Lipoprotein SPD_1609 of *Streptococcus pneumoniae* Promotes Adherence and Invasion to Epithelial Cells Contributing to Bacterial Virulence

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In most bacteria, iron plays a vital role in pathogenesis processes. To support survival and infection, *Streptococcus pneumoniae* has evolved three ABC transporters, PiaABC, PiuABC, and PitABC. Moreover, indirect evidence supports that operon 804 may be a novel ABC transporter in *S. pneumoniae*. We have recently described the identification of lipoprotein SPD_1609 in operon 804; however, whether the SPD_1609 protein affects the virulence of *S. pneumoniae* has not yet been studied. In the present work, alignment analysis showed that lipoprotein SPD_1609 is conserved in a variety of gram-positive bacteria, and deletion of the SPD_1609 gene led to a reduction in adherence and invasion of *S. pneumoniae* to host cells. RT-qPCR assays indicated that deletion of the SPD_1609 gene resulted in decreased expression of genes involved in colonization. Furthermore, decreased virulence in a mouse bacteremia infection model caused by the loss of the lipoprotein encoded by SPD_1609 was also demonstrated. Overall, these data provide insights into the functional role of lipoprotein SPD_1609 in pneumococcal pathogenesis, suggesting its possibility to be developed as a novel *S. pneumoniae* vaccine candidate.

**Keywords:** *Streptococcus pneumoniae*, SPD_1609, iron uptake, adherence, invasion, bacteremia infection

**INTRODUCTION**

*Streptococcus pneumoniae* (*S. pneumoniae*) is a gram-positive human pathogen and a frequent inhabitant of the upper airways in healthy hosts. *S. pneumoniae* can invade the lower airways when the immune system is weakened, causing a variety of serious diseases including pneumonia, bacterial meningitis, sinusitis, otitis media and bacteremia that are threats to public health (Van Der Poll and Opal, 2009). To survive and establish infection in the host, *S. pneumoniae* must acquire essential nutrients, including transition metals iron, manganese, zinc, and copper (Honsa et al., 2013). Iron concentrations ranging from $10^{-7}$ to $10^{-5}$ M are required by most bacteria for various biological processes, including glycolysis, oxygen transport, gene regulation, and DNA biosynthesis (Andrews et al., 2003; Schaible and Kaufmann, 2004; Cassat and Skaar, 2013). However, the
concentration of free iron in the host environment is extremely low (~10^{-17} M) (Andrews et al., 2003). As a consequence, many bacteria have developed multiple specialized iron uptake mechanisms to acquire iron from the host, such as the production of siderophores, heme acquisition systems, ferric or ferrous iron transporters, transferrin or lactoferrin receptors, and utilization of manganese instead of iron in metalloenzymes (in *Borrelia burgdorferi*) (Cassat and Skaar, 2013).

Iron plays a crucial role in the pathogenesis processes of *S. pneumoniae* (Honsa et al., 2013; Turner et al., 2017). *S. pneumoniae* can utilize heme, hemoglobin, ferrichrome, ferric and ferrous iron as iron sources, but not lactoferrin or transferrin (Romero-Espejel et al., 2013; Turner et al., 2017). To support survival and infection, *S. pneumoniae* has evolved three ABC transporters, PiaABC, PiuABC, and PitABC, with lipoproteins PiaA, PiuA, and PitA as substrate-binding proteins to uptake iron (Brown et al., 2001a; Torres et al., 2014). In a murine model of abscess formation (Torres et al., 2006; Pishchany et al., 2014). In *Staphylococcus aureus*, a mutant lacking IsdB (cell wall-anchored protein of iron-regulated surface determinant system Isd) shows a reduced virulence in a murine model (Hutchings et al., 2009; Kovacs-Simon et al., 2011; Kohler et al., 2016; Nguyen and Gotz, 2016; Yang et al., 2016). Moreover, indirect evidence supports that operon 804 may encode a novel ABC transporter in *S. pneumoniae* (Yang et al., 2016).

