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1 # «*In vitro* and *in vivo* efficacy of the combination of colistin and endolysins
2 # against clinical strains of Multi-Drug Resistant (MDR) pathogens»
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37 **ABSTRACT**

38 The multidrug resistance (MDR) among pathogenic bacteria is jeopardizing the worth of
39 antimicrobials, which had previously changed medical sciences. In this study, we used
40 bioinformatic tools to identify the endolysins ElyA1 and ElyA2 (GH108-PG3 family) present in the
41 genome of bacteriophages Ab1051Φ and Ab1052Φ, respectively. The muralytic activity of these
42 endolysins over MDR clinical isolates (*Acinetobacter baumannii*, *Pseudomonas aeruginosa* and
43 *Klebsiella pneumoniae*) was tested using the turbidity reduction assay. The minimal inhibitory
44 concentrations (MICs) of endolysin, colistin and their combination were determined using the
45 microdilution checkerboard method. The antimicrobial activity of the combinations was confirmed
46 by time kill curves and *in vivo* assays in larvae of *Galleria mellonella*. Our results showed that
47 ElyA1 displayed activity against all 25 strains of *A. baumannii* and *P. aeruginosa* tested and against
48 13 out of 17 strains of *K. pneumoniae*. No activity was detected when assays were done with
49 endolysin ElyA2. The combined antimicrobial activity of colistin and endolysin ElyA1 yielded a
50 reduction in the colistin MIC for all strains studied, except *K. pneumoniae*. These results were
51 confirmed *in vivo* in *G. mellonella* survival assays. In conclusion, the combination of colistin with
52 new endolysins such as ElyA1 could increase the bactericidal activity and reduce the MIC of the
53 antibiotic, thus also reducing the associated toxicity.

54 **IMPORTANCE**

55 The development of multiresistance by pathogen bacteria increases the necessity of the
56 development of new antimicrobial strategies. In this work, we combined the effect of the colistin
57 with a new endolysin, ElyA1, from a bacteriophage present in the clinical strain of *Acinetobacter*
58 *baumannii* Ab105. ElyA1 is a lysozyme-like family (GH108-GP3), whose antimicrobial activity
59 was described for first time in this work. Also, another endolysin, ElyA2, with the same origin and
60 family, was characterized but in this case no activity was detected. ElyA1 presented lytic activity
61 over a broad spectrum of strains from *A. baumannii, Pseudomonas aeruginosa*, and *Klebsiella*
62 *pneumoniae*. When colistin was combined with ElyA1 an increase of the antimicrobial activity was

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63 observed with a reduced concentration of colistin, and this observation was also confirmed *in vivo*
64 in *Galleria mellonella* larvae. The combination of colistin with new endolysins as ElyA1 could
65 increase the bactericidal activity and lowering the MIC of the antibiotic, thus also reducing the
66 associated toxicity.

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89 **INTRODUCTION**

90 The worldwide emergence of multidrug resistant (MDR) microorganisms that are refractory to
91 treatment with current therapeutic agents has emphasised the urgent need for new classes of
92 antimicrobial agents (1). Recently, the World Health Organization (WHO) published a list of
93 "priority pathogens" which includes those microorganisms that are considered a serious threat to
94 human health and for which new anti-infective treatments are urgently needed. The members of this
95 list are carbapenem-resistant *A.baumannii*, *P.aeruginosa* and *K.pneumoniae* clinical isolates (2).
96 A consequence of the emergence of the MDR bacteria is the return to the use of antimicrobials
97 whose use was reduced or abandoned. This was the case of colistin or polymixin E, a cationic
98 peptide which disturbs the stability of the outer membrane increasing its permeability through
99 electrostatic interactions and cationic displacement of the lipopolysaccharide. In spite of its
100 antimicrobial effects, this antibiotic presented nephrotoxicity effects and finally its use was
101 gradually dismissed and substituted by other more tolerable antibiotics (3, 4). The search of new
102 antimicrobial agents as well as their combination with old antibiotics such as colistin are new
103 strategies in seeking novel treatments against MDR microorganisms.
104 In recent years, a novel drug discovery approach has explored endolysin enzymes (also referred to
105 as enzybiotics) encoded by bacteriophages (viruses which infect bacteria) (5). Endolysins are
106 actively produced during the lytic cycle and exert antibacterial activity through degradation of
107 peptidoglycan in the bacterial cell wall (5, 6).
108 Endolysins are highly evolved enzymes produced by bacteriophages to digest the bacterial cell wall
109 at the end of their replication cycle and release the phage progeny. Endolysins target the integrity of
110 the cell wall and attack one of the major bonds in the peptidoglycan layer. They can be classified
111 into five groups according to the cleavage site: N-acetyl-β-D-muramidase (lysozymes); N-acetyl-β-
112 D-glucosaminidases (glycosidases); lytic transglycosylase; N-acetylmuramoyl-L-alanine amidases
113 and L-alanoyl-D-glutamate endopeptidases (6, 7).
114 Endolysins are good candidates as new antimicrobial agents against Gram-positive bacteria, in

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115 which the peptidoglycan layer of the cell wall is exposed to the medium. Several studies have
116 evaluated the potential use of endolysin against Gram-positive bacteria such as *Staphylococcus*
117 *aureus, Streptococcus agalactiae*, *Streptococcus pneumoniae* and *Streptococcus pyogenes* in animal
118 models of human infections and diseases (8-16). In Gram-negative bacteria, the outer membrane
119 acts as a barrier to many endolysins, and very few endolysins with exogenous activity against
120 Gram-negative bacteria have been described (many are biotechnologically engineered) (17-20).
121 Endolysins can attack Gram-negative bacteria when the outer membrane is previously
122 permeabilized with agents such as EDTA, which destabilizes the lipopolysaccharides of the outer
123 membrane; however, the combination of endolysin and EDTA is limited to a topical treatment of
124 localized infections (21, 22). In the search for alternative methods of killing MDR bacteria such as
125 *A. baumannii*, *P.aeruginosa* and *K.pneumoniae* various researchers have considered increasing the
126 muralytic activity of endolysins by combining them with different antibiotics to take advantage of
127 synergistic responses (22-25).

128 In this report, we identified and characterized an endolysin, named ElyA1, isolated from the
129 *A. baumannii* Ab105 (ROC0034a) bacteriophage Ab1051Φ. The endolysin displayed muralytic
130 activity against a broad spectrum of MDR organisms. In addition, combining endolysin ElyA1 with
131 colistin (polymyxin E) enhanced the susceptibility of the tested strains to colistin by at least four
132 times, thus highlighting the potential of endolysin ElyA1 as an antibacterial agent candidate. This
133 effect was confirmed by a test *in vivo*, in which the survival of the *G. mellonella* larvae increased
134 when colistin (¼ MIC) was supplemented with endolysin ElyA1. Moreover, another endolysin from
135 the same family, named ElyA2, was identified in the *A. baumannii* Ab105 bacteriophage Ab1052Φ,
136 but no muralytic activity was detected in this enzyme.

