dnaB and *dnaI* temperature-sensitive mutants of *Staphylococcus aureus*: evidence for involvement of DnaB and DnaI in synchrony regulation of chromosome replication

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DnaB and DnaI proteins conserved in low-GC content Gram-positive bacteria are apparently involved in helicase loading at the replication initiation site and during the restarting of stalled replication forks. In this study, we found five novel *dnaB* mutants and three novel *dnaI* mutants by screening 750 temperature-sensitive Gram-positive *Staphylococcus aureus* mutants. All of the mutants had a single amino acid substitution in either DnaB or DnaI that controlled temperature-sensitive growth, as confirmed by transduction experiments using phage 80 α . DNA synthesis as measured by [³H]thymine incorporation, origin-to-terminus ratios and flow cytometric analysis revealed that the *dnaB* and *dnaI* mutants were unable to initiate DNA replication at restrictive temperatures, which is similar to previous findings in *Bacillus subtilis*. Furthermore, some of the mutants were found to exhibit asynchrony in the initiation of DNA replication. Also, a fraction of the *dnaI* mutant cells showed arrested replication, and the *dnaI* mutant tested was sensitive to mitomycin C, which causes DNA lesions. These results suggest that DnaB and DnaI are required not only for replication initiation and but also for regulation of its synchrony, and they provide support for the involvement of DnaI activity in the restart of arrested replication forks *in vivo*.

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INTRODUCTION

The timing and synchrony of bacterial chromosome replication is strictly regulated at the initiation step. Initiation occurs only when the bacterium reaches initiation mass, a specific cell mass per chromosomal origin. To allow initiation, multiple origins within a bacterial cell are activated to open simultaneously once per cell cycle.

DnaA initiates DNA replication, binds to *oriC*, and mediates opening of the DNA strand. Control of the activity and amount of DnaA plays a critical role in the regulation of replication timing in *Escherichia coli* (Messer, 2002). Synchrony in *E. coli* cells is regulated by genes whose products have the common ability to associate with *oriC*

with involvement of *dnaA*, *dnaC*, *dam*, *seqA*, *hobH* and *fis* (Kornberg & Baker, 1992). Mutation of the *recA* and *rpoC* genes and the *datA* region for DnaA titration cause asynchrony.

There are significant differences in the sets of genes involved in replication initiation between Gram-positive *Bacillales*, such as *Bacillus subtilis* and *Staphylococcus aureus*, and the Gram-negative *E. coli*. Three priming proteins found in *Bacillales*, DnaD, DnaB and DnaI (which may load the DnaC helicase), have no homologues in *E. coli* (Bruand & Ehrlich, 1995; Bruand *et al.*, 1995; Moriya *et al.*, 1999; Ogasawara *et al.*, 1986). In addition, the *B. subtilis* YabA protein, which acts as a negative regulator for initiation at the *B. subtilis* *oriC*, has no homologue in Gram-negative *E. coli*. Conversely, SeqA, Dam and Hda, three *E. coli* proteins that negatively regulate the initiation of DNA replication, are absent in the genomes of the Gram-positive bacteria *B. subtilis* and *S. aureus*. Thus, the Gram-positive bacteria possess an alternative system for initiation control, although the molecular details remain unknown.

Both DnaB and DnaI, which are encoded in a single operon (Bruand & Ehrlich, 1995), are essential for the initiation of

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Abbreviation: EMS, ethylmethane sulfonate.

DNA replication in *B. subtilis* (Karamata & Gross, 1970; Mendelson & Gross, 1967). These two proteins interact directly but independently with the replicative DnaC helicase and are thought to deliver DnaC helicase to targeted regions of DNA. At these sites, DnaI acts as a helicase loader, whereas DnaB serves as an accelerator of loading (Imai *et al.*, 2000; Ioannou *et al.*, 2006; Soultanas, 2002; Velten *et al.*, 2003). In *B. subtilis*, DnaB and DnaI, together with PriA and DnaD, are required for primosome assembly, which is needed to restart replication (Bruand *et al.*, 2001, 2005). The *dnaB* gene is unique because its temperature-sensitive mutants simultaneously lose the attachment of *oriC* to the cell membrane and the ability to initiate a new round of replication after an increase to the restrictive temperature (Rokop *et al.*, 2004; Winston & Sueoka, 1980). DnaI is an AAA⁺ family protein (Neuwald *et al.*, 1999), with an N-terminal domain that can interact with replicative helicases and a C-terminal domain that contains ATPase and cryptic DNA-binding activities (Ioannou *et al.*, 2006). This protein is known to be a target of bacteriophage protein-mediated inhibition of DNA replication in *S. aureus* (Liu *et al.*, 2004). Despite this information, the exact function of DnaB and DnaI in replication initiation and its control are uncertain.

