Global Transcriptional Analysis of *clpP* Mutations of Type 2 *Streptococcus pneumoniae* and Their Effects on Physiology and Virulence

Gregory T. Robertson, Wai-Leung Ng, Joseph Foley, Raymond Gilmour, and Malcolm E. Winkler*

Infectious Diseases Research, Lilly Research Laboratories, Indianapolis, Indiana 46285

Streptococcus pneumoniae **is an important human pathogen that contains single copies of genes encoding the ClpP and FtsH ATP-dependent proteases but lacks the Lon and HslV proteases. We constructed and characterized the phenotypes of *clpP*, *clpC*, and *clpX* deletion replacement mutants, which lack the ClpP protease subunit or the putative ClpC or ClpX ATPase specificity factor. A Δ*clpP* mutant, but not a Δ*clpC* or Δ*clpX* mutant, of the virulent D39 type 2 strain of *S. pneumoniae* grew poorly at 30°C and failed to grow at 40°C. Despite this temperature sensitivity, transcription of the heat shock regulon determined by microarray analysis was induced in a Δ*clpP* mutant, which was also more sensitive to oxidative stress by H_2O_2 and to puromycin than its *clpP*⁺ parent strain. A Δ*clpP* mutant, but not a Δ*clpC* mutant, was strongly attenuated for virulence in the murine lung and sepsis infection models. All of these phenotypes were complemented in a Δ*clpP*/*clpP*⁺ merodiploid strain. Consistent with these complementation patterns, *clpP* was found to be in a monocistronic operon, whose transcription was induced about fivefold by heat shock in *S. pneumoniae* as determined by Northern and real-time reverse transcription-PCR analyses. Besides *clpP*, transcription of *clpC*, *clpE*, and *clpL*, but not *clpX* or *ftsH*, was induced by heat shock or entry into late exponential growth phase. Microarray analysis of Δ*clpP* mutants showed a limited change in transcription pattern (≈80 genes) consistent with these phenotypes, including repression of genes involved in oxidative stress, metal ion transport, and virulence. In addition, transcription of the early and late competence regulon was induced in the Δ*clpP* mutant, and competence gene expression and DNA uptake seemed to be constitutively induced throughout growth. Together, these results indicate that ClpP-mediated proteolysis plays a complex and central role in numerous pneumococcal stress responses, development of competence, and virulence.**

Four distinct classes of ATP-dependent proteases have been found in bacteria, including the multimeric Clp (ClpAP, ClpXP, or ClpCP) and HslUV (ClpYQ) proteases and the single-chain AAA (FtsH) and Lon family proteases (Lon) (19). The Clp proteases (caseinolytic proteases) were first identified in *Escherichia coli* and consist of an ATPase specificity factor (ClpA or ClpX in *E. coli* and ClpX or ClpC in *Bacillus subtilis*) and a proteolytic domain (ClpP) that contains a consensus serine protease active site (17, 19, 36). This multimeric enzyme complex assembles into a structure that is remarkably similar to that of the eukaryotic proteosome (7). In *E. coli*, ClpP-mediated proteolysis, which is regulated by heat shock, removes abnormal proteins that accumulate during stress conditions, recycles amino acids from nonessential proteins during starvation, and contributes to the clearance of truncated peptides produced from stalled ribosomes by the SsrA system (20, 47). In addition, ClpP-mediated proteolysis in *E. coli* controls the half-life of regulatory proteins, including the stationary phase sigma factor σˢ (59), the heterodimeric UmuD SOS protein (11), and several phage proteins (reviewed in reference 17). Despite the significant roles played by the ClpP system in stress responses in *E. coli*, the phenotype of *E. coli clpP* mutants is essentially identical to that of wild-type cells (5), espe-cially in the degradation of abnormal proteins (18). Presumably, this results from the presence of other redundant ATP-dependent proteases that likely play a more critical role in this process (i.e., Lon and HslUV).

ClpP-mediated proteolysis appears to be more central for stress tolerance and global regulation in bacteria other than *E. coli*. For example, *clpP* mutants of *B. subtilis* and *Lactococcus lactis* are impaired for survival during heat shock and other stress conditions and exhibit clear defects in the degradation of abnormal proteins (12, 14, 39). Microbial developmental processes, such as natural genetic competence and sporulation in *B. subtilis* (39), biofilm development in *Pseudomonas fluorescens* (41), and viability and cell cycle progression in *Caulobacter crescentus* (25), are dependent on ClpP-mediated proteolysis. Moreover, a growing number of studies demonstrate a requirement for ClpP-mediated proteolysis for virulence and disease progression of important bacterial pathogens. For example, ClpP function is required for virulence factor expression, intracellular survival, and pathogenesis of *Listeria monocytogenes* in mice (13). ClpP function has also been linked to the acid tolerance response and virulence of the intracellular pathogen *Salmonella enterica* serovar Typhimurium (21, 56) and to virulence factor expression in *Yersinia enterocolitica* (42). In some of these cases, the association between ClpP function and virulence may reflect the general role played by ClpP-mediated proteolysis in stress survival.

The *S. pneumoniae* genome has recently been shown to contain only single copies of *clpP* and *ftsH* genes and to lack *lon*

* Corresponding author. Mailing address: Lilly Research Laboratories, Drop Code 1543, Lilly Corporate Center, Indianapolis, IN 46285. Phone: (317) 433-0095. Fax: (317) 276-9159. E-mail: Winkler_Malcolm _E@Lilly.com.

and *hslUV* genes (23). In this regard, *S. pneumoniae* is unique among eubacteria, which contain both *clpP* and *lon* or multiple copies of *clpP* when *lon* is absent (7). *S. pneumoniae* contains putative orthologs of the ClpC, ClpE, ClpL, and ClpX ATPase specificity factors that presumably associate with the ClpP protease subunit (reviewed in reference 48). *S. pneumoniae* is a serious human pathogen that is a causal agent of bacterial pneumonia, bronchitis, meningitis, and otitis media and is particularly devastating to the elderly and the very young (55). Antibiotic-resistant clinical isolates of *S. pneumoniae* have recently become common, and multiple-antibiotic-resistant strains are now found worldwide (27, 52). Normal disease progression for *S. pneumoniae* begins with entry and colonization of the nasopharnyx followed by progression to other sites, such as the lung (55). The infection process exposes *S. pneumoniae* to numerous stress conditions, including temperature shift between the upper respiratory tract (30°C) and deeper tissues (37°C), pH changes, exposure to reactive oxygen species generated by host phagocytes, and nutritional deprivation (60).

The role of ClpP- or FtsH-mediated ATP-dependent proteolysis in the stress responses and virulence of *S. pneumoniae* is largely unknown. Recently, signature-tagged mutagenesis was used to identify ClpC as a putative virulence factor of *S. pneumoniae* in mice (45); however, ClpP did not turn up in this study. Pneumococcal ClpC was reported to regulate growth inhibition at high temperature, autolysis in the presence of antibiotics, transformation, and adherence (3); however, another report did not confirm these phenotypes (4). Chastanet and coworkers recently reported that ClpP and ClpE play a role in the thermotolerance of *S. pneumoniae* (4). Their biochemical and genetic evidence in a heterologous *B. subtilis* system suggest that CtsR regulates the heat-shock response of *clpC*, *clpL*, *clpE*, and *clpP* and unexpectedly *groESL* in *S. pneumoniae* (4). They further demonstrated that the expression of two competence genes is induced in a *clpP* mutant, suggesting a negative regulatory role for ClpP in competence development under inappropriate conditions (4). Here, we report further characterization of the phenotypes of *S. pneumoniae clpP*, *clpC*, and *clpX* mutants, including global microarray analysis of transcription patterns of *clpP* mutants and the heat shock regulon of *S. pneumoniae*. Our results show that ClpP-mediated proteolysis plays complex roles in the response of *S. pneumoniae* to thermal and oxidation stress, the regulation of the natural competence pathway, and virulence.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. pneumoniae* strains were cultivated statically in brain heart infusion (BHI) broth or on trypticase soy agar II blood agar plates (TSA-BA; Becton Dickson BBL) at 37°C in an atmosphere of 5% CO_2 unless otherwise stated. Chemically defined medium (CDM) for the growth of *S. pneumoniae* cultures (50) lacking methionine and glucose was formulated by JRH Biosciences (Denver, Pa.). CDM was supplemented with 1.0% (wt/vol) glucose, 0.1% (wt/vol) choline chloride, 0.075% (wt/vol) L-cysteine HCl, and 0.25% (wt/vol) $NaHCO_3$. For antibiotic selection, media was supplemented with 0.3 μg of erythromycin per ml, 100 μg of spectinomycin per ml, 2.5 μg of chloramphenicol per ml, or 2.5 μg of novobiocin per ml as appropriate.

