

# Characterization of a Unique ClpB Protein of *Mycoplasma pneumoniae* and Its Impact on Growth<sup>∇</sup>

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***Mycoplasma pneumoniae* accounts for 20 to 30% of all community-acquired pneumonia and has been associated with other airway pathologies, including asthma, and a range of extrapulmonary manifestations. Although the entire genomic sequence of *M. pneumoniae* has been completed, the functions of many of these genes in mycoplasma physiology are unknown. In this study, we focused on *clpB*, a well-known heat shock gene in other bacteria, to examine its role in mycoplasma growth. Transcriptional and translational analyses of heat shock in *M. pneumoniae* indicated that *clpB* is significantly upregulated, reinforcing its status as a critical responder to heat stress. Interestingly, *M. pneumoniae* ClpB does not use dual translational start points for ClpB synthesis, like other ClpB-characterized bacteria. Biochemical characterization of purified *M. pneumoniae* recombinant ClpB revealed casein- and lysine-independent ATPase activity and DnaK-DnaJ-GrpE-dependent chaperone activity. An *M. pneumoniae* mini-Tn4001-integrated, *clpB*-null mutant was impaired in its ability to replicate under permissive growth conditions, demonstrating the growth-promoting status of ClpB.**

*Mollicutes* are unusual prokaryotes that lack bacterial cell walls and exhibit limited metabolic capabilities due to their streamlined genomes. *Mycoplasma pneumoniae*, with a genome of 816 kb, is confined to a parasitic life-style in humans (16). The importance of *M. pneumoniae* as a causative agent of acute and chronic respiratory diseases and extrapulmonary pathologies has been well documented by epidemiological studies in many settings. *M. pneumoniae* accounts for 20 to 30% of all community-acquired pneumonia and has been implicated in asthma and chronic obstructive pulmonary disease (3, 36). This mycoplasma utilizes several attachment mechanisms to bind to sialylated and sulfated receptors on human target cells and to abundant host proteins, like fibronectin and surfactant protein A (2, 7, 18, 19). An understanding of how *M. pneumoniae* responds to in vivo environmental signals and successfully establishes residence in the host has been elusive. This is compounded by the absence of well-established prokaryotic regulatory signaling, such as alternative sigma factors and two-component systems. For example, the presence of only one sigma factor (*sigA*, mpn352) in *M. pneumoniae* suggests that its response to external stimuli is not controlled by the level of expression of alternative sigma factors. However, the presence of the conserved inverted repeat sequence (CIRCE [controlling inverted repeat of chaperone expression]) immediately in front of four heat shock genes (*dnaK*, *lonA*, *dnaJ*, and *clpB*) and the identification of the CIRCE-associated repressor (*hrcA*, mpn124) provide evidence for negative regulation of gene expression in *M. pneumoniae*.

Recent transcriptional studies of heat shock responses in *M. pneumoniae* (39), *Mycoplasma genitalium* (29), and *Mycoplasma hyopneumoniae* (6) have provided additional insights concerning the upregulation of specific heat shock genes. For example, in *M. pneumoniae*, *groE* retained remnants of the CIRCE-like element with four mutations within both arms of the inverted repeat and a deletion within the 9-nucleotide spacer sequence. Among the three copies of *dnaJ* (mpn002, mpn021, and mpn119), only mpn021 possessed a CIRCE-like element with three mutations in both arms of the inverted repeats. The CIRCE element of *dnaK* had one mutation in one arm of the inverted repeat. Evidence that CIRCE might act as a negative *cis* element was obtained by the observation that mutations in one or both arms of specific inverted repeats resulted in elevated transcription of the downstream genes even at a normal growth temperature (35, 43). In contrast, the CIRCE elements of *clpB* and *lonA* were undisturbed, indicating that *clpB* and *lonA* are the only heat shock response genes that are fully under the control of the CIRCE element. Studies with *M. genitalium* and *M. hyopneumoniae* confirmed CIRCE-mediated interactions (6, 29). Interestingly, CIRCE is reported mostly in association with *groE* or *dnaK* operons in other bacteria (43), suggesting that different modes of stress-related gene regulation exist in *M. pneumoniae*. Therefore, the most-relevant regulatory transcriptional and translational distinctions between *M. pneumoniae* and other gram-positive bacteria are (i) the absence of alternative sigma factors, (ii) the presence of ClpB alone as representative of the Clp family, and (iii) the occurrence of CIRCE sequences upstream of *clpB* and their absence upstream of *groESL*. In both *M. pneumoniae* and *M. genitalium* transcriptional studies, the ClpB gene was highly upregulated during heat shock compared to all other heat shock genes.

ClpB belongs to the AAA protein (ATPases associated with a variety of cellular activities) superfamily, which includes one category of the Clp/Hsp100 proteins with its members ClpA, ClpX, and ClpY, which facilitate the activities of ClpP, and another category where ClpB does not interact with ClpP. Clp

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(caseinolytic protease) was first identified as a heat shock-inducible, ATP-dependent protease complex capable of hydrolyzing casein. In spite of the significance of Clp ATPases in stress responses, *M. pneumoniae* has conserved only one ClpB, and a single gene in eubacteria encodes two differently sized proteins (79 and 93 kDa) via dual translational initiation sites within the *clpB* transcript (34).

ClpB mediates the full recovery of aggregated proteins with the cooperation of the DnaK chaperone system (DnaK, DnaJ, and GrpE; termed KJE) (27, 28, 42). In some organisms, the functional interdependence of ClpB and KJE is reflected in their gene organization and/or coordinated expression. For example, in *Mycoplasma capricolum* *clpB*, *dnaK*, *dnaJ*, and *grpE* genes are transcribed as a single operon (9), whereas in *M. pneumoniae* and *M. genitalium* the unlinked *clpB* and *dnaK* genes belong to the same CIRCE regulon (10, 16).

Here, we describe specific attributes of *M. pneumoniae* ClpB and show that a ClpB-deficient mutant demonstrates markedly impaired cell growth.

#### MATERIALS AND METHODS

***M. pneumoniae* strains and growth conditions.** Wild-type *M. pneumoniae* clinical strain S1 and the S1ΔClpB mutant strain were grown in SP-4 broth medium at 37°C for 72 to 96 h in 150-cm<sup>2</sup> tissue culture flasks as described earlier (7). For functional studies, *M. pneumoniae* S1 was inoculated into 300 ml SP-4 medium distributed equally into three 150-cm<sup>2</sup> tissue flasks, incubated at 37°C for 72 h, and then harvested, concentrated, pooled, and added to three liters of fresh SP-4 broth. The latter was immediately transferred equally into 30 150-cm<sup>2</sup> tissue culture flasks, which were divided into five groups of six flasks. One group was used for radiolabeling, and the remaining four groups were used for RNA isolation and immunoblotting.

**Biosynthetic radiolabeling of *M. pneumoniae* proteins.** For radiolabeling, mycoplasmas were biosynthetically labeled with [<sup>35</sup>S]methionine-cysteine as follows: surface-attached *M. pneumoniae* cells grown to mid-log phase were washed three times with Dulbecco's modified Eagle's medium (DMEM) and placed at 37°C or 43°C. Surface-attached cells were immersed in 1/10 of their original volume in DMEM without cysteine or methionine but supplemented with 10% fetal bovine serum. Then, 100 μCi of [<sup>35</sup>S]methionine-cysteine was added, and mycoplasmas were incubated at 37°C or 43°C for 2 to 6 h, pelleted, and washed four times with phosphate-buffered saline (PBS). Cell aliquots were harvested for protein estimation and for scintillation counts. Based on protein concentration, the remaining cell pellets were resuspended individually in sample buffer A (4% sodium dodecyl sulfate [SDS], 125 mM Tris [pH 6.8], 10% β-mercaptoethanol, 10% glycerol, 0.2% bromophenol blue) and heated to 95°C for 5 min. Then, equal amounts of whole-cell lysates were analyzed on 10% SDS-polyacrylamide gel electrophoresis (PAGE) gels and transferred to nitrocellulose membranes. Blots were then exposed to Kodak OMAT X-ray film for 1 to 2 weeks and autoradiograms analyzed. Quantitation of band intensities was performed by scanning autoradiograms and analyzing images with Kodak Image software. For immunoblot analysis, mycoplasma cells grown at 37°C or 43°C were washed two times, scraped into PBS, and characterized after protein quantification.

