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Regulation of *Streptococcus pneumoniae clp* Genes and Their Role in Competence Development and Stress Survival

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In vitro *mariner* transposon mutagenesis of *Streptococcus pneumoniae* chromosomal DNA was used to isolate regulatory mutants affecting expression of the *comCDE* operon, encoding the peptide quorum-sensing two-component signal transduction system controlling competence development. A transposon insertion leading to increased *comC* expression was found to lie directly upstream from the *S. pneumoniae clpP* gene, encoding the proteolytic subunit of the Clp ATP-dependent protease, whose expression in *Bacillus subtilis* is controlled by the CtsR repressor. In order to examine *clp* gene regulation in *S. pneumoniae*, a detailed analysis of the complete genome sequence was performed, indicating that there are five likely CtsR-binding sites located upstream from the *clpE*, *clpP*, and *clpL* genes and the *ctsR-clpC* and *groESL* operons. The *S. pneumoniae ctsR* gene was cloned under the control of an inducible promoter and used to demonstrate regulation of the *S. pneumoniae clpP* and *clpE* genes and the *clpC* and *groESL* operons by using *B. subtilis* as a heterologous host. The CtsR protein of *S. pneumoniae* was purified and shown to bind specifically to the *clpP, clpC, clpE*, and *groESL* regulatory regions. *S. pneumoniae* Δ*ctsR*, Δ*clpP*, Δ*clpC*, and Δ*clpE* mutants were constructed by gene deletion/replacement. ClpP was shown to act as a negative regulator, preventing competence gene expression under inappropriate conditions. Phenotypic analyses also indicated that ClpP and ClpE are both required for thermotolerance. Contrary to a previous report, we found that ClpC does not play a major role in competence development, autolysis, pneumolysin production, or growth at high temperature of *S. pneumoniae*.

The regulatory pathways leading to the development of competence for DNA uptake in the gram-positive bacteria *Bacillus subtilis* and *Streptococcus pneumoniae* are strikingly similar. Proteins required for DNA binding and transport, encoded by the so-called late competence genes, are well conserved in the two bacteria (12), and the initial regulatory events involve extracellular peptide-signaling systems in both cases (57). Competence of *S. pneumoniae* for DNA transformation is controlled by a peptide quorum-sensing signal transduction pathway including the ComC-derived competence-stimulating peptide, the ComD membrane-bound histidine kinase, and the ComE response regulator, all of which are encoded by the *comCDE* operon (5, 19, 42). In *B. subtilis*, an unrelated extracellular peptide derived from the ComX polypeptide activates the ComP/ComA two-component system that is encoded by the *comPA* operon lying directly downstream from *comX* (25, 57).

Major differences exist, however, in the intermediate steps between the quorum-sensing device and the specific synthesis of competence proteins involved in DNA uptake and processing. In *S. pneumoniae*, this link is provided by a specific sigma factor, ComX, whose synthesis is dependent on the ComD/ComE two-component system (26). The *S. pneumoniae* ComX sigma factor is, in turn, required for the competence-specific expression of late *com* genes (26). In *B. subtilis*, no competence-specific sigma factor exists and the link between quorum sensing and late competence gene expression instead requires the release of the ComK transcription activator from targeted proteolysis by the ClpCP ATP-dependent protease (34, 59).

Clp ATP-dependent proteases are involved in regulation by proteolysis in several bacteria (45) and consist of a proteolytic subunit, ClpP, on which substrate specificity is conferred through association with ATPase subunits (ClpA, ClpC, and ClpX), which include members of the ubiquitous Hsp100 family (52). ComK synthesis in *B. subtilis* involves a complex network of two-component systems and global regulators, in which the general stress response genes *clpC* and *clpP* play essential roles (34, 35, 37). *clpC* and *clpP* of *B. subtilis* are both members of the class III group of heat shock genes, whose expression is controlled by the CtsR repressor (11).

There is growing evidence indicating that Clp proteins play an important role in the survival and virulence of pathogens during host infection. The *clpP* gene was isolated during a signature-tagged mutagenesis screen for virulence genes of *Salmonella enterica* serovar Typhimurium (21). In *Yersinia enterocolitica*, ClpP has been shown to modulate transcription of the adhesion invasion locus (*ail*) (41). Clp ATPases have also been shown to be involved in virulence, including ClpX of *Staphylococcus aureus* (31) and ClpB of *Leishmania* sp. (52). Furthermore, patients with leprosy or tuberculosis have antibodies specifically directed against mycobacterial ClpC (33). In *Listeria monocytogenes*, ClpP and the ClpC and ClpE Hsp100

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TABLE 1. *B. subtilis* and *S. pneumoniae* strains used in this study

Strain	Relevant genotype	Source or reference[a]
B. subtilis		
QB4991	*trpC2* Δ*ctsR amyE*::(*'lacZ aphA*3)	11
QB8068	*trpC2* Δ*ctsR amyE*::(*'lacZ cat*)	pAC5→QB4991
QB8069	*trpC2* Δ*ctsR amyE*::(*'lacZ aphA*3) *thrC*::(P*xylA-ctsR spc*)	pxyl-*ctsR-Spn*→QB4991
QB8070	*trpC2* Δ*ctsR amyE*::(*clpP'-bgaB cat*) *thrC*::(P*xylA-ctsR spc*)	pDL-*clpP-Spn*→QB8069
QB8071	*trpC2* Δ*ctsR amyE*::(*clpC'-bgaB cat*) *thrC*::(P*xylA-ctsR spc*)	pDL-*clpC-Spn*→QB8069
QB8132	*trpC2* Δ*ctsR amyE*::(*groES'-bgaB aphA*3)	pDK-*groE-Spn*→QB8068
QB8133	*trpC2* Δ*ctsR amyE*::(*groES'-bgaB aphA3*) *thrC*::(P*xylA-ctsR spc*)	pxyl-*ctsR-Spn*→QB8132
QB8134	*trpC2* Δ*ctsR amyE*::(*clpE'-bgaB aphA3*)	pDK-*clpE-Spn*→QB8068
QB8135	*trpC2* Δ*ctsR amyE*::(*clpE'-bgaB aphA3*) *thrC*::(P*xylA-ctsR spc*)	pxyl-*ctsR-Spn*→QB8134
S. pneumoniae		
R6	Subclone of R36A original isolate	Laboratory stock
R800	R6 derivative	27
R348	*ebg*::*spc comC*::pXF520	29
R354	*ebg*::*kan comC*::pXF520	29
R461	*ebg*::*kan comC*::pXF520 *spc93*::*clpP*	This study
R638	*spc93*::*clpP*	R461→R800
SP2000	*ebg*::*spc comC*::pXF520 Δ*clpP*::*aphA*3	Δ*clpP* DNA→R348
SP2001	*ebg*::*spc comC*::pXF520 Δ*clpC*::*aphA*3	Δ*clpC* DNA→R348
SP2002	*ebg*::*spc comC*::pXF520 Δ*clpE*::*aphA*3	Δ*clpE* DNA→R348
SP2003	*ebg*::*spc comC*::pXF520 Δ*ctsR*::*aphA*3	Δ*ctsR* DNA→R348
R895	*ssbB*::pR424	pR424→R800
R1053	*ssbB*::pR424 Δ*clpE*::*aphA*3	SP2002→R895
R1054	*ssbB*::pR424 *spc93*::*clpP*	R461→R895
R1055	*ssbB*::pR424 Δ*ctsR*::*aphA*3	SP2003→R895
R1056	*ssbB*::pR424 Δ*clpC*::*aphA*3	SP2001→R895

[a] Arrows indicate construction by transformation with chromosomal or plasmid DNA or PCR-generated DNA fragments.

ATPases are all required for stress survival, growth at high temperature, and virulence (13, 40, 47, 48).

In *S. pneumoniae*, a gene encoding an Hsp100-type Clp ATPase was isolated during a large-scale identification of virulence genes using the signature-tagged mutagenesis technique (44). Virulence of the *S. pneumoniae* mutant was significantly affected, as shown by using a mouse septicemia model (44). However, despite their ubiquity in bacteria and their important role in virulence, little is known about the regulation and function of *clp* genes in pathogens other than *L. monocytogenes*, where many of the *clp* genes have been shown to be controlled by the CtsR repressor (39). Elucidation of the regulatory pathways controlling *clp* gene expression is therefore likely to be important for our understanding of the virulence of gram-positive pathogens.

We show here that ClpP of *S. pneumoniae* plays a role in the maintenance of low levels of *comCDE* expression under conditions that do not support competence development. We also show that expression of the *S. pneumoniae clpP* and *clpE* genes and *clpC* and *groESL* operons is heat inducible and controlled directly by the CtsR repressor. Phenotypic analyses indicate that, unlike in *B. subtilis* and contrary to a previous report (4), ClpC is not involved in control of the expression of *S. pneumoniae* competence genes.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and transformation. *Escherichia coli* K-12 strain TG1 (14) was used for cloning experiments, and *E. coli* strain BL21 λ DE3 (54) was used for protein overexpression and purification. *E. coli* strains were grown in LB medium (50) and transformed by electroporation with selection on LB plates supplemented with ampicillin (100 μg/ml).

The *B. subtilis* and *S. pneumoniae* strains used in this work are listed in Table 1. The *B. subtilis* strains used in this study were derivatives of *B. subtilis* 168 *trpC2* and were grown in LB medium. They were transformed and selected by using plasmid or chromosomal DNA as previously described (35).

S. pneumoniae strains were grown in brain heart infusion (BHI; Difco) or CAT (46) medium. Expression of late competence genes was examined during growth in C+Y medium (28) as described previously (1). Transformation of *S. pneumoniae* was performed as described previously (1, 28), by using precompetent cells treated with synthetic competence-stimulating peptide 1 (25 ng/ml) to induce competence. Transformants were selected by plating on D medium agar plates supplemented with 4% horse blood (1) with kanamycin at 250 μg/ml or spectinomycin at 100 μg/ml.

