

## A novel chimeric phage lysin with high *in vitro* and *in vivo* bactericidal activity against *Streptococcus pneumoniae*

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Received 31 October 2014; returned 2 December 2014; revised 9 December 2014; accepted 29 January 2015

**Objectives:** *Streptococcus pneumoniae* is becoming increasingly antibiotic resistant worldwide and new antimicrobials are urgently needed. Our aim was new chimeric phage endolysins, or lysins, with improved bactericidal activity by swapping the structural components of two pneumococcal phage lysozymes: Cpl-1 (the best lysin tested to date) and Cpl-7S.

**Methods:** The bactericidal effects of four new chimeric lysins were checked against several bacteria. The purified enzymes were added at different concentrations to resuspended bacteria and viable cells were measured after 1 h. Killing capacity of the most active lysin, Cpl-711, was tested in a mouse bacteraemia model, following mouse survival after injecting different amounts (25–500 µg) of enzyme. The capacity of Cpl-711 to reduce pneumococcal biofilm formation was also studied.

**Results:** The chimera Cpl-711 substantially improved the killing activity of the parental phage lysozymes, Cpl-1 and Cpl-7S, against pneumococcal bacteria, including multiresistant strains. Specifically, 5 µg/mL Cpl-711 killed  $\geq 7.5$  log of pneumococcal R6 strain. Cpl-711 also reduced pneumococcal biofilm formation and killed 4 log of the bacterial population at 1 µg/mL. Mice challenged intraperitoneally with D39\_IU pneumococcal strain were protected by treatment with a single intraperitoneal injection of Cpl-711 1 h later, resulting in about 50% greater protection than with Cpl-1.

**Conclusions:** Domain swapping among phage lysins allows the construction of new chimeric enzymes with high bactericidal activity and a different substrate range. Cpl-711, the most powerful endolysin against pneumococci, offers a promising therapeutic perspective for the treatment of multiresistant pneumococcal infections.

**Keywords:** *S. pneumoniae*, antimicrobial therapy, bacteriophages, bacterial biofilm, animal infections

### Introduction

*Streptococcus pneumoniae*, the pneumococcus, is a Gram-positive bacterium usually found in asymptomatic nasopharyngeal carriage, but is also the leading cause of many infections worldwide, ranging from very common and usually mild clinical conditions, such as acute otitis media and rhinosinusitis, to less frequent but potentially life-threatening invasive diseases, such as sepsis, community-acquired pneumonia and meningitis.<sup>1</sup> Classical treatment of these infections has been the use of certain well-tolerated drugs, especially  $\beta$ -lactam antibiotics, which significantly changed

the outcome of pneumococcal diseases and substantially reduced their clinical and economic impact. Unfortunately, in recent years the lack of new antibiotics and the emergence of MDR bacteria have led to a healthcare crisis frequently associated with the so-called 'post-antibiotic era'. This situation has provoked the US CDC to call for an aggressive and immediate action to halt the spread of drug-resistant pathogens.<sup>2</sup> In this context, phage therapy is becoming one of the most promising alternative weapons to fight dangerous multiresistant pathogens, with the use of not only isolated virions but also some of their products, like endolysins. These specialized enzymes are peptidoglycan hydrolases synthesized as

part of the tightly controlled late genes that provoke the lysis of the bacterial cells for phage progeny release. Endolysins have been shown to be very effective in killing a variety of Gram-positive bacteria when added exogenously as purified proteins.<sup>3</sup> Although the typical architecture consists of a two-domain structure, in which the N-terminal part is responsible for the catalytic activity (catalytic domain or CD) and the C-terminal part functions as a cell wall-binding domain (CWBD),<sup>4</sup> some peptidoglycan hydrolases possess multiple hydrolytic domains.<sup>5,6</sup>

Cpl-1<sup>7</sup> and Cpl-7<sup>8</sup> lysozymes are encoded by the virulent Cp-1 and Cp-7 pneumococcal bacteriophages, respectively, belonging to the *Podoviridae* family.<sup>9</sup> These enzymes are very similar in their CDs but differ completely in their CWBDs. The N-terminal CD of both enzymes belongs to the GH-25 family of glycosylhydrolases sharing 85.6% sequence identity (95.2% similarity). This domain folds into an irregular ( $\beta/\alpha$ )<sub>5</sub> $\beta$ <sub>3</sub> barrel, characteristic of lysozymes of the same family.<sup>7,10–12</sup> In its CWBD, Cpl-1 displays the typical choline-binding repeats that allow anchoring to the phosphocholine residues of (lipo)teichoic acids,<sup>7</sup> whereas Cpl-7 contains three perfect repeats forming three tandemly arranged helical bundles that recognize the cell wall in a choline-independent manner.<sup>8,13</sup> Linkers of both endolysins are different in amino acid sequence and length, suggesting distinct interactions between their respective modules, but both are highly hydrophilic and share a highly charged N-terminal motif. Recently, it has been demonstrated that a synthetic variant of Cpl-7, named Cpl-7S, was capable of improving the bactericidal effect of Cpl-7 not only against pneumococci but also against other important pathogens like *Streptococcus pyogenes* and *Enterococcus faecalis*.<sup>14</sup>

In this work, we have constructed chimeric proteins by shuffling the structural elements, i.e. the two functional domains and the linkers, from Cpl-1 and Cpl-7S endolysins, giving rise to four new enzymes. One of them, Cpl-711, turned out to be clearly more active than Cpl-1 in killing several pneumococcal strains grown in either planktonic or sessile (biofilm) conditions. The increased lethal action of Cpl-711 has been validated in a mouse model of bacteraemia, confirming that this new chimeric lysin is the most potent enzyme against pneumococci described so far.

## Materials and methods

### Ethics statement

All experiments were performed under strict adherence to the NIH guidelines for the ethical treatment of animals. Signs of infection were monitored three times daily throughout the experimental time course. Moribund mice were euthanized by cervical dislocation under isoflurane anaesthesia. Approval for these studies was granted by the Rockefeller University Institutional Animal Care and Use Committee (Protocol: 11005). Experiments were carried out at the Rockefeller University's animal housing facility, an AAALAC-accredited research facility, with all efforts made to minimize animal suffering.

### Bacterial strains, media and growth conditions

The bacterial strains used in this study are listed in Table S1 (available as Supplementary data at JAC Online). Pneumococcal strains were grown at 37°C in C medium supplemented with yeast extract (0.8 mg/mL; Difco Laboratories) (C+Y)<sup>25</sup> or on tryptic soy agar plates containing 5% defibrinated sheep blood.<sup>26</sup> The other Gram-positive bacteria were grown in

Todd–Hewitt broth containing 0.5% yeast extract (THY) at 37°C, without shaking. *Escherichia coli* strains, used for gene cloning and producing recombinant proteins, were grown in LB medium with shaking at 37°C.

### Synthesis and cloning of chimeric genes

The plasmids and oligonucleotides used in this work are listed in Table S1. The synthetic DNA fragments encoding Cpl-117, Cpl-177 and Cpl-711 were purchased from ATG:Biosynthesis (Merzhausen, Germany) as *E. coli* codon-optimized pUC derivatives. Codon optimization was performed using the web server *Optimizer* (<http://genomes.urv.es/OPTIMIZER>). The NdeI + PstI-digested fragments carrying the chimeric genes were sub-cloned into the pT7-7 expression vector, previously treated with the same restriction enzymes, and the resulting plasmids pTRD760, pTRD761 and pTRD762 were transformed into *E. coli* DH10B. *cpl-771* was constructed by a two-step process. The first required the incorporation of a conservative mutation in *cpl-7S* at the end of the linker using a modified PCR-driven overlap extension technique.<sup>27</sup> This mutation consists of the addition of a HindIII restriction site by two independent PCRs using A + B and C + D primers, respectively, and pTRD750 as template. Primers B and C are partially complementary and contain the HindIII restriction site. The resulting PCR products and the primers A and D were used for a third PCR to amplify *cpl-7S* that included the desired mutation. Parameters for the two first independent PCRs were: 94°C for 30 s (denaturation), 65°C for 30 s (annealing) and 72°C for 2 min (extension). The parameters for the third PCR were: 94°C for 30 s (denaturation), 65°C for 30 s (annealing) and 72°C for 4 min (extension). Then the modified *cpl-7S* was digested with NdeI and ClaI and cloned into pT7-7 treated with the same enzymes, rendering pHP200. The second step consisted of the fusion of the part of the *cpl-1* encoding CWBD of Cpl-1 (CWBD<sub>1</sub>) to that coding for the CD and linker region of Cpl-7S. To accomplish this, the portion of *cpl-1* encoding CWBD<sub>1</sub> was amplified from pCIP100 using E + F primers containing HindIII and ClaI restriction sites. PCR parameters used were: 94°C for 30 s (denaturation), 65°C for 30 s (annealing) and 72°C for 2 min (extension). The HindIII + ClaI-digested PCR product was cloned into pHP200, previously digested with the same enzymes, resulting in pHP771. The composition of chimeric genes was confirmed by DNA sequencing (Secugen, Madrid, Spain).

