The Journal of Microbiology (2010) Vol. 48, No. 2, pp. 229-235
DOI 10.1007/s12275-010-9300-0

Virulence Attenuation of *Streptococcus pneumoniae clpP* Mutant by Sensitivity to Oxidative Stress in Macrophages via an NO-Mediated Pathway

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(Received September 26, 2009 / Accepted October 27, 2009)

ClpP protease is essential for virulence and survival under stress conditions in several pathogenic bacteria. The *clpP* mutation in a murine infection model has demonstrated both attenuation of virulence and a sensitivity to hydrogen peroxide. However, the underlying mechanisms for these changes have not been resolved. Because macrophages play a major role in immune response and activated macrophages can kill microbes via oxygen-dependant mechanisms, we investigated the effect of the *clpP* mutation on its sensitivity to macrophage-mediated oxygen-dependant mechanisms. The *clpP* mutant derived from D39 (serotype 2) exhibited a higher sensitivity to oxidative stresses such as reactive oxygen intermediates, reactive nitrogen intermediates, and H_2O_2, but no sensitivity to osmotic stress (NaCl) and pH. Moreover, viability of the *clpP* mutant was significantly increased in murine macrophage cells by treatment with *S*-methylisothiourea sulfate, which inhibits inducible nitric oxide synthase (iNOS) activity and subsequently elicits lower level secretions of nitric oxide (NO). However, viability of wild type was unchanged. Taken together, these results indicate that ClpP is involved in the resistance to oxidative stresses after entrapment by macrophages and subsequently contributes to virulence via NO mediated pathway.

***Keywords*:** *S. pneumoniae*, *clpP*, oxidative stress, macrophage, NO

Streptococcus pneumoniae (pneumococcus), a Gram-positive bacteria with natural transformation capabilities, is the most common etiologic agent in community acquired pneumonia, bacteremia, and meningitis (Mandell *et al*., 2007). The bacteria can attach asymptomatically to the nasopharyngeal cells of healthy individuals via the interaction of bacterial surface adhesions with the epithelial cells. Bacteria cells then colonize and serve as a major reservoir for infection (Mandell *et al*., 2007). Once infection spreads into the bloodstream or other areas, it can induce severe diseases. During the spreading process, environmental changes in the host can stimulate bacterial gene expression and protein translocation, which may also contribute to the virulence of the bacteria.

Environmental stresses, which include temperature changes and nutrient limitation in the host, induce expression of a set of heat-shock proteins (HSPs) (Craig *et al*., 1993; Lee *et al*., 2006). Among the HSPs, the Hsp100/Clp family, which is ubiquitously present in prokaryotes and eukaryotes, contain an ATPase specificity factor (ClpA, ClpB, ClpC, ClpE, ClpL, etc.) and a protease domain (ClpP) that includes a consensus serine protease active site (Yu and Houry, 2007). A *clpP* mutant of *S. pneumoniae* showed sensitivity to high temperatures, H_2O_2 and puromycin (Robertson *et al*., 2002; Kwon *et al*., 2004). It also showed an increase in pneumolysin (*ply*) mRNA expression but not in the level and hemolytic activity of Ply after heat shock (Kwon *et al*., 2004). Moreover, after intranasal infection, viability of the *clpP* mutant in the lung was decreased compared to that of the wild type. After intraperitoneal injection, the infecting dose of *clpP* mutant needed to produce signs of morbidity in 50% of the test subjects (LD_{50}) was decreased and the survival time of subjects also declined (Robertson *et al*., 2002; Kwon *et al*., 2003). Expression of *clpP* in virulent strains was higher than in nonvirulent strains (Ko *et al*., 2006), and the *clpP* mutant was not required for nonvirulent R type strain (Robertson *et al*., 2003), demonstrating that ClpP is essential for virulence. ClpP is an effective vaccine for the prevention of pneumococcal diseases (Kwon *et al*., 2004; Cao *et al*., 2007).

