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Isolation and characterization of temperature-sensitive mutants of the *Staphylococcus aureus dnaC* gene

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Abstract

A protein encoded by the *Staphylococcus aureus dnaC* gene has 44% and 58% homology with *Escherichia coli* DnaB and *Bacillus subtilis* DnaC replicative DNA helicases, respectively. We identified five mutant strains whose temperature-sensitive colony formation phenotypes were complemented by the *dnaC* gene. DNA replication in these mutants has a fast-stop phenotype, indicating that the *S. aureus dnaC* gene encodes the replicative DNA helicase required for the elongation step. These mutants were also sensitive to UV irradiation, suggesting that the *dnaC* gene is involved in DNA repair. The number of viable mutant cells decreased at a non-permissive temperature, suggesting that *S. aureus* DnaC helicase is a promising target for antibiotics providing bactericidal effects.

Keywords: *dnaC* helicase; Temperature-sensitive mutant; DNA replication; DNA repair; *Staphylococcus aureus*

1. Introduction

DNA helicases cut the hydrogen bonds between bases in double stranded DNA, which serve as a single strand DNA template for DNA polymerases [1–3]. In *Escherichia coli*, the DnaB protein is the helicase that is essential for both the initiation and elongation steps of DNA replication [4]. DnaB helicase is loaded on single strand DNA aided by DnaC protein, a helicase loader. In *Bacillus subtilis*, a Gram-positive bacterium, DnaC protein is a counterpart of the *E. coli* DnaB helicase [5]. There is no known protein that has a high homology with the *E. coli* DnaC protein, but DnaI protein is suggested to also function as a helicase loader [5,6].

Staphylococcus aureus is a Gram-positive bacterium, a major pathogen against humans. Clinical strains of *S. aureus* that acquire multi-drug resistance cause serious opportunistic diseases. Recently, whole genome sequences with 2.8 Mb of two methicillin-resistant *S. aureus* strains were determined [7], and the putative genes involved in DNA replication were revealed, showing differences in some aspects of DNA replication between *S. aureus* and *E. coli*. For example, *S. aureus* has no homologous proteins to *E. coli* Dam methylase [8], SeqA protein [8], Hda protein [9–11], or DnaC protein, which are involved in the initiation step of DNA replication [7]. In addition, we recently reported two distinct DNA polymerase genes, the *dnaE* and the *polC* genes, that are essential for DNA replication in *S. aureus*, although only the *dnaE* gene is essential in *E. coli* [12].

Genome sequence analysis has revealed dozens of proteins with a helicase motif in *S. aureus* [7]. Among these proteins, the PcrA protein, a homologue to both UvrD and Rep proteins of *E. coli*, is essential for cell growth, although this protein is not required for DNA replication [13]. Recently, the *dnaC* gene encoding a homologue to *E. coli* DnaB and *B. subtilis* DnaC was reported to be required for *S. aureus* cell growth using antisense methods [14]. In the present study, we isolated temperature-sensitive *dnaC* mutants of *S. aureus*, and examined the function of DnaC protein in DNA replication and in DNA repair.

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2. Materials and methods

2.1. Bacterial strains and plasmids

The temperature-sensitive *S. aureus* strains TS0021, TS0052, TS0685, TS0686, and TS1424 were obtained by treating RN4220 (kindly provided by Dr. Keiichi Hiramatsu, Juntendo University) with ethylmethanesulfate (EMS; Sigma Chemical Co., St. Louis, MO, USA) as described previously [12]. Plasmid pND50 (kindly provided by Dr. Matsuhisa Inoue, Kitasato University) is a shuttle vector containing pUB110 replication origins, which allows proliferation in *S. aureus*, and pUC19, which acts in *E. coli*. *E. coli* JM109 (obtained from Takara Shuzo, Tokyo, Japan) was used for subcloning of DNA into pND50 for complementation analysis. PSB1, PSB2, PSB3, or PSB4 plasmids were obtained by insertion of DNA fragments, which was amplified by PCR using chromosomal DNA of RN4220 as a template, into the *Sma*I site in pND50. Subcloning of DNA for sequencing was performed with pBluescript II SK(+) (Stratagene, La Jolla, CA, USA) and *E. coli* JM109.

2.2. Culture of bacteria

S. aureus strains were cultured in Luria–Bertani (LB) medium (1% bactotryptone (BD, Sparks, MD, USA), 0.5% yeast extract (BD, Sparks, MD, USA), and 1% NaCl). Transformants of *S. aureus* and *E. coli* harboring pND50 or its derivatives were cultured in LB medium supplemented with 12.5 μg ml^{-1} chloramphenicol. Temperature-sensitive phenotypes of growth and of DNA replication were examined in NaCl-depleted LB medium. Transformants of *E. coli* containing pBluescript II SK(+) for sequencing were cultured in LB medium with 50 μg ml^{-1} ampicillin.