**Bacterial strains, plasmids, and DNA manipulations.** *Escherichia coli* Top10 (Invitrogen) and *E. coli* BL21(DE3) [F' *ompT hsdS* (*r<sub>B</sub>* *m<sub>B</sub>*) *gal dcm λ*(DE3) pLysS] (Stratagene) were grown in Luria-Bertani (LB) broth and used to clone and express the mycoplasma *clpB* gene (mpn531). For DNA manipulations, the following vectors were used: pCR2.1 (Ap<sup>r</sup> Km<sup>r</sup>, TA cloning vector; Invitrogen) and pET19b (Ap<sup>r</sup>, N-terminal His<sub>10</sub> tag, expression vector; Novagen). *M. pneumoniae* chromosomal DNA was isolated using an Easy DNA isolation kit (Invitrogen). Plasmid DNA was purified using the QIAprep spin protocol according to the manufacturer's instructions (Qiagen). The *M. pneumoniae clpB* locus was amplified by PCR, using strain S1 chromosomal DNA as a template. PCR amplification and UGA corrections were performed as described earlier for CARDS TX (18). The specific *clpB* oligonucleotide primers were as follows: ClpB F1, 5'-CATATGGATTTTGTAGTTTACACCAACACC-3'; ClpB R1, 5'-TTCTCCTTTTCCATTCGGCAGTTAGAG-3'; ClpB F2, 5'-CTCTAACTGC CGAATGGAAAAGGAGAAA-3'; and ClpB R2, 5'-GGATCCTAAGATTT AACCTTATTGGCTAC-3'.

The resulting ca.-2.0-kb PCR product was cloned into pCR2.1, generating a

plasmid designated pCR-ClpB, which was subsequently digested with NdeI and BamHI. Insert DNA was gel extracted and ligated into pET19b, which was digested with NdeI and BamHI to yield pET-ClpB. This plasmid was transformed into competent *E. coli* BL21(DE3) strains, and recombinant colonies were screened for resistance to ampicillin and expression of a protein product of the correct size. One recombinant clone was selected for further study. Verification of pET-ClpB was achieved by complete DNA sequencing (Department of Microbiology and Immunology Nucleic Acids Core Facility, The University of Texas Health Science Center at San Antonio). Sequences were analyzed using the BLAST program available in the NCBI database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).

**Expression and purification of *M. pneumoniae* rClpB protein.** The pET-ClpB plasmid in *E. coli* BL21(DE3) cells was expressed as described earlier (18), and fusion proteins were purified by nickel affinity chromatography under native conditions (Qiagen). Recombinant ClpB (rClpB) was eluted with 2.5-ml aliquots of lysis buffer containing 250 mM imidazole (elution buffer). Each collected fraction was desalted in 50 mM Tris-HCl buffer (pH 8.0) and 5% glycerol (TG) by using PD-10 columns, and individual fractions were analyzed by SDS-PAGE. Fractions with the highest purity of rClpB were pooled and applied to 1 ml Mono Q HR anion-exchange columns (Amersham Bioscience) preequilibrated with TG plus 0.25 M NaCl. rClpB was eluted with a linear gradient of NaCl (0.25 to 1.0 M in TG buffer) (flow rate, 0.5 ml · min<sup>-1</sup>; fractions, 1.0 ml). Protein concentrations were monitored at an absorbance of 280 nm, and peak fractions were analyzed by SDS-PAGE. Fractions with the highest purity of rClpB were concentrated, and 100 μl of the concentrated sample was applied to a 25-ml gel filtration Superdex 200 HR 10/30 column (GE Healthcare) equilibrated with TG plus NaCl buffer (AKTA purifier) and eluted with the same buffer (flow rate, 0.5 ml · min<sup>-1</sup>; fractions, 0.5 ml). Protein concentrations were monitored at an absorbance of 280 nm. Samples obtained at all stages were analyzed by SDS-PAGE and silver staining. Fractions containing pure rClpB protein were dialyzed in storage buffer and placed at -80°C.

**Generation of mouse anti-ClpB polyclonal antibodies.** Six-week-old BALB/c female mice (*n* = 5) were bled and screened by immunoblot analysis using rClpB to determine preexisting antibodies. As described earlier (18), mice were immunized with different concentrations of rClpB and later screened by immunoblot analysis using rClpB or whole *M. pneumoniae* cell lysates. All mice exhibited strong immune reactivity to rClpB and ClpB in whole-cell lysates. Sera were collected 2 weeks later, rescreened by immunoblot analysis and an enzyme-linked immunosorbent assay, and pooled.

**RNA isolation.** RNase-free reagents and plasticware were used to isolate total RNA from *M. pneumoniae* clinical strain S1. Surface-adherent cells were washed twice with sterile PBS, and Tri Reagent (Sigma) was added to each flask to facilitate cell lysis. To eliminate minute DNA contamination in RNA samples, isolated total RNA was treated with DNase I (Gibco-BRL) prior to use in cDNA synthesis, reverse transcription-PCR (RT-PCR), or primer extension (PE).

**PE.** For PE of *clpB*, oligonucleotide 5'-TGAGCACCTCATCGTTAATGCT A-3' was end labeled with [<sup>γ-32</sup>P]ATP by using polynucleotide kinase (United States Biochemical) and annealed to 25 μg of total mycoplasma RNA. PE was performed as previously described (30). PE products were phenol-chloroform extracted, precipitated with ethanol, and dissolved in 10 μl of H<sub>2</sub>O. Two microliters of each sample was analyzed in 6% sequencing gels alongside sequencing reactions (double-stranded-DNA cycle sequencing system; Gibco-BRL) generated with the same oligonucleotides and appropriate recombinant plasmids. The sequencing template for identification of the *clpB* transcriptional start site was generated by PCR amplification of the corresponding region in S1 by using primers 5'-TAGCGCGTAAAGATGTACCGTT-3' (532RTPCRP) and 5'-TGA GCACCTCATCGTTAATGCTA-3' (reverse primer used for PE of *clpB*). The amplified ~960-bp region was cloned in pCR2.1, and individual plasmids were purified from *E. coli* Top10 and sequenced.

**RT-PCR analysis.** One microgram of DNase I-treated total RNA isolated from *M. pneumoniae* cells was reverse transcribed using gene-specific antisense primers (Table 1) and the SuperScript first-strand synthesis system (Gibco-BRL). PCR amplification was performed using Platinum Taq DNA polymerase (Invitrogen), and generated products were analyzed by electrophoresis on 1.2% agarose. To prove the absence of DNA in RNA preparations, a parallel reaction was run without reverse transcriptase. The specificity of all primers was demonstrated by PCR amplification of the predicted regions, using chromosomal DNA isolated from *M. pneumoniae* clinical strain S1. Regions amplified from the clinical strain S1 template were sequenced and compared with reference strain M129.