S. aureus is a clinically important Gram-positive pathogenic bacterium. Comparison of the genomic sequences of *S. aureus* and *B. subtilis* reveals the presence of similar gene sets for DNA replication. In fact, many *S. aureus* plasmids can replicate in *B. subtilis* (Ehrlich, 1977). We have recently characterized several DNA replication mutants of *S. aureus* (Inoue *et al.*, 2001; Kaito *et al.*, 2002; Li *et al.*, 2004; Murai *et al.*, 2006). Herein, we report the isolation and characterization of five *dnaB* and three *dnaI* mutants in *S. aureus*. Our results suggest that DnaB and DnaI are required for the growth of staphylococcal cells. The results agree with the previous study in *B. subtilis* showing that these proteins are required for the initiation of chromosome replication. Our study suggests for the first time that *dnaB* and *dnaI* are involved in synchrony regulation of chromosome replication. The results also provide novel *in vivo* evidence that DnaI participates in the restart of replication from stalled replication forks.

METHODS

Bacterial strains and plasmids. The *S. aureus* strains used in this study are listed in Table 1. Temperature-sensitive strains of *S. aureus* were isolated by mutagenesis of RN4220 (Novick *et al.*, 1993) with ethylmethane sulfonate (EMS; Sigma), as described previously (Inoue *et al.*, 2001). Strains in which the erythromycin-resistant marker on pMutinT3 was integrated near the *dnaB* and *dnaI* operon were used as donors for phage transduction experiments (Table 1). In brief, the region between SA1505 and SA1506 (chromosomal location 1713953 to 1714765 on the *S. aureus* N315 genome) was amplified with primers 5'-GCCCCGAATTCTCACGCATAATCAACCCTGT-3' and 5'-GCCCCGATCCTCAAACGCGTTGTAACATG-3'. We used the complete sequence of the *S. aureus* N315 genome (Kuroda *et al.*, 2001) registered in the Genome Information Broker program in the DNA Data Bank of Japan (<http://gib.genes.nig.ac.jp/single/index>).

php?spid=Saur_N315). The amplified fragment was digested with *EcoRI* and *BamHI*, and cloned into the *EcoRI* and *BamHI* sites of pMutinT3, a suicide vector for *S. aureus* with a replication origin that functions in *E. coli* (Vagner *et al.*, 1998). The resulting plasmid, pMBI, was integrated into parent or mutant strains via homologous recombination, as confirmed by Southern blotting. *E. coli* JM109 (Takara Bio) was used for subcloning. Plasmids pND50 and pKE515 were used as shuttle vectors between *E. coli* and *S. aureus* (Li *et al.*, 2004; Yamagishi *et al.*, 1996). An *S. aureus* DNA replication gene library was prepared by cloning 17 PCR-amplified putative replication genes into the *SmaI* site of pND50 based on the N315 sequence. Plasmid pSdnaB contained the *dnaB* gene region (1736 bp; chromosomal location 1717095 to 1718830 on the N315 genome described above) in pKE515, which was derived from the RN4220 genomic DNA library (Li *et al.*, 2004) and was confirmed by sequencing analysis. Plasmid pSdnaI was pND50 with a 1155 bp portion of the *dnaI* gene (amplified using primers 5'-GGTCTTGATCCGGGTATTCA-3' and 5'-GCAAGATCGACAAGC-ATTTCT-3') inserted at the *SmaI* site. Each *SmaI* site on pKE515 and pND50 is the downstream position of the *cat* gene encoding chloramphenicol acetyltransferase and, in our experience, insertion of an open reading frame in the same direction as the *cat* gene is often critical for the cloned gene to function efficiently (data not shown). So, it is assumed that transcription from the *cat* gene on the pKE515 or pND50 vectors flows into the *SmaI* site and enables expression of an inserted open reading frame. This could allow the expression of DnaB or DnaI proteins from the pSdnaB or pSdnaI plasmids, respectively.

Chemicals and culture of bacteria. *S. aureus* strains were cultured in Luria-Bertani (LB) medium [1 % (w/v) bactotryptone (Becton Dickinson), 0.5 % (w/v) yeast extract (Becton Dickinson) and 1 % (w/v) NaCl] with 50 µg thymine ml⁻¹, 12.5 µg chloramphenicol ml⁻¹, 10 µg erythromycin ml⁻¹ or 50 µg kanamycin ml⁻¹. The restriction enzymes, ligation kits and random primer DNA-labelling kits were purchased from Takara Bio. [methyl-³H]Thymine (65.0 Ci mmol⁻¹; 2.4 TBq mmol⁻¹) was from Moravsek Biochemicals, and [α-³²P]dCTP (6000 Ci mmol⁻¹; 222 TBq mmol⁻¹) and [³⁵S]methionine (Redivue PRO-MIX; 1000 Ci mmol⁻¹; 37 TBq mmol⁻¹) were from Amersham Biosciences. *Pfu* polymerase for PCR was purchased from Stratagene.

