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Mycoplasmas regulate the expression of heat-shock protein genes through CIRCE–HrcA interactions

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Abstract

Mycoplasmas in general are rarely exposed to severe environmental changes except during its colonization and infection processes. Genomic analysis indicates that *Mycoplasma hyopneumoniae* possesses the genes of a single sigma factor and the HrcA repressor of negative regulation of the heat-shock response. A perfect inverted repeat sequence (5′-CTGGCACTT-N_9-AAGTGCCAA-3′) upstream of the DnaK gene has also been identified. In the present study, we demonstrate the functionality of HrcA–CIRCE interactions using the gel electrophoretic mobility shift assay. The presence of the unique sigma factor, HrcA repressor, and the CIRCE-like sequences reveals that mycoplasmal species may all use the negative regulatory mechanism in the heat-shock response. It is conceivable that mycoplasmas may have evolved a single HrcA repressor-based mechanism which might be the most simple and economical way of controlling HSP gene expression.

Keywords: CIRCE element; Heat-shock protein; HrcA repressor; *Mycoplasma hyopneumoniae*; Negative regulation; Electrophoretic mobility shift assay

The heat-shock response is a widespread phenomenon found in all living cells. In response to change in temperature and a variety of environmental stress conditions, a group of proteins called heat-shock proteins (HSPs) were induced [1]. Bacteria regulate the transcription of heat-shock genes by means of positive and negative mechanisms [2,3]. In *Escherichia coli*, the heat-shock genes are regulated by a positive mechanism depending on the relative levels and activities of sigma factor σ70 and σ32. Regular transcription processes are initiated through the recognition of promoters by the sigma factor σ70, a subunit of the RNA polymerase. Upon heat-shock, a different set of heat-shock promoters are recognized by the secondary sigma factor σ32 which was newly synthesized and stabilized. In *Bacillus subtilis*, the regulation consists of four classes and most heat-shock genes of *B. subtilis* are controlled by sigma factor B [4]. In another gram-positive bacteria *Streptomyces*, the synthesis of major HSPs is negatively controlled by at least three different repressors, namely the HrcA repressor, the HspR repressor/HAIR (HspR-associated repeat) regulon and the Rhea repressor [5]. Among the negative regulatory mechanisms, the most widespread pattern involves the HrcA repressor interacting with the conserved CIRCE (controlling inverted repeat of chaperon expression) elements [6]. The CIRCE elements dwell frequently in the upstream the *dnaK* and *groE* operons which encode molecular chaperons. The consensus sequence of the inverted repeat is 5′-TTAGCA CTC-N_9-

Abbreviations: CIRCE, controlling inverted repeat of chaperon expression; EMSA, electrophoretic mobility shift assay; HrcA, heat regulation at CIRCE; HSP, heat-shock protein; *hsp*, heat-shock protein gene; IPTG, isopropyl-β-D-thiogalactoside; IR, inverted repeat; nt, nucleotide; ORF, open reading frame; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

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GAGTGCTAA-3′, with 9 bp spacer and the repeats may extend in both directions by up to 3 bp [7].

Mycoplasma hyopneumoniae is the causative agent of porcine enzootic pneumonia and is a major contributor to the porcine respiratory disease complex [8–10]. Analysis of the genome sequence of *M. hyopneumoniae* has identified a few putative heat-shock proteins, such as DnaK (mhp072), DnaJ (mhp073), ClpB (mhp278), GrpE (mhp011), Lon (mhp541), and the putative heat-shock regulator HrcA (mhp010) [11]. To survey globally the heat-shock response of mycoplasmas, the transcriptional profiling performed on *M. hyopneumoniae* indicated that 47 genes were up-regulated and 30 genes were down-regulated during a temperature shift from 32 °C to 42 °C [12]. More recent studies with *M. hyopneumoniae* using microarray identified 91 genes, significantly regulated in response to heat-shock and 33 genes had a fold-change threshold of 1.5-fold or greater [13]. The transcriptional responses of *M. genitalium* to elevated temperature have also been investigated and the HrcA–CIRCE regulatory mechanism suggested [14].