The virulent type 2 encapsulated *S. pneumoniae* strain D39 and its unencapsulated derivative R6 (49) used in these studies were assigned unique strain designations to track isogenic derivatives (Table 1). Strains CPM7 [CP1250 *ssbB*::(pEVP3)::*ssbB*] and CP1500 were used as the *ssbB*::*lacZ* reporter strain and the source of genomic donor DNA in transformation experiments, respectively (Table 1) (34). *S. pneumoniae* mutants were constructed by transformation

of linear deletion replacement amplicons following treatment with competence stimulatory peptide (CSP) (34). Briefly, 1.0- to 1.5-kb DNA fragments corresponding to the 5′ and 3′ regions of target genes to be deleted (≈60 to 80% of internal coding sequences) and either the 1.0-kb *ermAM* (erythromycin resistance [Er^r]) (35) or *aad9* (spectinomycin resistance [Sp^r]) (33) cassettes were amplified by PCR using oligonucleotide primers containing short stretches of DNA sequences that were complementary to a DNA fragment designed to be adjoining (Table 2). The resulting three DNA fragments were mixed, allowed to anneal at the overlapping complementary sequences, and extended and amplified using a second round of PCR (22). Recombinant rTth polymerase and XL reaction buffer (Applied Biosystems) were used in these amplifications. Resultant linear gene amplicons containing deletion replacements were transformed into early exponential phase cultures of *S. pneumoniae* (OD_{620} of ~0.05 to 0.1) diluted 1 to 10 in BHI medium containing 10% heat-inactivated horse serum (Sigma), 10 mM glucose, and 100 ng of synthetic CSP-1 per ml and incubated for 15 min at 30 or 37°C to induce competence. Following transformation, bacteria were allowed to recover for 2 h to allow phenotypic expression of the antibiotic resistance marker, and recombinant clones were recovered on TSA-BA in the presence of selective antibiotics in nutrient broth soft-agar overlay (0.8% [wt/vol] Bacto Nutrient Broth, 0.4% [wt/vol] Bacto Agar) at 30 or 37°C.

Phenotypic characterization of *S. pneumoniae clpP* mutants. The ability of *S. pneumoniae* to grow at reduced and elevated temperatures was determined in triplicate on TSA-BA plates incubated at 30, 37, or 40°C for 24 h. The sensitivity of *S. pneumoniae* EL161 (D39 parent) and its derivatives to puromycin, H_2O_2, or HCl was evaluated using a disk diffusion assay, as EL59 (R6 parent) was incapable of forming an adequate lawn on TSA plates without blood supplementation. Bacteria were harvested from TSA-BA plates following overnight incubation at 37°C in an atmosphere of 5% CO_2 and suspended in sterile 0.9% (wt/vol) NaCl to yield an optical density at 620 nm (OD_{620}) of ~0.13 (~1 × 10^7 CFU per ml). One milliliter of suspension was mixed with 3 ml of nutrient broth soft agar and poured onto the surfaces of BHI agar plates. Sterile 8-mm-diameter disks were placed in the center of the lawns of test organisms and saturated with 10 μl of 4.8 mg of puromycin (Sigma) per ml, 4.9 M H_2O_2 (Mallinckrodt), or 1 N HCl (Red Bird Inc.). Average zones of inhibition were determined after 24 h of growth at 37°C under 5% CO_2, and statistical comparisons were made using one-way analysis of variance (ANOVA), where *P* values of <0.05 were considered significant. The effects of *clpP*, *clpC*, and *clpX* mutations on autolysis of pneumococcus was determined by measuring the loss of OD of 6-ml BHI cultures at mid-exponential phase (OD_{660} of ~0.13) over time following addition of a final concentration of 0.01% (wt/vol) Triton X-100.

Infection of ICR outbred mice. In conducting experiments with animals, the investigators adhered to the Guide for the Care and Use of Laboratory Animals, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (40a).

Bacteria grown overnight on TSA-BA plates were harvested into 0.9% (wt/vol) NaCl and adjusted to a MacFarland standard of 0.5 (~1.0 × 10^8 CFU per ml) which was further diluted fivefold in 0.9% NaCl. After brief isofluorane anesthetization, male ICR outbred mice (Harlan Sprague Dawley) were infected by intratracheal inoculation with 50 μl of bacterial suspension. At 0, 8, 24, and 48 h following inoculation, four mice per group were sacrificed by CO_2 asphyxiation and the lungs aseptically harvested and homogenized in 0.9% NaCl. Total numbers of viable bacteria per lung were determined by serial dilution and spreading onto TSA-BA plates. Statistical comparisons were made using ANOVA where *P* values of <0.05 were considered significant. For 50% lethal dose (LD_{50}) studies, bacteria were grown as described above with the exception that 0.5 MacFarland standard suspensions were serially diluted 10-fold in BHI medium to yield infection doses ranging from ~10^2 to 10^6 CFU per ml. Male ICR outbred mice were injected in the peritoneal cavity with 50 μl of bacterial suspensions and observed daily for clinical signs of disease. Moribund animals were sacrificed by CO_2 asphyxiation, and the LD_{50} was calculated based on the mean infecting dose required to promote evidence of morbidity in 50% of test subjects (10 mice per dose per strain) in a given period of time.

Extraction of total RNA from *S. pneumoniae*. Total RNA was extracted from *S. pneumoniae* by using a variation of the hot-phenol method described by Tsui et al. (53). Briefly, 10 ml of *S. pneumoniae* culture was mixed directly with 5 ml of 100°C lysis buffer containing 2% (wt/vol) sodium dodecyl sulfate (SDS) and 16 mM EDTA (pH 8.0) and held for 2 min at 100°C to induce cell lysis. The lysed culture was extracted with 10 ml of acidified phenol (Gibco BRL) at 65°C for 5 min with vigorous agitation. The aqueous phase was further extracted once with phenol-chloroform-isoamyl alcohol (25:24:1; Gibco BRL) and once with chloroform-isoamyl alcohol (Gibco BRL). Total RNA was concentrated from the aqueous phase by using Centriplus YM-30 cartridges (Millipore) and purified

TABLE 1. Bacterial strains and DNA amplicons used in strain construction

Strain	Genotype and/or phenotype[a]	Description	Source or reference
EL59	R6, avirulent unencapsulated parent	Derived from D39 isolate	49
EL161	D39, virulent encapsulated type 2 parent	Subclone of original clinical isolate	49
CP1500	*hex nov-r1 bry-r str-1 ery-r1 ery-r2* Nv[r]	Donor of novobiocin resistance point marker	34
CPM7	CP1250 *ssbB'*::(pEVP3)::*ssbB[+] ssbB*::*lacZ* Cm[r] SsbB[+]	Rx derivative with *ssbB*::*lacZ* reporter construct	34
EL539[b]	EL59 Δ*clpP*::*aad9* Sp[r]	EL59 transformed with linear Δ*clpP*::*aad9* amplicon	This study
EL555	EL59 Δ*bgaA*::*ermAM* Er[r]	EL59 bearing a Δ*bgaA*::*ermAM* mutation	D. LeBlanc strain collection
EL556	EL59 Δ*lytA*::*aad9* Sp[r]	EL59 transformed with linear Δ*lytA*::*aad9* amplicon	This study
EL677	EL161 Δ*clpP*::*aad9* Sp[r]	EL161 transformed with linear Δ*clpP*::*aad9* amplicon	This study
EL824	CPM7 Δ*clpP*::*aad9* Sp[r]	CPM7 transformed with linear Δ*clpP*::*aad9* amplicon	This study
EL854	EL59 Δ*clpC*::*ermAM* Er[r]	EL59 transformed with linear Δ*clpC*::*ermAM* amplicon	This study
EL856	EL161 Δ*clpC*::*ermAM* Er[r]	EL161 transformed with linear Δ*clpC*::*ermAM* amplicon	This study
EL873	EL59 Δ*clpX*::*ermAM* Er[r]	EL59 transformed with linear Δ*clpX*::*ermAM* amplicon	This study
EL923	EL677 Δ*bgaA*::*ermAM*::T1T2::*clpP[+]* Sp[r] Er[r] ClpP[+]	EL677 transformed with linear Δ*bgaA*::*ermAM*::T1T2::*clpP[+]*	This study
EL925	EL539 Δ*bgaA*::*ermAM*::T1T2::*clpP[+]* Sp[r] Er[r] ClpP[+]	EL539 transformed with linear Δ*bgaA*::*ermAM*::T1T2::*clpP[+]*	This study
EL961	EL161 Δ*bgaA*::*ermAM* Er[r]	EL161 transformed with linear Δ*bgaA*::*ermAM* from EL555	This study
Amplicons			
clpP-a		EL59 genomic DNA,[c] (clpPa1, clpPa2),[d] partial construct	This study
P-aad9-b		pDL278, (clpPb1, clpPb2), partial construct	This study
clpP-c		EL59 genomic DNA, (clpPc1, clpPc2), partial construct	This study
Δ*clpP*::*aad9*		clpP-a, P-aad9-b, clpP-c, (clpPa1, clpPc2), completed construct	This study
clpC-a		EL59 genomic DNA, (clpCa1, clpCa2), partial construct	This study
C-erm-b		pVA838, (clpCb1, clpCb2), partial construct	This study
clpC-c		EL59 genomic DNA, (clpCc1, clpCc2), partial construct	This study
Δ*clpC*::*ermAM*		clpC-a, C-erm-b, clpC-c, (clpCa1, clpCc2), completed construct	This study
clpX-a		EL59 genomic DNA, (clpXa1, clpXa2), partial construct	This study
X-erm-b		pVA838, (clpXb1, clpXb2), partial construct	This study
clpX-c		EL59 genomic DNA, (clpXc1, clpXc2)	This study
Δ*clpX*::*ermAM*		clpX-a, X-erm-b, clpX-c, (clpXa1, clpXc2), completed construct	This study
bgaA-a		EL59 genomic DNA, (bgaAa1, bgaAa2), partial construct	This study
B-erm-b		pVA838, (ermAMb1, ermAMb2), partial construct	This study
T1T2-c		pDL278, (terMc1, terMc2), partial construct	This study
clpP-d		EL59 genomic DNA, (clpPd1, clpPd2), partial construct	This study
bgaA-e		EL59 genomic DNA, (bgaAe1, bgaAe2), partial construct	This study
Δ*bgaA*::*ermAM*::T1T2::*clpP[+]*		bgaA-a, B-erm-b, T1T2-c, clpP-d, bgaA-e (bgaAa1, bgaAe2), completed construct	This study
lytA-a		EL59 genomic DNA, (lytAa1, lytAa2), partial construct	This study
L-aad9-b		pDL278, (lytAb1, lytAb2), partial construct	This study
lytA-c		EL59 genomic DNA, (lytAc1, lytAc2), partial construct	This study
Δ*lytA*::*aad9*		lytA-a, L-aad9-b, lytA-c, (lytAa1, lytAc2), completed construct	This study
Δ*bgaA*::*ermAM*		EL555 genomic DNA, (bgaAa1, bgaAe2), completed construct	This study

[a] Nv[r], resistant to novobiocin; Cm[r], resistant to chloramphenicol; Sp[r], resistant to spectinomycin; Er[r], resistant to erythromycin.
[b] Strain construction was carried out by transformation of indicated recipient with linear double stranded synthetic PCR amplicon.
[c] Template DNA for amplicon generation.
[d] Primer pair set used to generate a given amplicon (see Table 2).

using an RNeasy (Qiagen) column with on-column DNase I treatment performed according to the manufacturer's instructions.