DNA sequencing. DNA fragments containing the *dnaB* region were amplified by PCR from chromosomal DNA templates using primers 5'-CAAGTCTGCGACGTGTTA-3' and 5'-TCTTGTGAGAA-CGACCAGTT-3', purified with QIAquick gel extraction kits (Qiagen), and used as sequencing reaction templates. Thermal sequencing reactions were performed with PRISM BigDye terminator kits and analysed with an ABI 373A automated DNA sequencer (Applied Biosystems). For the *dnaI* region, the same primers were used as for pSdnaI construction.

Transformation and phage transduction of *S. aureus*.

Competent cells for electroporation were prepared as described previously (Inoue *et al.*, 2001). Briefly, plasmid DNA (100 ng) was delivered into competent cells by electroporation at 2.5 kV, 25 µF, and 100 Ω in a 0.2 cm cuvette (Gene Pulser II; Bio-Rad). Electroporated cells were grown in BHI medium (Becton Dickinson) containing 10 % sucrose for 1 h at 30 °C and spread on LB agar plates containing antibiotics for selection. Transduction using phage 80x was performed as described by Novick (1991).

Search for replication mutants. A pND50-based plasmid library, containing 17 putative DNA replication-related genes, was delivered by electroporation into the temperature-sensitive *S. aureus* mutants. Colonies grown at 43 °C on LB agar plates containing 12.5 µg chloramphenicol ml⁻¹ were isolated, and plasmid DNA was

Table 1. Strains used in this study

Strain	Relevant genotype	Construction
RN4220	<i>dnaB</i> ⁺ <i>dnaI</i> ⁺ <i>thyA</i> ⁺	Novick <i>et al.</i> (1993)
TS2203	RN4220 <i>dnaB2203</i>	Mutagenesis
TS2831	RN4220 <i>dnaB2831</i>	Mutagenesis
TS4374	RN4220 <i>dnaB4374</i>	Mutagenesis
TS8410	RN4220 <i>dnaB8410</i>	Mutagenesis
TS9803	RN4220 <i>dnaB9803</i>	Mutagenesis
TS6120	RN4220 <i>dnaI6120</i>	Mutagenesis
TS7302	RN4220 <i>dnaI7302</i>	Mutagenesis
TS8651	RN4220 <i>dnaI8651</i>	Mutagenesis
RNBI	RN4220 Ω (<i>sa1505-sa1506::pMutinT3erm</i>)	Insertion
BI2203	TS2203 Ω (<i>sa1505-sa1506::pMutinT3erm</i>)	Insertion
BI2831	TS2831 Ω (<i>sa1505-sa1506::pMutinT3erm</i>)	Insertion
BI4374	TS4374 Ω (<i>sa1505-sa1506::pMutinT3erm</i>)	Insertion
BI8410	TS8410 Ω (<i>sa1505-sa1506::pMutinT3erm</i>)	Insertion
BI9803	TS9803 Ω (<i>sa1505-sa1506::pMutinT3erm</i>)	Insertion
BI6120	TS6120 Ω (<i>sa1505-sa1506::pMutinT3erm</i>)	Insertion
BI7302	TS7302 Ω (<i>sa1505-sa1506::pMutinT3erm</i>)	Insertion
BI8651	TS8651 Ω (<i>sa1505-sa1506::pMutinT3erm</i>)	Insertion
LY2831	RNBI <i>dnaB2831</i>	Transduction
LY8410	RNBI <i>dnaB8410</i>	Transduction
LY6120	RNBI <i>dnaI6120</i>	Transduction
LY7302	RNBI <i>dnaI7302</i>	Transduction
NI8	RN4220 <i>thyA19::pSF151kan</i>	Insertion
LYTBI	NI8 Ω (<i>sa1505-sa1506::pMutinT3erm</i>)	Transduction
LYT2203	LYTBI <i>dnaB2203</i>	Transduction
LYT2831	LYTBI <i>dnaB2831</i>	Transduction
LYT4374	LYTBI <i>dnaB4374</i>	Transduction
LYT8410	LYTBI <i>dnaB8410</i>	Transduction
LYT9803	LYTBI <i>dnaB9803</i>	Transduction
LYT8651	LYTBI <i>dnaI8651</i>	Transduction

extracted. The nucleotide sequences of the DNA fragments inserted in the plasmids were determined.

Continuous labelling of DNA and protein synthesis in *S. aureus*. To monitor DNA synthesis, overnight cultures were diluted 1000-fold in LB medium containing 50 μ g unlabelled thymine ml⁻¹ and [³H]thymine and then grown at 30 °C until the OD₆₀₀ reached 0.1–0.15. The cells were then shifted to 43 °C or 30 °C and incubated for various amounts of time, after which a portion of the cells was treated with 10% (w/v) TCA containing 0.1 M sodium pyrophosphate, 0.5 M NaCl and 25 μ g thymine ml⁻¹. Acid-insoluble fractions were collected, and radioactivity was measured using a liquid scintillation counter. To monitor protein synthesis, [³H]thymine was replaced by [³⁵S]methionine.