### Production and purification of chimeric proteins

For overproduction of chimeric enzymes, transformed *E. coli* BL21(DE3) cells with pTRD760, pTRD761, pTRD762 or pHP771 were incubated in LB medium containing ampicillin (0.1 mg/mL) to an OD<sub>600</sub> of ~0.6. IPTG (0.1 mM) was then added, and the incubation was continued overnight at 30°C. Cells were harvested by centrifugation (10000 g, 5 min), resuspended in 20 mM sodium phosphate buffer (pH 6.0) (Pi buffer), disrupted in a French pressure cell and centrifuged (50000 g, 45 min) to remove cell debris. Streptomycin sulfate (Sigma; 2%, w/v) was added to the protein extract, incubating for 15 min at 4°C with slow stirring to facilitate DNA precipitation. The insoluble fraction was removed by ultracentrifugation (50000 g, 45 min) at 4°C. Cpl-711 and Cpl-771 proteins were purified by affinity chromatography using DEAE-cellulose, as previously described.<sup>24</sup> Cpl-117 and Cpl-177 were purified following the procedure reported for Cpl-7.<sup>8</sup> The purity of the isolated proteins was checked using 10% SDS-PAGE and MS (MALDI-TOF). Protein concentrations were determined spectrophotometrically using the corresponding molar absorption coefficients at 280 nm. Before use, all proteins were equilibrated by dialysis in Pi buffer.

### In vitro cell wall activity assay

Purified enzymes were checked *in vitro* for cell wall degradation using [*methyl*-<sup>3</sup>H]choline-labelled pneumococcal cell walls as the substrate and following a previously described method.<sup>28</sup> Briefly, 10 µL of enzyme

at the appropriate dilution was added to the reaction sample containing 240  $\mu$ L of Pi buffer and 10  $\mu$ L of radioactively labelled cell walls ( $\sim 35\,000$  cpm), as described previously.<sup>29</sup> After 15 min of incubation at 37°C, the reaction was stopped by adding 10  $\mu$ L of formaldehyde (37%, v/v) and 10  $\mu$ L of BSA (4%, w/v). The pellet was removed by centrifugation (12 000 g, 15 min), and the enzymatic activity was quantified by measuring the radioactivity in 200  $\mu$ L of the supernatant with a liquid scintillation counter (LKB Wallac). One unit of enzymatic activity (U) was defined as the amount of enzyme that catalyses the hydrolysis (solubilization) of 1  $\mu$ g of cell wall material in 10 min.<sup>28</sup>

### MIC determinations

MICs of Cpl-711, Cpl-771, Cpl-117 and Cpl-177 were determined by the microdilution method approved by the CLSI (2006) using CAMHB supplemented with 5% lysed horse blood. Modal values from three separate determinations were considered. Pneumococcal strain ATCC 49619 was used as a quality control strain for susceptibility testing (<http://www.lgcstandards-atcc.org/Products/All/49619.aspx>).

### Bacteriolytic and bactericidal assays

Bacteria were grown to logarithmic phase to an OD<sub>550</sub> of about 0.3, and then cultures were centrifuged and washed twice with PBS [137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub> and 1.8 mM KH<sub>2</sub>PO<sub>4</sub> (pH 6.0)], and the final OD<sub>550</sub> was adjusted to  $\sim 0.6$  in PBS, using a Helios Epsilon spectrophotometer (Thermo Scientific). Afterwards, resuspended bacteria were transferred into plastic tubes, and the tested enzyme was added. Samples were incubated at 37°C for 1 h, and the turbidity decrease (OD<sub>550</sub>) was measured at selected intervals. Controls replacing the added enzyme with Pi buffer were always run in parallel. Measurement of viable cells was carried out in C+Y or blood agar plates. For each sample, a 10-fold dilution series was prepared in PBS, and 10  $\mu$ L of each dilution was plated. Colonies were counted after overnight incubation at 37°C.

### Biofilm assays

Pneumococcal biofilms were produced using a P046 strain that is deficient in LytA and LytC autolysins.<sup>18</sup> Biofilms were grown in Costar 3595 96-well PST microtitre plates (Corning, New York, USA).<sup>18,30</sup> Briefly, cells were grown in C+Y medium to an OD<sub>595</sub> of 0.5–0.6, sedimented by centrifugation, resuspended in C+Y medium and aliquots of 200  $\mu$ L containing  $4.25 \times 10^6$  cfu were dispensed into each well in two microtitre plates. After 16 h of incubation at 34°C, the biofilm formed was washed with PBS and treated for 2 h at 37°C with either Pi buffer or different concentrations of enzymes in Pi buffer. Finally, one of the plates was stained with a 0.2% (w/v) crystal violet solution for 15 min and then washed three times with distilled water to remove non-adherent bacteria. After solubilizing the biofilm in 95% ethanol (200  $\mu$ L per well), the OD<sub>595</sub> was determined using a VersaMax microplate absorbance reader (Molecular Devices). The other plate was used to determine viability. To do this, a 10-fold dilution series was prepared in PBS and 10  $\mu$ L of each dilution was plated in blood agar plates. Colonies were counted after overnight incubation at 37°C. For observation by confocal laser scanning microscopy (CLSM), biofilms were grown at 34°C for 16 h on glass-bottomed WillCo-dish dishes (WillCo Wells). Then, they were washed with PBS and treated for 2 h at 37°C with either Pi buffer or different amounts of the corresponding enzymes. Biofilms were then washed three times with PBS to remove cell debris, and stained with the LIVE/DEAD BacLight bacterial viability kit L-13152 (Invitrogen-Molecular Probes) for monitoring the viability of bacterial populations. Cells with a compromised membrane—considered dead or dying—stained red, whereas those with an intact membrane stained green.<sup>18</sup> The biofilms were observed at  $\times 63$  magnification using a Leica TCS-SP5-AOBS-UV CLSM equipped with an argon ion laser. The

excitation/emission maxima were around 488/500 to 561 nm. Images were acquired using LCS software (Leica). Projections were obtained through the x–y plane (individual scans at 0.5  $\mu$ m intervals) and x–z plane (images at 5  $\mu$ m intervals).

### Murine bacteraemia model

The infection model was based on methods described previously,<sup>31,32</sup> using 4–6-week-old female BALB/c mice (weight range, 15–20 g) obtained from Charles River Laboratories (Wilmington, MA, USA). Briefly, after a period of acclimatization, mice were injected intraperitoneally (ip) with 0.5 mL of a  $5.5 \times 10^5$  cfu/mL suspension of *S. pneumoniae* strain D39\_IU cells. Bacterial inoculation titres were calculated by serial dilution and plating onto Columbia blood agar plates for each experiment. To confirm the bacteraemic state of mice at the time of treatment (i.e. 1 h after bacterial challenge), multiple organs, including spleen, liver, kidney and heart/blood, were removed aseptically from three mice, homogenized and plated for bacterial counts. One hour post-infection, the remaining animals were divided into four to five treatment groups per lysin and were injected ip with 0.5 mL containing 500, 200, 50 or 25  $\mu$ g of either Cpl-711 or Cpl-1 lysins, or PBS buffer as control. The survival rate for each experimental group was monitored every 12 h for the first 24 h and then every 24 h for up to 7 days post-infection. The results from four independent experiments were combined to evaluate a total of 20 mice for controls and 17 mice for each lysin-treated group.

### MS

Purified samples of chimeric proteins were analysed by MALDI-TOF, as described elsewhere.<sup>33</sup> A grid voltage of 93%, a 0.1 ion guide wire voltage, and a delay time of 350 ns in the linear positive ion mode were used. External calibration was performed with carbonic anhydrase (29024 Da) and enolase (46672 Da) from Sigma, covering an *m/z* range of 16000–50000 units.

