Novel Alleles of the *Escherichia coli dnaA* Gene Are Defective in Replication of pSC101 but Not of *oriC*

MARK D. SUTTON AND JON M. KAGUNI*

Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824-1319

Received 23 January 1995/Accepted 6 September 1995

Five novel alleles of the *Escherichia coli dnaA* gene that were temperature sensitive in maintenance of pSC101, a plasmid that is dependent on this gene for replication, were isolated. Nucleotide sequence analysis revealed that four of the five alleles arose from single base substitutions, whereas the fifth contained three base substitutions, two of which were silent. Whereas all five alleles were temperature sensitive in vivo for pSC101 maintenance, genetic and biochemical characterization indicated that only two were defective in replication from the chromosomal origin, *oriC*. As previously characterized mutations are defective in replication for both pSC101 and *oriC*, the *dnaA* mutations specifically defective in pSC101 maintenance represent a novel class. We speculate that one or more of these pSC101-specific mutants are defective in interaction with pSC101 RepA protein, which is also required for initiation of plasmid DNA replication.

The *dnaA* gene of *Escherichia coli* is essential for initiation of replication of the bacterial chromosome (23, 32, 37). A number of plasmids and bacteriophages also require this gene for DNA replication, as indicated by the inability of F (28, 36), R6K (55), and P1 (28, 54) to be maintained in a *dnaA* null mutant. Replication of pSC101 is also dependent on the *dnaA* gene (18, 20, 31). However, it is not capable of integrative suppression of a temperature-sensitive *dnaA* allele, in contrast to F (43), R6K (46), and P1 (12, 13). These findings suggest that many of the same functions of DnaA protein are required for replication of pSC101 and for the bacterial chromosome.

Biochemical characterization of DnaA protein indicates that it is multifunctional. It is a sequence-specific DNA-binding protein that recognizes 9-mer sequences (DnaA boxes) present in *oriC*, the chromosomal origin, as well as in replication origins of plasmids and bacteriophages that require this protein for replication (21). At *oriC*, DnaA protein, bound to ATP and stimulated by integration host factor (IHF) (or HU protein), is proposed to induce a structural alteration resulting in strand separation of the AT-rich region (7). As binding of DnaA protein to *oriC* is mutually exclusive and independent of IHF binding, stimulation of unwinding by IHF apparently occurs subsequent to DNA binding by DnaA protein (34). By contrast, at the pSC101 origin, enhancement of DnaA protein binding by either IHF or pSC101-encoded RepA protein is proposed to occur through formation of a looped structure (47).

The differences in mechanisms of initiation at *oriC* relative to the pSC101 origin likely relate to differences in their structural organization (Fig. 1). Whereas strong and weak DnaA protein binding sites in the pSC101 origin are similarly oriented (47), the four DnaA boxes in *oriC* are arranged with opposing polarities (21). Each replicon has an IHF binding site between DnaA boxes and 13-mer motifs (6, 19, 48). Iterons bound by RepA protein are important for enhancement of DnaA protein binding, leading to the speculation that RepA protein with IHF interacts directly with DnaA protein in the initiation process (47).

Although the *dnaA* gene was first identified nearly three decades ago (32, 37), the collection of conditionally defective alleles is limited to 14 (8, 17, 26, 30; reviewed in reference 40) (see Fig. 2A). In addition, the amino acid sequence has been deduced for 15 homologs of the *dnaA* gene from bacteria in the family *Enterobacteriaceae* (reviewed in reference 40). Their comparative analysis, revealing a remarkable degree of amino acid sequence conservation, suggests that conserved residues contribute to the structure or function of DnaA protein. If so, mutations that affect its functions are expected in conserved residues of this essential gene.

We embarked on the isolation and characterization of novel mutations of the *dnaA* gene by a genetic assay that measures pSC101 maintenance. As *oriC* and pSC101 appear to have different mechanisms of initiation, we felt it possible that mutations of *dnaA* defective in replication of pSC101 but not *oriC* may be identified. Some of the alleles described here are specifically defective for pSC101 maintenance. The substituted residues may affect a proposed interaction with pSC101 RepA protein.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmid DNAs used in this study are described in Table 1. pACYC*dnaA* was constructed by ligation of the 1.9-kb *ClaI*-*XhoI* fragment (Fig. 2A) containing the *dnaA* gene and its promoter from *pdnaA/dnaN* (10) to the 3.6-kb *ClaI*-*SalI* pACYC184 vector fragment (11). The *dnaA*⁺ gene of pACYC*dnaA* was replaced with mutant sequences by using the unique *Eco*NI and *Rsr*II restriction sites (encompassing the *dnaA* coding region but without the *dnaA* promoters) located internal to the *ClaI*-*XhoI* restriction sites (see Fig. 2A). Bacteriological methods were performed essentially as described by Miller (41). Bacterial transformation was done either by the calcium chloride method or by electroporation using a Bio-Rad Gene Pulser according to the manufacturer's recommendation. Bacterial strains and their plasmid-containing derivatives were routinely grown in Luria-Bertani (LB) medium supplemented, as indicated, with 50 µg of ampicillin per ml, 40 µg of kanamycin per ml, and 10 µg of tetracycline per ml. M9 minimal medium supplemented with 0.5% Casamino Acids, 0.2% glucose, 1 µg of thiamine per ml, and the indicated antibiotic(s) was used as noted below.

