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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

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Phage endolysins are murein hydrolases that break the bacterial cell wall to provoke lysis and release of phage progeny. Recently, these enzymes have also been recognized as powerful and specific antibacterial agents when added exogenously. In the pneumococcal system, most cell wall associated murein hydrolases reported so far depend on choline for activity, and Cpl-7 lysozyme constitutes a remarkable exception. Here, we report the improvement of the killing activity of the Cpl-7 endolysin by inversion of the sign of the charge of the cell wall-binding module (from −14.93 to +3.0 at neutral pH). The engineered variant, Cpl-7S, has 15 amino acid substitutions and an improved lytic activity against *Streptococcus pneumoniae* (including multiresistant strains), *Streptococcus pyogenes*, and other pathogens. Moreover, we have demonstrated that a single 25-μg dose of Cpl-7S significantly increased the survival rate of zebrafish embryos infected with *S. pneumoniae* or *S. pyogenes*, confirming the killing effect of Cpl-7S *in vivo*. Interestingly, Cpl-7S, in combination with 0.01% carvacrol (an essential oil), was also found to efficiently kill Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas putida*, an effect not described previously. Our findings provide a strategy to improve the lytic activity of phage endolysins based on facilitating their pass through the negatively charged bacterial envelope, and thereby their interaction with the cell wall target, by modulating the net charge of the cell wall-binding modules.

The major reservoir of *Streptococcus pneumoniae*, a Gram-positive encapsulated ovococcus, is found in asymptomatic nasopharyngeal carriers, whose prevalence varies by age and region (1). This human pathogen is the leading cause worldwide of community-acquired pneumonia and a major causative agent of invasive infections (meningitis and sepsis) and diseases affecting the upper (otitis media and sinusitis) and lower (pneumonia) respiratory tracts, among others (2). The disease burden is high, especially in developing countries, and the high-risk groups include children, elderly persons, and immunocompromised patients, with an estimate of 1.6 million deaths per year according to the World Health Organization (3). Therapeutics are hampered by insufficient vaccine coverage and an increase in antimicrobial resistance (4–6). In fact, resistance to traditional drugs may take treatment back to the preantibiotic era in many aspects, making necessary a radical change of strategy that should involve identification of new targets, development of new chemical compounds interacting with them, and the setup of procedures for early diagnosis and effective pathogen monitoring in biological fluids.

In this context, phage endolysins (lysins) constitute an alternative (or complementary) approach to classic antibiotics in the search for novel therapeutic strategies for fighting invasive pneumococcal disease. Endolysins are bacteriophage cell wall hydrolases that cleave the major bond types in the peptidoglycan and have been refined over millions of years for efficiently and specifically breaking the host cell wall, provoking cellular death. This lytic activity has been well known for nearly a century, and while entire virions have been used to control infection, their encoded lytic enzymes have not been exploited in their purified forms until recently for bacterial control *in vivo* (7–9). The sharp increase in antibiotic resistance among pathogenic bacteria is now fostering this approach, and bacteriolytic peptidoglycan hydrolases are also currently named "enzybiotics" (7). Current data indicate that these enzymes are effective primarily against Gram-positive bacteria, since when exogenously added, the outer membrane of the Gram-negative bacteria prevents their direct contact with the cell wall muropeptide. In contrast to antibiotics, which are usually broad spectrum and kill many different bacteria, most enzybiotics share characteristics such as their potency and specificity, since commonly they kill only the species (or subspecies) of bacteria from which they were produced. This stringent substrate specificity is usually linked to the acquisition of additional modules that specifically bind to structural motifs of the bacterial envelope distributed in a genus-specific or even species/strain-specific manner (10–12). There are some cases, however, where phage enzymes with broad lytic activity have been reported, e.g., the lysins PlyV12 and PlySs2 from bacteriophages of *Enterococcus faecalis* and *Streptococcus suis*, respectively (13, 14). Enzybiotics also exhibit low toxicity, moderate inhibition by the host immune response, and a low probability of resistance development (10, 12).

Many cell wall hydrolases reported so far in the pneumococcal system, from either host or phage origin, are choline-binding pro-

Received 25 June 2013 Returned for modification 21 July 2013
Accepted 9 August 2013
Published ahead of print 19 August 2013
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.01372-13.

doi:10.1128/AAC.01372-13

teins (CBPs) that depend on their attachment to the choline moieties of pneumococcal (lipo)teichoic acids, through specialized modules, for activity (15). There is a noticeable exception to this rule, i.e., the Cpl-7 lysozyme, encoded by the lytic pneumococcal phage Cp-7, whose cell wall-binding module (CWBM) is made of three identical CW_7 repeats (even at the nucleotide level) that are sequentially and structurally unrelated to the choline-binding motifs of the CBPs (16, 17). In contrast, its N-terminal catalytic module is 85.6% identical (90.9% similar) to that of Cpl-1 lysozyme. Interestingly, Cpl-7 is capable of hydrolyzing choline- as well as ethanolamine-containing pneumococcal cell walls (16), and it shows a specific activity on choline-containing purified cell walls that is comparable to that of Cpl-1 (17). Preliminary results strongly suggested that the CW_7 repeats recognize the peptidoglycan network as a target (18), an observation that could directly affect Cpl-7 antimicrobial capacity by broadening the putative range of susceptible pathogens. Indeed, CW_7-like motifs have been identified in a great variety of proteins that can be classified as probable cell wall hydrolases encoded mainly by Gram-positive bacteria and/or their prophages (17). To date, two phage lysins (Pal and Cpl-1) and the pneumococcal LytA autolysin have been successfully used as therapeutic agents in animal models of nasopharyngeal carriage, sepsis, or endocarditis triggered by *S. pneumoniae* strains and other bacteria containing choline-substituted teichoic acids (10, 19–21).

In this study, we have demonstrated that, in contrast with the restricted activity of Cpl-1, Cpl-7 lyses a variety of Gram-positive bacteria. Moreover, using protein engineering, we have enhanced its bactericidal activity by introducing 15 amino acid substitutions in the CWBM (5 per each repeat), which lowered its highly negative net charge by ca. 18 units at neutral pH. The modified enzyme, Cpl-7S, is highly effective against *S. pneumoniae*, including antibiotic-multiresistant strains, but also against other relevant Gram-positive pathogens, e.g., *Streptococcus pyogenes*, *E. faecalis*, and *Streptococcus mitis*. Furthermore, we have designed a protocol to destabilize the outer membranes of Gram-negative bacteria that renders these microorganisms susceptible to the action of Cpl-7S, as shown with *Escherichia coli* and *Pseudomonas putida* as proofs of concept. In addition, the *in vitro* bactericidal activity of Cpl-7S has also been validated *in vivo* employing a zebrafish embryo infection model.

