



A choline binding polypeptide of LytA inhibits the growth of *Streptococcus pneumoniae* by binding to choline in the cell wall

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Abstract

Streptococcus pneumoniae is a pathogen that mainly affects children and elderly individuals. The numerous serotypes and increased resistance to antibiotics make the treatment of pneumococcal infections sometimes difficult. Asymptomatic colonization is the main reservoir for *S. pneumoniae*, but no vaccine or antibiotic treatment is effective in eliminating this reservoir. Here, we show that a simulated choline binding polypeptide (ChBp) of LytA has antimicrobial activity against *S. pneumoniae*. ChBp showed specific antimicrobial activity against pneumococcal but not against non-streptococcal strains, and no cytotoxic effect was observed for 293t cell. The minimal inhibitory concentration (MIC) is between 10–25 µg/ml. In addition, we found ChBp functions by binding to the choline in the cell wall with a binding capacity between 3.25 and 7.5 × 10⁻⁶g/CFU. The binding cannot kill, but can inhibit the growth of pneumococcal cells for up to 12 h (50 µg/ml). Viable cells were decreased by 50% at 18 h, and eliminated at 36 h of incubation. These results show that ChBp has potential for the treatment of pneumococcal disease, or for eliminating nasopharyngeal colonization.

Introduction

Streptococcus pneumoniae is an opportunistic pathogen that causes diseases (sepsis, meningitis, and pneumonia) in people with reduced immunity such as young children and the elderly. *S. pneumoniae* colonizes the nasopharyngeal tract in adult and young children [1]. This carriage has been demonstrated as the main reservoir and as a source of horizontal spread of *S. pneumoniae*. *S. pneumoniae* is one of the most common bacterial respiratory pathogen; it is reported that pneumococcal infections cause approximately 500,000 deaths in children under the age of five, every year [2].

The elimination of nasopharyngeal colonization was considered as an effective way to reduce pneumococcal diseases [2]. However, no suitable intervention was available for this purpose. In recent years, protein conjugate vaccines (PCVs) have been used in several countries, such as PCV7, PCV10, PCV13, and PCV23 [3]. These vaccines are mainly composed of capsular polysaccharide (CPS), and provide strict serotype-specific protections [4]. PCVs have reduced the incidence of vaccine serotypes strains, but the incidence of serotype strains of those not included in the vaccine were increased [5]. Thus, more capsular polysaccharide types were included in the newly licensed 23-valent vaccine [6]. However, because of the large numbers of serotypes that are not covered by the vaccines and the resistance to multiple antibiotics [7], the treatment of *S. pneumoniae* remains a challenge.

Bacteriophages encoded lytic enzymes, such as Cpl-1 (lysozyme) Pal, Hbl and Ejl (amidase) have been proposed as agents against *S. pneumoniae* [8]. Cpl-1 and Pal have proven to be sufficient to hydrolyze the cell wall of *S. pneumoniae* [9]. However, because of practical considerations, like the high cost, these lytic enzymes have not been used in preventing *S. pneumoniae* infection or colonization. Sequence comparisons of these lytic enzymes revealed they have a similar choline binding domain (ChBD). Choline is an

The author Zhikun Zhang made the greatest contribution to this paper.

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ChBR1	T	G	W	Q	K	N	-	D	T	G	Y	W	Y	V	H	S	-	-	D	G	S	Y	P	K	
ChBR2	D	K	F	E	K	-	-	I	N	G	T	W	Y	Y	F	D	-	S	S	G	Y	M	L	A	
ChBR3	D	R	W	R	K	H	T	D	-	G	N	W	Y	W	F	D	-	N	S	G	E	M	A	-	
ChBR4	T	G	W	K	K	I	A	D	-	K	-	W	Y	Y	F	N	-	E	E	G	A	M	K	-	
ChBR5	T	G	W	V	K	Y	K	D	-	T	-	W	Y	Y	L	D	A	K	E	G	A	M	V	..	G
ChBp	T	G	W	V	K	-	-	D	N	G	S	W	Y	Y	L	N	-	L	S	G	Y	M	L	-	

