



## Medical Microbiology

# The virulence of *Streptococcus pneumoniae* partially depends on *dprA*



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## ABSTRACT

*Streptococcus pneumoniae* is one of the most frequent opportunistic pathogens worldwide. DNA processing protein A (DprA) is an important factor involved in bacterial uptake and DNA integration into bacterial genome, but its role in *S. pneumoniae* virulence remains unclear. The aim of this study was to characterize the effects of the pneumococcal *dprA* gene on the pathogenesis of *S. pneumoniae*. To construct a *dprA*-deficient pneumococcal strain, the *dprA* gene of the *S. pneumoniae* strain D39 was inactivated. The virulence of this *dprA*-deficient strain, designated  $\Delta$ D39, was compared with that of the wild-type strain by evaluating their respective capabilities to adhere to human pulmonary epithelial cells (PEC-A549) and by analyzing their choline-binding protein expression levels. In addition, the expression profiles of genes associated with virulence and host survival assays were also conducted with the mutant and the wild-type strain. Our results indicate that the capability of  $\Delta$ D39 to adhere to the PEC-A549 airway cells was significantly lower ( $p < 0.01$ ) compared with D39. Additionally, the 100-KD choline-binding protein was not detected in  $\Delta$ D39. The addition of competence-stimulating peptide (CSP) lead to a significantly reduction of *psaA* mRNA expression in the *dprA*-deficient mutant and an increased level of *psaA* transcripts in the wild-type strain ( $p < 0.01$ ). The median survival time of mice intraperitoneally infected with  $\Delta$ D39 was significantly higher ( $p < 0.01$ ) than that of mice infected with D39. The results of this study suggest that DprA has a significant effect on virulence characteristics of *S. pneumoniae* by influencing the expression of choline-binding protein and PsaA.

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## Introduction

*Streptococcus pneumoniae* is a Gram-positive, facultative anaerobic, alpha-hemolytic streptococci. This pathogenic bacteria is widely found throughout nature and can cause different types infections, such as bronchitis, rhinitis, acute sinusitis, otitis media, conjunctivitis, meningitis, bacteremia, sepsis, osteomyelitis, septic arthritis, endocarditis, peritonitis, pericarditis, cellulitis, and brain abscess.<sup>1</sup> Due to the rapid increase of drug-resistant bacteria, the curative effect of antibiotics is declining.<sup>2</sup> To find new ways of treating pneumococcal infections, new virulence genes need to be identified and the molecular mechanisms of pneumococcal virulence and drug resistance should equally be thoroughly investigated.

One of the remarkable features of *S. pneumoniae* is their natural capability to undergo genetic transformation. This mechanism of DNA uptake may result in important changes in the bacterial genetic background, which may lead to the emergence of bacterial subpopulations carrying different genetic traits in relation to the parental generation.<sup>3</sup> When *S. pneumoniae* enter in the competent state, the autolysin—considered an important *S. pneumoniae* virulence factor—is activated. As consequence, *S. pneumoniae* release cell-wall fragments and hemolysin.<sup>4</sup> Another study demonstrated that the virulence of *S. pneumoniae* may significantly decrease after mutations in some of their protein genes.<sup>5</sup> All together, these data suggest that transformation can directly influence the virulence of *S. pneumoniae*.

Natural transformation in *S. pneumoniae* is a complex process that requires at least 23 genes. To integrate new DNA into the genome, a bacterium must enter in a special competent state for binding, taking up, and recombining exogenous DNA. In *S. pneumoniae*, competence is induced by DNA-damaging agents such as mitomycin C, topoisomerase inhibitors, and the fluoroquinolone antibiotics norfloxacin, levofloxacin, and moxifloxacin. Transformation protects *S. pneumoniae* against the bactericidal effect of DNA-damaging agents.<sup>6</sup> Additionally, the induction of competence in *S. pneumoniae* was associated with increased resistance to oxidative stress and upregulation of RecA protein, which plays a key role in removing DNA damage.<sup>7</sup>

