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Screening and Identification of ClpE Interaction Proteins in *Streptococcus pneumoniae* by a Bacterial Two-Hybrid System and Co-immunoprecipitation[§]

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Hsp100/Clp proteins have crucial functions in the protein quality control, stress tolerance, and virulence of many pathogenic bacteria. ClpE is an important virulence factor involved in adherence and invasion in *Streptococcus pneumoniae*. To explore the underlying mechanism, we screened ClpE interaction proteins using a bacterial two-hybrid system and co-immunoprecipitation. We used ClpE as bait and constructed the pBT-ClpE bait plasmid for two-hybrid screening. Then, we constructed *ClpE::GFP* fusion for co-immunoprecipitation screening using anti-GFP monoclonal antibody. We obtained eight potential ClpE interaction proteins, including carbamoyl-phosphate synthase, pyruvate oxidase (SpxB), phosphoenolpyruvate-protein phosphotransferase, aminopeptidase N (pepN), L-lactate dehydrogenase, ribosomal protein S4, sensor histidine kinase (SPD_2019), and FtsW (a cell division protein). FtsW, SpxB, pepN, and SPD_2019 were confirmed to interact with ClpE using Bacterial Two-hybrid or Co-immunoprecipitation. Morphologic observations found that Δ*clpE* strain existed in abnormal division. β-Galactosidase activity assay suggested that ClpE contributed to the degradation of FtsW. Furthermore, FtsW could be induced by heat shock. The results suggested that ClpE might affect cell division by regulating the level of FtsW. These data may provide new insights in studying the role of ClpE in *S. pneumoniae*.

Keywords: *Streptococcus pneumoniae*, ClpE, interaction proteins, FtsW, bacterial two-hybrid, co-immunoprecipitation

Introduction

The HSP100/ClpATPase family is composed of highly conserved and widespread heat shock proteins in bacteria. Aside from their chaperone function, they can also form ATP-dependent protease complexes with the peptidase ClpP for the proteolysis of specific cellular substrates (Schirmer *et al*., 1996; Kress *et al*., 2009).

These family proteins are essential in bacterial infection. They are part of the protein quality control machinery. They can recognize, unfold, and translocate substrate proteins for degradation by ClpP (Kirstein *et al*., 2006). Another important activity of the HSP100/Clp protein family is transcription regulation. They can affect the activity of some transcription factors, making them conducive to the growth and dissemination of bacteria in the host, causing various infectious diseases (Ingmer and Brøndsted, 2009).

Similar to most members of the HSP100/Clp family, ClpE could affect the growth, proliferation, and virulence of bacteria. In *Listeria monocytogenes*, ClpE is required for prolonged survival at 42°C and is involved in virulence and cell division (Nair *et al*., 1999). ClpE can also help *L. monocytogenes* adapt to the stressful environment of the gastrointestinal tract (Jiang *et al*., 2010). In low-GC Gram-positive bacteria lacking the McsA/McsB complex, ClpE senses and responds to oxidative stress. It is involved in the inactivation of the transcription factor CtsR (Elsholz *et al*., 2011).

Our previous studies found that the Δ*clpE* strain shows reduced virulence and adherence ability in *S. pneumoniae* and that ClpE could modulate the expression of important virulence determinants and metabolism-related factors (Zhang *et al*., 2009). However, the underlying regulation mechanism remains unknown. Finding ClpE interaction proteins will help us reveal the molecular mechanisms of the role of ClpE in the growth and pathogenicity of *S. pneumoniae*.

In this report, eight ClpE interaction proteins were identified using a bacterial two-hybrid system and co-immunoprecipitation (IP). Among them, FtsW, SpxB, pepN, and SPD_2019 were confirmed to interact with ClpE. Based on the found that the Δ*clpE* strain existed in abnormal division through morphologic observations, β-galactosidase activity assay suggested that ClpE contributed to the degradation of FtsW. These findings suggested that ClpE can affect cell division by regulating the level of FtsW.

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Materials and Methods

Bacterial strains and plasmids

S. pneumoniae D39 was used as the parental strain for all genetic manipulations. *Escherichia coli* XL1-Blue MRF' Kan, BacterioMatch II two-hybrid system reporter strains derived from *E. coli* XL1-Blue MRF', and BacterioMatch II two-hybrid system vector kit (pBT, pTRG, pBT-LGF2, pTRG-Gal11^{P}) were purchased from Agilent.

Cloning and purification of recombinant ClpE-His$_6$ in *E. coli* and antisera preparation

The *clpE* gene was amplified by polymerase chain reaction (PCR) using D39 genomic DNA as a template with primers ClpE1 and ClpE2 (Table 1) containing *Eco*RI and *Sal*I restriction enzyme sites. The PCR product was digested by the two enzymes and cloned into pET28a to generate the plasmid pET28a-ClpE.

