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Chemotherapy with Phage Lysins Reduces Pneumococcal Colonization of the Respiratory Tract

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Running Head: Phage lysins against pneumococcal colonization

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Bacteriophage-encoded lytic enzymes, also named lysins, or enzybiotics, are efficient agents to kill bacterial pathogens. Colonization of the respiratory tract by *Streptococcus pneumoniae* is a prerequisite for the establishment of the infection process. Hence, we have evaluated the antibacterial activity 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 have 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 activity of these lysins was also evaluated using a mouse nasopharyngeal carriage model. Exposure of epithelial infected cells to Cpl-7S did not modify the killing of any of the pneumococcal strains investigated. However, treatment with Cpl-1 or Cpl-711 increased the killing of *S. pneumoniae* adhered to both types of human epithelial cells with Cpl-711 being more effective than Cpl-1, at sub-inhibitory concentrations. In addition, treatment with amoxicillin had no effect reducing the carrier state whereas mice treated by the intranasal route with Cpl-711 significantly reduced nasopharyngeal colonization with no detection of bacterial load in 20-40% of the mice. This study indicates that Cpl-1 and Cpl-711 lysins might be promising antimicrobial candidates for therapy against pneumococcal colonization.

47 *Streptococcus pneumoniae* is one of the major etiologic agents of acute otitis media,
48 community-acquired pneumonia, sepsis and bacterial meningitis causing high morbidity
49 and mortality rates worldwide (1, 2). Asymptomatic carriage is the prerequisite for all
50 these infections and is more frequently associated with early childhood (3). Successful
51 colonization of the upper respiratory tract is critical for horizontal spread of genes
52 involved in antibiotic resistance and/or virulence, and may lead to the development of
53 invasive pneumococcal disease (IPD), which is the most severe clinical manifestation
54 (3, 4). In addition, microbial colonization of the lower respiratory tract is associated
55 with chronic obstructive pulmonary disease (COPD), in which exacerbations and airway
56 inflammation are important aspects related to persistence (5).
57 Purified phage-encoded lysins (also known as endolysins and enzybiotics) represent a
58 promising alternative to current antibacterials as lysins kill susceptible bacteria much
59 faster than standard antibiotics (6, 7). Lysins are murein hydrolases that specifically
60 cleave different bonds of the bacterial peptidoglycan, thereby triggering osmotic lysis.
61 These specialized enzymes generally display a modular organization containing an N-
62 terminal catalytic domain (CD) and a C-terminal cell wall-binding domain (CWBD)
63 with a flexible linker region connecting both domains (7). This architecture has allowed
64 the successful swapping of functional domains to construct new chimeric proteins and
65 the engineering of wild type enzymes to improve catalytic or stability properties leading
66 to modification of the spectrum of susceptible bacteria (8-11).
67 In the pneumococcal system, several lysins have been characterized, like Pal amidase
68 (12) and Cpl-1, Cpl-7, Cpl-7S and Cpl-711 lysozymes (9, 10). The lysozymes, Cpl-1
69 and Cpl-7 are encoded by bacteriophages Cp-1 and Cp-7, respectively, and their CDs
70 belong to the glycosyl hydrolases family 25 (GH_25; PF01183); Cpl-7S is an
71 engineered variant derived from Cpl-7 in which 15 amino acid residues of the CWBD

72 were changed to enhance its bactericidal activity, and Cpl-711 is a synthetic chimera
73 that contains the CD Cpl-7, at the N-terminal region, and the linker and CWBD of Cpl-
74 1, at the C-terminal region (Fig. 1). In terms of lytic efficacy and specificity, Cpl-1 and
75 Cpl-711 require the presence of choline residues in the teichoic acids of the
76 pneumococcal cell wall to perform their antibacterial activity, whereas Cpl-7S is
77 choline-independent due to the presence of CW_7 repeats in its CWBD (9). Thus, Cpl-1
78 and Cpl-711 show specific antipneumococcal activity against planktonic and biofilm
79 cultures, in contrast with Cpl-7S that has a broader range of susceptible bacteria and
80 was also capable of killing other relevant pathogens including *Streptococcus pyogenes*
81 and *Enterococcus faecalis* (9). Moreover, this kind of lysin therapy has been shown to
82 be effective against a variety of severe pneumococcal infections including meningitis,
83 pneumonia and sepsis, with the advantage of a marked specificity (12-14).
84 It should be noted that although the protecting activity of these three lysins against
85 systemic pneumococcal infection is documented (9, 10, 12-15), evidence demonstrating
86 their efficacy against nasopharyngeal colonization by *S. pneumoniae* has not been
87 reported. In addition, the therapeutic use of enzybiotics against chronic bacterial
88 respiratory infections is relatively unexplored. This is important since it is generally
89 thought that nasopharyngeal carriage by *S. pneumoniae* is essential for the pathogenesis
90 process because it is a prerequisite for invasive disease (16). Although the introduction
91 of current anti-pneumococcal conjugate vaccines has decreased nasopharyngeal
92 colonization, more than 30% of children are still asymptomatic carriers of pneumococci
93 with multiple serotypes (17-19). In addition, *S. pneumoniae* is one of the most frequent
94 pathogens causing acute exacerbations and recurrent pneumonia episodes in COPD
95 patients (5). From the clinical perspective, this situation is even worse as multidrug
96 resistant pneumococcal isolates are a frequent cause of persistent infections in COPD

97 patients and antibiotic resistance compromises treatment outcome (20, 21). This
98 antimicrobial approach might be a potential strategy to diminish the colonization
99 process and even to clear persistent bacteria in chronic respiratory infections. As phage
100 lysins kill bacteria rapidly on contact, the use of these lytic enzymes may reduce
101 bacterial attachment to epithelial cells of the upper and lower respiratory tracts.

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

108

109 RESULTS

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

121 showing the highest MICs, whereas Cpl-711 had the best activity showing the lowest
122 MICs (Table 1).

123 **Killing of *S. pneumoniae* attached to human lung epithelial cells.** We first studied
124 the effect of Cpl-1, Cpl-7S and Cpl-711 on the killing of *S. pneumoniae* attached to lung
125 epithelial A549 cells. Treatment at 10 µg/ml with Cpl-1 or Cpl-711 significantly killed
126 pneumococci attached to the cells, whereas Cpl-7S showed no detectable effect (Fig.
127 2A). The chimeric enzyme Cpl-711 was more effective than Cpl-1 against the clinical
128 isolate 69 of serotype 19F ($P<0.05$). The effect of lower concentrations of enzybiotics
129 was evaluated only for Cpl-1 and Cpl-711. Incubation of infected cells with 5 µg/ml of
130 either compound reduced the viability of *S. pneumoniae* (Fig. 2B). At this dose, Cpl-711
131 showed a higher ability to kill attached pneumococci than Cpl-1 being statistically
132 significant for isolates 48 ($P<0.01$) and 3498 ($P<0.001$). At the lower dose tested (1
133 µg/ml), Cpl-1 was apparently ineffective, whereas the chimera Cpl-711 significantly
134 reduced the viability of the bacteria attached showing efficacy at sub-inhibitory
135 concentrations (Fig. 2C). From the antimicrobial perspective, Cpl-1 was active killing
136 the bacteria attached to lung cells only at concentrations equal or above the MIC (Fig. 2
137 and Table 1). Overall, Cpl-711 showed the highest efficacy against all isolates, at all
138 concentrations tested.