TABLE 1 Bacterial viability after treatment with three different enzybiotics[a]

Organism (serotype)	Viability after treatment with[b]: Cpl-1	Cpl-7	Cpl-7S	Source or reference[c]
Gram-positive bacteria				
S. pneumoniae				
R6 (none)	*	+++	++++	55
D39 (2)	*	++	+++	56
P007 (3)	*	++	++	57
P008 (4)[d]	*	++	+++	D. Llull
69 (19F)	*	++	+++	36
1515/97 (6B)	*	++	+++	36
Corynebacterium jeikeium^T	−	−	−	DSMZ 7171
E. faecalis^T	−	++	++	CECT 481
Lactococcus lactis subsp. *lactis*^{T}	−	−	−	CECT 185
Listeria monocytogenes^T	−	−	−	CECT 4031
Mycobacterium smegmatis mc^{2}155	−	−	−	58
S. agalactiae^T	−	−	−	CECT 183
Staphylococcus aureus^T	−	−	−	CECT 86
Streptococcus dysgalactiae[e]	−	+	+	CECT 926
S. iniae^T	−	+	+	CECT 7363
S. mitis				
SK598	−	++	++	37
Type strain	++++	+++	++++	NCTC 12261
Streptococcus mutans^T	−	−	−	CECT 479
S. pyogenes^T	−	+++	++++	CECT 985
Gram-negative bacteria[f]				
E. coli	−	+	+++	
P. putida KT2442	−	++	++	59

[a] Bacteria were incubated at 37°C in PBS ($OD_{550} \approx 0.6$) with the indicated enzybiotic at 5 μg · ml^{-1}. Viability was determined after 1 h of incubation.
[b] −, no effect; +, decrease of 1 log unit in viable cells; ++, decrease of 2 log units in viable cells; +++, decrease of 3 log units in viable cells; ++++, decrease of ≥4 log units in viable cells; *, <10 CFU · ml^{-1}.
[c] DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; CECT, Colección Española de Cultivos Tipo; NCTC, National Collection of Type Cultures.
[d] Constructed as for strain P007 but with DNA from a type 4 pneumococcal strain.
[e] Determined by fluorescence microscopy.
[f] Treated with 0.01% carvacrol prior to enzybiotic addition.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. They were tested as substrates for lytic enzymes using the standard protocol described below. Pneumococcal strains were grown in C medium supplemented with yeast extract (0.8 mg · ml^{-1}; Difco Laboratories) (C+Y) (22), with incubation at 37°C. The other Gram-positive bacteria were grown in brain heart infusion broth (BHI) (*Corynebacterium jeikeium*, *Streptococcus dysgalactiae*, and *Streptococcus iniae*), LB medium (*Mycobacterium smegmatis* mc^{2}155), or M17 medium (*Lactococcus lactis*) (23) at 37°C without shaking, except *S. iniae*, which was grown with shaking. *E. coli* and *P. putida* were grown in LB medium with shaking at 37°C and 30°C, respectively.

Synthesis of the Cpl-7S-coding gene. The synthetic DNA fragment encoding Cpl-7S was purchased from ATG:Biosynthetics (Merzhausen, Germany) as an *E. coli* codon-optimized pUC derivative recombinant plasmid. The gene synthesis was also used to break the nucleotide identity among the three repeats of the CW_7 by changing some codons without altering the respective amino acid residues. The resulting synthetic gene and its corresponding amino acids are shown in Fig. S1 in the supplemental material.

Cloning, expression, and purification of Cpl-7S. To optimize the expression of Cpl-7S, the relevant DNA fragment initially cloned in the pUC derivative plasmid was subcloned into pT7-7 (24) using NdeI and PstI, and the resulting plasmid (pTRD750) was transformed into *E. coli* strain BL21(DE3). For overexpression of Cpl-7S, transformed BL21(DE3) cells were incubated in LB medium containing ampicillin (0.1 mg · ml^{-1}) to an optical density at 600 nm (OD_{600}) of 0.6. Isopropyl-β-D-thiogalactopyranoside (0.1 mM) was then added, and incubation was continued overnight at 30°C. Cells were harvested by centrifugation (10,000 × *g*, 5 min), resuspended in 20 mM sodium phosphate buffer (pH 6.0), and disrupted in a French pressure cell. The insoluble fraction was separated by centrifugation (15,000 × *g*, 15 min), and Cpl-7S was purified from the supernatant following the procedure previously described for the wild-type enzyme (17). Cpl-7S eluted at a lower salt concentration (0.3 M NaCl) than the wild-type Cpl-7 in the DEAE-cellulose ion-exchange chromatography. The purity of the isolated protein was checked by SDS-PAGE (12% acrylamide-bisacrylamide) and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry before storage at −20°C in 20 mM phosphate buffer (pH 6.0). Purification of the other enzybiotics was performed as previously described (17, 25–27), and protein concentrations were determined spectrophotometrically using the

respective molar absorption coefficients at 280 nm (17, 25–27). Before use, all proteins were equilibrated in 20 mM sodium phosphate buffer, pH 6.0 (P_i buffer).

Computational calculations. Net charges of full-length proteins and modules at neutral pH were estimated from the respective sequences with the program Sendterp (28). The electrostatic potentials of the CW_7 surfaces were calculated from the CWBM model (17) using the Adaptative Poisson-Bolztmann Solver (APBS) software implemented in PYMOL (29). The free geometry-based algorithm Fpocket (30) was used to examine the CWBM three-dimensional (3D) model with the aim to identify potential binding sites for the CW_7 targets. Equivalent results were found by using the structure of a single repeat as input.

Analytical ultracentrifugation. Sedimentation velocity experiments were carried out in an Optima XL-A analytical ultracentrifuge (Beckman Coulter) at 20°C. Measurements were performed in P_i buffer at 45,000 rpm using cells with double-sector Epon-charcoal centerpieces. Differential sedimentation coefficients were calculated by least-squares boundary modeling of the experimental data with the program SEDFIT (28).

CD. Circular dichroism (CD) spectra were recorded at 20°C using a J-810 spectropolarimeter (Jasco Corporation) equipped with a Peltier cell holder. Measurements were performed in 1-mm- and 0.2-mm-pathlength cells (for far- and near-CD spectra, respectively) using the experimental conditions described previously (17). The buffer contribution was subtracted from the raw data, and the corrected spectra were converted to mean residue ellipticities using average molecular masses per residue of 112.30 (Cpl-7) and 112.76 (Cpl-7S).

Mass spectrometry. Purified samples of Cpl-7S were analyzed by MALDI-TOF mass spectrometry as described elsewhere (31). 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 (29,024 Da) and enolase (46,672 Da) from Sigma, covering an *m/z* range of 16000 to 50000.

***In vitro* cell wall activity assay.** Purified enzymes were checked for *in vitro* cell wall degradation using [*methyl*-^{3}H]choline-labeled pneumococcal cell walls as the substrate and following a previously described method (32). Briefly, 10 μl of enzyme at the appropriate dilution was added to the reaction sample containing 240 μl of P_i buffer and 10 μl of radioactively labeled cell walls (~15,000 cpm). After 15 min of incubation at 37°C, the reaction was stopped by adding 10 μl formaldehyde (37%, vol/vol) and 10 μl bovine serum albumin (BSA) (4%, wt/vol). The pellet was removed by centrifugation (12,000 × *g*, 15 min), and the enzymatic activity was quantified by measuring the radioactivity in the supernatant with a liquid scintillation counter (LKB Wallac).

