SPD_1495 Contributes to Capsular Polysaccharide Synthesis and Virulence in *Streptococcus pneumoniae*

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**ABSTRACT** *Streptococcus pneumoniae*, a Gram-positive human pathogen, causes a series of serious diseases in humans. SPD_1495 from *S. pneumoniae* is annotated as a hypothetical ABC sugar-binding protein in the NCBI database, but there are few reports on detailed biological functions of SPD_1495. To fully study the influence of SPD_1495 on bacterial virulence in *S. pneumoniae*, we constructed a deletion mutant (D39 Δspd1495) and an overexpressing strain (D39 spd1495/H11001). Comparative analysis of iTRAQ-based quantitative proteomic data of the wild-type D39 strain (D39-WT) and D39 Δspd1495 showed that several differentially expressed proteins that participate in capsular polysaccharide synthesis, such as Cps2M, Cps2C, Cps2L, Cps2T, Cps2E, and Cps2D, were markedly upregulated in D39 Δspd1495. Subsequent transmission electron microscopy and uronic acid detection assay confirmed that capsular polysaccharide synthesis was enhanced in D39 Δspd1495 compared to that in D39-WT. Moreover, knockout of spd1495 resulted in increased capsular polysaccharide synthesis, as well as increased bacterial virulence, as confirmed by the animal study. Through a coimmunoprecipitation assay, surface plasmon resonance, and electrophoretic mobility shift assay, we found that SPD_1495 negatively regulated cps promoter expression by interacting with phosphorylated ComE, a negative transcriptional regulator for capsular polysaccharide formation. Overall, this study suggested that SPD_1495 negatively regulates capsular polysaccharide formation and thereby enhances bacterial virulence in the host. These findings also provide valuable insights into understanding the biology of this clinically important bacterium.

**IMPORTANCE** Capsular polysaccharide is a key factor underlying the virulence of *Streptococcus pneumoniae* in human diseases. Thus, a deep understanding of capsular polysaccharide synthesis is essential for uncovering the pathogenesis of *S. pneumoniae* infection. In this study, we show that protein SPD_1495 interacts with phosphorylated ComE to negatively regulate the formation of capsular polysaccharide. Deletion of spd1495 increased capsular polysaccharide synthesis and thereby enhanced bacterial virulence. These findings further reveal the synthesis mechanism of capsular polysaccharide and provide new insight into the biology of this clinically important bacterium.

**KEYWORDS** *Streptococcus pneumoniae*, capsule, virulence

*Streptococcus pneumoniae* is a major pathogenic bacterium that causes various serious diseases in humans worldwide and may lead to high morbidity and mortality, especially among young children and elderly (1–3). The capsular polysaccharide (CPS) is recognized as one of the most important virulence factors of *S. pneumoniae* for infecting the host because nonencapsulated bacteria are almost completely harmless.
(4–6). The ability to regulate CPS production is crucial for the survival of *S. pneumoniae* in different hosts.

CPS is required for effective colonization in the nasopharyngeal tract of the host and invasive infections in the blood and lungs (6, 7). First, to colonize the pharynx nasalis, *S. pneumoniae* reduces the synthesis of CPSs to increase the exposure of pneumococcal surface structures, such as adhesins, which are necessary for initial colonization. When *S. pneumoniae* escapes the nasopharynx, it invades the lungs and subsequently invades the bloodstream. During invasion, CPS is highly expressed to mask the surface antigens in order to reduce complement deposition and protect the bacterium against opsonophagocytosis (4, 8, 9).

Until now, more than 90 antigenically distinct serotypes of *S. pneumoniae* CPS have been identified, most CPSs are comprised of the repeating units such as glucose (Glu), L-rhamnose (Rha), L-rhamnose, L-rhamnose, glucose, and glucuronic acid (GlcUA), and all the CPSs, except for serotypes 3 and 37, are synthesized in the Wzx/Wzy-dependent pathway (6, 10–12). The *cps* gene cluster participated in the formation of CPS; it is located at the same region of the chromosome between the *dexB* and *aliA* genes, which encodes *cps2E*, *cps2D*, *cps2L*, *cps2I*, etc. (10, 13–15). Further, several possible transcriptional regulators of the *cps* locus, such as RegM (16), ComX (17), CopY (18), CpsR (19), GlnR (20), RitR (21), and ComE (9), have also been reported to regulate CPS formation. In particular, ComE exists in *S. pneumoniae* in nonphosphorylated and phosphorylated forms, and only the latter form can negatively regulate the expression of the *cps* gene cluster via interaction with the upstream promoter of *cps in vivo* (9).

Our group focused on the relationship between iron transportation and bacterial virulence. We found that in the Δ*pitA* Δ*piaA* Δ*pitA* triple mutant, in which three major iron transporters were simultaneously deleted, the protein SPD_1495 was upregulated by 6.435- and 86.55-fold at the mRNA and protein levels, respectively, compared to that in D39-WT (22, 23). We speculated that SPD_1495 may also contribute to bacterial virulence. SPD_1495 is annotated as a sugar-binding protein in the NCBI database and is highly conserved in *Streptococcus* species. However, there are few reports on the detailed biological functions and the effects of SPD_1495 on bacterial virulence. Therefore, this study explores the biological function of SPD_1495 in *S. pneumoniae* using quantitative proteomics combined with biochemical validations. All of the experimental data indicated that SPD_1495 can interact with phosphorylated ComE to negatively regulate CPS formation. Our study provides novel insight regarding CPS formation, as well as the virulence ability of *S. pneumoniae*.

