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2 **Intracellular *Pseudomonas aeruginosa* persist**
3 **and evade antibiotic treatment in a wound infection model**
4

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22 resistance; macrophage; zebrafish; anti-infectious treatment

23

24 **Abstract**

25

26 Persistent bacterial infections evade host immunity and resist antibiotic treatments through various
27 mechanisms that are difficult to evaluate in a living host. *Pseudomonas aeruginosa* is a main cause
28 of chronic infections in patients with cystic fibrosis (CF) and wounds. Here, by immersing
29 wounded zebrafish embryos in a suspension of *P. aeruginosa* isolates from CF patients, we
30 established a model of persistent infection that mimics a murine chronic skin infection model. Live
31 and electron microscopy revealed persisting aggregated *P. aeruginosa* inside zebrafish cells,
32 including macrophages, at unprecedented resolution. Persistent *P. aeruginosa* exhibited adaptive
33 resistance to several antibiotics, host cell permeable drugs being the most efficient. Moreover,
34 persistent bacteria could be partly re-sensitized to antibiotics upon addition of anti-biofilm
35 molecules that dispersed the bacterial aggregates *in vivo*. Collectively, this study demonstrates that
36 an intracellular location protects *P. aeruginosa in vivo* from host innate immunity and antibiotics,
37 and provides new insights into efficient treatments against chronic infections.

38

39

40 **Introduction**

41 Many bacterial pathogens can persist inside their hosts for long periods of time, due to
42 immunodeficiency of the host, immune evasion by the bacteria and/or ineffective antibiotic
43 treatments. *Pseudomonas aeruginosa* is a Gram-negative bacterium ubiquitous in watery
44 environments and a major cause of a variety of nosocomial infections worldwide ¹, including
45 urinary tract, respiratory tract, wound/skin and blood infections. *P. aeruginosa* infections can be
46 either acute or chronic, implying different virulence strategies to evade host immunity ¹. Chronic
47 colonization is frequent during cystic fibrosis (CF), non-CF bronchiectasis or chronic obstructive
48 pulmonary disease (COPD), and also occurs on wounds ^{2,3}. In long-lasting lung infections in CF
49 patients, *P. aeruginosa* adopts a sessile lifestyle, with bacterial communities organized in a biofilm
50 structure, impeding bacterial clearance by the immune system ⁴.

51 *P. aeruginosa*, which is well known for its capacity to develop resistance to antibiotic treatments,
52 belongs to the threatening group of ESKAPEE pathogens, and is recognized by the World Health
53 Organization as a critical priority for new therapeutics ⁵. Chronic bacterial infections are
54 challenging to treat with antibiotics due to a combination of intrinsic, acquired, and adaptive drug
55 resistance, along with the specific bacterial lifestyle in infected tissues. The adaptive resistance to
56 antibiotics, which is a phenotypic non-heritable trait ⁶, is related to several factors, including
57 biofilm lifestyle, slow growth and low metabolic activity ⁷. Moreover, increasing evidence
58 supports the idea that *P. aeruginosa* undergoes an intracellular life cycle during infection ^{8,9},
59 including human pulmonary infection as shown with the recent observation of intracellular *P.*
60 *aeruginosa* in the airway epithelium of CF lung explants ¹⁰. Whereas intracellular localization was
61 shown to affect drug efficacy against *P. aeruginosa* in cell culture models ¹¹⁻¹³, this aspect has not
62 been addressed *in vivo*, especially in the context of a persistent infection.

63 While a plethora of *in vivo* models have been used to assess *P. aeruginosa* virulence ¹⁴, only a
64 small subset were aimed at studying this pathogen in the context of persistent colonization and
65 testing the efficacy of treatments on chronic infection. Current animal models to study *P.*
66 *aeruginosa* respiratory chronic pathogenesis mainly rely on lung administration of bacteria
67 embedded in agar/agarose beads ¹⁵. A murine chronic skin infection model has also been
68 developed, mimicking long-term colonization in humans ¹⁶. Zebrafish (*Danio rerio*), a vertebrate
69 that shows the advantages of invertebrates (moderate ethical issues, low cost, high production of

70 eggs), represents an appealing *in vivo* model for drug testing and high-resolution real-time
71 visualization of bacteria and host cells due to embryo transparency. More specifically, the
72 zebrafish innate immune system is very similar to that of mammals, notably regarding phagocytic
73 cells (neutrophils and macrophages) and soluble immune mediators such as cytokines and
74 complement proteins¹⁷. Real time *in vivo* imaging has previously allowed the visualization of the
75 interaction of *P. aeruginosa* with innate immune cells in the context of acute infections¹⁸⁻²⁰.

76 In this study, we established for the first time a model of persistent colonization of the zebrafish
77 embryo by *P. aeruginosa*, using a caudal wound infection protocol and *P. aeruginosa* CF clinical
78 isolates. The visualization of persisting bacteria revealed the importance of an intracellular phase,
79 notably inside macrophages, and the formation of bacterial aggregates *in vivo*. Persistent bacteria
80 became less responsive to treatment by several classes of antibiotics, and host cell permeable
81 antibiotics were found to be more efficient. In addition, antibiotic efficacy against persistent
82 bacteria could be potentiated by addition of anti-biofilm compounds. This work offers an *in vivo*
83 model to investigate the contribution of intracellular *P. aeruginosa* to chronic infection, with
84 unprecedented imaging capabilities, and paves the way to assess the efficacy of therapeutics in the
85 context of a persistent colonization.

86

87 **Results**

88 **Zebrafish embryo is an appropriate model to monitor a persistent infection with *P.*** 89 ***aeruginosa* CF isolates**

90 While reference laboratory strains causing acute infections (PAO1, PA14, and PAK) have been
91 widely used in the zebrafish model to gain insights into the host-*P. aeruginosa* interaction *in vivo*,
92 investigations of the pathogenesis of clinical isolates in this vertebrate model have remained very
93 scarce, and relied on the microinjection of single CF isolates^{21,22}. We recently developed a wound
94 infection protocol in zebrafish based on the immersion of tail fin-amputated embryos with *P.*
95 *aeruginosa* PAO1 strain, which caused an acute infection within 20 hpi²³. Here, we used this
96 infection mode to evaluate *in vivo* the virulence of three CF isolates (A6520, B6513 and C6490),
97 that were not tested earlier in any infection model, to ensure an unbiased approach. The survival
98 of zebrafish embryos was first monitored over 40 hours post infection (hpi) (Figure 1A). All three

99 CF isolates were highly attenuated, being associated with 90% (A6520, B6513) to 100% (C6490)
100 survival, when compared to the reference strain PAO1 (50% survival) (Figure 1B). Additional
101 experiments were next conducted on these CF isolates to assess whether their attenuated phenotype
102 was linked to the elimination or not of the bacteria by the host.

103 To discriminate between bacterial clearance and bacterial persistence, we assessed the evolution
104 of the bacterial load per embryo over 72 hpi. To easily recognize and count *P. aeruginosa* bacteria,
105 we used green-fluorescent strains harboring a chromosomally-integrated *gfp* gene with constitutive
106 expression, to avoid the risk of GFP signal loss upon time. For all strains, a notable variability
107 between individual larvae was noticed, with some differences being >100-fold (2 log₁₀). With
108 isolates A6520 and C6490, the majority of the embryos were observed to be free of bacteria at 67
109 or 43 hpi, respectively (Figure 1C). Conversely, with strain B6513, 90% of the bacteria were
110 eliminated between 1.5 to 18 hpi, but the remaining bacterial load (median of 630 CFU) stayed
111 relatively constant in numbers until 65 hpi (Figure 1C). Thus, isolate B6513 developed a persistent
112 infection in this immersion zebrafish model, whereby a subset of bacteria survived after 18 hpi
113 without killing the host. The residual CFU analysis for isolate B6513 was extended once up to 6
114 days post-infection (dpi) and a stable number of persistent bacteria per fish was observed.
115 Subsequent experiments were carried out only on embryos for up to 3 dpi (i.e. 5 days post-
116 fertilization, within the frame not regulated as animal experiments) to follow the 3R-principle and
117 limit a possible reinfection upon food ingestion.

118 To extend the relevance of this model, we assessed the virulence and persistence of the well
119 characterized isolate RP73, a late CF isolate capable of long-term colonization in mouse airways
120 ^{24,25}. Consistent with previous mouse studies, and similarly to strain B6513, the RP73 isolate failed
121 to induce embryo mortality and persisted in infected zebrafish embryos (Figures 1C and 1D). To
122 verify that the persistent phenotype reflected colonization of the wound and not the ability of the
123 isolates to adhere to the embryo's skin, we assessed the bacterial load over time in uninjured
124 embryos incubated with persistent strains B6513 and RP73. At 24 hpi, no CFU were detected for
125 either isolate (Figure S1), showing that the injury was required to drive a persistent infection.

126 We next addressed the behaviour of the three CF isolates A6520, B6513 and C6490 in a mouse
127 model of chronic infection that involves cutaneous abscesses ¹⁶. *P. aeruginosa* LESB58, a well-
128 characterized CF isolate that causes chronic lung infection and chronic skin abscess in mice ^{16,26},

129 was used as a reference to assess *in vivo* growth and virulence of the clinical isolates over a seven-
130 day period after subcutaneous injection in mice (with approx. 5×10^7 CFU). As previously
131 reported, *P. aeruginosa* LESB58 persisted at the infection site, being recovered at median densities
132 above 10^7 CFU/abscess at 3 and 7 dpi (Figure 1E). Similarly, the clinical isolate *P. aeruginosa*
133 B6513 was recovered at high median densities, $>10^8$ CFU/abscess at 3 and 7 dpi. In contrast,
134 clinical isolates *P. aeruginosa* A6520 and C6490 were mostly eliminated from the host when
135 compared to the other strains, as reflected by low median bacterial densities ($< 10^4$ CFU/abscess
136 at 3 dpi, and 10^3 or below the limit of detection at 7 dpi). Trends in abscess sizes were reflective
137 of bacterial recovery from the localized infection site at both time points, with abscess
138 (dermonecrotic cutaneous tissue) lesion areas of 15-39 mm² and 47-98 mm² for strains LESB58
139 and B6513, respectively, whereas strains A6520 and C6490 did not cause abscess formation
140 (Figure 1F). Healing of the cutaneous tissue appeared to occur at 7 dpi for both *P. aeruginosa*
141 LESB58 and B6513, since the sizes of abscesses had decreased (-24 and -51 mm² respectively)
142 relative to the abscesses of animals sacrificed 3 dpi. Accordingly, the mean clinical scores of
143 animals decreased during the time from 3 to 7 dpi across treatment groups (Figure S2).
144 Furthermore, clinical scores of animals remained low overall, with a maximum recorded total score
145 of 8/50 (in the *P. aeruginosa* B6513 treatment group), indicating that none of the strains caused
146 severe disease in animals.