In vitro *mariner* mutagenesis. Mutagenesis of *S. pneumoniae* chromosomal DNA was performed as previously described (29). Briefly, plasmid pR412 was used as the source for the 1,146-bp *spc mariner* minitransposon, which carries the *spc* spectinomycin resistance-encoding gene (29). Plasmid pR412 was incubated with chromosomal DNA from *S. pneumoniae* strain R800 in the presence of purified Himar1 transposase, leading to random insertion of the minitransposon within the chromosomal DNA (29). Gaps in the transposition products were repaired, and the resulting in vitro-generated transposon insertion library was used to transform *S. pneumoniae* (29).

DNA manipulations and general experimental procedures. Standard procedures were used to extract plasmids from *E. coli* (50). Chromosomal DNA of *B. subtilis* was isolated as previously described (35). Chromosomal DNA of *S. pneumoniae* was isolated by using the *B. subtilis* protocol, excepted for the lysis step, which was performed by resuspending cells in 0.1 ml of SEDS solution (NaCl at 0.15 M, EDTA at 0.15 M, deoxycholate [DOC] at 0.01%, sodium dodecyl sulfate at 0.02%) and incubating them for 5 min at 37°C. Amplification of DNA was performed by the PCR technique (38, 49) using *Pwo* polymerase (Roche) and *S. pneumoniae* R6 or R800 chromosomal DNA. Nucleotide sequences were determined by the dideoxy-chain termination method (51) using modified T7 DNA polymerase (55) (Amersham-Pharmacia).

Plasmids and plasmid constructions. The plasmids used in this study are listed in Table 2, and the oligonucleotides used are listed in Table 3. Plasmid pXT (10), a derivative of pDG1731 (18), was used to express genes under control of the xylose-inducible promoter P*xylA*. A *Bam*HI/*Eco*RI DNA fragment corresponding to the coding sequence of *S. pneumoniae* R6 *ctsR* was generated by PCR using oligonucleotides AC7(−25) and AC8(+469). Oligonucleotide positions are given relative to the translation initiation codon. This fragment was cloned into the respective sites of pXT to yield plasmid pxyl-*ctsR-Spn*, which allows *ctsR* expression under control of the xylose-inducible promoter, with integration as a

TABLE 2. Plasmids used in this study

Plasmid	Description	Source or reference
pDG1731	Plasmid allowing integration at the *thrC* locus	18
pXT	pDG1731 derivative allowing gene expression from the P*xylA* xylose-inducible promoter	10
pxyl-*ctsR-Spn*	pXT derivative carrying the *ctsR* coding sequence of *S. pneumoniae*	This study
pDL	Plasmid allowing integration at the *amyE* locus, and transcriptional fusion with *bgaB*	61
pAC5	Plasmid allowing integration at the *amyE* locus, with the pC194 *cat* gene	30
pAC7	Plasmid allowing integration at the *amyE* locus, with the *E. faecalis aphA3* cassette	60
pDK	pAC7 derivative carrying the *bgaB* gene	This study
pDL-*clpC-Spn*	pDL derivative carrying a *clpC′-bgaB* fusion	This study
pDL-*clpP-Spn*	pDL derivative carrying a *clpP′-bgaB* fusion	This study
pDK-*clpE-Spn*	pDK derivative carrying a *clpE′-bgaB* fusion	This study
pDK*groES-Spn*	pDK derivative carrying a *groE′-bgaB* fusion	This study
pET28a	Vector for overexpression of His-tagged proteins	Novagen
pET*CtsR-Spn*	pET28a derivative for overproduction of *CtsR*	This study
p5.00	Plasmid carrying the *luc* reporter gene	Martin Stieger
pEVP3	Plasmid containing the Cm^r-encoding gene	7
pR412	Plasmid carrying the *mariner* minitransposon	29
pR422	p5.00 derivative with the *luc* gene under control of the *ssbB* promoter	This study
pR424	pEVP3 derivative carrying the *ssbB′-luc* fusion from pR422, associated with the Cm^r-encoding gene	This study

single copy at the *thrC* locus. This plasmid was introduced into strain QB4991 or derivatives of QB8068, in which the endogenous *ctsR* gene is deleted, to give strains QB8069, QB8133, and QB8135 (see Table 1 for details). QB8068 is a derivative of strain QB4991 in which the *Enterococcus faecalis aphA3* Km^r gene (58) at the *amyE* locus was replaced with the pC194 *cat* chloramphenicol resistance gene by transformation with plasmid pAC5 (30).

Plasmid pDL (61) was used to construct transcriptional fusions between the promoter region of *clpC* (*clpC′-bgaB*) or *clpP* (*clpP′-bgaB*) and the *Bacillus stearothermophilus bgaB* gene, encoding a thermostable β-galactosidase (22), with subsequent integration at the *amyE* locus. Plasmid pDK is a derivative of pAC7 (60) in which the *E. coli lacZ* gene is replaced with the *B. stearothermophilus bgaB* gene from plasmid pDL. Plasmid pDK was used to construct transcriptional fusions between the promoter region of *clpE* (*clpE′-bgaB*) or the *groESL* operon (*groE′-bgaB*) and the *bgaB* gene. Transcriptional fusions in pDL or pDK were constructed by using *Eco*RI/*Bam*HI DNA fragments generated by PCR using oligonucleotides TM291(−345) and TM292(−15), TM289(−171) and TM290(−8), AC80(−260) and AC81(−3), and AC82(−210) and AC83(−9), corresponding to the *clpC*, *clpP*, *clpE*, and *groESL* promoter regions, respectively. Positions are given relative to the translation initiation codon. These fragments were cloned into the respective sites of plasmid pDL or pDK to produce plasmids pDL*clpC-Spn*, pDL*clpP-Spn*, pDK*clpE-Spn*, and pDK*groE-Spn*, respectively. Linearization of these plasmids at the unique *Pst*I site and transformation of the *B. subtilis* QB8068 or QB8069 strain with selection for chloramphenicol or Km^r yielded strains QB8071 (*clpC′-bgaB*), QB8070 (*clpP′-bgaB*), QB8134 (*clpE′-bgaB*), and QB8132 (*groES′-bgaB*).

CtsR was overexpressed by using pET*CtsR-Spn*, a derivative of pET28a (Novagen) in which a 478-bp *Bsa*I/*Xho*I DNA fragment corresponding to the *ctsR* coding sequence, generated by PCR using oligonucleotides AC6 and AC9, was cloned between the *Nco*I and *Xho*I sites of plasmid pET28a. This allows the creation of a translational fusion adding six histidine residues to the carboxy terminus of the protein and placing expression of the gene under the control of a T7 promoter.

clpC, *clpP*, *clpE*, and *ctsR* deletion/replacement mutants were constructed by first performing a ligation between DNA fragments (~500 bp) corresponding to the chromosomal DNA regions immediately upstream and downstream from each gene with an 877-bp *Eco*RI/*Bam*HI DNA fragment generated by PCR using oligonucleotides AC84 and AC85 that carries the *aphA3* Km^r gene deprived of its transcription initiation and termination signals. Fragments corresponding to the regions upstream and downstream from *clpC*, *clpP*, *clpE*, and *ctsR* were generated by PCR using oligonucleotides AC90 and AC91 and AC92 and AC93; AC94 and AC95 and AC96 and AC97; AC98 and AC99 and AC100 and AC101; and AC86 and AC87 and AC88 and AC89, respectively. Each resulting ligation was used as a template for PCR amplification using the external oligonucleotides (e.g., AC90 and AC93 for *clpC*). Products were purified following gel electrophoresis using the QIAquick Gel Extraction Kit (Qiagen). Purified DNA fragments were used directly for transformation of *S. pneumoniae* with selection for Km^r, and complete deletion of each gene was verified by PCR using additional oligonucleotides located further upstream and downstream from the original fragments.

Construction of an *ssbB′-luc* transcriptional fusion was carried out in two steps. The *luc* gene was placed under control of the *ssbB* promoter by construction of plasmid pR422 as follows. A DNA fragment overlapping the 5′ end of the *ssbB* gene was amplified from *S. pneumoniae* R800 chromosomal DNA by PCR using oligonucleotides MP122 and MP158 and digested with *Bam*HI-*Hin*dIII to generate a 246-bp fragment. This fragment was cloned into a 9,004-bp-long *Bam*HI-*Hin*dIII fragment from plasmid p5.00, which confers erythromycin resistance and carries the *Photinus pyralis luc* gene encoding firefly luciferase (53) to generate plasmid pR422 (Table 2). The *ssbB′-luc* transcriptional fusion was then associated with a chloramphenicol resistance-encoding gene to generate plasmid pR424 by cloning a 1,930-bp *Hin*dIII-*Sma*I fragment from plasmid pR422 containing the *ssbB′-luc* transcriptional fusion into a 2,022-bp *Hin*dIII-*Bsa*AI fragment from plasmid pEVP3 (Table 2). Transformation of *S. pneumoniae* cells with plasmid pR424, with selection for chloramphenicol resistance, leads to integration of the plasmid at the *ssbB* locus by a single-crossover event.

β-Galactosidase and luciferase assays. β-Galactosidase specific activities in *S. pneumoniae* were determined as described previously for *B. subtilis* (32, 35, 36), by using a Multiskan Ascent photometric microplate reader, and expressed as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside per minute per milligram of protein. Cell lysis was performed by adding 0.005% DOC–0.01% sodium dodecyl sulfate (final concentrations).