RESULTS AND DISCUSSION

Deletion of *spd1495* results in low utility of sugar source in *S. pneumoniae*. To elucidate the detailed biological function of SPD_1495 in *S. pneumoniae*, homologous replacement was used to obtain the deletion mutant D39Δ*spd1495*, and the plasmid pIB169-*spd1495* was transformed into D39-WT to construct the overexpression strain D39*spd1495*+. Western blot analysis showed that the expression of SPD_1459 in D39*spd1495*+ was significantly higher than that in D39-WT and that D39Δ*spd1495* hardly expressed SPD_1495. These results indicated that the mutant strains were successfully constructed (Fig. 1A and B).

Since SPD_1495 is annotated as an ABC sugar-binding protein in the NCBI database, the utility of sugar source of D39-WT and D39Δ*spd1495* was detected. Nutrition-restricted C+Y medium with or without a sugar source was used to obtain bacterial growth curves of the D39-WT and mutant strains (Fig. 1C). Without any sugar source in C+Y medium, the D39-WT, D39Δ*spd1495*, and D39-WT + *spd1495* strains almost did not grow. Therefore, *N*-acetylneuraminic acid (Neu5Ac), arabinose, xylose, glucose, fructose, lactose, sucrose, and maltose as the only sugar sources in C+Y medium promoted bacterial growth. The results showed that the addition of Neu5Ac, arabinose, and xylose as the only sugar sources in the culture medium almost rescued the growth of both the WT and mutant strains. The supplementation of glucose, fructose, lactose, sucrose, and maltose as the only sugar sources in C+Y medium can recover cell survival at different
FIG 1  Deletion of spd1495 affected the growth of S. pneumoniae D39 in medium containing different sugar sources. (A) Confirmation of gene deletion and overexpression of spd1495 using Western blotting. (B) SDS-PAGE results of whole-cell lysates of D39-WT, D39\Delta spd1495, and D39spd1495+ strains as loading controls for the Western blotting experiments of panel A. (C) Growth curves of D39-WT (red), D39\Delta spd1495 (blue), and D39spd1495+ (green) strains cultured in C+Y medium containing no sugar, Neu5Ac, arabinose, xylose, glucose, fructose, lactose, sucrose, and maltose. Statistical analysis was conducted using Prism 6.0.
levels. Compared to that of D39-WT, D39Δspd1495 showed obvious hysteresis of growth, especially when grown in C+Y medium supplemented with fructose and sucrose, whereas D39spd1495+ exhibited a marginal increase in growth. These results indicated that SPD_1495 may be a sugar-binding protein in S. pneumoniae and improve sugar utility of bacteria, which is consistent with the annotation in the NCBI database, but its detailed biological function was not reported.

Deletion of spd1495 promotes the biosynthesis of CPS. To further explore the detailed biological function of SPD_1495 in S. pneumoniae, we used iTRAQ-based proteomics to identify differentially expressed proteins between D39-WT and D39Δspd1495. A strong correlation (r² = 0.91374) (Fig. 2A) indicated a good reliability with two independent biological replicates. A total of 994 proteins were commonly detected (Fig. 2B). More than 88% of the proteins had <50% variation (Fig. 2C), and the fold change threshold used to define differentially expressed proteins was <0.67 or >1.50 with P < 0.05. The relative quantitative analysis revealed 54 differentially expressed proteins in D39Δspd1495, with 34 upregulated and 20 downregulated proteins, compared to those in D39-WT (see Table S1 in the supplemental material).

KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis revealed that the differentially expressed proteins were enriched in three biological processes, namely, polyketide sugar unit biosynthesis, streptomycin biosynthesis, and acarbose and validamycin biosynthesis (Fig. 2D and E). It is noteworthy that the biosynthesis of polyketide sugar unit was the most significant pathway with the smallest P value among the three pathways. Following deletion of the spd1495 gene, 34 proteins, including Cps2C, Cps2K, Cps2l, Cps2T, Cps2E and Cps2D etc., which are associated with CPS synthesis were obviously upregulated (Table 1). For example, Cps2L, Cps2M, Cps2N, Cps2T, Cps2F, Cps2G, Cps2I, and BgaA were involved in the synthesis of dTDP-L-rhamnose, a component in CPS synthesis (10). Furthermore, the STRING analysis (Fig. 2F and G) showed that the upregulated proteins in D39Δspd1495, such as Cps2C, Cps2K, Cps2l, Cps2T, Cps2E, and Cps2D, participate in the CPS synthesis network, and the raw materials, such as rhamnose, were used to promote CPS synthesis. The upregulation of cps gene was further validated by reverse transcription-quantitative PCR (RT-qPCR) analysis (Fig. 3A). Moreover, we also found that many upregulated proteins in D39Δspd1495, such as LacB, LacD, DexB, BglA-1, SPD_0559, SPD_1664, and SPD_0412, formed a small network. These proteins are involved in carbohydrate metabolism, which supplies some of the key raw materials for CPS synthesis, such as ATP and NAD+/NADPH, etc. (24). The 20 downregulated proteins were not enriched into any biological progress. Thus, both KEGG pathway enrichment analysis and STRING analysis indicated that the upregulated proteins are enriched in CPS synthesis. Therefore, we speculated that CPS synthesis in S. pneumoniae is negatively regulated by SPD_1495.

