



Inhibition of Autolysis by Lipase LipA in *Streptococcus pneumoniae* Sepsis

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More than 50% of sepsis cases are associated with pneumonia. Sepsis is caused by infiltration of bacteria into the blood via inflammation, which is triggered by the release of cell wall components following lysis. However, the regulatory mechanism of lysis during infection is not well defined. Mice were infected with *Streptococcus pneumoniae* D39 wild-type (WT) and lipase mutant ($\Delta lipA$) intranasally (pneumonia model) or intraperitoneally (sepsis model), and survival rate and pneumococcal colonization were determined. LipA and autolysin (LytA) levels were determined by qPCR and western blotting. *S. pneumoniae* Spd_1447 in the D39 (type 2) strain was identified as a lipase (LipA). In the sepsis model, but not in the pneumonia model, mice infected with the $\Delta lipA$ displayed higher mortality rates than did the D39 WT-infected mice. Treatment of pneumococci with serum induced LipA expression at both the mRNA and protein levels. In the presence of serum, the $\Delta lipA$ displayed faster lysis rates and higher LytA expression than the WT, both *in vitro* and *in vivo*. These results indicate that a pneumococcal lipase (LipA) represses autolysis via inhibition of LytA in a sepsis model.

Keywords: infection, LipA, LytA, sepsis, *S. pneumoniae*

INTRODUCTION

Sepsis is a medical emergency and requires immediate intervention to mitigate the various symptoms. Without treatment, the survival rate of sepsis patients lowers to close to zero at 36 h post-onset. Gram-positive bacterial sepsis accounts for 57% of all sepsis cases each year (Martin, 2012), and more than 50% of sepsis is associated with pneumonia (Munford and Suffredini, 2009). *Streptococcus pneumoniae* is a causative agent of pneumonia, bacteremia, meningitis, otitis media, and sinusitis (Liu et al., 2012), colonizes the human nasopharynx. (Berry et al., 1989; Lanie et al., 2007). *S. pneumoniae* contains many virulence factors that contribute to colonization and disease (Kadioglu et al., 2008; Orihuela et al., 2004). The autolysin LytA, an N-acetylmuramoyl-L-alanine amidase, cleaves the lactyl-amide bonds between the glycan strands of cell wall peptidoglycan (Berry et al., 1989). Furthermore, LytA is critical for *S. pneumoniae* pathogenesis during pneumonia and septicemia, as well as meningitis (Canvin et al., 1995; Hirst et al., 2008). However, the mechanism by which pneumococcal lysis is regulated in models of pneumonia and sepsis remains unclear. Therefore, an understanding of the mechanisms underlying sepsis and pneumonia, and the pathophysiology of sepsis by pneumococci would be required for the implementation of a new therapy for sepsis.

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Lipases (triacylglycerol hydrolase, E.C. 3.1.1.3), with a consensus catalytic triad Gly-x-Ser-x-Gly around the active site serine, are defined as glycerol ester hydrolases that hydrolyze triglycerides to free fatty acids and glycerols (Joseph et al., 2008). In gram-negative *Photorhabdus* and *Xenorhabdus* spp., a putative lipase enhances the secretion of the toxin complex (Yang et al., 2012). Moreover, when *Aeromonas* lipase is secreted, it alters the host plasma membrane (Pemberton et al., 1997). In gram-positive *Staphylococcus aureus* and *Staphylococcus epidermidis*, extracellular lipases are considered to be virulence factors (Gill et al., 2005). In a study on *S. aureus*, strains isolated from patients with deep or subcutaneous infections (septicemia, furunculosis, and pyomyositis) displayed higher lipase expression than those from patients with superficial infections (Rolof et al., 1987). In addition, purified *S. aureus* lipase alters the surface structure of granulocytes, thereby impairing bacterial uptake by this cell type (Rolof et al., 1988).

In this study, the Spd_1447 protein was characterized as a lipase and named LipA. Unlike other pathogen lipases, the *S. pneumoniae* LipA was not detected in pneumococcal culture, suggesting that this lipase may play a different role in virulence. Furthermore, in a murine sepsis model, a $\Delta lipA$ displayed higher virulence under serum-rich conditions than the wild type (WT) protein because of higher LytA expression in the presence of serum *in vitro* and *in vivo*. Furthermore, serum can induce LipA expression, which suppresses pneumococcal autolysis. In summary, in the presence of serum, LipA inhibits LytA-mediated autolysis in *S. pneumoniae*, thus attenuating virulence.

MATERIALS AND METHODS

Animal study and ethics statement

Four-week-old male CD-1 (ICR) mice were purchased (Orient Bio Inc., Korea). All animal experiments (protocol PH-530518-06) were performed according to the animal care guidelines of the Korean Academy of Medical Sciences. Experimental procedures were approved and monitored by the Animal Care and Use Committee of Sungkyunkwan University (Korea).

Intranasal infection: *S. pneumoniae* was cultured to $A_{550} = 0.3$ (1.5×10^8 CFU/ml). 1 ml of pneumococcal culture was harvested by centrifugation ($4,000 \times g$, 4°C, 10 min) followed by resuspension into 100 μ l of phosphate buffer saline (PBS). Mice received 10 μ l of suspension comprising 1.5×10^7 CFUs into nares with nares up for 3 min to allow complete absorption of the inoculum.

Intraperitoneal infection: *S. pneumoniae* was cultured to $A_{550} = 0.3$ (1.5×10^8 CFU/ml). One ml of pneumococcal culture was obtained by centrifugation ($4,000 \times g$, 4°C, 10 min) and then resuspended in 1 ml of phosphate buffer saline (PBS). The 100 μ l of the suspension was serially diluted with PBS to make 1.5 - 3×10^5 CFU/ml. The 100 μ l of the final suspension containing 1.5 - 3×10^4 CFUs was injected into peritoneal cavity.

Bacterial strains and cell culture conditions

The bacterial strains and plasmids used in this study are dis-

played in [Supplementary Table S1](#). All *S. pneumoniae* strains (WT and $\Delta lipA$) were cultured in Todd Hewitt broth (THY) (Gibco, USA), as described previously (Luong et al., 2015).