There are reports showing that loss of bacterial iron transporter systems often correlates with affected virulence in murine models (Brown et al., 2001a; Torres et al., 2006; Pishchany et al., 2014). In *Staphylococcus aureus*, a mutant lacking IsdB (cell wall-anchored protein of iron-regulated surface determinant system Isd) shows a reduced virulence in a murine model of abscess formation (Torres et al., 2006; Pishchany et al., 2014). In *S. pneumoniae*, Brown et al., 2001a have reported that the piaA-mutant exhibits reduced virulence in both mouse systemic and pulmonary infection models, and the piuB- and pitA- mutants exhibit reduced virulence only in a systemic infection model (Brown et al., 2001a, 2002).

Bacterial lipoproteins are a major category of membrane proteins with various functions. These proteins often have important effects on pathogen/host interactions during the development of bacterial infection, and thus some of them have been shown to be potential vaccines (Brown et al., 2001b; Hutchesons et al., 2009; Kovacs-Simon et al., 2011; Kohler et al., 2016; Nguyen and Gotz, 2016; Yang et al., 2016). In gram-positive bacteria, most of these lipoproteins are substrate-binding proteins of ABC transporters involved in the transport of a set of substrates such as metal ions, amino acids, peptides, sugars, lipids and vitamins that are necessary for virulence (Brown et al., 2001b; Kohler et al., 2016; Yang et al., 2016). We recently reported that lipoprotein SPD_1609 in operon 804 was involved in iron uptake; however, whether the SPD_1609 protein affects the virulence of *S. pneumoniae* has not yet been studied (Yang et al., 2016). Therefore, the aim of this work was to investigate the effects of lipoprotein SPD_1609 on pneumococcal virulence in vitro and in vivo.

**MATERIALS AND METHODS**

**Ethics Statement**

Balb/c mice were supplied by the Laboratory Animal Unit of Sun Yat-sen University. All animal experiments were approved by the Committee on the Use of Live Animals in Teaching and Research of Zunyi University and conformed to institutional and governmental guidelines and regulations.

**Bacterial Strains and Growth Conditions**

All *S. pneumoniae* strains used in this study were derivatives of the parental *S. pneumoniae* D39 strain. These *S. pneumoniae* strains were cultured in Todd-Hewitt broth (Oxoid, United Kingdom) containing 0.5% yeast extract (THY) or grown on Columbia agar (Difco, United States) containing 5% sheep blood (Ruite, China) at 37°C in a 5% CO₂ incubator. *Escherichia coli* DH5α was grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) at 37°C with shaking at 200 rpm. When required, growth media for *S. pneumoniae* were supplemented with tetracycline (Tet, 3.5 μg/mL) and chloramphenicol (Cm, 4 μg/mL), and media for *E. coli* were supplemented with ampicillin (Amp, 100 μg/mL) and chloramphenicol (Cm, 20 μg/mL). The iron-restricted medium was prepared by adding 5% Chelex-100 (Bio-Rad, United States) to THY for 8 h under continuous stirring, then filtering sterilization to remove the Chelex-100 and supplementing with 100 μM CaCl₂ and 2 mM MgCl₂. When necessary, 20 μM FeCl₃, hemin or ferrichrome (Fch) was added to the iron-restricted medium.

**Multiple Amino Acid Sequence Alignment**

Comparative analysis between the amino acid sequence of *S. pneumoniae* SPD_1609 protein without the signal peptide (35–355 AA) with multiple protein sequences of (iron) ABC transporter substrate-binding proteins from *Streptococcus pyogenes*, *Streptococcus mitis*, *Streptococcus infantis*, *Streptococcus suis*, *Clostridium cadaveris*, *Abiotrophia defectiva*, *Granulicatella adiacens*, *Huttagella hathewayi*, *Roseburia intestinalis*, *Bacillus cereus*, *Paenibacillus sp.* Y412MC10, *Bacillus sp.* FJAT-14515, *Trueperella pyogenes*, and *Lactobacillus heliongianginesis* and the PitA protein of *S. pneumoniae* TIGR4 were performed. The sequence similarity searches of SPD_1609 were initiated by a Position-Specific Iterated BLAST (PSI-BLAST) search and analyzed by Clustal X2.1. To reflect exactly the similarity of these proteins, the alignment was performed only with the mature part of the lipoprotein, without the signal peptide, and the signal peptide was predicted using Signal P 4.1 soft.