To validate the relationship between the CPS synthesis and SPD_1495 in S. pneumoniae, transmission electron microscopy (TEM) was used to detect the thickness of the CPS. The images of TEM showed that the thickness of CPS decreased in the following order: D39Δspd1495 > D39-WT > D39spd1495+. This indicates that SPD_1495 could negatively regulate CPS formation (Fig. 3B). The relative content of uronic acid, which is the main component of the capsule, was determined to further quantitatively compare the difference in CPS formation. As shown in Fig. 3C, the content of uronic acid decreased in the following order: D39Δspd1495 > D39-WT > D39spd1495+; this is consistent with the order of decrease in capsule thickness. Therefore, both the thickness of CPS and the content of uronic acid confirmed that, compared to the D39-WT, D39Δspd1495 produced higher quantities of CPS. These results jointly indicated that SPD_1495 negatively regulated capsule formation.

Deletion of spd495 increases virulence in S. pneumoniae. The CPS plays an important role in the virulence of S. pneumoniae (25). The survival rates of mice infected with D39-WT, D39Δspd1495, and D39spd1495+ were determined to uncover the contribution of SPD_1495 to bacterial virulence. As shown in Fig. 3D, the D39Δspd1495
mutant was more virulent than D39-WT; however, the virulence of SPD_1495-overexpressing strains was very similar to that of D39-WT in the mouse pneumonia model. This result indicated that SPD_1495 may increase the virulence of S. pneumoniae by decreasing the formation of the CPS and demonstrated that SPD_1495 affects the pathogenicity of S. pneumoniae during infection in vivo.

SPD_1495 affects the formation of S. pneumoniae CPS via interacting with phosphorylated ComE. In order to seek regulation network of SPD_1495, the SPD_1495 was expressed and purified by glutathione S-transferase (GST) tag affinity chromatography.
There was a band of ~45 kDa, corresponding to the calculated molecular weight of SPD_1495 in SDS-PAGE, the Western blotting with SPD_1495 antibody confirmed the expression of SPD_1495 (Fig. 4A and B). The proteomic data and detection of capsule showed that the formation of CPS was negatively regulated by SPD_1495. In *S. pneumoniae*, the *cps* gene cluster is mainly responsible for the CPS biosynthesis (19). We speculated that SPD_1495 may regulate the expression of the *cps* gene cluster via interaction with the CPS operon. However, electrophoretic mobility shift assay (EMSA) results revealed that

| Gene or protein       | Protein                          | Avg fold change | Avg –log10 P  |
|-----------------------|----------------------------------|-----------------|--------------|
| **Carbohydrate metabolic process** |                                  |                 |              |
| *bgaA*                | β-Galactosidase, putative         | 4.30            | 9.80         |
| *bglA-1*              | 6-Phospho-β-glucosidase          | 7.55            | 4.89         |
| *dexB*                | Glucan 1,6-α-glucosidase         | 19.24           | 4.24         |
| *lacD*                | Tagatose 1,6-diphosphate aldolase| 2.89            | 3.48         |
| *lacB*                | Galactose-6-phosphate isomerase  | 3.82            | 1.88         |

| **Capsule-associated proteins** |                                  |                 |              |
| Cps2L                 | Glucose-1-phosphate thymidylyltransferase | 10.86          | 6.94         |
| Cps2K                 | Cps2K                             | 14.65           | 0            |
| Cps2C                 | Polysaccharide export protein     | 9.18            | 4.85         |
| Cps2D                 | Cps2D                             | 4.66            | 1.50         |
| Cps2F                 | Glycosyl transferase, group 2 family protein | 10.87          | 5.70         |
| Cps2G                 | Glycosyl transferase, group 1 family protein | 8.57          | 2.50         |
| Cps2M                 | Cps2M                             | 11.95           | 2.27         |
| Cps2L                 | Cps2L                             | 7.14            | 2.26         |
| Cps2T                 | Cps2T                             | 11.92           | 6.56         |
| Cps2E                 | Cps2E                             | 6.15            | 2.19         |
| Cps2N                 | dTDP-glucose 4,6-dehydratase      | 17.01           | 7.51         |

There was a band of ~45 kDa, corresponding to the calculated molecular weight of SPD_1495 in SDS-PAGE, the Western blotting with SPD_1495 antibody confirmed the expression of SPD_1495 (Fig. 4A and B). The proteomic data and detection of capsule showed that the formation of CPS was negatively regulated by SPD_1495. In *S. pneumoniae*, the *cps* gene cluster is mainly responsible for the CPS biosynthesis (19). We speculated that SPD_1495 may regulate the expression of the *cps* gene cluster via interaction with the CPS operon. However, electrophoretic mobility shift assay (EMSA) results revealed that ...

**FIG 3** SPD_1495 negatively regulated capsular synthesis. (A) SPD_1495 negative regulate the *cps* genes expression in D39-WT and D39Δspd1495. (B) TEM results for strains D39-WT, D39Δspd1495, and D39spd1495+. (C) Uronic acid contents in strains D39-WT, D39Δspd1495, and D39esp1495+. (D) The virulence of strains D39-WT, D39Δspd1495, and D39esp1495+. Data were analyzed by two-tailed, unpaired Student t tests, and results are expressed as means ± the SD. ***, P < 0.001; **, P < 0.01; *, P < 0.05.
SPD_1495 cannot directly interact with cps operon (Fig. 4C). A previous study by Zheng et al. identified the transcriptional regulator in the upstream promoter region of cps by DNA-pulldown but did not find SPD_1495 (9). Therefore, we deduce that SPD_1495 may negatively regulate CPS formation indirectly.