For detection of luciferase activity, strains were first grown in CAT medium to an optical density at 550 nm (OD_{550}) of 0.4. Cells were then resuspended in fresh 15% glycerol-containing CAT medium and frozen at −80°C. For inoculation, frozen cultures were thawed and diluted 1,500-fold in CAT medium and 280 μl was distributed among the wells of a 96-well Corning NBS plate. The cultures were incubated at 37°C in an Anthos LucyI luminometer. Approximately 3.5 h after inoculation and injection of 20 μl of a 10 mM luciferin solution in CAT medium, relative luminescence units (RLU) and OD_{492} were measured at 8-min intervals.

Overexpression and purification of CtsR. pET-*CtsR-Spn* was introduced into the BL21 λ DE3 (plysE) strain (Novagen), in which the T7 RNA polymerase gene is under the control of the inducible *lacUV5* promoter. The resulting strain was grown in LB medium at 30°C, and expression was induced during the exponential growth phase by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; 0.1 mM). Purification of CtsR was performed by using immobilized metal affinity chromatography as previously described (11).

Gel mobility shift DNA-binding assays. *Eco*RI/*Bam*HI DNA fragments, corresponding to the promoter regions of the *clpP* and *clpE* genes and the *clpC* and *groESL* operons, were generated by PCR with oligonucleotides TM289 and TM290, AC80 and AC81, TM291 and TM292, and AC82 and AC83, respectively. Fragments were radioactively labeled with [α-^{32}P]dATP using the Klenow fragment of DNA polymerase I (Gibco-BRL). Radiolabeling, DNA binding, and gel electrophoresis mobility shift assays were performed as previously described (11).

DNase I footprinting. DNA fragments corresponding to the *clpC, clpP, clpE*, and *groE* promoter regions used for DNase I footprinting were prepared by PCR using 20 pmol of oligonucleotides AC24 and AC25, AC22 and AC23, AC112 and

TABLE 3. Oligonucleotides used in this study

Name	Sequence	Description
TM289	GAAGAATTCGAAAAAGAAGAATGACTTGG	P*clpP′-bgaB* fusion
TM290	GGAGGATCCTTTTGAGTTTTAATTTTGTTGG	P*clpP′-bgaB* fusion
TM291	GAAGAATTCCACGCTTGGTATCTTGAGATTCAC	P*clpC′-bgaB* fusion
TM292	GGAGGATCCTCTCTTTAAACCTTGACCTTG	P*clpC′-bgaB* fusion
AC6	CTCCTCGAGCTTCCCTTTTCTATCTACCTC	CtsR overproduction
AC7	GGAGGATCCGTTTAAAGAGAGAGGTGGGTTTGT	P*xylA′-ctsR* fusion
AC8	GAAGAATTCAATAGTTCATCTTACTTCCCT	P*xylA′-ctsR* fusion
AC9	GGTGGTCTCCCATGAGATTTAAAAATACATCGGATCATA	CtsR overproduction
AC22	TCGAAAAGAAGAATGACTTGG	P*clpP* footprint
AC23	TCCTTTTGAGTTTTAATTTTGTTGG	P*clpP* footprint
AC24	GAATTAGGCTTAGATAAGTAG	P*clpC* footprint
AC25	CCAGATTGATCTAAAATCGCC	P*clpC* footprint
AC72	CGTAAGAACGTTCTCCACGGCTTGTTTG	Primer extension on *clpP*
AC80	ATTGAATTCATCGCAATGGAAATTTACGAAC	P*clpE′-bgaB* fusion
AC81	AAAGGATCCATCTACCTCATTTCTTTCTTTAGCC	P*clpE′-bgaB* fusion
AC82	GTGAGAATTCTGCAGGCCAAGATTTGGCAG	P*groE′-bgaB* fusion
AC83	TCTGGATCCCTCCATAATGAGATAG	P*groE′-bgaB* fusion
AC84	GTTAGAATTCGCTTCTTGGGGTAT	*aphA3* cassette
AC85	TAGGGATCCAAATCTAGGTACTAA	*aphA3* cassette
AC86	TCCCCATGGACTTAGCGGTGGGATG	*ctsR* deletion
AC87	ATCGAATTCCAAACCCACCTCTCTC	*ctsR* deletion
AC88	AAGGGATCCAAGATGAACTATTCAAAAGC	*ctsR* deletion
AC89	GTACCATGGCGTTGGCGTAAAGCC	*ctsR* deletion
AC90	AAACCATGGAATGAATGTATCGAAAGTGCC	*clpC* deletion
AC91	GTACGAATTCGTTGGCGTAAAGCC	*clpC* deletion
AC92	TGGGGATCCGGATATTCGTTTTGACCAGG	*clpC* deletion
AC93	ATGCCATGGCAAATTTTAACTGGCCTGC	*clpC* deletion
AC94	TGACCATGGTTCCAGCTGCTAAAGTTGGC	*clpP* deletion
AC95	TTTGAATTCTAATTTTGTTGGTCAAATG	*clpP* deletion
AC96	ACTGGATCCGCGCCCAGGAAACACTTG	*clpP* deletion
AC97	GCTACCATGGCAAGCGCCACAAACGATAG	*clpP* deletion
AC98	CCTCCATGGTAAAATAGTAACGATAAG	*clpE* deletion
AC99	CCTGAATTCTTTAAAGGTCAAAAATAG	*clpE* deletion
AC100	GCAAGGGATCCATTCAGATTAAATCTGCC	*clpE* deletion
AC101	ATACCATGGATAATGCAAGATTCC	*clpE* deletion
AC112	TTTTCATTGTAACAACTTCTCAAAGC	P*clpE* footprint
AC113	CCATTGAGATTGGTGTAAAGATG	P*clpE* footprint
AC114	GATTTGGCAGATTTGGTCTTGG	P*groE* footprint
AC115	CGGTCCCCTAATGGTTTCAAC	P*groE* footprint
AC124	AGCCTGCAAGGACAAAGCCTCC	Primer extension on *groE*
MP122	CGCGGATCCGGTGTAGACGTTAAACGTCC	*ssbB′-luc* fusion
MP158	GCCGCGAAGCTTCTCAGGATATTGCAGATAC	*ssbB′-luc* fusion

AC113, and AC114 and AC115, respectively. Labeling and DNase I treatment were performed as previously described (11).

RNA extraction and primer extension. *S. pneumoniae* strains were grown in BHI medium at 37°C without shaking until the OD_{600} reached 0.6. Cells were pelleted and frozen immediately. Frozen cells were resuspended in 0.4 ml of water and disrupted with a FastPrep cell disintegrator (Bio 101, Inc.) for 30 s at 4°C by using 0.5 g of glass beads (106 μm; Sigma) in the presence of 0.4 ml of 4% Bentone MA (Rheox) and 0.5 ml of phenol-chloroform-isoamyl alcohol, pH 8.0 (Amresco). After centrifugation for 2 min at 20,817 × *g* supernatants were successively extracted with phenol-chloroform (1:1, vol/vol) and then chloroform-isoamyl alcohol (24:1, vol/vol). RNA was precipitated with isopropanol in the presence of 0.2 M NaCl and resuspended in 20 μl of water. RNA concentrations were determined by measuring the A_{260}, and samples were stored at −20°C. Primer extensions were performed by incubating 20 μg of RNA, 1 pmol of oligonucleotide (previously labeled with [γ-^{32}P]ATP [110 TBq/mmol] using T4 polynucleotide kinase), and 25 U of avian myeloblastosis virus reverse transcriptase (Roche). Oligonucleotides were chosen so as to hybridize approximately 30 bp downstream from the translation initiation codon (see Table 3). The corresponding DNA sequencing reactions were carried out by using the same oligonucleotides and PCR-amplified DNA fragments carrying the respective promoter regions.

Database comparisons and sequence analysis. Computations were performed with the Genetics Computer Group sequence analysis software package (version 10.1; Genetics Computer Group, Inc., Madison, Wis.). Sequence comparisons with the GenBank database were accomplished with the National Center for Biotechnology Information BLAST2 (2) network service with the default parameter values provided. The complete *S. pneumoniae* type 4 genome sequence (56) was kindly made available by The Institute for Genomic Research (http://www.tigr.org).

RESULTS

Inactivation of *clpP* leads to overexpression of the *comCDE* operon in *S. pneumoniae*. In vitro *mariner* transposon mutagenesis of *S. pneumoniae* chromosomal DNA was used to generate a library of mutants by transformation of strain R354, which carries a chromosomal *comC′-lacZ* transcriptional fusion (see Materials and Methods and reference 29). Mutants displaying a *comCDE*^{up} or *cup* phenotype (29), i.e., increased β-galactosidase activity on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)-T^{-} plates (29), on which competence genes are normally not expressed, were chosen for further study. Chromosomal DNA was isolated from each mutant and used for backcross experiments by transforming the R354 parental strain with selection for spectinomycin resistance to ensure linkage of the *cup* phenotype with the transposon insertion. A

total of 42 insertions were found to be distributed into nine different location groups, two of which have been previously characterized (29). A third class was represented by a single mutant, strain R461, in which the transposon was inserted directly upstream from a previously uncharacterized gene whose product shares 55% amino acid sequence identity with ClpP of *B. subtilis*, the proteolytic subunit of the Clp ATP-dependent protease (35). This suggested that ClpP may play a role in the early steps of competence development in *S. pneumoniae*. However, it remained to be determined whether the *cup* phenotype linked to the transposon insertion was due to overexpression or loss of expression of the *clpP* gene.