Origin-to-terminus ratio. Southern hybridization experiments were performed to determine the relative abundance of the chromosomal origin and terminus as described previously (Li *et al.*, 2004). Overnight cultures were diluted 1000-fold in LB medium with 50 μ g thymine ml⁻¹ and incubated at 30 °C until the OD₆₀₀ reached 0.3. Half of each culture was harvested as exponential-phase cells, and the rest was further incubated at 43 °C for 2 h. Genomic DNA was extracted, double-digested with *EcoRI* and *EcoRV*, separated by agarose (1.5%) gel electrophoresis, and transferred to a nitrocellulose membrane. The position of each ³²P-labelled probe, as defined by the *S. aureus* N315 genome sequence, was 0.08 min for the origin and

50.03 min for the terminus. Each region cloned into the *SmaI* site of pND50 was excised by digestion with *KpnI* and *BamHI*, separated by agarose gel electrophoresis, extracted, and labelled with [α -³²P]dCTP using a random primer DNA-labelling kit version 2 (Takara Bio). Hybridization intensities were determined using a BAS1500 image analyser (Fuji Film). The relative origin-to-terminus ratio was normalized using the intensity of the pSot plasmid, which contained both the origin and the terminus regions of the probe at the *SmaI* and *HincII* sites of pND50, respectively. *EcoRI/HindIII/BamHI*-digested pSot gave a theoretical molar ratio of 1.

Flow cytometric assay. The dynamics of the chromosomal DNA replication process was analysed using flow cytometry as described previously (Li *et al.*, 2004; Skarstad *et al.*, 1995). Briefly, exponentially growing cells at an OD₆₀₀ of 0.15 were harvested and either treated with 50 μ g rifampicin ml⁻¹ and 10 μ g cephalixin ml⁻¹ at 30 °C for an additional 2 h, or incubated at 43 °C with or without cephalixin for an additional 2 h. Harvested cells were fixed in 75% ethanol, treated with 20 μ g RNaseA ml⁻¹ at 50 °C for 1 h, sonicated, washed, and stained with 1 μ M SYTOXgreen (Molecular Probes). Samples were analysed using a FACSCalibur (Becton Dickinson). A single-cell suspension was generated by sonication as described previously (Li *et al.*, 2004; Murai *et al.*, 2006).

Mitomycin C sensitivity. Exponentially growing *S. aureus* cells (OD₆₀₀ 0.1–0.15) at 30 °C were incubated at 43 °C for 2 h in the

presence of 0, 50, 100, 200 or 300 ng mitomycin C ml⁻¹. The cells were then diluted and spread on LB agar plates, incubated at 30 °C for 36 h, and colony numbers were counted.

RESULTS AND DISCUSSION

Isolation of temperature-sensitive *dnaB* and *dnaI* mutants of *S. aureus*

To identify the essential genes for *S. aureus* cell growth, we isolated more than 750 temperature-sensitive mutants that grew at 30 °C but not at 43 °C (Inoue *et al.*, 2001; Li *et al.*, 2004). In this study, we searched for replication mutants using a pND50-based plasmid library that contains 17 putative DNA replication-related genes. The transformants of 10 strains grown at 43 °C carried the plasmid pSdnaBI containing the *dnaB* and *dnaI* gene operon. To determine which gene is responsible for complementation, the strains were transformed by electroporation with a pSdnaB or pSdnaI plasmid in shuttle vector pKE515 or pND50, respectively. Of the 10 mutant strains, five carrying pSdnaB

grew at 43 °C (TS2203, TS2831, TS4374, TS8410 and TS9803). An additional three strains carrying pSdnaI grew at 43 °C (TS6120, TS7302 and TS8651) (data not shown). The results suggest that these temperature-sensitive mutants are candidates for mutants of *dnaB* and *dnaI*, respectively.

Determination of mutation sites in the *dnaB* and *dnaI* genes

To confirm the mutations in the candidate *dnaB* or *dnaI* gene mutants, we determined the nucleotide sequences of their chromosomal *dnaB* or *dnaI* genes. We also compared them to the sequences from their parent strain, RN4220.

