## ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/15281840

## Molecular Characterization of the Gene Operon of Heat Shock Proteins HSP60 and HSP10 in Methicillin-Resistant Staphylococcus aureus

### ARTICLE in BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS · JULY 1993

Impact Factor: 2.3 · DOI: 10.1006/bbrc.1993.1686 · Source: PubMed

CITATIONS

reads **7** 

### 5 AUTHORS, INCLUDING:



### Makoto Kuroda

National Institute of Infectious Diseas...

111 PUBLICATIONS 4,318 CITATIONS

SEE PROFILE



Lupin EX1124 Page 1

Find authenticated court documents without watermarks at docketalarm.com.

### MOLECULAR CHARACTERIZATION OF THE GENE OPERON OF HEAT SHOCK PROTEINS HSP60 AND HSP10 IN METHICILLIN-RESISTANT Staphylococcus aureus

### Toshiko Ohta, Kyoko Honda, Makoto Kuroda, Keiko Saito, and Hideo Hayashi

Department of Microbiology, Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki, 305, JAPAN

Received April 24, 1993

SUMMARY: Methicillin-resistance of *S. aureus* (MRSA) was diminished or depressed at 44°C. In order to investigate whether bacterial heat shock response is correlated with mathicillin resistance, we examined the inducibility of the heat shock proteins (HSPs) in MRSA, and cloned and sequenced of their genes. A temperature shift from 37°C to 46°C enhanced the production of at least 8 kinds of cytoplasmic proteins as measured with two-dimensional PAGE. The induced protein profile was almost the same as methicillin sensitive *S. aureus*, and stress conditions due to ethanol, cadmium or low pH. Two of these proteins were HSP60 and HSP10. Their N-terminal amino acid sequences were 79%, and 83%, homologous with thermobacterium PS3, respectively. A positively hybridized 4.2kbp DNA fragment encoding both proteins was isolated from the chromosomal DNA of MRSA. The resulting sequence revealed two reading frames and showed high homology to those of *hsp60* (*groEL*) and *hsp10* (*groES*) of bacteria (*E. coli*) and several other species. The genes of HSP60 and HSP10 in *S. aureus* comprised an operon as in *E. coli*. The relationship between those HSPs and PBP2' is currently under investigation. (\* 1993 Academic Press, Inc.

Heat shock proteins are temporarily expressed when cells are exposed to sublethal heat shock or the other stresses (1,2). These proteins are highly conserved from bacteria to animals (3). Some HSPs are chaperonins, which are involved in the folding, refolding, assembly, and disassembly of polypeptide chains during protein translocation processes (1,4,5). Homologous proteins to the GroEL and GroES of *Escherichia coli* chaperonins (3) have been found in other bacteria (6,7). The groE genes comprise an operon under positive transcriptional control promoted by heat shock sigma factor,  $\sigma^{32}$  (8,9).

Saphylococcus aureus, a pathogenic gram-positive bacteria, can grow under various stress conditions including high temperature, low pH, high pH, high osmotic pressure, or at lethal

0006-291X/93 \$4.00 Copyright © 1993 by Academic Press, Inc. All rights of reproduction in any form reserved. 730

DOCKE.

RM

concentration of drugs. Methicillin-resistant *S.aureus* (MRSA), can resist most  $\beta$ -lactam compounds. This drug-resistance was depressed with an exposure to high temperature at 44°C (10), suggesting possible involvement of heat shock proteins in the resistance mechanism in *S.aureus*. In order to clarify whether the heat shock response correlates with expression of the resistance, we examined the inducibility of the HSPs in MRSA and sequenced their genes. Here we determined the structure of the gene operon encoding HSP60 and HSP10.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions:** Clinically isolated and identified S. aureus 912 (MIC for methicillin =  $100\mu$ g/ml) was used for the cloning experiments. Plasmid pUC119 was used as a vector for the construction of genomic libraries, *E.coli* JM109 was the host. T-vector, a DNA fragment derived from pCR1000 was the vector for polymerase chain reaction (PCR) products, purchased from Invitrogen Co.(U.S.A.). S. aureus was grown at 37 °C in a brain heart infusion broth (BHI broth). Plasmid-containing *E. coli* was routinely grown at 37 °C in Luria-

Bertani medium (LB broth) supplemented with ampicillin (50µg/ml).

