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SP0454, A Putative Threonine Dehydratase, Is Required For Pneumococcal Virulence In Mice

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Increasing pressure in antibiotic resistance and the requirement for the design of new vaccines are the objectives of clarifying the putative virulence factors in pneumococcal infection. In this study, the putative threonine dehydratase *sp0454* was inactivated by erythromycin-resistance cassette replacement in *Streptococcus pneumoniae* CMCC 31203 strain. The *sp0454* mutant was tested for cell growth, adherence, colonization, and virulence in a murine model. The Δ*sp0454* mutant showed decreased ability for colonization and impaired ability to adhere to A549 cells. However, the SP0454 polypeptide or its antiserum did not affect pneumococcal CMCC 31203 adhesion to A549 cells. The *sp0454* deletion mutant was less virulent in a murine intranasal infection model. Real-time RT-PCR analysis revealed significant decrease of the pneumococcal surface antigen A expression in the *sp0454* mutant. These results suggest that SP0454 contributes to virulence and colonization, which could be explained in part by modulating the expression of other virulence factors, such as *psaA* in pneumococcal infection.

Keywords: *Streptococcus pneumoniae*, *sp0454*, colonization, virulence, adherence, invasion

Introduction

Streptococcus pneumoniae is a common asymptomatic commensal of the nasopharynx in healthy individuals. However, upon dissemination to other sites of the body, this Gram-positive bacterium causes diseases, such as otitis media, pneumonia, septicemia, and meningitis. Children, elderly people, and immunocompromised patients are recognized as the populations at high risk of suffering from serious invasive pneumococcal diseases (Kwon *et al*., 2003; Hendriksen *et al*., 2008; Zhang *et al*., 2009).

Nasopharyngeal colonization is mainly mediated by the attachment of bacterial surface-exposed components to epithelial cells in the upper respiratory tract. This process may be a prerequisite for normal invasive pneumococcal infections. In addition, nasopharyngeal carriage has been proposed to serve as a main source of the transmission of pneumococci in the community (Bogaert *et al*., 2004; Bootsma *et al*., 2007). Examining the capacity of colonization appears an essential step in studying the contribution of a novel factor.

Many well-characterized components, such as capsule, choline-binding proteins, and pneumolysin, have been implicated in pneumococcal diseases (Bergmann and Hammerschmidt, 2006; Nelson *et al*., 2007). More recently, some novel factors were tested for their involvement in the pathogenesis of pneumococcal infections. These factors include, but are not limited to, surface-associated streptococcal lipoprotein rotamase A (SlrA), putative proteinase maturation protein A (PpmA) (Hermans *et al*., 2006; Cron *et al*., 2009), serine protease HtrA (Sebert *et al*., 2002), and two plasmin- and fibronectin-binding proteins PfbA and PfbB (Yamaguchi *et al*., 2008; Papasergi *et al*., 2010). All the abovementioned factors have contributed to the virulence of *S. pneumoniae* in distinct ways. These studies highlight the need for further research of other putative virulence factors that possibly play roles in pneumococcal infections.

The gene *sp0454* (GenBank: AE007356) is an *in vivo* inducible gene in a mouse sepsis model (Meng *et al*., 2008). This gene is important in infection, as identified by signature-tagged mutagenesis technique (Hava and Camilli, 2002). Previous studies revealed that *sp0454* is involved in pneumococcal infection, but does not integrate other necessary biological studies. Bioinformatic analysis predicted that SP0454 might be a threonine dehydratase in relation to the metabolism of branch-chain amino acids. Antigen identification study showed that SP0454 has highly immunogenic epitopes and is considered the most promising vaccine candidate (Giefing *et al*., 2007). It is therefore hypothesized that SP0454 might be a significant contributor in pneumococcal infections. In the present study, *sp0454* was inactivated in a CMCC 31203 pneumococcal strain background and tested for its contribution to pneumococcal virulence in terms of growth, adherence, and colonization.

Materials and Methods

Bacterial strains

S. pneumoniae strain CMCC 31203 (serotype 3) was obtained from China Medical Culture Collection (CMCC, Beijing, China). Pneumococci were routinely grown in C+Y medium or on blood agar (BA) plates under microaerophilic condi-

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tions at 37°C. Erythromycin (0.25 μg/ml) was added to the culture medium as needed.