**Synthesis of radiolabeled cDNA.** Hybridization probes were also generated using the SuperScript first-strand synthesis system (Gibco-BRL). Twenty micrograms of total RNA plus a mixture containing 0.2 μM of each gene-specific antisense oligonucleotide (Table 2) was heated to 70°C for 10 min and cooled to

TABLE 1. *M. pneumoniae* genes and their oligonucleotide primers used for transcriptional analysis by RT-PCR

Gene	Primer name <sup>a</sup>	Primer sequence	Amplicon length (bp)
mpn529	529RTPCRP 529RTPCRM	ACAACATCGAGTAAGCCATTGTC TAGTCTGCGTATTTTTAGCGTACC	317
mpn530	530RTPCRP 530RTPCRM	GGAACCAAATAATTTAAAAGAAGAGCTAG TCGTTAAGTAGTCAGCTATTTTTTCAG	404
<i>clpB</i>	531RTPCRP1 531RTPCRM2	AATTTATGAACTTTCGTTGTCTGG CATTTCTACAGCAGCTACCAAGGC	426
	531RTPCRP2 531RTPCRM2	TTTACTAACTGAAGCAGTCCGACG TCTCAACTATTACGTTGTAACATC	544
<i>licA</i>	532RTPCRP 532RTPCRM	TAGCGCGTAAAGATGTACCGTT CAGCAACACGTAATTACCGTTTC	557
<i>ackA</i>	533RTPCRP 533RTPCRM	GATTCGGTGATCGTAGATGCA AGCGAAAACATCACGCATATCC	558
mpn534	534RTPCRP 534RTPCRM	GAATACTTTTAAAGAAACCCTCTTTTCA ACCTTTCTAATTTGCTGTTTGG	408

<sup>a</sup> P, reverse primer; M, forward primer.

room temperature. Reverse transcription was performed with both [ $\alpha$ -<sup>32</sup>P]dCTP and [ $\alpha$ -<sup>32</sup>P]TTP (NEN) replacing the corresponding nucleotides in the reaction buffer (29). After unincorporated nucleotides were removed by gel filtration 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 the hybridization buffer.

**DNA macroarray construction, hybridization, and data analysis.** DNA regions of *M. pneumoniae* 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 a Bio-Dot SF micro-filtration 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 one with *recA* DNA, which served as the control for hybridization stringency. <sup>32</sup>P-labeled cDNAs generated using total mRNA from heat-shocked and control *M. pneumoniae* genes served as probes. Blots were prehybridized and hybridized as suggested by the manufacturer (Bio-Rad Laboratories). 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 exposure cassettes with storage phosphor screens.

Individual screens were scanned using Typhoon 9400 (Molecular Dynamics), and resulting image files were assessed by determining pixel density for each band after background subtraction (ImageQuant 5.2; Molecular Dynamics). Numeric files were exported into a Microsoft Excel spreadsheet for analysis. To determine the transcript amounts for different experimental conditions, we expressed them as percentages of the *nox* or *eno* housekeeping gene. We further calculated average intensities for each gene under applied experimental conditions. To evaluate changes in specific transcripts, we determined the ratio of the corresponding average percent intensities of each gene-specific slot. These ratios represent relative transcript levels (*n*-fold) of each gene under the two experimental conditions. Triplicate values from three separate experiments were averaged, and comparative data between experiments differed by less than 10%.

**ATPase activity of rClpB.** ClpB-ATPase activity was measured by estimating the concentration of inorganic phosphate produced from ATP by using the malachite green colorimetric assay (15, 21). Reactions were performed at 37°C for 15 min in assay buffer (100 mM Tris-HCl [pH 8.0], 10 mM MgCl<sub>2</sub>, 5 mM ATP, 1 mM EDTA, 1 mM dithiothreitol) containing different concentrations of rClpB (0.1 μM to 10 μM). ATPase activities were also determined in the presence of 0.25 mg/ml  $\alpha$ -casein (Sigma) or 0.04 mg/ml poly-L-lysine (Sigma).

**Chaperone activity of ClpB.** ClpB chaperone refolding activity was measured as reported previously (22). In brief, firefly luciferase (Promega) was diluted 300-fold into PBS buffer containing 1 mg/ml bovine serum albumin and incubated at 45°C for 15 min. After heat treatment, luciferase samples were cooled to room temperature and diluted 20-fold into renaturation buffer (30 mM

HEPES-KOH [pH 7.6], 120 mM KCl, 10 mM MgCl<sub>2</sub>, 5 mM ATP, 1 mM EDTA, 1 mM dithiothreitol, and 0.1 mg/ml bovine serum albumin). The reaction was initiated by addition of rClpB (0.1 to 10 μM), *Mycoplasma genitalium* rDnaK (1 to 5 μM) or rClpB (0.1 to 10 μM), and the KJE system (DnaK [0.5 to 5 μM], DnaJ [0.1 to 1 μM], and GrpE [0.05 to 0.5 μM]). DnaK, DnaJ, and GrpE were purchased from StressGen Biotechnologies (Victoria, BC, Canada). Refolded luciferase activities in refolding solution were determined using a Turner Bio-systems luminometer.

**Tn mutagenesis of *M. pneumoniae*.** We and others have found Tn4001 to be useful in studies with *M. pneumoniae* and *M. genitalium* (14, 32). To avoid multiple insertions, we used transposon (Tn) pMT85-miniTn4001, which has a *colE1* plasmid origin of replication and a selectable gentamicin/kanamycin marker within Tn4001 to allow for direct rescue of chromosomal sequences adjacent to the insertion site (41). pMT85-miniTn4001 was electroporated into competent mycoplasmas as reported previously (32). After growth in selective medium (two passages), the complete mixture of amplified Tn mutants was collected, and *M. pneumoniae* cell suspensions were passed through 25-gauge needles several times and filtered through membrane filter units with 0.45-μm pores (Millipore) to remove aggregates. Mycoplasma cells were diluted in SP-4 medium and plated on SP-4 agar plates containing 80 μg/ml gentamicin. Chromosomal DNAs from individual Tn-integrated colonies were HindIII digested, self-ligated, and transformed in *E. coli* Top10. Plasmid DNAs were isolated and subjected to DNA sequencing in order to identify the Tn integration site in the chromosomal DNA of *M. pneumoniae*. Sequencing across mini-Tn4001 Tn-plasmid junctions was facilitated by Tn4001 IS256 outer repeat sequences 5'-GAT AAAGTCCGTATAATTGTGTA AAAAG-3' (forward) and 5'-CTTTACACAA TTATACGGACTTTATC-3' (reverse), which anneal at the 5' and 3' ends of Tn4001, respectively. For each mini-Tn4001 insertion, a DNA sequence of 600 to 800 bp in each direction from the site of insertion was determined. To confirm the integration of Tn4001 within the *clpB* open reading frame, oligonucleotide primers clpB-F1 and clpB-R1 were used for PCR amplification, and chromosomal DNAs from S1 and the S1ΔClpB mutant were used as template DNAs.

**Immunoblot analysis.** Whole-cell lysates were prepared from *M. pneumoniae* S1 and the S1ΔClpB mutant grown to mid-log phase in SP-4 medium at 37°C and 43°C as described above. Equal amounts of whole-cell lysates from each strain were separated on 4 to 12% Nu-PAGE gel (Invitrogen) and transferred to nitrocellulose membranes. Immunoblots were then performed with a 1:2,000 dilution of mouse polyclonal anti-ClpB antibody, polyclonal anti-*M. genitalium* DnaK antibody (W. Zhang and J. B. Baseman, unpublished results), rabbit polyclonal anti-LonA antibody (a gift from Richard Herrmann), and mouse monoclonal antibodies raised against P1 and the His tag (7). Sera from *M. pneumoniae*-infected BALB/c mice, uninfected control mice, *M. pneumoniae*-infected patients, and uninfected human controls were used to detect the immune response against ClpB.

