JOURNAL OF BACTERIOLOGY, Aug. 1994, p. 4779–4783
0021-9193/94/$04.00+0

Vol. 176, No. 15

Molecular Cloning of Two New Heat Shock Genes Related to the *hsp70* Genes in *Staphylococcus aureus*

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Received 23 February 1994/Accepted 17 May 1994

We have identified two new heat shock protein genes, *orf37* and *orf35*, in *Staphylococcus aureus*, located upstream and downstream of *grpE*(*hsp20*), *dnaK*(*hsp70*), and *dnaJ*(*hsp40*) homologous genes in the order *orf37-hsp20-hsp70-hsp40-orf35*. The transcripts of both *orf37* and *orf35* were increased by thermal upshift of the culture from 37 to 46°C. The heat shock promoters were located upstream of *orf37* and upstream of *hsp40*. The deduced peptide of *orf37* showed similarity with those of *orfA* in *Clostridium acetobutylicum* and *orf39* in *Bacillus subtilis*. *orf35* was unique in *S. aureus* and has not yet been described in other bacteria.

Heat shock proteins (HSPs) are the proteins which are transitionally overexpressed when organisms are exposed to sublethal heat shock or stresses to protect themselves from otherwise lethal assaults (5, 7, 11, 22). The gene structures of

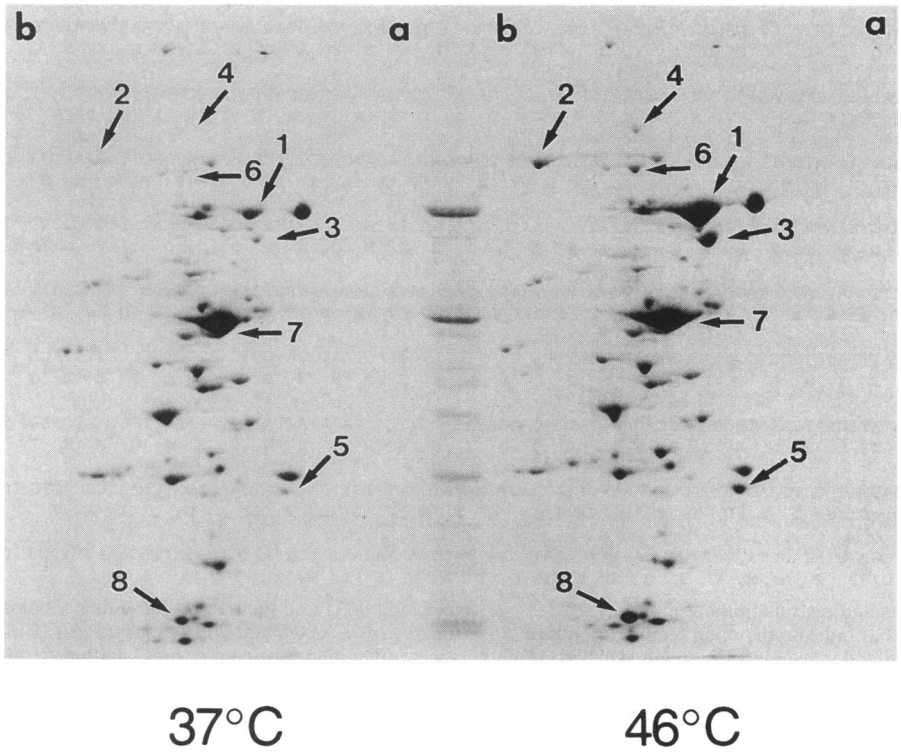

FIG. 1. Two-dimensional gel electrophoresis profiles of the cytosolic proteins of *S. aureus* 912 before and after the thermal upshift. Logarithmic-phase cells grown at 37°C were transferred to 46°C and kept at that temperature for 25 min, and then the cytosolic proteins were prepared. The pH range 4 to 9 for the first dimension; sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 10% gel was used for the second dimension. Numbered arrows indicate the positions of heat-induced proteins. a, acidic side; b, basic side. N-terminal amino acid sequences of the eight proteins indicated in the figure are, from the N terminus to the C terminus, as follows: 1, SKIIGIDLGTTNSXVTVLEG, homologous with Hsp70; 2, MLFGRLTERAQRVLAHAQEEAIRLNSKNIXTECL, homologous with ClpB; 3, VKQLKFSEDARQAMLRGVDQ, homologous with Hsp60; 4, MDINKITYAVQNAKQQAIEI, no homology; 5, TNKDESVEKNTESTVEETNIKQNIDDSVEQ, homologous with GrpE; 6 and 7, not determine 8, MLKPIGNRVIIEKKEQEQTTKSGIVLTDSAKEKS, homologous with Hsp10.

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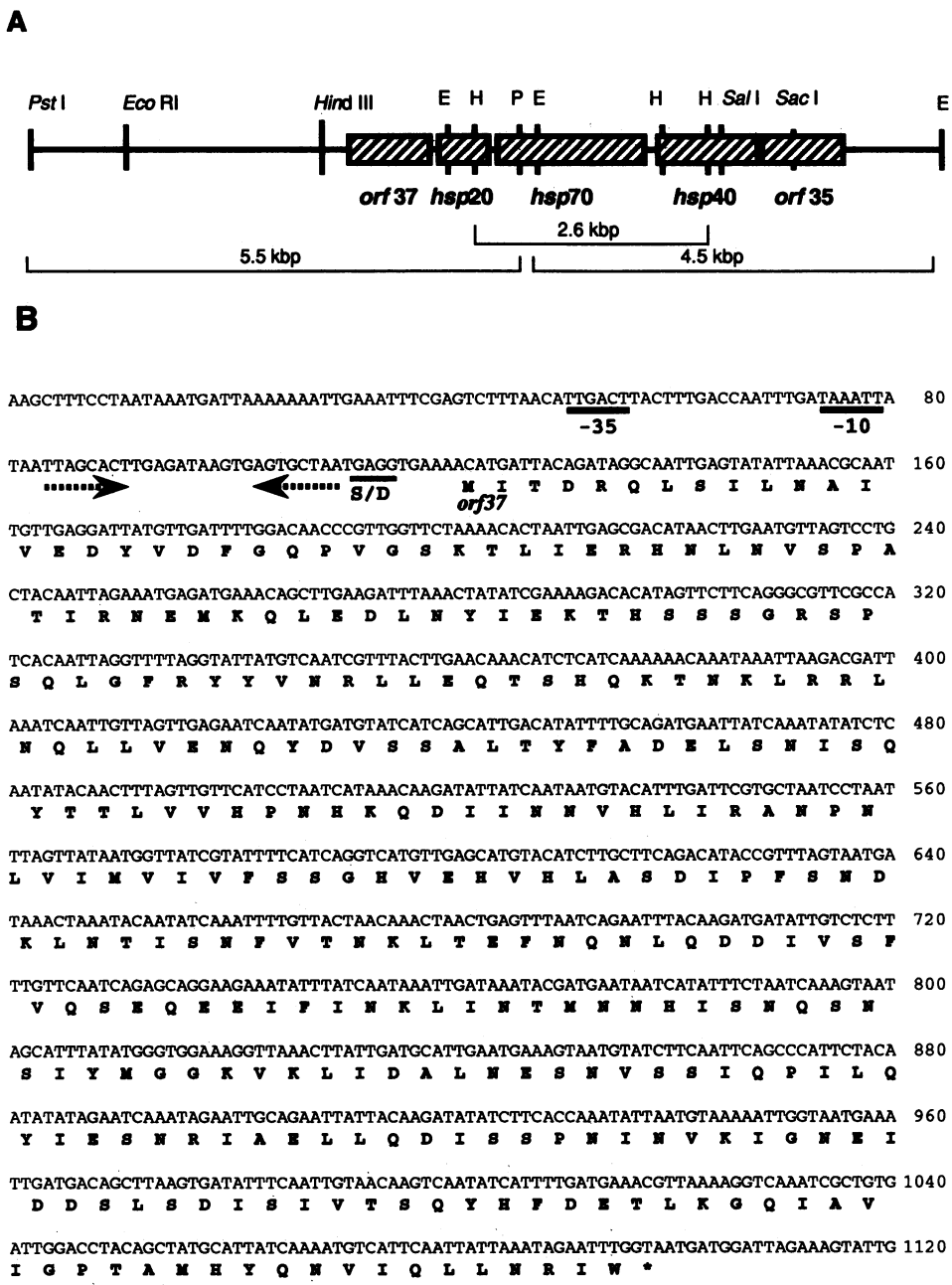

