pCL1921 (SpecR; Lerner and Inouye 1990).

Plasmid pHYD554 is a pCL1921 derivative carrying a 5-kb chromosomal fragment that includes the rep promoter and was obtained by subcloning of a KpnI-PstI fragment from the phage clone λ556 of the ordered genomic library of Kohara et al. (1987) into the corresponding sites of pCL1921.

DNA methods: Standard protocols were followed for experiments involving recombinant DNA (Sambrook et al. 1989). Intracellular content of test plasmids in different strains was estimated following agarose gel electrophoresis of plasmid preparations that were made from constant numbers of cells withdrawn from the cultures; prior to the plasmid isolation procedure, a fixed volume of culture of DH5α carrying plasmid pHYD762 (2.2 kb) was added as loading control to normalize for efficiency of plasmid recovery (Harinarayanan and Gowrishankar 2003). The dnaTC locus (see Figure 1) was PCR amplified from chromosomal DNA preparations of isogenic strains GJ1541 (splD) and GJ1556 (splA′) with the aid of primers DNACFP (5'-GGCAATGGCACAGCACAG-3') and DNASRT (5'-GTCGCTTATGCCATCGGT-3'); both primers were used also for sequencing the PCR products (each 1.5 kb long), along with a third internal primer, DNACIP (5'-CATGTCGCCAAGCCCGTTCGC-3').

Transposon tagging of the splD locus: Random transpositions of the Kanr encoding mini-Tn10 derivative Tn10Kan into the chromosome of the nusG splD (dnaCl331) strain GJ1538 were generated following infection with phage A1316, as described (Kleckner et al. 1991; Miller 1992). A P1 lysate prepared on a pool of 106 Kanr clones was used to transduce the nusG splD strain GJ1504 to Kanr. The Kanr transductants were pooled and transformed with plasmid pACYC184 to identify derivatives of GJ1504 that, by virtue of their coheriting the splD allele (from GJ1538) with a linked Tn10Kan insertion, survived transformation with pACYC184. One clone (GJ1539) so identified was subsequently shown to have a Kanr insertion (designated _{rep}C:A10Kan) ~90% cotransducible with the splD locus.

UV survival tests: Sensitivity of a strain to UV was determined essentially as described by Miller (1992). Cells from a 50-ml volume of LB culture (grown at 37°C to an A600 of ~0.5) were chilled on ice for 30 min and resuspended in an equal volume.
of 0.1 M MgSO<sub>4</sub>. Five-milliliter aliquots of the suspension were placed in 85-mm petri dishes and exposed to UV (at 0.4 J/ M<sup>2</sup>/sec) for different lengths of time, after which they were protected from visible light. Appropriate dilutions of each aliquot were then plated on LB for estimation of the viable count.

**Other methods:** All strain constructions were by P1 transduction (Gowrishankar 1985); when the recipient strain carried an Amp<sup>+</sup> plasmid, transductants were recovered at limiting dilutions to minimize the extent of Amp degradation by the recipient lawn on the selection plates. The mutator phenotype of a strain was scored by spotting ~10<sup>7</sup> cells of LB-grown