The genes encoding D39 *spd_1447* (GenBank ABJ55222.1) and WU2 *spd_1447* were cloned into *E. coli* BL21 using pET32b(+). *E. coli* strains were cultured in Luria broth.

The murine macrophage RAW 264.7 and lung carcinoma A549 epithelial cell lines were obtained from the American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS; Gibco) and 1X penicillin/streptomycin (PAA Laboratories GmbH) (Nguyen et al., 2014).

Cloning and purification of recombinant D39 *spd_1447* and WU2 *spd_1447*

The gene encoding *spd_1447* (*lipA*) was amplified from the D39 or WU2 genome using primers ([Supplementary Table S2](#)) that contained *Hind*III and *Bam*HI restriction enzyme sites. The PCR products were digested with *Hind*III and *Bam*HI (New England BioLabs, USA) and cloned into the corresponding restriction sites in the pET32b(+) vector (Novagen, USA). *E. coli* BL21 (DE3) were transformed with the plasmids, and ampicillin (100 μ g/ml) was used for selection of recombinants.

Because the recombinant Spd_1447 (LipA) formed an inclusion body, it was purified under denatured conditions and then refolded to a native form. At log phase, the protein was induced with 0.1 mM isopropyl- β -D-thiogalacto-pyranoside at 18°C for 18 h, followed by sonication in buffer A (50 mM NaH_2PO_4 , 300 mM NaCl, and 1 mM PMSF at pH 8.0) containing 10 mM imidazole and 0.1% Triton X-100. The culture was centrifuged at $13,000 \times g$ at 4°C for 30 min, and subsequently, the pellet was dissolved in denaturing buffer B (buffer A containing 8 M Urea and 10 mM imidazole) with agitation for 15 min. Following centrifugation, the supernatant was subjected to nickel-nitrilotriacetic acid column (Invitrogen, USA) chromatography according to the manufacturer's instructions. Following elution from the column, the fraction containing Spd_1447 was placed in a dialysis tube and refolded with a series of decreasing urea concentrations (4 M, 2 M, 1 M, and 0.5 M for 2 h each) in elution buffer at 4°C. Subsequently, the protein was further purified by HPLC using buffer A to obtain a pure fraction. The pure fraction was then concentrated using molecular sieve centrifugal filter units (Amicon Ultra-15; Millipore, USA). The protein was quantitated using the DC Protein assay (Bio-rad, USA). Unless otherwise specified, reagents were purchased from Sigma-Aldrich.

Construction of LipA S166A pneumococcal mutant strain

For construction of SPD_1447 (LipA) point mutant, *spd_1447* (*lipA*) in pET32b(+) vector was isolated from *E. coli* BL21 (DE3) and amplified by LipA S166A primers ([Supplementary Table S2](#)). The PCR conditions comprised of 95°C for 30 s as an initial denaturation step and 12 cycles of 95°C for 30 s, 55°C for 1 min, 68°C for 7min, and 68°C for 5 min as extension step. The PCR products which contain LipA S166A mutation were treated with *Dpn*I (New England Biolabs, USA) at 37°C for 2 h to remove the original un-

mutagenized LipA, and then transformed into *E. coli* XL1 Blue, and ampicillin resistant (50 µg/ml) strain selected. The transformant containing LipA S166A and pCR 2.1 vector (ThermoFisher scientific, USA) were digested with *NotI* and *XbaI* (New England BioLabs, USA) and then ligated to clone the LipA S166A into pCR 2.1 to generate pCR 2.1 Ω LipA S166A. Subsequently, kanamycin cassette was amplified by PCR using pDL 276 vector containing Kan^r cassette as a template, and Kan^r cassette-F and Kan^r cassette-R as primers (Supplementary Table S2), and subsequently digested with *EcoRI* and *NotI* followed by cloning into the recombinant pCR 2.1 Ω LipA S166A digested with the same restriction enzyme sites to generate pCR 2.1 Ω LipA S166A Kan^r. The final form of recombinant plasmid pCR 2.1 Ω LipA S166A-Kan^r-SPD_1447~8 containing the right flanking arm at the end of Kan^r was amplified by PCR using D39 genomic DNA (serotype 2) as a template and SPD_1447~8 as primers (Supplementary Table S2) and digested with *KpnI* and *Bam*-HI and then ligated to the respective enzyme sites of pCR 2.1 Ω LipA S166A Ω Kan^r. Recombinant plasmid pCR 2.1 Ω LipA S166A-Kan^r-SPD_1447~8 was transformed into D39 Δ *lipA*, and kanamycin (250 µg/ml) resistant colony was selected as recombinant. The transformants were confirmed by Western blot and DNA sequencing (CosmoGenetech) (data not shown).

Lipolytic activity assay

Lipolytic activity was determined by a colorimetric assay measuring the release of p-nitrophenol from a p-nitrophenyl laurate substrate (Pinsirodom and Parkin, 2001). Purified Spd_1447 (150 µg) was incubated with 400 µl of 420 µM p-nitrophenyl laurate and 400 µl of 0.1 M Tris-Cl (pH 8.2). Absorbance at 410 nm was recorded up to 8 h. The result was converted into the concentration of p-nitrophenol based on a p-nitrophenol solution standard curve (0-25 µM).

Purified Spd_1447 was incubated with p-nitrophenyl butyrate (C4), p-nitrophenyl dodecanoate (C12), or p-nitrophenyl palmitate (C16) for 2 h, and release of p-nitrophenol was determined as described above. All reagents were purchased from Sigma-Aldrich.

Tributylin agar plate

The tributyrin agar plate was used to screen lipase-producing microbes (Jørgensen et al., 1991). THY agar with 1% (v/v) tributyrin (MB Cell, USA) was prepared by emulsifying by sonication followed by autoclaving. The *S. pneumoniae* D39 WT or Δ *lipA* strain or LipA S166A strain was plated on a THY tributyrin agar plate and incubated at 37°C for 12 h under anaerobic conditions. Clear halos formed around lipase-producing bacterial colonies.

For the lipase inhibition studies, the lipase inhibitor (-)-tetrahydrolipstatin (THL; Santa Cruz, USA) was added to THY tributyrin agar at 45°C to produce final concentrations of 0, 5, or 25 µg/ml. *S. pneumoniae* D39 culture (10 µl) was plated on the agar plates, as described above.