The *dnaB* gene of *S. aureus* is predicted to encode a 466 aa protein with a molecular mass of 54.5 kDa and 28 % amino acid sequence identity with the corresponding protein of *B. subtilis* strain 168 (Fig. 1a). The predicted amino acid sequence contains an N-terminal hydrophobic region, a putative DNA-binding region (Ogasawara *et al.*, 1986) and a phage Rep-like motif in the middle of the C-terminal

Fig. 1. Mutational sites in *B. subtilis* DnaB (a), and *S. aureus* DnaB (b) and DnaI (c). In (a), arrows indicate temperature-sensitive mutations and lines indicate suppressor mutations for the *priA* mutation. For the DnaI protein in (c), A and B in the highly conserved region represent Walker-type ATPase motifs A and B, respectively.

region (Rokop *et al.*, 2004) (Fig. 1b). Two putative ATP-binding motifs deduced from the GKT motif in *B. subtilis* DnaB (Hoshino *et al.*, 1987) were not detected in *S. aureus* DnaB. Each of the DnaB mutants had a single transition mutation (C113T, G268A, G275A, C931T and C1082T), resulting in single amino acid substitutions (P38L, A90T, G92E, P311S and P361L in TS4374, TS2203, TS2831, TS9803 and TS8410, respectively). The mutants were named *dnaB4374*, *dnaB2203*, *dnaB2831*, *dnaB9803* and *dnaB8410*, respectively (Fig. 1b).

The *dnaI* gene of *S. aureus* is predicted to encode a 306 aa protein with a molecular mass of 35.6 kDa and 42 % amino acid sequence identity with the corresponding protein of *B. subtilis* strain 168. Walker A and B motifs for nucleotide binding were found in the middle region. Each of the mutants had a single transition or transversion mutation (G476A, G683A and G683T), resulting in single amino acid substitutions (G159D, G228E and G228V in TS6120, TS7302 and TS8651, respectively), and the mutations were named *dnaI6120*, *dnaI7302* and *dnaI8651*, respectively (Fig. 1c).

The mutations in the *S. aureus* *dnaB* mutants were located in the N- and C-terminal regions (Fig. 1b). This resembles the polarization of the *B. subtilis* *dnaB* mutations (Fig. 1a) and supports the hypothesis that DnaB contains two domains (Bruand *et al.*, 2001; Ogasawara *et al.*, 1986). Pro38, which was substituted in *dnaB4347*, is highly conserved among bacterial DnaB proteins and is located in the hydrophobic region (Fig. 1b), which may mediate association with and recruitment of *oriC* to the cell membrane. Thus, the Pro38 residue probably plays an important role in the membrane binding of DnaB by contributing hydrophobicity and by restricting the conformation of the membrane-binding domain. Both Ala90 and Gly92 were located in the turn portion of the helix–turn–helix motif, which participates in DNA binding (Fig. 1b). The alteration of the side-chains of these residues may affect the tertiary structure of the motif and reduce the ability of *B. subtilis* DnaB to bind single-stranded DNA (Bruand *et al.*, 2005). Pro311 is not conserved amongst DnaB proteins, suggesting that it does not have a common function in DnaB proteins. The reduction of DnaB activity at 43 °C in the P311S mutant is probably due to a change in the protein's conformation. The *dnaB8410* mutant has a P361L mutation, which corresponds to the S371 residue in *B. subtilis* DnaB. In *B. subtilis*, the S371P mutation of *dnaB75* suppresses the temperature-sensitive phenotypes of the *dnaD23* and *dnaB134* mutations as well as the poor growth phenotype of the *priA1* mutation. Furthermore, the S371P-mutated DnaB75 protein has a higher affinity for single-stranded DNA compared to wild-type DnaB (Bruand *et al.*, 2001, 2005; Rokop *et al.*, 2004). Thus, the Pro361 residue of *S. aureus* DnaB and the Ser371 residue of *B. subtilis* DnaB may be involved in a conformational change of the protein. The Gly159 and Gly222 residues, which are substituted in the *dnaI* mutation, are well conserved among DnaI proteins and are located in the

nucleotide-binding motifs Walker A and Walker B, respectively (Fig. 1c). Therefore, these two mutations might affect control of DnaI protein function through the binding of adenine nucleotides.

Phage transduction experiments

Because EMS-treated cells are thought to have multiple mutations in their chromosomal DNA, we examined the role of the mutations in the temperature-sensitive phenotype by transduction using phage 80 α . This method is analogous to a system using phage P1 to transduce *E. coli*. These general transducing phages carry a fraction of DNA from their previous bacterial host (donor) and inject it in the same manner as phage DNA into the next host (recipient). The exogenous DNA can participate in homologous recombination events along with the endogenous DNA, resulting in its integration into the recipient's genome. In this study, a drug-resistance gene was integrated near the *dnaBI* operon of each mutant strain, and the resulting strains were used as donors. The phages from donors were used to infect temperature-resistant parent strains RN4220 or NI8, and cells undergoing homologous recombination were selected by growth in the presence of drugs. In this assay, if the *dnaB* or *dnaI* mutation is responsible for the temperature-sensitive phenotype, a certain portion of the drug-resistant recombinants will show temperature-sensitive cell growth because of genetic linkage. Transductants of *thyA* mutant NI8 or RN4220 were selected at 30 °C by erythromycin resistance based on the integration of suicide plasmid pMBI, a pMutinT3 derivative harbouring a 813 bp fragment near the *dnaBI* operon and the erythromycin-resistance gene. The temperature sensitivity of all five *dnaB* mutants and all three *dnaI* mutants was highly linked with erythromycin resistance and at similar frequencies (63–75 % and 77–84 %, respectively; Table 2). Consistent with this, p*SdnaB* or p*SdnaI* complemented the temperature sensitivity of the respective transductants (Table 3). These results suggest that the temperature sensitivity of these mutant strains is due to mutations in DnaB or DnaI, respectively.