MICs. MICs of Cpl-7, Cpl-7S, and Cpl-1 were determined by the microdilution method approved by the Clinical and Laboratory Standards Institute (CLSI) (33) using cation-adjusted Mueller-Hinton II broth (Becton, Dickinson and Co., Le Pont-de-Claix, France) supplemented with 5% lysed horse blood (CA-MHB-LHB). 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).

Bactericidal assay. Bacteria were grown to logarithmic phase to an OD_{550} of 0.3, and then cultures were centrifuged and washed twice with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 [pH 6.0]), and the final OD_{550} was adjusted to ca. 0.6 in the same buffer. Afterwards, resuspended Gram-positive cells were transferred into plastic tubes, and the tested enzyme was added (1 to 3 μl in P_i buffer). Samples were incubated at 37°C for 1 h, and the turbidity decrease at 550 nm (OD_{550}) was measured at selected intervals. For Gram-negative bacteria, cells were resuspended in PBS supplemented with 0.01% carvacrol [2-methyl-5-(1-methylethyl)phenol] before processing as described for Gram-positive bacteria. Controls were always run in parallel, replacing the added enzyme with P_i buffer. Measurement of viable cells was carried out in C+Y or blood agar plates for Gram-positive bacteria and in LB agar plates for Gram-negative bacteria. For each sample, a 10-fold dilution series was prepared in PBS, and 10 μl of each dilution was plated. Colonies were counted after overnight incubation at 37°C.

Zebrafish embryo infection assay. Wild-type zebrafish embryos (ZF-Biolabs) were maintained according to standard protocols (34) and were dechorionated at 24 h postfecundation by treatment with pronase (2 mg · ml^{-1}) for 2 min. At 72 h postfecundation, embryos were individually distributed in 96-well plates and incubated in 50 μl of E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$ [pH 7]) at 28.5°C in the absence of the pathogen (controls) or in the presence of the pathogen (≈10^8 CFU/ml) for 7 h. Infected embryos were extensively washed with E3 medium to remove the bacteria and transferred, together with controls, to new 96-well microtiter plates containing the same autoclaved fresh medium supplemented with 25 μg (5 μl) of Cpl-7S or Cpl-1, or the same volume of P_i buffer (controls), and then incubated at 28.5°C under sterile conditions. Mortality was followed in all samples for 5 days, adding fresh E3 medium every day. Zebrafish embryos were considered dead when no movement was observed, even if a heartbeat was observed. Opacification of the larvae was always found to follow shortly. Each experiment was repeated at least 3 times, and 24 to 36 embryos were used per condition and experiment.

Immunochemistry and imaging analyses. Whole-mount immunochemistry was performed using standard zebrafish protocols (34). Zebrafish were anesthetized by immersion in tricaine (MS-222) (Sigma-Aldrich) at 200 mg · ml^{-1}. Animals were fixed overnight in BT fix (34). Permeabilization was carried out by freezing the embryos in acetone at −20°C for 7 min, followed by different washes in distilled water and a final wash in 0.1 M phosphate buffer (pH 7.3). Pneumococcal type 2 polyclonal antiserum (Staten Serum Institut) was used as the primary antibody, at a 1:200 dilution, whereas the secondary antibody was anti-rabbit–Alexa 568 diluted 1:25 (M. Probes). Unstained embryos and those stained only with the secondary antibody were used as negative controls. Confocal laser scanning microscopy (CLSM) images of embryos stained by immunochemistry were taken with a Leica TCS-SP2-AOBS optical inverted microscope (Leica Microsystems, Solms, Germany), and with HC PL APO CS 10×/0.40, 20×/0.70, and HCX PL APO CS 40×/1.25 to 0.75 oil immersion objectives. Images were processed with the LAS-AF (Leica) and NIH ImageJ.

Statistical analysis. All data are representative of results obtained from repeated independent experiments, and each value represents the mean ± standard deviations for 3 to 5 replicates. Statistical analysis was performed by using the two-tailed Student's *t* test (for two groups), whereas analysis of variance (ANOVA) was chosen for multiple comparisons. GraphPad InStat version 3.0 (GraphPad Software, San Diego, CA) was used for statistical analysis.

RESULTS

***In vitro* bactericidal activity of pneumococcal murein hydrolases.** Cpl-7 shows a specific activity on choline-containing purified cell walls comparable to that of Cpl-1 (17). In contrast, when these two lysozymes were tested exogenously using as the substrate live *S. pneumoniae* R6 cells suspended in phosphate-buffered saline (PBS) (see Materials and Methods), we found that the bacteriolytic activity of Cpl-7 was significantly lower than that of Cpl-1. Indeed, comparison with the three well-established pneumococcal enzybiotics showed that Cpl-1 and the autolysin LytA were very effective to kill and lyse the nonencapsulated strain, whereas Pal showed an intermediate activity and Cpl-7 was the least efficient enzyme (Fig. 1). Similar results were found when the encapsulated strains D39, P007, and P008 were tested with Cpl-1 and Cpl-7 (see Fig. S2 in the supplemental material).

Changing the net charge of Cpl-7. In an attempt to understand the reasons underlying the reduced lytic efficiency of Cpl-7 on intact pneumococcal cells when added externally, we per-

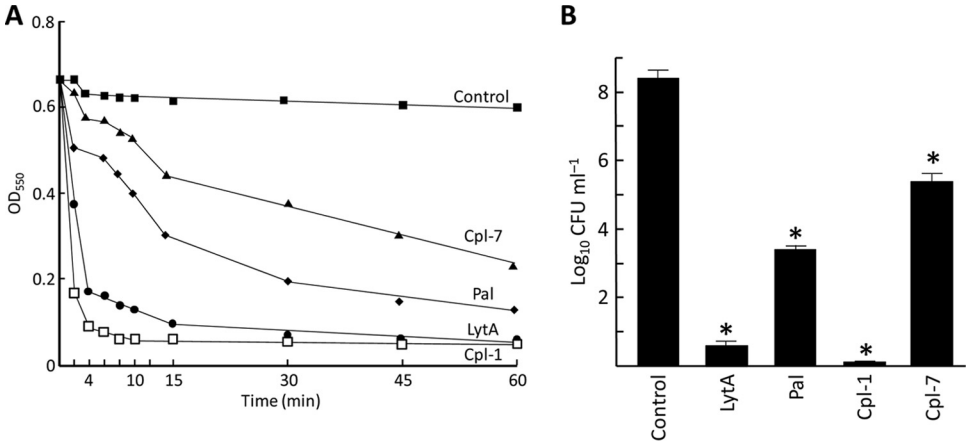

FIG 1 Bacteriolytic and bactericidal effects of different lytic enzymes against *S. pneumoniae* strain R6. (A) Exponentially growing pneumococci were washed, suspended in PBS at an OD_{550} of ≈0.6, and incubated in the absence or in the presence of the selected enzyme (5 μg · ml^{-1}) at 37°C. Decay of the bacterial suspension OD_{550} was followed for 60 min. Data are representative of four independent experiments. (B) Viable cells were determined on blood agar plates after 60 min of incubation under the same conditions. Data are means from four independent experiments. Error bars represent standard deviations, and asterisks mark results that are statistically significant compared to those for the controls in the absence of enzybiotics (one-way ANOVA with a *post hoc* Dunnet test; *, $P < 0.001$).

