A *dnaT* Mutant With Phenotypes Similar to Those of a *priA2::kan* Mutant in *Escherichia coli* K-12

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ABSTRACT

The ability to repair damaged replication forks and restart them is important for cell survival. DnaT is essential for replication restart *in vitro* and yet no definite genetic analysis has been done in *Escherichia coli* K-12. To begin, *dnaT822*, an in-frame six-codon (87–92) deletion was constructed. *DnaT822* mutants show colony size, cell morphology, inability to properly partition nucleoids, UV sensitivity, and basal SOS expression similar to *priA2::kan* mutants. *DnaT822 priA2::kan* double mutants had phenotypes similar to those of the single mutants. *DnaT822* and *dnaT822 priA2::kan* mutant phenotypes were fully suppressed by *dnaC809*. Previously, a dominant temperature-sensitive lethal mutation, *dnaT1*, had been isolated in *E. coli* 15T[–]. *DnaT1* was found to have a base-pair change relative to the *E. coli* 15T[–] and *E. coli* K-12 *dnaT* genes that led to a single amino acid change: R152C. A plasmid-encoded *E. coli* K-12 mutant *dnaT* gene with the R152C amino acid substitution did not display a dominant temperature-sensitive lethal phenotype in a *dnaT*⁺ strain of *E. coli* K-12. Instead, this mutant *dnaT* gene was found to complement the *E. coli* K-12 *dnaT822* mutant phenotypes. The significance of these results is discussed in terms of models for replication restart.

THE ability to reload the replication machinery at repaired replication forks, away from the site of regulated cell-cycle-dependent initiation of DNA replication, has become an important theme in the way prokaryotic and eukaryotic cells replicate their genomes (VON HIPPEL 2000; COX 2001; SHERRATT 2003). At the core of this process is the ability to recognize a broken replication fork, repair it, and then reload or restart the replication machinery. Since replication fork collapse can occur at any place on the chromosome, replication restart must be sequence independent and occur in such a way as to reload the replication machinery in the same direction in which it was replicating before the collapse.

Much of what is understood about this process at the biochemical level has come from the study of the *in vitro* loading of the DnaB replicative helicase by the DnaC protein on the single-stranded Φ X174 chromosome (reviewed in KORNBERG and BAKER 1992; MARIANS 1992, 1996). For this to occur, four proteins, originally called n, n', n'', and i, and now known to be the PriB, PriA, PriC, and DnaT proteins, respectively, need to be sequentially assembled onto the primosome assem-

bly site (PAS) of the single-stranded Φ X174 chromosome. These proteins target the DnaBC complex to the repaired replication fork and aid DnaC to load DnaB onto the chromosome. This model system has been expanded to show that the same proteins can load DnaB at recombinational intermediates and replication fork-like structures (LIU and MARIANS 1999; LIU *et al.* 1999; MARIANS 2000).

An early inference of the Φ X174 primosome assembly pathway was that PriA, PriB, PriC, and DnaT would be essential for DNA replication *in vivo*. This was first tested genetically by the construction of mutations in *priA* (LEE and KORNBERG 1991; NURSE *et al.* 1991). Such mutants were viable but extremely sick. *PriA* mutant phenotypes include sensitivity to UV irradiation and rich media; deficiency in recombination, stable DNA replication, and chromosome partitioning; and high basal levels of SOS expression (LEE and KORNBERG 1991; NURSE *et al.* 1991; MASAI *et al.* 1994; KOGOMA *et al.* 1996; SANDLER *et al.* 1996; MCCOOL and SANDLER 2001). In that these phenotypes seemed reminiscent of a recombination-deficient mutant, understanding these phenotypes has led to a better understanding of how DNA replication and recombination are linked in the cell (COX *et al.* 2000; KOWALCZYKOWSKI 2000). The Φ X174 system also incorrectly predicted that mutations in *priB* and *priC* by themselves would have no phenotypic consequence for an otherwise wild-type cell and that mutations in both of these genes would be synthetically lethal (SANDLER *et al.* 1999). These observations and others based on the synthetic lethality between *priA* and *priC* and the ability

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FIGURE 1.—A representation of the multiple pathways model for replication restart. The substrate for these reactions is shown on the left and is thought to be a repaired replication fork or recombination intermediate. The product of the reactions is a replication fork shown on the right. The proposed order of gene products acting in replication restart in the various pathways is shown from left to

right. Thick lines indicate what are thought to be the main pathways. Dashed line is representative of a pathway that can function in the absence of PriA and DnaT and requires DnaC809. Possible roles of the proteins are indicated.

of different *dnaC* mutations to indirectly suppress the absence of either just *priA* (*dnaC809*) or *priA* and *priC* (*dnaC809,820*) were fitted to a multiple pathway model for replication restart (SANDLER 2000; Figure 1). This model describes replication restart at repaired replication forks on the chromosome *vs.* loading of the replication machinery on a single-stranded DNA phage (SANDLER 2000, 2001; SANDLER and MARIANS 2000).