TABLE 2. *M. pneumoniae* genes and their oligonucleotide primers used for RT-PCR quantification of transcripts by DNA macroarray analysis<sup>a</sup>

Gene	Primer name	Primer sequence	Amplicon length (bp)	T <sub>a</sub> (°C)
<i>dnaJ1</i>	MPN002P MPN002M	AGCGGAGTATGACGCTATGCTGCGC TGTTGAGTTCGTACCACATTTGTCCAGGG	496	60
<i>dnaJ2</i>	MPN021P MPN021M	TTGCCCTATGATTTAGAGATTGCG ATTGGCTACTACATTATGGTCAACC	477	55
<i>dnaJ3</i>	MPN119P MPN119M	AATAAGGCACCGGATGCGGC TAACCGTACTCAGGATTACCAATCATTGC	589	57
<i>grpE</i>	MPN120P MPN120M	TTACCGAAATTCTCAGTTCCATCCG CGATACCTTACCACCGTATTAGCCG	553	55
<i>hrcA</i>	MPN124P MPN124M	ACCCGACAAGCCCAAATTCTCAAGG GCGATCATTAAGATCCTGACACATACC	536	55
<i>tig</i>	MPN331P MPN331M	TTGATGCCATCTTCCAGCCG TGATCTCTAACTGCTTAATTGCTTTGAGC	539	55
<i>lon</i>	MPN332P MPN332M	AGCGTTAGATAAGTTGTTGGAACGG TTTAATGTGCTCTGGATATGGG	495	55
<i>sigA</i>	MPN352P MPN352M	AAGGAGAGTGATGTACCAAAGAAGCGTCG GGAAGAATCGAGCGAACCTAGAAAGAAACG	455	57
<i>nox</i>	MPN394P MPN394M	TTTGGGTTGTGGAATTGCCTGG GGTTAATGCCCGCTTCTTCATGG	556	57
<i>dnaK</i>	MPN434P MPN434M	CTACCGCCGCTGCATTGGCTTATGG AAACATCCGAAATTGGGTTGCGAGTACG	446	60
<i>clpB</i>	MPN531P MPN531M	AATTTATGAACTTTCGTTGTCTGG CATTTCTACAGCAGCTACCAAGGC	426	55
<i>groEL</i>	MPN573P MPN573M	GCTGTATCTACCAATGATATTGCTGG TTCCATGCTACCTTCTAGTAACGG	492	55
<i>groES</i>	MPN574P MPN574M	AGTGCCCAAGGGAAGTGTGCG AAAGTAGATCGTGTACCCGATGCC	103	55
<i>eno</i>	MPN606P MPN606M	AACTACGCGATGGTGTATCCC GCTGCCTTAATTGCTTCGACC	553	55
<i>ftsH</i>	MPN671P MPN671M	GCCTACTACCAATTATTATCTTTGTCA TTTAAGGTTTGTTCGACCACCG	523	55

<sup>a</sup> Reverse primers (M) were used to synthesize cDNA for hybridization. T<sub>a</sub>, annealing temperature.

## RESULTS

**Primary sequence and conserved domains of *M. pneumoniae* ClpB (MPN531).** ClpB is the only member of the Clp family in *M. pneumoniae*, and we analyzed its primary sequence for the presence of specific motifs and essential amino acids involved in nucleotide and substrate binding as described for *E. coli* ClpB. An obvious difference is that *M. pneumoniae* ClpB comprises a 715-amino-acid sequence (Fig. 1A), compared to 857 amino acids for *E. coli* ClpB (16, 34). In *E. coli*, ClpB is synthesized in two forms, a 93-kDa (ClpB<sub>EC</sub>) and a 79-kDa (ClpB'<sub>EC</sub>) peptide (34), with ClpB'<sub>EC</sub> translation commencing at 149 codons downstream of the ClpB<sub>EC</sub> start site. Alignment of amino acid sequences showed that the translational start site of *M. pneumoniae* ClpB coincides with amino acid 143 of ClpB<sub>EC</sub>, which is near the start codon of *E. coli* ClpB'. As

predicted by Scan Prosite, *M. pneumoniae* ClpB contains two well-conserved AAA domain regions (also designated ATP-1 and ATP-2), comprising 234 and 192 amino acids that share almost 80% similar and 50% identical amino acids compared to the most distantly related prokaryotes. Like *E. coli*, both ATP domains of *M. pneumoniae* ClpB possess a core region that forms the nucleotide binding pocket, containing Walker A and Walker B motifs (Fig. 1A) (37). Within the ATP-1 domain, 34 amino acid residues downstream of Walker A and 16 amino acids upstream of Walker B are separated by a central pore (CP) that contributes to protein substrate binding, with the essential tyrosine residues at position 105. A similar CP is observed within the ATP-2 domain at 37 residues downstream of Walker A and 13 residues upstream of Walker B (33). In addition, *M. pneumoniae* ClpB possesses a C-terminal region

