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An insight into the significance of the DnaK heat shock system in *Staphylococcus aureus*

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ABSTRACT

The bacterium *Staphylococcus aureus* utilizes a variety of different mechanisms to survive unfavorable stress conditions that are critical for its persistence in the environment and for pathogenicity. The staphylococcal DnaK heat shock system functions as a major protein folding machine under stress conditions that cause aggregation and un-folding of proteins. In prior studies, *S. aureus* cells with a non-functional DnaK system showed reduced tolerance to heat, oxidative and antibiotic stresses, a lowered carotenoid production, and decreased survival in a murine host. This study provides insights that the altered phenotypes of the *dnaK* mutant cells are not due to decreased SigB activity in the mutant cell. Transcriptomic profiling studies provide evidence that a large number of genes encoding proteins involved in cell wall biogenesis, virulence and general stress tolerance, and genes encoding proteins involved in metabolic processes are differentially regulated in *dnaK* mutant cells relative to wild-type *S. aureus*. It was also determined that loss of functional DnaK caused a reduction in the ability of *S. aureus* to make biofilms and its adherence to eukaryotic cells. This study provides evidence of a global significance of DnaK heat shock system in *S. aureus*.

Introduction

Staphylococcus aureus is a leading cause of diseases from moderate skin infections to life-threatening afflictions, such as endocarditis, pneumonia and septicemia. Patients often suffer from recurrent *S. aureus* skin and soft tissue infections due to an absence of protective immunity (Kim et al., 2011). Treatment of staphylococcal infections can be problematic as the organism quickly acquires resistance to antibiotics. While, vancomycin was the drug of choice for treatment of infections caused by methicillin-resistant *S. aureus* strains, *S. aureus* clinical isolates with resistance to vancomycin have been discovered (Whitener et al., 2004). It is becoming increasingly important to find new ways to treat *S. aureus* infections.

S. aureus has not only survived but also managed to thrive over the years in the environment because of its adaptability and stress resistance capabilities (Clements and Foster, 1999). It is resistant to a variety of stresses, such as oxidative, pH, osmotic, and temperature stresses. Production of a number of heat shock proteins (Hsp) is the key to the survival of *S. aureus* at higher temperatures. Bacterial heat shock proteins assist in proper folding and assembly of cellular proteins, and also prevent misfolding and aggregation of proteins (Lund, 2009; Muga and Moro, 2008; Walter and Buchner, 2002). In bacterial species, although several classes of heat shock proteins have been identified, the GroESL and DnaK heat shock protein systems are considered to be the major heat shock response protein systems (Lund, 2009; Muga and Moro, 2008).

S. aureus dnaK is transcribed as a pentacistronic message comprising *hrcA–grpE–dnaK–dnaJ–prmA* (Singh et al., 2007). HrcA protein negatively regulates gene expression of this locus under normal growth conditions. The next three genes, *grpE*, *dnaK* and *dnaJ* encode proteins that collectively function as an important bacterial protein folding machinery (Muga and Moro, 2008). The DnaK proteins are ATP-dependent molecular chaperones composed of an N-terminal nucleotide-binding domain and a C-terminal protein substrate-binding domain (Chang et al., 2008). DnaK cycles between an ATP-DnaK (low-affinity state) and an ADP-DnaK (high-affinity state) state. DnaJ brings unfolded proteins to DnaK that remain bound to DnaK-ADP until a favorable physiological state is regained. GrpE functions as a nucleotide exchange factor that promotes dissociation of ADP from DnaK for ATP and all three proteins are essential for the DnaK system to be functional (Chang et al., 2008; Nakamura et al., 2010; Singh et al., 2007).

In a previous study, *S. aureus* strains with a non-functional DnaK system demonstrated poor growth at elevated temperatures, under oxidative and antibiotic stress conditions. Bacterial strains lacking the DnaK system produced less pigment and had reduced survival in a murine host (Singh et al., 2007). Some of these observations raised the question whether these effects can be attributed to

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Table 1
Bacterial strains used in this study.

Strains or plasmid	Characteristics	Reference
S. aureus SH1000	*S. aureus* strain 8325-4 with functional *rsbU*	Horsburgh et al. (2002)
SH1000:*dnaK*	*S. aureus* SH1000 with mutation in the *dnaK* gene (Kan^R)	Singh et al. (2007)
SH1000:*sigB*	SH1000 with mutation in the *sigB* gene (Tet^R)	This study
SH1000:*dnaK sigB*	SH1000 with mutation in the *dnaK* and *sigB* genes (Kan^R, Tet^R)	This study

alterations/reduction in SigB activity. SigB is required for *S. aureus* stress responses and it also down-regulates pigment production. Therefore, a *sigB* mutant was included to compare its phenotypic defects with that of the DnaK-defective *S. aureus*. Findings of this study suggest that lack of a functional DnaK in *S. aureus* impacts the expression of a large number of genes. Proteins encoded by these genes are likely responsible for the phenotypic defects observed in the *S. aureus dnaK* mutant.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains used in this study are described in Table 1. Strain SH1000 (Horsburgh et al., 2002) was used as the parent *S. aureus* in this study. Construction of a *dnaK::kan* mutant of SH1000 was described previously (Singh et al., 2007). A *sigB::tet* mutant of SH1000 was generated by transducing *sigB*::*tet* from PC400 (Chan et al., 1998). A *dnaK sigB* double mutant was constructed by transducing the *sigB*::*tet* mutation into the SH1000 *dnaK*::*kan* mutant strain. *S. aureus* cells were routinely grown aerobically at 37 °C in tryptic soy broth/agar (TSB/TSA; Beckton Dickinson). Broth cultures were grown in a shaking incubator (220 rpm). Kanamycin and tetracycline when needed were added at the concentrations of 100 μg ml^{-1} and 10 μg ml^{-1}, respectively

Growth kinetics of wild-type S. aureus, dnaK, sigB, and dnaK sigB mutants under stress

Early-exponential phase cultures (OD_{600} = 0.6) were diluted 50-fold in fresh TSB and cultures were placed at 37 °C in a shaking incubator and growth was measured as described previously (Singh et al., 2007). In parallel flasks, the following stress conditions were imposed by modifications of TSB or by changes in growth temperatures: H_2O_2 (1.1 mM) and high temperature (43 °C).