The *dnaT* gene codes for a 20-kD protein. By itself, this protein has no known intrinsic activity. It is, however, essential for the sequential loading of PriA, PriB, and PriC onto several DNA substrates so that DnaC can mediate the loading of the DnaB helicase (reviewed in KORNBERG and BAKER 1992; MARIANS 1992, 1996). In this reaction, the PriA protein first binds the DNA substrate. The PriB protein then facilitates the loading of the DnaT protein (LIU *et al.* 1996). Increasing the amount of DnaT in a reaction can circumvent the need for PriB in that reaction and rescue the defects of certain mutant PriA proteins (LIU *et al.* 1996). Once associated with the PriA-DNA complex, the DnaT protein is stably maintained in the complex through the remainder of the reaction (NG and MARIANS 1996b). The protein is thought to act in this reaction as a trimer (NG and MARIANS 1996b). While PriA, PriB, and DnaT are essential for this reaction, PriC plays a poorly understood, nonessential role. Elimination of PriC from this reaction decreases the loading of DnaB and thus DNA replication by a third (NG and MARIANS 1996a; XU and MARIANS 2003).

Given the differences between the predictions based on the Φ X174 *in vitro* DNA replication system and the observed phenotypes associated with *priA*, *priB*, and *priC* mutations, it was of importance to make a mutant of the *dnaT* gene and test its role in replication restart *in vivo*. The *dnaT* gene is located at 99 min on the *Escherichia coli* K-12 chromosome and resides in an operon with *dnaC* and two uncharacterized genes, *yjjA* and *yjjB* (Figure 2). While there are no published reports of *E. coli* K-12 *dnaT* null mutants, *dnaT1*, a temperature-sensitive dominant mutation has been isolated in *E. coli* 15T⁻ by LARK *et al.* (1978). The early interpretation of the function of the *dnaT* gene based on the study of

this mutant was that DnaT was involved in termination of DNA replication (LARK *et al.* 1978).

To begin an analysis of the *dnaT* gene in *E. coli* K-12, an 18-bp, six-codon (87–92) in-frame deletion of *dnaT* was created. This article describes the construction and initial phenotypic characterization of this mutant, *dnaT822*. It was found for each phenotype tested that the *dnaT822* mutant behavior was similar to that of a *priA2::kan* mutant. These similarities included poor growth and viability, sensitivity to UV irradiation, high basal levels of SOS expression, and two populations of cells when viewed microscopically: one wild type and one filamentous with poorly partitioning nucleoids. In some cases, the *dnaT822* mutant phenotype was slightly more severe than the *priA2::kan* mutant phenotype. A *priA2::kan dnaT822* double mutant had the same phenotype of either single mutant. The *dnaT822* single- and *priA2::kan dnaT822* double-mutant phenotypes were fully suppressed by the *priA2::kan* extragenic suppressor, *dnaC809*. Lastly, the *dnaT* sequence of the *E. coli* 15T⁻ strain carrying the *dnaT1* mutation was determined. This *dnaT* gene showed one difference from the predicted amino acid sequence of the wild-type *dnaT* from *E. coli* 15T⁻ or K-12: a change of an arginine residue for a cysteine residue at codon 152 (R152C). The construction of this mutant *dnaT* gene onto a plasmid and its introduction into *E. coli* K-12 failed to create a temperature-sensitive phenotype.

MATERIALS AND METHODS

Bacterial strains and plasmids: All bacterial strains used in this work are derivatives of *E. coli* K-12 or *E. coli* 15T⁻ (Table 1). Plasmids used in this work are shown in Table 2. Strains for all experiments were grown in either 56/2 minimal medium (WILLETTS *et al.* 1969) or Luria-Bertani medium. Minimal medium for UT205 and UT3062 was supplemented with 0.4 μ g/ml thymine. Antibiotics were added when appropriate to the following final concentrations: kanamycin (25 μ g/ml), tetracycline (10 μ g/ml), ampicillin (25 μ g/ml), and chloramphenicol (25 μ g/ml).

Construction of *dnaT*⁺ plasmids: pJDM14 was used during the initial cloning and mutagenesis of *dnaT*. PCR primers prSJS197 (5'-AATGGTCGCGCTGGTATCGG) and prSJS198 (5'-GCATTCATCTGCCCTTCACAC) were used to amplify the