To purify ClpE-His$_6$, the recombinant plasmid pET28a-ClpE was transformed into *E. coli* BL21. The protein was then induced by 0.3 mM isopropyl-β-D-thiogalactoside and purified using a nickel-nitrilotriacetic acid column (Millipore, USA) according to the manufacturer's instructions. Polyclonal anti-clpE was raised in mice or rabbit through routine immunogenic procedures (Wu *et al.*, 2010).

Bacterial two-hybrid library construction and screening

The bait plasmid pBT-ClpE was constructed by cloning *clpE* from D39 into pBT. The *clpE* gene (2,259 bp) was amplified by PCR using primers ClpE3 and ClpE4 (Table 1) from the genomic DNA of *S. pneumoniae* D39. After restriction digestion with *Eco*RI and *Not*I, the PCR fragment was cloned into vector pBT. The genomic DNA of *S. pneumoniae* D39 was digested with *Sau3A*I into approximately 500 bp to 2,000 bp fragments and then cloned into pTRG digested with *Bam*HI to produce a library of pTRG-DNA fragments. Then, the bait plasmid pBT-clpE and pTRG-DNA fragments were co-transformed into the bacterial two-hybrid reporter strain. The bacterial two-hybrid reporter strain was assayed for growth on either a nonselective screening medium (no 3-amino-1,2,4-triazole or 3-AT) plate (NSSM) or a selective screening medium (5 mM 3-AT) plate (SSM). The colonies that exhibited growth in an SSM plate were picked for PCR analysis and sequencing.

Table 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description or sequence	Source or use
Streptococcus pneumonia		
Strain		
D39	Serotype 2, virulent strain, NCTC 7466	NCTC
D39Δ*clpE*	erm^{r}	Zhang *et al.* (2009)
Plasmid		
pET28a	kan^{r}	protein expression
pBT	3.2 kb, Cm^{r}	Agilent
pTRG	4.4 kb, tet^{r}	Agilent
pBT-LGF2	positive control plasmid	Agilent
pTRG-Gal11^{P}	positive control plasmid	Agilent
pAE03	spec, ery, promoter-less gfp+ (C-terminal fusions)	J.W. Veening (Newcastle Univ.)
pEVP3	Nonreplicative vector (Cm^{r} lacZ)	D.A. Morrison (Univ. of Illinois at Chicago)
Primer (5′→3′)		
ClpE1	GCCGC*GAATTC*ATGCTTTGTCAAAACTGTAAAATTA	
ClpE2	GCCGC*GTCGAC*TTATTTTTCTTTTTCAGAACTTTTA	
ClpE3	AAGAAT*GCGGCCGC*AATGCTTTGTCAAAACT	
ClpE4	GCTCCCG*GAATTC*TTATTTTTCTTTTTCAG	
ClpE5	GCGCAGT*GAATTC*GCAACTCTTTCCAATCGCTACATCC	
ClpE6	ATAAGAAT*GCGGCCGC*ATTTTTCTTTTTCAGAAC	
ClpP1	ATAAGAAT*GCGGCCGC*AATGATTCCTGTAGTTATTGAACAAA	
ClpP2	GCGCAGT*GAATTC*TTAGTTCAATGAATTGTTGGCCATA	
FtsW1	GCTCCCG*GAATTC*AGATGAAGATTAGTAAGAGGCACTTAT	
FtsW2	CCG*CTCGAG*CTACTTCAACAGAAGGTTCATTGGT	
FtsW3	GCGCAGT*GAATTC*AACCATTCTGGCGCTCGTATCTGCC	
FtsW4	ATAAGAAT*GCGGCCGC*ACTTCAACAGAAGGTTCATTGGT	
FtsW5	G*AAGATCT*ATGAAGATTAGTAAGAGGCACTTAT	
FtsW6	TCC*CCCGGG*CTTCAACAGAAGGTTCATTGGT	
2019F	GCTCCCG*GAATTC*AGATGATAAAAAATCCTAAATTATTAA	
2019R	CCG*CTCGAG*CTACAAGCTAATCTTAAATTCCATA	
pepN1	CGC*GGATCC*ATGCAAGCAGTTGAACATTTTATTA	
pepN2	CCG*GAATTC*TTATGCATTTCCGTATTGAAGAACA	
SpxB1	CCG*GAATTC*AGATGACTCAAGGGAAAATTACTGCAT	
SpxB2	CCG*CTCGAG*TTATTTAATTGCGCGTGATTGCAAT	

Note, The italics portions refer to the restriction enzyme sites.