In *S. pneumoniae*, the ubiquitous recombinases, RecA and DprA are required for DNA integration into the bacterial genome.<sup>8</sup> RecA plays important roles in the homologous recombination pathway, catalyzing DNA strand invasion and homology search.<sup>4</sup> DprA was reported to be involved in binding to the internalized single-stranded DNA (ssDNA) and promoting the loading of RecA on ssDNA during natural transformation.<sup>9</sup> The inactivation of *dprA* results in >10<sup>4</sup>-fold reduction in transformation.<sup>10</sup> DNA internalized in *dprA*-deficient competent cells appears to be completely destroyed,<sup>10</sup> suggesting that DprA plays a prominent role in protecting incoming ssDNA from nuclease(s). DprA can also repress the early expression of competence genes—by interacting with ComE during competence regulation—but does not affect proteolysis of labile early gene products.<sup>11</sup>

Despite the knowledge about the involvement of DprA in the bacterial DNA uptake and integration, the specific

mechanism by which DprA influences the virulence of *S. pneumoniae* remains unclear. Choline-binding proteins (CBPs), pneumolysin (Ply), capsular polysaccharide biosynthesis locus (*Cps2A*), and pneumococcal surface protein A (*PsaA*) are factors associated with the virulence of *S. pneumoniae*.<sup>12,13</sup> To further explore the role of DprA in *S. pneumoniae* virulence, a *dprA*-deficient mutant was constructed, and the influence of DprA on *S. pneumoniae* virulence was investigated by assessing adherence rates, the expression levels of virulence associated genes, and mice survival after infections with the isogenic *dprA*-deficient mutant and the wild-type strain of *S. pneumoniae*.

## Materials and methods

### Bacterial strains and growth conditions

The wild-type *S. pneumoniae* strain used in this study was D39, which is a virulent encapsulated type 2 *S. pneumoniae* strain obtained from the National Institute for the Control of Pharmaceutical and Biological Products of China. The *S. pneumoniae* cells were grown at 37 °C in 5% CO<sub>2</sub> in THY medium containing (per liter) 36.4 g of Todd-Hewitt broth and 5.0 g of yeast extract. Kanamycin was added to the growth medium at a concentration of 350 µg/mL for the selection of pneumococcal transformants.

*Escherichia coli* DH5 $\alpha$  cells (stored in our lab) were grown on Luria-Bertani (LB) agar or broth. pGEM-Teasy vector based plasmid were introduced into *E. coli* by transformation as previously described by Hanahan.<sup>14</sup> For the selection of *E. coli* transformants, ampicillin (100 µg/mL) was added to the growth medium.

For the determination of growth curves, *S. pneumoniae* cells were grown in THY medium to an OD<sub>620</sub> of 0.4, and the culture fluid was subsequently diluted to an OD<sub>620</sub> of 0.01. The OD<sub>620</sub> value was determined each 30 min, and a growth curve was plotted.

### Construction of a partial deleted-*dprA* mutant

To create a mutation in the *dprA* gene in *S. pneumoniae*, a 1351-bp *Juat* cassette was amplified with *Juat1* (5'-ATTGGCGGCCA-CCGTTTGATTTTAAATGGATAATG-3') and *Juat2* (5'-ATTGGCCGGCCCCCTTCTTATGCTTTTGGAC-3') from kanamycin-resistant *E. coli* chromosomal DNA and used to disrupt *dprA*. A 1111-bp fragment (*dprA* up) containing part of *dprA* was amplified with *dap1* (5'-CGCGAGCTCGAAAAATCCTCTACTCTACTAATCCAC-3') and *dap3* (5'-ATTGGCCGGCCAGATTCCAACATCTGACAAAATAG-3') from *S. pneumoniae* DNA. An 872-bp fragment (*dprA* down) containing part of both the downstream *dprA* sequence and *licC* was amplified with *dap2* (5'-ATTGGCGCGCGCTTTCACACGAATCTCCTTTATT-3') and *dap4* (5'-TCCCCGCGGTTTTCTGGGGCTTGGGTCTA-3') from *S. pneumoniae* DNA. The resulting three PCR products were digested using restriction enzymes (*FseI*, *AscI*) known to cut at the corresponding sites within the primers, and the digested products were ligated together into a pGEM-T vector (Promega, USA). The pGEM-T target vector was subsequently introduced into *S. pneumoniae* D39 by transformation, and recipient bacteria that had successfully integrated the recombinant fragment

