Sublethal β-lactam antibiotics induce PhpP phosphatase expression and StkP kinase phosphorylation in PBP-independent β-lactam antibiotic resistance of *Streptococcus pneumoniae*

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Running title: Pneumococcal PhpP as a PP2C type protein phosphatase

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Abstract

StkP and PhpP of *Streptococcus pneumoniae* have been confirmed to compose a signaling couple, in which the former is a serine/threonine (Ser/Thr) kinase while the latter was annotated as a phosphotase. StkP has been reported to be involved in penicillin-binding protein (PBP)-independent penicillin resistance of *S. pneumoniae*. However, the enzymatic characterization of PhpP and the role of PhpP in StkP-PhpP couple remain poorly understood. Here we showed that 1/4 minimal inhibitory concentration (MIC) of penicillin (PCN) or cefotaxime (CTX), the representatives of β-lactam antibiotics, could induce the expression of *stkP* and *phpP* genes and phosphorylation of StkP in PCN/CTX-sensitive strain ATCC6306 and three isolates of *S. pneumoniae* (MICs: 0.02-0.5 μg/ml). The product of *phpP* gene hydrolyzed PP2C type Ser/Thr phosphotase-specific RRA(pT)VA phosphopeptide substrate with the Km and Kcat values of 277.35 μmol/L and 0.71 S^{-1}, and the hydrolytic activity was blocked by sodium fluoride, a PP2C type Ser/Thr phosphatase inhibitor. The phosphorylation levels of StkP in the four *phpP* gene-knockout (Δ*phpP*) mutants were significantly higher than that in the wild-type strains. In particular, the MICs of PCN and CTX against the Δ*phpP* mutants were significantly elevated as 4-16 μg/ml. Therefore, our findings confirmed that sublethal PCN and CTX act as environmental inducers to cause the increase of *phpP* and *stkP* gene expression and StkP phosphorylation. PhpP is a PP2C type Ser/Thr protein phosphatase responsible for dephosphorylation of StkP. Knockout of the *phpP* gene results in a high level of StkP phosphorylation and PBP-independent PCN/CTX resistance of *S. pneumoniae*.

Importance

Streptococcus pneumoniae is a common pathogen in human populations in many countries

and areas due to the prevalence of β-lactam antibiotic-resistant pneumococcal strains. Production of β-lactamases and mutation of penicillin-binding proteins (PBP) have been considered as the major β-lactam antibiotic-resistant mechanisms in bacteria, but *S. pneumoniae* has not been confirmed to produce any β-lactamases and many pneumococcal strains present PBP mutation-independent β-lactam antibiotic resistance. StkP is a Ser/Thr kinase of *S. pneumoniae* to compose a signal-couple with PhpP protein. The present study demonstrated that the PhpP is a PP2C-type phosphotase for dephosphorylation of StkP and the sublethal penicillin (PCN) or cefotaxime (CTX) acted as environmental signal molecules to induce the expression of PhpP. The knockout of PhpP-encoding gene caused the PCN/CTX resistance generation of PCN/CTX-sensitive pneumococcal strains. All the data indicate that StkP-PhpP couple of *S. pneumoniae* is involved in PBP mutation-independent β-lactam antibiotic resistance by phosphorylation of StkP.

Key words: *Streptococcus pneumoniae*; PhpP; PP2C type protein phosphatase; StkP-PhpP signaling couple; β-lactam antibiotics; Drug resistance

Introduction

Streptococcus pneumoniae is a major causative agent of bacterial pneumonia and tympanitis in children [1-3]. More importantly, in the recent years, *S. pneumoniae*-infected meningitis cases with high fatality have been frequently reported in many countries and areas [4-8]. Therefore, *S. pneumoniae* is a common pathogen for human beings with global importance.

β-lactam antibiotics are the first choice in clinic to cure *S. pneumoniae*-infected patients [9]. However, in the recent years, β-lactam antibiotic-resistance of *S. pneumoniae* isolates from patients is continuously increased and the antimicrobial-resistant *S. pneumoniae* strains became more epidemic in many countries and areas [10-15], which has been considered as the major reason for increased incidence of *S. pneumoniae*-infected diseases [15,16]. Bacterial β-lactamases have been confirmed to play a key role in generation of β-lactam antibiotic resistance in many bacteria including *S. pneumoniae* [17]. Mutation of penicillin-binding proteins (PBP), the receptors of β-lactam antibiotics located on surface of bacteria, has been reported as the major β-lactam antibiotic resistant mechanism in bacteria [18,19]. However, recent studies found that some of the *S. pneumoniae* strains had no PBP mutation but presented β-lactam antibiotic resistance [20-22], indicating that *S. pneumoniae* may have a PBP mutation-independent mechanism of β-lactam antibiotic resistance.

StkP is a sequence-conserved eukaryotic-type serine/threonine (Ser/Thr) kinase (STK) of *S. pneumoniae* that has been confirmed to be involved in PBP mutation-independent penicillin resistance [22]. In the chromosomal DNA of *S. pneumoniae*, StkP-encoding gene (*stkP*) and *phpP* gene compose a *stkP-phpP* operon and the product of *phpP* gene is annotated as a putative phosphatase [23,24]. A previous study demonstrated that the PhpP

and StkP of *S. pneumoniae* composed a StkP-PhpP signaling couple [25]. It has been reported that both prokaryotic and eukaryotic STKs are activated through phosphorylation at Ser/Thr sites and some certain protein phosphatases can inactivate STKs by hydrolysis of phosphoryl groups at the Ser/Thr residual sites in STKs [26]. In particular, a previous study revealed that penicillin (PCN) could cause the gene expression profile change of *S. pneumoniae* [27]. Therefore, we presume that β-lactam antibiotics may act as environmental inducers to cause the change of PhpP and StkP expression and StkP dephosphorylation of *S. pneumoniae* as well as the PhpP may be involved in the StkP-associated PBP mutation-independent penicillin resistance by dephosphorylation of StkP.

In the present study, we used PCN and cefotaxime (CTX) as the representatives of β-lactam antibiotics to detect their induction of *phpP* and *stkP* gene expression and then identified the product of *phpP* gene as a PP2C type Ser/Thr protein phosphatase by virtue of its ability to hydrolyze Ser/Thr phosphatase-specific substrates. Moreover, the *phpP* genes of *S. pneumoniae* strains were inactivated to determine the role of PhpP in dephosphorylation of StkP *in vivo* and the change of β-lactam antibiotic resistance. The results of this study confirmed that the product of *S. pneumoniae phpP* gene is a Ser/Thr protein phosphatase that involved in β-lactam antibiotic resistance-associated StkP-PhpP signaling couple by StkP dephosphorylation during induction of sublethal PNC and CTX.

Materials and Methods

Bacterial strains and culture.

S. pneumoniae ATCC6306 and three β-lactam antibiotic-sensitive *S. pneumoniae* isolates (No.: SP5, SP9 and SP14, belonging to serotype 3, 19F and 19A) from pneumonia children were kindly provided by the Department of Medical Microbiology and Parasitology, Zhejiang University School of Medicine. All the strains were cultured with Colombia blood agar (bioMerieux, France) or 0.5% yeast extract-containing Todd-Hewitt (TH) broth (Sigma, USA) at 37°C [28]. Besides, *Escherichia coli* EL21DE3 (Novagen, USA) was cultured in Luria-Bertani (LB) medium (Oxoid, England) at 37°C.

Animal.

New Zealand white rabbits (3.0 to 3.5 kg per animal) were provided by the Laboratory Animal Center of Hangzhou Medical College (Certificate No.: SCXK[zhe]2012-0173). All the animal experimental protocols were approved by the Ethics Committee for Animal Experiment of Hangzhou Medical College.

Drug susceptibility test.

Susceptibility of each of the four *S. pneumoniae* strains to PCN or CTX was detected by E-test (bioMerieux) according to the manufacturer's instruction. The minimal inhibitory concentrations (MIC) against *S. pneumoniae* strains, ≤ 2 or ≥ 8 μg/ml of PCN (Sigma) and ≤ 1 or ≥ 4 μg/ml of CTX (Sigma), were considered to be sensitive or resistant [29].

Detection of sublethal PCN- and CTX-induced expression of *phpP* and *stkP* genes.