139 To visualize the antimicrobial effect of these enzybiotics, confocal laser scanning
140 microscopy (CLSM) was used. A549 cells were infected with fluorescent clinical
141 isolates of *S. pneumoniae* (strains 48 and 69) and exposed to 10 µg/ml of Cpl-1 or Cpl-
142 711 (Fig. 3). Although only few cells were observed having bacteria attached after
143 treatment with the lysins, our results confirmed that the two lysins were effective killing
144 the numbers of attached bacteria for both isolates, although Cpl-711 was significantly

145 more effective (P< 0.05) than Cpl-1 killing the isolate 69 (serotype 19F) (Fig. 3C and
146 D).

147 **Antimicrobial activity of lysins against MDR pneumococci attached to human**
148 **nasopharyngeal cells.** Colonization of the nasopharynx is critical for the pathogenesis
149 of *S*. *pneumoniae* as this environment is the main reservoir of the pathogen. The use of
150 antimicrobial agents to kill pneumococci attached to epithelial cells of the upper
151 respiratory tract may be a suitable strategy to fight this early stage of IPD. Human
152 nasopharyngeal epithelial cells were infected with MDR pneumococci and treated with
153 Cpl-1, Cpl-7S and Cpl-711 as described above. Administration of 10 μg/mL of Cpl-1
154 and Cpl-711 significantly reduced the survival of all the investigated isolates, being
155 very efficient with strains 48 and 3498 — more than 2-log reduction was observed
156 whereas Cpl-7S showed no activity (Fig. 4A). Treatment with 5 μg/ml of either Cpl-1 or
157 Cpl-711 was still capable of reducing pneumococcal viability for strains 48 and 3498,
158 whereas only Cpl-711 showed efficacy against strain 69 (Fig. 4B). At the lowest dose
159 investigated (1 μg/ml), only Cpl-711 reduced the number of pneumococci attached to
160 nasopharyngeal cells (Fig. 4C). Overall, lysin Cpl-711 showed the highest activity
161 killing attached pneumococci to nasopharyngeal cells against the three clinical isolates
162 investigated.

163 These experiments were repeated using fluorescently-labeled bacteria to evaluate *in*
164 *vitro* the capacity of Cpl-1 and Cpl-711 to decrease the colonization of the human
165 nasopharynx (Fig. 5). Treatment with 10 μg/ml reduced the number of pneumococci
166 attached to the nasopharyngeal cells confirming the antimicrobial efficacy of these
167 compounds (Fig. 5). Among the two lysins investigated, Cpl-711 showed higher
168 efficiency than Cpl-1 reducing the attachment of isolate 69 (serotype 19F) to
169 nasopharyngeal cells ($P<0.05$) (Fig. 5D).

170 β-Lactams are the antibiotics of choice in the treatment of pneumococcal infections.
171 To check the ability of AMX to fight pneumococcal colonization, lung and
172 nasopharyngeal epithelial cell lines were infected with MDR *S. pneumoniae* isolates and
173 treated with different concentrations of AMX (Fig. 6). Bacterial killing using the
174 clinical isolate 48 (serotype 23F) was not reduced at any of the concentrations of AMX
175 tested (Fig. 6A). One possible explanation is that the higher dose used (10 μg/ml) was
176 below the MIC for this strain (Table 1). Survival of strain 69 (serotype 19F) was only
177 significantly reduced at the higher concentration tested (10 μg/ml) whereas 5 μg/ml (2.5
178 × MIC) was unsuccessful (Fig. 6B). Additionally, AMX significantly killed the AMX
179 susceptible strain 3498 (serotype 8) from the nasopharyngeal cell line, although it did
180 not show any significant effect when tested on lung epithelial cells (Fig. 6C). One
181 possible explanation for the limited effect of AMX in these assays might be due to the
182 short exposure of the infected cells to this antibiotic. This possibility might explain the
183 relatively minor effect observed for AMX since 1 h exposure may not be enough time to
184 induce cell death under these conditions which would confirm that the lysins kill
185 bacteria more rapidly than antibiotics. Overall, these results suggest that AMX is only
186 effective under certain settings and when the concentration administered is at least 5
187 times higher than the MIC.

188 ***In vivo* clearance of nasopharyngeal carriage by enzybiotics.** Eradication (or even
189 a significant reduction) of the carrier state is likely to have a critical impact in the
190 transmission of MDR *S. pneumoniae* strains and, consequently, in the incidence of IPD.
191 To test this possibility in a mouse model of colonization, we utilized strain 48 (serotype
192 23F), which is an MDR clinical isolate of *S. pneumoniae* with high levels of resistance
193 to β-lactams and macrolides. Nasopharyngeal colonization with this isolate was
194 established in groups of mice, and 40 h post-colonization, animals were treated by the

intranasal route with phosphate-buffered saline (PBS) (as placebo) or with 10 μg per mouse of Cpl-7S, Cpl-1 or Cpl-711 (Fig. 7A). 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 eradicate nasopharyngeal carriage in up to 20% of the mice, suggesting that this enzybiotic is the most effective in the clearance of bacteria colonizing the upper respiratory tract (Fig. 7A).

To reinforce these findings, nasopharyngeal carriage was repeated using a different clinical isolate 69 (serotype 19F) and AMX was included to evaluate the impact of a common antibiotic in our *in vivo* model of colonization. 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, administration of either of the three lytic enzymes reduced the nasopharyngeal colonization, being Cpl-711 the enzyme with the highest activity ($P < 0.05$ comparing Cpl711 *vs* 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 in the nasopharyngeal carriage of *S. pneumoniae*, three doses were administered at 48 h, 72 h and 96 h after colonization was established. Hence, 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 Kruskal Wallis test ($P<0.001$) showing a significant killing of *S. pneumoniae* attached to the nasopharynx by the different lysins being Cpl-711 the most effective (Fig 7).