2.3. Genetic engineering of S. aureus

Competent cells of *S. aureus* strains were prepared as described previously [12]. DNA (1 μg) and the competent cells (22 μl) were mixed and placed in a 0.2-cm cuvette (Bio-Rad, Hercules, CA, USA), and electroporation was performed (100 Ω, 25 μF, 2.5 V). Plasmid DNAs were extracted from *S. aureus* cells [11] and the *E. coli* strain JM109 was used for amplification and purification of the plasmids used for DNA sequencing.

2.4. Measurement of DNA and protein synthesis in S. aureus

Incorporation of radiolabeled thymidine or methionine into acid-insoluble fractions was monitored as described previously [12]. Pulse-labeling experiments were performed as follows. An overnight cell culture (100 μl) in a synthetic medium (0.05 mol l^{-1} potassium phosphate buffer (pH 7.3), 1 mmol l^{-1} $MgSO_4$, 20 mmol l^{-1} $(NH_4)_2SO_4$, 2 μmol l^{-1} $Fe(NH_4)(SO_4)_2$, 1% glucose, 0.5% casamino acids, 3 μg ml^{-1} thymine, 6.25 ng ml^{-1} biotin, 3.1 μg ml^{-1} sodium pantothenate, and 1.55 μg ml^{-1} nicotinic acid) was diluted in the same synthetic medium (5 ml), cultured at 30°C until cell growth reached the exponential phase, and then the temperature was shifted to 43°C. Aliquots (500 μl) were added to 4.38 μCi of [*methyl*-^{3}H]thymidine, and then incubated at 43°C for 3 min. Radioactivity incorporated into acid-insoluble fractions was measured in a liquid scintillation counter.

2.5. PCR and DNA sequencing

DNA fragments containing the wild-type *dnaC* gene were prepared by PCR methods and used for complementation analysis of the temperature-sensitive mutants. Primers designed to sandwich the region of the *dnaC* gene were 5′-TAACAATGGAAGCACTTGGTGGCGGTG-3′ and 5′-CCAATATCGAGACCATAATGCTAATA-3′ (PSB1), 5′-TACACAAGATGTTAAAGGTAAAGGTA-3′ and 5′-CCAATATCGAGACCATAATGCTAATA-3′ (PSB2), and 5′-TAACAATGGAAGCACTTGGTGGCGGTG-3′ and 5′-CGCAGTCTTACCTACAGATGGACG-3′ (PSB3). PCR was performed using *Pfu* polymerase (Stratagene, La Jolla, CA, USA) with chromosomal DNA from RN4220 as a template.

dnaC gene sequences of the temperature-sensitive mutants and RN4220, the parent strain, were determined using the ABI Prism Bigdye terminator cycle sequencing protocol (Applied Biosystems, Foster City, CA, USA) as described previously [12]. Sequence data were registered in GenBank under accession No. AB054590 (*dnaC* gene).

2.6. Examination of UV sensitivity

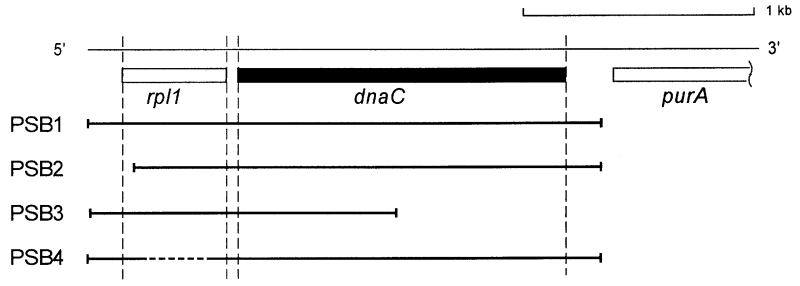
An overnight culture of the bacterial strain (10 μl) was diluted to 1 ml in LB medium, and cultured at 30°C to the exponential growth phase ($OD_{660} = 0.1$). The culture was shifted to 43°C for 10 min and the diluted aliquots (10^{-5}–10^{-3}-fold) were plated on LB agar plates. The plates were immediately irradiated with UV followed by incubation at 43°C for 3 h, and then at 30°C for 15 h. The number of colonies was then counted.