In vivo survival and colonization

For a pneumonia model, mice were infected intranasally (*i.n.*) with 1.5×10^7 colony-forming units (CFU) of *S. pneu-*

moniae D39 and TIGR4 or 4.5×10^7 CFU of WU2 (Luong et al., 2015). The survival rate was monitored daily. For a sepsis model, mice were infected intraperitoneally (*i.p.*) with 1.5×10^4 CFU of D39, WU2, or TIGR4, and survival was recorded every day.

For the colonization experiment, mice were infected with 1.5×10^7 CFU of *S. pneumoniae i.n.* or 1.5×10^4 CFU *i.p.* At indicating time point, the organs were collected, homogenized, serially diluted, and plated on THY blood agar. Gentamycin (10 µg/ml) or erythromycin (2.5 µg/ml) was added to the blood agar to count the D39 WT or Δ *lipA* bacteria, respectively. The counted viable bacterial CFUs were divided by the number of injected bacterial CFUs.

Western blotting

S. pneumoniae lysate was collected, as described previously (Luong et al., 2015). Protein samples were prepared using DC™ protein assay (Bio-Rad) and separated on 10-12.5% sodium dodecyl sulfate-polyacrylamide gel, then transferred to polyvinylidene difluoride membranes (Millipore) using a Trans-Blot SD semi-dry transfer cell (Bio-Rad). The membrane was blocked to prevent non-specific binding with 5% skimmed milk (Difco) for 2 h at 25°C, and then incubated with the primary antibody overnight at 4°C. Later, the membrane was incubated with anti-mouse IgG antibody conjugated with horseradish peroxidase (Promega, USA). Bands were detected using WEST-ZOL™ Plus (iNtRON) and a chemiluminescence imaging system (Davinchi-Chemi, Korea).

Cell cytotoxicity assay

S. pneumoniae [multiplicity of infection (MOI), 25] was infected to a confluent monolayer of RAW 264.7 or A549 cells for 2 h in the presence of 10% FBS. Cytotoxicity was determined by 3-(4,5-dimethylthiazole-2-yl)-2,5-dephenyl-tetrazolium bromide assay (Sigma-Aldrich), as previously described (Kang et al., 2002), and A₅₄₀ was determined using an enzyme-linked immunosorbent assay reader (Softmax).

RNA isolation and quantitative real-time polymerase chain reaction (qPCR)

Bacterial RNA was isolated using the hot-phenol method, as previously described (Kwon et al., 2003). All RNA samples were treated with DNase I (TaKaRa, Japan). RNA (1 µg) was converted to cDNA by Easy Script Reverse Transcriptase (Abm, Canada) and random primers (TaKaRa). qPCR was performed using a Step-One-Plus Real-time PCR System (Applied Biosystems, USA) according to the manufacturer's instructions, as previously described using the primers shown in Supplementary Table S2. The qPCR data was exported after dividing to 16s RNA level to control the loading amount. The value was displayed in Figs. 4A and 5C as increase fold of serum-induced pneumococci (serum) per no-serum pneumococci (control). A control bar (one fold value) was shown as a control level.

Deoxycholate- and Triton X-100-induced lysis

Sodium deoxycholate (DOC) and Triton X-100 are known to induce pneumococcal autolysis (Mellroth et al., 2012). Goat serum (10%; Life Technologies) was added to the bacterial

culture and incubated for 10 min. DOC or Triton X-100 was added to the bacterial culture at a final concentration of 0.05% or 0.1% (v/v), respectively, to induce bacterial lysis. For absorbance determination, A_{550} was monitored every 20 s for up to 5 min. For viable cell counting, the bacterial culture was serially diluted and plated onto THY blood agar plates.

Bacterial gene expression in a murine sepsis model

Mice were infected *i.p.* with 1.5×10^4 CFU of *S. pneumoniae*. Blood were collected at the most severely morbid stage (Hajaj et al., 2012) and subjected to centrifugation at $800 \times g$ at 4°C for 10 min to remove red blood cells. Subsequently, the bacteria were harvested by centrifugation at $15,000 \times g$ at 4°C for 10 min, and bacterial RNA was isolated by the hot-phenol method, as previously described (Tran et al., 2011). RNA was treated with DNase I (TaKaRa) and converted to cDNA using random primers (TaKaRa). qPCR was performed using the Step One Plus Real-time PCR System (Applied Biosystems) according to the manufacturer's instructions, as previously described (Tran et al., 2011). The value was displayed in Figs. 4C and 5C as increase fold of *in vivo*-collected pneumococci (*in vivo*) per non-infected pneumococci (control). A control bar (one fold value) was shown as a control level.

S. pneumoniae pellets at the log phase were harvested and incubated with whole mouse blood (CD-1, male) for 15 min. Subsequently, the bacteria in the whole blood were harvested by centrifugation at $15,000 \times g$ for 10 min. Total RNA was isolated and subjected to qPCR, as described above. The value was displayed in Figs. 4D and 5C as in-

crease fold of whole blood-induced pneumococci (whole blood) per non-induced pneumococci (control). A control bar (one fold value) was shown as a control level.

Statistical analysis

Western blotting band density was analyzed by ImageJ 1.43 (NIH, USA). Animal studies were statistically analyzed by the log-rank test (survival) or the Mann-Whitney rank sum test (colonization). All other analyses were performed using one-way analysis of variance (ANOVA). *p*-values were denoted as follows: **p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001.

RESULTS

Hypothetical protein Spd_1447 is a lipase

A hypothetical protein encoded by the *spd_1447* locus in *S. pneumoniae* D39 was found to contain the conserved catalytic triad (Gly-His-Ser-Leu-Gly) (CDD: 238382) of the lipase family (NCBI). Sequence alignment also demonstrated that Spd_1447 is a lipase homolog of gram-positive *Clostridium* sp. or *Lactobacillus* sp. (Supplementary Fig. S1). For confirmation, the Spd_1447 proteins in *S. pneumoniae* serotype 2 D39 were purified and used for lipase activity determination (Pinsirodom and Parkin, 2001). After 2 h incubation, the purified Spd_1447 protein produced higher p-nitrophenol levels than the control (Fig. 1A). Furthermore, the D39 Spd_1447 protein displayed time-dependent lipase activity, and the product reached a plateau at 6 h post-incubation (Fig. 1B) indicating that this Spd_1447 protein, named LipA, can be considered a lipase.