DISCUSSION

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

232 Since nasopharyngeal colonization by pneumococcus is a prerequisite for developing
233 IPD (3, 16), the use of certain antibiotics to reduce (or even eliminate) carriage has been
234 proposed, although the long-term use of this prophylactic strategy might contribute to
235 an increased carriage of non-susceptible pneumococcal isolates (28-32). Administration
236 of 10 μg of AMX (0.5 mg/kg) by the intranasal route did not reduce pneumococcal
237 colonization in our mouse model. The lack of activity of AMX was not unexpected
238 given the dose and route used as previous studies have shown that treatment with 100
239 μg of penicillin by the intranasal route was insufficient to decrease nasopharyngeal
240 colonization caused by pneumococcal resistant strains (33). The major advantages of
241 using phage lysins are the high degree of specificity of these compounds, their
242 effectiveness against MDR pathogens, their low toxicity, and the low probability of
243 resistance development (6, 22, 25). Our study shows that Cpl-1 and Cpl-711, the latter

244 in a higher extent, are promising enzybiotics to reduce the nasopharyngeal carriage
245 caused by antibiotic-susceptible and non-susceptible pneumococci. The chimera Cpl-
246 711 was the most efficient lysin reducing the colonization of *S. pneumoniae* not only
247 from nasopharyngeal cells but also from lung cells, which is of great relevance in terms
248 of public health as clearance of the pneumococcal carrier state might be beneficial for
249 certain groups at risk of suffering recurrent IPD episodes (31, 34-37). The results of the
250 present study demonstrate that local administration of enzybiotics successfully kills the
251 bacteria attached to nasopharyngeal and lung epithelial cells being effective in the
252 reduction of colonization of cell tissues and mucous sites, which might be very
253 important from a prophylactic perspective. In terms of chronic respiratory infections,
254 patients with COPD are at high risk of developing recurrent IPD (38, 39). The
255 importance of pneumococcal infection affecting patients with chronic medical
256 conditions resides in the difficulty of clearing persistent pneumococcal strains from
257 their lower respiratory tract (40, 41). In this sense, it would be reasonable that, in a
258 future work and following an approach similar to that of conventional inhalers for
259 human use, administration of Cpl-711 or Cpl-1 by the intranasal route could effectively
260 decrease the attachment of *S. pneumoniae* to human lungs. This hypothetical therapeutic
261 approach might be useful for the outcome of the infection in patients suffering recurrent
262 pneumonia episodes associated to COPD or other chronic respiratory conditions in
263 which *S. pneumoniae* is one of the major etiologic agents (42, 43). This is in agreement
264 with previous observations showing that phage therapy may be an attractive strategy to
265 fight pulmonary infections (44). As Cpl-711 and Cpl-1 were the most effective lytic
266 enzymes against pneumococcal infections associated to cell surfaces, they might be
267 promising candidates against airway colonization caused by clinical isolates of *S.*
268 *pneumoniae*. This antimicrobial alternative may also be useful to reduce the spread of

269 MDR pneumococcal strains as their emergence is of great concern worldwide. In
270 addition, the use of lysins may be a promising therapy to avoid the transmission
271 between children who are the main carrier of *S. pneumoniae* with several serotypes
272 colonizing simultaneously their nasopharynx (17-19). Furthermore, the use of these
273 enzymes might be important to abolish persistent and recurrent pneumococcal
274 respiratory infections affecting patients with chronic underlying diseases.

275

276 # MATERIALS AND METHODS

277 **Bacterial strains, culture conditions and susceptibility testing.** The *S. pneumoniae*
278 MDR clinical isolates used in this study included strain 48 (serotype 23F), strain 69
279 (serotype 19F) and strain 3498 (serotype 8). Pneumococcal isolates were cultured at
280 37°C under 5% CO_2 on reconstituted tryptose blood agar base (TSA) plates (Difco
281 Laboratories) supplemented with 5% defibrinated sheep blood (Thermo Scientific,
282 Hampshire, UK) or in Todd-Hewitt broth supplemented with 0.5% yeast extract to an
283 optical density at 580 nm (OD_{580}) of 0.4 ($\approx 10^8$ cfu/mL) and stored at −80°C in 10%
284 glycerol as single-use aliquots. *Escherichia coli* strains, used for gene cloning and
285 producing recombinant proteins, were grown in LB medium with shaking at 37°C.
286 Susceptibility tests to antibiotics PEN, ERY, LVX, TET, Chloramphenicol (CHL) and
287 AMX were assessed three times by the agar dilution technique (45) according to the
288 criteria of the Clinical and Laboratory Standards Institute (CLSI). MICs of Cpl-1, Cpl-
289 7S and Cpl-711were determined by the microdilution method approved by the CLSI
290 using cation-adjusted Mueller-Hinton broth supplemented with 5% lysed horse blood as
291 previously described (10).

292 **Production and purification of lysins.** Overproduction and purification of lysin
293 proteins were performed as previously described (9, 10). The purity of the isolated

294 proteins was checked using 10% SDS–PAGE and MALDI-TOF analysis. Protein
295 concentrations were determined spectrophotometrically using the corresponding molar
296 absorption coefficients at 280 nm. Before use, all proteins were equilibrated by dialysis
297 in 20 mM sodium phosphate buffer (pH 6.0).

298 **Interaction of *S. pneumoniae* with human epithelial cells.** Experimental
299 procedures with human epithelial cells were performed using Detroit-562 (D562)
300 nasopharyngeal cells (CCL138; ATCC) and A549 lung cells (CCL-185; ATCC) as
301 previously described (46, 47). Monolayers were cultured to 90–95% confluence in
302 tissue culture flasks containing RPMI 1640 medium supplemented with 1 mM sodium
303 pyruvate or 1% HEPES for D562 or A549 respectively (46, 47). For adhesion assays,
304 10^5 cells (D562 or A549) seeded in 24-well plates were infected with 2×10^6 CFU and
305 incubated at 37°C in a 5% CO_2 atmosphere for 1 h. For killing activity mediated by the
306 enzybiotics of the study or AMX as a comparator antibiotic, cells previously infected
307 for 1 h were washed five times with PBS and incubated for an additional hour in tissue
308 culture medium containing PBS (as placebo) or different concentrations of Cpl-1, Cpl-
309 7S or Cpl-711 as enzybiotics or AMX as antibiotic to kill attached bacteria. The
310 concentrations of the lytic enzymes assayed and AMX were 10 μg/ml, 5 μg/ml, and 1
311 μg/ml. Finally, cells were washed five times with PBS and lysed with 300 μl of a
312 solution containing 0.025% saponin-PBS for 10 min (48). Viable bacteria recovered
313 from infected cells were obtained by plating serial dilutions on blood agar plates.

314 **Confocal laser scanning microscopy.** *S. pneumoniae* strains described above were
315 fluorescently labeled by incubation with FAM-SE (Molecular Probes) as previously
316 explained (48, 49). D562 and A549 cells infected with FAM-SE-labeled bacteria were
317 seeded on 12-mm circular coverslips for immunofluorescence staining. Coverslips
318 containing the infected cells were washed twice in PBS containing 0.1% saponin (in

319 PBS), once in PBS and later incubated for 30 min with staining solution containing
320 Hoechst (Invitrogen) diluted 1/2500 for DNA staining and rhodamine-phalloidin
321 (Invitrogen) diluted 1:200 for actin cytoskeleton detection. Finally, coverslips were
322 washed twice in PBS containing 0.1% saponin, once in PBS, and once more in H_2O,
323 mounted with Aqua Poly/Mount (Polysciences), and analyzed with a Leica spectral SP5
324 confocal microscope using the software Leica (LAS-AF).