In vitro mutagenesis of the *dnaA* gene. A 30-µg sample of pDS596 DNA (33) was incubated for 60 min at 65°C in 1 ml of 1 M hydroxylamine-HCl (Sigma) and 0.1 M sodium phosphate, pH 6.5 (41). The sample was then dialyzed against 200 volumes of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA for 2 h, ethanol precipitated, washed with 70% ethanol, and resuspended in the above buffer. Hydroxylamine treatment reduced the transformation efficiency of the plasmid DNA to 1% of that of the untreated DNA. An *Eco*NI-*XhoI* restriction fragment containing the complete *dnaA* coding region from hydroxylamine-treated pDS596 was gel purified (Qiaex DNA extraction kit; Qiagen) and ligated to

* Corresponding author. Mailing address: Department of Biochemistry, Michigan State University, East Lansing, MI 48824-1319. Phone: (517) 353-6721. Fax: (517) 353-9334. Electronic mail address: (Internet) kaguni@pilot.msu.edu.

FIG. 1. Structural organization of *oriC* and the pSC101 replication origin. DnaA protein binding sites (DnaA boxes) are represented by pentagons with the indicated orientations. DnaA boxes containing at most one mismatch from the consensus are cross-hatched, whereas those with more than four or five mismatches are not. AT-rich 13-mer repeats (open circles), RepA binding sites (50, 52) (shaded boxes [direct repeats RS1 to RS3] or arrows [inverted repeats IR1 and IR2]), and IHF (19, 48) and Fis (25) binding sites are also shown.

gel-purified pING1 vector fragment from nonmutagenized pDS596 to form pDS596* (Fig. 2B). This construction places the *dnaA* gene without most of its promoter region distal to the *araB* promoter, which is regulated by the plasmid-encoded AraC protein.

Genetic assay to identify mutations of *dnaA* defective in pSC101 maintenance. A genetic screen was devised to identify plasmid-borne mutations of *dnaA* unable to complement the conditional phenotype of the *dnaA204*(Ts) allele for pSC101 maintenance. MS206 containing pSC101 was transformed with the mutagenized *dnaA*-containing plasmid pDS596* by selection for ampicillin resistance at 30°C on LB medium containing kanamycin and tetracycline (Fig. 3). The transformants were then grown in LB medium containing the above antibiotics at 30°C in 96-well microtiter plates and diluted to a cell density of approximately 10^4 CFU/ml. Aliquots (1 to 2 μ l) were replica plated in duplicate onto supplemented M9 medium with or without tetracycline and incubated at 30 or 41.5°C to screen for the inability of transformants to maintain pSC101 at the elevated temperature. The phenotypes conferred by the plasmid-borne mutations were confirmed by retransformation into MS206(pSC101) followed by incubation at 30 and 41.5°C on supplemented M9 medium. The vector only (pING1) or vector containing the *dnaA5*(Ts) (pDS105), *dnaA46*(Ts) (pDS215), *dnaA204*(Ts) (pDS319), or *dnaA*⁺ (pDS596) allele served as a control.

Chemical cleavage of mismatched bases. A chemical mismatch cleavage procedure was performed with heteroduplex DNA essentially as described previously (15). Since hydroxylamine modifies cytosine mismatches and osmium tetroxide modifies thymine mismatches (16, 42), analysis of both strands provides mapping information for all possible substitutions. The sizes of cleavage products detected by autoradiography were determined relative to molecular weight markers. By chemical cleavage, the nature of putative base changes was deduced by the base preference of hydroxylamine (C specific) or osmium tetroxide (T specific) and the template or coding strand that was cleaved. By taking into account the position of the labelled nucleotide relative to the coding sequence of the *dnaA* gene, the approximate locations of the mispaired nucleotides were determined.

DNA sequence analysis. On the basis of the results from chemical cleavage, relevant regions for each of the five alleles were sequenced by the chain-terminating enzymatic method. Oligonucleotide primers JK-7 (5'-GTGGAGTCCATATGTCACTTTTCGCTTTGG-3'), JK-21 (5'-ATAACCCGTTGTTCC-3'), JK-22 (5'-GACCACCTAACGGACCGCTC-3'), and JK-26 (5'-GAGCGCTTGTTCAGGACAT-3'), synthesized by an in-house facility with an Applied Biosystems model 394 synthesizer, were used with Sequenase (U.S. Biochemicals), essentially as described by the manufacturer, with plasmid DNAs purified by the Qiagen mini- or midi-column method. Samples were electrophoresed in a 6% DNA sequencing gel and autoradiographed. The following oligonucleotide primers used for sequencing are homologous to the indicated nucleotide positions: JK-7, residues -12 to 18 of the coding strand (also contains mismatches at positions -3, -2, -1, and 1); JK-21, residues 494 to 508 of the coding strand; JK-22, residues 1459 to 1478 of the template strand; and JK-26, residues 610 to 629 of the coding strand.

