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Genetic Method To Analyze Essential Genes of *Escherichia coli*▽

Katarzyna Hupert-Kocurek,† Jay M. Sage,‡ Magdalena Makowska-Grzyska, and Jon M. Kaguni*

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

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The genetic analysis of essential genes has been generally restricted to the use of conditional mutations, or inactivating chromosomal mutations, which require a complementing plasmid that must either be counterselected or lost to measure a phenotype. These approaches are limited because they do not permit the analysis of mutations suspected to affect a specific function of a protein, nor do they take advantage of the increasing abundance of structural and bioinformatics data for proteins. Using the *dnaC* gene as an example, we developed a genetic method that should permit the mutational analysis of other essential genes of *Escherichia coli* and related enterobacteria. The method consists of using a strain carrying a large deletion of the *dnaC* gene, which is complemented by a wild-type copy expressed from a plasmid that requires isopropyl-β-D-thiogalactopyranoside for maintenance. Under conditions in which this resident plasmid is lost, the method measures the function of a *dnaC* mutation encoded by a second plasmid. This methodology should be widely applicable to the genetic analysis of other essential genes.

The genetic study of cellular pathways that are essential for viability has utilized conditionally defective alleles, or chromosomally encoded null mutations that are complemented by a functional copy carried in a plasmid. These approaches can demonstrate the requirement of a specific gene for viability, but the penetrance of a mutation can vary depending on the mutation's effect on the activity of the respective gene product. For example, conditionally defective alleles often give rise to misfolded or partially unstable proteins, which retain limited function to cause a weak phenotype. The accumulation of extragenic suppressors may also affect the phenotype. The alternate approach of studying a phenotype caused by a large deletion relies on a complementing plasmid encoding the gene of interest. If the gene is expressed from a regulatable promoter, the plasmid may be inadequate if the level of expression under noninduced or repressed conditions continues to maintain viability. Mutants carrying complementing plasmids that can be counterselected or that are temperature sensitive for maintenance are useful but limited because the approach cannot address the importance of an individual activity of a multifunctional protein.

For essential proteins with several biochemical functions, site-directed mutagenesis followed by the in vitro characterization of the corresponding mutant protein can reveal the importance of a protein's individual activities if either structural information for a protein is available or conserved amino acids have been identified. However, a genetic method to correlate the biochemical defect to a phenotype is generally not available.

Almost all DNA replication genes are essential, including *dnaC*, which acts during the initiation of DNA replication (reviewed in reference 16) and the reassembly of collapsed replication forks (7, 19). Like DnaA and subunits of the DnaX or clamp loader complex, as well as their functional counterparts in eukaryotic cells, DnaC is a member of the AAA+ family of ATPases (reviewed in references 10 and 15). Koonin noted a significant similarity in amino acid sequence between *Escherichia coli* DnaA and DnaC and suggested that they share a common ancestry (17). As DnaC functions at a different step in initiation than DnaA, it appears that each protein has evolved to perform separate functions. However, in contrast to the measurable activities of DnaA protein during initiation, DnaC action at *oriC* requires the formation of the DnaB-DnaC complex, in which six DnaC monomers interact on one face of the DnaB hexamer (2, 24). Interestingly, despite the strict requirement for the DnaB-DnaC complex in the entry of DnaB at *oriC*, the complex is not catalytically active (9, 28, 29). To reveal the helicase and ATPase activities of DnaB, DnaC must first dissociate from DnaB in a process requiring the hydrolysis of ATP bound by DnaC. By itself, DnaC binds, albeit weakly, to ATP (K_d [dissociation constant] of 8 μM) (3, 9, 13, 28). Its interaction with single-stranded DNA (3, 9, 18) suggests that DnaC binds to the unwound region of *oriC* opened by DnaA, which leads to the binding of DnaB. Inasmuch as DnaB's single-stranded DNA binding activity may suffice, this activity of DnaC may not be involved.

Because of our interest in the role of DnaC protein during initiation in bacteria, we sought to obtain novel mutations in the *dnaC* gene in order to gain insight into DnaC function from the biochemical study of corresponding mutant proteins. In this report, we describe a genetic method for identifying inactivating missense mutations in *dnaC*. The approach can be used for more-extensive mutational analysis of *dnaC* and for any other essential gene of *E. coli* or another closely related bacterium. Using the *dnaC* gene as an example, the method relies on complementing a null *dnaC* mutant with a *dnaC*-carrying plasmid whose maintenance depends on isopropyl-β-D-thiogalactopyranoside (IPTG) in the culture medium. With a separate plasmid that does not depend on IPTG for its maintenance, the host strain cannot survive without IPTG if this

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

† Present address: Department of Biochemistry, University of Silesia, Jagiellonska 28, 40032 Katowice, Poland.

‡ Present address: Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA 01655-0116.

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TABLE 1. *E. coli* K12 strains and plasmids