Fig. 1 ChBp is designed with two hairpins (square) and a connecting loop (line). Five choline binding repeats (ChBRs) of LytA were compared by Mega5.0. The relative consistent amino acids (gray) were retained, and the variable sites on connecting loop are selected with linear amino acids for ChBp

integral part of the pneumococcal cell wall, it is present in teichoic acid [10], and teichoic acid is bound to the N-acetylmuramic acid residue of peptidoglycan by a phosphodiester bond. Choline is important in the life cycle of *S. pneumoniae*, the bacteria stops growing in medium without choline [11]. The choline-modified repeats serve as targets of murein hydrolases and surface proteins. The affinities of these enzymes to choline regulate their function efficiently and in an orderly manner [12].

In addition to exogenous lytic enzymes, the *S. pneumoniae* encodes several autolysins. LytA is such an amidase, which is known as the major pneumococcal autolysin [13]. LytA is composed of an N-terminal N-acetylmuramoyl L-alanineamidase domain, and a C-terminal ChBD with six choline binding repeats (ChBRs) [14]. The crystal structure of ChBD bound to LytA reveals the ChBRs are β -hairpins and form a left-handed superhelix. The secondary structures of ChBRs (repeats 1–5) are β -hairpins with a connecting loop. The choline binding sites are found in the hydrophobic interface between consecutive ChBR pairs. Three binding sites are located in the hairpins and one is in the connecting sequences; they form a shallow cavity to bind choline with aromatic rings [15].

In *S. pneumoniae*, the function of LytA is strictly controlled. Firstly, the sequence of LytA has no signal sequence, the large extracellular molecule of LytA can be detected only in stationary phase [16]. Secondly, *S. pneumoniae* cells are protected from lysis during exponential growth, but not in the stationary phase. This protection in exponential phase may be provided by the plasma membrane (PM)-associated penicillin-binding proteins (PBPs) [16]. In this study, we initially designed a small choline binding polypeptides (ChBp) which may be used in the rapid detection of *S. pneumoniae*. To our surprise, this polypeptide showed anti-*S. pneumoniae* activity. To our knowledge, this is the first report investigating the antibacterial function of ChBp.

Materials and methods

polypeptides design and synthesis

The sequences of the ChBRs were analyzed by MEGA5.0. The polypeptide was designed according to the conserved

sequence using the sequence deduced by Jalview as reference [12]. The amino acid before the choline binding site on the connecting loop is aromatic residues (Tyr), the rest of the residues were without aromatic rings (Fig. 1). The aromatic residues surrounding the site are known to be important in choline binding [15].

The polypeptide was synthesized by Guoping Pharmaceutical (China) using the bivalirudin-2-Cl-Resin method. The polypeptide was purified with C18 (10 μ m) chromatographic column. Quality analysis of the polypeptide was validated using ultra performance liquid chromatography (UPLC) and mass spectrometry (MS).

Bacterial strains

Six *Streptococcus pneumoniae* strains, including ATCC49619, were used for testing antimicrobial activity. The six pneumococcal clinical isolates were isolated in Sichuan, China. They are multidrug-resistant harboring PBP variants and tn2010 transposons. ATCC49619 is a reference culture isolated in the U.S. with no antibiotic resistance. Strains of *E. cloacae*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *A. baumannii*, and *S. aureus* were also used to determine the specificity of ChBp for pneumococci.

Antimicrobial activity assay

The MIC of ChBp against ATCC49619 was determined by using a broth dilution protocol [17]. Briefly, ATCC49619 was plated on blood agar plate at 37 °C for 16–20 h. Bacterial colonies were transferred to brain heart infusion medium (BHI) and cultured to a concentration about 1×10^6 CFU/ml. The grown medium was transferred into centrifuge tubes. Then, 50 μ l liquids containing ChBp at different concentrations were added to these tubes (final concentrations: 100, 50, 25, 12.5, 6.25, and 3.12 μ g/ml), the tubes were incubated for another 16–20 h at 37 °C. The OD595 of cultured mediums were measured at 200 μ l in 96-well plates every 6 h. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of polypeptide that completely inhibited growth.