Heat shock of *S. pneumoniae*. *S. pneumoniae* cells were cultivated statically overnight in CDM at 37°C in an atmosphere of 5% CO_2. Cultures were diluted to an OD_{620} of ~0.03 in 40 ml of prewarmed CDM and allowed to incubate without shaking at 37°C until the culture reached early to middle exponential growth phase (OD_{620} of ~0.3). Cultures were divided into three 10-ml portions which were heated at 37°C or for 5 and 15 min at 45°C, after which RNA was extracted as described above.

Northern analysis and quantitative real time reverse transcription (RT)-PCR. Aliquots of 5 μg of total *S. pneumoniae* RNA, purified as described above, were separated by formaldehyde agarose gel electrophoresis and transferred using a Northern Max kit (Ambion, Inc.) according to the manufacturer's instructions. A 0.5-kb internal fragment of *clpP* generated by PCR with the primer set clpP-5'

and clpP-3' (Table 2) was labeled with psoralen-biotin by using a Brightstar psoralen-biotin nonisotopic labeling kit (Ambion) and used as a probe for Northern analysis according to the conditions given for the Northern Max kit. Bands on the exposed audioradiographic film were quantified using Sigma Scan version 2.0 (Jandel). Relative increases (*n*-fold) in *clpP* band intensities of RNA isolated from cultures following a shift from 37 to 45°C for 5 and 15 min were compared to that of the 37°C control.

S. pneumoniae clpP mRNA was quantified by a real-time, 5' exonuclease PCR (TaqMan) assay (31) using a primer-probe combination that recognized a portion of the *S. pneumoniae clpP* gene. For comparison purposes, primer-probe sets for *fabK* and 16S rRNA were also employed. The primers and probes are listed in Table 2. Primer-probe sets were selected using Primer Express software (PE Biosystems, Foster City, Calif.) and were obtained from PE Biosystems. The primers were used in concentrations of 300 nM and the probes were used in

TABLE 2. Synthetic oligonucleotides used in these studies

Name of primer	Sequence (5′ → 3′)[a]
Primer set for *clpP* probe generation for Northern blotting	
clpP-5′	GCCGTGGAGAACGTTCTTA
clpP-3′	GGGCGCTCATCCAGTTA
Primer/probe sets used for quantitative real-time RT-PCR	
S.pn. 16S-forward	GACGATACATAGCCGACCTG
S.pn. 16S-reverse	GAGTCTGGGCCGTGTCTCA
S.pn. 16S-probe	6FAM-TCCCAGTGTGGCCGATCACCCT-TAMRA
S.pn. clpP-forward	GGCTGCATCTATGGGAACAGTC
S.pn. clpP-reverse	GCTTTTGTATCATTTAGCATCGCA
S.pn. clpP-probe	6FAM-TCGCATCAAGTGGAGCAAAAGGCA-TAMRA
S.pn. fabk-forward	CTGCAGGAGGAATTGCGG
S.pn. fabk-reverse	CAGCCTCTGCACCTAGCATAAA
S.pn. fabk-probe	6FAM-TGGTGAAGGTGCTGCGGCTGG-TAMRA
Primer sets used for deletion replacement cassette construction by overlap-extension PCR	
clpPa1	GCGAATCGTTCCACTTGTA
clpPa2	<u>CGAACGAAAATCGATC</u>TCCACGGCTTGTTTGTT
clpPb1	<u>AACAAACAAGCCGTGG</u>AATCGATTTTCGTTCGTGAAT
clpPb2	<u>TTCTGCATCTGCATGGACT</u>TAGAATGAATATTTCC
clpPc1	<u>GGAAATATTCATTCTAAGTCCATGCAGATGCAGAA</u>
clpPc2	CAGCAGTTGCTTGTTGTA
clpCa1	CCCGAGCGGATAAAATT
clpCa2	<u>TTATTTCCATAACTTCTTTT</u>ATACGAGTCGCCAAGGCA
clpCb1	<u>TGCCTTGGCGACTCGTATA</u>AAAGAAGTTATGGAAATAA
clpCb2	<u>TCAAAGAGGAGAACGGAAT</u>AGTTAGCTCCTTGGAAGCTGT
clpCc1	<u>ACAGCTTCCAAGGAGCTAAC</u>TATTCCGTTCTCCTCTTTGAT
clpCc2	GGTTTTGTCACATGATCAT
clpXa1	CCGTTACTTGTCAGAACTTT
clpXa2	<u>TTATTTCCATAACTTCTTTT</u>GTGGTTCAAGATATGGA
clpXb1	<u>TCCATATCTTGAACCACAAAA</u>GAAGTTATGGAAAT
clpXb2	<u>TAAGAAAGCAAGGTTTGGTACT</u>TTAGCTCCTTGGAAGCTGT
clpXc1	<u>ACAGCTTCCAAGGAGCTAAA</u>GTACCAAACCTTGCTTTCTTA
clpXc2	CTCTGCGATGACTTCAATT
bgaAa1	TCAATTGATTACTCACTCTACTCTTG
bgaAa2	<u>CAGCTTCCAAGGAGCTAAT</u>AAACCTAGTTCTGCTGCGATTTGCAA
ermAMb1	<u>TCGCAGCAGAACTAGGTTT</u>ATTAGCTCCTTGGAAGCTGTCAGTA
ermAMb2	<u>CGTTTTATTTGATGC</u>AAGAAGTTATGGAAATAAGACTTAG
terMc1	<u>TTTCCATAACTTCTT</u>GCATCAAATAAAACGAAAGGCTCAGT
terMc2	<u>CGTGACTGGAAAYAC</u>TTTTCCAAGTCGTAGAAACGCAAAAAGGCCAT
clpPd1	GCCTTTTTGCGTTTCTACGACTTGGAAAAGTATTTCCAGTCACGAAA
clpPd2	<u>ATGAGAAAGTAAGTTCTT</u>AAGAAATCTCTCCCTATTATA
bgaAe1	<u>GGAGAGATTTCTT</u>AAGAACTTACTTTCTCATAAACCAGTT
bgaAe2	GCTAAAGAATGTCAGGCGCTTTTATCT
lytAa1	GGCATGGGGATTGTCATT
lytAa2	<u>CGAACGAAAATCGATG</u>TTGAGTGTGCGTGTA
lytAb1	<u>TACACGCACACTCAAA</u>TCGATTTTCGTTCGTGAAT
lytAb2	<u>TTTGAGGTAGTACCAGCCTGT</u>TAGAATGAATATTTCC
lytAc1	<u>GGAAATATTCATTCTAA</u>CAGGCTGGTACTACCTCAAA
lytAc2	CCCTTAGCTACCAACTGA

[a] Abbreviations: 6FAM, 6-carboxyfluorescein; TAMRA, *N, N, N′, N′*-tetramethyl-6-carboxyrhodamine. The underlined portion of each oligonucleotide represents short heterologous extensions that are complementary to the fragments of DNA to be linked by overlap-extension PCR.

concentrations of 200 nM in 25-μl reaction mixtures. TaqMan reactions were set up using an EZ RT-PCR kit (PE Biosystems). Each reaction contained twofold serial dilutions of either 200 ng or 20 ng of total RNA, depending on the primer-probe set used. The quantitative RT-PCRs were performed on a ABI PRISM 7700 (PE Biosystems) and included the following steps: 2 min treatment with uracil *N*-glycosylase at 50°C, 30 min of reverse transcription at 60°C, 5 min at 95°C, and then 40 cycles of amplification using 95°C for 15 seconds followed by 60°C for 60 s. Three replicates were performed for each sample. The results were calculated using the comparative critical threshold ($\Delta\Delta C_T$) method (user bulletin no. 2 [http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf]; Applied Biosystems), in which the amount of target is normalized to a reference (non-heat shock control) relative to an internal calibrator target RNA (16S rRNA). Transcript amounts from a control target gene (*fabK*) were analyzed using the same parameters. Data are presented as the relative changes in exper-

imental target amounts compared to those of the 37°C control following 5- and 15-min shifts from 37 to 45°C or in the *clpP* mutant (EL539).