Strain or plasmid	Genotype and/or properties (reference); source
Strains	
PC2	*leuB6 thyA47 dnaC2 dnaT12 deoC3 rpsL153* λ^- (5); laboratory stock
SS1020	λ^- *rph-1 zjj202*::*Tn*10 *dnaC2*; Steven Sandler
HME5	F^- λ^- IN (*rrnD-rrnE*) Δ*lacU169* λcI*857* Δ(*cro-bioA*) (32); Donald Court
MC1061	*araD139* Δ(*ara*, *leu*)*7697* Δ*lacX74 galU galK rpsL hsdR2* (r_{K-} m_{K+}) *mcrB1*; laboratory stock
KH1061	*araD139* Δ(*ara*, *leu*)*7697* Δ*lacX74 galU galK rpsL hsdR2* (r_{K-} m_{K+}) *mcrB1* Δ*dnaC*::*cat*; this work
MF1061	*araD139* Δ(*ara*, *leu*)*7697* Δ*lacX74 galU galK rpsL hsdR2* (r_{K-} m_{K+}) *mcrB1* Δ*dnaC*::*cat recA*::Kan^r; Magdalena M. Felczak of this laboratory by P1 transduction from BW26355 (relevant genotype, Δ*recA635*::Kan^r) (8) into MC1061, followed by selection for kanamycin resistance; the transductant and BW26355 were confirmed to be UV sensitive
Plasmids	
pACYC184	*cat*, Tet^r (6)
pACYC184*dnaC*	Tet^r, *dnaC* (this work)
pAM34	Amp^r, IPTG-dependent maintenance (32); Kaymeuang Cam
pAM34*dnaC*L1-2	Amp^r, IPTG-dependent maintenance, *dnaC* (this work)
pAM34*dnaC*L1-4	Amp^r, IPTG-dependent maintenance, *dnaC* (this work)
$pINC_{SSD}$	Kan^r, pBR322 derivative, *dnaC* under *araBAD* promoter control and downstream from the ribosome binding site for gene 10 of T7 phage (1)
p*dnaC113*	Amp^r, pBR322 derivative, *dnaC* under *araBAD* promoter control (20)
p*dnaC*97 (Pro108Ser)	Kan^r, $pINC_{SSD}$ derivative, missense mutation encoding Pro108Ser substitution (20)
p*dnaC*116 (Leu44Pro)	Kan^r, $pINC_{SSD}$ derivative, missense mutation encoding Leu44Pro substitution (20)
p*dnaC*250 (Leu11Gln)	Kan^r, $pINC_{SSD}$ derivative, missense mutation encoding Leu11Gln substitution (20)
pHK-*dnaC*	Amp^r, pET11a derivative (Novagen), *dnaC* joined at the 5′ end to sequence encoding protein kinase A phosphorylation site and six histidine residues (9); Mike O'Donnell
pHK-*dnaCK112R*	Amp^r, derivative of pHK-*dnaC* encoding a Lys112Arg substitution (9); Mike O'Donnell

plasmid encodes a nonfunctional *dnaC* allele but remains viable if it carries an active *dnaC* gene.

MATERIALS AND METHODS

Bacteriological methods, strains, and plasmids. Bacteriological methods were performed essentially as described previously (22). Bacterial transformation was done either by the calcium chloride method or by electroporation using a Bio-Rad Gene Pulser according to the manufacturer's recommendations. Bacterial strains and their plasmid-containing derivatives (Table 1) were routinely grown in Luria-Bertani (LB) medium supplemented with ampicillin (100 μg/ml), kanamycin (40 μg/ml), tetracycline (10 μg/ml), and/or chloramphenicol (35 μg/ml) as appropriate. To construct one set of *dnaC* plasmids, the fragment carrying the *dnaC* gene from p*dnaC113* (20) was isolated after cleavage with BamHI nuclease and ligated into the BamHI cleavage site of pAM34 to construct pAM34*dnaC*L1-4 and pAM34*dnaC*L1-2, which, respectively, contain the inserted DNAs in the clockwise and counterclockwise orientations relative to the physical map of pBR322. Because the orientation of the *dnaC* gene in pAM34 did not affect complementation of the Δ*dnaC*::*cat* mutant (see Results), pAM34*dnaC*L1-2 was used in the experiments described unless otherwise stated and is noted as pAM34*dnaC* for simplicity. A derivative of pBR322, pAM34 initiates DNA replication by extension of the primer transcript from the *lac* promoter, which replaces the natural primer promoter of pBR322 (14). Maintenance of pAM34 requires IPTG in the culture medium. To construct pACYC184*dnaC*, the *dnaC* gene carried in $pINC_{SSD}$ (1) was PCR amplified with oligonucleotide primers AATATTTGCCCATGGATACCGCCAGAAAC and AATGCTCATCCGGAATTCTGTGCCATAAGC; cleavage sites for NcoI and EcoRI endonuclease are underlined. The amplified DNA was digested with the respective endonucleases, and the DNA was ligated into the EcoRI and NcoI sites of pACYC184, disrupting the chloramphenicol resistance gene. DNA sequence analysis of the *dnaC* gene confirmed the presence of the wild-type allele in the plasmids constructed (Table 1).

Construction of Δ*dnaC*::*cat* mutants. A null *dnaC* mutant was constructed using the *E. coli* λ Red targeted-mutagenesis system (8, 32). Briefly, a chloramphenicol resistance cassette was PCR amplified from pACYC184 DNA with oligonucleotide primers (GACAGCCAAATTCCACCAGGATTCAGAGGGTAACGAGCGGCTATTTAACGACCCTG and CATCATCATTACTCAAGGTGGAATTGTGTCGCAGTATACCTGTGACGGAAGATCAC) that are complementary to the *dnaC* coding region, as indicated by underlined sequences, and the gene encoding chloramphenicol acetyltransferase (*cat*) carried in pACYC184. Following transformation with 50 ng of the PCR product into *E. coli* HME5 carrying the *dnaC* plasmid, $pINC_{SSD}$, or without a plasmid as a negative control, selection for chloramphenicol-resistant colonies led to the substitution of the *dnaC* gene with the *cat* gene by homologous recombination. The mutation was then transduced via bacteriophage P1 into MC1061 carrying p*dnaC113*. This strain was transformed by pAM34*dnaC*, followed by growth in medium lacking ampicillin but supplemented with 0.5 mM IPTG to obtain the strain carrying only the latter plasmid. In these strains, the presence of the chromosomal Δ*dnaC*::*cat* mutation was confirmed by PCR analysis with 25 pmol of oligonucleotide primers (AGCCAAATTCCACCAGGATTC and CATCATCATTACTCAAGG TGG) that are complementary to *yjjA* and *dnaT* bordering the chromosomal *dnaC* gene and 25 ng of bacterial DNA, which was isolated using a DNeasy tissue kit (QIAGEN). To construct the *asnA*::*cat* strain, oligonucleotides AGC GGGCGATAGCGAAAG and CGGTCAGCTTAAACGTGG, which are complementary to *asnA*, were used to amplify a DNA fragment from an *oriC* minichromosome carrying an insertion of the *cat* gene in *asnA* (25). After transforming the amplified DNA into HME5, the Δ*asnA*::*cat* mutation was verified by PCR analysis with 25 ng of bacterial DNA and 25 pmol of the oligonucleotide primers as described above.