**Construction of the 1609- Mutant Strain and the 1609 Complement Strain**

A long flanking homology-polymerase chain reaction (LFH-PCR) process was used to generate the spd-1609- mutant strain (1609- mutant) (Wach, 1996). Briefly, the 500 bp region upstream of spd-1609 was amplified using primers spd-1609-P1 and spd-1609-P3, while the 500 bp region downstream of spd-1609 was amplified using primers spd-1609-P2 and spd-1609-P4. The tetracycline gene was amplified using primers *tet-F* and *tet-R*. The three PCR fragments generated were

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joined together by overlap extension PCR using primers spd-1609-P1 and spd-1609-P2 to form an approximately 2.5-kb final linear DNA construct containing the deletion fragment. Then, linear DNA construction was used for homologous recombination and transformed into S. pneumoniae D39. Transformants were selected with agar plates containing 3.5 µg/mL tetracycline, and mutants were confirmed by PCR and Western blotting. All mutations were stable after six sequential passages in 0.5% THY without antibiotic selection. The primers used for the construction of the mutants are listed in Supplementary Table S1.

To confirm the link between the 1609- mutant strain and the phenotype exhibited by the S. pneumoniae 1609-mutant, a complement strain (1609 complement) that contains a recombinant shuttle plasmid pIB169 (Biswas et al., 2008) with the coding sequence of the full-length spd-1609 gene in the isogenic 1609- mutant background was used. The full spd-1609 gene, including a C-terminal 6 × His-tag, was amplified with primers pIB169-1609-F and pIB169-1609-R by PCR from the D39 genomic DNA, digested with Sac I and Kpn I and then ligated into the digested vector to generate the complementation plasmid, pIB169-1609. The recombinant plasmid was screened by using 20 µg/mL Cm in E. coli and 4 µg/mL Cm in S. pneumoniae and confirmed by PCR, DNA sequencing and Western blotting.

**Determination of Growth Curves**

*Streptococcus pneumoniae* D39 wild type, 1609- mutant and 1609 complement strains were inoculated into normal THY medium or iron-restricted Chelex-THY medium at equal inoculation at 37 °C with 5% CO₂, OD₆₀₀ was monitored every 2 h for 12 h. All data was conducted using GraphPad Prism 5.0.

**Purification of the SPD_1609 Protein, Preparation of the SPD_1609 Polyclonal Antibody and Western Blotting Analysis**

The spd_1609 gene encoding the SPD_1609 protein without a signal peptide in the *S. pneumoniae* D39 strain was amplified by PCR and ligated into the prokaryotic expression vector pGEX-4T-1 to generate the recombinant plasmid pGEX-4T-1609. The fusion protein GST-1609 was induced by IPTG expression in Escherichia coli BL21 (DE3) and purified by GST affinity chromatography. After the GST-tag was cleaved by thrombin, the SPD_1609 protein was obtained using GST affinity chromatography. Balb/c mice were immunized with the purified SPD_1609 protein without a GST tag to generate SPD_1609 polyclonal antibodies according to the previous study (Zhang et al., 2015). For Western blotting analysis, bacteria were collected when the OD₆₀₀ reached 0.5 and lysed in SDS lysis buffer with sonication. After separation on a 12% SDS-polyacrylamide gel (SDS-PAGE), protein samples were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, United States). The membranes were then blocked with 5% (w/v) skim milk and probed with the mouse primary antibody SPD_1609. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Promega, United States) was used as the secondary antibody, and the results were visualized using Clarity Western ECL Substrate (Bio-Rad, United States). SDS-PAGE of total proteins was used as the loading control for the Western blotting experiments.

**Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) Analysis**

*Streptococcus pneumoniae* D39 wild type, 1609- mutant and 1609 complement strains were grown in normal THY medium or iron-restricted Chelex-THY medium, respectively. The logarithmic phase cultures were centrifuged and washed three times with prechilled phosphate-buffered saline (PBS) that had been treated with Chelex-100. The cell pellets were dried in a Scanvac Freeze Dryer (Labgene Scientific, Switzerland), and the dry weights were measured. The dried cells were digested in neat trace metal-grade nitric acid for 20 min at 95°C. The acid solution was diluted with ultrapure water and centrifuged at 13,200 g for 30 min. The supernatants were collected and submitted for ICP-MS analysis. The results were expressed as ng of Fe per mg dry weight of cells (Jacobsen et al., 2011; Waterman et al., 2012). Three independent biological experiments were repeated.