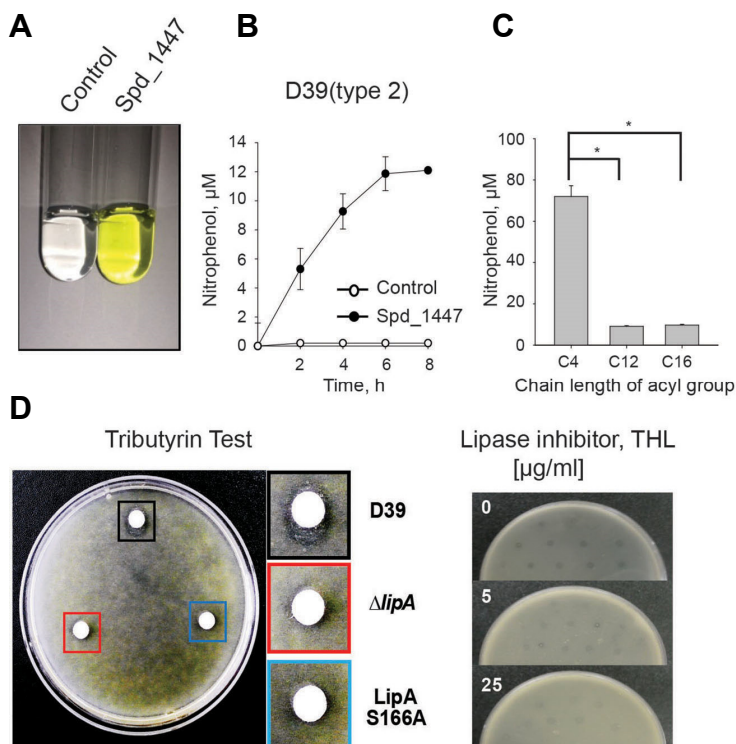


Fig. 1. *S. pneumoniae* Spd_1447 (LipA) lipase activity.

(A) *S. pneumoniae* D39 (type 2) Spd_1447 hydrolyzed p-nitrophenyl laurate and released p-nitrophenol (yellow color) after a 2-h incubation. (B) *S. pneumoniae* D39 (type 2) Spd_1447 hydrolyzed p-nitrophenyl laurate to p-nitrophenol in a time-dependent manner. (C) *S. pneumoniae* D39 Spd_1447 hydrolyzed p-nitrophenyl butyrate (C4), p-nitrophenyl dodecanoate (C12), and p-nitrophenyl palmitate (C16), and released p-nitrophenol after a 2-h incubation. The values were calibrated with a blank control and expressed as released p-nitrophenol concentration. Statistical analyses were performed using one-way ANOVA (**p* ≤ 0.05). (D) *S. pneumoniae* D39 WT (Black square) had a clearer zone on the tributyrin THY agar plate than the $\Delta lipA$ (Red square) or LipA S166A (Blue square). The lipase inhibitor THL impaired formation of the clear zone around the *S. pneumoniae* D39 WT colonies on tributyrin THY agar plates. The results displayed represent data from three independent experiments.

Next, lipase activity was analyzed using various lipid substrates, including p-nitrophenyl butyrate (C4), p-nitrophenyl dodecanoate (C12), and p-nitrophenyl palmitate (C16). The amount of p-nitrophenol released from the C4 substrate was dramatically higher than that from the C12 and C16 substrates (Fig. 1C), indicating that *S. pneumoniae* LipA preferentially hydrolyzes short-chain rather than long-chain lipid substrates.

A lipase-producing microbe can hydrolyze tributyrin and form a clear halo around the colony (Jørgensen et al., 1991). D39 WT displayed a larger clear zone around the colonies on a tributyrin agar plate than $\Delta lipA$ or LipA S166A, indicating LipA has lipase activity and serine residue at amino acid position 166 plays an important role (Fig. 1D). Although LipA was not secreted from D39 WT, our subcellular components fractionation experiment showed that substantial amount of LipA was localized at the cell wall and membrane (data not shown), thus allowing contact to substrate lipid and produce the clear zone in tributyrin assay. Moreover, when a lipase inhibitor (THL) was supplemented, the halo around the colony became fainter as lipase inhibitor concentrations increased, indicating inhibition of lipase activity (Fig. 1D). Thus, these data demonstrate that LipA possesses lipolytic activity.

LipA does not affect *S. pneumoniae* virulence in a pneumonia model

In some pathogens, lipases are considered to be secreted virulence factors (Gill et al., 2005; Stehr et al., 2004). However, LipA was not secreted from *S. pneumoniae* serotype 2 (D39; data not shown). For the pneumonia model, mice were infected *i.n.* with *S. pneumoniae*, and survival rates and bacterial colonization were determined. The survival rate of the mice infected with the $\Delta lipA$ was not significantly different from that in those infected with WT in three sero-

types (Fig. 2A). In addition, the bacterial colonization in the lung, nasopharynx, and blood at 48 h post-infection did not differ between D39 WT and $\Delta lipA$ (Fig. 2B), demonstrating that LipA does not affect *S. pneumoniae* virulence following *i.n.* infection.

LipA inhibits *S. pneumoniae* virulence during sepsis

For the sepsis model, mice were infected *i.p.* with 3×10^4 CFU of *S. pneumoniae*. The infection by $\Delta lipA$ or LipA S166A led to a dramatically higher mortality rate than D39 WT infection. Specifically, $\Delta lipA$ infection killed all the mice within 40 h post-infection, whereas 20% of D39 WT infected mice survived over 50 h post-infection (Fig. 3A). Furthermore, mice infected with WU2 $\Delta lipA$ and TIGR4 $\Delta lipA$ displayed a significantly higher mortality rate (90% and 30%, respectively) at 25 h post-infection than WT-infected mice (60% and 0% respectively; Fig. 3A), demonstrating the higher virulence of the $\Delta lipA$ with lack of lipase activity compared to the WT in a sepsis model.