Mutagenesis and screening. Mutagenesis of the *dnaC* gene was carried out by error-prone PCR amplification with *Taq* DNA polymerase (Promega), $pINC_{SSD}$ as the DNA template, and the primers used for the construction of pACYC184*dnaC*. The reaction conditions were essentially as described by the manufacturer, but assays were supplemented with 0.1 to 0.5 mM Mn^{2+}. The amplified DNA was electrophoresed on an agarose gel, purified using a QIAGEN gel extraction kit, and then digested with EcoRI and NcoI endonuclease. After gel purification, the DNA carrying the mutagenized *dnaC* gene was ligated into the EcoRI and NcoI endonuclease sites of pACYC184 with T4 DNA ligase (New England Biolabs). The ligation mixture was then transformed into KH1061 (Δ*dnaC*::*cat*) carrying pAM34*dnaC* and plated on LB medium supplemented with 0.5 mM IPTG, 10 μg/ml tetracycline, and 100 μg/ml ampicillin. Transformants obtained after overnight incubation at 37°C were picked by hand or with a GeneMachine Mantis colony picker and transferred to microtiter plates containing the medium described above. In the absence of IPTG to maintain pAM34*dnaC*, cells carrying pACYC184, which carries an inactivating *dnaC* mutation, will fail to grow. To identify such transformants, the cultures after overnight growth at 37°C were diluted with LB medium about 10^4-fold into microtiter plates, and about 2.0 μl was transferred with a pronged replica plater onto the antibiotic-supplemented medium with or without IPTG. From transformants that showed a growth dependence for IPTG, plasmid DNA was isolated by using

TABLE 2. Plasmids carrying *dnaC* complement the temperature-sensitive phenotype of *dnaC* mutants

Strain	Plasmid	Relative frequency of colony formation[a]
SS1020 (*dnaC2*)	None	$<10^{-8}$
SS1020 (*dnaC2*)	pBR322	1.2×10^{-7}
SS1020 (*dnaC2*)	p*dnaC113*	7×10^{-2}
SS1020 (*dnaC2*)	$pINC_{SSD}$	2×10^{-2}
PC2 (*dnaC2*)	None	$<10^{-8}$
PC2 (*dnaC2*)	pACYC184*dnaC*	~1
SS1020 (*dnaC2*)	None	$<10^{-8}$
SS1020 (*dnaC2*)	pAM34	$<10^{-8}$
SS1020 (*dnaC2*)	pAM34*dnaC*L1-2	~1[b]
SS1020 (*dnaC2*)	pAM34*dnaC*L1-4	~1[b]

[a] Cultures of *E. coli* PC2 (*dnaC2*) or SS1020 (*dnaC2*) carrying the indicated plasmids were grown at 30°C in LB media supplemented with 10 μg/ml tetracycline, 100 μg/ml ampicillin or 50 μg/ml kanamycin to select for the respective plasmid. After serial dilution, the samples were plated on antibiotic-supplemented LB media followed by incubation at 30°C or 42°C for about 14 h. Transformants carrying pAM34 and its derivatives were plated on medium with or without 0.5 mM IPTG. The relative frequency of colony formation is the ratio of the number of colonies detected at 42°C to that at 30°C.

[b] Colony formation, which was observed at 42°C for the transformants carrying pAM34*dnaC*L1-2 and pAM34*dnaC*L1-4, required IPTG in the culture medium.

an Autogen 850 DNA purification robot, followed by DNA sequence analysis with a high-throughput Applied Biosystems 3730XL genetic analyzer and oligonucleotide primers (AATGCTCATCCGGAATTCTGTGCCATAAGC and ATCACAGACGGCATGATGAAC) that are complementary to pACYC184 and border the *dnaC* gene in pACYC184*dnaC*.

RESULTS

An efficient genetic screen to isolate mutations in *dnaC*, an essential gene of *E. coli*. To summarize our general approach, the strategy was to construct a null *dnaC* mutant whose deficiency is complemented by a *dnaC* plasmid that is conditionally maintained. If we introduce a separate plasmid carrying a mutagenized *dnaC* gene into the null mutant, the strain should remain viable after the loss of the resident plasmid if the incoming plasmid encodes a functional *dnaC* gene. If the incoming plasmid bears a nonfunctional gene, the strain cannot survive when it lacks the resident plasmid, thus providing a method to identify inactivating *dnaC* mutations. Our long-term objective is to study mutant DnaC proteins at the biochemical level to gain new insight into the role of DnaC during initiation or replication fork restart.

Construction of a null *dnaC* mutant. A plasmid that encodes the wild-type *dnaC* gene should sustain a null *dnaC* mutant. As a control experiment to identify suitable plasmids, we measured the complementation of *dnaC2*(Ts) mutants at a nonpermissive temperature by *dnaC* plasmids named p*dnaC113* and $pINC_{SSD}$. Both DNAs carry *dnaC* downstream from the *araBAD* promoter and were examined under noninduced conditions, but $pINC_{SSD}$ also bears the ribosome binding site of T7 bacteriophage gene 10 ahead of the *dnaC* gene (Table 1). These plasmids, as well as pAM34*dnaC*, are derivatives of pBR322. However, maintenance of pAM34*dnaC* requires IPTG in the culture medium for reasons described in detail below. We also constructed a derivative of pACYC184 encoding the *dnaC* gene, named pACYC184*dnaC*. As shown in Table 2, all of the *dnaC* plasmids complemented the temperature sensitivity of the *dnaC2* mutants, although the basal level of *dnaC* expression by plasmids that carry *dnaC* downstream from the *araBAD* promoter was only partially effective.

We then constructed a null *dnaC* strain by replacing the *dnaC* coding region with a chloramphenicol resistance cassette via the λ recombination system (8, 32) (see Materials and Methods). In the construction, we tested if we could construct the *dnaC* mutant with a strain carrying the *dnaC* plasmid, $pINC_{SSD}$, despite its incomplete complementation of the *dnaC2*(Ts) mutants (Table 2), because we plan to examine later if a limiting level of DnaC compared to the natural level can exacerbate the bias in the loading of DnaB that moves leftward from *oriC* relative to DnaB that moves to the right (4). We obtained chloramphenicol-resistant colonies only when the strain (HME5) carried the *dnaC* plasmid, suggesting the substitution of the chromosomal *dnaC* locus with the chloramphenicol resistance cassette. As a control, we obtained antibiotic-resistant recombinants when we used a chloramphenicol resistance cassette that should replace the chromosomal *asnA* gene regardless of the presence of the *dnaC* plasmid. The *asnA* gene is not essential for growth in rich medium. Although we obtained fivefold more recombinants of Δ*asnA*::*cat* than Δ*dnaC*::*cat* for the plasmid-bearing strain, we do not know if variability in the efficiency of electroporation or another factor, such as a limiting level of DnaC, causes the difference.