TABLE 1
Strains used in this work

Strain	<i>priA</i>	<i>dnaT</i>	<i>dnaC</i>	Other relevant genotypes	Source or reference
DM4000 ^a	+	+	+		M. Volkert
JC18983	2	+	+		SANDLER <i>et al.</i> (1996)
JC19008	2	+	809		SANDLER <i>et al.</i> (1996)
JC19257	+	+	809, 820		SANDLER <i>et al.</i> (1996)
JC13509 ^b	+	+	+		Lab stock
CAG184250	+	+	+	<i>zji-202::Tn10</i>	SINGER <i>et al.</i> (1989)
CAG18442	+	+	+	<i>thr-34::Tn10</i>	SINGER <i>et al.</i> (1989)
M7	+	+	+		Lab stock
PN105	2	+	+		NURSE <i>et al.</i> (1991)
SS156	+	+	+	<i>thr-34::Tn10</i>	SANDLER <i>et al.</i> (1999)
SS170	2	+	+	<i>thr-34::Tn10</i>	JC19008 → SS156 ^d
SS171	2	+	809		JC19008 → SS170 ^e
SS219	+	+	+	<i>thr-34::Tn10</i>	SANDLER <i>et al.</i> (1999)
SS257	+	+	+	<i>thr-34::Tn10</i>	CAG18442 → M7 ^f
SS292	+	+	809		JC19008 → SS257 ^e
SS291	+	+	809, 820		JC19257 → SS257 ^e
SS302	+	+	809, 820		pJDM21 → SS291
SS306	+	822	809, 820		This work
SS447	2	821	825	<i>argE</i> ⁺	Lab stock
SS453	2	822	809, 820		PN105 → SS306 ^d
SS458	2	822	809, 820	<i>zji-202::Tn10</i>	CAG18430 → SS453 ^f
SS463	+	822	+	<i>zji-202::Tn10</i>	SS458 → DM4000 ^f
SS464	+	822	+	<i>zji-202::Tn10</i>	SS463 → DM4000 ^f
SS466	2	822	809	<i>zji-202::Tn10</i>	SS463 → JC19008 ^f
SS467	2	822	+	<i>zji-202::Tn10</i>	SS463 → JC19008 ^f
SS475	+	822	+	<i>zji-202::Tn10</i>	SS463 → JC13509 ^f
SS476	+	822	809	<i>zji-202::Tn10</i>	SS466 → DM4000 ^f
SS1091	+	+	809		JC19008 → SS1213 ^b
SS1213	+	+	+	<i>thr-34::Tn10</i>	CAG18442 → JC13509 ^c
SS1462	+	822	809	<i>zji-202::Tn10</i>	SS466 → JC13509 ^c
SS1460	+	+	+	<i>thr-34::Tn10</i>	CAG18442 → JC13509 ^c
SS1468	+	+	+		pACYC184 → JC13059
SS1478	2	822	809		SS447 → SS1462 ^e
SS1489	+	822	+		pJDM57 → SS475
SS1495	+	+	+		pJDM57 → JC13059
UT205 ^c	+	1	+	<i>thy</i>	LARK <i>et al.</i> (1978)
UT3062 ^c	+	+	+	<i>thy</i>	LARK <i>et al.</i> (1978)

^a DM4000 has the following partial genotype: $\Delta(lac-pro)XIII$ *hisG4 argE3 ara-14 xyl-5 mtl-1 rpsL31 sulA::Mu-d* (Ap, *lac*, B::Tn9). This strain is identical to AC402 in CHAUDHURY and SMITH (1985).

^b JC13509 has the following partial genotype: *F*⁻ *lacMS286* $\phi 80dIII$ *lacBK1* *sullB103 argE4 his-4 thi-1 xyl-5 mtl-1* Sm^R T6^R. JC13509 is derived from SK362 (ZIEG and KUSHNER 1977).

^c UT205 and UT3062 are *E. coli* 15 T⁻ strains (LARK *et al.* 1978).

^d Select for Kan^R.

^e Select for Thr⁺.

^f Select for Tet^R.

dnaT coding region and native *dnaT* promoter (Figure 2). The 1.1-kb PCR product was inserted into the *Sma*I site of pUC18, resulting in pJDM4. The DNA sequence of this 1.1-kb PCR fragment was verified by DNA sequencing. A *Sph*I fragment containing a gene encoding spectinomycin resistance (Spec^R) from plasmid pSJS985 was inserted at the *Hpa*I site of pJDM4, resulting in pJDM14. pJDM59 is a pACYC184 derivative and was used for complementation studies. PCR primers prSJS524 (5'-GGTGCCTTATCTAGAGGTCTTTCCATTCC) and prSJS523 (5'-GGAAGACATACTGTTTCTCATATATGG AATCATGAACGAGAAGGG) were used to amplify the *dnaT*

gene with its native promoter (*dnaTp*). The PCR product was inserted into pCR2.1 (Invitrogen, Carlsbad, CA) by TOPO TA cloning methods (Invitrogen; pJDM58). A *Bam*HI-*Xho*I fragment from this plasmid was mixed with a *Bam*HI-*Sa*I fragment of pACYC184 and treated with T4 DNA ligase. The resulting plasmid was called pJDM59.