Signature-tagged mutagenesis, microarray, and genome-based analysis have all shown that *S. pneumoniae* expresses different genes upon systemic infection than by intraperitoneal injection, intravenous injection, or nasal infection through mucosal membranes (Polissi *et al*., 1998; Throup *et al*., 2000; Adamou *et al*., 2001; Paton and Giammarinaro, 2001; Wizemann *et al*., 2001; Hava and Camilli, 2002; Orihuela *et al*., 2003). Specific gene expression depends on the tissues (nasopharynx, lungs, blood, brain) through which *S. pneumoniae* passes after infection (Hava and Camilli, 2002; Orihuela *et al*., 2004). Also, *S. pneumoniae* in the blood expresses different genes from those expressed in tissue (Oggioni *et al*., 2006). Therefore, intravenous infection might modulate gene expression different than that from intranasal or intraperitoneal infection. However, the underlying mechanism of how *clpP* modulates virulence and its effects on intravenous infection remains unknown.

Macrophages play a central role in the innate as well as the acquired immune response against invading bacteria (Mac-

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Micking *et al*., 1997). In the innate immune system, macrophages play pivotal roles in the defense against infection (Calandra and Roger, 2003). Phagocytosis, bactericidal activity, and digestion of the bacteria by the phagocytes trigger a series of intracellular events. This includes production of reactive oxygen species (ROS) and nitric oxide (NO), and induction of inflammatory gene expression, which can contribute to bacterial killing (Marriott *et al.*, 2004). To elucidate the underlying mechanism of *clpP*, the effect of *clpP* mutation on virulence and macrophage killing was investigated. The *clpP* mutant was sensitive to oxidative stresses via an NO mediated pathway in macrophage, suggesting that ClpP contributes to resistance of pneumococci to oxidative stresses, and thus contributes to the pathogenesis of *S. pneumoniae*.

Materials and Methods

Bacterial strains and cell culture

S. pneumoniae encapsulated type 2 strain D39 (NCTC 7466) and its isogenic *clpP* derivative (HYK302) were cultured in Todd-Hewitt broth as described previously (Kwon *et al*., 2003). For selection of erythromycin resistant *clpP* mutants, erythromycin was added to the growth medium at a concentration of 2.5 μg/ml.

A murine macrophage RAW264.7 cell line was obtained from the American Type Culture Collection and cultured in Dulbecco modified Eagle Medium (DMEM) with 10% fetal bovine serum (Gibco BRL, USA), 100 U of penicillin G/ml, and 100 μg of streptomycin/ml. 0.2% $NaHCO_3$ was used as the basic medium, and the culture was kept at 37°C in the presence of 95% air-5% CO_2.

Survival in RAW264.7 cells

Cell monolayers were infected with 10^7 CFU of pneumococci (bacterium/cell ratio, 10:1) in DMEM culture medium without antibiotics and then incubated at 37°C. The cells were washed three times with phosphate buffered saline (PBS), and fresh medium containing 10 μg of penicillin/ml. 200 μg of gentamicin/ml was added to kill extracellular bacteria (time zero of the assay). To enumerate intracellular pneumococci at different times after infection, the supernatants were removed and cells were washed three times with PBS followed by lysis with 400 μl of Triton X-100 (0.025% in H_2O). Appropriate dilutions were plated on blood agar to determine the numbers of viable bacteria. The number of CFU was determined after 24 h of incubation at 37°C. Three independent assays were carried out (in triplicate) for each bacterial strain.

Colonization studies

Before challenge, bacteria were cultured at 37°C overnight on blood agar (supplemented with erythromycin where appropriate) and then inoculated into serum broth, consisting of 10% (v/v) sheep serum in THY broth. Bacteria were then statically grown for 3 h at 37°C to give approximately 10^9 CFU/ml. Five-week-old CD1 or BALB/c mice were challenged with 1×10^3 CFU/100 μl of pneumococci intraperitoneally, 1×10^7 CFU/10 μl of pneumococci intranasally, or with either 1×10^3 or 1×10^6 CFU/100 μl of pneumococci intravenously. At 1, 2, and 3 days postinfection, four mice from each group were sacrificed randomly to estimate the carriage of each strain. Samples were serially diluted as appropriate in sterile PBS and plated in duplicate on blood agar containing the appropriate antibiotic(s). Plates were incubated for approximately 16 h at 37°C in an atmosphere of 95% air-5% CO_2, after which colonies were counted and averaged between replicates.

