S2-4

Effect of *clpL, clpP* on *Streptococcus pneumoniae* Virulence

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Spread of *Streptococcus pneumoniae* from nasopharynx to other host tissues would require the organism to adapt to a variety of environmental conditions. Since heat shock proteins (HSPs) are induced by environmental stresses, we investigated the effect of heat shock on ClpL and ClpP synthesis and the effect of *clpL* and *clpP* mutation on the expression of key pneumococcal virulence genes. Pulse-labeling with [^{35}S]-methionine and chase experiments as well as immunoblot analysis demonstrated that ClpL, DnaK, and GroEL were stable. Purified recombinant ClpL refolded urea-denatured rhodanese in a dose dependent manner, demonstrating ClpL's chaperone activity. Although growth of the *clpL* mutant was not affected at 30 or 37°C, growth of the *clpP* mutant was severely affected at these temperatures. However, both *clpL* and *clpP* mutants were sensitive to 43°C. Although it was further induced by heat shock, the level of expression of ClpL in the *clpP* mutant was high at 30°C, suggesting that ClpP represses expression of ClpL. Furthermore, the *clpP* mutation attenuated virulence of *S. pneumoniae* significantly in a murine intraperitoneal infection model, whereas the *clpL* mutation did not. Interestingly, immunoblot and real-time reverse transcription PCR analysis demonstrated that pneumolysin and pneumococcal surface antigen A (PsaA) were induced by heat shock in wild type *S. pneumoniae*. Other virulence genes were also affected by heat shock and *clpL* and *clpP* mutations. Taken together, virulence gene expression seems to be modulated not only by heat shock but also by ClpL and ClpP proteases.

We further investigated the underlying mechanism of virulence attenuation by the *clpP* mutation. Although the same amount of capsular polysaccharide synthesis was detected in the *clpP* mutant and the wild type, the half-lives of pneumolysin (ply) and capsule synthesis (*cps2A*) mRNAs in the *clpP* mutant were >2 fold longer than those of the parent after heat shock, suggesting that the mRNA species were regulated posttranscriptionally by ClpP. In addition, the *clpP* mutant was defective in colonization of the nasopharynx and lungs of mice after intranasal challenge and was killed faster than the parent strain in the RAW264.7 mouse murine macrophage cell line, indicating that ClpP is required for colonization and intracellular survival in the host. Furthermore, fractionation studies demonstrated that ClpP was translocated into the cell wall after heat shock, and immunization of mice

with ClpP provided protective immunity against fatal challenge with D39 *S. pneumoniae*, making it a potential vaccine candidate against pneumococcal disease.

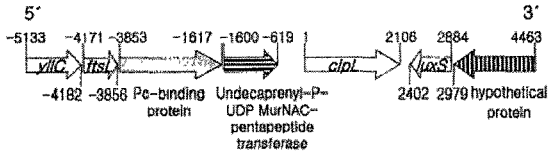

FIG 1. Physical map of *S. pneumoniae clpL* locus.

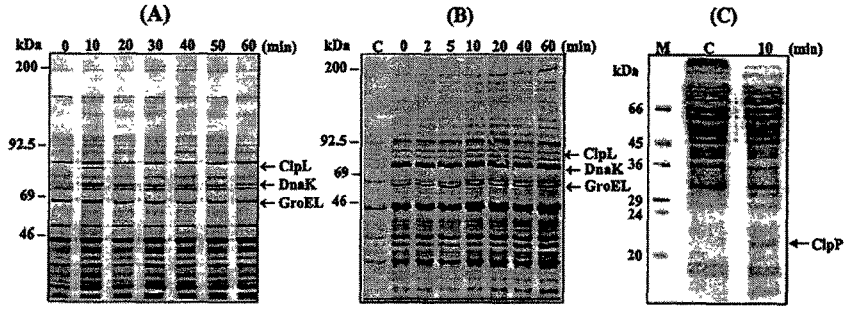

FIG. 2. Transient induction and stability of *S. pneumoniae* ClpL after heat shock.

(A) To determine induction kinetics, exponentially growing CP1200 cells were pulse labeled for 10 min with [^{35}S]methionine starting from the indicated time after the shift to 42°C. (B) To determine the stability of heat shock proteins, exponentially growing CP1200 cells were stressed at 42°C for 10 min and pulse labeled with [^{35}S]methionine at that time, and then the cell cultures were returned to 30°C, followed by chasing with excess nonradioactive methionine for the indicated times. (C) To determine induction of ClpP, exponentially growing CP1200 cells were pulse labeled for 10 min with [^{35}S]methionine starting from the indicated time after a shift to 42°C.

