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Transcriptional Heat Shock Response in the Smallest Known Self-Replicating Cell, *Mycoplasma genitalium*

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Mycoplasma genitalium **is a human bacterial pathogen linked to urethritis and other sexually transmitted diseases as well as respiratory and joint pathologies. Though its complete genome sequence is available, little is understood about the regulation of gene expression in this smallest known, self-replicating cell, as its genome lacks orthologues for most of the conventional bacterial regulators. Still, the transcriptional repressor HrcA (heat regulation at CIRCE [controlling inverted repeat of chaperone expression]) is predicted in the *M. genitalium* genome as well as three copies of its corresponding regulatory sequence CIRCE. We investigated the transcriptional response of *M. genitalium* to elevated temperatures and detected the differential induction of four *hsp* genes. Three of the up-regulated genes, which encode DnaK, ClpB, and Lon, possess CIRCE within their promoter regions, suggesting that the HrcA-CIRCE regulatory mechanism is functional. Additionally, one of three DnaJ-encoding genes was up-regulated, even though no known regulatory sequences were found in the promoter region. Transcript levels returned to control values after 1 h of incubation at 37°C, reinforcing the transient nature of the heat shock transcriptional response. Interestingly, neither of the *groESL* operon genes, which encode the GroEL chaperone and its cochaperone GroES, responded to heat shock. These data suggest that *M. genitalium* selectively regulates a limited number of genes in response to heat shock.**

Cellular response to elevated temperatures is reflected in the transient induction of a subset of proteins called heat shock proteins (Hsps), which protect cells from damage caused by the accumulation of misfolded and unfolded proteins. Often, Hsps are induced by other stresses, such as cold, starvation, pH, oxidative insult, etc., and are essential for normal growth and protein homeostasis. Generally, Hsps belong to the families of molecular chaperones or proteases that are associated with the folding, assembly, transport, and degradation of newly synthesized or denatured proteins (48). Two major cytoplasmic chaperone systems that facilitate the proper folding of newly synthesized proteins are DnaK/DnaJ/GrpE and GroES/GroEL (17). DnaK (also known as Hsp70) binds to short unfolded hydrophobic regions of newly synthesized proteins, while the GroES/GroEL system interacts with larger proteins. DnaK activity is controlled by DnaJ (also known as Hsp40) (25) and a nucleotide exchange factor designated GrpE (16). Trigger factor (Tig) and ClpB are also important cytoplasmic chaperones. Tig prevents the misfolding and aggregation of nascent chains as they are translated by the ribosome (17). ClpB, in cooperation with the DnaK system, causes the resolubilization of aggregates (26). Cytoplasmic proteases (i.e., Lon, Clp family of ATPases, FtsH, and others) function mainly to degrade misfolded or aggregated proteins (12, 13).

The expression of Hsps in prokaryotes is under transcriptional, posttranscriptional, and translational regulation. Transcriptional regulatory mechanisms of Hsps have been studied in detail in gram-negative *Escherichia coli* and gram-positive *Bacillus subtilis* isolates (18, 32). While positive regulation by σ^{32} is the most typical mechanism in *E. coli*, three regulatory mechanisms have been identified in *B. subtilis*: class I (negative control by heat-inducible transcription repressor HrcA), class II (positive control by σ^{B} factor), and class III (negative regulation by CtsR [**c**lass **t**hree **s**tress **r**esponse regulator]) (28). The transcriptional regulation by the HrcA repressor (**h**eat **r**egulation at **C**IRCE [**c**ontrolling **i**nverted **r**epeat of **c**haperone **e**xpression]) occurs through its binding to the regulatory DNA element CIRCE (49). The latter is a negative *cis* element consisting of a conserved 9-bp inverted repeat (IR) that is separated by a 9-bp spacer (TTAGCACTC-N_9-GAGTGCT AA). Mutational analysis has confirmed that the conservation of nucleotides in the IR of the CIRCE determines the efficiency of repression. The regulation occurs when HrcA binds to CIRCE and prevents the transcription of downstream genes. Recently, the CIRCE region has been shown to control gene expression by increasing the stability of transcripts (21) and the transcription of *hrcA* itself appears to be controlled by several mechanisms, including autoregulation. Repressor HrcA activity is influenced by its interaction with the GroES/GroEL system (30, 45). In numerous organisms, CIRCE is located upstream to genes of the *dnaK* and *groESL* operons (49). In contrast to HrcA, CtsR regulates the *clpC*, *clpE*, and *clpP* operons by binding specifically to a direct heptanucleotide repeat in their promoter regions whose consensus sequence is A/GGTCAAA NAN A/GGTCAAA. In addition to *B. subtilis*, the CtsR repressor has been detected in a limited number of organisms, including *Listeria monocytogenes*, *Lactococcus lactis*, and *Streptoccoccus pneumoniae* (9, 10). HspR, another repressor of *hsp* gene expression in bacteria, binds to the HspR-associated inverted repeat element that is located in the upstream promoter region of *dnaK* operons in *Streptomyces* (6)

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TABLE 1. *M. genitalium* genes selected for analysis of transcriptional response to mild heat shock

Locus[a]	Name	Product
MG002	*dnaJ1*	DnaJ-like protein
MG019	*dnaJ2*	Heat shock protein
MG200	*dnaJ3*	DnaJ-like protein
MG201	*grpE*	Heat shock protein
MG205	*hrcA*	Transcription repressor
MG238	*tig*	Trigger factor
MG239	*lon*	ATP-dependent protease La
MG305	*dnaK*	Heat shock protein 70
MG355	*clpB*	ATP-dependent Clp protease
MG392	*groEL*	Heat shock protein
MG393	*groES*	Heat shock protein
MG457	*ftsH*	Cell division protein
MG001	*dnaN*	DNA polymerase III, subunit beta
MG003	*gyrB*	DNA gyrase subunit B
MG020	*pip*	Proline iminopeptidase
MG202		Hypothetical protein
MG391	*lap*[b]	Cytosol aminopeptidase
MG249	*rpoD*	RNA polymerase sigma-43 factor
MG275	*nox*	NADH oxidase
MG407	*eno*	Enolase

[a] Loci are numbered as in www.tigr.org.
[b] Designation of the MG391 locus is from www.stdgen.lanl.gov.

TABLE 2. Oligonucleotide primers for transcriptional analysis by RT-PCR[a]

Primer name	Primer sequence	PCR product (bp)
001RTPCRP	TCAATGGATCCAATGGAAAAG	531
001RTPCRM[b]	CTATGTGATCTAATAGAGAACTTGGG	
002RTPCRP	TGGAAGAACTCGAACTTCGTAAGACC	618
002RTPCRM[b]	TGTTTATCGTTTGGAACAAGGAACC	
003RTPCRP	CTTGAAGGACTTGAGGCTGTTAGA	531
003RTPCRM[b]	GACTGTGGGTTTTGTTTACGA	
019RTPCRP	ACAAATTGTTGTTCCCTATAACC	594
019RTPCRM[b]	CTGTTCGCCACTCTTAGTGCC	
200RTPCRP	TGGTTGGTGATACTAGGTATGGG	598
200RTPCRM[b]	TTCAGGTTGGGTGATGAAGG	
201RTPCRP	ACGCCATTAGAACCTTAGTTGTCAAGAAC	593
201RTPCRM[b]	GCTGACAAATACTGATGCAAAGCGG	
202RTPCRP	AGAAGAACAATCCAAATTGCGATAAGTG	346
202RTPCRM[b]	TTGATGACGAGTTATTATCGTCATCATTT	
205RTPCRP	GAATTTAACGCCACGGCAAGC	564
205RTPCRM[b]	AAAGCGGGTATCAATGATGCG	
238RTPCRP	CCATTGATTTCAAAACGATCA	476
238RTPCRM[b]	ATCCATTGGGGTGAATTCCA	
239RTPCRP	TTAAACTGGCTAATCTTGTCCC	673
239RTPCRM[b]	AAACTTCTTATCTAATGCTTCTGC	
249RTPCRP	TCTTGATACAAAGCCCGTTCTGC	511
249RTPCRM[b]	GAGCACATCCATGAATTTCGTGC	
275RTPCRP	CTTATGATAGAAACACAAACATCTCG	468
275RTPCRM[b]	CAAGCTGCTTCAGCAAGTTCC	
305RTPCRP	ATTGAGATGCGAAGGTGAGGG	620
305RTPCRM[b]	TTCCATTACAGAAACACAAGAATTGG	
355RTPCRP	AATAATCAATCTTGAGACAGAGTATGC	561
355RTPCRM[b]	AGAACCAATAGGTTTGTTTGGG	
391RTPCRP	TTTCCTGCTTTCAACCGTGC	471
391RTPCRM[b]	TAACAAGTAAACGTGCTTCTCTTTCAGAGC	
392RTPCRP	ATTTAAGTCAAGGTGTTGCTGTTATCCG	468
392RTPCRM[b]	GTAATTAATGAACTAGCTACAGAAGCAGCT	
393RTPCRP	TTGTAATTGCTCTTGGTGCTGGTCC	499
393RTPCRM[b]	TTGTGCTAATATGGTAGCTGTTGTTGTACC	
407RTPCRP	GATTAAACTAGACAATACTCCCAACAAAGC	457
407RTPCRM[b]	TGGCAATAGCAATATCATCTCAAGG	
457RTPCRP	TTCCATTACCTTTGATGGTTT	482
457RTPCRM[b]	TCCCATCTGGGCATATTTCA	
339RTPCRP	AAGCATTAGGGTCTGGTGGTCTACC	476
339RTPCRM[b]	CTTCGTTATTTCCAAACATCACTCC	