Transcriptional profiling of S. aureus cells lacking a functional DnaK system

Total RNA was extracted from wild-type *S. aureus* SH1000 and its isogenic *dnaK* mutant cultures grown in TSB to OD_{600} = 0.7. Bacterial cells were harvested by centrifugation, resuspended in 1.0 ml RNA*later*® (Ambion), and incubated overnight at 4 °C. Bacteria were recovered by centrifugation, resuspended in Trizol (Tel-Test, Inc., Friendwood, TX), and lysed using 0.1 mm silica/zirconium beads in a Mini-Beadbeater (BioSpec). Total RNA was extracted from the lysate as per manufacturer's recommendations. The extracted RNA was DNase treated and purified using an RNeasy kit (Qiagen). A Microbe mRNA enrichment kit (Ambion) was used for mRNA enrichment. The mRNA-enriched samples were used to generate labeled cDNA in a reverse transcription reaction using SuperScript™ III reverse transcriptase (Invitrogen), random hexamers, and Cy3- or Cy5-dUTP (GE Healthcare). To control for variations in label incorporation, dye-flip experiments were also carried out for each sample. Equivalent amounts of Cy3- and Cy5-labeled cDNAs representing either *S. aureus* wild-type or *dnaK* mutant samples were mixed, purified using a Qiagen MinElute kit (Qiagen), and concentrated with Amicon YM 10 columns. Mixed and concentrated probes were eluted in a buffer containing 50% formamide, 0.1% SDS and 5× SSC, and denatured at 94 °C for 3 min. The *S. aureus* PFGRC 6 array slides were prehybridized with 0.1% BSA solution in 5× SSC and 0.1% SDS for 30 min at 42 °C. Denatured probes were then placed on these slides for hybridization for 16–20 h at 42 °C, under a cover slip. The slides were then washed at room temperature sequentially in 2× SSC/0.1% SDS, 1× SSC, 0.2× SSC and 0.05× SSC, respectively, for 1 min each and dried by centrifugation. Slides were imaged using a GenePix 4000B scanner (Molecular Devices). Cy-3 and Cy-5 intensities for each spot were quantified, followed by median background correction, and normalization using GenePix Pro 6.0 software. *S. aureus* genes exhibiting a two-fold or greater difference in fluorescence following dye-flip consistency evaluations were considered to be altered in the *dnaK* mutant compared to wild-type *S. aureus*. Mean values of gene expression ratios based on two independent samples and four different hybridization experiments (including the dye-flip) were used to determine the genes with altered expression in the *dnaK* mutant.

Quantitative real-time RT-PCR (qRT-PCR) assays

qRT-PCR assays were used to validate microarray results. All primers used in real-time assays are shown in Supplementary Table S1. Total RNA from cultures grown to OD_{600} = 0.7 in TSB was extracted as described above. cDNA was synthesized using 0.5 μg of total RNA in a 20-μl reverse transcription reaction containing random hexamers and SuperScript™ III reverse transcriptase (Invitrogen). Control reactions lacking reverse transcriptase were performed to confirm the absence of contaminating genomic DNA in all samples. All real-time PCR reactions were carried out with Bio-Rad iCycler (iQ5 system). Transcript levels of genes were normalized to DNA gyrase mRNA based on a previous report (Eleaume and Jabbouri, 2004; Goerke et al., 2000). Fold-changes in gene expression were calculated using the formula $2^{-\Delta\Delta CT}$ as described in Livak and Schmittgen (2001).

qRT-PCR was also used for a selected number of genes identified as differentially expressed in microarray experiments to determine if the alterations in their expression were growth phase dependent. For these studies, cells from wild-type and *dnaK* mutant cultures were harvested from early- (OD_{600} = 0.7), mid- (OD_{600} = 1.8), and late-exponential (OD_{600} = 3.0) as well as from stationary (OD_{600} = 4.2) phase. Expression patterns of some of these genes were also determined in *sigB* and *dnaK sigB* mutant cells grown to OD_{600} = 0.7.

Levels of protein A in S. aureus cells

Total protein was extracted from lysostaphin-treated *S. aureus* cells as described previously (Singh et al., 2008). Membrane bound proteins were extracted by five cycles of freeze thaw of *S. aureus* cells as described previously (Singh et al., 2008). Total and membrane-bound proteins were separated by SDS-PAGE, and transferred to nitrocellulose membranes (Pierce). Membranes were blocked with 5% skimmed milk and incubated with rabbit antibodies conjugated to horseradish peroxidase (Bio-Rad). Blots were visualized for protein A using an Opti-4CN substrate kit (Bio-Rad).

Adherence of the dnaK mutant to A549 human lung epithelial cells

Adherence of *S. aureus* strain SH1000 and its derivative *dnaK* mutant was determined by infection of A549 human lung epithelial