Quantitative immunoblot analysis of DnaA protein. Cultures were grown in supplemented M9 medium (41), unless otherwise indicated, containing the appropriate antibiotics at 30°C. At mid-log growth phase (optical density at 595 nm [OD₅₉₅] of 0.5 to 0.7), 1 OD₅₉₅ of cells were collected by centrifugation and resuspended in 50 mM Tris (pH 7.6) and 20% (wt/vol) sucrose. An equal volume of 50% glycerol, 0.2 M Tris-HCl (pH 7.6), 1% sodium dodecyl sulfate (SDS),

0.1% bromophenol blue, and 40 mM dithiothreitol was then added. The final concentration of each sample was equivalent to 10^{-2} OD₅₉₅ units/ μ l. Samples were frozen in liquid nitrogen, stored at -70°C prior to electrophoresis in 10% polyacrylamide gels containing SDS, and then subjected to immunoblot analysis (51). Detection was done with a monoclonal antibody (M43, which recognizes residues 133 to 141) to DnaA protein incubated in 10 mM Tris (pH 7.4), 154 mM NaCl, 0.05% Tween 20, and 2% nonfat milk. ¹²⁵I-labelled goat anti-mouse immunoglobulin G (ICN) was used as the second antibody. Immunoreactive species were quantitated with a Packard Instant Imager or with a Millipore Bio Image Visage 110, as indicated below.

Assay of in vitro *oriC* replication. Reactions (25- μ l reaction volumes) were performed with a crude protein fraction deficient in DnaA protein activity prepared from WM433 (22). Purified DnaA⁺ protein was used as a control. Lysate supernatants (fraction I) were prepared by using a gentle lysis procedure as described elsewhere (33) with cell pellets obtained from 1-liter cultures of MS3897 harboring the mutant alleles in pING1. Expression of the respective gene products was induced by addition of L-arabinose (Sigma) to 0.7%, followed by growth for 3 h at 30°C.

RESULTS

Isolation of five novel *dnaA* mutations. To obtain novel mutations of the *dnaA* gene, a genetic assay was developed (Fig. 3). This method measured the inability of a plasmid-borne mutation to complement the conditional defect of the *dnaA204*(Ts) allele for pSC101 maintenance. To measure pSC101 maintenance at an elevated temperature, the host strain (MS206) was also integratively suppressed by a mini-R1 plasmid. Whereas this strain was viable up to 41.5°C because of integrative suppression, an isogenic strain lacking the mini-R1 plasmid was viable only up to 38°C (data not shown). When the integratively suppressed strain was transformed by pSC101, this plasmid was not maintained at the elevated temperature unless it was coresident with another containing the *dnaA*⁺ gene (pDS596) (Table 2), whose expression is under control of the *araB* promoter but was not induced (Fig. 2B). Sufficient levels of DnaA protein were expressed from this construct in the absence of arabinose induction to permit the establishment and maintenance of pSC101. Other controls were done with this strain containing the latter plasmid but bearing other alleles (*dnaA5*, *dnaA46*, and *dnaA204*) temperature sensitive for replication from *oriC* (reviewed in reference 40). These *dnaA* alleles were also temperature sensitive for pSC101 maintenance (Table 2), confirming that pSC101 maintenance is *dnaA* dependent (18, 20, 31).

By this method in combination with hydroxylamine mutagenesis of the *dnaA* gene (Fig. 2 and 3), five alleles de-

TABLE 1. Bacterial strains and plasmids^a

Strain or plasmid	Genotype ^b or characteristics	Source, construction, or reference
Strains		
MC1061	<i>araD139 Δ(ara-leu)7696 ΔlacX74 galU galK hsdR2</i> (<i>r_K⁻ m_K⁺</i>) <i>mcrB1 rpsL</i> (<i>F⁻</i>)	Laboratory stock
EH3894	<i>asnB32 relA1 spoT1 thi-1 fuc-1 lysA ilv-192 zia::pKN500</i> (<i>pKN500</i> = mini-R1) <i>ΔdnaA mad-1^c tnaA::Tn10</i> (<i>F⁻</i>)	M. Yarmolinsky (28)
MS3895	EH3894 but lacking <i>Tn10</i>	Selection for Tc ^s of EH3894 on Bochner medium (4)
MS3896	MS3895, <i>mad-2^c</i>	Selection for growth of MS3895 at 41.5°C
KL16-99	<i>recA1</i> HfrKL16	L. Snyder
MS3897	MS3896, <i>recA1 lysA⁺ fuc⁺</i>	(MS3896) × (KL16-99), select Lys ⁺ , and screen for RecA ⁻ by UV ^s
TH433	<i>leu-19 pro-19 trp-25 his-47 thyA59 arg-58 met-55 deoB23 lac-11 gal-11 strA56 sul-1 hsdS_{K-12} dnaA204 tnaA::Tn10</i> (<i>F⁻</i>)	This laboratory
MS204	MS3896, <i>dnaA204 tnaA::Tn10</i>	(MS3896) × P1(TH433), select Tc ^r , and screen for Ts pSC101 maintenance
MS205	MS204 but lacking <i>Tn10</i>	Selection for Tc ^r of MS204 on Bochner medium (4)
MS206	MS205, <i>recA1 lysA⁺ fuc⁺</i>	(MS205) × (KL16-99), select Lys ⁺ , and screen for RecA ⁻ by UV ^s
Plasmids		
pING1	Ap ^r ; pBR322 <i>oriV</i> , expression vector, <i>Salmonella typhimurium</i> <i>araB</i> promoter and <i>araC</i>	D. Ray (35)
pDS105	<i>dnaA5</i> cloned into pING1	This laboratory (33)
pDS215	<i>dnaA46</i> cloned into pING1	This laboratory (33)
pDS319	<i>dnaA204</i> cloned into pING1	This laboratory (33)
pDS596	<i>dnaA⁺</i> cloned into pING1	This laboratory (33)
pDS596*	pDS596 containing a hydroxylamine-treated <i>EcoNI-XhoI</i> fragment of the <i>dnaA</i> gene	This work
pA31T	A31T cloned into pING1	This work
pV198M	V198M cloned into pING1	This work
pG287S	G287S cloned into pING1	This work
pT301I	T310I cloned into pING1	This work
pL447W	L447W cloned into pING1	This work
pACYC184	Cm ^r Tc ^r ; p15A <i>oriV</i> , cloning vector	Laboratory stock (11)
pACYC <i>dnaA</i>	Cm ^r ; <i>dnaA⁺</i> cloned into pACYC184	This work
pACYC <i>dnaA46</i>	Cm ^r ; <i>dnaA46</i> cloned into pACYC184	This work
pACYCA31T	Cm ^r ; A31T cloned into pACYC184	This work
pACYCV198M	Cm ^r ; V198M cloned into pACYC184	This work
pACYCG287S	Cm ^r ; G287S cloned into pACYC184	This work
pACYCT301I	Cm ^r ; T301I cloned into pACYC184	This work
pACYCL447W	Cm ^r ; L447W cloned into pACYC184	This work
pSC101	Tc ^r ; DnaA-dependent low-copy-number replicon	S. Cohen (14)
pKN505	Ap ^r ; pBR322 <i>oriV</i> , expresses R1 <i>copA</i> activity	K. Nordström (24)