[a] The same primers were used in amplification of gene-specific regions from genomic DNA blotted on membranes.
[b] Reverse primers were also used in first-strand cDNA synthesis.

and *Mycobacterium* (35). The HspR-associated inverted repeat element is again an inverted repeat similar to that of CIRCE with the DNA sequence of CTTGAGT-N_7-ACTCAAG. Another distinct repressor is RheA, which binds to an inverted repeat TCTCATC-N_5-GATGACA and serves as the thermosensor of the 18-kDa heat shock-responsive protein of *Streptomyces albus* (33, 34). Thus, the transcription of *hsp* genes in bacteria is under strict control by different types of regulators.

Mycoplasma genitalium, a cell wall-less bacterial species of the class *Mollicutes*, was first isolated from male patients with nongonococcal urethritis (38) and subsequently from patients with pneumonia (4) and polyarthritis following primary pneumonia (37) and from male urethral and female vaginal/cervical specimens (3, 23). Furthermore, specific PCR, serological detection assays, and analysis of clinical symptoms have implicated *M. genitalium* in female reproductive tract diseases, including cervicitis and endometritis (5, 24, 29, 36). It appears that *M. genitalium* is a host-dependent prokaryotic pathogen with a very limited genome that targets the mucosal epithelium of both genital and respiratory tissues. Although *M. genitalium* exhibits significant morphological similarities and antigenic cross-reactivities with *Mycoplasma pneumoniae*, the genome size of *M. genitalium* (580 kb) is markedly smaller than that of *M. pneumoniae* (816 kb). In fact, *M. genitalium* is the smallest self-replicating cell known to date and this distinct property has attracted scientists to define the minimal set of essential genes required for life (15, 20). Surprisingly, *M. genitalium* reveals little similarity with other bacterial species with regard to the regulation of gene expression, as its genome lacks genes encoding orthologs for most of the conventional bacterial regulators, such as alternative sigma factors and two-component systems (14). However, *M. genitalium* possesses orthologs for *hsp* genes, including the gene encoding the regulatory protein HrcA. Therefore, we set out to characterize *M. genitalium hsp* orthologs by determining their promoter regions and measuring the transcriptional expression of these genes during mild heat shock.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Wild-type *M. genitalium* strain G37 (13th passage) was grown in 150-cm^2 tissue culture flasks in 100 ml of SP-4 medium at 37°C for 72 h. To study transcriptional response after heat shock, replicate cultures were subjected to increased temperatures (40°C, 42°C, and 44°C) for 30 and 60 min. *E. coli* cells were grown in LB broth or LB agar at 37°C, and *E. coli* carrying specific plasmids were grown in LB broth or LB agar containing ampicillin (50 μg/ml).

Design of primers and DNA amplification. Genomic DNA was isolated from *M. genitalium* strain G37 by using the Easy-DNA kit (Invitrogen), and plasmids containing *hsp* sequences were isolated by using the QIAprep spin kit (QIAGEN). Specific *M. genitalium* genes were selected from NCBI databases to monitor transcriptional heat shock response patterns (Table 1). Oligonucleotide primers for the amplification of *hsp* genes (Table 2) and for primer extension (PE) analysis (Table 3) were designed using the PC Gene program. Primers for real-time reverse transcriptase PCR (RT-PCR) were designed using Primer Express version 2.0. (Table 4).

RNA isolation. RNase-free reagents and plasticware were used to isolate total RNA from *M. genitalium*. Surface-adherent *M. genitalium* cells were washed twice with sterile phosphate-buffered saline, and Tri Reagent (Sigma) was added to each flask to facilitate cell lysis. To eliminate minute DNA contamination in

TABLE 3. Oligonucleotide primers for primer extension analysis

Primer name	Primer sequence	Nucleotide position within the gene
MG001PE	GACAGAAAAGTATTCATTGTTAGC	91–114
MG002PE	GGGTGATAACGCTTTGCTAA	73–92
MG003PE	TCTAACAGCCTCAAGTCCTTCAAG	52–72
MG019PE	GTGATATTGCATTGCAAGCTTTCTAAAAGC	76–105
MG020PE	CCGGTTTACCATTAGGATTTCCTTGTG	68–94
MG200PE	CGTTGTTACGATCAGGATGGT	101–121
MG201PE	TTCTTGACAACTAAGGTTCTAATGGCG	53–79
MG202PE	TCATAGTGGTCTTCACTTATCGCAATTTGG	27–55
MG205PE	TAATTTACTGCCAACAGGGATAGC	67–90
MG238PE	TTGGGTAGCTTGTCAGTAATTTGG	70–93
MG239PE	TTGATGATCTTACGCGAACGG	90–110
MG305PE	CCAAGGTCAATGCCAAT	25–41
MG355PE	ATCTCTTCCAATAATAGGATCGACC	90–115
MG391PE	GCACGGTTGAAAGCAGGAA	131–149
MG392PE	AGTAATTAATGGGCATCCAAATTTTCTCTC	117–148
MG393PE	TTACAATCCCTTTATTAG	122–139
MG457PE	GACTCATCAAAAGACTTTTCAAGCAG	67–92

TABLE 4. Oligonucleotide primers for RT-PCR quantification of transcripts by SYBR green chemistry

Primer name	Primer sequence	Gene
200SYBRP	TCAACAGTCAAGCAACCCCTAAT	*dnaJ3*
200SYBRM	TTTGCAATTCACCATCGGTTT	
205SYBRP	TCAATCGTTTTGTGTTGCTGG	*hrcA*
205SYBRM	ACACTTCTTAAGCGGGTTTTCAGA	
239SYBRP	GAGATAGCAAACCGATTGGAAAA	*lon*
239SYBRM	TAAATCTTCAATCCCTGCTTGGA	
305SYBRP	TACCGCTTCTAACTTTATTCCAGTTG	*dnaK*
305SYBRM	AATTGTGTTGCACCTTCCCC	
355SYBRP	AAGTTTCTGAAAATGAAATTGCTGAA	*clpB*
355SYBRM	GAATTCCTGTTGTTTGTGAAATAACTTC	
393SYBRP	AAGGTTATTTCAGTTGCTGCAGTG	*groES*
393SYBRM	ACCATCCCCAGCAATGTCAT	
275SYBRP	CACTGATGATCTTTTCTATTCCAACC	*nox*
275SYBRM	GCGCCCATCTGTTTCAACTC	
407SYBRP	ATGAAGGTGGATTTGCGCC	*eno*
407SYBRM	TGTCAAGTGCATCTTCTGCAAGTT	

RNA samples, we treated total RNA with DNase I (Gibco-BRL) prior to use in cDNA synthesis, RT-PCR, or PE.

RT-PCR analysis. One microgram of isolated and DNase I-treated RNA was reverse transcribed using gene-specific antisense primers (Table 2) and the SuperScript first-strand synthesis system (Gibco-BRL). PCR amplification was carried out using Platinum *Taq* DNA polymerase (Invitrogen), and the generated products were analyzed on agarose.