3. Results

3.1. Complementation of temperature-sensitive phenotype by the dnaC gene

We isolated more than 200 strains of temperature-sensitive mutants from 2×10^5 colonies of *S. aureus* RN4220 treated with EMS [12]. These mutants formed colonies on an LB agar plate at 30°C, but not at 43°C. Among them, we identified 17 mutants whose incorporation of

A



B

Number of transformants x 10^{-4} (30℃/43℃)					
	Strain				
Plasmid	TS0021	TS0052	TS0685	TS0686	TS1424
PSB1	0.7 / 1.0*	0.3 / 0.3	0.6 / 0.9	1.3 / 1.5	1.0 / 1.5
PSB2	0.2 / 0.2*	0.1 / 0.02	0.2 / 0.2	0.9 / 0.002	0.2 / 0.2
PSB3	0.9 / <0.001*	0.2 / 0.001	1.0 / 0.05	1.3 / 0.003	1.5 / <0.001
PSB4	-**	0.1 / 0.1	-**	0.7 / 0.7	-**
pND50	3.0 / <0.001*	0.1 / <0.001	1.0 / <0.001	2.5 / <0.001	1.2 / <0.001

*48h incubation

-**; not determined

Fig. 1. A: Schematic representation of the *dnaC* gene region of *S. aureus*. Open and filled boxes show open reading frames. The direction of transcription is from left to right for the three open reading frames presented. The filled box indicates the *dnaC* gene. The thick lines under the boxes indicate the DNA region amplified by PCR that was inserted into plasmids PSB1, PSB2, PSB3, and PSB4. The thick dotted line in PSB4 indicates the deleted DNA region. B: Complementation of temperature-sensitive mutants by a plasmid containing the *dnaC* gene. Plasmids were transformed into five temperature-sensitive DNA replication mutants. The colony numbers at 30°C and 43°C on LB agar plates containing chloramphenicol are presented. Data represent mean numbers of colonies obtained from three different experiments when 22 μl of competent cells and 1 μg of DNA were used.

[^{3}H]thymidine into acid-insoluble fractions was inhibited at 43°C, but that of [^{35}S]methionine was not inhibited (data not shown). These mutants were considered to be temperature-sensitive DNA replication mutants.

DnaC protein in *S. aureus*, whose amino acid sequence has 44% and 58% homology with *E. coli* DnaB and *B. subtilis* DnaC replicative DNA helicases, respectively, was identified by the *S. aureus* genome project [7]. Based on the sequence information of the *S. aureus* genome, we designed primers and amplified the *dnaC* gene from RN4220. The fragment was inserted into the *Sma*I site of pND50, resulting in PSB1 (Fig. 1A). During cloning of the *dnaC* gene in the RN4220 strain, we found that glutamic acid 356 of DnaC protein encoded in the N315 and Mu50 strains was changed to Lys (GenBank accession No. AB054590). Complementation analysis of the 17 isolated temperature-sensitive DNA replication mutants indicated that the temperature-sensitive phenotype of TS0021, TS0052, TS0685, TS0686, and TS1424 was suppressed by PSB1 (Fig. 1B). We further constructed PSB3, in which the 248 amino acid residues were deleted from the C-terminal end of the DnaC protein (Fig. 1A), and electroporated it into these five mutants. The results indicate that the temperature-sensitive phenotypes of these five mutants were not complemented by PSB3.

To determine whether the *rpl1* gene, which encodes 50S ribosomal protein L9 in the PSB1 plasmid, is necessary for complementation, we constructed PSB2 in which the N-terminal 18 bp of the *rpl1* ORF (444 bp) was deleted from PSB1 (Fig. 1A), and transformed it into these five mutants. The temperature-sensitive phenotypes of TS0021, TS0685, and TS1424 were complemented by PSB2 at the same level as by PSB1, whereas the complementation efficiency of PSB2 for TS0052 and TS0686 was much less than that of PSB1 (Fig. 1B). Considering the possibility that the decreased efficiency was caused by decreased expression of the *dnaC* gene at PSB2, we then constructed PSB4 in which the middle region of *rpl1* (230 bp, the region between the *Bst*PI site and the *Bln*I site) was deleted (Fig. 1A), and transformed it into TS0052 and TS0686. The temperature-sensitive phenotypes of TS0052 and TS0686 were efficiently complemented by PSB4 at the same level as by PSB1. These results indicate that the temperature-sensitive phenotypes of these five mutants are complemented by the *dnaC* gene.

3.2. Identification of mutations in the dnaC gene of mutant strains

Next, we determined whether mutations occur in the chromosomal *dnaC* gene of TS0021, TS0052, TS0685, TS0686, and TS1424. Sequencing analysis indicated that each mutant has only one mutation site, resulting in a substitution of an amino acid within the coding region