formed a careful comparative inspection of available data. We observed that the net charge of Cpl-7 was extremely negative (−29.77 at neutral pH) compared either to those of the other three pneumococcal enzybiotics (−14.82 for Cpl-1, −14.57 for LytA, and −10.57 for Pal) or to those of nonpneumococcal endolysins (35). The strong negative charge of Cpl-7 is scattered along the molecule but is particularly remarkable on the CWBM, compared to the corresponding modules of the other pneumococcal enzybiotics (see Table S1 in the supplemental material). Interestingly, Low and coworkers recently noticed a correlation between the charges of catalytic domains of phage lysins and their dependence on CWBMs for bacteriolytic activity, as the cell walls of Gram-positive bacteria generally have a negative charge (35). In line with this, we hypothesized that charge disparity on CWBMs might account, in particular, for the distinct bacteriolytic activities of Cpl-7 and Cpl-1, considering the high similarity of their catalytic modules and their comparable specific activities on choline-containing purified cell walls (17). To test this hypothesis, and aiming to produce a Cpl-7 variant with enhanced antimicrobial activity, the sequence of the CW_7 repeats was examined for residues whose mutation allowed inversion of the net charge while affecting neither the fold nor cell wall recognition. To do this, five amino acid changes per repeat (15 mutations in the whole CWBM) were made (Fig. 2A): three basic residues (either Lys or Arg) were introduced at positions not conserved within the CW_7 family (PF08230) (L216K, D225K, and A230R; numbering corresponds to the first CW_7 repeat), whereas two partially conserved aspartic acid residues were mutated to asparagines (D233N and D239N), changing the total charge of the module from −14.93 to +3.0. As shown in Fig. 2 all mutations were located outside the cavities (one per repeat) identified as potential binding sites on the CWBM model surface by the Fpocket software. This Cpl-7 variant, named Cpl-7S here, has a total net charge of −11.84, which is comparable to those of the other three pneumococcal lysins.

Evaluation of Cpl-7S structural conservation. The recombinant Cpl-7S lysozyme showed high expression levels in *E. coli* and was purified using the protocol established for the wild-type en-

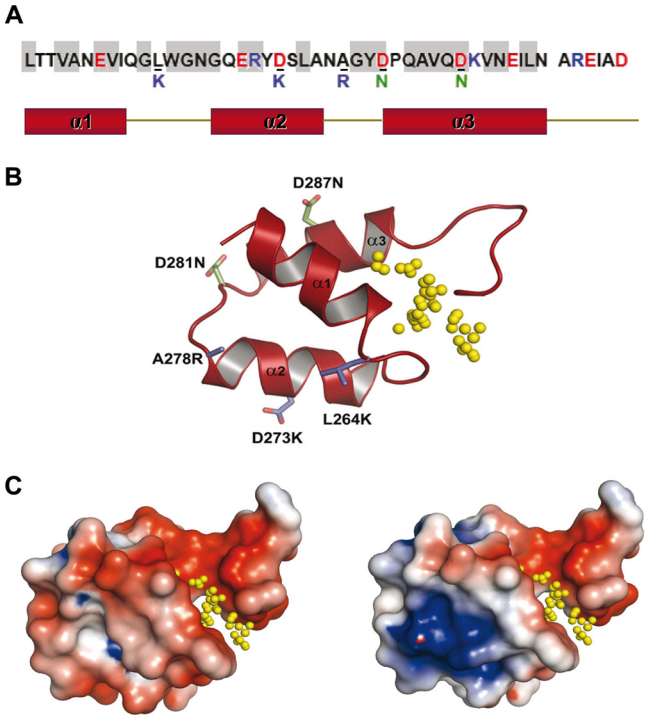

FIG 2 Modification of the net charge of the CWBM of Cpl-7. (A) Distribution of amino acid substitutions along the sequence of CW_7 repeats. The upper row shows one repeat and linker sequences of Cpl-7, with acidic and basic residues depicted in red and blue, respectively. Positions mutated are underlined, with substitutions indicated below (basic amino acids in blue and neutral polar residues in green). Gray boxes show conserved regions in the CW_7 family (PF08230), and α-helical segments are shown as purple rectangles. (B) Cartoon representation of the three-dimensional structure of the second repeat from the model by Bustamante et al. (17). In blue are residues replaced by basic amino acids, and in green are replacements of aspartic acid residues by asparagines (stick mode representation). (C) Molecular surface of the same repeat colored according to its electrostatic potential in Cpl-7 (left) and Cpl-7S (right) (red for acidic and blue for basic). The binding cavity identified by Fpocket software is shown by the small yellow spheres.

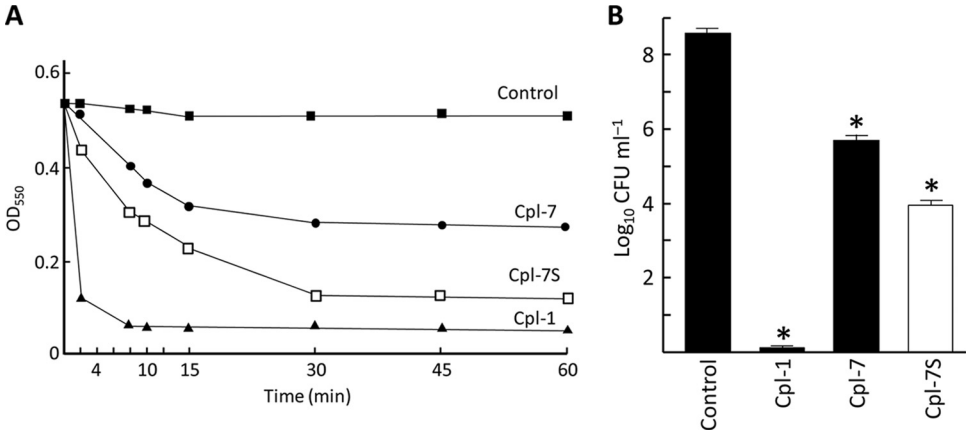

FIG 3 Bacteriolytic and bactericidal effects of phage lytic lysozymes against *S. pneumoniae* strain R6. (A) Bacterial cells were treated as for Fig. 1, and the time course of bacterial suspension turbidity was followed. Data are representative of four independent experiments. (B) Viable cells were determined on blood agar plates after 60 min of treatment with the enzybiotics. Data are means from four independent experiments. Error bars and asterisks have the same meaning as in Fig. 1. Differences between Cpl-7 and Cpl-7S activities are statistically significant ($P < 0.05$).

zyme (17). Protein samples were found to be homogeneous according to SDS-PAGE and MALDI-TOF analyses, which yielded a molecular mass of 38,419 Da, in good agreement with the sequence-based molecular mass (38,450.5 Da, with the initial methionine processed). Conservation of the folded state was checked by CD spectroscopy and analytical ultracentrifugation. As shown in Fig. S3 in the supplemental material, the CD spectra of Cpl-7S and Cpl-7 are almost superimposable, both in the far- and near-UV regions, confirming that their secondary and tertiary structures were comparable. In addition, ultracentrifugation experiments showed that Cpl-7S, like the wild-type enzyme, sediments as a single species ($s_{20,w}$ = 2.93 S) corresponding to the monomer (≈38.8 kDa). The specific activities of Cpl-7 and Cpl-7S were also similar, as determined using radioactively labeled pneumococcal cell walls (Cpl-7, 6.2×10^4 U · mg^{-1}; Cpl-7S, 6.5×10^4 U · mg^{-1}). In contrast, Cpl-7S was considerably more active than Cpl-7 on whole R6 cells (see below). These results confirmed that Cpl-7S maintained the structural features of the wild-type form while its killing capacity on pneumococcal cells was significantly enhanced, in agreement with our hypothesis. It is worth noting that Cpl-7S keeps most of its bactericidal effect even after 7 days at 37°C (3 log units instead of the 4-log-unit decrease on R6 viable cells produced by fresh Cpl-7S in the standard assay described above) (see Fig. S4 in the supplemental material). This enzymatic robustness might be extremely convenient for further pharmacological or biotechnological applications.