Primer extension analysis. We performed PE analysis to determine the transcriptional start points of *M. genitalium* heat shock genes. For PE, oligonucleotide primers (Table 3) were end labeled with [γ-^{32}P]ATP by using polynucleotide kinase (Gibco-BRL). Twenty-five μg of total mycoplasma RNA and 1 pmol of radiolabeled oligonucleotides were heated to 70°C for 10 min and cooled to allow primer annealing with corresponding regions of the transcripts. After annealing, reverse transcription was performed using the SuperScript first-strand synthesis system (Gibco-BRL). PE reactions were terminated by incubation at 70°C for 15 min, and *E. coli* RNase H (2 units per sample) was added and incubation continued at 37°C for 20 min. Individual PE products were phenol-chloroform extracted, precipitated with ethanol, and dissolved in 10 μl of H_2O and 5 μl of loading buffer. Three microliters of each sample was analyzed in 6% sequencing gels (United States Biochemical) alongside sequencing reactions that were generated with the same ^{32}P-labeled oligonucleotides and plasmid DNA template. For sequencing, all corresponding regions were generated by TA cloning of sequences that were PCR amplified using high-fidelity *AccuTaq* LA DNA polymerase (Sigma). Sequencing was performed using a double-stranded DNA cycle sequencing system (Gibco-BRL).

Synthesis of radiolabeled cDNA. Hybridization probes were also generated using the SuperScript first-strand synthesis system (Gibco-BRL). Twenty micrograms of RNA plus a mixture containing 0.2 μM of each gene-specific antisense oligonucleotide (except *mg339*) (Table 2) were heated to 70°C for 10 min and cooled to room temperature. Reverse transcription was performed with both [γ-^{32}P]dCTP and [γ-^{32}P]TTP (NEN) replacing corresponding nucleotides in the reaction buffer. Reactions were heat inactivated at 70°C for 15 min, and *E. coli* RNase H (2 units per sample) was added prior to a 20-min incubation at 37°C. After unincorporated nucleotides were removed by gel filtration by using G-25 Sephadex column chromatography (Roche), an equal volume of formamide was added. Following denaturation (boiling for 5 min), individual probes were added to hybridization buffer.

DNA macroarray construction, hybridization, and data analysis. DNA regions of *M. genitalium* genes were amplified using specific primers (Table 2). All PCR products were purified by gel extraction, and 200 ng of each product was blotted in triplicate on Zeta probe membranes by using Bio-Dot SF microfiltration apparatus as suggested by the manufacturer (Bio-Rad Laboratories). Each array contained two controls: one with no DNA, which served as the control for reagent contamination, and the other with *recA* DNA, which served as the control for hybridization stringency. ^{32}P-labeled cDNAs that were generated by using total mRNA from heat-shocked and control *M. genitalium* served as probes. Blots were prehybridized for 4 h at 42°C in prehybridization solution (50% formamide, 120 mM Na_2HPO_4, 250 mM NaCl, 7% sodium dodecyl sulfate [SDS], 1 mM EDTA) and hybridized (prehybridization solution containing appropriate probes) at 42°C for 18 h. Membranes were washed at room temperature for 15 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS, 30 min in 0.5× SSC–0.1% SDS, and 30 min in 0.1× SSC–0.1% SDS. After posthybridization washes, all membranes were sealed in plastic bags and placed in a PhosphorImager cassette.

Individual PhosphorImager screens were analyzed using PhosphorImager 400E (Molecular Dynamics) with a pixel size of 176 μm, and resulting image files were assessed by determining pixel density for each band using ImageQuant (version 5.0) (Molecular Dynamics). Numeric files were exported into a Microsoft Excel spreadsheet for analysis. After background subtraction, the intensity of each band was expressed as the percentage of total intensity of all bands for each membrane (normalization with regard to specific activity of individual probes). The correlation coefficients of percent intensities, determined individually for triplicate slots, ranged from 0.917 to 0.997. To measure data reproducibility in three repeats of each experiment, we calculated individual ratios of percentage intensities for all membranes. These ratios (a value of 1 indicated that data generated in two compared experiments were identical) ranged from 0.834 to 1.154, with coefficients of variation ranging from 15.0 to 37.6%. To analyze transcript amounts for different experimental conditions, we expressed them as a percentage of a housekeeping gene (*nox* or *eno*). We further calculated averaged intensities for each gene under experimental conditions and used the two-tailed *t* test for two samples to compare differences. To evaluate increases in specific transcripts, we determined the ratio of corresponding averaged percent intensities of each gene-specific slot. These ratios represent relative transcript levels (*n*-fold) of each gene under the two experimental conditions.

Relative quantification of gene expression by real-time RT-PCR. To verify results generated by DNA array for *dnaK*, *lon*, *clp*, *dnaj3*, and *grpE* genes, we used real-time RT-PCR. Analysis was performed with the ABI Prism 7900 sequence detection system and SYBR green chemistry (Applied Biosystems). Primers positioned within the same gene-specific regions (Table 2) were designed using Primer Express version 2.0 for *dnaK* (MG205), *lon* (MG239), *clpB* (MG355), *dnaJ3* (MG200), *grpE* (MG201), *eno* (MG407), and *nox* (MG275) (Table 4). Regions that were previously amplified for DNA arrays were cloned, and isolated plasmid DNA was used to generate standard curves and determine the efficiencies of all target amplifications as recommended in user bulletin no. 2. Serial dilutions of template were prepared, and amplifications were performed under default conditions using SYBR green PCR master mix. After confirming that efficiencies of all individual amplifications were approximately equal, we applied the comparative threshold cycle method to compare amounts of transcripts in different experimental conditions. One microgram of RNA was reverse transcribed by using a mixture of all of the reverse primers that were previously described in PCR amplification steps. All genes were analyzed individually to confirm the specificity of generated amplicons. Transcripts of *eno* or *nox* were utilized as normalizers, and changes (*n*-fold) in amounts of other transcripts were expressed of the corresponding amounts in cultures at zero time.

Electrophoretic gel mobility shift assay. DNA probes for the electrophoretic gel mobility shift assay (EMSA) were generated by PCR. Sizes of probe DNA and primers for amplifications were as follows: For *M. genitalium dnaK*, 270 bp, amplified by primers MGDNAK1 (5′-TGTTTTGTTGTGTGAATTGTTCAAG

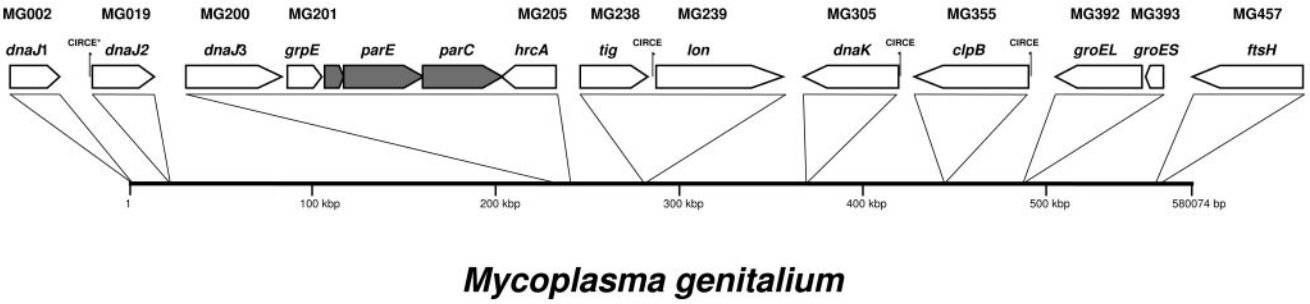

FIG. 1. Schematic diagram of the position and organization of heat-stress related genes in the *M. genitalium* genome. Presumed heat shock-related genes are pictured as clear open arrows, and their orientations represent the corresponding coding strand. Closed arrows show genes separating *hrcA* from *dnaJ3* and *grpE*. Genes are designated by their locus numbers and names as listed at www.tigr.org. Also, their position on the chromosome is illustrated. Copies of DnaJ-like protein coding genes are numbered 1 through 3 for convenience. All copies of CIRCE regulatory elements are shown; the least-conserved copy is labeled with asterisk.