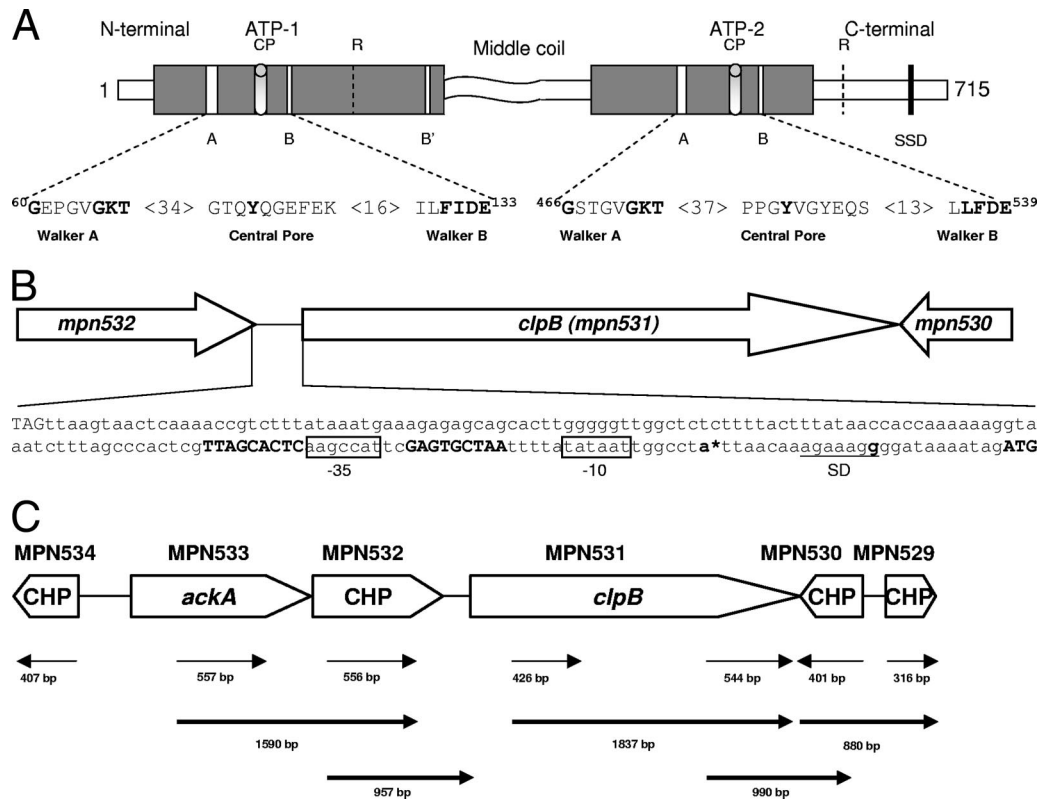


FIG. 1. *M. pneumoniae* ClpB organization and transcription. (A) Proposed domain organization of *M. pneumoniae* ClpB and conserved motifs involved in ATP binding and ATP hydrolysis. *M. pneumoniae* ClpB retains only a shortened N-terminal domain, two nucleotide binding domains (ATP-1, positions 115 to 265; and ATP-2, positions 392 to 583) which are separated by the middle region, and a C-terminal region. Both nucleotide binding domains contain the Walker A (<sup>60</sup>GX<sub>4</sub>GKT<sup>69</sup> and <sup>466</sup>GX<sub>4</sub>GKT<sup>473</sup>) and Walker B (<sup>130</sup>Hy<sub>2</sub>DE<sup>133</sup> and <sup>536</sup>Hy<sub>2</sub>DE<sup>539</sup>) motifs, where X<sub>n</sub> represents *n* amino acids of any kind and Hy<sub>n</sub> represents *n* hydrophobic amino acids. ATP-1 has one additional nucleotide binding motif, termed Walker B' (<sup>249</sup>Hy<sub>2</sub>DE<sup>252</sup>). CPs (positions 102 to 111 and 511 to 519) contribute to protein substrate binding. Arg (R) residues (positions 187 and 609) are proposed to serve as Arg fingers, contacting ATP bound to an adjacent subunit. The sensor and substrate domain (SSD) (<sup>666</sup>GAR<sup>668</sup>) potentially senses the nucleotide status of ATP-2. The middle coiled-coil region is predicted to be involved in protein-protein interactions. (B) Promoter region of *clpB*. The bold ATG (far right) indicates the putative translational start site of *clpB* (*mpn531*). Inverted repeats on each side of the 9-nucleotide spacer sequence of the CIRCE element are shown in bold. The promoter region contains a Pribnow box (−10 site), which is an exact match for the consensus sequence (TATAAT) for the housekeeping sigma factor ( $\sigma$ 70). A putative −35 site (boxed) was also identified upstream of the Pribnow box. The stop codon of *mpn532* (TAG, far left) is 175 nucleotides upstream of the start codon of *clpB*. An asterisk designates the transcriptional start site (bold letter) as determined by PE. A Shine-Dalgarno (SD) sequence (underlined) is located downstream of the putative transcriptional start site. (C) Transcriptional analysis of genes surrounding *clpB* of *M. pneumoniae*. RT-PCR was performed on total RNA isolated from *M. pneumoniae* cells as described in Materials and Methods. A schematic of *clpB* and its surrounding genes is presented. Conserved hypothetical proteins (CHP) are indicated. Thin arrow lines represent regions that were amplified to confirm transcription of individual genes. Bold arrow lines represent RT-PCR products to indicate cotranscription of corresponding genes. Numbers represent the sizes of RT-PCR-amplified products.

and a highly variable middle sequence as reported for other bacteria (11) but differs by exhibiting a shortened N terminus, as revealed by alignment with ClpB<sub>EC</sub>. The middle coiled-coil sequence is predicted to be involved in protein-protein interactions, and recent studies confirm its role in DnaK-dependent shuffling of aggregated proteins (13, 24). Besides sensing the nucleotide status of the core ATPase domain, the C-terminal domain of the ATP-2 domain has been proposed to mediate substrate interaction with the sensor and substrate discrimination domain (26). The essential Lys residues of the Walker A motif (K-68 and K-472), the Glu residues of the Walker B motif (E-133 and E-539), and the Arg residue of the GAR motif (<sup>666</sup>GAR<sup>668</sup> in ClpB) are conserved in *M. pneumoniae* ClpB (Fig. 1A). However, the conserved Ser residue at amino acid 407, a putative phosphorylation site for casein kinase II with

the consensus pattern SKPE in ClpB<sub>EC</sub>, is not detected in *M. pneumoniae* ClpB.

Analysis of the *clpB* promoter region of *M. pneumoniae* clinical strain S1 revealed the presence of conserved CIRCE, suggesting that *clpB* is transcribed from its own promoter. Promoter mapping of S1 revealed a single transcriptional start site 17 nucleotides downstream of the CIRCE element (Fig. 1B), whereas *clpB* transcriptional studies with the *M. pneumoniae* M129 type strain revealed the presence of an additional transcriptional start site (38). *clpB* is preceded by genes encoding an acetate kinase (*ackA*, *mpn533*) and a hypothetical protein (*mpn532*) (Fig. 1B and C). Downstream of *clpB* are genes encoding a conserved hypothetical protein (*mpn530*) and a histone-like protein (*mpn529*). Genes *mpn529* to *mpn533* are transcribed in the same orientation, except for

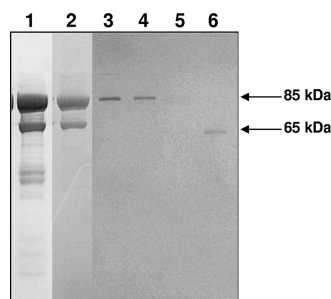


FIG. 2. Purification of ClpB of *M. pneumoniae*. rClpB protein was purified through different columns as described in Materials and Methods, separated on SDS-PAGE gel, and Coomassie blue stained. Protein fractions were from a Ni-nitrilotriacetic acid column (lane 1), a Mono Q anion exchange column (lane 2), and a Superdex 200 gel filtration column eluted using a linear NaCl gradient (lanes 3 to 6).

mpn530, which is transcribed on the complementary strand. To analyze the organization and expression of these genes in strain S1, we performed RT-PCR analysis using primers based on the available *M. pneumoniae* M129 genomic sequences (Table 1). For all reactions, genomic DNA isolated from strain S1 was used to ensure that all targeted regions were present and could be amplified with the designed primers. Evidence indicated that all genes (mpn529 to mpn534) are transcribed in the predicted orientations. Further, bicistronic transcriptional studies showed that *ackA* and mpn532 (Fig. 1C) were cotranscribed as predicted from sequence analysis (short intergenic region: 7 nucleotides). Even in the presence of the CIRCE element, RT-PCR demonstrated cotranscription of *clpB* with mpn532. Additionally, mpn530 was found to be transcribed in both orientations, and a possible cotranscription of *clpB* and mpn529 was demonstrated.

**Cloning, expression, and purification of the *M. pneumoniae* ClpB protein.** To obtain sufficient amounts of *M. pneumoniae* ClpB for functional characterization and generation of anti-serum, we utilized the His tag expression system. ClpB featured a single UGA-encoded tryptophan at amino acid position 322, which corresponds to the TGA codon at nucleotide positions 964 to 966. The UGA was corrected, permitting *clpB* to be expressed as a 715-amino-acid recombinant protein fused at the NH<sub>2</sub>-terminal end with the His<sub>10</sub> tag. The purified rClpB protein preparation was analyzed on SDS-PAGE gel, revealing a predicted 85-kDa protein; however, a 65-kDa protein and some low-molecular-weight peptides were also observed (Fig. 2, lane 1). The 85- and 65-kDa proteins were further separated by Mono Q anion exchange (Fig. 2, lane 2) and Superdex 200 gel filtration columns (Fig. 2, lanes 3 to 6) and analyzed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis and immunoblotting. MALDI-TOF analysis and immunoblots with anti-mouse rClpB and His-tagged antibodies confirmed that the 85- and 65-kDa proteins were indeed ClpB, and both employed similar translational start points (data not shown). However, MALDI-TOF analysis did not identify the C-terminal region (amino acid residues 600 to 715) in the 65-kDa ClpB peptide.