into their chromosomes by homologous recombination were selected by their resistance to kanamycin. Primers *dap1* and *dap4* were used to screen for the appropriate deletion, and the resulting PCR products were confirmed by sequencing. *S. pneumoniae* containing the correct deletion within *dprA* was named  $\Delta D39$  and was used for further studies.

#### Adherence of the wild-type and *dprA*-deficient *S. pneumoniae* strains to PEC-A549 airway cells

The capability of the *dprA*-deficient mutant to adhere to the human pulmonary epithelial cell line, PEC-A549 (Shanghai Institute of Cell Biology, China) was assessed in 12-well tissue culture plates. The PEC-A549 cells were cultured in DMEM/Ham's F-12 medium (pH 7.5) with 5% fetal calf serum for 2–3 days. The PEC-A549 cell monolayers were then rinsed twice with phosphate-buffered saline (PBS), and *S. pneumoniae* cells suspended in DMEM-Ham's F-12 medium ( $2.0 \times 10^7$  CFU/mL; 1 mL/well) were added to the cells on 12-well plates. After 1 h-incubation at 37 °C, the PEC-A549 cells were washed five times with PBS (pH 7.5) to remove any remaining non-adherent bacteria. The mixture (epithelial and bacterial cells) was treated with 0.25% trypsin with 0.02% EDTA (200  $\mu$ L/well) at 37 °C for 5 min. After, 400  $\mu$ L of 0.025% cold TritonX-100 was added and the mixture was repeatedly pipetted to lyse the epithelial cells. The mixture was diluted with PBS, and 100  $\mu$ L of trypticase soy agar plates containing 5% defibrinated sheep serum were inoculated into each well. The bacterial cells were cultured for 16 h and the number of bacterial colonies counted. Adherence was determined by dividing the number of adherent bacteria per 100 epithelial cells. The values reflect averages of four wells from independent experiments.

#### Real-time RT-PCR

To investigate if *dprA* plays a role in the expression of some important virulence genes during bacterial transformation, both the wild-type (D39) strain and *dprA*-deficient ( $\Delta D39$ ) pneumococcal mutant (grown at a pH of 8.0) were induced for competence by adding competence-stimulating peptide (CSP; 20 ng/mL) at the optimal bacterial cell density (OD<sub>550</sub> of 0.1) to induce competence. For the real-time quantitative reverse transcription-PCR (real time RT-qPCR) analysis, the bacteria were collected at 0 and 20 min intervals after the addition of CSP to the culture.

Total RNA was obtained using the hot acid phenol method as previously described.<sup>15</sup> Levels of mRNA for *pneumolysin* (*ply*), *psaA*, *cbpA*, and *cps2A* genes were quantified by one-step real-time RT-PCR with the Takara SYBR RT-PCR system (Takara Bio). The specific primers used for the real time qRT-PCR assays have been previously described elsewhere<sup>16</sup> and were used at a final concentration of 100 nM per reaction. Specific primers for the 16S rRNA were included as an internal control. The real-time qPCR was performed in an ABI7500 Real-Time cyclor (Applied Biosystems, USA). The RT-PCR cycling conditions were set as instructed by the manual for the Takara SYBR RT-PCR system. Amplification data were acquired at the extension step and analyzed using the ABI Software version 2.0 with comparative critical threshold values. The  $\Delta\Delta Ct$  calculation for the relative quantification of target was used as follows

