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Cloning, sequencing, and transcriptional analysis of the *dnaK* heat shock operon of *Listeria monocytogenes*

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ABSTRACT The complete *dnaK* operon of *Listeria monocytogenes* was isolated by chromosome walking using the previously cloned *dnaK* gene as a probe. Molecular analysis of the locus identified 6 genes in the order *hrcA*, *grpE*, *dnaK*, *dnaJ*, *orf35*, and *orf29.* Primer extension analysis revealed 3 transcription start sites—S1, S2, and S3—upstream of the *hrcA*, *grpE*, and *dnaJ*, respectively. The transcription from S1 was heat inducible. Analysis of the sequences revealed the consensus promoter sequences of gram-positive bacteria, P1 and P2 upstream of the *hrcA* and *dnaJ*, respectively. The *hrcA* gene and a regulatory sequence, designated CIRCE (controlling inverted repeat of chaperone expression), play a role in the regulation of expression of the *dnaK* locus in response to heat shock in several gram-positive bacteria. Their presence upstream of the *dnaK* locus in *L monocytogenes* suggested a similar regulatory mechanism for the transcription initiated at the promoter, P1. Northern blot analysis led to the detection of 4 mRNA species of 4.9 kb, 3.6 kb, 3.6 kb, and 1.2 kb; the first 2 species were heat inducible. The current results indicate that 4 distinct transcripts directed by 3 promoters are involved in the expression of the *dnaK* operon of *L. monocytogenes.*

INTRODUCTION

Listeria monocytogenes is a foodborne gram-positive bacterium that can cause lethal infections in immunocompromised persons. *L. monocytogenes* belongs to the facultative intracellular bacteria, which are able to survive and replicate in professional phagocytes such as macrophages and monocytes and in unprofessional phagocytic mammalian cells (Dabri et al 1990; Gaillard et al 1987; Portnoy et al 1988). The intracellular environment, especially that of professional phagocytes, imposes hostile conditions on invading bacteria. The diverse bactericidal factors include toxic oxidative products, lysosomal and granular peptides, low pH, and low levels of essential nutrients. It is therefore expected that intracellular bacteria require specific products to cope with intracellular stress conditions. Recent evidence shows that stress proteins play an important role in intracellular survival and growth by facultative bacteria within macrophages (Johnson et al 1991; Köhler et al 1990; Phillips et al 1995; Rouquette et al 1996; Yamamoto et al 1996).

The stress proteins are induced by a variety of different stimuli, such as heat shock, oxidative products, nutrient limitation, acidic condition, and damage caused by toxic chemicals and physical agents. We previously characterized the heat shock response of *L. monocytogenes* (Hanawa et al 1995). Among the stress proteins identified were homologues of DnaK, GroEL, and GroES. DnaK is well known to have an important role on cellular metabolism as a molecular chaperone. We have recently demonstrated that the DnaK chaperone of *L. monocytogenes* is involved in stress tolerance and efficient phagocytosis with macrophages through molecular cloning of the *dnaK* locus, introduction of an insertion in the locus, and characterization of the mutant (Hanawa et al 1999). Early studies in *Escherichia coli* revealed that the *dnaK* operon comprises the genes *dnaK* and *dnaJ* (for review, see Georgopoulos et al 1990) and that the expression of the genes is under the positive control of an alternative factor, σ^{32}, which is a sigma subunit of RNA polymerase (for review, see Yura

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et al 1993). In contrast, recent studies in gram-positive bacteria revealed the *dnaK* operon is more complex and more variable in its molecular organization. Genes associated with the *dnaK* operon usually include *hrcA, grpE, dnaK,* and *dnaJ* (Eaton et al 1993; Narberhaus et al 1992; Ohta et al 1994; Wetzstein et al 1992). A further 3 genes, *orf35, orf28,* and *orf50*, are also associated with the *dnaK* operon in *Bacillus subtilis* to form a heptacistronic operon (Homuth et al 1997). Furthermore, the regulatory mechanisms of the expression in gram-positive bacteria are quite different from those of *E coli.* In *B. subtilis* and *Streptococcus mutans,* the regulation of expression of *dnaK* and other class I heat shock genes appears to be negatively controlled by an HrcA protein, which binds at a conserved cis-acting inverted repeat (a CIRCE element: controlling inverted repeat of chaperone expression) located near vegetative promoters (Zuber and Schumann 1994; Jayaraman et al 1997; Ballard et al 1998).

Our recent cloning and sequencing of the *dnaK* locus of *L. monocytogenes* have revealed that the *hrcA, grpE,* and *dnaJ* genes are associated with this region, suggesting that the operon consists of the genes *hrcA, grpE, dnaK,* and *dnaJ* (Hanawa et al 1999). However, the disruption of the *dnaK* gene by insertion of a fragment did not impair the production of a DnaJ protein, suggesting the independent expression of the *dnaJ* gene (Hanawa et al 1999). Furthermore, no obvious promoters were detected upstream of the *dnaK* translational start site. To define the molecular structure and the expression of the *dnaK* locus in *L monocytogenes,* we decided to isolate the complete *dnaK* operon and analyze the transcriptional regulation of the genes. In this report, we show that 6 genes are in the *dnaK* operon of *L. monocytogenes* and that 4 distinct transcripts are produced from the operon through cloning, sequencing, and transcriptional analysis.