The transposon insertion upstream from *clpP* (*spc93*::*clpP*) was introduced into the wild type *S. pneumoniae* R800 strain by transformation with chromosomal DNA from strain R461 to give strain R638. Primer extension experiments were carried out to examine *clpP* expression by using total RNA isolated from strain R638 or the original wild-type parental strain, R800. As shown in Fig. 1A, *clpP* is expressed in strain R800 during growth in BHI medium and this expression is abolished in strain R638. Analysis of the nucleotide sequence of the region preceding the transcription initiation sites revealed likely −10 and −35 sequences for the lower signal (TTGACC N17 TATAAT; see Fig. 7B) sharing strong similarities with the consensus sequences of promoters recognized by the vegetative form of RNA polymerase holoenzyme, $E\sigma^A$. The *mariner* minitransposon was inserted with the duplication of a GA dinucleotide 65 bp upstream from the *clpP* translation initiation codon, between the −35 and −10 sequences (see Fig. 7B), consistent with the fact that *clpP* expression is correspondingly abolished. No consensus promoter-type sequences could be identified upstream from the uppermost signal, suggesting that this signal could be due to transcription from a promoter recognized by a minor sigma factor or to processing from a larger transcript.

In order to confirm that ClpP negatively regulates *comCDE* expression in *S. pneumoniae*, a Δ*clpP* mutant (SP2000) was constructed by chromosomal replacement of the entire *clpP* coding sequence with the *E. faecalis aphA3* Km^r gene through a double-crossover event. This was carried out by transforming *S. pneumoniae* strain R348, which carries a *comC′-lacZ* transcriptional fusion, with a PCR-generated DNA fragment containing the *aphA3* Km^r-encoding gene and flanked by two 500-bp segments corresponding to the chromosomal regions immediately upstream and downstream of the *clpP* gene (see Materials and Methods).

As shown in Fig. 1B, expression of *comC′-lacZ* in strain R348 is very low during growth in BHI medium (approximately 4 nmol of ONP min^{-1} mg of $protein^{-1}$) and is strongly increased (up to 24-fold) in strain SP2000 (Δ*clpP*::*aphA3*), confirming that ClpP negatively regulates *comCDE* expression.

Inactivation of *clpC* or *clpE* does not affect competence development or expression of the *comCDE* and *ssbB* genes in *S. pneumoniae*. The fact that ClpP acts to negatively regulate expression of *comCDE* suggested that one of the Clp ATPase subunits may also act as a repressor. Analysis of the complete *S. pneumoniae* type 4 genome sequence (56) indicates that there are four genes encoding Clp ATPases, which we have designated *clpC*, *clpE*, *clpL*, and *clpX*, in accordance with established nomenclature (9, 52). ClpC, ClpE, and ClpL all belong to the Hsp100 family of Clp ATPases (9, 52).

In order to test whether the ClpC or ClpE ATPase plays a role in *comCDE* expression, Δ*clpC* and Δ*clpE* mutants of *S. pneumoniae* (strains SP2001 and SP2002, respectively) were constructed by transformation of strain R348 by the method described above for the Δ*clpP* mutant strain. As shown in Fig. 1B, *comC′-lacZ* expression in the Δ*clpC* and Δ*clpE* mutants was very low during growth in BHI medium and not significantly different from that in the R348 parental strain, indicating that, in contrast to ClpP, neither ClpC nor ClpE negatively regulates *comCDE* expression.

In order to examine the effects of ClpP, ClpC, and ClpE on late competence gene expression, chromosomal DNAs from strains R461 (*spc93*::*clpP*), SP2001 (Δ*clpC*::*aphA3*), and SP2002 (Δ*clpE*::*aphA3*) were used to introduce the corresponding mutations into *S. pneumoniae* strain R895 by transformation. The transposon insertion upstream from *clpP*, which practically abolishes expression of the gene (Fig. 1A), was used instead of the Δ*clpP*::*aphA3* mutation, since strain SP2000 (Δ*clpP*::*aphA3*) was unable to grow in C+Y competence medium, a phenotype similar to that reported for the *B. subtilis* Δ*clpP* mutant (35), whereas strain R461 (*spc93*::*clpP*) was able to grow, albeit poorly (Fig. 2A), suggesting that residual expression of *clpP* occurred despite the transposon insertion within the promoter region.

The resulting derivatives of strain R895 carry a chromosomal transcriptional fusion between the promoter of the *ssbB* gene, encoding single-stranded DNA-binding protein and known to be specifically induced during competence development (3, 43), and the *P. pyralis luc* gene, encoding firefly luciferase. The *ssbB* gene, also known as *cilA* (3), is specifically transcribed by RNA polymerase holoenzyme containing the ComX competence-specific sigma factor (26). Expression of *ssbB′-luc* has been shown to be directly correlated with the kinetics of transformation during competence development in *S. pneumoniae* (M. Prudhomme and J. P. Claverys, unpublished results).

As shown in Fig. 2A, no significant difference in the expression of *ssbB′-luc* was observed during growth in C+Y medium between the different *clp* mutants and the otherwise isogenic R895 reference strain, indicating that ClpP, ClpC, and ClpE are not required for competence development in *S. pneumoniae* under these conditions. Indeed, transformation assays during growth in C+Y medium for the *S. pneumoniae* Δ*clpC*::*aphA3* mutant (strain SP2001) were not significantly different from that of the R348 parental strain (data not shown).

However, when cells were grown in CAT medium, in which derivatives of the R800 laboratory strain do not develop spontaneous competence, expression of *ssbB′-luc* was increased approximately 50-fold in strain R1054 (*spc93*::*clpP*), confirming the negative role of ClpP on competence gene expression in *S. pneumoniae* (Fig. 2B). Furthermore, unlike otherwise isogenic parental strain R354, the *spc93* mutant (strain R461) developed spontaneous competence for transformation in CAT medium (data not shown), which is fully consistent with the isolation of the mutant on the basis of a *cup* phenotype on X-Gal-T^- plates. The fact that the only transposon insertion at the *clpP* locus leading to a *cup* phenotype was found upstream from the gene and not within the coding sequence is not un-

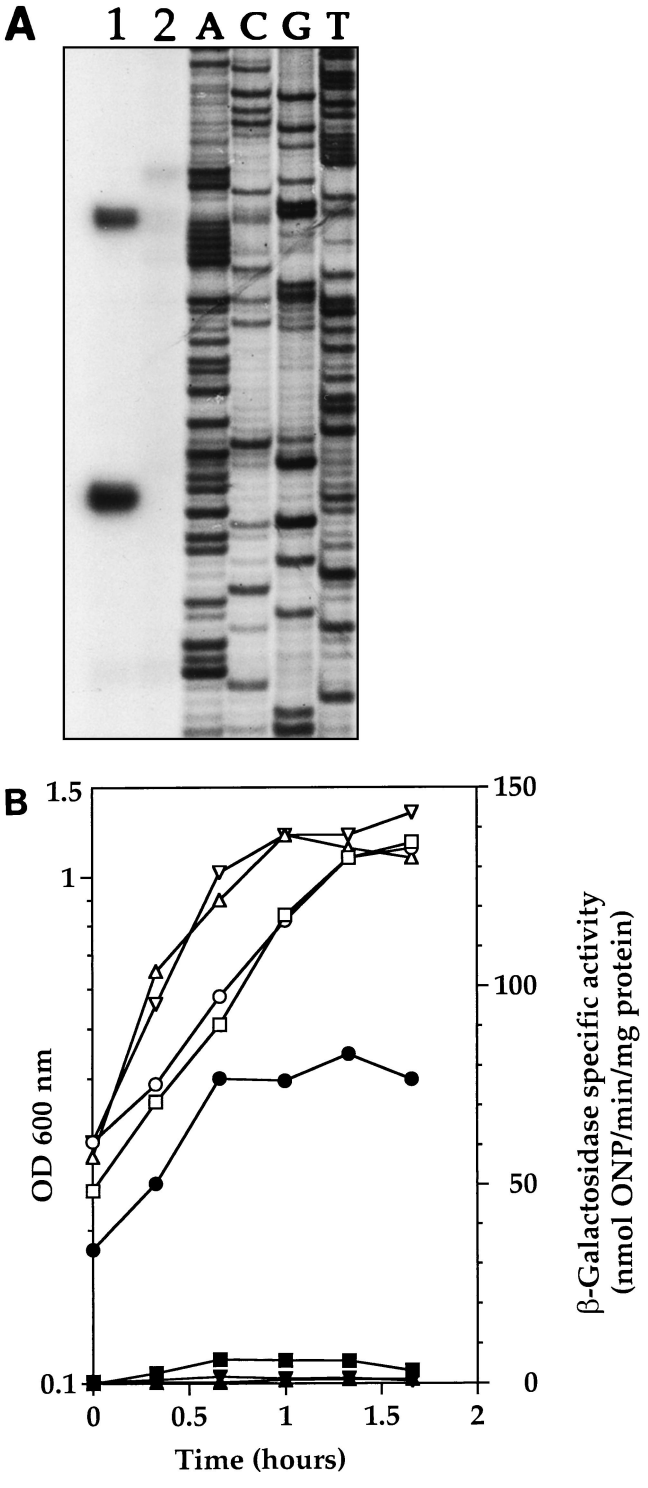

FIG. 1. (A) *clpP* expression in strain R638 is drastically reduced. Primer extension analysis of *clpP* mRNA was performed with total RNAs isolated from parental strain R800 (lane 1) and *spc93*::*clpP* mutant strain R638 (lane 2). The corresponding DNA sequence is shown on the right. (B) Expression of a *comC'-lacZ* fusion is strongly increased in a Δ*clpP* mutant. Strains SP2000 (Δ*clpP*::*apha3*) (○, ●), SP2001 (Δ*clpC*::*apha3*) (▽, ▼), and SP2002 (Δ*clpE*::*apha3*) (△, ▲) and parental strain R348 (□, ■) were grown in BHI medium. Open symbols indicate the OD_{600}, and solid symbols indicate β-galactosidase specific activity, expressed as nanomoles of ONP per minute per milligram of protein.

expected, since insertions disrupting the gene would be associated with a growth defect on X-Gal T^- plates (29), a medium comparable to CAT, in which the Δ*clpP*::*aphA3* mutant is also unable to grow.

