Role for dnaK locus in tolerance of multiple stresses in *Staphylococcus aureus*

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Heat-shock proteins are essential for stress tolerance and allowing organisms to survive conditions that cause protein unfolding. The role of the *Staphylococcus aureus* DnaK system in tolerance of various stresses was studied by disruption of dnaK by partial deletion and insertion of a kanamycin gene cassette. Deletion of dnaK in *S. aureus* strain COL resulted in poor growth at temperatures of 37 °C and above, and reduced carotenoid production. The mutant strain also exhibited increased susceptibility to oxidative and cell-wall-active antibiotic stress conditions. In addition, the mutant strain had slower rates of autolysis, suggesting a correlation between DnaK and functional expression of staphylococcal autolysins. Deletion of dnaK also resulted in a decrease in the ability of the organism to survive in a mouse host during a systemic infection. In summary, the DnaK system in *S. aureus* plays a significant role in the survival of *S. aureus* under various stress conditions.

INTRODUCTION

*Staphylococcus aureus* is an aggressive pathogen that is responsible for a wide array of diseases ranging from pyogenic skin infections to complicated life-threatening diseases, such as bacteremia, central nervous system infections, endocarditis, osteomyelitis, pneumonia, sepsis, septic arthritis, urinary tract infections, and chronic lung infections associated with cystic fibrosis (Lyczak *et al.* 2002; Projan & Novick, 1997). Additionally, toxigenic strains of *S. aureus* are responsible for diseases such as toxic shock syndrome and scalded skin syndrome (Projan & Novick, 1997). Of additional concern, the organism is resistant to most available antibiotics, and there are very limited therapeutic options for the treatment of staphylococcal infections (Bal & Gould, 2005; Chopra, 2003; Cui *et al.*, 2006; Schito, 2006).

Heat-shock proteins (Hsps) that are molecular chaperones play important roles in bacterial stress tolerance. The molecular genetics and expression of these conserved Hsps have been most extensively studied in *Escherichia coli* and *Bacillus subtilis*. In *E. coli*, expression of Hsps is regulated by the alternative sigma factor 32 (σ32), which is encoded by the rpoH gene (Mogk *et al.*, 1999; Yura *et al.*, 2000). Hsps provide major protection against cytoplasmic protein damage. In addition to σ32 in *E. coli*, an alternative sigma factor, σE, also protects proteins from misfolding in the extracytoplasmic compartment under extreme heat stress (Yura *et al.*, 2000).

In *Bac. subtilis*, at least four classes of Hsps have been identified based on their regulatory mechanisms. Class I Hsps comprise the classical Hsps that are encoded by genes of the groES/EL and dnaK operons, and cellular levels of these proteins are controlled by the HrcA repressor (Schulz & Schumann, 1996). Class II Hsps are σB dependent, and are induced by heat and other stresses (Schulz & Schumann, 1996). Class III Hsps are Clp proteases/ATPases, and are typically under the control of the transcriptional repressor CtsR (Derre *et al.*, 1999). The class IV Hsps are encoded by genes that are not controlled by HrcA, σB or CtsR (Helmann *et al.*, 2001).

In *S. aureus*, the Hsp100/Clp ATPases have been extensively studied in recent years, and they have been shown to play important roles in stress tolerance, intracellular replication in eukaryotic epithelial cells, biofilm formation, expression of extracellular toxins, and pathogenicity in a murine model of infection (Chatterjee *et al.*, 2005; Frees *et al.*, 2003, 2004; Michel *et al.*, 2006). Surprisingly, in contrast, the *S. aureus* GroES/EL and DnaK proteins have not been studied to this depth. DnaK and GroES/GroEL belong to a family of Hsps found in all organisms (Craig, 1985); these Hsps have been highly conserved during evolution, indicating their important role in cellular metabolism (Maguire *et al.*, 2002). These Hsps are molecular chaperones and proteases that are synthesized in elevated

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Abbreviation: Hsp, heat-shock protein.

Several studies have indicated an altered expression of staphylococcal GroEL and DnaK homologues under various environmental growth conditions (Qoronfleh et al., 1990). These proteins are induced during infection of epithelial cells under conditions that do not involve thermal stress (Qoronfleh et al., 1998), suggesting involvement of these proteins in the infection process. Additionally, sera from endocarditis patients have antibodies against these Hsps (Qoronfleh et al., 1993). These Hsps have also assumed significance in antibiotic action, as they are induced under cell-wall-active antibiotic stress in S. aureus (Singh et al., 2001a; Utaida et al., 2003).

*S. aureus dnaK* has been predicted to be part of an operon consisting of at least five genes: *hrcA-grpE-dnaK-dnaJ-prmA* (Gill et al., 2005; Kuroda et al., 2001; Ohta et al., 1994). This genomic organization is similar to the five genes of a heptacistronic message observed in *Bac. subtilis* (Homuth et al., 1997). It has been recently reported that the *S. aureus* dnaK, as well as the groEL operon, are subjected to dual negative regulation by CtsR and HrcA (Chastanet et al., 1998), suggesting involvement of these proteins in the infection process. Additionally, sera from endocarditis patients have antibodies against these Hsps (Qoronfleh et al., 1993). These Hsps have also assumed significance in antibiotic action, as they are induced under cell-wall-active antibiotic stress in *S. aureus* (Singh et al., 2001a; Utaida et al., 2003).