MATERIALS AND METHODS

Bacterial strains and plasmids

L. monocytogenes serotype 1 strain 10403S (Bishop and Hinrichs 1987) was used and its cultivation was previously described (Hanawa et al 1995). The *E. coli* JM109 (Yanisch-Perron et al 1985) was cultured in brain-heart infusion medium (Nissui, Tokyo, Japan) supplemented with 100 μg/ml ampicillin, 25 μg/ml chloramphenicol, or 25 μgml tetracycline as required. The plasmid pACYC184 (Chang and Cohen 1978) was used in the construction of a partially genomic library, while the pBluescript SK/KS (Strategene, La Jolla, CA, USA) was used for subcloning experiments.

DNA isolation, manipulation, and sequencing

The preparation of chromosomal DNA from *L. monocytogenes* was described previously (Hanawa et al 1999). The plasmid DNA purification, restriction analysis, ligation, and agarose gel electrophoresis were performed according to the methods described by Sambrook et al (1989). For Southern hybridization, the digested DNAs were electrophoresed through a 0.8% agarose gel and then transferred to Gene Screen Plus membrane (NEN, Boston, MA, USA). The DNA fragment as a probe was labeled by using the Gene Image random prime labeling and detection system (Amersham, Buckinghamshire, UK), and hybridization was performed according to the manufacturer's instructions. The sequencing was carried out with Sequenase (USB, Cleveland, OH, USA) and T7 or T3 DNA primers for pBluescript plasmid or synthetic primers.

Preparation of RNA and Northern hybridization

Total cellular RNA was isolated using the RNeasy kit from Qiagen (Hilden, Germany). RNA for Northern blot analysis was separated in formaldehyde gels and transferred to Gene Screen Plus membrane. Size determination was done by using an RNA ladder (281, 623, 955, 1383, 1908, 2604, 3638, 4981, and 6583 bp; Promega, Madison, WI, USA) as the standard. The following DNA fragments were used as hybridization probes specific to *hrcA* covering the sequence from nt 303 to 773, *grpE* covering nt 1446 to 1819, *dnaK* covering nt 2086 to 2821, *dnaJ* covering nt 4027 to 4702, *orf35* covering nt 5154 to 6056, and *orf29* covering nt 6106 to 6767. These sequences were generated through polymerase chain reaction amplification. The hybridization was performed by using the hybridization buffer from the Gene Image random prime labeling and detection system (Amersham) at 65°C for 18 to 20 hours. The washing of the membrane after hybridization and detection of signal were performed according to the manufacturer's instructions.

Determination of the transcription start site

To define the transcription start sites, primer extension analysis was used. Synthetic oligonucleotides, 0294 (5′-CGATGATGGCACGGAAAATCAAAAG-3′), 1342 (5′-TTTCTCAGACACTTTAGCCA-3′), and 3976 (5′-GCTGAGGCGCTTTTGGAAAT-3′), which are complementary to nt 294 to 270 of the *hrcA* coding sequence, to nt 1342 to 1323 of the *grpE* coding sequence, and to nt 3976 to 3956 of the *dnaJ* coding sequence, respectively, were end labeled with [γ-^{32}P]-adenosine triphosphate and annealed to 25 μg of total cellular RNA. The primers were extended with 100 units of avian myeloblastosis virus reverse transcriptase (Superscript II, BRL) and deoxyribonucleotide thiophosphates (Sigma, St. Louis, MO, USA) at 42 °C for 30 minutes. Nucleic acid was precipitated with ethanol, dried, resuspended in TE buffer (pH 8.0), and extracted by Microspin column S200 (Pharmacia, Uppsala,