Construction of *clpE::GFP* fusion and *ftsW::GFP* fusion

To construct the plasmid pAE03-ClpE or pAE03-FtsW carrying the gfp$^+$ fused to the *S. pneumoniae clpE* gene or *ftsW* gene, the *clpE* gene or *ftsW* gene was amplified by PCR using D39 genomic DNA as a template with primers ClpE5 and ClpE6 or primers FtsW3 and FtsW4 containing *Eco*RI and *Not*I restriction enzyme sites. The primers used are listed in Table 1. The PCR product was digested by the two enzymes and cloned into pAE03 to generate the plasmids pAE03-ClpE and pAE03-FtsW.

To construct the plasmid pEVP3-FtsW carrying the lacZ$^+$ fused to the *S. pneumoniae ftsW* gene, the *ftsW* gene was amplified by PCR using D39 genomic DNA as a template with primers FtsW5 and FtsW6 (Table 1) containing *Bgl*II and *Sma*I restriction enzyme sites. The PCR product was digested by the two enzymes and cloned into pEVP3 to generate the plasmid pEVP3-FtsW.

The plasmids pAE03-ClpE and pAE03-FtsW were transformed into the *S. pneumoniae* D39 strain, and the plasmid pEVP3-FtsW was transformed into the D39 and D39Δ*clpE* strains as described previously (Zhang *et al*., 2009).

Co-IP screening and identification

The lysates of D39 and D39 (*clpE::GFP*) or D39 (*ftsW::GFP*) were precleared with 600 μl protein G-anchored agarose beads at 4°C for 1 h with gentle agitation. Subsequently, 3 mg of the precleared protein lysates were incubated with anti-GFP monoclonal antibody (clontech) and 600 μl protein G-anchored agarose beads at 4°C overnight with gentle agitation. The beads were collected and washed five times with lysis buffer. Immunoprecipitated proteins were detected from the beads by adding 50 μl of 5× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer and boiling for 5 min. The supernatants were analyzed by SDS-PAGE. The difference bands between the D39 and D39 (*clpE::GFP*) strains were sent to Beijing Genomics Institute (BGI, China) for Matrix-assisted laser desorption ionization-time of flight mass spectrometry MALDI TOF/TOF MS (Bruker Daltonics, Germany) analysis. The MS and MS/MS spectra were combined using the BioTools 3.0 software and search with Mascot software 2.3.02 against the NCBI *S. pneumoniae* protein database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=protein&term=txid1313%5BOrganism%5D&cmd=search). Protein candidates provided by this combined PMF and MS/MS search were considered as valid when the global Mascot score was greater than *S. pneumoniae* protein database with a significance level of e-value<0.05. The procedures were performed as described previously (Zi *et al*., 2012). The supernatants of D39 and D39 (*ftsW::GFP*) were analyzed by western blot with anti-ClpE polyclonal antibody.

Western blot

SDS-PAGE (10% polyacrylamide gel) was performed as described previously (Tran *et al*., 2011). A bacterial pellet was collected by centrifugation at 13,000×g for 2 min and washed twice with phosphate-buffered saline (PBS; pH 7.4). Then, the cells were sonicated for 3 min, and cell debris was removed by centrifugation at 13,000×g for 15 min. The supernatant was collected and used for further studies. The proteins (10 μg) were transfected onto polyvinylidene difluoride membranes, blocked with 5% skim milk (Difco), and then probed with a 1:500 dilution of anti-GFP (Beyotime), anti-ClpE, or anti-codY polyclonal antibody (prepared by our own lab). The secondary antibody was a 1:5,000 dilution of goat