Bactericidal activity of Cpl-7S against pneumococcal strains. The antimicrobial capacity of Cpl-7S was tested against several pneumococcal strains using the protocol described in Materials and Methods, which measures the turbidity decrease at 550 nm (OD_{550}) and bacterial survival after 60 min of incubation with and without lysin at 37°C. Direct comparison of the Cpl-7, Cpl-7S, and Cpl-1 killing capacities pointed out the improved activity of Cpl-7S compared to the wild-type Cpl-7 (Fig. 3). However, Cpl-7S was not as lethal as Cpl-1; i.e., a decrease of 7 log units on R6 culture viability was caused by 1 μg · ml^{-1} Cpl-1, 20 μg · ml^{-1} Cpl-7S, or 50 μg · ml^{-1} Cpl-7. Interestingly, Cpl-7S showed similar bactericidal action against other encapsulated pneumococci tested, including the multiresistant clinical strains 1515/97 (serotype 6B) and 69 (serotype 19F) (see Fig. S5 in the supplemental material). The latter strain is resistant to tetracycline, erythromycin, chloramphenicol, and amoxicillin, among other antibiotics (36). These results demonstrated that the Cpl-1, Cpl-7, and Cpl-7S lysozymes did not display the same bacteriolytic properties when acting from the outside of live cells, in spite of having rather similar specific activities. In addition, the observed differences in bactericidal activity were confirmed by the respective MICs measured with *S. pneumoniae* strain ATCC 49619: 256 ± 50 μg · ml^{-1} for Cpl-7, 64 ± 10 μg · ml^{-1} for Cpl-7S, and 16 ± 4 μg · ml^{-1} for Cpl-1.

Bactericidal activity of Cpl-7S against nonpneumococcal species. As Cpl-7 was active on choline- and ethanolamine-containing (lipo)teichoic acids and preliminary results pointed to the peptidoglycan network as the CW_7 target, it was conceivable that the Cpl-7 and Cpl-7S lysozymes could lyse other Gram-positive pathogens apart from pneumococcus. Thus, the bacteriolytic and bactericidal activities of Cpl-7 and Cpl-7S were tested on various streptococcal and nonstreptococcal bacteria. As the data in Table 1 reveal, acquisition of CW_7 repeats has conferred to Cpl-7 and Cpl-7S the ability to efficiently kill several nonpneumococcal bacteria, with the synthetic Cpl-7S enzyme being the most powerful lysin. In particular, Cpl-7S decreased the viability of three other important human pathogens, i.e., *S. pyogenes*, *S. mitis*^{T}, and *E. faecalis*, by 4, 4, and 2 log units, respectively, at very low enzyme concentration (5 μg · ml^{-1}) within 1 h (Fig. 4 and Table 1). In addition, *S. mitis* SK598, a strain that contains ethanolamine instead of choline in the cell wall (37), was also efficiently killed, whereas 90% of *Streptococcus iniae* and *Streptococcus dysgalactiae* cells were killed after 60 min of enzybiotic treatment (Table 1). It is worth noting that measurement of viable *S. dysgalactiae* cells was performed using optical microscopy with a fluorescent live/dead cell reagent (BacLight Kit; Invitrogen), since these bacteria form long chains and direct counting of CFU on agar plates was inaccurate. On the other hand, Cpl-1 was completely unable to perceptibly destroy bacteria lacking choline-containing cell walls and killed the choline-containing *S. mitis* type strain as efficiently as Cpl-7S (Table 1).

Bactericidal activity of Cpl-7S against Gram-negative bacteria. In an attempt to broaden further the antimicrobial spectrum

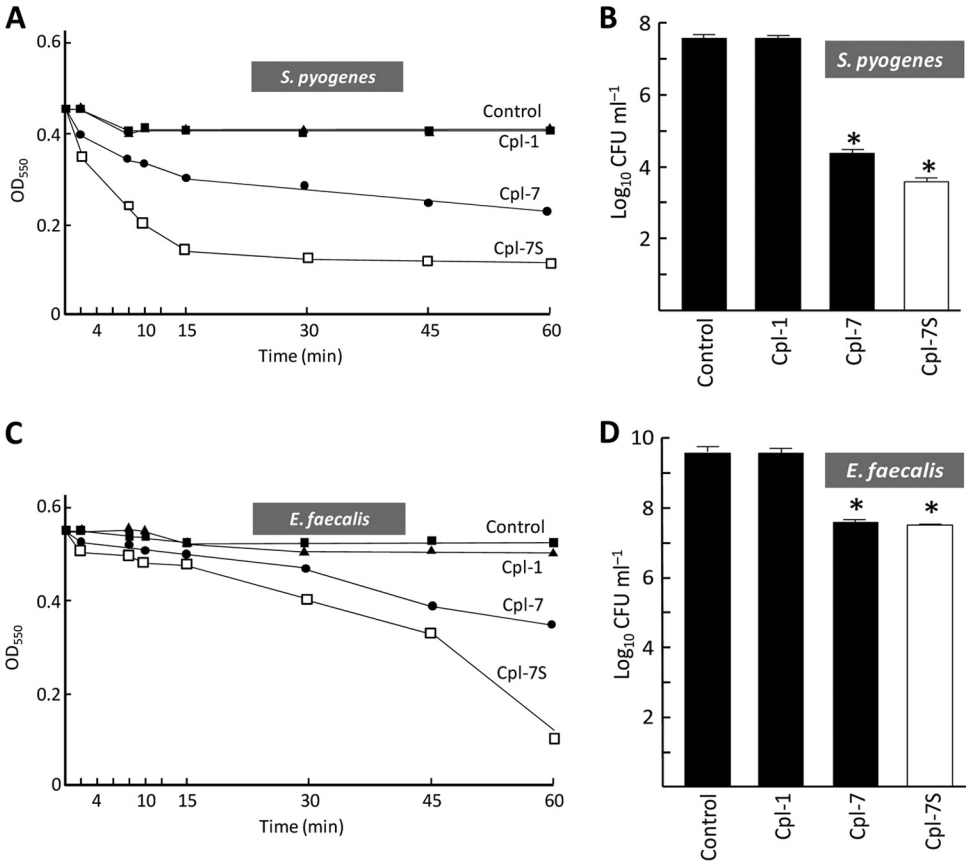

FIG 4 Bacteriolytic and bactericidal effects of pneumococcal phage lysozymes against *S. pyogenes* and *E. faecalis*. (A and C) Exponentially growing bacterial cultures were washed, suspended in PBS, and incubated in the absence or presence of the assayed enzyme (5 μg · ml^{-1}) at 37°C. Variation of the OD_{550} of the cultures was followed for 60 min. Data are representative of four independent experiments. (B and D) Bacterial viability was determined after 60 min of treatment with Cpl-1, Cpl-7, and Cpl-7S. Data are means from four independent experiments. Error bars and asterisks have the same meaning as in Fig. 1. Differences between Cpl-7 and Cpl-7S activities are statistically significant ($P < 0.05$).