DNA synthesis in the *dnaB* and *dnaI* mutants

We next examined whether DNA replication is blocked at the restrictive temperature in the transductants (LYT mutants). Using transductants significantly reduces the possible effects of second mutations in EMS-treated cells. In addition, the *thyA* mutation in these cells blocks *de novo* synthesis of dTMP, which is required for improved detection of [³H]thymine incorporation into DNA. Shifting exponentially growing LYT2831 and LYT8651 cells from 30 °C to 43 °C resulted in a halt in DNA synthesis as monitored by [³H]thymine incorporation into acid-insoluble fractions, whereas protein synthesis continued (Fig. 2). A similar cessation of DNA synthesis with a residual increase was observed for the four other *dnaB* mutants and two of the *dnaI* mutants (data not shown).

Table 2. Phage transduction analyses

Donor*	Recipient†	Selected Erm ^r growth‡ (no. tested)	Unselected TS growth (no.)	Co- transduction frequency§ (%)
BI2203	NI8	96	60	63
BI2831	NI8	94	70	75
BI4374	NI8	96	60	63
BI8410	NI8	88	56	64
BI9803	NI8	95	60	63
BI6120	RN4220	96	81	84
BI7302	RN4220	96	76	79
BI8651	NI8	66	51	77
RNBI	NI8	24	0	0

*Erythromycin-resistant markers were inserted in the donor strains near the *dnaB* and *dnaI* gene operon, and their presence was confirmed by Southern blotting.

†Transduction experiments using phage 80 α were carried out with RN4220 or NI8 (RN4220 Δ thyA) as the recipient.

‡Erythromycin-resistant (Erm^r) colonies were selected and purified, and a temperature-sensitivity (TS) test was performed at 30 °C and 43 °C using LB agar plates containing 10 μ g erythromycin ml⁻¹.

§Co-transduction frequency is defined as the linkage of the temperature-sensitive and erythromycin-resistant phenotypes in tested erythromycin-resistant transductants.

Such a cessation of DNA synthesis is usually associated with replication-initiation mutants, and the patterns are similar to those observed in cells treated with chloramphenicol, which blocks replication at the initiation step. Decreased DNA synthesis at the restrictive temperature was rescued in the five *dnaB* mutants by p*SdnaB* and in the

Table 3. Complementation of temperature-sensitive cell growth

Strain	10 ⁻⁴ × Transformants (43 °C/30 °C)* with:		
	p <i>SdnaB</i>	p <i>SdnaI</i>	Vector
LYT2203	4.0/4.5		0.0/4.8
LYT2831	4.6/3.9		0.0/4.6
LYT4374	6.7/7.6		0.0/7.2
LYT8410	1.1/1.7		0.0/1.0
LYT9803	3.0/4.8		0.0/4.6
TS6120		1.1/1.2	0.0/1.2
TS7302		1.2/1.1	0.0/1.0
TS8651		3.2/3.6	0.0/2.8

*Competent cells were transformed by electroporation with 100 ng plasmid p*SdnaB*, p*SdnaI*, or the respective empty vector (pKE515 or pND50, respectively), and incubated at 30 °C or 43 °C for 40 h on LB plates containing 12.5 μ g chloramphenicol ml⁻¹. The numbers of transformants were counted.

three *dnaI* mutants by p*SdnaI* (data not shown). These results suggest that in the *dnaB* and *dnaI* mutants of *S. aureus*, DNA replication stops at the restrictive temperature, possibly at the initiation step.

Relative abundance of chromosomal origin and terminus

To examine whether the *dnaB* and *dnaI* mutants have a defect in the initiation step of DNA replication, we determined the relative abundance of the chromosomal origin and the terminus locus (origin-to-terminus ratio) (Li *et al.*, 2004). If DnaB and DnaI are required for

Fig. 2. DNA and protein synthesis of *dnaB* and *dnaI* mutants. LYTBI, LYT2831 (*dnaB2831*) and LYT8651 (*dnaI8651*) cells were grown at 30 °C to an OD₆₀₀ of 0.1–0.15 in LB medium (5 ml) containing 50 μ g thymine ml⁻¹ and [³H]thymine or [³⁵S]methionine. Cultures were shifted to 43 °C (filled symbols) or left at 30 °C (open symbols) and then incubated for the indicated time. Incorporation of [³H]thymine (squares) or [³⁵S]methionine (circles) into acid-insoluble fractions was measured. Incorporation at each time point is expressed relative to incorporation at time 0. Data are means $\pm$ SE of at least three independent experiments (error bars not shown where smaller than symbols).

