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Characterization of *dnaK* Mutants in *Streptococcus pneumoniae*

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DnaK is a major heat shock protein and known to be highly conserved in all species. Previously, the *dnaK* in *Streptococcus pneumoniae* was cloned and the immunogenic nature characterized. In this study, *dnaK* mutants were generated by insertion of duplication mutagenesis and their characteristics examined. They had defective growths at all temperatures (20°C-42°C) and cell divisions, and formed filaments after a temperature shift from 30 to 42. A unique feature of the *dnaK* mutants of *S. pneumoniae*, unlike those of *E. coli* and *B. subtilis*, was the growth capability at high temperature (42°C) without producing the putative GroEL. Our results suggest that DnaK may serve as a regulator and/or modifier in GroEL gene expression.

Keywords: DnaK, GroEL, *Streptococcus pneumoniae*, Thermotolerance.

Introduction

The heat shock response is characterized by the *de novo* production of a small number of proteins (heat shock proteins: HSPs) after heat and other environmental stresses (Neidhardt and VanBogelen, 1987; Gross, 1996; Baek *et al.*, 1999; Park *et al.*, 1999). Bacterial HSPs are produced during infection, especially when the bacteria are inside the phagocytes. The HSP has been known to be highly conserved on an amino acid level and to function as chaperones. As part of the major heat shock proteins, DnaK promotes proper folding and translocation of proteins (Georgopoulos and Welch, 1993; Parsell and Lindquist, 1993) and is a candidate for major antigens in several pathogens (Kaufmann *et al.*, 1991; Kaufmann and Schoel, 1994). Although substantial progress has been made in the action mechanism of DnaK, interaction of DnaK with other proteins is still undetermined (Teter *et al.*, 1999).

In *Streptococcus pneumoniae* (pneumococcus), the elucidation of the HSP's role in the pathogenesis seems crucial in understanding pathogenesis and host response. *S. pneumoniae,* (a causative agent of pneumonia, meningitis, and middle ear infection) is present in nasopharynx as a part of normal flora (Willet, 1992), and it experiences a change in temperature, pH, and ethanol concentration. Invading pneumococci encounter additional environmental stresses. Macrophages, and other phagocytic cells, detect and engulf pneumococci soon after infection, exposing them to an extremely hostile environment (Bryun *et al.*, 1992; Salyers and Whitt, 1994). This hostile environment may act as a strong stress to *S. pneumoniae* and influence gene expression of heat shock proteins, since environmental stress can trigger or enhance expression of virulence genes (Buchmeier and Heffron, 1990; Dorman *et al.*, 1990; Mekalanos, 1992). Although some part of pneumococcal DnaK is highly conserved, and has immunodominant antigenic nature, anti-pneumococcal DnaK antiserum did not crossreact with DnaK homologues in *E. coli, Staphylococcus aureus* and human cells. This suggests that *S. pneumoniae* DnaK may be a good candidate as a vaccine (Hamel *et al.*, 1997; Kim *et al.*, 1998). Still, the role of DnaK in pathogenesis remains unknown.

Previously, we characterized general properties of the heat shock response of *S. pneumoniae* to heat and ethanol stress. Heat shock induced the synthesis of about 6 heat shock proteins (10-, 54-, 67-, 73-, 84-, and 115-kDa), and ethanol shock induced a protein with a molecular weight of about 104-kDa. Further purification and N-terminal amino acid sequence determination of 67- and 73-kDa proteins, as well as Western blot analysis, suggested that the 67- and 73-kDa proteins were GroEL and DnaK homologue (Choi *et al.*, 1997, 1998, 1999; Hamel *et al.*, 1997). To assess the role of heat shock response in pneumococcus, one of the most prominent heat shock protein genes, *dnaK*, was cloned, sequenced and characterized (Kim *et al.*, 1998). The cloned 3.4-kb fragment contained complete ORF of *dnaK*. The partial nucleotide sequence of 5′ and 3′ end region of this cloned 3.4-kb fragment was highly homologous to *grpE* and *dnaJ* gene of *L. lactis*, respectively. The genomic organization of *dnaK* locus of *S. pneumoniae* differs from that of *E. coli*, because the *grpE* gene is part of the *dnaK-dnaJ* operon in *S.*

