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EXPERIMENTAL THERAPEUTICS

Chemotherapy with Phage Lysins Reduces Pneumococcal Colonization of the Respiratory Tract

Bruno Corsini,[a] Roberto Díez-Martínez,[b]* Leire Aguinagalde,[a] Fernando González-Camacho,[a] Esther García-Fernández,[b]* Patricia Letrado,[b]* Pedro García,[b,c] Jose Yuste[a,c]

[a]Centro Nacional de Microbiología, Instituto de Salud Carlos III (ISCIII), Majadahonda, Madrid, Spain
[b]Centro de Investigaciones Biológicas, CSIC, Madrid, Spain
[c]Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES), Madrid, Spain

ABSTRACT Bacteriophage-borne lytic enzymes, also named lysins or enzybiotics, are efficient agents for the killing of bacterial pathogens. The colonization of the respiratory tract by *Streptococcus pneumoniae* is a prerequisite for the establishment of the infection process. Hence, we have evaluated the antibacterial activities of three different lysins against pneumococcal colonization using human nasopharyngeal and lung epithelial cells as well as a mouse model of nasopharyngeal colonization. The lysins tested were the wild-type Cpl-1, the engineered Cpl-7S, and the chimera Cpl-711. Moreover, we included amoxicillin as a comparator antibiotic. Human epithelial cells were infected with three different multidrug-resistant clinical isolates of *S. pneumoniae* followed by a single dose of the corresponding lysin. The antimicrobial activities of these lysins were also evaluated using a mouse nasopharyngeal carriage model. The exposure of the infected epithelial cells to Cpl-7S did not result in the killing of any of the pneumococcal strains investigated. However, the treatment with Cpl-1 or Cpl-711 increased the killing of *S. pneumoniae* organisms adhered to both types of human epithelial cells, with Cpl-711 being more effective than Cpl-1, at subinhibitory concentrations. In addition, a treatment with amoxicillin had no effect on reducing the carrier state, whereas mice treated by the intranasal route with Cpl-711 showed significantly reduced nasopharyngeal colonization, with no detection of bacterial load in 20 to 40% of the mice. This study indicates that Cpl-1 and Cpl-711 lysins might be promising antimicrobial candidates for therapy against pneumococcal colonization.

KEYWORDS pneumococcus, phage lysins, nasopharyngeal colonization, mouse model

Streptococcus pneumoniae is one of the major etiologic agents of acute otitis media, community-acquired pneumonia, sepsis, and bacterial meningitis, causing high morbidity and mortality rates worldwide (1, 2). Asymptomatic carriage is the prerequisite for all these infections and is more frequently associated with early childhood (3). The successful colonization of the upper respiratory tract is critical for the horizontal spread of genes involved in antibiotic resistance and/or virulence and may lead to the development of invasive pneumococcal disease (IPD), which is the most severe clinical manifestation (3, 4). In addition, microbial colonization of the lower respiratory tract is associated with chronic obstructive pulmonary disease (COPD), in which exacerbations and airway inflammation are important aspects related to persistence (5).

Purified phage-borne lysins (also known as endolysins and enzybiotics) represent a promising alternative to current antibacterials, as lysins kill susceptible bacteria much faster than standard antibiotics (6, 7). Lysins are murein hydrolases that specifically cleave different bonds of the bacterial peptidoglycan, thereby triggering osmotic lysis.

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Address correspondence to Pedro García, pgarcia@cib.csic.es, or Jose Yuste, jyuste@isciii.es.

* Present address: Roberto Díez-Martínez, The Zebrafish Lab, Noáin, Navarra, Spain; Esther García-Fernández, Centro Nacional de Biotecnología, CSIC, Madrid, Spain; Patricia Letrado, The Zebrafish Lab, Noáin, Navarra, Spain.

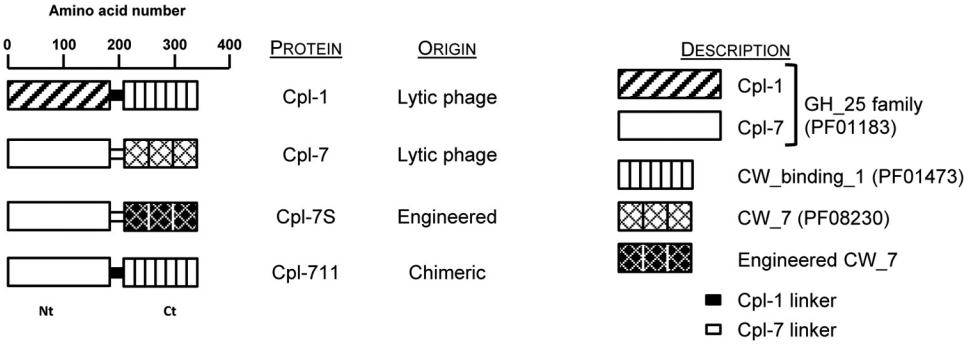

FIG 1 Schematic representations and descriptions of parental Cpl-1, Cpl-7, engineered Cpl-7S, and chimeric Cpl-711 lysozymes. Cpl-711 contains the CD of Cpl-7S, the linker of Cpl-1, and the CWBD of Cpl-1. CDs belong to the GH_25 family of glycosyl hydrolases and share 159 of 186 amino acid residues between Cpl-1 (hatched bars) and Cpl-7 (open bars). Linkers of Cpl-1 (13 amino acid residues) and Cpl-7 (16 amino acid residues) are not depicted at scale. Nt, N-terminal domain; Ct, C-terminal domain.

These specialized enzymes generally display a modular organization, containing an N-terminal catalytic domain (CD) and a C-terminal cell wall-binding domain (CWBD), with a flexible linker region connecting both domains (7). This architecture has enabled the successful swapping of functional domains to construct new chimeric proteins and the engineering of wild-type enzymes to improve the catalytic or stability properties, leading to a modification of the spectrum of susceptible bacteria (8–11).

