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The global transcriptional regulator MgaSpn affects the virulence of *Streptococcus pneumoniae* by regulating PcpA

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Abstract

The global transcriptional regulator MgaSpn is a significant virulence factor of *Streptococcus pneumoniae*. In our previous study, we found that MgaSpn is a regulator of bacterial virulence by modulating the levels of phosphorylcholine (ChoP) and capsular polysaccharides (CPS) on the surface of *S. pneumoniae*. Here, we report for the first time that *pcp*A expression was significantly increased in *mgaSpn* deletion strains and significantly decreased when *mgaSpn* was overexpressed. Electrophoretic mobility-shift and DNase I footprinting assays confirmed that MgaSpn interacts with the *pcp*A promoter (P_{pcpA}) at two specific binding sites. Virulence experiments demonstrated that the interaction between MgaSpn and PcpA is necessary for pneumococcal colonization and invasive infection. Western blot analysis indicated that iron concentration can influences the regulation of PcpA expression via MgaSpn. In summary, these results revealed that MgaSpn regulates PcpA and plays a significant role in pneumococcal pathogenesis.

Keywords *Streptococcus pneumoniae*, MgaSpn, *pcpA*, Iron levels

Introduction

Streptococcus pneumoniae is both the primary pathogen of community-acquired pneumonia and the major pathogen responsible for mortality in children under 5 years old [1–3]. Globally, approximately 800,000 children die from pneumococcal diseases annually, with over 90% of these deaths occurring in developing nations where few children have access to life-saving serotype-based vaccines [4, 5]. As a common opportunistic pathogen, *S. pneumoniae* usually asymptomatically colonizes in human nasopharyngeal mucosa. Under specific selective pressures, the expression of certain bacterial virulence genes is upregulated or downregulated [6, 7], enhancing pathogenic potential and resulting in severe invasive diseases such as invasive pneumonia or even sepsis [8–12]. This conditional invasive pathogenesis of *S. pneumoniae* indicates that its virulence is regulated by environmental signals. However, the mechanism of invasive pathogenicity caused by such environmental changes, such as metal ion homeostasis, host immune responses, and mucosal microbiota interactions, have not been fully elucidated [13–16]. Therefore, further research on pathogenic mechanisms of *S. pneumoniae* is needed to guide prevention and treatment protocols.

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S. pneumoniae infections require that this bacterium adapt to different host environments and this process is performed *via* a highly coordinated expression of virulence factors. In Gram-negative bacteria such as *Salmonella* or *Escherichia coli*, pathogenicity islands (PAIs) often encode cohesive virulence modules, which are acquired through horizontal gene transfer and localized to specific loci [17–19]. In contrast, *S. pneumoniae* lacks such large-scale virulence gene clusters. The virulence genes of *S. pneumoniae*, such as choline-binding proteins (CBPs), capsule biosynthesis genes (*cps*), and surface proteins (e.g., PspA, CbpA), are not clustered together and regulated by single-component regulatory systems including *lux*S, *rlr*A, *reg*M/R, and *mgr*A or by 14 two-component systems (TCS) [20, 21]. Since virulence is a multi-factorial process, the challenge ahead will be to further elucidate how virulence factors coordinate to cause disease.

A crucial element of the infection process is host cell interactions that are mediated by capsular polysaccharides (CPS), cell wall and surface proteins. The latter are considered potential virulence factors due to their direct contribution to host-pathogen interactions, although their roles in local and invasive infections are not yet fully understood. There are four major groups of surface proteins in *S. pneumoniae*, including lipoproteins, proteins carrying the amino acid sequence LPXTG, choline-binding protein family (CBP) and non-classical surface proteins (NCSP). In particular, the cell wall associated choline phosphate (ChoP) is a mediator of host-pathogen interactions [22–24] *via* non-covalent binding to CBPs [25].

CBPs can interact with the host during colonization or invasion, and they include the *S. pneumoniae* surface protein A (PspA) and *S. pneumoniae* surface protein C (PspC; CbpA) as well as the *S. pneumoniae* choline-binding protein A (PcpA) [21, 26, 27]. Expression of the *psp*A gene is regulated by binding of the TCS protein phospho-VicR to the upstream region of *pspA* [28]. Similarly, the RR06/HK06 TCS directly regulates *psp*C transcription [29] and *pcp*A is regulated by the Mn^{2+} dependent regulatory factors PsaR and CodY, and TCS01 system components [30–32].

MgaSpn is another global transcriptional regulator that responds to environmental changes and is orthologous to Mga in the group A *Streptococcus* (GAS) [33–35]. MgaSpn contains two conserved helix-turn-helix (HTH) domains that function to bind the protein to DNA. Our laboratory had previously confirmed that MgaSpn is a transcriptional suppressor of CPS and ChoP biosynthesis [36]. In this study, we found that MgaSpn altered adhesion functions by specific binding to *pcpA*. For the first time, we also found that MgaSpn protein can regulate PcpA expression in response to alterations in environmental iron concentration. These findings provide new experimental evidence for understanding the pathogenesis of *S. pneumoniae*. In the current work, we provide evidence that MgaSpn is a regulator of PcpA expression.

Materials and methods

Bacterial strains and growth conditions

Strains and plasmids used in this research are listed in Table 1. *S. pneumoniae* strain D39 derivatives were incubated in C + Y medium (5% yeast extract, pH 7.0) at 37 °C in a 5% CO_2 atmosphere or in C + Y medium containing glucose at 2, 4, 8 and 16 mM, respectively, as the sole carbon source. Bacteria were also incubated in THY medium that was treated with 1% Chelex 100 (BioRad, Hercules, CA, USA) to render it iron-deficient and used with Fe^{2+} added back to 0, 0.2 and 0.4 mM in the form of $FeSO_4 \cdot 7H_2O$. *S. pneumoniae* was transformed following the method described before [37].

Construction of unencapsulated mutant strains

PCR amplification was used to synthesize gene cassettes using gene-specific primers (Table 2) and strain D39 as the source of DNA genomic template. The unencapsulated strains JH0002, JH1102 and JH1107 as the target strains chosen were used for genomic modifications [36]. A two-step transformation process was used to construct *pcp*A -deficient strains [38]. PcpA-P1/PcpA-JC F and PcpA-JC R/PcpA-P4 primer pairs were used to amplify upstream (565 bp) and downstream (549 bp) homologous arms of the *pcp*A locus. Primers Pr1332 and Pr1333 were then used to amplify the Janus cassette (1471 bp) from genomic DNA of strain ST588. The upstream arm, Janus cassette and downstream arm were then used in a fusion PCR reaction with primers PcpA-P1 and PcpA-P4 to generate the mutational cassette (2564 bp) that was then transformed into JH0002 strain (WT) to construct Δ*pcp*A::JC1 strain [39]. The upstream and downstream primer pairs PcpA-P1/PcpA-P2 and PcpA-P3/PcpA-P4 were used to create a fusion PCR deletion cassette (1093 bp) that was then transformed into Δ*pcp*A::JC strain to produce the *pcp*A unmarked deletion strains (JH9708 and JH9709).