### Circular dichroism

Circular dichroism spectra were recorded at 20°C in a J-810 spectropolarimeter (Jasco Corporation) equipped with a Peltier cell holder. Measurements were performed in 1 mm and 1 cm path-length cells (far- and near-circular dichroism spectra, respectively) using the experimental conditions previously described.<sup>13</sup> Buffer contribution was subtracted from protein spectra and corrected data were converted into mean residue ellipticities using the corresponding average molecular mass per residue. Choline titration curves were obtained by measuring the increase in ellipticity at 225 nm as a function of the ligand concentration. The dissociation constants were calculated by fitting the Hill equation to the experimental curves.<sup>34</sup>

### Analytical ultracentrifugation

Sedimentation velocity experiments were run at 45000 rpm using cells with double sector Epon-charcoal centrepieces. Differential sedimentation coefficients were calculated by least-squares boundary modelling of the experimental data with the program SEDFIT and corrected to *s*<sub>20,w</sub> values.<sup>35</sup> Sedimentation equilibrium experiments were carried out at different rotor speeds, from 10000 to 17000 rpm, as described previously,<sup>34</sup> and weight-average molecular weights were calculated using the Heteroanalysis program, version 1.1.2 (<http://www.biotech.uconn.edu/auf/>). All measurements were performed in Pi buffer at 20°C using protein concentrations of  $\sim 0.2$  mg/mL in an Optima XL-A analytical ultracentrifuge (Beckman Coulter) with an AN50-Ti rotor. The ratio of the frictional translational coefficients of the protein particle and the equivalent rigid sphere sedimenting like the protein (*f/f*<sub>0</sub>) and Stockes's radio (*R*<sub>s</sub>), related

to the protein hydrodynamic shape, were calculated from the protein molecular masses and  $s_{20,w}$  values using the partial specific volumes and hydration coefficients estimated from the amino acid sequence with the SEDNTERP program.<sup>36</sup>

Modelling of the Cpl-711 structure

The three-dimensional model of the Cpl-711 chimera was built automatically using the Cpl-1 structure as a template (PDB access code; 1H09) with the Swiss-Model server in the alignment mode (<http://swissmodel.expasy.org>). Model quality was stereochemically and energetically validated with Procheck<sup>37</sup> and VERIFY 3D,<sup>38</sup> respectively, comparing well with Cpl-1. Structure figures were made with PYMOL (Schrödinger LLC, Cambridge MA, USA).

Statistical analysis

All *in vitro* results are representative of data obtained from repeated independent experiments, and each value represents the mean  $\pm$  SD for three or four replicates. Statistical analysis was performed using the two-tailed Student *t*-test (for two groups), whereas analysis of variance (ANOVA) was chosen for multiple comparisons. For all *in vivo* data, the log-rank (Mantel–Cox) and Gehan–Breslow–Wilcoxon tests were used to draw, analyse and compare the survival curves. GraphPad InStat version 5.0 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis.

Results

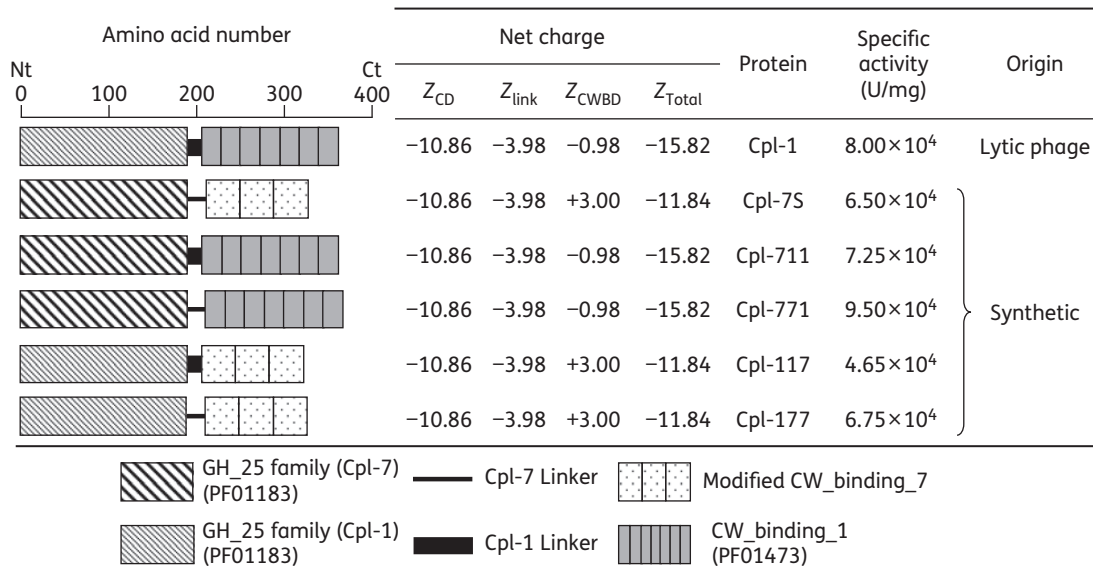
Design of chimeric proteins

With the aim of creating endolysins with improved bactericidal activity against pneumococcal and non-pneumococcal species when added exogenously to bacterial cultures, we decided to shuffle and combine the three structural elements (CD, linker and CWBD) of Cpl-1 and Cpl-7S lysozymes. Cpl-1 is strictly dependent on the presence of choline residues in teichoic acid to fully hydrolyse the bacterial cell wall, whereas Cpl-7S is an

engineered variant of the WT Cpl-7 (the exact construct is described below), which degrades susceptible cell walls in a choline-independent manner. Cpl-7S has an improved antimicrobial activity compared with Cpl-7, due to the inversion of the net charge of the CWBD.<sup>14</sup> Figure 1 shows a schematic representation of the four synthetic chimeric proteins (Cpl-711, Cpl-771, Cpl-117 and Cpl-177) and their parental proteins (Cpl-1 and Cpl-7S).

Cloning, overproduction and purification of the new enzymes were carried out as detailed in the Materials and methods section. The three-number code of these chimeric lysozymes corresponds to the origin of the CD (first number), linker region (second number) and CWBD (third number) of both enzymes: 1 from Cpl-1 and 7 from Cpl-7S. Thus, Cpl-711 and Cpl-771 contain the CD of Cpl-7S at the N-terminal region, and the CWBD<sub>1</sub> at the C-terminal region. The only difference between them lies in the origin of the linker connecting both domains, coming from either Cpl-1 (in Cpl-711) or Cpl-7S (in Cpl-771). Following the same rule, proteins Cpl-117 and Cpl-177 share the CD of Cpl-1 and the CWBD of Cpl-7S (CWBD<sub>7S</sub>), the difference being in the linkers coming from either Cpl-1 (in Cpl-117) or Cpl-7S (in Cpl-177).

The amino acid substitutions introduced in Cpl-7S with respect to Cpl-7 (five residues in each one of the three CW\_7 repeats of the CWBD<sub>7</sub>) led to a drastic change in total charge of the domain ( $Z_{CWBD}$ ) from  $-14.93$  to  $+3.00$ .<sup>14</sup> It is worth noting that, despite the sequence differences, the net charges of the CDs ( $Z_{CD}$ ) of Cpl-1 and Cpl-7S are identical, as are those of their linkers ( $Z_{link}$ ), meaning that the differences in the net charge of the four chimeric proteins reside in the CWBD. Therefore, the chimeras Cpl-711 and Cpl-771 present a net charge identical to that of Cpl-1, whereas those of chimeras Cpl-117 and Cpl-177 are identical to that of Cpl-7S (Figure 1). Charge has been demonstrated to be a key factor in the optimal lytic activity of endolysins when added exogenously.<sup>14,39</sup> Nevertheless, the specific activities of the four purified chimeras and parental proteins were very similar,



**Figure 1.** Schematic representation and characteristics of chimeric and parental murein hydrolases. The synthetic chimeric proteins Cpl-711, Cpl-771, Cpl-117 and Cpl-177, and their parental proteins Cpl-1 and Cpl-7S, are shown together with their origin, specific activity (U/mg) and net charge. Nt, N-terminal; Ct, C-terminal;  $Z_{CD}$ , catalytic domain charge;  $Z_{link}$ , linker charge;  $Z_{CWBD}$ , cell wall-binding domain charge;  $Z_{Total}$ , total charge.



ranging from  $4.65 \times 10^4$  to  $9.50 \times 10^4$  U/mg, when tested on radioactively labelled pneumococcal cell walls (Figure 1).

### Bactericidal activity of chimeric enzymes against pneumococcal strains

The bacteriolytic/bactericidal activity of the four chimeras was tested against several pneumococcal strains. Initially, the antibacterial activity was analysed against *S. pneumoniae* R6 cells using 5 µg/mL of each enzyme. Experiments were performed measuring the OD<sub>550</sub> for 60 min and plating for bacterial counting. Cpl-711 and Cpl-771, which possess the CWBD<sub>1</sub> characteristic of a choline-dependent enzyme, showed dramatic bacteriolytic effects with a rapid decrease in cell density, similar to that observed with Cpl-1. On the other hand, Cpl-117 and Cpl-177, which contain the CWBD<sub>7S</sub>, displayed lower bacteriolytic activity than Cpl-711 and Cpl-771, but comparable to that of the parental Cpl-7S lysozyme (Figure 2a). These results correlated well with data for bacterial survival after endolysin treatment. Cpl-711 and Cpl-771 nearly sterilized the cultures, causing a decrease in R6 viability of  $\geq 7.5$  log, whereas Cpl-117 and Cpl-177 reduced viability by 4.7 log (Figure 2b).