### Methods

#### Bacterial strains, plasmids and growth conditions.

The bacterial strains and plasmids constructs used in this study are shown in Table 1. *S. aureus* and *E. coli* strains were routinely grown aerobically at 37 °C in tryptic soy broth/agar (TSB/TSA; Beckton Dickinson) and Luria–Bertani broth/agar, respectively. Broth cultures were grown in a shaking incubator (250 r.p.m.), unless stated otherwise. When needed, ampicillin (50 μg ml⁻¹), kanamycin (30 μg ml⁻¹ for *E. coli*; 100 μg ml⁻¹ for *S. aureus*) and chloramphenicol (10 μg ml⁻¹) were added to the growth medium.

#### DNA isolation and manipulations.

Plasmid DNA was isolated using the Qiaprep kit (Qiagen); chromosomal DNA was isolated using DNAzol kit (Molecular Research Center) from lysostaphin-treated *S. aureus* cells, as per the manufacturer’s instructions. All restriction and modification enzymes were purchased from Promega. DNA manipulations, and Southern blot and Northern blot analyses, were carried out using standard procedures. PCR was performed with the GeneAmp PCR system (Perkin-Elmer). Oligonucleotide primers were obtained from Integrated DNA Technology.

#### Construction of a dnaK mutant in *S. aureus*.

To create a dnaK mutant, primers P1 (5’-GCTTAGTTCAAGATGATAACCC-3’) and P2 (5’-CAGAGCCATTGAATCCTTGTC-3’) were used to amplify a

### Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
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<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Reference</th>
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<tr>
<td><em>S. aureus</em> RN4220</td>
<td>A restriction minus derivative of <em>S. aureus</em> strain 8325-4</td>
<td>Kreiswirth et al. (1983)</td>
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<tr>
<td><em>S. aureus</em> 8325-4</td>
<td>A laboratory strain of <em>S. aureus</em> cured of all the prophages</td>
<td>Novick (1991)</td>
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<td><em>S. aureus</em> COL</td>
<td>Homogeneous* in meticillin-resistance expression</td>
<td>Peltz et al. (2000)</td>
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<td><em>S. aureus</em> SH1000</td>
<td><em>S. aureus</em> strain 8325-4 with functional <em>rbbU</em></td>
<td>Horsburgh et al. (2002)</td>
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<td>COL::dnaK</td>
<td><em>S. aureus</em> COL with mutation in the dnaK gene (KanR)</td>
<td>This study</td>
</tr>
<tr>
<td>SH1000::dnaK</td>
<td><em>S. aureus</em> SH1000 with mutation in the dnaK gene (KanR)</td>
<td>This study</td>
</tr>
<tr>
<td>DC1</td>
<td>COL::dnaK complemented with 6.4 kb dnaK locus (KanR, CamR)</td>
<td>This study</td>
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<td><em>E. coli</em> JM109</td>
<td>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F’ (traD36 proAB lacPAM15)</td>
<td>Yanisch-Perron et al. (1985)</td>
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*Plasmids*