We also constructed mutant strains containing mutated *pcp*A promoter elements. In brief, $\Delta P_{pcpAp1+p2}$::JC1 strain was constructed using the Janus cassette fragment linking the homologous arm (2786 bp) that was amplified with primers Pr9725-$P_{pcpAp1+p2}$::JC F/Pr9729-$P_{pcpAp1+p2}$::JC R and transformed into strain JH0002. Subsequently, we constructed nine mutated P_{pcpAp1} fragments and eight mutated P_{pcpAp2} fragments that were then transformed into $\Delta P_{pcpAp1+p2}$::JC1 strain to construct the *pcp*A promoter mutant strains JH9714 and JH9715. The P_{pcpA} fragment (335 bp) was then amplified using

Table 1 Strains and plasmids used in this study

Strains or plasmids	Genotype and relevant features	Resistance (^{R})
E. coli		
DH5α		-
BL21		-
Streptococcus pneumoniae strains		
JH1900	*Streptococcus pneumoniae* D39 strain, Capsulated strainserotype 2,*rpsl* K56T	Sm^R
JH0001	JH1900, Δ*dexB-cps2A*::JC1	Kan^R
JH0002	JH1900, Δ*dexB-cps2A*	Sm^R
JH1101	JH1900, Δ*mgaSpn*::Erm	Erm^R
JH1102	JH0002,Δ*mgaSpn*	Sm^R
JH1106	JH0002,JH0002Δ*mgaSpn*::*mgaSpn*	$Spec^R$
JH1203	JH1900, Δ*mgaSpn*::JC1	Kan^R
JH7001	JH1900, Δ*mgaSpn*	Sm^R
JH7002	JH1900,JH1900Δ*mgaSpn*::*mgaSpn*	$Spec^R$
JH9700	JH1900,Δ*pcpA*::JC1	Kan^R
JH9701	JH7001,Δ*pcpA*::JC1	Kan^R
JH9702	JH0002,Δ*pcpA*::JC1	Kan^R
JH9703	JH1102,Δ*pcpA*::JC1	Kan^R
JH9706	JH1900,Δ*pcpA*	Sm^R
JH9707	JH1900,Δ*mgaSpn*Δ*pcpA*	Sm^R
JH9708	JH0002,Δ*pcpA*	Sm^R
JH9709	JH0002,Δ*mgaSpn*Δ*pcpA*	Sm^R
JH9710	JH0002,pEVP3-luc-P_{pcpA}	Chl^R
JH9711	JH1102,pEVP3-luc-P_{pcpA}	Chl^R
JH9712	JH1106,pEVP3-luc-P_{pcpA}	Chl^R
JH9713	JH0002,ΔP_{pcpA}p1+p2::JC1	Kan^R
JH9714	JH0002,P_{pcpA}p1 multisite mutation	Sm^R
JH9715	JH0002,P_{pcpA}p2 multisite mutation	Sm^R
Plasmids		
pEVP3	*E. coli-Streptococcus pneumoniae* integrative vector	Chl^R
pEVP3-luc	pEVP3 derivative carrying the luc reporter gene	Chl^R
pEVP3-P_{pcpA}-luc	P_{pcpA} cloned into insertion vector	Chl^R
pPEPZ-P_{lac}	Integrative plasmid with P_{lac} promoter	$Spec^R$
pPEPZ-*mgaSpn*	pPEPZ-P_{lac} derivative carrying	$Spec^R$
pET-28a	protein expression vector	Kan^R
pET-28a-*pcpA*-28-390AAs	pET-28a derivative carrying the *pcpA*-28-390AAs fused to a (C-ter) His6 tag	Kan^R

primers P_{pcpA}-XhoI/P_{pcpA}-BamH1. The obtained gel-purified amplicon was then digested with *Bam*HI and *Xho*I and inserted into suicide plasmid pEVP3 to generate the pEVP3-P_{pcpA}-luc plasmid that was then transferred into JH0002, JH1102 and JH1107 strains. This process generated P_{pcpA} luciferase (*luc*) reporter strains.

Quantitative Real-time PCR

Total RNA was obtained from 3 mL of log-phase bacterial culture in C + Y medium. RNA was extracted using an RNA prep pure Cell/Bacteria Kit (Tiangen, Beijing, China). A PrimeScript first-strand cDNA synthesis kit (Takara, Japan) was used to reverse transcribe RNA to cDNA. Quantitative Real-time PCR (qRT-PCR) was performed on CFX Connect TM (BIO-RAD, Singapore) and reactions included gene-specific primers (Table 2) and reactions were performed in triplicate and listed as the mean ± standard deviation. mRNA levels are expressed relative to that of *gyrB*.

Monitoring luc transcriptional fusion activity during Pneumococcal growth

The pEVP3-P_{pcpA}-luc plasmid was transformed into JH0002, JH1102 and JH1107 strains to obtain P_{pcpA} reporter gene strains JH9710, JH9711 and JH9712, respectively. Luc reporter activity was measured using a commercial instrument (GloMax Multi Jr Single Tube Detection System, Promega) using JH9710, JH9711 and JH9712 strains cultured in C + Y medium.

Transcriptome sequencing

Transcriptome sequencing was performed as described previously [40]. Briefly, strain for transcriptome sequencing were collected in the OD620 of 0.5 period in C + Y medium. Pneumococcal cultures were pre-treated with ammonium sulfate to terminate protein-dependent transcription and degradation, then were centrifuged at 10,000 g for 10 min. Bacterial sediment was transported at -20℃ temperature to the Beijing Novogene Company laboratory for transcriptome sequencing and differential expression analyzation. The DESeq software was used for differential expression analysis between the two comparison combinations (two biological replicates per group). P value was adjusted by Benjamini and Hochberg methods, and P value < 0.05 after correction and|log2foldchange|>1 were used as the threshold of significant differential expression.

Western blotting

Protein extracts used for Western blotting experiments were generated from *S. pneumoniae* cultured in either C + Y medium or deferrated THY (see above) containing Fe^{2+} added to 0, 0.2 and 0.4 mM. Cells (5 mL) were harvested at 0.3 (OD 600 nm) and lysed in 200 µL 150 mM NaCl, 0.1% deoxycholate, 15 mM EDTA and 0.2% SDS pH 8. The samples were separated by 10% SDS-PAGE and subsequently transferred to PVDF membranes that were then exposed to primary antibodies overnight at 4 °C and then secondary antibodies at 37 °C for 1 h using standard conditions. Primary polyclonal antibodies to PcpA were generated in C57BL/6 mice using purified PcpA protein.

Table 2 PCR primers used in this study

Primers	Sequence(5′ to 3′)
PcpA F	CCATCGGGTGTAGTCGTGTT
PcpA R	CTGGTTGGGTTAAAGTTTCTGG
CbpC F	CACAGGTGGCCGATTTGTTG
CbpC R	TGCCAACCGACAACCATCTC
Ply F	TCATCCAGGCTTGCCGATTT
Ply R	ATTGGGCAACATAGGCACCA
SPD_0803 F	AAACTTTGGACTTGGCGTGAT
SPD_0803 R	TTACGTGGGCCTGTTCCGTA
MgaSpn F	AGTTGCTCCTAGTTACGAACC
MgaSpn R	ACCTTCTATTCCTTCTGCCTGC
gyrB F	GTTCGTATGCGTCCAGGGAT
gyrB R	ATACCACGCCCATCATCCAC
PcpA-JC F	TTCAGCATTATCCTCTAGAGTATCCATTAGCTAAATCT
PcpA-JC R	AGCATAAGGAAAGGCTCGAGTTAAATTTCCTTACATATTT
Pr1332	TCTAGAGGATAATGCTGAAAACTCCTTGAAG
Pr1333	CTCGAGCCTTTCCTTATGCTTTTGGAC
PcpA-P1	CAAACGTAACACTCCAGACCG
PcpA-P2	AAATATGTAAGGAAATTTAAGTATCCATTAGCTAAATCT
PcpA-P3	AGATTTAGCTAATGGATACTTAAATTTCCTTACATATTT
PcpA-P4	GCAGGGAGTCTATTGACTCTTTG
P_{pcpA}-XhoI	CCGCTCGAGGCGAATTGAATTTCGAGAG
P_{pcpA}-BamH1	CGCGGATCCCCGCAGCTGTAGTTAATGAT
Pr9725	CCGCAAATGCAAAAGTACCG
P_{pcpA}p1+p2::JC F	TTCAGCATTATCCTCTAGAAAGCTGGTCAAGATGGAAAACC
P_{pcpA}p1+p2::JC R	AGCATAAGGAAAGGCTCGAGGGCCACTCTTCAATATGTGCG
Pr9729	CGTAATCCGAAGCGTTTACGAT

The mouse anti-MgaSpn antibodies were previously described [36]. Primary antibodies were used at 1:5000 and the secondary antibody was 1:10000 goat anti-mouse IgG (KPL, Lenexa, KS, USA).