In the pneumococcal system, several lysins have been characterized, such as Pal amidase (12) and Cpl-1, Cpl-7, Cpl-7S, and Cpl-711 lysozymes (9, 10). The lysozymes Cpl-1 and Cpl-7 are harbored by bacteriophages Cp-1 and Cp-7, respectively, and their CDs belong to the glycosyl hydrolase family 25 (GH_25; PF01183). Cpl-7S is an engineered variant derived from Cpl-7, in which 15 amino acid residues of the CWBD were changed to enhance its bactericidal activity, and Cpl-711 is a synthetic chimera that contains the CD from Cpl-7, at the N-terminal region, and the linker and CWBD of Cpl-1, at the C-terminal region (Fig. 1). In terms of lytic efficacy and specificity, Cpl-1 and Cpl-711 require the presence of choline residues in the teichoic acids of the pneumococcal cell wall to perform their antibacterial activities, whereas Cpl-7S is choline independent due to the presence of CW_7 repeats in its CWBD (9). Thus, Cpl-1 and Cpl-711 show specific antipneumococcal activities against planktonic and biofilm cultures, in contrast with Cpl-7S that has a broader range of susceptible bacteria and was also capable of killing other relevant pathogens, including *Streptococcus pyogenes* and *Enterococcus faecalis* (9). Moreover, this kind of lysin therapy has been shown to be effective against a variety of severe pneumococcal infections, including meningitis, pneumonia, and sepsis, with the advantage of a marked specificity (12–14).

It should be noted that although the protection activity of these three lysins against systemic pneumococcal infection is documented (9, 10, 12–15), evidence demonstrating their efficacy against nasopharyngeal colonization by *S. pneumoniae* has not been reported. In addition, the therapeutic use of enzybiotics against chronic bacterial respiratory infections is relatively unexplored. This is important since it is generally thought that nasopharyngeal carriage by *S. pneumoniae* is essential for the pathogenesis process, because it is a prerequisite for invasive disease (16). Although the introduction of current antipneumococcal conjugate vaccines has decreased nasopharyngeal colonization, more than 30% of children are still asymptomatic carriers of pneumococci with multiple serotypes (17–19). In addition, *S. pneumoniae* is one of the most frequent pathogens causing acute exacerbations and recurrent pneumonia episodes in COPD patients (5). From the clinical perspective, this situation is even worse, as multidrug-resistant pneumococcal isolates are a frequent cause of persistent infections in COPD patients and antibiotic resistance compromises treatment outcome (20, 21). Thus, antimicrobial approaches that utilize phage lysins might be a potential strategy to diminish the colonization process and even to clear persistent bacteria in

TABLE 1 Antimicrobial susceptibility of three MDR pneumococcal isolates

Treatment	MIC (μg/ml) of strain (serotype):		
	48 (23F)	69 (19F)	3498 (8)
PEN	8	2	0.015
ERY	>128	>128	>128
LVX	2	1	16
TET	64	4	64
CHL	4	4	4
AMX	16	2	0.06
Cpl-1	4	8	2
Cpl-7S	64	128	64
Cpl-711	1	4	2

chronic respiratory infections. As phage lysins kill bacteria rapidly on contact, the use of these lytic enzymes may reduce bacterial attachment to epithelial cells of the upper and lower respiratory tracts.

In this study, we investigated the antimicrobial activity of Cpl-1, Cpl-7S, and Cpl-711 lysins against multidrug-resistant (MDR) pneumococcal strains attached to human nasopharyngeal and lung epithelial cells. In addition, we studied their ability to clear pneumococci from the nasopharynx by using a mouse model of infection. Our results suggest that Cpl-1 and Cpl-711 can be used as novel therapeutic strategies to fight persistent colonizations of the respiratory tract by *S. pneumoniae*.

RESULTS

Determination of MICs for clinical *S. pneumoniae* isolates. Since the susceptibility patterns of the three MDR *S. pneumoniae* clinical isolates to the different lysins were unknown, we tested them against common antibiotics and Cpl-1, Cpl-7S, and Cpl-711 lysins (Fig. 1 and Table 1). According to EUCAST breakpoints for *S. pneumoniae*, the results revealed that the strains selected had different degrees of susceptibility, showing resistance to at least three different antibiotics, including erythromycin (ERY) and tetracycline (TET) for all the strains. In addition, isolate 48 (serotype 23F) had high MICs to penicillin (PEN) and amoxicillin (AMX). Isolate 69 (serotype 19F) was resistant to PEN and AMX, whereas isolate 3498 (serotype 8) showed high resistance to levofloxacin (LVX). Among the three different lysins, Cpl-7S was the enzyme with lowest antimicrobial activity, showing the highest MICs, whereas Cpl-711 had the best activity, showing the lowest MICs (Table 1).

Killing of *S. pneumoniae* attached to human lung epithelial cells. We first studied the effects of Cpl-1, Cpl-7S, and Cpl-711 on the killing of *S. pneumoniae* attached to lung epithelial A549 cells. The effect of the treatment at 10 μg/ml with Cpl-1 or Cpl-711 on the killing of pneumococci attached to the cells was significant, whereas Cpl-7S showed no detectable effect (Fig. 2A). The chimeric enzyme Cpl-711 was more effective than Cpl-1 against the clinical isolate 69 of serotype 19F ($P < 0.05$). On the basis of these data, the effects of lower concentrations of enzybiotics were evaluated only for Cpl-1 and Cpl-711. The incubation of infected cells with 5 μg/ml of either compound reduced the viability of *S. pneumoniae* (Fig. 2B). At this dose, Cpl-711 showed a greater ability to kill attached pneumococci than Cpl-1, being statistically significant for isolates 48 ($P < 0.01$) and 3498 ($P < 0.001$). At the lowest dose tested (1 μg/ml), Cpl-1 was apparently ineffective, whereas the chimera Cpl-711 significantly reduced the viability of the attached bacteria, showing efficacy at subinhibitory concentrations (Fig. 2C). From the antimicrobial perspective, Cpl-1 was active in the killing of the bacteria attached to lung cells only at concentrations equal to or higher than the MIC (Fig. 2 and Table 1). Overall, Cpl-711 showed the highest efficacy against all isolates at all concentrations tested.

To visualize the antimicrobial effect of these enzybiotics, confocal laser scanning microscopy (CLSM) was used. A549 cells were infected with fluorescent clinical isolates of *S. pneumoniae* (strains 48 and 69) and exposed to 10 μg/ml of Cpl-1 or Cpl-711