Δ*sp0454* mutant

The gene *sp0454* was deleted from the parental CMCC 31203 strain by isogenic gene replacement with erythromycin (erm) cassettes as described elsewhere (Wach, 1996; Meng *et al*., 2008). Briefly, a 780 bp *erm* cassette was amplified with *erm*a1 and *erm*a2 from the chromosomal DNA of erythromycin-resistant *S. pneumoniae* CPM8 (provided by D. A. Morrison from the University of Illinois, Chicago). A 339 bp upstream fragment was amplified with *sp0454*a1 and *sp0454*a3 from *S. pneumoniae* CMCC 31203 chromosomal DNA. A 421 bp downstream fragment of *sp0454* was amplified with *sp0454*a2 and *sp0454*a4 from *S. pneumoniae* CMCC 31203 chromosomal DNA. The *erm* and upstream and downstream fragments of *sp0454* were used as template for generating an *up-erm-down* fusion fragment using primers *sp0454*a1 and *sp0454*a2. The fusion fragment was transformed into *S. pneumoniae* CMCC 31203 with 100 ng of synthetic CSP-1 peptide per ml (Pozzi *et al*., 1996). Viable mutants were selected on BA plates supplemented with 0.25 μg/ml erythromycin, and confirmed for *sp0454* deletion by PCR. All primers are listed in Table 1. RT-PCR was carried out to detect the expression of the flanking region genes *sp0453* and *sp0455* and to rule out the polarity of erythromycin-resistance cassette replacement.

Virulence studies

All animal experiments were conducted according to relevant national and international guidelines and approved by the ethics committee of Chongqing Medical University, China [Permission Number: SYXK (yu) 2007-0001]. Female Balb/c mice (6 weeks to 8 weeks old) were used for the infection experiments. Bacteria were cultured overnight at 37°C on BA plates (supplemented with erythromycin as required), and then grown in C+Y medium at 37°C to OD_{600}≈0.4. Twenty mice were randomly assigned into two groups receiving a challenge of wild-type or mutant pneumococcal strain. Mice were lightly anesthetized with 1.5% pentobarbital sodium and then received inoculation of a 30 μl sample of PBS containing approximately 5×10^8 CFU of *S. pneumoniae* via the nostrils. The infection dose was confirmed by a viable count on blood agar plates. Survival of the challenged mice was monitored 4 times daily for the first 5 days, twice per day for the subsequent 5 days, and daily until 21 days after the challenge. At the end of the experiment, the mice were sacrificed via excessive inhalation of CO_2.

Recombinant SP0454 polypeptide and its antiserum

Based on bioinformatic analysis, an extra-membrane loop of SP0454 was overexpressed and purified. The encoding sequence (70 bp to 807 bp) was amplified by PCR from *S. pneumoniae* CMCC 31203 with primers *sp0454b1* and *sp0454b2*, which incorporated flanking *Nde*I and *Bam*HI restriction sites (Table 1). After digestion with *Nde*I and *Bam*HI, the resulting fragment was then cloned into pCold® TF (TaKaRa, China) and transformed into *E. coli* BL21. The SP0454 polypeptide was induced with the addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to increase the phase cultures of *E. coli* BL21 exponentially. After incubation at 20°C for 15 h, cells were harvested by centrifugation and lysed by sonication. The SP0454 polypeptide was purified by affinity chromatography on Ni nitrilotriacetic acid resins according to manufacturer's instructions. Human rhinovirus 3C protease (HRV 3C protease, produced by our own laboratory) was used to remove the tag. The expression and purification of HRV 3C protease was performed as described previously (Wen *et al*., 2005). The fusion protein SP0454 was digested by incubating with HRV 3C protease