Microarray analysis. Fragmentation and labeling of RNA was performed as instructed by Affymetrix (Santa Clara, Calif.). Briefly, 60 μg of total RNA was fragmented in 1.1× T4 polynucleotide kinase buffer (New England Biolabs) for 10 min at 95°C. The fragmented RNA was then end-labeled with 0.1 mM [γ-S]ATP by using T4 polynucleotide kinase for 1 h at 37°C. Labeled RNA was precipitated and allowed to react with 2 mM PEO-iodoacetyl-biotin (Pierce) in 30 mM MOPS (morpholinepropanesulfonic acid) (pH 7.5) for 1 h at 37°C. The biotinylated RNA was purified using a DNA/RNA midi kit (Qiagen) according to the manufacturer's instructions. RNA was concentrated by precipitation with isopropanol. An aliquot of 10 μg of fractionated biotin-labeled RNA was used for hybridization with each *S. pneumoniae* R6 microarray, which were custom designed and manufactured by Affymetrix. The custom arrays represent >95%

of *S. pneumoniae* R6 predicted open reading frames (ORFs) (23) tiled onto two low-density 50-μm feature gene arrays. The genes that were not represented on the chip included one copy of those from identical or redundant ORFs (i.e., *comX1* and *comX2*), ORFs that were <200 bp in length, and ORFs encoding tRNAs. Each gene on the microarray was represented by a probe set which consisted of 20 pairs of perfect-match and mismatch oligonucleotides. Hybridization, staining with R-phycoerythrin (Molecular Probes), and washing conditions were performed as suggested by Affymetrix. Stained microarrays were scanned in a Hewlett-Packard GeneArray Scanner at 570 nm. Data were analyzed using Affymetrix Microarray Suite 4.0. The absence, presence, and relative expression level of a given gene is subject to multiple standardized algorithms, which can be viewed in detail at the Affymetrix website. The hybridization intensity of each probe pair was calculated based on the difference in signal between the perfect-match and the mismatch oligonucleotides. The relative change (*n*-fold) of the normalized hybridization intensity of the experimental (e.g., R6 Δ*clpP* mutant) probe set is relative to that of the control (e.g., R6 parent) probe set. Only probe pairs having hybridization intensities that were within the standard deviation of the mean of the hybridization intensity of the entire probe set were included in the relative change (*n*-fold) calculation. For microarray analysis of heat shock gene induction, the results represent a single microarray analysis and only changes in predicted heat-inducible genes are reported.

Competence and β-galactosidase assays. For an assay of natural competence development, bacterial cultures of similar ODs after overnight incubation in BHI broth at 37°C were diluted 1 to 10 in 8 ml of BHI medium or CDM to yield an OD_{620} of ~0.01. Cultures were incubated at 37°C in an atmosphere of 5% CO_2, and growth was monitored by direct measurement of changes at OD_{620}. At 30-min intervals, 100 μl of cell suspension was removed and mixed with 0.2 μg of CP1500 genomic DNA carrying a marker for novobiocin resistance (Table 1). Cultures were incubated for 15 min at 37°C and then treated with 20 U of DNase I (Sample Buffer II; Genomic Solutions) to remove remaining extracellular DNA. Cultures were incubated at 37°C for an additional 45 min to allow phenotypic expression of the antibiotic marker. Nv^r clones were recovered from dilutions of the cell suspensions in novobiocin-containing nutrient broth soft-agar overlays poured onto TSA-BA plates. The number of Nv^r colonies per milliliter of culture was recorded following overnight incubation at 37°C in an atmosphere of 5% CO_2.

For *ssbB*::*lacZ* expression studies and experimental induction of competence with CSP-1, bacteria were grown overnight in BHI broth at 37°C and diluted into 3 ml of fresh BHI medium to an OD_{620} of ~0.002. Cultures were incubated until the OD_{620} reached 0.07, at which point genomic DNA from donor strain CP1500 (Table 1) was added to 0.2 μg per ml with and without 50 ng of synthetic CSP-1 per ml. Cultures were incubated for 20 min at 37°C and then treated with 20 U of DNase I to remove remaining extracellular DNA. Thirty minutes after addition of donor DNA, 1 ml of cell suspension was removed, lysed with 0.1% (vol/vol) Triton X-100 at 37°C for 2 min, and assayed for β-galactosidase activity by using ONPG (*o*-nitrophenyl-β-D-galactopyranoside) as the substrate. Results of β-galactosidase assays are expressed as Miller units normalized to the OD_{620} of the culture when the assay was initiated. The remaining culture was allowed to incubate in BHI medium at 37°C for an additional 90 min to allow phenotypic expression of the antibiotic resistance marker, and Nv^r clones were recovered as described above.

Database searches for HrcA and CtsR DNA binding motifs. The 5′ leader regions of genes whose transcriptions were induced in the absence of *clpP* were searched using Vector NTI version 5.0 to find the consensus nucleotide binding sequences for CtsR (RGTCAAWnAnRGTCARn) (9) and HrcA (TTAGCACT C-N9-GAGTGCTAA) (40), where R equals G or A, W equals A or T, and n equals any base. Additional searches were performed on the genome sequence of *S. pneumoniae* R6 by using the nucleic acid pattern searching program fuzznuc, developed and distributed through the European Molecular Biology Open Software Suite (EMBOSS [http://www.uk.embnet.org/Software/EMBOSS/index .html]).

RESULTS

Temperature requirements for construction of *S. pneumoniae* clp mutants. The lack of multiple classes of ATP-dependent proteases in *S. pneumoniae* R6 led to investigation of the contribution of ClpP to stress response and pathogenesis of this bacterium. Genes predicted to encode homologs of ClpP, ClpX, and ClpC were readily identifiable in the *S. pneu-*

moniae R6 genome. The predicted *S. pneumoniae clpP* and *clpX* gene products share >76% amino acid similarity with their counterparts in *B. subtilis* and *E. coli* (14, 18, 37). The gene encoding the ClpC protein was previously cloned and characterized in *S. pneumoniae* (3) and shares 63% amino acid similarity with its ortholog from *B. subtilis* (28). Individual deletion replacement mutations of *clpP*, *clpX*, and *clpC* were constructed by transforming *S. pneumoniae* with linear deletion cassettes for each gene bearing a selective antibiotic marker; thus, mutations were confined to the limited regions in the amplicons used for transformation. Δ*clpC* mutants were obtained using standard transformation conditions at 37°C for both the R6 (EL59) and D39 (EL161) parents. In contrast, isolation of Δ*clpP* strains was significantly enhanced following transformation and recovery at 30°C in the R6 background and was absolutely essential for isolation of these mutants in the D39 background (data not shown). Δ*clpX* mutants of R6 were obtained at a lower-than-expected frequency at 37°C, and the isolation of a Δ*clpX* mutation in D39 was achieved only by high-efficiency transformation using genomic DNA isolated from the R6 Δ*clpX* strain (EL539). At present, the basis for the apparent decrease in the recovery of Δ*clpX* and Δ*clpP* transformants in the D39 background is not known. All mutants were confirmed by diagnostic PCR analysis of genomic DNA from isolated clones (data not shown).

ClpP, but not ClpX or ClpC, is required for growth at reduced and elevated temperatures. The requirement for a reduced temperature for the efficient recovery of *clpP* mutants of *S. pneumoniae* suggested that ClpP proteolysis may be needed for growth under certain stress conditions. To determine whether ClpP-mediated proteolysis was required for growth under heat stress conditions, pneumococcal *clp* mutants or parental strains were tested for their ability to grow on solid media at 30, 37, and 40°C. Unlike the R6 (EL59) or D39 (EL161) parent strains (Fig. 1) (data not shown), the Δ*clpP* mutants did not grow at 40°C. In contrast, *clpC* and *clpX* single mutants were not restricted for growth at 40°C. These findings are consistent with those reported recently by Chastanet and coworkers for *S. pneumoniae clpP* mutants in liquid media (4).

We observed a slight reduction in colony size of Δ*clpP* strains on TSA-BA plates and a somewhat slower doubling time (78 min versus 63 min) in CDM for the R6 Δ*clpP* (EL539) mutant compared to its parent (EL59) at 37°C. Surprisingly, the D39 Δ*clpP*::*aad9* mutant (EL677) showed a marked reduction in colony size at 30°C on TSA-BA plates, despite the fact that 30°C incubation was required for efficient initial recovery of the D39 Δ*clpP* mutant (see above). Taken together, these findings suggest that loss of the pneumococcal *clpP* gene product effects growth at temperature extremes and reducing growth rate may enhance the initial recovery of these mutants. Last, complementation of *clpP* mutant phenotypes had not been demonstrated previously (4). Therefore, we introduced an ectopic copy of the *clpP*$^+$ gene and its promoter into the dispensable (58) *bgaA* locus of Δ*clpP* mutants (EL925 and EL923; Table 1). Growth of the Δ*clpP*/*clpP*$^+$ merodiploids was indistinguishable from that of the R6 and D39 parent strains on different media at 30, 37, and 40°C (Fig. 1; data not shown). Thus, loss of *clpP* function alone is responsible for all of the observed growth defects at reduced and elevated temperatures.
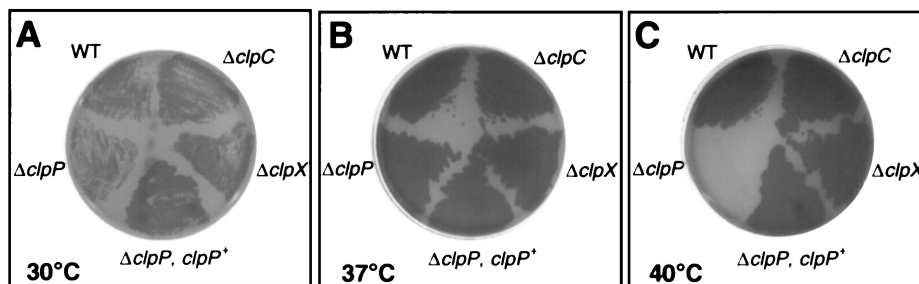
FIG. 1. Impaired growth of *S. pneumoniae* R6 Δ*clpP* mutant at reduced and elevated temperatures. WT (R6 parent [EL59]), Δ*clpP* (R6 Δ*clpP*::*aad9* [EL539]), Δ*clpC* (R6 Δ*clpC*::*ermAM* [EL854]), Δ*clpX* (Δ*clpX*::*ermAM* [EL873]), or Δ*clpP*, *clpP*+ (R6 Δ*clpP*::*aad9*/Δ*bgaA*::*ermAM*:: T1T2::*clpP*+ [EL925]) were streaked in quadrants of TSA-BA plates (see Materials and Methods), which were incubated for 24 h at 30°C (A), 37°C (B), or 40°C (C).