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**TABLE 1**

List of *E. coli* K-12 strains

| Strain<sup>a</sup> | Genotype<sup>b</sup> |
|-------------------|----------------------|
| CAG18619          | *zji-3188::Tn10kan*  |
| DH5α              | Δ(argF-lac)U169 supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 (φ80lacZΔM15) |
| MC4100            | Δ(argF-lac)U169 rpsL150 relA1 araD139 fbgB3501 deoC1 ptsE25 |
| SS135             | argE3 his-4 thi-1 rpsL31 lacBKI ΔpriB302 |
| GJ11              | MC4100 proU22E::lac |
| GJ1313            | GJ11 nusG-G146D *zji-900::Tn10<sup>c</sup> |
| GJ1354            | GJ3127 dncG1331(A84T)<sup>e</sup> |
| GJ3127            | GJ1558 *zji-901::Tn10dKan |
| GJ1341            | GJ1504 dncG1331(A84T) *zji-901::Tn10dKan |
| GJ3127            | MC4100 dncG1331(A84T) mdoB::Tn10<sup>g</sup> |
| GJ3128            | MC4100 mdoB::Tn10 |
| GJ3185            | MC4100 gal3p3 penB1 znd-981::Tn10dKan |
| GJ5201<sup>f</sup> | GJ3127 Δrefc::Cam |
| GJ5202            | GJ3128 Δrefc::Cam |
| GJ5203            | MC4100 Δrefc::Cam |
| GJ5204            | MC4100 priC303::Kan |
| GJ5205<sup>e</sup> | *thr leu arg his sulA mdoB::Tn10 (λsulA::lac) |
| GJ5206            | GJ5205 dncG1331(A84T) |
| GJ5207            | GJ5205 recA::Kan |
| GJ5208            | GJ5206 recA::Kan |
| GJ5209            | GJ5205 lexA3 mdoB::Tn9 |
| GJ5210            | GJ5206 lexA3 mdoB::Tn9 |
| GJ5211            | GJ3127 dnc::Tn9 |
| GJ5212            | GJ3127 mutH471::Kan |
| GJ5213            | GJ5212 dnc::Tn9 |
| GJ5220            | GJ5212 priA::Kan |
| GJ5221            | GJ5212 priA::Kan |
| GJ5222            | GJ3127 recA::Kan |
| GJ5223            | GJ3128 recA::Kan |
| GJ5224<sup>f</sup> | GJ11 dncG1331(A84T) recB268::Tn10 |
| GJ5225            | GJ11 recB268::Tn10 |

<sup>a</sup> The following strains have been described earlier: CAG18619 (Berlyn et al. 1996); DH5α (Sambrook et al. 1989); MC4100 and GJ11 (Gowrishankar 1985); SS135 (Sandler et al. 2001); GJ1313 (Jayashree and Gowrishankar 1995), wherein the nusG-G146D allele was designated as rpoB1964; and GJ1504 and GJ3185 (Harinarayanan and Gowrishankar 2003). All other strains were obtained or constructed in this study.

<sup>b</sup> Genotype designations are as in Berlyn et al. (1996). All strains are F<sup>−</sup>. The following mutations were transduced from the parenthetically indicated strains described in SaiSree et al. (2000): lexA3 mdoB::Tn9 (GJ1999) and Δrefc::Cam (GJ1217); Reddy and Gowrishankar (2000): dnc::Tn9 (GJ2282), mutH471::Kan (GJ2284), and recA::Kan (GJ2293); and Sandler et al. (2001): priC303::Kan (SS145). Mutation priA::Kan was transduced from strain PN105 obtained from the *E. coli* Genetic Stock Center and recB268::Tn10 from strain JJC315 obtained from B. Michel.

<sup>c</sup> The *dnaC1331(A84T)* mutation has also been referred to as spl-16 in this study.

<sup>d</sup> The *mdoB::Tn10* insertion was previously identified as *zji-202::Tn10* (Berlyn et al. 1996; Nichols et al. 1998) and was transduced from strain CAG18430. Although the *Tn10* insertion in CAG18619 was reported to be at the same site as the *Tn10* insertion in CAG18430 (Berlyn et al. 1996), our transductional mapping data suggest otherwise (see text and data not shown).

<sup>e</sup> GJ5201 was recovered and maintained as a transformant derivative with the plasmid pHYD554 carrying *refc*.

<sup>f</sup> GJ5205 is a derivative of strain GJ1992 (SaiSree et al. 2000). Not all mutations in the strain have been listed here.

<sup>g</sup> GJ5224 was constructed in several steps of P1 transduction from strain GJ1538.
cultures on LB-Rif plates; typically, the mut<sup>+</sup> and mut strains yielded, respectively, <3 and 50–100 Rif<sup>+</sup> colonies per spot. Procedures for nitrosoguanidine mutagenesis and β-galactosidase assays were as described (Miller 1992).