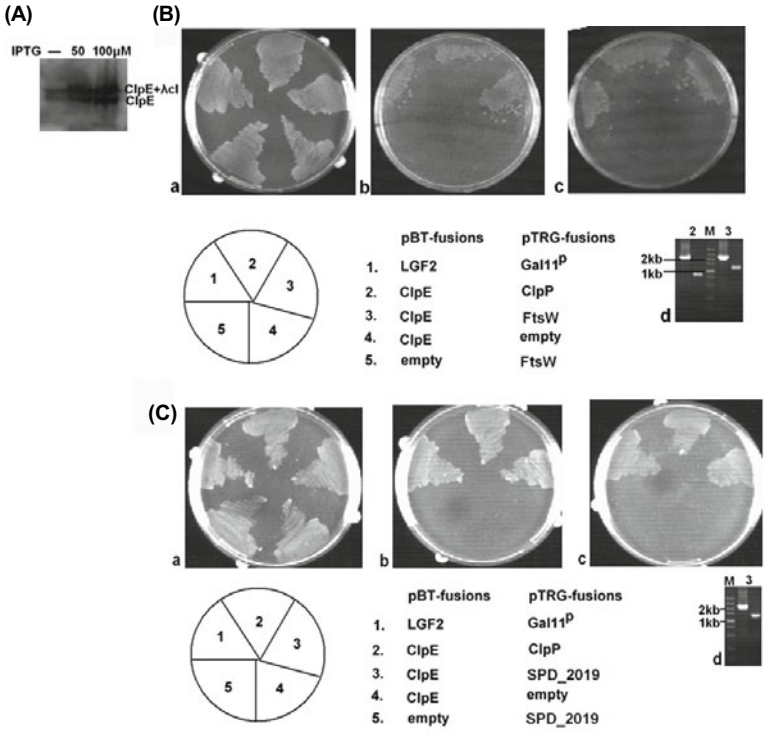

Fig. 1. ClpE interacted with FtsW or SPD_2019. (A) Western blot analysis was carried out with anti-ClpE polyclonal serum to verify the expression of pBT-ClpE. (B, C) The bacterial two-hybrid reporter strain expressing different fusion proteins was assayed for growth on an NSSM plate (a), or SSM plate (b), or DSSM plate (c). The interaction between pBT- LGF2 and pTRG-Gal11^P or pBT-ClpE and pTRG-ClpP was used as positive control. PCR for identifying the results of co-transformation of pBT-ClpE and pTRG-ClpP or pTRG-FtsW, pBT-ClpE and pTRG-SPD_2019 (d). M, DNA Marker.

anti-mouse or goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (HRP; Promega). Chemiluminescence was used to detect HRP-conjugated secondary antibody used in western blots.

Bacterial two-hybrid identification procedure

The plasmids pTRG-ClpP, pTRG-FtsW, pTRG-SPD_2019, pTRG-pepN, and pTRG-SpxB were generated by amplifying the *clpP* gene (591 bp) with primers ClpP1 and ClpP2, the *ftsW* gene (1,230 bp) with primers FtsW1 and FtsW2, the *spd2019* gene (1,332 bp) with primers 2019F and 2019R, the *pepN* gene (2,547 bp) with primers pepN1 and pepN2, or the *spxB* gene (1,776 bp) with primers SpxB1 and SpxB2, followed by insertion into the plasmid pTRG. The primers used are listed in Table 1. The bait plasmids pBT-clpE and pTRG-ClpP, pTRG-FtsW, pTRG-SPD_2019, pTRG-pepN, or pTRG-SpxB was co-transformed into the bacterial two-hybrid reporter strain. The bacterial two-hybrid reporter strain expressing different fusion proteins was assayed for growth on an NSSM, SSM, or DSSM (5 mM 3-amino-1,2,4-triazole (3-AT) + streptomycin) plate. The preparation of NSSM, SSM, or DSSM plates was according to the instruction of Bacterial two-hybrid kit from Agilent.

β-Galactosidase assay

S. pneumoniae D39 and D39Δ*clpE* containing ftsW::LacZ fusion were grown in C+Y until OD_{600}=0.5. Gentamicin used to stop translational elongation was added to the cultures to a final concentration of 50 μg/ml (Purcell *et al.*, 2012). Samples (2 ml) were collected at subsequent time points of 10, 20, 30, and 50 min at 37°C or 50°C. The samples were resuspended in 100 μl of PBS-0.1% Triton X-100 and then lysed for 15 min at 37°C (Lee and Morrison, 1999). The β-galactosidase assay with o-nitrophenyl-β-D-galactopyranoside as a substrate was conducted following the manufacturer's protocol (Beytotime). β-Galactosidase activity was given in optical density at OD_{405}.

Statistical analysis

Statistical analysis was performed using unpaired Student's t-test (two-tailed). All statistical analyses were performed using GraphPad Prism version 5.0.

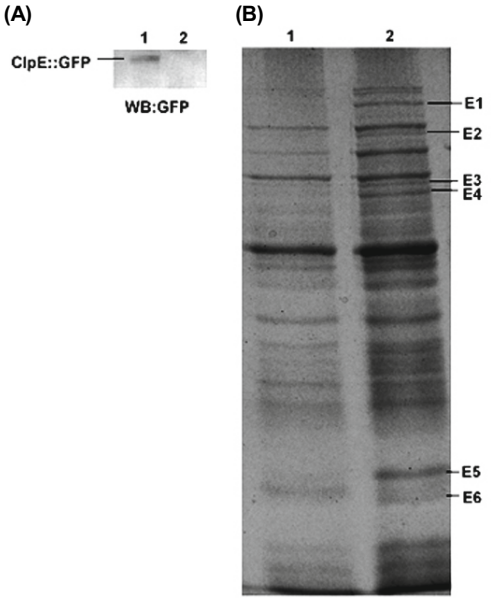

Fig. 2. Different bands between D39 and D39(*ClpE::GFP*) identified by co-IP. (A) The *clpE::GFP* fusion could be normally expressed in D39. Lanes: 1, D39(*ClpE::GFP*); 2, D39. (B) SDS-PAGE showed the different bands of co-IP samples from the lysate of D39 and D39(*ClpE::GFP*). Lanes: 1, D39; 2, D39(*ClpE::GFP*).