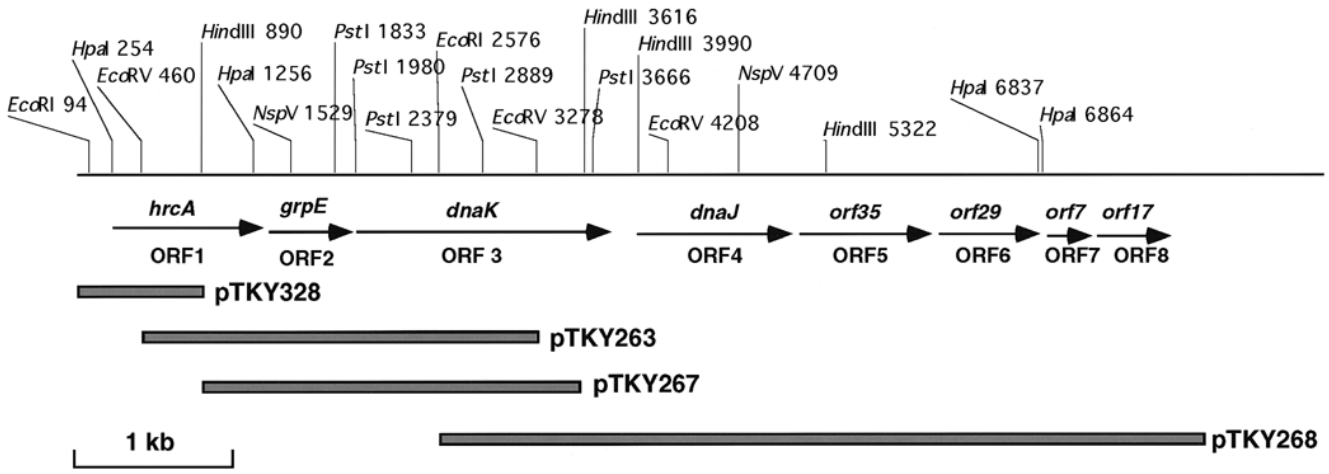

Fig 1. Restriction map of the *Listeria monocytogenes dnaK* locus and recombinant plasmids. Locations of the identified genes are indicated with arrows.

Sweden). The samples with formamide loading dye were subjected to electrophoresis in 5% polyacrylamide sequencing gels. DNA sequencing reactions, using the same primer that was used in the primer extension analysis and a plasmid carrying the appropriate region of the *hrcA* locus, *grpE* locus, or the *dnaJ* locus as a temperate, were run adjacent to the primer extension reactions to identify the transcription initiation site.

Nucleotide sequence accession number

The sequence data of complete operon reported here will appear in the DDBJ/EMBL/GenBank nucleotide sequence database under accession number AB023064. Sequence data of the *dnaK* gene have been submitted to the DDBJ database by us and have been assigned the accession number AB007771.

RESULTS

Isolation of the complete *dnaK* operon of *L. monocytogenes*

To determine the nucleotide sequence of the *dnaK* gene of *L. monocytogenes,* we have previously cloned the 2.9-kb *Eco*RV, 2.5 kb *Hin*dIII, and 6.0 kb *Eco*RI fragments that gave clear hybridization signals to the *dnaK* gene from *E. coli,* as previously reported (Hanawa et al 1999). Sequencing of part of the 2.9-kb insert of pTKY263 (Fig 1) revealed the presence of the *hrcA* homologue near one end, but the gene was incomplete, missing its 5′ end. By using the 2.9 kb *Eco*RV fragment containing 828 bp of the 3′ end of the *hrcA* gene, we cloned the complete *hrcA* gene in the current study. Hybridization of this fragment to chromosomal DNA digested with different restriction enzymes revealed specific signals, including a 1.9-kb *Hin*dIII fragment (results not shown). According to the restriction cleavage map and sequence of the 2.9-kb insert of pTKY263 (Fig 1), the 1.9-kb *Hin*dIII fragment probably carried the 5′ end of *hrcA* gene. Thus, a partially genomic library containing *Hin*dIII fragments of *L. monocytogenes* DNA in pACYC184 was constructed and screened by hybridization with the 2.9 kb *Eco*RV fragment. A positive clone, pTKY328, carrying the 1.9-kb *Hin*dIII fragment was isolated. The restriction cleavage map of the cloned fragments containing whole genes related to possible *dnaK* operon of *L. monocytogenes* is shown in Figure 1.

Sequencing and genetic organization of the *dnaK* operon of *L. monocytogenes*

A total of 7800 bp was sequenced and analyzed. Sequencing revealed the presence of 8 Orfs (Figs 1, 2).

Orf1 is 1038 bp long (nt 252–1287) encoding a peptide of 345 aa with a predicted molecular mass of 39.7 kDa, with a Shine-Dalgalno–like sequence preceding the initiation codon by 8 bp. The aa sequence was similar to HrcA of *B subtilis* (Wetzstein et al 1992), HrcA of *S mutans* (Jayaraman et al 1997), OrfA of *Clostridium acetobutylicum* (Narberhaus et al 1992), Orf37 of *Staphylococcus aureus* (Ohta et al 1994), and Orf1 of *Lactococcus lactis* (Eaton et al 1993), as shown in Table 1. Therefore, we designated the gene *hrcA.*

Orf2 is 576 bp long (nt 1331–1904) encoding a peptide of 191 aa with a predicted molecular mass of 21.8 kDa, with a putative ribosomal binding site 8 bp 5′ to the start codon. This protein showed significant homology to GrpE of other bacteria (Table 1), and thus the gene was designated *grpE.*

Orf3 is 1842 bp long (nt 1940–3779) encoding a peptide of 613 aa with a predicted molecular mass of 66.2 kDa,