Electrophoretic mobility shift assay (EMSA)

EMSA assays utilized 5′ -biotin-tagged P_{pcpA}, $P_{pcpA\text{p1}}$ and $P_{pcpA\text{p2}}$ amplicons generated from strain JH1900 strain. In brief, reactions were carried out in 10 µL volumes containing binding buffer, 0–6 µg protein, 0.5 µg poly (dI-dC) and 0.5 ng biotin-labeled probe. The reactions were incubated at 25 °C for 20 min and processed using the guidelines for the commercial LightShift R Chemiluminescence EMSA kit) (Thermo Fisher, Pittsburg, PA, USA).

DNase I footprinting assay

DNase I footprinting assays were performed as previously described [41]. In brief, fluorescent FAM-labeled probes for P_{pcpA} were amplified using strain JH1900 genomic DNA templates. The probes (335 ng) were incubated with differing levels of purified MgaSpn protein in 40 µL reaction volumes at 25 °C for 30 min and a solution containing 0.015 U / 10 µL of DNase I (Promega, Madison, WI, USA) was then added along with freshly prepared 100 nM $CaCl_2$. The reactions were incubated at 37 °C for 1 min and 140 µL terminator solution was then added to stop the reaction. The samples were extracted using phenol and precipitated with ethanol and the DNA-protein pellets were dissolved in 30 µL nuclease-free water. Electrophoresis process that included the GeneScan-LIZ600 size standard (Applied Biosystems, Foster City, CA, USA) was then performed under standard conditions.

Adhesion assays

Bacterial adhesion assays utilized human adenocarcinomic alveolar epithelial cell (A549) cells seeded at a density of 2×10^5 per well in 24-well plates and incubated overnight at 37 °C. Bacterial suspensions were diluted to 1×10^8 colony-forming units (CFU)/mL in DMEM media (Thermo Fisher) and 500 µL were used to obtain a multiplicity of infection (MOI) of 100. The cells were co-incubated at 37 °C for 30 min [42] and the wells were then washed 5 × with PBS. For adhesion assays, cells were lysed in sterile ddH_2O. The lysates were serially diluted and plated on blood agar plates to identify intracellular and extracellular CFU [42]. For invasion assays, extracellular bacteria were treated with 10 µg/mL penicillin and 100 µg/mL gentamicin for 15 min prior to lysis and CFU plating. Intracellular CFUs were then determined as described above.

Bacterial cell staining

Visualization of bacterial cell adhesion to A549 cells was performed by placing slide cover slips in the 24-well plates in advance of co-culture. In brief, A549 cells (2×10^5/ well) were allowed to attach using an overnight incubation and then washed 5–6 × with PBS. *S. pneumoniae* cells were labeled with fluorescein isothiocyanate at 37℃ for 30 min and added to the A549 cells in DMEM using 500 µL to achieve an MOI of 100 and incubated at 37 °C for 30 min. The wells were then gently rinsed 5 × with PBS and 200 µL 4% paraformaldehyde was added to each well, fixed at room temperature for 30 min and then washed 3 × with PBS. PBST (PBS – 0.5% Triton-100) 400 µL was then added to each well and allowed to permeabilize at room temperature for 5 min and then washed 3 × with PBS. DAPI (200 µL) was then added to each well to allow nuclear staining (10 min). The cells were then washed as per above and viewed using a confocal laser scanning microscope (Leica, Germany).

Animal experiments

Female C57BL/6 mice (6–8 weeks, 20–21 g) were purchased from the Laboratory Animal Center of Chongqing Medical University that were randomly divided into four groups ($n = 6$). All animal experiments described in this study were approved by the Animal Care and Use Committee of Chongqing Medical University (reference number: IACUC-CQMU-2024-0512) and were performed in strict accordance with the regulations of the Guide for the Care and Use of Laboratory Animals. The animals were infected using 1×10^8 CFU bacteria via the nasal cavities using a standard protocol. Nasal lavage fluid and lung tissues were collected and crushed using a mechanical mortar and pestle and diluted samples were plated on blood agar plates for CFU calculations.

A second group of 12 mice randomly divided into four groups ($n = 3$) were treated as per above but the lung tissues were processed for hematoxylin-eosin (H&E) staining. In brief, lungs were fixed with 4% paraformaldehyde for 48 h at 4 °C and then embedded in paraffin and cut into 5 mm sections. H&E staining was performed as previously described [43].

Table 3 Virulence genes in JH1101 strain

Locus tag	Fold Change	Gene name	Gene description
Increased			
SPD_0345	-2.0	*cbpC*	choline-binding protein CbpC
SPD_0803	-4.3	-	PspC domain-containing protein
SPD_1726	-5.9	*ply*	cholesterol-dependent cytolysin pneumolysin
SPD_1965	-13.9	*pcpA*	choline-binding protein PcpA

Statistical analysis

To compare statistical differences between groups, Student's *t*-test, non-parametric Mann-Whitney or Wilcoxon tests were employed using GraphPad Prism 8.0.2 (GraphPad Software, San Diego, CA, USA). Statistical significance was defined as $P < 0.05$.

Results

Mga*Spn* is involved in the negative regulation of PcpA

MgaSpn has high homology with the Mga protein from GAS strains and acts as a transcriptional activator of virulence genes in *S. pyogenes* [44, 45]. Our laboratory has previously reported that MgaSpn can activate transcription from both the *cps* (capsule) gene cluster and the *lic*1 teichoic acid synthesis-related gene cluster [36]. We therefore explored whether MgaSpn can regulate other surface virulence factors. We compared the transcriptomes of *mgaSpn*-deficient strain JH1101 and WT JH1900 cultured in C + Y medium [40]. We identified 168 upregulated and 98 downregulated genes in JH1101 that fell within the cutoff of Δ 2-fold (Table S1). The upregulated genes mainly involved genes related to PTS sugar transporters, bacteriocin and pneumococcal surface proteins, the downregulated genes mainly involved ribosomal proteins and ABC transporters. What attracted our attention were the virulence genes *pcpA*, *ply*, *SPD_0803* and *cbpC*, which were significantly upregulated in the *mgaSpn*-deficient strain JH1101 (Table 3).

We more closely examined the expression of these virulence factors by direct mRNA measurements using qRT-PCR. We utilized the WT strain JH1900, the *mgaSpn*-deficient strain JH7001 and the *mgaSpn* overexpression strain JH7002. We found that when compared to WT, only *pcp*A was significantly upregulated in the *mgaSpn*-deficient strain (2- to 3-fold) and significantly downregulated ~ 2-fold in the overexpression strain (Fig. 1). Our previous research also indicated that MgaSpn can alter *cps* expression [36], so we examined unencapsulated versions of WT, *mgaSpn*-deficient and *mgaSpn* overexpressing strains. The same trend was observed for the expression of *pcpA* in unencapsulated strains (Fig. 1).