Each of the four *S. pneumoniae* strains was inoculated into TH broth for a 200 rpm shaking incubation at 37°C. When the value of optical density at 600 nm (OD_{600}) of pneumococcal culture turbidity reached 0.5, 1/4 MIC PCN or CTX was added and then incubated for 0.5, 1, 2, 4, 8 or 12 h as above. After centrifugation and washing with phosphate buffered saline (PBS), total RNA of each of *S. pneumoniae* strains was extracted using a TRIzol® Max™ Bacterial RNA Isolation kit (Invitrogen) plus a gDNA Eraser Kit (TaKaRa, China) and then quantified by ultraviolet spectrophotometry [30]. Subsequently, cDNA from each of the total RNAs was synthesized using a PrimeScript™ RT Reagent Kit (TaKaRa). Using each of the cDNAs as template, the *phpP*- or *stkP*-mRNA level was assessed by real-time fluorescence quantitative reverse transcription polymerase chain reaction (qRT-PCR) with the primers P-1F/P-1R or S-1F/S-1R (Table 1) using a SYBR® Premix Ex-Taq™ Kit (TaKaRa) in an ABI 7500 Real-Time PCR System (ABI, USA). The primers used were designed using Primer Premier 5.0 software according to the *phpP* or *stkP* gene sequences in GenBank (accession No.: NC_003098 and NC_003028). In the qRT-PCR, 16S rRNA gene of *S. pneumoniae* was used as the internal reference [31], while the PCN- or CTX-untreated *S. pneumoniae* strains were used as the controls. The obtained qRT-PCR data were analyzed using the ΔΔCt model and randomization test in REST 2005 software [32].

Amplification and sequencing of *PhpP* and *stkP* gene segments.

Genomic DNA of each of the four *S. pneumoniae* strains was extracted using a Bacterial Genomic DNA Preparation Kit (Axygen). By using a High Fidelity PCR Kit (TaKaRa), the

157 entire *phpP* or *stkP* gene segments were amplified from the DNA templates by PCR using the
158 primers P-2F/P-2R or S-2F/S-2R (Table 1). The PCR products were examined by 1.5%
159 ethidium bromide-stained agarose gel electrophoresis and then cloned into pMD19-T plasmid
160 using a T-A Cloning Kit (TaKaRa) to form recombinant pMD19-T^{phpP} and pMD19-T^{stkp}
161 plasmids for sequencing by Invitrogen Co. in Shanghai of China.
162
163 **Bioinformatic analysis of *phpP* and *stkP* genes.**
164 Since the nucleotide and amino acid sequence identities of *phpP* and *stkP* genes from the four
165 *S. pneumoniae* strains were as high as 98.7%-100%, the *phpP* and *stkP* genes of *S.*
166 *pneumoniae* strain ATCC6306 were analyzed using TMHMM and NCBI Database Conserved
167 Domain Database (CDD) software [33].
168
169 **Generation of prokaryotic expression systems of *phpP* and *stkP* genes.**
170 The pMD19-T^{phpP} or pMD19-T^{stkP} plasmid from *S. pneumoniae* strain ATCC6306 and
171 pET42a vector (Novagen) were digested with both Nde I and Xho I or Nde I and Hind III
172 (TaKaRa). The recovered *phpP* or *stkP* gene segment was linked with the linearized pET42a
173 using T4 DNA ligase (TaKaRa) and then transformed into *E. coli* BL21DE3 by $CaCl_2$
174 transformation method to form *E. coli* BL21DE3$^{pET42a\text{-}phpP}$ or *E. coli* BL21DE3$^{pET42a\text{-}stkP}$. The
175 engineered strains were cultured in LB medium containing 50 μg/ml kanamycin (Sigma) and
176 the pET42a-*phpP* and pET42a-*stkP* were extracted from the strains using a Plasmid
177 Extraction Kit (Axygen) for sequencing again.
178

Expression of *phpP* and *stkP* genes and extraction of expressed products.

The engineered strains, *E. coli* BL21DE3 $^{pET42a\text{-}phpP}$ and *E. coli* BL21DE3$^{pET42a\text{-}stkP}$, were cultured in kanamycin-containing LB liquid medium to express the target recombinant proteins (rPhpP and rStkP) under induction of 0.5 mM isopropy-β-D-thiogalactoside (IPTG, Sigma). After ultrasonic breakage on ice and a 13,800×g centrifugation for 10 min (4°C), the supernatants of cultures were collected to extract soluble rPhpP and rStkP using a Ni-NTA affinity chromatographic column (BioColor, China). The extracted rPhpP or rStkP was quantified using a BCA Protein Assay Kit (Thermo Scientific, USA). Both the expressed and extracted rPhpP and rStkP were examined by sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) plus a Gel Image Analyzer (Bio-Rad, USA).

Preparation of rPhpP-IgG and rStkP-IgG

New Zealand rabbits were intradermally immunized on days 1, 14, 21 and 28 with Freund's adjuvant-mixed 2 mg rPhpP or rStkP per animal. Fifteen days after the last immunization, the sera were collected to separate rStkP-IgG or rPhpP-IgG by ammonium sulfate precipitation plus a DEAE-52 column chromatography using 10 mM phosphate buffer (pH 7.4) for elution [34]. The titer of rPhpP-IgG or rStkP-IgG binding to rStkP or rPhpP was detected by immunodiffusion test.

Phosphatase activity assays.

The enzymatic activity of rPhpP was detected using a pNPP-Phosphate Assay Kit (BioAssay Systems, USA) and a PP2C-specific Ser/Thr Phosphatase Assay Kit (Promega, USA) [35,36].

Briefly, 0.5, 1, 2.5, 5, 10 or 20 μg rPhpP was mixed with 500 nM para-nitrophenyl phosphate (p-NPP), an universal Ser/Thr phosphatase substrate, in 100 μl reaction buffer. After a 30-min incubation at 37°C, the OD_{405} values reflecting p-NPP hydrolysis were detected using type M5 spectrophotometer (Bio-Rad). On the other hand, 0.5, 1, 2.5, 5, 10 or 20 μg rPhpP was mixed with 100 μM RRA(pT)VA phosphopeptide, a PP2C type Ser/Thr protein phosphatase-specific substrate, in 100 μl reaction buffer. After incubation as above, the OD_{600} values reflecting RRA(pT)VA hydrolysis were detected by spectrophotometry.

Phosphatase inhibition test.

Okadaic acid (OA) is an inhibitor of PP1, PP2A and PP2B type Ser/Thr phosphatases while sodium fluoride (NaF) is a PP2C type Ser/Thr phosphatase inhibitor [37,38]. Briefly, 5 μg rPhpP was mixed with 0.1, 0.5, 1, 5 or 10 μM OA (Sigma) or 0.1, 0.5, 1, 5 or 10 mM NaF (Sigma) in 100 μl reaction buffer and then incubated at 37°C for 30 min. The activity of OA- or NaF-treated rPhpP to hydrolyze RRA(pT)VA substrate was detected as described above.

Determination of Km and Kcat values.

According to the results of phosphatase activity assays, 5 μg rPhpP was mixed with 50, 100, 150, 200 or 250 μM RRA(pT)VA substrate and then detected by spectrophotometry as described above. According to the OD_{600} values reflecting RRA(pT)VA hydrolysis and free phosphate concentration standard curve, the Km and Kcat values of rPhpP hydrolyzing the substrate were calculated by double reciprocal Lineweaver-Burk plot [39].

Generation and identification of *phpP* gene-knockout mutant.

pEVP3 plasmid has been used to generate the target gene-knockout mutant of *S. pneumoniae* [40,41]. Briefly, a 495-bp *phpP* gene segment (*phpP*-495) from *S. pneumoniae* strain ATCC6306 was amplified using a High Fidelity PCR Kit (TaKaRa) with the primers P-3F/P-3R and then cloned into pMD19-T to form pMD19-T$^{phpP\text{-}495}$ for sequencing as above. The pMD19-T$^{phpP\text{-}495}$ and pEVP3 plasmid were digested with both Xho I and BamH I (TaKaRa). The recovered *phpP*-495 segment was linked with the linearized pEVP3 using T4 DNA ligase (TaKaRa) to form suicide plasmid pEVP3^{phpP495} for sequencing again. Each of the four *S. pneumoniae* strains was inoculated in TH broth containing 0.01% $CaCl_2$ and 0.2% BSA (Sigma) for a 250-rpm incubation at 37°C to the OD_{600} value as 0.25 and then collected by centrifugation. The competent pneumococcal cells were suspended in 200 μl TH broth containing 0.01% $CaCl_2$, 0.2% BSA and 200 ng/ml competence stimulation peptide (CSP, AnaSpec, USA) and then added with 200 ng pEVP3$^{phpP\text{-}495}$ for transformation. The mixtures were smeared on 5% sheet blood Columbia agar (bioMerieux) plates containing 2.5 μg/ml chloromycin (CM, Sigma) for a 48-h incubation at 37°C in a 5% CO_2 atmosphere to obtain *phpP* gene-knockout colonies [42]. The strategy for generating *phpP* gene-knockout mutants (Δ*phpP*-6306, Δ*phpP*-SP5, Δ*phpP*-SP9 and Δ*phpP*-SP14) is summarized in Fig. 1.