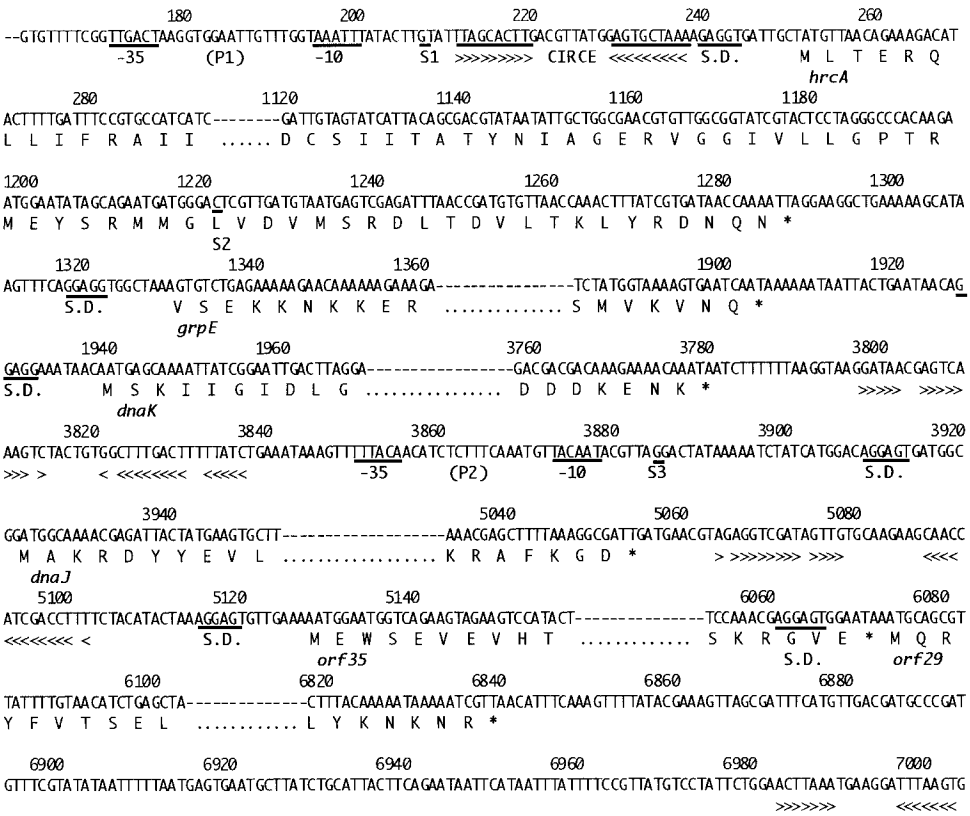

Fig 2. Nucleotide sequences, amino acid sequences, and potential regulatory elements of the *Listeria monocytogenes dnaK* locus. The nucleotide and deduced amino acid sequences of each 5′ end and 3′ end of *hrcA, grpE, dnaK, dnaJ, orf35* and *orf29* genes are detailed. The deduced amino acid sequences are shown below the DNA sequences with single-letter codes. The translation stop signals are marked by asterisks below the respective codons. Predicted ribosome-binding sites are underlined and labeled as SD (Shine-Dalgalno). Breaks in the sequence presented are indicated by a dashed line. The −35 and −10 regions of putative promoter sequences are underlined. The transcriptional initiation sites that were mapped by primer extension analyses are underlined and designated S1, S2, and S3, respectively. Arrowheads below the DNA sequence show regions of dyad symmetry. The CIRCE-element–like inverted repeat (see text) is indicated with arrowheads.

Table 1 Comparison of the amino acid sequences of HrcA, GrpE, DnaK, DnaJ, Orf35, and Orf29 from *L monocytogenes* with their homologues in bacterial species[a]

	Identity (%)						
Organism	**HrcA**	**GrpE**	**DnaK**	**DnaJ**	**Orf35**	**Orf29**	**Accession no.**
Bacillus subtilis	51.0	56.4	80.7	69.2	55.1	48.4	M84964
Lactococcus lactis	26.9	37.8	78.0	61.5	NA	NA	X76642, M99413
Staphylococcus aureus	28.6	43.3	77.8	60.9	43.4	NA	D30690
Streptococcus mutans	26.6	35.6	NA	NA	NA	NA	U78296
Clostridium acetobutylicum	32.2	39.6	66.1	50.4	43.5	NA	M74569, X69050
Streptomyces coelicolor	NA	24.2	59.7	38.9	NA	NA	L08201
Mycobacterium tuberculosis	NA	26.7	59.6	42.1	NA	NA	X58406
Brucella ovis	NA	NA	59.2	45.5	NA	NA	M95799
Escherichia coli	NA	29.7	56.7	49.6	NA	NA	X07863, K01298, M12565
Borrelia burgdorferi	NA	31.4	58.5	38.1	NA	NA	M96847, M97914
Chlamidia trachomatis	24.3	33.7	55.5	NA	NA	NA	L25105, M27580
Homo sapiens	NA	NA	44.9	35.0	NA	NA	M11717, L08069

NA, sequences either not applicable or not available.

[a] The results expressed as percentage identity in alignment. Sequences were obtained through the GenBank database using the accession numbers listed.

with a putative ribosomal binding site 9 bp 5′ to the initiation codon. As already mentioned, this protein is highly homologous to DnaK of various species (Hanawa et al 1999), and thus this gene was called *dnaK.*

Orf4 is 1134 bp long (nt 3923–5054) encoding a peptide of 377 aa with a predicted molecular mass of 41.1 kDa, with a putative ribosomal binding site 9 bp 5′ to the initiation codon. Homology searches indicated that this protein was similar to DnaJ of other bacteria (Table 1), and thus the gene was designated *dnaJ.*

Orf5 is 945 bp long (nt 5129–6071) encoding a peptide of 314 aa with a predicted molecular mass of 34.8 kDa, with a putative ribosomal binding site 9 bp 5′ to the initiation codon. Homology searches indicated that this protein was similar to Orf35 of *B. subtilis* (Homuth et al 1997), Orf35 of *S. aureus* (Ohta et al 1994), and OrfB of *C. acetobutylicum* (Behrens et al 1993). These proteins showed significant homology to the ribosomal protein L11 metyltransferase encoded by *prmA* of *E. coli* (Vanet et al 1993).