After demonstrating the extreme effect of 5 µg/mL Cpl-711 and Cpl-771 on R6 cells, we tested their efficacy at lower concentrations to determine the differences in bactericidal activity compared with Cpl-1, considered to date as the most active endolysin against pneumococcal infections.<sup>40</sup> To accomplish this, bactericidal assays were repeated using the three lysins at 0.1 and 0.01 µg/mL. Cpl-711 and Cpl-771 produced greater decreases in OD<sub>550</sub> than Cpl-1 at both concentrations (Figure 2c and e). Furthermore, cell viability analysis after 1 h of treatment revealed that chimera Cpl-711 showed the highest bactericidal activity against R6, reducing the number of viable cells by 2 and 2.6 log at 0.01 and 0.1 µg/mL, respectively. This reduction was about 1 log more than Cpl-771 at both concentrations. This large lethal effect, achieved with such a low concentration of chimeric enzyme, largely exceeded the killing capacity of Cpl-1, which at 0.1 µg/mL reduced cell viability by 90% and at 0.01 µg/mL by only 15% (Figure 2d and f).

To check whether Cpl-711 was also the most active chimera against encapsulated pneumococci, similar experiments were done using the *S. pneumoniae* strains D39 (serotype 2), P007 (serotype 3) and P008 (serotype 4), and the multiresistant clinical strains 1515/97 (serotype 6B) and 48 (serotype 23F). As in the previous assays, the difference in terms of bactericidal activity among chimeric and parental proteins was clearer when the tests were performed with reduced concentrations of the enzymes. A summary of the bactericidal experiments with these strains using 0.1 µg/mL of each protein is shown in Table 1. Cpl-711 was also the most potent bactericidal agent against P007 and P008 strains, decreasing cell viability by 2 log, whereas the chimera Cpl-771 and the parental protein Cpl-1 reduced viability by  $\leq 1$  log when tested with these strains. Furthermore, Cpl-711 was the only endolysin that showed a marked effect on the multiresistant strain 48, reducing viability by 1 log. As for the other strains tested, D39 and the multiresistant 1515/97, both Cpl-711 and Cpl-771 chimeras exhibited a similar bactericidal effect, which was 1 log higher than that of Cpl-1. These results confirmed that, despite their similar specific activities *in vitro*, the four chimeras do not display the same bactericidal properties when acting from the outside of living bacteria.

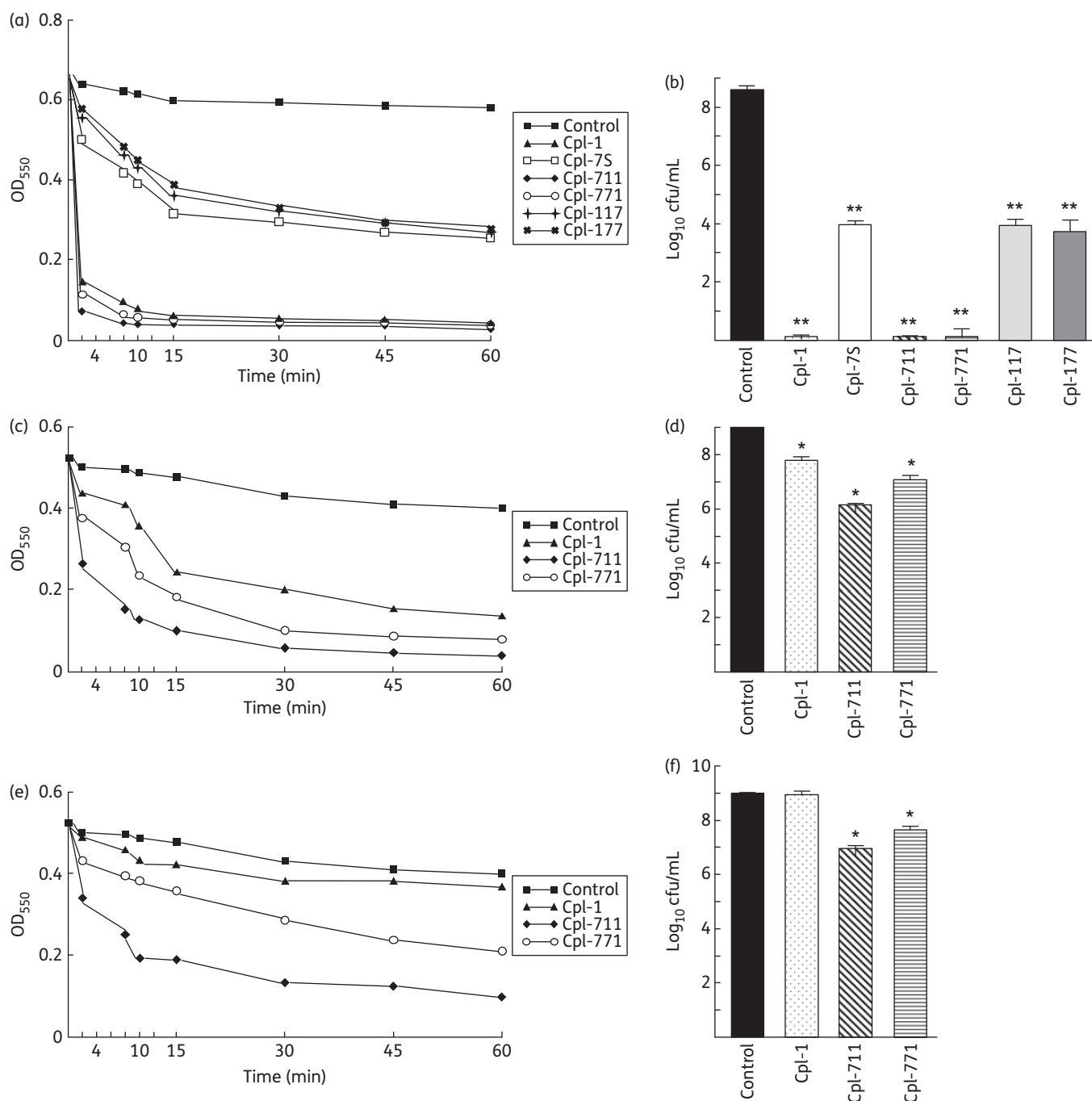
Moreover, Cpl-711 was the most lethal enzyme independent of the size and composition of the pneumococcal capsule. In addition, results derived from these bactericidal assays were confirmed by MICs determined for the *S. pneumoniae* ATCC 49619 strain, which were  $4.5 \pm 0.5$  mg/L for Cpl-711,  $8.5 \pm 0.5$  mg/L for Cpl-771,  $255 \pm 5$  mg/L for Cpl-117 and  $350 \pm 35$  mg/L for Cpl-177, whereas those of Cpl-1 and Cpl-7S were  $16 \pm 4$  and  $54 \pm 10$  mg/L, respectively.<sup>14</sup> To visualize the extraordinary killing effect of the chimeric Cpl-711 lysin on pneumococcal strains, we recorded a video under the microscope (see Video S1), which clearly demonstrates in real time the rapid lytic process of purified Cpl-711 on strain R6.

### Bactericidal activity of chimeric proteins against other streptococcal species

As previously described, the presence of CW<sub>7</sub> repeats in Cpl-7 and Cpl-7S confers the ability to efficiently kill several non-pneumococcal bacteria, with the engineered variant Cpl-7S being the most powerful lysin against them.<sup>14</sup> To test the capacity of the four newly constructed chimeras to kill other streptococcal species, bactericidal assays against the human pathogens *S. pyogenes* and *Streptococcus mitis* SK137 were performed. In the case of *S. pyogenes*, 0.1 µg/mL of any chimera showed a poor bactericidal effect (data not shown), so the experiments were repeated using 5 µg/mL protein. Under these conditions, Cpl-7S displayed the highest lethal effect (4 log decrease in viability) followed by Cpl-117 and Cpl-177 with 2 log lethality (Figure S1A). These values confirmed that the chimeras harbouring the CWBD<sub>7S</sub> did not improve the bactericidal activity of the parental Cpl-7S enzyme. Cpl-1 did not kill *S. pyogenes*, in accordance with previous data<sup>14</sup> and, similarly, the same negative results were obtained for Cpl-711 and Cpl-771 chimeras (Figure S1A). As expected from the presence of choline in its cell wall,<sup>21</sup> *S. mitis* SK137 revealed a greater susceptibility to Cpl-1, Cpl-711 and Cpl-771, with Cpl-711 being the most active, killing 4.7 log of bacteria, whereas Cpl-771 and Cpl-1 reduced viability by 3.6 log (Figure S1B). In this case, Cpl-7S did not produce the same lethal effect as with *S. pyogenes*, causing a decrease in viability of 3.3 log, similar to Cpl-1. Chimeras Cpl-117 and Cpl-177 were the least active enzymes against this species, as they killed about 2.3 log of cells (Figure S1B). These results reinforce the idea that differences in susceptibility to the action of these endolysins are directly related to the specificity and affinity of the corresponding CWBDs, although sequence composition of CDs and modular organization are also relevant, as well as the composition and structure of the whole bacterial envelope.