Table 1. Primers used in this study

Primer	Sequence
*erm*a1	5′-CCGGGCCCAAAATTTGTTTGAT-3′
*erm*a2	5′-AGTCGGCAGCGACTCATAGAAT-3′
*sp0454*a1	5'- AGACCTAAGGGCAGGGACTA -3'
*sp0454*a3	5'-ATCAAACAAATTTTGGGCCCGGTTCACGATAACAGAGGAGACAGT-3'
*sp0454*a2	5'- CATCTGCGTTGGGCACTCAT -3'
*sp0454*a4	5'-ATTCTATGAGTCGCTGCCGACTCGTAGGTGGCGGTATTATCG -3'
sp0454b1	5'-GGAATTC**CATATG**ATAAACTCTTATCAAGGGGATTTGT -3'
sp0454b2	5'-CGC**GGATCC**TTAAAGTAGTTGTTTACTTTGATCTTTT -3'
16S rRNA a1	5′- GTAGTCCACGCTGTAAACGATGAGT-3′
16S rRNA a2	5′- CTGTCCGAAGGAAAACTCTATCT -3′
psaA a1	5′- CTAAAGCCTATGGTGTCCCAAG -3′
psaA a2	5′- CGGTCATCCACATTGATTCTAC -3′
nanA a1	5′-AGTCTCTGAAGAAGGCTGGCTCT-3′
nanA a2	5′-GAGCAGTGGTATTTGGAACGTC-3′
sp0453 a1	5′-GTCGGAACCATCATAGGTCTCA-3′
sp0453 a2	5′-GCAGCCAGTGTACGGTCAAG-3′
sp0455 a1	5′-AGACCTACCATAGACTGA-3′
sp0455 a2	5′-GTTTAGATAACACTATTGAC-3’

Note: The underline portions refer to the reverse complementary sequence of *erm*a1 and *erm*a2 respectively.
The bold parts denote the flanking *Nde*I and *Bam*HI sequence of *sp0454b1* and *sp0454b2*

[5:1 (mass ratio)] at 4°C for 12 h, followed by further purification with Hiprep 16/60 Sephacryl S-100 HR (GE healthcare) according to the manufacturer's recommendations. Polyclonal anti-SP0454 polypeptide antiserum was raised in mice by routine immunogenic procedures (Gong *et al*., 2010; Wu *et al*., 2010).

Colonization studies

The enumeration of bacteria in different organs after the intranasal challenge was performed as described previously (Jeong *et al*., 2009). Briefly, 60 Balb/c mice were randomly divided into 2 groups and intranasally inoculated with either the wild-type strain or the Δ*sp0454* mutant at 1×10^8 CFU. Mice were sacrificed by cervical dislocation at 12, 24, 36, 48, and 72 h post-infection. Nasal washes were collected. Lungs were isolated aseptically, washed three times with PBS (pH 7.3), and homogenized in PBS with a tissue homogenizer on ice. Serial dilution in sterile PBS was made before plating onto blood agar. Plates were incubated for approximately 16 h at 37°C in an atmosphere of 95% air-5% CO_2 to obtain viable colonies.

Adherence and invasion assays

Adherence and invasion assays were performed as described previously (Pracht *et al*., 2005). Briefly, human lung epithelial carcinoma A549 (Shanghai Rui Qi Biotechnology Co., Ltd., China) was grown to 90% confluence in 24-well tissue culture plates and washed 3 times with PBS (pH 7.3). Culture medium (1 ml, without antibiotics) was then added to each well. Exponential-phase cultures of CMCC31203 and Δ*sp0454* mutant were harvested by centrifugation, washed with PBS, and re-suspended in Dulbecco's Modified Eagle's Medium. Monolayers were infected with 2×10^7 bacteria (bacterium/cell ratio=100:1), followed by another 1 h of incubation at 37°C. Cells were trypsinized, lysed, and plated to obtain viable colonies to determine the total numbers of adherent and intracellular bacteria. For invasion experiments, cells were treated with fresh medium containing 100 units/ml penicillin and 100 μg/ml gentamicin to kill extracellular bacteria. After additional incubation for 1 h, the monolayers were washed with PBS. Cells were detached from plates by treatment with 0.25% trypsin-0.02% EDTA solution and then lysed by through the addition of cold distilled water. Appropriate dilutions were plated on Columbia BA plates to determine the numbers of viable bacteria.

A549 cells were pretreated with SP0454 polypeptide or medium alone at 37°C for 1 h to test whether SP0454 contribute directly to the adherence. Then, wild-type CMCC 31203 bacteria were added to A549 cells. The culture was incubated for 1 h at 37°C in a 5% CO_2-95% air atmosphere. Further blocking experiments were performed using anti-SP0454 polypeptide antiserum. Wild-type pneumococci were pre-incubated with anti-SP0454 polypeptide antiserum (1:100 dilution) or negative control (normal serum, 1:100 dilution) for 30 min prior to exposure to A549 cells. All experiments were carried out in triplicate, and each assay was repeated at least three times.