Identification of *phpP* gene-knockout mutants.

Growth of the Δ*phpP*-6306, Δ*phpP*-SP5, Δ*phpP*-SP9 or Δ*phpP*-SP14 mutant was assessed by spectrophotometry. The *phpP* gene knockout in the Δ*phpP* mutants was determined by PCR using the primers P-4F/P-4R and P-5F/P-5R (Table 1) and sequencing of the 5'-*phpP*-pEVP3

and pEVP3-3'-*phpP* segment amplification products. Using 1:200 diluted rabbit anti-rPhpP-IgG as the primary antibody and HRP-conjugated goat anti-rabbit-IgG (Abcam, USA) as the secondary antibody, Western blot assay was performed to detect the PhpP form the Δ*phpP* mutants, in which the wild-type strains were used as the controls.

Detection of sublethal PCN- or CTX-induced StkP phosphorylation.

The Δ*phpP* mutants and their wild-type strains were treated with 1/4 MIC PCN or CTX for 1, 2, 4 or 8 h at 37°C. After centrifugation and washing with PBS, the pneumococcal pellets were suspended in deionized water and then ultrasonically broken on ice. The lysates were centrifuged at 14,000×g for 10 min (4°C) and then the supernatants were collected to detect protein concentrations using a BCA Protein Assay Kit (Thermo Scientific). 200 μg of each of the total pneumococcal proteins was mixed with 20 μg rabbit anti-rStkP-IgG for a 2-h incubation in a 90-rpm rotator (4°C). The mixture was added with 600 μg protein-A-coated agarose beads (Millipore, USA), followed by a 60-min incubation as above. After a 14,000×g centrifugation for 5 min and washing thoroughly with PBS, the precipitated beads were suspended in 200 μl 50 mM Tris-HCl buffer (TB, pH7.5) and then mixed with 200 μl 2M NaOH solution for a 30-min water-bath at 65°C to release phosphate groups from the captured StkP according to the instruction of Phosphoprotein Phosphate Detection Kit (Sangon Biotech, Canada). The mixture was neutralized with 200 μl 4.7 M HCl solution and then added with 200 μl detection buffer for a 30-min incubation at room temperature. The OD_{620} value reflecting phosphate group concentration released from IgG-captured StkP were detected using a spectrophotometer (Molecular Devices, USA) [43]. In the detection, the

PCN- or CTX-untreated Δ*phpP* mutants and their wild-type strains were used as the controls.

Detection of β-lactam antibiotic resistance of Δ*phpP* mutants.

Susceptibility of the Δ*phpP*-6306, Δ*phpP*-SP5, Δ*phpP*-SP9 and Δ*phpP*-SP14 mutants to PCN or CTX was detected by E-test as described above. In the detection, the wild-type *S. pneumoniae* strains were used as the controls.

Statistical analysis

Data from a minimum of three experiments were averaged and presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test were used to determine significant differences. Statistical significance was defined as $p < 0.05$.

Results

Increase of *stkP*- and *phpP*-mRNA levels induced by sublethal PCN and CTX.

The *phpP*- and *stkP*-mRNA levels of each of the four tested *S. pneumoniae* strains were relatively lower. When the strains were treated with 1/4 MIC PCN or CTX, the *phpP*- and *stkP*-mRNA levels were rapidly elevated (Fig. 2A-2D). The data suggested that sublethal PCN and CTX can act as the stimulators to induce the expression of *S. pneumoniae phpP* and *stkP* genes.

Characterization of *phpP* and *stkP* genes.

The product of *S. pneumoniae phpP* or *stkP* gene was predicted as a secretary cytosolic or a transmembrane protein (Fig. 3A and 3B). The *phpP* gene contains a PP2Cc type protein phosphatase domain (6-238 aa) with five enzymatic active sites (Fig. 3C). The *stkP* gene contains a C type STK domain belonging to STKc_PknB superfamilay containing twelve ATP-binding sites, six dimer interface sites, two activation loops, eight polypeptide substrate-binding sites and sixteen enzymatic active sites as well as four penicillin-binding protein and STK-associated (PASTA) superfamily domains (Fig. 3D).

Effects of expression and extraction of *stkP* and *phpP* genes.

The nucleotide and amino acid sequence identities of *phpP* or *stkP* genes from the four *S. pneumoniae* strains were 99.3%-100% and 99.1%-100% or 99.2%-99.7% and 98.7%-99.9%, compared to the same two genes in GenBank (accession No.: NC003098) (data not shown). The two engineered strains expressed the target recombinant proteins rPhpP and rStkP,

302 respectively, and the extracted rPhpP or rStkP was showed as a single band in gels (Fig.
303 4A-4D).
304
305 **Powerful Ser/Thr protein phosphatase activity of rPhpP.**
306 The rPhpP from *S. pneumoniae* ATCC6306 hydrolyzed p-NPP, an universal Ser/Thr
307 phosphatase substrate, and RRA(pT)VA, a PP2C type Ser/Thr protein phosphatase-specific
308 substrate, with concentration-dependent manners (Fig. 5A). The Km and Kcat values of
309 rPhpP hydrolyzing RRA(pT)VA substrate were 277.35 μmol/L and 0.71 S^{-1}, respectively (Fig.
310 5B). Moreover, the PP2C type Ser/Thr protein phosphatase inhibitor NaF, but not the PP1,
311 PP2A or PP2B type Ser/Thr phosphatase inhibitor OA, inhibited the hydrolytic activity of
312 rPhpP (Fig. 5C). The data suggested that the product of *S. pneumoniae phpP* gene is a PP2C
313 type Ser/Thr protein phosphatase.
314
315 **PCN/CTX-induced StkP phosphorylation and PhpP-caused StkP dephosphorylation.**
316 The Δ*phpP* mutants could grow persistently in the TH broth similarly to the wild-type strains
317 (Fig. 6A). The PCR plus sequencing and Western Blot assay confirmed the *phpP* gene
318 knockout and no PhpP expression in all the four Δ*phpP* mutants (Fig. 6B and 6C). 1/4 MIC
319 PCN or CTX could cause the increase of StkP phosphorylation levels in the Δ*phpP* mutants
320 and their wild-type strains (Fig. 6D and 6E). However, the Δ*phpP* mutants presented
321 significantly higher PCN- or CTX-induced StkP phosphorylation levels than the wild-type
322 strains (Fig. 6D and 6E). The data suggested that sublethal PCN and CTX can induce
323 phosphorylation of StkP and PhpP can cause dephosphorylation of StkP *in vivo*.

Increased PCN and CTX resistance of Δ*phpP* mutants.

The MICs of PCN and CTX against wild-type ATCC6306, SP5, SP9 and SP14 strains of *S. pneumoniae* were 0.02-0.5 μg/ml, indicating all the strains were sensitive to both PCN and CTX. However, the MICs of PCN and CTX against the four Δ*phpP* mutants were significantly elevated as 4-16 μg/ml (Table 2). The data suggested that PhpP is involved in β-lactam antibiotic resistance of *S. pneumoniae*.

Discussion

Kinases play key roles in signal transduction in both eukaryotes and prokaryotes and are activated by either phosphorylation or dephosphorylation [44]. Unlike eukaryotes, histidine kinases but not serine/threonine/tyrosine (Ser/Thr/Tyr) kinases act as the major signal sensors and transducers in prokaryotic bacteria [45]. Previous studies confirmed that StkP of *S. pneumoniae* is an eukaryotic-type Ser/Thr protein kinase containing PBP-like domains in its extracellular region and Ser/Thr phosphorylation sites in its intracellular region [46], and PhpP and StkP of *S. pneumoniae* compose a StkP-PhpP signaling couple [25]. Operon is a genic unit for transcription in prokaryotic genome that composed of promoter, operator and function-associated structural genes [47]. Our bioinformatic analysis showed that PhpP- and StkP-encoding genes of *S. pneumoniae* are located in the same one operon for co-expression, implying the close functional association between the two genes. As described in the introduction, StkP of *S. pneumoniae* is involved in PBP mutation-independent penicillin resistance [22]. Therefore, the product of *phpP* gene, PhpP, may act as a protein phosphotase to down-regulate StkP phosphorylation level by dephosphorylation to participate in StkP-involved β-lactam antibiotic resistance.