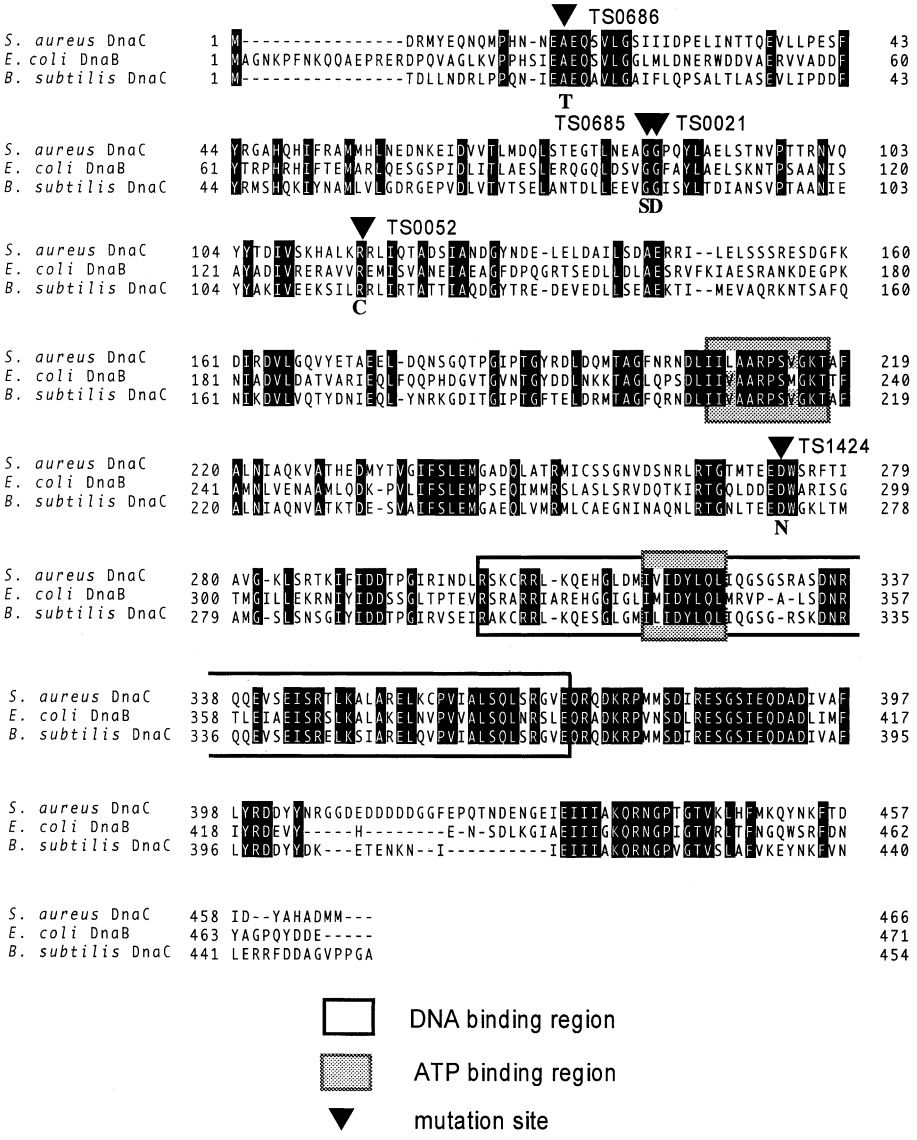


Fig. 2. Mutation sites of the temperature-sensitive mutants in the *dnaC* gene. Amino acid sequences of *S. aureus* DnaC, *E. coli* DnaB, and *B. subtilis* DnaC are aligned. White characters indicate identical amino acid residues within the three species. The DNA binding and ATP binding regions in *E. coli* DnaB are indicated by the open and shaded boxes, respectively. Mutation sites identified by sequencing are indicated by arrowheads, and the substituted amino acid residues are indicated by bold letters at the bottom.

of the *dnaC* gene. As shown in Fig. 2, TS0686, TS0685, TS0021, TS0052, and TS1424 have amino acid substitutions of Ala16Thr, Gly84Ser, Gly85Asp, Arg116Cys, and Asp273Asn, respectively, via transition mutations from G:C to A:T (GCT to ACT, GGT to AGT, GGC to GAC, CGT to TGT, and GAT to AAT, respectively).

3.3. DNA replication stops immediately in the dnaC mutants

We examined DNA replication of these temperature-sensitive mutants after increasing the temperature to the non-permissive temperature using continuous labeling experiments with [^{3}H]thymidine. The incorporation of [^{3}H]thymidine into acid-insoluble fractions by RN4220 continued after shifting the temperature from 30°C to 43°C, whereas those by TS0021, TS0052, TS0685, and TS0686 stopped immediately after the increase in temperature (Fig. 3). [^{3}H]Thymidine incorporation in TS1424 continued for 60 min after the temperature shift and then stopped. We then tested whether DNA synthesis in these mutants would have a fast-stop phenotype by pulse labeling with [^{3}H]thymidine. DNA synthesis was blocked within 5 min by the addition of 100 μg ml^{-1} norfloxacin, whereas it continued for 15 min and then decreased following the addition of 100 μg ml^{-1} chloramphenicol (Fig. 4). The former inhibits the swivelase activity of DNA gyrase, which is required for progression of the replication fork, whereas chloramphenicol inhibits protein synthesis leading to an inhibition of the initiation of DNA replication [15]. The rate of DNA synthesis in TS0021, TS0052, TS0685, and TS0686 decreased to less than 30% within