$\Delta\Delta Ct = (Ct, \text{ target gene} - Ct, 16S \text{ rRNA})_{\chi} - (Ct, \text{ target gene} - Ct, 16S \text{ rRNA})_{\gamma}$ , where  $\chi = S. pneumoniae$  strain  $\Delta D39$  with and without CSP and  $\gamma = S. pneumoniae$  strain D39 with and without CSP. After validation of the method, results for each sample were expressed in N-fold changes in  $\chi$  target gene copies, normalized to 16S rRNA relative to the copy number of the target gene, according to the following equation: amount of target =  $2^{-\Delta\Delta Ct}$ .<sup>17</sup> All experiments were carried out in quadruplicate.

#### Analysis of choline-binding proteins (CBPs) expression

For CBPs analysis experiments, wild-type (D39) and *dprA*-deficient ( $\Delta D39$ ) *S. pneumoniae* isogenic cells were grown in HYT medium to an OD<sub>620</sub> of 0.6, centrifuged at 5000 rpm for 20 min, and washed twice with PBS. The cells were then harvested, resuspended in a PBS solution containing 2% choline chloride at  $10^{10}$  cells/mL and equilibrated for 20 min at room temperature. The cells were subsequently centrifuged at 5000 rpm for 20 min, and resuspended in 100 mL of TBS. The cell suspension was subjected to ultrasonication with 15 5-second pulses with 10-second intervals between pulses at 400W (Misonix XL2020, Farmingdale, NY) on ice. SDS-PAGE (12% polyacrylamide gel) was carried out as previously described by Laemmli,<sup>18</sup> and the proteins in the gel were visualized by performing Coomassie brilliant blue staining.

#### Virulence studies

Intraperitoneal challenge with a highly virulent strain of *S. pneumoniae* (D39) or its isogenic *dprA*-deficient mutant ( $\Delta D39$ ) was performed to evaluate the role of *dprA* on *S. pneumoniae* virulence. Male 6- to 8-week-old BALB/c mice weighing 18–22 g were used in this experiment. Inoculation of the mice with pneumococci was conducted according to the previously described procedure.<sup>13</sup> The wild-type strain and *dprA* mutant were tested in two separate experiments, involving each a total of 21 mice. To assess *S. pneumoniae* virulence in blood, the BALB/c mice were intraperitoneally injected with 0.1 mL of  $5 \times 10^6$  CFU/mL of a suspension of pneumococcal cells. The numbers of surviving animals were then recorded daily for 14 days. All experimental procedures were conformed with the guidelines for the care and use of laboratory animals of PLA General Hospital, Beijing, China (No PLAGH2014078).

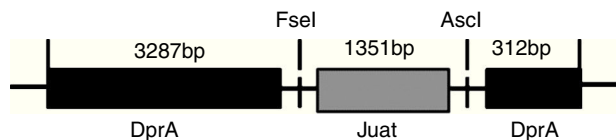
#### Statistics

Statistical analyses were performed using unpaired Student's t-tests. Data are presented as means  $\pm$  the standard deviation of the mean for four independent experiments. Differences in the mean survival time between groups were analyzed using two-tailed Mann-Whitney U test.