RESULTS

Identification of dnaC1331(A84T) as a suppressor of nusG-pACYC184 lethality: We have recently described a novel lethality phenotype associated with runaway replication (that is, uncontrolled increase in copy number) of ColE1-like plasmids such as pACYC184 in E. coli nusG or rho mutants that are defective in factor-dependent transcription termination (Harinarayanan and Gowrishankar 2003). Following nitrosoguanidine mutagenesis and β-galactosidase assays were as described (Miller 1992).

We have shown previously that a rho-A243E mutant is also killed following transformation with pACYC184 (Harinarayanan and Gowrishankar 2003). With the aid of the linked Tn10dKan marker, the dnaC1331 allele was introduced into the rho strain, and here, too, it was able to suppress the plasmid-mediated lethality (data not shown).

dnaC1331(A84T) reduces copy number of Rom- ColE1-like plasmids: Harinarayanan and Gowrishankar (2003) have shown that lethality in pACYC184 transformants of the nusG and rho strains was accompanied by a substantial increase in plasmid copy number that is most prominent in the early stationary phase of growth; furthermore, mutations in several loci (including hns, rpoB, penB, and polA), as well as in multicopy recG<sup>+</sup> or rom<sup>+</sup>, were identified as suppressors of lethality that were all also associated with reversal of the copy number alteration in the mutants.

The dnaC1331(A84T) suppressor mutation, characterized in the present study, also behaved like the suppressors previously described in that it was able to reverse the increase in pACYC184 content otherwise observed in cultures of the nusG strain in the early-stationary growth phase (Figure 2). Densitometric analysis of the band intensity of pACYC184 (relative to that of the loading control) in each of the lanes in Figure 2 indicated that the pACYC184 content (per unit of biomass) in the nusG mutant in the exponential and stationary phases of growth was, respectively, two- and ninefold higher than that in the corresponding culture aliquots of either the wild-type strain or the nusG dnaC1331 double mutant. The latter two strains themselves exhibited an approximate doubling in pACYC184 content as the cultures progressed from the log phase to the stationary phase.

Two mechanisms have been identified by which the different suppressors act to suppress nusG or rho-pACYC184 lethality. The first is by reversing the transcription termination defect in the mutants, and the second is by a primary effect on plasmid copy number even in the nusG<sup>+</sup> rho<sup>+</sup> strain (Harinarayanan and Gowrishankar 2003). The dnaC1331(A84T) mutation did not alleviate the defect in transcription termination in the nusG and rho strains, as judged by the extent of transcriptional polarity relief at different loci such as galP3 (an IS2 insertion in the interval between the promoter and the first structural gene of the gal operon) or trpE (Oc; a nonsense mutation in the first gene of the trp operon; data not shown). On the other hand, the mutation was associated with a pronounced decrease in plasmid pACYC184 content even in the nusG<sup>+</sup> rho<sup>+</sup> background (Figure 3).

As mentioned in the Introduction, the plasmid-encoded Rom protein serves to downregulate the copy number of ColE1-like plasmids by stabilizing the interaction between RNA-II and its antisense inhibitor RNA-I. When plasmids such as pUC19, pBR329, or pBR322 were ex-
Amino acid differences in isogenic dnaC+ and dnaC1331(A84T) mutants, it was observed that the content of the first two (which, like pACY184, are Rom−) is also markedly reduced in dnaC relative to dnaC+, whereas that of the third (which is Rom+) is only marginally affected in the mutant (Figure 3). Densitometric analysis of the test plasmid band intensities in Figure 3 (after normalization against the corresponding loading controls) indicated that the ratio of plasmid content in the dnaC1331 mutant to that in dnaC+ was, respectively, 0.19, 0.06, and 0.27 for the plasmids pACYC184, pBR329, and pUC19 and that it was 0.77 for plasmid pBR322. These results also established that there is no correlation between plasmids conferring lethality in the nusG or rho mutants and those whose copy number is reduced by the dnaC1331(A84T) mutation [since pBR329 is an example of a plasmid that does not kill the nusG/rho strains (Harinarayanan and Govrishankar 2003) and yet whose copy number is reduced in the dnaC strain].