**Transcriptional and translational analysis of the *M. pneumoniae* ClpB gene at an elevated temperature.** In order

to further clarify ClpB properties, we assessed the effects of a temperature shift from 37°C to 43°C on the expression of ClpB at the transcriptional and translational levels and compared these effects with those for other heat shock and specific housekeeping genes (Table 2). Using the *clpB* probe, we detected *clpB*-specific mRNA in cells grown at 37°C, with a very strong signal (~22-fold increase) in cells grown for 2 h at 43°C; *lonA* transcription was equally increased (Table 3). Transcription of other CIRCE-regulated genes, such as *dnaK* and *dnaJ2*, increased much less dramatically (Table 3). Except for *dnaJ1* (~7-fold increase), all other non-CIRCE regulated hsp genes (*groEL*, *groES*, and *hrcA*) demonstrated various responses, ranging from three- to sixfold, under the same conditions (six-, three-, and three-fold increases, respectively) (Table 3). Parallel analysis of [<sup>35</sup>S]methionine-cysteine biosynthetically labeled proteins showed enhanced syntheses of three heat shock proteins (Fig. 3A). Densitometer analysis revealed 3.3-, 4.5-, and 2.1-fold increases in the syntheses of the 65-, 82-, and 94-kDa proteins, respectively. However, these proteins were not visible in parallel Coomassie blue-stained gels (Fig. 3B).

We used hsp-specific antibodies (see Materials and Methods) to further identify the upregulated *M. pneumoniae* protein patterns. The abundant 82-kDa *M. pneumoniae* heat shock protein was immunoreactive with antibodies raised against purified rClpB (Fig. 3C). The prominent 65-kDa *M. pneumoniae* protein was immunoreactive with antibodies against DnaK protein (Fig. 3C). The 94-kDa *M. pneumoniae* protein was also identified by immunoblot analysis using LonA protease-specific antibodies (data not shown).

**ATPase activities of ClpB.** Amino acid sequence analysis predicted the presence of two well-conserved ATP domains in *M. pneumoniae* ClpB (Fig. 1A). To corroborate their role in ATPase activities, we analyzed rClpB in the absence or presence of the artificial substrates  $\alpha$ -casein and poly-lysine, which are known to stimulate hydrolysis of ATP by ClpB (22, 40). *M. pneumoniae* rClpB exhibited ATPase activity over a range of

TABLE 3. Transcriptional response of *M. pneumoniae* during heat shock<sup>a</sup>

Gene	Ratio (fold)
<i>dnaJ1</i> .....	6.83
<i>dnaJ2</i> .....	1.68
<i>dnaJ3</i> .....	2.21
<i>gpeE</i> .....	2.11
<i>hrcA</i> .....	2.82
<i>tig</i> .....	1.12
<i>lon</i> .....	21.71
<i>sigA</i> .....	2.39
<i>nox</i> .....	1.00
<i>dnaK</i> .....	5.96
<i>clpB</i> .....	22.28
<i>groEL</i> .....	5.54
<i>groES</i> .....	2.82
<i>eno</i> .....	1.44
<i>ftsH</i> .....	1.97

<sup>a</sup> *M. pneumoniae* S1 cells grown in DMEM were placed at 43°C for 2 h. The housekeeping gene *nox* was used as a normalizer. Ratios represent changes in transcript amounts (at 43°C/37°C). Intensities were determined by measuring spots in individual circles. Each value represents the average for three separate experiments. Similar transcriptional responses were observed when *eno* was used as a normalizer.

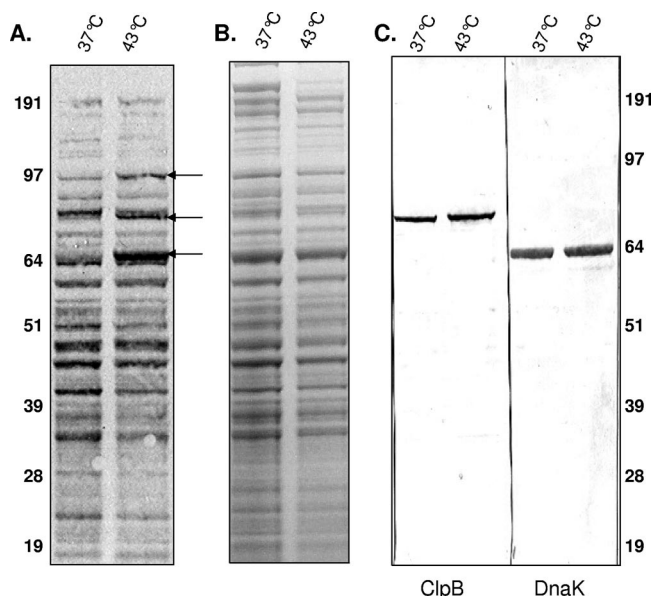


FIG. 3. Translational response of *M. pneumoniae* during heat shock. [ $^{35}\text{S}$ ]methionine-cysteine was added to *M. pneumoniae* S1 cells grown in DMEM at 37°C and 43°C, and levels of radiolabeled proteins were analyzed after heat shock as described in Materials and Methods. *M. pneumoniae* proteins were separated on 4 to 12% Nu-PAGE gel and transferred to nitrocellulose membranes and exposed to X-ray film (A), Coomassie blue stained (B), and transferred to nitrocellulose membranes and immunoblotted using anti-ClpB and anti-DnaK antisera (C). Arrows indicate the upregulated heat shock proteins.

concentrations. However, at low concentrations, there were small differences in ATPase activity in the absence and presence of casein or lysine, while at a 10  $\mu\text{M}$  concentration of rClpB, we observed a 20% increase in ATPase activity in the presence of casein or lysine (Fig. 4). However, these increases were modest compared to the 5- to 20-fold stimulations observed with ClpB<sub>EC</sub> under similar conditions (22, 40).

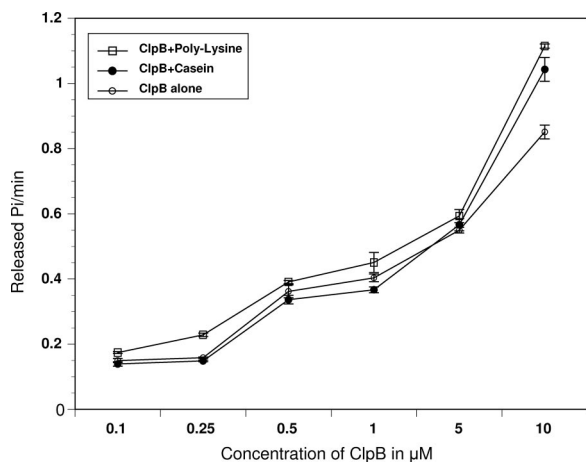


FIG. 4. ATPase activities of ClpB. ATPase activities of ClpB were measured at different ClpB protein concentrations as described in Materials and Methods in the absence or presence of  $\alpha$ -casein (0.25 mg/ml) or poly-L-lysine (0.04 mg/ml).

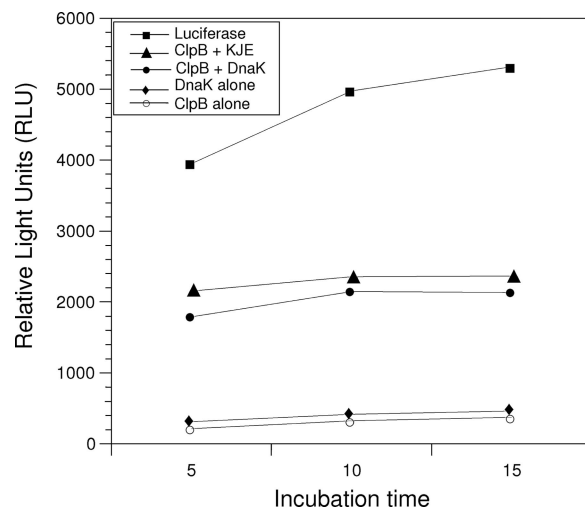


FIG. 5. Chaperone activities of ClpB. The activities of firefly luciferase during its chaperone-assisted reactivation were monitored using luminescence (see Materials and Methods). Activities of rClpB alone; rClpB with DnaK; rClpB with DnaK, DnaJ, and GrpE (KJE); DnaK alone; and unheated luciferase alone are compared. The protein concentrations used were 33 nM luciferase, 2.0  $\mu\text{M}$  ClpB, 2.0  $\mu\text{M}$  *M. genitalium* rDnaK, 1.0  $\mu\text{M}$  *E. coli* DnaK, 1.0  $\mu\text{M}$  DnaJ, and 1.0  $\mu\text{M}$  GrpE.