CtsR of *S. pneumoniae* negatively regulates expression of *clpP*, *clpE*, and the *clpC* operon. Analysis of the nucleotide sequence of the *S. pneumoniae clpP* promoter region revealed the existence of a likely operator site for the CtsR repressor of stress response genes, whose existence in *S. pneumoniae* was previously reported (11), suggesting that, as in *B. subtilis*, the *clpP* gene may belong to the CtsR regulon. A detailed DNA motif analysis of the complete *S. pneumoniae* type 4 genome sequence (56), carried out using the consensus CtsR heptad direct repeat operator sequence (A/GGTCAAA NAN A/GG TCAAA; 11), revealed only five candidate CtsR-binding sites.

These were located upstream from the *clpE*, *clpL*, and *clpP* genes, as well as the *ctsR-clpC* and *groESL* operons. In order to investigate regulation of the *S. pneumoniae clp* genes, the model gram-positive bacterium *B. subtilis* was used as a heterologous host. Regulation by CtsR of *S. pneumoniae* was studied in derivatives of *B. subtilis* strain QB4991, in which the entire *B. subtilis ctsR* gene is deleted (11).

The resulting strains contain the *S. pneumoniae ctsR* gene cloned under control of the P*xylA* xylose-inducible promoter and integrated as a single copy at the *thrC* locus, as well as transcriptional fusions between the promoter regions of the *S. pneumoniae clpC* (strain QB8071), *clpP* (strain QB8070), and *clpE* (strain QB8135) genes and the *bgaB* gene of *B. stearothermophilus*, which encodes a thermostable β-galactosidase, integrated as single copies at the *amyE* locus (see Materials and Methods).

Strains QB8071, QB8070, and QB8135 were grown at 37°C in LB medium in the presence or absence of xylose, and β-galactosidase activities were assayed (reported as nanomoles of ONP per minute per milligram of protein). As shown in Fig. 3A, *clpC'-bgaB* was weakly expressed (~20 U of enzyme activity) in the presence of xylose when CtsR was produced and its expression was increased approximately eightfold in the absence of xylose. Expression of *clpP'-bgaB* and *clpE'-bgaB* fusions followed similar patterns (Fig. 3B and C), with basal levels of 40 U for *clpP* and 80 U for *clpE* in the presence of xylose, increasing approximately eightfold in the absence of xylose when *ctsR* was not expressed. These results clearly indicate that CtsR negatively regulates the *S. pneumoniae clpC*, *clpP*, and *clpE* genes.

CtsR binds specifically to the regions upstream from *clpP*, *clpE*, and the *clpC* operon. An in vitro approach was used to demonstrate the direct interaction of *S. pneumoniae* CtsR with its target sites. For this purpose, the *S. pneumoniae ctsR* coding sequence was cloned into the pET28a vector, generating a carboxy-terminal translational fusion with six histidine residues. The resulting His-tagged CtsR protein (approximately 19 kDa) was then overproduced in *E. coli* and purified with an Ni-nitrilotriacetic acid agarose column (see Materials and Methods).

Purified *S. pneumoniae* CtsR was used in gel mobility shift DNA-binding assays with DNA fragments corresponding to the promoter regions of *clpC*, *clpP*, and *clpE*. Radiolabeled, PCR-generated DNA fragments corresponding to positions −139 to +65 (*clpC*), −171 to −9 (*clpP*), and −137 to +62 (*clpE*), relative to the respective translation initiation codons,

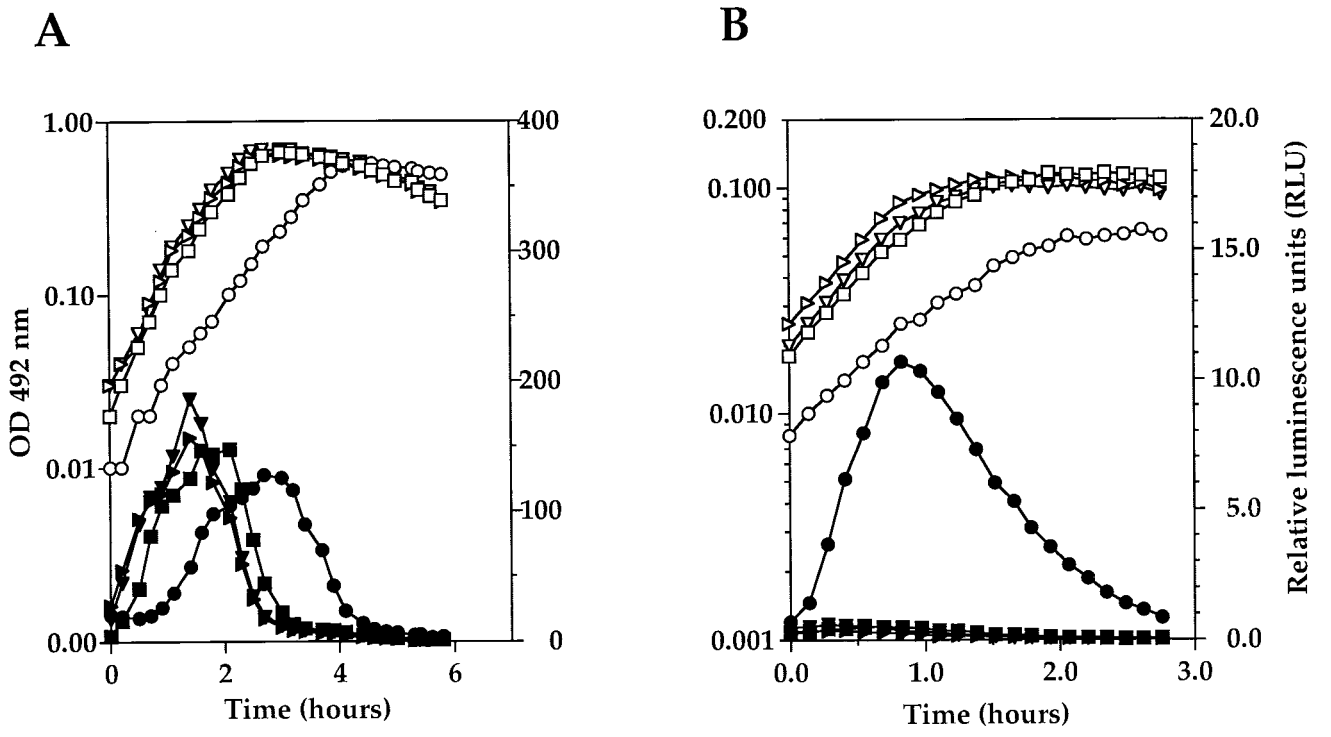

FIG. 2. Expression of *ssbB'-luc* in strains R1053 (Δ*clpE*::*apha3*) (▷, ▶), R1054 (*clpP*::*spc93*) (○, ●), and R1056 (Δ*clpC*::*apha3*) (▽, ▼) and parental strain R895 (□, ■). Strains were grown in C+Y competence-permissive medium (A) or CAT non-competence-permissive medium (B). Open symbols indicate the OD_{492}, and solid symbols indicate RLU expressed as a function of time.

were incubated with increasing amounts of CtsR. All DNA-binding assays were performed in the presence of an excess of nonspecific competitor DNA [1 μg of poly(dI-dC)]. As shown in Fig. 4A, CtsR bound specifically to all three radiolabeled promoter fragments, forming a single protein-DNA complex in each case, with complete displacement of the DNA fragments at the highest CtsR concentrations. These results indicate that CtsR of *S. pneumoniae* negatively regulates the expression of the *clpC*, *clpP*, and *clpE* genes by binding directly to their promoter regions.

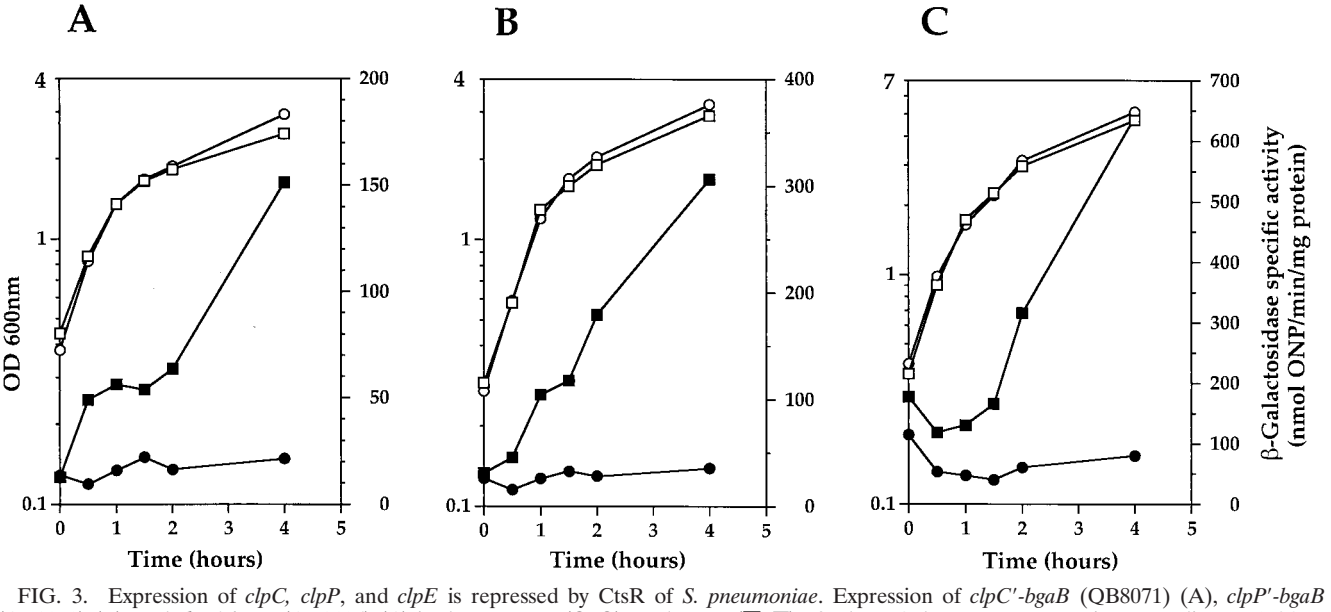

FIG. 3. Expression of *clpC, clpP*, and *clpE* is repressed by CtsR of *S. pneumoniae*. Expression of *clpC'-bgaB* (QB8071) (A), *clpP'-bgaB* (QB8069) (B), and *clpE'-bgaB* (QB8135) (C) in the presence (○, ●) or absence (□, ■) of xylose. Cultures were grown in LB medium at 37°C to an OD_{600} of 0.3, and xylose was added to one-half of the culture at a final concentration of 20 mM. Open symbols indicate the OD_{600}, and solid symbols indicate β-galactosidase specific activity, expressed as nanomoles of ONP per minute per milligram of protein.