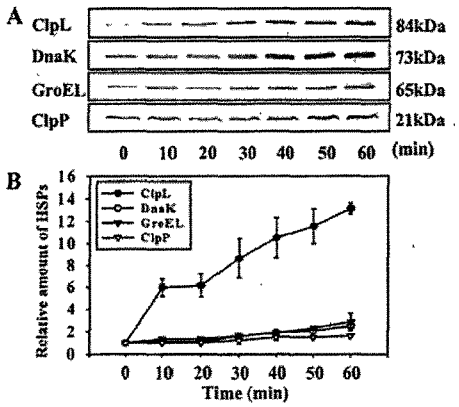

Fig. 3. Steady accumulation of ClpL after heat shock

[A]. Immunoblot analysis of whole cell lysates of growing *S. pneumoniae* CP1200 cells exposed to 42°C. *S. pneumoniae* cells grown at 30°C until A_{550}=0.3 were heat shocked at 42°C for the indicated times. [B]. Densitometric analysis of relative levels of ClpL, ClpP, DnaK and GroEL after heat shock as shown in panel A shock as shown in panel A.

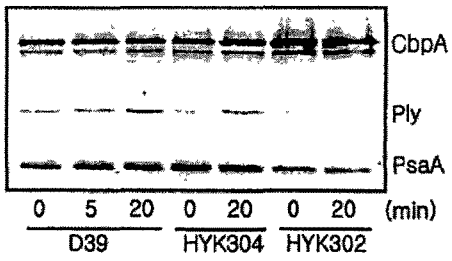

FIG. 4. Induction of virulence-associated genes by heat shock. Exponentially growing encapsulated *S. pneumoniae* D39 and its isogenic *clpP* (HYK302) and *clpL* (HYK304) deriva tives were heat shocked at 42°C for 20 min. Subsequently, cell lysates were subjected to immunoblot analysis with a mixture of polyclonal antisera raised against CbpA, Ply, and PsaA.

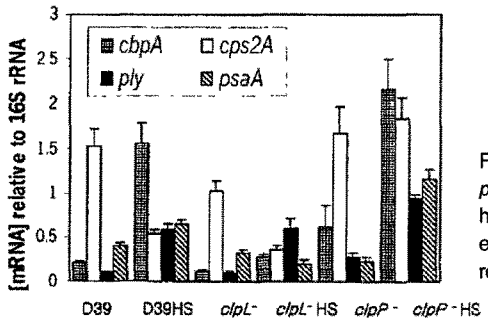

FIG. 5. Relative mRNA concentrations of *cbpA*, *cps2A*, *ply*, and *psaA* in D39 and the *clpL* and *clpP* mutants before and after heat shock as determined by real-time RT-PCR. Between RNA extracts, levels of individual mRNA species were corrected with reference to that obtained for the internal 16S rRNA control..

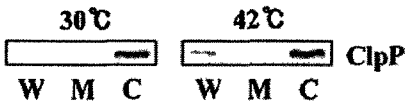

FIG.6. Translocation of ClpP after heat shock. Exponentially growing *S. pneumoniae* at 30°C was heat shocked at 42°C for 30 min. Cells were collected by centrifugation, and the proteins were fractionated and subjected to SDS-PAGE. Subsequently, ClpP was visualized by immunoblot analysis with polyclonal ClpP specific antiserum. W, cell wall; M, membrane; C, cytoplasm

TABLE 1. Effect of heat shock on half-lives of *cps2A* and *ply* mRNA[a].

Temperature	Half-life, min			
	D39		ClpP mutant	
	cps2A	*ply*	*cps2A*	*ply*
30°C	3.8±1.23	2.75±0.16	5.0±0.62	5.8±2.71
42°C	2.0±0.45[b]	1.75±0.33[b]	4.1±0.55[b, c]	3.75±0.79[d]

For measuring mRNA half-lives, rifampicin (100 μg/ml) was added and aliquots of 1.5 ml culture suspension were collected at 10 min intervals from which total RNA was extracted using the hot acid phenol method. Subsequently, mRNA levels were determined by real-time reverse transcription PCR.

The mRNA half-lives were analyzed by non-linear least squares fitting to the sum of exponentials. All experiments were carried out in quadruplicate.

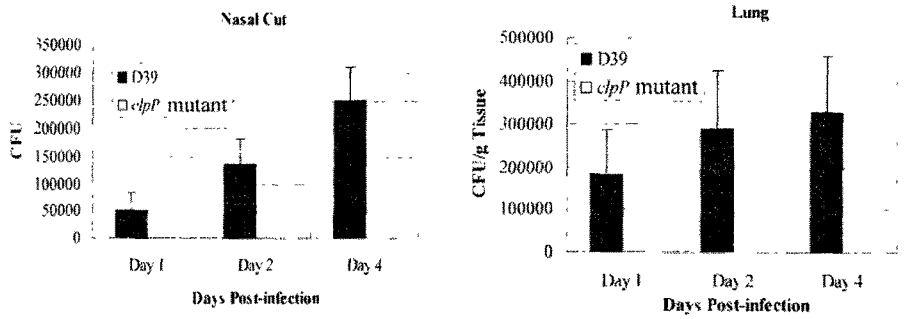

FIG. 7. Bacterial recovery from the nasopharynx of CD1 mice after intranasal challenge with D39 and its isogenic *clpP* derivative over a 4- day period.

References

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4. Molecules and Cells 11(3): 360-368 (2001).