Antimicrobial specificity to *S. pneumoniae*

Six isolates of *S. pneumoniae* were cultured with BHI in 1.5 ml centrifuge tubes containing 25 μ g/ml ChBp at 37 °C for 14–16 h (using BHI cultured as control). The final concentrations of these growth media were measured at OD595. The antimicrobial activity of ChBp (25 μ g/ml) was also tested against *E. cloacae*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *A. baumannii* and *S. aureus*. The method was the same as described above.

Binding capacity of ChBp to *S. pneumoniae*

The binding ability of ChBp to choline was first determined by incubation with DEAE Sefinose (Sangon, China). DEAE is an analog of choline [18]. In brief, 50 μ l ChBp (500 μ g/ml) was mixed with 10 μ g DEAE Sefinose, and incubated at 37 °C for 30 min. The medium was centrifuged for 3 min at 4000 \times g, and then the supernatant was analyzed by tricine-PAGE using ChBp (500 μ g/ml) as control.

The binding capacity of ChBp to *S. pneumoniae* was measured by incubating bacterial cells with diluted ChBps. 400 μ l cultured cells were harvested by centrifugation and the cells were incubated with 40 μ l ChBp (4, 2, and 1 mg/ml, 500, 250, 125, 62.5, and 32 μ g/ml). The number of cells was counted by overnight culture on blood agar plate with 10³ times dilution.

Inhibition recovery and time killing assay

The recovery assay was conducted by adding ChBp (25 μ g/ml, \sim 1 \times MIC) to suspensions of *S. pneumoniae* from logarithmic cultures grown at 37 °C. The ChBp in the medium was removed by centrifugation 1 h post incubation, followed by addition of an equal volume of fresh medium. The optical densities (OD₅₉₅) of the growing cultures were measured using a microplate reader. In addition, 200 μ l of logarithmic growth suspension was supplemented with ChBp of 50 μ g/ml. 20 μ l suspension was removed at various times (1, 2, 4, 8, 12, 18, 24, and 36 h), and plated on blood agar plate immediately. The carry-over of ChBp on to the plates was low (0.0128 μ g/cm²). The colony forming units in the treated suspensions were calculated after incubation at 37 °C for 16–20 h.

Cytotoxicity against 293t

293t cells were cultured in Dulbecco's modified Eagle's medium, high glucose (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were seeded into six wells of 96-well plates at about 2×10^4 cells (100 μ l) per well; the three experimental wells were supplemented with 50 μ g/ml ChBp. All the plates were incubated at 37 °C under 5% CO₂ for 24 h and cell growth was visualized using an inverted microscope.

Results

Polypeptides design and synthesis

In order to bind choline, the polypeptide was designed with all four choline binding residues. The designed sequence

may fold independently and form a choline binding cavity (for structure of ChBp see ref. 15). The choline binding polypeptide was designed as TGWVKDNGSWYYLNLNLS-GYML (Fig. 1). The final purity of the synthesized polypeptide was about 95.050%; the UPLC and MS results were shown in figure S1.

Antimicrobial activity assay

Antimicrobial activity of ChBp was first tested by the broth dilution assay. ChBp showed antibacterial activity against ATCC49619; the MIC of ChBp was between 10 and 25 μ g/ml (4.2–10.5 μ M). At ChBp concentrations of 10 μ g/ml, which is lower than the MIC, it was still possible to reduce the maximum OD of ATCC49619 (Fig. 2).

ChBp was designed to bind choline, thus it is expected to be active only against *S. pneumoniae*. To test the specific antimicrobial activity of ChBp, six pneumococcal clinical isolates together with six non-streptococcal strains were treated with 25 μ g/ml ChBp at the beginning of bacterial cultures, using untreated ones as control. As shown, the final ODs of controls were different, which is mainly influenced by the expression of capsular polysaccharide. As expected, the growth of pneumococcal isolates were suppressed to different degrees, and the growth of non-streptococcal strains were not affected (Fig. 3). These results indicated that the ChBp inhibits growth by binding to choline. In addition, the effects on the growth of *S. pneumoniae* isolates were different, which is probably influenced by the type and quantity of capsular polysaccharide on the cell wall. The growth of SWU09 with low expression of capsular polysaccharide is entirely inhibited. In addition, no cytotoxicity was found in the culture of 293t system supplemented with 50 μ g/ml ChBp (Fig. S2).