137 MATERIALS AND METHODS

138 Strains and culture conditions

139 The bacterial strains and plasmids used in this study included 25 *A. baumannii* MDR strains
140 belonging to 22 different sequence types (STs) (Table 1). The strains were isolated from colonized

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141 or infected patients within the framework of the II Spanish Multicentre Study which counted with
142 the participation of 45 Spanish hospitals (GEIH-REIPI *Acinetobacter baumannii* 2000-2010,
143 Genbank Umbrella Bioproject accession number PRJNA422585)(26). They included 25 MDR
144 clinical strains of *P. aeruginosa* (many included in CC274), all of which were isolated from cystic
145 fibrosis patients, and 17 carbapenemase-producing strains of *K. pneumoniae*, which were isolated in
146 20 Spanish hospitals during the EuSCAPE project (27, 28). Moreover, *Escherichia coli* DH5α and
147 Rosetta strains were used in cloning assays (Table 1).

148 All strains were cultured in LB (Luria Bertani) at 180 rpm and 37ºC. For solid medium, 2% of agar
149 was added to LB broth. When transformation assays were done the medium was supplemented with
150 50µg/ml of ampicillin.

151 **Identification and Purification of the endolysins ElyA1 and ElyA2**

152 Endolysin gene prediction, from the genome of the bacteriophage Ab105Φ1 (GenBank:
153 KT588074.1) and Ab105Φ2 (GenBank: KT588075.2) (26) (Figure 1), was performed with the
154 bioinformatic tools PHASTER (Phage Search Tool Enhanced Release) and RAST (Rapid
155 Annotation Using Subsystem). Protein homology analysis was performed by BLAST (Basic Local
156 Alignment Search Tool), Clustal Omega and MView. Protein families were assigned using
157 InterProScan and the domain graphic was assigned with PROSITE MyDomains.

158 The endolysin genes were amplified by PCR from the genomic DNA of *A. baumannii* Ab105,
159 which contains the DNA of the prophages Ab105Φ1 and Ab105Φ2, and cloned into the expression
160 vector pET-28a (Novagen). The recombinant plasmids were transformed into competent *E. coli*
161 DH5α cells (Novagen) for DNA production and purification, and the integrity of both constructs
162 was verified by sequencing. All of the primers used are listed in Table 1. Finally, the plasmids were
163 transformed into *Escherichia coli* Rosetta pLys-S cells (Novagen) for expression of the protein.

164 After induction with 1 mM IPTG, the culture (1 l) was grown at 30°C for 5 h. The bacterial cells
165 were recovered by centrifugation (in a JLA 81000 rotor, Beckman-Coulter, at 6 Krpm for 15 min)
166 and disrupted by sonication (VibraCell 75042 sonicator, Bioblock Scientific, tip model CV33). The

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167 sample was centrifuged in a JA 25-50 rotor (Beckman-Coulter) at 20 Krpm for 30 min. The
168 recovered supernatant was filtered using 0.45 µm syringe-driven filters (Jet Biofil) and loaded in a
169 His-Trap column (GE Healthcare) equilibrated with 350 mM NaCl, 50 mM Tris pH 7.5, 1 mM
170 TCEP and 10 mM Imidazole. The proteins were eluted with 350 mM NaCl, 50 mM Tris pH 7.5, 1
171 mM TCEP and 150 mM Imidazole. After concentration in an Amicon Ultracel 10,000 MCWO
172 concentrator (Millipore), the sample was loaded into a Superdex 75 16/60 column (GE Healthcare),
173 equilibrated with 150 mM NaCl, 20 mM Tris pH 7.5 and 1 mM TCEP. The protein was eluted in a
174 single peak. Finally, the pooled peak fractions were concentrated to 40 mg/ml, as previously
175 described. The purification process was carried out at 4º C, and the purity was determined by SDS-
176 PAGE (Figure 1).

177 **Determination of the muralytic activity of endolysins ElyA1 and ElyA2**

178 Muralytic activity was determined using the Gram-negative overlay method described by Schmitz
179 et al (29). Briefly, two clinical isolates of *A. baumannii*, MAR001 and PAU002, were grown to
180 stationary phase (10^9 CFU/ml) in LB, pelleted and resuspended in PBS buffer pH 7.4. Agar was
181 added directly to the bacteria suspension suspension at a concentration of 0.8% and autoclaved for
182 15min at 120ºC. The medium, containing the disorganized cells with the peptidoglycan exposed,
183 was solidified in Petri dishes and aliquots (50 µg) of endolysin, or the endolysin buffer as negative
184 control were spotted on the surface.

185 The muralytic activity was measured using as target a culture of *A. baumannii* Ab105 treated with
186 EDTA in order to permeabilize the outer membrane. An overnight culture of *A. baumannii* Ab105
187 was diluted 1:100 in LB medium and grown to exponential phase (0.3-0.4 OD600nm). The culture
188 was centrifuged (3000g, 10min), and the resulting pellet was resuspended in 20mM Tris-HCl buffer
189 at pH 8.5 with 0.5 mM EDTA before being incubated for 30 min at room temperature. The pellet
190 was recovered by centrifugation and washed twice in Tris-HCl buffer pH 8.5. Finally, the cells were
191 resuspended in 20 mM Tris-HCl 150 mM NaCl pH8.5 and 25 µg/ml of endolysin ElyA1. The
192 activity was measured by the turbidity reduction assay, as a decrease in the OD600nm after

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193 incubation with after with shaking at 37ºC (17). The optical density was measured at intervals of 5
194 minutes for a period of 20 minutes and the time point of the higher activity was established. Also,
195 the optimal pH and temperature for the endolysin activity were determined employing the turbidity
196 reduction assay. The reaction was done as described previously, with the Tris-HCl at different pH
197 (range 6.5 to 9) and temperature (room temperature, 30ºC and 37ºC).