<table>
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<th>Characteristics</th>
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<td>Promega</td>
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<td>Cloning vector for <em>E. coli</em> (AmpR, KanR)</td>
<td>Invitrogen</td>
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<td>pTZ18R</td>
<td>Cloning vector for <em>E. coli</em> (AmpR)</td>
<td>Mead et al. (1986)</td>
</tr>
<tr>
<td>pCU1</td>
<td>Shuttle vector (AmpR in <em>E. coli</em> and CamR in <em>S. aureus</em>)</td>
<td>Augustin et al. (1992)</td>
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<td>pTZ18R-dnaK</td>
<td>Plasmid pTZ18R containing a 2.1 kb DNA fragment encompassing the <em>S. aureus</em> dnaK gene</td>
<td>This study</td>
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<td>pTZ-dnaK-Kan</td>
<td>A 1.4 kb kanamycin resistance gene was used to replace the EcoRV fragment of construct pTZ18R-dnaK</td>
<td>This study</td>
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<tr>
<td>pCU1-dnaK</td>
<td>6.4 kb fragment containing all five genes of the dnaK locus</td>
<td>This study</td>
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*Most cells in the population of homogeneous strains grow in the presence of high concentrations of meticillin (Sutherland & Rolinson, 1964).
2.4 kb DNA fragment, using chromosomal DNA from *S. aureus* strain COL as the template. The amplicon encompassing the entire dnaK gene, 164 bp upstream, and 391 bp downstream, was cloned into plasmid pCR2.1 (Invitrogen). A HindIII fragment of approximately 2.2 kb was excised from this construct, and subcloned into the vector pTZ18R (Mead et al., 1986). A 432 bp DNA fragment was excised from the resultant construct pTZ18R-dnak by digestion with EcoRV, and replaced with a 1.4 kb kanamycin-resistance cassette (Gutierrez et al., 1996). The vector pTZ18R cannot replicate in Gram-positive bacteria, and the plasmid-disrupted dnaK construct pTZ-dnak-kan was used as a suicide vector to transform *S. aureus* strain RN4220 via electroporation (Schenk & Laddaga, 1992). The transformants were selected on TSA plates containing kanamycin. The selection resulted in a single crossover, and integration of the entire construct into the *S. aureus* chromosome. Phage 80α was propagated on these transformants, and used to resolve the mutation in the dnaK gene in *S. aureus* by performing transduction outcrosses, as described previously (Singh & Moskovitz, 2003; Singh et al., 2001b). The transductants were confirmed for a mutation in the dnaK gene using PCR and Southern blotting. For genetic complementation of the dnaK mutant, a 6.4 kb DNA fragment was PCR amplified using primers P5 (5′-GGTAACCTCTTCATGACTGAGAAATTG-3′) and P6 (5′-TCTAGAAGCAACATCATATTGGCA-3′). *S. aureus* COL genomic DNA as a template, and EX1 DNA Polymerase (Stratagene). Restriction sites (underlined) were included in the primers to facilitate subcloning of the amplified fragments. The amplicon represents a fragment starting 340 nt upstream of the *hrcA* gene and 285 nt downstream of the *prmA* gene that was cloned to the KpnI and Xbal sites of a shuttle plasmid pCU1 (Augustin et al., 1992), and subsequently transferred to the dnaK mutant of *S. aureus* strain COL.

**Growth kinetics of the wild-type *S. aureus* and its isogenic dnaK mutant under stress.** Mid-exponential-phase cultures (OD<sub>600</sub> 0.6) were diluted 50-fold in a nephelo culture flask (Wheaton) containing 50 ml fresh TSB, with a flask-to-medium volume ratio of 6:1. In parallel flasks, the following stress conditions were imposed through appropriate modifications of TSB: 8.8 mM H<sub>2</sub>O<sub>2</sub>, 4 % (v/v) ethanol, low pH (pH 4.5), high pH (pH 10.0) and NaCl (2.5 M). Growth was then followed by measurement of OD<sub>600</sub> spectrophotometrically. These stress conditions were chosen based on prior studies (Singh & Moskovitz, 2003; Singh et al., 2001a, 2001b; Uotaiada et al., 2003), or on pilot studies where significant growth inhibition was observed for wild-type bacteria.

**Viability and thermotolerance of *S. aureus* exposed to different stresses.** For induction of thermotolerance, mid-exponential-phase cultures (OD<sub>600</sub> 0.6) were diluted 50-fold in fresh TSB, and placed at 37°C until the OD<sub>600</sub> reached 0.3. The cultures were then divided into two parts, and one was maintained at 37°C, while the other was incubated at 48°C for 30 min. Both flasks were then shifted to 60°C, and after 0, 5, and 30 min, aliquots of the cultures were quickly transferred to ice, diluted in TSB, plated on TSA, incubated at 37°C for 48 h, and counted for viable colonies.

**Determinations of susceptibility of the dnaK mutant to antibiotics and H<sub>2</sub>O<sub>2</sub>.** The MICs for the wild-type and the dnaK mutant of *S. aureus* strain COL were determined as described (Pfeltz et al., 2000), using 96-well microtiter plates containing twofold serial dilutions of oxacillin, meticillin and H<sub>2</sub>O<sub>2</sub>. Mid-exponential-phase wild-type and dnaK mutant staphylococcal cells were then added to these dilutions, to a final concentration of 5×10<sup>5</sup> c.f.u. ml<sup>-1</sup>. The plates were incubated at 37°C for 48 h, and the lowest concentration of antibiotic or H<sub>2</sub>O<sub>2</sub> with no apparent visible growth was considered to be the MIC.