Construction of plasmids containing *dnaT822* and placement of *dnaT822* on the chromosome: pJDM14 was restricted with *Nco*I and *Bsp*EI and treated with Klenow and T4 DNA ligase to produce pJDM16. This protocol deleted 18 nucleotides in the middle of the *dnaT* coding region corresponding

TABLE 2
Plasmids used in this work

Plasmid	Parent	Relevant gene(s)	Source
pACYC184			Lab stock
pSJS985		<i>aadA</i>	SANDLER and CLARK (1994)
pUC18			Lab stock
pBR322			Lab stock
pCR2.1			Invitrogen
pKO3			LINK <i>et al.</i> (1997)
pJDM4	pUC18	<i>dnaT</i> ⁺	
pJDM14	pJDM4	<i>dnaT</i> ⁺ <i>aadA</i>	
pJDM16	pJDM14	<i>dnaT822 aadA</i>	
pJDM20	pBR322	<i>dnaT822 aadA</i>	
pJDM21	pKO3	<i>dnaT822 aadA</i>	
pJDM54	pCR2.1	<i>dnaT</i> R(152)C	
pJDM57	pACYC184	<i>dnaT</i> R(152)C	
pJDM59	pACYC184	<i>dnaT</i> ⁺	

to amino acids 87–92 (GKFAMY). pJDM16 was restricted with *EcoRI-BamHI* and treated with DNA polymerase I Klenow fragment in the presence of dNTPs to blunt the ends. The 2.4-kb *EcoRI-BamHI* fragment from pJDM16 was then combined with pBR322 restricted with *SphI* and treated with DNA polymerase I Klenow fragment as above. The two fragments were then treated with T4 DNA ligase to produce pJDM20. pJDM20 was restricted with *BamHI* and *SalI* and the fragment that contained the *dnaT* region was combined with similarly restricted pKO3. The fragments were then treated with T4 DNA ligase to produce pJDM21. This plasmid was used to transfer *dnaT822* to the chromosome according to the method of LINK *et al.* (1997). Briefly, this involves the formation of a co-integrate created by a recombination event between the *dnaT* locus on the chromosome and the *dnaT* region that has been inserted on the plasmid by the selection of chloramphenicol resistance at 42° (pKO3 encodes the *cat* gene and a temperature-sensitive replication gene). Resolution of the co-integrate occurs by recombination. This time, however, the recombination event is on the other side of the *dnaT822* mutation. This is done by growing the cells containing the co-integrate in the absence of chloramphenicol at 30° and selecting individual colonies on solid Luria media containing 5% sucrose (pKO3 also encodes the counterselectable gene, *sacB*). The presence of *dnaT822* on the chromosome of SS306 was confirmed by PCR and restriction analysis of the PCR product. The 18-bp deletion creates a *BglI* site at the site of the deletion that is diagnostic

of *dnaT822*. The sequence of *dnaT822* in this strain was confirmed by DNA sequence analysis.

Construction of plasmids containing the *E. coli* K-12 *dnaT* gene encoding an amino acid change R152C: The gene sequence directing the expression of a mutant *dnaT* protein with the R152C amino acid change was cloned into pACYC184 using PCR. The crossover PCR strategy (COPPI *et al.* 2001) required first the amplification of two fragments of the *dnaTC* region such that each fragment had an overlapping region of 20 bp that included the introduction of the coding sequence for the R152C amino acid change. PCR primers prSJS524 (5'-GGTGGCGTTATCTAGAGGTCTTTCCATTCC) and prSJS526 (5'-CCGCCGTTGCTGGCACAACCGATTTCG) were used to amplify the first fragment. This was a portion of the *dnaT* region spanning just upstream of the *dnaT* promoter to ~460 bp within the *dnaT* gene. The second fragment was amplified using primers prSJS525 (5'-CCGCCGTTGCTGGCACAACCGATTTCG) and prSJS523 (5'-GGAAGACATACTGTTTCTCATATATGGAATCATGAACGAGAAGGG). This fragment spanned from approximately the 440 bp position to the middle of the *dnaC* gene. The two fragments were then mixed and 10 rounds of thermocycling were performed in the absence of primers, followed by 30 rounds in the presence of prSJS524 and prSJS523. The full-length PCR product was inserted into pCR2.1 using the TOPO TA cloning protocol (Invitrogen). This sequence was verified by DNA sequencing. A *BamHI-XhoI* fragment from this vector was combined with *BamHI-SalI*

FIGURE 2.—Map of the *yjiB-dnaT-dnaC-yjiA* region. The promoters and terminators as determined by MASAI and ARAI (1988) are shown. The positions of *dnaT822* and *dnaT1* as determined in this report are shown in their relative positions.

TABLE 3
Summary of phenotypes tested

Genotype			Phenotype		
<i>priA</i>	<i>dnaT</i>	<i>dnaC</i>	Colony size ^a	UV survival ^b	Levels of SOS ^c
+	+	+	+++	0.678 ± 0.160	110 ± 20
2	+	+	+	0.005 ± 0.003	840 ± 60
+	822	+	+	0.002 ± 0.001	910 ± 90
2	+	809	+++	0.443 ± 0.256	140 ± 20 ^d
+	822	809	+++	0.946 ± 0.269	80 ± 20
2	822	809	+++	0.659 ± 0.249	100 ± 20

^a Colony size of a strain grown on minimal medium at 37° for 3 days. +, small colony size; +++, large colony size.