TTATATGG-3′) and MGDNAK2 (5′-ACCTTCCATTACAGAAACACAAGA ATTGGTAG-3′); For *M. genitalium dnaJ2*, 210 bp, amplified by primers MGD NAJ1 (5′-CAAGAACTTGTTAAAAAAACCTTGGTTGAG-3′) and MGD NAJ2 (5′-TAAAAGCTCTTTTTATGTCTTGAGAACTAG-3′); and for *Chlamydia trachomatis dnaK*, 240 bp, amplified by primers CTDNAK1 (5′-CTATTGGATT GGTGCTCTAAAAATCTTCC-3′) and CTDNAK2 (5′-ACCCTCTAGGTAG AGCTTAGTGGCCATAAG-3′). Reverse primers for each probe were end labeled with [γ-^{32}P]ATP (6,000 Ci/mmol; Dupont-NEN) before PCR amplification. Genomic DNA of *M. genitalium* or *C. trachomatis* was used as template for the generation of probe DNA. Also, unlabeled primers were generated by PCR for competition assays. All PCR-generated probes or DNA fragments were purified (QIAGEN PCR purification kit) before use, and EMSA was performed as previously reported (11). The reaction mixture contained 20,000 cpm of probe and 150 nM of recombinant chlamydial His_6-HrcA (kindly provided by M. Tan, University of California at Irvine) in 10 μl binding buffer [25 mmM Tris-HCl (pH 8.0), 6 mM MgCl2, 0.5 mM EDTA, 20 mM KCl, 0.5 mM dithithreitol, 100 μg poly(dI-dC) per ml, 100 μg salmon sperm DNA per ml, and 5% glycerol]. For specific experiments, the reaction mixture also contained 250 μg of competitor DNA (unlabeled probe DNA). Reaction mixtures were incubated at room temperature for 10 min, products were separated by 4% native polyacrylamide gel electrophoresis for 2 h, and gels were dried and subjected to autoradiography.

RESULTS AND DISCUSSION

Heat shock-related genes in *M. genitalium*. Despite its small genome, *M. genitalium* possesses an array of genes associated with heat shock response, which are dispersed throughout its genome (Fig. 1). Interestingly, *M. genitalium* possesses three paralogous genes encoding putative DnaJ-like proteins: the first gene is MG002 which encodes DnaJ1. Genes that are upstream and downstream of MG002 encode the β subunit of DNA polymerase III (*dnaN*, locus MG001) and subunit B of DNA gyrase (*gyrB*, MG003) and are associated with both DNA replication and repair (1). The second gene (*dnaJ2*, locus MG019) is flanked by the gene encoding helicase (*mot1*, MG018; which is involved in RNA processing) and the proline iminopeptidase-coding gene (*pip*, MG020; which is involved in the degradation of proteins, peptides, and glycopeptides). The third gene (*dnaJ3*, locus MG200) is the only gene with an apparent linkage to another *hsp* gene that encodes putative nucleotide exchange factor GrpE (*grpE*, MG201). Very close to this region is another heat shock-related gene that encodes the heat shock regulatory protein HrcA (*hrcA*, locus MG205). However, *hrcA* exhibits a direction of transcription that is opposite to that of *grpE* (locus MG201). Other heat shock-related genes are *lon* (MG239), *dnaK* (MG305), and *clpB* (MG355), which encode ATP-dependent protease La (Lon protease), Hsp70 (or DnaK), and the ATPase subunit of ATP-dependent Clp protease (ClpB), respectively. Interestingly, the putative promoter regions of these genes include the CIRCE element, possibly indicating that these genes are regulated by HrcA. In addition, the genes encoding the heat shock chaperone GroEL (locus MG393) and cochaperone GroES (MG392) are located adjacent to each other as observed in most bacterial species. Besides these conventional *hsp* genes, *M. genitalium* possesses several other related genes. For example, MG238 encodes the trigger factor (Tig) that is associated with protein folding and was previously shown to catalyze protein folding in association with ribosomes (2, 39). It is noteworthy that *tig* is located directly upstream of the MG239 locus, which encodes Lon protease. Also, the last *hsp*-related locus is MG457, which encodes the heat shock-associated protease FtsH.

Although *M. genitalium* represents the smallest self-replicating cell, it has the same set of heat shock-related genes as those of *M. pneumoniae*, *Mycoplasma gallisepticum*, and *Mycoplasma penetrans*, which have relatively larger genome sizes (Table 5). Surprisingly, based on comparative genome analyses, *M. genitalium* possesses more heat shock-related genes than *Mycoplasma pulmonis*, which lacks genes encoding the chaperones GroES, GroEL, and ClpB ATPase, and *Mycoplasma capricolum*, *Mycoplasma hyopneumoniae*, *Mycoplasma mobile*, and *Ureaplasma urealyticum*, which lack the genes for GroES and GroEL. In contrast, *Mycoplasma mycoides*, *M. capricolum*, *M. pulmonis*, *M. hyopneumoniae*, and *M. mobile* possess Hsp33, a cytoplasmically localized protein with highly reactive cysteines that respond quickly to changes in the redox environment (22), which is absent in *M. genitalium*.

As mentioned above, three paralogous *dnaJ* genes were identified in the *M. genitalium* genome. Multiple copies of *dnaJ* genes are found in only *M. pneumoniae* and *M. gallisepticum* (Table 5). The presence of several DnaJ-like proteins is not unusual since DnaJ homologs selectively associate with defined subgroups of DnaK substrates or lead to the translocation of DnaK into different cell compartments (41). All three DnaJ-like proteins of *M. genitalium* possess the J domain within N-terminal regions (Fig. 2A). This domain mediates the interaction of DnaJ with Hsp70 (DnaK) and has been described for bacterial DnaJ proteins (19, 41). All three *M. genitalium* J

TABLE 5. Characteristics of heat shock-related genes among *Mycoplasmataceae*

Organism	Genome size (kb)	No. of genes	Status of genes in organism[a]									
			HrcA	DnaK	DnaJ	GrpE	GroES	GroEL	ClpB	Tig	Lon	FtsH	Hsp33
M. penetrans	1,359	1,120	+	+	+	+	+	+	+	+	+	+	
M. mycoides	1,212	1,176	+	+	+	+	+	+	+	+	+	+	+
M. capricolum	1,010	827	+	+	+	+			+	+	+	+	+
M. gallisepticum	996	865	+	+	+ (6)	+	+	+	+	+	+	+	
M. pulmonis	964	818	+	+	+	+				+	+	+	+
M. hyopneumoniae	893	784	+	+	+	+			+	+	+	+	+
M. pneumoniae	816	779	+	+	+ (3)	+	+	+	+	+	+	+	
M. mobile	777	673	+	+	+	+			+	+	+	+	+
U. urealyticum	752	629	+	+	+	+			+	+	+	+	
M. genitalium	580	484	+	+	+ (3)	+	+	+	+	+	+	+	

[a] Values in parentheses indicate copy numbers of DnaJ-like proteins. +, present in organism.

domains display the following characteristic features (Fig. 2B) (41): secondary structure analysis predicts four helices (I to IV), helix II is rich in basic residues, the loops between helices II and III contain the conserved motif HPD, and in both helices II and III, hydrophobic residues required for stabilization of helices are conserved. Further analysis of amino acid sequences reveals that proteins DnaJ1 and DnaJ3 possess only the J domain and, therefore, these two proteins belong to the type III functionally distinct group of J proteins (41). Type III J proteins do not bind to nonnative polypeptides, and it is unlikely that these proteins function as molecular chaperones. Some type III J proteins assist in the recruitment of a select isoform of Hsp70 to a discrete site (27). In other cases, type III J proteins are recruited independently of the site of action, where they productively stimulate ATP hydrolysis by the partner Hsp70 (7). Only protein DnaJ2 of *M. genitalium* possesses two additional domains as described for *E. coli* chaperone DnaJ (type I of J proteins) (41). The central Zn finger domain of *M. genitalium* DnaJ2 encloses three complete and one partial repeat CxxCxGxG. This domain has been implicated in substrate binding (8). The region between the J domain and Zn finger domain, as with *E. coli* DnaJ protein, also possesses high numbers of glycine residues (Fig. 2A). Lastly, the DnaJ C-terminal domain is present in *M. genitalium* DnaJ2 protein, suggesting that DnaJ2 (MG019) is a true homologue of DnaJ chaperone.

