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1 **Contamination of a hospital plumbing system by persister cells of a copper-**
2 **tolerant high-risk clone of *Pseudomonas aeruginosa***

3
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20 **Running title:** Copper-tolerant *P. aeruginosa* ST308 in hospital drinking water

21

22 **Keywords:** *Pseudomonas aeruginosa*; persisters; copper; premises plumbing; One Health

23

24 **Abstract**

25 **Background.** *Pseudomonas aeruginosa* (PA) is an important opportunistic pathogen that
26 thrives best in the distal elements of plumbing and waste-water systems. Although
27 nosocomial outbreaks of PA have been associated with water sources, the role of the
28 plumbing system of healthcare premises as a reservoir for this pathogen is still unclear.

29 **Materials and methods.** We collected water samples from 12 technical areas, distant from
30 any medical activity, in a teaching hospital in France once a week for 11 weeks. We used a
31 method that resuscitates persister cells because of the nutrient-poor conditions and the
32 presence of inhibitors (*e.g.* chlorine and copper ions). Briefly, water was sampled in sterile
33 bottles containing 100 μ M of the copper-ion chelating agent diethyldithiocarbamate (DDTC).
34 A portion of the samples was immediately filtered through 0.45- μ m membranes, deposited
35 on R2A agar plates, and incubated seven days at 22°C (following European
36 recommendations). The remaining water was incubated 14 days at 22°C and then filtered
37 and cultured on R2A, blood-, or cefrimide-containing agar plates. PA isolates were identified
38 by MS MALDI-TOF, genotyped by PFGE and WGS, and tested for survival in a 150 μ g/L
39 copper (II) sulphate solution.

40 **Results.** Although the 12 water sampling points always tested negative with the
41 recommended method, 67% were positive at least once for PA with the adapted method
42 (*i.e.* with DDTC). The 14 PA persister isolates found throughout the plumbing system were
43 clonal and belong to the high-risk clone ST308. Their genome harbours a 37-kb genomic
44 island (GI-7) containing 13 genes linked to copper resistance. ST308 survived better in the
45 copper solution than comparators that did not harbour GI-7 (*P. aeruginosa* strains PAO1,
46 PA14, and ST235). The deletion of GI-7 in ST308 abrogated its tolerance to copper. The GI-7
47 nucleotide sequence shares 98% and 72% identity with sequences from the environmental

48 species *Pseudomonas putida* and the phytopathogenic species *Pseudomonas syringae*,
49 respectively.

50 **Conclusion.** We report the contamination of the plumbing system of a healthcare premises
51 by persister cells of the high-risk clone *P. aeruginosa* ST308. New recommendations for the
52 monitoring of water contamination should consider persister cells. The genomic island GI-7,
53 which confers tolerance to copper, probably originates from *Pseudomonas* species found in
54 copper-contaminated soils and plants. Agricultural practices may have an unexpected
55 consequence, allowing copper-tolerant pathogens to survive in the hospital environment
56 and contaminate fragile patients.

57 **1. Introduction**

58 *Pseudomonas aeruginosa* is an important opportunistic pathogen in immunocompromised
59 or critically ill patients (Cuttelod et al., 2011; Loveday et al., 2014). It is ubiquitous in soil,
60 water, and other moist environments, including hospital water networks and water points
61 (Bédard et al., 2016; Loveday et al., 2014). *P. aeruginosa* thrives best in the distal elements
62 of plumbing and waste-water systems, such as taps, sinks, U-bends, and toilets (Bédard et
63 al., 2016; Varin et al., 2017), which in turn can act as a source of patient contamination.
64 Hence, 15 to 50% of *P. aeruginosa* healthcare-associated infections originate from regularly
65 used contaminated water points (Blanc et al., 2004; Cholley et al., 2008; Rogues et al., 2007;
66 Trautmann et al., 2006). *P. aeruginosa* strains transmitted from contaminated tap water to
67 patients are usually considered to be of environmental origin. However, Kotay *et al.* showed
68 that faucets and aerators are easily contaminated by strains coming from the U-bends,
69 themselves seeded by clinical strains (Kotay et al., 2017). Hence, the hospital environment
70 may act as a reservoir and amplifier of clinical strains of *P. aeruginosa*.