^a Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Tc, tetracycline; UV^s, sensitivity to UV light; Ts, temperature sensitive; P1, P1 lysate; ×, genetic cross.

^b See the report of Bachmann (3) for genetic symbols.

^c *mad-1* is a mutation that confers cold-resistant growth on minimal media and temperature-sensitive growth on rich media to a strain that contains a *dnaA* null mutation and is integratively suppressed by a mini-R1 plasmid (*zia::pKN500*) (28). It is unlinked by P1 transduction to both the integrated mini-R1 and *dnaA* null mutation and is thought to be a large chromosomal inversion (28). For consistency, we have assigned the *mad-2* designation to derivatives of *ΔdnaA zia::pKN500 mad-1* strains that spontaneously acquired temperature-resistant growth on both rich and minimal media. We speculate that *mad-2* is also a chromosomal inversion resulting in a more optimal placement of *zia::pKN500* relative to *terC* (39). Replication of both the *mad-1* and *mad-2* mutants remains dependent on the integrated mini-R1 plasmid (*zia::pKN500*), as demonstrated by the cold-sensitive phenotype of the strains following their transformation to ampicillin resistance with pOU420, a plasmid encoding an R1 *copA*(Ts) allele (28) (data not shown).

fective for pSC101 maintenance at high temperature (41.5°C) were identified from a collection of 1,790 transformants (Table 2). At the elevated temperature, mutants G287S (with a G-to-S mutation at position 287), T301I, and L447W displayed small or minute colonies relative to those at 30°C or in comparison with the *dnaA⁺* transformant (pDS596). A31T and V198M each showed a modest defect, having reductions in plating efficiency to 0.11 and 0.09, respectively, at the elevated temperature (Table 2).

Determination of nucleotide sequences of mutant *dnaA* alleles. The respective mutation(s) of each allele was mapped by a chemical modification method (data not shown) (15), and the appropriate regions for each were sequenced (Table 3). A single nucleotide substitution was identified for four of the five alleles. L447W was found to contain three nucleotide substi-

tutions, two of which are silent. In all, each mutant allele was found to contain a single deduced amino acid substitution. All mutations were consistent with hydroxylamine mutagenesis (C→T transitions) (16, 42) except for the transversion (T→G) of L447W, which we presume arose spontaneously.

Compared with the *dnaA* alleles previously described (reviewed in reference 40), these are novel mutations (Fig. 2A). Amino acids V-198, T-301, and L-447 are highly conserved among 15 *dnaA* homologs (Table 3). A-31 and G-287 are less well conserved among all homologs but are fairly well conserved among gram-negative bacteria. As hydroxylamine treatment should produce nonsense mutants (9), the failure to obtain these or to reisolate previously identified mutants may be due to the small number of transformants screened (1,790). Indeed, *dnaA5*, *dnaA46*, and *dnaA204* mutations were temper-

FIG. 2. (A) Map of the *dnaA* gene. Relevant restriction enzyme sites, promoters (pentagons), the DnaA box, and positions of mutations for already-known *dnaA* alleles (listed below) (8, 17, 26, 30; reviewed in reference 40) as well as those described here (listed above) are indicated. Extragenic suppressors of *dnaX2016*(Ts) that reside in the *dnaA* gene (Cs,Sx) (26) are indicated. Positions are not indicated for intragenic suppressor mutations (*dnaA46cos* and *dnaA508cos*) of the *dnaA46* and *dnaA508* alleles. Numbers refer to the first and last amino acid residues of DnaA protein. (B) pDS596* was constructed (see Materials and Methods) by insertion into vector pING1 of an *Eco*NI-*Xho*I restriction fragment containing the hydroxylamine-treated *dnaA* coding region. The recombinant plasmid carries the mutagenized *dnaA* gene without most of the promoter region downstream from the *araB* promoter, which is regulated by AraC protein, also encoded by the plasmid vector.

ature sensitive for pSC101 maintenance as measured by this genetic screen (Table 2). Furthermore, the residual activity of the *dnaA204* allele at 42°C (29) may have restricted the types of mutations detected, as those able to function in concert with the *dnaA204* gene product would not have been obtained.