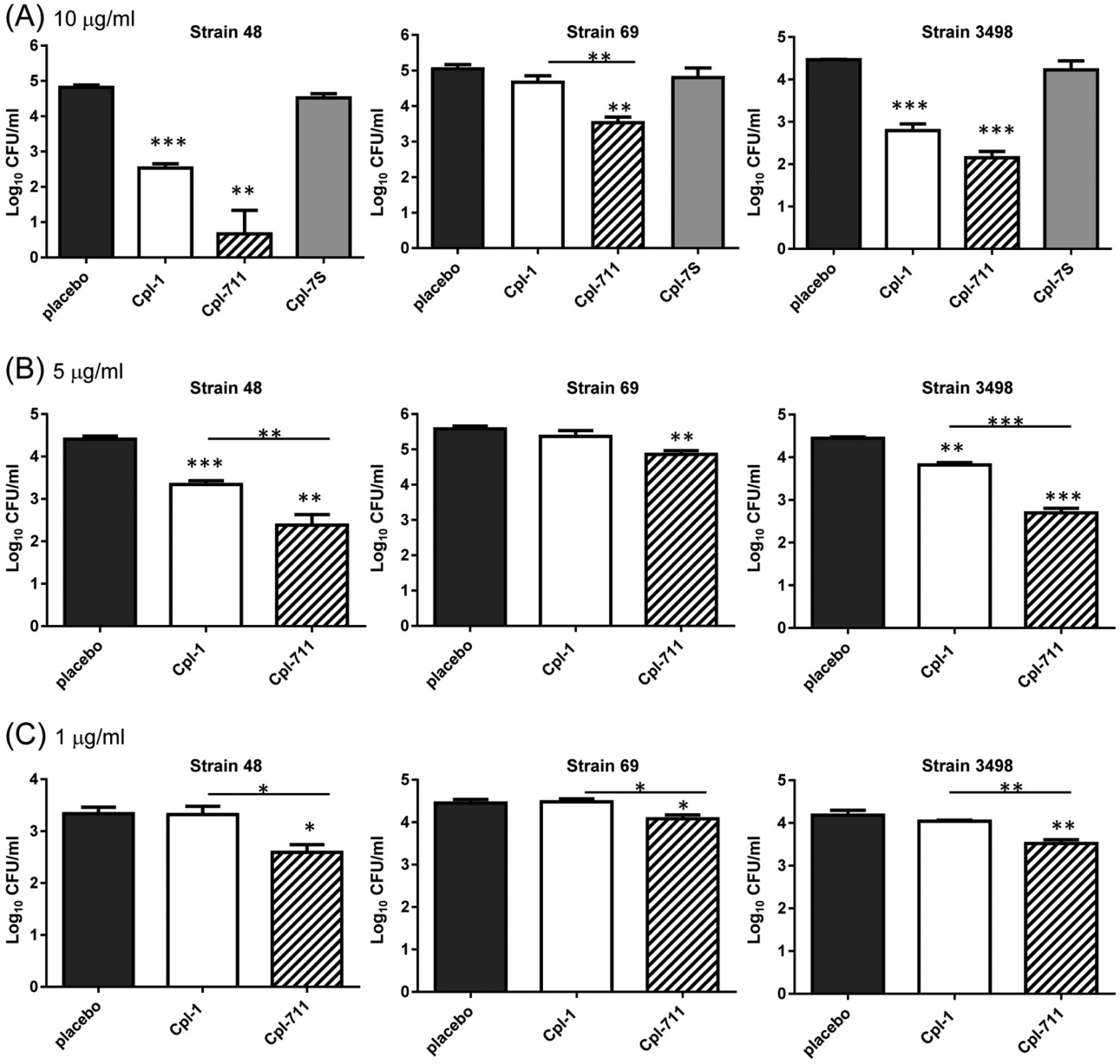

FIG 2 Killing by lytic enzymes of MDR isolates of *S. pneumoniae* attached to human lung epithelial cells. A549 cells colonized with pneumococcal strains were exposed to PBS (placebo) or to 10 μg/ml (A), 5 μg/ml (B), or 1 μg/ml (C) of either Cpl-1, Cpl-711, or Cpl-7S. Error bars represent the SDs and asterisks indicate statistical significance of the lytic enzyme investigated compared to the placebo group; asterisks on the horizontal lines indicate significance when Cpl-1 and Cpl-711 were compared.

(Fig. 3). Although only few cells were observed having bacteria attached after treatments with the lysins, our results confirmed that the two lysins were effective in killing the attached bacteria of both isolates, although Cpl-711 was significantly more effective ($P < 0.05$) than Cpl-1 in killing isolate 69 (serotype 19F) (Fig. 3C and D).

Antimicrobial activity of lysins against MDR pneumococci attached to human nasopharyngeal cells. The colonization of the nasopharynx is critical for the pathogenesis of *S. pneumoniae*, as this environment is the main reservoir of the pathogen. The use of antimicrobial agents to kill pneumococci attached to epithelial cells of the upper respiratory tract may be a suitable strategy to fight this early stage of IPD. Human nasopharyngeal epithelial cells were infected with MDR pneumococci and treated with Cpl-1, Cpl-7S, and Cpl-711 as described above. The administration of 10 μg/ml of Cpl-1 and Cpl-711 significantly reduced the survival of all the investigated isolates, being very