In the colonization experiments, after *i.p.* infection, $\Delta lipA$ infection produced higher bacterial loads in the blood at 24 h post-infection ($p = 0.029$; Fig. 3B) than D39 infection. Unlike *i.p.* infection, in the colonization experiments, after *i.n.* infection, $\Delta lipA$ infection did not produce any significant difference in bacterial loads from D39 infection (Fig. 2B).

Additionally, to determine effect of LipA on pneumococcal virulence *in vitro*, cells cytotoxicity was measured after pneumococcal infection at the indicated time. $\Delta lipA$ infection produced higher cytotoxicity in RAW 264.7 or A549 cell (MOI, 25) than D39 or the complemented strain infection (Fig. 3C) in the presence of 10% fetal bovine serum. These results demonstrate that $\Delta lipA$ possesses a higher virulence than WT in sepsis model.

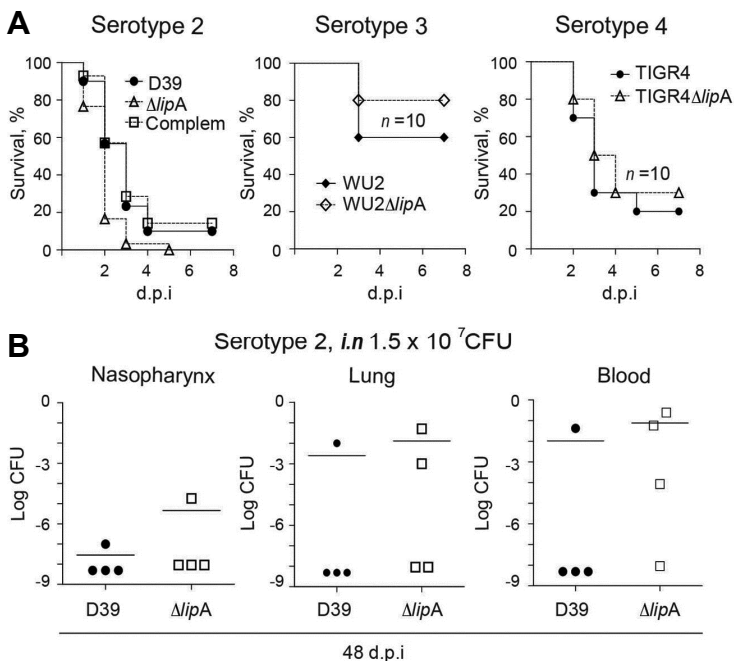


Fig. 2. LipA did not significantly attenuate virulence in a pneumonia model.

(A) Mice ($n = 15$) were infected *i.n.* with 1.5×10^7 CFU of the type 2 and 4 strains or 4.5×10^7 CFU of type 3, and survival time was recorded until 7 days post-infection (d.p.i.). (B) Mice ($n = 4$) were infected with 1.5×10^7 CFU of type 2 strain *i.n.*, and viable cells in the nasopharynx, lung, and blood were determined by plating the homogenized samples on THY blood agar. The experiment was performed twice and representative data was shown. Significant differences were analyzed by the log-rank test (A) or the Mann-Whitney rank sum test (B).

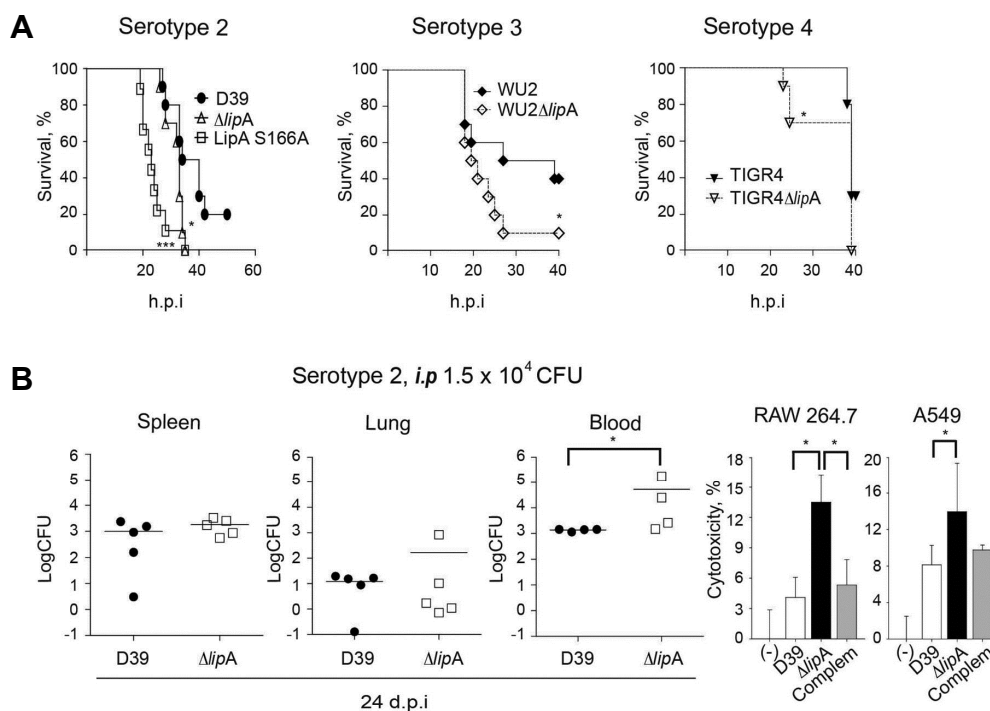


Fig. 3. Virulence attenuation by LipA in a sepsis model. (A) Mice ($n = 10$) were infected *i.p.* with 3×10^4 CFU of type 2 strains or 1.5×10^4 CFU of type 3 and 4 pneumococcal strains, and survival time was recorded until 60h post-infection (h.p.i.). (B) Mice ($n = 4-5$) were infected with 1.5×10^4 CFU of the type 2 strain *i.p.*, and viable cells in spleen, lung, and blood were determined by plating the homogenized samples on THY blood agar. The experiment was performed in twice and representative data was from one experiment. (C) The $\Delta lipA$ displayed higher cytotoxicity in the presence of serum than that of D39 and the complemented strain. RAW 264.7 or A549 cells were infected with pneumococcal strains (MOI, 25) for 2h in the presence of 10% FBS, and cytotoxicity was determined by 3-(4,5-dimethylthiazole-2-yl)-2,5-dephenyl-tetrazolium bromide assay. Results represent data from three independent experiments. Significant differences were analyzed by the log-rank test (A), the Mann-Whitney rank sum test (B), or one-way ANOVA (C) (* $p \leq 0.05$).