Sensitivity of *S. pneumoniae* clpP mutant to H₂O₂ and puromycin stress. The ClpP proteases participate in the degradation of abnormal or damaged proteins that arise during times of stress or in response to chemical or antibiotic treatment (12, 18, 29). The failure of *clpP* mutants of *S. pneumoniae* to grow at elevated temperatures is consistent with an absolute requirement for ClpP proteolysis to deal with conditions that are known to cause proteins to misfold. To test this idea further, we determined the sensitivity of *S. pneumoniae* D39 Δ*clpP*::*aad9* (EL677) to the translational inhibitor puromycin, which binds to the peptidyl transferase site in ribosomes and prematurely truncates translation to generate randomly folded puromycyl peptides (16). Compared to its *clpP*+ D39 parent (EL161), the Δ*clpP* mutant was more sensitive to puromycin in disk diffusion assays (Table 3). Consistent with this result, treatment of *S. pneumoniae* R6 (EL59) with subinhibitory concentrations of puromycin caused a strong induction of the pneumococcal heat shock regulon as determined by DNA microarray analysis (unpublished result). The Δ*clpP* strain was also more sensitive to H₂O₂ challenge than its *clpP*+ parent (Table 3). Both strains were equally sensitive to mild acid stress (Table 3). Again, the sensitivity of the Δ*clpP* mutant to puromycin and H₂O₂ was complemented in the Δ*clpP*/*clpP*+ merodiploid strain (EL923; Table 3).

Autolysis is not perturbed in clpP mutants. ClpC was implicated in the autolysis of *S. pneumoniae* following drug treatment or entrance into stationary phase (3). This defect was thought to have arisen because of the loss of expression of *lytA*, which encodes the major pneumococcal autolysin. In contrast to this earlier conclusion, it was recently reported that mutations in *ctsR*, *clpC*, *clpE*, or *clpP* did not alter penicillin- or desoxycholate-induced autolysis of *S. pneumoniae* (4). To resolve this apparent discrepancy, we tested whether *S. pneumoniae* strains with individual mutations in *clpP*, *clpX*, or *clpC* were defective in nonionic detergent-induced autolysis. We found that autolysis of the mutants was indistinguishable from that of the parent strain and occurred almost immediately when 0.01% (vol/vol) Triton X-100 was added to cultures (see Materials and Methods; data not shown). In contrast, cultures of a Δ*lytA* mutant (EL556) stopped growing but did not significantly decrease in optical density after treatment with Triton X-100 (data not shown). Our results confirm and extend those of Chastanet and coworkers (4) and argue that the nor-

mal LytA autolytic pathway is not perturbed in the absence of the *clpP*, *clpC*, or *clpX* genes.

The ClpP protease contributes to survival of pneumococcus in ICR outbred mice. The sensitivity of the D39 *clpP* mutant to H₂O₂, puromycin, and temperature suggested that ClpP may either directly or indirectly affect the capacity of this catalase-negative bacterium to act as a respiratory pathogen. To test this hypothesis, male ICR outbred mice were infected by intratracheal challenge with the D39 *clpP*+ parent (EL161), D39 Δ*clpP*::*aad9* (EL677), and the merodiploid D39 Δ*clpP*::*aad9* Δ*bgaA*::*ermAM*::T1T2::*clpP*+ (EL923). Mice were also infected with D39 Δ*bgaA*::*ermAM* (EL961) and D39 Δ*clpC*::*ermAM* (EL856) as controls for insertion in the *bgaA* locus and to assess the effects of the lack of ClpC, which was reported to contribute to pneumococcal virulence (45), respectively. In repeated experiments, the Δ*clpP* mutant failed to colonize the lungs of infected animals to significant levels and produced no mortality in these animals through 48 h of infection (Fig. 2A). In contrast, the D39 parent (EL161), *clpP*+ Δ*bgaA*::*ermAM* control strain (EL961), and the Δ*clpP*/*bgaA*::*clpP*+ merodiploid (EL923) were highly virulent, replicated to high levels, and yielded no surviving infected animals by 48 h (Fig. 2B). Both the D39 Δ*bgaA*::*ermAM* (EL961) and the Δ*clpP*/*bgaA*::*clpP*+ merodiploid (EL923) strains showed slight, but significant, decreases in lung colonization at 8 h compared to that of the D39 parent (EL161) (Fig. 2B). This decrease likely reflected the absence of functional *bgaA*, which has been suggested to participate in pneumococcus pathogenesis (58). In contrast to the strong attenuation exhibited by the Δ*clpP* mutant (EL677), the

TABLE 3. Increased sensitivity of *S. pneumoniae* D39 Δ*clpP* mutant to puromycin and hydrogen peroxide

Strain[b]	Diameter of zone of inhibition (mm) (mean ± SD[a]) on:		
	Puromycin	Hydrogen peroxide	HCl
EL161	22.7 ± 0.9	46.6 ± 1.0	9.9 ± 0.6
EL677	36.6 ± 0.6**[c]	57.5 ± 1.5**	9.5 ± 0.6
EL923	22.9 ± 0.8	45.1 ± 0.9	NT

[a] Results represent the average of four determinations and were repeated in at least two separate experiments. NT, not tested.
[b] Genotypes: EL161 (D39 parent); EL677 (D39 Δ*clpP*::*aad9*); EL923 (D39 Δ*clpP*::*aad9* Δ*bgaA*::*ermAM*::T1T2::*clpP*+).
[c] **, P < 0.001 (compared to D39).

A

B
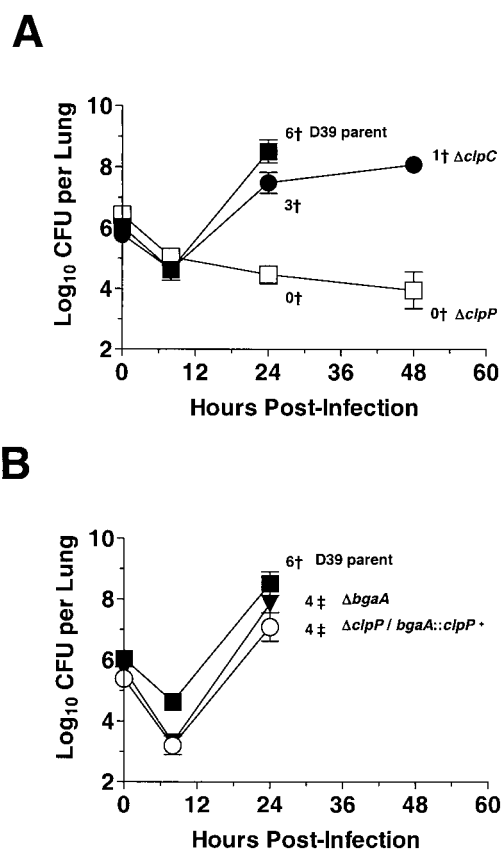

FIG. 2. Impaired virulence of *S. pneumoniae* D39 Δ*clpP* mutant in lung model of infection. Male ICR outbred mice were infected by intratracheal inoculation with the indicated strains (see Materials and Methods). At each time point, four mice per group were sacrificed, and the CFU per lung were determined by serial dilution and plating on TSA-BA (see Materials and Methods). Symbols: ■, D39 parent (EL161); □, D39 Δ*clpP*::*aad9* (EL677); ●, D39 Δ*clpC*::*ermAM* (EL856); ○, D39 Δ*clpP*::*aad9*/Δ*bgaA*::*ermAM*::T1T2::*clpP*+ merodiploid (EL923); ▼, D39 Δ*bgaA*::*ermAM* (EL961); vertical bars, standard deviations; †, number of moribund animals at each time point; ‡, number of animals that became moribund between the 24- and 48-h time points.

Δ*clpC*::*ermAM* mutant (EL856) was only marginally attenuated, replicated to high levels, and produced significant morbidity in infected ICR mice (Fig. 2A). The attenuation of the Δ*clpP* mutant (EL677) was further evaluated using a murine model of septicemia. In these studies, we determined the average infecting dose of the D39 parent (EL161) and the Δ*clpP* mutant (EL677) required to produce signs of morbidity in 50% of the test subjects (LD_{50}) within 24 h following direct intraperitoneal injection. The LD_{50}s of the D39 parent (EL161) and the Δ*clpP* mutant were 10 to 100 and >10^6, respectively (data not shown), again indicating the strong requirement for *clpP* function in the pathogenicity of type 2 encapsulated pneumococcus in a murine host.

clpP transcript amounts increased in *S. pneumoniae* in response to heat shock. To date, all reported *clpP* gene products from nonphotosynthetic bacteria are regulated by heat shock (46). Chastanet et al. (4) located a consensus binding site for CtsR (class three stress gene repressor) (9) 67 base pairs upstream of the pneumococcal *clpP* ATG start codon (Fig. 3A;