## Results

#### Construction and growth rate of the *dprA* mutant strain ( $\Delta D39$ )

For the construction of the *dprA* mutant, a DNA fragment containing a *NotI* cassette insertion was amplified by PCR



**Fig. 1 – Mutated region of *dprA*. A schematic of the *dprA* mutation that was created by the insertion of a 1351-bp *Juat* cassette.**

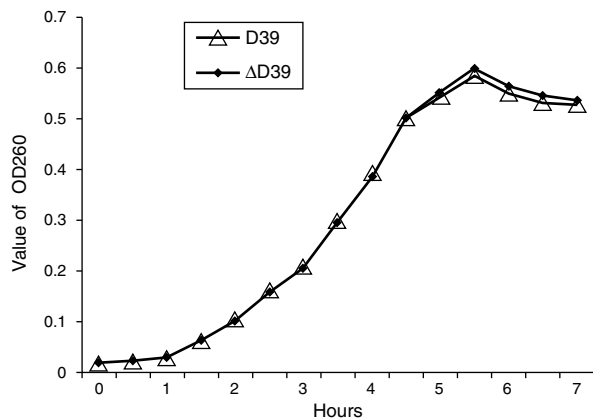
and incorporated into the chromosome by transformation as described in Materials and Methods (Fig. 1). The construction of the mutant was confirmed by PCR demonstrating the absence of the *dprA* segment. The growth rate of *dprA* mutant at 37 °C was similar to that of the parental strain D39, with a doubling time of 55 min, compared with about 54 min for D39 (Fig. 2). Thus, *dprA* does not seem to be essential for the growth of *S. pneumoniae* at 37 °C.

#### Effect of a *dprA* mutation on the *S. pneumoniae* adherence rate

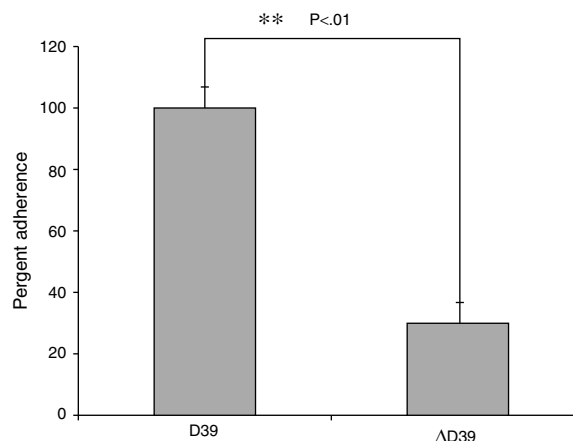
The effect of the *dprA* gene on the adherence rate of *S. pneumoniae* strain D39 was determined by comparing the capability of the wild-type strain D39 and the isogenic *dprA* mutant ( $\Delta$ D39) to adhere to pulmonary epithelial cell line PEC-A549 *in vitro*. In comparison with D39, the adherence rate of  $\Delta$ D39 to PEC-A549 cells was significantly lower ( $p < 0.01$ ). Specifically, the adherence rate of  $\Delta$ D39 was only 30% of the adherence rate achieved by the parent strain, D39 (Fig. 3). This result suggests that *dprA* plays an important role in the adherence of *S. pneumoniae* to host cells.

#### Effect of *dprA* mutation on the expression of choline-binding proteins (CBPs)

CBPs are major *S. pneumoniae* virulence factors that exist on the bacterial surface. Changes in CBPs expression can influence the adherence rate and virulence of *S. pneumoniae*. Unlike the observed for the wild-type strain,<sup>12,13</sup> we did not detect the



**Fig. 2 – Growth curves of *S. pneumoniae* strains D39 and  $\Delta$ D39. Growth curves of *S. pneumoniae* wild-type (D39) strain and *dprA*-insertional ( $\Delta$ D39) mutant. Data are representative of four independent experiments.**



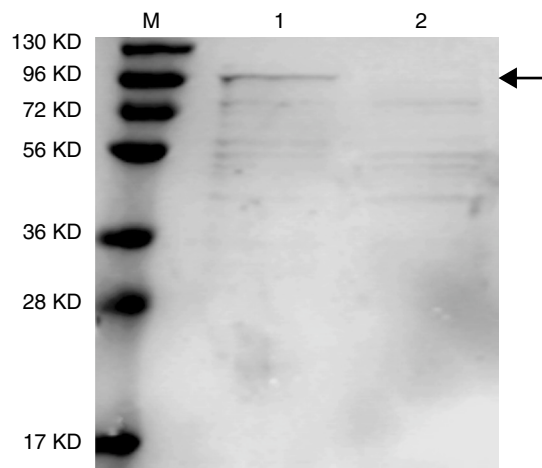
**Fig. 3 – Adherence of *S. pneumoniae* strains D39 and  $\Delta$ D39 to PEC-A549 cells. Adherence was expressed as the number of pneumococci attached per 100 PEC-A549 cells.**