Serum induces LipA

In the sepsis model, $\Delta lipA$ significantly increased virulence (Fig. 3). Therefore, the impact of serum on LipA expression was determined. The addition of serum to the *S. pneumoniae* culture significantly induced *lipA* mRNA expression in D39 WT and the complemented strain (Fig. 4A). Moreover, serum supplementation also induced LipA level increases in D39 WT (Fig. 4B), indicating that serum can induce increases in levels of both *lipA* mRNA and LipA protein in *S. pneumoniae* *in vitro*.

To check *lipA* induction by serum, mice were infected *i.p.* with *S. pneumoniae* ($n = 3$), and bacterial RNA was isolated from the blood and analyzed by qPCR. *lipA* expression in the D39-infected mice was significantly higher than in the non-infected control (Fig. 4C), and *lipA* was undetectable in the $\Delta lipA$ -infected samples. Moreover, when pneumococci were incubated with whole mouse blood for 15 min, *lipA* expression in D39 WT was significantly induced (Fig. 4D), indicating *lipA* induction by serum in the sepsis model.

Serum inhibits autolysis in a LipA-dependent manner

Human serum suppresses antibiotic-mediated autolysis in gram-positive *S. aureus* (Stratton et al., 1986). However, the role of serum in *S. pneumoniae* autolysis remains unknown.

To identify whether serum can affect pneumococcal autolysis, DOC-induced autolysis were determined. Serum inhibited DOC-induced-autolysis of D39 WT and the complemented strain compared with $\Delta lipA$ (Fig. 5A). However, without serum, the autolysis rates of D39 WT, the complemented strain, and $\Delta lipA$ were similar (Fig. 5A). Moreover, in the presence of serum, after DOC treatment, the viability of the D39 WT was significantly higher than that of $\Delta lipA$ (Fig. 5A). However, without serum, the viability of the D39 WT was similar to that of $\Delta lipA$ (Fig. 5A).

Additionally, in the presence of serum, D39 WT Triton-X100-induced autolysis was abrogated compared with that of $\Delta lipA$ (Fig. 5B). In contrast, without serum, the autolysis rates of D39 WT and $\Delta lipA$ were not significantly different (Fig. 5B). Moreover, in the presence of serum, the viability of D39 WT was significantly higher than that of $\Delta lipA$ (Fig. 5B), while without serum, no significant difference in viability was observed (Fig. 5B). These data suggest that serum can inhibit *S. pneumoniae* autolysis via LipA.

Serum represses *S. pneumoniae* LytA levels via LipA

Among autolysins, LytA plays an important role in pneumococcal lysis (López and García, 2004). In the presence of serum, the *lytA* fold changes (serum/control) in D39 and the

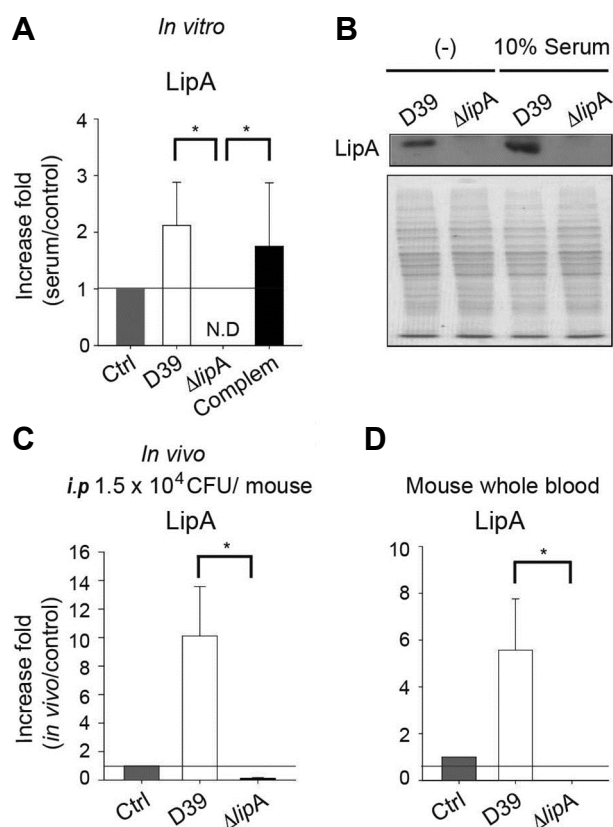


Fig. 4. LipA induction by serum. (A, B) *S. pneumoniae* strains were cultured until log phase. Goat serum was added to a final concentration of 10% and then incubated for 5 min, followed by *lipA* mRNA quantitation by qPCR (A), or incubated for 10min, followed by western blot analysis (B). SDS-PAGE results following Coomassie Blue staining was displayed and served as a loading control. The experiment was performed three times independently. Statistical analyses were performed using one-way ANOVA (* $p \leq 0.05$). (C) Mice ($n = 3$) were infected with 1.5×10^4 CFU of *S. pneumoniae* D39 or $\Delta lipA$ intraperitoneally (*i.p.*). At the severely morbid stage, mouse blood was collected, and *lipA* mRNA expression was determined by qPCR. (D) Pneumococci at log phase in THY broth were incubated with whole mouse blood for 15min, and *lipA* mRNA expression was determined by qPCR. (C, D) Each mouse sample was analyzed in triplicate, and the Mann-Whitney rank sum test was used for statistical analysis. N.D: not detected. (* $p \leq 0.05$). (A, C and D) Control bar (Ctrl) was displayed one fold value (control/ control).

complemented strain were lower than 1, indicating that *lytA* expression after the addition of serum was lower than in the control (Fig. 5C). However, in the presence of serum, the *lytA* fold change in the $\Delta lipA$ mutant was approximately 2, indicating that *lytA* expression after the addition of serum was significantly increased relative to that in the control (Fig. 5C). Therefore, these data suggest that serum can inhibit *lytA* expression in *S. pneumoniae* D39 WT but not in the $\Delta lipA$ mutant.