**RNA extraction and Northern hybridization.** An overnight culture of *S. aureus* strain COL was diluted 100-fold in TSB, and grown at 37°C until the OD<sub>600</sub> reached 0.3. The cultures were divided into four parts, and subjected to various stress conditions. For heat stress, the culture was shifted to 43°C. Oxacillin stress was imposed by the addition of this antibiotic at a final concentration of 1.2 mg ml<sup>-1</sup>. Oxidative stress was imposed by the addition of H<sub>2</sub>O<sub>2</sub> to a final concentration of 15 mM. The control culture was maintained at 37°C. Cultures were stressed for 10 min. Bacterial cells were harvested by centrifugation, resuspended in 1.0 ml RNA later (Ambion), and incubated overnight at 4°C to inactivate RNases. Cells were washed with PBS, recovered by centrifugation, resuspended in 1.0 ml RNA-Be (Tel-Test), and broken using 0.1 mm silica/zirconium beads in a BioSpec Mini-Beadbeater by subjecting the cell suspension to three cycles of bead beating (30 s each, with 1 min breaks of cells on ice). The method was adapted from a procedure to extract RNA from *Mycobacterium tuberculosis* cells, as described (Talaat et al., 2002). Total RNA was chloroform extracted from the cell-free supernatant, and precipitated by the addition of 2-propanol (50% v/v). The resulting RNA was treated with DNase I (Ambion), and purified by affinity chromatography using the RNeasy kit (Qiagen). Subsequently, equal amounts of RNA (10 μg) from each sample were separated during 1.2% denaturing agarose gel electrophoresis, and transferred to a nylon membrane. The 6.4 kb DNA fragment from the construct pCU-dnak was gel purified, radio-labelled using the Prime-a-Gene labelling system (Promega) in the presence of [α-<sup>32</sup>P]dATP [specific activity >3000 Ci mmol<sup>-1</sup> (>111 TBq mmol<sup>-1</sup>); MP Biochemicals], and used to probe the membrane. The scanned Northern blot as a JPEG image was analysed with ImageJ 1.6 software, which is a public domain Java image processing program (http://rsb.info.nih.gov/ij) (Abramoff et al., 2004).

**Persistence of *S. aureus* strain SH1000 and its isogenic dnaK mutant during oxacillin treatment.** Overnight cultures of the wild-type *S. aureus* strain SH1000 and its isogenic dnaK mutant were diluted 1:1000 in fresh TSB, and incubated at 37°C with shaking (150 r.p.m.) to an OD<sub>600</sub> of 0.5. Both cultures were subsequently treated for 16 h at 37°C with 10 μg oxacillin ml<sup>-1</sup>. Total viable counts in both cultures before and after oxacillin treatment were performed by serial dilution and plating. The surviving fraction was calculated by dividing the number of viable organisms subsequent to oxacillin treatment by the number of viable organisms before antibiotic treatment.

**Whole-cell autolysis assays.** Autolysis assays were performed as previously described (Pfeltz et al., 2000). Briefly, wild-type and dnaK mutant cultures of *S. aureus* COL were grown to an OD<sub>600</sub> of 1.0 at 37°C in PYK medium (0.5% bacto peptone, 0.5% yeast extract, 0.3% K<sub>2</sub>HPO<sub>4</sub>, pH 7.2). After one wash with cold water, cells were resuspended to an initial density of 1.2 in 0.05 M Tris/HCl buffer, pH 7.2, containing 0.05% Triton X-100. The flasks were incubated at 37°C with shaking (150 r.p.m.), and subsequent readings were taken every 30 min for 6 h.

**Carotenoid production in wild-type *S. aureus* and its isogenic dnaK mutant.** Overnight cultures (5.0 ml) were pelleted by centrifugation, and the pellets were washed twice in 5.0 ml sterile water. The final supernatant was removed, and the pellets were then weighed. The pigments were extracted with methanol, as described (Marshall & Wilmoth, 1981). The volume of the final carotenoid-containing extract from 0.25 g of cells was placed in petroleum ether, and the A<sub>462</sub> was obtained with a Beckman DU-70 spectrophotometer.

**Survival of the wild-type and the dnaK mutants in a murine systemic infection model.** Wild-type *S. aureus* strain COL and its isogenic dnaK mutant were grown to mid-exponential phase (OD<sub>600</sub> 0.6) in TSB. Bacterial cells from each culture were harvested by centrifugation, washed three times with 1% TSB, and resuspended in 1% TSB. Cell viability in the suspension was determined by serial
dilution and plating. A mixture of wild-type and dnaK mutant cells, containing a total of 5 × 10⁶ c.f.u. ml⁻¹, was prepared (76/24% mixture of mutant/wild-type) in 1% TSB, and 0.2 ml of this suspension was injected into the peritoneal cavity of Swiss white Hla(ICR)/CVF female mice (16–20 g) (Hilltop Lab Animals Inc.) with a 26 gauge needle fitted to a 1 ml syringe, following published procedures with appropriate modifications (Schwan et al., 2006). At 4, 8 and 30 h, the mice were euthanized by CO₂ asphyxiation. The peritoneum was lavaged with 1 ml 1% TSB, and the liver and spleen were aseptically removed from the peritoneal cavity. Excised livers and spleens were homogenized in 2 and 1 ml 1% TSB, respectively, using a glass tissue grinder fitted with a glass pestle. Peritoneal lavages and the tissue homogenates were serially diluted, plated on TSA, and allowed to grow overnight by incubation at 37 °C. The bacterial colonies were subsequently patched on TSA plates containing kanamycin. The number of bacterial colonies growing in the presence of kanamycin was used to calculate the fraction of mutants relative to wild-type in the bacterial population in the infected tissues. The ratio of surviving mutant was subsequently compared with the percentage of mutants in the mixed culture used to inject mice.

