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

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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 bacteraemia, central nervous system infections, endocarditis, osteomyelitis, pneumonia, septicemia, 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 conditions.

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 bodies against these Hsps (Qoronfleh et al., 1990) are induced during infection of mouse host.

S. aureus dnaK has been predicted to be part of an operon consisting of at least five genes: hrcA–grpE–dnaK–dnaJ–prnA (Gill et al., 2005; Kuroda et al., 2001; Ohta et al., 1994). This genomic organization is similar to the first 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.

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.

S. aureus strains and plasmids used in this study are shown in Table 1. S. aureus and E. coli cells 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.

<table>
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<th>Table 1. Bacterial strains and plasmids used in this study</th>
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<td><strong>Strain or plasmid</strong></td>
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<tr>
<td>S. aureus RN4220</td>
</tr>
<tr>
<td>S. aureus 8325-4</td>
</tr>
<tr>
<td>S. aureus COL</td>
</tr>
<tr>
<td>S. aureus SH100</td>
</tr>
<tr>
<td>COL::dnaK</td>
</tr>
<tr>
<td>SH100::dnaK</td>
</tr>
<tr>
<td>DCI</td>
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<td>E. coli JM109</td>
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**Plasmids**

<table>
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<th><strong>Strain or plasmid</strong></th>
<th><strong>Characteristics</strong></th>
<th><strong>Reference</strong></th>
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<tr>
<td>pGEMT</td>
<td>Cloning vector for E. coli (AmpR)</td>
<td>Promega</td>
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<tr>
<td>pCR2.1</td>
<td>Cloning vector for E. coli (AmpR, KanR)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pTZ18R</td>
<td>Cloning vector for E. coli (AmpR)</td>
<td>Mead et al. (1986)</td>
</tr>
<tr>
<td>pCU1</td>
<td>Shuttle vector (AmpR in E. coli and CamR in S. aureus)</td>
<td>Augustin et al. (1992)</td>
</tr>
<tr>
<td>pTZ18R-dnaK</td>
<td>Plasmid pTZ18R containing a 2.1 kb DNA fragment encompassing the S. aureus dnaK gene</td>
<td>This study</td>
</tr>
<tr>
<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>
</tr>
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</table>

*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 cross-over, and integration of the entire construct into the *S. aureus* chromosome. Phage 80z 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 & Moshkovitz, 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’-GTAACCTCTTCTTCACTAGAGAAGTG-3’) and P6 (5’-CTCAGAAGCAACCTCACATTTGTCTA-3’). *S. aureus* COL genomic DNA as a template, and EXL 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 *dnaK* gene and 285 nt downstream of the *prnA* 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 (OD600 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 H2O2, 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 OD600 spectrophotometrically. These stress conditions were chosen based on prior studies (Singh & Moshkovitz, 2003; Singh et al., 2001a, 2001b; Utaida 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 (OD600 0.6) were diluted 50-fold in fresh TSB, and placed at 37 °C until the OD600 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 H2O2.** The MICs for the wild-type and the *dnaK* mutant of *S. aureus* strain COL were determined as described (Pfeitz et al., 2000), using 96-well microtitre plates containing twofold serial dilutions of oxacillin, meticillin and H2O2. Mid-exponential-phase wild-type and *dnaK* mutant staphylococcal cells were then added to these dilutions, to a final concentration of 5 × 10^8 c.f.u. ml⁻¹. The plates were incubated at 37 °C for 48 h, and the lowest concentration of antibiotic or H2O2 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 OD600 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⁻¹. Oxidative stress was imposed by the addition of H2O2 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 RNAiso (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-32P]dATP [specific activity >3000 Ci mmol⁻¹ (>111 TBq mmol⁻¹)]; 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 OD600 of 0.5. Both cultures were subsequently treated for 15 h at 37 °C with 10 μg oxacillin ml⁻¹. 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 autoysis assays.** Autoysis assays were performed as previously described (Pfeitz et al., 2000). Briefly, wild-type and *dnaK* mutant cultures of *S. aureus* COL were grown to an OD600 of 1.0 at 37 °C in PYK medium (0.5 % bacto peptone, 0.5 % yeast extract, 0.3 % K2HPO4, 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 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 A462 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 (OD600 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 $5 \times 10^8$ 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 CO$_2$ 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 dnaK 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
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 persisters 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

Fig. 2. (a) Northern blot hybridization of the dnaK gene. Total RNA isolated from the cultures of S. aureus strain COL was separated on a 1.2 % denaturing agarose gel, as described in Methods, and stained with ethidium bromide to ensure that equivalent amounts of total RNA were present in each lane (top panel). S. aureus strain COL was grown in TSB to an OD600 of 0.3. The liquid culture was divided into equal volumes in four tubes, and stressed for 10 min before processing for RNA isolation. Lanes: 1, control; 2 and 4, cells stressed with 15 mM H2O2 and 1.2 mg oxacillin ml⁻¹, respectively; 3, cells exposed to 43 °C. Lanes 5 and 6, dnaK mutant and mutant complemented with entire dnaK locus on a high-copy plasmid, respectively. Cells were processed using methods similar to those used for the wild-type cells in lane 1. The arrows indicate the transcripts reacting with the probe corresponding to the 6.4 kb fragment of construct pCU-dnaK. (b) Quantitative data of the intensity of two apparent bands in (a). The 5.8 kb transcript is shown by the shaded bar, and the 3.5 kb transcript is shown by the black bar. Lane 6, both bands were saturated, and the intensity exceeded the scale shown on the y-axis; thus, the bands are shown by broken bars.
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 persist.

### Table 2. Susceptibilities of *S. aureus* parental and *dnaK* mutant strains to oxacillin, meticillin and H$_2$O$_2$

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<tr>
<th>Bacterial strain</th>
<th>MIC Oxacillin (µg ml$^{-1}$)</th>
<th>MIC Meticillin (µg ml$^{-1}$)</th>
<th>MIC H$_2$O$_2$ (mM)</th>
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<tr>
<td><em>S. aureus</em> strain COL</td>
<td>400</td>
<td>800</td>
<td>2.0</td>
</tr>
<tr>
<td>COL : <em>dnaK</em> mutant</td>
<td>100</td>
<td>200</td>
<td>0.25</td>
</tr>
<tr>
<td>Complemented strain</td>
<td>400</td>
<td>800</td>
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</table>

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 H$_2$O$_2$. Values indicate the mean of two independent experiments.

### 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
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.
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 monocyctogenes 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 3825-4, 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, in which the SigB defect has been corrected (Horsburgh et al., 2002). Additional stress response defects in a *dnaK* mutant of *S. aureus* strain 3825-4 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 to 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 carboxylation 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 Brucella 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 VraSR two-component regulatory system (Kuroda et al., 2003). Inactivation of VraSR results in reduced oxacillin MICs in meticillin-resistant *S. aureus* (Boyle-Vavra et al., 2006; Gardete et al., 2006). VraSR 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 also 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.
ACKNOWLEDGEMENTS

This work was supported by start-up funds from the Kirksville College of Osteopathic Medicine (KCOM) to V. K. S., a grant from KCOM Biomedical Sciences Graduate Program to L. S. J., grant AI43970 to B. J. W., and grant GM071363 to R. K. J. from the National Institutes of Health.

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stress response, autolysis, and DNA repair.


Edited by: T. Msadek