### Dispersing and lethal activity of Cpl-711 against pneumococcal biofilms

As with many bacterial species, *S. pneumoniae* is capable of growing as stable biofilms, a microbial community associated with up to 80% of all chronic infections,<sup>41</sup> including those recalcitrant to antibiotics.<sup>42,43</sup> Several bacterial and phage murein hydrolases (LytA, Ejl, Pal, Cpl-1 and Cpl-7) efficiently destroy pneumococcal biofilms.<sup>30</sup> Thus, we also examined the ability of Cpl-711 to disperse pneumococcal cells when grown as biofilms. At 1 µg/mL Cpl-711 reduced biofilm formation by 92%, whereas Cpl-1 and Cpl-7S reached 50% and 60%, respectively (Figure 3a). Moreover, the loss of viable cells in the same experiment was more dramatic



**Figure 2.** Bacteriolytic and bactericidal effects of different chimeric and parental enzymes against *S. pneumoniae* strain R6. Enzymes were added at 5 µg/mL (a and b), 0.1 µg/mL (c and d) and 0.01 µg/mL (e and f). Decay of the bacterial suspension (OD<sub>550</sub>) after addition of the selected enzyme (a, c and e). Incubation was performed at 37°C for 60 min. Controls with PBS are included. Data correspond to a representative experiment. Viable cells determined on blood agar plates after 60 min of incubation at 37°C (b, d and f). Data are means from four independent experiments. Error bars represent standard deviations, and asterisks indicate that results are statistically significant compared with the control in the absence of enzymes (one-way ANOVA with a post hoc Dunnet test; \**P* < 0.01, \*\**P* < 0.001).

since Cpl-711 killed about 4 log of the bacterial population, in contrast to the 1.5 log drop caused by Cpl-1 and Cpl-7S (Figure 3b). The different degree of biofilm disintegration was fully confirmed by CLSM, which clearly showed the thickness of corresponding biofilms as well as the remaining live and dead cells (Figure 3c).

#### Activity of Cpl-711 in a mouse infection model

To validate the *in vitro* bactericidal results of Cpl-711 on pneumococcal cells, we employed a mouse bacteraemia model and compared the *in vivo* activity of the enzyme with the parental Cpl-1. The most appropriate bacterial challenge dose that was lethal

**Table 1.** Bactericidal activity of the different chimeras and their parental enzymes against *S. pneumoniae*

Strain (serotype) <sup>a</sup>	Viability decrease after treatment with <sup>b</sup>					
	Cpl-1	Cpl-7S	Cpl-711	Cpl-771	Cpl-117	Cpl-177
<i>S. pneumoniae</i>						
R6 (none)	+	–	+++	++	–	–
D39 (2)	+	–	++	++	–	–
P007 (3)	–	–	++	+	–	–
P008 (4) <sup>c</sup>	+	–	++	+	–	–
1515/97 (6B)	–	–	+	+	–	–
48 (23F)	–	–	+	–	–	–

<sup>a</sup>Bacteria were incubated at 37°C in PBS (OD<sub>550</sub> ~0.6) with the indicated enzyme at 0.1 µg/mL. Viability was determined after 1 h of incubation by plating samples onto blood agar plates and analysing cfu.

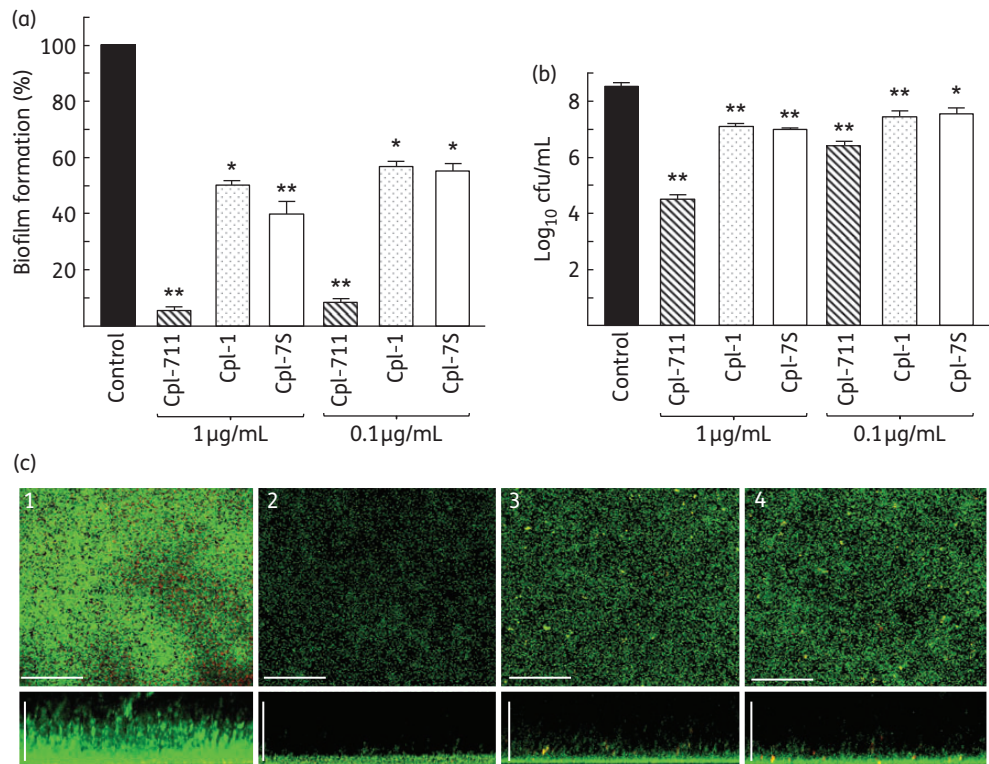
<sup>b</sup>– indicates no effect, and +, ++ and +++ indicate a decrease in viability of 1, 2 or 3 log, respectively.

<sup>c</sup>Constructed as strain P007, but with DNA from a serotype 4 pneumococcal strain.

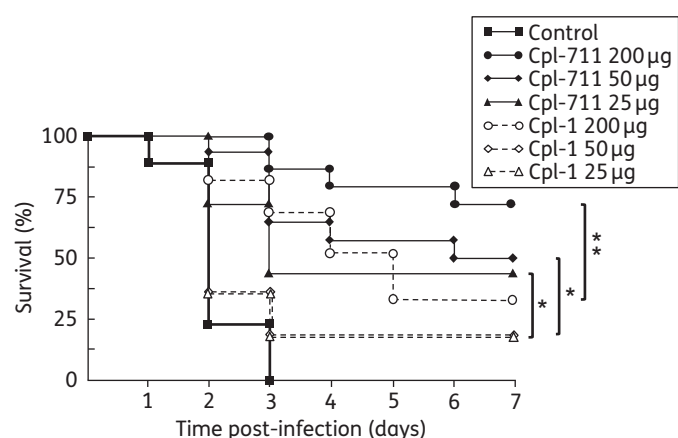
to mice in 2–3 days was found to be  $5.5 \times 10^5$  cfu/mL for pneumococcal strain D39\_IU, when administered by ip injection. One hour after bacterial challenge, animals were treated with a single ip injection containing different amounts of either Cpl-711 or Cpl-1 (in the range of 25–500 µg per mouse), or PBS as control. Mouse survival was followed over 7 days and the results of four different experiments were combined and represented as a Mantel–Cox curve. Rescue of the animals treated with Cpl-711 varied depending on the concentration used: 100% survival (20/20) when treated with 500 µg of the enzyme (not shown); 70% survival (14/20) when treated with 200 µg; 50% survival (10/20) when treated with 50 µg; and 45% survival (9/20) when treated with 25 µg. These results show that treatment with the Cpl-711 chimera resulted in about 50% greater protection than treatment with Cpl-1. For example, only 20% of mice (4/20) survived when treated with 50 µg of Cpl-1 compared with 50% survival with the same amount of Cpl-711 (Figure 4).