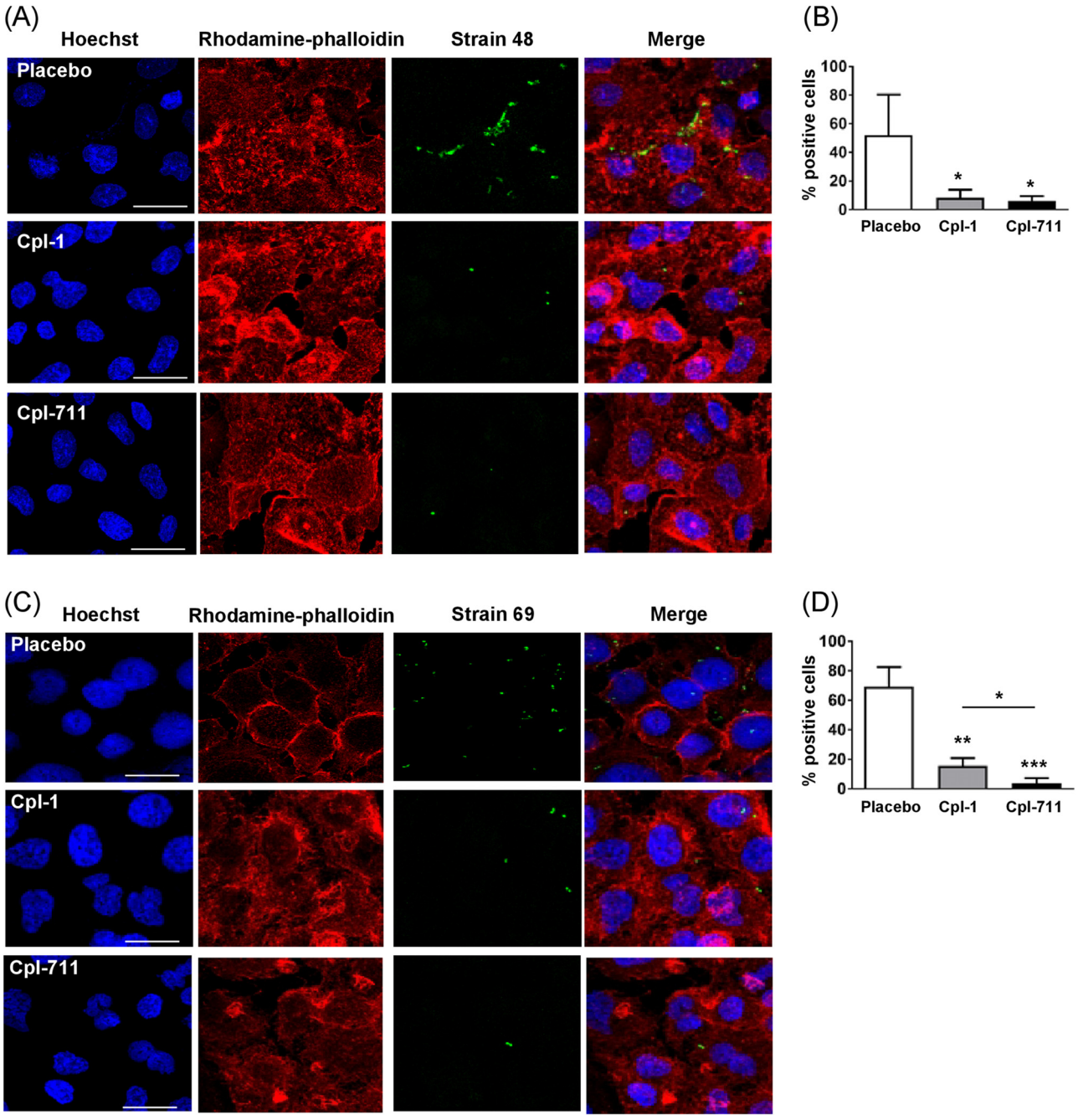

FIG 3 Detachment of MDR pneumococcal strains from human lung cells. A549 cells were infected with the indicated pneumococcal strains, and 1 h later, cells were exposed to PBS (as placebo) or to 10 μg/ml of Cpl-1 or Cpl-711. (A) CLSM images of cells infected with clinical isolate 48. (B) Percentages of epithelial cells associated with at least one fluorescent bacterial cell. (C) CLSM images of cells infected with clinical isolate 69. (D) Percentages of epithelial cells associated with at least one fluorescent bacterial cell. DNA was stained by Hoechst, actin cytoskeleton was visualized with rhodamine-phalloidin staining, and bacterial isolates were fluorescently labeled with FAM-SE. For quantification, at least 100 epithelial cells were counted. Error bars represent the SDs and asterisks indicate statistical significance of the lytic enzyme investigated compared to the placebo group. Asterisk on the horizontal line indicates significance when Cpl-1 and Cpl-711 were compared.

efficient with strains 48 and 3498; a more than 2-log reduction was observed, whereas Cpl-7S showed no activity (Fig. 4A). The treatment with 5 μg/ml of either Cpl-1 or Cpl-711 was still capable of reducing pneumococcal viability for strains 48 and 3498, whereas only Cpl-711 showed efficacy against strain 69 (Fig. 4B). At the lowest dose, Cpl-711 was effective against strains 48 and 3498 only (Fig. 4C). Overall, lysin Cpl-711 showed the highest activity for killing the pneumococci of the three clinical isolates attached to nasopharyngeal cells.