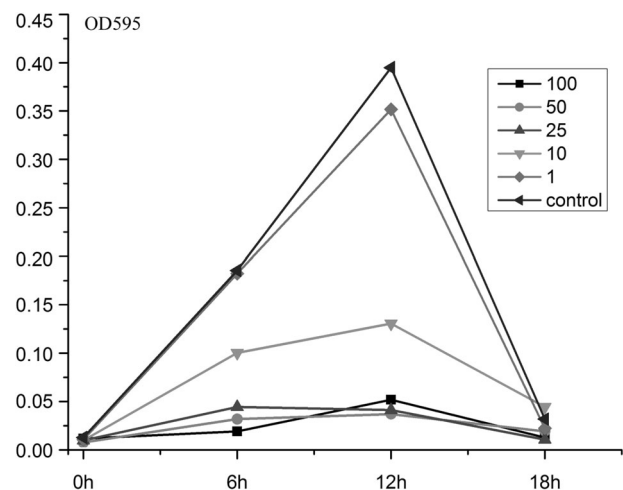


Fig. 2 Antimicrobial activity of ChBp against ATCC49619, a simply constructed growth curve of ATCC49619 in liquid culture system supplemented with ChBp of different concentrations (μ g/ml)

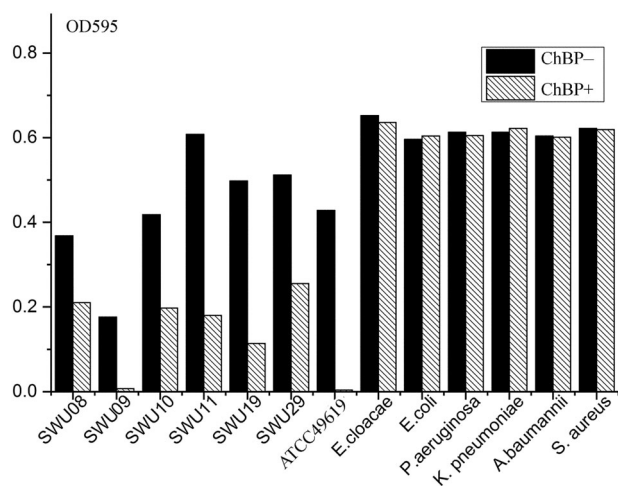


Fig. 3 Effects of ChBp on antimicrobial growth. Clinical *S. pneumoniae* strains (SWUs) and non-streptococcal strains were cultured in medium supplemented with (+) or without (-) ChBp (25 μ g/ml). The final concentrations of these mediums were measured at 14–16 h post incubation

Binding ability of ChBp

ChBp was incubated with DEAE Sefinose in order to validate the binding ability to choline. As shown, ChBp was completely absorbed by DEAE (Fig. 4a), which indicates that it could bind to choline molecule. In order to further calculate the binding capacity of ChBp, pneumococcal cells were incubated with diluted ChBps. ChBps of 62.5 but not 125 μ g/ml (40 μ l) was completely absorbed by 4×10^6 CFU/ml (0.4 ml) cells (Fig. 4b). The calculated binding capacity was between 1.56 and 3.12×10^{-6} μ g/CFU, which equals to 3.96 – 7.92×10^8 ChBp per bacterial cell.

ChBp inhibits the growth of pneumococcal cells

The binding of ChBp likely results in the inhibition of cell growth, but this may not have direct bactericidal activity. The growth of ATCC49619 quickly recovered after removing ChBp, and the final concentration became the same as the untreated control. In addition, liquid cultures with 50 μ g/ml of ChBp were plated on blood agar plate at different time post-incubation. Compared with untreated cultures, no significant differences were detected in colony forming units up to 12 h of exposure. The colony forming cells were down to 50% at about 18 h of incubation; no colony forming cells were detected at 36 h (Fig. 5). These results indicated that long term exposure of ChBp to pneumococcal cells may activate the autolysis of *S. pneumoniae*, which is essential for killing of pneumococcal cells.