100-KD CBP in the *dprA* mutant (Fig. 4). This result suggests that the expression of CBPs seems to be dependent on the expression of the *dprA* gene.

#### Modulation of the expression of virulence-associated factors by CSP-induced competence

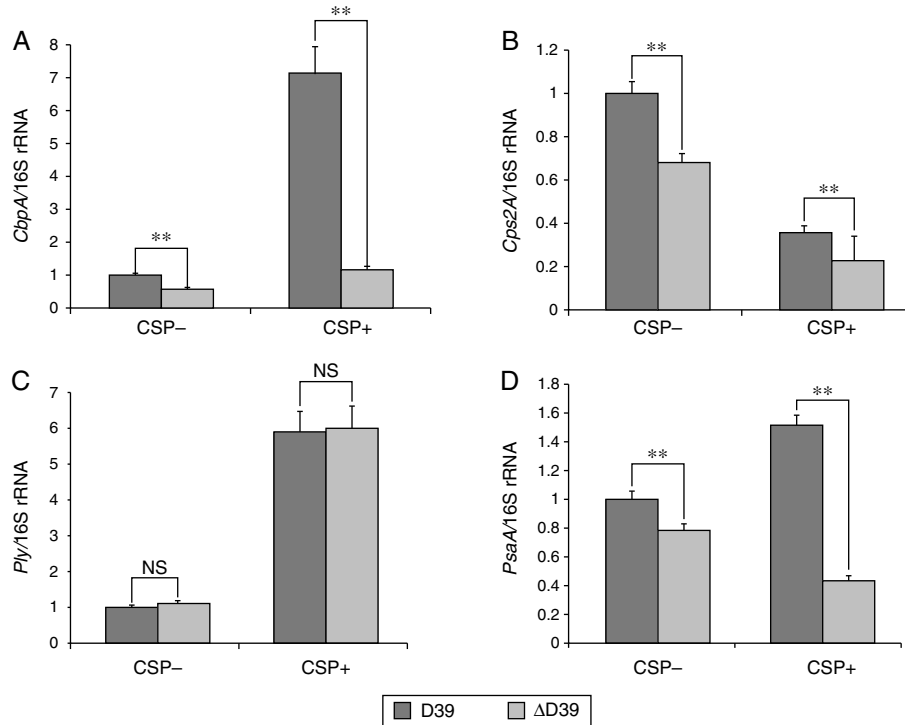
Competence, induced by CSP, can affect the expression of virulence factors.<sup>19</sup> Thus, the modulation of virulence gene expression was examined at the mRNA level in the wild-type *S. pneumoniae* strain and in the *dprA*-mutant. The mRNA levels of *ply*, *cbpA*, *psaA*, and the capsule synthesis gene *cps2A* were determined by real-time RT-PCR (Fig. 5).