RESULTS

Construction of the dnaK mutant

The genetic organization of the dnaK locus in S. aureus is shown in Fig. 1(a). Altogether, five genes have been proposed to constitute the dnaK cluster in S. aureus (Kuroda et al., 2001; Ohta et al., 1994). The first gene of this cluster is proposed to encode a protein HrcA that negatively regulates the expression of the dnaK locus (Chastanet et al., 2003). A study by others, and nucleotide sequence analysis of the dnaK locus in S. aureus, have suggested the presence of two putative promoters; one upstream of hrcA, and another one upstream of dnaJ (Ohta et al., 1994). Potential transcription start sites have been mapped for both promoters, suggesting dual regulation of the dnaK locus in S. aureus (Ohta et al., 1994). In the present Northern analysis studies, two transcripts (approx. 5.8 kb and 3.5 kb) hybridized with the gene probe specific to dnaK locus genes (Fig. 2a); this is consistent with a report by others (Ohta et al., 1994). Based on the genetic organization and the findings of Ohta et al. (1994), it was considered that the 5.8 kb transcript represented a poly-cistronic message with all five genes shown in Fig. 1(a). To construct a mutation, the EcoRV fragment of the dnaK gene was deleted, and replaced by a kanamycin-resistance gene cassette. The mutation was subsequently confirmed by Southern blot analysis (data not shown) and PCR (Fig. 1b). Although the dnaK mutation was constructed in three different strains of S. aureus (COL, SH1000 and 8325-4), most experiments described in this study were carried out with the dnaK mutant of strain COL.

DnaK contributes to heat and oxidative stress tolerance

Comparison of the growth kinetics shows that the growth of the dnaK mutant and the wild-type S. aureus in liquid

Fig. 1. (a) Diagrammatic representation of the dnaK locus comprising five genes in S. aureus. A deletion mutant of this locus was constructed by replacing the EcoRV fragment of the dnaK gene with a 1.4 kb kanamycin-resistance cassette. The mutant was subsequently complemented in trans with a 6.4 kb DNA fragment (indicated by arrows). (b) PCR verification of a mutation in the dnaK gene in S. aureus. Primers P3 (5’-CCTACACGGCAAGCAATG-3’) and P4 (5’-AATATAGCTGCACGTTTGCTG-3’) were used in the PCR that allowed amplification of a 677 bp product (lane 1) when genomic DNA from wild-type S. aureus strain COL was used. These primers amplified a fragment of approximately 1.7 kb when genomic DNA from the dnaK mutant of S. aureus strains SH1000 and COL was used as a template (lanes 2 and 3, respectively). Lanes 4 and 5, PCR product when genomic DNA from the dnaK mutants of S. aureus strains COL and SH1000, respectively, were complemented in trans with the 6.4 kb dnaK locus that was used as a template. The larger PCR product was only faintly visible because of complementation with the wild-type gene on a high-copy plasmid pCU1. M, DNA ladder (HindIII digest of λ DNA).
culture was the same at 30 °C (Fig. 3a) and lower temperatures (data not shown). However, the dnaK mutant showed a longer lag period and slower growth at 37 °C (Fig. 3b), and failed to grow at 43 °C (Fig. 3c). The dnaK mutant also showed slower growth when grown on TSA plates at 37 °C. To verify if this was indeed the lack of a functional dnaK gene that impaired its growth at higher temperatures, the dnaK mutant was complemented with the entire dnaK locus in trans on plasmid pCU1. As evident in Fig. 3(c), thermal stress tolerance was partially restored in the complemented strain. This partial restoration of thermal tolerance may be due to complementation of the mutant with the genes of the dnaK locus on a high-copy plasmid leading to higher-than-desired production of the proteins of this locus, which in turn may reduce the growth rate. The response of the dnaK mutant was also tested during its growth under different stress conditions compared with wild-type S. aureus. No apparent difference in the growth was observed in the presence of 4% (v/v) ethanol, or in acidic (pH 4.5), alkaline (pH 10.0) or high salt (2.5 M NaCl) conditions (data not shown). However, in the presence of 8.8 mM H₂O₂, the wild-type S. aureus was able to grow after a long lag period (Fig. 3d), but the dnaK mutant cells failed to grow after 3 days’ incubation. MIC determinations also indicated an eightfold lower H₂O₂ MIC for the dnaK mutant compared with wild-type S. aureus (Table 2).

Involvement of dnaK in induction of thermotolerance

Mid-exponential-phase cells of the dnaK mutant and its isogenic S. aureus wild-type strain COL were shifted to 60 °C for a lethal shock, either directly from 37 °C or after pre-exposure to 48 °C for 30 min. As shown in Fig. 4 (right), pre-exposure to 48 °C increased the resistance to lethal heat shock by approximately 1000-fold after 5 min, and approximately 45-fold after 10 min exposure to 60 °C, respectively. However, when untreated dnaK mutant cells were exposed to 60 °C for 5 or 10 min, no survivor cells were recovered (Fig. 4, left). Pre-exposure to 48 °C for 30 min helped some of the mutant bacteria survive the lethal shock. In view of this finding, it is suggested that DnaK may have a partial role in the induction of thermotolerance in S. aureus.