198 **Antibacterial assays**

199 The antibacterial activity of the endolysin was assayed with all of the 67 clinical strains of *A.*
200 *baumannii, P. aeruginosa* and *K. pneumoniae* (Table 1). The activity was determined using the
201 turbidity reduction assay, as previously described, at pH 8.5 and 37ºC. The incubation times in the
202 presence of EDTA varied according to the species assayed: 30 min for *A. baumannii* and *K.*
203 *pneumoniae* and 15 min for *P. pneumoniae.*

204 **Broth microdilution checkerboard assay and microdilution test to determine minimum**
205 **inhibitory concentrations (MICs)**

206 This assay was done in those strains with the higher and the lower susceptibility to endolysin. All
207 the strains tested were susceptible to colistin except three strains that were colistin resistant: *A.*
208 *baumannii* SOF004b, *P. aeruginosa* AUS034 and *K. pneumoniae* KP2. The effect of the interaction
209 between endolysin and colistin was done by the chequerboard MIC assay. In a 96-well microtiter
210 plate with Mueller-Hinton Broth (MHB), 7 serially double dilutions were done for endolysin and 6
211 for colistin. The wells were then inoculated with the test culture at a final concentration of 10^5
212 colony forming units (cfu/ml). The MIC of colistin (0 to 2 μgml^{-1}) and concentration of endolysin
213 ElyA1 protein (3.125 to 200 μgml^{-1}) was assayed independently in the same plate. The MIC was
214 determined as the concentration of antimicrobial agent in the well where no visible growth of
215 bacteria was observed after incubation for 24h at 35ºC. The MIC_{AB} was defined as the MIC of drug
216 A in the presence of drug B, and the MIC_{BA} was defined as the MIC of drug B in the presence of
217 drug A.

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219 **Time kill curve assay**

220 Time kill curve assays were carried out with those strains in which the MIC of colistin in ElyA1-
221 colistin combinations was decreased by at least fourfold in checkerboard assays. The assay was
222 conducted according to previously described techniques (30). Flasks of LB containing colistin and
223 colistin plus endolysin at the concentration corresponding to the checkerboard assay were
224 inoculated with a dilution 1:100 of an overnight culture in stationary phase of the tested strain and
225 incubated at 37ºC and 180 rpm in a shaking incubator. Aliquots were removed after 0, 6 and 24 h
226 and were serially diluted and plated to produce colony forming units (cfu). Synergy was established
227 when a ≤2 -log_{10} decrease in cells counts at 6 or 24 h in the antimicrobial combination relative to the
228 most active single agent was observed. No effect was considered to have occurred when the counts
229 were <2-log_{10} lower or higher relative to the culture with the single agent. Antagonism was defined
230 when the counts in the culture with antimicrobial combination were ≥ 2-log_{10} higher than in the
231 culture with single most active antimicrobial agent.

232 The reduction in the MIC of the colistin in combination with endolysins was also assayed by
233 combining colistin with another endolysin ElyA2, isolated from the bacteriophage Ab105Φ2. The
234 curve was done for the same strains and under the same conditions as for colistin-ElyA1.

235 ***Galleria mellonella*** **Infection Model**

236 The *Galleria mellonella* model was an adapted version of that developed by Peleg et al (31, 32).
237 The procedure was as follows: twelve *G. mellonella* larvae, acquired from TruLarvTM (Biosystems
238 Technology, Exeter, Devon, UK), were each injected with 10 µl of a suspension of *A. baumannii*
239 PON001, diluted in sterile phosphate buffer saline (PBS) containing 1×10^5 CFU (± 0. 5 log). The
240 injection was performed with a Hamilton syringe (volume 100 µl) (Hamilton, Shanghai, China). An
241 hour after infection, larvae were injected with 10 µl of colistin in combination with endolysin
242 ElyA1, colistin in combination with endolysin ELyA2, and colistin alone as a control group, all at
243 the concentrations used in the time kill curve. After being injected, the larvae were placed in Petri
244 dishes and incubated in darkness at 37°C. The number of dead larvae was recorded for 5 days. The

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245 larvae were considered dead when they showed no movement in response to touch (31).
246 The mortality curves corresponding to the *in vivo Galleria mellonella* infection model were
247 constructed using GraphPad Prism v.6 and the data were analysed using the Graham-Breslow-
248 Wilcoxon test. In both cases, p-values <0.05 were considered statistically significant, and the data
249 were expressed as mean values.

250 **RESULTS**

251 **Identification of endolysins ElyA1 and ElyA2**

252 The gene of 546 bp coding for endolysin ElyA1 was identified as an ORF (Open Reading Frame)
253 encoding a protein of 181 aa (GenBank: ALJ99090.1) and 20.22 kDa. The protein sequence was
254 analysed with InterProScan and classified as a lysozyme (N-acetylmuramidase) with a C-terminal
255 domain corresponding with the Glycosyde hydrolase 108 superfamily and also a Peptidoglycan
256 Binding domain PG3 at the N-terminal end.
257 Protein homology analysis revealed a high level of homology (more than 80%) with a group of 9
258 endolysins from *A. baumannii* bacteriophages belonging to the same protein family as ElyA1 (20).
259 Endolysin ElyA2 gene of 543 bp was identified as an ORF encoding a protein of 180 aa (GenBank:
260 ALJ99174.1) and with a MW of 20.19 kDa. The sequence analysis revealed that ElyA2 protein is
261 also a lysozyme (N-acetylmuramidase) with a C-terminal domain corresponding with the
262 Glycosyde hydrolase 108 superfamily and also a Peptidoglycan Binding domain PG3 at the N-
263 terminal end.
264 As ElyA1 protein, this enzyme presented a high homology (more than 80%) with the same group of
265 9 endolysins and also a homology of 90% with ElyA1 protein (20).

266 **Endolysins muralytic activity characterization**

267 In the first screening of the muralytic activity of the purified endolysin ElyA1 in the Gram-negative
268 overlay plates, a lysis halo appeared in both strains of *A. baumannii* tested (Figure 2a).
269 The muralytic activity of this enzyme was characterized using the Gram-negative bacteria *A.*
270 *baumannii* Ab105 as substrate, as this is the host strain for the phage Ab105Φ1. The enzymatic