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pneumoniae as in other gram positive bacteria (Kim *et al.*, 1998). Also, the expression of *B. subtilis dnaK* operon is regulated by inverted repeat sequences (Zuber and Schumann, 1994); whereas *E. coli* DnaK is negatively regulated by titration of s^{32} (Gross, 1996). The differences between gram positive, and gram-negative bacteria, suggest that the regulation mechanism of heat shock response of gram-positive bacteria (especially DnaK function of gram-positive bacteria) is quite different than *E. coli* and other gram-negative bacteria. In this study, we further explored the action mechanism of DnaK by construction and characterization of *dnaK* mutants, and found a novel feature of DnaK in gene expression of *groEL* and thermotolerance.

Materials and Methods

Bacterial strains, plasmids, and growth conditions The bacterial strains and plasmids used in this work are listed in Table 1. All *S. pneumoniae* strains were grown in broth derived from casein-trypton (CAT) based medium either in liquid, or on solid plates containing 1.5% agar (Kim *et al.*, 1996). Cultures were grown in broth at 37°C without aeration. Complete transformation medium (CTM), prepared from CAT broth by addition of (per liter) 110 mg of $CaCl_2$ and 2 g of bovine serum albumin (fraction V; Sigma, St. Louis, USA), was used to transform *S. pneumoniae* (Kim *et al.*, 1998). For selection in pneumococcus, erythromycin was added to growth medium to a concentration of 1 μg/ml. Transformation of *S. pneumoniae,* was performed, as described elsewhere (Rhee and Morrison, 1988).

DNA isolation and manipulation Chromosomal DNA of *S. pneumoniae* was isolated, as previously described (Rhee and Morrison, 1988). DNA was manipulated by standard methods (Sambrook *et al.*, 1989).

Construction of *dnaK* insertion mutant of *S. pneumoniae* To construct *dnaK* mutant in *S. pneumoniae,* insertion duplication mutagenesis (Mejean *et al.*, 1981; Morrison *et al.*, 1984; Chandler and Morrison, 1987), using pJDC9 (Chen and Morrison, 1987) as a vector, was performed. The erythromycin resistance gene (*ermB*) from pJDC9 was used as a marker. Initially the recombinant plasmid pSKK01 (Kim *et al.*, 1998: Fig. 1), containing PCR product of *S. pneumoniae dnaK,* was double digested with *Sph*I and *Sal*I. The resulting *dnaK* insert fragment was ligated to pJDC9 vector, digested with the same restriction enzymes. The ligated vector was then crossed into the *S. pneumoniae* CP1200 chromosomal DNA by transformation, and erythromycin-resistant mutants were selected.

Protein-labeling *S. pneumoniae* wild type and mutant were cultured at 30°C and 4×10^8. Cells were then harvested and resuspended in 1 ml of semisynthetic labeling medium (Choi *et al.*, 1999), prewarmed at 30°C and incubated for 10 min at 30°C. 1μ l of L-[^{35}S]-methionine (555MBq/ml; Amersham, Richmond, USA) was added to labeling media and then the culture was transferred to a 42°C water bath for heat shock. The cells were harvested and lysed by repeating freezing-thawing cycles in 20 μl of lysis buffer (5 mM Tris-Cl, pH 8.0, 30 mM EDTA, 0.1% Triton X-100, 0.25 mg/ml PMSF, 1 mM DTT). The supernatant of lysates was analyzed by SDS-PAGE and autoradiography.

