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.

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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, septicæmia, 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 (σ^{32}), 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 σ^{32} in *E. coli*, an alternative sigma

factor, σ^E , 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 σ^B 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, σ^B 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.

amounts during sublethal heat and other stress conditions. They prevent misfolding and aggregation of proteins, and promote their refolding and proper assembly under normal and stress conditions (Checa & Viale, 1997; Craig, 1985; Diamant & Goloubinoff, 1998; Hartl, 1996; Hubbard & Sander, 1991; Walter & Buchner, 2002). The Hsps bind and release hydrophobic segments of an unfolded polypeptide chain in an ATP-hydrolytic reaction cycle (Hartl, 1996; Walter & Buchner, 2002).

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 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

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference
<i>S. aureus</i> RN4220	A restriction minus derivative of <i>S. aureus</i> strain 8325-4	Kreiswirth <i>et al.</i> (1983)
<i>S. aureus</i> 8325-4	A laboratory strain of <i>S. aureus</i> cured of all the prophages	Novick (1991)
<i>S. aureus</i> COL	Homogeneous* in meticillin-resistance expression	Pfultz <i>et al.</i> (2000)
<i>S. aureus</i> SH1000	<i>S. aureus</i> strain 8325-4 with functional <i>rsbU</i>	Horsburgh <i>et al.</i> (2002)
COL: <i>dnaK</i>	<i>S. aureus</i> COL with mutation in the <i>dnaK</i> gene (Kan ^R)	This study
SH1000: <i>dnaK</i>	<i>S. aureus</i> SH1000 with mutation in the <i>dnaK</i> gene (Kan ^R)	This study
DC1	COL: <i>dnaK</i> complemented with 6.4 kb <i>dnaK</i> locus (Kan ^R , Cam ^R)	This study
<i>E. coli</i> JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F'</i> (<i>traD36 proAB⁺ lacI^ΔAM15</i>)	Yanisch-Perron <i>et al.</i> (1985)
Plasmids		
pGEMT	Cloning vector for <i>E. coli</i> (Amp ^R)	Promega
pCR2.1	Cloning vector for <i>E. coli</i> (Amp ^R , Kan ^R)	Invitrogen
pTZ18R	Cloning vector for <i>E. coli</i> (Amp ^R)	Mead <i>et al.</i> (1986)
pCU1	Shuttle vector (Amp ^R in <i>E. coli</i> and Cam ^R in <i>S. aureus</i>)	Augustin <i>et al.</i> (1992)
pTZ18R- <i>dnaK</i>	Plasmid pTZ18R containing a 2.1 kb DNA fragment encompassing the <i>S. aureus dnaK</i> gene	This study
pTZ- <i>dnaK</i> -Kan	A 1.4 kb kanamycin resistance gene was used to replace the <i>EcoRV</i> fragment of construct pTZ18R- <i>dnaK</i>	This study
pCU1- <i>dnaK</i>	6.4 kb fragment containing all five genes of the <i>dnaK</i> locus	This study

*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 *Hind*III 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 *Eco*RV, 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 80 α 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'-GGTACCTTCTTTGACTGAGAGAATGG-3') and P6 (5'-TCTAGAGCAACCACTCATATTTGTCA-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 *hrcA* gene and 285 nt downstream of the *prnA* gene that was cloned to the *Kpn*I and *Xba*I 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 & Moskovitz, 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 10 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 (Pfeltz *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 × 10⁵ 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 RNAlater (Ambion), and incubated overnight at 4 °C to inactivate RNases. Cells were washed with PBS, recovered by centrifugation, resuspended in 1.0 ml RNA-Bee (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 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 (Pfeltz *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 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 5×10^8 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 *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

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'-CCTACACGCCAAGCAATG-3') and P4 (5'-AATACTAGACTGTCAGCTTCGT-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 (*Hind*III digest of λ DNA).

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.

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

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.

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

Bacterial strain	MIC		
	Oxacillin (µg ml ⁻¹)	Meticillin (µg ml ⁻¹)	H ₂ O ₂ (mM)
<i>S. aureus</i> strain COL	400	800	2.0
COL: <i>dnaK</i> mutant	100	200	0.25
Complemented strain	400	800	2.0

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

Fig. 4. Survival of the *dnaK* mutant and its isogenic wild-type *S. aureus* strain COL to lethal heat stress. Left, cultures were shifted from 37 °C to 60 °C for 5 (shaded bars) and 10 min (bars with horizontal lines); black bars, number of bacteria at 37 °C. Right, cultures were shifted from 48 °C to 60 °C for 5 (shaded bars) and 10 min (bars with horizontal lines); black bars, number of bacteria at 48 °C. Values indicate the mean of three independent experiments. WT, wild-type. Error bars represent SEM.

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

Fig. 5. (a) Growth of the *dnaK* mutant (□) and its isogenic wild-type *S. aureus* strain COL (■) in the presence of 100 µg oxacillin ml⁻¹. (b) Autolysis of the *dnaK* mutant (□) compared with autolysis of the isogenic wild-type *S. aureus* strain COL (■). Values indicate the mean of two independent experiments.

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 ± 0.148 vs 0.178 ± 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

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.

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 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 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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