Replication activity of the novel *dnaA* alleles in a Δ *dnaA* strain. To assess the phenotypes conferred by the novel alleles without the possible contribution of a chromosomal *dnaA* allele, plasmids bearing the mutant alleles were transformed into an integratively suppressed strain lacking the *dnaA* gene (MS3897). Except for the *dnaA* null mutation, this strain is isogenic to that used to isolate the novel mutants. Plasmids bearing each of the novel alleles were taken up by this strain as efficiently as the controls of vector alone (pING1) or the vector bearing the wild-type (pDS596) or *dnaA46*(Ts) (pDS215) allele (data not shown). The *dnaA* null strain carrying each of the novel mutant alleles was then transformed by pSC101. At 30°C, their transformation efficiencies were comparable to those of controls with the *dnaA*⁺ or *dnaA46* allele in pING1 (data not shown). At 41.5°C, the transformation efficiency with plasmids carrying the novel mutations or the *dnaA46* allele was greatly reduced relative to that of the plasmid carrying *dnaA*⁺ (data not shown). The temperature-sensitive phenotype for pSC101 maintenance was confirmed by plating representative transformants at 30 and 41.5°C on supplemented M9 medium (Table 4).

At the elevated temperature, an increased plating efficiency was observed with these plasmid-borne mutations coresident with the chromosomal *dnaA204* allele relative to the *dnaA* null allele in the isogenic strain (Table 2 compared with Table 4). This may be due to contribution of the *dnaA204* allele, which maintains residual activity at 42°C (29).

An elevated level of DnaA protein compared with that required for pSC101 maintenance is required for *oriC* replication. We discovered that the *dnaA*⁺-containing plasmid pDS596 was unable to maintain an *oriC* plasmid (data not shown), whereas pSC101 was maintained under comparable conditions (Table 4, line 1). This was in the absence of arabinose induction in the *dnaA* null strain (MS3897).

By contrast, the *dnaA*⁺ gene expressed from its own promoter contained in pACYC184 was sufficient to maintain an *oriC* plasmid (data not shown) or to support chromosomal replication from *oriC* upon inhibition of integrative suppression (Table 5) (see below). These results suggest that expression of DnaA protein, when under *araB* promoter control and not induced, is limiting for *oriC* replication. Quantitative immunoblot analysis supported this interpretation (Fig. 4). The level of DnaA⁺ protein expressed from pACYC*dnaA* contained in the null strain was about 40-fold higher than the uninduced level expressed from pDS596 (Fig. 4, inset). In the latter plasmid, the level of expression was not dramatically affected by the growth medium. The level of DnaA⁺ protein

FIG. 3. Genetic assay to identify *E. coli* *dnaA* mutants defective in pSC101 maintenance at 41.5°C. See Materials and Methods for a description. Abbreviations: Ap, ampicillin; Km, kanamycin; Tc, tetracycline.

expressed from pDS596, barely detectable by autoradiography, concurs with the observation of Abeles et al. (1). Together, these results indicate that the level of DnaA protein required for pSC101 replication in vivo is less than that required for replication of an *oriC* plasmid or the bacterial chromosome.

In the null strain, the levels of expression of DnaA⁺ and DnaA46 proteins encoded in pACYC184 were about 2.1- and 2.9-fold higher, respectively, than the chromosomally encoded level of MC1061 (Fig. 4). The calculated cellular abundance of chromosomally encoded DnaA protein (1.9×10^3 molecules per 10^{-9} OD₅₉₅; see Fig. 4 legend) is slightly greater than previously published estimates (1×10^3 to 1.5×10^3 molecules per 10^{-9} OD₅₉₅) (45). In this experiment, DnaA protein was not detected in the null strain lacking a plasmid or harboring pACYC184, confirming its genotype.

Replicon-specific defect of the novel *dnaA* alleles. The method used to obtain novel mutations screened for the in-

TABLE 2. Inability of plasmid-borne *dnaA* alleles to confer temperature-resistant growth to *E. coli* MS206(pSC101)

MS206(pSC101) transformant ^a	<i>dnaA</i> allele	Plating efficiency ^b
pING1	None	8.1×10^{-6}
pDS596	<i>dnaA</i> ⁺	1.4
pDS105	<i>dnaA5</i>	5.1×10^{-5}
pDS215	<i>dnaA46</i>	2.1×10^{-5}
pDS319	<i>dnaA204</i>	5.6×10^{-5}
pA31T	A31T	0.11
pV198M	V198M	9.1×10^{-2}
pG287S	G287S	1.3 ^c
pT301I	T301I	0.82 ^c
pL447W	L447W	1.3 ^d

^a MS206(pSC101) transformants contain the indicated *dnaA* alleles in pING1 and expressed from the *araB* promoter.

^b Transformants were grown at 30°C in LB medium containing kanamycin, ampicillin, and tetracycline before being plated. Plating efficiency is expressed as the number of CFU observed at 41.5°C relative to that at 30°C on supplemented M9 medium containing the above antibiotics. Plating efficiency was determined in the absence of arabinose induction. On media without tetracycline, the colony morphology of MS206(pSC101) with the different plasmid-borne mutants was similar to that of the *dnaA*⁺ transformant. By contrast to these results with synthetic media, pSC101 maintenance was not temperature sensitive on LB medium with these plasmid-encoded *dnaA* mutations or with temperature-sensitive alleles (*dnaA5*, *dnaA46*, and *dnaA204*).