Sensitivity to stress

D39 and the *clpP* mutant were cultured in THY broth until OD=0.3. Subsequently, 1 ml of bacterial culture (approximately 1×10^8 CFU) was harvested by centrifugation at 3,000×g for 5 min, and the bacterial pellet was washed gently once with 1 ml of PBS and resuspended in 1 ml of PBS. To determine sensitivity of the *clpP* mutant to reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI), and osmotic stress, final concentrations of 200 mM paraquat, 1 mM $NaNO_2$, and 1 mM NaCl were used. For $NaNO_2$ sensitivity, pH was adjusted to 4.5, which produces antibacterial activity of nitric oxide (MacMicking *et al.*, 1997). For determination of sensitivity to paraquat and osmotic stress, the pH was adjusted to 7.5. Sensitivity to osmotic stress was determined by mixing 500 μl of THY broth containing 2 M NaCl with 500 μl of bacterial suspension. pH stress was determined by using THY broth adjusted to pH 4.5 or pH 7.5. Sensitivity to stress was determined by counting viable cells after stress followed by serial dilution and pour plating on THY agar. Experiments were performed at least 3 times.

NO sensitivity assay

To determine sensitivity to NO secreted by macrophages, cell-based NO sensitivity was used as described previously (MacMicking *et al*., 1997). RAW 264.7 cells (2×10^5 cells/well) were treated either with LPS alone or LPS and SMT simultaneously for 18 h. After 18 h, a small aliquot of the supernatant was used to determine NO and TNF-α levels, and the remaining culture was used to infect pneumococci and sensitivity to NO. After infecting the cells with 1×10^7 of the D39 or the *clpP* mutant (Raw 264.7:bacteria MOI=1:50), the culture was incubated at 37°C, 5% CO_2, for 1 or 3 h. One hundred microliter of 0.5% Triton X100 was added to the culture to lyse the cells, and viable cell number in the lysate was determined by plating on THY agar after serial dilution.

TNF-α assay

The TNF-α assay was performed as described previously (Tu *et al*., 2007). RAW 264.7 cells (2×10^5 cells/well) were infected with 1×10^7 CFU of pneumococci (MOI, 50). After infection, supernatant was collected and centrifuged at 5,500×g for 20 min to remove the bacterial pellet, and the level of secreted TNF-α was measured using an enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences) as recommended by the supplier. Curves were fitted to sigmoidal dose-response curves and compared using an F test (GraphPad Prism, version 4.0; GraphPad Software).

Nitrite determination

The amount of NO_2^- accumulated in the culture supernatants was measured as described previously (Ding *et al*., 1988). Briefly, 100 μl of the supernatant was removed from each well and placed into an empty 96-well plate. After adding 100 μl of Griess reagent to each well, the absorbance was measured at 550 nm using a Molecular Device microplate reader. NO_2^- concentration was calculated from a $NaNO_2$ standard curve, and are indicative of the amount of NO production. The Griess reagent was prepared by mixing 1 part of 0.1% naphthylethylene diamine dihydrochloride in distilled water with 1 part of 1% sulfanilamide in 5% concentrated H_3PO_4.

Statistics

Statistical analysis was performed using paired or unpaired Student's *t* tests. Data are presented as Mean±SD of the mean for two or three independent experiments. Statistically significant differences were defined as $P<0.05$.

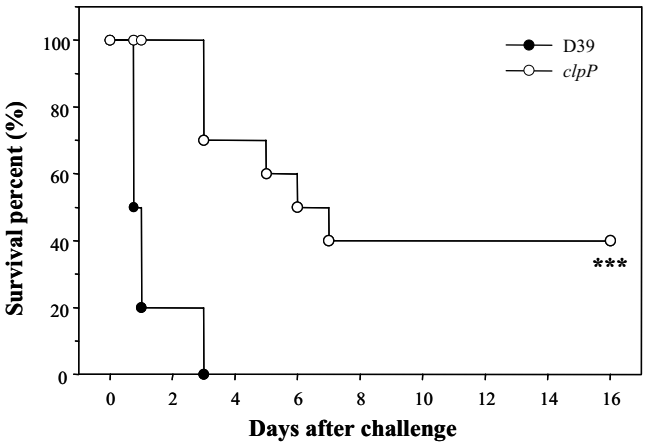

Fig. 1. Mouse survival after intravenous challenge. Bacterial strains were grown in serum broth to an A550 of 0.3. Groups of 10 BALB/c male mice were injected intravenously with 0.1 ml of diluted cultures containing approximately 1×10^6 CFU of D39 or the *clpP* mutant. *** $P<0.001$ compared with D39 group.