A

B

C

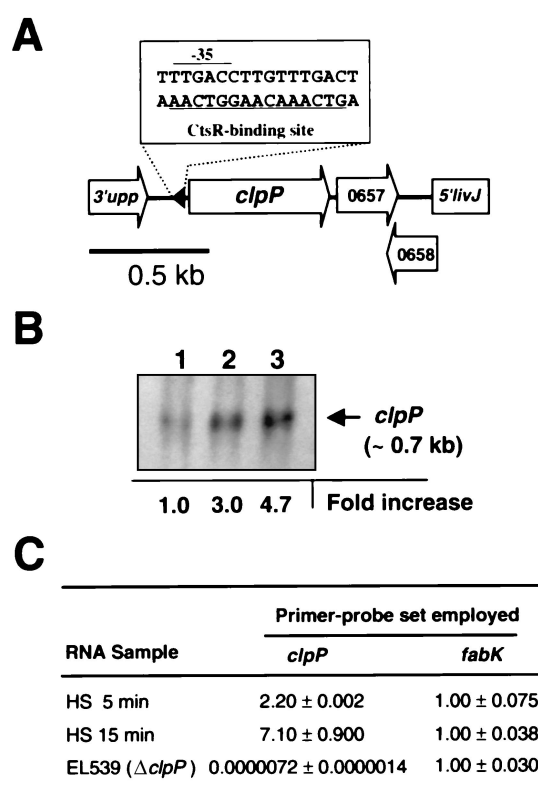| RNA Sample	Primer-probe set employed	
	clpP	*fabK*
HS 5 min	2.20 ± 0.002	1.00 ± 0.075
HS 15 min	7.10 ± 0.900	1.00 ± 0.038
EL539 (Δ*clpP*)	0.0000072 ± 0.0000014	1.00 ± 0.030

FIG. 3. Transcription of *clpP* in *S. pneumoniae* R6 at 37°C and after heat shock at 45°C. (A) Structure of the *clpP* locus of *S. pneumoniae* R6 (drawn to scale). Arrows indicate predicted directions of transcription. The predicted −35 and CtsR-binding sites (underlined) are indicated. See text for details. (B) Northern blot of total RNA isolated from *S. pneumoniae* R6 (EL59) probed for *clpP* (see Materials and Methods). Relative expression levels are indicated below the autoradiograph (see Materials and Methods). Lanes: 1, 37°C; 2, 45°C for 5 min; 3, 45°C for 15 min. (C) Quantitative real-time RT-PCR of *clpP* and *fabK* transcription following heat shock (HS) at 45°C. Data represent the relative change (*n*-fold) in mRNA amounts compared to that of the 37°C control, calibrated to 16S rRNA amounts (see Materials and Methods). *fabK* transcript amounts were included as a control of a gene not known to be regulated by heat shock. No *clpP* transcript was detected from the Δ*clpP* mutant (R6 *clpP*::*aad9*) (EL539).

Table 5), and they showed that purified CtsR binds to this region (4). They also reported a 30-fold induction of a *S. pneumoniae clpP-bgaB* fusion in a heterologous *B. subtilis* system in response to heat shock at 48°C (4). The magnitude of this heat shock response was considerably greater than that reported for other stress-response proteases (15, 18). Therefore, we determined the extent of *clpP* heat shock induction by Northern analysis and real-time quantitative RT-PCR (TaqMan) in the R6 parent strain (EL59) after a shift to 45°C for 5 and 15 min (Fig. 3B and 3C). Transcription of pneumococcal *clpP* increased in response to heat shock by 2.2- to 7.1-fold by real-time RT-PCR (Fig. 3C) and 3.0- to 4.7-fold by Northern analysis (Fig. 3B). In addition, the Northern analysis indicated that *clpP* is a monocistronic operon in *S. pneumoniae*. The transcript length of 0.7 kb corresponds to that expected for the 5′-leader and *clpP* reading frame alone and not a bicistronic transcript of >0.9 kb containing both the *clpP* and *orf-0657* reading frames (Fig. 3A).

Global transcriptional profile of Δ*clpP* mutant of strain R6. The phenotypes of *clpP* mutants suggest that the ClpP protease plays fundamental roles in the normal physiology, stress survival, and pathogenesis of *S. pneumoniae*. To begin to address the genetic basis for these phenotypes, we performed an analysis of global transcription of the R6 Δ*clpP* mutant compared to the R6 parent during exponential growth in CDM by using a custom Affymetrix oligonucleotide microarray (Materials and Methods). As an internal control of this method, *clpP* transcripts were not detected (called absent, with a decrease of at least 22-fold) from the R6 Δ*clpP* mutant (EL539) compared to the R6 parent strain (EL59) (Table 4). Furthermore, the level of transcription from the *upp* and *orf-0657* genes, which flank *clpP* (Fig. 3A), was the same in the R6 Δ*clpP* and its *clpP*+ parent, indicating a lack of polarity on the surrounding genes.

Changes in relative transcript amounts of ≥2.0-fold were observed for 150 and 200 genes, respectively, in two separate and independent experiments. Of these transcriptional changes, 79 were common to both experiments and thus were considered reproducible (see Materials and Methods). Some of the identified genes were linked in the chromosome and showed similar changes in expression; however, additional experiments would be necessary to demonstrate polycistronic operons for these genes. The majority of altered genes could be clustered into several major classes (Table 4), including the majority of the known competence and heat shock regulons (all induced), genes required for virulence and oxidative stress resistance (all repressed), a large group of hypothetical genes of unknown functions, and several genes associated with metabolism and cell surface components. Repressed genes that mediate resistance to oxidative stress included *tpx* (thioredoxin-linked thiol peroxidase) and *sodA* (manganese-dependent superoxide dismutase). Repressed genes that influence pneumococcal virulence included *sodA* and *psaABC* (manganese transport) (2, 57). It is noteworthy that the transcription of several cell surface proteins that may mediate pathogenesis were induced or repressed in the R6 *clpP* mutant, including *lytA* (major autolysin), *dlytC* (D-alanyl carrier protein), and *cbpD* (choline-binding protein D) (Table 4).

Altered competence gene expression in *clpP* mutants. Chastanet and coworkers recently reported that expression of fusion constructs of the early *comDCE* and late *ssbB* competence genes was increased in a *clpP* insertion mutant (4). This observation led to the conclusion that ClpP may act as a negative regulator that prevents competence gene expression under inappropriate conditions. Our global transcription analysis showed that transcription of the majority of genes in the *S. pneumoniae* competence regulon is highly induced in a Δ*clpP* mutant during mid-exponential growth phase (Table 4). To test whether this induction was transient, we assayed the assimilation of donor genomic DNA in a Δ*clpP* mutant derived from the competence indicator strain CPM7 (Table 1) (34). In agreement with the microarray studies, the Δ*clpP* mutant (EL824) became competent for transformation in CDM at a much lower cell density than did its parental control (CPM7) and remained competent throughout logarithmic growth (Fig. 4A). In contrast, competence in the parental CPM7 strain was transient, resulting in typical pneumococcal transformation kinetics (44) (Fig. 4A). We also found that natural genetic com-

petence did not develop for CPM7 grown in BHI but that this block in competence development could eventually be overcome in a Δ*clpP* strain (EL824) (Fig. 4B). Independent experiments with BHI medium revealed strong *ssbB*::*lacZ* expression (fivefold increase above the parental control) in the Δ*clpP* mutant or after addition of synthetic CSP-1 to the *clpP*+ strain, and this was accompanied by a comparable increase in the transformation frequency (data not shown). Maximum expression of *ssbB*::*lacZ* (ninefold increase compared to the untreated parental control) and transformation frequency occurred in the Δ*clpP* mutant treated with synthetic CSP-1 (data not shown). Thus, our microarray and direct determinations of competence development support and extend the conclusion of Chastanet et al. (4) that ClpP proteolysis plays a negative regulatory role in natural competence development of *S. pneumoniae*. Our results further suggest that ClpP proteolysis plays a role in the termination of competence as well.

Transcriptional induction of major heat shock genes in *S. pneumoniae* R6. Microarray analysis of the *S. pneumoniae clpP* strain during normal exponential growth revealed a strong induction of genes that classically comprise the bacterial heat shock regulon (Table 4). Computer searches had previously demonstrated the presence of consensus HrcA binding sites (putative class I heat shock genes) (40) immediately upstream of the putative *groES-groEL* and *hrcA-grpE-dnaK-dnaJ* operons and CtsR consensus binding sites (putative class III heat shock genes) (9) preceding the *clpP*, *ctsR-clpC*, *clpE*, *clpL*, and *groES-groEL* loci (Table 5) (4, 26). Putative HcrA and CtsR binding sites were not identified upstream of other pneumococcal genes in the R6 genome (data not shown). Microarray analysis revealed significant transcriptional induction of these pneumococcal classes I and III heat shock genes in bacteria shifted to 45°C (Table 5). In contrast, transcription of *clpX* (putative specificity factor for ClpP) and *ftsH* (ATP-dependent protease) were not induced by thermal shock (Table 5), despite the fact that they are class IV heat shock genes in *B. subtilis* (48). Last, de Saizieu and coworkers (8) found that expression of the *clpL* and *groEL* heat shock genes was induced as pneumococcus entered the late exponential phase of growth. We found that transcription of the remaining class I and class III heat shock genes were induced in late exponential compared to early exponential cultures of *S. pneumoniae* R6 grown in CDM (Table 5). Unlike the heat shock genes, expression of *clpX* and *ftsH* was not affected by growth phase (Table 5).