RNA isolation and real-time reverse transcription (RT)-PCR

Total RNA was extracted and reverse-transcribed according to manufacturer's instructions (Omega Bio-Tek, Inc., USA). The *sp0453*, *sp0455*, *psaA*, and *nanA* were quantitated by One-Step Real-Time RT-PCR with SYBR Premix Ex Taq (TaKaRa) using the following procedure: 95°C for 10 sec; 95°C 5 sec for 40 cycles; and 55°C for 20 sec. The sequences of the primers are listed in Table 1. A standard curve was generated by plotting the threshold cycle values against the input complementary DNA concentration for each gene. The concentrations of the experimental samples were determined by interpolating the threshold cycle values onto the standard curve. Expression levels were computed by normalizing the concentration of each virulence gene to the concentration of the 16S ribosomal RNA in the sample. All experiments were carried out in quadruplicate.

Statistical analysis

Statistical analysis was performed using unpaired Student's t tests (two-tailed). Data presented are means±standard devi-

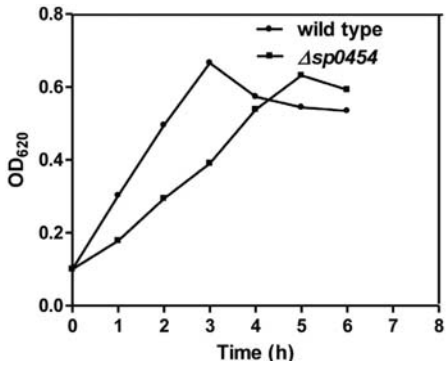

Fig. 1. Comparison of the *in vitro* growth of the *Δsp0454* mutant with CMCC 31203 wild-type strain. *In vitro* growth curves of wild-type strain and *Δsp0454* mutant during incubation at 37°C. The absorbance at 620 nm was measured at intervals.

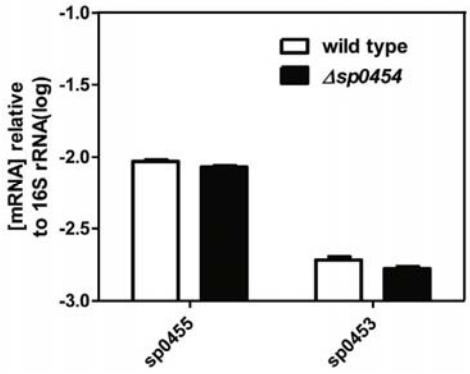

Fig. 2. Expression of *sp0453* and *sp0455* in the *sp0454* deletion mutant. Relative mRNA concentrations of *sp0453* and *sp0455* from CMCC 31203 and Δ*sp0454* mutant were determined by real-time RT-PCR. Data represent means±standard deviations of quadruplicate samples from each RNA extract. The expression of two genes had no difference between the wild-type and Δ*sp0454* mutant strain.

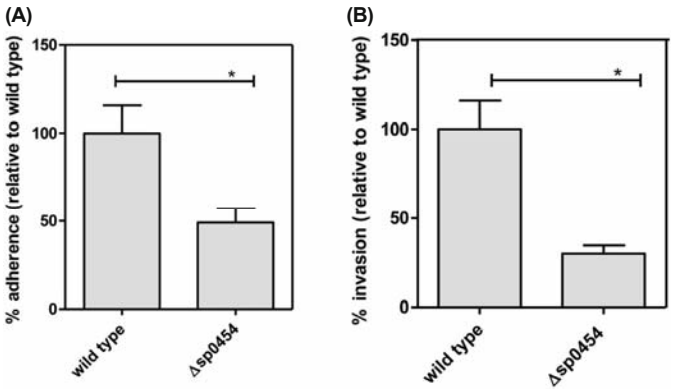

Fig. 3. Adherence and invasion of the wild type and Δ*sp0454* mutant to A549 cells. Adherence (A) and invasion (B) of *Δsp0454* mutant were significantly less than that of the wild type (**P*<0.05).

ations of three to four independent experiments. Differences in survival rate between groups were analyzed using the Mann-Whitney *U* test (two-tailed). All statistical analyses were performed using GraphPad Prism version 5.0.