Antisera preparation Pneumococcal GroEL was purified by DEAE-Sephacel and ATP-agarose chromatography and used for antiserum preparation, as previously described (Choi *et al.*, 1998). Antiserum against PAGE-purified DnaK was generated as follows. The exponential culture of *S. pneumoniae* CP1200 was incubated at 42°C for 30 min, and the total lysate proteins were separated by electrophoresis on an 7.5% SDS-polyacrylamide gel, then lightly stained with Coomassie brilliant blue. The p73 (DnaK) bands were cut out and electroeluted. These eluted proteins were transferred to polyvinylidene difluoride (PVDF) membrane. One hundred micrograms of p73 per 1 ml of PBS were mixed with 1 ml of Freunds incomplete adjuvant. This mixture was then injected intramuscularly and subcutaneously into rabbits. Two booster shots were given every 2 weeks, and antiserum was collected after 6 weeks.

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

Strain or plasmid	Relevant property(ies)	Reference or source
E. coli Strains		
XL1-Blue	recA1 lac[F'proABlacI^{q}ZΔM15Tn10(Tet^r)]	Stratagene
S. pneumoniae Strains		
CP1200	*malM511 str1*	Kim *et al.*, 1998
SKK9515	*malM511 str1 dnaK::pJDC9::ermB*	This study
SKK9522	*malM511 str1 dnaK::pJDC9::ermB*	This study
SKK9523	*malM511 str1 dnak::pJDC9::ermB*	This study
Plasmids		
pJDC9	7.0-kb, Em^r, *lacZ'*	Chen & Morrison, 1987
pBluescript SK(-)	3.0-kb, Ap^r	Stratagene
pGEM-7Zf(+)	3.0-kb, Ap^r	Promega
pGEM-T	3.0-kb, Ap^r, TA cloning vector	Promega
pSKK01	3.7-kb, Ap^r, 0.65-kb *dnaK* PCR product cloned in PGEM-T	Kim *et al.*, 1998
pSKK02	7.7-kb, Em^r, 0.65-kb *dnaK* fragment cloned in pJDC9	This study

Western Blotting Proteins separated on a 10% SDS-polyacrylamide gel were electroeluted onto a PVDF membrane and developed. The PVDF membranes were exposed to 1:200 - 1:1,000 dilutions of a rabbit antisera raised against GroEL or DnaK of *S. pneumoniae,* then a 1:2,000 dilution of anti-rabbit immunoglobulin G antibodies was conjugated to horseradish peroxidase and developed with 4-choro-1-naphthol and hydrogen peroxide.

Determination of induced thermotolerance The effect of *dnaK* mutation on induced thermotolerance was assessed by growing cells at 30°C, then subjected to a 20 min heat shock at 42°C, before exposure to a lethal temperature of 45°C. Following heating at 45°C, samples were removed and transferred to an ice-cold media, and immediately plated on agar to measure colony-forming capacity.

Microscopy Cells from exponentially growing cultures at 30°C or 42°C were prepared on glass microscope slides and examined under the microscope (Optiphot-2, Nikon Co., Japan). All micrographs were prepared with the same magnification (1,000).

Results

Identification of the gene product of *dnaK* mutant To construct *dnaK* mutant, mutagenesis was performed by transformation of competent cells with chimeric DNA, formed by the ligation of vector pJDC9 to 0.65-kb PCR product (the forward and reverse PCR primer sequences were 5′-CARGCNACNAARGAYGCNGG-3′ and 5′-GCNACNGC YTCRTCNGGRTT-3′, where Y = T or C, R = A or G, and

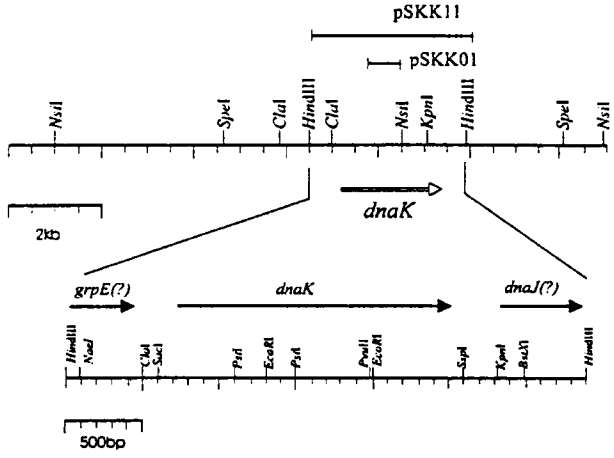

Fig. 1. Restriction map and genetic organization of the *dnaK* gene of *S. pneumoniae.*