71 Although nosocomial outbreaks of *P. aeruginosa* have been associated with water sources
72 (Abdouchakour et al., 2018; Garvey et al., 2017; Quick et al., 2014), the role of the plumbing
73 system of the healthcare premises as a reservoir for this pathogen is still unclear. Water
74 contamination in hospitals is monitored by immediate filtration and culture on agar media
75 (Health Technical Memorandum 04-01, 2016; NF EN ISO 16266, 2008). However, water
76 distribution and plumbing systems are hostile environments for bacterial development, with
77 nutrient-poor conditions and inhibitors (*e.g.* chlorine and copper-ions). These conditions
78 favour the development of persister cells (also called VBNC – viable but not culturable),
79 which are not culturable on media upon which they were usually able to grow (Bédard et al.,

80 2014a; Kim et al., 2018; Li et al., 2014). Hence, culture-based methods can miss water
81 samples containing *P. aeruginosa* DNA (Bédard et al., 2015).

82 The biocidal properties of copper are exploited for the distribution of water and copper-
83 containing materials are widely used for pipes and fixtures of plumbing systems, leading to
84 the release of copper ions into the drinking water (Dwidjosiswojo et al., 2011). In addition,
85 copper is widely used as a biocide in agriculture and as a growth promoter in animal
86 production. This metal thus heavily contaminates soil directly treated with copper sulphate
87 or exposed to copper-containing manure (Nunes et al., 2016).

88 Here, we screened the plumbing system of a healthcare premises for *P. aeruginosa* using a
89 method that resuscitates persister cells. We used pulsed-field gel electrophoresis, whole-
90 genome sequencing, and gene inactivation to determine the population structure of the
91 bacterial isolates found in the water network and identify the biological features that may
92 have helped the strains to survive in this harsh environment.

93

94 **2. Materials and methods**

95 **2.1 Setting.** We sampled the plumbing system of the 1200-bed University Hospital of
96 Besançon (UHB, France), consisting of four buildings. The grey, orange, green, and blue
97 buildings were built in 1982, 1999, 2012, and 2015, respectively. UHB has two independent
98 water inlets. One inlet serves the blue building, whereas the other inlet serves the three
99 other buildings (Figure 1). All pipes of the drinking-water installation are made of copper.

100 **2.2 Water sampling.** We exclusively sampled faucets distant from any medical activity to
101 avoid any contamination with *P. aeruginosa* isolates originating from patients or the
102 environment of care units. Indeed, sampled faucets were in technical areas, such as crawl
103 spaces, medical equipment storage areas, and technical facilities. One-litre water samples
104 were obtained weekly for 11 weeks (from May to August 2017) from 12 sampling points.
105 Specifically, we sampled the two main water inlets and technical areas of the grey ($n = 4$),
106 orange ($n = 3$), green ($n = 2$), and blue ($n = 1$) buildings (Figure 1). Water was sampled,
107 according to the EN ISO 19458 guidelines (NF EN ISO 19458, 2006), in sterile polypropylene
108 bottles containing 20 mg/L sodium thiosulphate (VWR, Fontenay-sous-Bois, France) after
109 aerator removal, disinfection of the outlet with a gas burner or a swipe soaked with the
110 disinfectant Aniosurf (ANIOS, Lille-Hellemmes, France), and a 1-min pre-flush (Wang et al.,
111 2017; NF EN ISO 19458, 2006). In addition, we adapted the EN ISO 19458 guidelines by
112 immediately supplementing all samples with 100 μ M of the copper-ion chelating agent
113 diethyldithiocarbamate (DDTC; Sigma-Aldrich, Saint-Quentin Fellavier, France), which can
114 resuscitate copper-impregnated bacterial cells (Dwidjosiswojo et al., 2011). Bottled sterile
115 water (Fresenius Kabi, Sevres, France) was also sampled in triplicate as a negative control.