Transcriptional start of *M. genitalium hsp* genes. In general, *hsp* genes in bacteria are transcriptionally controlled by different positive and/or negative regulators, and the promoter regions of *hsp* genes carry specific sequences to bind to these regulators. To determine whether *hsp* genes of *M. genitalium* possess similar sequences, we analyzed the transcriptional start

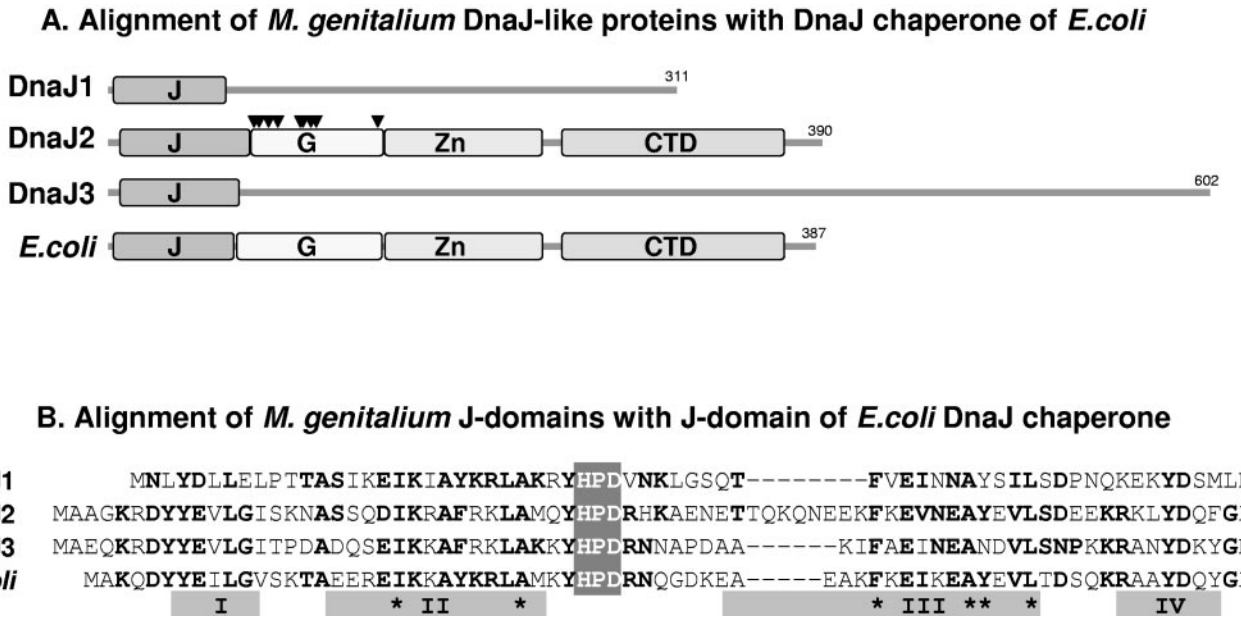

FIG. 2. Comparison of *M. genitalium* DnaJ-like proteins with *E. coli* chaperone DnaJ. (A) Structural comparison of *M. genitalium* DnaJ-like proteins DnaJ1, DnaJ2, and DnaJ3 with chaperone DnaJ of *E. coli*. The J domains (J), glycine-rich region (G; arrow heads indicate glycine residues), zinc-finger domain (Zn), and carboxy-terminal domain (CTD) are illustrated. (B) Alignment of J domains. Gray boxes illustrate positions of predicted helices (I to IV). Asterisks show hydrophobic residues that are required for the stabilization of helices. The conserved motif HPD in the loop between helices II and III is indicated. Bold letters represent basic residues.

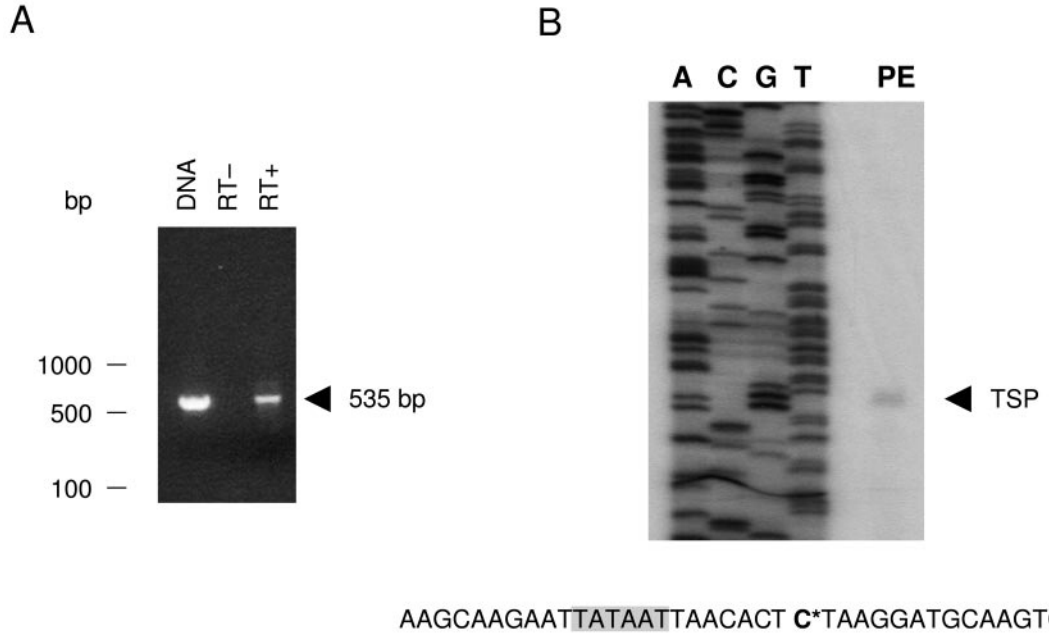

FIG. 3. Transcriptional analysis of *dnaJ2*. (A) Reverse transcription PCR was performed on total RNA isolated from *M. genitalium* cells, which generated product (RT+) as visualized on 1.2% agarose. To prove the absence of DNA in RNA preparations, a parallel reaction was run without reverse transcriptase (RT−). Genomic DNA was used to demonstrate the specificity of primers by amplification of a single 535-bp product. A 100-bp DNA ladder (Gibco BRL) was run alongside. (B) Identification of *dnaJ2* TSP. PE and DNA sequencing products (lanes A, C, G, and T) were generated by end-labeled primer MG019PE and separated on 6% urea-polyacrylamide sequencing gel. The promoter region of *dnaJ2* is at bottom, with the shaded area representing the putative −10 region, the asterisk designating the transcriptional start (bold C), and the bold ATG indicating the putative translational start.

point (TSP) of each *hsp* gene by PE using gene-specific primers (Table 3). After confirming the transcription of all genes by RT-PCR (illustrated by analysis of *dnaJ2* transcription) (Fig. 3A), we identified TSPs for genes *dnaJ2* (Fig. 3B and 4A), *dnaJ3*, *hrcA*, *tig*, *lon*, *dnaK*, *clpB*, *groES*, and *ftsH* (Fig. 4A). However, PE yielded no discernible TSPs for *dnaJ1*, *grpE*, and *groEL* when corresponding specific primers were used, although RT-PCR clearly demonstrated the presence of all transcripts.

The 5′ untranslated regions (the stretch of transcript between transcriptional and translational starts that is not translated into protein) of *M. genitalium hsp* genes differed widely, from a minimum of 2 nucleotides in the *groES* transcript to a maximum of 51 to 52 nucleotides in the *ftsH* transcript (Fig. 4A). In the case of *ftsH*, a heterogeneous TSP was observed. Further, the regions that were immediately upstream of TSPs displayed a highly conserved −10 region with nucleotides TA (A/T)AAT (Fig. 4A) as recently described for *M. pneumoniae* and *M. genitalium* (31, 40, 43). However, no conserved −35 region (43) could be identified for these genes, consistent with our earlier report for the adhesin gene promoters of *M. genitalium* (31). We also analyzed the 5′ untranslated regions of the transcripts for sequences that were complementary to the 3′ end of 16S rRNA. Again, as with numerous previously published data (43), no obvious Shine-Dalgarno sequence (ribosome binding site) could be identified for the analyzed genes.

As mentioned earlier, we detected CIRCE sequences that were associated with *dnaK*, *lon*, and *clpB* (Fig. 1 and 4A). In all three cases, the CIRCE element is located upstream of each corresponding TSP. The distance between the CIRCEs and TSPs ranged from 16 (*clpB*) to 20 nucleotides (*lon*). Additionally, we identified a CIRCE-like sequence in the promoter region of *dnaJ2* but this element is least conserved (TTAaCA CTC-N_9-aAGTGaTAA; nonconserved nucleotides are presented in lowercase letters). Furthermore, this element is positioned closer to the *dnaJ2* translational start. Hence, the TSP of this gene lies within the CIRCE-like region (Fig. 4A). An analysis of the upstream sequences of other *hsp* genes did not reveal any other known regulatory elements or IRs. This, particularly the absence of any regulatory element upstream of the *groES* and *groEL* genes, is unlike many bacterial *hsp* genes.