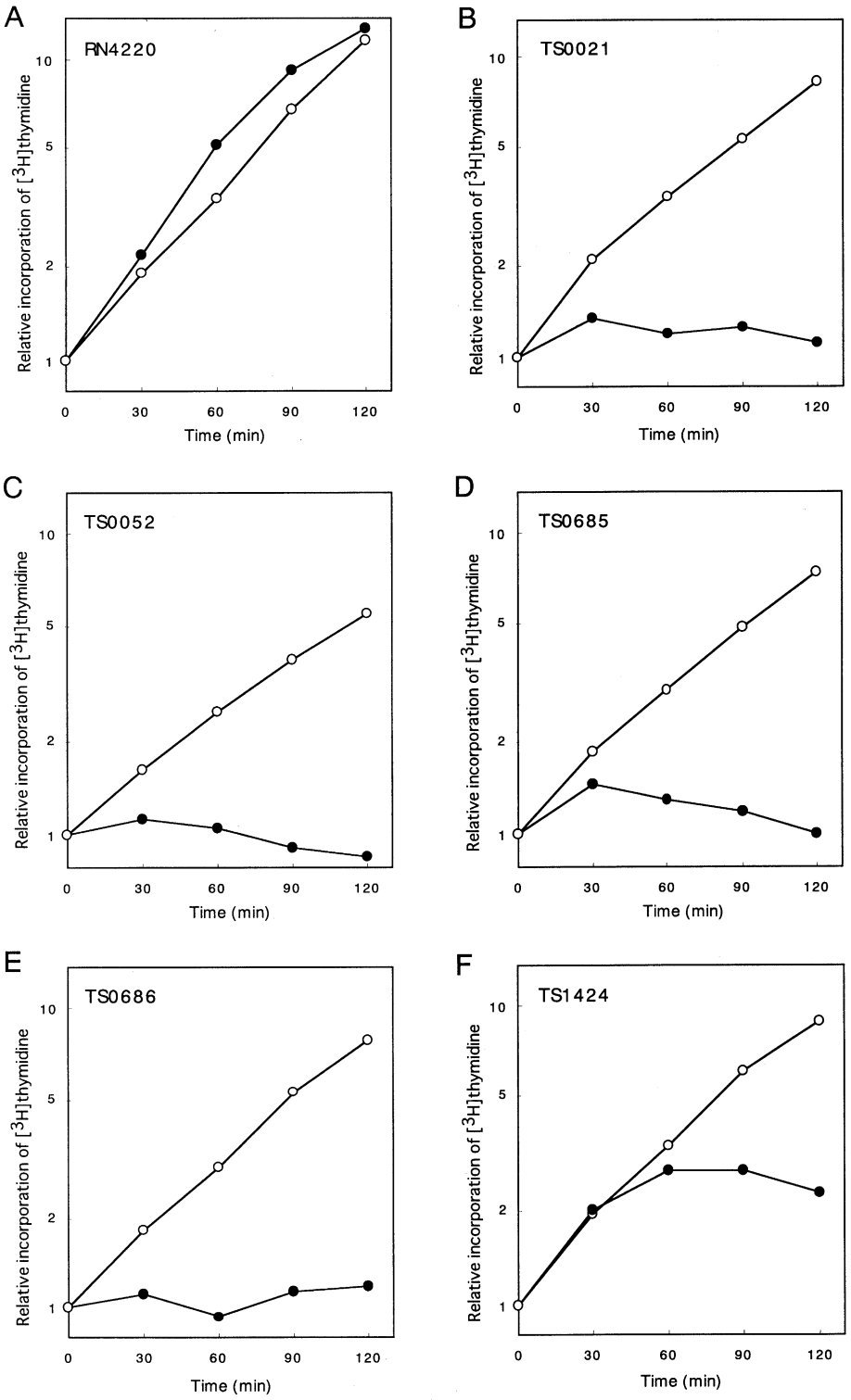


Fig. 3. DNA synthesis in the temperature-sensitive *dnaC* mutants. Exponentially grown cell cultures (OD_{660} = 0.1) pre-labeled for two to three cycles of cell division at 30°C were shifted to 43°C (●) or 30°C (○), and incorporation of [^{3}H]thymidine into the acid-insoluble fraction was measured. The vertical axis indicates the relative values of the [^{3}H]thymidine incorporation plotted on a log scale. A: RN4220; B: TS0021; C: TS0052; D: TS0685; E: TS0686; F: TS1424. Incorporated values at time zero are 959, 610, 1397, 1555, 436, and 847 cpm, respectively.