A coimmunoprecipitation (Co-IP) assay was performed to identify the proteins interacting with SPD_1495. Compared to that of IgG in the control group, SPD_1495 specifically captured some proteins of molecular weight between 20 and 37 kDa (Fig. 4D). These proteins were cut and digested with trypsin and then were identified by mass spectrometry. The identification results showed that SPD_1495 can interact with several proteins, including RpsC, ComE, SPD_1495, RecA, CshB, and CoaC (Table 2). Notably, ComE exists in two forms (nonphosphorylated and phosphorylated) in S. pneumoniae, and only phosphorylated form ComE negatively regulates the expression of the cps gene cluster via an interaction with the upstream promoter of cps (9). Thus, to further uncover the function of SPD_1495 in the formation of CPS, a phosphomimetic form (ComED58E) was expressed and purified to check its interaction with

TABLE 2 Proteins interacting with of SPD_1495 identified in a Co-IP assay

| Protein | Function | Coverage (%) |
|---------|----------|--------------|
| RpsC    | 30S ribosomal protein S3 | 73.7 |
| ComE    | Response regulator | 50.4 |
| SPD_1495| Sugar ABC transporter, sugar-binding protein | 58.8 |
| RecA    | Protein RecA | 54.6 |
| CshB    | DEAD-box ATP-dependent RNA helicase CshB | 58.8 |
| CoaC    | Phosphopantothenoylcysteine decarboxylase | 53.6 |
SPD_1495 by surface plasmon resonance (SPR). The result of SPR studies also showed that ComED58E can interact with SPD_1495 directly in vitro (Fig. 4E), and their dissociation constant is 1.55 × 10⁻⁶ M. Moreover, EMSA showed that ComED58E interacting with SPD_1495 has a stronger binding ability than ComED58E on its own for the same Bio-cps probe (Fig. 4F), indicating that SPD_1495 contributes to the binding between ComED58E and the cps operon and thus regulates CPS synthesis. Furthermore, RT-qPCR results demonstrated that the expression levels of CPS-related genes were upregulated in D39Δspd1495 (Fig. 3A). Moreover, the proteomics data showed the consistent result that many proteins involved in CPS synthesis were upregulated in D39Δspd1495. In summary, our data suggest that SPD_1495 can interact with phosphorylated-ComE in S. pneumoniae to negatively regulate CPS formation.

Amino acid sequence homologous alignment and protein evolutionary tree analysis. To assess the conservation of SPD_1495 in bacteria, amino acid sequence alignment and the phylogenetic tree construction were carried out (Fig. 5A and B). The result showed that the SPD_1495 protein is highly conserved in Gram-positive bacteria, especially in the genus Streptococcus. A database search showed that several highly homologous proteins of SPD_1495, for example, B7692_06535, YESO_2, and SPAR10_0271 are also annotated as sugar ABC transporter substrate-binding proteins. The detailed biological function of this protein family has not been reported. This is the preliminary report of the
| Organism          | Strain     |
|-------------------|------------|
| S. pneumoniae     | SPD_1495   |
| S. pneumoniae     | SPCG 1656  |
| S. iniae          | C7385_11262|
| S. pneumoniae     | yesS_2     |
| S. enterica       | AT700_22835|
| S. milis          | B7692_06535|
| P. lacticilis     | HMPREF1234_16725|
| S. infantis       | SPAR10_0271|
| Robinsonella      | RHS 5684   |
| S. oralis         | HMPREF8579_1739|
| S. anilatus       | SPAR10_0277|
| S. mitis          | yesS_0     |
| Enterococcus      | A5883_003599|
| S. pyogenes       | EGG78_01410|
| S. iniae          | IUSA1_01165|
| S. corcuri        | yesC_0     |
| S. oralis         | HMPREF8579_1739|
| S. cristatus      | TW70_07033 |
| S. constellatus   | RN79_07030 |
| B. japonicum      | BJT 65520   |
| S. eritiri        | THUE       |
| Y. pestis         | toGB       |

**FIG 5 (Continued)**
function of SPD_1495 in \textit{S. pneumoniae} CPS synthesis. Hence, the regulation of capsule formation and bacterial virulence of SPD_1495 observed in the present study may represent a novel biological function of this cluster of proteins.

Previous research has shown that the CPS of \textit{S. pneumoniae} is one of the key virulence factors required for effective infection host (26, 27), and many vaccines targeting CPS have been developed to treat or prevent bacterial infections in recent years (28). In this work, convincing evidence was obtained indicating that SPD_1495 can negatively regulate CPS synthesis by interacting with phosphorylated ComE in \textit{S. pneumoniae}. Further, SPD_1495 is highly conserved among Gram-positive bacteria. Our data suggest that SPD_1495 is a vaccine or drug target candidate because of its contribution to virulence during bacteremia. Accordingly, our further studies will investigate whether SPD_1495 can be an efficacious vaccine antigen for preventing \textit{S. pneumoniae} infection.