**Cell Culture, Adherence and Invasion Assays**

A549 human alveolar epithelial cell lines (ATCC; CCL-185) were maintained in DMEM medium (Life Tech Technologies, United States) supplemented with 10% FBS (Gibco, United States) and incubated at 37 °C in 5% CO₂. Cells were transferred to 24-well plates and cultivated to confluent cell layers (∼2 × 10⁵ cells/well) for adherence and invasion assays. Bacterial adherence and invasion assays were performed essentially as previously described with minor modifications (Quin et al., 2007; Johnson et al., 2015). Briefly, *S. pneumoniae* wild type, 1609- mutant and 1609 complement strains were grown to an optical density (OD₆₀₀) of approximately 0.3 and then diluted to 2 × 10⁷ CFU/mL with phenol red-free 1640 medium containing 1% FBS before adding to the monolayer at a multiplicity of infection (MOI) = 100 bacteria/cell and incubated for 1 h (adherence assays) or 2 h (invasion assays).

For adherence assays, the unbound bacteria were removed by washing with 1 × PBS three times, and the number of adhering bacteria was counted by lysing the A549 cells with 0.025% Triton X-100 and plating the lysate. For invasion assays, the monolayers were initially treated as the process for the adherence assay, but after removing the medium and washing with 1 × PBS three times, 1 mL of phenol red-free 1640 medium with 1% FBS containing 100 µg/mL gentamicin was added to the monolayers to kill bacteria that attached to the surfaces of the A549 cells. The plates were incubated for 1 h at 37°C in 5% CO₂. After this step, the monolayers were washed three times with 1 × PBS, and the number of invading bacteria was counted by lysing the A549 cells with 0.025% Triton X-100 and plating the lysate.

All adherence and invasion experiments were performed in triplicate and repeated three times, and the adherence and invasion abilities were expressed by determining the number of adherent or invasive bacteria per number of host cells.
Virulence of the spd-1609- Mutant in the Mouse Model of Bacteremia Infection

Six-week-old female Balb/c mice were used for animal infection experiments. For the bacteremia infection model, mice were challenged with $5 \times 10^8$ CFU of bacteria by intravenous (i.v.) injection through the tail vein and monitored for mortality over the next 14 days. Blood samples were collected via tail venous puncture at 24 h postinfection and properly diluted and plated in replicates on Columbia blood agar plates.

Real-Time Quantitative PCR (RT-qPCR)

When wild-type S. pneumoniae, 1609- mutant and 1609 complement strains were grown to logarithmic growth phase (OD$_{600}$ = 0.3), total RNA from each strain was extracted with TRIzol reagent (Invitrogen, United States) following the manufacturer’s protocol. The purity and concentration of the isolated RNA were determined using a NanoDrop 2000 UV-VIS Spectrophotometer (Thermo Scientific, United States). cDNA was generated from 1 µg RNA without DNA contamination using the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen Biotech, China) according to the kit’s specifications. RT-qPCR was carried out using EvaGreen Dye (Bio-Rad, United States) in a MiniOpticon Real-Time PCR System (Bio-Rad, United States). The cycle threshold (Ct) value was recorded, and the relative quantification of specific gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), with gyrB as an internal control. The results are shown as the 1609- mutant or 1609 complement against the wild-type (WT) strain. The primer sequences are shown in Supplementary Table S1. All data were evaluated with three independent biological experiments.

Histological Analysis

To characterize histopathology following bacteremia infection, lung biopsy samples were collected from mice infected with S. pneumoniae WT, 1609- mutant and 1609 complement strains. Mice were sacrificed at 48 h postinfection, and samples were collected and fixed in 4% paraformaldehyde. Fixed lung samples were embedded in paraffin according to procedures used for routine histology. Stained samples were examined using an Olympus microscope (Olympus, Japan).