The baseline expression levels of *cbpA*, *cps2A*, and *psaA* were lower in  $\Delta$ D39 than in D39 ( $p < 0.01$ ), but the baseline



**Fig. 4 – Detection of choline-binding proteins (CBPs) in *S. pneumoniae* strains D39 and  $\Delta$ D39. A representative SDS-PAGE gel of the corresponding cell lysis products is shown. Left to right: slot M, protein molecular weight marker; slot 1, D39; slot 2,  $\Delta$ D39. Arrow indicates the choline-binding proteins (CBPs) band.**





**Fig. 5 – Relative quantity of *cbpA*, *cps2A*, *ply*, and *psaA* mRNA in *S. pneumoniae* strains D39 and  $\Delta$ D39 with and without CSP. Real-time qRT-PCR was performed to assess the relative mRNA concentrations of *cbpA* (A), *cps2A* (B), *ply* (C), and *psaA* (D) in *S. pneumoniae* strains D39 and  $\Delta$ D39 in the absence or presence of CSP. The levels of individual mRNA species were normalized using as reference the internal control (16S rRNA). \*\*  $p < 0.01$ , NS = no significance.**

expression levels of *ply* were not statistically different between the wild-type and the isogenic *dprA* mutant. Following the addition of CSP, the relative *cbpA* mRNA level increased 7.15-fold ( $p < 0.01$ ) in D39, but the level of *cbpA* expression only increased 2.1-fold ( $p < 0.01$ ) in the *dprA* mutant (Fig. 5A). Additionally, the mRNA levels of *ply* were increased 5.83- and 5.4-fold in D39 and the *dprA* mutant, respectively, after CSP was added (both  $p < 0.01$ ) (Fig. 5C). Furthermore, expression of *cps2A* in the *dprA* mutant was significantly decreased following CSP addition ( $p < 0.01$ ) (Fig. 5B). The expression of *psaA* was significantly increased ( $p < 0.01$ ) in D39 after CSP was added. In contrast, CSP treatment decreased the mRNA level of *psaA* by 1.8-fold in the *dprA* mutant ( $p < 0.01$ ) (Fig. 5D). Together, our results suggest that the *dprA* mutation may negatively affect the expression of *psaA*.

#### Effect of a *dprA* mutation on *S. pneumoniae* virulence

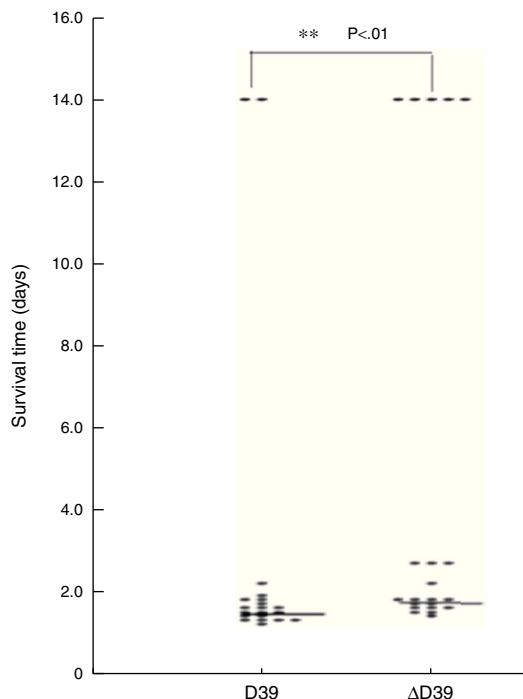
To further investigate the effect of the *dprA* mutation on the virulence of *S. pneumoniae*, the survival time of mice after intraperitoneal infection with approximately  $10^5$  CFU of D39 or  $\Delta$ D39 pneumococci was measured. The median survival time (36 h) for mice infected with the parental strain (D39) was significantly lower than that (44 h) for mice infected with the *dprA* mutant (Fig. 6), as was the overall survival rate ( $p < 0.01$ ). This result indicates that *dprA* function is critical for virulence factor expression in *S. pneumoniae*.

## Discussion

CPBs are proteins that were previously associated with cell lysis<sup>20</sup> and contain a number of short choline-binding repeats that anchor them non-covalently to teichoic and lipoteichoic acids in the pneumococcal cell wall.<sup>21</sup> We found that *S. pneumoniae* cells with mutated *dprA* lack 100-KD CBPs, suggesting that *dprA* plays a key role in CBPs expression and thus might influence the CBPs competence-induced cell lysis. However, further studies are needed to precisely determine the protein(s) affected and the correspondent mechanisms.