According to the differences of amino acid sequences and structures, Ser/Thr protein phosphatases are classified into MPP and PPP families as well as the former contains Mg^{2+} or Mn^{2+}-dependent PP2C type phosphatases while the latter includes PP1, PP2A and PP2B type phosphatases [48]. PP2C type phosphatases has an extensive distribution in bacteria, yeasts, plants and mammalian cells to play various and complicated functions such as signaling transduction by dephosphorylation, cellular generation cycle regulation, monitoring DNA

353 damage and RNA splicing [48-50]. Our bioinformatic prediction showed that *phpP* gene of *S.*
354 *pneumoniae* contains a PP2C type protein phosphatase domain. The recombinant expression
355 product of *phpP* gene (rPhpP) hydrolyzed both the general Ser/Thr phosphatase substrate
356 p-NPP and PP2C type Ser/Thr protein phosphatase-specific substrate RRA(pT)VA in a
357 concentration-dependent manner but its RRA(pT)VA-hydrolyzed ability was blocked by the
358 PP2C type Ser/Thr protein phosphatase-specific inhibitor NaF. Previous studies confirmed
359 that StkP and PhpP of *S. pneumoniae* compose a StkP/PhpP signaling couple in which StkP is
360 activated by Ser/Thr residual phosphorylation and PhpP could hydrolyze phosphoryl groups
361 in StkP *in vitro* [25,51]. However, we confirmed that the sublethal PCN and CTX induced the
362 phosphorylation of StkP and the StkP phosphorylation levels in the Δ*phpP* mutants were
363 significantly higher than that in the wild-type strains. All the data suggested that the product
364 of *S. pneumoniae phpP* gene is a PP2C type Ser/Thr protein phosphatase to play a reverse
365 regulation role in StkP/PhpP signaling couple by dephosphorylation of StkP.

366 Previous studies reported that penicillin-binding protein and STK-associated (PASTA)
367 superfamily domains in some proteins from many Gram-positive bacteria have a potential
368 ability to bind to β-lactam antibiotics [52,53], and our bioinformatic analysis also revealed
369 that StkP of *S. pneumoniae* possesses four PASTA domains located its carboxyl terminal,
370 implying that the StkP may served as the β-lactam antibiotic receptor and β-lactam antibiotics
371 may cat as environmental activators of StkP-PhpP signaling couple. In the present study, the
372 sublethal PCN or CTX (1/4 MIC) caused the significant increase of *stkP*- and *phpP*-mRNA
373 levels and StkP phosphorylation. The data suggested that StkP-PhpP couple is a β-lactam
374 antibiotic-associated signaling pathway of *S. pneumoniae*.

375 Bacterial PBP is a group of peptidoglycan biosynthesis-associated transpeptidases,
376 carboxypeptidases and endopeptidases that located on surface of bacteria [54]. β-lactam
377 antibiotics can bind to the peptidases to cause them inactivation by due to enzymatic
378 molecular allosterism but PBP mutation can cause β-lactam antibiotic resistance due to the
379 decrease of β-lactam antibiotic-binding ability of PBP [55,56]. However, some *S.*
380 *pneumoniae* strains presented PBP mutation-independent β-lactam antibiotic resistance
381 [20-22]. In the present study, all the tested *S. pneumoniae* strains were sensitive to both PCN
382 and CTX (MICs: 0.02-0.5 μg/ml). When the *phpP* genes were knockout, the Δ*phpP* mutants
383 became resistant to the two antibiotics (MICs: 4-16 μg/ml). The data imply that StkP
384 phosphorylation promotes but PhpP-based StkP dephosphorylation inhibits the generation of
385 PBP mutation-independent β-lactam antibiotic resistance.
386

387 ## Conclusions

388 Sublethal PCN and CTX can act as environmental inducers to cause the increase of *stkP* and
389 *phpP* gene expression and StkP phosphorylation of *S. pneumoniae*. The product of *phpP* gene
390 is a PP2C type Ser/Thr protein phosphatase to cause dephosphorylation of StkP that plays a
391 reverse regulating role in StkP/PhpP signaling couple. Sublethal β-lactam antibiotics can act
392 as environmental inducers of *phpP* and *stkP* gene expression and knockout of *phpP* gene
393 caused the significant increase of β-lactam antibiotic resistance of *S. pneumoniae*.
394
395

Additional file

This manuscript has no additional files.

Abbreviations

S. pneumoniae: *Streptococcus pneumoniae*; PNC: penicillin; CTX: cefotaxime; quantitative reverse transcription polymerase chain reaction: qRT-PCR; Δ*phpP*: *phpP* gene-knockout mutant; MIC: minimal inhibitory concentration; mRNA: messenger ribonucleic acid; PBP: penicillin-binding proteins; STK: serine/threonine kinase; DNA: deoxyribonucleic acid; TH: Todd-Hewitt; LB: Luria-Bertani; OD: optical density; PBS: phosphate buffered saline; RNA: ribonucleic acid; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electropheresis; CDD: conserved domain database; IPTG: isopropy-β-D-thiogalactoside; OA: Okadaic acid; NaF: sodium fluoride; SD: standard deviation; PASTA: penicillin-binding protein and STK-associated; TB: Tris-HCl buffer.

Acknowledgements

We are grateful to Dr. Morrisson (University of Illinois, Chicago, USA) for kindly providing the pEVP3 plasmid used in this study.

Funding

This work was supported by a grant from the National Natural Science Foundation of China (81772232) and a grant from Zhejiang Provincial Program for the Cultivation of High-Level Innovative Health Talents (2012-241).

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions

A.H.S. and J.Y. conceived and designed the experiments. Y.Y.H., Y.H.S. and P.D. performed the experiments. Y.Y.H., Y.H.S. and X.X.L. analyzed the data. Y.Y.H., A.H.S. and J.Y. wrote the manuscript. A.H.S. obtained the funding. All authors have read and approved the manuscript.

Ethics approval and consent to participate

This study has no medical ethic problems. All the animal experimental protocols were approved by the Ethics Committee for Animal Experiment of Hangzhou Medical College.

Consent for publication

Not applicable.

Competing interests

All the authors have no potential conflict of interest to declare.

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616

617 # Legend

618 Fig. 1 Strategy for generation of *S. pneumoniae* Δ*phpP* mutant. See section of materials and
619 methods in text for details.

620

621 Fig. 2 Increase of *phpP*- and *stkP*-mRNAs after treatment with sublethal PCN and CTX.
622 (A). Increase of *phpP*-mRNA induced by 1/4 MIC PCN, detected by qRT-PCR. Bars show
623 the mean ± SD of three independent experiments. The *phpP*-mRNA levels in the
624 PCN-untreated *S. pneumoniae* strains (before treatment) were set as 1.0. *: $p<0.05$ *vs* the
625 *phpP*-mRNA levels in the PCN-untreated strains.
626 (B). Increase of *phpP*-mRNA induced by 1/4 MIC CTX, detected by qRT-PCR. The legend is
627 the same as shown in A but for CTX-induced *phpP*-mRNA detection.
628 (C). Increase of *stkP*-mRNA induced by 1/4 MIC PCN, detected by qRT-PCR. Bars show the
629 mean ± SD of three independent experiments. The *stkP*-mRNA levels in the PCN-untreated *S.*
630 *pneumoniae* strains (before treatment) were set as 1.0. *: $p<0.05$ *vs* the *stkP*-mRNA levels in
631 the PCN-untreated strains.
632 (B). Increase of *stkP*-mRNA induced by 1/4 MIC of CTX, detected by qRT-PCR. The legend
633 is the same as shown in C but for CTX-induced *stkP*-mRNA detection.

634

635 Fig. 3 Predicted characteristics of *S. pneumoniae phpP* and *stkP* genes.
636 (A). Structure and location of *phpP* gene product, predicted using TMHMM software.
637 (B). Structure and location of *stkP* gene product, predicted using TMHMM software.
638 (C). PP2C type phosphatase domain in *phpP* gene product (PhpP), predicted using NCBI

639 Database CDD software.

640 (D). STK and PASTA domains in *stkP* gene product (StkP), predicted using NCBI Database

641 CDD software.

642

643 Fig. 4 Amplification and expression of *S. pneumoniae phpP* and *stkP* genes.

644 (A). Amplification fragments of *phpP* genes from *S. pneumoniae* strains, determined by PCR.

645 Lane M: DNA marker. Lanes 1-4: amplicoms of *phpP* genes from *S. pneumoniae* strains

646 ATCC6306, SP5, SP9 and SP14 (738 bp). Lane 5: blank control.

647 (B). Amplification fragments of *stkP* genes from *S. pneumoniae* strains, determined by PCR.