Results and Discussion

Reduced viability of the *clpP* mutant in mouse organs

Previously, intranasal challenges in mice using a highly virulent capsular type 2 strain (D39) and it's isogenic *clpP* mutant (HYK302) revealed that the *clpP* mutant was unable to colonize the nasopharynx or the lungs during a 4-day infection period (Kwon *et al.*, 2004). Also, the *clpP* mutant was cleared rapidly in murine macrophage RAW264.7 cells (Kwon *et al.*, 2004). These results suggest that the *clpP* mutant failed to colonize the lungs of mice at significant levels after intranasal infection, and the *clpP* mutant is stress-sensitive or susceptible to macrophages. However, the number of *clpP* mutants in each organ has not been determined. To confirm that the *clpP* mutant was sensitive to immune response in the mouse, we determined viability of the mutant in several organs such as the lungs, spleen, and brain. The lungs are the major infection site and the location of alveolar macrophage, the primary elements in host defense against invasion by *S. pneumoniae* (Knapp *et al.*, 2003), the spleen is where immune stimulation occurs via B and T cell differentiation during pathogen invasion in the host, and the brain is the site of pneumococcal meningitis. After intravenous infection with 1×10^3 CFU, survival of mice infected with the *clpP* mutant was significantly longer than that of the wild type (Fig. 1). Also, after intravenous infection with 1×10^6 CFU, the *clpP* mutant was cleared one day later and no viable cells were detected in the spleen, lungs, or brain after 2 and 3 days of infection (Fig. 2). In contrast, the viable cell numbers of wild type continuously increased over time after intravenous infection in all the organs examined (Fig. 2). Similarly, after intraperitoneal infection with 1×10^3 CFU, the *clpP* mutant was cleared more rapidly