^c Small CFU observed at 41.5°C.

^d Tiny CFU observed at 41.5°C.

ability of a plasmid-encoded allele to maintain pSC101 in a host strain harboring the *dnaA204*(Ts) allele at the nonpermissive temperature (Fig. 3). Consequently, it is possible that the mutants obtained were not defective in replication from *oriC*. To address this issue, chromosomal replication from *oriC* was measured by inhibition of replication from the integrated R1 origin in the *dnaA* null strain (MS3897). Inhibition was achieved by introduction of a plasmid containing the R1 *copA* gene (pKN505), which encodes an RNA antisense to the transcript for R1 RepA protein (24). Formation of the RNA duplex interferes with expression of RepA protein required for R1 replication (49).

The level of DnaA protein, when encoded by pACYC184

TABLE 3. Nucleotide and deduced amino acid alterations of five *dnaA* alleles^a

<i>dnaA</i> allele	Nucleotide position ^b	Nucleotide substitution ^c	Amino acid position	Deduced amino acid substitution (sequence conservation) ^d
A31T	91	G→A	31	Ala→Thr (6/15)
V198M	592	G→A	198	Val→Met (13/15)
G287S	859	G→A	287	Gly→Ser (6/15)
T301I	902	C→T	301	Thr→Ile (12/15)
L447W	1335	G→A	445	Glu→Glu
	1338	G→A	446	Gln→Gln
	1340	T→G	447	Leu→Trp (12/15)

^a Chemical modification of the template and coding strand to map the approximate positions of mutations (data not shown) was performed as described in Materials and Methods. As controls, the *dnaA*⁺ and *dnaA46* alleles were analyzed. The (cytosine) substitutions of the *dnaA46* allele at residues 551 and 754 (30) were confirmed by this method. No mispairs in either strand of the *dnaA*⁺ gene were detected. Appropriate regions of each mutant allele based on the chemical modification mapping results were sequenced by the chain-terminating enzymatic method.

^b Numbers refer to the *dnaA* coding region, with position 1 corresponding to the first nucleotide of the coding sequence (27, 44).

^c Nucleotide substitutions are expressed as those in the coding strand.

^d Sequence conservation is indicated by the frequency of residues conserved at respective positions among the 15 *dnaA* homologs (reviewed in reference 40).

TABLE 4. Plasmid-borne *dnaA* mutations are temperature sensitive for pSC101 maintenance

MS3897 transformant ^a	<i>dnaA</i> allele	Plating efficiency ^b
pDS596	<i>dnaA</i> ⁺	0.91
pDS215	<i>dnaA46</i>	9.0×10^{-5}
pA31T	A31T	2.8×10^{-3}
pV198M	V198M	1.2×10^{-3}
pG287S	G287S	1.9×10^{-2}
pT301I	T301I	8.1×10^{-4}
pL447W	L447W	1.1×10^{-4}

^a MS3897 transformants contain the indicated *dnaA* alleles in pING1 and expressed from the *araB* promoter.

^b Transformants were grown at 30°C in LB medium containing kanamycin, ampicillin, and tetracycline before being plated. Plating efficiency is expressed as the ratio of the number of CFU observed at 41.5°C to the CFU at 30°C on supplemented M9 medium containing the above antibiotics. Plating efficiency was done in the absence of arabinose induction.

and expressed from its own promoter, was sufficient to support *oriC* replication (data not shown) (Table 5). To examine the *dnaA* mutants for *oriC* replication activity, derivatives of pACYC184 containing these alleles were constructed. They were introduced into the *dnaA* null strain, and then the R1 *copA*-containing plasmid (pKN505) was introduced to inhibit integrative suppression (Table 5). In contrast to the other mutant alleles, the A31T, G287S, and L447W alleles supported replication from *oriC* as effectively as the wild-type control (pACYC-*dnaA*). This observation suggests that the defect of the A31T, G287S, and L447W alleles is specific for pSC101 replication. Transformants harboring the *copA*-containing plasmid (pKN505) and either the plasmid-borne *dnaA46* or T301I allele were obtained at 30°C but were extremely unstable, preventing an assessment of their replicon specificity. This observation is not understood but is unlikely to be due to insufficient levels of DnaA protein (Fig. 4). DnaA46 and T301I proteins were expressed at levels comparable to those of the other mutant proteins.

As a control, pSC101 maintenance in the integratively sup-

TABLE 5. A subclass of *dnaA* mutations is specifically defective in pSC101 maintenance

MS3897 transformant ^a	<i>dnaA</i> allele	Plating efficiency ^b	
		pSC101	pKN505
pACYC <i>dnaA</i>	<i>dnaA</i> ⁺	1.17	1.16
pACYC <i>dnaA46</i>	<i>dnaA46</i>	5.9×10^{-3}	NA ^c
pACYCA31T	A31T	1.01	0.89
pACYCV198M	V198M	1.30	6.3×10^{-2}
pACYCG287S	G287S	1.6×10^{-2}	0.91
pACYCT301I	T301I	7.8×10^{-2}	NA
pACYCL447W	L447W	4.2×10^{-2}	1.03

^a MS3897 transformants contain the indicated *dnaA* alleles in pACYC184 and expressed from the *dnaA* promoter.