DISCUSSION

Unlike most other bacterial species, *S. pneumoniae* contains only two ATP-dependent proteases, ClpP and FtsH, which are encoded by single-copy genes (23). We report here that *clpP* plays complex, pleiotropic roles in pneumococcal stress resistance, gene expression, regulation of genetic competence, and virulence in a murine host. *clpP* mutants of virulent *S. pneumoniae* strain D39 exhibited a very limited range of growth centered around 37°C. As noted before (4), *clpP* mutants of *S. pneumoniae* do not grow at 40°C, and we show further that this temperature sensitivity is complemented by a wild-type copy of *clpP*+ (Fig. 1). We found that D39 *clpP* mutants were cold sensitive and grew poorly at 30°C (see Results), whereas R6

TABLE 4. Transcription profile of *S. pneumoniae* R6 Δ*clpP* mutant growing exponentially in CDM at 37°C[a]

Role and gene name	Description/putative function	Fold change relative to R6 *clpP*⁺ parent[b]
Heat shock and proteolysis		
clpC	ATP-dependent Clp protease, ATP-binding subunit	2.6/3.8
ctsR	Transcriptional regulator, class III heat shock genes	3.7/3.4
clpL	ATP-dependent Clp protease, ATP-binding subunit	24.9/13.2
clpE	ATP-dependent Clp protease, ATP-binding subunit	2.4/1.8
hrcA	Transcriptional regulator, class I heat shock genes	4.2/8.6
grpE	Heat shock cochaperone	2.7/4.9
dnaK	Heat shock chaperone HSP70	2.9/6.4
dnaJ	Heat shock cochaperone	3.7/≈27.5
groEL	Heat shock chaperone	2.8/7.8
groES	Heat shock cochaperone	2.7/5.4
nisP	Subtilisin-like serine protease	≈4.2/4.4[c]
clpP*	ATP-dependent Clp protease, proteolytic subunit	(≈−22.0)/(−21.8)[d]
Competence and DNA metabolism		
comD	Competence factor dependent histidine kinase	≈17.0/≈15.1
comE	Competence factor dependent response regulator	24.7/31.6
comX1	Competence-specific sigma factor	3.5/3.4
spr1859	ComYA-like protein	20.5/12.1
cglD	ComG-like protein	6.8/6.8
cglC	Pilin-like wall structure	1.6/5.3
cglB	Prepilin transport ATPase	52.8/28.9
cglA	Prepilin transport pore	34.1/≈31.8
comF	Competence protein	2.4/3.0
celA	DNA binding	≈5.7/4.9
celB	DNA uptake pore	2.4/2.5
ssbB, cilA	ssDNA binding	10.6/10.7
dinF	Efflux pump	4.8/3.1
recA	DNA recombination	5.4/2.4
cinA	Unknown	4.7/4.1
comA	Transport, ATP binding protein	4.1/1.5
comB	Transport	≈23.3/11.9
Peptide/amino acid transport		
aliA	ABC transporter, oligopeptide transport	(−2.2)/(−2.7)
pncP	ABC transporter, unknown substrate	≈11.4/≈3.3
spr0100	ABC transporter, amino acid transport	2.8/≈2.9
spr1773	ABC transporter, ATP-binding protein	≈8.9/≈12.6
Sugar transport and metabolism		
malR	Transcriptional repressor, maltose operon	≈3.8/2.1
manN	PTS-EIIAB, mannose specific	2.1/2.2
bglA	6-Phospho-β-glucosidase	(−12.8)/(−2.9)
spr0277	Conserved hypothetical	(−7.6)/(−3.2)
spr0278	PTS-EIIB, sugar specific	(−7.6)/(−2.4)
bglG	Transcriptional antiterminator, BglG family	(−6.0)/(−2.3)
spr0280	PTS-EIIA, sugar specific	(−6.7)/(−2.6)
spr0281	Hypothetical	(−7.9)/(−3.1)
spr0282	PTS-EIIC, sugar specific	(−7.4)/(−2.9)
Gene regulation		
rr06	Unknown, response regulator	≈24.3/2.8
hk06	Unknown, sensor histidine kinase	9.7/3.1
plcR	Transcriptional regulator	≈4.2/3.5
Cell surface		
lytA	Major autolysin	2.7/1.7
dlytC	D-alanyl carrier protein	≈8.3/3.0
cbpD	Choline-binding protein D	≈9.4/4.0
htrA	Putative cell surface serine protease	(−2.0)/(−4.5)
Oxidative stress resistance		
sodA	Superoxide dismutase, manganese dependent	(−2.1)/(−2.0)
tpx	Thiolredoxin-linked thiol peroxidase	(−2.3)/(−3.1)
Metal transport/metabolism		
copY	Transcriptional regulator, copper transport	≈11.1/3.7
ctpA	P-type ATPase, copper transport	≈13.4/2.7
psaB	ATP-binding protein, Mn^{2+} transport	(−2.4)/(−2.0)
psaC	Membrane spanning permease, Mn^{2+} transport	(≈−2.5)/(−2.4)

Continued on following page

TABLE 4—*Continued*

Role and gene name	Description/putative function	Fold change relative to R6 clpP+ parent[b]
Transposon or insertion sequence		
spr1984	Transposase H, truncation	≈5.5/3.8
Hypothetical/unassigned		
spr1856	Unknown	23.6/13.8
spr1858	Unknown	9.5/14.8
spr1996	Probable integral membrane protein	2.3/2.3
spr0020	Unknown	≈8.2/2.9
spr0030	Unknown	≈9.5/≈8.3
spr0031	Unknown	4.0/≈8.7
spr0141	Unknown	(≈−86.9)/(−27.7)
spr0142	Unknown	(−13.0)/(−13.9)
spr0143	Unknown	(−9.2)/(−8.0)
spr0144	Conserved hypothetical	(−8.6)/(−9.9)
spr0182	Unknown	2.1/2.5
spr0328	Conserved hypothetical	2.3/2.3
spr0785	Unknown	(≈−4.9)/(−2.9)
spr0912	Unknown	(≈−5.6)/(−3.1)
spr0946	Conserved hypothetical	4.1/3.5
spr1549	Unknown	(−7.3)/(−2.8)
spr1629	Probable DNA binding protein	≈5.9/≈16.1
spr1630	Unknown	≈8.0/4.2
spr1762	Unknown	≈4.3/≈4.0
spr1765	Unknown	≈24.3/≈34.1
spr1767	Probable component, lantibiotic production	≈8.9/≈12.1
spr1768	Probable component, lantibiotic production	9.6/6.2
spr1772	Unknown	7.5/6.2

[a] Affymetrix microarray analysis of purified RNA was performed as described in Materials and Methods.
[b] Data from each of the two independent experiments.
[c] ≈, relative difference (n-fold) calculated relative to background instead of the RNA signal for clpP+ parent.
[d] Decreased transcription relative to the control is bracketed and indicated with a minus sign.

clpP mutants were only moderately impaired for growth at 30°C (Fig. 1). *clpX* and *clpC* mutants, which lack ATPase specificity factors that bind to ClpP and that have been implicated in stress tolerance in other bacteria (14, 28), were not impaired for growth compared to their parent strains (Fig. 1; data not shown).

Chastanet and coworkers (4) recently reported that *clpE* mutants of *S. pneumoniae* are temperature sensitive, implying a role for ClpE or possibly ClpEP in resistance to thermal shock. Microarray analysis showed that transcription of *clpP*, *clpC*, *clpL*, and *clpE*, but not *clpX* or *ftsH*, are subject to heat shock regulation (Table 5). Consistent with this result, *clpP*, *clpC*, *clpL* and *clpE* were members of class III heat shock operons regulated by CtsR protein (Table 5) (4). This finding distinguishes *S. pneumoniae* from *B. subtilis*, in which *clpX* and *ftsH* are class IV heat shock genes whose transcription is induced by elevated temperature (reviewed in reference 48). Combinations of *clpC*, *clpL*, and *clpX* mutations have not been tested for temperature sensitivity.

S. pneumoniae clpP mutants were temperature sensitive despite the fact that transcription of the class I and class III heat shock regulons was induced compared to that of the *clpP*+ parent in bacteria growing exponentially at 37°C (Table 5). The class I and class III heat shock regulons were also induced as *S. pneumoniae* R6 entered late exponential growth phase, which precedes the onset of autolysis (Table 5). Misfolded proteins, which likely accumulate in *clpP* mutants, and possibly during late phase growth of the R6 parent, are thought to be a signal for heat shock induction (40). Consistent with this in-

terpretation, *clpP* mutants were more sensitive to puromycin than their *clpP*+ parents (Table 3). Similar temperature and puromycin sensitivity were reported for a *clpP* mutant of *L. lactis*, which is a close relative of *S. pneumoniae* (12). Alternatively, ClpP could regulate the heat shock response directly. In *B. subtilis*, the McsA and McsB proteins, which are cotranscribed with *clpC*, modulate the targeting of CtsR by the ClpCP protease during stress (30). However, this exact mechanism is unlikely to occur in *S. pneumoniae*, because the *mcsA* and *mcsB* genes are absent by BLAST analysis (1).