FIG. 2. Arrangements and restriction map of *orf37*, *hsp20*, *hsp70*, *hsp40*, and *orf35* and the nucleotide sequences of *orf37* and *orf35*. (A) Five open boxes on the solid bar indicate the open reading frames of *orf37*, *hsp20*, *hsp70*, *hsp40*, and *orf35*. Selected *Pst*I (P), *Eco*RI (E), *Hin*dIII (H), *Sal*I, and *Sac*I restriction endonuclease sites are shown. Two promoters were identified upstream of *orf37* and upstream of *orf40*. Characteristic palindrome structures were found upstream of *orf37*, at the junction of *orf70* and *orf40*, and downstream of *orf35*. (B and C) Nucleotide sequences of *orf37* (B) and *orf35* (C). The nucleotide sequence data of the five genes, including intergenic regions, will appear in the DDBJ/Gen Bank/EMBL nucleotide sequence data library. S/D, Shine-Dalgarno sequence.

HSPs have been studied extensively in *Escherichia coli* and certain gram-positive bacteria (8, 12, 18, 20, 26). In *E. coli*, those genes of DnaK, DnaJ, GroES, GroEL, and GrpE (3, 9) constitute a certain cluster with independent promoters, e.g., *dnaK* and *dnaJ* form *dnaKJ* and *groES* and *groEL* form *groE* (13, 23), respectively. In gram-positive bacteria, the cluster of HSP genes is different from that of gram-negative organisms, e.g., *Clostridium acetobutylicum* forms a cluster of *orfA-grpE-dnaK-dnaJ* in that order (18) and *Bacillus subtilis* forms a group of *orf39-grpE-dnaK-dnaJ* (8, 12, 30), while the products of *orf39* or *orfA* have not yet been characterized (31). In *Staphylococcus aureus*, however, neither the HSPs nor the gene structure has been identified. We suspect that HSPs are playing important roles in the regulation of toxin production and the acquisition of antibiotic resistance because much evidence has been found indicating that toxin production and antibiotic resistance are depressed at higher culture temperatures. Examples are that the methicillin resistance of *S. aureus* was depressed at a