### Cpl-711 and Cpl-771 retain the global structure and choline-binding capacity of parental Cpl-1

In Cpl-1 folding, the relative positions of the CD and the CWBD<sub>1</sub> are restrained by the interactions between both modules and the



**Figure 3.** Degradation of pneumococcal biofilms upon addition of Cpl-711. (a) Percentage of crystal violet staining remaining in the *S. pneumoniae* P046 biofilm after treatment for 2 h at 37°C with 1 or 0.1 µg/mL Cpl-711 and the parental proteins Cpl-1 and Cpl-7S. The values of biofilm were normalized to the highest absorbance reading, and the percentage was calculated in relation to untreated strain P046. (b) Viable cells of *S. pneumoniae* P046 growing as biofilms determined on blood agar plates after treatment for 2 h at 37°C with 1 and 0.1 µg/mL Cpl-711, Cpl-1 and Cpl-7S. (c) CLSM image of the viability of biofilm-grown *S. pneumoniae* P046 treated for 2 h with 0.1 µg/mL Cpl-711 (panel 2), Cpl-1 (panel 3) or Cpl-7S (panel 4) or without any enzyme (panel 1). In all panels with calculated data, the data represent the mean of four independent experiments. Error bars represent standard deviations, and asterisks indicate that results are statistically significant compared with the control in the absence of enzymes (one-way ANOVA with a post hoc Dunnet test; \* $P < 0.05$ , \*\* $P < 0.01$ ). Bar, 25 µm.



**Figure 4.** Mice were rescued from lethal *S. pneumoniae* D39\_IU infection by Cpl-711. Mice were injected ip with  $5.5 \times 10^5$  cfu/mL *S. pneumoniae* D39\_IU strain (time 0). One hour later, mice received one ip injection of either PBS (control) or lysin. The lines represent the survival of mice treated (or not) with different concentrations of Cpl-711 or Cpl-1. Mice were monitored for survival over a period of 7 days and results plotted as Kaplan–Meier survival curves. Survival curves were compared with the log-rank (Mantel–Cox) and Gehan–Breslow–Wilcoxon tests (\* $P < 0.01$ ; \*\* $P < 0.001$ ).

tightness of the linker, which contains a DDEEDD motif that could be responsible for the enzyme's extended conformation (Figure S2). In addition, the fold adopted by the region of CWBD<sub>1</sub> that interacts with the CD also determines the total number of choline-binding functional sites.<sup>7</sup> The CWBD and linker of Cpl-1 and Cpl-711 are identical and their CDs differ in only 27 residues, but those involved in the interface with the CWBD<sub>1</sub> (Ala160, Phe162, Glu163, Phe165, Trp174, Tyr176, Ile185 and Leu187 in strands  $\beta 6$ – $\beta 8$ ) are all conserved (Figure S2). Therefore, the modular structure and the choline-binding capacity of Cpl-1 are likely preserved in Cpl-711, whose three-dimensional structure has been modelled using the X-ray structure of Cpl-1 as template (Figure S2C). The same probably applies to the Cpl-771 chimera, whose linker conserves five of the six initial acidic residues (Figure S2A), although some differences might be expected in the linker conformation because of its length (16 residues in Cpl-771 versus 11 in Cpl-711). To check these assumptions, the binding of choline to Cpl-711 and Cpl-771 was measured and the structural organization of both chimeras examined with and without choline using analytical ultracentrifugation and circular dichroism. As shown in Figure S3(A and B), the circular dichroism spectra of Cpl-1, Cpl-711 and Cpl-771 are very similar, both in the far- and near-UV regions, confirming that their secondary and tertiary structures are comparable in the free and choline-bound forms. The small differences in the regions sensitive to the contribution of aromatic side chains are probably due to the presence of one more tryptophan (Trp63) and the change F77Y in the CD of Cpl-7. Indeed, the spectra of the two chimeras are identical within experimental error. Variations of the circular dichroism spectra induced by choline addition were also alike and the titration curves obtained by monitoring the ellipticity changes at 225 nm as a function of ligand concentration showed that choline-binding affinities were also comparable (Figure S3C). The dissociation constants estimated by fitting the Hill equation to the titration data varied from  $4.1 \pm 0.2$  mM (Cpl-711) to  $3 \pm 1$  mM (Cpl-771) and

$3.6 \pm 0.2$  mM (Cpl-1), and the Hill coefficients were also similar (Table S2).

The association state and the hydrodynamic features of Cpl-711 and Cpl-771 and their complexes with choline were examined by analytical ultracentrifugation in comparison with Cpl-1. In the absence of choline, all of the enzymes sediment as single species, whose almost identical sedimentation coefficients correspond to protein monomers (Figure S3D), as verified by sedimentation equilibrium experiments (Table S2). Like in Cpl-1, choline binding regulates the self-association of the two chimeras that sediment as dimers under choline-saturating conditions (Figure S3D and Table S2), a characteristic that has been shown to be relevant for LytA and Cpl-1 activities.<sup>34,44–46</sup> Additionally, neither the linker nor the CD exchange has perceptible consequences on the overall protein shape since the hydrodynamic parameters ( $f/f_0$  and  $R_s$ ) calculated from sedimentation data for each association (monomer or dimer) state were alike for the three proteins (Table S2). Together these results confirmed that the overall structural features of Cpl-1 are preserved in Cpl-711 and Cpl-771, in agreement with our hypothesis.

## Discussion

Endolysins, like all phage-encoded proteins, have been subjected to a functional optimization process through joint evolution with their obligate bacterial hosts over millions of years. The final results are enzymes selected to cause rapid lysis of the host for efficient release of phage progeny, thus assuring phage survival. This evolutionary process has generated a wide variety of lysins that originate from the continuous recombination of different functional modules, from bacteria and phages, and their subsequent horizontal gene transfer.<sup>47</sup> Many of the natural enzymes encoded by these chimeric genes have been well studied, the putative origin of each module has been suggested and different fusions or deletions constructed and tested to determine their activities against the susceptible bacteria.<sup>48</sup> Additionally, functional synthetic endolysins have been engineered at the genetic level by module recombination to suit specific lytic requirements. For example, fusion of the lysostaphin gene from *Staphylococcus simulans* to the B30 endolysin gene from a *Streptococcus agalactiae* phage, or to its endopeptidase-coding module, created new enzymes active against *S. agalactiae*, *Streptococcus uberis* and *Staphylococcus aureus*, the main pathogens responsible for mastitis in cows.<sup>49</sup> It has also been demonstrated that pneumococcal murein hydrolases from either the host or phages harbour a CWBD essential for activity<sup>50</sup> that can be exchanged with other pneumococcal or clostridial CWBDs to recreate functional chimeric lysin.<sup>51,52</sup> Nonetheless, the overall published data suggest that natural endolysins are optimal enzymes to lyse and kill certain bacteria via their natural route. However, it is plausible to consider the possibility for enzymatic improvement through genetic engineering, at least when endolysins are added exogenously.

Detailed knowledge of the structural and functional properties of lysozymes Cpl-1 and Cpl-7 prompted us to explore the effect of combining their CDs and CWBDs as well as the linkers connecting them. To this aim, we took into account the information provided by the crystal structure of the entire Cpl-1 (free and ligand-bound) and the available structural models of Cpl-7.<sup>7,8,53</sup> We chose the synthetic Cpl-7S lysozyme instead of the natural Cpl-7 to construct the



new chimeric enzymes because of its superior bactericidal activity.<sup>14</sup> The most remarkable (and unexpected) result of our approach was the notable bactericidal capacity of Cpl-711, which was clearly greater than that exhibited by the natural endolysin Cpl-1, considered to date as the most powerful lytic enzyme against pneumococcal infections. This improved activity of Cpl-711 was evident not only against several pneumococcal strains grown as planktonic cultures, including some antibiotic-resistant strains, but also against pneumococcal biofilms *in vitro*, and gave superior protection compared with other lysins in a mouse model of bacteraemia. Therefore, the eventual application of this potent chimeric protein to treat multiresistant pneumococcal infections deserves future investigation.

The noticeable improvement of the bactericidal activity of Cpl-711, compared with that of Cpl-1, is interesting in itself since, to our knowledge, despite the numerous endolysin fusion constructs reported so far, only the Ply187-KSH3 chimera showed a higher bactericidal effect over its parental enzyme.<sup>54</sup> Nevertheless, Ply187, encoded by a phage infecting *S. aureus*, was atypical among endolysins from Gram-positive bacteria because of the poor activity displayed by the recombinant enzyme, which was probably due to the absence of a CWBD. Thus, the addition of an SH3b domain from LysK endolysin, known to act as a CWBD, enhanced its lytic activity.<sup>54</sup> In contrast, Cpl-711 is the result of swapping of catalytic and substrate-binding domains from natural bimodular lysozymes that already behave as very potent bactericidal agents.