116 **2.3 Microbiological analysis of the water.** Within 2 h after sampling, we filtered 250 mL
117 water through 0.45- μ m membranes, placed them on R2A agar (Biokar Diagnostics, Allonne,
118 France), and incubated them for seven days at 22°C, according to the method recommended
119 by the European and French technical guidelines (Health Technical Memorandum 04-01,
120 2016; NF EN ISO 16266, 2008). The remaining 750 mL were stored for 14 days at 22°C in the
121 dark (Dwidjosiswojo et al., 2011; Li et al., 2014). Then, we filtered three subsamples of 250
122 mL, placed the membranes either on R2A agar and incubated them for seven days at 22°C,
123 ceftrimide-containing agar (Biorad, Marne la Coquette, France) and incubated them for 48 h
124 at 37°C, or Columbia agar supplemented with 5% horse blood (Thermofisher Oxoid, Dardilly,
125 France) and incubated them for 48 h at 37°C. *P. aeruginosa* colonies were detected by
126 standard microbiological methods (*i.e.* colony morphology, positive oxidase reaction, and
127 pigment production) and further identified by MALDI-TOF mass spectrometry (Microflex LT;
128 Bruker Daltonik GmbH, Bremen, Germany) according to the manufacturer's
129 recommendations. We stored each isolate in brain-heart infusion broth supplemented with
130 30% glycerol at -80°C until further analysis at the Centre de Ressources Biologiques-Filière
131 Microbiologique de Besançon (Biobank number BB-0033-00090).

132 **2.4 Determination of antibiotic susceptibility.** We tested the activity of ten clinically
133 important antibiotics (ceftazidime, cefepime, piperacillin-tazobactam, imipenem,
134 meropenem, aztreonam, amikacin, tobramycin, ciprofloxacin, and fosfomycin) against all
135 *P. aeruginosa* isolates by the disk diffusion method (EUCAST, 2017).

136 **2.5 Genotyping analysis.** The clonality of *P. aeruginosa* isolates was first investigated by
137 pulsed-field gel electrophoresis (PFGE) with *DraI* digestion, as previously described (Slekovec
138 et al., 2012). The restriction profiles were analysed using GelCompar software (Applied

139 Maths, Kortrijk, Belgium). Pulsotypes and clusters were defined according to international
140 recommendations (Tenover et al., 1995).

141 We sequenced the genome of *P. aeruginosa* isolates using Illumina NextSeq technology
142 (pair-end, 150 bp, and coverage > 240 X) to decipher the genetic relationship between
143 isolates and identify specific genetic elements. Reads were subsampled to a coverage of 80X
144 before genome assembly using Spades v3.9.0 with read correction and k-mer sizes of 22, 33,
145 55, and 77 (Bankevich et al., 2012). The sequence type (ST) of isolates was determined *in*
146 *silico* from WGS data (Larsen et al., 2012).

147 **2.6. Phylogenetic analysis.** A previously described subset of 3,767 core genes was extracted
148 from each assembled genome and other representative genomes using a home-made
149 python script for a core genome MLST (cgMLST; <https://github.com/bvalot/wgMLST>)
150 (Mellmann et al., 2016). These genes were concatenated for Bayesian phylogenetic analysis
151 using MrBayes v3.2.6 with a GTR+G substitution model (Ronquist et al., 2012). We ran
152 1,000,000 iterations of two Markov Chain Monte Carlo simulations. The convergence of the
153 two chains was confirmed by a calculated PSRF (Potential Scale Reduction Factor) close to
154 1.0. The posterior probability of each branch was > 99%.

155 **2.7. Determination of the Cu²⁺ concentration in water samples.** The Cu²⁺ concentration was
156 assessed in triplicate in the *P. aeruginosa*-positive water outlets (Figure 1). We used an
157 inductively coupled plasma (ICP) with a GC system 7900 (Agilent technologies, Massy,
158 France) coupled to a triple quadrupole mass spectrometer (7000 ICP-MS/MS model, Agilent
159 technologies, Massy, France).