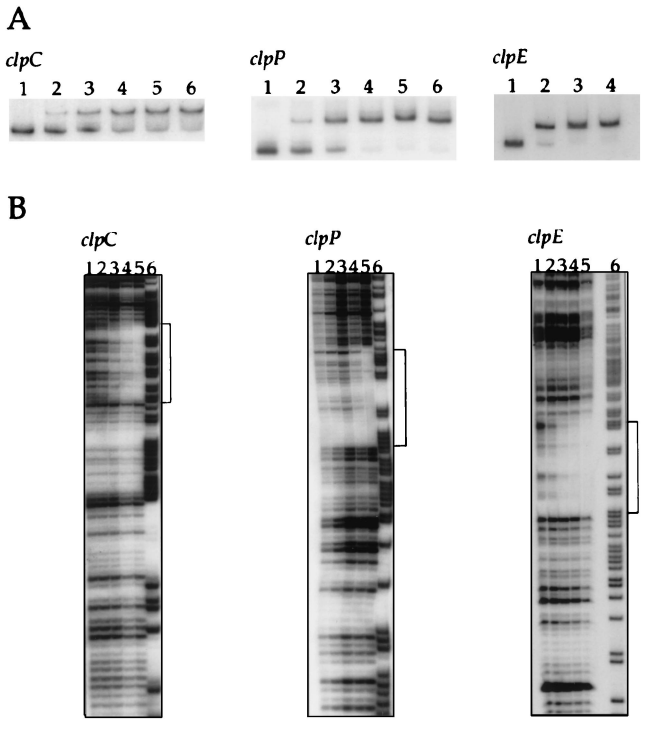

FIG. 4. CtsR binds specifically to the *clpC*, *clpP*, and *clpE* promoter regions. In gel mobility shift experiments (A), radiolabeled DNA fragments (10,000 cpm) corresponding to the *clpC, clpP*, and *clpE* promoter regions were incubated with increasing amounts of purified CtsR as follows: for *clpC* and *clpP*, lanes 1 to 6, 0, 10, 20, 40, 60, and 80 ng of CtsR, respectively; for *clpE*, lanes 1 to 4, 0, 20, 40, and 80 ng of CtsR, respectively. In DNase I footprinting analyses of CtsR binding (B), 50,000 cpm of each radiolabeled DNA fragment corresponding to the *clpC, clpP*, or *clpE* promoter region was incubated with increasing amounts of purified CtsR as follows: lanes 1 to 5, 0, 100, 200, 400, and 800 ng of CtsR, respectively; lane 6, G+A Maxam and Gilbert reaction of the corresponding DNA fragments. Regions protected by CtsR are shown by brackets.

In *B. subtilis*, CtsR binds to a highly conserved directly repeated sequence (A/GGTCAAA NAN A/GGTCAAA) that often overlaps the −35 and −10 sequences or the transcriptional start site of the controlled promoters (11). DNase I footprinting assays were performed on *S. pneumoniae* DNA fragments carrying the *clpC*, *clpP*, and *clpE* promoter regions to determine the extent of the protected region and the precise location of the CtsR-binding sites (Fig. 4B). When the nontemplate strand of the *clpC* DNA fragment was end labeled, CtsR protected a region extending from position −46 to position −21 (Fig. 4B). CtsR protected regions on the nontemplate strands extending from position −86 to position −62 for *clpP* and from position −58 to position −35 for *clpE* (Fig. 4B). All positions are given relative to the respective translational start sites.

The protected regions within the *clpC*, *clpP*, and *clpE* promoter sequences each contain the direct repeat CtsR operator site, in agreement with sequence analysis predictions (see Fig. 7B).

The *groESL* operon of *S. pneumoniae* is a novel member of the CtsR regulon. As mentioned above, genome sequence analysis also revealed the existence of a potential CtsR-binding site upstream from the *groESL* operon, which encodes the classical chaperonins GroES and GroEL. This was somewhat surprising, since in *B. subtilis* and many other low-G+C gram-positive bacteria, the *groESL* operon belongs to the class I family of heat shock genes, known to be controlled by the HrcA repressor through its interaction with the highly conserved CIRCE operator sequence (TTAGCACTC-N9-GAGT GCTAA) (20). The CtsR regulons of *B. subtilis* and *L. monocytogenes* consist of genes encoding subunits of the Clp ATP-dependent protease (11, 39), and this would be the first example of a *groESL* operon regulated by CtsR. Inspection of the nucleotide sequence of the *groESL* upstream region revealed the presence of a highly conserved CIRCE operator sequence as well, located 16 bp downstream from the potential CtsR-binding site (see Fig. 7B). This tandem operator arrangement suggests that the *S. pneumoniae groESL* operon may be dually regulated by both HrcA and CtsR.

CtsR-dependent regulation of *groESL* expression was examined by using *B. subtilis* as a heterologous host as described above for the *clp* genes. Strain QB8133 carries a *groES′-bgaB* transcriptional fusion integrated at the *amyE* locus and the *S. pneumoniae ctsR* gene cloned under control of the P*xylA* xylose-inducible promoter at the *thrC* locus (see Materials and Methods). As shown in Fig. 5, *groES′-bgaB* was weakly expressed (~100 U) in the presence of xylose when CtsR was produced and its expression was increased approximately

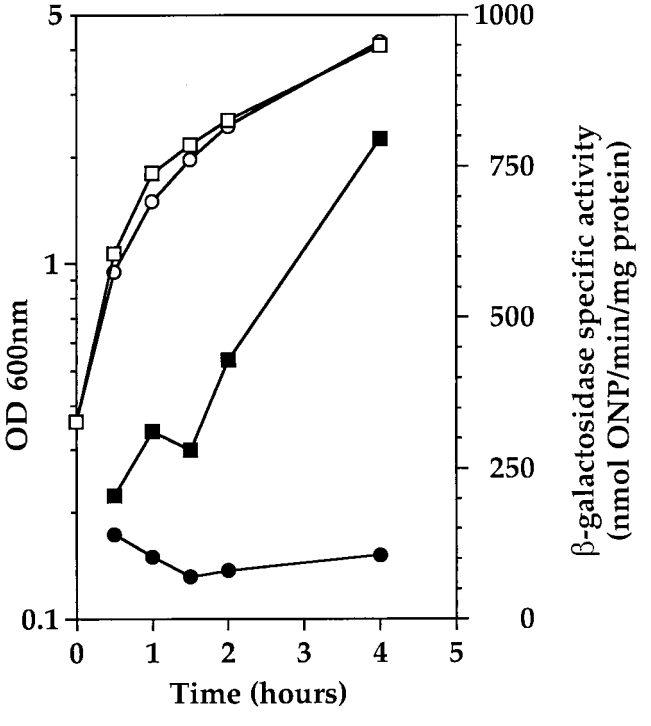

FIG. 5. *groESL* is under negative control of CtsR in vivo. Expression of *groE′-bgaB* (QB8133) in the presence (○, ●) or absence (□, ■) of xylose. Cultures were grown in LB medium at 37°C to an OD_{600} of 0.3 and divided in two, and xylose was added to one of the cultures at a final concentration of 20 mM. Open symbols indicate the OD_{600}, and solid symbols indicate β-galactosidase specific activity, expressed as nanomoles of ONP per minute per milligram of protein.

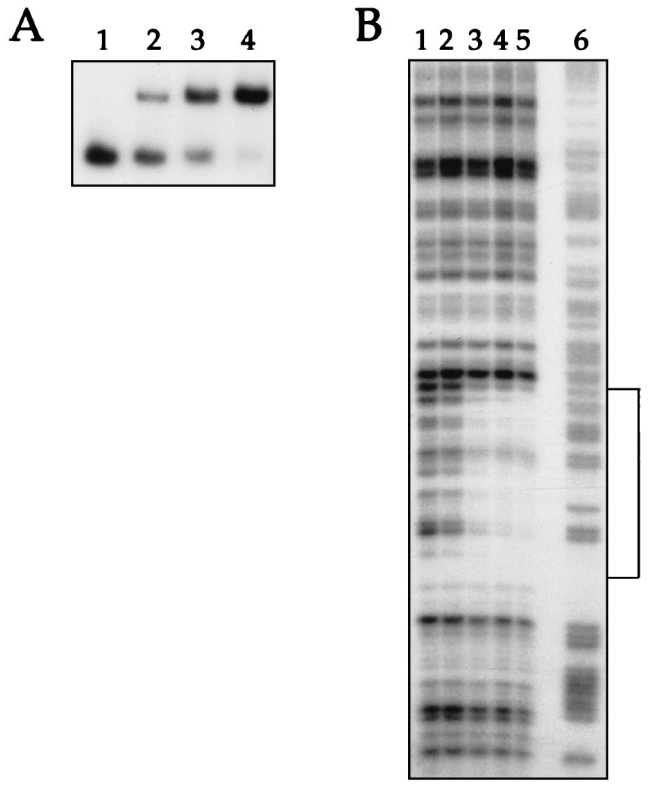

FIG. 6. CtsR binds specifically to the *groESL* promoter region. In gel mobility shift experiments (A), radiolabeled DNA fragments (10,000 cpm) were incubated with increasing amounts of purified CtsR. Lanes 1 to 4, 0, 20, 40, and 80 ng of CtsR, respectively. In DNase I footprinting experiments (B), 50,000 cpm of the radiolabeled DNA fragment corresponding to the promoter region was incubated with increasing amounts of purified CtsR. Lanes: 1 to 5, 0, 100, 200, 400, and 800 ng of CtsR, respectively; 6, G+A Maxam and Gilbert reaction of the corresponding DNA fragment. The region protected by CtsR is indicated by a bracket.

eightfold in the absence of xylose, confirming the prediction that CtsR negatively regulates *groESL* expression.