To verify that MgaSpn directly regulates *pcp*A, we also constructed *pcp*A promoter-luciferase fusion reporter strains where *pcp*A transcriptional activity could be directly monitored by quantification of luminescence. These strains were cultured in C + Y medium and sampled throughout growth. In the logarithmic growth phase, P_{pcpA} transcriptional activity for the *mgaSpn*-deficient strain JH9711 was significantly higher than that of the WT strain JH9710 (Fig. 1). This indicated that MgaSpn was directly involved in the negative regulation of *pcp*A. In addition, the expression of PcpA protein in the unencapsulated strains JH0002, JH1102 and JH1107

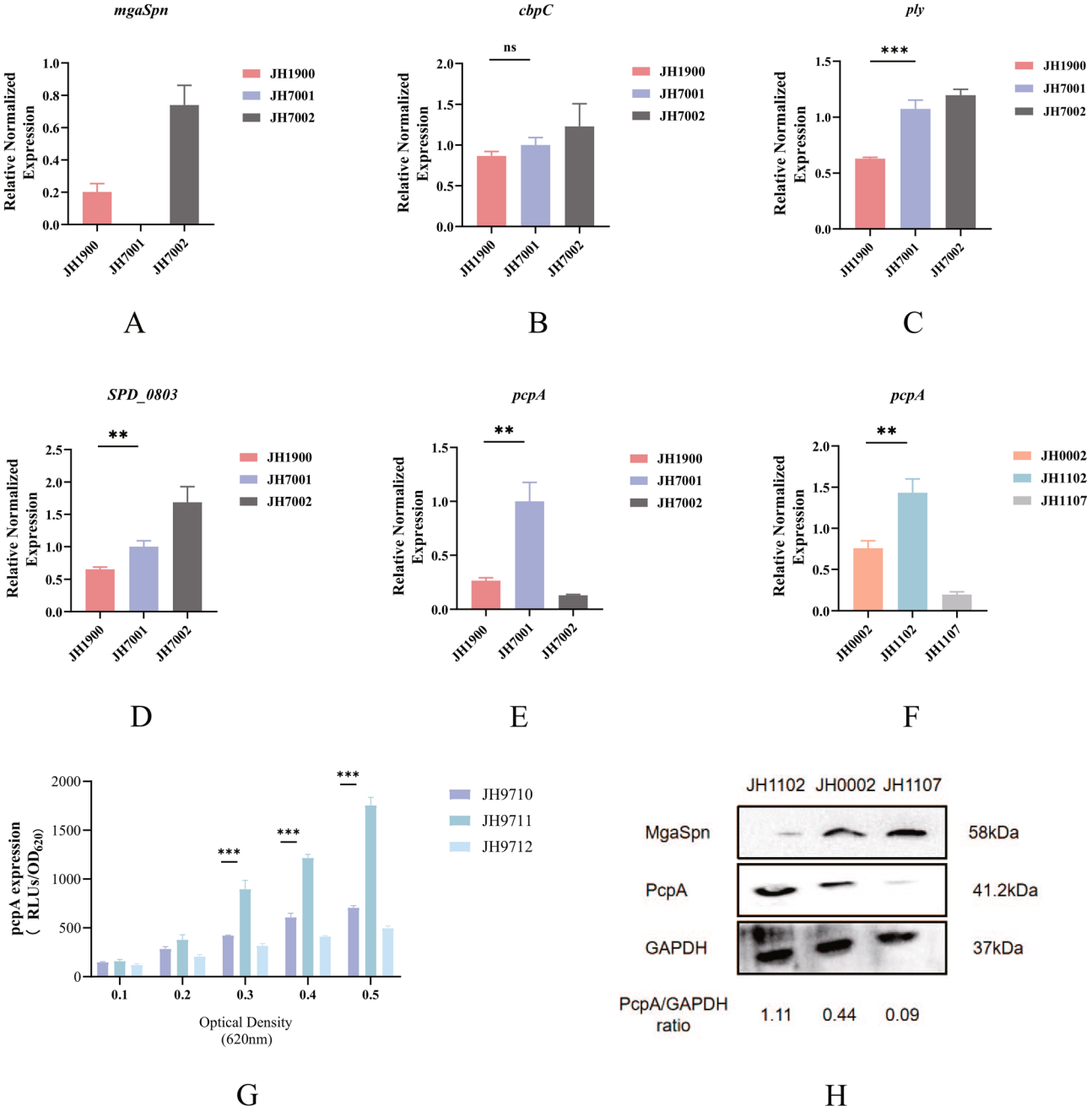


Fig. 1 Expression of virulence genes. **A**. Relative mRNA levels for *mgaSpn* in the WT (JH1900), *mgaSpn*-deficient (JH7001) and *mgaSpn*-overexpressed (JH7002) strains as indicated. mRNA levels are expressed relative to that of *gyrB*. **B**, **C**, **D** and **E**. Relative mRNA levels for *cbpC*, *ply*, *SPD_0803* and *pcpA* in the WT (JH1900), *mgaSpn*-deficient (JH7001) and *mgaSpn*-overexpressed (JH7002) strains as indicated. mRNA levels are expressed relative to that of *gyrB*. **F**. Relative mRNA levels for *pcpA* in the unencapsulated WT (JH0002), *mgaSpn*-deficient (JH1102) and *mgaSpn*-overexpressed (JH1107) strains as indicated. mRNA levels are expressed relative to that of *gyrB*. **G**. Promoter activity of *pcp*A in the unencapsulated WT (JH9710), *mgaSpn*-deficientand (JH9711) and *mgaSpn*-overexpressing (JH9712) strains. Data are presented as the mean ± SD from three independent experiments with biological duplicates. ***$P < 0.001$, **$P < 0.01$, and not significant (NS) as analyzed by unpaired two-tailed Student's *t*-test. **H**: Western blotting for PcpA using protein extracts from JH0002, JH1102 and JH1107 unencapsulated strains

was detected by Western Blot. The results showed that the expression of PcpA protein was significantly upregulated following *mgaSpn* deletion, and downregulated upon *mgaSpn* overexpression (Fig. 1). These results indicate that MgaSpn could directly and negatively regulate PcpA expression.

MgaSpn binds to the *pcpA* promoter region

MgaSpn possesses two conserved HTH DNA-binding motifs that indicated this protein is most likely involved in direct DNA interactions [32]. We therefore used EMSA to explore whether the MgaSpn protein can directly bind to the *pcp*A promoter. A gradually increased concentration of MgaSpn protein with the *pcp*A promoter fragment revealed a positive and concentration-dependent correlation between protein added and the corresponding gel shift during electrophoresis (Fig. 2A). We also included an unlabeled probe competition lane and the unlabeled probes competed to bind to the MgaSpn- and *pcpA*-labeled probes. These results indicated that MgaSpn was specifically bound to the *pcp*A promoter region (Fig. 2A).

We additionally performed DNase I footprinting analysis to identify specific contact sites of MgaSpn in the P_{pcpA} promoter probe (Fig. 2B). We identified 35 bp (5'-acattttataaattgtcatctctccataaacacag-3') and 23 bp (5'-gtcaaggaaaattaacccacttt-3') regions (Fig. 2C). These findings indicated that MgaSpn is a regulator or co-regulator of *pcp*A.

Two binding sites are functional sites for MgaSpn transcriptional regulation

The binding sites in the *pcp*A promoter identified above were further examined for in vivo binding activity to determine if these were functional and necessary for MgaSpn transcriptional regulation. We therefore constructed mutations in these P_{pcpA} sites. In particular, we introduced 9 mutations into the first binding site (strain JH9714) and 8 into the second binding site (strain JH9715) (Fig. 3A). EMSA results using these mutated probes indicated a weakened binding activity to MgaSpn (Fig. 3B and C).

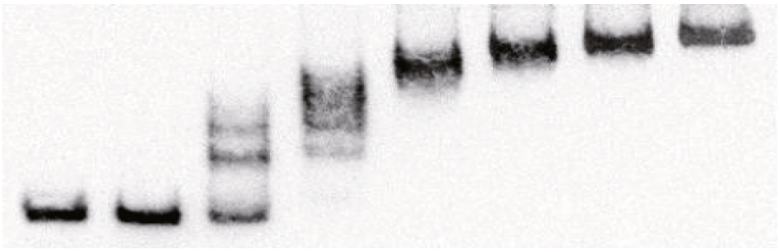
It is known that the PcpA protein is primarily involved in bacterial adhesion and aggregation [46, 47]. Adhesion and invasion assays were performed using the unencapsulated strains JH0002, JH1102, JH9708 (*pcp*A-deficient strain), JH9714 and JH9715, respectively, to A549 cells to investigate the virulence of promoter mutant strains. Previous studies have suggested that *pcp*A mutants displayed reduced adhesion ability compared to the WT strain [48]. We found that strain JH9708 had significantly reduced invasion and adhesion abilities compared to other strains. In addition, strains JH9714 and JH9715 had stronger invasion and adhesion abilities compared to the strain JH0002 (Fig. 3D, E and F). These findings indicated that the two binding sites were functional sites for MgaSpn transcriptional regulation.