Suppression by dnaC1331(A84T) or priB of the Ts growth phenotype of pUC19 transformants: Lin-Chao et al. (1992) have shown that (i) the very-high-copy-number phenotype of plasmid pUC19 is the result of a mutation in its gene encoding RNA-II, which destabilizes the latter’s interaction with the inhibitor RNA-I; (ii) the interaction of pUC19-encoded RNA-II with RNA-I is rendered even less stable at 42°C, so that cultures that have been shifted from 30°C to 42°C exhibit a further increase in plasmid copy number and a reduction in growth rate; and (iii) Rom protein expression rescues the slow-growth phenotype at 42°C.

We confirmed in this study that pUC19 transformants of the wild-type strain GJ3128 exhibit a pronounced increase in plasmid copy number upon temperature upshift to 42°C (Figure 4A). The transformants also exhibited a Ts growth phenotype on LB agar medium (Figure 4B). The isogenic dnaC1331(A84T) mutant GJ3127 exhibited neither the plasmid copy number increase at 42°C (Figure 4A) nor the Ts growth phenotype (Figure 4B).

As expected, the pUC19-associated Ts phenotype was also suppressed in a penB strain [in which plasmid copy number is reduced because of an increased stability of the inhibitor RNA-I (Xu et al. 1993; Figure 4B)]. When
null mutations in priB or priC were tested, the former but not the latter suppressed the Ts phenotype (Figure 4B). [In the absence of pUC19, the wild type and all the mutant strains were unaffected for growth at 42° (Figure 4B).] A priB missense mutant with reduced copy number of R0m–ColE1-like plasmids has previously been reported (Berges et al. 1997).

**Chronic SOS induction in the dnaC1331(A84T) mutant and synthetic lethality with rep or priC mutations:** The growth rates of the wild-type strain GJ3128 and its dnaC1331(A84T) derivative GJ3127 were indistinguishable in both LB (2.47 and 2.44 hr\(^{-1}\), respectively, at 37°; see also Figure 4B and Figure 6) and glucose-minimal A (0.87 and 0.88 hr\(^{-1}\), respectively, at 37°), suggesting that the mutant protein is unaffected in its ability to load DnaB helicase at replication forks initiated at oriC. At the same time, the mutation conferred a moderate level of chronic SOS induction with a threefold increase of sulA-lac expression in the dnaC1331(A84T) strain; the increase was abolished, as expected, upon introduction of recA or lexA3 mutations that render the SOS regulon noninducible (Walker 1996; Figure 5). These observations suggested that the dnaC1331 mutant might be affected for replication fork assembly at restart primosomes.

As a test of this possibility, we looked for synthetic lethal interactions between dnaC and other genes previously implicated in recombinational repair and replication restart pathways. In the first approach, double mutants of dnaC1331(A84T) with each of the other mutations were sought to be constructed by P1 transduction. As shown in Figure 6, viable double mutants were recovered with recA::Kan, recB::Tn10, or priA::Kan; likewise, double mutants were also obtained at the expected frequencies for three other loci: recG258::Kan, Δrep ABC::Cm, and ΔpriB302 (data not shown). [Consistent with an earlier report (Gregg et al. 2002), the priA single mutant was itself very sick and gave rise to many fast-growing suppressor clones (see Figure 6).] Growth of each of the double mutants was in general no more significantly impaired than that of the respective single locus mutants, although the recB dnaC strain alone did exhibit a 40% slower growth rate than recB (see Figure 6). On the other hand, no transductants were obtained when we sought to introduce the rep::Kan, Δrep::Cm, or priC::Kan alleles into the dnaC1331(A84T) strain (data not shown).

Two additional experiments were undertaken to establish that rep is synthetically lethal with dnaC1331(A84T). The strategy for the first was analogous to that employed by Sandler (2000) for demonstrating rep-priA lethality and involved an attempt to introduce dnaC1331(A84T) in a nonselective fashion into the Δrep strain. A P1 lysate prepared on a strain (GJ3127) carrying the dnaC1331 mutation along with the closely linked mdoB::Tn10 marker was used to transduce a pair of isogenic rep\(^+\) and Δrep::Cm strains (MC4100 and GJ5203, respectively) to Tet\(^r\). The recipient strains also carried plasmid pUC19 that rendered them Ts for growth (see above). As expected, the vast majority (39/40) of the Tet\(^r\) transductants in the cross into the rep\(^+\) strain had become temperature resistant, indicating coinheritance of dnaC1331(A84T), whereas none of 40 transductants in the
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**Figure 6.** Viability of double mutants of dnaC1331(A84T) with recA::Kan, recB::Tn10, or priA::Kan. Growth was scored on LB agar plates of the following pairs of strains (in the order, dnaC and dnaC1331): with no additional mutation (Nil), GJ3128 and GJ3127; recA, GJ5223 and GJ5222; recB, GJ5225 and GJ5224; and priA, GJ5221 and GJ5220.