These profiles prompted us to study the general regulatory mechanisms of heat-shock response employed by mycoplasmas. In analyzing the P65 antigen gene which encodes a heat-shock protein DnaK of *M. hyopneumoniae*, a perfect inverted repeat sequence (5′-CTGGCACTT-N_9-AAGTGCCAA-3′) spanning from nt-101 to -85 upstream from the start codon of the DnaK gene was identified [15]. Sequence analysis also pointed out the presence of a HrcA gene and only a single sigma factor in the genome of *M. hyopneumoniae* [11]. These observations imply that these small free-living cells may retain only the negative regulatory mechanism to face heat-shock and stress conditions. In the present study, the interactions of the potential CIRCE element with the unique HrcA protein were examined using the gel electrophoretic mobility shift assay. The possibility of other mycoplasmal species in sharing the same negative regulatory mechanism in heat-shock response was also analyzed in silicon.

Materials and methods

Bacterial strains and plasmids. The strains used in the study include *M. hyopneumoniae* 232 (passage 28; obtained from Dr. R.F. Ross, Iowa State University USA), *E. coli* XL1-blue (*sup*E44*hsd*R17*rec*A1*end*A1 *gyr*A46*thi rel*A1*lac*$^-$F′[*proAB*$^+$ *lac*I^q *lac*ZΔ M15 Tn10 [Tet^R]) for gene cloning, and *E. coli* M15 [pREP4] (Kan^R *lac*$^-$*ara*$^-$*gal*$^-$*mtl*$^-$*rec*A$^+$*uvr*$^+$ F$^-$) for gene expression [16]. Plasmid pQE30 for the over expression and the Ni-NTA columns for protein purification [17] were purchased from QIAGEN Inc. (USA), pGEM-T cloning vector (pGEM-T Easy Vector System kit) was from Promega Inc. (USA).

Cloning and site-directed mutagenesis of HrcA gene. The general molecular biology experiments were performed using the protocols described previously [18]. The HrcA gene of *M. hyopneumoniae* was amplified by polymerase chain reaction (forward primer: 5′-GGCGCGG ATCCATG CTAATTTGCTTTATTTTTTG-3′, reverse primer: 5′-TCCC CCGGGCTA TCCCTTT CTAATCTTCGTTTTTG-3′) from the genomic DNA of *M. hyopneumoniae* strain232, ligated to the EcoRI–ApaI site of pGEM-T vector and obtained plasmid pHRC-A. Then we used the Stratagene's QuickChange Site-Directed Mutagenesis kit to mutate the TGA codon of the HrcA gene so that the full length of gene can be expressed in *E. coli*. The TGA (nt 751–753) codon coding for tryptophan in *M. hyopneumoniae* was mutated to TGG using the mutagenic primers (forward primer: 5′-GAAAAAAAATCA ATTTGGG AAATGCTTGA AGAT CG AACAAC-3′; reverse primer: 5′-GTTGTTCGATCTTCAA GC ATTTCCCAAAT TGATT TTTTTTC-3′). The *E. coli* XL1-blue was transformed by the reaction mixture to produce plasmid pHRC-M. The nucleotide sequence was confirmed by DNA sequencing [19].

Over expression and purification of HrcA proteins. The plasmid pHRC-M was transformed to *E. coli* strain M15 for over expression and purification of the HrcA protein. The overnight culture was inoculated (1:100 dilution) to 2.5 L of prewarmed LB (per liter contains: 10 g tryptone, 10 g NaCl, and 5 g yeast extract) with 25 μg/mL kanamycin and 100 μg/mL ampicillin at 37 °C until OD_{600} reached approximately 0.6. Induction was initiated and continued for 4 h after the addition of 2 mM IPTG. The cells were harvested, washed with buffer A (25 mM Tris–HCl, pH 7.5, 2 mM Na_2EDTA, 1 mM dithiothreitol, 15% w/w glycerol) and lysed with the lysis buffer (buffer A with 8 M urea). To purify the HrcA protein, 10 mL of Ni–NTA agarose resin (Qiagen) was packed in a BioRad column (1.5 × 6 cm) and equilibrated with buffer A. The lysate was applied to the column and washed with 100 mL of buffer B (same as buffer A, except the pH was adjusted to 6.0) at 1.5 mL/min. HrcA protein was eluted with a gradient of 0–0.5 M imidazole in 30 mL buffer A at 0.8 mL/min. The collected protein was transferred to the molecular porous membrane MWCO-3500 dialysis tubing (Spectrum Inc., USA). The protein was dialyzed at 4 °C in 500 mL of denature buffer (8 M urea in 1 × PBS buffer) followed by the addition of PBS (1.2 mL/min) until the urea concentration dropped to 0.3 M. The protein was then transferred to 1× PBS buffer and dialyzed for 8 h for refolding the protein. The protein was flash frozen and stored at −80 °C in 20% v/v glycerol. The protein concentration was determined by the Lowry method. The SDS–PAGE analysis was performed and the protein bands were visualized with Coomassie blue.