160 **2.8 Chromosomal deletion of the GI-7 genomic island in *P. aeruginosa*.** We constructed a
161 GI-7 deletion mutant by using overlapping PCR and recombination events as previously
162 described (Kaniga et al., 1991). Briefly, 750 bp located up and downstream of the GI-7 region
163 was amplified by PCR using the specific primer pairs Gi7_5'_Fw
164 (AGCCAAGCACCAGGTCTATT)/Gi7_5'_Rev (AGGCAGATGATCTGGCGGTAAGTAGGCT) and
165 Gi7_3'_Fw (GCCAGATCATCTGCCTTGGGTCATGTCA)/Gi7_3'_Rev (ACCAAATTGACCTGCGC-
166 AT) (annealing at 60°C, extension 0.5 min). Subsequently, an overlapping PCR amplified the
167 previous amplicons with the external pair of primers Gi7_5'_Fw/Gi7_3'_Rev (annealing at
168 63°C, 1 min extension), using the BioRad iProof HF Master Mix (Biorad) according to the
169 manufacturer's instructions. We cloned the resulting blunt-end PCR product into the plasmid
170 pCRTM-Blunt using the Zero Blunt[®] PCR Cloning Kit (Invitrogen, Carlsbad, United States) and
171 subcloned it into the suicide vector pKNG101 in the *E. coli* CC118 λ pir strain, previously
172 digested with *Bam*HI/*Ap*I restriction enzymes. We transferred the recombinant suicide
173 vector in the *P. aeruginosa* PA-8 isolate by triparental conjugation. Transconjugants were
174 selected on *Pseudomonas* Isolation Agar supplemented with 2,000 mg/L streptomycin.
175 Homologous recombination was obtained after selection of transconjugants on M9 minimal
176 medium (42 mM Na₂HPO₄, 22 mM KH₂PO₄, 19 mM NH₄Cl, and 8.5 mM NaCl) supplemented
177 with 5% sucrose. The deletion of 27,288 bp in the *P. aeruginosa* PA-8 Δ GI-7 isolate was
178 confirmed by PCR and sequencing.

179 **2.9 Survival in copper solution.** For all tested *P. aeruginosa* isolates, an overnight culture in
180 Luria-Bertani broth (LBB, Biorad) was diluted 1/200 into fresh LBB and further incubated at
181 37°C under gentle shaking until mid-log phase. The bacterial suspension was then
182 centrifuged at 4,000 x *g* for 20 min. The pellet was washed once and resuspended in sterile

183 deionized water. Bacteria were suspended in a 150 µg/L copper (II) sulphate solution (Sigma-
184 Aldrich) to obtain a concentration of $\sim 10^8$ bacteria/ml, which was further incubated for 24 h
185 at 22°C in the dark. We chose a concentration of 150 µg/L as it was the highest value
186 retrieved at a *P. aeruginosa*-positive faucet. Initial and final bacterial concentrations were
187 determined by plating the appropriate dilutions on LB agar (Biorad) and incubating overnight
188 at 37°C. The ratios of the final and initial bacterial concentrations determined the survival
189 rates. We repeated each experiment six times independently for all isolates. We used the
190 *P. aeruginosa* reference strains PAO1 and PA14 and a clinical isolate of ST235 (strain PA1646;
191 Pitié Salpêtrière University Hospital, Paris, France) as controls.

192 **2.10 Statistical analysis.** We used RStudio software version 3.2.2 (Boston, USA) for all
193 statistical analysis. The significance of the differences in bacterial survival in copper (II)
194 sulphate solution between the various tested *P. aeruginosa* isolates was determined by the
195 analysis of variance (ANOVA). A multiple comparison test, Tukey's HSD (honestly significant
196 difference) Test, was performed when the ANOVA detected a significant statistical
197 difference. The chosen significance threshold was 0.05 for all tests.

198

199 **3. Results and discussion**

200 **3.1 Contamination of the hospital plumbing system by *P. aeruginosa*.** None of the 132
201 water samples tested was found to be positive for *P. aeruginosa* using the method
202 recommended by the English and French technical guidelines (Health Technical
203 Memorandum 04-01, 2016; NF EN ISO 16266, 2008; NF EN ISO 19458, 2006). In contrast, the
204 use of the copper-ion chelating agent associated with a sample incubation of 14 days
205 (adapted method) resuscitated 14 persister isolates of *P. aeruginosa* from 11 samples
206 (8.3%). Persister isolates found with the adapted method grew on R2A agar ($n = 8$), Columbia
207 agar supplemented with 5% horse blood ($n = 4$), or ceftrimide-containing agar ($n = 2$). Overall,
208 eight of 12 sampling points were positive at least once for *P. aeruginosa*, with the four
209 buildings and two water inlets being positive for the pathogen (Table 1).