Orf6 is 768 bp long (nt 6074–6839) encoding a peptide of 255 aa with a predicted molecular mass of 28.9 kDa, with a putative ribosomal binding site 8 bp 5′ to the initiation codon. Homology searches indicated that this protein named Orf29 was similar to Orf28 of *B. subtilis* (Homuth et al 1997) which exhibits significant homology to the 29.6 kDa protein YggJ of *E. coli.*

Orf7 is 174 bp long (nt 7039–7210) encoding a peptide of 57 aa with a predicted molecular mass of 6.9 kDa. This protein, named Orf7, exhibits significant homology to the 30S ribosomal protein S21 of *B. subtilis* (87.3 % identify SWISS-PROT. Rel. 34).

Orf8 is 456 bp long (nt 7223–7676) encoding a peptide of 151 aa with a predicted molecular mass of 16.8 kDa. The aa sequence was compared with sequences in the SWISS-PROT (Release 34) database. This analysis revealed that this protein, named Orf17, has a sequence homology to the transcriptional regulatory proteins TstD and PhoP of *Salmonella typhimurium.*

Regulatory sequences in the *L monocytogenes dnaK* operon

Analysis of sequences upstream of *hrcA* revealed sequences similar to the consensus promoter sequences of gram-positive bacteria (Graves and Rabinowitz 1986). The −35 sequence was 5′-TTGACT beginning at position 171, and the −10 sequence was 5′-TAAATT, with 17 nt in between (P1 in Fig 2 and Fig 5). Sequence analysis also revealed the presence of an inverted repeat sequence between the putative promoter element and the initiation codon of *hrcA* that had considerable sequence similarity with the CIRCE element found in the regulatory region of the *dnaK* operon and the *groE* operon of several bacteria (Zuber and Schumann 1994; Jayaraman et al 1997; Ballard et al 1998). The inverted repeat sequence of TTAGCACTt-nt 9-GAGTGCTAa differed from the consensus sequence of the CIRCE element at 1 position.

No sequences resembling −35/−10 transcription signals were observed upstream of *grpE* and *dnaK.* The start codon of *dnaJ* is 141 nt downstream from the stop codon of *dnaK.* In this intergenic region, a second promoter (P2 in Fig 2 and Fig 5) was observed with a −35 box of 5′-TTTACA, separated 17 nt from a −10 box of 5′-TACAAT. No obvious promoter elements were observed upstream of either *orf35* or *orf29.* However, upstream of *orf7,* which is 197 nt downstream from the termination codon of the *orf29,* a third promoter was detected with a −35 box of TTGACG separated 17 nt from a −10 box of TATAAT whose sequences are available in DDBJ/EMBL/GenBank database. No obvious promoter elements were observed upstream of Orf17. The validity of the promoters P1 and P2 was confirmed by primer extension analysis (see below).

Four palindromic structures were identified. As shown in Figures 2 and 5, the first stem and loop structure spanning nt 3798 to 3837 is located immediately downstream of the termination codon of *dnaK.* The second stem and loop structure was found in the region (nt 5064–5102) separating *dnaJ* and *orf35.* The third stem and loop structure was located in the region (nt 6983–7003) separating *orf29* and *orf7.* The fourth structure spanning nt 7705–7752 was located downstream of the termination codon of *orf17.* The sequences of this region are available in the DDBJ/EMBL/GenBank database.

Transcriptional analysis of the *L. monocytogenes dnaK* operon

Sequence analysis of the *dnaK* region of *L. monocytogenes* suggested that the transcriptions are initiated at various sites. To identify the functional promoters responsible for transcription of these genes, primer extension was performed on RNA prepared from either *L. monocytogenes* grown at 30°C or bacteria heat shocked at 45°C for 5 minutes, 10 minutes, or 20 minutes. Synthetic oligonucleotides 0294, 1342, and 3976 corresponding to the regions immediately 5′ of *hrcA, grpE,* and *dnaJ,* respectively, were used as primers. The results are shown in Figure 3.

The oligo 0294 yielded a extension product identifying S1 at nt 207. S1 was located immediately downstream of the promoter element P1, which had been identified upstream of *hrcA* by sequence analysis. Primer extension using the oligo 1342 gave an extension product identifying S2 at nt 1221. S2 is not preceded by sequences resembling any known promoter. Furthermore, primer extension using the oligo 3976 yielded a product identifying S3 at nt 3885. S3 was located immediately downstream