To obtain direct evidence that we were able to replace the chromosomal *dnaC* gene with the chloramphenicol resistance cassette, we isolated bacterial DNA from 17 randomly chosen recombinants of HME5 that also carried the *dnaC* plasmid, $pINC_{SSD}$, as described in Materials and Methods. We then performed PCR analysis using oligonucleotide primers that are complementary to sequences that flank the chromosomal *dnaC* locus (Fig. 1A) and are known not to anneal to any of the *dnaC* plasmids described in this work (data not shown; see below). If the *dnaC* gene remains intact, PCR amplification should produce a DNA fragment of about 0.85 kb, which we observed with bacterial DNA isolated from HME5 carrying $pINC_{SSD}$ (Fig. 1A, lane 3). In comparison, the failure to detect a PCR product for the reaction lacking a bacterial DNA template (lane 2) shows that PCR amplification of any DNA depends on the addition of a DNA template. If the recombinants contain the Δ*dnaC*::*cat* mutation, the PCR product should be about 1.1 kb in size. Of the isolates analyzed, the expected DNA fragment was observed in 13 recombinants. PCR analysis of three isolates revealed a DNA fragment of about 0.85 kb (lanes 6, 8, and 9), indicating that the chloramphenicol resistance cassette had not replaced the chromosomal *dnaC* gene and may have inserted elsewhere in the chromosome. No PCR product was detected in one isolate (lane 15), suggesting that PCR amplification failed. Because no other PCR products except those described above were detected in any of the reactions, the results confirm our expectation that the oligonucleotide primers are specific for the chromosomal *dnaC* locus. Hence, Fig. 1 shows only the relevant portion of the gel. Analogous PCR analysis confirmed the construction of the Δ*asnA*::*cat* strain (data not shown; see Materials and Methods).

We have moved the Δ*dnaC*::*cat* mutation into various strains by P1 transduction with reasonable transduction frequencies but only when a *dnaC* plasmid was present. As a representative example, we constructed the strain named KH1061 by trans-

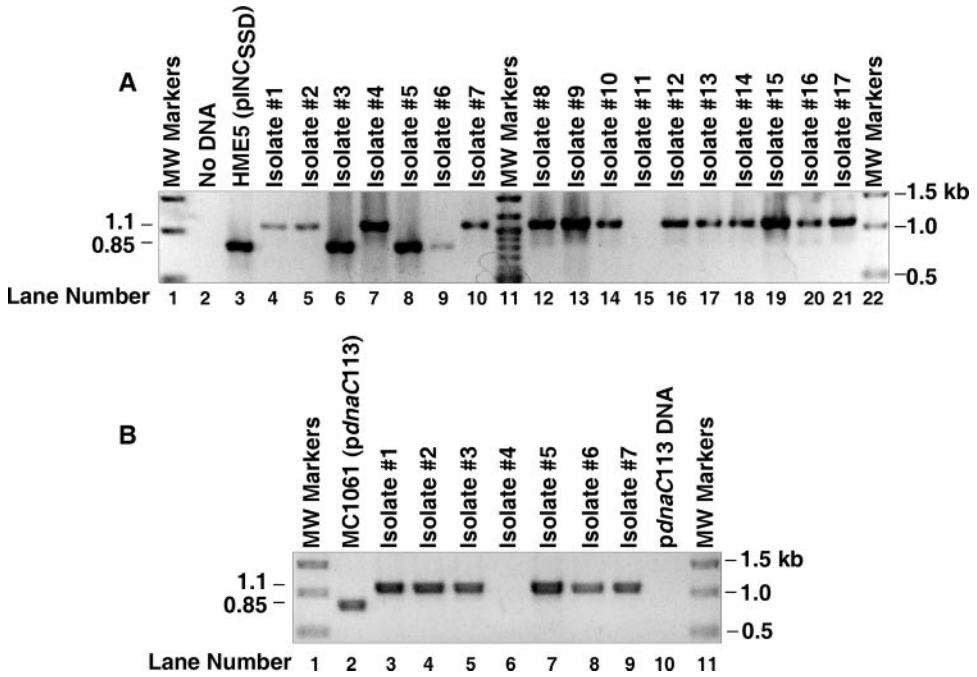

FIG. 1. Construction of a Δ*dnaC*::*cat* mutant. *E. coli* HME5 carrying the *dnaC* plasmid, $pINC_{SSD}$, was transformed as described in Materials and Methods with a DNA fragment encoding the *cat* gene, which is flanked by DNA that is homologous to the 5′ and 3′ ends of the *dnaC* gene. PCR analysis of genomic DNA isolated from individual chloramphenicol-resistant colonies was performed with oligonucleotide primers that are complementary to sequences in *yjjA* and *dnaT* that border the chromosomal *dnaC* locus. In panel A, the lanes at the extreme left and right contain DNA fragments whose sizes are indicated at the right of the figure. Lane 11 contains DNA fragments of 1.5, 1.2, 1.0, 0.9, 0.8, 0.7, 0.6, and 0.5 kb. A PCR lacking bacterial DNA corresponds to lane 2. Lanes 3 to 10 and 12 to 21 contain bacterial DNA from HME5 carrying $pINC_{SSD}$ or from the indicated chloramphenicol-resistant recombinants that also contain the *dnaC* plasmid. The estimated sizes of the amplified DNA are indicated at the left of the figure. In panel B, the Δ*dnaC*::*cat* mutation was transferred into *E. coli* MC1061(p*dnaC*113) by P1 transduction, using isolate 4 in panel A as the donor strain, followed by selection for chloramphenicol-resistant transductants. PCR analysis was performed as described for panel A either with bacterial DNA isolated from seven independent transductants, with bacterial DNA isolated from MC1061 carrying p*dnaC*113 (lane 2), or with p*dnaC*113 DNA (25 ng; lane 10). Lanes 1 and 10 contain DNA fragments whose sizes are indicated at the right of the figure. The estimated sizes of the amplified DNA are noted at the left.