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271 activity was measured after different incubation at different temperatures and pH. The maximum
272 activity was obtained after incubation for 10 min at pH8.5 and 37ºC (Figure 2b-c). In addition, the
273 muralytic activity on the cells of Ab105 was assayed directly or after treatment of the cells with
274 EDTA to permeabilize the outer membrane. However, no activity was detected when the enzyme
275 was added directly to the cells whose outer membrane was not previously permeabilized with
276 EDTA, and also in this case the cells tended to aggregate (data not shown).
277 The antibacterial assays showed a broad lytic spectrum of activity against the strains of the three
278 species tested (Figure 3). As expected because of the origin of the *A. baumannii* endolysin, the
279 highest activity observed was among the 25 *A. baumannii* strains tested. Although the activity was
280 more variable in *P. aeruginosa*, muralytic activity was detected against all of the strains tested.
281 Finally, endolysin ElyA1 displayed activity against 13 of the 17 *K. pneumoniae* strains, although at
282 lower levels than in *A. baumannii* and *P. aeruginosa*. The strains tested in the three species
283 belonged to different STs, but no correlation between the susceptibility to endolysin ElyA1 and the
284 ST was detected.
285 When muralytic activity of endolysin ElyA2 was tested no activity was detected under any
286 condition assayed. On the contrary, this enzyme induces the aggregation of the cells at all the
287 enzyme concentration tested both in those cells treated previously with EDTA and in those with an
288 intact outer membrane.
289 **Endolysin ElyA1 and colistin combination activity Assays**
290 As endolysin ElyA1 is only active when the outer membrane of the target bacterial cell is
291 solubilized, the MIC of the endolysin was not able to be determined by the microdilution
292 checkerboard test. We therefore aimed to detect any decrease in the colistin MICs when used in
293 combination with endolysin ElyA1. The addition of endolysin ElyA1 yielded a fourfold reduction
294 in the colistin MICs in four of the six strains tested (*A. baumannii* GMA001 and PON001, *P.*
295 *aeruginosa* AUS531 and *K. pneumoniae* KP17) (Figure 4). By contrast, only a twofold reduction in
296 the colistin MIC was observed with *P. aeruginosa* AUS601 and no decrease with *K. pneumoniae*

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297 KP16. The latter was consistent with the lack of enzymatic activity observed in the antibacterial
298 assays (Figure 4). Finally, no antimicrobial activity was detected when the combination was tested
299 in the colistin resistant isolates (data not shown).
300 The results of the time kill curve assay confirmed the results obtained by the microdilution
301 checkerboard test (Figure 4). A 2 log reduction was shown in the growth of both the *A. baumannii*
302 strains and *P. aeruginosa* AUS531 after 6 hours in the culture with 1/4 of colistin and endolysin
303 ElyA1, indicating a synergetic reaction between colistin and endolysin ElyA1. By contrast, no
304 reduction was observed in the *K. pneumoniae* KP17 culture.
305 **Mortality in the *in vivo Galleria mellonella* Model**
306 Larvae of the wax moth were infected with the clinical strain of *A. baumannii* PON001. When
307 infected larvae were treated with colistin (¼MIC) in combination with endolysin ElyA1 (25 µg/ml),
308 a significative increase ($P<0.05$) in the survival was observed in the treated larvae respect to those
309 treated only with colistin (¼ MIC) (Figure 5a). When colistin (¼ MIC) was combined with ElyA2
310 (25 µg/ml) no significative differences ($P>0.05$) were obtained when compared with the treatment
311 with colistin, as no muralytic activity was previously detected with ElyA2 (Figure 5b).
312 **DISCUSSION**
313 The discovery and development of novel antimicrobial agents to treat infections caused by the
314 "priority" group of pathogens has occurred in the medical and research community (2).
315 Enzybiotics have become a focus of attention of many research groups worldwide. Endolysins, one
316 type of enzybiotic, are species or genus-specific enzymes that act by hydrolysing the peptidoglycan
317 layer of the bacterial cell wall. There are no reports of bacteria developing resistance to endolysins,
318 which is a great problem in both antibiotic therapy and also in phage therapy (16). Moreover,
319 endolysins have been recognized by the United States of America in the National Action Plan for
320 Combating Antibiotic-resistant Bacteria (33), which identified the use of "phage-derived lysins to
321 kill specific bacteria while preserving the microbiota" as a key strategy to reduce the development
322 of antimicrobial resistance (34) due to the absence of toxicity in human cells (35).

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323 The outer membrane of Gram-negative bacteria acts as a barrier preventing access of many
324 endolysins to their natural target, the peptidoglycan layer. This problem has been approached by
325 different strategies, including solubilization of the outer membrane with EDTA, modification of the
326 endolysin PGs by deletion or substitution and by the development of fusion proteins such Artilysin-
327 175, made by fusing the endolysin with a peptide to enable the enzyme to pass through the outer
328 membrane which has been a successful strategy (18, 36, 37). In *P.aeruginosa* strains, Briers et al.
329 (18) worked in the development of Art-175 constituted by antimicrobial peptide (AMP) sheep
330 myeloid 29-amino acid peptide (SMAP-29) fused with endolysin KZ144. Art-175 showed
331 muralytic activity in *P.aeruginosa* isolate and continuous exposure to Art-175 did not elicit the
332 development of resistance. On its own, SMAP-29 is cytotoxic to mammalian cells (38); however,
333 Art-175 exhibited little toxicity in L-292 mouse connective tissue.

334 As a new strategy we combined the membrane destabilizing effect of the colistin ("last-line"
335 treatment for these bacteria due to the concerns about its nephrotoxicity and neurotoxicity (39)),
336 which is a cationic peptide used as an active outer membrane agent, and two endolysins identified
337 by our research group that belong to a lysozyme-like family (GH108-PG3) never before used as
338 antimicrobial treatment.

339 In this work we identified two endolysins, ElyA1 and ElyA2, obtained from *A. baumannii*
340 bacteriophage Ab1051Φ and Ab105Φ2, available in a collection of clinical strains of *A. baumannii*
341 isolated during the II Spanish Multicentre Study GEIH/REIPI-*A.baumannii* 2000-2010 (Accession
342 number, Genbank Umbrella Bioproject PRJNA422585) (26, 40).

343 Endolysins ElyA1 and ElyA2 are lysozyme-like proteins with a catalytic domain and a cell wall
344 binding domain (CBD), responsible for recognition of the cell surface ligands and affinity for the
345 bacterial substrate (41, 42). This structure is most commonly found in endolysins from
346 bacteriophages that target Gram-positive bacteria. However, the PG_3 domain present in endolysins
347 ElyA1 and ElyA2 has been identified in some Gram-negative bacteria and in a group of nine
348 endolysins isolated from bacteriphages of *A. baumannii*; the domain shows high homology with