^b Value shown is the average of three measurements of the surviving fraction of cells at 20 J compared with that at 0 J.

^c Miller units of β-galactosidase activity per OD₆₀₀. Strains tested harbor a *sulAp-lacZ* fusion [*Mu-d*(Ap, *lacB*:Tn9)]. The value is the average of three measurements.

^d Data taken from SANDLER *et al.* (1996).

digested pACYC184 and treated with T4 DNA ligase to form pJDM57.

Phenotypic analyses of mutant strains: The phenotypic analyses of cell viability, UV resistance, and SOS expression have been described elsewhere (SANDLER *et al.* 1999). Cells were prepared for microscopy as described previously (McCOOL and SANDLER 2001). Microscopy was carried out using a Nikon E600 microscope. An ORCA-ER cooled CCD camera (Hamamatsu) and Openlab software (Improvision) were used for all image acquisition and processing. The lengths of 500 individual cells were measured. Filaments are defined as a cell >6 μm in length (McCOOL and SANDLER 2001).

RESULTS

Construction of the *dnaT822* mutant: The *dnaT* gene is the second of four genes (*yjiB*, *dnaT*, *dnaC*, and *yjiA*) that share an overlapping pattern of transcription regulation (MASAI and ARAI 1988). Promoters and termination sites have been mapped. The first and last genes in this group, *yjiB* and *yjiA*, have not yet been genetically characterized and are of unknown function. The third gene, *dnaC*, is an essential gene in *E. coli*. DnaC loads the DnaB helicase at two different types of protein-DNA complexes: DnaA at *oriC* (BRAMHILL and KORNBERG 1988) and assemblages of combinations of PriA, PriB, PriC, Rep, and DnaT at repaired replication forks (see BRAMHILL and KORNBERG 1988; MASAI and ARAI 1988). To create a mutation of *dnaT* that was not polar on *dnaC* expression, an in-frame deletion of 18 bp was constructed. This procedure removed codons 87–92 (see MATERIALS AND METHODS). This deletion mutation, called *dnaT822*, was transferred to the chromosome of a *dnaC809,820* strain using the method of LINK *et al.* (1997). As mentioned above, *dnaC809,820* indirectly suppresses the absence of proteins in both the PriA-dependent and the PriA-independent pathways (SANDLER 2000). A *dnaC priA2::kan* suppressor was used be-

cause it was predicted that a *dnaT* null mutant would have a phenotype like that of a *priA2::kan* mutant and would be difficult to recover in a *dnaC*⁺ strain. *DnaC809,820* was used instead of *dnaC809* in the event that *dnaT* may have an unpredicted role in the PriA-independent pathway. The sequence of *dnaT822* on the chromosome was verified by DNA sequencing (data not shown). The *dnaT822* mutation was then separated from *dnaC809,820* by recombination after P1 transduction (see Table 1).

Phenotypes of *dnaT822*: The multiple pathways model (Figure 1) predicted that mutations in *dnaT* would have a phenotype similar to that of *priA2::kan* mutants. As mentioned above, *priA2::kan* mutants have pleiotropic phenotypes. Some of these include: small colony size, high basal levels of SOS expression, sensitivity to UV irradiation, and two types of cells when viewed microscopically (wild-type-like cells and filaments with poorly segregating nucleoids). To assess whether *dnaT822* phenotypes are like those of a *priA2::kan* mutant, a strain containing only *dnaT822* was subjected to a series of tests. The data (Table 3; Figure 3) show that a *dnaT822* mutant, like a *priA2::kan* mutant, has a small colony phenotype, about eight-fold higher basal levels of SOS expression, and is sensitive to UV irradiation. Finally, microscopic analysis revealed that cells from a culture of a *dnaT822* strain, again like a *priA2::kan* strain, could be separated into two classes, wild-type cells and filaments with poorly partitioning nucleoids, in almost the same proportions as had been reported for *priA2::kan* cultures. In the cases of colony size and UV sensitivity (Table 3), the phenotypes of *dnaT822* appeared slightly more severe (Figure 3). It is concluded that except for small quantitative differences, the phenotypes produced by *dnaT822* are very similar to those produced by *priA2::kan*.

FIGURE 3.—Typical colony, cell, and nucleoid morphologies of the following strains: (A and B) JC13509 (wild type), (C and D) JC19021 (*priA2::kan*), (E and F) SS475 (*dnaT822*), and (G and H) SS1499 (*dnaT822* on the chromosome and *dnaT*⁺ on plasmid pJDM59). Colonies were photographed following 3 days of incubation at 37° on minimal medium. Cells were stained with 4',6-diamidino-2-phenylindole and prepared for microscopy as outlined in MATERIALS AND METHODS. The percentages to the right of the figure indicate the portion of a typical population of cells consisting of filaments (see MATERIALS AND METHODS for details).