\textbf{Conclusion.} Taken together, our research revealed the biological function of SPD_1495 in capsule formation in \textit{S. pneumoniae}. This protein negatively regulates CPS synthesis by interacting with phosphorylated ComE, a key transcriptional regulator located in the cps locus. Thus, deletion of the spd1495 gene will increase the production of dTDP-\(\alpha\)-rhamnose, thereby promoting the biosynthesis of CPS. Furthermore, the CPS is recognized as one of the most important virulence factors in \textit{S. pneumoniae} for infecting the host. Deletion of spd1495 will upregulate the CPS to improve the virulence factor related to the host, thereby enhancing the ability of \textit{S. pneumoniae} to infect host cells (Fig. 6). Considering the importance of CPS in bacterial adherence and entry into the host, regulation of SPD_1495 is essential in \textit{Streptococcus} infection. Moreover, the animal assay indicated that SPD_1495 is an important protein in regulating the virulence in \textit{S. pneumoniae}. Therefore, SPD_1495 may be a vaccine or drug target candidate for the development of novel antibacterial to treat the \textit{Streptococcus} infections.

\textbf{MATERIALS AND METHODS}

\textbf{Bacterial growth conditions.} The \textit{S. pneumoniae} D39 was cultured in THY medium (Todd-Hewitt broth; Oxoid, UK) with 0.5\% yeast extract (Oxoid) or on Columbia agar (Difco, USA) containing 5\% sheep
blood (Ruite, China) at 37°C in a 5% CO2 incubator (MCO-170AICUVHL-PC; Panasonic, Japan). *Escherichia coli* DH5α and BL21 (Invitrogen, USA) were both cultured in Luria-Bertani (LB) medium at 37°C in an incubator shaker (Yi Heng, China). All strains were stored at –80°C in 15% glycerol. Erythromycin (Erm; Sigma, USA) at 0.25mg/ml, chloramphenicol (Cm; Sigma) at 4mg/ml, or ampicillin (Amp; Sigma) at 100μg/ml was added to the medium when needed for selective culture. All of the bacterial strains and plasmids used are listed in Table 3.

**Construction of mutant *S. pneumoniae* D39 strains.** Long-flanking-homology PCR (LFH-PCR) was used to construct strain D39Δspd1495 (29, 30), and the primers used in this study are listed in Table 4. The target gene, spd1495 (1,329bp), was replaced by an antibiotic resistance cassette gene (erm). The reconstructed region, including erm (829bp), upstream (817bp) and downstream (624bp) of spd1495 was ligated by LFH-PCR using primers P3 to P8. The products of LFH-PCR were transferred into strain D39-WT, and the positive transformant was selected by Erm-containing Columbia sheep blood agar plate. D39Δspd1495 strains were stocked after seven sequential passages in the THY medium with 0.25μg/ml Erm. To construct spd1495-overexpressing strains, the recombinant plasmid pIB169-spd1495 was constructed by inserting the spd1495 gene (1,329bp) into pIB169 plasmid using a ClonExpress II

### Table 3: Bacterial strains and plasmids used in this study

| Strain or plasmid          | Relevant characteristic(s) | Source or reference |
|----------------------------|----------------------------|---------------------|
| **Strains**                |                            |                     |
| *S. pneumoniae*            |                            |                     |
| D39                        | Wild type                  | ATCC (USA)          |
| Δspd1495 mutant            | In-frame spd1495 mutant strain derived from D39; Ermt | This study          |
| D39/pBl169-spd1495         | D39 strain transformed with pBl169-spd1495; Cmrt | This study          |
| **E. coli**                |                            |                     |
| BL21                       | Wild type                  | Invitrogen (USA)    |
| BL21/pGEX-4T-1-spd1495     | BL21 strain transformed with pGEX-4T-1-spd1495; Amprt | This study          |
| **Plasmids**               |                            |                     |
| pGEX-4T-1                  | pGEX vector contained tac promoter; Ampp | Invitrogen (USA)    |
| pGEX-4T-1-spd1495          | *S. pneumoniae* D39 spd1495 (33–442 AA) fragment cloned into pGEX-4T-1; Ampp | This study          |
| pBl169                     | Shuttle plasmid contained Pveg promoter; Cmrt | 14                 |
| pBl169-spd1495             | *S. pneumoniae* D39 spd1495 (1–442 AA) fragment cloned into pBl169; Cmrt | This study          |
| pET-28b(+)                 | pET-28b(+) vector contained T7 promoter; Kanrt | Novagen (USA)       |
| pET-28b(+)–comED58E         | *S. pneumoniae* D39 comE (1–250 AA) fragment cloned into pBl169; Cmrt | This study          |

*Ermt, erythromycin resistance; Cmrt, chloramphenicol resistance; Amprt, ampicillin resistance. Kanrt, kanamycin resistance; AA, amino acids.