147 Taken together, our results showed that the injured embryo immersion model of zebrafish infection
148 is suitable to assess persistence of *P. aeruginosa* clinical isolates. Importantly, the persistence
149 profiles of strains in zebrafish are consistent with the phenotypes observed in a murine chronic
150 infection model. We next took advantage of the unique imaging opportunities offered by the
151 zebrafish embryo to visualize persisting bacteria at high resolution.

152

153 **Dynamic interaction of persistent *P. aeruginosa* with host immune cells *in vivo***

154 Macrophages and neutrophils have been shown to rapidly phagocytose *P. aeruginosa* upon
155 zebrafish infection^{20,27-29}. We took advantage of the optical transparency of embryos to image
156 GFP-expressing isolate B6513 throughout the infection, assess bacterial morphology, localization
157 and interaction with recruited macrophages, using Tg(*mfap4::mCherry-F*) zebrafish embryos that
158 harbor red fluorescent macrophages. Consistent with CFU counts, we observed an important

159 decrease in the bacterial load between 1.5 and 24 hpi (Figure 2A). At 1.5 hpi, most bacteria
160 appeared free living, while at 24 and 72 hpi, they mainly appeared as aggregates localized nearby
161 the injury site (Figure 2A). Intra-macrophagic *P. aeruginosa* were present throughout the course
162 of infection (Figures 2A and 2B). The presence of bacterial aggregates, with putative localization
163 inside macrophages, was not specific to the B6513 isolate since similar findings were visualized
164 with the RP73 strain (Figure S3). Bacterial aggregates were retained within macrophages for up to
165 10 h, as shown using time-lapse confocal microscopy (Figure 2C). The size distribution of GFP
166 positive spots was stable over time, with a volume mainly comprised between $1.5 \mu\text{m}^3$ (individual
167 bacteria) and $100 \mu\text{m}^3$ (clusters) in size (Figure 2D). Larger aggregates ($>100 \mu\text{m}^3$) were observed
168 in some embryos. Infection seemed to delay macrophage recruitment to the wound at the early
169 time point (1.5 hpi, Figure 2E). Macrophage recruitment in infected embryos peaked at 24 hpi and
170 decreased slowly, being close to the level of uninfected controls at 72 hpi (Figures 2E and S4).

171 The interaction of persistent bacteria with neutrophils using the Tg(*LysC:dsRed*) transgenic line
172 harboring red fluorescent neutrophils was subsequently investigated (Figure S5). In agreement
173 with previous experiments done on microinjected embryos²⁷⁻²⁹, numerous intra-neutrophilic *P.*
174 *aeruginosa* were observed shortly after colonization (Figures 2F and 2G). However, this frequency
175 was strongly reduced at 24 and 48 hpi, and no intra-neutrophil bacteria were observed at 72 hpi.
176 Thus, the kinetics of appearance of intra-neutrophil and intra-macrophage bacteria were very
177 different (Figure 2G), suggesting that the interaction with neutrophils was restricted to the early
178 steps of colonization and/or that the killing activity of neutrophils and/or their short half-life
179 prevented any intra-neutrophilic persistence of *P. aeruginosa*. Cumulatively, this live imaging
180 analysis revealed that persistence likely occurs as bacterial clusters and within an intra-
181 macrophage niche.

182

183 **High resolution imaging of the intracellular niche of persistent bacteria**

184 As described above, live imaging revealed the presence of persistent *P. aeruginosa* bacteria, both
185 inside and outside macrophages at 24 and 48 hpi (Figures 2A and 2G), but likely not within
186 neutrophils (Figures 2F and 2G). Since *P. aeruginosa* can also enter and reside within non-
187 phagocytic cells³⁰⁻³², we investigated their presence within non-phagocytic cells at the infection
188 site. To this aim, we used Tg(*rcn3:Gal4/UAS:mCherry*) transgenic embryo harboring red

189 fluorescent mesenchymal cells. Intracellular bacteria (individual or clusters) were clearly
190 visualized at 24 and 48 hpi (Figure S6), demonstrating that *P. aeruginosa* also uses this niche *in*
191 *vivo*, supporting pioneer work carried out with mice corneal infections^{9,32}.

192 We used electron microscopy (EM) to highlight the ultrastructural context of persistent bacteria in
193 the tail fin of 24 or 48 hpi fixed infected Tg(*mfap4::mCherry-F*) embryos (Figure 3). Bacteria
194 were largely seen inside cells, either isolated (Figure 3A) or in clusters (Figure 3B). A detailed
195 analysis indicated that some bacteria were localized in vacuoles, as shown by the shape of clusters
196 and/or the visualization of a vacuolar membrane, whereas other appeared in the cytoplasm (Figures
197 3A and 3B). Some cells with intracellular bacterial cluster appeared damaged (Figure 3C, left
198 panel) and bacteria could be visualized in apparently cell remnants or in the extracellular space
199 (Figure 3C, right panels). Since identifying the host-cell type only by ultrastructural criteria was
200 challenging, we also performed correlative Light-Electron Microscopy (CLEM) (Figures 3D and
201 3E). A single large cluster of bacteria inside a macrophage was found by light microscopy (Figure
202 3D). The same embryo was processed for EM and serial lateral sections were prepared and imaged
203 by array tomography (Figure 3E). The cluster showed a high number of bacteria inside a cell
204 identified as a macrophage by the corresponding fluorescence image.

205 Taken together, live and electron microscopy analyses indicated that *P. aeruginosa* bacteria
206 persisting at the infection site were largely intracellular, in either macrophages or non-phagocytic
207 cells. Moreover, CLEM imaging allowed visualization of a large cluster of *P. aeruginosa* inside a
208 macrophage. These findings support the uniqueness of the zebrafish model in deciphering how *P.*
209 *aeruginosa* interacts with host cells during infection.

210

211 **Strains with a persistent profile in zebrafish survived inside cultured macrophages and** 212 **formed biofilms *in vitro***

213 In our *in vivo* model, persistent *P. aeruginosa* were visualized as intramacrophage bacterial
214 clusters at the zebrafish wound site (Figures 2 and 3). We addressed the *in vitro* features of the CF
215 isolates by investigating their phenotypes in a macrophage cell line and in a biofilm assay. We first
216 monitored the intra-macrophagic survival of the different clinical strains by performing a
217 gentamicin protection assay in murine J774 cells infected at a multiplicity of infection (MOI) of

218 10, as done previously with PAO1^{33,34}. After 1.5 h of internalization, quantification of intracellular
219 bacteria revealed that strain A6520 was largely eliminated, while the number of C6490 and RP73
220 cells were only modestly reduced (Figure 4A). On the other hand, B6513 was not eliminated and
221 survived inside macrophages for at least 1.5 h after phagocytosis (Figure 4A). Phagocytic rates of
222 the different strains were not significantly different (Figure 4B). Time-lapse microscopy
223 performed during 3 h after phagocytosis correlated with CFU measurements, with a striking
224 clearance of strain A6520, but no clearance of macrophage cells, while B6513 was still residing
225 within macrophages (Figure 4.C). The visualization of intra-vacuolar clusters with B6513 showed
226 that this bacterium can reside in closed compartments within phagocytic cells (Figure 4D). We
227 next evaluated the ability of the diverse strains to form biofilms. While A6520, B6513 and RP73
228 produced a pellicle covering the broth medium, C6490 failed to form such a biofilm at the air-
229 liquid interface (Figure 4E).

230 Overall, these data show that the two isolates recognized as persistent in the zebrafish embryo
231 model combined the ability to resist killing by macrophages upon phagocytosis and the ability to
232 form biofilms, whereas the two non-persistent isolates lacked one of these two traits.

233

234 **Persistent *P. aeruginosa* bacteria exhibit adaptive resistance to antibiotic treatment**

235 Antibiotic adaptive resistance is a major issue in the treatment of chronic infections. We
236 investigated antibiotic efficacy on embryos infected by the isolate B6513, by applying a 30 min
237 treatment at 1.5, 24 and 48 hpi (Figure 5A). Three clinically-used antibiotics to fight *P. aeruginosa*
238 infections were tested, namely tobramycin (an aminoglycoside), colistin (a polymyxin) and
239 ciprofloxacin (a fluoroquinolone)³⁵. Antibiotics were used at 40 times the minimum inhibitory
240 concentration (MIC; 40 µg/mL for tobramycin and 20 µg/mL for ciprofloxacin), except for colistin
241 that was toxic for embryos and thus used at 2.5 x MIC (10 µg/mL). All drugs caused a sharp
242 reduction in bacterial loads when applied soon after the initiation of infection (1.5 hpi), from
243 approx. 20-fold (ciprofloxacin and tobramycin) to complete eradication (colistin) (Figure 5B).
244 However, their efficacy was strongly reduced when used at 24 or 48 hpi, with colistin and
245 ciprofloxacin decreasing CFU counts by less than 7-fold and tobramycin having no significant
246 effect (Figure 5B). The ability of bacteria to withstand antibiotic challenges occurred at around the
247 time of establishment of a persistent infection in the embryos, associated with intracellular and

248 aggregated bacteria. This phenotype was not linked to the acquisition of mutations conferring
249 resistance, since colonies isolated following ciprofloxacin challenge at 48 hpi remained fully
250 sensitive to the antibiotic *in vitro*.