N = A, G, T, or C) of *S. pneumoniae dnaK* (Fig. 1). A recombinant plasmid, containing *dnaK* insert, cannot replicate in the host cell. Instead this plasmid is recombined with chromosomal DNA, using the insert DNA as a target, and inserted into the host chromosome. The resulting transformants have insertion of recombinant plasmid flanked by duplicated target sequences (Mejean *et al.*, 1981; Morrison *et al.*, 1984; Chandler and Morrison, 1987) using pJDC9 (Chen and Morrison, 1987). The erythromycin-resistant *dnaK* mutants were analyzed by SDS-PAGE. DnaK present in the wild type was not present in the mutant. Also, in *dnaK* insertion mutants, the overall protein pattern was changed:

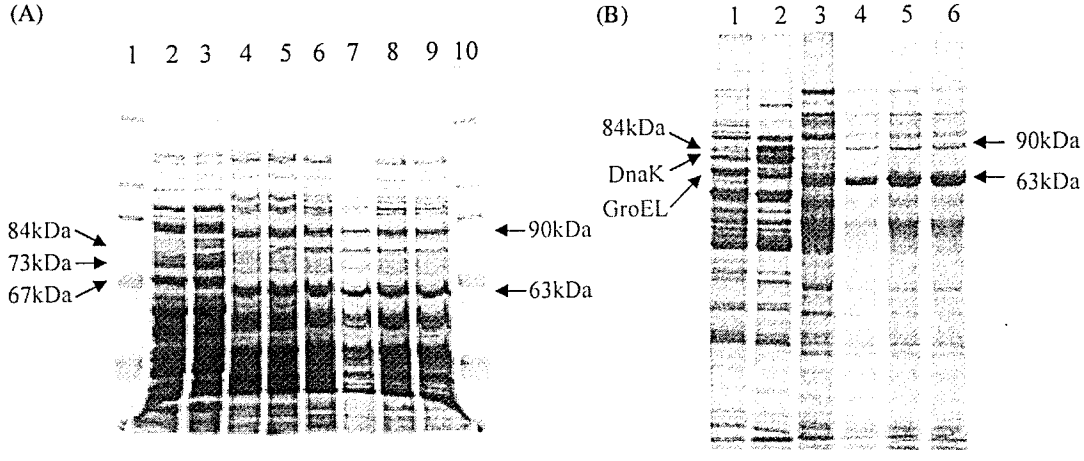

Fig. 2. Protein patterns of the wild type and the *dnaK* mutants after heat shock. For heat shock, a logarithimically growing culture at 30°C was shifted to 42°C. Total cellular proteins were analyzed by 10% SDS-polyacrylamide gel electrophoresis. Proteins were visualized by Coomassie blue staining [A], or proteins were labeled with [^{35}S]-methionine and visualized by autoradiography [B]. Panel [A]: Even numbered lanes were not heat-shocked and odd numbered lanes were heat-shocked for 30 min. Lanes 1 and 10, molecular weight markers; lanes 2 and 3, CP1200 (*dnaK*$^{+}$); lanes 4 and 5, SKK9515 (*dnaK*$^{-}$); lanes 6 and 7, SKK9522 (*dnaK*$^{-}$); lanes 8 and 9, SKK9523 (*dnaK*$^{-}$). The arrows on the right indicate overproduced proteins in the *dnaK* mutants. Molecular weight markers are, from the top, myosin (206,000 Da), -galactosidase (116,000 Da), phosphorylase b (97,400 Da), bovine albumin (66,000 Da), ovalalbumin (45,000 Da), and carbonic anhydrase (29,000 Da). Panel [B]: Lane 1, CP1200 (wild type) at 30°C; lane 2, CP1200 heat-shocked for 30 min; lane 3, SKK9522 (*dnaK*$^{-}$) at 30°C; lane 4, SKK9522 heat-shocked for 1 hour; lane 5, SKK9522 heat-shocked for 5 hours ; lane 6, SKK9522 heat-shocked for 10 hours. The arrows on the right indicate overproduced proteins in the *dnaK* mutant.