Transcriptional analysis reveals operon organization of *hsp* genes. Although TSPs for *dnaJ1*, *grpE*, and *groEL* were undetectable by PE, analysis of these genes by RT-PCR yielded expected products, clearly indicating that these genes were transcribed (Fig. 4B). *dnaJ1* (MG002) is located immediately downstream of the gene encoding the β subunit of DNA polymerase III (*dnaN*, MG001), and the stop codon of *dnaN* overlaps with the ATG codon of *dnaJ1*, suggesting possible cotranscription of both genes. RT-PCR encompassing the regions of both genes (Fig. 4B) confirmed this hypothesis. Further, a TSP was identified upstream of *dnaN*. Interestingly, further analysis demonstrated that the *dnaJ1* transcript is part of an even longer message, as no TSP was detected for *gyrB* (MG003), which encodes subunit B of DNA gyrase (Fig. 4B).

In contrast to *dnaJ1*, both *grpE* and *groEL* are located immediately downstream of heat shock-related genes *dnaJ3* and *groES*, respectively (Fig. 1). Therefore, it was presumed that

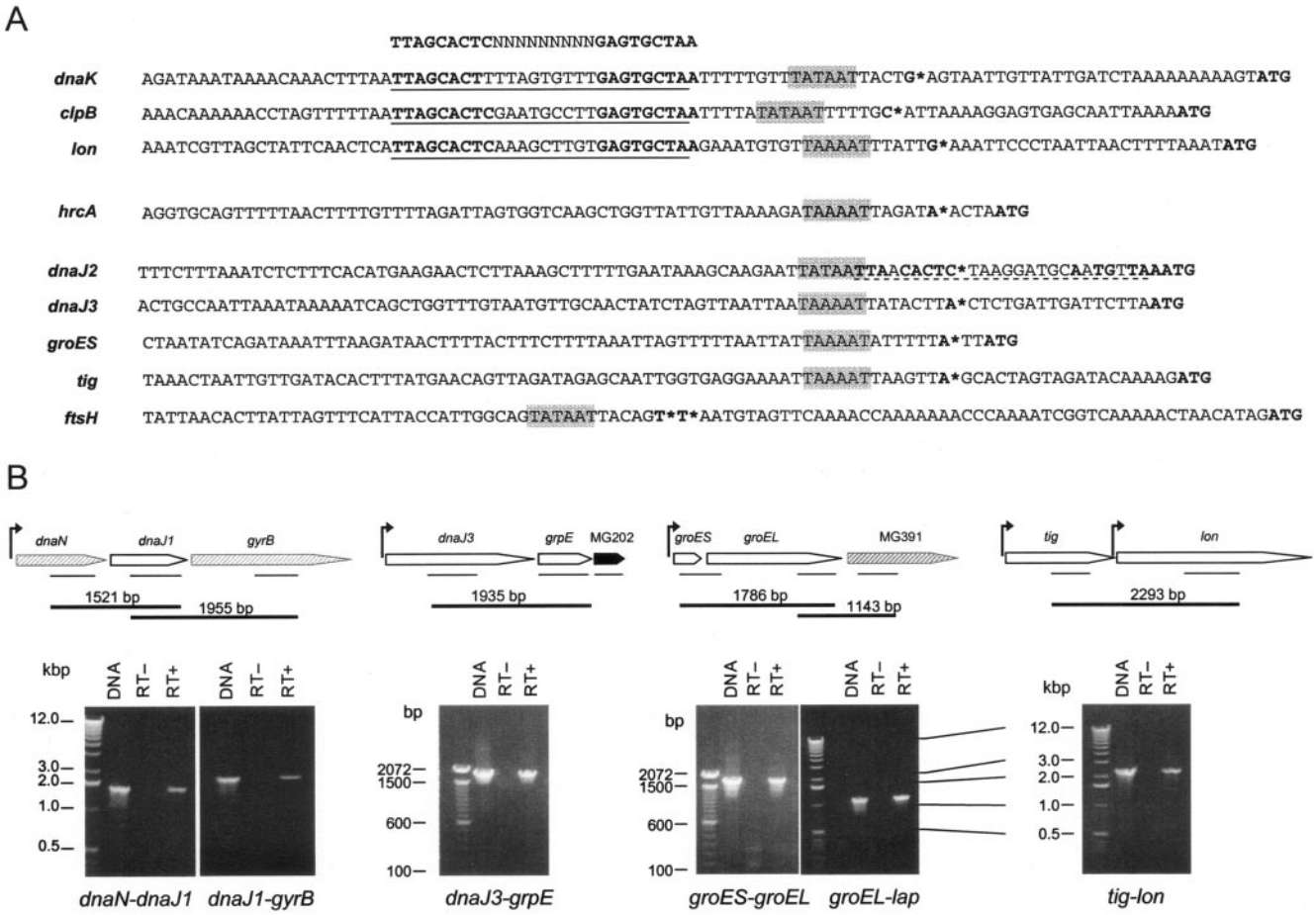

FIG. 4. Transcriptional analysis of *M. genitalium* heat shock genes. (A) Promoter regions and transcriptional start points. Asterisks designate transcriptional starts (bold letters) that were determined by PE. Shaded areas of sequences indicate putative −10 regions. The bold ATG (far right) in each sequence indicates the putative translational start. Underlined sequences (solid lines) represent CIRCE elements. IRs of CIRCE elements are shown in bold. The dashed underline indicates a CIRCE-like sequence upstream of *dnaJ2* (MG019), and only conserved nucleotides of the IR are bolded. The CIRCE sequence is indicated above the *dnaK* promoter region. (B) Analysis of transcription by RT-PCR. Gene schematics with identified transcriptional starts are presented. Thin lines represent regions that are amplified to confirm the transcription of individual genes. Bold lines represent RT-PCR products indicating the cotranscription of corresponding genes. Numbers represent the size of RT-PCR-amplified products separated on 1% agarose (RT+ lanes), alongside corresponding reactions without reverse transcriptase (RT− lanes) and PCRs using *M. genitalium* genomic DNA (DNA lanes) to demonstrate specificity of primers.

these genes would be cotranscribed with their corresponding upstream genes. RT-PCR amplification of the *dnaJ3-grpE* (1,935 bp) and *groES-groEL* (1,786 bp) intergenic regions indeed confirmed the presence of bicistronic transcripts (Fig. 4B). Further analysis demonstrated that the transcript, which started upstream of *groES* (MG393), comprised the *groEL* transcript as well as the transcript of the adjacent downstream gene (locus MG391 encoding leucyl aminopeptidase, *lap*) (Fig. 4B). We employed a similar approach to determine whether gene MG202 was part of the *dnaJ3-grpE* operon. However, repeated RT-PCR amplification using several different sets of primers failed to generate a product in support of this hypothesis.

A particularly interesting finding was the transcriptional linkage between *tig* and *lon* genes (adjacent loci MG238 and MG239, respectively), which was reinforced by the presence of a bicistronic message in RT-PCR amplification (Fig. 4B) (2,293-bp product). This is somewhat surprising because *lon* itself has its own promoter for transcription. However, recent analyses of *M. genitalium* and *M. pneumoniae* adherence-related operons also revealed multiple promoters driving the transcription of functionally related genes (31, 40).

Our observation that all putative *hsp* genes of *M. genitalium* are transcribed during growth at 37°C agrees with the abundant expression of several chaperones (DnaK, DnaJ3, GroES, and GroEL) in *M. genitalium* cultured under similar conditions (42).