251 During infection, *P. aeruginosa* may benefit from intracellular niches to escape antibiotics^{8,11}.
252 Our microscopy analysis revealed the intracellular localization of persistent *P. aeruginosa*, which
253 could *de facto* be protected from antibiotics (Figures 2A-C, Figure 3 and Figure S6).
254 Antipseudomonal antibiotics differ in their ability to penetrate into eukaryotic cells, and their
255 activity vary against intracellular *P. aeruginosa* in cultured phagocytes¹¹. To assess if the
256 intracellular niche was responsible for the treatment failure in our model, we tested the *in vivo*
257 activity of another fluoroquinolone, ofloxacin, that is known to be host cell permeable and to
258 demonstrate better diffusion into human tissues than ciprofloxacin³⁶. Persistent bacteria at 24 and
259 48 hpi exhibited some tolerance towards ofloxacin (used at 40 x MIC, 20 µg/mL), when compared
260 to bacteria treated very early after the start of infection. However, among the four antibiotics tested,
261 ofloxacin was the most efficient molecule at the persistent stages (24 and 48 hpi), thus reinforcing
262 the notion that eradication of intracellular persistent bacterial reservoirs requires administration of
263 antibiotics able to reach these niches (Figure 5C). To complete this finding, we imaged
264 Tg(*mfap4::mCherry-F*) embryos with persistent bacteria after treatment with ofloxacin and
265 compared with images of non-treated embryos. The percentage of larvae with intra-macrophage
266 bacteria dropped to 30% in ofloxacin-treated fishes, whereas it remained around 70% in non-
267 treated controls.

268 Taken together, our results showed that the persistence of *P. aeruginosa* in zebrafish embryos is
269 associated with an adaptive phenotype of resistance to antibiotics. Importantly, the observation
270 that the most cell-permeant antibiotic was also the most effective one, suggests that reservoirs
271 tolerant to other antibiotics are located within cells.

272

273 **Anti-biofilm compounds potentiate antibiotic efficacy on persistent bacteria**

274 In our zebrafish model, persistent *P. aeruginosa* formed aggregates, which may display a higher
275 tolerance to antibiotics than free-living bacteria⁷. We hypothesized that anti-biofilm compounds
276 would help to disassemble bacterial aggregates, thereby re-sensitizing persistent *P. aeruginosa* to

277 antibiotics, potentially promoting their eradication with the assistance of the immune system.
278 Among commercially available molecules with appealing activity against *P. aeruginosa* biofilm,
279 we first used the human Atrial Natriuretic Peptide (hANP), an efficient biofilm-disperser *in vitro*
280 that potentiates different antibiotics including tobramycin³⁷. Though devoid of antibacterial
281 activity by itself, peptide hANP was able to reduce the formation of biofilms by strain B6513 *in*
282 *vitro* (Figure S7). We then assessed its capacity to potentiate tobramycin and colistin treatments in
283 infected embryos at 48 hpi, a time point at which bacteria were partly tolerant to antibiotics (Figure
284 6A). When applied alone, hANP modestly reduced bacterial loads by 2.5-fold, suggesting that it
285 somehow assisted the innate immune system to eliminate bacteria. Strikingly, when used in
286 combination with antibiotics, a more significant reduction of the pathogen burden was recorded
287 i.e., 7-fold for tobramycin and 16-fold for colistin, when compared with the controls (Figure 6B).
288 We performed similar experiments with a structurally different anti-biofilm compound, the fatty
289 acid *cis*-2-*decenoic* acid (CDA). CDA, a molecule produced by *P. aeruginosa*, promotes biofilm
290 dispersal and potentiates clearance of pre-established biofilms in combination with antibiotics^{38,39}.
291 Moreover, CDA was proposed as a suitable molecule to target intra-macrophagic biofilms of
292 pathogenic *Escherichia coli*⁴⁰. When used alone at 48 hpi, CDA had no detectable effects on
293 persistent *P. aeruginosa* in zebrafish embryos, but when added in combination with colistin,
294 bacterial loads per embryo decreased by 5-fold, thus increasing the antibiotic efficacy by >3-fold
295 (Figure 6C).

296 We next took advantage of the optical transparency of embryos to visualize *in vivo* the effect of
297 these anti-biofilm molecules on persistent bacteria. After a 5 h exposure to hANP or CDA, a
298 reduction of the size of some bacterial clusters and concomitant increased number of isolated
299 bacteria were observed, which was not the case in the H₂O control conditions (Figures 6D and S8).

300 Cumulatively, these results are consistent with the notion that persistent *P. aeruginosa* bacteria
301 present in aggregated structures, which are dispersible upon addition of anti-biofilm molecules,
302 contribute to the adaptive antibiotic-resistant phenotype. Moreover, these findings validate the
303 pertinence of our *in vivo* model to identify compounds that could potentiate the effects of
304 conventional antibiotics.

305

306 **Discussion**

307 Modeling bacterial chronic infection *in vivo* is essential to understand pathogenesis and evaluate
308 treatment efficacy in a context that includes responsive host innate immunity and tissues. Here, we
309 propose a novel *in vivo* model, complementary to mouse models, based on the infection of
310 wounded zebrafish embryos with *P. aeruginosa* clinical strains. The zebrafish embryo model
311 provides unique opportunities for intravital imaging and highlights the importance of an
312 intracellular phase for establishing bacterial persistence in a vertebrate host. Moreover, this model
313 recapitulates a major feature of chronicity, i.e. adaptive antibiotic resistance, and allows a simple
314 screen for compounds that re-sensitize bacteria to antibiotics.

315 To establish this first model of persistent *P. aeruginosa* infection in zebrafish, we combined
316 different parameters that have not been tested with clinical isolates to date: (i) parallel analysis of
317 several *P. aeruginosa* isolates from CF patients, including a reference isolate known to establish a
318 long-term infection in a murine model of chronic pneumonia²⁴, (ii) experiments over a period of
319 up to 3 days after infection, and (iii) wound infection. The bacterial isolates could not be
320 differentiated in this model based on the mortality rate of embryos since, in contrast to PAO1
321 strain, the mortality was very low or absent with the four isolates tested. However, the analysis
322 over a 3 day period allowed us to clearly distinguish two classes of strains, those being persistent
323 and those that were eliminated. Notably, the virulence and bacterial load profiles of clinical isolates
324 in zebrafish perfectly matched the profiles observed in a mouse model of chronic wound infection,
325 further validating the physiological relevance of our non-mammalian model.

326 The zebrafish embryo offers unique advantages to track bacterial infections in real time, and to
327 study the interaction between bacteria and host cells. In keeping with previous studies using other
328 infection routes, intravital microscopy revealed that infecting *P. aeruginosa* introduced at the tail
329 injury were phagocytosed by both neutrophils and macrophages shortly after infection^{20,41}. While
330 neutrophils were still present at the injury site at later time points, they rarely interacted with
331 persistent *P. aeruginosa*, as opposed to macrophages. Unexpectedly, persistent bacteria were often
332 visualized inside macrophages, which are long-living cells, indicating that *P. aeruginosa* displays
333 strategies to resist macrophage killing and can use macrophages as a persistence niche. Such
334 strategies are hallmarks of diverse intracellular pathogens, and were not expected for *P.*
335 *aeruginosa*, which is considered an extracellular bacterium. This is however consistent with our

336 findings highlighting an intramacrophage stage in cellular models and during *P. aeruginosa* acute
337 infection^{20,33,34}. In addition, we recently reported that strain PAO1 can persist at low levels in
338 microinjected larvae that overcome or do not develop an acute infection, and some persistent
339 bacteria were also visualized inside macrophages⁴². Here high-resolution imaging (EM) of
340 infected zebrafish confirmed that persistent bacteria often reside inside host cells. Whereas the
341 intracellular location of *P. aeruginosa* during infection was first reported three decades ago³², its
342 relevance *in vivo* has been a matter of debate and its role in persistence has not to date been
343 addressed. Importantly, recent studies using intravital confocal imaging of infected mouse corneas
344 and confocal imaging of human lung explants from CF patients support the intracellular location
345 of *P. aeruginosa* during infection, as individual bacterial or small bacterial clusters^{10,43}. The nature
346 of cells harboring *P. aeruginosa* was however not ascertained in these studies and, in this regard,
347 the zebrafish model is a unique model, complementary to mice and human studies, that enables
348 monitoring of the dynamics of intracellular *P. aeruginosa* *in vivo*. Intra-animal high resolution
349 correlative microscopy (CLEM) in zebrafish allowed us to image for the first time an
350 intramacrophage *P. aeruginosa* cluster. Moreover, real time imaging demonstrated that such
351 clusters can last for more than 10 hours inside macrophages. In infected zebrafish, intracellular
352 persistent bacteria were also visualized in non-phagocytic cells, supporting the importance of
353 invasion of non-phagocytic cells during infection^{43,44}.

354 Our previous studies with PAO1 derived strains infecting cultured macrophages showed diverse
355 outcomes, including intracellular bacteria that reside in vacuoles or in the cytoplasm^{33,42}. We
356 observed here a vacuolar location for isolate B6513 upon infection of a macrophage cell line, and
357 high-resolution EM imaging from infected zebrafish confirmed that individual or clustered
358 persistent bacteria can reside inside host cell vacuoles *in vivo*. This is in line with the recent finding
359 of a vacuolar persistent subpopulation of intracellular *P. aeruginosa* in cultured epithelial cells and
360 the intravital imaging of colored puncta considered as vacuolar bacterial clusters during corneal
361 infection⁴³. A vacuolar location should contribute to clustering of bacteria, which may retain a
362 similar cluster structure in case of vacuolar rupture. Some clusters of persistent *P. aeruginosa*
363 visualized in zebrafish had sizes above 10 μm , which is in line with aggregates found in CF lungs
364 and chronic wounds^{45,46} that are considered to represent non-attached biofilm-like structures⁴⁷.
365 Of note, the bacterial aggregates observed in zebrafish could be dispersed, with a concomitant
366 increase in isolated bacteria, upon addition of two different anti-biofilm molecules, hANP or CDA.

367 Real time imaging of the dispersion of bacterial aggregates in a living animal demonstrates the
368 unprecedented benefits of zebrafish embryo to follow the dynamic of bacterial clusters *in vivo*.