ferring the Δ*dnaC*::*cat* mutation by P1 transduction from isolate 4 of Fig. 1A into *E. coli* MC1061 carrying p*dnaC*113. As a control, we used the strain lacking the *dnaC* plasmid and obtained chloramphenicol-resistant transductants only when the strain carried the *dnaC* plasmid. When we analyzed the bacterial DNA from seven randomly chosen transductants, we found that six contained the Δ*dnaC*::*cat* mutation, as indicated by the presence of a PCR product of about 1.1 kb (Fig. 1B, lanes 3 to 5 and 7 to 9). We did not detect a PCR product in lane 6; the reason for this negative result was not determined. By comparison, we observed a 0.85-kb DNA when we analyzed the genomic DNA isolated from MC1061 carrying p*dnaC*113 (lane 2) to correlate with the strain's $dnaC^+$ genotype. No amplified DNA was detected in the reaction containing the *dnaC* plasmid as the template for PCR analysis (lane 10), indicating that the oligonucleotide primers are specific for the chromosomal *dnaC* locus.

Isolation of novel *dnaC* mutations. To obtain mutations in *dnaC*, we used a derivative of pBR322 that requires IPTG for plasmid DNA replication. To explain the role of this inducer of the *lac* operon for plasmid maintenance, we first summarize the mechanism of pBR322 replication. After synthesis of the pBR322 primer transcript (RNA II), RNase H processes RNA II in the absence of a small antisense RNA (RNA I). DNA polymerase I then extends the primer transcript during the initial steps of plasmid DNA replication. Gil and Bouché have described pAM34, a pBR322 derivative that has its primer transcript regulated by the *lac* promoter region. The plasmid also encodes $lacI^Q$, which causes repression of the *lac* promoter in the absence of IPTG (14). Repression of the primer transcript leads to rapid loss of the plasmid such that only 0.1% of cells in culture contained the plasmid after 14 generations (less than an overnight incubation).

To confirm the properties of pAM34, we observed that the plasmid carrying the $dnaC^+$ gene (pAM34*dnaC*) complemented the Δ*dnaC* strain (KH1061) also bearing pACYC184 but only when the growth medium contained IPTG (Table 3). The transformation frequency was more than 10^5-fold lower in the absence of IPTG than in its presence. Thus, viability of the Δ*dnaC* strain depended on IPTG. With the strain originally bearing pAM34*dnaC*, we showed that it remained viable on medium lacking IPTG after selection for antibiotic-resistant transformants carrying pACYC184 encoding $dnaC^+$ but without selection for antibiotic resistance conferred by the resident pAM34*dnaC* plasmid. Apparently, the incoming plasmid complements the Δ*dnaC* strain when the resident plasmid is lost.

Interestingly, under the conditions that fail to maintain the resident pAM34*dnaC* plasmid, derivatives of $pINC_{SSD}$

TABLE 3. In absence of IPTG required to maintain the resident *dnaC* plasmid, pAM34*dnaC*, viability of *E. coli* KH1061 (Δ*dnaC*::*cat*) depends on separate *dnaC* plasmid

Plasmid	Relative plating efficiency[a]
pACYC184	$<2 \times 10^{-6}$
pACYC184*dnaC*	0.95
$pINC_{SSD}$	2.4×10^{-2}
p*dnaC*97 (Pro108Ser)	5×10^{-2}
p*dnaC*116 (Leu44Pro)	1.5×10^{-2}
p*dnaC*250 (Leu11Gln)	2.3×10^{-2}

[a] The indicated plasmids were transformed into KH1061 (Δ*dnaC*::*cat*) carrying the plasmid pAM34*dnaC*. Various dilutions of the transformation mixture were plated on LB medium lacking or supplemented with 0.5 mM IPTG and either tetracycline or kanamycin to select for the respective plasmids. Ampicillin was included in the medium containing IPTG to select for colonies carrying pAM34*dnaC*. Incubation was carried out at 37°C overnight. Relative plating efficiency is the ratio of the number of colonies detected in the absence of IPTG to that in its presence.

(p*dnaC*97, p*dnaC*116, and p*dnaC*250; Table 1) encoding mutant DnaCs with defects in binding to ATP or forming a complex with DnaB (20) complemented the Δ*dnaC* strain as effectively as the wild-type *dnaC* plasmid ($pINC_{SSD}$) (Table 3). These observations suggest that the primer transcript of these plasmids can act in *trans* by annealing to the homologous region of pAM34*dnaC* to prime plasmid DNA replication. Because of these results, we chose pACYC184 as the incoming plasmid to carry the DNA fragment encoding *dnaC*. To introduce mutations in the *dnaC* gene, we PCR amplified the DNA under error-prone conditions. Transformants obtained on medium supplemented with IPTG and antibiotics for both resident (pAM34*dnaC*) and incoming (pACYC184*dnaC*) plasmids were picked manually or with a robotic instrument and grown in microtiter plates. We then screened the isolates on solid medium supplemented with or lacking 0.5 mM IPTG, as described in Materials and Methods, but with selection for antibiotic resistance conferred by pACYC184 to identify those that either did not grow or grew poorly. At this step, we diluted the cultures 10^4-fold. One reason for the dilution is that we had found that the frequency of colony formation of KH1061 (Δ*dnaC*::*cat*) carrying pAM34*dnaC* was reduced by only about twofold at 0.05 mM IPTG and by (1 to 2) $\times 10^2$-fold at 0.005 mM IPTG. Thus, an undiluted culture or a lesser dilution contains an amount of IPTG that is almost sufficient to maintain the resident pAM34*dnaC* plasmid. A second reason for diluting the cultures was to increase the sensitivity of the assay. By reducing the number of viable bacterial cells of each culture ([0.5 to 1] $\times 10^9$/ml) to approximately 100 to 200 per screened sample of about 2 μl, we should be able to identify *dnaC* mutations that are partially active.