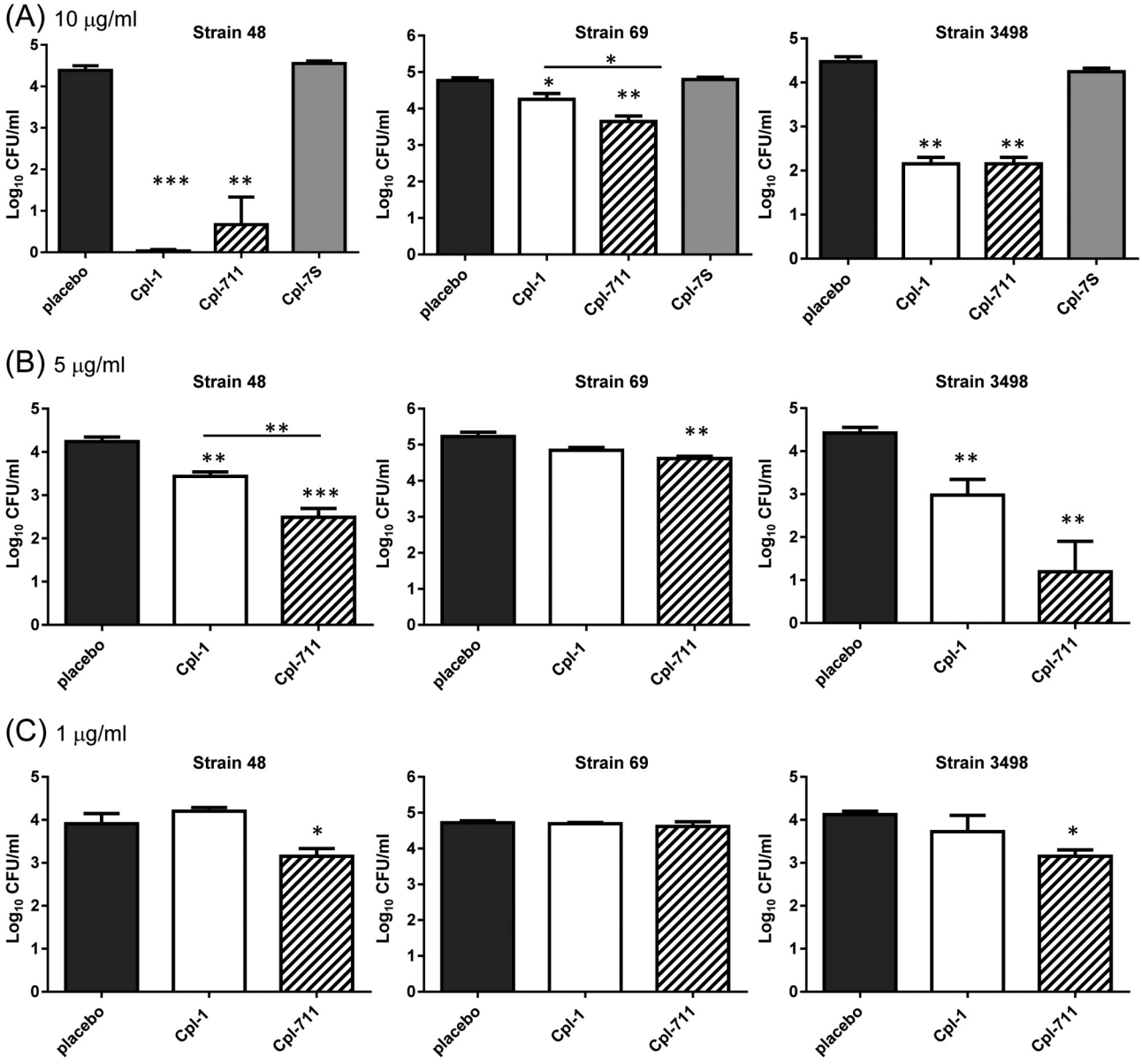

FIG 4 Bacterial killing by lytic enzymes of MDR isolates of *S. pneumoniae* attached to human nasopharyngeal epithelial cells. Detroit 562 cells were infected with pneumococcal strains, and 1 h later, cells were exposed to PBS as placebo or to 10 μg/ml (A), 5 μg/ml (B), or 1 μg/ml (C) of either Cpl-1, Cpl-711, or Cpl-7S. Error bars represent the SDs and asterisks indicate statistical significance of the lytic enzyme investigated compared to the placebo group; asterisks on the horizontal lines indicate significance when Cpl-1 and Cpl-711 were compared.

These experiments were repeated using fluorescently labeled bacteria and 10 μg/ml of Cpl-1 and Cpl-711 to visualize the capacity of these enzymes to decrease the colonization of the human nasopharynx (Fig. 5). The treatments with 10 μg/ml reduced the numbers of pneumococci attached to the nasopharyngeal cells, confirming the antimicrobial efficacy of these compounds (Fig. 5). Among the two lysins investigated, Cpl-711 showed a higher efficiency than Cpl-1 in reducing the attachment of isolate 69 (serotype 19F) to nasopharyngeal cells ($P < 0.05$) (Fig. 5D).

β-Lactams are the antibiotics of choice in the treatment of pneumococcal infections. To check the ability of AMX to fight pneumococcal colonization, lung and nasopharyngeal epithelial cell lines were infected with MDR *S. pneumoniae* isolates and treated with different concentrations of AMX (Fig. 6). We did not observe bacterial killing for clinical isolate 48 (serotype 23F) at any concentration of AMX tested (Fig. 6A). One possible explanation is that the higher dose used (10 μg/ml) was below the MIC for this

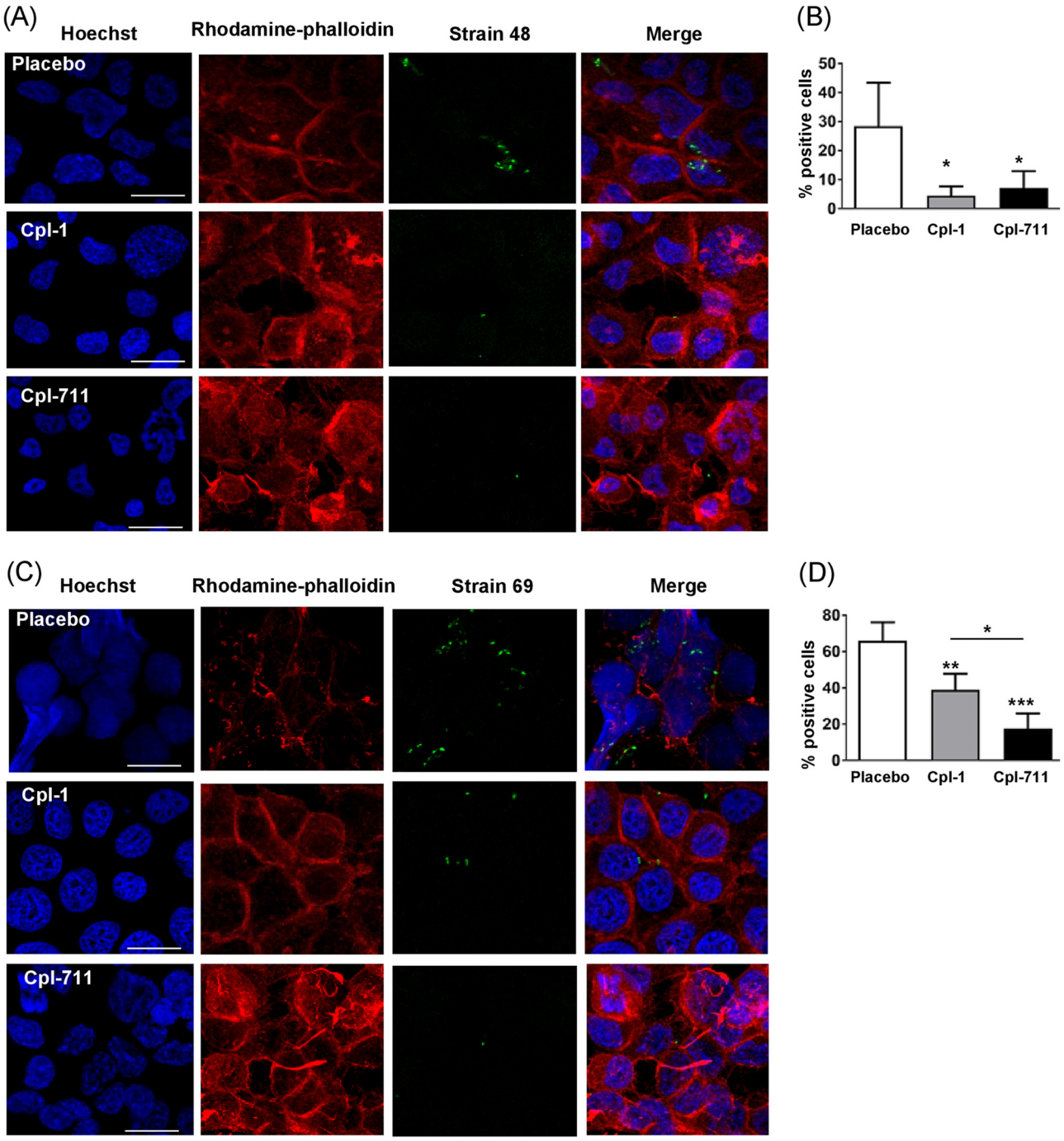

FIG 5 Detachment of MDR pneumococcal strains from nasopharyngeal cells. Detroit 562 cells were infected with the indicated pneumococcal strains, and 1 h later, cells were exposed to PBS (as placebo) or to 10 μg/ml of Cpl-1 or Cpl-711. (A) CLSM images of cells infected with clinical isolate 48. (B) Percentages of positive epithelial cells associated with at least one fluorescent bacterial cell. (C) CLSM images of cells infected with clinical isolate 69. (D) Percentages of positive epithelial cells associated with at least one fluorescent bacterial cell. DNA was stained by Hoechst, actin cytoskeleton was visualized with rhodamine-phalloidin staining, and bacterial isolates were fluorescently labeled with FAM-SE. For quantification, at least 100 epithelial cells were counted. Error bars represent the SDs and asterisks indicate statistical significance of the lytic enzyme investigated compared to the placebo group. Asterisk on the horizontal line indicates significance when Cpl-1 and Cpl-711 were compared.