The *mgaSpn* influences adhesion and pathogenicity of *S. pneumoniae* via *pcp*A regulation

The preceding results indicated that mgaSpn is a negative regulator of *pcp*A. We therefore constructed an unencapsulated *pcp*A and *mgaSpn* double-deletion strain (JH9709) (Fig. S1). To verify the influence of *pcpA* and *mgaSpn* on pneumococcal growth, the growth curves of JH0002, JH1102, JH9708 and JH9709 strains were examined. The growth rates and maximum biomass for these four strains did not significantly differ (Fig. S2). We therefore performed adhesion and invasion assays for these strain since previous studies had indicated that MgaSpn negatively regulates both adhesion and invasion ability of bacteria while PcpA is an essential element in adhesion and invasion [36, 48]. When compared with the results of the WT JH0002 strain, the *mgaSpn*-deficient strain JH1102 strain had a greater adhesion and invasion ability while strain JH9708 (*pcp*A null) was weaker in both these capacities (Fig. 4). In addition, expression of *pcp*A in JH1102 was significantly increased. (see Fig. 1, above). In order to confirm that the enhanced adhesion and invasion ability of JH1102 was related to *pcp*A, we observed the adhesion and invasion ability of JH9709 to A549 cells. Compared with JH1102, the adhesion and invasion ability of JH9709 was significantly reduced (Fig. 4). Therefore, this implicated *mgaSpn* in both adhesion and pathogenicity of *S. pneumoniae* by regulating *pcp*A.

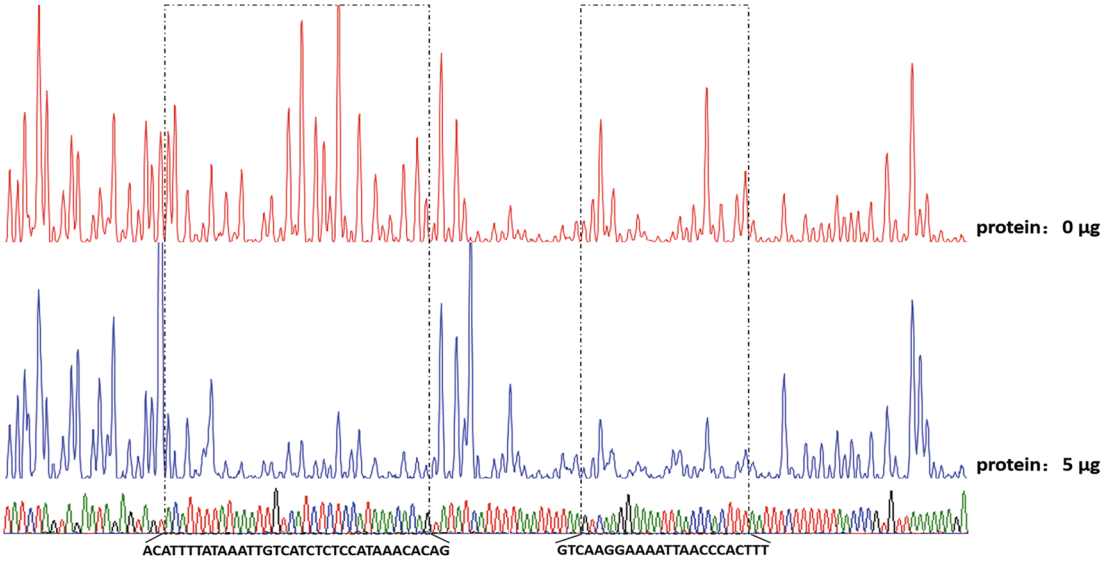
The *mgaSpn* affects lung infection of *S. pneumoniae* by regulating *pcpA*

Previous studies of *S. pneumoniae* indicated that PcpA is necessary for lung colonization and infection while having no impact on nasopharyngeal colonization [49]. *In vivo* virulence experiments were performed to observe changes in virulence of mutant strains. We nasally challenged mice with strains JH0002, JH1102, JH9708 and JH9709 and performed nasal lavages after 12 h to determine bacterial loads. CFU counts for JH1102 and JH9709 groups did not statistically differ (Fig. S3). In contrast and contrary to expectations, the lung colonization ability of JH1102 did not increase. In contrast, bacterial loads in the lung tissues in the JH1102 infection group was higher than that for the JH9709 group (Fig. 5A). In addition, compared with JH0002 infected group, lung H&E staining depicted increased inflammatory cell infiltration and pathological injury in the JH1102 infection group and decreased inflammatory cell infiltration and pathological injury in the JH9708 and JH9709 groups (Fig. 5B). These results indicated that *mgaSpn* affects lung infection of



Protein	(ug):	6	0	1	2	3	4	5	6
P pcpA	(ng):	1	1	1	1	1	1	1	1
Unlabled	(ng):	100	0	0	0	0	0	0	0

A



B

CCGCAGCTGTAGTTAATGATAATATTGTAGTTTTTTTCATTTAAATTTCCTTACATATT
TATTTTCTAATAGATATAAAATTATAACTTTGCTAGCCGTTGTTATAAAAAGTTTTATT
CAATCATTATCTAGTAGAATAGAGTAGTACATTTTATAAATTGTCATCTCTCCATAAA
CACAGTATATCATTTTTAAATTTTAAGTCAAGGAAAATTAACCCACTTTAATTTCTTT
TTTAACCCGTGTTAAAAAAGAAATTAAAGTGGGTTAAAAAAGAAATTAAAGTGGG
TTAAAAAAGAAATTAACTGAGTAAAAACTCTCGAAATTCAATTCGC

C

Fig. 2 MgaSpn binds to the P_{pcpA}. **A**. EMSA of P_{pcpA} with the MgaSpn proteins. **B**. DNase I footprinting protection assay of MgaSpn. **C**. Binding sites for MgaSpn (in yellow background)