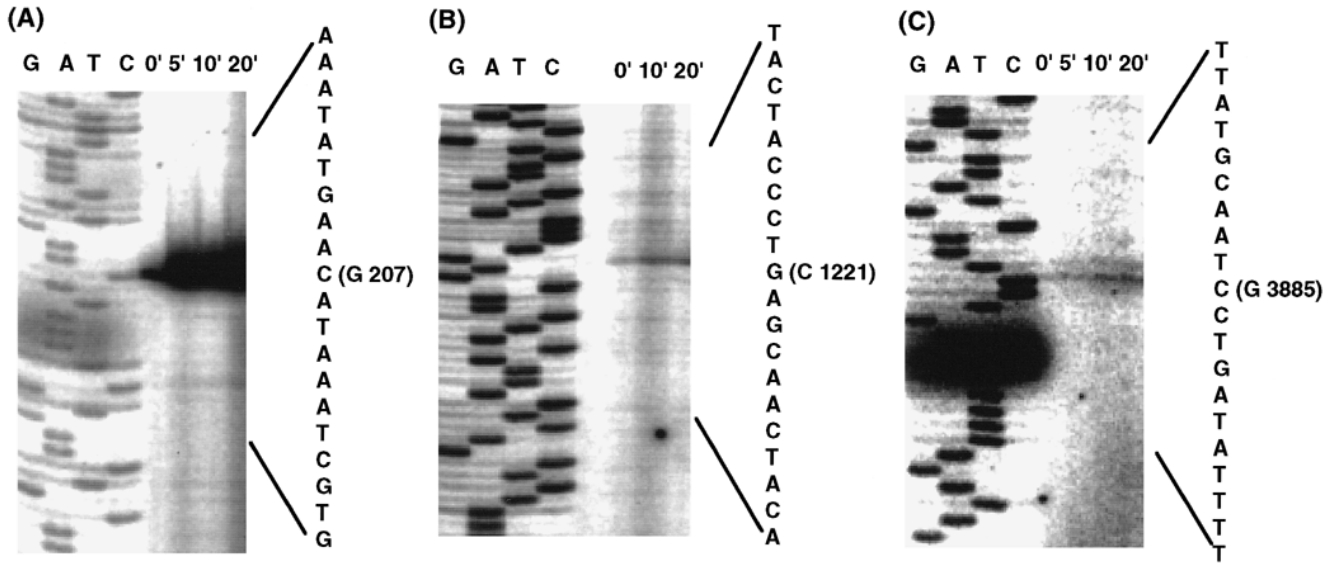

Fig 3. Primer extension analysis of the *Listeria monocytogenes dnaK* locus. Total RNAs (25 μg per lane) from *L. monocytogenes* 10403S grown at 30°C (0 minutes) and heat shocked at 45°C for 5 minutes, 10 minutes, or 20 minutes, were subjected to primer extension using oligonucleotides 0294 specific for *hrcA* (A), 1342 specific for *grpE* (B), and 3976 specific for *dnaJ* (C). The sequence of oligonucleotides used are shown in Materials and Methods. Products were compared with a sequencing ladder using the corresponding oligonucleotide primer. The sequences obtained are listed to the right of each panel and the first nucleotide of each transcript is shown with nucleotide position on the sequences.

of the promoter element P2, identified upstream of *dnaJ.* These results suggest that several distinct transcripts are produced from the *dnaK* operon of *L. monocytogenes.*

The in vivo transcripts of the *dnaK* operon were detected using Northern blot analysis with total RNAs prepared from *L. monocytogenes* grown at 30°C and from heat-shocked bacteria. On Northern blots hybridized with *hrcA*-, *grpE*-, and *dnaK*-specific probes, 2 identical RNA species of 4.9 kb and 3.6 kb each were detected; they represent transcripts of the total operon with and without the *dnaJ* gene, respectively (Fig 4A–C). It is obvious that the transcription was induced by a heat shock. With the *dnaJ*-specific probe, as expected, the transcript of 4.9 kb was detected (Fig 4D). In addition, a 3.6-kb RNA and a 1.2-kb RNA that were not induced by heat shock appeared. We assumed that a small amount of the 3.6-kb transcript was initiated at S2 and represents a polycistronic transcript encompassing the 3 genes, *grpE, dnaK,* and *dnaJ.* To distinguish this 3.6-kb species for *grpE, dnaK,* and *dnaJ* from the 3.6-kb transcript for *hrcA, grpE,* and *dnaK,* 4 transcripts were labeled a, b, c, and d, as shown in Figure 5. Because of its low abundance, the 3.6 kb mRNA-c could be appeared as a doublet with 3.6 kb mRNA-b on hybridization blots with the probes for *grpE* and *dnaK.* The transcript of 1.2 kb could be a monocistronic *dnaJ* mRNA. Some blots exhibited hybridization signals at the 2 positions where the 16S and 23S rRNAs migrate.

Analysis of the nucleotide sequence suggested that the *orf35* and *orf29* are part of the *dnaK* operon, because both are not separated by a large noncoding region and because no apparent promoters are observed upstream of both genes. We examined the possibility by the Northern blot analysis of total RNA with the *orf35*-specific and *orf29*-specific probes but failed to detect any band in Northern blots (data not shown; see Discussion). The transcriptional organization of the complete *dnaK* operon as deduced from the Northern analysis and from the primer extension is shown diagramatically in Figure 5.