Characterization of the *dnaC* mutations by DNA sequence analysis and by relative plating efficiencies of plasmid-bearing strains. DNA sequence analysis of the pACYC184 derivatives from isolates identified in the genetic screen was then performed (Table 4). The results confirm the presence of *dnaC* mutations, substantiating the genetic method.

To characterize the plasmid-borne mutations genetically, we measured the relative plating efficiencies of strains initially

TABLE 4. Novel *dnaC* mutations and their phenotypes

Mutant plasmid	Nucleotide substitution(s)	Amino acid substitution(s)	Relative plating efficiency[a]	
			KH1061 (*recA*$^+$ Δ*dnaC*::*cat*)	MF1061 (*recA*::Kan^r Δ*dnaC*::*cat*
pACYC184*dnaC*	None	None	1.2	1.3
pACYC184	Not relevant	Not relevant	1.8×10^{-3}	1.2×10^{-5}
31-f3	G289A, T318A (silent)	Gly97Ser	1.0×10^{-3}	2.6×10^{-5}
157-c2	A428G	Lys143Arg	1.4×10^{-3}	1.3×10^{-5}
36-c4	G289A	Gly97Ser	2.1×10^{-4}	1.4×10^{-5}
29-d3	A534G (silent), G716T	Arg239Leu	1.6×10^{-4}	2.6×10^{-5}
55-c6	T425C, A521G	Met142Thr, Gln174Arg	7.7×10^{-5}	9.3×10^{-6}
97-h9	T129C (silent), A218G	Asn73Ser	2.0×10^{-4}	2.0×10^{-5}
107-g12	A40G, T178C	Met14Val, Ser60Pro	3.3×10^{-4}	2.8×10^{-5}
125-c1	G359A	Cys120Tyr	2.1×10^{-4}	7.9×10^{-6}
145-f1	T89C, A237G (silent), A394T, G565A	Leu30Pro, Ile132Phe, Asp189Asn	4.1×10^{-4}	2.0×10^{-5}
151-a2	T86C, A248G	Leu29Pro, Asn83Ser	2.2×10^{-4}	1.7×10^{-5}
239-e10	T178A, T213C (silent), A336G (silent), C504A (silent), A632G	Ser60Thr, Lys211Arg	3.1×10^{-4}	1.2×10^{-5}
164-g2	G82A, T207C (silent), A676T	Glu28Lys, Ser226Cys	6.0×10^{-4}	1.9×10^{-5}
40-g4	A71G, A107G, T189C (silent), T254A, A354T (silent), A400G, C483T (silent)	Lys24Arg, Gln36Arg, Leu85Stop, Thr134Ala	1.6×10^{-5}	1.1×10^{-5}
4-g1	A712T	Ser238Cys	1.2[b]	1.2[b]
38-e4	T68C, T207C (silent)	Phe23Ser	1.7[b]	1.2[b]
54-b6	A84T, T706A	Glu28Val, Tyr236Asn	1.0[b]	0.7[b]
234-h9	T358C, T686A	Cys120Arg, Val229Glu	1.4[b]	0.7[b]
35-b4	T650A	Val217Glu	6.1×10^{-6}	1.8×10^{-5}
246	T131C	Leu44Pro	4.6×10^{-6}	2.3×10^{-5}
256-f12	A451C, G659A	Thr151Pro, Arg220His	1.0×10^{-5}	2.1×10^{-5}

[a] Cultures of the indicated strains carrying pAM34*dnaC* and the pACYC184 derivative encoding the respective *dnaC* mutation(s) were serially diluted and plated as described in footnote *a* of Table 3, but the plating medium was additionally supplemented with chloramphenicol. Relative plating efficiency is defined in footnote *a* of Table 3.

[b] On medium lacking IPTG, the colonies were either heterogeneous in size or uniformly smaller (for Phe23Ser).

carrying both pAM34*dnaC* and the respective pACYC184 derivatives on media with and without IPTG (Table 4). The relative plating efficiency is the ratio of the number of colonies observed on medium lacking IPTG to the number of colonies observed on IPTG-supplemented media. This experiment utilizing strains with coresident plasmids differs from that for Table 3, which measures the relative plating efficiency after transformation to reflect the establishment of the incoming plasmid. We also compared isogenic *recA*$^+$ and *recA*::Kan^r strains to determine the effect of homologous recombination on the genetic method. Via DNA recombination, substitution of the mutant sequence carried in the multicopy pACYC184 derivative by the wild-type *dnaC* sequence from pAM34*dnaC* produces a mixed population of the pACYC184 derivative. If the mutation is recessive and insensitive to gene dosage, the bacterial cell encoding both the wild-type gene and the *dnaC* mutation should be phenotypically *dnaC*$^+$ in the absence of IPTG to select against pAM34*dnaC*. Alternatively, because DnaC function requires the assembly of the DnaB-DnaC complex with a stoichiometry of six DnaC monomers per DnaB hexamer, the formation of an active DnaB-DnaC complex should depend on the relative ratio of wild-type to mutant plasmid encoding a recessive mutation. Either circumstance may lead to a relative increase in colony formation depending on the frequency of DNA recombination with the *recA*$^+$ strain compared with the *recA* mutant when the pAM34*dnaC* plasmid has been lost. As for the behavior of dominant-negative mutations, we describe them below and in the Discussion.