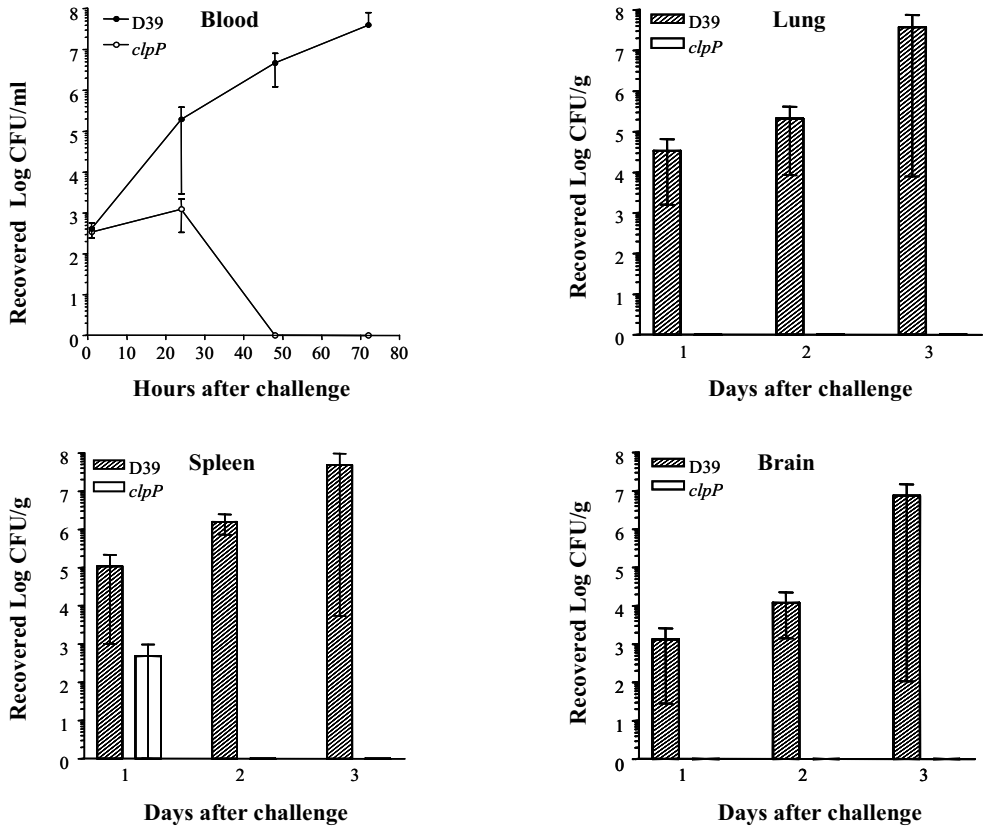

Fig. 2. Viability of the *clpP* mutant in various tissues after intravenous challenge. Bacterial strains were grown in serum broth to an A550 of 0.3. Groups of 5 ICR male mice were injected intravenously with 0.1 ml of diluted cultures containing approximately 1×10^6 CFU of D39 or the *clpP* mutant. Mice were sacrificed after 1, 24, 48, and 72 h and the number of bacteria in tissue was determined by plating on a 5% blood agar plate.

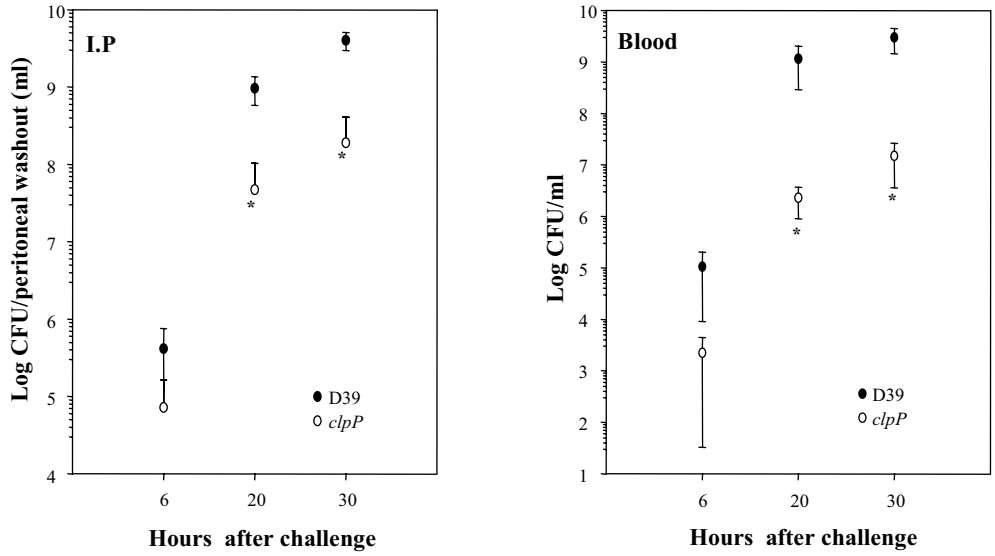

Fig. 3. Viability of the *clpP* mutant in intraperitoneal cavity and blood after intraperitoneal challenge. Bacterial strains were grown in serum broth to an A550 of 0.3. Groups of 4 BALB/c male mice were injected intraperitoneally with 0.1 ml of diluted cultures containing approximately 1×10^3 CFU of D39 or the *clpP* mutant. Mice were sacrificed after 6, 20, and 30 h and the number of bacteria in the peritoneal washout solution and blood were determined by plating on 5% blood agar plate. * $P<0.05$ compared with the D39 group.

than wild type at 6, 20, and 30 h post-infection in the blood and intraperitoneal cavity (Fig. 3). Intranasal infection also decreased the viability of the *clpP* mutant at 3 h post-infection (data not shown). These results corroborate that ClpP protease plays an important role in mouse virulence as well as survival in the intraperitoneal cavity, blood, lung, brain, and spleen.