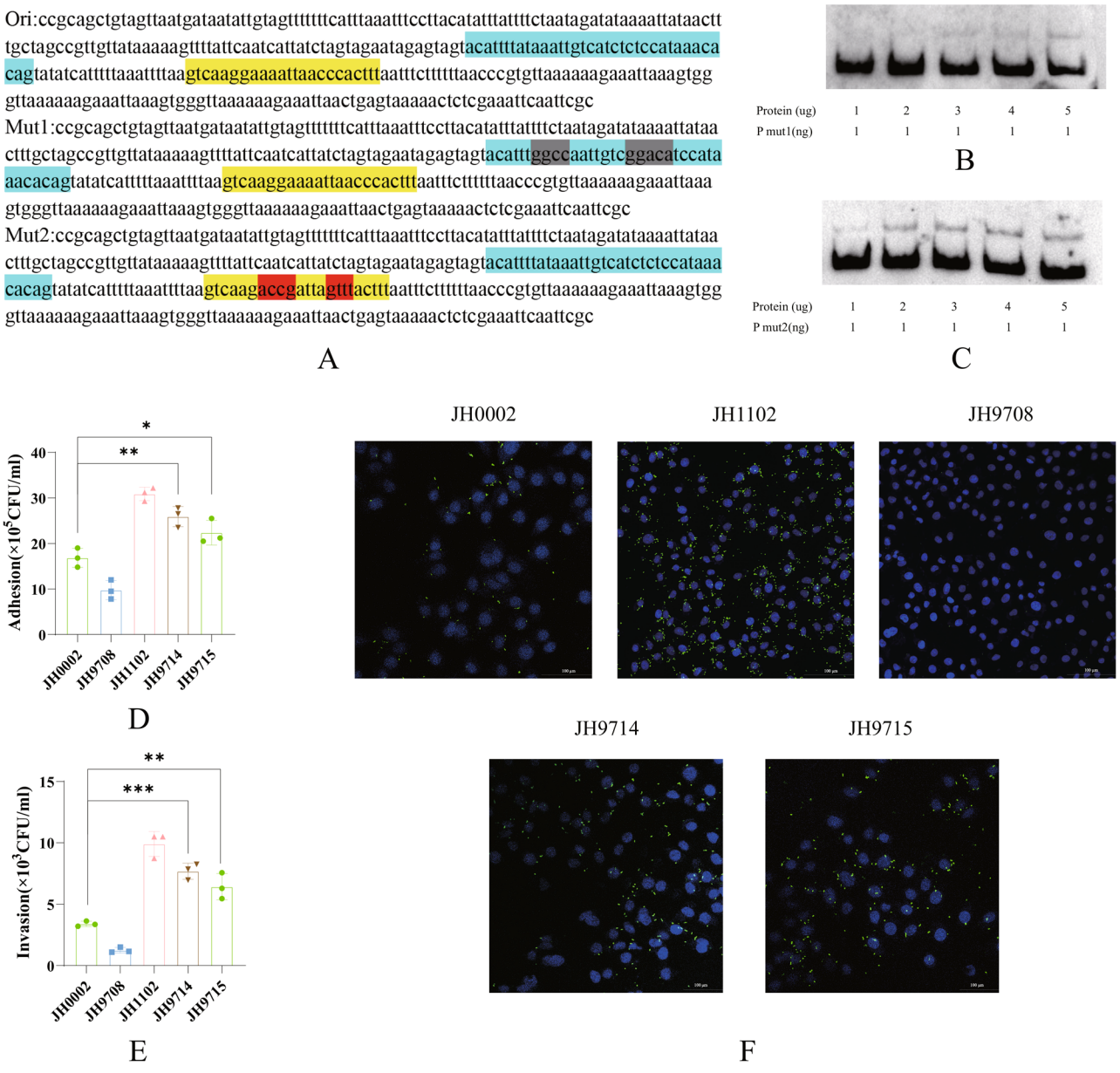


Fig. 3 Detection of functional binding sites for MgaSpn transcriptional regulation. **A**. Ori is the promoter fragment of pcpA; blue background is the first binding region of MgaSpn and pcpA; yellow background is the second binding region of MgaSpn and pcpA; Mut1 is the first binding site mutation fragment of the pcpA promoter; gray background is mutation region of the first binding region; Mut2 is the second binding site mutation fragment of the pcpA promoter; and red background is mutation region of the second binding region. **B**: Binding of promoter region Mut1 to MgaSpn. **C**. Binding of promoter region Mut2 to MgaSpn. D and E. A549 cells were infected with the unencapsulated strains JH0002, JH1102, JH9708, JH9714 and JH9715 at an MOI = 100. **D**. Adherent and **E**. invasive bacteria were counted by plating from serial dilutions. ***$P < 0.001$, **$P < 0.01$, not significant (NS) as analyzed by unpaired two-tailed Student's *t*-test. **F**. Photomicrographs of bacteria adhering to A549 epithelial cells using confocal microscopy

S. pneumoniae by regulating *pcpA* and other virulence factors.

Iron concentration alters MgaSpn regulation of PcpA

The preceding experimental results demonstrated that the global transcriptional regulator MgaSpn is a negative regulator of *pcp*A. Mga in GAS is a known sensor of external environmental signals [50]. We therefore examined whether MgaSpn regulates the expression of PcpA *via* environmental signal sensing. We found that PcpA levels were not significantly altered between strain JH0002 and JH1102 when cultured under differing temperatures, CO_2 levels, pH or glucose concentrations (Fig. S4). We had previously found that the H391D/A mutations altered the regulation of ChoP by MgaSpn in response to glucose, suggesting a link to PTS

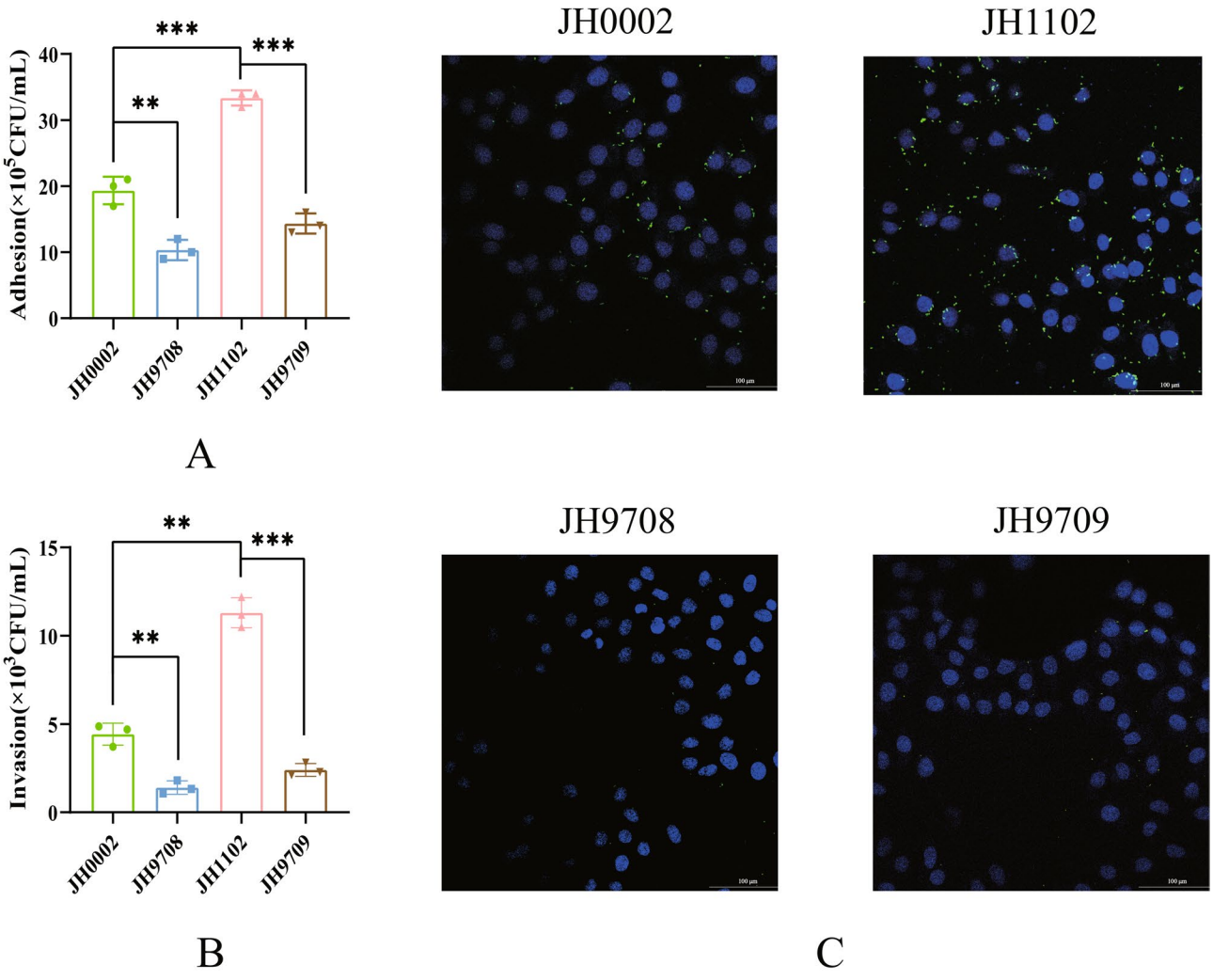


Fig. 4 Infection of A549 epithelial cells. **A**, **B**, and **C**. A549 cells were infected with the unencapsulated strains JH0002, JH1102, JH9708 and JH9709 at an MOI of 100. **A**. Adherent and **B.** invasive bacteria were counted by plating from serial dilutions. ***$P < 0.001$, **$P < 0.01$, and not significant (NS) as analyzed by unpaired two-tailed Student's t-test. **C.** Photomicrographs of bacteria adherent to A549 epithelial cells using confocal microscopy

phosphorylation [51]. We used our luciferase reporter strains to assess P_{pcpA} activity at differing glucose concentrations. However, there were no significant differences in P_{pcpA} activity at differing glucose concentrations (Fig. S4). Since host iron levels can directly influence virulence factor production in many bacterial pathogens [52–54], we found that PcpA expression was inversely related to iron level in the medium. In particular, in strain JH0002, PcpA expression decreased with increasing iron concentration, but there was no significantly difference of PcpA expression in strain JH1102 (Fig. 6). These results indicated that iron concentration can alter the regulation of PcpA expression via MgaSpn.