of Cpl-7S, a distinct strategy was applied to overcome the physical barrier imposed by the outer membranes of Gram-negative bacteria. The approach was to use sensitization of the outer membrane with compounds that, at the employed concentration, could facilitate enzyme passage without having bactericidal effects by themselves. This goal was achieved by incubation of Gram-negative bacteria with 0.01% carvacrol (an aromatic oily liquid obtained from oregano and thyme oils) prior to addition lysins. It is known that carvacrol and related compounds disintegrate the outer membranes of Gram-negative bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane (38).

We examined the sensitivity of *E. coli* and *P. putida* as representatives of Gram-negative bacteria. Both species became susceptible to the lytic action of Cpl-7S upon preincubation of bacteria with 0.01% carvacrol (Fig. 5). At this concentration, incubation in carvacrol-containing buffer barely decreased cell viability (0.32 ± 0.09 log unit in 60 min [mean for three independent experiments]), whereas subsequent addition of 5 μg · ml^{-1} Cpl-7S caused cell survival to plummet, reducing it by 3 log units. The combined bactericidal action of an essential oil and a lysin was functional only for Cpl-7S and Cpl-7, since other enzybiotics tested, namely, Cpl-1, Pal, and LytA, were totally ineffective (Table 1 and unpublished results). This observation further supports the notion that the capacity of Cpl-7S for killing Gram-negative bacteria arises from the ability of CW_7 repeats to recognize and bind the cell wall muropeptide, a structural element shared by Gram-positive and Gram-negative bacteria.

Bactericidal activity of Cpl-7S using an infection animal model. The results described above demonstrated that Cpl-7S is highly efficient in killing a variety of Gram-positive bacteria, particularly *S. pneumoniae*, *S. pyogenes*, *S. mitis*, and *E. faecalis*. To validate these data in an animal model of infection, we chose an alternative and relatively new model for studying streptococcal pathogenesis, i.e., zebrafish (*Danio rerio*) embryos (39). Thus, at 72 h postfecundation, zebrafish embryos were brought in contact with each tested pathogen (typically the D39 pneumococcal strain or *S. pyogenes*^{T} strain, adjusted to 1×10^{8} CFU · ml^{-1} of either bacterium) by immersion in E3 medium. Incubation was prolonged for 7 h at 28.5°C, using heat-killed (10 min at 65°C) D39 cells as negative control. Afterwards, embryos were extensively washed with the same medium and treated with 25 μg Cpl-7S or the corresponding volume of P_i buffer, as explained in Materials and Methods. The mortality rate of embryos in the bacterium-containing samples was significant (28.7% for *S. pneumoniae* and 35% for *S. pyogenes*) (Fig. 6), although the time course of the process and morphological deformations were apparently pathogen dependent. Embryos exposed to pneumococci showed in-

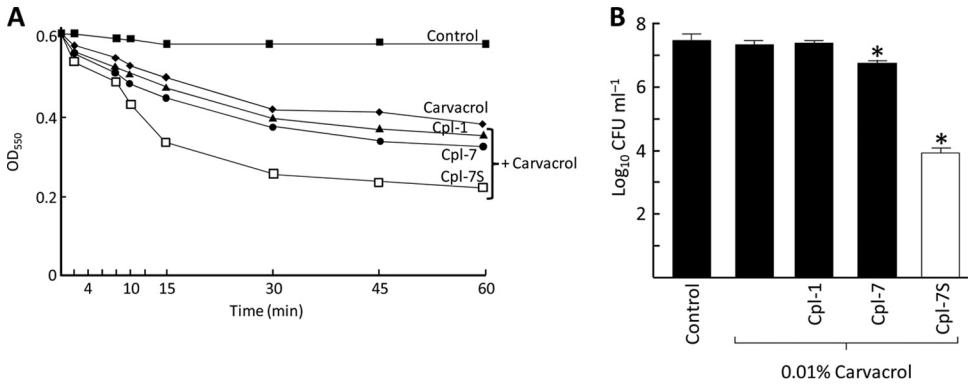

FIG 5 Bacteriolytic and bactericidal effects of pneumococcal phage lysozymes against *E. coli* in the presence of carvacrol. (A) Exponentially growing *E. coli* DH10B cells were washed, suspended in PBS at an OD_{550} of ≈0.6, and incubated at 37°C in the presence of 0.01% carvacrol and the assayed enzymes (5 μg · ml^{-1}). The time course of bacterial suspension turbidity was followed. Data are representative of four independent experiments. (B) Viable cells were determined on blood agar plates after 60 min of treatment with the same enzymes. Data are means from four independent experiments. Error bars and asterisks have the same meaning as in Fig. 1. Differences between Cpl-7 and Cpl-7S activities are statistically significant ($P < 0.05$).

flammation of different parts of the body (mainly heart and liver), and death occurred at ca. 96 h postinfection, while *S. pyogenes*-treated embryos showed an apparent necrotization without any visible deformation and died at about 24 h after infection. The use of higher bacterial inoculums compromises embryo viability, since the turbidity increase affects the zebrafish embryonic development (34). Addition of a single 25-μg dose of Cpl-7S to bacterium-infected embryos protected them from death, with noticeable survival rates being reached (99% for pneumococcus- and 95.3% for *S. pyogenes*-infected samples) (Fig. 6). Among the various pneumococcal strains tested (either encapsulated or not), D39 was the most lethal strain. It is interesting to note that the nonencapsulated R6 strain was virtually avirulent, confirming that, as in humans and animal models of pneumococcal infection reported to date, the capsule is also an essential virulence factor in the zebrafish model. Embryos treated with Cpl-1 showed the same level of protection as those treated with Cpl-7S for *S. pneumoniae*-infected embryos, but no protection at all was found for those infected with *S. pyogenes* (unpublished results). Finally, to ascertain that bacterial infection was the real cause of embryo death, we localized the pneumococcal cells into the embryos by whole-mount immunochemistry, using a polyclonal antibody recognizing the capsular polysaccharide of strain D39 as the primary antibody. As shown in Fig. 7 and in Fig. S6 in the supplemental material, the specific fluorescent signals corresponding to pneumococci appeared around the gills, although basal fluorescence was detected in the eyes of embryos, probably due to their high content of pigment cells. Confocal laser scanning microscopy (CLSM) also allowed confirmation that *S. pneumoniae* cells were internalized into the embryo body.