325 **Animal experiments.** All the experiments involving the use of animals were
326 performed following the guidelines of the Bioethical and Animal Welfare Committee of
327 Instituto de Salud Carlos III (ISCIII) that reviewed and approved protocol CBA PA 52-
328 2011-v2 and PROEX 218/15. Animals were bred at ISCIII animal facility following
329 institutional guidelines for animal use and care. Infection experiments conformed to the
330 Spanish legislation (RD 53/2013) and European Union regulations (2010/63/EU).
331 C57BL/6 mice (8–16 weeks old) were used for the carriage model. To investigate
332 nasopharyngeal colonization, groups of at least five mice under anaesthesia with
333 isofluorane were inoculated intranasally with 10^7 cfu (in a volume of 10 μl) of the
334 pneumococcal strain 48 (serotype 23F), as previously described.(46) Administration of
335 PBS or 10 μg per mouse of Cpl-1, Cpl-7S or Cpl-711 by the intranasal route was
336 initiated as a single dose 40 h after the induction of pneumococcal colonization
337 following the schedule described previously (25). Five hours after the treatment, all
338 animals were killed, and the nasopharyngeal lavage fluid was collected, diluted and
339 plated for determination of viable bacteria (25, 46). To extend the results to a different
340 pneumococcal strain, the infection model described above was repeated using the
341 clinical isolate 69 (serotype 19F), including an extra group of mice treated with AMX
342 (10 μg per mouse). To investigate the possibility of enhanced activity by treating with
343 different doses of the different compounds, groups of 5 mice were colonized for 40 h

344 with 10 μL of bacterial suspension containing 10^7 cfu of strain 69. Administration of
345 PBS or 10 μg per mouse of AMX, Cpl-1, Cpl-7S or Cpl-711 by the intranasal route was
346 initiated at 48 h, 72 h and 96 h after bacterial colonization. Mice were killed at 120 h
347 and bacterial counts were determined in the nasopharyngeal lavage fluid.

348 **Statistical analysis.** Data are representative of results obtained from repeated
349 independent experiments, and each data point represents the mean and standard
350 deviations (SD) for 3 to 5 replicates. Statistical analysis was performed by using two-
351 tailed Student´s *t*-test (for two groups) and Kruskal-Wallis test was also used for the
352 mouse model data. For statistical analysis, GraphPad InStat version 7.02 (GraphPad
353 Software, San Diego, CA, USA) was used. Differences of the various treatments in
354 comparison to placebo or between the different lysins were considered statistically
355 significant with P <0.05 (*) and highly significant with P <0.01 (**) and P <0.001
356 (***).

357

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365

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563

564 **FIGURE LEGENDS**

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

571

572 **FIG 2** Killing by lytic enzymes against MDR isolates of *S. pneumoniae* attached to
573 human lung epithelial cells. A549 cells colonized with pneumococcal strains were
574 exposed to PBS (placebo) or to 10 μg/ml (A), 5 μg/ml (B) or 1 μg/ml (C) of either Cpl-
575 1, Cpl-711 or Cpl-7S. Error bars represent the SDs and asterisks indicate statistical
576 significance of the lytic enzyme investigated compared to the placebo group and
577 asterisks on the horizontal line indicate significance when Cpl-1 and Cpl-711 were
578 compared.

579

580 **FIG 3** Detachment of MDR pneumococcal strains from human lung cells. A549 cells
581 were infected with the indicated pneumococcal strains and 1 h later cells were exposed
582 to PBS (as placebo) or to 10 μg/ml of Cpl-1, or Cpl-711. (A) CLSM images of cells
583 infected with the clinical isolate 48. (B) Percentage of epithelial cells associated to at
584 least one fluorescent bacterial cell (C) CLSM images of cells infected with the clinical
585 isolate 69. (D) Percentage of epithelial cells associated to at least one fluorescent
586 bacterial cell. DNA was stained by Hoechst, actin cytoskeleton was visualized with
587 rhodamine-phalloidin staining and bacterial isolates were fluorescently labeled with
588 FAM-SE. For quantification, at least 100 epithelial cells were counted. Error bars

589 represent the SDs and asterisks indicate statistical significance of the lytic enzyme
590 investigated compared to the placebo group. Asterisks on the horizontal line indicate
591 significance when Cpl-1 and Cpl-711 were compared.
592
593 **FIG 4** Bacterial killing by lytic enzymes against MDR isolates of *S. pneumoniae*
594 attached to human nasopharyngeal epithelial cells. Detroit 562 cells were infected with
595 pneumococcal strains and 1 h later cells were exposed to PBS as placebo or to 10
596 μg/mL (A), 5 μg/mL (B) or 1 μg/mL (C) of either Cpl-1, Cpl-711 or Cpl-7S. Error bars
597 represent the SDs and asterisks indicate statistical significance of the lytic enzyme
598 investigated compared to the placebo group and asterisks on the horizontal line indicate
599 significance when Cpl-1 and Cpl-711 were compared.
600
601 **FIG 5** Detachment of MDR pneumococcal strains from nasopharyngeal cells. Detroit
602 562 cells were infected with the indicated pneumococcal strains and 1 h later cells were
603 exposed to PBS (as placebo) or to 10 μg/ml of Cpl-1, or Cpl-711. (A) CLSM images of
604 cells infected with the clinical isolate 48. (B) Percentage of positive epithelial cells
605 associated to at least one fluorescent bacterial cell. (C) CLSM Images of cells infected
606 with the clinical isolate 69. (D) Percentage of positive epithelial cells associated to at
607 least one fluorescent bacterial cell. DNA was stained by Hoechst, actin cytoskeleton
608 was visualized with rhodamine-phalloidin staining and bacterial isolates were
609 fluorescently labeled with FAM-SE. For quantification, at least 100 epithelial cells were
610 counted. Error bars represent the SDs and asterisks indicate statistical significance of
611 the lytic enzyme investigated compared to the placebo group. Asterisks on the
612 horizontal line indicate significance when Cpl-1 and Cpl-711 were compared.
613

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

620

621 **FIG 7** Antimicrobial activity of lytic enzymes Cpl-1, Cpl-711 or Cpl-7S administered
622 by the intranasal route against nasopharyngeal colonization in mice. (A) Colonization
623 with isolate 48 and a single dose of 10 µg/ml of the different lytic enzymes (or PBS as
624 placebo) administered at 40 h post-infection (pi). (B) Colonization with isolate 69 and a
625 single dose of 10 µg/ml of the different lytic enzymes, AMX (or PBS as placebo)
626 administered at 40 h post-infection (pi). (C) Colonization with isolate 69 and
627 administration of 10 µg/ml of the different lytic enzymes, AMX or PBS (as placebo) at
628 48 h, 72 h and 96 h post-infection (pi). Results are expressed as bacterial counts
629 obtained from the nasopharyngeal lavage fluid. Error bars represent the SDs and
630 asterisks indicate statistical significance of the lytic enzyme investigated compared to
631 the placebo group (Student *t* tests). Analysis using Kruskal-Wallis test showed a *P* value
632 < 0.001 for panels A, B and C. Asterisks on the horizontal line indicate significance
633 between Cpl-711 and lysins and dots line represents the limit of bacterial detection.