Differential expression of heat shock-related *M. genitalium* genes subjected to temperature shifts. A characteristic feature of *hsp* genes is their response to elevated temperatures. In order to analyze the response of *M. genitalium hsp* genes, we determined the levels of transcript by using DNA arrays. DNA regions of *dnaK*, *dnaJ2*, *dnaJ3*, *groES*, *lon*, *clpB*, and *hrcA*, as well as regions of the normalizing genes (*nox* and *eno*) were amplified using specific primers (Table 2) and blotted onto membranes as described in Materials and Methods. Total RNA was isolated from heat-shocked and control (37°C) *M.*

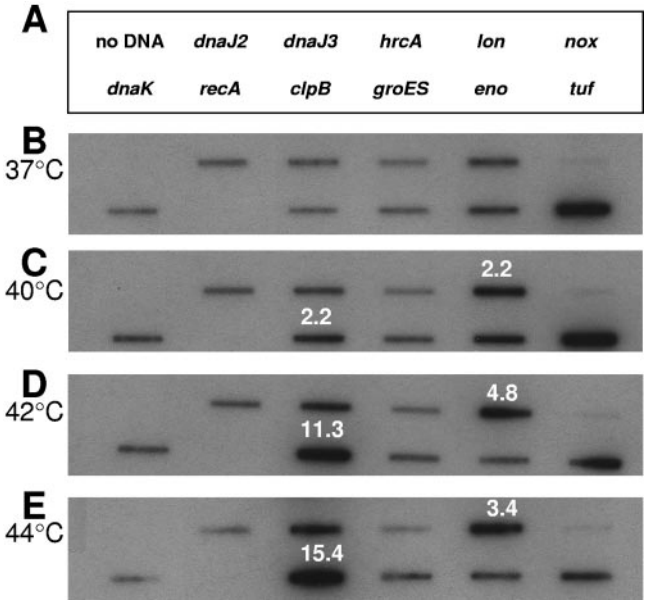

FIG. 5. Autoradiograph of DNA blot arrays for selected *M. genitalium* genes. (A) Designations of gene-specific PCR products blotted onto Zeta probe membrane. Membranes were hybridized with radiolabeled cDNAs that were generated from *M. genitalium* incubated at 37 (B), 40 (C), 42 (D), and 44°C (E) for 30 min. Synthesis of cDNA and other procedures are described in Materials and Methods. Numerical values indicate an increase in the amount of transcripts after temperature shift (value [*n*-fold] of transcript amount at 37°C).

genitalium cells, and corresponding cDNAs were generated and hybridized to membranes (Fig. 5). Exposing *M. genitalium* cultures to increased temperatures for 30 min led to differential changes in transcript amounts. We observed strongly increased levels of the *clpB* transcript (2.2-, 11.3-, and 15.4-fold) (Fig. 5) and lesser, but noticeable, increases of *lon* (2.2-, 4.8-, and 3.4-fold). No substantial changes were observed for the *dnaJ2*, *dnaJ3*, *dnaK*, or *groES* transcripts in this experiment.

We performed more detailed studies by exposing cells to 42°C (mild heat shock) for 15, 30, and 60 min (Fig. 6). Simultaneously, a set of replicate cultures was heat shocked for 60 min at 42°C and then returned to 37°C for 60 min. Control cells were kept at 37°C continuously without disturbance. While 15 min of incubation at 42°C did not produce conclusive responses, we detected significant increases in the levels of *lon* and *clpB* transcripts after 30 min and even higher values after 60 min of heat shock. In addition to these two genes, the *dnaK* gene was up-regulated after 60 min. Further, both *dnaJ3* and *grpE* transcripts showed increased levels after 60 min of incubation at 42°C (Fig. 6). It is noteworthy that cells that were heat shocked for 60 min at 42°C and then returned to 37°C for 60 min exhibited transcript values similar to those of control cells (Fig. 6; Table 6). This indicated that the observed heat induction in *M. genitalium* was selective and reversible. All three genes with the strongest heat shock-related responses

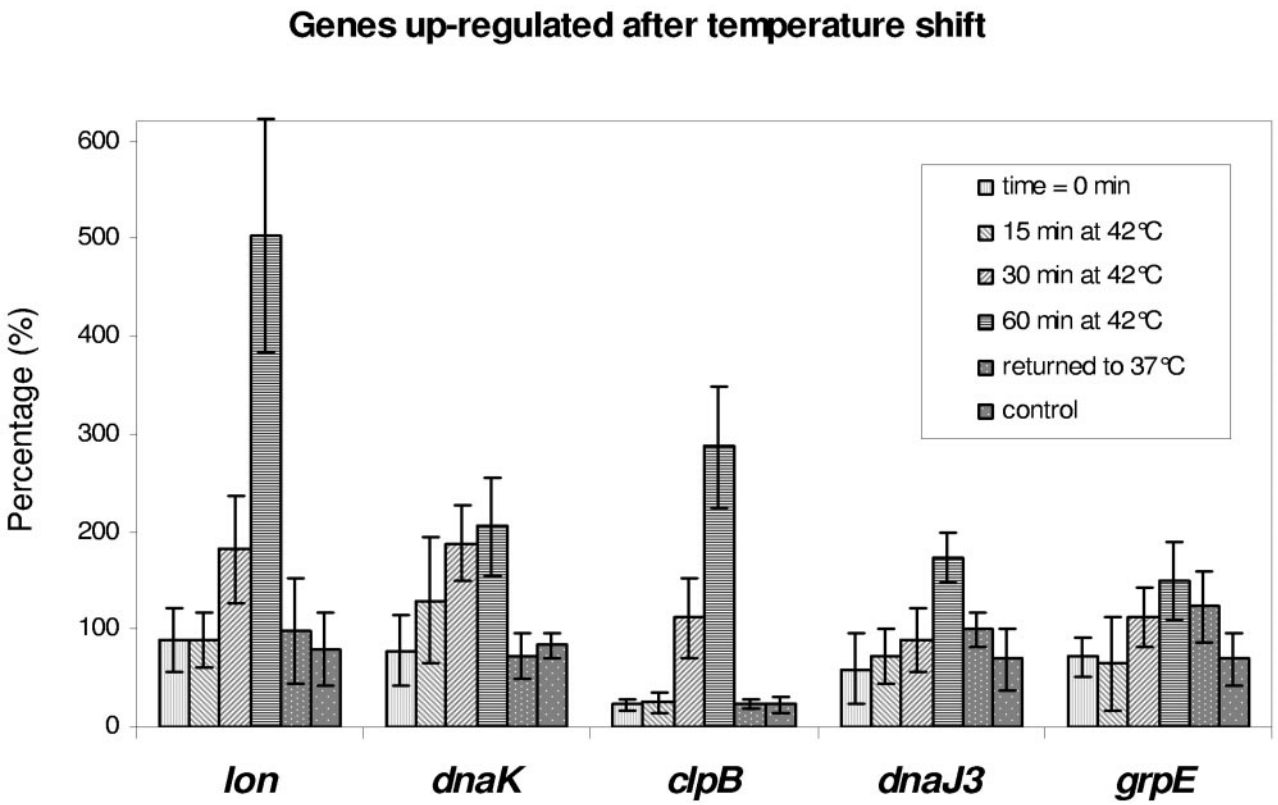

FIG. 6. Histogram of up-regulated heat shock genes of *M. genitalium* incubated at 42°C for different times. Housekeeping gene *eno* was used as the normalizer, and values represent each mRNA as the percentage of *eno* transcript. Data are presented as means ± standard deviations (error bars) (three experiments, each performed in triplicate). "Returned to 37°C" represents cells that were subjected to 42°C for 60 min and returned to 37°C for 60 min before RNA isolation. "Control" represents cells that were incubated at 37°C for 120 min before RNA isolation.

TABLE 6. Changes in transcripts levels after temperature shift to 42°C[a]

Gene	15 min	30 min	60 min	Returned[b]	Control[c]
Up-regulated after temp shift					
lon	1.01	2.06 (8.00)[d]	5.71 (38.09)[d]	1.12 (3.74)	0.91 (0.98)
dnaK	1.66	2.40 (5.37)[d]	2.62 (8.85)[d]	0.93 (2.10)	1.06 (1.71)
clpB	1.11	4.99 (12.94)[d]	12.86 (43.38)[d]	1.05 (2.35)	1.00 (1.11)
dnaJ3	1.22	1.50 (2.27)	2.92 (5.00)[d]	1.68 (2.56)	1.17 (1.65)
grpE	0.91	1.58 (1.88)	2.09 (4.85)[d]	1.72 (0.99)	0.98 (1.30)
Unaffected or down-regulated by temp shift					
dnaJ1	0.73	0.49	0.10	0.00	1.56
dnaJ2	1.13	1.04	1.06	0.70	1.11
groES	1.60	1.20	1.28	1.19	1.07
groEL	1.11	1.04	0.86	0.76	0.73
tig	1.16	1.67	1.85	1.44	1.12
ftsH	1.02	0.93	0.87	0.98	0.96
hrcA	1.16	1.08	1.17	1.04	1.01
rpoD	0.95	0.76	0.92	2.90	2.54
tuf	1.09	0.72	0.61	0.83	1.07

[a] All values represent transcript levels (*n*-fold) of each gene relative to the level at zero time; values in parentheses represent increases (*n*-fold) as detected by real-time RT-PCR.
[b] "Returned" *M. genitalium* cultures subjected to 42°C for 60 min were returned to 37°C for 60 min and RNA isolated.
[c] "Control" *M. genitalium* cultures were incubated at 37°C through experiment.
[d] Significantly increased.