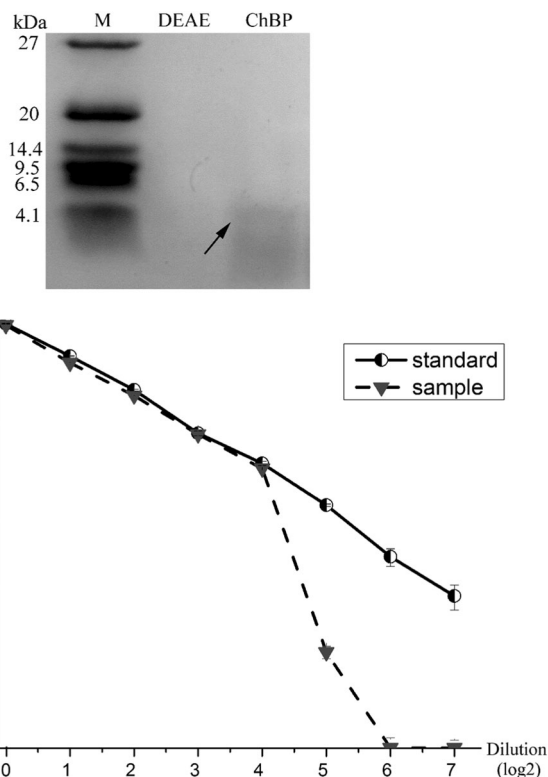


Fig. 4 a Tricine-PAGE of ChBp before and after incubation with DEAE-Sefinose. DEAE-Sefinose was removed by centrifugation post incubation. The supernatant was detected by Tricine-PAGE; as shown ChBp was completely absorbed by DEAE-Sefinose. **b** Concentrations of diluted ChBps (initial concentration: 4 mg/ml) before and after incubation with pneumococcal cells. Pneumococcal cells were removed by centrifugation post incubation. As shown, 40 μ l ChBp of 62.5–125 μ g/ml was completely absorbed by 1.6×10^6 ATCC49619 cells

Discussion

LytA is the only N-acetylmuramoyl L-alanine amidase which exists in *S. pneumoniae*. Since its discovery in 1970s, the function of N-terminal amidase has been well studied. It cleaves the peptidoglycan by hydrolyzing the lactyl-amide bond that links the polypeptides and the glycan strands. During the logarithmic growth only a small fraction of LytA is associated with the extracellular cell wall, and this binding plays a role in cell division [19]. The extracellular LytA gradually increases as a result of cell lysis at stationary phase. This ability to induce autolysis was considered as the function of the N-terminal amidase. However, independent binding experiments of the N-terminal amidase and ChBD reveal that the amidase is restricted to nascent peptidoglycan, which is rich only in the logarithmic period [16, 20]. In this study, we have demonstrated that the simulated choline binding polypeptides of LytA shows growth inhibitory functions. Thus, the binding of