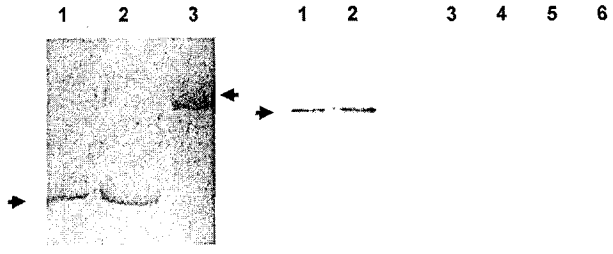

Fig. 3. Western blot analysis of the wild type and the *dnaK* mutants. The culture was grown at 30°C and, if needed, heat-shocked at 42°C for 30 min, then total cellular proteins were probed with anti-pneumococcus heat shock protein antibodies. [A] Total protein extract from the heat-shocked culture was probed with anti-DnaK. Only the relevant portions of the blots are shown; lane 1, SKK9522 (*dnaK*⁻); lane 2, SKK9523 (*dnaK*⁻); lane 3, CP1200 (*dnaK*⁺). The arrowhead on the left indicates 63-kDa truncated DnaK protein. The arrowhead on the right indicates the 73-kDa DnaK protein. [B] Probed with anti-GroEL. Lane 1, CP1200 (*dnaK*⁺) incubated at 30°C; lane 2, CP1200 heat-shocked; lane 3, SKK 9522 (*dnaK*⁻) incubated at 30°C; lane 4, SKK9522 heat-shocked; lane 5, SKK9523 (*dnaK*⁻) incubated at 30°C; lane 6, SKK9523 heat-shocked. The arrowhead on the left indicated the 65-kDa GroEL protein.

dnaK mutants did not synthesize heat shock protein p67 (GroEL) (Fig. 2 [A]). On the other hand, few proteins (about 90-kDa and 63-kDa) were overproduced. Protein labeling with [^{35}S]-methionine revealed the same result (Fig. 2 [B]). When whole cell extracts were subjected to Western blot analysis with antibodies, raised against purified *S.pneumoniae* Dnak, Dnak protein of a normal size (73-kDa) was synthesized in the wild type, but a premature DnaK protein with a molecular weight of about 63-kDa was detected in the *dnaK* mutant (Fig. 3). The results showed that the *dnaK* mutation is a non sense mutation resulting in premature termination of the peptide. However, when cell lysates were subjected to Western blot with the antibody raised against pneumococcus p67, the GroEL protein of a normal size (67-kDa), shown in the wild type, disappeared (Fig. 3 [B]). These results demonstrated that the disruption of the *dnaK* gene in the insertion mutants was accompanied by the disappearance of the *groEL* gene product.

Poor growth of the *dnaK* mutant To characterize the *dnaK* mutants of *S. pneumoniae*, the growth rate was determined, and their morphology was observed. The *dnaK* mutants of *S. pneumoniae*, SKK9522 and SKK9523, grew very poorly and their generation time was approximately 10 hours at 30°C, 37°C, and 42°C; whereas the generation time of the wild type was approximately 45 min at 37°C. Their maximum optical density at 550 nm was about 0.1 (Fig. 4). These features indicate that the DnaK protein has important functions in cellular metabolism, not only during heat shock, but also under normal, nonstressed conditions.