The *dnaC* gene is immediately downstream of the *dnaT* gene. As mentioned above, *dnaC* is essential for initiation of DNA replication at *oriC* and replication restart. Even though the *dnaT822* mutation was designed not to be polar on *dnaC*, it is still possible that the phenotypic effects elicited by this mutation could be produced by some unknown effect of *dnaT822* on the expression of *dnaC*. Additionally, the *dnaT822* deletion mutation removes only six codons from the middle of the *dnaT* gene. It is therefore likely that this mutant gene produces a polypeptide that is similar to the *dnaT*⁺ protein. DnaT performs its role in the cell as a member of a complex of proteins. It is possible that *dnaT822* produces its phenotypic effect either by inactivating the *dnaT* gene product so that it cannot interact with the other primosomal proteins during replication restart or by allowing a partially active DnaT protein to interact with the other replication restart proteins and thereby somehow poison the complex. If the former is true, then *dnaT822* would be recessive to *dnaT*⁺. If the latter is true, then *dnaT822* would be dominant to *dnaT*⁺. To

test both of these ideas, *dnaT*⁺ was cloned into pACYC-184 (see MATERIALS AND METHODS for construction of pJDM59) and used to transform a strain with *dnaT822* on the chromosome. Figure 3 shows that this strain has wild-type colony size and cell morphology. Therefore, *dnaT*⁺ on a plasmid is able to complement *dnaT822* for the mutant phenotypes tested.

We conclude that it is likely that the phenotypes seen in a *dnaT822* mutant are not due to any polar effect on *dnaC* and that *dnaT822* behaves as a mutation that is recessive to *dnaT*⁺. Other interpretations of this result are discussed below.

PriA and *dnaT* are in the same pathway: The phenotypic analyses of *dnaT822* and *priA2::kan* mutants show that these two mutants have similar phenotypes. Liu and Mariani have shown that PriA-PriB-DnaT and PriA-DnaT complexes can assemble on PAS *in vitro* and that either complex is competent for Φ X174 replication (LIU *et al.* 1996). Taken together, these findings suggest that *priA* and *dnaT* may be in the same pathway for replication restart *in vivo*. This hypothesis makes two predictions. First, a *priA2::kan dnaT822* double-mutant strain should have phenotypes like either of the single-mutant strains. Second, a *dnaC* mutation that suppresses *priA2::kan* phenotypes should also suppress *dnaT822* phenotypes.

To test the first prediction, a *priA2::kan dnaT822* double mutant (SS467) was constructed (Figure 4). The donor in the cross was *zji-202::Tn10 dnaT822* and the recipient was *priA2::kan dnaC809*. Tetracycline-resistant transductants were selected. It was predicted that, in most transductants, *dnaC809* in the recipient would be replaced by *dnaC*⁺ from the donor since *dnaT* and *dnaC* are adjacent genes and tightly linked with *zji-202::Tn10*. Transductants of two different sizes (large and small) were seen. PCR analysis revealed that, of the small colony isolates tested, all were *dnaT822 priA2::kan (dnaC*⁺*)* double mutants (crossovers at 1 and 4 in Figure 4). Analysis of some of the large colony isolates indicated that these were *dnaT822 priA2::kan dnaC809* (crossovers at 1, 2, 3, and 4 in Figure 4). One of the resulting *dnaT822 priA2::kan* double mutants (SS467) was inspected for colony size (Figure 4) and cell morphology (data not shown). These phenotypes were exactly like those of either single mutant (Figure 3), supporting the notion that *dnaT* and *priA* function in the same pathway of replication restart.

To test the second prediction of whether *dnaC809* could suppress the phenotypes of a *dnaT822* by itself, *dnaT822* and *dnaC809* (the donor was SS466) were introduced simultaneously into DM4000 and JC13509 (see Table 1). From the data summarized in Table 3 and Figure 5, it is clear for the phenotypes tested (colony size, UV survival, and basal levels of SOS expression) that *dnaC809* rescued the *dnaT822*-associated phenotype in each case. It was additionally tested whether or not *priA* was required for this suppression by analysis of

FIGURE 4.—Construction of a *dnaT822 priA2::kan* double mutant. (A–D) Colonies produced by DM4000 (wt), JC18983 (*priA2::kan*), SS464 (*dnaT822*), and SS467 (*priA2::kan dnaT822*), respectively, as they appeared following 60 hr of incubation on 56/2 minimal medium containing X-Gal (see MATERIALS AND METHODS). (E) A diagram of the P1 transductional crosses employed to construct a *dnaT822 priA2::kan* double mutant. The combination of crossovers 1 and 4 results in a *dnaT822 priA2::kan dnaC809* genotype (SS467). Isolates were screened by PCR amplification [primers prSJS197 (5'-AATGGTCGCGCTGGTATC TGG) and prSJS379 (5'-GATCACCCACAACT GTT)] and restriction digestion. The presence of an extra *Bgl*I site is diagnostic for *dnaT822* and the absence of a single *Hin*II is diagnostic for *dnaC809* (data not shown). The same method was used to construct a *dnaT822 priA2::kan dnaC809* by screening for recombinants that had crossovers at 1, 2, 3, and 4 (SS466).