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349 ElyA1 and belongs to the same family (Figure 1) (20, 40, 43). Our molecular features and
350 comparative genomes results in bacteriophage endolysins were confirmed through the work of
351 Oliveira et al (44).
352 Furthermore, the bacteriophages from which these endolysins were isolated, Ab1051Φ and
353 Ab105Φ2, occur in a large number of clinical isolates of *A. baummanii* (26). The cell wall binding
354 domain has been shown to be responsible for the specificity and affinity of the endolysins for its
355 substrate (43). However, we observed that endolysin ElyA1 displays a broader spectrum of activity
356 against strains of *A. baumannii* and also many strains of *P. aeruginosa* belonging to the same order,
357 *Pseudomonadales*, and to lesser extent some strains of *K. pneumoniae* from another
358 gammaproteobacterial order, *Enterobacteriales.* In this case, the target of endolysin ElyA1,
359 identified in Peptidoglycan binding domains (PGs) as a D-Asn (43), is probably conserved among
360 the *Pseudomonadales*, thus explaining the broad spectrum of action of this enzyme. Interestingly,
361 we were not able to detect muralytic activity in endolysin ElyA2 because this enzyme induces the
362 aggregation of the cells *in vitro,* probably preventing the activity of the enzyme over all the cells.
363 In the present study, we used the cationic polymyxin antibiotic colistin to overcome the
364 impenetrability of the outer membrane to endolysin ElyA1. Colistin disturbs the outer membrane
365 via an electrostatic interaction with lipopolysaccharides and phospholipids present in the outer
366 membrane (4). The synergistic effect of endolysin LysABP-01 (Lysozyme-like protein from the
367 GH19 family) and colistin on *A. baumannii* has previously been described (25). Although endolysin
368 ElyA1 does not display exogenous activity, because of its inability to cross the outer membrane,
369 this problem was largely overcome when the enzyme was used in combination with colistin, and the
370 antimicrobial activity of the combined therapy was higher than for both substances used alone in all
371 the tested strains except for those of *K. pneumoniae*. A reduction in the colistin MIC of at least
372 fourfold occurred for all of the *A. baumannii* strains tested and for *P. aeruginosa* strain AUS531,
373 and a corresponding twofold reduction was observed for *P. aeruginosa* strain AUS601. A reduction
374 in the colistin MIC was also obtained for the *K. pneumoniae* strain KP17, the most susceptible to

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375 endolysin ElyA1. The increased antimicrobial activity with endolysin ElyA1 and colistin was
376 confirmed with the almost 3 log reduction in growth after 6 h in all strains tested, except KP17 *K.*
377 *pneumoniae*. Growth of the culture reached the same level as in the control after 24 h, probably due
378 to degradation of the enzyme and colistin, as also reported by Thummeepak *et al.* (22). In all of the
379 strains tested the reduction in the colistin MIC was consistent with the muralytic activity of
380 endolysin ElyA1 obtained for those strains. No antimicrobial activity was observed when this assay
381 was conducted in colistin-resistant strains, probably because of the inability of the enzyme to access
382 the peptidoglycan layer, as the necessary destabilization of the outer membrane by the colistin was
383 not produced in these isolates. However, there are several resistance mechanisms to colistin
384 described which include those in which the lipopolysaccharide is modified or is not produced
385 preventing the joint of the colistin to the outer membrane, but there are other mechanisms in the
386 literature which include efflux pumps described in *A. baumannii* and inhibition of respiratory
387 enzymes as NADH oxidase in Gram positives as *Bacillus spp* and NADH quinone oxidoreductase
388 in *E. coli.* It is probable that the activity of the enzyme would be higher in the bacteria with colistin
389 resistance mechanisms different from that who modify the lipopolysaccharides (45-51). Future
390 studies will be done over a range of different colistin resistant bacteria in order to reduce the MIC
391 for colistin in combination with endolysin ElyA1.
392 The results obtained *in vitro* were confirmed with the assays done *in vivo*, as the survival of the
393 infected larvae of *G. mellonella* was higher when the worms were treated with a combination of a
394 reduced fourfold MIC of colistin and endolysin ElyA1 than with colistin alone. As a control the
395 same assay was performed with endolysin ElyA2, in which the muralytic activity was not detected,
396 and no differences were observed with the treatment with colistin.
397 In conclusion, this is the first study *in vitro* and *in vivo* (*G.mellonella*) where colistin is
398 combined with endolysin ElyA1 (Glycosyde hydrolase 108 superfamily) against clinical MDR
399 pathogens, which may enable the concentration of colistin used in antimicrobial treatments to be
400 reduced, thus also reducing the toxic side effects of the antibiotic. The broad spectrum of action of

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401 endolysin ElyA1 would enable more MDR Gram-negative bacteria to be included as targets for this
402 combined antimicrobial treatment.
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441 **TRANSPARENCY DECLARATIONS**

442 All authors declare no conflict of interest

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612 **FIGURES AND TABLES**

613 **Table 1.** Description of the bacterial strains, plasmids and primers used in this study.

614 **Figure 1**. Genome of the bacteriophages Ab105Φ1 (GenBank: KT588074.1) and
615 Ab105Φ2 (GenBank: KT588075.2) by figure modified from PHAST software. .SDS-
616 PAGE purification of the endolysins ElyA1 and ElyA2 (chromatography study).

617 **Figure 2.** Characterization of enzymatic activity: a) Muralytic activity of ElyA1 was
618 done by spots of ElyA1 and endolysin buffer as negative control in a Gram-negative
619 overlay of two *Acinetobacter baumannii* clinical isolates, MAR001 and PAU002; b) pH
620 range and c) Temperature range, were determined by the specific activity measured as
621 the difference in optical density of the culture per µg of enzyme and minute.

622 **Figure 3.** Specific activity of endolysin ElyA1 tested in clinical isolates from different
623 mutlilocus sequence types (STs) of three Gram-negative members of the ESKAPE
624 group: *Acinetobacter. baumannii, Pseudomonas aeruginosa and Klebsiella*
625 *pneumoniae*.

626 **Figure 4.** Bactericidal activity of colistin in combination with endolysin ElyA1
627 measured by MIC and time kill curves in *Acinetobacter baumannii* strains GMA001 (a)
628 and PON001 (b); *Pseudomonas aeruginosa* strains AUS531(c) and AUS601(d);
629 *Klebsiella pneumoniae* strains KP17(e) and KP16 (f). The time kill curves were only
630 constructed for strains in which there was a fourfold reduction in colistin MICs (red
631 square) when used in combination with the endolysin ElyA1(yellowsquare).

632 **Figure 5.** Survival curves of *G. mellonella* larvae infected with *A. baumannii* clinical
633 strain PON001. a) Treated with colistin (1/4 MIC) and with colistin (1/4 MIC)
634 combined with endolysin ElyA1 (25µg/ml); b) Treated with colistin (1/4 MIC) and with
635 colistin (1/4 MIC) combined with endolysin ElyA2 (25µg/ml). This experiment was
636 carried out with an adequate survival control.