Our results also demonstrated that the adherence to PEC-A549 cells of pneumococcal strain D39 was reduced by mutation in *dprA*. Because PspA, PspC, CbpA are associated with bacterial adherence to host cells,<sup>22–24</sup> it seems logical to examine the relationship between *dprA* and these proteins. Here, we assessed the expressions of *cbpA*, *cps2A*, and *psaA* in the *S. pneumoniae* D39 strain and in the isogenic *dprA* mutant by real-time qRT-PCR. Our data demonstrate that the  $\Delta$ *dprA* has lower levels of *cbpA*, *cps2A*, and *psaA* expression compared with the parental wild-type strain.

After CSP was added to induce competence, the expression level of *cps2A*, which is the first gene in the capsule biosynthesis locus, was reduced in both the wild-type strain and the *dprA* mutant, what might result in a potentially lower resistance to the host immune system.<sup>25</sup> However, the expression level of *cps2A* in the *dprA* mutant was lower



**Fig. 6 – Intraperitoneal challenge with *S. pneumoniae* strains D39 and  $\Delta$ D39. Groups of BALB/c mice were intraperitoneally injected with  $5 \times 10^8$  CFU of *S. pneumoniae* strains D39 and  $\Delta$ D39. The survival time of each of the mice is indicated ( $p$  value  $< 0.01$ ). Transverse lines denote median survival times for each of the groups.**

than that of wild-type strain, which may probably has some consequence for the bacterial virulence. In the presence of CSP, the expression level of *cbpA* increased in both wild-type and *dprA* mutant, but the expression level of *cbpA* in the *dprA* mutant was much lower compared with the wild type. This result seems to indicate that *dprA* upregulates *cbpA* mainly during the competence state.

Notably, the expression of *psaA* was increased in the D39 following the addition of CSP, but it was decreased in the *dprA* mutant, suggesting that *dprA* plays an important role in the regulation of *psaA* expression. Therefore, virulence gene regulation might be modulated by DprA. Previous studies have shown that the combination of low virulence with high resistance to host immune cells may lead to the establishment of chronic bacteremia, in which the bacteria are able to evade the host immune system and survive in the host but are unable to cause fulminant disease, a phenomenon that has been previously demonstrated in a *pneumolysin*-negative mutant of *S. pneumoniae* strain R6.<sup>4,5</sup> Although we did not test that hypothesis, it is possible that the *dprA* mutant might exhibit a wild-type level of resistance to host macrophages upon stress challenge, even though the overall virulence was decreased.

In fact, the mouse infection model indicates that mutation in *dprA* can significantly attenuate the virulence of a pneumococcal strain. This seems to suggest that factors related with competence and transformation mechanisms are possibly linked with pathogenic process of *S. pneumoniae*. However, this connection also appear to rely, at least in part, on

the genetic background of the bacterium. For example, the virulence of another pneumococcal strain was reduced by approximately 8000-fold in a systemic infection model with a *comD* gene mutant compared with the isogenic wild-type strain.<sup>5</sup> Whether or not these factors are categorically involved in competence and transformation is a matter that requires further investigation.

In summary, DprA is an essential recombination-mediated protein that is involved in the natural transformation of *S. pneumoniae*. This protein mainly protects the intracellular plasmid or DNA that were taken up by the bacteria from degradation by DNase. Here, we report that a *dprA* mutation leads to a decreased virulence of *S. pneumoniae* that was paralleled by the absence of CBPs. Because natural transformation is closely correlated with the emergence of drug resistance, the finds that *dprA* participates in both transformation and virulence suggests that DprA may be an effective target for inhibiting drug resistance and reducing *S. pneumoniae* virulence.

### Conflicts of interest

The authors declare no conflicts of interest.

### Acknowledgments

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