the *dnaT822 priA2::kan dnaC809* triple mutant (SS466) constructed above. SS466 formed wild-type-sized colonies and had wild-type nucleoid organization and wild-type basal levels of SOS expression (Table 3 and data not shown). We conclude that *dnaC809* suppresses the effects of *dnaT822* and that this does not depend on the presence or absence of the *priA* gene product.

FIGURE 5.—Percentage survival of several strains after treatment with increasing dosage of UV irradiation. The isogenic strains are JC13509 (*dnaT⁺ dnaC⁺*, diamonds), SS475 (*dnaT822*, squares), and SS1462 (*dnaT822 dnaC809*, triangles).

A *dnaT* missense mutant (R152C) does not cause temperature sensitivity in *E. coli* K-12: LARK *et al.* (1978) isolated the first mutation in *dnaT* in a strain of *Escherichia coli* 15T⁻. *DnaT1* produced a temperature-sensitive dominant lethal phenotype. At the time, DnaT was hypothesized to have a function important for termination of DNA replication. Given that *dnaT822* and *priA2::kan* mutants are very similar phenotypically and

TABLE 4

Nucleotide sequence comparison for the *dnaT* regions of different strains of *E. coli*

Position	Nucleotides at indicated position		
	<i>E. coli</i> K-12	UT3062	UT205
300	T	C	C
333	G	A	A
414	G	A	A
454	C	C	T (R152C)
501	C	T	T

Full sequences for the *dnaT* regions are from either GenBank AE000507 or deposited files AY331182 and AY331181 for the *dnaT* regions from *E. coli* K-12, UT3062, and UT205, respectively. Position refers to nucleotide numbering from the "A" in the start codon. All changes are in third positions and do not change the amino acid sequence except the one at position 454 as indicated.

that DnaT is essential for replication restart *in vitro*, it can be hypothesized that *dnaT1* may somehow stop the process of replication restart at the nonpermissive temperature. To begin to test this idea, the *dnaT* genes from UT3062 (*dnaT*⁺) and UT205 (*dnaT1*) were determined to ascertain the mutation associated with *dnaT1*. Table 4 shows that there was one nucleotide difference between *dnaT*⁺ (from *E. coli* 15T⁻) and *dnaT1*. The C-to-T change at base number 454 caused an arginine residue at codon 152 to be replaced by a cysteine residue (R152C). The fact that this base-pair change was the only difference between the two genes suggests that the cause of the temperature-sensitive dominant phenotype of *dnaT1* mutant strains was this amino acid change. Comparison with the *dnaT*⁺ gene from *E. coli* K-12 reveals that the *dnaT1* mutation from *E. coli* 15T⁻ would also cause the same change in amino acid sequence. There were four other nucleotide positions within the *dnaT* coding region where the *dnaT*⁺ gene from *E. coli* 15T⁻ was different from the *E. coli* K-12 *dnaT*⁺ gene (Table 4). None of these differences, however, led to a corresponding change in amino acid sequence.

To test whether the R152C amino acid change would create a temperature-sensitive dominant lethal phenotype in *E. coli* K-12, this single change was combined with the *E. coli* K-12 *dnaT* gene and inserted into pACYC184 (see MATERIALS AND METHODS). The resultant plasmid, pJDM57, was used to transform *dnaT*⁺ and *dnaT822* strains. It was found that pJDM57 (*dnaT*R152C) complemented the defects of the *dnaT822* strain at 30° and 42° (data not shown). Additionally, it did not adversely affect the viability of an *E. coli* K-12 *dnaT*⁺ strain at 42° compared to 30° (data not shown).

We conclude that the only mutation in the *dnaT* gene of the *E. coli* 15 T⁻ strain carrying the temperature-sensitive dominant mutation, *dnaT1*, is a single base-pair change that causes an arginine residue to be replaced by a cysteine residue at codon 152. When this mutation was combined with an *E. coli* K-12 *dnaT* gene on a plasmid, it did not cause temperature-sensitive growth in a *dnaT*⁺ or *dnaT822* strain of *E. coli* K-12. This mutation appears to cause no adverse affects to the *dnaT* gene since it was able to complement the *dnaT822* mutant.