### Table 4: Primers used in this study

| Primer                  | Sequence (5’-3’)                                      |
|-------------------------|-------------------------------------------------------|
| P1(spd1495-F)           | GATCTGGTTCCGCGTGATCTCTCAAGTGCTATGGTGAGGGGCAAACAG     |
| P2(spd1495-R)           | TCGTCATAGCAGATGGCGCCGCTATTGCTATAGCCTTTTTTG           |
| P3(spd1495-up-F)        | GCCTGTTTAGCGATGTACCATAGTG                             |
| P4(spd1495-up-R)        | ATTCTATGAGTCCGCTCAGCTCTTTAGTATCTATATAAAAAGT          |
| P5(erm-F)               | AGTGGCCGACGACTAGAATAT                                 |
| P6(erm-R)               | CCGGGCCTAAAAATGTGTTGAT                                |
| P7(spd1495-down-F)      | ATCAAAACAAAAAATGGGCCCCGGTTGTATTTCTCTATGTAATAAAGC    |
| P8(spd1495-down-R)      | GTGAGGTCTAAATCACTTCTTCCAAC                           |
| P9(pBl169-spd1495-F)    | GGAACGGCCCGGCTCCGAGATCCATGAAATTTAATAGTGTACGATAC     |
| P10(pBl169-spd1495-R)   | GTGCGAATCCGGAGGGATCCGGATGATGATGATGTGTTCTATCAGCTTTTTTGTTG |
| P11(ComE-F)             | GCATCTTTCAGAGATCGATCAGTTAAAAAGTCTTTAATAGTGTATG     |
| P12(ComE-R)             | CTGCCGTTGCATGCGATCAGCTTTAGTATCAGCTTTTATCAATACAT    |
| P13(ComED58E-F)         | GTAATCGGTTCTTTATATCACATATCTGATTAGTTA                 |
| P14(ComED58E-R)         | GAATATCGGTTCTTTATATCACATATCTGATTAGTTA               |
| P15 cpsF Bio-           | TACATCATGCTTCTTCTTTATATTTG (labeled by biotin)       |
| P16 cpsR                | TTAAGGCTTACTCTAGATTAAACA                             |
| P17 cps2A-F             | TAGCCCCATGGAGACCGTGAC                                  |
| P18 cps2A-R             | TCTGCCATGTCATATAAGAAAGGAGA                             |
| P19 cps 2C-F            | ACAGTACCTTCTTCTTCCTAAATGT                              |
| P20 cps 2C-R            | CAAATCTGGAAGCGCCCTCTTATTTATTTATTTGAGAGGCTCAAGAC      |
| P21 csp 2D-F            | ACAGGATCCGACGACTTTCTTCT                                 |
| P22 csp 2D-R            | TCACCTGCTCCGGTACTAAAA                                 |
| P23 csp 2E-F            | TTGGTGGGAGATTGTTGAGCGC                                 |
| P24 csp 2E-R            | TGCCATCTACAGGAATGTCCTT                                  |
| P25 csp 2L-F            | GATACGGTTCTTTAATCGTGGG                                 |
| P26 csp 2L-R            | GAGGGCGAGCAGAGCTTTTCTTTTTTTTTCTTTTCTTTTCTTTTCTTTTCT  |
| P2716S rRNA-F           | TCCGTTTGATTAAGCTACTGATTG                              |
| P2816S rRNA-R           | TCCGTTTGATTAAGCTACTGATTG                              |

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one-step cloning kit (Vazyme, China). The recombinant plasmid pH169-spd1495 containing an Cm resistance cassette was then transferred into the D39-WT strain. The transformants were screened in Cm-containing Columbia sheep blood agar plate. Finally, all of the mutant strains, including D39Apdpd and D93pdp1495Δ, were confirmed by Western blotting (31).

Cloning, expression, and purification of SPD_1495 and ComE\textsuperscript{D58E}. The spd1495 gene was amplified from \textit{S. pneumoniae} D39 genomic DNA (the primers are listed in Table 4). The product of PCR and pGEX-4T-1 plasmid were digested by restriction enzymes BamHI and Not I (TaKaRa, Japan), and then the digested fragments were ligated by the CloneExpress II kit. The constructed plasmid pGEX-4T-1-spd1495 was verified by sequencing (Sangon Biotech, China) and then transferred into \textit{E. coli} BL21. For SPD_1495 expression, BL21/pGEX-4T-1-spd1495 was cultured in LB medium with 100 \(\mu\)g/ml Amp in a shaking incubator at 37°C. When the optical density at 600 nm \((OD_{600})\) reached 0.6 to 0.8, protein expression was induced by adding 0.5 mM isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG; Sigma) with a further 5 h of incubation. The bacteria were harvested by centrifugation at 8,000 \(\times\) g for 10 min at 4°C and washed with 0.1 M phosphate-buffered saline (PBS) three times. The harvested cells were suspended in 0.01 M PBS and lysed by sonication. GST-SPD_1495 fusion protein was isolated by glutathione-Sepharose 4B (GE Healthcare Life Science, Sweden) and digested with thrombin in 0.01 M PBS buffer according to the manufacturer’s instructions to remove the GST tag. The purified SPD_1495 was then used as an antigen for antibody preparation (Tianjin Sun gene Biotech Co., China). The BALB/c mice (6 and postfixed in 1% osmium tetroxide for 2 h at 4°C. Subsequently, 2% uranyl acetate was added to dye

Western blot assay. From each sample, 25 \(\mu\)g of protein was loaded into 12% SDS-PAGE gels and then transferred onto a polyvinylidene fluoride (polyvinylidene difluoride) membrane (Millipore, USA). Anti-SPD_1495 antibody was incubated with polyvinylidene difluoride membrane at 4°C, and horseradish peroxidase-conjugated goat anti-mouse was used as a secondary antibody. The results were visualized with Clarity Western ECL Substrate (Bio-Rad, USA) and captured using ImageMaster 2D Platinum 6.0 (GE Healthcare, USA). Meanwhile, SDS-PAGE gels stained with Coomassie brilliant blue G250 were used as a loading control.

Growth curve analysis. The D39-WT and mutant strains were cultured in C+Y medium (no sugar) with Neu5Ac, arabinose, xylose, glucose, fructose, lactose, sucrose, and maltose as only sugar source, respectively, at equivalent inoculation doses at 37°C in a 5% CO\textsubscript{2} incubator. The OD\textsubscript{600} was continuously measured every hour by UV-visible spectroscopy (Evolution 300; Thermo Fisher Scientific, USA) for 12 h, and the data were analyzed by Prism 6.0 (GraphPad Software, USA).