210 **3.2 Recommended methods failed to detect persister cells of *P. aeruginosa*.** All
211 *P. aeruginosa* isolates were only able to grow after a 14-day incubation with the copper
212 chelator DTTC, which relieves bacteria of copper inhibition (Dwidjosiswojo et al., 2011).
213 Persister cells are not immediately culturable on media upon which the species is usually
214 able to growth. However, resuscitated cells recover their full virulence, as shown by cell and
215 murine models (Dwidjosiswojo et al., 2011; Li et al., 2014). The guidelines for water testing
216 recommend water sampling in thiosulphate-containing bottles, followed by immediate 0.45-
217 μm filtration and culture on agar media (Health Technical Memorandum 04-01, 2016; NF EN
218 ISO 16266, 2008). In our hands, this method did not allow the growth of persister cells of
219 *P. aeruginosa*. Hence, all the water sampling points ($n = 12$) tested negative with the
220 recommended techniques, whereas eight (67%) were positive for *P. aeruginosa* with the
221 adapted method. Four of the 12 sampling sites in the plumbing system, two unused
222 showers, and two unused faucets were not operated according to the acknowledged rules

223 (Health Technical Memorandum 04-01, 2016). Three were positive for *P. aeruginosa*,
224 confirming that water stagnation in backwater degrades the microbiological quality of water
225 (Health Technical Memorandum 04-01, 2016). In addition, although the treatment of water
226 with a copper chelator allowed the detection of *P. aeruginosa* persister cells in the plumbing
227 system of the premises, this method was not quantitative. However, as a *P. aeruginosa*
228 count of ≥ 1 CFU/100 ml is already considered to be unsatisfactory (Health Technical
229 Memorandum 04-01, 2016; NF EN ISO 16266, 2008), the quantification of the pathogen is of
230 minor importance. Enhancing the performance of the detection of *P. aeruginosa* in the
231 plumbing system of healthcare premises will expedite decontamination measures of the
232 drinking water installation and would reduce the risk of contamination of regularly used
233 water points, their colonization by the bacterial pathogen, and subsequent transmission to
234 patients.

235 **3.3 A single ST308 *P. aeruginosa* clone colonized the entire UHB plumbing system.** The 14
236 *P. aeruginosa* isolates of the present collection displayed a typical morphology and were
237 fully susceptible to all antibiotics tested (data not shown).

238 We tested the genetic relatedness of the 14 *P. aeruginosa* isolates using PFGE and found
239 that they all shared the same pulsotype (Supplementary Figure S1). Four isolates were
240 selected for genome sequencing (Table 1). PA-3 and PA-6 were isolated from the two main
241 water inlets, and isolates PA-8 and PA-12 came from the water network of the blue and grey
242 buildings, respectively. Their genomes were constructed by *de novo* assembly. *In silico* MLST
243 revealed that the four sequenced isolates belong to clone ST308. The PFGE data were
244 confirmed by cgMLST, which found < 20 different alleles between the four isolates, thus
245 confirming the clonality of the collection (Mellmann et al., 2016). We also assessed the

246 genetic distance between our local ST308 clone and representatives of the *P. aeruginosa*
247 species. This collection included the genomes of (i) other ST308 isolates, (ii) isolates of high-
248 risk clones ST111, ST175, ST233, ST235, ST277, and ST395, responsible for outbreaks in
249 healthcare settings, and the two reference strains PAO1 and PA14 (Supplementary Table S1).
250 As expected, our local ST308 clone clustered with the four other ST308 representatives
251 (Figure 2). The other tested genomes were distributed between two major phylogenetic
252 groups, one including PAO1 and the other PA14, as previously shown (Freschi et al., 2015).
253 The ST308 and ST235 isolates belong to the PA14 group and the high-risk clones ST111,
254 ST175, ST233, ST277, and ST395 belong to the PAO1 group.