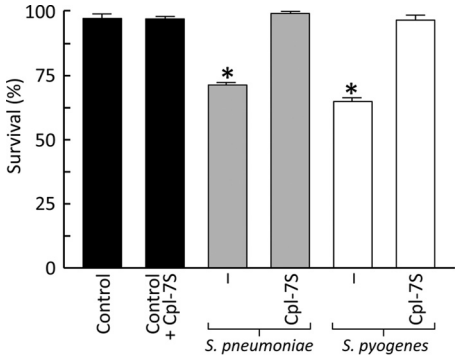

FIG 6 Pathogen infection in the zebrafish embryo model. Survival of zebrafish embryos infected with *S. pneumoniae* or *S. pyogenes* and treated or not at 7 h after infection with 25 μg Cpl-7S ($n = 24$ to 36 embryos/condition) is shown. Data are means from four independent experiments. Error bars represent standard deviations, and asterisks mark the results that are statistically significant for the overall comparison of infected or Cpl-7-treated embryos versus the controls (one-way ANOVA with a *post hoc* Dunnet test; *, $P < 0.001$).

DISCUSSION

Phage lysins may constitute a promising weapon to kill multiresistant bacterial pathogens, and they are currently also known as enzybiotics (protein antibiotics). Recently, it has been proposed that the concept of enzybiotics should be extended and refer to all the enzymes, regardless of their origin, exhibiting antibacterial and/or antifungal activity (40). Experimentally proved results, both *in vitro* and *in vivo*, are required to be included in a database that compiles the enzybiotics reported so far in the literature (41). In the last updated version, there are 21 examples of such lysins, including those specifically directed against pneumococcal strains: Cpl-1 phage lysozyme, Pal phage amidase, and LytA bacterial amidase.

Regarding the Cpl-7 lysozyme, a pneumococcal murein hydrolase that does not contain a choline-binding module, massive genome sequencing has boosted the number of bacterial genomes known to contain homologues of its C-terminal CW_7 repeats. Currently, it appears that this cell wall-binding motif is scattered in a variety of bacterial genes with different formats: CW_7 may exist as a single or double motif or as 1 to 3 tandem repeats fused to different putative functional modules. The PFAM database version 27.0 (last date accessed, 19 May 2013) (42) describes CW_7 repeats in 202 protein sequences (corresponding to 126 species) that are organized in 31 different architectures. Another conclusion drawn from database searches was that many, but not all, of the putative murein hydrolases containing CW_7 motifs would belong to phage lysins, as the corresponding genes appear to form parts of phage lytic cassettes (17). To date, besides Cpl-7, only the endolysin from the λSA2 prophage of *Streptococcus agalactiae*

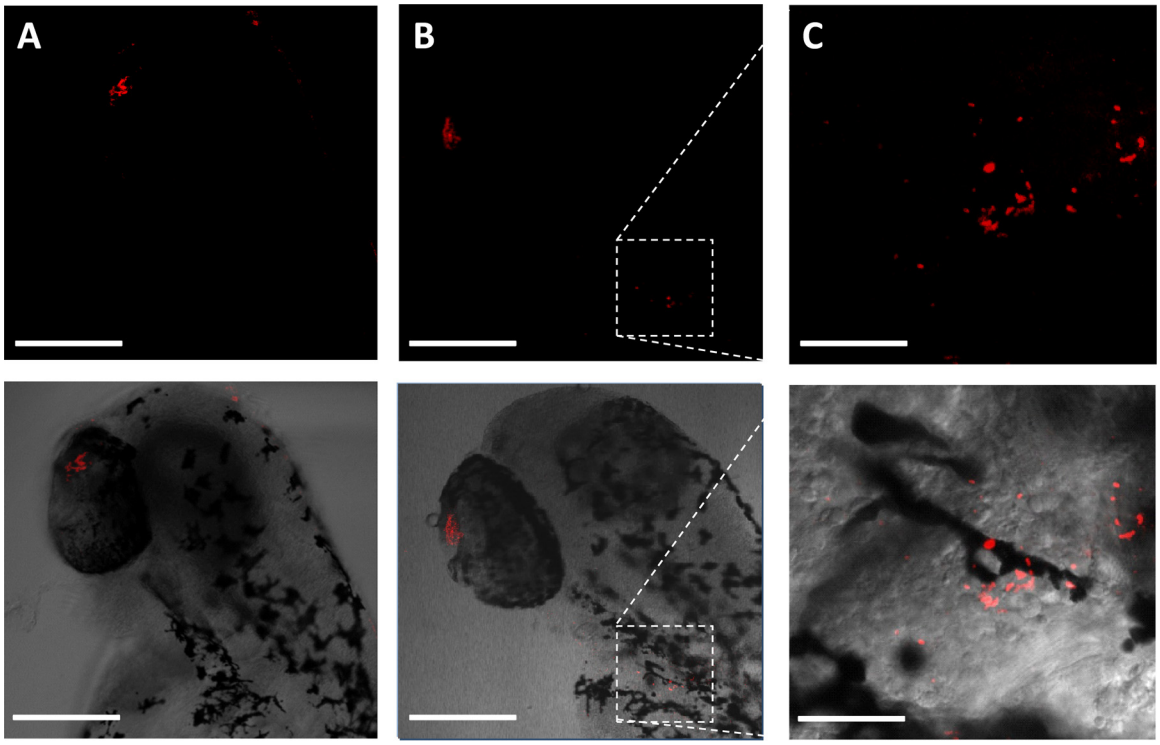

FIG 7 Localization of *S. pneumoniae* in infected zebrafish embryos. Representative whole-mount immunofluorescence of zebrafish embryos at 48 h postinfection (5 days postfecundation) with *S. pneumoniae* was analyzed by CLSM. D39 cells (red) were labeled with a polyclonal antibody recognizing pneumococcal type 2 capsular polysaccharide. Maximal projections from 15 z-stacks were constructed from fluorescence and differential interference contrast confocal images. Top panels, red (bacterial) fluorescence; bottom panels, transmitted light and red (bacterial) fluorescence overlay. (A) Pneumococcus-free embryos at 5 days postfecundation were used as a control (20× objective; details of the gills are shown in Fig. S6 in the supplemental material). (B) Embryo head infected by *S. pneumoniae* (20× objective). (C) Details of *S. pneumoniae* infection around the gills (40× objective). Exposure settings were identical for all samples. Bars, 250 μm (A and B) or 25 μm (C).

(strain 2603 V/R) and its close homologue LySMP from the *S. suis* SMP bacteriophage have been demonstrated to have cell wall-degrading activity (43, 44). In addition, acquisition of CW_7 repeats has conferred to Cpl-7 the ability to degrade pneumococcal cell walls containing either choline or ethanolamine. This unusual characteristic, together with the wide distribution of the CW_7 motif in bacterial genomes and recent evidence on specific muropeptide targeting by CW_7 repeats (18), strongly indicated that Cpl-7 could recognize and degrade other bacterial peptidoglycans apart from that of pneumococci.