strain (Table 1). The survival of strain 69 (serotype 19F) was only significantly reduced at the higher concentration tested (10 μg/ml), whereas 5 μg/ml (2.5$\times$ MIC) was unsuccessful (Fig. 6B). Additionally, the effect of AMX was significant for killing the AMX-susceptible strain 3498 (serotype 8) from the nasopharyngeal cell line, although no significant effect was shown when tested on lung epithelial cells (Fig. 6C). One

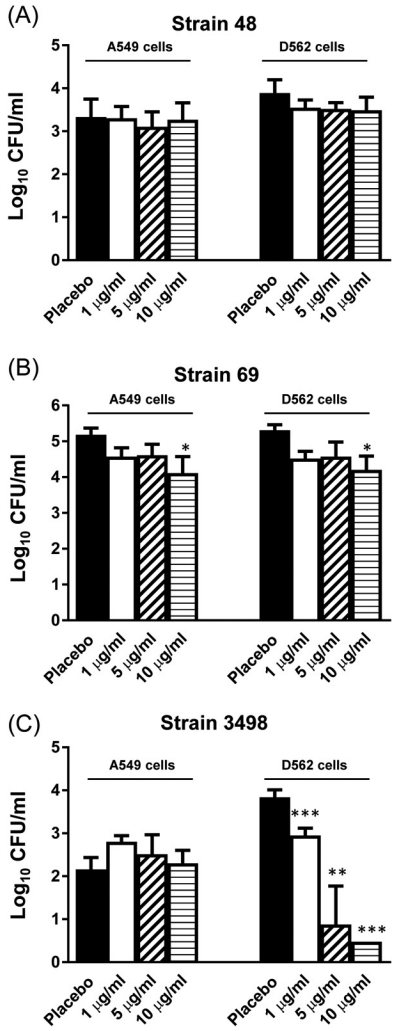

FIG 6 Bacterial killing by AMX of MDR isolates of *S. pneumoniae* on lung (A549) or nasopharyngeal (D562) epithelial cells. Cells colonized with pneumococcal strains were exposed to PBS (placebo) or AMX (10 μg/ml, 5 μg/ml, 1 μg/ml). (A) Strain 48; (B) strain 69; (C) strain 3498. Error bars represent the SDs and asterisks indicate statistical significance of the treatment with AMX compared to the placebo group.

possible explanation for the limited effect of AMX in these assays might be the short exposure of the infected cells to this antibiotic. This possibility might explain the relatively minor effect observed for AMX, since a 1-h exposure may not be enough time to induce cell death under these conditions, which would confirm that the lysins kill bacteria more rapidly than antibiotics. Overall, these results suggest that AMX is only effective under certain settings and when the concentration administered is at least 5 times higher than the MIC.

***In vivo* clearance of nasopharyngeal carriage by enzybiotics.** Eradication (or even a significant reduction) of the carrier state is likely to have a critical impact on the transmission of MDR *S. pneumoniae* strains and, consequently, on the incidence of IPD. To test this possibility in a mouse model of colonization, we utilized strain 48 (serotype 23F), which is an MDR clinical isolate of *S. pneumoniae* with high levels of resistance to β-lactams and macrolides. Nasopharyngeal colonization with this isolate was established in groups of mice, and at 40 h postcolonization, animals were treated by the intranasal route with phosphate-buffered saline ([PBS] as a placebo) or with 10 μg per mouse of Cpl-7S, Cpl-1, or Cpl-711 (Fig. 7A). The administration of a single dose of these enzybiotics significantly reduced colonization, demonstrating the *in vivo* antimicrobial activity of all these enzymes. Among the lysins tested, only Cpl-711 was able to