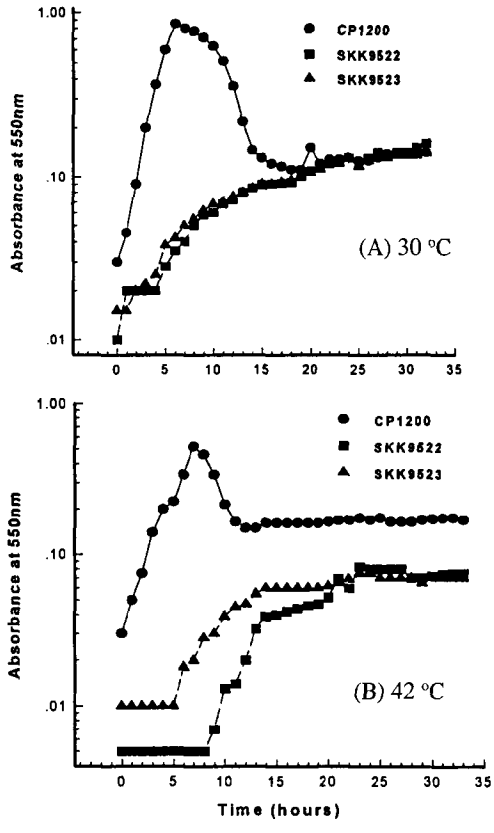

Fig. 4. Growth rate of the wild type and *dnaK* mutants at 30 and 42°C. Wild type (CP1200), and *dnaK* mutant SKK9522, were incubated at 30 [A] and 42°C [B], and optical density at 550nm was measured every hour.

Thermotolerance of the *dnaK* mutant The *dnaK* mutants were slightly more resistant to heat shock than the wild type, and they showed induced thermotolerance (Fig. 5). These results showed that *dnaK* mutants became defective in cell division after heat shock. It was reported previously that mutations in *dnaK, groE,* and *htpR* genes led to inhibition of cell division after a shift of the cells to nonpermissive temperatures (Georgopoulos and Eisen, 1974; Donachie *et al.*, 1984; Tsuchido *et al.*, 1986; Paek and Walker, 1987), suggesting that these proteins were required for cell division after heat shock.

Filamentation of the dnaK mutant When the *dnaK* mutants were examined under the microscope, before and after a temperature shift from 30°C to 42°C, wild type cells showed uniform cell length before and after the temperature shift. However, *dnaK* mutants produced filaments vigorously after a temperature shift up (Fig. 6).

Discussion

The cellular function of the DnaK protein is thought to play a role as a molecular chaperone. It sustains cellular function through association with components of synthetic machines

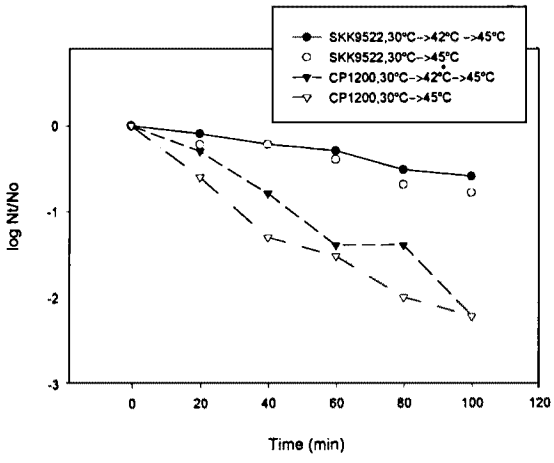

Fig. 5. Induced thermotolerance in the wild type and *dnaK* mutant of *S. pneumoniae*. For determination of basal thermotolerance, a logarithmically growing culture at 30°C was heated at 45°C. For determination of induced thermotolerance, cultures were preincubated at 42°C for 20 min prior to exposure to 45°C. ●, direct inactivation of CP1200 (*dnaK*$^+$) at 45°C; ■, inactivation of CP1200 at 45°C after pre-exposure to 42°C for 20 min; ▲, direct inactivation of SKK 9522 (*dnaK*$^-$) at 45°C; ▼, inactivation of SKK9522 at 45°C after pre-exposure to 42°C for 20 min. Nt/No denotes a ratio of number viable cells at time t/number of viable cells at time zero.