In strong contrast with their similar specific activities on purified cell walls (17), *in vitro* tests of activity by exogenous addition of Cpl-7 to pneumococcal cultures revealed a bacteriolytic capacity much lower than that of Cpl-1. One obvious difference between these two assays of activity was the way of access to the peptidoglycan layer. In purified cell wall preparations, substrate fragmentation facilitates the accessibility and cleavage of susceptible bonds, as also do phage-encoded holins when endolysins act *in vivo* from the inside of the cell (45, 46). However, from the outside of intact cells, accessibility and diffusion can be controlled, among other factors, by muropeptide cross-linking, membrane- and cell wall-attached lipoteichoic and teichoic acids, and capsular polysaccharides. All these elements dramatically alter the appearance and charge of the outer envelope of Gram-positive bacteria, providing, at the same time, a continuum of negative charge (47). In this context, the most distinctive feature of Cpl-7, in comparison to other cell wall hydrolases, was the high negative charge of its CWBM, which extended the negative electrostatic potential harbored by its catalytic module to the whole molecule. This extremely negative net charge could severely hamper, via unfavorable electrostatic interactions, the accessibility of Cpl-7 to the peptidoglycan network and account (at least partially) for its minor antipneumococcal activity.

This hypothesis has been experimentally confirmed by engineering the variant Cpl-7S, whose net charge was increased from −29.77 to −11.84, by reversing the sign of the net charge of the CWBM without affecting the native protein fold. Cpl-7S shows stronger bacteriolytic activity than Cpl-7 against most pneumococcal strains tested, including the multiresistant clinical isolates, and also against *S. mitis*^{T} and *S. pyogenes*^{T}. The intermediate to moderate activities against *E. faecalis*, *S. mitis* strain SK598, *S. iniae*, and *S. dysgalactiae* are, however, similar to those of Cpl-7, while the other Gram-positive bacteria tested were refractory to the lytic activity of both enzymes. The correlation between the degree of susceptibility of a given Gram-positive bacterium to Cpl-7S and the detailed architecture of its cell surface warrants further study. However, it is tempting to speculate that the specificity and final bacteriolytic activity of Cpl-7S against a particular substrate could be due to a complex process initially mediated by the composition and charges of the two partners engaged: the endolysin and the bacterial envelope. Thus, the inversion of charge engineered in the CWBM would have facilitated the initial

approach of Cpl-7S and its diffusion through the capsule and/or peptidoglycan networks, thereby helping the positioning and correct attachment through the CWBM and the efficient cleavage of cognate bonds. Indeed, our results suggest that in *S. pneumoniae*, the acquisition of the polysaccharidic capsule hampers the bactericidal activity of Cpl-7 and Cpl-7S, and specifically, replacement of the type 3 capsule of strain P007 by the type 4 capsule in the otherwise identical P008 strain increases the activity of Cpl-7S by 1 log unit (Table 1). This is in contrast with data reported for other enzybiotics, for example, PlySs2 (14) or PlyG (8), whose respective activities against *S. pyogenes* and *Bacillus anthracis* showed no difference for unencapsulated or thickly capsulated variants.

The results reported here showed, for the first time, a correlation between the net charge of the CWBM of one endolysin and its bacteriolytic activity. Moreover, they constitute a good example of enhancing endolysin lethal activity by structure-based protein engineering, since tailor-made substitution of specific amino acids has reduced 4-fold the MIC value of the parental lysin against pneumococci. A similar approach was employed with the XlyA lysin from *Bacillus subtilis*, where reversion of the net charge of the catalytic module from −3 to +3, by introducing five amino acid changes, eliminated its dependence of the CWBM for activity (35). Notably, inversion of the CWBM net charge of the Cpl-7S lysozyme has required a total of 15 amino acid substitutions that increased the net charge by ≈18 units without affecting either the protein fold or the CW-7 binding cavities, according to biophysical and computational studies. Of note, Cpl-7S can efficiently kill, with carvacrol as adjuvant, two model Gram-negative bacteria, *E. coli* and *P. putida*. Carvacrol is one of the compounds that group as "essential oils" with proven antibacterial activity at concentrations ranging from 0.2% to 1% that, alone or in combination with antibiotics (48, 60), are exploited nowadays as preservatives in the food industry (38). In our study, the combined action of 0.01% carvacrol and 5 $\mu g \cdot ml^{-1}$ Cpl-7S has revealed a novel behavior of the synthetic lysozyme among experimentally demonstrated enzybiotics (41), since none of them, including those that showed a broadened range of susceptible bacteria, were effective against Gram-negatives pathogens. Recently, the *in vitro* ability of EL188 endolysin to efficiently kill *Pseudomonas aeruginosa* when combined with EDTA, a Ca^{2+} chelator that permeabilizes the outer membranes of Gram-negative bacteria, has also been reported (49). In a different approach, a hybrid lysin built by fusion of the T4 lysozyme to the FyuA-targeting domain of pesticin (a bacteriocin with peptidoglycan-degrading activity) was shown to kill *Yersinia pestis* as well as clinical *E. coli* isolates expressing the FyuA outer membrane receptor (50). However, its bactericidal activity against *E. coli* cells was rather moderate (≈20% survival after 60 min of treatment at 100 $\mu g \cdot ml^{-1}$).

Confirmation of the killing effect of Cpl-7S *in vivo* has been achieved using a zebrafish embryo model of infection, which could foresee the application of Cpl-7S against life-threatening pathogens as relevant as *S. pneumoniae* and *S. pyogenes*, especially focused on multiresistant bacteria of these species, without affecting the normal microbiota. In this context, it has been anticipated that the mechanism of action of enzybiotics, that is, cleaving specific bonds of a very well-conserved polymer among bacteria, makes the appearance of mutants resistant to these enzymes unlikely (51), a theoretical assumption corroborated to date in practice (52). In addition, endolysins are efficient enzybiotics on mucous membranes (53, 54), which are major reservoirs and routes of infection of those pathogens.

All these results allow the conclusion that modulation of the net charge of cell wall-binding motifs might be a general way of improving the enzymatic efficiency and selectivity of putative or actual enzybiotics, in the same way that introduction of a positive net charge in the catalytic module might confer CWBM-independent activity to phage lysins (35), thereby expanding the range of susceptible pathogens. In this respect, currently available results still support the notion that even lysins with a wider range of antimicrobial activity would exert a less dramatic effect on the normal microbiota than conventional antibiotics.

ACKNOWLEDGMENTS

We are indebted to P. Sánchez-Testillano, M. T. Seisdedos, and G. E. Serrano for helping us with the immunochemistry analysis. We thank M. García-Franco and L. Araújo for advice with the zebrafish embryo experiments and D. V. Laurents for revising the English version. We are also grateful to E. Cano, G. García, and V. López for excellent technical assistance.

This research was funded by grants from the Ministerio de Ciencia e Innovación (MICINN) to P. García (SAF2009-10824) and E. García (SAF2012-39444-C02-01) and from the MICINN (BFU2009-10052 and BF42012-36825) and the Consejería de Educación de la Comunidad de Madrid (S2010/BMD/2457) to M. Menéndez. Additional funding was provided by the CIBER de Enfermedades Respiratorias (CIBERES), an initiative of the Instituto de Salud Carlos III (ISCIII). Roberto Díez-Martínez was the recipient of a fellowship from the MICINN (FPI program).

We declare a competing financial interest. We are coinventors on a Spanish patent application (no. P201330777) covering the results contained in this article. Any potential income generated by exploitation of the patent rights and received by our employers, the CSIC, and CIBERES shall be shared with us according to Spanish law.

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