**Chaperone activities of ClpB.** Since ClpB has the biologically important capacity to rescue proteins from an aggregated state by mediating the disaggregation of stress-damaged proteins, we tested the chaperone activity of ClpB in vitro by following ClpB/DnaK-dependent reactivation of heat-aggregated firefly luciferase (Fig. 5). rClpB exhibited significant disaggregation activity in the presence of KJE as shown by the reactivated luciferase activity. Surprisingly, ClpB also exhibited a significant disaggregation activity in the presence of mycoplasma rDnaK alone. However, mycoplasma ClpB or DnaK alone did not enhance the refolding of luciferase under similar experimental conditions (Fig. 5). KJE alone gave the same results as DnaK alone.

#### ClpB mutation impairs cell growth and alters morphology.

To explore the role of ClpB in *M. pneumoniae*, we performed Tn mutagenesis. Chromosomal DNAs from individual mutants were isolated and plasmids generated. Sequencing of one of the plasmids revealed that the Tn was integrated in direct orientation within the coding region of *clpB* (76 nucleotides downstream of the start codon). By using primers specific for *clpB*, we reconfirmed mini-Tn4001 integration (Fig. 6A) within the mutant S1 $\Delta$ ClpB. The ClpB mutant colony sizes were similar to those of the wild type on SP-4 agar plates, but in liquid culture, the ClpB mutant grew slowly and displayed obvious differences in cell morphology over time (Fig. 6B). Interestingly, subsequent passage of the S1 $\Delta$ ClpB mutant in broth resulted in no growth. However, whole-cell lysates of the first passage of liquid-grown cultures were analyzed for the presence of ClpB by immunoblotting with mouse polyclonal antibodies raised against rClpB. As expected, the ClpB mutant lacked ClpB protein whereas whole-cell lysates of the wild-type S1 strain recognized a single band at 82 kDa. For comparative purposes, we analyzed the ClpB mutant and the wild-type S1

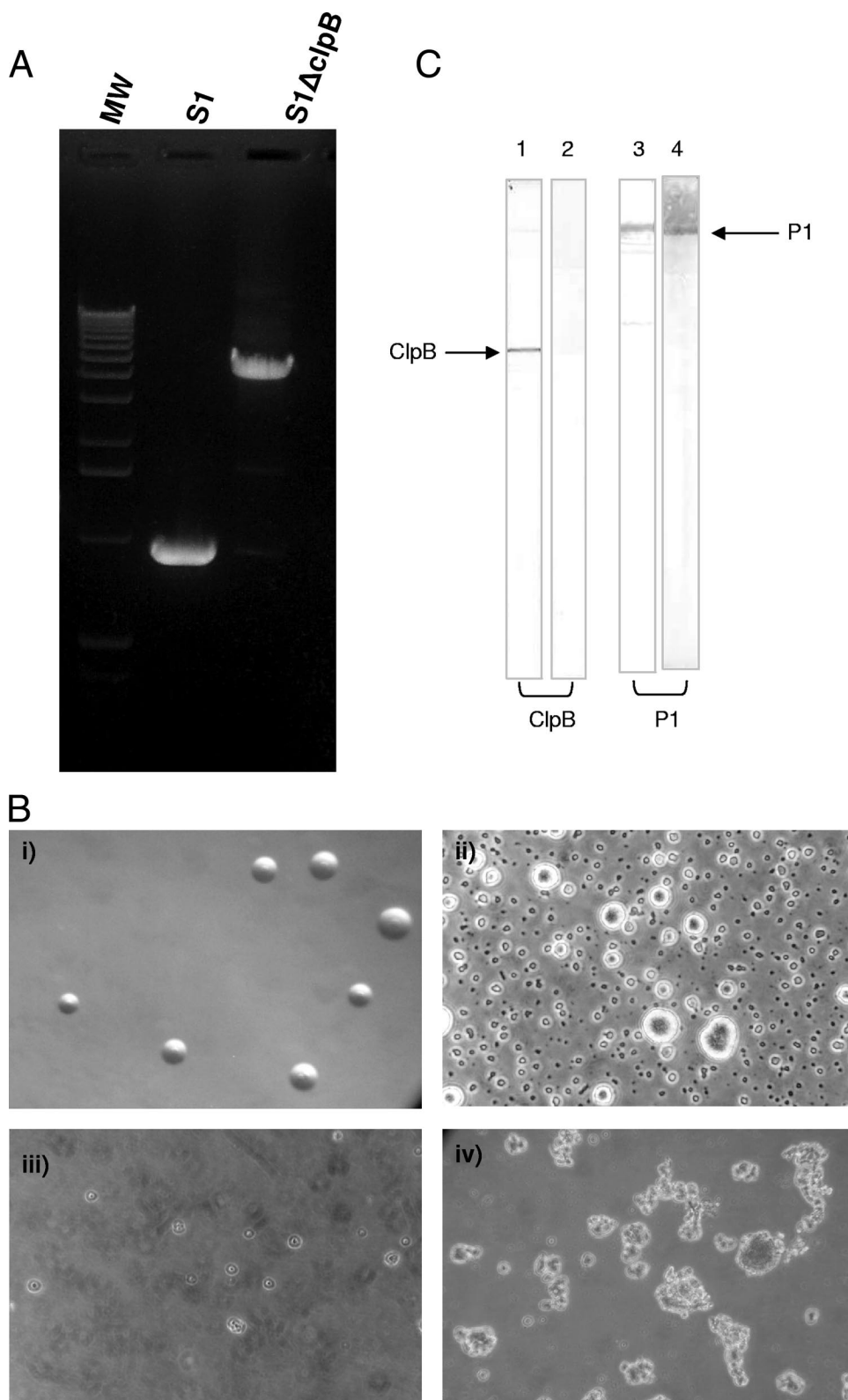


FIG. 6. Construction of ClpB-null mutant *M. pneumoniae* S1ΔClpB. (A) PCR amplification of *clpB* in ClpB mutant and wild-type *M. pneumoniae* S1. After sequence confirmation of integration of mini-Tn4001 in *clpB*, the integrated region was amplified by region-specific mpn531 primers. Due to integration of Tn, an extra DNA fragment, 3 kb larger than that in wild-type *M. pneumoniae* S1, was amplified in mutant S1ΔClpB. MW, molecular weight. (B) Morphology of ClpB mutant of *M. pneumoniae* S1 strain. (i) ClpB mutant on SP-4 agar plate (similar to wild-type result [not shown]). (ii to iv) Morphologies of wild-type and ClpB mutant cells grown in SP-4 broth: (ii) *M. pneumoniae* S1 after 48 h (showing well-established adhering cells/colonies), (iii) ClpB mutant after 1 week (showing mostly floating cells/colonies), and (iv) ClpB mutant after 3 weeks (showing large, floating clumps). (C) ClpB expression in *M. pneumoniae*. Total cell lysates from wild-type *M. pneumoniae* strain S1 and the ClpB mutant (S1ΔClpB) were separated on SDS-PAGE gel, transferred to a nitrocellulose membrane, and treated with anti-ClpB and anti-P1 adhesin antibodies. Lanes: 1, S1 anti-ClpB antibodies; 2, S1ΔClpB anti-ClpB antibodies; 3, S1 anti-P1 antibodies; 4, S1ΔClpB anti-P1 antibodies.



strain for the synthesis of adhesin protein P1 (MPN141) and detected P1 in both by immunoblotting (Fig. 6C).