369 Persistent bacteria in the zebrafish model were refractory to antibiotic challenge, which mimics
370 bacterial behavior during chronic infection in mammals and humans⁴⁸. Such adaptive antibiotic
371 resistance of bacteria has been attributed to several features, including biofilm structures, which
372 are well documented *in vitro* for *P. aeruginosa*⁷. Here, the aggregated bacteria visualized *in vivo*
373 appeared to contribute to adaptive resistance since anti-biofilm compounds could significantly re-
374 sensitize persistent bacteria to antibiotic treatment. The bacterial intracellular stage is another
375 feature allowing evasion of antibiotic challenge that recently attracted interest in the case of
376 extracellular pathogens as *P. aeruginosa*^{8,49}. For example, the residence inside cultured lung
377 epithelial cells reduced antibiotic efficiency^{12,50} and intracellular *P. aeruginosa* in bladder
378 epithelial cells promoted bacterial persistence and antibiotic tolerance in a mouse model of urinary
379 tract infection¹³. In our zebrafish model, the observation that the most efficient cell-permeant
380 antibiotic, ofloxacin, demonstrates the highest efficacy supports the idea that the intracellular
381 location provides a niche where persisting bacteria are less accessible to antibiotics. Ofloxacin,
382 remains however only partially efficient against persistent bacteria, which may correlate with a
383 poor efficacy against bacteria with a vacuolar location, which would further protect bacteria from
384 antibiotics⁴³.

385 Finding new therapeutics to tackle chronic infections is critical and we provide a new platform to
386 screen and evaluate *in vivo* the efficacy of treatments against persistent *P. aeruginosa*. Our model
387 underscores the importance of strategies efficiently targeting the protective intracellular niche,
388 which plays a crucial role in sustaining persistence within the host animal. This was not obvious
389 for a pathogen such as *P. aeruginosa* which has been considered to be largely extracellular, but
390 was consistent with the importance of an intramacrophage stage during infection for other major
391 pathogens considered as extracellular pathogens, including *Acinetobacter baumannii* and
392 *Streptococcus pneumoniae*^{51,52}. Additionally our *in vivo* model reinforces the relevance of anti-
393 biofilm strategies that disperse bacterial aggregates to re-sensitize *P. aeruginosa* to antibiotic
394 treatments. This zebrafish model offers the possibility of insightful therapies, especially
395 combination treatments, that could be transposed and refined in mammalian models, including
396 man. Integrating studies in zebrafish and mouse models should greatly accelerate the identification

397 of efficient treatments against *Pseudomonas*. It will also allow better understanding of the
398 contribution of intracellular stages prior to the formation of biofilm-like structures in models of *P.*
399 *aeruginosa* chronic infection.

400

401 **Materials and Methods**

402 **Clinical strains used in the study and growth conditions**

403 *P. aeruginosa* clinical strains were routinely grown at 37°C in lysogeny broth (LB) with shaking
404 at 180 rpm. Clinical strains A6520, B6513 and C6490 were isolated at the teaching hospital of
405 Besançon (France) from three individual cystic fibrosis patients aged 4, 1 and 17 years,
406 respectively showing a transient (A6520, B6513) or chronic (C6490) lung colonization with *P.*
407 *aeruginosa*. All these patients were treated with antibiotics to control the infection (clinical data
408 not available). Genome sequencing was carried out to classify the strains. Sequence Types (STs)
409 were determined according to the MLST scheme available at PubMLST (<https://pubmlst.org>).
410 Strains A6520, B6513 and C6490 were found to belong to the same phylum as reference strain
411 PAO1⁵³, to harbor the exotoxin-encoding gene *exoS*, and to be genotypically distinct (ST274,
412 ST27 and ST633, respectively). This Whole Genome Shotgun project has been deposited at
413 DDBJ/ENA/GenBank under the accession JAWDIG000000000 (A6520), JAWDIH000000000
414 (B6513) and JAWDII000000000 (C6490). RP73 is a well described CF *P. aeruginosa*²⁵.

415

416 **Chemicals**

417 All antibiotics were purchased from Sigma-Aldrich. Ciprofloxacin was dissolved at 25 mg/mL in
418 0.1 M hydrochloric acid. Ofloxacin was dissolved at 20 mg/mL in 1 M NaOH. Colistin sulfate salt
419 and Tobramycin were dissolved at 8 mg/mL in ultrapure water. The human Atrial Natriuretic
420 Peptide (hANP) was purchased from Tocris Bioscience (Bio-Techne) and was dissolved at
421 1 mg/mL in ultrapure water and stored at -20 °C. The cis-2-Decenoic acid (CDA) was purchased
422 from Sigma-Aldrich and was dissolved at 5.8 mM in 100% DMSO and stored at -20°C.

423

424 **Minimum inhibitory concentration (MIC) assays**

425 Susceptibility levels of strain B6513 to the fluoroquinolones ciprofloxacin (0.5 µg/mL) and
426 ofloxacin (0.5 µg/mL), the polymyxin colistin (4 µg/mL), and the aminoglycoside tobramycin (1
427 µg/mL) were determined by the standard microdilution method in 96-well plates. Overnight
428 cultures in LB broth were diluted in the same medium to an initial inoculum of $OD_{600nm} = 0.1$.
429 MIC was defined as the lowest concentration that inhibited bacterial growth.

430

431 **Construction of stable fluorescent *P. aeruginosa* strains**

432 Strains with chromosomally encoded GFP were obtained by triparental mating by using a
433 recombinant integrative plasmid miniCTX carrying the PX2-GFP fusion⁵⁴ (from Ina Attree,
434 Université Grenoble Alpes, France). Following overnight growth in LB broth with appropriate
435 antibiotics, *E. coli* TOP10 carrying either helper plasmid pRK2013³⁴ or miniCTX-PX2-GFP were
436 mixed (20 µL of each culture) and deposited as drops onto the surface of LB plates for 2 h at 37°C.
437 In parallel, the overnight cultures of *P. aeruginosa* strains were incubated at 42°C. 20 µL fractions
438 of these stationary phase cultures were then individually added to the *E. coli* dry drops and left at
439 37°C for 5 h. The bacterial spots were then scrapped off and directly plated on LB plates containing
440 Irgasan (25 µg/mL) and tetracycline (from 50 to 200 µg/mL) to select *P. aeruginosa*
441 transconjugants. After 18 h at 37°C, several colonies were streaked out on LB medium to verify
442 the stable GFP expression, and an individual GFP⁺ colony was cultured in liquid LB to obtain a
443 glycerol stock.

444

445 **Zebrafish lines**

446 Wild-type AB and Golden lines or AB/Golden mixed genetic background zebrafish were used for
447 survival and CFU experiments. For live imaging, lines carrying fluorescent macrophages
448 Tg(*mfap4:mCherry-F*)ump6TG⁵⁵, neutrophils Tg(*LysC:dsRed*)nz50⁵⁶ or mesenchymal cells
449 Tg(*rcn3:Gal4/UAS:mCherry*)^{57,58} were used. Fish maintenance, staging and husbandry were
450 performed as described⁵⁹. Eggs were obtained by natural spawning, collected in petri dishes and
451 incubated at 28°C in fish water composed of 60 µg/mL sea salts (Instant Ocean) in distilled water
452 supplemented with 0.4 mM NaOH and 0.1% methylene blue. Embryos and larvae were staged
453 according to⁶⁰. For experiments, larvae were used at 2-day post-fertilization (dpf) until 5 dpf.

454

455 **Infection by immersion of injured zebrafish embryos**

456 The protocol was essentially as described earlier²³, with few modifications. Overnight bacterial
457 cultures were diluted at 1:20 in fresh LB broth and incubated until the OD_{600nm} reached approx.
458 0.8-1. Cultures were centrifuged at 4000 rpm for 10 min and re-suspended in fish water at approx.
459 10⁷ bacterial/mL. The bacterial load was determined by subsequent plating onto LB agar after
460 dilution into phosphate-buffered saline (PBS). 2 dpf embryos, previously dechorionated, were
461 anesthetized with tricaine (300 µg/mL, Sigma-Aldrich) and injured at the tail fin using 25-gauge
462 needles under a stereomicroscope (Motic). Embryos were distributed into a 6-well plate containing
463 4 mL of bacterial suspension (or fish water as a control) immediately after the injury, and incubated
464 at 28°C for 1.5 h. Two washes in fish water were subsequently performed (30 min in 10 mL and a
465 few minutes in 4 mL, respectively) to eliminate bacteria in the bath. Finally, larvae were transferred
466 individually into a 96-well plate (survival experiment) or a 24-well plate (CFU measurement and
467 microscopy) containing fish water, and incubated using a light/dark cycle at 28°C. For survival
468 curves, death was determined based on the absence of heart beat after visual inspection.

469

470 **Bacterial load measurement in infected embryos**

471 Before CFU quantification, infected embryos were washed for a few minutes in 4 mL of fish water
472 and subsequently crushed individually using a pestle in 100 µL of PBS. Then 100 µL of Triton X-
473 100 (1% final concentration) were added for 10 min to liberate bacteria from residual cells/tissues.
474 Following lysis, several dilutions in PBS were spotted on LB agar plates and incubated approx. 18
475 h at 37°C. Only GFP⁺ colonies were considered for counting. For CFU measurement following
476 treatment, embryos were washed before the treatment and not prior the crushing (see below).

477

478 **Embryo imaging**

479 After reaching the 50% epiboly stage, embryos were maintained in fish water supplemented with
480 the melanization inhibitor Phenylthiourea (PTU) to prevent pigmentation. Before imaging, larvae
481 were anesthetized with 300 µg/mL tricaine and placed in 35 mm glass-bottom dishes (FluoroDish,
482 World Precision Instruments) and immobilized with 1% low-melting-point agarose (Sigma-

483 Aldrich), covered with fish water after solidification. Zebrafish larvae were imaged at indicated
484 hpi using the ANDOR CSU-W1 confocal spinning disk on an inverted NIKON microscope (Ti
485 Eclipse) with ANDOR Zyla 4.2 sCMOS camera (40x water/NA 1.15 objective). Exposure time
486 for all channels (green, red and DIC) was set at 50 ms. The 3D files generated by multi-scan
487 acquisitions were processed using Image J and compressed into maximum intensity projections.
488 Brightness and contrast were adjusted for better visualization. Deconvolution of images was done
489 to improve resolution with Huygens (SVI).

490

491 **Quantification of macrophage recruitment and bacterial volumes**

492 As a proxy to quantify macrophage recruitment at the injury site following spinning disk confocal
493 imaging, the area formed by positive pixels (mCherry⁺) was measured at the median plan of each
494 stack. To that aim, using Fiji, a threshold of positivity was applied using the ‘‘IsoData’’ method.
495 3D reconstructions and volume quantification of GFP⁺ events were performed using the Imaris
496 ‘‘surface’’ tool.