The synthetic lethality of (A84T) mutant:

The synthetic lethality of (A84T) mutant can be explained by the needs for replication restart and to test this possibility we introduced a _dam_ mutation into the dnaC1331 strain. Mutants deficient in the Dam methylase suffer an increased frequency of double-strand breaks (DSBs) by a process that is dependent on a functional mismatch repair system encoded by the _mutHLS_ genes (Radman and Wagner 1986; Wang and Smith 1986; Kuzminov 1999; Marinus 2000). In general, the need for replication restart is also increased during growth of cultures in rich medium (when the time interval between successive replication forks is considerably reduced) when compared to that in minimal medium, and _priA_ mutants that are defective in replication restart or _priB_ gyrB strains exhibit rich-medium sensitivity (Kogoma 1997; Gregg et al. 2002; Grompone et al. 2003).

Transductants recovered from the cross in which a _dam::Tn9_ mutation was introduced into the dnaC1331 (A84T) strain were very sick on LB but not on glucose-minimal agar plates (see Figure 7) and gave rise to spontaneous fast-growing suppressors. Five of eight such suppressors that were tested had acquired a mutator phenotype, suggestive of the loss of mismatch repair functions in them. _dam::Tn9_ transductants that were recovered in a dnaC1331(A84T) _mutH_ strain GJ5212 also did not exhibit the rich-medium-sensitive phenotype (Figure 8).

These data provide strong support for the notion that the _dnaC1331_ mutant is rendered growth sensitive by the increased frequency of replication-directed DSBs that are generated in the absence of Dam methylase. On the other hand, the _dnaC1331_ (A84T) single mutant was no more sensitive to UV irradiation than was its isogenic _dnaC_ parent (Figure 9), suggesting that the mutant protein is able to function as effectively as the wild type to satisfy the increased need for replication restart on UV-damaged DNA templates.

**DISCUSSION**

The _dnaC1331_ (A84T) mutation was identified in this study by its ability to suppress the lethality associated with runaway replication of Rom^-ColE1-like plasmids in transcription termination-defective _nusG_ and _rho_ mutants and was shown to do so by a primary effect on plasmid copy number even in otherwise wild-type strains. The amino acid alteration in the mutant protein has occurred in a short stretch of identified sequence similarity with the AP protein (which is functionally analogous to DnaC in phage λ replication; Nakayama et al. 1987). The _dnaC1331_ (A84T) allele was also synthetically lethal with the _rep_ or _priC_ null mutations. These results are interpreted below in terms of a model in which the former may be compromised for one or more pathways of replication restart on the chromosome. We reasoned that the mutation may also consequently be unable to tolerate conditions that increase the need for replication restart, and to test this possibility we introduced a _dam_ mutation into the _dnaC1331_ strain. Mutants deficient in the Dam methylase suffer an increased frequency of double-strand breaks (DSBs) by a process that is dependent on a functional mismatch repair system encoded by the _mutHLS_ genes (Radman and Wagner 1986; Wang and Smith 1986; Kuzminov 1999; Marinus 2000). In general, the need for replication restart is also increased during growth of cultures in rich medium (when the time interval between successive replication forks is considerably reduced) when compared to that in minimal medium, and _priA_ mutants that are defective in replication restart or _priB_ gyrB strains exhibit rich-medium sensitivity (Kogoma 1997; Gregg et al. 2002; Grompone et al. 2003).