255 The survival and persistence of *P. aeruginosa* in the harsh environment of the plumbing
256 system of the healthcare premises presumably relies on its ability to form persister cells
257 within biofilms and colonize free-living amoebae, which offer the bacterial pathogen
258 protection against hostile conditions and act as a vehicle of dissemination (Bédard et al.,
259 2016, 2014b; Hota et al., 2009; September et al., 2007; Wingender and Flemming, 2011). The
260 presence of a single ST308 clone in the drinking water installation could either be due to
261 monoclonal inoculation from the water supply system of the city or intraspecific competition
262 (through the production of pyocins) won by this particular clone (Michel-Briand and Baysse,
263 2002).

264 **3.4 *P. aeruginosa* ST308 is an emerging high-risk clone.** Although no outbreak of
265 *P. aeruginosa* ST308 has occurred thus far among patients hospitalized in the UHB,
266 carbapenem-resistant isolates of this clone have already been retrieved in the U-bends of
267 the haematology unit (Varin et al., 2017). However, two hospital outbreaks of *P. aeruginosa*
268 ST308 involving the environment have been recently described in Europe. First, a multidrug-

269 resistant ST308 clone that produces the metallo- β -lactamase IMP-8 spread throughout the
270 haematological-oncological unit of a German hospital over three years, where the
271 environment was a reservoir for patient contamination (Willmann et al., 2015). Second,
272 *P. aeruginosa* ST308 caused a five-year outbreak in patients in a French hospital and
273 persisted for an additional four years in the hospital environment, causing sporadic
274 infections (Abdouchakour et al., 2018). Here again, the environment was identified as the
275 likely source and reservoir of the outbreak. In addition, clone ST308 can also produce
276 metallo- β -lactamases of Ambler class A (GES-7) and class B (IMP-1, IMP-8, IMP-13, IMP-45,
277 IMP-63, and VIM-2) (Oliver et al., 2015; Liapis et al., 2018). Overall, *P. aeruginosa* ST308 is a
278 high-risk clone, which can locally acquire resistance determinants that possibly originated
279 from the water-distribution system.

280 **3.5 ST308 isolates are tolerant to copper due to genomic island GI-7.** The concentration of
281 Cu^{2+} in the water at the eight sampling points that tested positive for *P. aeruginosa* ranged
282 from 0.6 ± 0.006 to 143.9 ± 1.9 $\mu\text{g/L}$ (Table 1). These concentrations are below the maximum
283 acceptable concentration of 2,000 $\mu\text{g/L}$ defined by French and European standards and the
284 World Health Organization. However, the presence of ST308 in this copper-containing water
285 led us to test its survival in such hostile conditions. We compared the survival of isolate PA-8
286 (representative of the ST308 clone that contaminates the UHB water network) with that of
287 the *P. aeruginosa* reference strains PAO1 and PA14 and strain PA1646, a representative of
288 the major epidemic high-risk clone ST235, in a copper-containing solution. PCR experiments
289 confirmed that the genomes of PAO1, PA14, and PA1646 (ST235) do not harbour GI-7 (data
290 not shown). The PA-8 isolate survived better in a 150 $\mu\text{g/L}$ copper solution than the
291 reference strains PAO1, PA14, and PA1646 (ST235) (Figure 3).