634

635
636 **Table 1. Antimicrobial susceptibility of three MDR pneumococcal isolates**
637
638

S. pneumoniae isolates	MIC (μg/ml) of the following strains (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

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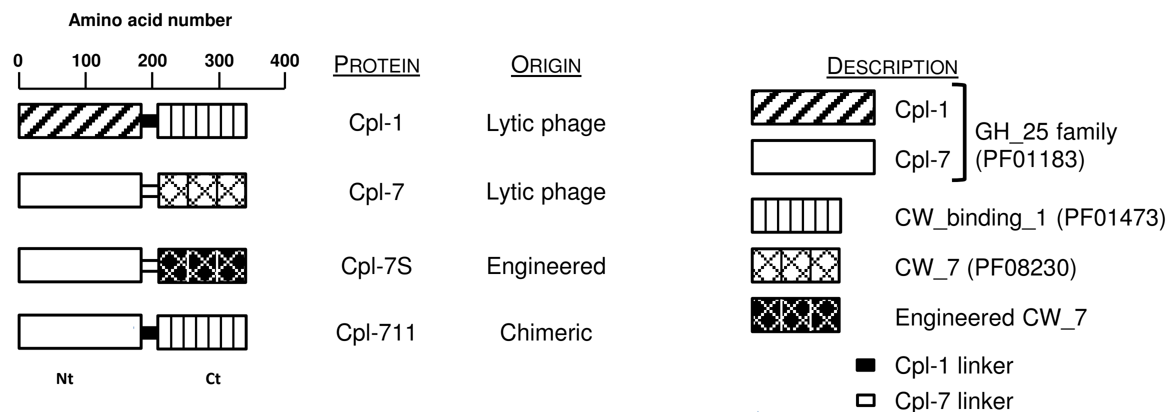

FIG 1 Schematic representation and description of parental Cpl-1, Cpl-7, engineered Cpl-7S and chimeric Cpl-711 lysozymes. Cpl-711 contains the CD of Cpl-7S, the linker of Cpl-1 and the CWBD of Cpl-1. CDs belong to the GH_25 family of glycosyl hydrolases and share 159 out of 186 amino acid residues between 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; Ct, C-terminal.

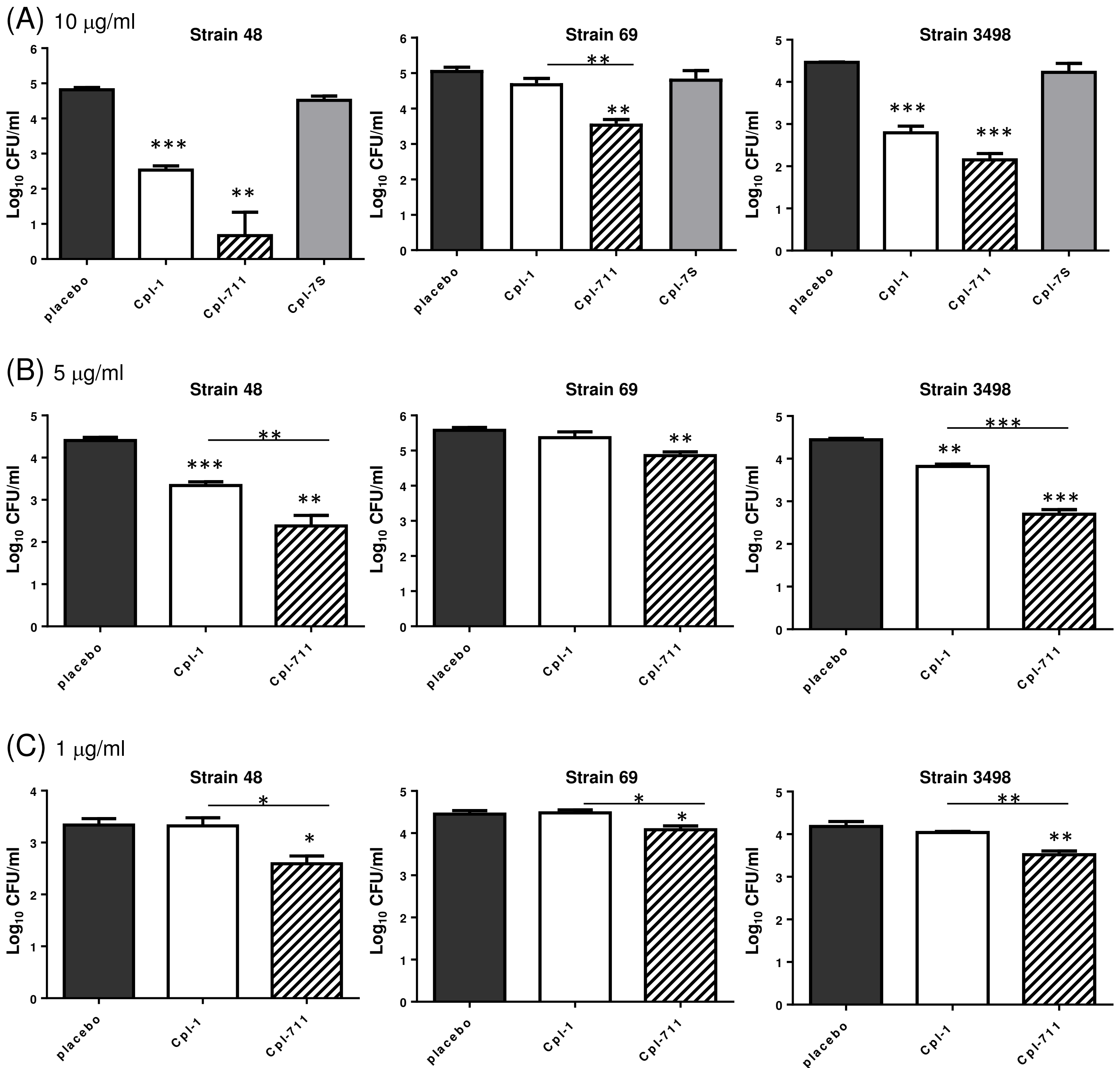

FIG 2 Killing by lytic enzymes against MDR isolates of *S. pneumoniae* attached to human lung epithelial cells. A549 cells colonized with pneumococcal strains were exposed to PBS (placebo) or to 10 µg/ml (A), 5 µg/ml (B) or 1 µg/ml (C) of either Cpl-1, Cpl-711 or Cpl-7S. Error bars represent the SDs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