(*lon*, *clpB*, and *dnaK*) possess CIRCE regulatory elements, implying that HrcA is involved in their regulation.

Interestingly, *dnaJ2*, which encodes a J protein that is the most homologous to *E. coli* chaperone DnaJ (Fig. 2A), possesses a CIRCE-like sequence (Fig. 4A). However, no response to increased temperature was detected in the corresponding transcript (Table 6). This may be due to the following reasons: (i) Relative to the CIRCE element that is located upstream of *dnaK*, *lon*, and *clpB*, the CIRCE-like sequence that is upstream of *dnaJ2* has three mismatches within the 18 nucleotides that constitute the IR. Possibly this could lead to decreased binding of HrcA and, therefore, no regulation, or (ii) the position of CIRCE appears to be critical to the regulation of expression. In other words, the CIRCE elements that are associated with *dnaK*, *lon*, and *clpB* are localized upstream to TSPs, relative to *dnaJ2*, in which the TSP is located within the CIRCE-like sequence.

We also observed a 2.92-fold up-regulation of *dnaJ3* transcript after 60 min at 42°C (Fig. 6; Table 6). The mechanism leading to higher expression of the *dnaJ3-grpE* operon is difficult to explain because no known regulatory elements were found in the promoter region. Still, it appears that only DnaJ3 of the DnaJ-like proteins is involved in the heat shock response of *M. genitalium*.

As mentioned above and demonstrated in Table 6, no other transcripts were induced under heat stress. Most unpredictably, no changes were observed for *groES* and *groEL*, which encode major heat shock proteins. The absence of a *groES-groEL* response to mild heat may indicate that these genes are not under the control of any regulator molecule. This also suggests that in mycoplasmas, the GroES/GroEL system may play a less important role in protein homeostasis than that in other prokaryotes. Such an assumption is further supported by the fact that not all mycoplasma species possess *groESL* genes (Table 5). Generally, GroESL is important for the proper synthesis of large proteins with hydrophobic regions. Interestingly, *M. genitalium* synthesizes large proteins, but they are more hydrophilic and may not require the GroES/GroEL system (47). Under stress conditions, the unchanged amounts of GroES/GroEL system chaperones could actually lead to higher induction of HrcA-CIRCE-controlled genes as reported recently (30, 45) since the GroES/GroEL system becomes occupied by large misfolded proteins and is incapable of preserving HrcA in the active state.

DnaK, Lon, and ClpB, products of the three strongest up-regulated genes in *M. genitalium*, are preferably involved in the degradation of misfolded proteins. Together with the above-mentioned lack of GroES/GroEL system response to temperature shift and the observed tendency for decreased amounts of *tuf* transcript at 42°C (Table 6), it is reasonable to expect decreased de novo synthesis of proteins in *M. genitalium* that are exposed to elevated temperatures. We repeatedly attempted to biosynthetically label proteins in heat-shocked cells but always observed markedly reduced protein synthesis relative to unstressed control cultures (data not shown).

Our observations partly agree with the transcriptional response patterns to heat shock that were reported for *M. pneumoniae* genes (44). After the heat shock of *M. pneumoniae* cells (shift from 32 to 43°C for 30 min) the up-regulation of 47 genes and down-regulation of 30 genes was detected. Genes encoding DnaK, Lon, and ClpB were up-regulated as in *M. genitalium*, and these three *M. pneumoniae* genes have CIRCE elements located in their upstream regions. Similarly, no up-regulation of the *dnaJ2* ortholog (MPN021) was reported (44), which supports our observation that the presence of CIRCE-like elements in mycoplasmas is not sufficient to elicit transcriptional induction. In contrast, the up-regulation of *groES* (MPN574) and the gene encoding putative leucine aminopeptidase (*lap*, MPN572) was detected. The organization of the *groESL* region in *M. pneumoniae* is similar to that of *M. genitalium* (Fig. 4B) with *lap* positioned immediately downstream to *groEL* (MPN573). We showed that both *groES* and *groEL* are cotranscribed from the single promoter together with the adjacent downstream gene *lap* (Fig. 4B), and we observed no

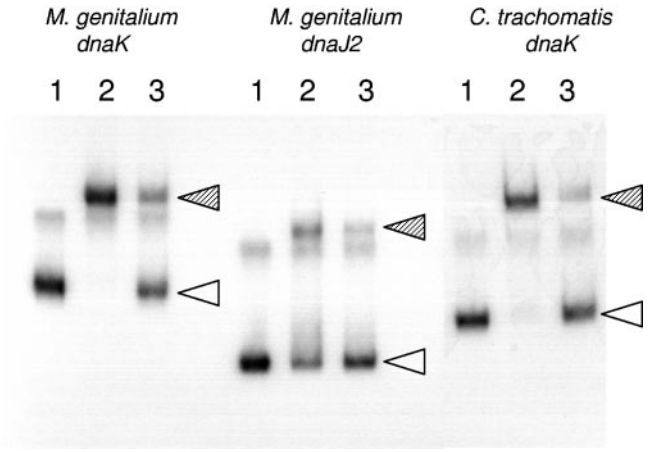

FIG. 7. Interaction between HrcA- and CIRCE-containing promoter regions. DNA probes for *M. genitalium dnaK* and *dnaJ2* and *C. trachomatis dnaK* promoter regions (lanes 1) were amplified and interacted with chlamydial HrcA protein (lanes 2). The presence of unlabeled competitive probe in reaction mix (lanes 3) confirmed specificity of interaction.

transcriptional upregulation of the *groESL* operon genes of *M. genitalium*. Additional experimental data could help to better understand these observed differences. The cotranscription of *groES*, *groEL*, and *lap* genes appears very likely in *M. pneumoniae* but was not confirmed experimentally. In *M. genitalium*, we could not detect any additional promoter in the *groESL* operon, although the existence of another promoter, which drives the transcription of *lap*, cannot be excluded without experimental proof.

Differential heat shock expression of *hsp* genes in *M. genitalium* was further evaluated by real-time RT-PCR (Table 6), and significant transcript changes for *lon*, *clpB*, *dnaK*, and *dnaJ3* were confirmed.

Interaction of chlamydial HrcA with promoter regions of *dnaK* and *dnaJ2* of *M. genitalium*. Although the CIRCE sequences in front of *dnaK*, *clpB*, and *lon* showed significant identity with other species, we wanted to determine whether *M. genitalium* HrcA directly bound to CIRCE in order to regulate expression. Unfortunately, we were unable to achieve the expression of *M. genitalium* HrcA protein in *E. coli* by using several expression systems. Therefore, we examined the interaction between recombinant HrcA from *C. trachomatis* (kindly provided by M. Tan, University of California at Irvine) and the *M. genitalium* CIRCE element. As indicated in Fig. 7, chlamydial HrcA readily interacts with the promoter region of *dnaK* of *M. genitalium* (lane 2), which was comparable to the interaction between chlamydial HrcA and its own *dnaK* promoter (lane 2) (46). Moreover, the addition of unlabeled competitors markedly reduced the DNA-protein complex formation (lanes 3). Since the promoter region of *dnaJ2* possessed a CIRCE-like element (Fig. 4A), we tested this region for its interaction with chlamydial HrcA and observed a relatively lower but specific interaction with HrcA (lanes 2 and 3). These results suggest that the HrcA protein of *M. genitalium* is involved in the transcriptional regulation of *lon*, *clpB*, and *dnaK* expression. However, the poor binding of HrcA to the upstream region of *dnaJ2*, without concomitant heat shock-related increases in transcription (Table 6), may implicate other factors in HrcA-CIRCE-mediated regulation, such as the position of the CIRCE sequence related to the TSP of a transcript.

Whatever might be the precise mechanisms of stress-related responses in *M. genitalium*, this smallest of cells is capable of transcriptional regulation, which had been thought nonexistent. For the first time, we demonstrate the functionality of HrcA-CIRCE interactions in control of transcription in *M. genitalium* and raise the possibility that other transcription regulatory mechanisms exist, as indicated by *dnaJ3-grpE* upregulation.

ACKNOWLEDGMENT

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