292 We therefore searched for genetic elements that could confer tolerance to copper and aide
293 in the implantation of ST308 in the plumbing system. Analysis of the sequenced genomes of
294 four UHB-contaminating ST308 isolates retrieved a 36,905-bp genomic island that contains
295 13 genes encoding copper resistance mechanisms (Figure 4). Among them, the genes *czcC*,
296 *czcB*, and *czcA* encode an outer membrane protein, an adaptor subunit, and an efflux pump,
297 respectively. These proteins form the heavy metal RND efflux pump CzcABC that has already
298 been described in *P. aeruginosa* and *P. putida*. This zinc- and copper-induced pump confers
299 resistance to heavy metals (Chong et al., 2016; Leedj arv et al., 2008). The gene *copG* encodes
300 a hypothetical export protein. CopM is a cytochrome C oxidase involved in electron
301 transport (Behlau et al., 2011). CopA, CopB, and CopZ are a copper binding protein, an
302 exporting ATPase, and a chaperone, respectively (Chong et al., 2016; Navarro et al., 2016).
303 The *CopA* and *CopB* genes constitute an operon encoding a putative metal-binding protein
304 and a copper oxidase described in clinical isolates of *P. aeruginosa* and *Acinetobacter*
305 *baumannii* and the environmental species *Pseudomonas syringae* and *Xanthomonas* sp.
306 (Behlau et al., 2011; Teitzel et al., 2006; Teixeira et al., 2008; Williams et al., 2016). CopA and
307 CopB bind copper-ions in the periplasm and outer membrane, respectively (Teitzel et al.,
308 2006). GI-7 also encodes two copper-binding plastocyanins and a copper ABC transporter,
309 involved in copper homeostasis and/or transport (Chong et al., 2016; Quaranta et al., 2007).
310 Finally, the two regulator genes *copS* and *copR* encode proteins that form a two-component
311 signal transduction system (Behlau et al., 2011).

312 We confirmed the role of GI-7 in the tolerance of ST308 to copper in solution by deleting the
313 entire genomic island by chromosomal recombination and testing the survival of the deleted
314 mutant (named PA-8ΔGI-7) under the same conditions as those previously used. As

315 expected, the deletion of GI-7 significantly decreased the tolerance of ST308 in a 150 µg/L
316 copper (II) sulphate solution relative to its wildtype parent (Figure 3). Moreover, the PA-
317 8ΔGI-7 mutant had a tolerance to copper similar to that of the other comparators (*i.e.* PAO1,
318 PA14, PA1646 – ST235). Thus, GI-7 plays an important role in the copper tolerance of ST308,
319 which may have helped the implantation of *P. aeruginosa* in the plumbing system and could
320 further promote contamination of the water outlets, waste-water system, and patients. In
321 addition, the survival of copper-tolerant bacteria in the water-distribution and plumbing
322 systems may be favoured by their enhanced resistance to amoebic predation. Hence, Hao *et*
323 *al.* (Hao *et al.*, 2016) showed that copper resistance determinants in *P. aeruginosa* play
324 crucial roles in bacterial resistance to predation by amoeba, which are prevalent in water
325 distribution systems (Delafont *et al.*, 2013).

326 **3.6 GI-7 probably originated from environmental *Pseudomonas* and spread among high-**
327 **risk clones of *P. aeruginosa*.** GI-7 has the features of a transposable element, with a
328 transposase-encoding gene, together with direct and indirect repeats flanking the
329 transposon (Figure 4). We blasted GI-7 against the NCBI database and found it in the
330 genomes of 11 *P. aeruginosa* clinical isolates, with 99% identity (Supplementary Table S2).
331 Two ST308 isolates from the USA and Mexico harboured GI-7. This genomic island was also
332 found in the genomes of three isolates of *P. aeruginosa* ST395, amongst which DHS01 was
333 the representative of a clone responsible for a long-term outbreak in the UHB, with
334 identified environmental reservoirs (Bertrand *et al.*, 2000; Petitjean *et al.*, 2017). GI-7 was
335 also found in the genomes of the sporadic ST198 and ST309 clones, as well as isolates of the
336 high-risk international clones ST111 and ST253. Interestingly, an isolate of the environmental
337 species *Pseudomonas putida* displayed a sequence that shared 98% identity with that of

338 ST308. *P. putida* typically inhabits soil and water and is occasionally isolated from
339 hospitalized patients (Molina et al., 2013). GI-7 was also found in the genome of the
340 phytopathogenic *Pseudomonas syringae* pv. *actinidiae* strain SR198 (Monteil et al., 2016),
341 with 72% identity. These two environmental species are constantly exposed to this heavy
342 metal. Copper-derived biocides (e.g. copper sulphate) have been extensively used in wood
343 impregnation stations and agriculture and as an animal feed additive since the mid-18th
344 century (Hobman and Crossman, 2015; Nunes et al., 2016), making copper one of the most
345 common soil contaminants. For example, copper contamination can reach 57.5 mg/kg dry
346 weight of soil in Europe (Heijerick et al., 2006). Plant- and soil-associated species have thus
347 accumulated resistance determinants to tolerate the presence of copper (Hobman and
348 Crossman, 2015; Nunes et al., 2016). Overall, GI-7 likely emerged in environmental
349 *Pseudomonas* species before its transfer to the high-risk clones of *P. aeruginosa* ST308,
350 ST395, ST111, and ST253.