HrcA-CIRCE interaction by electrophoretic mobility shift assay. To perform the electrophoretic mobility shift assay experiments [20], the 110 bp fragment (2 nmol) containing the potential CIRCE element (including the inverted repeats IR-1 and IR-2; forward primer: 5′-CCGC TCGAGCTTG ACTTGCAGTTAGTTTGTTG-3′; reverse primer: 5′-C AAAACAAACTATACCATAA TTTGGC-3′), or the 260 bp DNA fragments carrying only the invert repeat IR-2 region (forward primer: 5′-TCATAAATTCTG GCACTTTTTAACTTTAAG-3′; reverse primer: 5′-GGTATCCTTTCTCTAATTT TTTTGAATTTTG-3′), were produced by PCR amplification, respectively. The PCR products were purified with the GFX PCR DNA and Gel Band Purification kit (Amersham Pharmacia Biotech, USA), treated with calf intestinal alkaline phosphatase (NEB, New England Biolab) to remove the 5′-phosphate, and labeled with the [r-^{32}P] ATP using the T4 polynucleotide kinase (New England Biolab, USA). The DNA fragments (5 pmol) were mixed with the recombinant HrcA protein (30 pmol or more, premixed with competitive DNA of high concentration) in the binding assay buffer (40 mM Tris–HCl, pH 8.0, 4 mM $MgCl_2$, 70 mM KCl, 135 μM EDTA, 100 μM dithiothreitol, 7.5% glycerol) and incubated for 10–15 min at room temperature. The reaction mixtures were then analyzed on a 5% native polyacrylamide gel prepared with TSE buffer (6.7 mM Tris–HCl, pH 7.5, 3.3 mM sodium acetate, 1 mM EDTA). The gel was prerun for 2 h at 20 mA. After loading the sample, then the gel was run at 4 °C and 70 V for 1.5–3 h. The gel was transferred to Whatman 3 MM paper, dried with gel dryer, and visualized by exposure to the X-ray film.

Bioinformatic analysis. In searching for the consensus and the non-consensus CIRCE element, we used the EMBOSS program (http://emboss.sourceforge.net) to detect the inverted repeat sequences of the HSP genes. Using the palindrome execution file in EMBOSS, and the input sequences selected from EMBL (with the form such as "embl: L43967"), the following parameters were set: the minimum length of palindromes: 10; maximum length of palindromes: 15; maximum gap/spacer between the inverted repeats: 15; number of mismatches allowed in palindrome: 1–3. The mycoplasmal genomes were obtained in NCBI using the keyword:txid2093[orgn]. Then we checked the positions of every known HSP gene, and compared with the data we created by EMBOSS

for inverted repeats upstream (0–300 nt) of the HSP genes. The potential CIRCE elements were also searched using the PatternMatch program of SDSC Biology Workbench (http://workbench.sdsc.edu). We scanned the upstream 500 nucleotides of each HSP for the consensus CIRCE using the sequence <5′-TTAGCACTC N_9 GAGTGCTAA-3′>, allowing 3 mismatches within each arm of the inverted repeat, and 6–12 spacer nucleotide as the pattern.