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The _dnaC1331_ (A84T) mutation was identified in this study by its ability to suppress the lethality associated with runaway replication of Rom^-ColE1-like plasmids in transcription termination-defective _nusG_ and _rho_ mutants and was shown to do so by a primary effect on plasmid copy number even in otherwise wild-type strains. The amino acid alteration in the mutant protein has occurred in a short stretch of identified sequence similarity with the AP protein (which is functionally analogous to DnaC in phage λ replication; Nakayama et al. 1987). The _dnaC1331_ (A84T) allele was also synthetically lethal with the _rep_ or _priC_ null mutations. These results are interpreted below in terms of a model in which the
**dnaC** mutant is specifically defective in the PriA-PriB pathway of replication restart.

**DnaC and assembly of replication forks by different pathways:** As mentioned above, DnaC delivers the DnaB helicase for replication fork assembly during the processes of (i) initiation of chromosome replication at oriC, (ii) replication restart following the collapse of previously assembled forks, and (iii) plasmid replication. The dnaC1331(A84T) mutant exhibits chronic SOS induction, which suggests some perturbation in chromosomal DNA replication in the strain. The fact that the mutant has a normal growth rate in both rich and minimal media argues that it is not affected for replication fork assembly at the ABC primosome (that is, DnaA-bound oriC). That, on the other hand, it may be affected for replication restart (that is, at the φX-type primosome at D-loops) is suggested by the findings that (i) dnaC1331(A84T) confers rich-medium sensitivity in combination with dam (that is, when the requirement for replication restart functions is expected to be increased because of frequent DSBs) and (ii) this inviability is suppressed by mutation in the gene mutH for mismatch repair.

Sandler and co-workers have obtained genetic data to suggest that three alternative pathways exist for replication restart at D-loops in wild-type cells, which have been designated (on the basis of respective gene product requirements) as the PriA-PriB, PriA-PriC, and Rep-PriC pathways (Sandler et al. 1999, 2001; Sandler 2000). Of these, it is only for the PriA-PriB pathway that supporting biochemical evidence is available at present (Xu and Marians 2003).

Many phenotypes associated with the dnaC1331(A84T) mutation (for example, that it is synthetically lethal with priC but not with either priA or priB) may be explained by this multiple-pathway model of replication restart by making the additional assumptions that (i) the ability to restart replication at D-loops is important for viability and (ii) in the dnaC1331 strain, the PriA-PriB pathway of replication restart at D-loops is rendered largely, if not completely, nonfunctional; consequently, the mutant is dependent on the functionality of the PriA-PriCand Rep-PriC pathways for its survival. The fact that the dnaC1331 mutant is sensitive to Dam methylase deficiency (Figure 8), but not to UV irradiation (Figure 9), would then suggest that there is a much greater need for the PriA-PriB pathway of replication restart on DNA templates with DSBs than on those with single-strand lesions. McGlynn and Lloyd (2000) have also provided evidence that replication-generated DSBs are infrequent in UV-irradiated E. coli.

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**Figure 7**—Synthetic lethality of dnaC1331(A84T) with Δrep::Cm or priC::Kan. Lanes in the three panels represent individual Tet<sup>+</sup> transductants obtained from crosses into the recipient strains wild type (MC4100), Δrep::Cm (G5203), or priC303::Kan (G5204), as indicated, with a P1 lysate grown on G3127 (dnaC1331 linked 95% to mdoB::Tn10). Chromosomal DNA from each transductant was subjected to PCR of and Bsm<sub>I</sub> polymorphism testing at the dnaC locus, as described in the text and in the legend to Figure 1. R, chromosomal DNA from the corresponding recipient strain. Marked beneath each lane is the dnaC genotype (+, dnaC<sup>+</sup>; −, dnaC1331) of the strain, as inferred from the Bsm<sub>I</sub> polymorphism analysis.

**Figure 8**—Rich-medium sensitivity in dnaC1331(A84T) dam mutant and its suppression by mutH. Strains G3127 (dnaC), G5211 (dam dnaC), and G5213 (dam dnaC mutH) were scored for growth on glucose-minimal A (Min A) and LB agar plates.

