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, 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

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–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., 2003). However, *S. aureus* is able to overcome this repression during growth at higher temperatures and in other stress conditions (Chastanet et al., 2003). Beyond these studies, the roles of proteins encoded by the dnaK locus in *S. aureus* physiology are poorly understood. To begin to elucidate these roles, a knockout mutant of the dnaK gene in *S. aureus* was constructed. The findings of this study suggest critical roles for the staphylococcal dnaK locus in protection from heat, oxidative and antibiotic stress, in autolysis and pigmentation, and in survival in a mouse host.

### METHODS

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmid constructs 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.

**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’–GCTGTAGTTCAAGATGATAACCC–3’) and P2 (5’–CAGAGCCATTGAATCCTTGTC–3’) were used to amplify a

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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Reference</th>
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</thead>
<tbody>
<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>
</tr>
<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>
</tr>
<tr>
<td><em>S. aureus</em> COL</td>
<td>Homogeneous* in meticillin-resistance expression</td>
<td>Peltz et al. (2000)</td>
</tr>
<tr>
<td><em>S. aureus</em> SH1000</td>
<td><em>S. aureus</em> strain 8325-4 with functional rsbU</td>
<td>Horsburgh et al. (2002)</td>
</tr>
<tr>
<td>COL* dnaK</td>
<td><em>S. aureus</em> COL with mutation in the dnaK gene (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>SH1000* dnaK</td>
<td><em>S. aureus</em> SH1000 with mutation in the dnaK gene (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>DC1</td>
<td>COL*dnaK complemented with 6.4 kb dnaK locus (Kan&lt;sup&gt;R&lt;/sup&gt;, Cam&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em> JM109</td>
<td>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F&lt;sup&gt;−&lt;/sup&gt; (traD36 proAB&lt;sup&gt;+&lt;/sup&gt; lacPAM15)</td>
<td>Yanisch-Perron et al. (1985)</td>
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**Plasmids**

| pGEMT | Cloning vector for *E. coli* (Amp<sup>R</sup>) | Promega |
| pCR2.1 | Cloning vector for *E. coli* (Amp<sup>R</sup>, Kan<sup>R</sup>) | Invitrogen |
| pTZ18R | Cloning vector for *E. coli* (Amp<sup>R</sup>) | Mead et al. (1986) |
| pCU1 | Shuttle vector (Amp<sup>R</sup> in *E. coli* and Cam<sup>R</sup> in *S. aureus*) | Augustin et al. (1992) |
| pTZ18R-dnaK | Plasmid pTZ18R containing a 2.1 kb DNA fragment encompassing the *S. aureus* dnaK gene | This study |
| pTZ-dnaK-Kan | A 1.4 kb kanamycin resistance gene was used to replace the EcoRV fragment of construct pTZ18R-dnaK | This study |
| pCU1-dnaK | 6.4 kb fragment containing all five genes of the dnaK locus | This study |

*Most cells in the population of homogeneous strains grow in the presence of high concentrations of meticillin (Sutherland & Rolinson, 1964).*
2.2 kb was excised from this construct, and subcloned into the vector plasmid pCR2.1 (Invitrogen). A gene, 164 bp upstream, and 391 bp downstream, was cloned 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 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 & 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’-GGTACCTTTGACTGAGAGAATGG-3’) and P6 (5’-CTTGAAGGACCACTCATTTTGTCA-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 was propagated in E. coli cells, 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 16 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 autolysis assays. Autolysis assays were performed as previously described (Pfetlz 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 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 A₄₅₀ 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
The genetic organization of the 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 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 mutant 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 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 thermostolerance**

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 thermostolerance 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.

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

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Oxacillin (μg ml⁻¹)</th>
<th>Meticillin (μg ml⁻¹)</th>
<th>H₂O₂ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus strain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COL</td>
<td>400</td>
<td>800</td>
<td>2.0</td>
</tr>
<tr>
<td>COL: dnaK mutant</td>
<td>100</td>
<td>200</td>
<td>0.25</td>
</tr>
<tr>
<td>Complemented strain</td>
<td>400</td>
<td>800</td>
<td>2.0</td>
</tr>
</tbody>
</table>

**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 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, 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 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 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 stimulon (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.
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