Results

Bacterial two-hybrid screening

Full-length *clpE* was amplified using PCR, cloned into the expression vector pET28a, and then over-expressed in *E. coli* BL21 (DE3). The purified protein ClpE is shown in Supplementary data Fig. S1. Then, full-length *clpE* was inserted into pBT to produce ClpE-λcI fusion. Western blot analysis revealed the expression of the ClpE-λcI fusion protein using polyclonal anti-ClpE (Fig. 1A). The fusion protein was unstable and inclined to break down, producing ClpE and λcI proteins. The same situation existed in the AMPK α2-λcI fusion protein (Fu and Gao, 2009).

Before the screening, we successfully established a bacterial two-hybrid method using the empty plasmid as a negative control and the pBT-LGF2 and pTRG-Gal11^{P} plasmids in

Table 2. The results of MALDI TOF/TOF MS analysis of IP screening experiments

Sample name	Protein ID	Protein mass	Protein score	Significance score	Isoelectric point	Coverage rate (%)	Description
E1	gi|116516501|ref|YP_816600.1|	116498.8	174	70	4.52	22.12	*carB* gene product
E2	gi|116515610|ref|YP_816194.1|	95404.98	188	70	4.54	23.00	*pepN* gene product
E3	gi|395589749|gb|EJG50065.1|	64734.97	86.4	70	4.44	32.88	*ptsI* gene product
E4	gi|116516119|ref|YP_816132.1|	65327.13	386	70	4.81	34.52	*spxB* gene product
E5	gi|298238379|gb|ADI69510.1|	35897.6	296	70	4.97	26.81	*l-ldh* gene product
E6	gi|116516581|ref|YP_815552.1|	23014.41	294	70	10.73	65.52	*rpsD* gene product

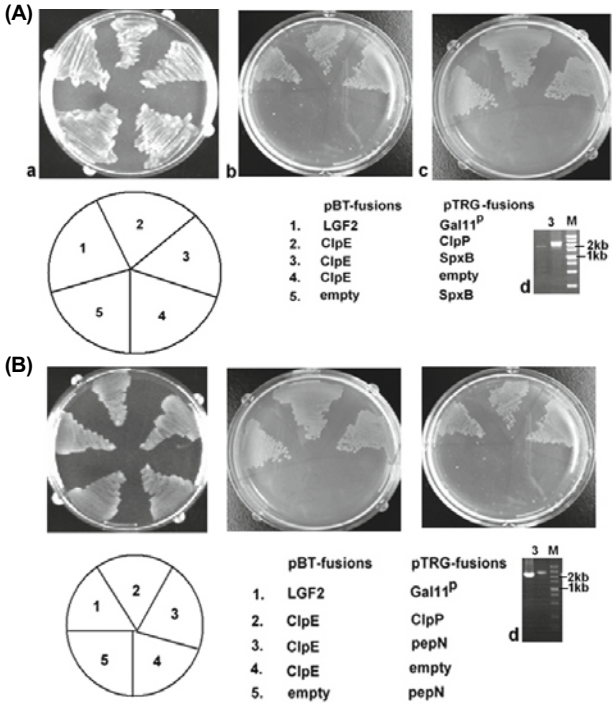

Fig. 3. Identifying the interaction of ClpE and spxB (A) or pepN (B). The plasmid pBT and pTRG fusion with different genes were co-transformed into bacteria two-hybrid reporter strains. Their growth was assayed on NSSM plate (a), or SSM plate (b), or DSSM plate (c). PCR for identifying the results of co-transformation of pBT-ClpE and pTRG-pepN (d).

the kit and ClpP, which is reported to interact with ClpE (Kress *et al*., 2009), as a positive control.

Subsequently, we performed interaction protein screening. When constructing the library, we only obtained 800 clones containing the plasmid pTRG connected with different sizes of DNA fragments because of low transformation efficiency. These mixing plasmids and the bait plasmid pBT-ClpE were co-transformed into the bacterial two-hybrid reporter strain. The physical interaction of ClpE and potential interaction proteins was monitored by the activation of the *HIS3* reporter gene as indicated by growth in the absence or presence of 3-AT.