Results

Δ*sp0454* mutant

Viable Δ*sp0454* CMCC 31203 was obtained through the replacement of *sp0454* with erythromycin-resistance cassette and selected on BA plates supplemented with erythromycin. The erythromycin-resistance cassette was stable in *Δsp0454* mutant. The *sp0454* fragment could not be PCR-amplified from the *Δsp0454* mutant (data not shown). No loss of resistance occurred following growth *in vitro* and *in vivo*. The mutant was identical to the wild-type strain with respect to colony morphology. The Δ*sp0454* CMCC 31203 grew a bit slower than the wild-type strain, as determined by measurement of optical density (Fig. 1). The two flanking genes, *sp0453* and *sp0455*, were not altered by the erythromycin-resistance cassette replacement (Fig. 2).

SP0454 involved in the attachment and invasion by *S. pneumoniae* of lung epithelial cells

Loss of *sp0454* in *S. pneumoniae* caused a significant reduction in the capacity to colonize the lung epithelial A549 cells. The proportion of bacterial adherence appeared to be slightly lower (*P*<0.05) for the Δ*sp0454* mutant than the wild-type strain (Fig. 3A). The Δ*sp0454* mutant was also impaired in invading the A549 cells compared with the wild-type strain (Fig. 3B).

Role of SP0454 polypeptide and its antiserum on pneumococcal adherence

Cells or bacteria were incubated with SP0454 polypeptide or its antiserum, respectively, to investigate the possible contribution of *sp0454* in the adherence and invasion of lung cells. Pre-incubation of A549 cells with SP0454 polypeptide did not substantially influence pneumococcal attachment to A549 cells (Fig. 4A). Likewise, SP0454 polypeptide anti-sera failed to block the adherence between pneumococci and A549 cells (Fig. 4B).

Lack of *sp0454* is associated with impaired pneumococcal colonization and pneumonia

Pneumococcal nasopharyngeal colonization is a prerequisite for invasive diseases. The *S. pneumoniae* CMCC 31203 mutant lacking *sp0454* showed an impaired colonizing capacity. At each time point, the proportion of bacterial colonization was slightly lower (*P*<0.05) for Δ*sp0454* mutant than for the wild-type strain (Fig. 5A). The bacterial number of Δ*sp0454*

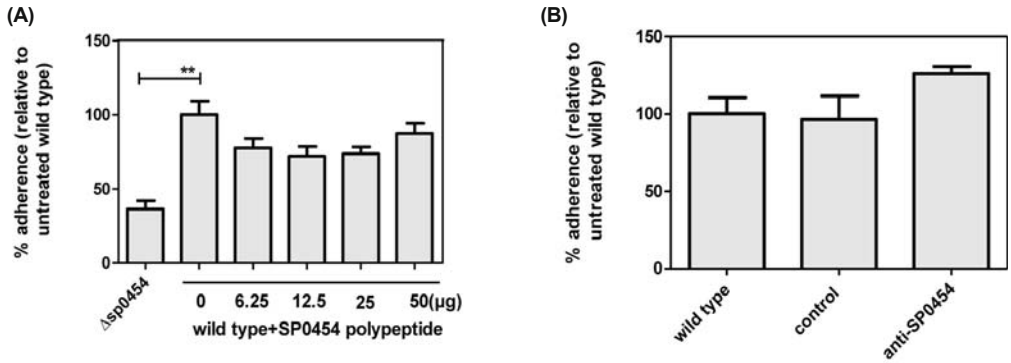

Fig. 4. Effects of SP0454 polypeptide or its antiserum on pneumococcal adherence. A549 cells were exposed to SP0454 in different concentrations (A) and then incubated with wild type strain; Wild type strain were firstly incubated either with normal sera (control) or anti-SP0454 and then incubated with A549 cells (B). Either SP0454 polypeptide or its antisera has no effect on pneumococcal adherence. (***P*<0.01)