497

498 **Treatment of infected embryos with antibiotics and anti-biofilm molecules**

499 Prior any treatment, embryos were systematically washed for a few minutes in 4 ml of zebrafish
500 water. Infected larvae were incubated with 40 x MIC of antibiotics, except for colistin (2.5 x MIC).
501 Depending of the antibiotic, controls were done with fish water alone or supplemented with NaOH
502 or HCl at the proper concentration. At least 5 embryos of the same condition were treated together
503 for 30 min in 1 mL at room temperature. For CDA (20 μ M) and hANP (10 μ M), treatments were
504 applied individually for 5 h at 43 hpi in 200 μ L at 28°C. Control conditions were fish water alone
505 (hANP) or supplemented with 0.34% DMSO (CDA). Independent experiments were systemically
506 performed 3 times, with at least 5 larvae per condition (i.e minimum 15 embryos in total).

507

508 **Cutaneous infection in mice**

509 Animal experiments were performed in accordance with the Canadian Council on Animal Care
510 (CCAC) guidelines and were approved by the University of British Columbia Animal Care
511 Committee protocol (A23-0030). Mice used in this study were outbred CD-1 mice (female). All

512 animals were purchased from Charles River Laboratories, Inc. (Wilmington, MA, United States)
513 and were 7-8 weeks of age at the time of experiments. Mice weighed 25 ± 2 g at the experimental
514 start point and standard animal husbandry protocols were employed.

515 We tested the virulence of *P. aeruginosa* clinical isolates (A6520, B6513, and C6490) and the
516 Liverpool Epidemic Strain LESB58 in a nuanced model of cutaneous high-density infection as
517 previously described¹⁶ with minor modifications. All strains were sub-cultured at 37°C with
518 shaking (250 rpm) to an $OD_{600nm} = 1.0$ in LB. Cells were washed twice with sterile phosphate
519 buffered saline (PBS) and resuspended to a final $OD_{600nm} = 0.5$ or 1.0 for clinical isolates or
520 LESB58 strains, respectively. Strains were used to form high-density abscess infections to model
521 invasive or chronic infections. Abscesses were formed by injection of 50 μ L of bacteria on the left
522 dorsum of mice for 3 or 7 days. Disease progression and overall welfare of animals was monitored
523 daily up to day three, and weekly thereafter. At experimental endpoint, animals were euthanized
524 using carbon dioxide followed by cervical dislocation, and abscess lesion size was measured using
525 a caliper. Abscesses were harvested in PBS and homogenized using a Mini-Beadbeater (BioSpec
526 Products, Bartlesville, OK, United States) for bacterial enumeration on LB. Two independent
527 experiments containing 2-4 biological replicates each were performed.

528

529 **Electron microscopy**

530 Samples were chemically fixed using 2.5% glutaraldehyde (Electron Microscopy Sciences #
531 16216) in fish water. After fixation, embryos were stored at 4°C in the fixation solution until
532 subsequent processing. The procedure for embedding in resin was adapted from a previous method
533⁶¹. All the procedure, except the overnight incubation in uranyl acetate, was performed using a
534 Pelco Biowave® PRO+ Microwave processing systems (TED Pella) following the program
535 indicated in Table S1. Samples were post-fixed with 2% osmium tetroxide (OsO_4) in 0.1M
536 cacodylate buffer pH 7.4 containing 5mM $CaCl_2$, immediately followed by 1.5% K-ferrocyanide
537 in the same buffer. After washing, samples were treated with 1% thiocarbohydrazide at 60°C,
538 washed with distilled water before a second incubation in 2% OsO_4 . After washing, samples were
539 then incubated overnight in 1% uranyl acetate at 4°C. Samples were next heated at 40°C and
540 further processed in the microwave (Table S1), washed and incubated in lead aspartate pre-heated
541 at 50°C. Dehydration was performed with growing concentrations of acetonitrile. Samples were

542 then impregnated in Epon Hard⁺™ resin, and polymerized 48 h at 60°C. All chemicals were from
543 EMS.

544 Thin serial sections were made using an UCT ultramicrotome (Leica) equipped with a Jumbo ultra
545 35° diamond knife (Diatome). Section ribbons were collected on silicon wafers (Ted Pella) with
546 the help of an ASH2 manipulator (RMC Boeckler). Sections were imaged with a Zeiss Gemini
547 360 scanning electron microscope on the MRI EM4B platform under high vacuum at 1.5 kV. Final
548 images were acquired using the Sense BSD detector (Zeiss) at a working distance between 3.5 and
549 4 mm. Mosaics were acquired with a pixel size of 5 nm and a dwell time of 3.2 μs.

550

551 **Macrophage infection and quantification of intracellular bacteria**

552 J774 cells (murine macrophage cell line) were maintained at 37°C in 5% CO₂ in Dulbecco's
553 modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS,
554 Gibco). The infection of J774 macrophages by *P. aeruginosa* was carried out essentially as
555 described previously³⁴. J774 macrophages (5x10⁵ cells/well) were infected by mid-log phase
556 bacteria in PBS, at a MOI of approx. 10. Infection synchronization was done by a 5 min
557 centrifugation at 1000 rpm of the 24-well plate, and bacterial phagocytosis was allowed to proceed
558 for 25 min. Cells were then washed three times with sterile PBS and fresh DMEM medium
559 supplemented with 400 μg/mL gentamicin was added, which was retained throughout the infection
560 to kill non-phagocytosed bacteria. Macrophages were lysed after 20 min (T0) or 2 h (T1) of
561 gentamicin treatment, by using 0.1% Triton X-100 and the number of viable bacteria was
562 determined by subsequent plating onto LB agar plates. Survival rate at T1 was compared to the
563 number of internalized bacteria at T0.

564

565 **Live microscopy on cultured macrophages**

566 J774 macrophages were seeded in **glass bottom 8 wells μ-slide (Ibidi #80827)** in DMEM medium
567 supplemented with 10 % FBS and infected with *P. aeruginosa* strains expressing GFP as described
568 above. Imaging started after 30 min of phagocytosis, when the media was changed to DMEM
569 supplemented with 400 μg/ml gentamicin until 3 hrs post phagocytosis. Cells were imaged using
570 an inverted epifluorescence microscope (AxioObserver, Zeiss), equipped with an incubation

571 chamber set-up at 37°C and 5% CO₂ and a CoolSNAP HQ2 CCD camera (Photometrics). Time-
572 lapse experiments were performed, by automatic acquisition of random fields using a 63X
573 Apochromat objective (NA 1.4). The frequency of acquisition is indicated in figure legends. Image
574 treatment and analysis were performed using Zen software (Zeiss).

575

576 **Biofilm formation**

577 Biofilm formation was assessed in low magnesium medium in glass tubes 24 h at 30°C under static
578 condition as described previously⁶². After 24h, bacterial growth was measured by OD_{600nm} and
579 tubes were carefully washed with water. The biofilm at the air-liquid interphase was stained using
580 crystal violet (CV) 0.1% during 15 min at room temperature. After staining, tubes were washed
581 with water and CV was extracted using acetic acid (30%) and quantified by measuring the OD_{570nm}.

582

583 ***In vitro* efficiency of hANP**

584 The flow cell system, which allows for continuous bacterial biofilm formation, is assembled,
585 prepared and sterilized as described earlier⁶³. For studying the impact of hANP peptide on
586 established strain B6513 biofilm, we used an established protocol³⁷. Briefly, bacterial cells from
587 an over-night culture, were recovered by centrifugation (10 min, 7,500 rpm) and washed with
588 sterile physiological water (0.9% NaCl). Each channel of the flow cell (1 mm x 4 mm x 40 mm,
589 Bio centrum, DTU) was inoculated with 300 µL of bacterial suspension prepared at an optical
590 density of OD_{580nm}=0.1. Bacterial adhesion was allowed without any flow for 2 h at 37°C. After 2
591 h of adhesion, the LB medium was pumped with a flow rate of 2.5 mL/h at 37°C for 24 h. Next,
592 the 24 h-old biofilm was exposed for 2 h to 300 µL of hANP (0.1 µM) or 300 µL of ultra-pure
593 distilled water (control condition), added to each channel of the flow cell and without flow. Prior
594 to image acquisition, biofilm cultures were then rinsed with LB medium using a 2.5 mL/h flow
595 rate for 15 min. Finally, bacterial cells were marked with 5 µM of SYTO9 green-fluorescent dye
596 (Invitrogen) and observed using confocal laser scanning microscopy (CLSM). CLSM observations
597 of biofilms were performed using a Zeiss LSM710 microscope (Carl Zeiss Microscopy) using a
598 x40 oil immersion objective. In order to capture the entire biofilm depth, images were taken every
599 millimeter. For visualization and processing of three-dimensional (3D) images, the Zen 2.1 SP1

600 software (Carl Zeiss Microscopy) was used. Using the COMSTAT software
601 (<http://www.imageanalysis.dk/>), quantitative analyses of image stacks were carried out⁶⁴.

602

603 **Statistical analysis**

604 GraphPad Prism 8.3.0 was used to perform all statistical tests and create graphs. The indicated test
605 used to analyze each dataset was chosen depending on the normality of the data. Multiple
606 comparisons were done by one-way ANOVA or Kruskal-Wallis test, followed by Tukey's or
607 Dunn's pairwise comparison, respectively. The Dunnett's many-to-one comparison was used
608 following a one-way ANOVA. Mann-Whitney U test was used to compare two groups.

609

610 **Ethics statement for zebrafish**

611 All zebrafish experiments described in the present study were conducted at the University of
612 Montpellier by following the 3rs -Replacement, Reduction and Refinement- principles according
613 to the European Union guidelines for handling of laboratory animals
614 (https://environment.ec.europa.eu/topics/chemicals/animals-science_en) and were approved by
615 the "Direction Sanitaire et Vétérinaire de l'Hérault" and the "Comité d'Ethique pour l'utilisation
616 d'animaux à des fins scientifiques" under reference CEEA-LR-B4-172-37. Breeding of adult fish
617 adhered to the international guidelines specified by the EU Animal Protection Directive
618 2010/63/EU. All experiments were performed before the embryos free feeding stage (5 dpf) and
619 did not fall under animal experimentation law according to the EU Animal Protection Directive
620 2010/63/EU. Embryos were euthanized using the anesthetic tricaine up to a lethal dose before
621 bleach treatment. Embryo manipulation, handling, and euthanasia were performed by well-trained
622 and authorized staff. Embryos were euthanized using an anesthetic overdose of buffered tricaine
623 before bleach treatment.