^b Transformants of pSC101 were grown at 30°C in LB medium containing kanamycin, chloramphenicol, and tetracycline before being plated. Transformants of pKN505 were grown on supplemented M9 agar medium, picked, and diluted in M9 salts before being plated. Plating efficiency is expressed as the number of CFU observed at 41.5°C relative to the CFU at 30°C on supplemented M9 medium containing kanamycin, chloramphenicol, and either tetracycline (for pSC101) or ampicillin (for pKN505).

^c Plating efficiency is not available (NA) because of the instability of the transformant containing both the plasmid-encoded *dnaA* allele and pKN505.

pressed null strain was examined (Table 5). The G287S, T301I, and L447W alleles were temperature sensitive for maintenance of pSC101. By contrast, the A31T and V198M alleles were not defective for pSC101 maintenance at the elevated temperature.

Suppression of the temperature-sensitive phenotype for pSC101 maintenance by elevated levels of expression. When contained in pACYC184, all of the mutant *dnaA* alleles except the G287S allele, which was unchanged, showed an increase in plating efficiency of 2 to 3 orders of magnitude at the elevated temperature relative to that for pING1 (compare Tables 4 and 5). The A31T and V198M alleles were not temperature sensitive for pSC101 maintenance when contained in pACYC184. Although we have not measured the cellular abundance of these or the other novel alleles when contained in pING1, we believe that their elevated levels of expression when in

FIG. 4. Quantitative immunoblot analysis of DnaA protein. The level of DnaA protein encoded by the various recombinant plasmids in the *dnaA* null strain (MS3897) was determined as described in Materials and Methods. "None" refers to the MS3897(pACYC184) transformant. The levels were quantified with a Bio-Rad Instant Imager. For each sample, the counts detected during a 100-min exposure minus background (right ordinate) were normalized relative to the chromosomally encoded level of wild-type strain MC1061 (left ordinate). In the inset, scanning densitometry of an autoradiogram (not preflashed) was required because of the low levels of expression that could not be detected with the Instant Imager. Optical densities of DnaA protein encoded by pDS596 grown in either LB medium (0.24 U) (right ordinate, inset) or M9 minimal medium (0.15 U) were normalized (left ordinate, inset) relative to the level encoded by pACYC*dnaA* (9.43 U). Relative to known amounts of purified DnaA protein analyzed in parallel, the cellular abundance of DnaA protein encoded by MC1061 was determined.

FIG. 5. In vitro *oriC* replication activity of novel DnaA mutant proteins. Replication assays were performed as described in Materials and Methods at 30°C (●) or 42°C (○). Crude lysate supernatants containing the indicated mutant DnaA proteins used in these in vitro replication assays were comparable in total protein concentration as determined by the dye-binding method (5) and in DnaA protein abundance as judged by immunoblot analysis (data not shown). DnaA protein is expressed either in nanograms (purified DnaA⁺ protein) or microliters of fraction I (mutants). As determined by immunoblot analysis, 1 μ l of the respective fraction I corresponds to ~150 ng of DnaA protein.

pACYC184 suppress, sometimes partially, the temperature-sensitive phenotype for pSC101 maintenance. This interpretation is based on two observations. First, immunoblot analysis showed that the level of DnaA⁺ protein was approximately 40-fold elevated in the pACYC184 construct relative to that in pING1 (Fig. 4, inset). Second, the viability of many *dnaA*(Ts) mutants increases when the alleles are present on both the chromosome and a pBR322 derivative (30), presumably by a gene dosage effect.

Also in this analysis (Fig. 4), the levels of the mutant proteins encoded in pACYC184 were found to be between 1.9- and 2.7-fold higher than that of the chromosomally encoded DnaA⁺ control (MC1061) and similar to or slightly elevated in comparison with the control with DnaA⁺ protein encoded by the comparable plasmid (pACYCdnaA). The latter difference may possibly relate to differences in protein stability. Alternatively, the difference in expression levels of the pACYC184 constructs may relate to their relative abilities to autoregulate their own expression. The *dnaA* promoter region containing a DnaA box is autoregulated by the binding of DnaA protein (2, 28, 38, 53).

We do not know whether the A31T or V198M allele, when expressed at the chromosomal level, will be temperature sen-

TABLE 6. Summary of mutant *dnaA* phenotypes^a

<i>dnaA</i> allele	pSC101 maintenance		<i>oriC</i> replication	
	Low level of DnaA ^b	High level of DnaA ^c	In vivo (high level of DnaA ^c)	In vitro
A31T	Ts	+	+	+/- Ts
V198M	Ts	+	Ts	Ts
G287S	Ts	Ts	+	+
T301I	Ts	Ts	NA	Ts
L447W	Ts	Ts	+	+

^a This table is based on results presented in Fig. 5 and Tables 4 and 5. Abbreviations: Ts, temperature sensitive; +, temperature insensitive; +/- Ts, moderately temperature sensitive; NA, data not available.

^b Level of expression observed when the mutant alleles were contained in pING1 and under control of the *araB* promoter in the noninduced state (Fig. 4, inset).

^c Level of expression observed when the mutant alleles were contained in pACYC184 and expressed from the *dnaA* promoter (Fig. 4).

sitive for pSC101 maintenance or whether the A31T allele will be temperature sensitive for replication from *oriC*. This issue may be addressed by substituting the chromosomal copy with each allele or by modulating their expression from the *araB* promoter to the chromosomal level.