With pACYC*dnaC* as a control, we observed essentially no difference in the relative plating efficiencies between the *recA*$^+$ and *recA*::Kan^r strains (Table 4). With pACYC184, the ratios of 1.8×10^{-3} and 1.2×10^{-5} for the *recA*$^+$ strain and the *recA* mutant, respectively, confirm the requirement of the IPTG-dependent plasmid for viability, but we were expecting comparable ratios for the two strains. We observed ratios within a twofold range (the range of error for the assay) with a separate isolate of the *recA* mutant originally bearing pACYC184 and pAM34*dnaC*, and with two other independent isolates of the *recA*$^+$ strain carrying pACYC184 and the *dnaC* plasmid, indicating that the results are reproducible (data not shown). For the *recA*$^+$ strain, we presume that the difference in the relative plating efficiency compared with the ratio given in Table 3 relates to the different methods. To attempt to explain the results with pACYC184, we considered the possibility that a single crossover event between pACYC184 and pAM34*dnaC* joins the two plasmids. Based on comparative DNA sequence analysis of pACYC184 and pAM34*dnaC*, the plasmids share short regions of homology in the replication origins and a longer region of 219 bp that corresponds to a DNA sequence downstream from the tetracycline resistance gene in pACYC184 (23, 30). The sequence remained in pAM34*dnaC* after the tetracycline resistance gene in the parental plasmid was replaced with a DNA fragment containing the *lacI*^{Q} gene and the *lac* promoter (14). If formed, the cointegrant would maintain the *dnaC*$^+$ gene by virtue of DNA replication from the pACYC184 replication origin to preserve viability on medium lacking IPTG, explaining the higher ratio for the *recA*$^+$ strain than for the *recA*::Kan^r mutant. Whereas the values for the *recA*$^+$ strain carrying plasmids 31-f3 and 157-c2 were within a twofold range of the ratio for the strain carrying pACYC184, supporting this interpretation, the *recA*$^+$ strain carrying most of the remaining *dnaC* mutant plasmids showed more greatly reduced ratios of plating efficiency compared with pACYC184 (Table 4, rows 5 to 15 and 20 to 22). These last results undermine the suggestion of cointegrate formation. Other exceptions are described below. We do not have an alternate explanation for the results obtained with pACYC184, but this lack of understanding does not weaken the method.

Comparing the relative plating efficiencies, the ratios for the *recA*$^+$ strain carrying most of the mutant *dnaC* plasmids are roughly one to two orders of magnitude greater than the ratios for the *recA* mutant bearing the respective plasmids (Table 4). Since homologous recombination should lead to a population of the pACYC184 derivative carrying both the wild-type *dnaC* sequence and the *dnaC* mutation, their proportion in individual cells and the associated phenotype depend on when recombination occurred in an earlier cell cycle or the present cycle and on the random probability of replicating the plasmid carrying wild-type *dnaC* compared to the mutant *dnaC* plasmid. In support, dissimilar ratios were observed for the *recA*$^+$ strain but not the *recA* mutant with two plasmids encoding a Gly97Ser substitution. The greater relative plating efficiencies in the *recA*$^+$ strain suggest the RecA-dependent formation of cointegrates and/or the substitution of the *dnaC* mutation by the wild-type *dnaC* sequence in these pACYC184 derivatives. However, the latter possibility appears to be infrequent, because DNA sequence analysis did not indicate the presence of both wild-type and mutant sequences (data not shown). In summary and despite the exceptions discussed below, the results in Table 4 indicate that homologous recombination does not interfere with the method.

Exceptions were observed with two groups of plasmids. One group encodes the Ser238Cys and Phe23Ser substitutions and the Glu28Val-Tyr236Asn and Cys120-Val229Glu double substitutions where the ratios are near 1 regardless of the host strain (Table 4). Relative to the colonies of the *recA*$^+$ strain carrying pACYC*dnaC*, which were similar in size on medium with or without IPTG, colony sizes for the other plasmid-bearing strains were comparable on medium supplemented with IPTG. On medium lacking IPTG, the colonies were either heterogeneous in size or uniformly smaller (for Phe23Ser), which suggests that these *dnaC* alleles encode partially active proteins that ineffectively support DNA replication, affecting colony size. Because we diluted the cultures prior to screening to improve the sensitivity of the assay, these observations support the conclusion that the genetic method can identify *dnaC* mutations that encode proteins having partial activity.

The second group encodes Val217Glu, Leu44Pro, and Thr151Pro Arg220His substitutions. The ratios for these plasmids in the *recA*$^+$ strain were lower by 34-fold, 20-fold, and 2-fold, respectively, than for the *recA* mutant (Table 4). For the latter plasmid, the difference is within the experimental error. The reduced plating efficiency caused by plasmids encoding the Val217Glu and Leu44Pro substitutions in the *recA*$^+$ strain relative to that of the *recA* mutant when pAM34*dnaC* has been lost suggests that these *dnaC* mutations are weakly dominant-negative compared with the wild-type *dnaC* gene in a mixture of plasmid molecules in a bacterial cell. Mutations that are strongly dominant-negative to *dnaC*$^+$ are not expected because they may be lethal.