DISCUSSION

Several biochemical experiments have implicated the DnaT protein of *E. coli* K-12 in replication restart. Previously, the genetic requirement for *dnaT* in replication restart had not been tested. This article describes an 18-bp, six-codon in-frame deletion mutation in the *dnaT* gene that causes phenotypes similar to those caused by *priA2::kan* mutations. These include poor growth, UV sensitivity, high basal levels of SOS expression, and two populations of cells when viewed microscopically: one wild type and one with filaments that show poorly parti-

tioning nucleoids. In some cases, the phenotypes caused by *dnaT822* were found to be slightly more severe than the corresponding *priA* phenotypes. The parallels in phenotypes of *dnaT822* mutants, *vs.* slight quantitative differences, suggest an overwhelming similarity to *priA2::kan* mutants. These similarities are further demonstrated by the observation that *dnaC809*, an indirect suppressor of *priA2::kan*, also suppresses the absence of *dnaT* (and *priA* in the cell). The mechanistic repercussions of these observations will be discussed below. These data suggest not only that *dnaT* has an important role in replication restart, but also that its role is similar to that of *priA*.

DnaT822 is a deletion mutation removing six codons from the middle of the *dnaT* gene. The mutation was constructed so as to disturb the expression of the essential downstream gene, *dnaC*, as little as possible. The presence of *dnaT*⁺ on a multicopy plasmid in the *dnaT822* mutant completely restored the wild-type phenotype. The simplest interpretation of these results is that *dnaT822* is a recessive, complete loss-of-function mutation. More complicated interpretations of the data are also possible. Since *dnaT822* deletes only six codons, it is possible that *dnaT822* could be a partial activity mutant. If so, then it is possible that a mutant that removed all activity would result in cell death instead of the poor viability and the other phenotypes seen. As mentioned above, it is likely that DnaT acts in the cell as part of a complex with PriA and PriB, and it was considered that *dnaT822* may exert its phenotypic effect as a dominant mutant poisoning the activity of the complex. While this was tested by the complementation analysis, it is possible that the complementation seen is due to the multiple copies of the *dnaT*⁺ gene on a plasmid and would not be observed if *dnaT*⁺ and *dnaT822* were in equal copy in the cell. Further experiments can test the validity of these more complicated explanations.

Preliminary results available at the time of the writing of the article that introduced the multiple pathways model of replication restart (SANDLER 2000) suggested that DnaT acted in the PriA-PriB and PriA-PriC pathways and would not be required in the PriC-Rep-dependent, PriA-independent pathway. The data within showing that *dnaT822* alone and *dnaT822 priA2::kan* have the same phenotype and that both mutants are fully suppressed by *dnaC809* support this model with respect to *dnaT*'s contributions to the different pathways (see Figure 1). The model in Figure 1 suggests that DnaT functions after PriA and PriB (PriA-PriB pathway) or PriA and PriC (PriA-PriC pathway). This suggestion is made on the basis of several biochemical studies by Marians and colleagues of the loading of PriA, PriB, and DnaT into the primosome (see Introduction). While the genetic evidence presented here is consistent with this model, it is also consistent with a model in which DnaT and PriA form a complex and then interact with PriB or PriC. There is, however, only limited biochemical

evidence to support this model (LIU *et al.* 1996). Future experiments will distinguish between these models.

The isolation and characterization of *dnaT1* by Lark and colleagues has been an intriguing facet of the DNA replication field for some time. While the DnaT gene product was originally interpreted as having a role in termination of DNA replication (LARK *et al.* 1978), biochemical analysis has clearly implicated this protein in replication restart. It was therefore attractive to hypothesize that *dnaT1* may be a mutation that inhibits replication restart rather than causing termination of DNA replication at the nonpermissive temperature. To begin to test this idea, the sequence of the *dnaT1* gene from the original *E. coli* 15T⁻ was determined and found to have a single amino acid change (R152C) from the wild-type gene in either *E. coli* 15T⁻ or *E. coli* K-12. A *dnaT* gene on a plasmid containing this mutation was constructed to test whether this *dnaT* mutant would create a temperature-sensitive dominant lethal phenotype in *E. coli* K-12. It was found that this plasmid did not confer a temperature-sensitive dominant lethal phenotype and instead complemented a *dnaT822* mutant. While these data show that a plasmid-encoded *dnaT* gene with a mutation causing the R152C amino acid change is not sufficient to cause the temperature-sensitive dominant lethal phenotype in *E. coli* K-12 (as it did in *E. coli* 15T⁻), they do not definitively test whether the R152C mutation is the single cause of the temperature-sensitive dominant lethal phenotype found in the UT205 strain. A simple explanation for this series of observations is that the temperature-sensitive dominant lethal phenotype of this mutation is dependent on other aspects of the *E. coli* 15T⁻ physiology compared to the *E. coli* K-12 physiology. Several differences have been reported between these physiologies (ALIKHANIAN *et al.* 1966; ARBER and WAUTERS-WILLEMS 1970). Another explanation is that some other closely linked mutation is required, possibly in addition to the R152C change, to produce the temperature-sensitive phenotype. Other models are also possible.

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