We obtained 25 clones that could grow in an SSM (5 mM 3-AT) plate after screening. Given the randomness of Sau3AI digestion, we only obtained two potential ClpE interaction proteins (SPD-2019 and FtsW) with the correct expression in the plasmid pTRG after PCR identification and sequencing. Then, we cloned full-length SPD_2019 or FtsW into pTRG for further validation of the interaction between ClpE and FtsW or SPD_2019. Their interaction was also verified using the *aadA* gene, which confers streptomycin resistance, as a secondary reporter (Figs. 1B and 1C).

Co-IP screening

Taking into account the limitations of the bacterial two-hybrid method, such as small library capacity and frame shift caused by the random digestion of *Sau3A*I, we performed the co-IP screening assay.

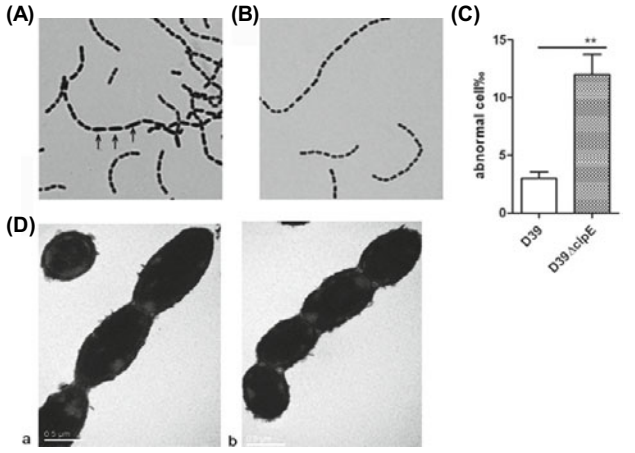

Fig. 4. Effects of ClpE on the cell division of *S. pneumoniae* after 30 min heat stress at 50°C. The morphology of D39 and D39Δ*clpE* were observed under oil immersion objective after Gram stain. (A) Abnormal division was shown in D39Δ*clpE* (arrow). (B) D39: normal control. (C) The proportion of abnormal cells in D39 and D39Δ*clpE* according to (A) and B). (D) The morphology of D39Δ*clpE* and D39 under electron microscope (40,000×). a, D39Δ*clpE*; b, D39.

The plasmid pAE03-ClpE was transformed into *S. pneumoniae* D39. The C-terminal fusion protein GFP of ClpE showed normal expression after western blot detection using anti-GFP polyclonal antibody (Fig. 2A). Furthermore, we performed co-IP for D39 and D39 (*ClpE::GFP*) using anti-GFP monoclonal antibody. The difference bands between these two strains were used for MS analysis. We obtained six potential ClpE interaction proteins: CarB, SpxB, PtsI, PepN, Ldh, and RpsD (Table 2 and Fig. 2B).

Verification of the interaction between ClpE and the potential interaction proteins

Previous studies reported that PepN and SpxB are related to virulence (Spellerberg *et al*., 1996; Hava and Camilli, 2002). Hence, we used both genes for further studies. We cloned the gene *pepN* or *spxB* to pTRG to validate their interaction with ClpE using the bacterial two-hybrid assay. The results showed that these two proteins could interact with ClpE (Fig. 3).

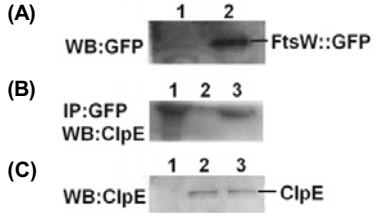

Fig. 5. Interaction of ClpE with FtsW was verified by co-IP. (A) The cell lysate of D39 and D39(*ftsW::GFP*) were detected for WB using anti-GFP antibody. Lanes: 1, D39; 2, D39(*ftsW::GFP*). (B) The immunoprecipitated products were used for Western blotting (WB) using anti-ClpE polyclonal antibody. Lanes: 1, The rClpE protein was used as a positive control; 2, D39; 3, D39(*ftsW::GFP*). (C) The cell lysate of D39 and D39 (*ftsW::GFP*) were detected for WB using anti-ClpE polyclonal antibody before co-IP. Lanes: 1, negative control (irrelevant protein); 2, D39; 3, D39 (*ftsW::GFP*).

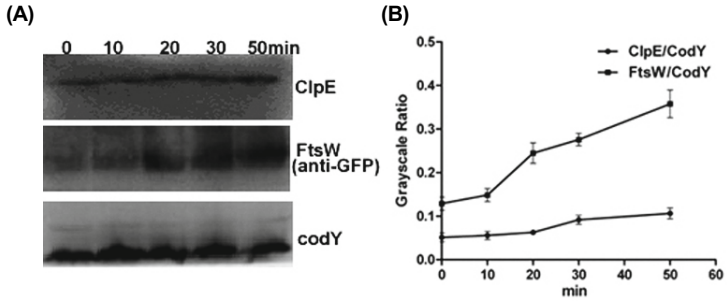

Fig. 6. The expression of FtsW was increased after heat shock. (A) D39(*ftsW::GFP*) were cultured at 37°C until OD_{600}=0.5. The cell lysates were collected at 0, 10, 20, 30, 50 min after heat shock at 50°C, which were used for Western blotting (WB) using anti-ClpE or anti-GFP antibody. The CodY was used as an internal control. (B) Grayscale analysis of the expression of ClpE and FtsW using Quantity one.