Any explanation of the observed differences in activity among the four constructed chimeras and the two parental enzymes must consider the concept of modular evolution of proteins<sup>55</sup> and the fact that the activity of the complete enzyme will be determined by the specific features of the isolated domains as well as by those conferred by the interactions established between them and with the optimized linkers. In addition, the lytic action of endolysins from the outside of intact cells is probably a complex process controlled by many factors, like the composition and charges of the endolysin and the bacterial envelope. In this respect, the lack of correlation between the activities measured using purified cell wall fragments or intact cultures of R6 strain as substrates, as well as the influence of capsule composition on the killing activity of Cpl-1, Cpl-711 and Cpl-771 (Table 1), clearly demonstrated that substrate fragmentation and/or the inherent removal of components attached to the bacterial envelope minimize the potential impact of sequence differences on the enzymatic activity. This lack of correlation may be due to differences in substrate accessibility and bond cleavage.

Another general conclusion drawn from this study is that the type of CWBD is a major factor in the lethality of endolysins, i.e. Cpl-117 and Cpl-177 are very similar to the parental Cpl-7S, whereas Cpl-711 and Cpl-771 are rather similar to Cpl-1. It is well established that the nature of the CWBD influences substrate specificity and cell-wall binding affinity as well as the overall endolysin structure. In this respect, Cpl-1 structure differs from that of Cpl-7, which, according to a SAXS-based model, adopts an extended structure with the CWBD<sub>7</sub> horizontally packed to the catalytic barrel.<sup>7,8</sup> Such divergence, enhanced by choline-mediated dimerization of Cpl-711, Cpl-771 and Cpl-1, would likely modify protein diffusion as well as substrate recognition and lytic efficiency. Interestingly, a covalently stabilized dimer of Cpl-1 was shown to have increased antipneumococcal activity and decreased plasma clearance.<sup>46</sup>

Since Cpl-1 and Cpl-711 only differ in the CD, the first clue to help explain the large increase in lethal activity of Cpl-711 may be found in the 27 distinct residues of both domains (Figure S2). These amino acid differences affect neither the catalytic residues, which are sequentially and structurally conserved,<sup>7,8</sup> nor the modular organization and choline-binding capacity. Non-conservative changes are located mostly between the loop connecting strand  $\beta$ 3 to helix-3 and the beginning of helix-4, which includes the region containing the proton donor Glu94 (Figure S2). However, Cpl-1 and Cpl-711 have the same overall net charge. Consequently, this factor, so relevant for the activity of Cpl-7S and other endolysins,<sup>14,39</sup> does not explain the improved activity of Cpl-711 compared with natural Cpl-1. Nevertheless, the amino acid changes modify the electrostatic potential of the catalytic surface (Figure S4), which could affect the unfavourable electrostatic interactions with the negative charge of the cell wall. This can also slightly alter the pattern of the contacts observed in the model proposed for the complex between Cpl-1 and a pentasaccharide dipentapeptide.<sup>8,56</sup> On the other hand, it seems unlikely that the glycopeptide chain bound to the catalytic cleft could make contact with other regions of the CD. However, the possibility that endolysins could interact with adjacent muropeptide chains (or other molecules present in the intact cell wall) cannot be completely ruled out. It might help explain their different behaviour with cell wall fragments or intact cells, as well as the distinct bactericidal activities reported here. Interestingly, the structure of the Cpl-1 E94D mutant complexed with a disaccharide pentapeptide showed an additional molecule of the substrate analogue bound outside the catalytic groove (PDB access code: 2J8F). For similar reasons, a certain rearrangement of a longer linker in the Cpl-771 chimera could account for its decrease in lethality and MIC increase compared with Cpl-711.

The absence of a structural model of the entire Cpl-7 at atomic resolution precludes a thorough analysis of the differences found among Cpl-7S, Cpl-177 and Cpl-117, although the SAXS-based model of Cpl-7 suggests that, like in Cpl-1, the hydrophobic cavity formed by strands  $\beta$ 6– $\beta$ 8 might interact with the CWBD<sub>7S</sub>.<sup>8</sup> Of note, the increase in the MIC values of Cpl-177 and Cpl-117 with respect to Cpl-7S is comparable to the reduction observed on passing from Cpl-1 to Cpl-711 or Cpl-771 (i.e. the inverse modification) and further supports the notion that sequence divergences make the CD of Cpl-7 more efficient. Elucidation of the three-dimensional structures of the full-length chimeras together with that of Cpl-7S and their complexes with peptidoglycan analogues could provide interesting, although partial, insights into the molecular basis of the differences in catalytic activity and/or substrate binding of this collection of lysozymes.

In summary, this study discloses the complexity of mechanisms underlying the exogenous bacteriolytic/bactericidal action of endolysins and demonstrates the feasibility of constructing new and more lethal phage lysins from WT or modified preexisting enzymes. Taking into account the high diversity within bacterial and bacteriophage populations, there are great opportunities for designing novel antimicrobials that might reduce the increasing incidence of antibiotic resistance obstructing therapeutic efforts worldwide.

## Acknowledgements

We thank Ernesto García for helpful comments and for critically reading the manuscript prior to submission, and E. Cano, S. Ruiz and V. López for

skillful technical assistance. We are indebted to M. Domenech, M. Seisdedos and G. E. Serrano for their advice and help with CLSM imaging, and to G. García for help in protein purification.

## Funding

This research was funded by grants from the Ministerio de Economía y Competitividad (MINECO) to P. G. (SAF2009-10824 and SAF2012-39444-CO2-01), and from the MINECO (BFU2009-10052 and BF4U2012-36825) and the Consejería de Educación de la Comunidad de Madrid (S2010/BMD/2457) to M. M. and Rockefeller University laboratory funds to V. A. F. Additional funding was provided by the CIBER de Enfermedades Respiratorias (CIBERES), an initiative of the Instituto de Salud Carlos III (ISCIII). R. D.-M. was the recipient of one fellowship from the MINECO (FPI programme). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

## Transparency declarations

None to declare.

## Supplementary data

Table S1, Table S2, Video S1 and Figures S1 to S4 are available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