A31T, V198M, and T301I are thermolabile in in vitro replication of an *oriC* plasmid. To corroborate the genetic studies, in vitro replication activity of the mutant DnaA proteins was measured at both 30 and 42°C (Fig. 5). The V198M and T301I mutant proteins were thermolabile in vitro for *oriC* plasmid replication. Whereas G287S protein appeared comparable to the wild-type protein in replication activity at both temperatures, A31T protein was moderately thermolabile. The replication activity of L447W protein was not thermolabile. However, the extents of nucleotide incorporation with A31T and L447W proteins were lower than those observed for both the wild-type and G287S proteins. The crude protein fractions used in this assay contained comparable levels of mutant protein, as judged by immunoblot analysis (data not shown). Whether a contaminating factor in the samples may have affected the results obtained can be addressed when these proteins are purified. The marginal effect of temperature on the replication activity of A31T, G287S, and L447W proteins is in agreement with the in vivo phenotypes of these mutants for chromosomal replication (Table 5), suggesting that these alleles are specifically defective for pSC101 maintenance.

DISCUSSION

dnaA mutants specifically defective in pSC101 maintenance.

The method used to identify mutations of the *E. coli dnaA* gene relied on use of an integratively suppressed strain (MS206) containing the *dnaA204*(Ts) allele. Because of the integrated mini-R1 plasmid, the *dnaA* gene and *oriC* are not essential for bacterial growth (28). Incubation at a temperature nonpermissive for the *dnaA204* allele provided a means of assessing the phenotype of the plasmid-encoded *dnaA* gene for maintenance of pSC101 (Fig. 3). Whereas bacterial growth in the absence of tetracycline was observed at 30 and 41.5°C (unpublished results), pSC101 was not maintained at the elevated temperature in the absence of a functional *dnaA* gene product (Table 2). With this approach, five alleles unable to complement the conditional phenotype of the *dnaA204* allele for pSC101 maintenance were obtained. Additionally, these alleles were temperature sensitive for pSC101 maintenance in a strain lacking the *dnaA* gene (Table 4). Compared with the 14 other missense mutations of the *dnaA* gene (8, 17, 26, 30;

reviewed in reference 40), these are novel (Fig. 2A). Furthermore, V198M, T301I, and L447W have substitutions in amino acid residues that are highly conserved in *dnaA* homologs of the family *Enterobacteriaceae* (Table 3).

Of the five alleles, A31T, G287S, and L447W were not temperature sensitive for *oriC* replication under conditions in which replication from the integrated R1 origin was inhibited (Table 5). Biochemical studies corroborated these genetic results (Fig. 5). Assays using lysate supernatants enriched with the respective mutant proteins indicated that G287S and L447W, and to a lesser degree A31T, were also temperature resistant in *in vitro* replication of an *oriC* plasmid. Collectively (Table 6), these results suggest that the G287S and L447W mutations, and possibly A31T, are specifically defective in replication of pSC101.

We do not understand the instability of the *dnaA* null strain bearing both the *copA* plasmid and a plasmid-borne *dnaA46* or T301I allele (Table 5). That these alleles were plasmid-borne and not chromosomally encoded may be of relevance. Suppressors of *dnaX2016*(Ts) that reside in the *dnaA* gene are dominant to plasmid-borne *dnaA*⁺ when chromosomally located but recessive in the converse construction (26). A similar difference in activity depending upon the mutant allele location (chromosome versus plasmid) has been reported for *dnaA*(Ts) alleles encoding an alanine-to-valine substitution at residue 184 (30). These alleles, expressed from the *dnaA* promoter, exhibit a cold-sensitive phenotype when plasmid encoded and in a strain containing a chromosomal *dnaA*⁺ allele. However, cold sensitivity was not observed when the thermolabile allele was present on the chromosome with the wild-type allele residing in a plasmid.

pSC101 maintenance requires a lower level of DnaA protein than the level needed for replication from *oriC*. With the *dnaA*⁺ gene in pDS596, our inability to establish an *oriC* plasmid in a *dnaA* null mutant (unpublished results) contrasts with the establishment and maintenance of pSC101 under comparable conditions (Table 4). With the *dnaA*⁺ gene in pACYC184 in the *dnaA* null strain, we were able to establish an *oriC* plasmid (unpublished results), as well as to sustain chromosomal replication from *oriC* on inhibition of replication from the integrated R1 origin (Table 5). This result, suggesting that the level of DnaA protein required for pSC101 replication is less than that required for replication of an *oriC* plasmid or the bacterial chromosome, was confirmed by quantitative immunoblotting (Fig. 4).

DnaA protein may interact with RepA protein of pSC101. Among the plasmids of *E. coli*, many require DnaA protein for replication. Of these, F and P1 are capable of integrative suppression of strains containing the *dnaA46*(Ts) allele (12, 13, 43) but not a *dnaA* null allele (28, 36). By comparison, the strict dependence of pSC101 on DnaA protein for maintenance (18, 20, 31) was interpreted to suggest that it requires many of the same functions of *dnaA* that are required for initiation from *oriC*. The mutations G287S and L447W, and possibly A31T, obtained in this study that appear to have a specific defect in pSC101 replication are particularly interesting, for they suggest a unique role of DnaA protein in pSC101 replication. One or more may be defective in the proposed interaction of DnaA protein with pSC101-encoded RepA protein, which facilitates the binding of DnaA protein to the pSC101 replication origin (47). Further examination of the binding of these mutant proteins to the pSC101 replication origin and the development of an *in vitro* system to study pSC101 replication should provide a greater understanding of the interplay between these proteins and of whether the amino acids that have been substituted normally interact with pSC101 RepA protein.

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