DISCUSSION

This paper describes the first complete *dnaK* operon of *L. monocytogenes.* The *dnaK* region of *L. monocytogenes* contains at least 4 heat shock genes with the arrangement *hrcA-grpE-dnaK-dnaJ,* followed by 2 Orfs, *orf35* and *orf29.* The chromosomal organization of these 6 genes at a single locus and the presence of the *hrcA* homologue along with the CIRCE element resemble the arrangement seen in other gram-positive bacteria, including *C. acetobutylicum* (Narberhaus et al 1992), *B. subtilis* (Wetzstein et al 1992), and *S. aureus* (Ohta et al 1994), though slight differences are observed. In *C. acetobutylicum* and *S. aureus, orf29* has not been identified downstream of *dnaJ.* In *B. subtilis,* the *dnaK* operon contains a seventh large Orf, *orf50,* to form a heptacistronic operon (Homuth et al 1997). On the contrary, in *L. monocytogenes, orf29* was followed by 2 Orfs, *orf7* and *orf17*, which appeared to form a separate transcriptional unit from the *dnaK* operon (Fig 1).

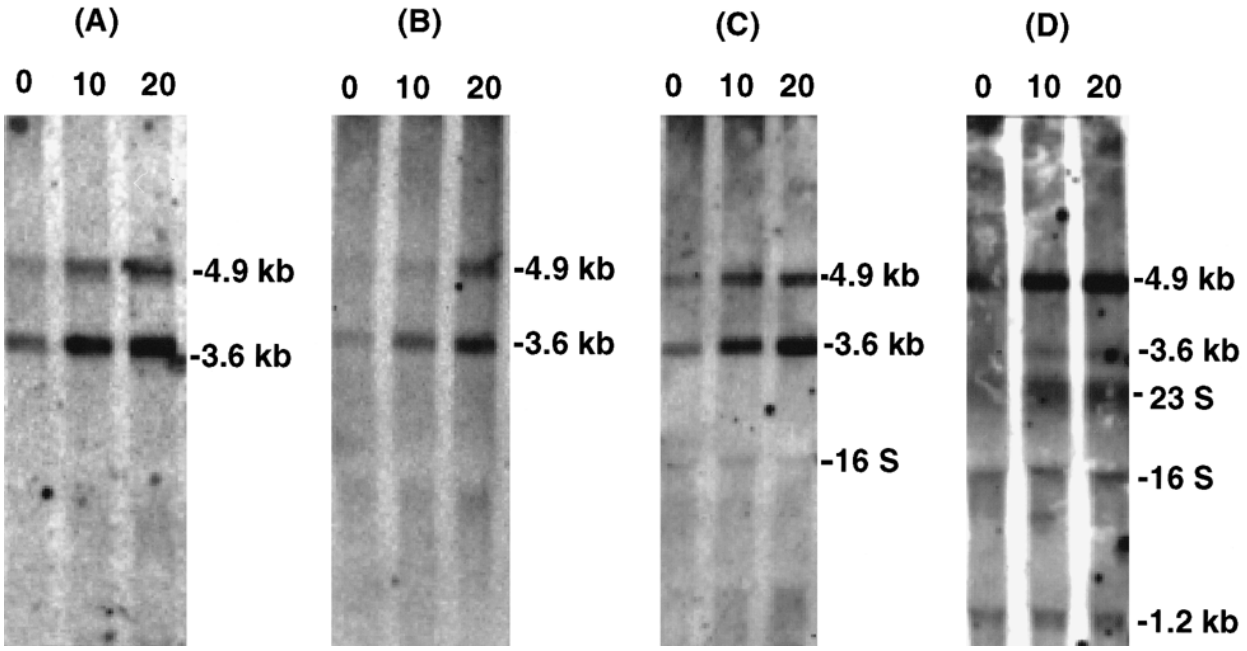

Fig 4. Northern blot analysis of *hrcA*, *grpE*, *dnaK*, and *dnaJ* mRNAs in response to heat shock. RNA was isolated from *Listeria monocytogenes* 10403S grown at 30°C (0 minutes) and at 10 or 20 minutes after a temperature shift to 45°C. The filters were hybridized with labeled DNAs corresponding to the *hrcA* (A), *grpE* (B), *dnaK* (C), and *dnaJ* (D). The size of each transcript was determined by comparison with RNA size markers.

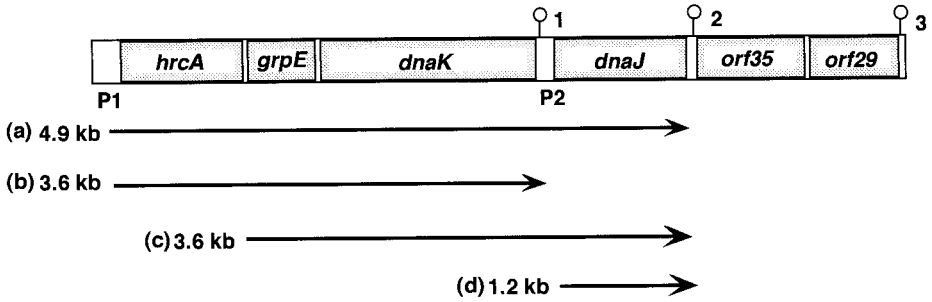

Fig 5. Genetic and transcriptional map of the *dnaK* operon of *Listeria monocytogenes.* This schematic representation is based on the determination of transcription start sites and Northern blot analysis. The promoters P1 and P2 were deduced from the sequences and the corresponding transcription start sites as determined by primer extension analysis. Potential stem-loop structures numbered 1 to 3 are indicated.