Sensitivity of the *clpP* mutant to oxidative stresses but not to osmotic stress and pH *in vitro*

Although the *clpP* mutant was sensitive to H_2O_2 (Robertson *et*

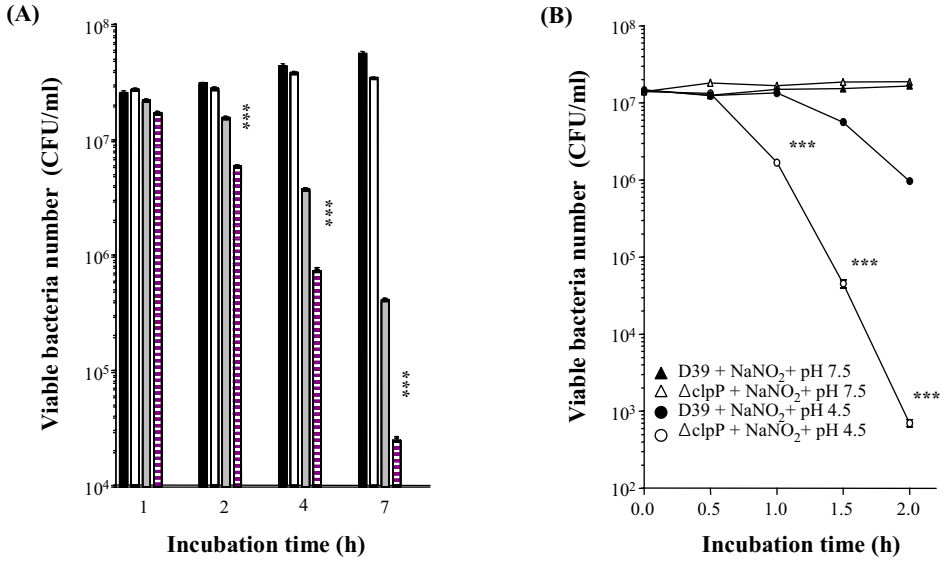

Fig. 4. Sensitivity of the *clpP* mutant to reactive oxygen species and reactive nitrogen species. (A) Bacterial strains were grown in THY broth to an A550 of 0.3. Approximately 2×10^7 CFU of D39 or the *clpP* mutant were added to THY broth containing paraquat (200 mM), and incubated for the indicated time at room temperature. Viable number of bacteria in broth was then determined by plating on THY agar plate. Closed bar, Open bar, Dotted bar, and Lined bar represent D39 (-paraquat), *clpP* mutant (-paraquat), D39 (+paraquat), and *clpP* mutant (+paraquat), respectively. *** $P<0.001$ compared with D39 group. (B) Bacterial strains were grown in THY broth to an A550 of 0.3. Approximately 1×10^7 CFU of D39 or the *clpP* mutant were added to either THY broth (pH 4.5) containing $NaNO_2$ (1 mM) or THY broth (pH 7.5) without $NaNO_2$, and incubated for the indicated time at room temperature. The viable number of bacteria in broth was then determined by plating on a THY agar plate. Closed triangle, Open triangle, Closed circle, and Open circle represent D39 (-$NaNO_2$, pH 7.5), *clpP* mutant (-$NaNO_2$, pH 7.5), D39 (+$NaNO_2$, pH 4.5), and *clpP* mutant (+$NaNO_2$, pH 4.5), respectively. *** $P<0.001$ compared with D39 group.

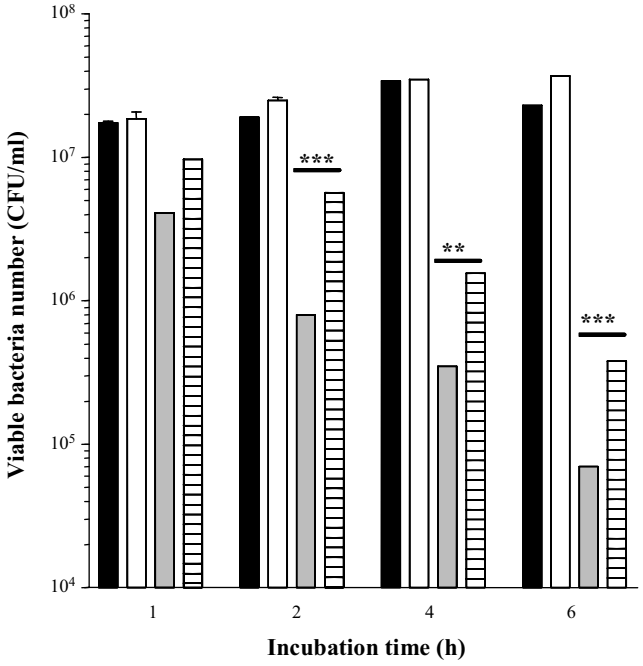

Fig. 5. *clpP* mutants are not pH sensitive. Bacterial strains were grown in THY broth to an A550 of 0.3. Approximately 2×10^7 CFU of D39 or the *clpP* mutant were added to THY broth adjusted to pH 7.5 or pH 4.5, and incubated for the indicated time at room temperature. The viable number of bacteria in broth was then determined by plating on a THY agar plate. Closed bar, Open bar, Dotted bar, and Lined bar represent D39 (pH 7.5), *clpP* mutant (pH 7.5), D39 (pH 4.5), and *clpP* mutant (pH 4.5), respectively.