## References

- Andrade AL, Toscano CM, Minamisava R et al. Pneumococcal disease manifestation in children before and after vaccination: what's new? *Vaccine* 2011; **29** Suppl 3: C2–14.
- McCarthy M. CDC calls for urgent action to combat rise of drug resistant pathogens. *BMJ* 2013; **347**: f5649.
- Fischetti VA. Bacteriophage lysins as effective antibacterials. *Curr Opin Microbiol* 2008; **11**: 393–400.
- Loessner MJ. Bacteriophage endolysins—current state of research and applications. *Curr Opin Microbiol* 2005; **8**: 480–7.
- Rigden DJ, Jedrzejewski MJ, Galperin MY. Amidase domains from bacterial and phage autolysins define a family of  $\gamma$ -D,L-glutamate-specific amidohydrolases. *Trends Biochem Sci* 2003; **28**: 230–4.
- Nelson DC, Schmelcher M, Rodriguez-Rubio L et al. Endolysins as antimicrobials. *Adv Virus Res* 2012; **83**: 299–365.
- Hermoso JA, Monterroso B, Albert A et al. Structural basis for selective recognition of pneumococcal cell wall by modular endolysin from phage Cp-1. *Structure* 2003; **11**: 1239–49.
- Bustamante N, Campillo NE, García E et al. Cpl-7, a lysozyme encoded by a pneumococcal bacteriophage with a novel cell wall-binding motif. *J Biol Chem* 2010; **285**: 33184–96.
- López R, García E. Recent trends on the molecular biology of pneumococcal capsules, lytic enzymes, and bacteriophage. *FEMS Microbiol Rev* 2004; **28**: 553–80.
- Rau A, Hogg T, Marquardt R et al. A new lysozyme fold. Crystal structure of the muramidase from *Streptomyces coelicolor* at 1.65 Å resolution. *J Biol Chem* 2001; **276**: 31994–9.
- Porter CJ, Schuch R, Pelzek AJ et al. The 1.6 Å crystal structure of the catalytic domain of PlyB, a bacteriophage lysin active against *Bacillus anthracis*. *J Mol Biol* 2007; **366**: 540–50.
- Hermoso JA, García JL, García P. Taking aim on bacterial pathogens: from phage therapy to enzybiotics. *Curr Opin Microbiol* 2007; **10**: 461–72.
- Bustamante N, Rico-Lastres P, García E et al. Thermal stability of Cpl-7 endolysin from the *Streptococcus pneumoniae* bacteriophage Cp-7; cell wall-targeting of its CW<sub>7</sub> motifs. *PLoS One* 2012; **7**: e46654.
- Díez-Martínez R, de Paz H, Bustamante N et al. Improving the lethal effect of Cpl-7, a pneumococcal phage lysozyme with broad bactericidal activity, by inverting the net charge of its cell wall-binding module. *Antimicrob Agents Chemother* 2013; **57**: 5355–65.
- Hoskins J, Alborn WE Jr, Arnold J et al. Genome of the bacterium *Streptococcus pneumoniae* strain R6. *J Bacteriol* 2001; **183**: 5709–17.
- Lanie JA, Ng W-L, Kazmierczak KM et al. Genome sequence of Avery's virulent serotype 2 strain D39 of *Streptococcus pneumoniae* and comparison with that of unencapsulated laboratory strain R6. *J Bacteriol* 2007; **189**: 38–51.
- Domenech M, García E, Moscoso M. Versatility of the capsular genes during biofilm formation by *Streptococcus pneumoniae*. *Environ Microbiol* 2009; **11**: 2542–55.
- Moscoso M, García E, López R. Biofilm formation by *Streptococcus pneumoniae*: role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion. *J Bacteriol* 2006; **188**: 7785–95.
- Yuste J, Jado I, Fenoll A et al.  $\beta$ -Lactam modification of the bacter-aemic profile and its relationship with mortality in a pneumococcal mouse sepsis model. *J Antimicrob Chemother* 2002; **49**: 331–5.
- Soriano F, Cafini F, Aguilar L et al. Breakthrough in penicillin resistance? *Streptococcus pneumoniae* isolates with penicillin/cefotaxime MICs of 16 mg/L and their genotypic and geographical relatedness. *J Antimicrob Chemother* 2008; **62**: 1234–40.
- Bergström N, Jansson PE, Kilian M et al. Structures of two cell wall-associated polysaccharides of a *Streptococcus mitis* biovar 1 strain. A unique teichoic acid-like polysaccharide and the group O antigen which is a C-polysaccharide in common with pneumococci. *Eur J Biochem* 2000; **267**: 7147–57.
- Sambrook J, Russell DW. *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 2001.
- Tabor S. Expression using the T7 RNA polymerase/promoter system. In: Ausubel FM, Brent R, Kingston RE et al., eds. *Current Protocols in Molecular Biology*. New York: Green and John Wiley & Sons, 1990; 16.2.1–11.
- Sanz JM, García JL. Structural studies of the lysozyme coded by the pneumococcal phage Cp-1. Conformational changes induced by choline. *Eur J Biochem* 1990; **187**: 409–16.
- Lacks S, Hotchkiss RD. A study of the genetic material determining an enzyme activity in *Pneumococcus*. *Biochim Biophys Acta* 1960; **39**: 508–18.
- MacFaddin JF. *Media for Isolation–Cultivation–Identification–Maintenance of Medical Bacteria*. Baltimore, MD: Williams & Wilkins, 1985.
- Ho SN, Hunt HD, Horton RM et al. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 1989; **77**: 51–9.
- Höltje JV, Tomasz A. Purification of the pneumococcal N-acetylmuramyl-L-alanine amidase to biochemical homogeneity. *J Biol Chem* 1976; **251**: 4199–207.
- Mosser JL, Tomasz A. Choline-containing teichoic acid as a structural component of pneumococcal cell wall and its role in sensitivity to lysis by an autolytic enzyme. *J Biol Chem* 1970; **245**: 287–98.
- Domenech M, García E, Moscoso M. In vitro destruction of *Streptococcus pneumoniae* biofilms with bacterial and phage peptidoglycan hydrolases. *Antimicrob Agents Chemother* 2011; **55**: 4144–8.
- Jado I, López R, García E et al. Phage lytic enzymes as therapy of antibiotic-resistant *Streptococcus pneumoniae* infection in a murine sepsis model. *J Antimicrob Chemother* 2003; **52**: 967–73.

- 32 Gilmer DB, Schmitz JE, Euler CW *et al.* Novel bacteriophage lysin with broad lytic activity protects against mixed infection by *Streptococcus pyogenes* and methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2013; **57**: 2743–50.
- 33 Moreno FJ, Quintanilla-López JE, Lebrón-Aguilar R *et al.* Mass spectrometric characterization of glycosylated  $\beta$ -lactoglobulin peptides derived from galacto-oligosaccharides surviving the *in vitro* gastrointestinal digestion. *J Am Soc Mass Spectrom* 2008; **19**: 927–37.
- 34 Monterroso B, Saiz JL, García P *et al.* Insights into the structure-function relationships of pneumococcal cell wall lysozymes, LytC and Cpl-1. *J Biol Chem* 2008; **283**: 28618–28.
- 35 Schuck P. Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and Lamm equation modeling. *Biophys J* 2000; **78**: 1606–19.
- 36 Laue TM, Shah BD, Ridgeway TM *et al.* Computer-aided interpretation of analytical sedimentation data for proteins. In: Harding SE, Rowe AJ, Horton JC, eds. *Analytical Ultracentrifugation in Biochemistry and Polymer Science*. Cambridge: Royal Society of Chemistry, 1992; 90–125.
- 37 Laskowski RA, MacArthur MW, Moss DS *et al.* PROCHECK: a program to check the stereochemical quality of protein structures. *J Appl Crystallogr* 1993; **26**: 283–91.
- 38 Lüthy R, Bowie JU, Eisenberg D. Assessment of protein models with three-dimensional profiles. *Nature* 1992; **356**: 83–5.
- 39 Low LY, Yang C, Perego M *et al.* Role of net charge on the catalytic domain and the influence of the cell-wall binding domain on the bactericidal activity, specificity, and host-range of phage lysins. *J Biol Chem* 2011; **286**: 34391–403.
- 40 Grandgirard D, Loeffler JM, Fischetti VA *et al.* Phage lytic enzyme Cpl-1 for antibacterial therapy in experimental pneumococcal meningitis. *J Infect Dis* 2008; **197**: 1519–22.
- 41 Wolcott RD, Ehrlich GD. Biofilms and chronic infections. *JAMA* 2008; **299**: 2682–4.
- 42 Moscoso M, Domenech M, García E. Vancomycin tolerance in Gram-positive cocci. *Environ Microbiol Rep* 2011; **3**: 640–50.
- 43 Domenech M, García E, Moscoso M. Biofilm formation in *Streptococcus pneumoniae*. *Microb Biotechnol* 2012; **5**: 455–65.
- 44 Varea J, Saiz JL, López-Zumel C *et al.* Do sequence repeats play an equivalent role in the choline-binding module of pneumococcal LytA amidase? *J Biol Chem* 2000; **275**: 26842–55.
- 45 Romero P, López R, García E. Key role of amino acid residues in the dimerization and catalytic activation of the autolysin LytA, an important virulence factor in *Streptococcus pneumoniae*. *J Biol Chem* 2007; **282**: 17729–37.
- 46 Resch G, Moreillon P, Fischetti VA. A stable phage lysin (Cpl-1) dimer with increased antipneumococcal activity and decreased plasma clearance. *Int J Antimicrob Agents* 2011; **38**: 516–21.
- 47 Hendrix RW. Bacteriophages: evolution of the majority. *Theor Popul Biol* 2002; **61**: 471–80.
- 48 Sheehan MM, García JL, López R *et al.* The lytic enzyme of the pneumococcal phage Dp-1: a chimeric lysin of intergeneric origin. *Mol Microbiol* 1997; **25**: 717–25.
- 49 Donovan DM, Dong S, Garrett W *et al.* Peptidoglycan hydrolase fusions maintain their parental specificities. *Appl Environ Microbiol* 2006; **72**: 2988–96.
- 50 García JL, Díaz E, Romero A *et al.* Carboxy-terminal deletion analysis of the major pneumococcal autolysin. *J Bacteriol* 1994; **176**: 4066–72.
- 51 Díaz E, López R, García JL. Chimeric phage-bacterial enzymes: a clue to the modular evolution of genes. *Proc Natl Acad Sci USA* 1990; **87**: 8125–9.
- 52 Croux C, Ronda C, López R *et al.* Interchange of functional domains switches enzyme specificity: construction of a chimeric pneumococcal-clostridial cell wall lytic enzyme. *Mol Microbiol* 1993; **9**: 1019–25.
- 53 Silva Martín N. Biología estructural de las interacciones patógeno - hospedador en *Streptococcus pneumoniae*: mecanismos de reconocimiento y lisis de la pared bacteriana. PhD Thesis. Madrid: Universidad Autónoma de Madrid, 2011.
- 54 Mao J, Schmelcher M, Harty WJ *et al.* Chimeric Ply187 endolysin kills *Staphylococcus aureus* more effectively than the parental enzyme. *FEMS Microbiol Lett* 2013; **342**: 30–6.
- 55 Copley RR, Ponting CP, Schultz J *et al.* Sequence analysis of multidomain proteins: past perspectives and future directions. *Adv Protein Chem* 2003; **61**: 75–98.
- 56 Pérez-Dorado I, Campillo NE, Monterroso B *et al.* Elucidation of the molecular recognition of bacterial cell wall by modular pneumococcal phage endolysin CPL-1. *J Biol Chem* 2007; **282**: 24990–9.