624

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644

645 **Author contributions:**

646 Conceptualization: S.P., F.N. and A.B.B.P.
647 Methodology: S.P., F.N., R.E.W.H, P.P. and A.B.B.P.
648 Investigation: S.P., F.N., L.B., A.B., M.A.A., M.L., P.N.
649 Visualization: S.P., F.N., L.B., A.B., M.A.A., M.L.
650 Supervision: A.B.B.P.
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652 Writing—review & editing: S.P., R.E.W.H, O.L., P.P. and A.B.B.P.
653 Funding acquisition: A.B.B.P.

654

655 **Competing interests:**

656 The authors declare no competing interests.

657

658 **Data and materials availability:**

659 All data needed to evaluate the conclusions of this study are presented in the paper and/or the
660 Supplementary Materials. Further information for resources and reagents should be directed to and
661 will be fulfilled by A.B.B.P. at LPHI, CNRS, France (anne.blanc-potard@umontpellier.fr). All
662 unique/stable reagents generated in this study are available from A.B.B.P without restriction,
663 except for *P. aeruginosa* clinical isolates and fluorescent derivatives that are available under
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665

666

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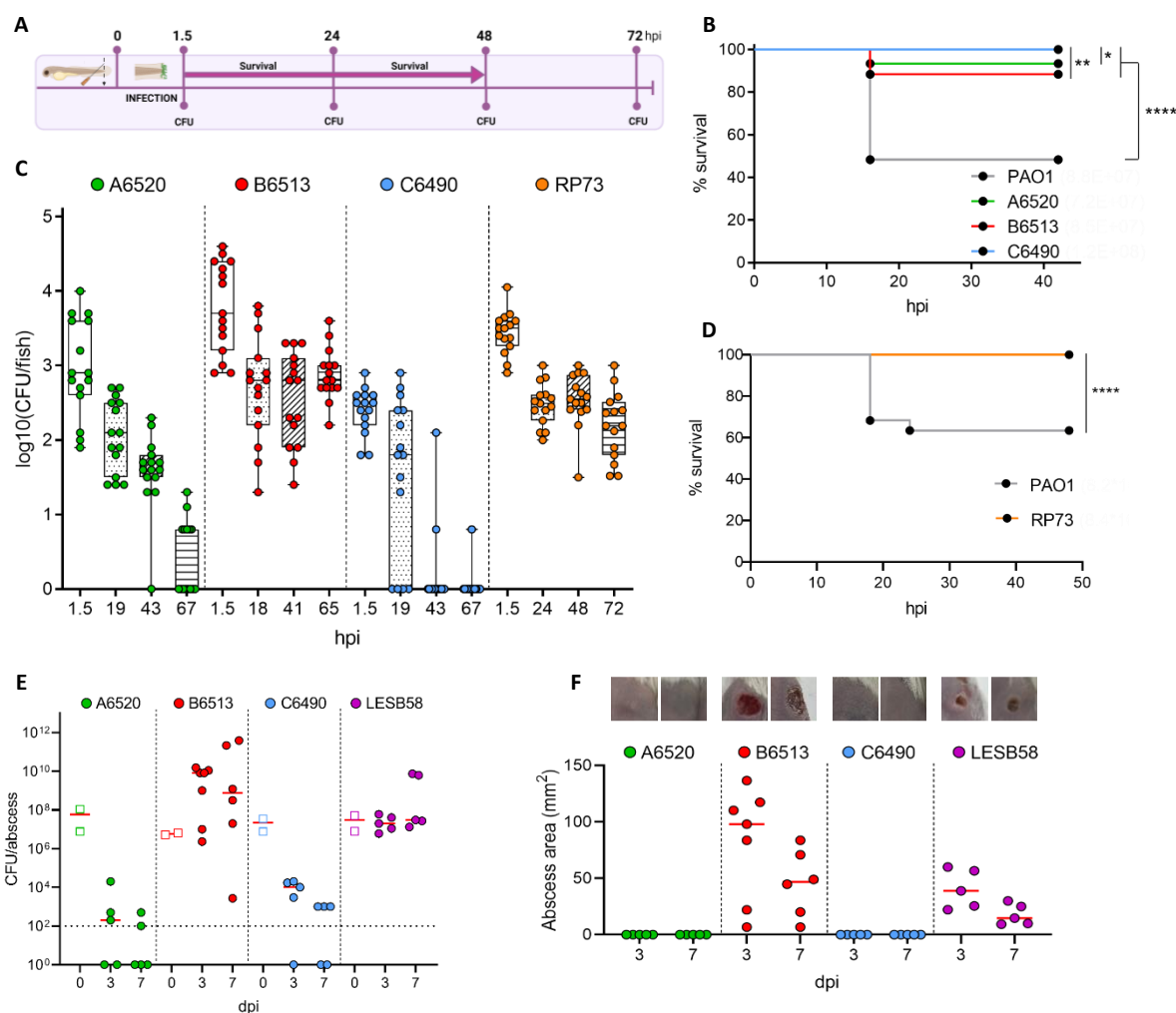
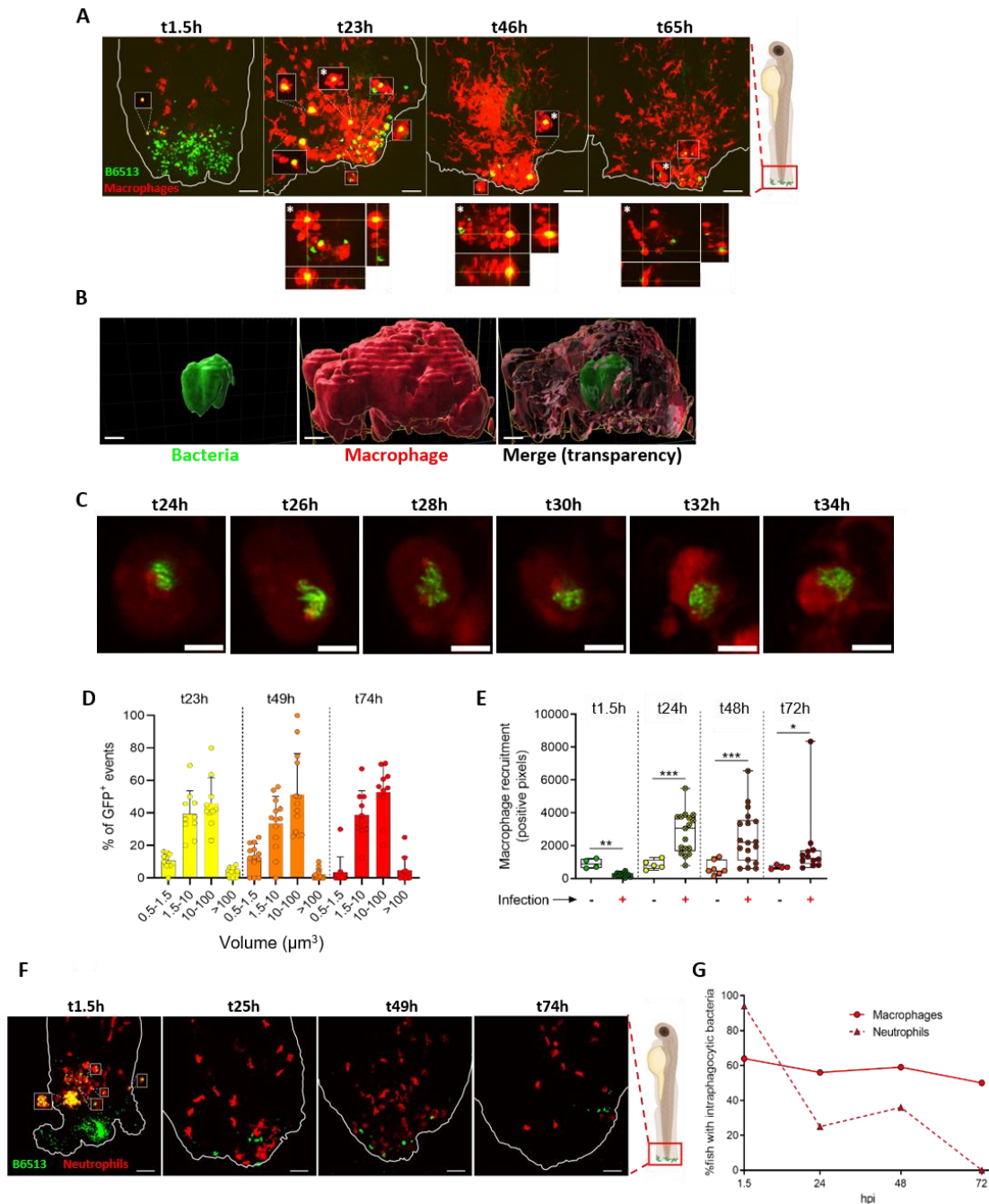


Figure 1. CF isolates can establish a persistent infection in zebrafish embryos, with parallel profiles to a murine model of infection.