Chemicals: Pharmalytes (pH3-7 and pH7-9) were obtained from Pharmacia LKB(Sweden).

Adenosine  $[\gamma^{-3^2}P]$  triphosphate (6,000 Ci/ m mol) was obtained from Amersham (1 Ci=37 GBq). Lysostaphin, DNase I, RNase A, and proteinase K were purchased from Sigma Chemical Co.(St.Louis). Primers for DNA sequencing, DNA polymerase Klenow fragment, phosphokinase, and Ampli Taq-DNA polymerase were obtained from Takara Shuzo(Kyoto), and dideoxynucleoside triphosphates was from Boehringer Mannheim Inc.(Germany). Other chemicals used were of biochemical grade.

**Two-dimensional polyacrylamide gel electrophoresis:** Two-dimensional gel electrophoresis was carried out as described by Hirabayashi *et al.* (11).

Amino acid sequencing analysis: N-terminal sequences of the heat-inducible proteins obtained from two-dimensional gel electrophoresis were analyzed by a gas-phase protein sequenator (Model 470A connected to a Model 120A phenylthiohydantoin-amino acid analyzer, and a Model 900A Integrator, Applied Biosystems Inc.) after being transblotted onto a poly(vinyl-diphenyl)floridone (PVDF) membrane. In this method, the Coomassie-Blue-stained spots corresponding to the heatinducible proteins were cut out from the membrane and applied to the sequenator without Polybrene treatment, as described previously (12,13).

**Preparation of DNA:** Chromosomal DNA was prepared from *S. aureus* in the late logarithmic phase in L-B broth by the method of Matsuhashi *et al*. (14) with some modifications. Large-scale or small-scale preparation of plasmid DNA was carried out by the alkaline-SDS method (15).

**Construction, screening of gene libraries and DNA sequencing:** Gene libraries for cloning of the *hsp*60 were constructed with completely digested chromosomal DNA of *S. aureus* 912. The positively hybridized DNA fragments were ligated to the appropriately digested and dephosphorylated vectors (pUC119). Transformation in *E. coli* JM109 was carried out by the method of Norgard *et al.*(16). Positive clones were checked by restriction endonuclease digestion and were sequenced by the method of Sanger *et al.*(17).

Southern blot hybridization analysis: Total chromosomal and plasmid DNA fragments were isolated on agarose gels. Southern blots on nylon membranes (Gene Screen Plus, Du Pont) were carried out by the blotter (STRATAGENE, Funakoshi Co., Tokyo) and hybridization was performed as described previously (18), according to the manufacturer's instructions. DNA fragment of 600 bp for the probe was amplified by the PCR using chromosomal DNA of *S. aureus* as template. The

DOCKET

oligonucleotide primers synthesized for the PCR were 5'-GG(AGCT)GA(CT)GG(AGCT)AC(AG CT)AC(AGCT)AC(AGCT)GC(AGCT)AC(AGCT)GT-3' corresponding to the codons down stream of the sequence Gly<sup>85</sup> of *Pseudomonus aeruginosa* GroEL and the complementary primer, 5'-TC(A GCT)CC(AG)AA(AGCT)CC(AGCT)GG(CT)GC(AGCT)TT(AGCT)AC(AGCT)GC-3' corresponding to the codons upstream of Asp<sup>282</sup> (19). PCR was performed for 30 cycles of 95°C for 1min, 45°C for 2min, and 72°C for 2.5min after denaturation at 95°C for 3min in the DNA thermal cycler (PJ2000, Perkin Elmer Cetus) employing the following conditions: 200mM dNTPs, 25mM TAPS (N-tris(hydroxymethyl)methyl-3-aminopropane-sulfonic acid), pH9.3, 50mM KCl, 2mM MgCl<sub>2</sub>, 1mM 2-mercaptoethanol, 0.25 mg/ml activated salmon sperm DNA, chromosomal DNA (about 100 ng), the primers (100 pmoles), and Ampli Taq DNA polymerase (2.5 units). The amplified DNA fragments were cloned into pCR1000 using a TA Cloning kit (Invitrogen Co., U.S.A.), and sequenced as described above. These DNA probes were labeled with digoxigenin using the DNA labeling kit (Boehringer Mannheim).