The sigma factor and the HrcA repressor genes were searched in NCBI/genome using keyword "txid2093[orgn]", and then traced every protein product in the protein coding table for sigma factor and HrcA repressor using the keywords: HrcA, heat, repressor and sigma factor. The uniqueness of the sigma factor and HrcA genes were also confirmed through searching the gene database of NCBI. The amino acid sequences of HrcA proteins were multiple-aligned using the Clustal W program (http://www.ebi.ac.uk/clustalw/) and the secondary structures were predicted by the GOR IV program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html).

Results and discussion

Many bacteria have established sophisticated regulatory networks combining positive and negative mechanisms to control gene expression in an environmentally responsive way. For many pathogens, heat-shock has also been considered a stress condition during pathogen–host interactions. As we searched the 12 completely sequenced mycoplasmal genomes (http://www.ncbi.nlm.nih.gov) for the potential genes involved in regulating the heat-shock response, only a single general sigma factor could be identified [11,21–28]. Coincidentally, the 12 published mycoplasmal genome sequences all contain the predicted *hrcA* gene. Therefore, it is very likely that these mycoplasmas use a negative regulatory mechanism based on the HrcA–CIRCE interaction.

To perform the electrophoretic mobility shift assay, the recombinant HrcA protein was prepared. As shown in Fig. 1A, the recombinant HrcA protein of approximately 40 kDa was induced, purified by the Ni–NTA column and refolded. As shown in Fig. 1B, the purification step was not very efficient (lane 3) and the renatured HrcA protein is approximately 70% in purity (lane 4).

As we searched for the potential CIRCE element on the upstream of the *dnaK* gene of *M. hyopneumoniae*, a perfect inverted repeat sequence, IR-1 (5′-CTGGC ACTT-N_9-AAGTGCCAA-3′) was found at nt −101 to −85 from the *dnaK* gene [15]. As shown in Fig. 2, another palindrome-like sequence, IR-2 (5′-TTTTGTCTA-N_8-AG ACAAAA), spanning from nt −68 to −44 overlapping with the proposed promoter region of the gene was also identified (4). The EMSA experiments were then designed to distinguish whether IR-1 or IR-2 or both of the inverted repeats acted as the cis-elements with the HrcA protein. As shown in Fig. 3A, the band position of the 110 bp DNA fragment carrying the IR-1 and IR-2 regions was gradually shifted upward as the concentration of HrcA protein increased. However, the band of the 260 bp DNA fragment containing only the IR-2 region remained in the same position in the presence of 60 pmol or excess amounts of HrcA protein (Fig. 3B). Similarly, the HrcA protein did not drag other nonspecific plasmid DNAs (data not shown). The results indicate unambiguously that the HrcA protein interacts specifically with the 110 bp DNA fragment containing the IR-1 and IR-2 regions and not the IR-2 region in the 260 bp fragment.