648 The legend is the same as shown in A but for *stkP* gene amplification (1977 bp).

649 (C). Expression and extraction effects of rPhpP, detected by SDS-PAGE. Lane M: protein

650 marker. Lane 1: wild-type *E. coli* BL21DE3. Lane 2: the expressed rPhpP (~28.3 kDa). Lane

651 3: the extracted rPhpP by Ni-NTA affinity chromatography.

652 (D). Expression and extraction effects of rStkP, detected by SDS-PAGE. The legend is the

653 same as shown in C but for rStkP expression and extraction (~75.8 kDa).

654

655 Fig. 5 Powerful protein phosphatase activity of rPhpP from *S. pneumoniae*.

656 (A). Ability of rPhpP hydrolyzing phosphatase substrates p-NPP and RRA(pT)VA,

657 determined by spectrophotometry. Bars show the means ± SD of three independent

658 experiments. p-NPP is a universal Ser/Thr phosphatase substrate while RRA(pT)VA is a

659 PP2C type Ser/Thr protein phosphatase-specific substrate.

660 (B). Km and Kcat values of rPhpP hydrolyzing RRA(pT)VA substrate, determined by

661 spectrophotometry. 5 μg rPhpP and 50, 100, 150, 200 or 250 μM RRA(pT)VA
662 phosphopeptide were used.
663 (C). Enzymatic activity of rPhpP after treatment with phosphatase inhibitors, determined by
664 spectrophotometry. NaF is a PP2C type Ser/Thr protein phosphatase inhibitor while OA is an
665 inhibitor of PP1, PP2A or PP2B type Ser/Thr phosphatases.
666
667 Fig. 6 PCN/CTX-induced StkP phosphorylation and PhpP-caused StkP-dephosphorylation.
668 (A). Growth curves of the Δ*phpP* mutants and wild-type strains in TH broth, determined by
669 spectrophotometry. Bars show the means ± SD of three independent experiments.
670 (B). Schematic diagram of PCR and sequencing results of the Δ*phpP* mutants.
671 (C). PhpP absence in the Δ*phpP* mutants, determined by Western Blot assay. Lanes 1-4:
672 immunoblotting bands of PhpP proteins from the wild-type *S. pneumoniae* strain ATCC 6306
673 and *S. pneumoniae* isolates SP1, SP5 and SP9. Lanes 5-8: no immunoblotting bands of PhpP
674 proteins in the Δ*phpP* mutants.
675 (D) Sublethal PCN-induced increase of StkP phosphorylation and decrease of StkP
676 phosphorylation in the Δ*phpP* mutants, detected by spectrophotometry. Bars show the means
677 ± SD of three independent experiments. *: $p<0.05$ *vs* the StkP phosphorylation levels of the
678 PCN-untreated wild-type strains and Δ*phpP* mutants. $^{\#}$: $p<0.05$ *vs* the StkP phosphorylation
679 levels of the wild-type strains.
680 (E) Sublethal CTX-induced increase of StkP phosphorylation and decrease of StkP
681 phosphorylation in the Δ*phpP* mutants, detected by spectrophotometry. The legend is the
682 same as shown in D but for detection of CTX-induced StkP phosphorylation.
683

684 Table 1. Sequences of primers used in this study.

Primer	Sequence (5' to 3')	Purpose	Size (bp)
P-1	F: CTTCTGCCTSCTTCTGGTGT	*phpP*-mRNA detection	119
	R: TATTGGTGATTCGCGTATC		
S-1	F: TCGCTCGGTTTCAGAGATGTATGT	*stkP*-mRNA detection	190
	R: TGGGTTTTTGATTGATTTGTGTTC		
16S	F: CGACGATACATAGCCGACCTG	16S RNA segment as	145
	R: AAAACCTTCTTCACTCACGCG	qRT-PCR inner reference	
P-2	F: CGCCATATG(Nde I)GAAATTTCATTATTA	*phpP* gene detection and	738
	R: CGCCTCGAG(Xho I)TTCTGCATCCTCCTCGTTC	expression	
S-2	F: GCGCATATG(*Nde* I)ATCCAAATCGGCAAG	*stkP* gene detection and	1977
	R: GCGAAGCTT(Hind III)AGGAGTAGCTGAAGTTGT	expression	
P-3	F: CGCCTCGAG(Xho I)ATATCGCTAGTGAAATGG	*phpP* gene segment for	495
	R: CGCGGATCC(BamH I)CACTGGTTACAATATCAC	knockout	
P-4	F: ACATGAATGTAAGGATATCATG	Δ*phpP* mutant identification	895
	R: GGATGTGCTGCAAGGCGATTA		
P-5	F: GCTTTCTTCATTAGAATCAATCC	Δ*phpP* mutant identification	907
	R: TCACTGCCACTTCTTCCCCATC		

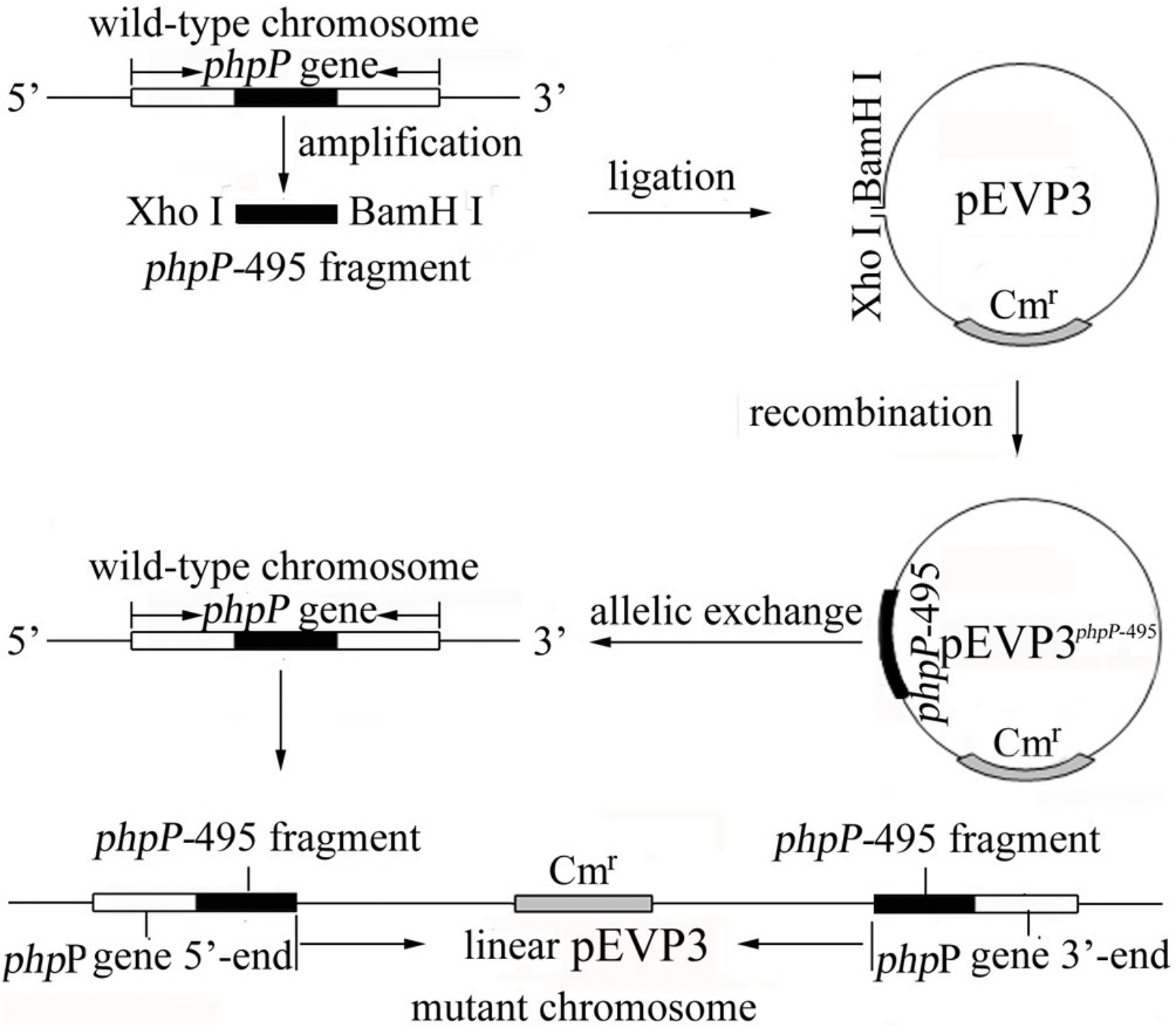
685 F: forward primer. R: reverse primer. Underlined areas indicate the sites of endonucleases.