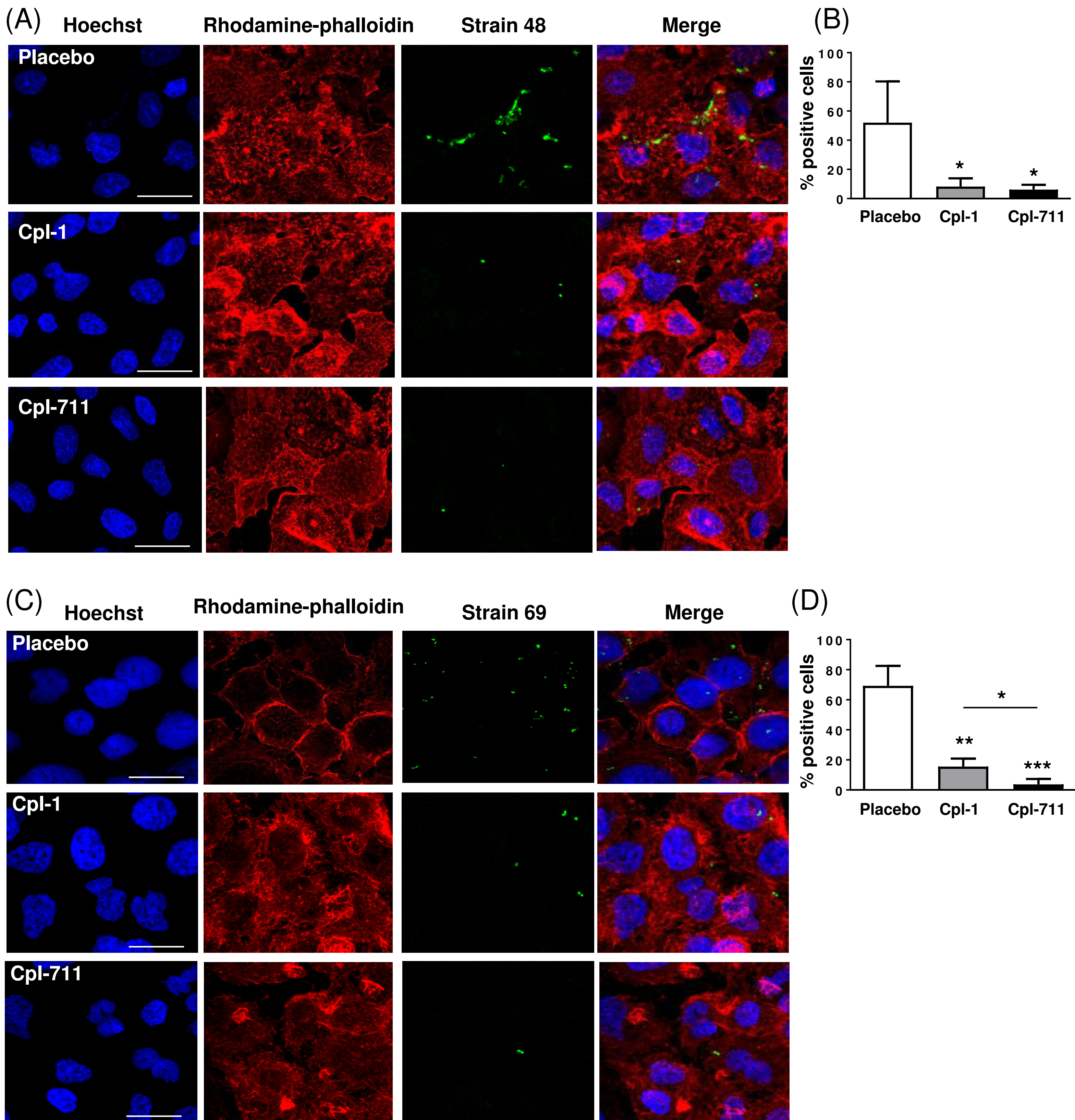

FIG 3 Detachment of MDR pneumococcal strains from human lung cells. A549 cells were infected with the indicated pneumococcal strains and 1 h later cells were exposed to PBS (as placebo) or to 10 μg/ml of Cpl-1, or Cpl-711. (A) CLSM images of cells infected with the clinical isolate 48. (B) Percentage of epithelial cells associated to at least one fluorescent bacterial cell (C) CLSM images of cells infected with the clinical isolate 69. (D) Percentage of epithelial cells associated to 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. Asterisks on the horizontal line indicate significance when Cpl-1 and Cpl-711 were compared.