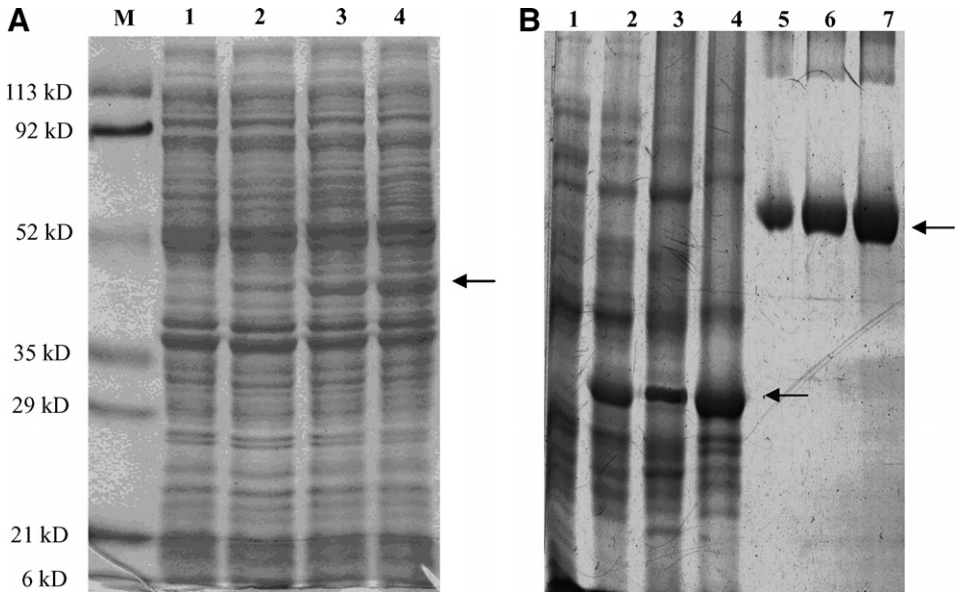

Fig. 1. Expression and purification of the HrcA protein. (A) The SDS–PAGE analysis of *M. hyopneumoniae* proteins. Lane M, protein size marker 113, 92, 52, 35, 29, 21, and 6 kDa. Lane 1, the total protein profile without induction; lanes 2–4, total protein after induction for 1, 3, and 5 h, respectively. The arrow indicates the band position of the induced HrcA protein. (B) The purification and refolding of the HrcA protein. Lanes 1 and 2 represent the total mycoplasmal protein before and after the induction for 4 h. Lane 3 represents the protein profile after Ni–NTA column purification and refolding by dialysis. Lane 4 represents the aggregated proteins after dialysis. Lanes 5–7 represent 0.5 μg, 1.0 μg, and 2.0 μg of bovine serum albumin for estimating the amount of purified HrcA protein.

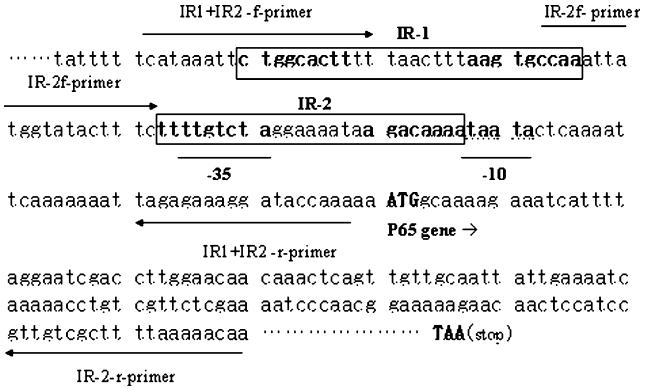

Fig. 2. The regulatory regions of the *dnaK* gene of *M. hyopneumoniae*. IR-1 and IR-2 represent the sequences of the predicted invert repeats in the upstream region. The arrows above and below the DNA sequences represent the forward and reverse PCR primers designed for amplifying the IR-1 + IR-2 and IR-2 regions. The codon ATG and TAA represent the start and stop codons of the DnaK gene. The underlined −35 and −10 regions are the predicted promoter regions of the *dnaK* gene (15). The possible Shine–Dalgarno sequence, 5′-AAAGGA-3′, just in front of the start codon can also be predicted.

To look for the possible CIRCE element sequences which may appear on the upstream of the HSP genes of other mycoplasmal species, ten of the twelve published mycoplasmal genomes were analyzed. As shown in Table 1, most HSP genes such as *dna*K, *lon*, *clp*B and *gro*ES/*gro*EL appear to have CIRCE-like elements which share very high similarities with the consensus 5′-TTAGC ACTC-N9-GAGTGCTAA-3′. However, the distance of the predicted CIRCE element from the start codons of the HSP genes vary from −20 (the negative sign means that the CIRCE element dwell within the genes) to 102 nt. This implies that the DNA sequence fragments involved in the HrcA–CIRCE interaction may be looped to interfere with the actions of RNA polymerase. Other than the consensus, some non-consensus inverted repeats with diversified sequences were also identified on the upstream of some HSP genes (Table 1). These unusual inverted repeats may explain why the *gro*ESL operon genes of *M. genitalium*, do not responds to heat-shock [14]. A multiple alignment of the published mycoplasmal HrcA sequences indicated that the protein is highly conserved and that the amino acid sequences of the HrcA proteins from the strain 7448, strain 232 and strain J of *M. hyopneumoniae* are exactly the same (data not shown). How a single HrcA repressor molecule recognizes multiple CIRCE-like sequences and even very different inverted repeat sequences within a mycoplasmal cell deserves further studies. In the RCSB Protein Data Bank, the structure 1STZ from *Thermotoga maritime* is the only published HrcA crystal structure [29]. A unique helix–turn–helix–turn–helix secondary structure was noticed at its N-terminal region. The contiguous helix–turn–helix motifs can also be predicted in the N-terminus of the HrcA protein of *M. hyopneumoniae* using the GOR-IV secondary structure prediction method (data not shown). Based on these observations, we speculate that the alternative combination of the contiguous helix–turn–helix motifs may provide multiple DNA-binding domains to recognize different CIRCE-like sequences within each species of mycoplasmas. Other than the helix–turn–helix motifs, the surrounding residues may also contribute to the recognitions of the very distinct CIRCE elements by the HrcA proteins.