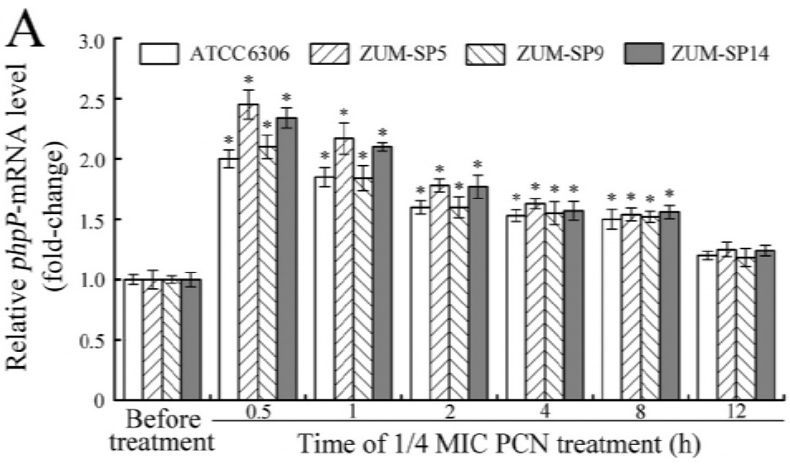
686

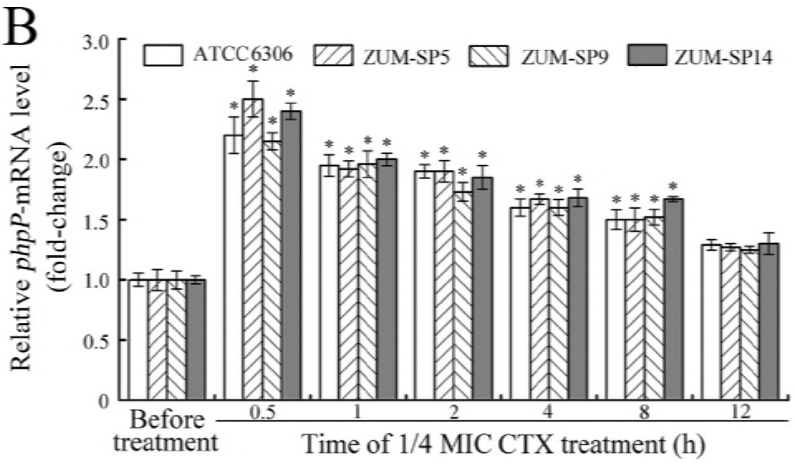
687 Table 2 MICs of PCN and CTX against *S. pneumoniae* strains.

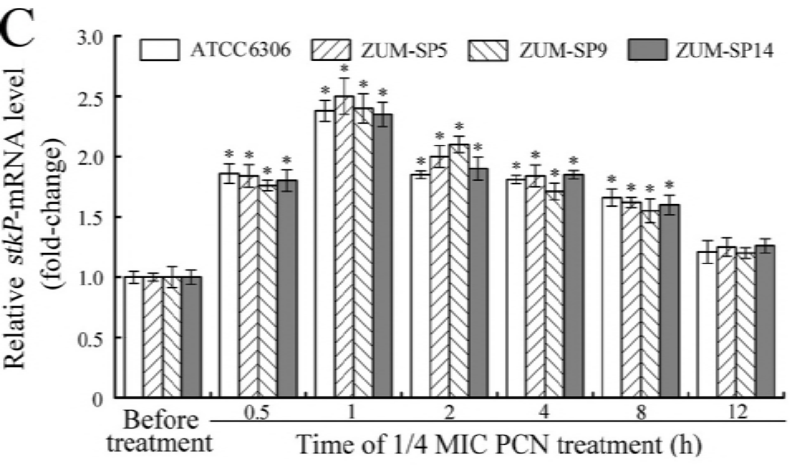
Strain	MIC (μg/ml)	
	PCN	CTX
Wild-type ATCC6306	0.05	0.12
Δ*phpP*-6306	8	8
Wild-type SP5	0.05	0.25
Δ*phpP*-SP5	8	16
Wild-type SP9	0.25	0.5
Δ*phpP*-SP9	16	16
Wild-type SP14	0.02	0.03
Δ*phpP*-SP14	4	4

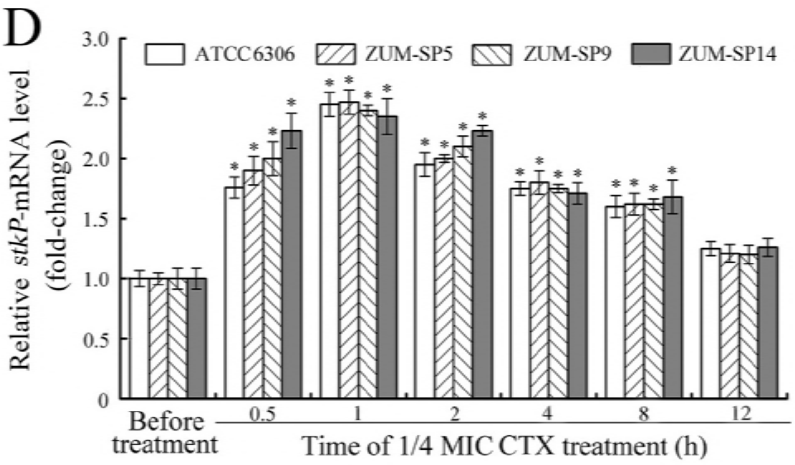
688

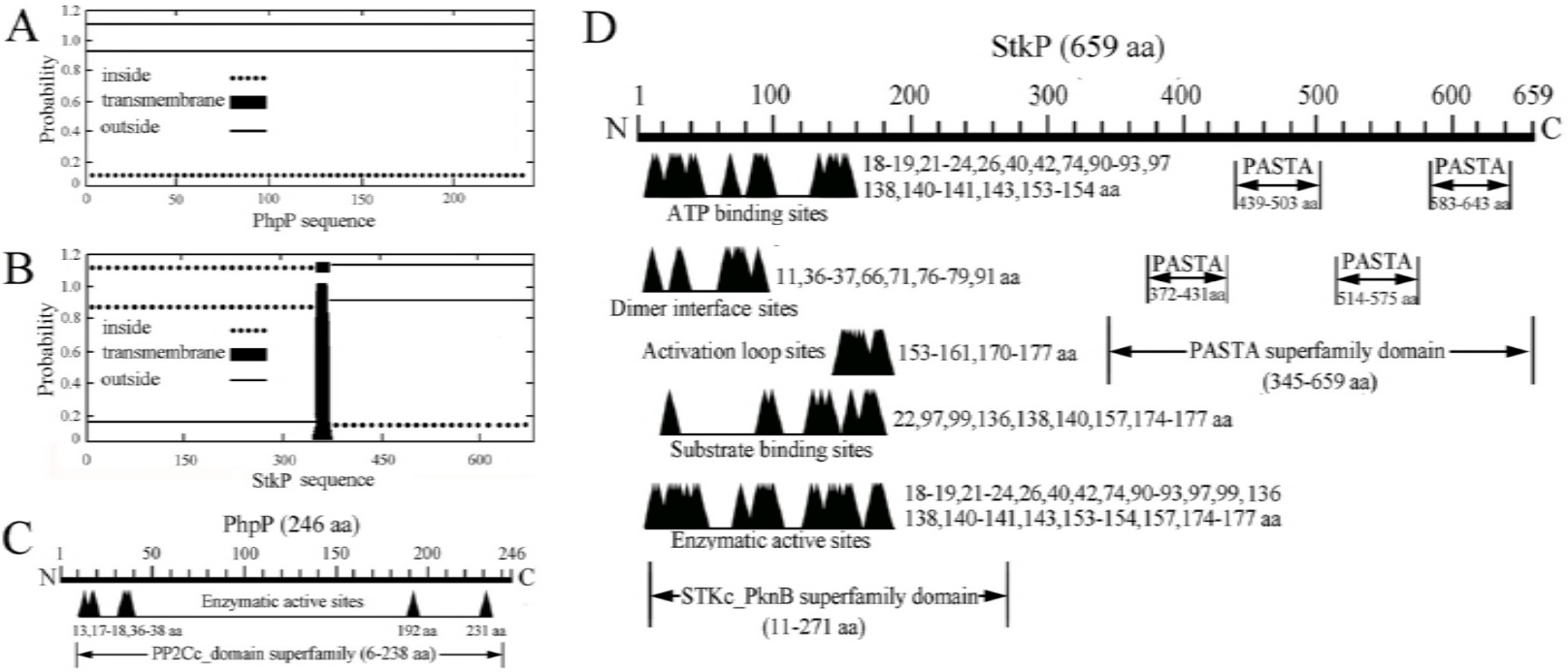

wild-type chromosome
phpP gene
5'
3'
amplification
Xho I
BamH I
phpP-495 fragment
ligation
Xho I
BamH I
pEVP3
Cm^r
recombination
wild-type chromosome
phpP gene
5'
3'
allelic exchange
phpP-495
pEVP3^phpP-495
Cm^r
phpP-495 fragment
Cm^r
phpP-495 fragment
phpP gene 5'-end
linear pEVP3
phpP gene 3'-end
mutant chromosome