Fig. 3. Flow cytometric analyses of *dnaB* and *dnaI* mutants. Results are shown for exponentially growing RNBI (a, b, c, d), LY6120 (e, f, g, h), LY7302 (i, j, k, l), LY2831 (m, n, o, p) and LY8410 (q, r, s, t) cells grown at 30 °C in LB medium (OD₆₀₀ 0.15) and sampled directly for flow cytometry (a, e, i, m, q), after a 2 h incubation at 30 °C in the presence of rifampicin (Rfp) and cephalaxin (Cex) (b, f, j, n, r), after a 2 h incubation at 43 °C in the presence of cephalaxin (c, g, k, o, s), or after a 2 h incubation at 43 °C (d, h, l, p, t). Harvested cells were fixed, stained with SYTOXgreen, and analysed for chromosome content using a FACSCalibur. Data are representative of more than three independent experiments.

initiation but not for elongation, the ongoing replication of the mutant cells at the non-permissive temperature will complete, and the origin-to-terminus ratio in exponentially growing cells containing multiple replication forks will decrease from a value greater than 1.0 to a theoretical value of 1.0. On the other hand, if DnaB and DnaI are required for elongation, the origin-to-terminus ratio will remain at the higher value. The origin-to-terminus ratio of the five *dnaB* mutants in exponential phase ranged from 2.0 to 2.2 at the permissive temperature and decreased to 1.4 or 1.5

after the temperature increase. These values were similar to those obtained for LYTBI cells treated with chloramphenicol or in the stationary phase (1.5 and 1.4, respectively; Table 4). The chloramphenicol-treated cells complete ongoing replication without the initiation of chromosome replication. As for *dnaI* mutants, the values also decreased to 1.7 from higher values after the temperature increase. These results suggest that shifting to the restrictive temperature causes DNA replication to stop at the initiation step in all of the *dnaB* and *dnaI* mutants.

Flow cytometric analysis

To further confirm that the defect in these *dnaB* and *dnaI* mutants was in the initiation step, we performed flow cytometric analysis. Exponentially growing RNBI, LY6120, LY7302, LY2831 and LY8410 cells showed broad peaks (Fig. 3a, e, i, m and q, respectively), indicating that the cells were a mixture in various phases of the cell cycle. The cells were then treated for 2 h with cephalexin and rifampicin at 30 °C, which inhibits cell division and the initiation but not the elongation step of DNA replication. RNBI, LY6120 and LY8410 cells showed major 4N and minor 2N chromosome equivalents (Fig. 3b, f and r), whereas LY7302 and LY2831 cells exhibited 3N in addition to 2N and 4N peaks (Fig. 3j and n). The appearance of a 3N peak suggests that the *dnaB2831* and *dnaI7302* mutant cells lose synchrony regulation of chromosomal DNA replication initiation.

Incubation of mutant cells for 2 h at 43 °C in the presence of cephalexin showed peaks at chromosome equivalents (Fig. 3g, k, o and s) similar to cells treated with cephalexin and rifampicin at 30 °C. The same treatment of RNBI cells resulted in the broadening of the peak and an increase in DNA content (Fig. 3c). Furthermore, in *dnaB* and *dnaI* mutant cells incubated at 43 °C for 2 h without antibiotics, there was a major 1N and a minor 2N peak (Fig. 3h, l, p and t). This indicates that most of the cells complete an ongoing round of replication and subsequent cell division without initiation of the following round of replication. In each case, loss of the initiation of DNA replication was suppressed by p*SdnaB* or p*SdnaI* (data not shown). Other mutant cells, including *dnaB2203*, *dnaB4374*, *dnaB9803* and *dnaI8651*, showed patterns similar to mutants *dnaB8410* and *dnaI6120*. These results suggest that *dnaB* and *dnaI* mutants have defects in the initiation step of chromosome replication.

Mitomycin C sensitivity

It has been suggested that *B. subtilis* DnaI, together with DnaB and DnaD, functions in replication fork reassembly by a PriA-dependent and independent mechanism (Bruand *et al.*, 2001; Marsin *et al.*, 2001). Loss of PriA protein results in increased sensitivity to UV irradiation, suggesting that replication restart, which is mediated by PriA, participates in DNA repair (Kogoma *et al.*, 1996; Polard *et al.*, 2002). The *dnaB75* mutation has been shown to suppress the effects of a *B. subtilis* *priA* deletion mutation (Bruand *et al.*, 2001), and we previously observed that the *S. aureus* *dnaD* mutant is sensitive to mitomycin C and UV irradiation (Li *et al.*, 2004). Therefore, *S. aureus* DnaI may also be involved in the restart of replication and DNA repair.