Deletion of dnaK decreases oxacillin resistance in meticillin-resistant S. aureus strain COL and persists in meticillin-susceptible strain SH1000

Oxacillin resistance in the dnaK mutant was reduced to an MIC of 100 µg ml⁻¹ compared with 400 µg ml⁻¹ for its isogenic wild-type S. aureus strain COL (Table 2). A fourfold reduction in the meticillin MIC was also observed for the dnaK mutant when compared with the wild-type
COL (Table 2). Additionally, in shaking liquid cultures in the presence of oxacillin, growth inhibition was more apparent for the dnaK mutant compared with the wild-type parent strain (Fig. 5a). These results suggest that the lack of a functional DnaK reduces oxacillin and meticillin tolerance in S. aureus. However, no increase in antibiotic susceptibility was observed in the case of meticillin-susceptible S. aureus with a disrupted dnaK locus.

Persistent bacterial cells are a small subset of cells within a bacterial population that are able to survive clinically effective antibiotic concentrations. The persister cells do not contain genetic traits that confer resistance to antibiotics, and, as a result, the progeny of these persisters are just as sensitive to the antibiotic used to treat the parental cells (Korch & Hill, 2006; Stewart, 2002). Although oxacillin tolerance was reduced in the dnaK mutant of meticillin-resistant S. aureus strain COL, no such reduction in the oxacillin MIC was observed in the dnaK mutant of meticillin-susceptible S. aureus strain SH1000. The MIC for both strains was determined to be 0.975 µg ml⁻¹. In addition, the oxacillin MICs of the wild-type SH1000 and its derivative dnaK mutant survivors after oxacillin treatment in liquid culture did not change. This suggests that the bacteria, though not resistant to the antibiotic, were able to survive oxacillin treatment, albeit at a very low level (0.54%). However, when the percentage survival of the persister wild-type and the isogenic dnaK mutant were compared, the mutant had a rate of persistence that was approximately 26-fold lower (0.021%) than the wild-type strain.

Whole-cell autolysis of the dnaK mutant
The effect of mutation in the dnaK gene on autolytic activity was also examined. The autolysis experiments showed a slower rate of autolysis of the dnaK mutant cells compared with wild-type S. aureus strain COL (Fig. 5b). The wild-type S. aureus strain COL was more resistant to oxacillin in liquid culture compared with the dnaK mutant, and was able to grow to a higher cell density (Fig. 5a). However, the wild-type S. aureus culture started to lyse, and the rate of cell lysis was more pronounced compared with the derivative dnaK mutant cells (Fig. 5a). After

### Table 2. Susceptibilities of S. aureus parental and dnaK mutant strains to oxacillin, meticillin and H₂O₂

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<th>Bacterial strain</th>
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</tr>
<tr>
<td>COL: dnaK mutant</td>
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<tr>
<td>Complemented strain</td>
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**Fig. 3.** Growth of the dnaK mutant (□) and its isogenic wild-type S. aureus strain COL (■). (a) growth at 30 °C; (b) growth at 37 °C; (c) growth at 43 °C, shaded squares represent growth of the mutant complemented with a 6.4 kb wild-type dnaK fragment on plasmid pCU1; (d) growth in the presence of 8.8 mM H₂O₂. Values indicate the mean of two independent experiments.
3 days’ incubation, the oxacillin-treated wild-type *S. aureus* COL culture was completely clear, while the dnaK mutant culture stayed relatively turbid (data not shown). The data suggest altered autolysin production/processing in the dnaK mutant compared with the wild-type *S. aureus*.

**Transcriptional analysis of the *S. aureus* dnaK locus**

Comparison of the growth kinetics of the dnaK mutant with wild-type *S. aureus* suggests that DnaK plays a role in the tolerance of heat, oxidative and antibiotic stress. To determine if any of these stress conditions induced the expression of the dnaK gene, Northern blot analysis experiments were carried on total RNA from the control and *S. aureus* cells exposed to these stress conditions. A probe representing the entire dnaK locus genes hybridized with two transcripts of approximately 5.8 and 3.5 kb. Both bands were induced in response to heat and oxacillin stresses (Fig. 2a, lanes 3 and 4; Fig. 2b). An increased production of dnaK locus transcripts in response to heat shock is consistent with an earlier report (Ohta *et al.*, 1994). However, these transcripts were not detected at an elevated level in a more recent global transcription profiling study aimed to characterize the staphylococcal heat shock, cold shock, stringent and SOS responses (Anderson *et al.*, 2006). In the present study also, stress conditions such as oxidative stress had no effect on the expression of dnaK locus genes (Fig. 2a, lane 2; Fig. 2b). As expected, the corresponding bands were absent in the mutant bacteria (Fig. 2a, lane 5; Fig. 2b), but the level of expression of both transcripts was considerably higher in the case of the mutant complemented with dnaK locus genes on a high-copy plasmid, even under normal growth conditions (Fig. 2a, lane 6; Fig. 2b).