C

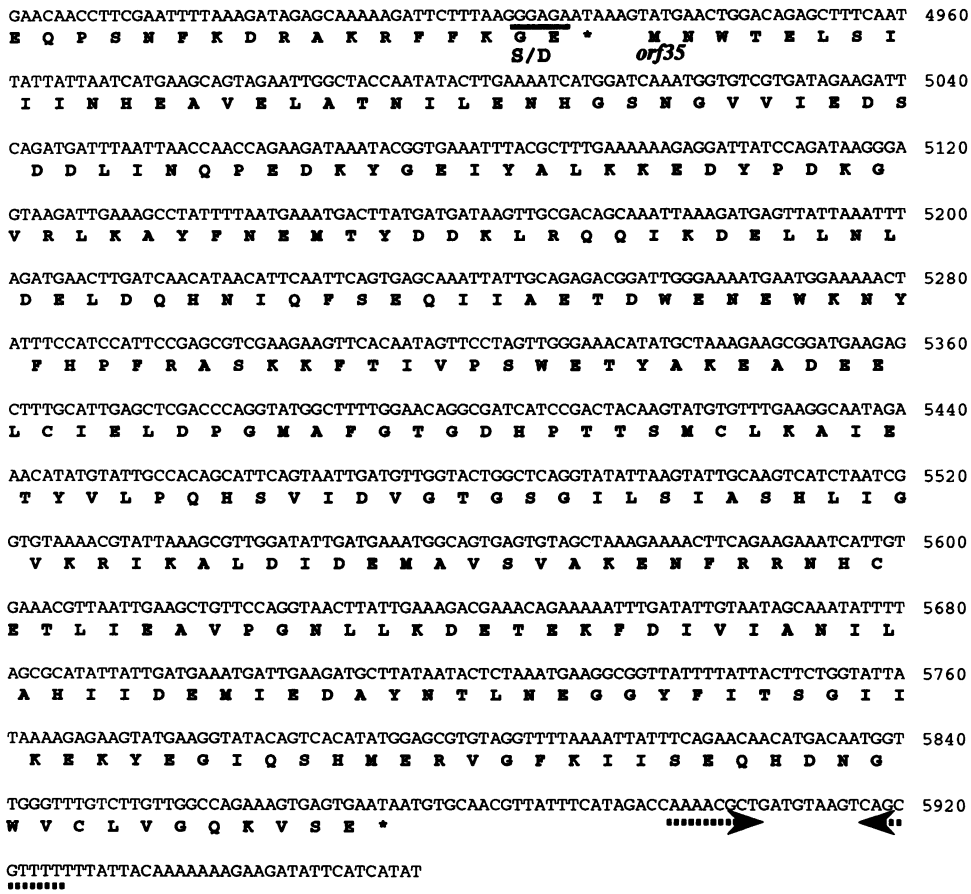

FIG. 2—*Continued.*

temperature above 35°C (29) and the production of some exotoxins was depressed at 42°C. Since the properties of *S. aureus* HSPs have not been well characterized, we investigated whether *S. aureus* has such HSPs and cloned the HSP genes by using a methicillin-resistant strain, *S. aureus* 912.

Induction of HSPs. When an early-logarithmic-phase culture (in brain heart infusion broth) of strain 912 (MIC of methicillin, 100 μg/ml) or a standard laboratory strain, *S. aureus* 209P, was transferred from 37 to 46°C, the growth rate was depressed by about 50% of that of the control culture (37°C). The cytosol protein compositions were examined by two-dimensional gel electrophoresis by the method of Nakamura et al. (10, 16) as shown in Fig. 1. The expression of at least eight kinds of proteins was clearly enhanced at 46°C, and these proteins were confirmed to be synthesized de novo upon thermal upshift by a pulse-labeling method using [^{35}S]methionine (data not shown). The N-terminal amino acid sequences of these HSPs were analyzed as described in the legend to Fig. 1 by a method described previously (20, 21). Point 1 of Fig. 1, named Hsp70, was 75% homologous to DnaK of *E. coli*; point 2, not yet named, was 25% homologous to ClpB of *E. coli* (27); point 3, named Hsp60, was 63% homologous to GroEL of *E. coli* (21). Point 5 seemed not to be homologous to GrpE of *E. coli* but proved to be a GrpE homolog from the DNA sequencing data and the deduced amino acid sequence (stated below) and was named Hsp20. Hsp20 contained 19-amino-acid (19-aa) sequence in addition to the N terminus of *E. coli* GrpE. Point 8, named Hsp10, was 55% homologous to GroES of *E. coli*. Points 6 and 7 were unable to be determined, and point 4 did not show any homology with any known proteins by homology search.

Cloning and sequencing of two new heat shock genes. An oligonucleotide primer set for *hsp70*, 5′-CCIGA(TC)GA(AG)GTIGTIGC-3′ and 5′-TCIGC(AG)TC(TC)TTIACCAT-3′, was synthesized, and the DNA probe of 449 bp was then prepared by PCR using staphylococcal chromosomal DNA as the template. The total chromosomal DNA isolated by the method of Matsuhashi et al. (14) was digested with *Hin*dIII, and Southern blot hybridization was performed with the probe (26). A fragment of 2.6 kbp hybridized with the probe, and it was cloned into the *Hin*dIII site of plasmid pUC119 (4, 19). In the same manner, the 5.5-kbp fragment of *Pst*I digestion and the 4.5-kbp fragment of *Eco*RI digestion hybridized with the probe, and these fragments were cloned to the *Pst*I and *Eco*RI sites of plasmid pUC119, respectively. The restriction map and orientation of these isolated clones are depicted in Fig. 2.