Interaction between ClpE and FtsW was verified using co-IP

The growth of D39Δ*clpE* strain was much slower than that of the wild-type strain (Zhang *et al.*, 2009). Morphologic observations found that the Δ*clpE* strain existed in abnormal division after heat stress at 50°C (Figs. 4A, 4B, 4C, and Supplementary data Fig. S2). The length of D39Δ*clpE* was also much longer than that of the wild-type D39 under electron microscope (Fig. 4D). This might because that it was difficult for D39Δ*clpE* to go through normal cell division so that we could easily capture dividing cells. FtsW, an essential membrane protein involved in pneumococcal cell division, has 10 transmembrane segments in *S. pneumoniae* (Gérard *et al.*, 2002). Thus, we used FtsW for further studies. To confirm further the result of *in vivo* interaction, the co-IP assay was undertaken. Considering that FtsW is a membrane protein, we could not obtain the recombinant FtsW. Thus, we constructed the *ftsW::GFP* fusion, which was transformed into *S. pneumoniae* D39. The *ftsW::GFP* fusion could be normally expressed in D39 (Fig. 5A). The cell lysates of the D39 WT and D39 (*ftsW::GFP*) strains were immunoprecipitated with anti-GFP antibody and reprobed with anti-ClpE antibody. The IP samples from the strain expressing *ftsW::GFP* contained one prominent band absent in the control samples of D39 (Fig. 5B). The band at the same position of the positive control should be ClpE. Their cell lysates confirmed that they all contained ClpE before the IP assay (Fig. 5C). The result showed that FtsW could be co-immunoprecipitated with ClpE. These data were in agreement with the bacterial two-hybrid result that FtsW could interact with ClpE.

FtsW was regulated by heat shock

The transcription of ClpE was induced by heat shock (Robertson *et al.*, 2002). We analyzed the relationship between FtsW and heat shock. The result suggested that the expression of ClpE was elevated after being induced for 50 min at 50°C. This finding is consistent with the result of a previous report (Robertson *et al.*, 2002). The result also suggested that the expression of FtsW was increased after heat shock (Figs. 6A and 6B), indicating that FtsW was regulated by heat shock.

ClpE was involved in the degradation of FtsW

Given that ClpE could interact with FtsW, we speculated that it targets FtsW for ClpEP-catalyzed proteolysis. To test this speculation, the β-galactosidase assay was carried out. We constructed the *ftsW::LacZ* fusion, which was transformed into the *S. pneumoniae* D39 and D39Δ*clpE* strains. Their β-galactosidase activities were evaluated at 37°C after adding gentamicin. Adding gentamicin is expected the bacteria itself to stop the synthesis of FtsW Protein. Further we observed the amount changes of the protein FtsW within the time delay in the thermal stress. The results showed that β-galactosidase activity always maintains a high level at 37°C (Fig. 7A). After the strain D39 (*ftsW::LacZ*) exhibited growth at 50°C, β-galactosidase activity was rapidly reduced. This finding indicated that FtsW was degraded under heat shock (Fig. 7B). However, the decline in the β-galactosidase activity of D39Δ*clpE* (*ftsW::LacZ*) was slower under the same condition (Fig. 7B). This result suggested that ClpE contributed to the degradation of FtsW although it might not be the main

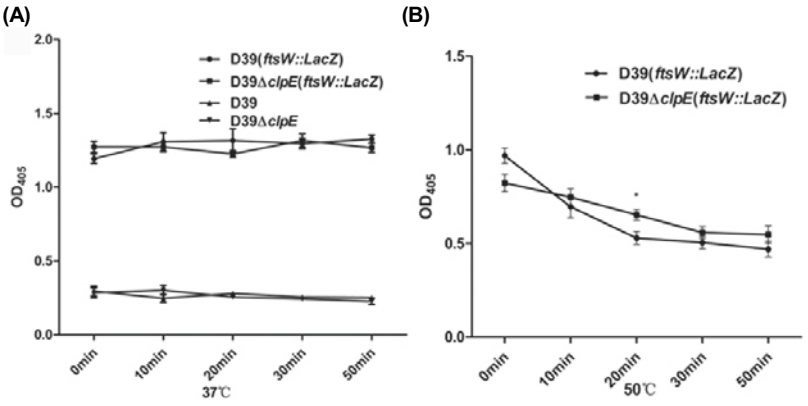

Fig. 7. Analysis of β-galactosidase activity of D39 and D39ΔclpE harboring a *ftsW::lacZ* reporter fusion. The β-galactosidase activity was measured at 0 min (prior to the addition of gentamicin) and 10–50 min after addition of gentamicin in D39 and D39Δ*clpE* grown at 37°C (A) and 50°C (B). β-Galactosidase activity was given in optical density at OD_{405} and was the mean of three independent experiments.

protein involved in this process.