Statistical Analysis

Data from the ICP-MS, RT-qPCR, adhesion and invasion assays were analyzed using two-tailed unpaired Student’s t-test and expressed as the mean ± SEM. Data on the survival of mice for the virulence experiment were analyzed using the log-rank (Mantel-Cox) test, and the numbers of CFUs in the different experimental groups were compared using the Mann-Whitney test. Statistical analysis was carried out using GraphPad Prism 5.0, and significant differences were considered when $p < 0.05$.

RESULTS

Multiple Amino Acid Sequence Alignment Analysis

To discover whether the SPD_1609 protein is conserved in gram-positive bacteria, an alignment was performed using (iron) ABC transporter substrate-binding proteins from S. pseudopneumoniae, S. mitis, S. infantis, S. suis, C. cadaveris, A. defective, G. adiacens, H. hathewayi, R. intestinalis, B. cereus, Paenibacillus sp., Bacillus sp., T. pyogenes, and L. heilongjiangensis with PSI-BLAST search and Clustal X2.1 soft. The alignment analysis indicated that the sequences are homologous with more than 56% similarities (Table 1). As shown in Figure 1, 14 amino acids in the SPD_1609 protein are invariant residues in all included homologous proteins, with two glutamic acid (E), a tyrosine (Y) and an aspartic acid (D) being the iron-coordination residues in all well-characterized iron binding proteins (Sun et al., 2009; Cheng et al., 2013). In addition, SPD_1609 has no significant similarity with PiaA or PiuA but shares 23% identity and 40% similarity with SPD_1609.

### Table 1

| Bacteria                  | Accession numbers | Identities with SPD_1609 | Positives with SPD_1609 |
|---------------------------|-------------------|--------------------------|-------------------------|
| S. pseudopneumoniae       | WP_023941553.1    | 99%                      | 100%                    |
| S. mitis                  | WP_000738379.1    | 96%                      | 96%                     |
| S. infantis               | EF054387.1        | 92%                      | 95%                     |
| S. suis                   | WP_01463980.1     | 56%                      | 75%                     |
| C. cadaveris              | WP_027639640.1    | 51%                      | 73%                     |
| A. defective              | WP_023392505.1    | 51%                      | 72%                     |
| G. adiacens               | WP_005605248.1    | 53%                      | 71%                     |
| H. hathewayi              | WP_034534116.1    | 46%                      | 70%                     |
| R. intestinalis           | CBL12437.1        | 41%                      | 66%                     |
| B. cereus                 | WP_000802851.1    | 43%                      | 67%                     |
| Paenibacillus sp.         | WP_015738642.1    | 37%                      | 61%                     |
| Bacillus sp.              | WP_028389965.1    | 38%                      | 62%                     |
| T. pyogenes               | WP_038567276.1    | 38%                      | 56%                     |
| L. heilongjiangensis      | WP_045101852.1    | 36%                      | 60%                     |
| S. pneumoniae TIGR4       | AAK74422.1        | 23%                      | 40%                     |
positive with PitA when aligned with the BLAST 2 sequence program (Table 1 and Figure 1).

**Effect of the SPD_1609 Protein on Bacterial Growth**

To explore the function of the SPD_1609 protein in *S. pneumoniae*, we constructed a 1609- mutant strain and a 1609 complement strain. The 1609- mutant and complement strains were confirmed by PCR and Western blotting (Figure 2 and Supplementary Figure S1). First, we detected the growth curves of the WT, 1609- mutant and 1609 complement strains under iron-abundant (THY) and iron-depleted conditions (Chelex-THY). As shown in Figure 3A, under the iron abundant (THY) condition, the growth curves showed almost no difference among the WT, 1609- mutant and 1609 complement strains. However, under iron-depleted conditions (Chelex-THY), compared with WT, the 1609- mutant strain grew slowly and...
had a lower maximal OD value, while the 1609 complement strain showed similar growth curves to WT. Then, we tested the growth of the 1609- mutant strain in normal THY media, Chelex-100 treated iron-restricted media (Chelex-THY), and iron-restricted media with the addition of 20 µM FeCl₃, hemin or ferrichrome (Fch). The growth of the 1609- mutant strain in iron-restricted media was reduced compared with that in normal THY media, and the addition of hemin could restore growth to a similar normal level; however, the addition of FeCl₃ or Fch did not restore growth (Figure 3B). These results indicated that lipoprotein SPD_1609 may be involved in FeCl₃ or ferrichrome uptake but not hemin uptake.