637

Strain, Plasmid, Primer Strain	Description, Characteristics and Sequence	Origin and Reference
Strains		
Acinetobacter baumannii	25 clinical isolates (22 STs) from the II Spanish Multicentre Study (GEIH-*REIPI Acinetobacter baumannii* 2000-2010) (Accession number Genbank PRJNA422585)	27
Pseudomonas aeruginosa	25 clinical isolates (ST274 [n = 15]; ST1089 [n = 3]; ST not known [n = 7])	28
Klebsiella pneumoniae	17 clinical isolates belonging to 16 different STs	29
Escherichia coli DH5α	Strain using for cloning	Novagen
Escherichia coli Rosetta pLys-S	Strain for protein expression	Novagen
Plasmid		
pET-28a	Km^{r}, T7*lac*, His-Tag, T7-Tag, thrombine protease site	Novagen
Primers		
Forward	5´-AGTTCTGTTCCAGGGGCCCCATATGAA CATTGAACAATATCTTGATGAA-3	This study
Reverse	5´-AGTGGTGGTGGTGGTGGTGCTCGAG**T** **CA**CATTGATACTCGATTAGCAAT-3´	This study

638

639 *Abbreviations:* ST; multilocus sequence type

640 **Table 1.**

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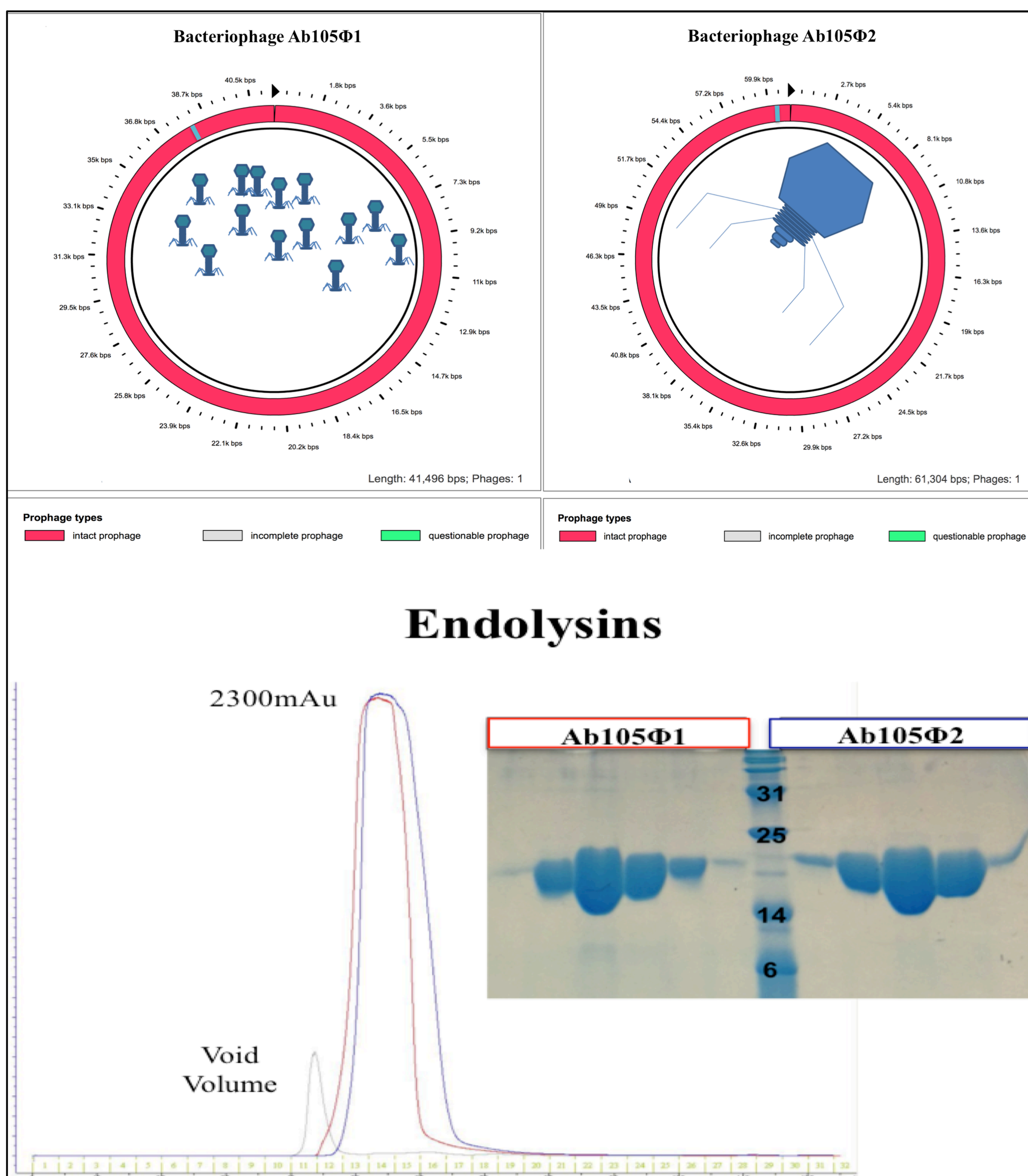

641

642 **Figure 1.**

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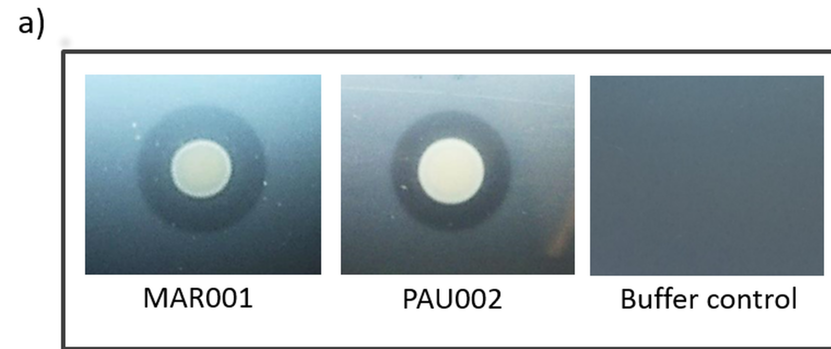