al., 2002), the underlying mechanism of virulence attenuation by the *clpP* mutation remains unknown. Therefore, we determined the viability of the mutant under oxidative stress conditions such as exposure to ROS and reactive nitrogen intermediate, which are generated in the macrophages. When the superoxide generator, paraquat, was added to the culture media, the viability of the *clpP* mutant decreased significantly to 40%, 20%, and 6% of the wild type after 2, 4, and 7 h of infection, respectively (Fig. 4A). Furthermore, sodium nitrite in acidic (pH 4.5) reduced the viability of the *clpP* mutant significantly to 12.5%, 0.8%, and 0.07% of the wild type after 1, 1.5, and 2 h, respectively (Fig. 4B). In contrast, the *clpP* mutant did not respond to osmotic stress (1 M NaCl) (data not shown) or low pH (Fig. 5). Therefore, the sensitivity to sodium nitrite is not due to low pH, but due to inherent sensitivity to oxidative stress. Similarly, previous work showed that the *clpP* mutant was not sensitive to reactive nitrogen intermediates at pH 7.5 but was sensitive at pH 4.5 (MacMicking *et al*., 1997). Thus, oxidative stress rather than low pH or osmotic stress could affect survival of the *clpP* mutant in mice.

Although the *Salmonella clpP* mutant was sensitive to osmotic stress and acidic stress (Thomsen *et al*., 2002), the *S. pneumoniae clpP* mutant was not, potentially because of differences in their natural niche: normally, *Salmonella* can survive in natural environments and in a variety of hosts such as chicken, animal, and human, whereas humans are the only natural host for pneumococci. Therefore, *clpP* could also play a different role depending on the microorganism.

Sensitivity of the *clpP* mutant to oxidative stresses in macrophage via NO dependent pathway