Effect of the SPD_1609 Protein on Iron Uptake
Although our previous study reported that the piaA/piuA/1609 triple mutant resulted in impaired iron acquisition compared to the piaA/piuA double mutant (Yang et al., 2016), the effect of the 1609- single mutant on iron uptake is unknown. Therefore, the intracellular levels of iron among the WT, 1609- mutant and 1609 complement strains were detected using ICP-MS. In normal THY media, the iron contents were not significantly different among the WT, 1609- mutant and 1609 complement strains (Figure 3C). However, in the iron-depleted media, the iron content in the 1609- mutant strain was obviously lower than that in the WT and 1609 complement strains (Figure 3C).

Effect of the SPD_1609 Protein on Adherence and Invasion of the Bacterium to Host Cells
In addition to pneumolysin and the phagocytosis-inhibiting polysaccharide capsule, the virulence of S. pneumoniae is enhanced by the capacity of bacteria to adhere to and invade host cells and then diffusion into host tissue (Andrews et al., 2003; Zakrzewicz et al., 2016). Many surface proteins of S. pneumoniae have been shown to be involved in adherence and invasion processes (Balachandran et al., 2002; Uchiyama et al., 2009; Keller et al., 2013). Considering that SPD_1609 is a surface lipoprotein, we investigated whether SPD_1609 could affect pneumococcal adherence and invasion to human alveolar epithelial cells (A549) in vitro, and piuA- mutant as a positive control. As shown in Figure 4, the 1609- mutant strain exhibited an almost 60% decrease in A549 adherence and a 70% decrease in A549 invasion compared with the WT parent strain, and the defect was rescued upon complementation of the mutant with spd-1609 on a plasmid vector: 1609 complement strain. This result indicates that SPD_1609 is important for S. pneumoniae colonization of human lung carcinoma cells.

Gene Expression of S. pneumoniae Regulated by SPD_1609
To further explore the reason that the 1609- mutation reduced the adherence and invasion of the bacterium to host A549 cells, the gene expression of a range of surface proteins including adhesins choline binding protein A (pcpA and cbpA), neuraminidase A (nanA) and piuA involved in colonization (Sanchez-Beato et al., 1998; Kadioglu et al., 2008) was detected using RT-qPCR. The RT-qPCR results indicated that the pcpA and cbpA and nanA genes were downregulated in the 1609- mutant strain compared with the WT parent strain (Figure 5A), consistent with the observation that the spd-1609 deletion reduced bacterial adherence and invasion to host A549 cells. However, piuA was induced in the 1609- mutant strain compared to WT; one possible reason for the high expression of piuA in the 1609- mutant strain could be to compensate for iron uptake. In contrast, these genes showed
no significant change between the WT and 1609 complement strains (Figure 5B).

**Importance of the SPD_1609 Protein for *S. pneumoniae* Virulence in vivo**

We also dissected the importance of the SPD_1609 protein in *vivo*, and a mouse model of bacteremia infection was employed. For bacteremia infection, a group of six mice was inoculated i.v. with 5 × 10⁶ CFU of the WT, the 1609-mutant or the 1609 complement strain through the tail vein and monitored for mortality over a 14-day period. Mice infected with the 1609- mutant had significantly lower bacterial CFU in the blood at 24 h following infection than the mice infected with the WT or the 1609 complement strains (Figure 6A). Moreover, the mice infected with the 1609- mutant displayed significantly higher survival rates compared with the mice infected with the WT strain (Figure 6B). When the 1609- mutant strain was complemented with the *spd_1609* gene, no significant difference was observed in survival (Figure 6B) or bacterial blood titers (Figure 6A) compared to the results for infection with the WT strain.

To further observe the effect of the SPD_1609 protein on bacteremia infection, histology analysis was performed. The lung biopsy samples were collected from the mice uninfected with bacteria (control), and the mice infected with wild-type *S. pneumoniae*, 1609- mutant and 1609 complement strains after 48 h of infection. Lung histological examination revealed that mice infected with WT, 1609-mutant or 1609 complement strains displayed alveolar disruption, with more inflammatory cell infiltration than control (Figures 6C–F). However, less alveolar damage and inflammatory cell infiltration was observed in mice infected with the 1609- mutant when compared with mice infected with the WT or 1609 complement strains (Figures 6D–F).