**Figure 9**—UV tolerance of dnaC<sup>+</sup> (G3128, △) and dnaC1331 (G3127, ○) strains. The percentage fraction of surviving cells, following exposure to UV for the indicated time periods, was determined. Two independent experiments yielded similar results, and the data from one of them are plotted. The initial culture densities for the two strains were, respectively, 1.2 × 10<sup>8</sup> and 9.2 × 10<sup>7</sup>/ml.
Consistent with the model that the PriA-PriB pathway is rendered nonfunctional in the dnaC1331(A84T) mutant is the finding that the dnaC1331 strain shares several phenotypic features in common with the null priB mutant (Sandler et al. 1999; Sandler 2000; Grompone et al. 2003; this study): minimal or no growth phenotype as single mutants, no change in UV tolerance, reduced copy number of ColE1-like plasmids, viability in combination with priA, and severe sickness or lethality in combination with rep or priC. On the other hand, the dnaC1331 mutant, but not the priB mutant, is SOS induced, which suggests that the former might harbor some reversible trapped intermediates of the PriA-PriB pathway that activate the cotranscribe activity of RecA but do not compromise growth.

One finding of ours that is not readily accounted for by the three-pathway replication restart model of Sandler and co-workers is that of rep-dnaC1331 synthetic lethality (because the PriA-PriC pathway would still be expected to be functional in the double mutants). One possibility is that the PriA-PriC pathway requires the Rep helicase (as has also been speculated earlier; Sandler and Marians 2000); in such a case, however, rep would be expected to exhibit synthetic lethality with priB, but the double mutants have been reported to be viable (Sandler 2000). The alternative possibility is that PriA-PriC may not represent a major pathway of replication restart, as is further discussed below.

Two lines of genetic evidence were earlier interpreted as support for a PriA-PriC pathway of replication restart: (i) priB or priC single mutants are reasonably healthy whereas a priBC double mutant is even more sick than priA (Sandler et al. 1999) and (ii) the priA300 mutation, which specifies an altered PriA protein that is competent for ΦX-type primosome assembly but is defective for the 3′−5′ DNA helicase activity, behaves like the priA knockout when combined with a priB but not a priC null mutation; it was proposed that the mutant protein is proficient for replication restart via the PriA-PriB but not via the PriA-PriC pathway (Sandler et al. 2001). However, both these findings may also be explained by an alternative model in which the helicase activity of PriA participates in an additional, PriB- and PriC-independent pathway of replication restart (see Figure 10). Indeed, a pathway of direct resetting of regressed replication forks (that can occur following blockage of normal fork progression; Seigneur et al. 1998; McGlynn and Lloyd 2000) that is mediated by the combined actions of the RecG (or RecBCD) and PriA proteins (including the latter’s 3′−5′ helicase activity) has been proposed by Lloyd and co-workers (McGlynn and Lloyd 2000; Gregg et al. 2002; Jakta and Lloyd 2003). Furthermore, Flores et al. (2002) have shown that in a strain with increased frequency of replication fork regression because of a DNA polymerase III ψ subunit mutation, replication restart is compromised by the priA300 mutation but not by a priC null mutation [suggesting that the pathway rendered defective in the priA300 strain is one that does not require PriC, which is contrary to the proposal made by Sandler et al. (2001)].

On the basis of these arguments, therefore, we suggest that replication restart events occur primarily by the PriA-PriB and Rep-PriC pathways at D-loops, as well as by the direct resetting of regressed replication forks (Figure 10) and that, in the dnaC1331(A84T) mutant, the PriA-PriB pathway is rendered nonfunctional.