Discussion

The premise of bacterial survival is to adapt to the environmental conditions of different niches, such as the host nasopharynx, lung, blood and brain [55, 56]. Therefore, global transcriptional regulators that respond to changes in the external environment are critical during infection. MgaSpn is such a regulator. We previously found that MgaSpn could negatively regulate capsular and phosphocholine-related genes *via* direct promoter binding to the *cps* and *lic*1 (a gene related to phosphocholine biosynthesis) promoters. Interestingly, deletion of the *mgaSpn* gene resulted in an increase of virulence [36]. However, MgaSpn possesses a wide range of regulatory targets in a complex virulence regulatory network. The present study was focused on a genome wide screening of target genes regulated by MgaSpn to explore additional MgaSpn functions.

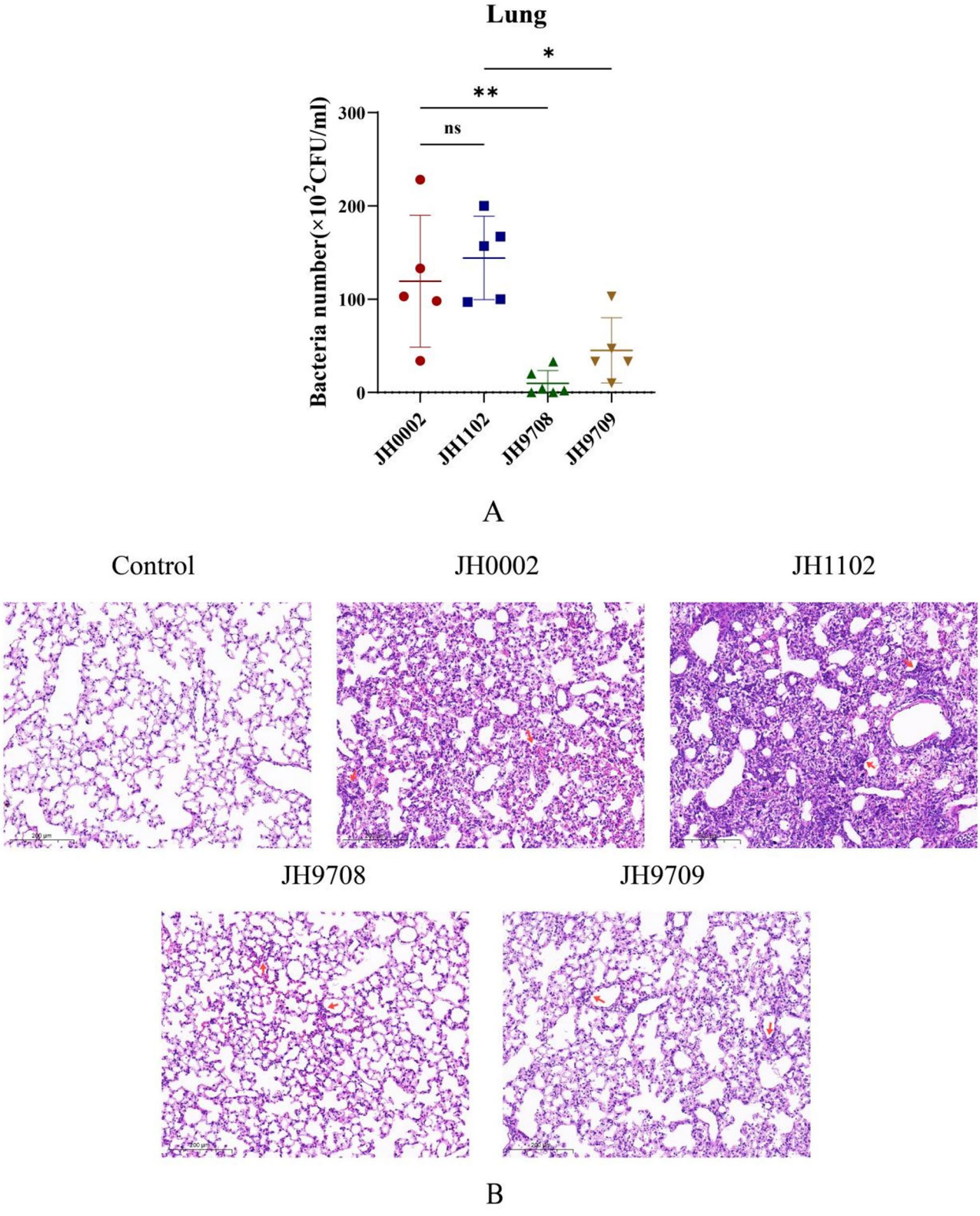


Fig. 5 The lung colonization and infection of *S. pneumoniae*. **A**. Lung colonization indicated by CFU in lung homogenates. ***$P < 0.001$, **$P < 0.01$ and not significant (NS) as analyzed by unpaired two-tailed Student's *t*-test. **B**. Photomicrographs of mouse lung histopathology

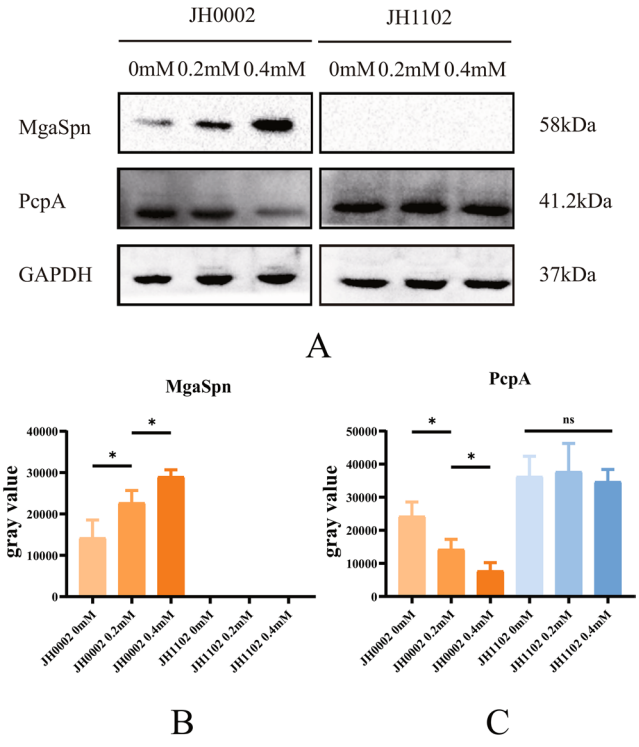


Fig. 6 Detection of PcpA and MgaSpn expression under different iron levels. **A**. Western blotting for MgaSpn and PcpA at the indicated iron levels in the medium. **B** and **C**. Densitometry values for protein bands of the accompanying blots. ***$P < 0.001$, **$P < 0.01$ and not significant (NS) as analyzed by unpaired two-tailed Student's t-test

In our previous studies, we constructed a *mgaSpn*-deficient strain and transcriptome sequencing results indicated that MgaSpn had a negative regulatory effect on some CBPs expression [40]. Subsequently, we screened out the CBP gene, *pcpA*, through qRT-PCR, luciferase reporter assays, and Western blots (Fig. 1). PcpA has been established as a virulence factor in murine models of pneumonia and sepsis, primarily facilitating adhesion to epithelial cells [48, 57]. While PcpA is known to be regulated by the Mn^{2+}-dependent regulator PsaR, the regulator CodY and the two-component signal transduction system TCS01 [31, 32, 58, 59], this study identifies MgaSpn as a novel regulator of PcpA. The EMSA demonstrated that MgaSpn specifically binds to P_{pcpA}, and the DNase I footprinting analysis identified potential binding sites (Fig. 2). Analysis of mutating two binding sites in the promoter region further showed that disrupting these sites restored adhesion to A549 cells (Fig. 3), implicating that both binding regions of MgaSpn and *pcp*A promoter region were functional binding sites.