Protein preparation, iTRAQ labeling, and proteomics analysis. From each sample, 100 \(\mu\)g of protein was extracted from D39-WT and D39Apdpd1495 strains in the exponential growth phase, digested with trypsin (Promega, USA) at 37°C for 16 h, and lyophilized. Then, a iTRAQ reagent multiplex kit (AB Sciex, USA) was used to label peptide samples according to the manufacturer’s protocol. D39-WT and D39Apdpd1495 strains were labeled with 114 and 116 isobaric tags for 1 h at room temperature, respectively. The labeled peptides from the two groups were mixed in equal proportions and dried. High-performance liquid chromatography (WuFeng, China) with a TechMate C18-ST column (4.6 mm \(\times\) 250 mm, 5 \(\mu\)m; TechMate, China) was used to separate the labeled peptides. In brief, an iTRAQ-labeled peptide mixture was eluted with a gradient of 2 to 80% acetonitrile in 20 mM ammonium formate (pH 10.0) for 65 min at a flow rate of 0.8 ml/min. The peptide elution was monitored at 214 nm. The eluted peptides were collected every minute, pooled into six fractions, and then lyophilized. MS analyses were performed with a TripleTOF 5600 (AB Sciex) mass spectrometer. The acquired raw data files (wiff) of each fraction were combined to search against the protein database of \textit{S. pneumoniae} D39, and ProteinPilot software 4.5 (AB Sciex) was used to identify and quantify the proteins. The parameters used for mass spectrometry and quantitative analysis were set as follows: sample type, iTRAQ 4plex (peptide quantification, and peptides with a global false discovery rate of 0.67% were considered for further analysis. The fold change was calculated as the 1.5/1.30 (95.0%) (23, 35). The iTRAQ was then used for protein quantification, and peptides with a global false discovery rate of \(\leq\)1% were considered for further analysis. The fold change was calculated as the 116/114 ratio for differentially expressed proteins. In this study, the cutoff threshold for upregulation and downregulation of the differentially expressed proteins were defined using the population statistics applied to the biological replicates reported by Gan et al. (36). The finalized cutoffs for upregulation (>1.5) and downregulation(<0.67) of proteins were used.

KEGG was used for the pathway enrichment analysis of 54 differentially expressed proteins in the “Wu Kong” platform (37, 38). Protein-protein interaction network analysis (score >0.4) using the STRING (39) database (version 10.5) was performed, and the results were visualized with Cytoscape (v3.5.1) (40). The interaction network was further analyzed using Molecular Complex Detection (MCODE) (41).
the cell pellets for 2 h at room temperature. After the dyeing step, the sample was dehydrated with 50% acetone for 15 min, 70% acetone for 15 min, 90% acetone for 15 min, and twice in 100% acetone for 20 min. All samples were then fixed in epoxy resin and left to polymerize at 37°C for 24 h, 45°C for 24 h, and 60°C for 48 h. Each sample was cut into approximately 50- to 80-nm thin slices and then dyed with 3% uranyl acetate and lead citrate. Finally, the prepared samples were detected using TEM (JEOL 2100F; Japanese Electronics Company).

**Determination of uronic acid content.** The D39-WT, D39Δspd1495, and D39Δspd1495+ strains in the exponential growth phase were harvested by centrifugation at 8,000 \( \times g \) for 10 min at 4°C and washed with 0.01 M PBS three times. Next, the bacteria were resuspended in the Tris-HCl/MgSO_4 buffer (Genebase and Sigma, USA) and centrifuged at 8,000 \( \times g \) for 5 min at 4°C to remove the supernatant and then resuspended with 500 \( \mu l \) of Tris-HCl/MgSO_4 buffer again. Next, 200 \( \mu l \) of bacterial solution and 1.2 ml of 12.5 mM tetraborate solution were mixed and heated at 100°C for 5 min. All of the samples were placed in ice water, and 20 \( \mu l \) of 15% m-hydroxybiphenyl solution was added to each sample, followed by thorough mixing. Meanwhile, the same volume 0.5% NaOH was added in control group. Finally, the concentration of each sample was detected in UV-visible spectrophotometry at 520 nm (42–44). All of the experimental results were processed by GraphPad Prism 6.0.

**Real-time quantitative PCR.** For D39-WT and D39Δspd1495, total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer’s protocol following treatment with 100 mg/ml lysozyme. The RNA concentration was determined by using a NanoDrop 2000 UV-VIS spectrophotometer (Thermo Fisher Scientific). A 1- \( \mu l \) mRNA sample was reverse transcribed using a transScript one-step gDNA removal and cDNA synthesis SuperMix kit for qPCR (TransGen Biotech, China) according to the manufacturer’s instructions. RT-qPCR was performed using the StepOne system (Applied Biosystems, USA) with the TransStart Tip Green qPCR supermix kit (TransGen Biotech); 16S RNA was used as an internal control. The cycle threshold (CT) value was recorded, and the relative quantification of gene expression was calculated using the 2^{-ΔΔCT} method (45). The results are presented as the gene expressions in the spd1495 mutant against those in the D39-WT strain. All data were obtained from three independent biological experiments. The primers used for RT-qPCR are shown in Table 4.

**Animal experiments.** All of the animals used in this study were purchased from the Department of Experimental Animal (Beijing HFK Bioscience); animal experiments were approved by the Ethics Committee for Animal Experiments of Jinan University. The 6- to 8-week-old female BALB/c mice were cared for according to the institutional guidelines for animal care under standard conditions.