The occurrence of CIRCE element in cyanobacteria, gram-positive bacteria and α-group of proteobacteria reve-

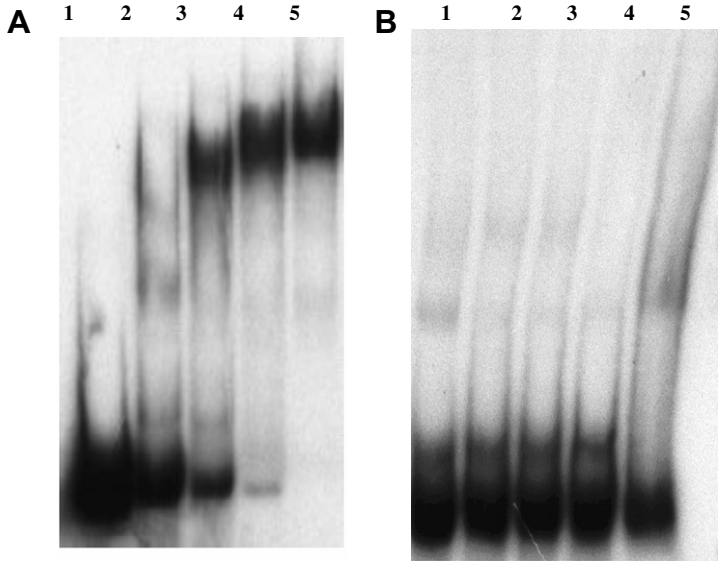

Fig. 3. The analysis of HrcA–CIRCE interactions by electrophoretic mobility shift assay. (A) Interaction of the 110 bp DNA fragments (containing the predicted IR-1 + IR-2 regions) with the purified recombinant HrcA protein. Lanes 1–5 represents the gel mobility patterns of the 110 bp DNA fragment (5 pmol) performed on 5% PAGE interacted with 0, 30, 60, 120, and 240 pmol HrcA protein, respectively. (B) Interaction of the 260 bp DNA fragments (carrying only the IR-2 region) with the HrcA protein. Lanes 1–5 represent the gel patterns of the 260 bp DNA fragment (5 pmol) interacted with 0, 30, 60,120, and 240 pmol HrcA protein, respectively.