**Immune response against ClpB during *M. pneumoniae* infection.** Analysis of *M. pneumoniae*-infected mice showed a strong immune response against rClpB at 35 days postinfection that was undetected in control mice. Similarly, patients infected with *M. pneumoniae* demonstrated a strong immune response against rClpB in contrast to uninfected controls.

## DISCUSSION

The heat shock response in mycoplasmas illustrates diversity in prokaryotic gene regulation, highlighted in *M. pneumoniae* by the presence of the CIRCE element and the absence of alternative sigma factors. Since relatively little is known about the mechanisms underlying stress response in *Mollicutes*, we initiated studies to characterize transcriptional and translational regulation in *M. pneumoniae*, focusing on the heat shock protein ClpB. We chose ClpB because of its fundamental importance in regulation and the fact that it is the only representative of the Clp AAA protein superfamily in *M. pneumoniae* (Fig. 1A). Further, the shortened *M. pneumoniae* ClpB variable N-terminal region differs from those of other bacterial ClpB proteins (8, 12, 17, 34).

Unlike *M. pneumoniae* reference strain M129, which uses two transcriptional start sites to express the full-length ClpB protein (38), clinical strain S1 used only one transcriptional start site, indicating the possibility of expression differences among clinical isolates (Fig. 1B). Nucleotide sequence analysis of the S1-ClpB open reading frame and its upstream promoter did not reveal any sequence variation from M129. Thus, the factors that determine the transcriptional start site of this gene remain unidentified.

We observed cotranscription of four genes of disparate functions (mpn533, mpn532, mpn530, and mpn529), along with *clpB* (Fig. 1C). Surprisingly, we detected transcription of *clpB* with the antisense strand of mpn530 (Fig. 1C). In addition, RT-PCR analysis showed a relatively small amount of transcription from the antisense strands between mpn529-530 and mpn531-530, suggesting that these genes are transcribed in single long polycistronic mRNAs. Cotranscription of functionally unrelated genes, along with antisense strands of downstream genes, has been reported for *M. genitalium* and *M. pneumoniae*, possibly due to the lack of transcriptional terminators (5).

The *M. pneumoniae* rClpB protein is expressed in two forms (85-kDa and 65-kDa proteins) in *E. coli* (Fig. 2). Monoclonal His-tagged antibodies recognized the NH<sub>2</sub>-terminal regions in both rClpB forms, indicating that the NH<sub>2</sub> terminus is not processed in *E. coli*. However, MALDI-TOF analysis revealed C-terminal deletion of *M. pneumoniae* rClpB, possibly by *E. coli* proteases.

In *M. pneumoniae*, the conserved CIRCE element was observed only with heat-shock genes *dnaJ2* (mpn021), *lonA* (mpn332), *dnaK* (mpn434), and *clpB* (mpn531) and not with *groESL* (mpn573 and mpn574). Even though a remnant of the CIRCE element has been observed upstream of *groESL*, it did not favor the upregulation of these products during heat shock (Fig. 3A). The absence of CIRCE-controlled GroE raises the question as to how the HrcA (MPN124) repressor is transiently

inactivated to induce *clpB*, *dnaJ*, *dnaK*, and *lonA* transcription in *M. pneumoniae*. For example, earlier studies with *Bacillus* and *Bradyrhizobium* species suggest that the GroE-CIRCE-HrcA complex plays an important role in monitoring protein folding (23, 25). On the other hand, a nonpolar  $\Delta$ *dnaK* mutant of *Lactococcus lactis* exhibited elevated levels of hsp, raising the possibility that DnaK-DnaJ chaperones could be involved in the functionality of the HrcA repressor (20). In *M. pneumoniae*, only the CIRCE-controlled heat shock proteins ClpB, DnaK, and LonA exhibited upregulation during heat shock (Fig. 3A). Although several other heat shock genes were increased at transcriptional levels upon heat shock (Table 3), the encoded proteins were not detected during pulse-chase experiments (Fig. 3A). Therefore, in *M. pneumoniae*, it appears that DnaK and ClpB chaperones assist in protein folding and renaturation and that the affinities of these chaperone systems for the respective HrcA proteins may determine their relative levels of expression and functional effectiveness in *M. pneumoniae*. This possibility is supported by in vitro chaperone activities of DnaK and ClpB on the refolding of luciferase (Fig. 5). The increased heat shock-driven synthesis of the CIRCE-controlled LonA protease, along with ClpB and DnaK, suggests that degradation of dissociated proteins is also an important function of these specific heat shock proteins (Fig. 3A). In addition to translational upregulation during heat shock, the display of a single ClpB band in immunoblots of whole-cell lysates of wild-type *M. pneumoniae* confirmed that *M. pneumoniae* ClpB was translationally processed in only one form (Fig. 3C and 6C), possibly due to the absence of the extended variable N-terminal region observed in bacterial ClpBs (8, 12, 17, 34).

We also observed that heat shock genes not under the control of the CIRCE element were induced transcriptionally (Table 3). However, we did not detect increases in protein levels for these upregulated genes (Fig. 3A), suggesting that a complex control network of interactions exists between specific heat shock genes and chaperones that regulate stress response.

Casein stimulates the ATPase activity of ClpB by binding to the N domain, which is abbreviated in *M. pneumoniae*. Even at high concentrations of ClpB, casein and lysine had limited effects on ATPase activity (Fig. 4), which is consistent with the role that the full-length N terminus of ClpB plays in other bacteria (1, 4), in contrast to the role of the shortened ClpB protein of *M. pneumoniae*. However, the chaperone function of *M. pneumoniae* ClpB appears intact, as it restores luciferase activity in the presence of KJE, which agrees with the results for ClpB $\Delta$ N of *Thermus thermophilus* (4) and contrasts with the result for ClpB $\Delta$ N of *E. coli* (1).

Although our ability to obtain a *clpB*-null mutant suggests that ClpB is not essential for the initial growth of *M. pneumoniae* in SP-4 agar and broth media, we observed that, upon clone selection, mutant cells lacking ClpB were fragile and aggregated during next-passage growth in SP-4 liquid culture. Like *M. pneumoniae*, cyanobacterium mutants lacking ClpB differ in growth from wild-type cells (31). Therefore, the absence of ClpB alone in *M. pneumoniae* triggers impaired growth, with subsequent failure to passage sequentially, suggesting that yet-uncharacterized critical regulatory circuits are

perturbed and metabolic pathways altered by the absence of ClpB. This is consistent with the lack in *M. pneumoniae* of other Clp-related proteins that have diverse functions often associated with stress adaptation in bacteria. Based on our data, ClpB appears to be a key chaperone involved in resolubilization of protein aggregates in *M. pneumoniae*. It is possible that the slow and eventual aborted growth of the ClpB mutant may arise from its incompetence to adequately compensate for the loss of ClpB and to refold proteins for normal growth-related functions. Interestingly, ClpB elicits immune responsiveness in experimentally infected mice and *M. pneumoniae*-infected patients, indicating that it is both expressed and immunogenic during infection. Clearly, understanding how each heat shock protein is regulated under a range of stress conditions will contribute to an appreciation of mycoplasma survival and pathogenicity during acute and chronic stages of disease.

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