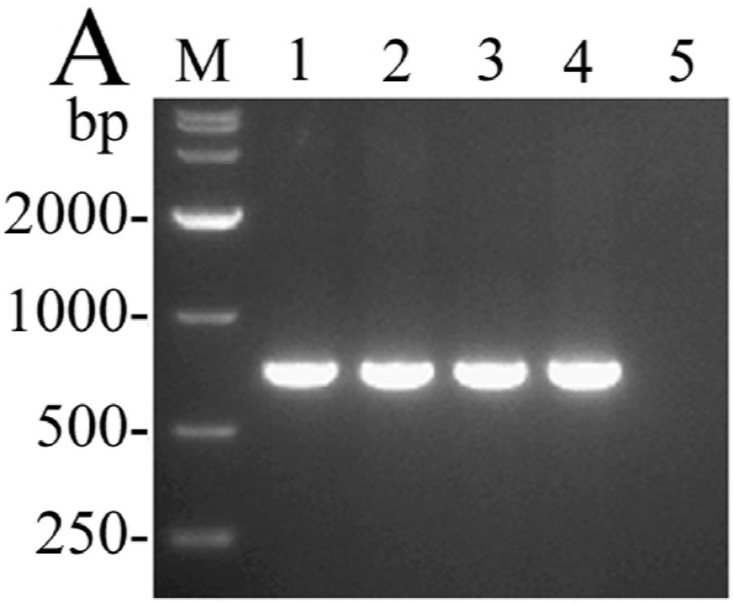

A
ATCC6306
ZUM-SP5
ZUM-SP9
ZUM-SP14
Relative phpP-mRNA level
(fold-change)
3.0
2.5
2.0
1.5
1.0
0.5
0
Before treatment
0.5
1
2
4
8
12
Time of 1/4 MIC PCN treatment (h)

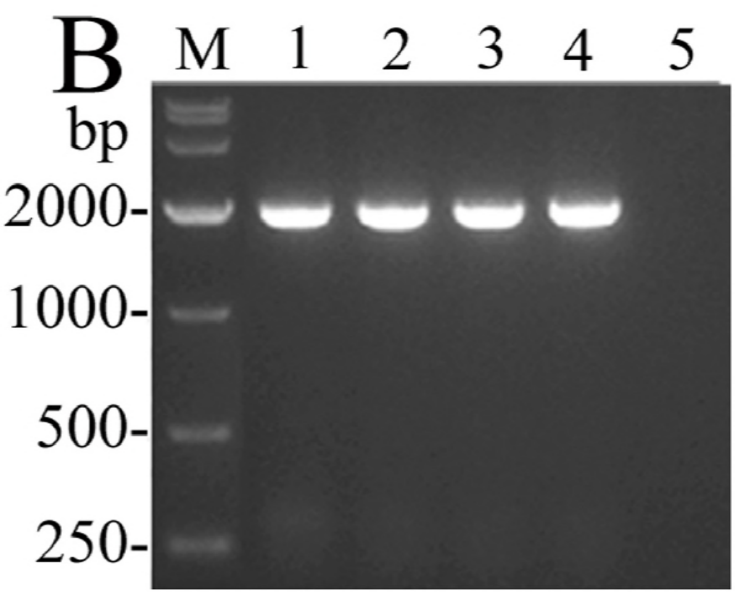

B
ATCC6306
ZUM-SP5
ZUM-SP9
ZUM-SP14
Relative phpP-mRNA level
(fold-change)
3.0
2.5
2.0
1.5
1.0
0.5
0
Before treatment
0.5
1
2
4
8
12
Time of 1/4 MIC CTX treatment (h)

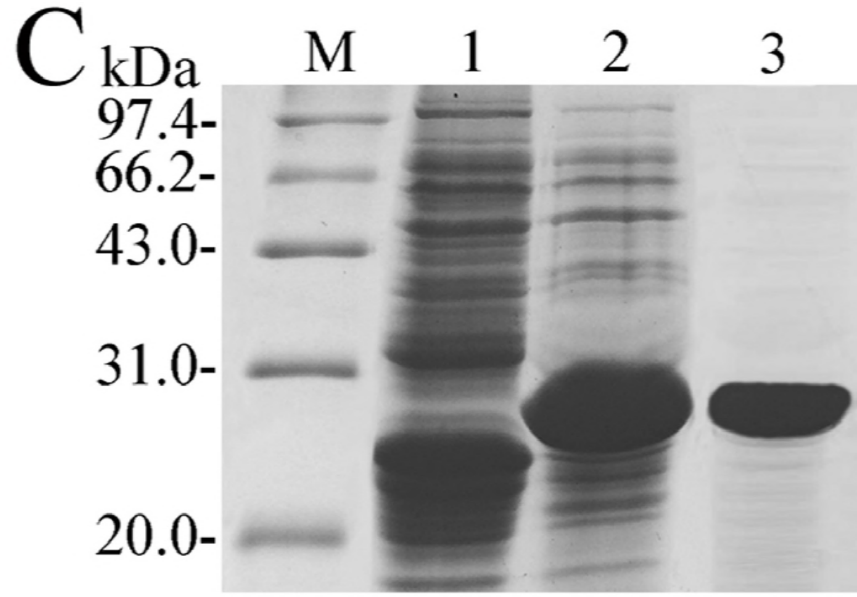

C
ATCC6306
ZUM-SP5
ZUM-SP9
ZUM-SP14
Relative stkP-mRNA level
(fold-change)
3.0
2.5
2.0
1.5
1.0
0.5
0
Before treatment
0.5
1
2
4
8
12
Time of 1/4 MIC PCN treatment (h)

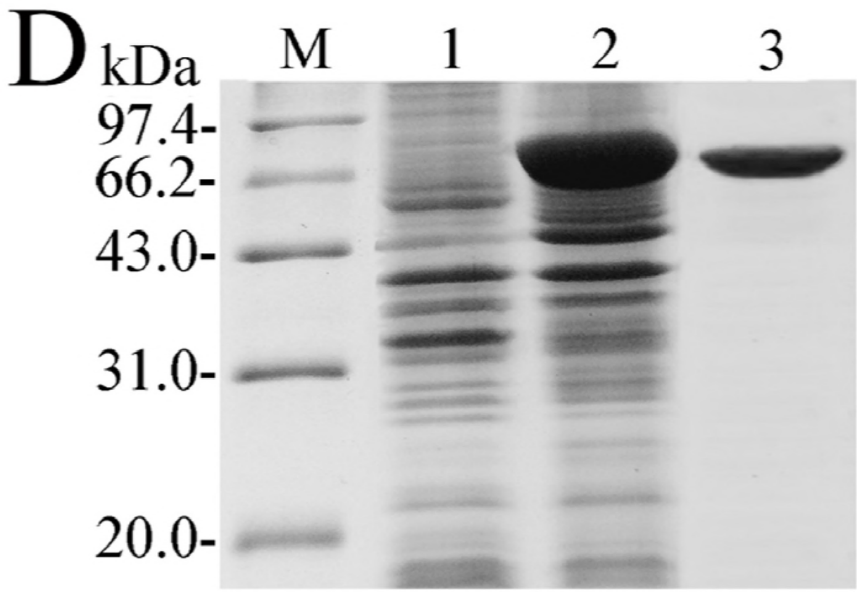

D
ATCC6306
ZUM-SP5
ZUM-SP9
ZUM-SP14
Relative stkP-mRNA level
(fold-change)
3.0
2.5
2.0
1.5
1.0
0.5
0
Before treatment
0.5
1
2
4
8
12
Time of 1/4 MIC CTX treatment (h)