These results highlight the importance of the SPD_1609 protein for virulence in a mouse model of bacteremia infection, indicating that the SPD_1609 lipoprotein is important for bacteremia infection during *in vivo* growth.
DISCUSSION

Iron acquisition is key for the growth and infection of *S. pneumoniae*. Three well-known ABC transporters found in *S. pneumoniae*, PiaABC, PiuABC and PitABC, are involved in iron uptake. We recently reported that lipoprotein SPD_1609 in operon 804 is involved in iron uptake. In the present study, we demonstrated that lipoprotein SPD_1609 is conserved in a variety of gram-positive bacteria (Figure 1). ICP-MS data indicated that the 1609- mutant strain impaired iron uptake only in iron-depleted media, and growth curve assays indicated that lipoprotein SPD_1609 may be involved in FeCl₃ or ferrichrome uptake, but not hemin uptake (Figure 3).

Further experiments revealed that the 1609- mutant strain has a significantly lower ability to adhere to and invade host cells than the WT strain (Figure 4). RT-qPCR assays indicated that deletion of the *spd_1609* gene resulted in decreased gene expression involved in colonization. Moreover, at 24 h after bacteremia infection, the bacteria in the blood of mice infected with the 1609-mutant strain were markedly reduced compared to the infection with the WT strain (Figure 6A). This result corresponds to a significant increase in the survival of mice infected with the 1609-mutant strain compared with the survival of mice infected with the WT or 1609 complement strains (Figure 6B). Taken together, these data revealed that lipoprotein SPD_1609 contributes to bacterial virulence during bacteremia infection.

Previous reports have suggested that the *piaA*- mutation attenuated bacterial virulence in both systemic and pulmonary infection models, and both *piuB*- and *pitA*- mutants exhibited reduced virulence only in the systemic infection model (Brown et al., 2001a, 2002). In this study, the 1609- mutation showed decreased bacterial virulence in the bacteremia infection model, which once again confirmed that the iron acquisition systems are associated with pneumococcal virulence.
Several bacterial surface proteins and lipoproteins of iron acquisition systems have elicited protective immunity in mice and thus are used as candidate vaccine antigens (Jomaa et al., 2006; Schmaler et al., 2009; Kim et al., 2010; Zhang et al., 2017). Jomaa et al. (2006) reported that immunization with PiaA and PiuA elicits specific antibody responses that prevent respiratory infection with *S. pneumoniae*. In *S. aureus*, cell wall-anchored surface proteins IsdA and IsdB of the iron-regulated surface determinants (Isd) system antibodies protect mice against abscess formation and lethal challenge (Kim et al., 2010). The staphylococcal surface lipoprotein FhuD2 (ferric-hydroxamate uptake D2), a component of an Fe ABC transporter, was considered to be a potential vaccine candidate as a protective antigen in a murine staphylococcal infection model.
In *S. pyogenes*, vaccination of surface protein Shp of the heme acquisition system protects mice against skin formation and lethal challenge (Zhang et al., 2017). In this connection, lipoprotein SPD_1609 may be a vaccine candidate because of its participation in iron acquisition and its contribution to virulence during bacteremia infection. Accordingly, our future work would investigate whether SPD_1609 can be a useful vaccine antigen for preventing *S. pneumoniae* infection and whether it can be more effective in combination with PiaA and PiuA or other novel *S. pneumoniae* vaccine candidates.

**DATA AVAILABILITY**

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

**AUTHOR CONTRIBUTIONS**

X-YY designed the research, performed the experimental work, and wrote the manuscript. NL performed the experimental work. J-YX and XS analyzed the data. Q-YH provided the initial idea and designed the research.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01769/full#supplementary-material

**FIGURE S1 | RT-qPCR expression analysis of the genes spd_1608, spd_1609, spd_1610 in WT and 1609- mutant strain.**

**TABLE S1 | The primer sequences of genes from *Streptococcus pneumoniae* D39 (GenBank: CP0000410.2).**
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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