15 min after the increase in temperature, indicating fast-stop phenotypes for DNA synthesis in these mutants (Fig. 4). In TS1424, incorporation of [^{3}H]thymidine continued for 90 min after the temperature shift (data not shown).

3.4. UV sensitivity of the dnaC mutants

Recent studies revealed that DnaB helicase in *E. coli* is involved in DNA recombination repair [16,17]. We ex-

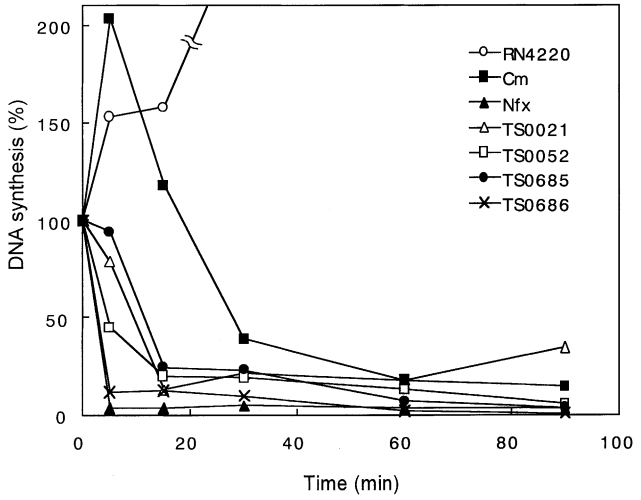


Fig. 4. The fast-stop phenotype of DNA synthesis in the *dnaC* mutants. Cultures grown exponentially (OD_{660} = 0.1) at 30°C were shifted to 43°C and incorporation of [^{3}H]thymidine for 3 min was measured at the indicated time. Relative percentage values of incorporation are shown. The 100% values were 268 279 (RN4220), 93 829 (TS0021), 174 457 (TS0052), 180 258 (TS0685), and 310 158 (TS0686) cpm, respectively.

amined whether temperature-sensitive *dnaC* mutants of *S. aureus* had a UV-sensitive phenotype. Exponentially growing cells were irradiated with UV, and further incubated at 43°C for 3 h, and at 30°C for 15 h. Viable cell numbers of four mutants, TS0021, TS0052, TS0686, and TS1424, decreased after exposure to UV, whereas transformation with PSB1 suppressed the UV-sensitive phenotype (Fig. 5). These results suggest that mutations in the *dnaC* gene are responsible for the UV-sensitive phenotype, although we cannot exclude the possibility that the sensitivity to UV irradiation in the case of the TS0686 mutant is an indirect effect caused by the decreased survival of TS0686/pND50 after incubation at 43°C.

3.5. Decrease in cell viability of the dnaC mutants at a non-permissive temperature

To determine if the DnaC protein is a valuable target of antibiotics with a bactericidal effect, it is important to first determine whether inactivation of the DnaC protein causes cell death. Viable cell numbers of the *dnaC* mutants after the temperature shift were examined. The number of viable cells of TS0052, TS0685, TS0686, and TS1424 decreased at 43°C, whereas those of wild-type RN4220 continued to increase under these conditions (Fig. 6). Those of TS0021 did not decrease after 12 h at 43°C. The difference of viability at 43°C among the mutants might occur due to the different effect of the individual amino acid substitution mutations with respect to the association kinetics of DnaC protein with each other or with other proteins.

4. Discussion

The *S. aureus dnaC* gene encodes a homologue of *E. coli* DnaB and *B. subtilis* DnaC replicative DNA helicases. We