Three transcriptional start sites, S1, S2, and S3, were identified upstream of *hrcA, grpE*, and *dnaJ,* respectively, by primer extension analysis (Fig 3). S1 and S3 mapped were consistent with the presence of promoter sequences P1 and P2 predicted from the sequence data. However, S2 is not preceded by sequences resembling any known promoter. It could be initiated by an alternative sigma factor of *L. monocytogenes.* Northern blot analysis revealed 4 mRNA species of various lengths. The 4.9-kb mRNA-a in all blots (Fig 4A–D), clearly enhanced by heat shock, could correspond by length to the transcript initiated at S1 and extending from *hrcA* through *dnaJ.* The 3.6-kb mRNA-b (Fig 4A–C), whose transcription was also induced by heat shock, could correspond by length to the genes of *hrcA, grpE,* and *dnaK.* As any additional starting sites in the region 5′ to *hrcA* coding sequence were not found, this transcription was initiated at S1 and could be terminated at the stem-loop structure 1 in the intergenic region between *dnaK* and *dnaJ.* Alternatively, the 3.6-kb mRNA-b could be a processing product of the 4.9 kb species as the stem-loop structure between *dnaK* and *dnaJ* could be a processing site. The following observation favors the processing hypothesis. The 3.6-kb mRNA-c (Fig 4D) that corresponds to the transcript extending to *dnaJ* was initiated at S2 by the length to the genes, *grpE, dnaK,* and *dnaJ.* If the stem-loop structure 1 acts as a transcriptional terminator, the 2.4 kb that was initiated at S2 and could be terminated at this structure should be observed in the blots with probes for *grpE* (Fig 4B) and *dnaK* (Fig 4C). However, the possible band was not detected in the hybridizations. Therefore, we assumed that the stem-loop structure 1 serves a potential processing site. The 1.2-kb mRNA-d, which was detected only by the hybridization with *dnaJ* probe, could be a monocistronic mRNA initiated at S3. We have recently reported that the production of DnaJ protein was not completely inhibited by the dis-

ruption of the *dnaK* gene by insertion of the foreign DNA (Hanawa et al 1999). This result could be due to the presence of 1.2-kb transcript.

Analysis of the nucleotide sequence suggested that *orf35* and *orf29* are part of the *dnaK* operon, because both are not separated by large noncoding regions and because no apparent promoters are observed. However, transcripts encompassing all 6 genes or demonstrating read through from *hrcA* to *orf29* could not be demonstrated. Even signals suggesting a monocistronic mRNA from each gene were not detected, when the probes specific to *orf35* or *orf29* genes were used for the hybridization (data not shown). In *B. subtilis,* the full-length transcript for *hrcA-grpE-dnaK-dnaJ-orf35-orf28-orf50* was not detected by Northern blot analysis (Wetzstein et al 1992). Homuth et al (1997) were able to detect the 8-kb transcript extending all 8 genes by Northern blot analysis using riboprobes instead of oligonucleotides for probe. Our failure to demonstrate the RNA species with full length during Northern analysis may be based on its low abundance and/or rapid posttranscriptional processing. Some of the Northern blots shown here exhibit hybridization signals at the 2 positions where the 16S and 23S rRNAs migrate. These bands might occur because of unspecific hybridization or the trapping of degradation products by the rRNAs.

Results from sequence analysis of the *dnaK* locus revealed the presence of a negative cis-acting CIRCE element (Fig 2) that differed by only 1 bp from the consensus CIRCE sequence (Hecker et al 1996) between the promoter Pl and the initiation codon of *hrcA.* Furthermore, the transcriptional analysis revealed a significant increase in the amount of the transcript initiated at S1 on heat induction. We found no convincing evidence for an additional CIRCE-like element in the locus. These findings, together with the identification of the gene encoding HrcA homologue (Fig 2), suggest that the *L. monocytogenes dnaK* operon is regulated, at least in part, in a manner similar to class I heat shock genes in *B. subtilis* (for review, see Hecker et al 1996). The CIRCE regulon is known to be under negative control of *hrcA.* Only the promoter P1 becomes active after temperature upshift, probably as a result of inactivation of the HrcA repressor. In addition to the promoter P1, a second internal promoter, P2, was identified between *dnaK* and *dnaJ* (Fig 2, Fig 5) and most probably is regulated by the vegetative sigma subunit. The genomic organization of the *dnaK* operon of *B. subtilis* (Homuth et al 1997), *C. acetobutylicum* (Behrens et al 1993), and *S. aureus* (Ohta et al 1994) is mostly comparable to that of *L. monocytogenes.* However, in *C. acetobutylicum* and *S. aureus,* there is no vegetative promoter located between *dnaK* and *dnaJ.*

In conclusion, the *dnaK* operon of *L. monocytogenes* consists of 6 genes and at least 4 distinct transcripts are involved in the expression.

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