**Mutation in dnaK alters pigmentation of the *S. aureus* cells**

The *S. aureus* dnaK mutant colonies appeared less pigmented compared with the wild-type COL in broth culture, and during its growth on TSA plates. The *S. aureus* strain SH1000 produces more pigment than the *S. aureus* strain COL, and the role of dnaK in the production of pigment was more apparent in strain SH1000. In a
quantitative assay, the wild-type *S. aureus* strain SH1000 was estimated to produce over threefold more pigment than the *dnaK* mutant strain (mean $A_{462}$ \(0.546 \pm 0.148\) vs \(0.178 \pm 0.008\), respectively; values are means of three independent experiments).

**Mutation in dnaK alters survival of *S. aureus* in vivo**

To determine the role of *dnaK* in *S. aureus* pathogenesis, mice were injected intraperitoneally with a mixture of wild-type COL and *dnaK* mutant bacteria. In these studies, the *dnaK* mutant fraction was deliberately kept at a higher level than the wild-type *S. aureus* to determine subtle changes in the ability of the mutant to survive in vivo. The infected mice were killed at 4, 8 and 30 h after injection. No visible abscess was detected in any of the mice, and the bacterial cell number [total c.f.u. (g tissue)$^{-1}$] showed a gradual decrease with time (Fig. 6a). Interestingly, the fraction of *dnaK* mutant cells recovered from the spleen and liver in infected mice decreased with time; however, the fraction of wild-type cells increased with time (Fig. 6b, c). Since both types of bacteria were gradually removed, the increased recovery of the wild-type cells compared with the *dnaK* mutant cells is suggestive of a role for DnaK in survival of *S. aureus* in vivo, rather than it being a mere advantage of growth of the wild-type compared with the *dnaK* mutant at 37°C.

**DISCUSSION**

Despite *S. aureus* being one of the most stress-tolerant bacterial pathogens, the mechanism of its adaptation to stress remains incompletely understood. Heat-stress tolerance is important for bacterial pathogens, as it signals a possible host environment, and helps the organism to prepare against the host immune onslaught. A locus comprising five genes (*hrcA–grpE–dnaK–dnaJ–prmA*) encoding proteins that are synthesized in elevated amounts subsequent to sublethal heat stress has been identified (Ohta et al., 1994). The first gene of this locus encodes a protein HrcA, which, under normal growth conditions, negatively regulates the expression of genes in this locus. The subsequent three genes of this locus (*grpE, dnaK* and *dnaJ*) encode proteins that function as major protein-folding machinery under stress conditions that cause aggregation and unfolding of proteins, and these are collectively referred to as the DnaK system (Mogk et al., 1999; Winter & Jakob, 2004). DnaK cycles between an ATP-DnaK state (low-affinity state) and an ADP-DnaK state (high-affinity state). DnaJ brings unfolded proteins to DnaK, and this leads to the hydrolysis of ATP bound to DnaK, leading to a conformational change to its high-affinity state. The unfolded protein remains bound to DnaK-ADP until a favourable physiological state is regained. GrpE, in turn, functions as a nucleotide-exchange factor, and promotes dissociation of ADP from DnaK for ATP (Harrison, 2003; Liberek et al., 1991; McCarty et al., 1995; Winter & Jakob, 2004). Thus, all three proteins are essential for the DnaK system to be functional.

![Fig. 6. Survival of the *dnaK* mutant and the isogenic wild-type *S. aureus* strain COL in the mouse model. Approximately 1.0×10^8 c.f.u. (24% wild-type, and 76% *dnaK* mutant) were injected intraperitoneally into mice. At each time point of 4, 8 and 30 h post-injection, three mice were killed. (a) Total number of bacteria recovered from murine livers (●) and spleens (○) at different time points post-infection. (b, c) Percentage survival of the *dnaK* mutant (●) and the isogenic wild-type strain (○) recovered from infected liver and spleen, respectively. The points represent mean values (three replicates); error bars represent SEM.](http://mic.sgmjournals.org)
To study the physiological roles of the DnaK system in S. aureus, a dnaK deletion mutant was constructed. Insertional inactivation of the dnaK gene slowed the growth of S. aureus at 37 °C (Fig. 3b), and a temperature of 43 °C was observed to be lethal for the mutant, but not for the wild-type S. aureus (Fig. 3c). These observations are consistent with findings in Bac. subtilis, E. coli, Brucella suis, Listeria monocytogenes and other bacterial species, where inactivation of dnaK has been shown to result in poor growth at higher temperatures (Bukau & Walker, 1990; Hanawa et al., 1999; Kohler et al., 2002; Schulz et al., 1995). In addition to heat, the DnaK system has also been implicated to have a role in other stress conditions, such as ethanol, low pH, salt and oxidative stress (Mogk et al., 1998; Echave et al., 2002; Periago et al., 2002). However, no difference in response to ethanol, acid, alkali or osmotic stress was observed in the case of a dnaK mutant compared with the wild-type S. aureus strain COL (data not shown). Interestingly, a dnaK mutant of the S. aureus strain 8325-4, which is defective in SigB (Kullik et al., 1998), was more sensitive to acid stress compared with its isogenic wild-type (data not shown). This response, however, was not observed in the case of the dnaK mutants of S. aureus strains SH1000 and COL (data not shown). S. aureus strain SH1000 is a derivative of strain 8325-4, which is defective in SigB (Kullik et al., 1998), more sensitive to acid stress compared with its isogenic wild-type (data not shown). This response, however, was not observed in the case of the dnaK mutants of S. aureus strains SH1000 and COL (data not shown). S. aureus strain SH1000 is a derivative of strain 8325-4, in which the SigB defect has been corrected (Horsburgh et al., 2002). Additional stress response defects in a dnaK mutant of S. aureus strain 8325-4 are probably suggestive of a coordination of various staphylococcal stress factors in dealing with adverse environmental conditions.