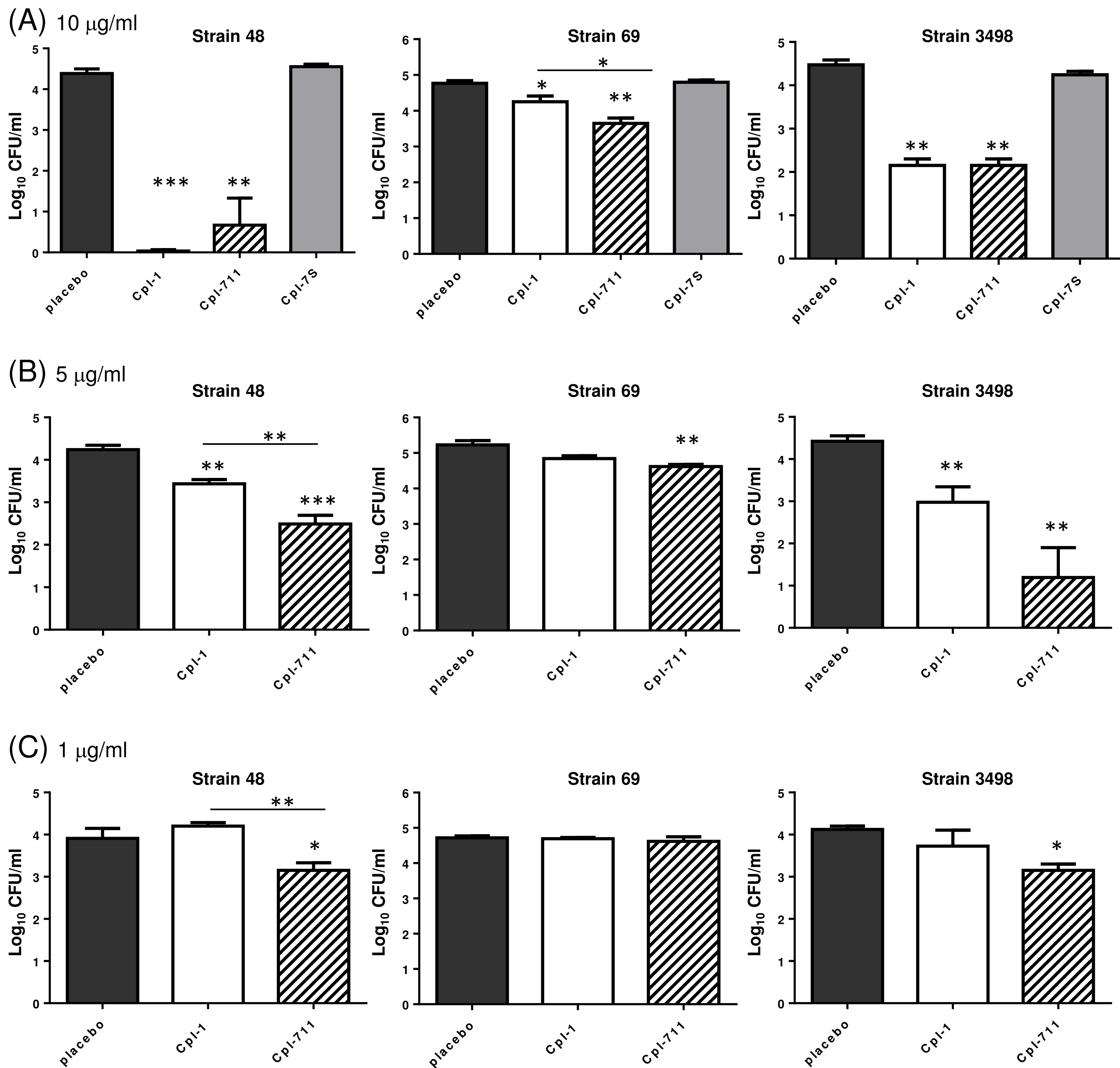

FIG 4 Bacterial killing by lytic enzymes against MDR isolates of *S. pneumoniae* attached to human nasopharyngeal epithelial cells. Detroit 562 cells were infected with pneumococcal strains and 1 h later cells were exposed to PBS as placebo or to 10 μg/mL (A), 5 μg/mL (B) or 1 μg/mL (C) of either Cpl-1, 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 and asterisks on the horizontal line indicate significance when Cpl-1 and Cpl-711 were compared.

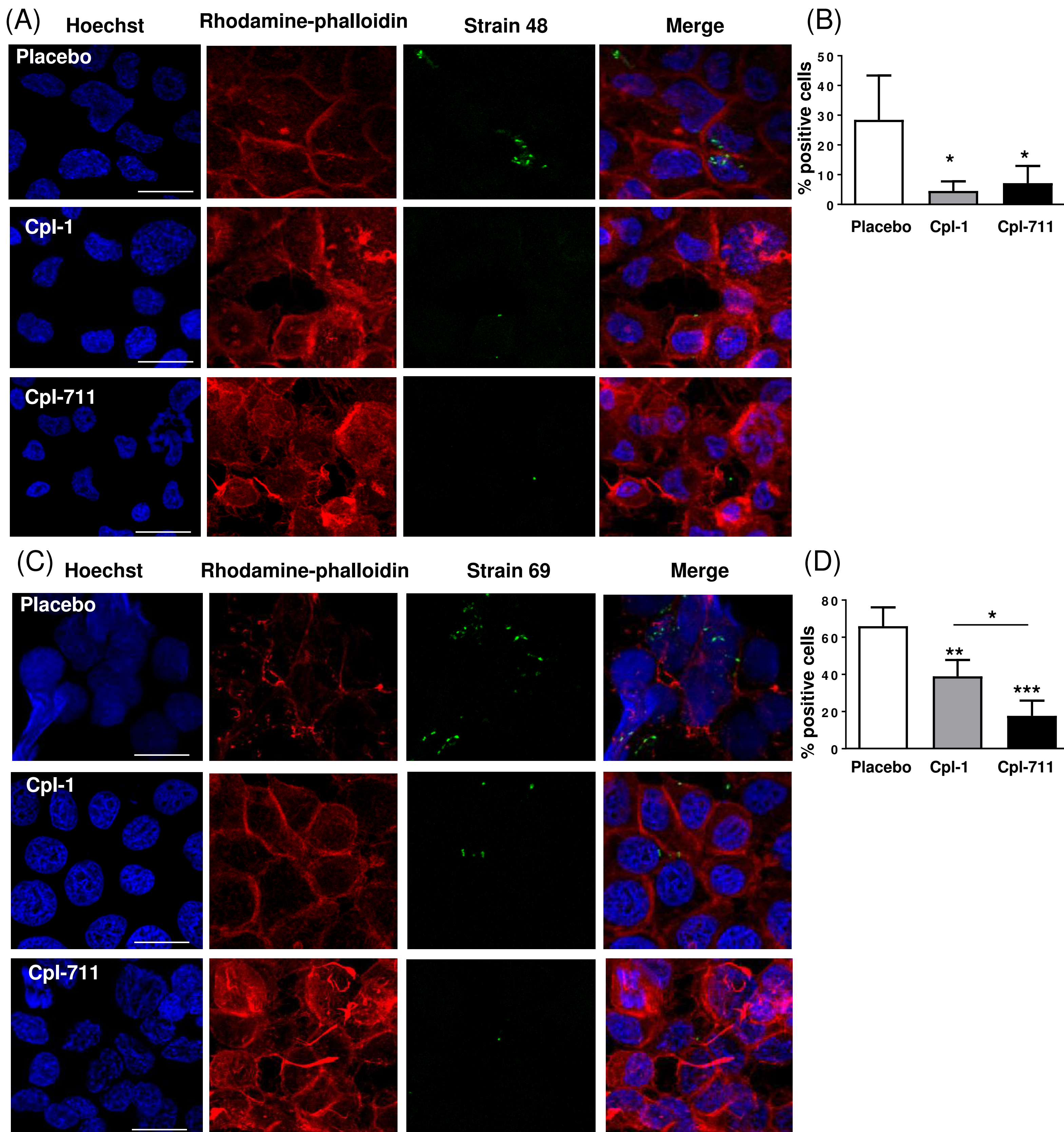

FIG 5 Detachment of MDR pneumococcal strains from nasopharyngeal cells. Detroit 562 cells were infected with the indicated pneumococcal strains and 1 h later cells were exposed to PBS (as placebo) or to 10 μg/ml of Cpl-1, or Cpl-711. (A) CLSM images of cells infected with the clinical isolate 48. (B) Percentage of positive epithelial cells associated to at least one fluorescent bacterial cell. (C) CLSM Images of cells infected with the clinical isolate 69. (D) Percentage of positive epithelial cells associated to 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. Asterisks on the horizontal line indicate significance when Cpl-1 and Cpl-711 were compared.