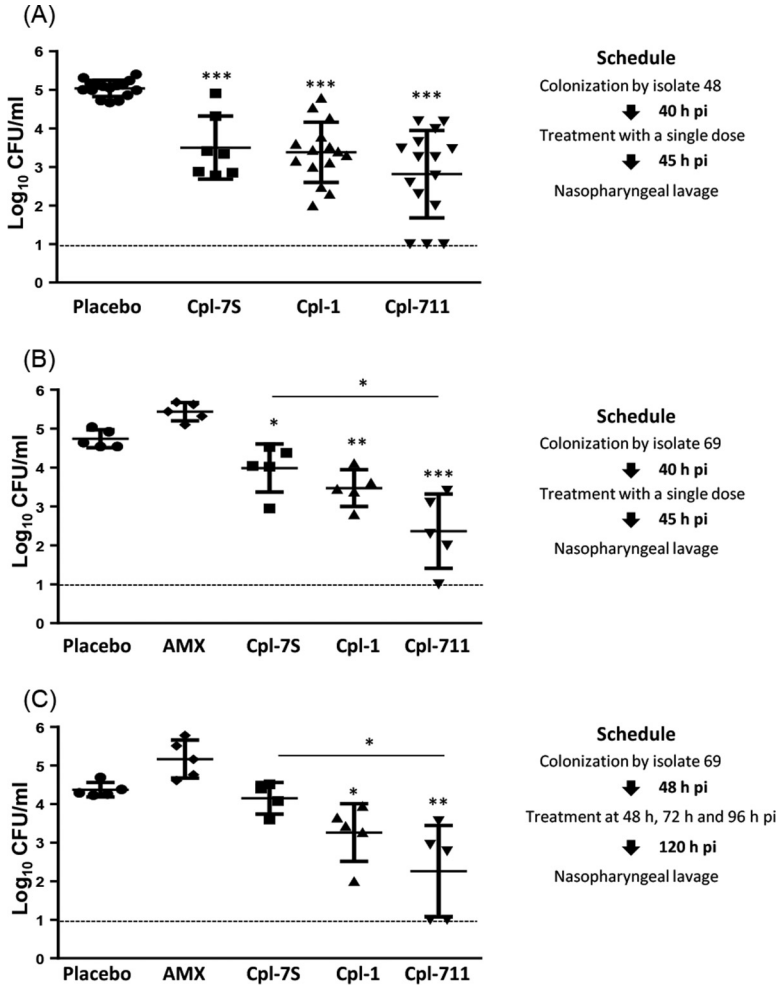

FIG 7 Antimicrobial activity of lytic enzymes Cpl-1, Cpl-711, and Cpl-7S administered by the intranasal route against nasopharyngeal colonization in mice. (A) Colonization with isolate 48 and a single dose of 10 μg/ml of the different lytic enzymes (or PBS as placebo) administered at 40 h postinfection (pi). (B) Colonization with isolate 69 and a single dose of 10 μg/ml of the different lytic enzymes, AMX, or PBS (as placebo) administered at 40 h postinfection (pi). (C) Colonization with isolate 69 and administration of 10 μg/ml of the different lytic enzymes, AMX, or PBS (as placebo) at 48 h, 72 h, and 96 h postinfection (pi). Results are expressed as bacterial counts obtained from the nasopharyngeal lavage fluid. Error bars represent the SDs and asterisks indicate statistical significance of the lytic enzyme investigated compared to the placebo group (Student's *t* tests). Analysis using Kruskal-Wallis test showed *P* values of <0.001 for panels A, B, and C. Asterisks on the horizontal lines indicate significance between Cpl-711 and lysins, and dotted lines represent the limit of bacterial detection.

eradicate nasopharyngeal carriage in up to 20% of the mice, suggesting that this enzybiotic is the most effective in the clearance of the bacteria colonizing the upper respiratory tract (Fig. 7A).

To reinforce these findings, nasopharyngeal carriage was repeated using a different clinical isolate, isolate 69 (serotype 19F), and AMX was included to evaluate the impact of a common antibiotic in our *in vivo* model of colonization. The administration of a single dose (10 μg/ml) of AMX, a dose 5 times higher than the MIC, did not affect the colonization by this strain (Fig. 7B). However, the administration of either of the three lytic enzymes reduced the nasopharyngeal colonization, with Cpl-711 being the enzyme with the highest activity ($P < 0.05$ comparing Cpl-711 versus Cpl-7S or Cpl-1), not only in bacterial killing but also in the nasopharyngeal clearance, as 20% of the mice had no detectable pneumococci (Fig. 7B). To evaluate the impact of giving repeated doses on the nasopharyngeal carriage of *S. pneumoniae*, three doses were administered, at 48 h, 72 h, and 96 h after colonization. Here, Cpl-711 was the most effective

enzybiotic investigated, showing complete eradication of the carrier state in 40% of the mice (Fig. 7C). The overall log reduction for the three lysins was compared using the Kruskal-Wallis test ($P < 0.001$), showing significant effects of the different lysins on the killing of *S. pneumoniae* attached to the nasopharynx, with Cpl-711 being the most effective (Fig. 7).

DISCUSSION

The use of purified bacteriophage-borne lytic enzymes is an alternative therapeutic strategy for the prevention and control of diseases caused by Gram-positive bacteria, including *S. pneumoniae* (6, 12–14, 22). The majority of the studies reporting the therapeutic potential of phage lysins are focused on the treatment of severe invasive infections, such as sepsis or meningitis (9, 10, 12, 13, 15), as well as surface-associated infections, including those from biofilms (23–26). An alternative application of phage lysins could be for diminishing the colonization process by bacterial pathogens. The use of lytic enzymes to remove bacterial colonization of the vagina by group B streptococci (24) or the oropharynx by *S. pyogenes* (27) has been proposed, but to our knowledge, there is only one report showing efficacy of a lysin (i.e., Pal amidase) against nasopharyngeal colonization by *S. pneumoniae* (25). Furthermore, the bacterial killing activity of phage lysins against pneumococcal isolates attached to epithelial cells located in the upper and lower respiratory tracts and their potential as decolonizing drugs were unknown.

Since nasopharyngeal colonization by pneumococcus is a prerequisite for developing IPD (3, 16), the use of certain antibiotics to reduce (or even eliminate) carriage has been proposed, although the long-term use of this prophylactic strategy might contribute to an increased carriage of nonsusceptible pneumococcal isolates (28–32). The administration of 10 μg of AMX (0.5 mg/kg) by the intranasal route did not reduce pneumococcal colonization in our mouse model. The lack of activity of AMX was not unexpected given the dose and route used, as previous studies have shown that treatment with 100 μg of penicillin by the intranasal route was insufficient to decrease nasopharyngeal colonization by pneumococcal resistant strains (33).

The major advantages of using phage lysins are the high degree of specificity of these compounds, their effectiveness against MDR pathogens, their low toxicity, and the low probability of resistance development (6, 22, 25). Our study shows that Cpl-1 and, in particular, Cpl-711, are promising enzybiotics to reduce nasopharyngeal carriage by antibiotic-susceptible and nonsusceptible pneumococci. The chimera Cpl-711 was the most efficient lysin, reducing the colonization by *S. pneumoniae* not only of nasopharyngeal cells but also of lung cells. This is of great relevance in terms of public health, as the clearance of the pneumococcal carrier state might be beneficial for certain groups at risk of suffering recurrent IPD episodes (31, 34–37). The results of the present study demonstrate that the local administration of enzybiotics successfully kills the bacteria attached to nasopharyngeal and lung epithelial cells, being effective in the reduction of colonization of cell tissues and mucous sites, which might be very important from a prophylactic perspective.

In terms of chronic respiratory infections, patients with COPD are at a high risk of developing recurrent IPD (38, 39). The importance of pneumococcal infections affecting patients with chronic medical conditions resides in the difficulty of clearing persistent pneumococcal strains from their lower respiratory tracts (40, 41). In this sense, it would be reasonable that, in a future work and by following an approach similar to that of conventional inhalers for human use, the administration of Cpl-711 or Cpl-1 by the intranasal route could effectively decrease the attachment of *S. pneumoniae* to human lungs. This hypothetical therapeutic approach might be useful with regard to the outcome of the infection in patients suffering recurrent pneumonia episodes associated with COPD or other chronic respiratory conditions in which *S. pneumoniae* is one of the major etiologic agents (42, 43). This is in agreement with previous observations showing that phage therapy may be an attractive strategy to fight pulmonary infections (44). As Cpl-711 and Cpl-1 were the most effective lytic enzymes against pneu-

mococcal infections associated with cell surfaces, they might be promising candidates against airway colonizations by clinical isolates of *S. pneumoniae*.