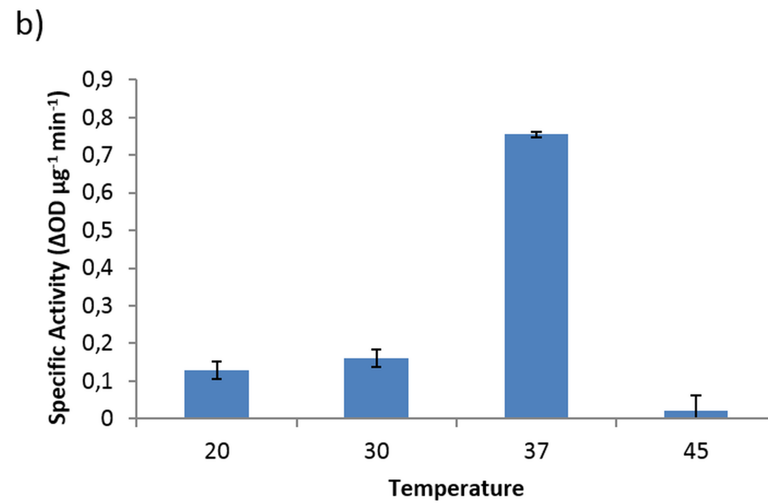

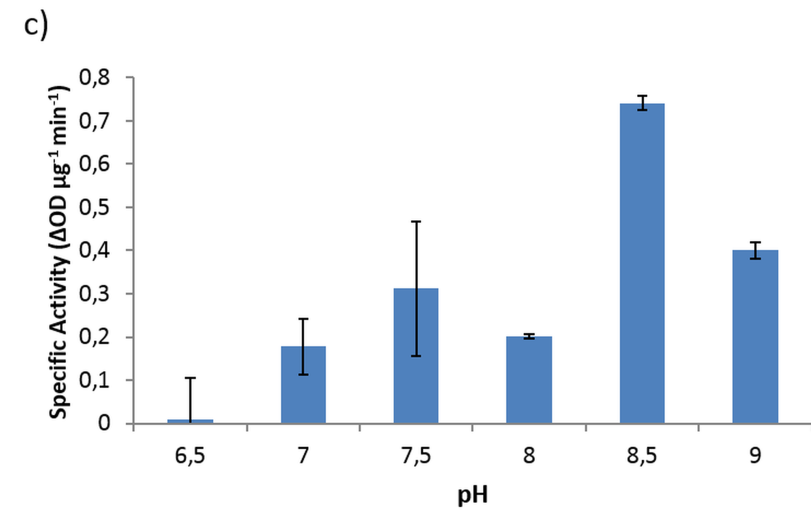

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651 **Figure 2.**

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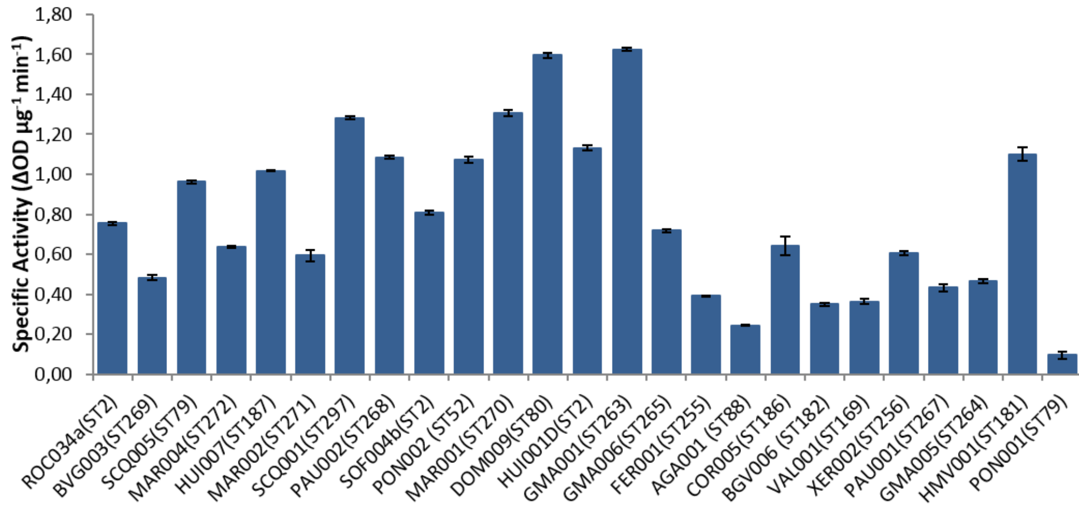