Discussion

The HSP100 protein family is part of the protein quality control machinery involved in the degradation of some denatured or unfolded proteins. Many studies demonstrated that ClpE is essential for the thermotolerance and virulence of *S. pneumoniae* (Chastanet *et al*., 2001; Zhang *et al*., 2009). However, the underlying regulation mechanism is unclear. In this study, we attempted to identify the target binding proteins of ClpE to provide possible clues.

The BacterioMatch II two-hybrid system is an effective tool for studying protein–protein interactions. This method has unique advantages such as faster growth rate, screening library for transmembrane proteins, and small molecules and weak interaction proteins (Joung *et al*., 2000). Therefore, this method was selected for the screening of ClpE interaction proteins. However, we only found two ClpE interaction proteins, SPD_2019 and FtsW, using this method. Furthermore, we confirmed that FtsW could interact with ClpE using the co-IP assay. We also obtained many false positive clones with reverse insertion or frame shift in plasmid pTRG. This result can be attributed to the fact that we used random digested genomic DNA to construct libraries, which led to too much frameshift translation when these DNA fragments integrated into the plasmid pTRG. To overcome the frameshift mutation of the DNA library, a cDNA library of full-length mRNA might be a good choice. However, given the rapid and efficient metabolism of bacterial mRNA, its half-life was only a few minutes, and its 3′ terminal poly (A) tail was less, short, and unstable. Thus, the purification of the mRNA and reverse transcription with oligo (dT) to obtain full length fraction encountered more and greater difficulties (Hu *et al*., 2002). Another reason is that our library capacity was too small because of low transformation efficiency in the experimental conditions.

Taking into account the limitations of the bacterial two-hybrid method, we also performed the co-IP assay. This method is more effective in detecting protein interactions in the natural state. Through this method, we obtained six potential ClpE interaction proteins: CarB, SpxB, PtsI, PepN, Ldh, and RpsD.

The above results suggest that the proteins could maintain the native conformation in these two methods. Therefore, they are both suitable for the identification of interaction proteins. Compared with the bacterial two-hybrid system, the co-IP assay is more suitable in screening unknown interaction proteins. Our experimental results showed that the protein screened by these two methods did not overlap because the co-IP assay has shortcomings. That is, it could not detect low affinity and transient protein–protein interactions. Therefore, using more than one screening method to obtain more comprehensive target interaction proteins is better.

Through the validation of these two methods, we finally confirmed four ClpE interaction proteins. In *S. pneumoniae*, pyruvate oxidase is a determinant of virulence (Spellerberg *et al*., 1996) and is involved in spontaneous transformation (Bättig and Mühlemann, 2008) and nasopharyngeal colonization (Regev-Yochay *et al*., 2007). Our previous study also showed that the expression of SpxB was down-regulated in the Δ*clpE* strain using two-dimensional electrophoresis assay (Zhang *et al*., 2009). The gene *pepN* was also reported to be related to the bacterial virulence of *S. pneumoniae* (Hava and Camilli, 2002). SPD_2019, which is a putative sensor histidine kinase, might be part of the two-component system of *S. pneumoniae*.

FtsW, which is an essential membrane protein involved in pneumococcal cell division, has 10 transmembrane segments in *S. pneumoniae* (Gérard *et al*., 2002). FtsW could form an apparent ring co-localized with the septal high-molecular-weight penicillin-binding proteins throughout the cell cycle of wild-type cells (Morlot *et al*., 2004). We found that, similar to ClpE, the expression level of FtsW was increased after heat shock. Using the β-galactosidase assay, we proved that ClpE was involved in the degradation of FtsW. This result prompted that the heat shock protein ClpE can affect cell division by regulating the level of FtsW. *In vitro* degradation experiments could provide evidence of the involvement of ClpE in the degradation of FtsW. However, difficulties were encountered in obtaining recombinant FtsW because of its 10 transmembrane segments. Hence, *in vitro* degradation experiments were not performed.

In summary, the four interaction proteins were all related to the virulence or pathogenicity of the bacteria, indicating that clpE can affect the virulence of the bacteria by regulating these proteins. The precise physiological relevance of these interactions has yet to be investigated.

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