By DNA sequencing (24), five open reading frames were defined. The deduced amino acid sequences were compared with the database, and three of these were defined as *hsp20*, *hsp70*, and *hsp40* (Fig. 2). Two new open reading frames, named *orf37* and *orf35*, which were located upstream of *hsp20* and downstream of *hsp40*, were found (Fig. 2). The nucleotide

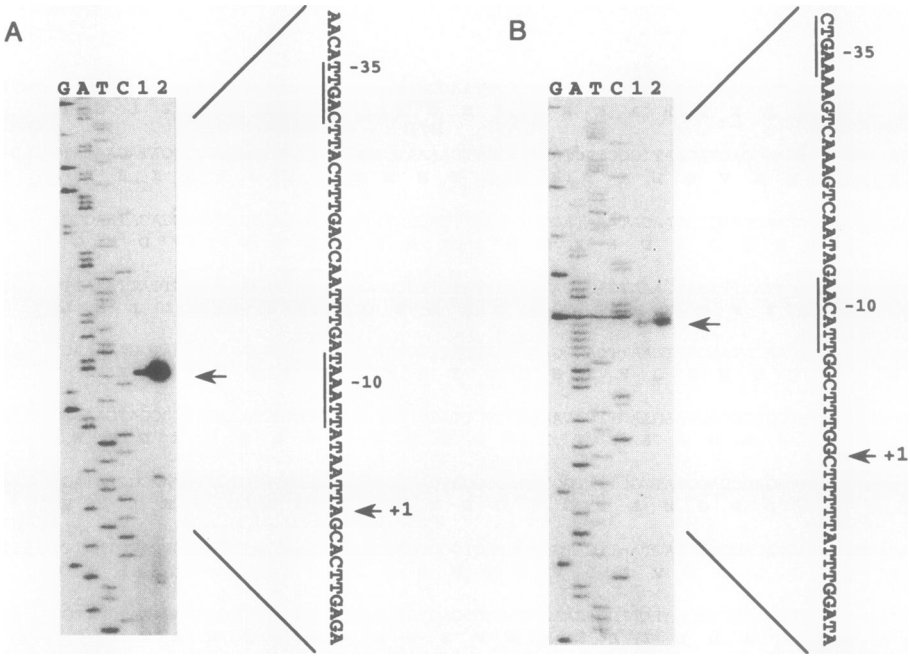

FIG. 3. Determination of the start point of *orf37* (A) and *orf35* (B) by primer extension analysis. Total RNA (5 μg) from *S. aureus* isolated before (lane 1) and 10 min after (lane 2) the temperature shift from 37 to 46°C was hybridized with ^{32}P-labeled primer. The primer extension products were electrophoresed in parallel with the sequencing products (G, A, T, and C) with the same labeled primer on a sequencing gel. The major product and its position on the sequence are indicated by arrows. The sequence to the right of the gel is the complementary strand of the sequencing. *orf37* started at the 37th base upstream of *orf37*, and *orf35* started at the 68th base upstream of *hsp40*.

sequence data for these five genes will appear in the DDBJ/GenBank/EMBL nucleotide sequence data library. Putative heat shock promoter sequences of −35 (TTGACT) and −10 (TAAATT) regions were located, one upstream of *orf37* and the other upstream of *hsp40*, by using the consensus sequences in several bacterial heat shock genes (17). The heat shock promoter sequences were common among gram-positive bacteria but different from those of *E. coli*. Three putative stem-loop structures were identified upstream of *orf37*, at the junction of *hsp70* and *hsp40*, and downstream of *orf35*. The sequence seems to be a common feature in gram-positive bacteria (32), although the functional roles of the structure need to be elucidated. The coding frames had a G+C content of 35%.