Table 1
Prediction of the CIRCE-like sequences of mycoplasmal HSP genes*

Species	Gene/operon	CIRCE sequences			D*
Consensus		5′-TTAGCACTC	N9	GAGTGCTAA-3′	
M. genitalium G37	*dnaK*	TTAGCACTC	N9	aAGTGCTAA	48
	dnaJ	TTAaCACTC	N9	aAGTGaTAA	0
	lon	TTAGCACTC	N9	GAGTGCTAA	45
	clpB	TTAGCACTC	N9	GAGTGCTAA	40
M. pneumoniae M129	*dnaK*	TTAGCACTt	N9	GAGTGCTAA	32
	groEL-groES	TTAGCAgTt	N9	aAGTGCaAA	29
	dnaJ	TTAaCACTC	N9	aAGTGaTAA	0
	lon	TTAGCACTC	N9	GAGTGCTAA	47
	clpB	TTAGCACTC	N9	GAGTGCTAA	42
M. penetrans HF-2	*dnaK*	TTAGCAaat	N9	agaTGCcAA	102
	grpE-dnaJ	TTAtCACat	N9	GAGTGCTAA	26
	lon	TTAGCACTt	N9	aAGTGaTAA	58
	groES-groEL	TTAGCAgTt	N9	aAGTGaTAA	33
M. gallisepticum R	*groEL*	TTAGCAact	N9	aAtTacTtA	85
	dnaK	TTAGCACTt	N9	GAtTGCTAA	86
	lon	TTAGCACTt	N9	GAGTGCTAA	21
M. hyopneumoniae 232	*dnaK*	ctgGCACTt	N9	aAGTGCcAA	85
	clpB	TTgGCACTC	N9	aAGTGCcAA	55
M. hyopneumoniae 7448	*dnaK*	cTgGCACTt	N9	aAGTGCcAA	84
	clpB	TTgGCACTt	N6	GAGTGCcAA	40
M. hyopneumoniae J	*dnaK*	cTgGCACTt	N9	aAGTGCcAA	84
	lon	TaAaaAaTC	N9	aAtTGCTAA	74
	clpB	TTgGCACTt	N9	GAGTGCcAA	−20[a]
M. mobile 163K	*dnaJ*	TTAGCACaa	N9	GAGTGCTAA	10
	dnaK	TTAGCACTt	N9	GAGTGCTAA	66
	lon	TTAGCACTt	N9	GAGTGCTAA	101
	clpB	TTAGCACTC	N9	aAGTGCTAA	62
M. capricolum ATCC 27343	*clpB-hrcA-grpE*	TTAGCACTC	N9	aAtTGCTAA	56
	lon	TTAGCACTt	N9	aAGTGCTAA	44
M. synoviae 53	*dnaK-grpE-hrcA*	TTAGCAaat	N9	GAGTGCTAA	31
	dnaJ	TTAGCAtat	N9	attTGCTAA	12
Nonconsensus					
M. genitalium G37	*groEL –groES*	aaaatatttt	N9	aaactaattt	4
M. hyopneumoniae 232	*hrcA-grpE*	tttgctttatt	N9	aatttaccaaa	1
	dnaJ	ttaacaaaat	N6	aatgtgataa	66
	lon	aaatttaatt	N13	aaagaaattt	91
M. hyopneumoniae 7448	*dnaJ*	taaattttccggttt	N13	aatcaggaaaattta	63
	hrcA- grpE	agataacaaa	N14	tttgtttttt	17
	lon	aaatttaatt	N13	aaagaaattt	86
M. hyopneumoniae J	*dnaJ*	aaattccttaa	N9	ttttggaattt	77
	hrcA-grpE	agataacaaa	N14	tttgtttttt	17
M. mobile 163K	*grpE*	ttctaaaaaaa	N9	tttatttataa	8
M. capricolum ATCC 27343	*dnaJ*	tataaaaactaaca	N8	tttttgtttttata	16
M. synoviae 53	*lon*	taaattaatt	N12	aaataatcta	35

*In the consensus block, the nucleotides differing from the perfect consensus sequence are shown in the lowercase.
The D* represents the distance (in nucleotides) of the predicted the CIRCE elements from the start codon of the HSP genes.
[a] The negative signs represent that the CIRCE-like sequences are within the genes.

ales that the negative regulatory mechanisms in heat-shock response may have emerged early in evolution [2]. It has long been suggested that mycoplasmas may have arisen by a degenerate evolution process from a branch of the eubacterial phylogenetic tree containing gram-positive eubacteria with DNA having low G + C content [29]. Beside the HrcA–CIRCE system, gram-positive bacteria with low G + C content also use the CtsR repressor to regulate some HSP genes. However, the HAIR/HspR regulon present in gram-positive bacteria with high G + C content

has not been found in gram-positive bacteria with a low G + C content [30]. In contrast, none of the 12 published mycoplasmal genomes carries the CtsR repressor or the HspR repressor genes. Therefore, it is conceivable that mycoplasmas may have evolved a single repressor mechanism which might be the most simple and economical way of controlling HSP gene expression.

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