Next, mice were infected *i.p.* with *S. pneumoniae* ($n = 3$),

and *lytA* mRNA levels in the blood were determined. The *lytA* fold change (*in vivo*/control) in D39 was markedly lower than 1, indicating that *lytA* expression in a sepsis model was much lower than in the control. However, the *lytA* fold change in the $\Delta lipA$ mutant was approximately 4, suggesting that *lytA* expression in $\Delta lipA$ was significantly higher than in the control (Fig. 5C). Thus, in a sepsis model of infection, *lytA* expression was inhibited in D39 WT but not in the $\Delta lipA$ mutant.

In addition, when pneumococci were incubated with whole mouse blood, the *lytA* fold change (whole blood/control) in D39 was lower than 1, whereas the *lytA* fold change in the $\Delta lipA$ mutant was approximately 3 (Fig. 5C). These data indicate that *lytA* expression in D39 was lower than in the control, whereas *lytA* expression in $\Delta lipA$ was higher than in the control. Therefore, mouse blood inhibited *lytA* expression in D39 but not in the $\Delta lipA$ mutant.

At the protein level, pneumococcal culture at the log phase was supplemented with serum, and the total cell lysate was used for western blotting analysis. Serum inhibited LytA expression in D39 WT and the complemented strains at 5 and 10 min post-incubation, but did not inhibit in the $\Delta lipA$ mutant (Fig. 5D), suggesting that LipA suppresses LytA expression in the presence of serum.

DISCUSSION

Most studies on microbial lipase have focused on the broad applications of lipases in industrial biotechnology (Jaeger et al., 1994). In addition, the role of lipases in pathogenesis has been preferentially characterized in fungi rather than in bacteria. Deletion of lipase genes in *Candida parapsilosis*, a fungal pathogen, inhibited fungal growth and biofilm formation and resulted in virulence repression (Gácsér et al., 2007). Furthermore, the lipase mutant in *Fusarium graminearum* increased mycotoxin production (Voigt et al., 2007). Bacterial lipase studies have been limited to the secretion of the lipase and damage to host cells during infection (Nawabi et al., 2008; Rollof et al., 1988). Gram-positive *S. aureus* lipase contains a signal peptide (295 amino acids), which is cleaved during secretion (Rollof and Normark, 1992). *S. pneumoniae* lipase (LipA) has no signal peptide sequence, and no LipA was detected in the bacterial culture supernatant (data not shown), indicating that pneumococcal LipA is not secreted. Additionally, the pneumococcal LipA did not affect virulence significantly following *i.n.* infection but inhibited virulence in a sepsis model of infection. These differences may be attributed to the presence of serum. Pneumococci in *i.n.* route infection (pneumonia model) would experience transition from nasal cavity to pulmonary and to blood and might adapt slowly in due course of infection whereas *i.p.* route infection (sepsis model) would directly be exposed to blood in such a short time and adapt to blood milieu and host immune system rapidly. Serum can induce LipA expression at both the mRNA and protein levels. Additionally, pneumococcal bacteremia patients displayed decreased levels of serum triglycerides, cholesterol, and high density lipoprotein cholesterol (Kerttula and Weber, 1987), indicating that serum and serum compounds play a role during