### **RESULTS AND DISCUSSION**

At 46 °C, the growth of *S.aureus* was depressed to 50%, and the production of coagulase and methicillin resistance were diminished whereas DNase, enterotoxin,  $\beta$ -lactamase were intact (data not shown). Two dimensional PAGE (Fig.1) revealed that a temperature shift from 37°C to 46°C



Fig. 1. Two-dimensional gel electrophoresis of cytosolic proteins. Logarithmic-phase cells of MRSA were soaked at  $37^{\circ}$ C and  $46^{\circ}$ C for 25min and the cytosolic proteins from the cell lysate were prepared as in MATERIALS AND METHODS. The samples were subjected to isoelectric focusing using a pH range of 4-9 for the first dimension, and then to a SDS-PAGE with 10% gel for the second dimension. Numbers with an arrow indicate the positions of heat-inducible proteins.

732

enhanced the production of at least 8 kinds of proteins. The production of heat-inducible-proteins in MRSA, like the Gram-positive bacterium, thermobacterium PS3 (20), was much stronger than that of Gram-negative *E.coli*. The *de novo* synthesis of these proteins detected by pulse-labeling were significantly inducible (data not shown). These heat-inducible proteins were also enhanced under 10% ethanol, 200µM cadmium, and pH5. Two proteins numbered as 5 and 8 were identified to be HSP60 (60kDa) and HSP10 (10kDa), respectively. The N-terminal amino acid sequences of VKQLKFSEDARQAMLRGVDQ(HSP60) and MLKPIGNRVIIEKKEQEQITKSGIVLTDSAKEKS (HSP10) were 79% and 83% homologous to those of thermobacterium PS3 (20), respectively. An oligonucleotide primer set for HSP60 was synthesized and a DNA probe was then prepared by PCR (polymerase chain reaction) using chromosomal DNA of *S.aureus* as template shown in MATERIALS AND METHODS. The DNA sequence of the amplified DNA fragment revealed that it was highly homologous to HSP60 genes of thermobacterium PS3 (20) and *E.coli* (3). *Hin*dIII and *Pst* I digested DNA fragment of 4.2kbp was found to be positive with the above probe in total Southern hybridization. The sequencing strategy of the isolated positive clone of HSP60 is outlined in Fig.2.

Corresponding nucleotide sequences of the partial amino acid sequences of HSP10 and HSP60 were found in the cloned DNA sequence (the broken lines in Fig.3), and the DNA fragment comprised two genes encoding HSP10 and HSP60 (Fig.3). The putative heat-shock-promoter sequences of -35 (TTGAAA) and -10 (TATTAT) were identified only upstream of the HSP10 gene, by their homology to the consensus sequences in several heat shock genes of other bacteria (7).



Fig. 2. Sequencing strategy of HSP10 and HSP60 genes. Two open boxes on the solid bar (named as pKHS-137 clone) indicate the open reading frames of HSP10 and HSP60, respectively. Selected *Hind* III, *Hinc* II, and *Pst* I sites are shown. The arrows below the restriction map indicate the length of insert to a vector plasmid (pUC119) and the extents of the sequenced fragments.

# DOCKET



## Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

## **Real-Time Litigation Alerts**



Keep your litigation team up-to-date with **real-time** alerts and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

## **Advanced Docket Research**



With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

## **Analytics At Your Fingertips**



Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

### API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

### LAW FIRMS

Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

### **FINANCIAL INSTITUTIONS**

Litigation and bankruptcy checks for companies and debtors.

### **E-DISCOVERY AND LEGAL VENDORS**

Sync your system to PACER to automate legal marketing.