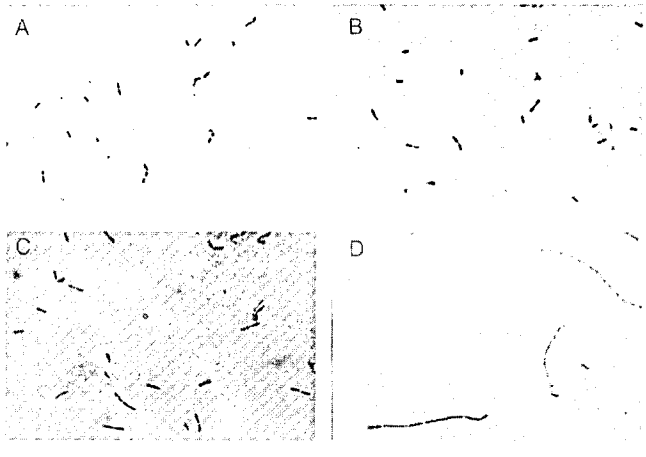

Fig. 6. Microscopic observation of the wild type and the *dnaK* mutant. Cells from exponentially growing cultures at 30, or 3 hours after a shift to 42°C, were prepared on the glass slide and then examined under the microscope. All micrographs were prepared with the same magnification (×1,000) as that for the wild type. [A] CP1200 (*dnaK*$^+$), 30°C; [B] CP1200, 42°C; [C] SKK9522 (*dnaK*$^-$), 30°C; [D] SKK9522, 42°C.

that form their functional structures, in addition to having a regulatory function in the heat shock response, especially in DNA replication (Yochem *et al.*, 1978; Sakakibara, 1988) and chromosomal segregation (Bukau and Walker, 1989).

Disruption of the *dnaK* locus was achieved by insertion-duplication mutagenesis. The vector, pJDC9, is an *E. coli* plasmid containing erythromycin-resistance gene, and cannot replicate in *S. pneumoniae*. Since the plasmid can be replicated only when it is inserted into *S. pneumoniae* (via homologous recombination using *S. pneumoniae* chromosomal insert DNA as a target and *S. pneumoniae* CP1200 is sensitive to erythromycin) there is no other way to produce erythromycin resistant transformants in *S. pneumoniae*. Therefore, all erythromycin-resistant transformants were supposed to be insertion mutants. Western blot analysis showed that anti-pneumococcal DnaK antibody cross-reacted with the p63 protein, which is nearly the same size as the overproduced protein in *dnaK* mutants. Therefore, the *dnaK* mutants of *S. pneumoniae* seemed to produce truncated DnaK protein terminated within pJDC9. This result suggested that the promoter region, and ribosome binding site of *dnaK* of *S. pneumoniae,* were not disrupted. Therefore, the transcription and translation start of *dnaK* gene was normal, but their termination occurred at the transcription termination sequences in pJDC9, which flanks the multiple cloning sites. The truncated DnaK consists of N-terminal (1-342) amino acids, in which 126-342 amino acids was used as a target fragment, and a part of *lacZ* derived from pJDC9 vector.

Our data demonstrate that the *dnaK* mutant did not produce the GroEL protein, although the mutant produced increased amounts of *dnaK* gene product at normal temperatures. This result suggests that *dnaK* mutation suppresses the expression of the *groEL* gene, or accelerates the degradation and/or modification of the GroEL protein in unknown ways. This is a unique feature of pneumococcus considering the fact that the *dnaK::cat* mutant in *B. subtilis* was not impaired in the induction profile of both its own operon and of the *groESL* operon (Schulz *et al.*, 1995). Also *E. coli dnaK* mutant showed an increase at the basal level of heat shock proteins, including the GroEL (Straus *et al.*, 1990). Since the DnaK protein in *E. coli* modulates the heat shock response negatively; thereby mutants in this gene were unable to turn off induced synthesis of heat shock proteins. This resulted in overexpression. In *S. pneumoniae*, lack of C-terminal half of the DnaK seemed to be unable to turn off the expression of *dnaK* and resulted in the overexpression of the truncated *dnaK* gene, which in turn lead to repression of the *groEL* gene. However, whether or not *groEL* is directly controlled by *dnaK* remains to be elucidated.