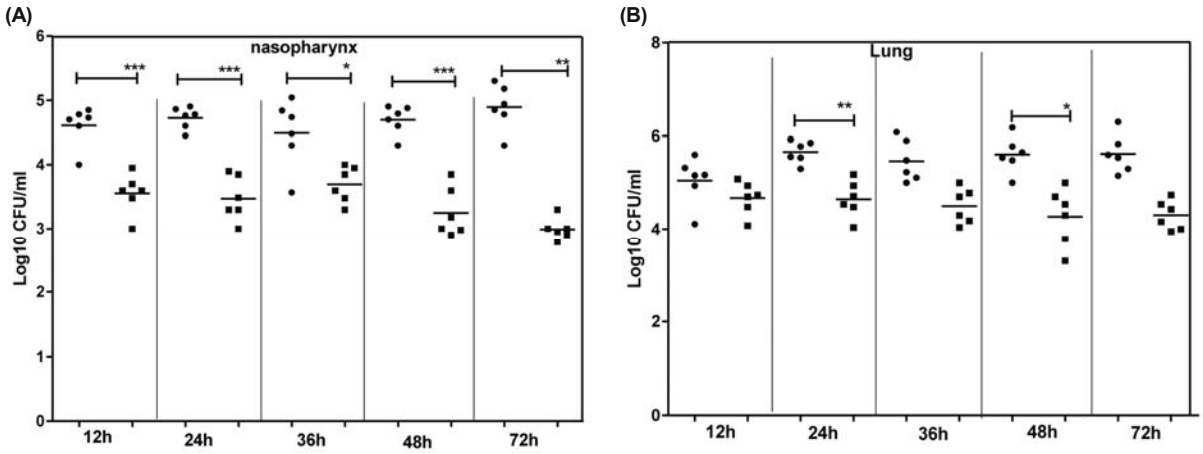

Fig. 5. Effect of the *sp0454* deletion mutation on bacterial recovery from nasopharynx and lungs of BalB/c mice. Thirty Balb/c mice/group were challenged intranasally with either wild type CMCC 31203 (●) or the Δ*sp0454* mutant (■) at 1×10^8 CFU/mouse. At the indicated times, the number of recovered bacteria from nasopharynxes (A) and lungs (B) was determined by plating on blood agar. Asterisks denote values significantly different from that for the wild type (*P<0.05; **P < 0.01; ***P < 0.001).

mutant was apparently maintained at a relatively lower level. Similarly, the bacteria recovered from the lungs infected with Δ*sp0454* mutant were significantly lower than those from the wild-type strain at each time point (Fig. 5B).

SP0454 contributes to pneumococcal intranasal infection

Mice were intranasally infected with either wild-type strain or *sp0454* mutant strain to investigate the effect of *sp0454* mutation on the virulence of *S. pneumoniae*. Survival time was recorded to compare their virulence (Fig. 6). Mice infected with the wild-type strain eventually succumbed to death. Eighty percent of the mice survived from the challenge with the Δ*sp0454* mutant, demonstrating that the loss of *sp0454* impairs virulence significantly compared with the wild-type strain.

The effect of SP0454 mutation on the expression of other surface-exposed components

The transcriptional alternations of pneumococcal surface antigen A (*psaA*) and neuraminidases (*nanA*) occurring in the *sp0454* mutant were examined to identify any differences that explain the strongly attenuated colonization of this mutant. A downregulation of *psaA* in the Δ*sp0454* mutant was found compared with the wild-type strain (Fig. 7). Downregulation of *nanA* was not observed in the *sp0454* mutant.By contrast, *nanA* was slightly increased.

Discussion

Streptococcus pneumoniae is a major pathogen of community-acquired pneumonia. *sp0454* is one of the genes in-

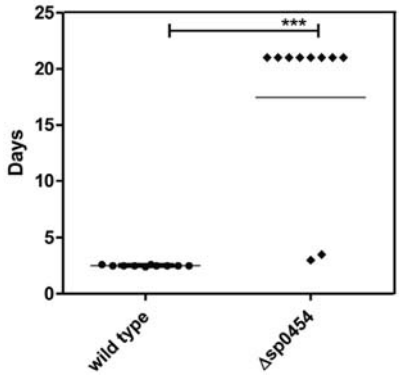

Fig. 6. Assessment of the virulence of the Δ*sp0454* mutant in mouse models of infection. Balb/c mice were challenged intranasally with approximately 5×10^8 CFU of wild type CMCC31203 (●) or the Δ*sp0454* mutant (◆). Results are shown as the survival time of each strain. Asterisks denote values significantly different from that for the wild-type (***P < 0.001).