Interestingly, mutation in dnaK led to significant impairment in the oxidative stress response of the mutant compared with the wild-type S. aureus strain COL. Oxidative stress is known to cause lethal damage to cellular DNA, lipids and proteins. In the case of proteins, oxidative stress leads to carbonylation of amino acids and oxidation of methionine and cysteine-bound sulfur atoms (Requena et al., 2001; Stadtman et al., 2003), and this may lead to protein unfolding (Berlett & Stadtman, 1997). This study suggests that functional DnaK protein refolding machinery is critical for the staphylococcal oxidative stress response. There was no apparent induction in the expression of the dnaK locus under the conditions of oxidative stress (Fig. 2, lane 3). This suggests that the basal level production of DnaK is sufficient to provide protection for cells against oxidative damage. Similar results have been reported in Bru. suis, where DnaK has been shown to be important in the resistance of the organism to oxidative stress, as well as to acidic pH (Kohler et al., 2002). In L. monocytogenes however, a dnaK mutant has been shown to be more susceptible to acidic conditions than the wild-type strain, but not more susceptible to oxidative stress (Hanawa et al., 1999).

The mutation in dnaK increased the susceptibility of meticillin-resistant strain COL to the cell-wall-active antibiotics oxacillin and meticillin (Table 2). In the case of the meticillin-susceptible strain SH1000, deletion of dnaK did not reduce the oxacillin MIC, but it led to a significantly reduced survival after oxacillin treatment. Challenge of meticillin-susceptible S. aureus by cell wall-active antibiotics induces a cell wall stress stimulus (Kuroda et al., 2003; Utaida et al., 2003; Wilkinson et al., 2005). Genes induced include genes encoding enzymes involved in cell-wall biosynthesis, and molecular chaperones and proteases. A number of these genes are controlled by the VraRS two-component regulatory system (Kuroda et al., 2003). Inactivation of VraRS results in reduced oxacillin MICs in meticillin-resistant S. aureus (Boyle-Vavra et al., 2006; Gardete et al., 2006). VraRS has been proposed to be a sentinel sensing perturbations in peptidoglycan biosynthesis, functioning to control the expression of genes involved in generation of a resistance phenotype. The decreased oxacillin MIC of the dnaK mutant of strain COL, and the decreased persistence of the dnaK mutant of strain SH1000, suggest that protein damage does occur as a result of challenge with cell-wall-active antibiotics, and that DnaK plays a role in dealing with these damaged proteins.

Surprisingly, the dnaK mutants of S. aureus produced fewer carotenoids during growth on TSA plates, and also in liquid culture, compared with their wild-type counterparts. Reduced pigmentation was more evident in case of the S. aureus strain SH1000. Pale yellow orange colonies were observed for the dnaK mutant, compared with bright golden-yellow colonies for the wild-type cells, on TSA plates. Although it is unclear how the DnaK deletion is affecting pigmentation in the S. aureus mutant cells, a recent study has shown that S. aureus mutants with disrupted carotenoid biosynthesis are more susceptible to oxidative killing and killing by neutrophils, and are less pathogenic (Liu et al., 2005).

When the dnaK mutants of S. aureus were tested for their survival in mice, the mutants showed significantly reduced survival compared with their wild-type counterpart. Several features of the dnaK mutant may account for this reduced survival. Their susceptibility to oxidative stress may account for their increased vulnerability to the host phagocytic cells. Reduced pigmentation may in part, be responsible for reduced survival in a host, in view of the findings of a correlation between carotenoid production and staphylococcal pathogenesis (Liu et al., 2005). Additionally, autolysins have also been demonstrated to be important in staphylococcal pathogenesis (Mani et al., 1994). At this point, although it is debatable whether DnaK should be viewed as a staphylococcal virulence factor, this study suggests that its presence confers an advantage during the initial stages of infection in a host.

In conclusion, inactivation of dnaK in S. aureus impairs the growth and survival of S. aureus at elevated temperatures, under oxidative stress, in the presence of cell-wall-active antibiotics and inside a host. Mutation also impairs carotenoid production and cell autolysis.
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