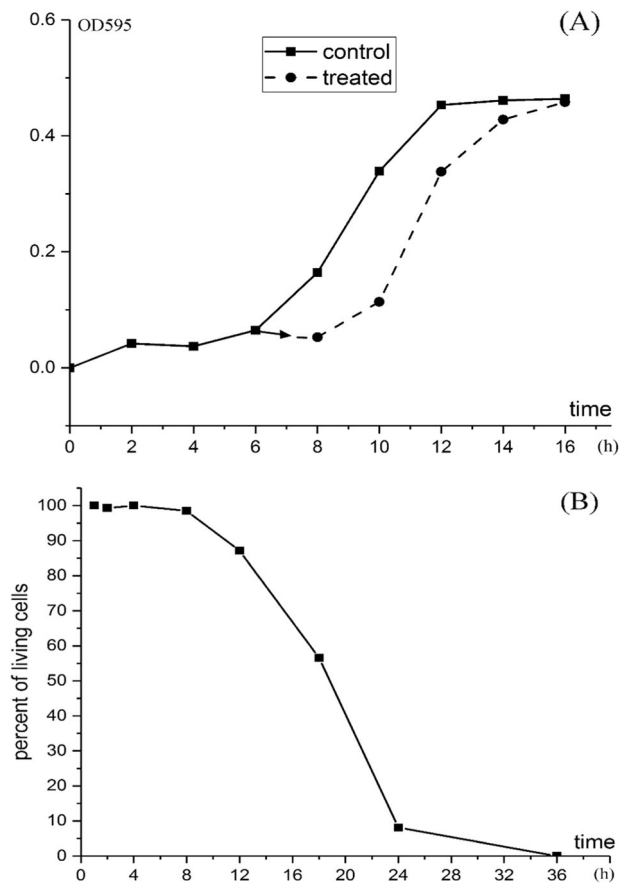


Fig. 5 **a** Growth recovery assay of ATCC49619. ChBp of 25 µg/ml was added to the culture medium of ATCC49619 at 6 h, and removed 1 h post incubation (indicated by arrow). The growth of ATCC49619 was recovered after the removal of ChBp. **b** Time-killing assay of ChBp. ATCC49619 cells were incubated with ChBp of 50 µg/ml (>2× MIC). The living cells were down to 50% at about 18 h and wiped out at 36 h post incubation

C-terminal ChBD of LytA may first inhibit the growth of *S. pneumoniae*, and then induce autolysis by the N-terminal amidase.

S. pneumoniae also encodes several other choline binding proteins, such as LytB, LytC, CbpD, and CbpF. Some of these proteins are exported by the signal peptide pathway, but they do not show significant impact on the growth of *S. pneumoniae*. The choline-modified teichoic acids are presented on the cell wall with different repeats [10]; the structure of ChBDs may restrict its binding to certain cell wall structures. The hook-shaped conformation restricts the LytC to hydrolyze non-cross-linked PG chains [21]. On the other hand, the number of ChBRs may increase the interspace diffuse resistance to reach cholines in the cell wall, while the ChBDs may reach the binding site under certain circumstance. ChBD of LytA showed the ability to bind to choline, and bound to the whole cell wall of *S. pneumoniae* [14]. But the autolysis activity of LytA is only activated when binding to choline occupies all of the

hydrophobic interfaces of the consecutive hairpins [22], which is easy to achieve without PM-associated PBPs on the cell wall.

As noted, ChBp was able to bind the cell wall via the choline binding activity (Fig.4). But the role of ChBp in antibacterial function is not clear. In liquid culture system, growth stops soon after addition of high concentrations of ChBp (not shown) with no accompanying morphological changes. However, it was noted that the cells were intact but stained gram-negative with ChBp of 10 µg/ml (<MIC) (Fig.S3) indicating cell wall changes. Together with these results, we infer that the binding of ChBp blocked cell wall synthesis. In gram-positive bacteria teichoic acid is synthesized in the cytoplasm, translocated across the cytoplasmic membrane, and covalently linked to nascent peptidoglycan [23]. PBP2b and PBP2a were found located at the equatorial cell wall during growth in *S. pneumoniae* for peripheral peptidoglycan synthesis [24]. The binding of ChBp to peripheral peptidoglycan may influence the function of PBPs, resulting in the inefficient polymerization of the newly synthesized units and incorporation into the peptidoglycan [25].

The function of ChBp provides a target of self-encoded surface-binding domains to help in the design of strain-specific drugs. In other bacterial species, the GW module in *Listeria* and repetitive sequence in *Staphylococcus epidermidis* are both surface binding structures that could function similar to that of ChBp in pneumococcus [26, 27]. In recent years, the extensive use of antibiotics has led to the increased resistance of pneumococci to multiple antibiotics. The genome of *S. pneumoniae* is highly recombinogenic, which enables it to harbor drug-resistant genes. Interestingly, the ChBp described in this study may serve as a new method in the treatment of multidrug-resistant pneumococci, and ChBp could be used for eliminating or decreasing the nasopharyngeal colonization of pneumococci in younger children.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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