(A) Experimental timeline to assess bacterial virulence and persistence in zebrafish embryo. (B) and (D) Embryo survival following infection by immersion with indicated *P. aeruginosa* strains at bacterial concentrations ranging from 7.2×10^7 to 1.2×10^8 CFU/mL. Survival was monitored for >40h following the infection (n=3, 60 larvae). Log-rank test: * $P < 0.05$, ** $P < 0.01$ and **** $P < 0.0001$. (C) Evolution of the bacterial load per embryo over time (until 72 hpi). Following infection by immersion with GFP⁺ *P. aeruginosa* clinical isolates, embryos were crushed at the indicated time points and plated for CFU counting (n=3, 15 larvae). (E) and (F) Virulence and persistence of CF isolates in a murine model of high-density cutaneous infection. Bacterial load (E) and size of abscesses (F) formed for three or seven days in CD-1 mice subcutaneously injected with indicated *P. aeruginosa* strains in the right dorsum were quantified. The CFUs reported at 0 dpi (empty squares) correspond to the injected inoculum. At 3 dpi and 7 dpi, abscesses were measured and harvested in phosphate buffered saline (PBS), homogenized and plated on lysogeny broth (LB) for bacterial enumeration. Data from two independent experiments containing 2–4 biological replicates each (n = 5–7) are displayed as the

997 median. The limit of detection (LOD) is displayed as a dashed line at 10^2 CFU/abscess. The photo
998 insets above the graph are representative images from treatment groups.
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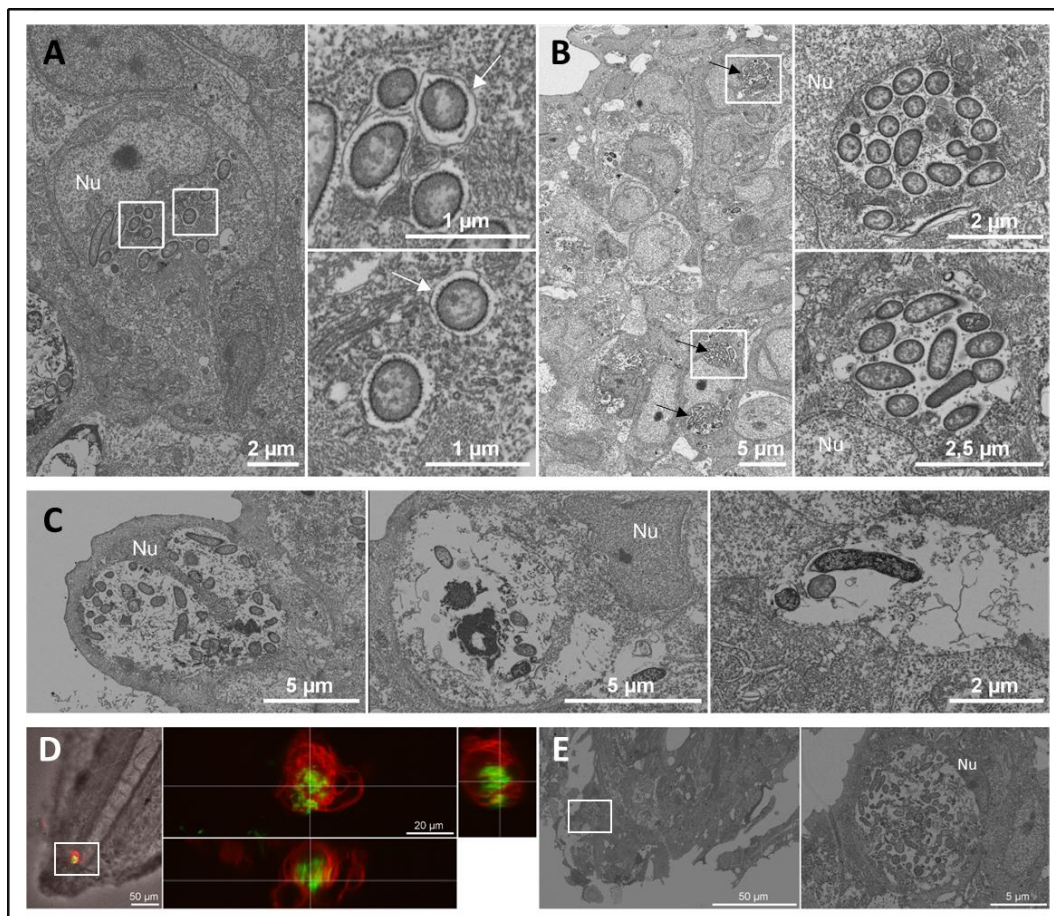
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Figure 2. Upon persistent colonization, *P. aeruginosa* B6513 form aggregates which can be visualized inside macrophages but not neutrophils. (A) Representative maximal projections of confocal images, showing interactions between bacteria (green) and recruited macrophages (red) in Tg(*mfp4:mCherry-F*) larvae at different time points. White rectangles denote images extracted from a single optical section of macrophages with intracellular *P. aeruginosa*. Below the images, orthogonal representations of the (*) events are shown, confirming that bacteria were inside macrophages. Scale bar: 40 μm . Note that pictures come from different embryos imaged for each indicated times. (B) 3D reconstruction allowing to confirm the intramacrophagic localization of a bacterial aggregate. Scale bar: 7 μm . (C) Zoomed single optical sections of an intramacrophagic

1011 bacterial aggregate tracked from 24 hpi to 34 hpi using time-lapse confocal imaging. Image
1012 acquisition was done every 30 min and images corresponding to 2 h intervals are shown.
1013 Brightness/contrast settings were modified comparatively to (A) for a better visualization of the
1014 aggregate organization after a deconvolution. Scale bar: 5 μm . (D) Volume repartition of GFP⁺
1015 events per larvae quantified following 3D reconstruction (10 to 13 embryos were imaged at each
1016 time point). (E) Macrophage quantification at the wound, measured by the number of red pixels at
1017 the median plan of the stack, in presence or absence of bacteria at various time points (4 to 8
1018 control and 8 to 18 infected embryos were imaged at each time point). Mann-Whitney test:
1019 * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. (F) Representative maximal projections of confocal images,
1020 showing interactions between bacteria (green) and recruited neutrophils (red) in Tg(*LysC:dsRed*)
1021 larvae. White rectangles denote images extracted from a single optical section of neutrophils with
1022 intracellular *P. aeruginosa*. Scale bar: 40 μm . Note that pictures come from different embryos
1023 imaged for each indicated times. (G) Evolution of the proportion of Tg(*mfap4:mCherry-F*) and
1024 Tg(*LysC:dsRed*) larvae with at least one event of intra-macrophage or intra-neutrophil bacteria,
1025 respectively, over time (10 to 16 larvae were imaged at each time point).
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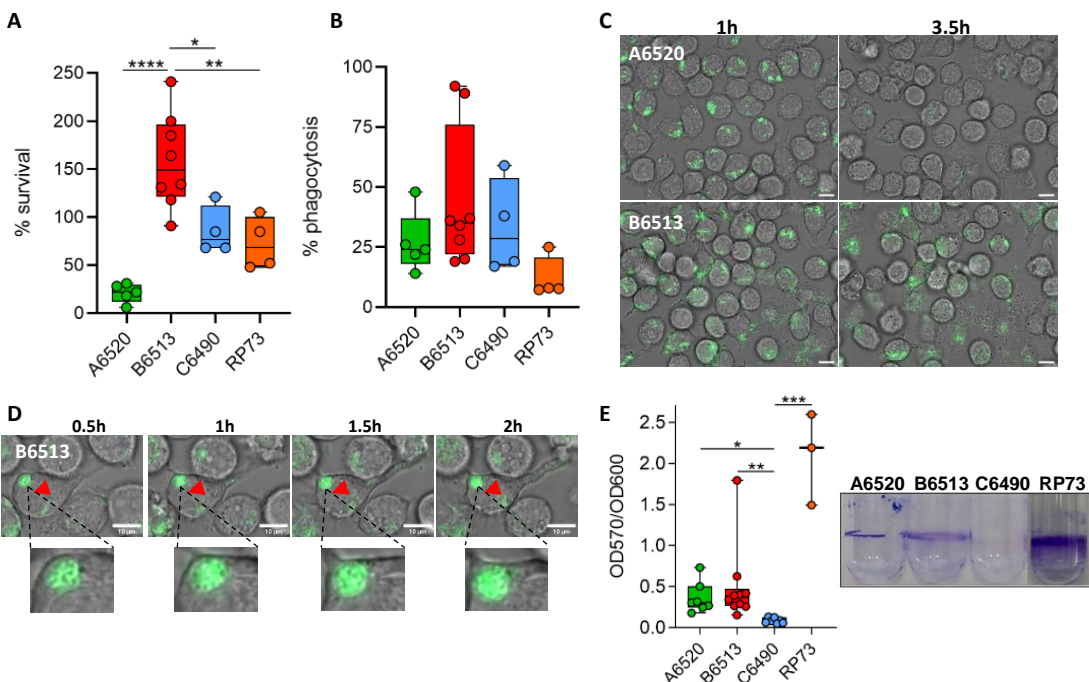
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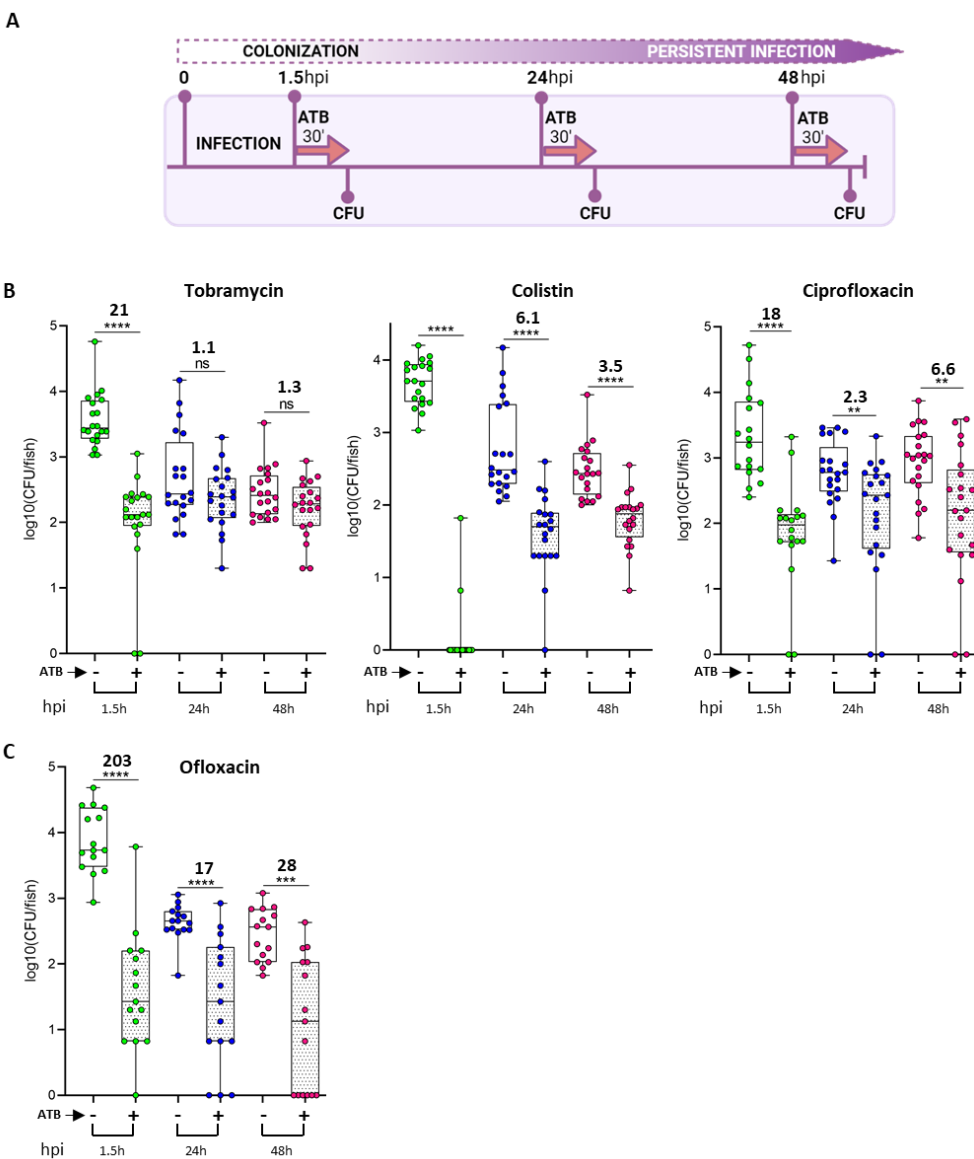
Figure 3. Electron micrographs of persistent *P. aeruginosa* B6513 in zebrafish. Representative images were acquired from a thin section of infected embryos at 24 hpi (D-E) or 48 hpi (A-C). (A) High magnification images of isolated intracellular bacteria near the tail fin edge (pixel size 5 nm). Right panels are zoom of the white squares. Nu: cell nucleus. A membrane is visualized around some bacteria (white arrow). (B) High magnification images of clustered intracellular bacteria with vacuolar shape (black arrows) at the tail fin. Right panels are zoom of the white squares. (C) High magnification images of intracellular bacteria in a damaged cell (left panel), or bacteria in apparently cell remnants (middle panel) or in the extracellular space (right panel). (D) and (E) Correlative Light-electron Microscopy (CLEM). (D) A z-stack of a live zebrafish embryo was acquired. The left panel shows a single fluorescent plane overlaid with the central DIC image of the fish tail (macrophages are seen in red and bacteria in green). A single large cluster containing dozens bacteria inside a macrophage is shown (white square). The right panel shows the orthogonal projection of the portion of the stack containing the cluster. (E) After processing the same fish for EM, lateral serial thin sections were prepared and imaged by EM. One section was acquired at a pixel size of 25 nm. Left panel shows an overview of the tail region. The large cluster observed in light microscopy is shown in its physiological context in the left panel (white square). The portion of the image containing the cluster is enlarged on the right panel, showing a high number of bacteria, filling almost all the cytoplasm of the cell and leaving the nucleus intact on one side.