CtsR binds specifically to the regions upstream from the *groESL* operon. Purified CtsR was used in gel mobility shift DNA-binding assays with a radiolabeled, PCR-generated DNA fragment corresponding to the promoter region of the *groESL* operon (positions −20 to +23 relative to the translation initiation codon). As shown in Fig. 6A, CtsR bound specifically to the radiolabeled promoter fragment, forming a single protein-DNA complex, with complete displacement of the DNA fragment at the highest CtsR concentration. DNase I footprinting assays were performed on the same DNA fragment to determine the extent of the protected region and the precise location of the CtsR-binding sites (Fig. 6B). When the nontemplate strand of the *groESL* DNA fragment was end labeled, CtsR protected a region extending from position −97 to position −73, relative to the translational start site, which contains the predicted direct repeat CtsR operator site (Fig. 6B and 7B).

Expression of the *clpP* and *clpE* genes and the *clpC* and *groESL* operons is induced by heat shock. Expression of CtsR-dependent genes is known to be induced under general stress conditions, including heat shock (9, 11, 23, 35, 39). In order to test whether the repression by *S. pneumoniae* CtsR also responds to heat shock, expression of the *clpC′-bgaB*, *clpP′-bgaB*, *clpE′-bgaB*, and *groES′-bgaB* transcriptional fusions was tested by using *B. subtilis* as a heterologous host in strains QB8071, QB8070, QB8135, and QB8133 during growth in LB medium in the presence of xylose at 37 or 48°C.

As shown in Table 4, all four genes were expressed at a low level at 37°C and strongly induced, from 20- to 30-fold, after a shift to 48°C, which is consistent with a CtsR-dependent stress response. This was confirmed in *S. pneumoniae* for the *groESL* operon at the mRNA level by primer extension experiments (data not shown), in agreement with a previous report showing that synthesis of GroEL is induced in response to heat shock (6).

Genes of the CtsR regulon are derepressed in an *S. pneumoniae* Δ*ctsR* mutant. To confirm CtsR-dependent regulation of the *clpP* and *clpE* genes and the *clpC* and *groESL* operons in *S. pneumoniae*, a Δ*ctsR* mutant of *S. pneumoniae* (strain SP2003) was constructed by transformation of strain R348 by chromosomal replacement of the entire *ctsR* coding sequence with the *aphA3* Km^r-encoding gene through a double-crossover event. Primer extension experiments were performed to examine expression of CtsR-dependent genes by using total RNA isolated from Δ*ctsR*::*aphA3* mutant strain SP2003 or otherwise isogenic parental strain R348. As shown in Fig. 7A, expression of the *clpP* and *clpE* genes and the *clpC* and *groESL* operons is increased in the Δ*ctsR*::*aphA3* mutant during growth in BHI medium at 37°C, confirming their repression by CtsR in *S. pneumoniae*. The transcription start sites, the potential promoter sequences, the CtsR and HrcA operator sequences, and the regions protected by CtsR in DNase I footprinting experiments are indicated in Fig. 7B. The Δ*ctsR*::*aphA3* mutation had no effect on *comCDE* or *ssbB* expression in *S. pneumoniae* (data not shown), suggesting that competence regulation is not strongly affected by derepression of CtsR-dependent genes.

ClpP and ClpE are required for growth at high temperature. Mutations inactivating *clp* genes in *B. subtilis* are known to be highly pleiotropic, affecting cell motility, growth at high temperature, competence development, and sporulation (35, 37). A phenotypic analysis of the *S. pneumoniae* Δ*ctsR*, Δ*clpC*, Δ*clpP*, and Δ*clpE* mutants and the R348 parental strain was carried out. All four mutants grew as did parental strain R348 as standard diplococcal cells in BHI medium at 37°C, with no cell filamentation or long-chain formation. No differences in penicillin- or DOC-induced autolysis were observed for the four mutants compared to the R348 parental strain, as shown in Fig. 8A for strain SP2001 (Δ*clpC*::*aphA3*), suggesting that production of the major autolysin, LytA, is not affected. All four mutants presented characteristic hemolytic halos on D-horse blood agar plates, comparable to that of the parental strain, suggesting that pneumolysin production is not affected.

Growth at different temperatures was examined in BHI medium. Cultures growing exponentially at 37°C were divided and incubated at different growth temperatures. As shown in Fig. 8B, the R348 parental strain and the Δ*ctsR* and Δ*clpC* mutants grew normally at 37 and 40°C, poorly at 42°C, and not at all at 44°C. In contrast, the Δ*clpE* and Δ*clpP* mutants presented a temperature-sensitive growth phenotype at 40, 42, and 44°C, suggesting that ClpE and ClpP are essential for adaptation to high temperatures. Our results also indicate that ClpC does

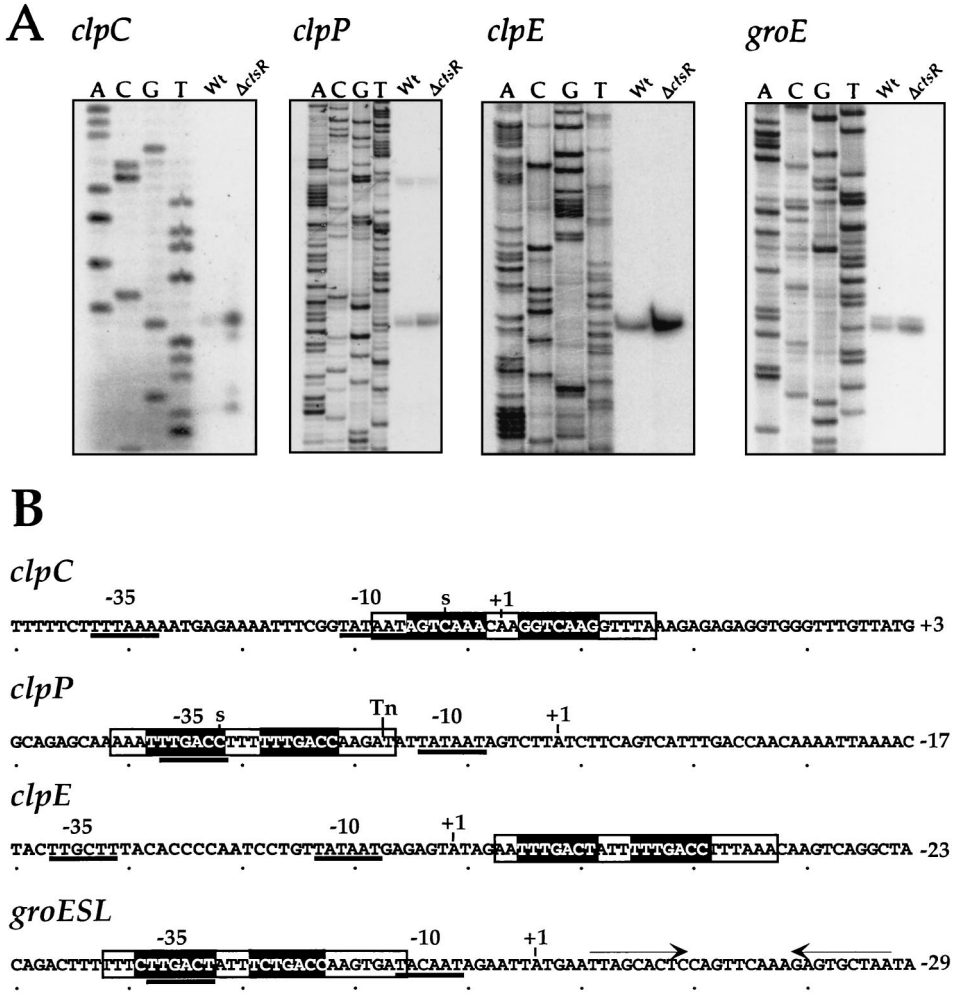

FIG. 7. (A) Primer extension analysis of *clpC*, *clpP*, *clpE*, and *groE* mRNAs in the Δ*ctsR* mutant. Total RNA isolated from wild-type (Wt) parental strain R348 or the Δ*ctsR* mutant (SP2003) was used as the template for reverse transcriptase. The corresponding DNA sequences are shown on the left. (B) Nucleotide sequences of the *clpC*, *clpP*, *clpE*, and *groE* promoter regions. Potential −35 and −10 sequences are underlined, transcriptional start points are indicated by S and +1, CtsR heptad direct repeat operator sequences are shaded, the CIRCE operator sequence is indicated by inverted arrows, and regions protected by CtsR in DNase I footprint experiments are boxed. Tn indicates the transposon insertion site upstream from *clpP* in strain R461. Positions are numbered relative to the translation initiation codon.

not affect competence development in *S. pneumoniae* and that it is not involved in autolysis, cell filamentation, pneumolysin expression, or growth at high temperature, contrary to a recent report (4).