The *dnaK* mutants of *S. pneumoniae* showed very poor growth at 20°C, 30°C, 37°C and 42°C. These features indicate that the DnaK protein has important functions in cellular metabolism, not only during heat shock, but also under normal, nonstressed conditions. The *dnaK* mutants were also defective in cell division, and hence grew as filaments. It was reported previously that mutants in *dnaK*, *groE*, and *htpR* gene led to the inhibition of cell division after a shift of the cells to nonpermissive temperatures (Georgepoulos and Eisen, 1974; Tsuchido *et al.*, 1986; Paek and Walker, 1987). This suggests that these proteins were required for cell division after heat shock.

The result reported here demonstrates that the *dnaK* mutant

can grow between 20°C and 42°C, although the growth rate decreased slightly at low (20°C) and high (42°C) temperatures. The wild type, however, grows much slower at low (20-30°C) and high temperatures than at 37°C. The very poor growth of the mutant at all temperatures might be, at least in part, due to the lack of the putative *groEL* gene product. But at 45°C, a lethal temperature, both the wild type and the mutants pneumococcus died. Therefore there is virtually no temperature sensitivity between 20°C and 42°C in pneumococcus. However, the *dnaK* mutation in *B. subtilis* could form colonies between 16°C and 52°C, where growth stopped and formed filaments above 52°C, while the wild type could grow up to 56°C (Schulz *et al.*, 1995). Also, in *E. coli*, *dnaK* mutants, both the insertion and deletion mutants had altered growth properties (Paek and Walker, 1987). The mutants grew more slowly than the wild type at low and optimum temperatures (30 and 37°C), but at a higher temperature (42°C), the *dnaK* mutants died and were unable to form colonies. This temperature sensitivity of *dnaK* null mutants was caused totally by the absence of *dnaK* function. We concluded that the DnaK protein is needed only at very high temperatures in *E. coli* and *B. subtilis*; whereas it is not strictly required in pneumococcus.

Stress induced proteins were documented to contribute to the stabilization of proteins upon exposure to a variety of stresses (Georgopoulos and Welch, 1993; Parsell and Lindquist, 1993). In this sense, induced thermotolerance is correlated with the function of the stress induced proteins. In *E. coli, dnaK*756 mutant cells were inactivated at 52°C faster than the control cells (Ramsay, 1988). This suggests that the intact *dnaK* gene product plays a role in protecting the cell from lethal damage at 52°C. However, when the *dnaK* mutants were pretreated with heat shock at 42°C prior to exposure to lethal temperature (52°C), viability of the cells pretreated at 42°C were no different than the control cells, which were directly challenged with lethal temperatures. This result suggests that the DnaK protein has little or no effect on induced thermotolerance, although DnaK was produced in large amounts in response to thermal stress (Ramsay, 1988). The *S. pneumoniae dnaK* mutant showed similar results. Both wild type and mutant showed induced thermotolerance. This result, as well as the requirement of hsp104 family (Clp protein in bacteria) at extreme temperatures (Sanchez and Lindquist, 1990; Squires *et al.*, 1991), suggests that the intact DnaK is not essential for thermotolerance. Therefore, it could be postulated that the DnaK protein of *S. pneumoniae* did not contribute to the induced thermotolerance, but other heat shock protein, especially hsp104 homologue, may play a significant role in the thermotolorance.

Taken together, our analysis of cellular defects of *dnaK* mutant provides evidence that DnaK has multiple cellular functions. The erythromycin resistant *dnaK* mutant can provide very useful information on a variety of studies; for example, the role of hsp70 in translocation of protein as well as competence, which appears at a specific cell concentration of 10^8/ml as a kind of stress. Furthermore, the *dnaK* mutant can be used to resolve the cellular function of hsp70 in either the disease pathogenesis, or its diagnostic and vaccination potential, after introduction into the virulent is introduced into the virulent S type pneumococcus strain by transformation.

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