We found that LY6120 cells incubated for 2 h at 43 °C without antibiotics had a major 1N peak with a tail between the 1N and 2N peaks (Fig. 3h). Similar patterns were observed for LY7302 (Fig. 3l) and LY8651 cells (data

Table 4. Relative abundance of chromosomal origin and terminus

Strain	Conditions*	Ori/ter†	Doubling time (min)
Expt 1			
LYTBI	Exp. cells	2.1	56
	CM, 2 h	1.5	–
	NFX, 2 h	2.0	–
	Stationary phase	1.4	–
LYT2203	Exp. cells	2.0	56
	43 °C, 2 h	1.5	–
LYT2831	Log cells	2.0	56
	43 °C, 2 h	1.4	–
LYT4374	Exp. cells	2.2	56
	43 °C, 2 h	1.5	–
LYT9803	Exp. cells	2.2	56
	43 °C, 2 h	1.5	–
LYT8410	Exp. cells	2.1	50
	43 °C, 2 h	1.5	–
LYT8651	Exp. cells	2.5	50
	43 °C, 2 h	1.7	–
Expt 2			
RNBI	Exp. cells	3.0	43
	CM, 2 h	1.5	–
	NFX, 2 h	2.8	–
LY6120	Exp. cells	2.8	43
	43 °C, 2 h	1.7	–
LY7302	Exp. cells	3.0	43
	43 °C, 2 h	1.7	–

*Cells exponentially growing in LB medium at 30 °C were harvested at exponential phase (Exp.) or further incubated at 43 °C for 2 h. In addition, wild-type LYTBI cells were supplied with 100 µg chloramphenicol (CM) ml⁻¹ or 100 µg norfloxacin (NFX) ml⁻¹ and further incubated for 2 h at 30 °C. Experiments 1 and 2 were carried out using *thyA* and *thyA*⁺ cells as the parent strain, respectively. The *thyA* strain grew more slowly than the *thyA*⁺ strain, resulting in reduced origin-to-terminus ratios in exponential-phase cells.

†The replication origin-to-terminus ratio (Ori/ter) of harvested cells was determined by Southern hybridization. The ratio was normalized using a p*Sot* plasmid, which contained both the origin and the terminus regions used for probes. Data represent mean values of three experiments.

not shown). These results suggest that the population includes cells that do not complete the elongation step of a round of DNA replication from 1N to 2N and thus represent replication arrests in the *dnaI* mutants. A similar phenotype was reported for an *E. coli* *dnaC* helicase loader mutant (Maisnier-Patin *et al.*, 2001). Therefore, we examined whether the *dnaI* mutant has a defect in DNA repair. In LYT8651 cells, mitomycin caused a decrease in the viable cell number that was complemented by the p*SdnaI* plasmid (Fig. 4). This suggests that the *dnaI8651* mutation is sensitive to mitomycin C, a compound known to cause DNA lesions. This finding suggests that DnaI participates in DNA repair.

Conclusions

In this study, we identified five novel *dnaB* mutants and three novel *dnaI* mutants by screening temperature-sensitive replication mutants in *S. aureus*. These eight mutants possess single amino acid substitutions in DnaB or DnaI. Phage transduction experiments confirmed the role of each mutation in temperature sensitivity. Three different experiments, including a DNA synthesis assay using [3 H]thymine (Fig. 2), determination of the origin-to-terminus ratio (Table 4), and flow cytometric analysis (Fig. 3), revealed that at the non-permissive temperature, the initiation step of DNA replication was halted in all the mutants. Thus, in agreement with previous findings in *B. subtilis*, our results show that DnaB and DnaI are essential for the initiation step of chromosomal DNA replication in *S. aureus*.

Flow cytometric analysis of the *dnaB2831* and *dnaI7302* mutant cells cultured at 30 °C in the presence of cephalexin and rifampicin showed 3N in addition to 2N and 4N peaks (Fig. 3). Bacterial cells contain $2n$ ($n=0, 1, 2$, etc.) origins because of the synchronous initiation at *oriC* (Skarstad *et al.*, 1995). Thus, DnaB and DnaI appear to be involved in the synchrony of initiation of DNA replication. To our knowledge, this is the first time that this has been described in Gram-positive bacteria. How the synchrony of the initiation step is controlled is currently unknown. Because

Fig. 4. Mitomycin C sensitivity of the LYT8651 mutant. Exponentially growing cells at 30 °C were incubated for 2 h at 43 °C in the presence of the indicated concentration of mitomycin C. Colony numbers of harvested cells were counted and are expressed relative to the number in the absence of mitomycin C. The data shown are representative of two independent experiments. The strains used were LYTBI (parent, ●), LYT8651 (*dnaI8651*, ○), LYT8651 harbouring pND50 (△) and LYT8651 harbouring pSdnaI (▲).

the period for replication initiation is strictly controlled, the lower ability of DnaB2831 and DnaI7302 to load the helicase at *oriC* could result in an asynchronous phenotype.

Finally, the present results (Fig. 3h and l, Fig. 4) support the involvement of DnaI in the restart of replication from arrested replication forks *in vivo*.

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