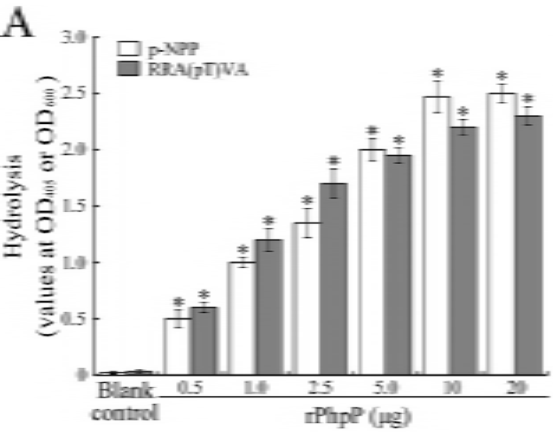

A
Probability
inside
transmembrane
outside
PhpP sequence
B
Probability
inside
transmembrane
outside
StkP sequence
C
PhpP (246 aa)
N
C
Enzymatic active sites
13,17-18,36-38 aa
192 aa
231 aa
PP2Cc_domain superfamily (6-238 aa)
D
StkP (659 aa)
N
C
18-19,21-24,26,40,42,74,90-93,97
138,140-141,143,153-154 aa
ATP binding sites
PASTA
439-503 aa
PASTA
583-643 aa
11,36-37,66,71,76-79,91 aa
Dimer interface sites
PASTA
372-431aa
PASTA
514-575 aa
Activation loop sites
153-161,170-177 aa
PASTA superfamily domain
(345-659 aa)
22,97,99,136,138,140,157,174-177 aa
Substrate binding sites
18-19,21-24,26,40,42,74,90-93,97,99,136
138,140-141,143,153-154,157,174-177 aa
Enzymatic active sites
STKc_PknB superfamily domain
(11-271 aa)

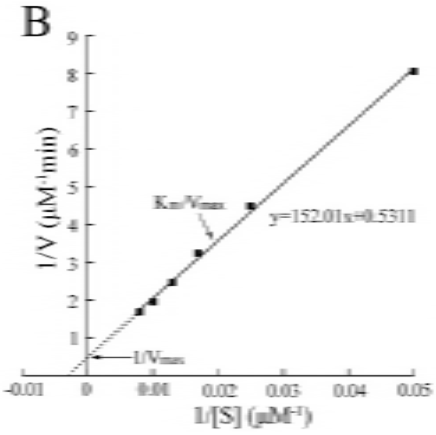

A
M 1 2 3 4 5
bp
2000-
1000-
500-
250-

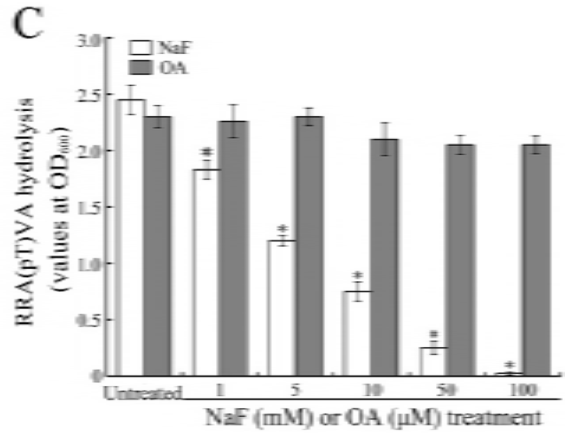

B
M 1 2 3 4 5
bp
2000-
1000-
500-
250-

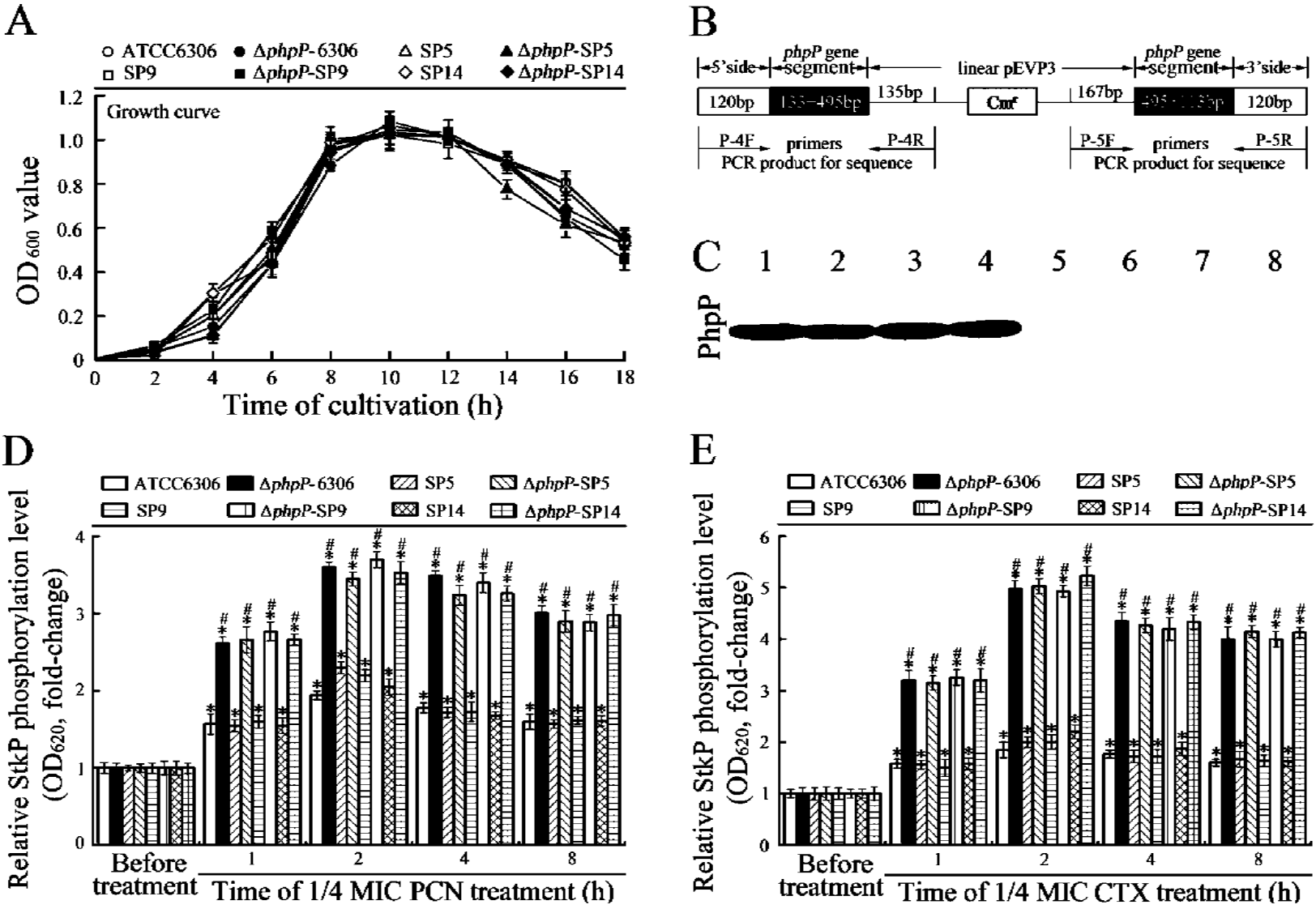

C
kDa
M 1 2 3
97.4-
66.2-
43.0-
31.0-
20.0-


D
kDa
M 1 2 3
97.4-
66.2-
43.0-
31.0-
20.0-


A
p-NPP
RRA(pT)VA
Hydrolysis
(values at OD405 or OD600)
3.0
2.5
2.0
1.5
1.0
0.5
0
Blank control
0.5
1.0
2.5
5.0
10
20
rPhpP (μg)


B
1/V (μM-1min)
9
8
7
6
5
4
3
2
1
Km/Vmax
y=152.01x+0.5311
1/Vmax
-0.01
0
0.01
0.02
0.03
0.04
0.05
1/[S] (μM-1)


C
NaF
OA
RRA(pT)VA hydrolysis
(values at OD600)
3.0
2.5
2.0
1.5
1.0
0.5
0
Untreated
1
5
10
50
100
NaF (mM) or OA (μM) treatment


A
ATCC6306
ΔphpP-6306
SP5
ΔphpP-SP5
SP9
ΔphpP-SP9
SP14
ΔphpP-SP14
Growth curve
OD600 value
Time of cultivation (h)
B
phpP gene segment
5'side
linear pEVP3
3'side
120bp
135bp
Cmr
167bp
P-4F
primers
P-4R
PCR product for sequence
P-5F
P-5R
C
PhpP
D
Relative StkP phosphorylation level (OD620, fold-change)
Before treatment
Time of 1/4 MIC PCN treatment (h)
E
Relative StkP phosphorylation level (OD620, fold-change)
Before treatment
Time of 1/4 MIC CTX treatment (h)