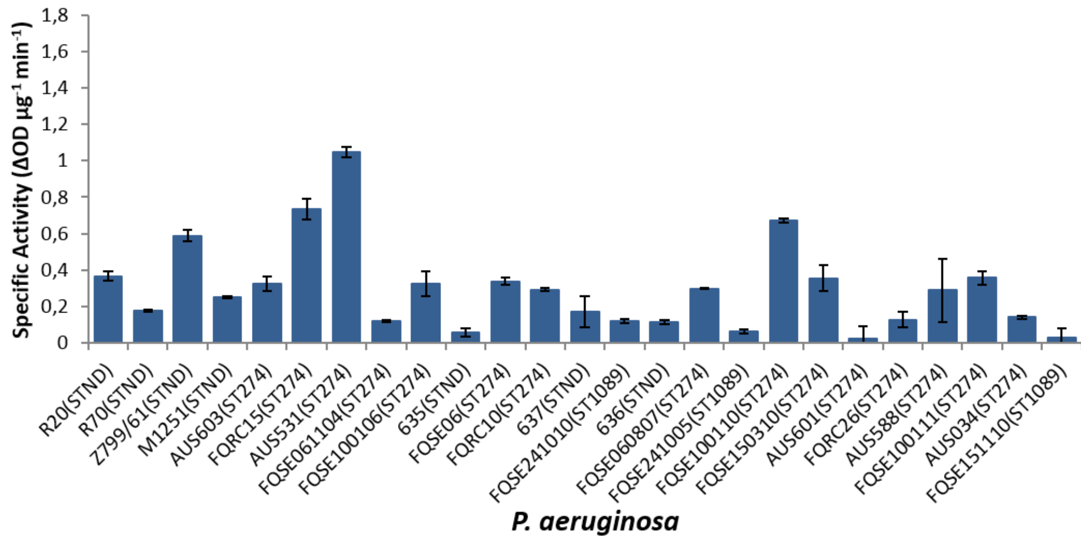

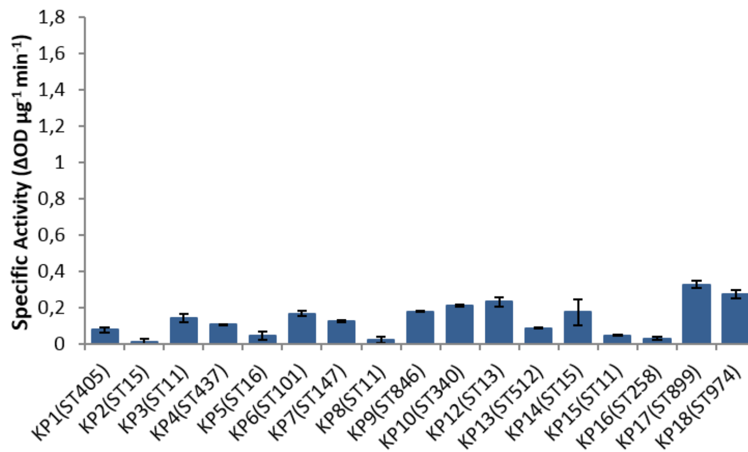

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653 **Figure 3.**

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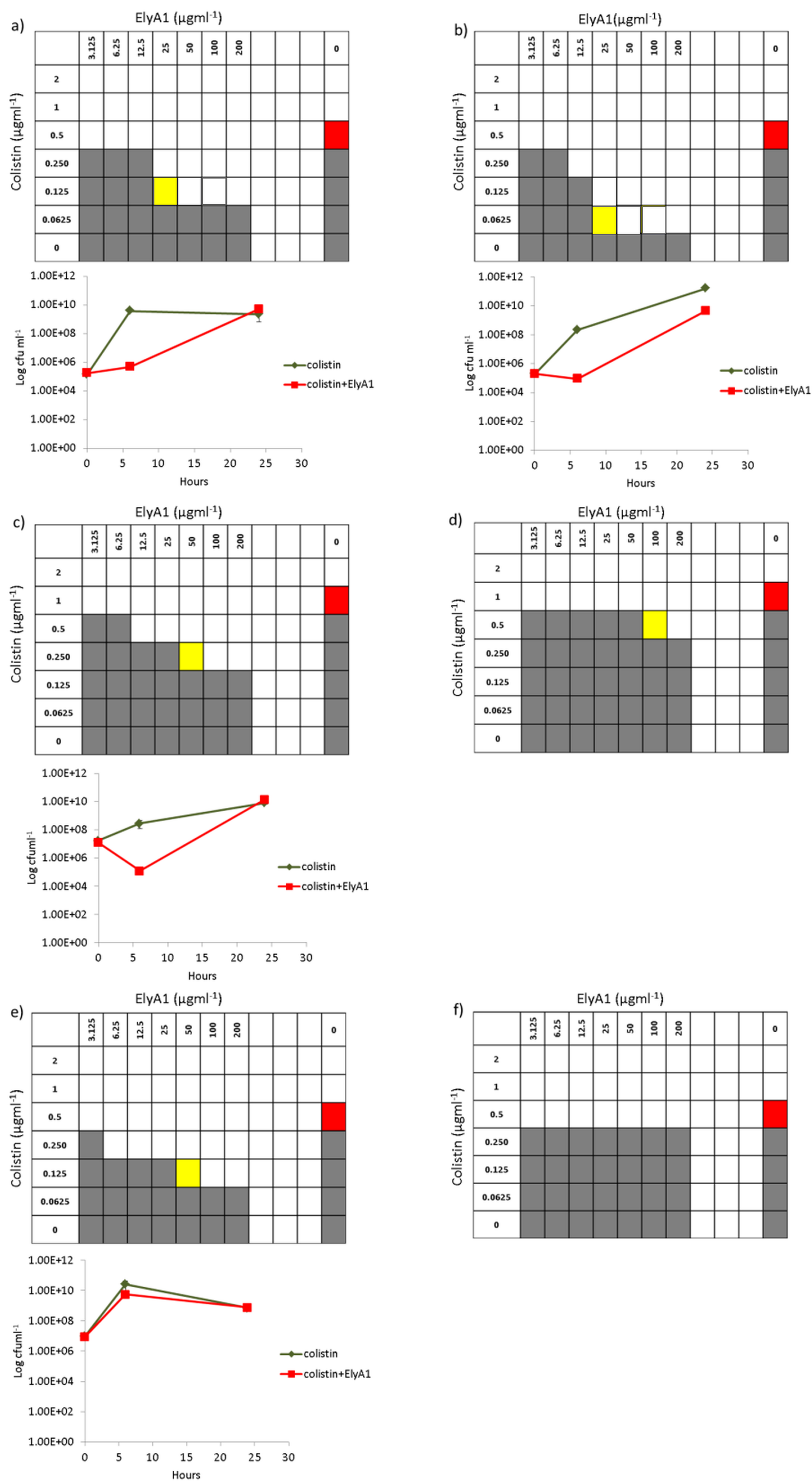

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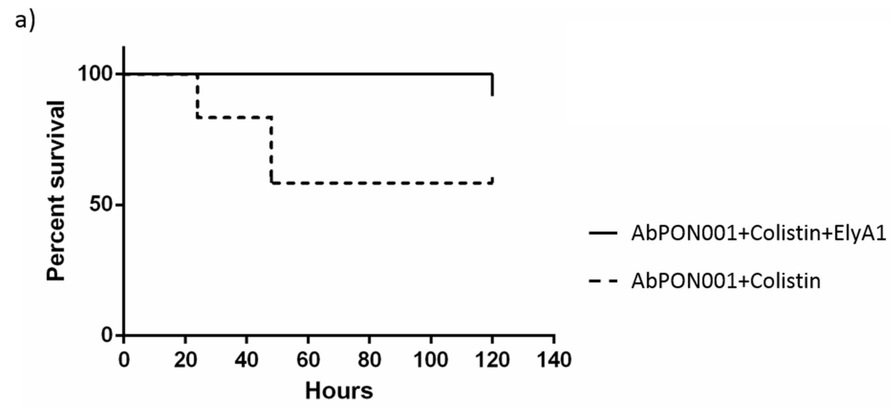
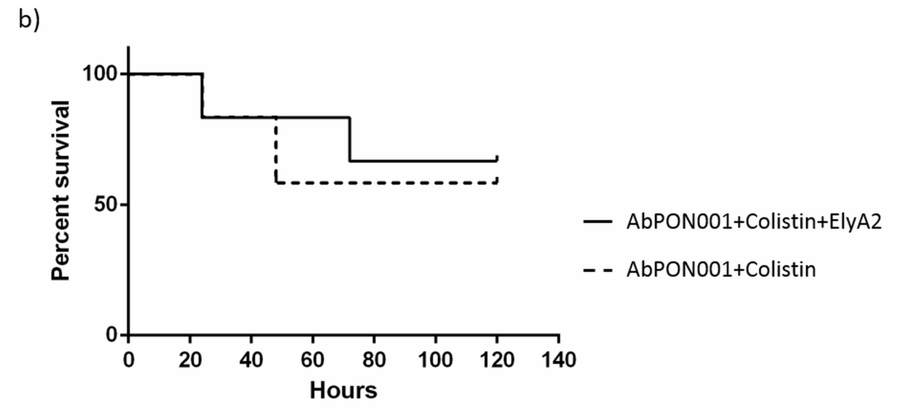
656 **Figure 4.**





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658 **Figure 5.**