The homologies of the deduced amino acid sequences of the five open reading frames of the cloned DNA were as follows. Hsp20 showed 30.1% homology with GrpE of *E. coli* (14); Hsp70 was 56.0% homologous to DnaK of *E. coli* (2); and Hsp40 was 47% homologous to DnaJ of *E. coli* (3). The homologies of Hsp20, Hsp70, and Hsp40 to proteins of *B. subtilis* were 48.8, 78.4, and 59.1%, respectively. Orf37 was 31% homologous to Orf39 of *B. subtilis* and 27% homologous to OrfA of *C. acetobutylicum*. Orf35 was unique to *S. aureus*, and to date, no homologous gene or protein has been found. The numbers of amino acids and molecular masses of these five putative polypeptides were as follows: Orf37, 326 aa and 36,988 Da; Hsp20, 208 aa and 24,022 Da; Hsp70, 610 aa and 66,346 Da; Hsp40, 397 aa and 41,623 Da; Orf35, 312 aa and 35,526 Da.

Expression of *orf37* and *orf35*. The transcription of *orf37* and *orf35* gene were increased upon thermal upshift by Northern (RNA) blot analysis (data not shown). The transcription start point was determined by primer extension analysis using the oligonucleotide complementary to the 5′-terminal region in the coding frame: the 20-mer of 5′-TTTCAATTATTATTA ATCAT-3′ that complements nucleotides 18 to 38 of *orf35* and the 26-mer of 5′-AATTGTTGAGGATTATGTTGATTTTG-3′ that complements nucleotides 38 to 46 of *orf37*. Total RNA was prepared from the culture before and 10 min after the temperature upshift. As shown in Fig. 3, a strong signal was observed after the temperature upshift, and the primer extension product was enhanced about 10-fold compared with that of the control. The transcriptions were started at the 37th base upstream of *orf37* for *orf37* (Fig. 3A) and at the 68th base upstream of *hsp40* for *orf35* (Fig. 3B). The promoter region (−10 and −35 sequences) was identical with the deduced consensus sequence by computer search. The product of *orf37* had a molecular mass of 37 kDa, and that of *orf35* had one of 35 kDa, as expressed in *E. coli* (data not shown).

At amino acid residues 61 to 67 of Orf37, there is a serine-rich consensus sequence, SSSGRSPS. A similar structure is found in *htpY*, a heat shock gene of *E. coli* encoding a 21-kDa polypeptide, which is located 700 bp upstream of the *dnaK-dnaJ* operon (15). *orf37* of *S. aureus* might correspond to this gene, although the deduced amino acid sequence and the molecular weight of *orf37* are different from those of *htpY*.

The deduced protein sequence of *orf35* showed 32% homology with a partial sequence of *orf2* located upstream of the *fis* gene in *E. coli* (1), but, because the sequence of *orf2* is still incomplete, the correlation still remains unclear. Northern blot analysis revealed that *orf35* was clearly a heat shock gene whose transcription was enhanced upon the temperature upshift. Orf35 should be a new staphylococcal heat shock protein, although its function has yet to be elucidated.

S. aureus, like another gram-positive thermophilic bacterium, PS3 (28), produces a much larger amount of HSPs than

gram-negative organisms, such as *E. coli*, do. This may suggest that the expression of *hsp* in gram-positive bacteria could be regulated by factors or through signal transduction systems different from those of gram-negative organisms. The distinct features of the HSPs in *S. aureus* as well as other gram-positive bacteria are that (i) at the −35 and −10 regions, the 6-bp TTGACT and the TAAATT, respectively, are well conserved, and (ii) characteristic palindrome structures are located at or close to the start point of the transcription. We are now focusing on elucidating the functional roles of the sequence in the induction of transcription by a heat shock by introducing point mutations at the site.

Nucleotide sequence accession number. The nucleotide sequence data for *orf37*, *hsp20*, *hsp70*, *hsp40*, and *orf35* have been deposited in the DDBJ/GenBank/EMBL data base under accession number D30690.

We thank J. Miyazaki (University of Tsukuba) for technical support of the two-dimensional electrophoresis. We also thank J. I. Vernon-Edo, University of Tsukuba, for critically reading the manuscript.

This work was supported by grants 05454188, 05670246, and 05268203 from the Ministry of Education, Science and Culture of the Japanese government and a grant from University of Tsukuba.

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