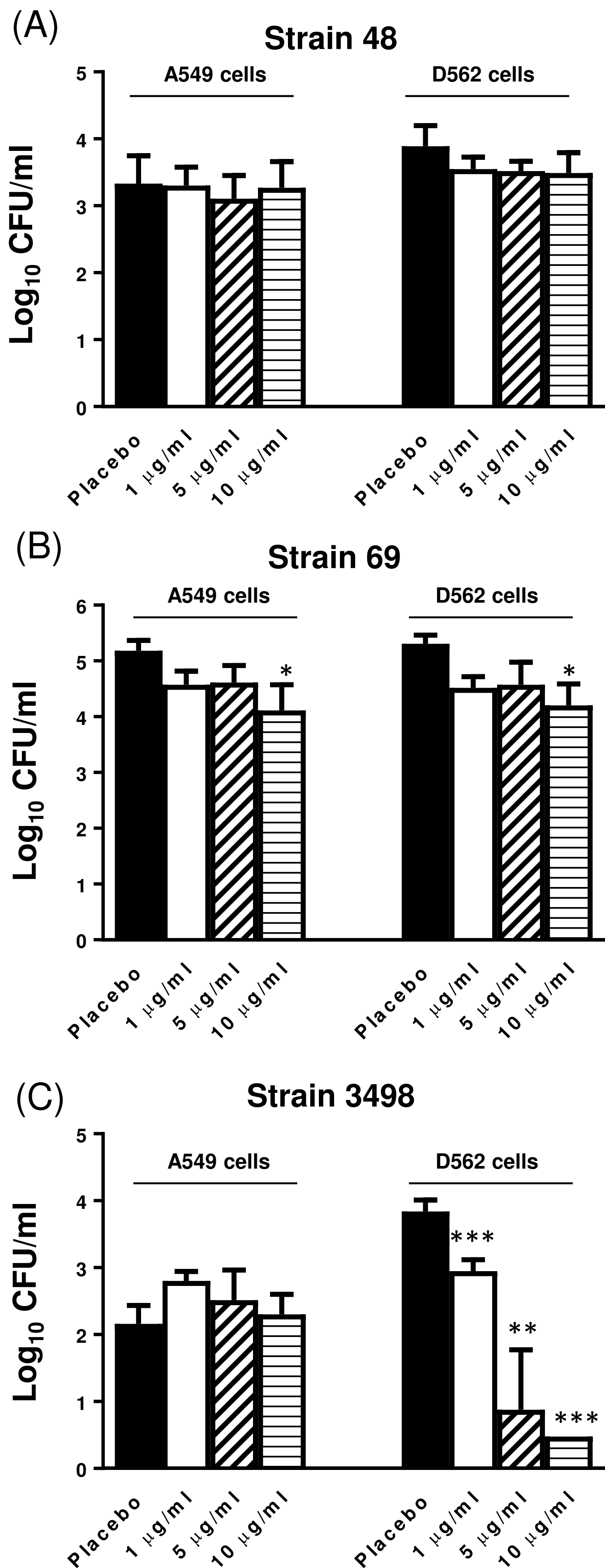

FIG 6 Bacterial killing of MDR isolates of *S. pneumoniae* by AMX on lung (A549) or nasopharyngeal (D562) epithelial cells (D562). Cells colonized with pneumococcal strains were exposed to PBS (placebo) or AMX (1 µg/ml; 5 µg/ml; 10 µg/ml). (A) Strain 48, (B) Strain 69, (C) Strain 3498. Error bars represent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

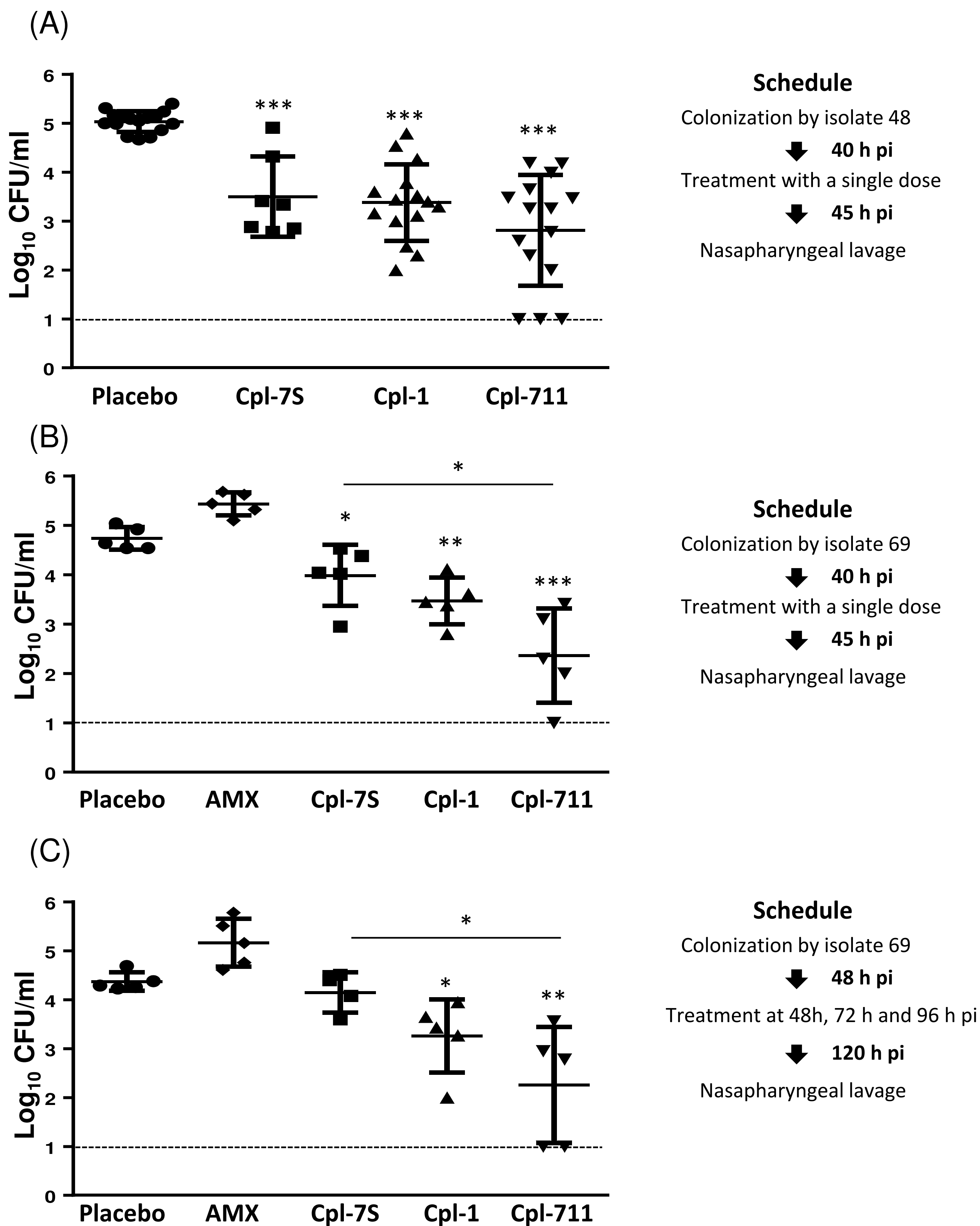

FIG 7 Antimicrobial activity of lytic enzymes Cpl-1, Cpl-711 or Cpl-7S administered by the intranasal route against nasopharyngeal colonization in mice. (A) Colonization with isolate 48 and a single dose of 10 μg/ml of the different lytic enzymes (or PBS as placebo) administered at 40 h post-infection (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 post-infection (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 post-infection (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 *t* tests). Analysis using Kruskal-Wallis test showed a *P* value < 0.001 for panels A, B and C. Asterisks on the horizontal line indicate significance between Cpl-711 and lysins and dots line represents the limit of bacterial detection.