Lipopolysaccharide (LPS) activates immune cells, including

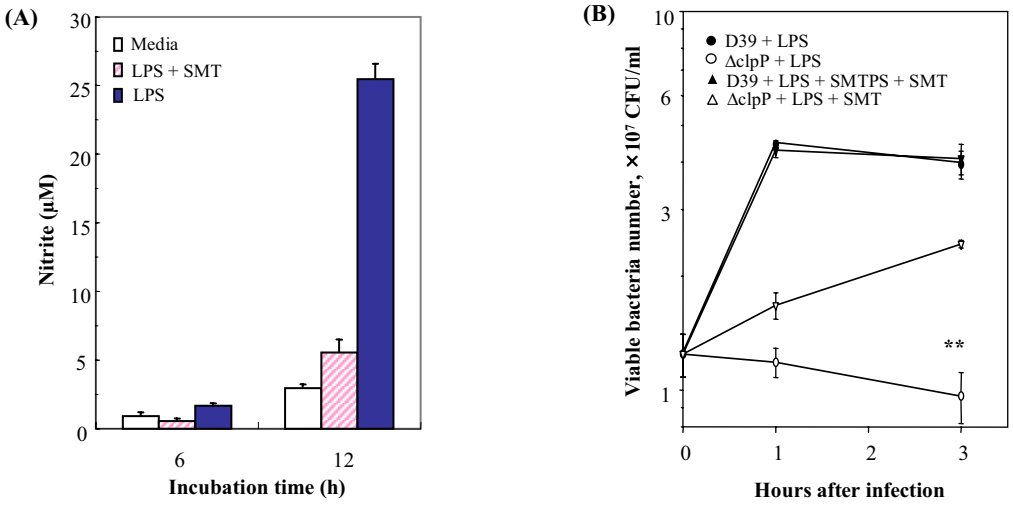

Fig. 6. Sensitivity of the *clpP* mutant to oxidative stresses in macrophage via NO dependent pathway. (A) Inducible NOS inhibition by *S* -methylisothiourea sulfate (SMT). RAW264.7 cells were incubated with LPS (1 μg/ml) only or LPS (1 μg/ml) plus SMT (100 μM). Supernatants were harvested after 6 and 12 h, and the level of nitric oxide in the supernatant was determined by an NO assay. (B) Viability increase after SMT treatment in the *clpP* mutant. RAW264.7 cells (2×10^5 cells/well) were treated with LPS (1 μg/ml) only or LPS (1 μg/ml) plus SMT (100 μM) for 16 h. After treatment, RAW264.7 cells were infected with 10^7 CFU of D39 or the *clpP* mutant. After incubation of the culture for the indicated time, RAW264.7 cells were harvested by detergent lysis of the cells and bacteria were quantified by plating the cell lysate on THY agar plates. Secreted NO levels after LPS or LPS plus SMT prior to pneumococcal infection were 49 μM and 8 μM, respectively. Closed circle, Open circle, Closed triangle, and Open triangle represent D39 (LPS only), *clpP* mutant (LPS only), D39 (LPS plus SMT), and *clpP* mutant (LPS plus SMT), respectively. $^{**}P<0.01$ compared with D39 group.

macrophages. The activated macrophages overproduce inflammatory cytokines such as tumor necrosis factor-α, interleukin (IL)-1, and IL-6, to trigger progressive activation of immune cells (Nathan, 1987). Subsequently, activated phagocytes generate and release ROS and reactive nitrogen intermediates via the TLR-4 pathway (Wright *et al*., 2004; Akira *et al*., 2006). We therefore tested the susceptibility of mutants to $NaNO_2$ in macrophages. To induce NO production, LPS was added to RAW264.7 murine macrophage cells. In parallel, RAW264.7 cells were treated with LPS and *S*-methylisothiourea sulfate (SMT), which inhibits inducible NO synthase (iNOS) and subsequently reduces NO production (Szabó *et al*., 1994), followed by measuring supernatant NO levels. To confirm activation of macrophages by LPS, NO levels and TNF-α secretion were determined. LPS significantly increased both NO production and TNF-α secretion (data not shown). SMT treatment for 12 h decreased NO levels 5-fold over LPS alone (Fig. 6A), from 49 μM to 8 μM at 16 h (data not shown). RAW264.7 cells were pretreated with either LPS alone or LPS and SMT for 16 h and then infected with pneumococci. These treatments did not affect the viability of the wild type (Fig. 6B), but cotreatment with LPS and SMT increased viability of the *clpP* mutant to 1.4- and 2.5-fold of LPS alone at 1 and 3 h post-infection, respectively (Fig. 6B). Thus, the sensitivity of the *clpP* mutant to oxidative stresses is mediated by NO. Since NO plays important role in mouse survival against pneumococcal infection (Kerr *et al*., 2004), the *clpP* mutation becomes sensitive to NO and virulence attenuation.

Flow cytometry analysis showed that ClpP was localized at the surface. ClpP protein is immunogenic in healthy children and is expressed during pneumococcal infection, since ClpP antibody levels were elevated in infected individuals. In addition, an anti-ClpP antibody could kill *S. pneumoniae* by polymorphonuclear leukocytes in a complement-dependent way. Therefore, ClpP may be a candidate for a mucosal pneumococcal vaccine (Cao *et al*., 2008).

Several hypotheses have been proposed for the sensitivity of the *clpP* mutant: 1) sensitivity to low temperature and nutrient starvation (Robertson *et al*., 2002; Frees *et al*., 2003), 2) susceptibility to the host immune system, especially to attack by peritoneal macrophages and blood complement (Borezee *et al*., 2001; Gaillot *et al*., 2001; Yamamoto *et al*., 2001; Robertson *et al*., 2002; Frees *et al*., 2003), 3) reduced ability to damage host cells. Here, we show for the first time that virulence attenuation of the *S. pneumoniae clpP* mutant could be due to, at least in part, sensitivity to oxidative stresses in macrophages via an NO-mediated pathway.

Acknowledgements

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (KRF-2008-313-E00727).

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