Previous studies have indicated that PcpA, which contains leucine-rich repeats (LRRs), plays an important role in bacterial adhesion to nasopharyngeal and lung epithelial cells [46, 47, 60]. When *pcp*A was deficient, the adhesion of *S. pneumoniae* to epithelial cells was decreased [48], which was consistent with our findings (Fig. 4). Previous studies have also indicated that adhesion and invasion of *mgaSpn*-deficient strain to A549 cells was increased compared to WT strain [36]. Based on the increase expression of *pcpA* observed in the *mgaSpn*-deficient strain, we hypothesize that the *mgaSpn* and *pcp*A double deletion mutant strain has decreased adhesion and invasion ability to epithelial cells. The results are consistent with the expectations (Fig. 4). Taken together, these results indicated that MgaSpn can affect the adhesion ability of *S. pneumoniae* by downregulating PcpA.

Previous studies have shown that PcpA promotes lung infection, and the ability of lung colonization is significantly decreased after *pcpA* deficient [49, 61], so we further performed in vivo assays to observe bacterial colonization ability in mice. Compared with WT, the significantly increased nasopharyngeal colonization ability of *mgaSpn*-deficient strain was likely through PcpA-independent pathways (Fig. S3). This may be attributed to the inhibition of PcpA expression in the high Mn^{2+} environment of the nasopharyngeal environment, as being suggested previously [49]. We hypothesized that MgaSpn not only regulates PcpA but also influences other virulence factors, such as the pneumococcal hemolysin Ply, the biofilm-regulating peptide BriC, and the quorum-sensing system pheromone BlpC, thereby affecting the nasopharyngeal colonization of *S. pneumoniae* (Table S1) [10, 62–64], indicating the need for further studies. Additionally, we observed that the increased lung colonization

ability of JH1102, compared to the WT, was not statistically significant (Fig. 5A). We considered that colonization ability of *S. pneumoniae* is also related to expression of the pneumococcal hemolysin Ply, choline-binding proteins, lipoproteins and CPS [8, 65]. However, the lung infection of *pcp*A and *mgaSpn* double-deletion strain was significantly reduced (Fig. 5). These findings suggest that MgaSpn can decrease lung infection through negative regulation of PcpA expression.

MgaSpn is orthologous to Mga in *S. pyogenes* that can regulate virulence factors though environmental sensing of osmotic pressure, temperature, iron and carbon dioxide [33, 66–68]. We cultured JH0002 and JH1102 strains under different conditions; only iron levels affected the level of PcpA, and this regulation was signiffcantly weakened following MgaSpn deffciency (Fig. S4 and Fig. 6). This indicated that the iron regulation of PcpA expression relies on the presence of MgaSpn and therefore implicates the latter in sensing the iron levels encountered in the host environment. This could involve indirect crosstalk with canonical iron regulators like PsaR or direct sensing via structural iron-binding motifs, though the precise pathway remains unresolved. Meanwhile, Mn^{2+} and Zn^{2+} antagonistically regulate PcpA through PsaR [59], raising the possibility that MgaSpn integrates multiple metal signals. These interesting questions requires further experimentation in future research.

While we have indicated that MgaSpn plays a role in sensing iron levels, there are notable limitations to consider. Firstly, the iron-sensing mechanism remains speculative, and structural studies are necessary to identify the metal-binding domains of MgaSpn. Secondly, in vitro assays may not fully replicate host-pathogen interactions, particularly in nasopharyngeal niches where Mn^{2+} represses *pcpA* [49]. Future work should validate these findings in *vivo* assays. Thirdly, MgaSpn, as a global transcriptional regulator, may have other potiential regulatory targets that may synergize with PcpA in immune evasion.

PcpA is a known virulence factor. We found for the first time that MgaSpn, a global transcriptional regulator, affects *S. pneumoniae* virulence by regulating PcpA. We also found that MgaSpn could sense iron concentration and regulate PcpA accordingly. This study is of great significance for understanding the virulence regulation and pathogenic mechanism of *S. pneumoniae*, providing possible strategies for reducing *S. pneumoniae* infections and theoretical guidance for drug design.

Abbreviations

CPS	Capsular polysaccharides
ChoP	Phorylcholine
EMSA	Electrophoretic mobility shift assay
GAS	Streptococcus pyogenes
S.pneumoniae	Streptococcus pneumoniae
TCS	Two-component signal transduction systems
CBPs	Choline binding proteins
Sm	Streptomycin
Kan	Kanamycin
Erm	Erythromycin
Chl	Chloramphenicol
Spec	Spectinomycin

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-04047-8.

Supplementary Material 1

Supplementary Material 2: Additional file 1: Figure S1. Construction of ***pcpA*** mutant strains. A: Schematic diagram of ***Janus cassette*** principle. B: Agarose electrophoresis strips of JH9706、JH9707、JH9708 and JH9709. C: Determinutesation of ***pcpA*** expression in JH1900、JH9706、JH9707、JH0002、JH9708 and JH9709 by quantitative PCR. mRNA levels are expressed relative to that of ***gyrB***. Additional file 2. Figure S2. Growth curves of JH0002 (circles), JH1102 (squares), JH9708 (positive triangle) and JH9709 (inverted triangle) growing in C+Y medium. The growth rates (h^{-1}) for each strain are indicated in the graph. Additional file 3: Figure S3. The bacterial ability of nasal colonization. Nasopharyngeal colonization of mice (n = 6 per group) infected with the indicated strains at 1×10^8 CFU intranasally in nasopharyngeal lavage fluid collected 12 h postinfection. ***P < 0.001, **P < 0.01, and not significant (NS) as analyzed by unpaired two-tailed Student's ***t***-test. Additional file 4: Figure S4. Expression of MgaSpn under different environmental conditions. A: Detection of MgaSpn content at different pH by Western blot. B: Detection of MgaSpn content at different temperatures by Western blot. C: Detection of MgaSpn content at different CO_2 concentrations by Western blot. D: Detection of MgaSpn content at different glucose concentrations by Western blot. E: Detection of P***pcpA*** content at different glucose concentrations by Luciferase reporter assay. ***P < 0.001, **P < 0.01, and not significant (NS) as analyzed by unpaired two-tailed Student's ***t***-test

Supplementary Material 3: Additional file 5. Table S1. Differential gene expression detected by transcriptome sequencing[a]

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Not applicable.

Author contributions
Yuqiang Zheng, Xuemei Zhang, and Yibing Yin conceived and designed the experiments. Shuhui Wang, Tianyi Xu, Ye Tao and Li Lei performed the experiments. Shuhui Wang analyzed the data and wrote the paper. Tianyi Xu, Ye Tao and Li Lei contributed the reagents, materials, and analysis tools. All authors read and approved the final manuscript.

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Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
All animal experiments described in this study were approved by the Animal Care and Use Committee of Chongqing Medical University (reference number: IACUC-CQMU-2024-0512) and were performed in strict accordance with the regulations of the Guide for the Care and Use of Laboratory Animals. We euthanized the mice using cervicaldislocation. With correct manipulation techniques, the mice were killed by rapidly dislodging their cervical vertebrae from the spine and mice were anesthetized before execution. Mice were anesthetized by intraperitoneal injection of 1% sodium pentobarbital at a dose of 50 mg/kg.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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