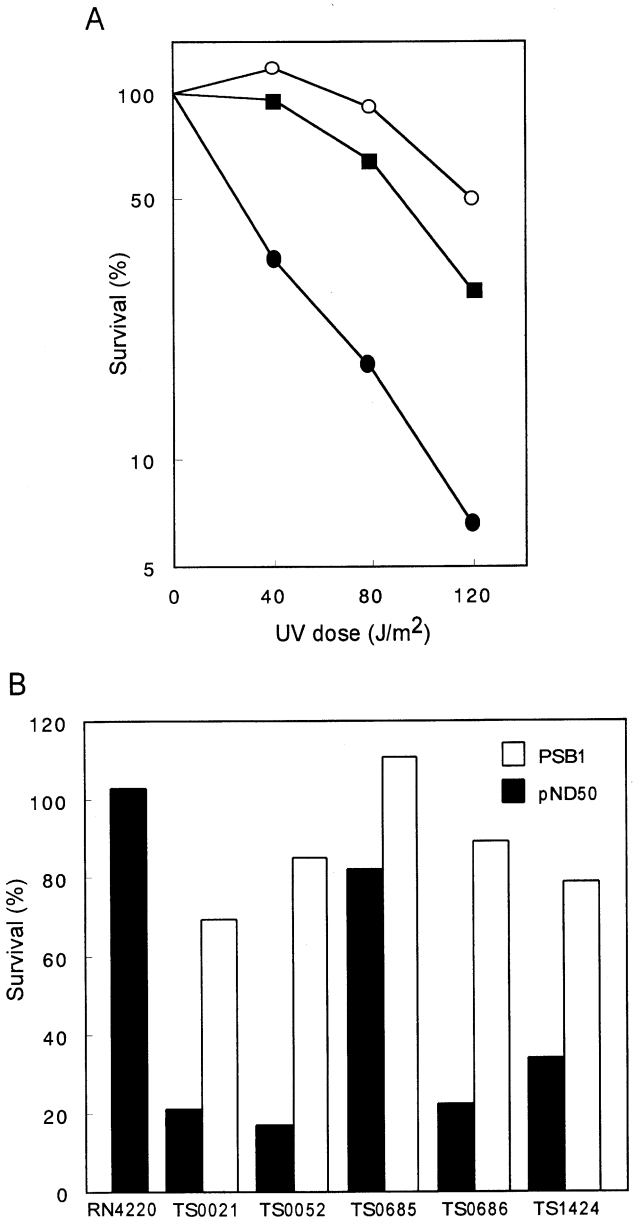


Fig. 5. UV sensitivity of the *dnaC* mutants. A: UV sensitivity of TS0686. Cultures grown exponentially (OD_{660} = 0.1) at 30°C were shifted to 43°C for 10 min and the cells were irradiated with various dosages of UV and further incubated at 43°C for 3 h. The viable cell numbers were examined followed by incubation at 30°C for 15 h. ●, TS0686/pND50; ■, TS0686/PSB1; ○, RN4220/pND50. The 100% values were 8.0×10^5, 8.9×10^7, and 1.2×10^8 cells ml^{-1}, respectively. B: UV sensitivity of the *dnaC* mutants. The decrease in viable cell numbers after UV irradiation (65.5 J m^{-2}) of the *dnaC* mutants harboring PSB1 or pND50 was examined. Relative percentage values of the viable cell numbers to those without irradiation are presented. The 100% values were 1.2×10^8 (RN4220/pND50), 7.9×10^7 (TS0021/pND50), 7.1×10^7 (TS0021/PSB1), 9.4×10^7 (TS0052/pND50), 9.8×10^7 (TS0052/PSB1), 1.4×10^8 (TS0685/pND50), 1.0×10^8 (TS0685/PSB1), 9.4×10^5 (TS0686/pND50), 8.9×10^7 (TS0686/PSB1), 1.1×10^8 (TS1424/pND50), and 9.3×10^7 (TS1424/PSB1) cells ml^{-1}, respectively.