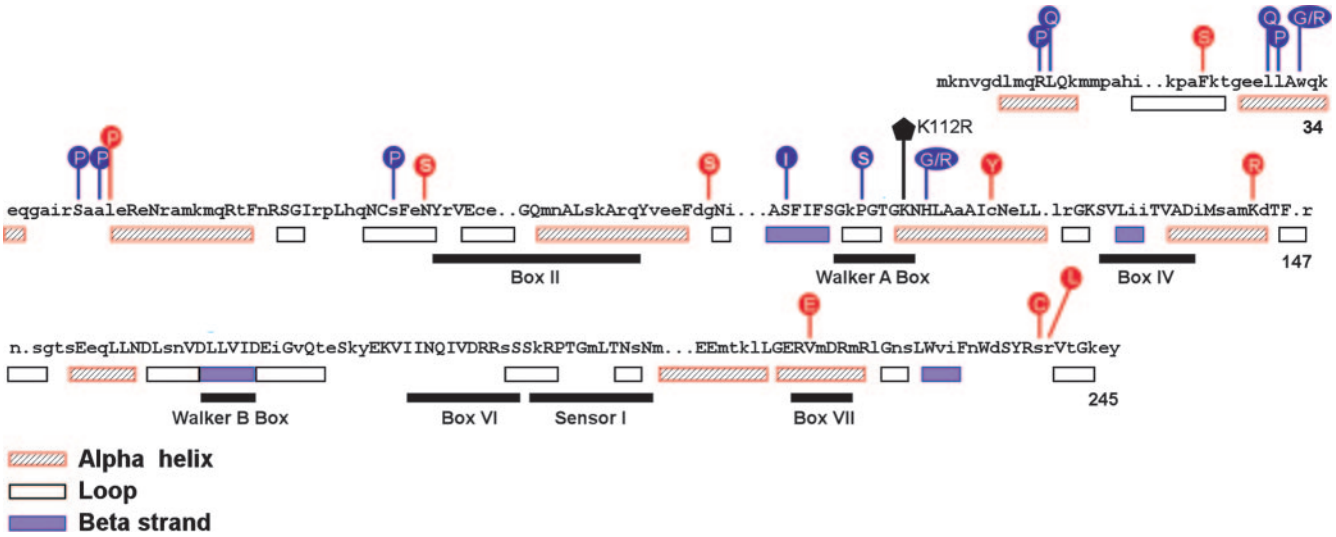

FIG. 2. Amino acid substitutions in mutant DnaC proteins. The amino acid sequence of *E. coli* DnaC shows conserved residues found in five of nine homologous DnaC proteins (uppercase letters) and gaps in the alignment (20). The numbers at the ends refer to the coordinates for the *E. coli* DnaC protein. Its predicted secondary structure is indicated by the open (loop), hatched (alpha-helix), or filled (beta-strand) boxes. Above the consensus sequence, the locations of the unique single amino acid substitutions in mutant DnaCs are also shown. Those in red were identified in the present work; substitutions in blue were previously described (20). The filled pentagon represents the Lys112Arg substitution encoded by pKH-*dnaCK112R* (9). AAA+ motifs are also shown. (Adapted from reference 20 with permission of the publisher.)

DISCUSSION

Method for analyzing functions of essential genes. In this work, we describe the construction of a null *dnaC* mutant and a method to identify *dnaC* mutations that disrupt or partially inactivate DnaC function. Our intention is to obtain a large collection of missense mutations from which we can identify those that are potentially interesting. Further study may reveal activities of DnaC that are poorly understood, such as the mechanism of the cooperative binding of DnaC to DnaB (12, 31), the significance of its interaction with single-stranded DNA (3, 9, 18), and its role in the entry of DnaB at the unwound region of *oriC* (9, 21).

The method can be adapted to the phenotypic analysis of site-directed mutations in other essential genes, but it is restricted to bacteria that can maintain pAM34 and its derivatives. Because of the abundance of structural and bioinformatics data on a variety of essential proteins, the approach permits the genetic analysis of specific amino acids to complement structure-function studies. It also can correlate in vivo effects with biochemical results.

Mutational analysis of *dnaC*. Lacking a three-dimensional structure for DnaC to provide a framework for correlating its biochemical activities, current structural information relies on cryoelectron microscopy (2, 24) and on sequence alignment of DnaC proteins from various bacteria (20). The latter analysis reveals motifs shared by AAA+ proteins (Fig. 2). As with other oligomeric AAA+ proteins that form a bipartite nucleotide binding pocket between adjacent monomers (reviewed in references 10 and 15), the Walker A box and other AAA+ motifs of DnaC are known or presumed to function in ATP binding or in coupling of the relative movement of domains with ATP hydrolysis, respectively. As direct evidence on the functional importance of the Walker A box motif, Davey et al. showed that a Lys112Arg substitution inhibited ATP binding (9). Based on this information, the mutant DnaC proteins in Table 4, with amino acid substitutions such as G97S, C120Y, and K143R near the Walker A box (Fig. 2), may be defective in ATP binding. The proviso on interpreting the mutant DnaCs is that these and other mutant proteins are as stable to proteases as wild-type DnaC in vivo. The Val217Glu substitution is in the box VII motif of AAA+ proteins. Because conserved residues in this element in other proteins are suggested to differentiate ATP from ADP (10, 15) or to form an oligomer (11, 26), we speculate that the substitution affects one or both activities.

Of the other single missense mutations in Table 4, those encoding the Phe23Ser and Leu44Pro substitutions were isolated previously using genetic selection to obtain mutant proteins that fail to interact with DnaB (20). Because the Leu44Pro substitution and others that map near the N terminus were shown biochemically to impair the binding of DnaC to DnaB, we speculate that Phe23Ser shares this defect. The Asn73Ser substitution alters a highly conserved residue, whereas the Ser238Cys and Arg239Leu substitutions near the carboxyl terminus (residue 245) change nonconserved residues. Because the function of these regions is unknown, the biochemical characterization of these mutant proteins may provide new insight.

Dominant-negative *dnaC* mutations. With two plasmids coresident in a null *dnaC* mutant, we were initially concerned that the assembly of mutant and wild-type DnaC to form an inactive $DnaB_6$-$DnaC_6$ complex might lead to a failure to duplicate the bacterial chromosome and inviability. If so, the genetic method might select against nonfunctional *dnaC* mutations. Fostering this concern, Davey et al. reported that combining the wild-type DnaC protein with increasing amounts of the mutant DnaC with the Lys112Arg substitution proportionally inhibited *oriC* plasmid replication (9). At an equivalent

ratio of mutant DnaC and DnaC$^+$, the DnaB-DnaC complex was inactive. To address this issue, we compared a plasmid encoding the Lys112Arg substitution (pHK-*dnaCK112R*) with the corresponding *dnaC*$^+$ plasmid (pHK-*dnaC*) (9) by introducing the DNAs into isogenic strains [MC1061 (*dnaC*$^+$) and KH1061 (Δ*dnaC*)]. Both strains carried p*dnaC113*, which encodes *dnaC* downstream from the *araBAD* promoter, and were examined under noninduced conditions. With both strains, we observed less than a twofold reduction in transformation frequency for the mutant *dnaC* plasmid compared with the *dnaC*$^+$ plasmid (K. Hupert-Kocurek and J. Kaguni, unpublished results). These findings indicate that the nonfunctional *dnaC* allele marginally affects viability. Thus, the genetic method should not yield a biased collection of *dnaC* mutations.

As a control, we showed that the *dnaC*$^+$ plasmid (pHK-*dnaC*) complemented a *dnaC2*(Ts) mutant (SS1020) at the nonpermissive temperature whereas the empty vector was inactive (Hupert-Kocurek and Kaguni, unpublished). Because the *dnaC* alleles of these plasmids are downstream from a T7 RNA polymerase promoter and the strains in the above experiments lack T7 RNA polymerase, read-through transcription from an upstream promoter apparently adequately complements the *dnaC2* mutant. If the cellular abundance of DnaC is comparable to the 10 to 20 DnaB monomers estimated per cell (27), an extremely low expression level appears to suffice.

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