For mouse infections, D39-WT, D39Δspd1495, and D39Δspd1495+ mutants were grown in THY medium and harvested in the exponential growth phase. The bacteria were resuspended in 0.01 M PBS and adjusted to the concentration of 1 \times 10^7 CFU/50 \( \mu l \). Then, 50- \( \mu l \) portions of the D39-WT, D39Δspd1495, and D39Δspd1495+ strains were injected into 6- to 8-week-old female BALB/c mice (\( n = 8 \)) via tail vein injection. The survival of the mice was recorded every 12 h for 4 days.

**Coimmunoprecipitation assay.** D39-WT in the exponential growth phase was harvested by centrifugation at 10,000 \( \times g \) for 10 min at 4°C and washed three times with 0.01 M PBS. D39-WT was resuspended in fresh cell lysates (1 mM phenylmethylsulfonfyl fluoride, 50 mM Tris HCl [pH 8.0], 150 mM NaCl, 1% NP-40) until the cell lysates were fully dissolved. The supernatant was collected by centrifugation at 10,000 \( \times g \) for 10 min at 4°C and then incubated with 40 \( \mu l \) of protein A/G-agarose (GE Healthcare, USA) and 5 \( \mu l \) of anti-mouse IgG for 1 h at 4°C, respectively, to remove the nonspecific binding protein, which can bind to IgG, followed by centrifugation at 2,500 \( \times g \) for 5 min at 4°C. Protein A/G-agarose was added, and the supernatants were incubated again. After centrifugation, the concentrations of supernatants were measured by a BCA protein assay kit. SPD_1495 antibody (2 \( \mu g \)) and IgG (2 \( \mu g \)) were added to the supernatants, followed by incubation for 16 h at 4°C, respectively. Then, 40 \( \mu l \) of protein A/G-agarose was added to bind the proteins at 4°C for 6 h, which were collected by centrifugation at 2,500 \( \times g \) for 5 min at 4°C and then resuspended in 800- \( \mu l \) of lysozyme. The isolated proteins were separated by SDS-PAGE, was stained by AgNO3, and then digested and identified by mass spectrometry (LTQ Orbitrap XL; Thermo Fisher Scientific).

**Surface plasmon resonance.** For SPR analysis, SPD_1495 protein (30 \( \mu g/ml \)) was fixed on a NanoGold-COOH sensor chip by capture coupling, and then recombinant ComE and ComE<sup>ESTR</sup> in 0.01 M PBS running buffer were injected sequentially into the chamber at different concentrations (50, 100, 200, and 400 nmol/liter for ComE and 100, 200, 400, 800, and 1,600 nmol/liter for ComE<sup>ESTR</sup>). The interaction of SPD_1495 with ComE was detected by using Open-SPR (Nicoya, Canada) at 25°C. The binding time and dissociation time were both 300 s, the flow rate was 20 \( \mu l/s \), and the chip was regenerated with hydrochloric acid (pH 2.5). A one-to-one diffusion-corrected model was fitted to the wavelength shifts corresponding to the various protein concentrations. The data were retrieved and analyzed by using TraceDrawer software.

**Electrophoretic mobility shift assay.** For EMSA, the 218-bp upstream promoter region of the cps gene cluster was amplified by PCR (for the primers, see Table 4) to produce the 5’-biotin-labeled probe Bio-cps from D39-WT genomic DNA. The amplified fragment was purified by gel extraction. EMSA was carried out according to the LightShift R chemiluminescent EMSA kit protocol (Thermo Fisher Scientific). Proteins of interest (2, 4, 6, or 8 \( \mu g \)) and 100 ng of labeled probe were incubated at 37°C for 30 min in 20 \( \mu l \) of reaction buffer, including 1 X binding buffer, 1 \( \mu g \) of poly(dI-dC), 2.5% glycerol, 0.05% NP-40, and 5 mM MgCl_2. The cps without binding protein was used as a negative control. After incubation, electrophoresis in 6% native Tris-borate-EDTA polyacrylamide gels was performed at 100 V for 60 min to analyze the binding reactions. The polyacrylamide gels were transferred to nylon membranes at 380 mA for 40 min. The membranes were cross-linked for 10 min using a UV lamp equipped with 254-nm bulbs.
and subsequently biotin-labeled DNA was detected by chemiluminescence according to supplier’s instructions (Thermo Fisher Scientific).

**Evolutionary analysis.** The amino acid sequence of SPD-1495 from *S. pneumoniae* D39 was used to query the NCBI database using the protein BLAST tool. The multiple sequence alignment and cluster analysis of the high-scoring proteins were performed by the software package Clustal-X 2.1. MEGAS.05 was applied to construct the protein evolutionary tree.

**Statistics.** Data were analyzed by two-tailed, unpaired Student t tests, and are expressed as means ±

the standard deviations (SD). Statistical analysis was conducted using Prism 6.0 (GraphPad Software, USA). Results were considered significant at $P < 0.05$.

**Data availability.** The raw proteomic data and search results have been deposited to the Proteome-Xchange Consortium via the PRIDE (46) partner repository and can be accessed with the reviewer account (website, http://www.ebi.ac.uk/pride; username, reviewer70538@ebi.ac.uk; password, x5Zecn7h).

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**TABLE S1**, PDF file, 0.02 MB.

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X.S., R.G., and Q.-Y.H. designed the project and revised the paper. Y.-D.Z., Y.P., K.H., and G.-F.D. performed the experiment and data-analysis. Y.-D.Z. and X.S. wrote the manuscript.

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