1049 **Figure 4. Persistent isolates are able to resist macrophages and form biofilm *in vitro*.** (A)
 1050 Bacterial survival within J774 macrophages (n=4 to 8). The number of intramacrophagic bacteria
 1051 was determined 20 min (T0) or 2 h after gentamycin treatment (T1). Survival is expressed as the
 1052 ratio between the numbers of bacteria recovered at T1 versus T0. One-way ANOVA:
 1053 **** $P < 0.0001$; Tukey post-hoc test: * $P < 0.05$, ** $P < 0.01$ and **** $P < 0.0001$. (B) Phagocytosis
 1054 efficiency of each strain (n=4 to 8). The number of intracellular bacteria recovered at T0 was
 1055 compared to the initial inoculum. (C) Representative initial and final images of time-lapse
 1056 microscopy to follow the behavior of GFP⁺ intramacrophagic bacteria (isolates A6520 and B6513)
 1057 for 2h30. Scale bar: 10 μ m. (D) Time lapse imaging of an intra-vacuolar bacterial cluster (red
 1058 arrowhead) visualized within a macrophage (a zoom is shown below). In (C) and (D), times after
 1059 the start of gentamycin treatment are indicated. (E) Biofilm formation at 24h assessed by crystal
 1060 violet (CV) assay on glass tubes (n=3 to 10). Quantification of the CV-labelled biofilm rings (a
 1061 representative picture is shown on the right) is normalized with bacterial growth (OD_{600nm}).
 1062 Kruskal-Wallis test: **** $P < 0.001$; Dunn post-hoc test: * $P < 0.05$, ** $P < 0.01$ and **** $P < 0.001$.

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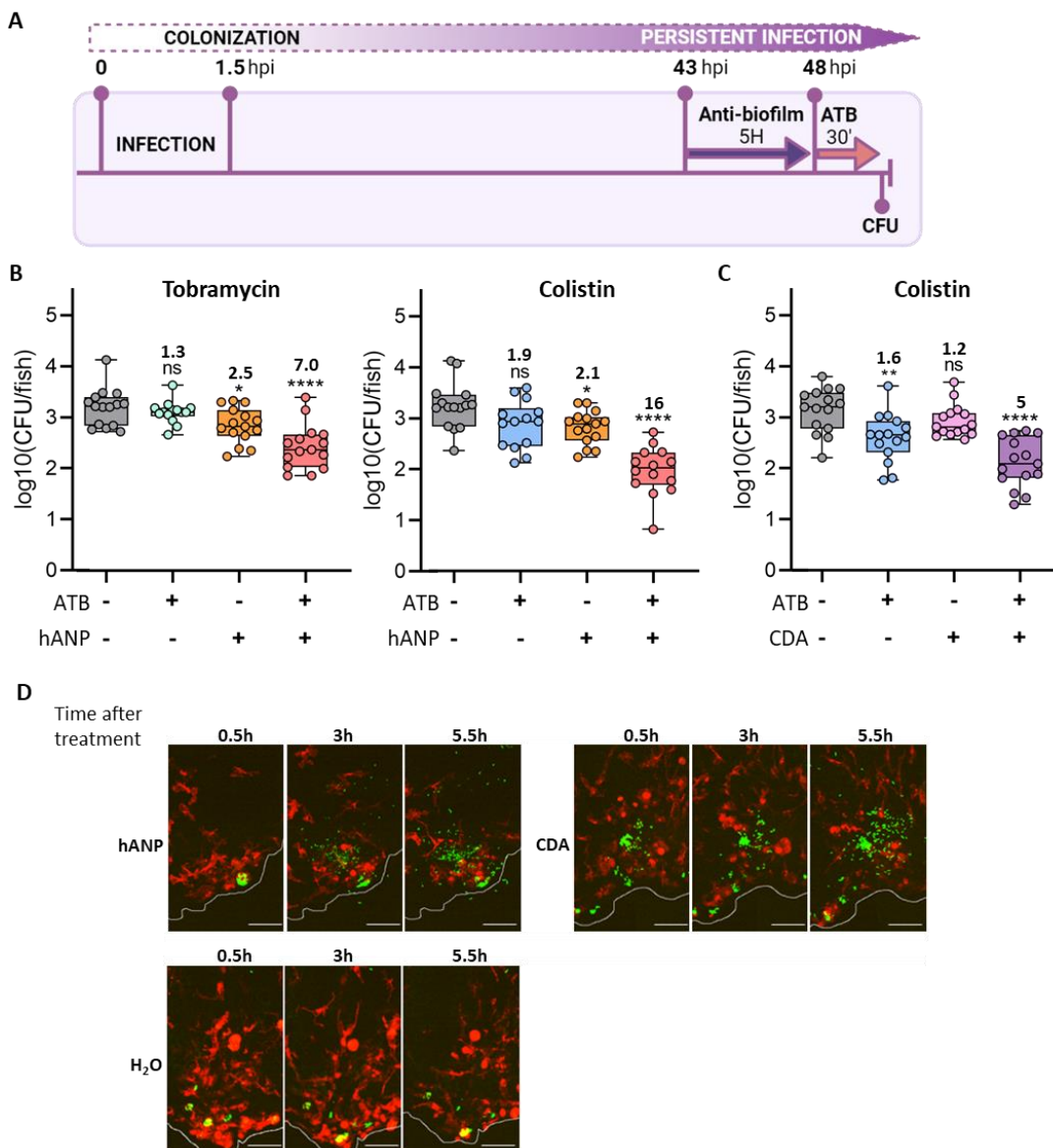
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1069 **Figure 5. Antibiotics have a reduced efficacy on persistent *P. aeruginosa* in infected**
1070 **zebrafish.** (A) Experimental procedure used to assess the efficacy of antibiotic treatments on
1071 infected embryos. ATB 30': antibiotic treatment for 30 min. (B) Efficacy of various antibiotics on
1072 strain B6513 with respect to the time post-infection (n=3 to 4, 15 to 21 larvae). Embryos colonized
1073 for 1.5, 24 or 48 h were subjected to the indicated antibiotic challenge, or incubated in water for
1074 the control condition. Following this 30 min treatment, bacterial load per embryo was determined
1075 in both groups. Mann-Whitney test: ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. Ratios were
1076 calculated regarding the median of the data set; thus there is no values for colistin at t1.5 h as the
1077 median of the treated group is zero. (C) Efficacy of ofloxacin, an antibiotic known to enter
1078 eukaryotic cells with high efficiency, on strain B6513 (same analysis as in (B)).

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1081 **Figure 6. Treatment with anti-biofilm compounds can re-sensitize persistent *P. aeruginosa***
 1082 **bacteria to antibiotics in infected zebrafish.** (A) Experimental procedure to assess the combined
 1083 effect of anti-biofilm molecules and antibiotics. ATB 30': antibiotic treatment for 30 min. (B) and
 1084 (C) Antibiotic potentiation by 10 μ M hANP (B) and 20 μ M CDA (C) at 48 hpi (n=3, 14 to 15
 1085 larvae). Anti-biofilm compounds were added for 5 h to embryos infected by B6513-gfp strain,
 1086 followed by the indicated antibiotic treatment, before CFU counting per embryo. One-way
 1087 ANOVA: ****p<0.0001; Dunnett post-hoc test (comparison with the control condition): *P<0.05,
 1088 **P<0.01 and ****P<0.0001. Ratios were calculated regarding the median of the data set. (D)
 1089 Maximal projections of confocal images, showing the effect of hANP or CDA following the
 1090 treatment used in (B) and (C), without antibiotic treatment. Pictures of Tg(*mfap4:mCherry-F*)
 1091 larvae infected by strain B6513-gfp were tan, from the beginning of the treatment, every 30 min
 1092 for 5.5 h. A selection of images at 3 time points is shown. Scale bar: 40 μ m.