The *clpP* operon is monocistronic on the basis of Northern analysis (Fig. 3) and the observation that Δ*clpP* insertion mutations did not diminish the amount of transcript synthesized from the downstream *orf-0657* (see Results). Moreover, Δ*orf-0657* insertion mutants did not exhibit the phenotypes of the Δ*clpP* insertion mutants (data not shown), and the Δ*clpP* phenotypes were complemented by an ectopic copy of wild-type *clpP*+ (Table 3; Fig. 1), indicating that these phenotypes were due solely to lack of *clpP* function. *clpP* is regulated by heat shock in all of the nonphotosynthetic bacteria reported to date (46). Previous work established that *S. pneumoniae clpP* is regulated by heat shock and is a member of the class III CtsR regulon (Table 5) (4). We found that the extent of *clpP* heat shock regulation in *S. pneumoniae* was considerably less than the 30-fold reported for heterologous expression in *B. subtilis* (4). Northern analysis and quantitative real-time RT-PCR indicated that *clpP* transcription was induced two- to threefold or five- to sevenfold in *S. pneumoniae* shifted to 45°C for 5 or 15 min, respectively (Fig. 3). In contrast, transcription of the *fabK*
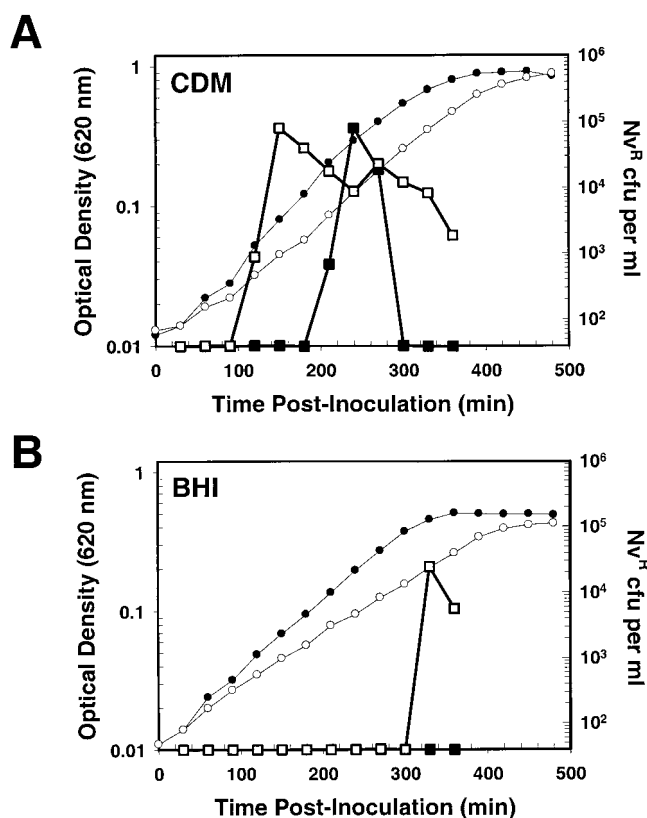
A

B

FIG. 4. ClpP negatively regulates competence development in *S. pneumoniae*. Cells grown in CDM (A) or BHI medium (B) were monitored for changes in OD_{620} and for the capacity to assimilate genomic donor DNA encoding resistance to novobiocin (see Materials and Methods). ●, OD_{620} of the *ssbB::lacZ* parent strain (CPM7); ○, OD_{620} of the Δ*clpP::aad9 ssbB::lacZ* strain (EL824); ■, Nv^r transformants recovered from the *ssbB::lacZ* parent strain (CPM7); □, Nv^r transformants recovered from the Δ*clpP::aad9 ssbB::lacZ* strain (EL824). The data are representative of two independent experiments.

gene, which is involved in fatty acid biosynthesis (23) and is not part of a heat shock regulon (Table 5), was not induced by this temperature shift (Fig. 3).

ClpP proteases do not generally seem to mediate the degradation of oxidatively damaged proteins (6). Therefore, it was somewhat unexpected to find that a *S. pneumoniae* Δ*clpP* mutant is more sensitive to H_2O_2 compared to its *clpP*$^+$ parent (Table 3). Global transcriptional analysis of this catalase-lacking bacterium showed that expression of the *tpx* (thioredoxin-linked thiol peroxidase) and *sodA* (manganese-dependent superoxide dismutase) genes was decreased in the Δ*clpP* mutant compared to its *clpP*$^+$ parent (Table 4). This repression could possibly account for the H_2O_2 sensitivity of the Δ*clpP* mutant. Further experiments are required to understand the link between ClpP function and sensitivity to H_2O_2.

The microarray and direct determination of competence development reported here support and extend the general conclusion of Chastanet and coworkers that ClpP acts as a negative regulator of competence expression (4). Moreover, our results are consistent with a role of ClpP in the termination of competence, which is normally transient in *S. pneumoniae* (44). We found that transcription of the majority of the known

competence regulon is strongly induced in the Δ*clpP* mutant at a time during exponential growth when the pathway is normally repressed (Table 4). Expression of an *ssbB-lacZ* reporter gene (data not shown) and DNA uptake (Fig. 4) were derepressed without the addition of CSP-1, and addition of synthetic CSP-1 further increased derepression and DNA uptake (data not shown). These results could be explained by increased expression of the ComDE two-component system, which senses CSP-1 levels and indirectly activates transcription of late genes in the competence pathway, or increased expression of the CSP-1 pheromone, which is cotranscribed in the *comCDE* operon (43). A direct role for ClpP-mediated proteolysis in regulating the cellular amounts of ComDE is a testable hypothesis, although the effect of ClpP could also be indirect. There is a precedent for ClpP-mediated proteolysis of a competence transcription factor in *B. subtilis* in which a complex forms containing the MecA adaptor protein, the ComK competence transcription factor, and ClpCP (54). *S. pneumoniae* encodes a MecA homolog (73% amino acid similarity to *B. subtilis*), but lacks other factors, such as ComK and ComS, involved in the *B. subtilis* MecA switch. Furthermore, our data combined with that of Chastanet and coworkers (4) suggest that ClpC and ClpE do not singly mediate the effect of ClpP on competence induction. The potential role of MecA and ClpX or ClpL in the regulation of *S. pneumoniae* competence by ClpP will be tested in future experiments.

Finally, we found that a D39 Δ*clpP* mutant was strongly attenuated for virulence in the mouse lung (Fig. 2) and sepsis models (>10^4 to 10^5 increase in LD_{50}; see Results). This attenuation phenotype was complemented by an ectopic copy of the *clpP*$^+$ gene (Fig. 2). In contrast, attenuation by a Δ*clpC* mutation was mild compared to that of the Δ*clpP* mutation (Fig. 2). It is unlikely that this attenuation is due to the temperature sensitivity of the D39 Δ*clpP* mutant, because the body temperature of mice ranges between 37.1 to 37.4°C (24), which supports the growth of the Δ*clpP* mutants in vitro (Fig. 1). Visual inspection of colonies of the D39 Δ*clpP* mutant on plates did not suggest any diminution of capsule production, which is strongly linked to *S. pneumoniae* virulence (38). Global transcription analysis showed that the transcription levels of several likely pathogenicity factors was reduced in the Δ*clpP* mutant compared to that of the *clpP*$^+$ strain, including *sodA* (manganese-dependent superoxide dismutase), *tpx* (thioredoxin-linked thiol peroxidase), and *psaABC* (manganese transporter) (Table 4). *sodA* and *psaABC* have previously been linked with the virulence of *S. pneumoniae* (2, 57), and antioxidizing enzymes, such as Tpx and Sod, have been linked to phagocyte resistance and virulence in several pathogens (10, 32). In addition, the transcription of several genes encoding cell surface proteins, which have been suggested to play possible roles in virulence (38), and proteins of unknown functions (23) are altered in Δ*clpP* mutants. Knockout mutants of the *hk06/rr06* two-component system, whose transcription is induced in the Δ*clpP* mutant (Table 4), are attenuated for virulence in our lung and sepsis animal models (data not shown) and those reported elsewhere (51). Together, these data imply that the large attenuation in virulence of Δ*clpP* mutants is a complex phenotype with many contributing factors. It remains to be determined how the loss of this major ATP-dependent

TABLE 5. Organization and transcriptional induction of R6 heat shock genes by temperature shift to 45°C, deletion of *clpP*, and entry of cultures into late exponential phase[a]

Regulatory element and operon organization[d]	Fold induction of[b]:			
	Heat shock (shift to 45°C)		$\Delta clpP$[c]	Entry into late exponential phase
	5 min	15 min		
HrcA-binding site (CIRCE)				
TTAGCACTCcagttcaaaGAGTGCTAA—30 nt—ATG—*groES*—*groEL*	5.6	5.8	2.7/5.4	3.2
TTAGCACTCtttataaaaGAGTGCTAA—109 nt—ATG—*hrcA*—*grpE*—*dnaK*—*dnaJ*	9.6	12.1	≈4.2/8.6	1.6
TTAGCACTCnnnnnnnnnGAGTGCTAA (consensus)[e]				
CtsR-binding site				
GGTCAAAAAAGGTCAAA (rev)[f]—67 nt—ATG—*clpP*	1.3	1.6	(≈−22.0)/(−21.8)	1.7
AGTCAAACAAGGTCAAg—25 nt—ATG—*ctsR*—*clpC*	1.7	2.5	3.7/3.4	1.7
GGTCAAAAATAGTCAAA (rev)—40 nt—ATG—*clpE*	2.0	3.2	2.4/1.8	2.8
GGTCAAAGATAGTCAAt—36 nt—ATG—*clpL*	10.9	38.1	24.9/13.2	9.2
GGTCAgAAATAGTCAAg (rev)—79 nt—ATG—*groES*—*groEL*	5.6	5.8	2.7/5.4	3.2
RGTCAAAnAnRGTCAAA (consensus)[g]				
NA—*clpX*	(−1.6)	(−2.0)	1.2/(−1.1)	(−1.1)
NA—*ftsH*	(−1.1)	(−1.6)	1.2/(−1.3)	1.1

[a] Growth of cultures, RNA extraction, and Affymetrix microarray analysis were performed as described in Materials and Methods.

[b] Relative induction (*n*-fold) was calculated for the first gene of each operon. ≈, relative change (*n*-fold) calculated relative to that of the background instead of 37°C control.

[c] Data are from each of two independent experiments.

[d] Number of nucleotides (nt) between the 3′ end of the putative regulatory element and the start codon for the first gene of each operon is given. NA, neither regulatory element was detected for *clpX* and *ftsH*.

[e] The consensus sequence was previously determined (9). Capitalized bases are strictly conserved. Lower case bases are not conserved.

[f] (rev), listed sequence is located on the noncoding strand.

[g] The consensus sequence was previously determined (39). (R), conservation of either A or G.

protease affects gene expression in *S. pneumoniae* at the level of protein stability and amount.

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