DISCUSSION

The Clp ATP-dependent protease plays an important role in regulation through proteolysis, in both *E. coli* (15, 17) and *B. subtilis* (34). We show here that ClpP of *S. pneumoniae* negatively regulates competence development by preventing expression of the *comCDE* operon under inappropriate conditions. Interestingly, the roles played by ClpP in competence development of *B. subtilis* and *S. pneumoniae* appear to be very different.

ClpP of *B. subtilis* provides a link between the initial quorum-sensing ComP/ComA signal transduction system and late *com* gene expression through targeted proteolysis of the ComK transcription activator (34, 59). ClpP is thus essential for competence gene expression, since in its absence, ComK is sequestered in an inactive form by the MecA/ClpC complex, preventing it from activating its own synthesis, as well as transcription of late *com* genes (34, 35, 59). In *S. pneumoniae*, however, the situation is quite different, since, as shown here, ClpP is not required for expression of late competence genes or competence development but, instead, acts negatively at the earliest

TABLE 4. Induction of *clp′-bgaB* and *groES′-bgaB* expression by heat shock

Strain	Promoter	β-Galactosidase sp act (nmol of ONP/min/mg of protein)		Ratio
		37°C	48°C	
QB8070	P*clpP*	35	1,200	34
QB8071	P*clpC*	20	520	26
QB8135	P*clpE*	80	1,600	20
QB8133	P*groES*	110	3,500	32

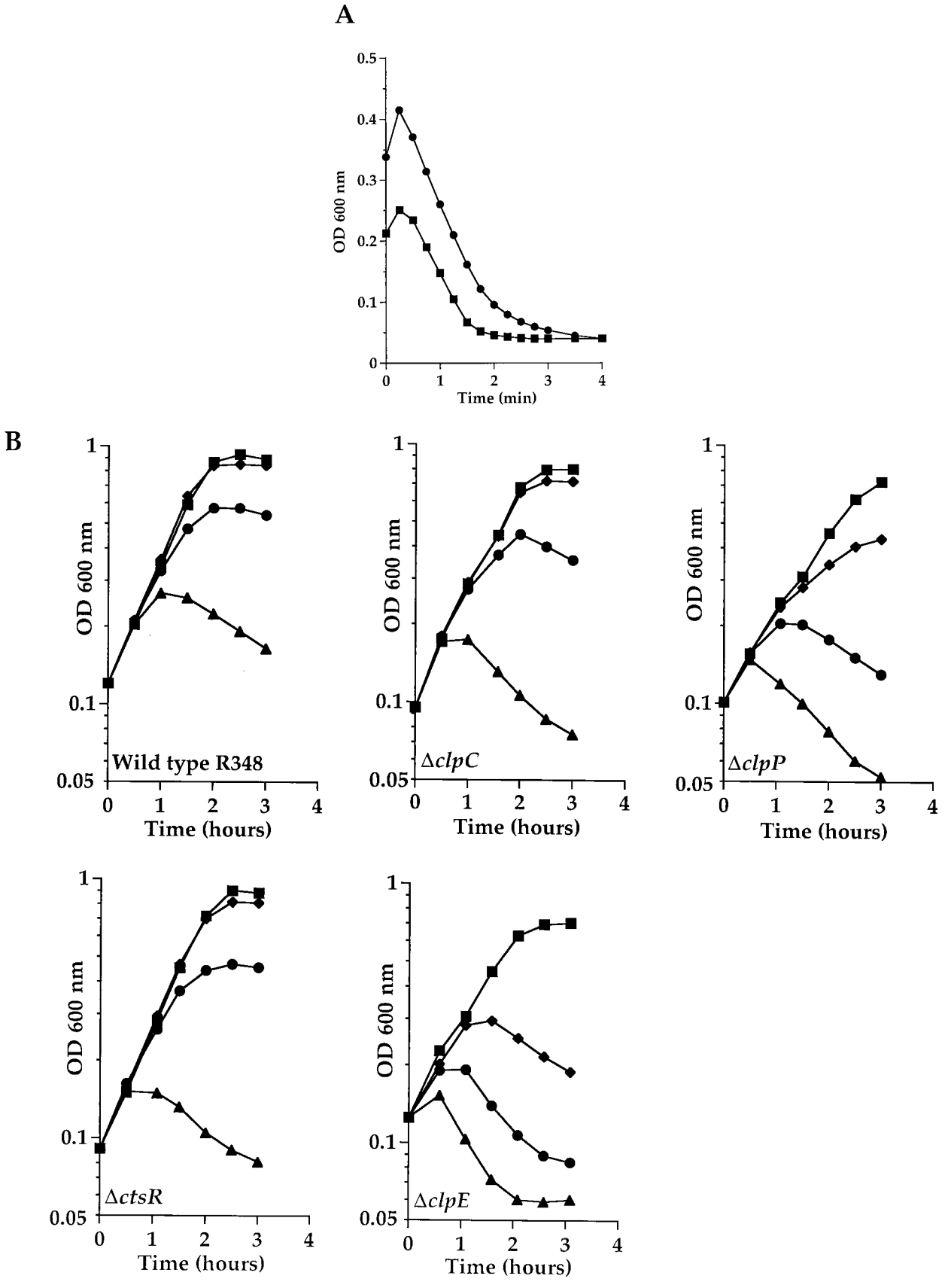

FIG. 8. (A) DOC-triggered autolysis is not affected in the Δ*clpC* mutant. Strain SP2001 (Δ*clpC*::*aphA3*) (●) and parental strain R348 (■) were grown in BHI medium until mid-exponential phase before induction of autolysis by addition of DOC (0.05% final concentration). (B) Growth of the *S. pneumoniae* R348 wild-type strain and Δ*clpC* (SP2001), Δ*clpP* (SP2000), Δ*ctsR* (SP2003), and Δ*clpE* (SP2002) mutant strains at 37°C (■), 40°C (◆),42°C (●), and 44°C (▲).

stages of the competence regulatory pathway to prevent inappropriate expression of the genes encoding the peptide quorum-sensing system. This is consistent with the idea that competence in *S. pneumoniae* is induced in response to changes in environmental conditions (8).

Many of the Clp proteins (ClpA, ClpX, and ClpC) act as ATPase subunits of the ATP-dependent Clp protease by associating with the ClpP proteolytic subunit, on which they confer substrate specificity (16, 17, 59). As shown in this report, neither ClpC nor ClpE plays a role in controlling competence gene expression, suggesting that one of the remaining Clp ATPases present in *S. pneumoniae*, ClpX or ClpL, may associate with ClpP instead.

Apart from *L. monocytogenes*, little is known about *clp* gene regulation in pathogenic bacteria, despite the fact that many of these genes play important roles in virulence (39). A detailed analysis of the complete *S. pneumoniae* type 4 genome sequence (56; http://www.tigr.org) indicates that only three of the four different types of heat shock response regulatory mechanisms originally defined in *B. subtilis* (11, 20) coexist in *S. pneumoniae*. Among them are the class I heat shock genes, defined as the HrcA regulon (the *dnaK* and *groESL* operons). There are no class II heat shock genes, since the σ^B stress sigma factor is not present in *S. pneumoniae*. We previously identified the *ctsR* gene of *S. pneumoniae* and several potential target genes from the genome sequence (11) and show here that class III regulation is present. Finally, class IV genes in *B. subtilis* are those whose induction by heat shock is not dependent on HrcA, σ^B, or CtsR. Many of these genes are present in *S. pneumoniae*, such as *clpX* and *ftsH*, and one can speculate that they will also prove to be heat shock genes.

We have shown that expression of the *S. pneumoniae clpP* and *clpE* genes and *clpC* and *groESL* operons is heat inducible and controlled directly by the CtsR repressor. Although ClpP acts negatively on competence gene expression, this role appears to be restricted to growth conditions under which competence genes are not expressed. Indeed, in a Δ*ctsR* mutant in which *clpP* expression is derepressed, competence gene expression is unaffected during growth in C+Y competence medium, suggesting that negative regulation by ClpP can no longer take place. Among the members of the CtsR regulon, the *groESL* operon of *S. pneumoniae* belongs to a new class of heat shock genes under dual regulation by both CtsR and HrcA and is preceded by operator sequences for both repressors. Accordingly, in a Δ*ctsR* mutant, expression of *groESL* is not strongly increased, consistent with repression by HrcA.

Phenotypic analyses also indicate that, unlike in *B. subtilis* and contrary to a previous report (4), ClpC is not involved in controlling competence development, nor does it play a role in autolysis, pneumolysin production, or growth at high temperature of *S. pneumoniae*.

ClpP and ClpE of *S. pneumoniae*, on the other hand, have both been shown to be required for growth at high temperature, suggesting they may interact to form a Clp ATP-dependent protease. This is in contrast to *B. subtilis*, in which ClpC is required for growth at high temperature but ClpE is not (9, 37). As in *L. monocytogenes*, the ClpE and ClpC ATPases both appear to play a role in the virulence of *S. pneumoniae*. Indeed, the *clpE* gene encoding an Hsp100-type Clp ATPase was isolated during a large-scale identification of virulence genes using the signature-tagged transposon mutagenesis technique (44). An insertion inactivating the *S. pneumoniae ctsR* gene was isolated by using a similar approach (24), and the corresponding mutant was found to be highly attenuated in a murine respiratory tract infection model, which the authors attributed to a polar effect of the transposon insertion on the expression of the *clpC* gene that lies directly downstream. The precise role of the *S. pneumoniae* Clp proteins in competence development and virulence, however, remains to be determined and will be the subject of further investigation.

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