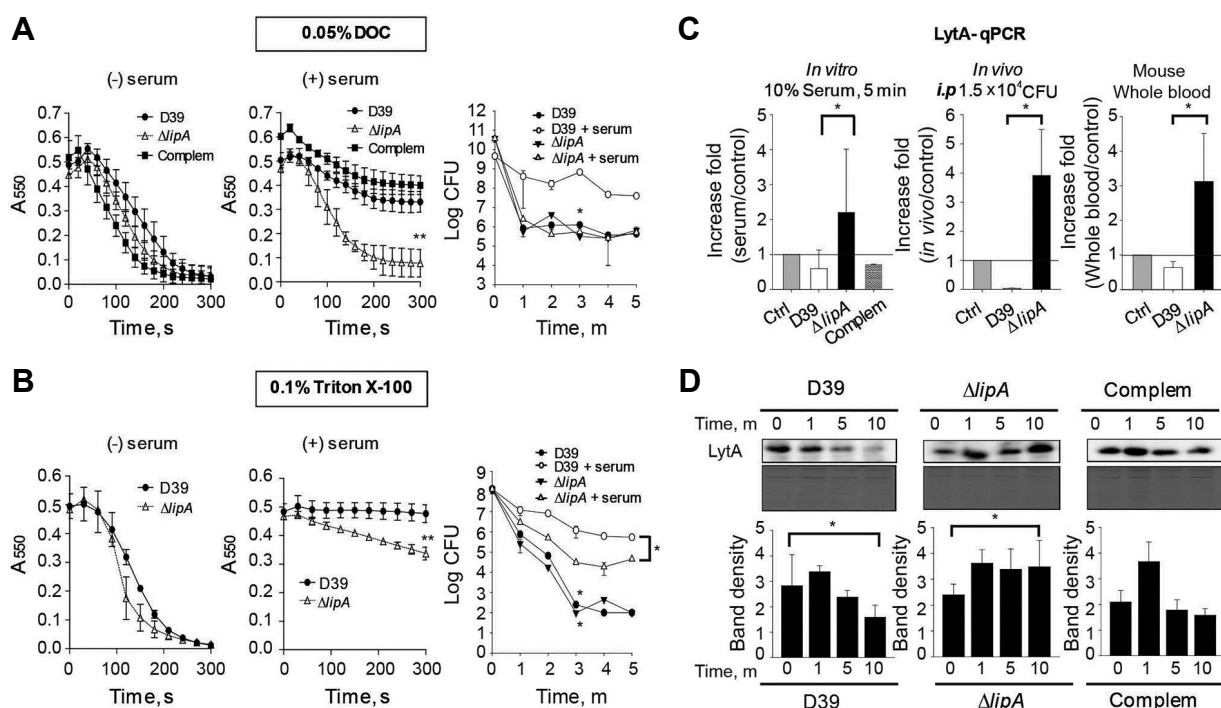


Fig. 5. Inhibition of autolysis and LytA expression by LipA *in vitro* and *in vivo*. Pneumococcal strains cultured in THY broth until log phase were incubated with 10% goat serum for 10 min. (A, B) 0.05% DOC (A) and 0.1% Triton X-100 (B) were added to the bacterial culture and absorbance at 550 nm or viable cell number was determined. (C) (left) Pneumococcal strains cultured in THY broth to the log phase were incubated with 10% goat serum for 5 min, and *lytA* mRNA was analyzed by qPCR (middle). Mice were infected with 1.5×10^4 CFU of pneumococcal strain intraperitoneally (*i.p.*). At the severely morbid stage, mouse blood was collected, and *lytA* RNA expression was analyzed by qPCR (right). The pneumococcal strain at the log phase was incubated with whole mouse blood for 15 min, and *lytA* mRNA was analyzed by qPCR. Each mouse sample was analyzed in triplicate, and the Mann-Whitney rank sum test was used for statistical analysis ($*p \leq 0.05$). Control bar (Ctrl) was displayed one fold value (control/control). (D) Pneumococcal strains cultured in THY broth to the log phase were incubated with 10% goat serum for 0, 1, 5, and 10 min. The LytA level was determined by western blotting. SDS-PAGE results following Coomassie Blue staining are displayed and served as a loading control. Band density was analyzed by ImageJ. (A, B, C-left, D) Representative data are displayed from three independent experiments and analyzed by one-way ANOVA ($*p \leq 0.05$, $**p \leq 0.01$).

pneumococcal sepsis. However, the correlation between the pneumococci and the host serum remains unclear. Here, we found that serum can affect bacterial lipase expression and pathogenesis.

Human serum inhibits antibiotic-mediated *S. aureus* autolysis (Stratton et al., 1986). Serum decreased DOC- or Triton X-100-induced pneumococcal autolysis. Moreover, serum impaired autolysis in the WT but not in the $\Delta lipA$, suggesting that serum-dependent inhibition of pneumococcal autolysis is mediated by LipA.

In addition, higher *lytA* expression in $\Delta lipA$ seems to decrease CFUs than the WT, however, $\Delta lipA$ shows higher CFUs than the D39 WT in the blood (Fig. 3B). DOC- or Triton X-100-induced pneumococcal autolysis would occur only until a limited number of cells and seems to be subject to feedback regulation resulting in normal growth stage at 5 min of serum exposure (Figs. 5A and 5B). We assumed that immediately after serum exposure, the $\Delta lipA$ induces *lytA* and subsequently lysis and release of cell wall and other cellular components, which trigger both proinflammatory cyto-

kines secretion from the host and nutrients procurement from the host resulting in inflammation (pneumonia and sepsis) as well as higher bacterial CFUs. Moreover, growth rate of the $\Delta lipA$ was recovered 5 h post-inoculation in the presence of serum (data not shown) suggesting that the $\Delta lipA$ seems to adapt to the serum environment and subject to feedback regulation resulting in repression of *lytA* expression. Therefore, the $\Delta lipA$ is more virulent immediately after the *i.p.* infection.

Short-chain fatty acids (SFAs), such as butyrate, increase the chemotactic response of neutrophils and neutrophil migration (Vinolo et al., 2009) or acetate and propionate bound to the murine GPR43 receptor, and enhance calcium flux, reactive oxygen species release, and the phagocytic activity of neutrophils (Maslowski et al., 2009). Butyrate also suppresses cytokines such as TNF- α and IL-6, but increases IL-10 (Chakravorty et al., 2000; Park et al., 2007) by inhibition of histone deacetylase activity (Waldecker et al., 2008) resulting in an increase in histone acetylation and modulation of the expression of these genes. Thus, butyrate and SFAs

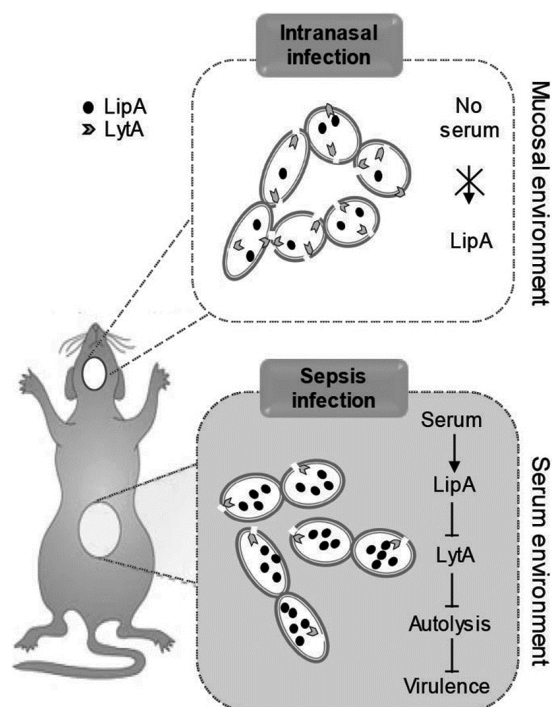


Fig. 6. Schematic model of serum-dependent autolysis inhibition by LipA in *S. pneumoniae* during sepsis. During intranasal infection, *S. pneumoniae* D39 WT and the $\Delta lipA$ did not display any significant differences in virulence. However, during sepsis infection, serum induced *lipA* expression, which subsequently inhibited LytA, leading to an inhibition of pneumococcal autolysis and virulence.

might modulate host defense inflammation. *S. pneumoniae* LipA preferentially releases butyrate (C4), rather than dodecanoate (C10) or palmitate (C12). Therefore, LipA can modulate inflammation and host defense mechanisms by producing SFAs and other fatty acids during pneumococcal infection. How LipA affects virulence through preferential substrate hydrolysis, and how LipA-produced fatty acids modulate host immunity during infection requires further investigation.

In summary, in a sepsis model, autolysis is inhibited to promote pneumococcal survival in the bloodstream prior to spreading into other niches. Pneumococcal autolysis suppression during septic infection is mediated by lipase LipA (Fig. 6). An investigation of pneumococcal LipA would provide a greater understanding of the mechanism by which *S. pneumoniae* modulates virulence to adapt to various host environments and maintain its survival until transfer to the next host.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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