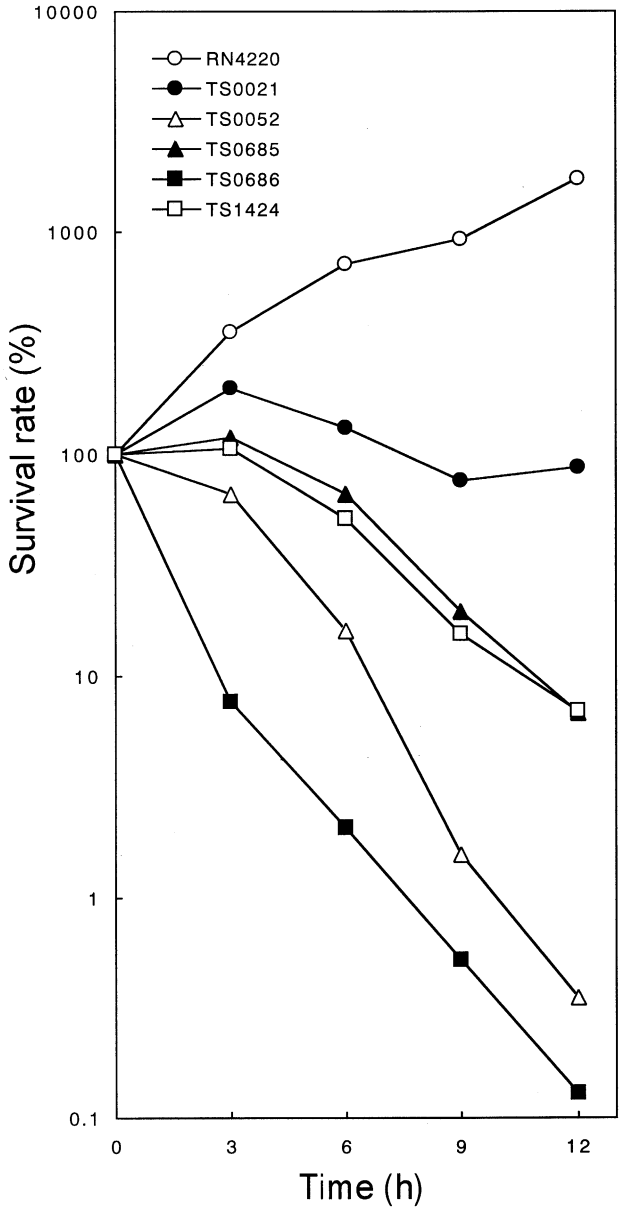


Fig. 6. Decrease in viable cell numbers of the *dnaC* mutants at the non-permissive temperature. Cultures grown exponentially ($OD_{660} = 0.1$) at 30°C were shifted to 43°C and the viable cell numbers were examined [12]. Relative percentage values of the viable cell numbers are presented. The 100% value was approximately 5.0×10^8 cells ml^{-1} in each mutant.

isolated five temperature-sensitive DNA replication mutants of *S. aureus* whose temperature-sensitive colony formation phenotypes were complemented by the *dnaC* gene. DNA replication measured by the incorporation of [^{3}H]thymidine in these mutants stopped immediately at 43°C, whereas protein synthesis continued. We further identified the mutation sites of these mutants, which cause one amino acid substitution within the *dnaC* gene. The results demonstrate that the DnaC helicase is essential for DNA replication in *S. aureus*.

The amino acid sequence of the DnaC protein of *S. aureus* (466 amino acids) has 44% identity with the *E. coli* DnaB and 58% identity with the *B. subtilis* DnaC (Fig. 2). The ATP binding and DNA binding regions identified in *E. coli* DnaB protein [18] are well conserved in the DnaC protein of *S. aureus* (Fig. 2). Four mutation sites were identified in the N-terminal region of *S. aureus* DnaC protein (Ala16Thr, Gly84Ser, Gly85Asp, Arg116Cys). Because the N-terminal region of *E. coli* DnaB is responsible for the helicase activity that is essential for DNA replication [18], helicase activity might be inactivated in these four mutants at the non-permissive temperature. The mutation site in TS1424 (Asp273Asn) is located between the two ATP binding motifs in the DnaC protein. This amino acid residue might contribute to the three-dimensional structure needed for hydrolysis of ATP, which is essential for helicase activity.

DNA replication in the temperature-sensitive *dnaC* mutants of *S. aureus* had a fast-stop phenotype (Fig. 4), indicating that the *S. aureus* DnaC protein is involved in the elongation step of DNA replication. Most temperature-sensitive mutants of the *E. coli dnaB* gene and of the *B. subtilis dnaC* gene have a fast-stop phenotype for DNA replication, whereas others have a slow-stop phenotype [3,19,20]. Therefore, these helicases might function at both the initiation and elongation steps of DNA replication. Among the *dnaC* mutants isolated in this study, TS1424 showed a residual DNA synthesis phenotype (Fig. 3) which led us to assume that *S. aureus* DnaC is involved in the initiation step of DNA replication.

In Gram-positive bacteria, there is little direct evidence that the DNA helicase responsible for DNA replication is involved in DNA repair. The present study demonstrated that the temperature-sensitive *dnaC* mutants are sensitive to UV irradiation (Fig. 5). This finding suggests that DnaC protein is involved in DNA repair. There are three distinct repair pathways for UV damage: photorepair, nucleotide excision repair, and recombination repair. *E. coli* DnaB helicase is thought to be involved in restarting the replication fork in recombination repair [16]. Progression of the replication fork stops at a thymine dimer formed by UV irradiation, resulting in a double strand break. A double strand break is mainly recovered by recombination repair. Thus, DnaC helicase in *S. aureus* might also be involved in recombination repair to protect cells from UV irradiation damage.

Viable cell numbers of four temperature-sensitive *dnaC* mutants decreased after the temperature was increased to 43°C (Fig. 6). Double strand breaks are formed in the temperature-sensitive *dnaB* mutants of *E. coli* at a non-permissive temperature [21]. Thus, double strand breaks might be formed in the temperature-sensitive *dnaC* mutants of *S. aureus* at 43°C. Our results suggest that inhibitors targeting the *dnaC* gene product might decrease viable cell numbers, resulting in bactericidal effects. DnaC protein inhibitors might kill bacteria in a synergistic manner with DNA damaging agents, which are provided by host immune systems. Therefore, we suggest that DnaC

protein is a promising target for new antibiotics against methicillin-resistant *S. aureus*, which causes serious clinical problems.

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