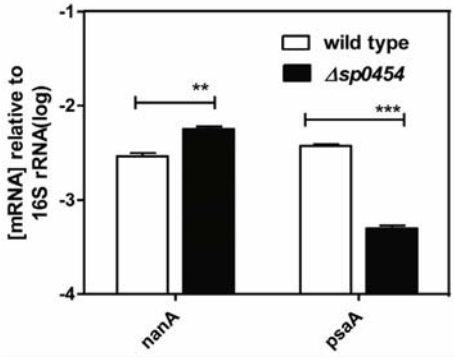

Fig. 7. Expression of *psaA* and *nanA* in the *sp0454* deletion mutant. Relative mRNA concentrations of *psaA* and *nanA* from CMCC31203 and Δ*sp0454* mutant were determined by real-time RT-PCR. Data represent means±standard deviations of quadruplicate samples from each RNA extract. The values obtained for the *sp0454* deletion mutant are significantly different from that of the wild-type strain (**P<0.05; ***P<0.001).

duced *in vivo* and obtained by our lab and others (Hava and Camilli, 2002; Meng *et al.*, 2008). It is hypothesized *sp0454* might contribute to pneumococcal virulence. In a preliminary experiment, viable *sp0454* mutants were successfully obtained by erythromycin-cassette replacement. This deletion mutant was used to examine the role of SP0454 in pathogenesis. The results demonstrated that *sp0454* was necessary for the establishment of pneumococcal colonization and pneumonia. From the experimentation, the deletion of *sp0454* clearly affected the virulence potential of *S. pneumoniae* in the mouse model, which was consistent with previous studies reported by Hava and Camilli demonstrating attenuation in a mouse model of pneumonia (Hava and Camilli, 2002). This effect was not the result of the polar effect caused by the flanking genes, because the flanking genes *sp0453* and *sp0455* were not altered following erythromycin cassette replacement. These observations provide evidence that SP0454 is a virulence factor.

We tried to prove the direct involvement of *sp0454* in adherence, however, either the SP0454 or its anti-sera could not block these effects mediated by the wild-type strain. There are two explanations for this observation: one is that SP0454 in *S. pneumoniae* CMCC 31203 (serotype 3) is not surface-accessible due to the capsule. The other is that the reduced adherence or invasion is not the direct result of *sp0454*.

The antigen *psaA* was negatively impacted following the deletion of *sp0454* in pneumococci. PsaA, a pneumococcal virulence factor, was proposed to be a pneumococcal adhesin responsible for pneumococcal binding to mammalian cells *in vitro* (Kadioglu *et al.*, 2008). This observation might explain in part that SP0454 mutant showed attenuated virulence as well as adherence. Fuethermore, this observation was very interesting and important because only the mutant of the two-component regulatory system (TCS04) system has been linked to reduced expression of *psaA* in TIGR4 strain using microarray analysis (McCluskey *et al.*, 2004). *Sp0454* is another gene associated with the regulation of *psaA*.

NanA was a secreted protein anchored to cell wall peptidoglycan, and was thought to cleave terminal sialic acid residues from host glycoproteins. It was reported that NanA was important for pneumococcal survival in the bloodstream (Manco *et al.*, 2006). Also, NanA could promote streptococcus pneumoniae to invison of human brain microvascular endothelial cells (Uchiyama *et al.*, 2009). *NanA* is upregulated by the deletion of *sp0454* which seems to promote bacterial attachment and invasion. This result could explain in part that SP0454 mutant showed a little increased virulence in mouse sepsis model (data not shown). However, NanA also contributed to the colonization of the nasopharynx (Orihuela *et al.*, 2004), but, the *Δsp0454* showed the decreased adhesive ability in general. Therefore, we speculated that *psaA* might play more important role in the *S. pneumoniae* to adherence to nasopharynx epithelial cells. Of course, there are many other factors involved in pneumococcal adherence, such as CbpA (Kadioglu *et al.*, 2008), PavA (Pracht *et al.*, 2005), ClpL (Tu *et al.*, 2007), PfbB mentioned above and some other unknown proteins. It is still unclear whether sp0454 could regulate these factors, which led to the increased or decreased expression of some factors, resulting in a synergistic effect.

Taken together, it could be proposed that the impaired virulence of the Δ*sp0454* mutant strain is the synergistic outcome of the up- or down-regulation of other virulence factors due to the deletion of gene *sp0454*. These speculations still need to be further comfirmed.

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