Antimicrobial alternatives using phage lysins may also be useful to reduce the spread of MDR pneumococcal strains, as their emergence is of great concern worldwide. In addition, the use of lysins may be a promising therapy to avoid transmission between children, who are the main carrier of *S. pneumoniae* and can have several serotypes colonizing their nasopharynges simultaneously (17–19). Furthermore, the use of these enzymes might be important for abolishing persistent and recurrent pneumococcal respiratory infections affecting patients with chronic underlying diseases.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and susceptibility testing. The *S. pneumoniae* MDR clinical isolates used in this study included strain 48 (serotype 23F), strain 69 (serotype 19F), and strain 3498 (serotype 8). Pneumococcal isolates were cultured at 37°C under 5% CO_2 on reconstituted tryptose blood agar base (TSA) plates (Difco Laboratories) supplemented with 5% defibrinated sheep blood (Thermo Scientific, Hampshire, UK) or in Todd-Hewitt broth supplemented with 0.5% yeast extract to an optical density at 580 nm (OD_{580}) of 0.4 ($\approx 10^8$ CFU/ml) and stored at −80°C in 10% glycerol as single-use aliquots. *Escherichia coli* strains, used for gene cloning and producing recombinant proteins, were grown in LB medium with shaking at 37°C. Susceptibility tests to antibiotics PEN, ERY, LVX, TET, chloramphenicol (CHL), and AMX were assessed three times by using the agar dilution technique (45) according to the criteria of the Clinical and Laboratory Standards Institute (CLSI). MICs of Cpl-1, Cpl-7S, and Cpl-711were determined by the microdilution method approved by the CLSI using cation-adjusted Mueller-Hinton broth supplemented with 5% lysed horse blood, as previously described (10).

Production and purification of lysins. Overproduction and purification of lysin proteins were performed as previously described (9, 10). The purity of the isolated proteins was checked using 10% SDS-PAGE and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis. Protein concentrations were determined spectrophotometrically using the corresponding molar absorption coefficients at 280 nm. Before use, all proteins were equilibrated by dialysis in 20 mM sodium phosphate buffer (pH 6.0).

Interaction of *S. pneumoniae* with human epithelial cells. Experimental procedures with human epithelial cells were performed using Detroit 562 (D562) nasopharyngeal cells (CCL-138; ATCC) and A549 lung cells (CCL-185; ATCC), as previously described (46, 47). Monolayers were cultured to 90 to 95% confluence in tissue culture flasks containing RPMI 1640 medium supplemented with 1 mM sodium pyruvate or 1% HEPES for D562 or A549, respectively (46, 47). For adhesion assays, 10^5 cells (D562 or A549) seeded in 24-well plates were infected with 2×10^6 CFU and incubated at 37°C in a 5% CO_2 atmosphere for 1 h. For killing activity mediated by the enzybiotics of the study or AMX as a comparator antibiotic, cells previously infected for 1 h were washed five times with PBS and incubated for an additional hour in tissue culture medium containing PBS (as placebo) or different concentrations of Cpl-1, Cpl-7S, or Cpl-711 as enzybiotics or AMX as an antibiotic to kill attached bacteria. The concentrations of the lytic enzymes assayed and AMX were 10 μg/ml, 5 μg/ml, and 1 μg/ml. Finally, the cells were washed five times with PBS and lysed with 300 μl of a solution containing 0.025% saponin-PBS for 10 min (48). The viable bacteria recovered from infected cells were obtained by plating serial dilutions on blood agar plates.

Confocal laser scanning microscopy. The *S. pneumoniae* strains described above were fluorescently labeled by incubation with 6-carboxyfluorescein-succinimidyl ester ([FAM-SE] Molecular Probes), as previously explained (48, 49). D562 and A549 cells infected with FAM-SE-labeled bacteria were seeded on 12-mm circular coverslips for immunofluorescence staining. The coverslips containing the infected cells were washed twice in PBS containing 0.1% saponin (in PBS) and once in PBS and were later incubated for 30 min with staining solution containing Hoechst (Invitrogen) diluted 1/2,500 for DNA staining and rhodamine-phalloidin (Invitrogen) diluted 1:200 for actin cytoskeleton detection. Finally, the coverslips were washed twice in PBS containing 0.1% saponin, once in PBS, and once more in H_2O and were mounted with Aqua-poly/mount (Polysciences) and analyzed with a Leica spectral SP5 confocal microscope using the Leica LAS-AF software.

Animal experiments. All the experiments involving the use of animals were performed in accordance with the guidelines of the Bioethical and Animal Welfare Committee of Instituto de Salud Carlos III (ISCIII) that reviewed and approved protocols CBA PA 52-2011-v2 and PROEX 218/15. The animals were bred at the ISCIII animal facility in accordance with institutional guidelines for animal use and care. The infection experiments conformed to the Spanish legislation (RD 53/2013) and European Union regulations (2010/63/EU). C57BL/6 mice (8 to 16 weeks old) were used for the carriage model. To investigate nasopharyngeal colonization, groups of at least five mice under anesthesia with isoflurane were inoculated intranasally with 10^7 CFU (in a volume of 10 μl) of the pneumococcal strain 48 (serotype 23F), as previously described (46). The administration of PBS or 10 μg per mouse of Cpl-1, Cpl-7S, or Cpl-711 by the intranasal route was initiated as a single dose 40 h after the induction of pneumococcal colonization according to the schedule described previously (25). Five hours after the treatment, all animals were killed, and the nasopharyngeal lavage fluid was collected, diluted, and plated for the determination of viable bacteria (25, 46). To extend the results to a different pneumococcal strain, the infection model described above was repeated using the clinical isolate 69 (serotype 19F), including an extra group of mice treated with AMX (10 μg per mouse). To investigate the possibility of enhanced

activity by treating with different doses of the different compounds, groups of 5 mice were colonized for 40 h with 10 μl of a bacterial suspension containing 10^7 CFU of strain 69. The administration of PBS or 10 μg per mouse of AMX, Cpl-1, Cpl-7S, or Cpl-711 by the intranasal route was initiated at 48 h, 72 h, and 96 h after bacterial colonization. Mice were killed after 120 h, and bacterial counts in the nasopharyngeal lavage fluid were determined.

Statistical analysis. Data are representative of results obtained from repeated independent experiments, and each data point represents the mean and standard deviation (SD) from 3 to 5 replicates. Statistical analyses were performed by using two-tailed Student's *t* tests (for two groups), and Kruskal-Wallis tests were also used for the mouse model data. For statistical analysis, GraphPad InStat version 7.02 (GraphPad Software, San Diego, CA, USA) was used. Differences of the various treatments in comparison to placebo or between the different lysins were considered statistically significant at a *P* value of <0.05 (*) and highly significant at *P* values of <0.01 (**) or <0.001 (***).

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