dnaC1331(A84T) and replication of ColE1-like plasmids: The dnaC1331(A84T) mutation was associated with a reduction in content of the Rom+ ColE1-like plasmids pACYC184, pBR329, or pUC19 (and consequently with suppression of the Ts phenotype of pUC19 transformants), but not with that of the Rom− plasmid pBR322. One way to explain these results is to assume that DnaB loading by the mutant DnaC protein at the D-loop formed in the plasmid ori region occurs with reduced efficiency and that it consequently becomes the rate-limiting step for replication of Rom+ plasmids, but that for Rom− plasmids, it is the number of replication primers that remains rate limiting for replication [since the Rom protein reduces the proportion of RNA-II molecules that are processed to generate them (Cesareni et al. 1991; Del Solar et al. 1998)]. Consistent with this explanation, we have found that the dnaC1331 mutation also does not affect the copy number of Rom+ plasmids in a pcnB strain [in which the half-life of the inhibitor RNA-I is increased (Xu et al. 1993), so that once again fewer replication primers are generated from RNA-II (data not shown)]. Our hypothesis is very similar to that proposed by Berges et al. (1997), who showed that a missense priB mutant harbors reduced copy numbers of Rom− but not of Rom+ ColE1-like plasmids.

The effects on plasmid replication of dnaC1331(A84T), priB null, or priC null mutations, as inferred from the data obtained in this study (Figure 4), are also in accord with a model in which replication fork assembly on the plasmid template is served primarily by the components of the PriA-PriB pathway of replication restart.
Need for and relative efficiency of the different replication restart pathways: When one considers the multiple replication restart pathways (Figure 10), two major questions are: What fraction of replicating chromosomes in wild-type cells (in the absence of exogenous DNA-damaging agents) suffers fork collapse necessitating replication restart? And what is the relative efficiency of each of the pathways in the process? The estimated values, in answer to the first question, have varied from 18 to 50 to 100% by different approaches, as discussed below.

i. The lower-end value was obtained in experiments involving growth of dnaC(Ts) mutants at the restrictive temperature (Maisnier-Patin et al. 2001). However, our own observations with dnaC1331(A84T) suggest that dnaC mutants may be differentially affected for DNAB loading at different fork-precursor structures, and it is therefore possible that, in the work of Maisnier-Patin et al. (2001), all pathways of replication restart were not inactivated at the restrictive temperature. The dnaC(Ts) mutants employed in their study were “slow stop” for DNA synthesis (Wechsler 1975), suggestive of a block mainly for fork assembly at oriC.

ii. The midrange value was the estimate obtained from the proportion of viable cells in cultures of recA strains defective in recombinational repair (reviewed in Kuzminov 1999; Cox et al., 2000), but once again there is evidence for the existence of RecA-independent pathways of replication restart (McGlynn and Lloyd 2001, 2002; Miranda and Kuzminov 2003).

iii. Finally, the argument that the capacity for replication restart is essential in every cell was based on both the extreme sickness or inviability of priA, priBC, or priAC mutants and the fact that these phenotypes could be suppressed by dnaC mutations (Sandler et al. 1999, 2001; Sandler 2000). As mentioned above, our finding in this study that the combination of dnaC1331(A84T) with priC is inviable also supports the notion that replication restart is required in every cell.

A related question in this context has been on the role of the Rep helicase in E. coli chromosome replication. Simultaneous deficiency of Rep and PriA is associated with inviability, but two alternative explanations have been offered for it. According to one model, Rep deficiency leads to increased frequency of replication fork collapse so that PriA becomes essential for survival (Seigneur et al. 1998). According to the second model, Rep and PriA participate in redundant pathways for replication restart (Sandler 2000). According to the second model, therefore, cell viability is again absolutely dependent on the capacity to restart replication, and our finding that rep dnaC1331(A84T) double mutants are inviable is in accord with it. Maisnier-Patin et al. (2001) showed also that 100% of rep dnaC(Ts) cells failed to complete replication initiated at oriC following a temperature upshift, but whether this was because of increased fork collapse or decreased restart associated with the rep mutation is not clear.

With regard to the relative efficiency of different pathways for restart, the extreme sickness of priA mutants has again been taken to mean that the PriA-independent pathways are at best minor contributors to replication restart. On the other hand, our finding in this study that the dnaC1331(A84T) single mutant is quite normal for growth but is inviable in combination with priC [which, unlike priA and priB, has so far been implicated only in “minor” pathway(s) of replication restart (Sandler 2000; Sandler et al. 2001; Flores et al. 2002; Grompone et al. 2003)] strongly suggests that the Rep-PriC pathway may indeed potentially be as efficient as the “major” PriA-PriB pathway.

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