351 **4. Conclusion**

352 We report the contamination of the plumbing system of a healthcare premises by persister
353 cells of *P. aeruginosa* ST308. New recommendations for the monitoring of water
354 contamination should account for persister cells to trigger decontamination measures of the
355 drinking water installation and reduce the risk of contamination of regularly used water
356 points. The genomic island GI-7, which confers copper tolerance to this emerging high-risk
357 clone, probably originated from soil- and plant-associated *Pseudomonas* species. Agricultural
358 practices may have unexpectedly selected copper-tolerant pathogens that can survive in
359 drinking-water installations of hospitals, resulting in the potential contamination of fragile
360 patients.

361

362 **Declarations of interest.** None

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366

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538

539 **Figure legends**

540 **Figure 1. Schematic representation of the buildings of the University Hospital of Besançon**
541 **(France) and the location of water samples.** For each building, the numbers on the left
542 indicate the floor. The circles and squares indicate the locations of the water sampling in the
543 healthcare plumbing system and water inlets. The sampling points where the copper
544 concentration was measured are underlined.

545

546 **Figure 2. Phylogenetic relationship between the *P. aeruginosa* ST308 isolates of the**
547 **drinking-water network of the University Hospital of Besançon (France) and high-risk**
548 **clones and reference strains PA14 and PAO1.** The phylogenetic tree is based on cgMLST
549 (3,767 genes). The scale bar indicates the genetic distance, in number of substitutions per
550 site. The prefix in the name of the strains indicates the sequence type.

551

552 **Figure 3. Survival of *P. aeruginosa* ST308 in a copper-containing solution.** Proportion of
553 living bacteria after 24 h of incubation in a 150 µg/L copper (II) sulphate solution
554 represented by a decimal logarithm scale. Values are shown as the mean ratio ± SD from six
555 independent experiments. ANOVA (analysis of variance) with a Tukey HSD (honestly
556 significant difference) test. (a) and (b) represent significant statistical difference, $p < 0.05$.

557

558 **Figure 4. Structure of the 37-kb genomic island, GI-7, associated with tolerance to copper**
559 **in *Pseudomonas aeruginosa* ST308.** The genes and ORFs are shown as arrows, with their
560 orientation of transcription indicated by the arrow. The colours of the arrows indicate the
561 function of the encoded proteins (see colour legend). Base-pair numbering is from the first
562 base of the left direct repeat.

563 **Supplementary data**

564

565 **Table S1.** Characteristics of *P. aeruginosa* isolates compared to the isolates of *P. aeruginosa*
566 ST308 found in the water network of the University Hospital of Besançon (France).

567

568 **Table S2.** Characteristics of *P. aeruginosa* strains harbouring GI-7 (with > 99% homology with
569 GI-7 of isolate ST308 *P. aeruginosa* PA-8 retrieved from the water system of the University
570 Hospital of Besançon, France).

571

572 **Figure S1.** Pulsed-field gel electrophoresis profiles of *DraI*-digested DNA from *P. aeruginosa*
573 ST308 isolates that were recovered from the drinking-water system of the University
574 Hospital of Besançon (France).

Orange building

2	A Unused faucet
1	B Technical facility
0	
-1	C <u>Unused shower</u>

Blue building

3	
2	
1	
0	
-1	
-2	J <u>Storage area</u>

Grey building

8	G Storage area
7	
6	
5	F Storage area
4	
3	
2	
1	
0	
-1	
-2	E <u>Unused faucet</u>
-3	D <u>Crawl space</u>

Water inlets

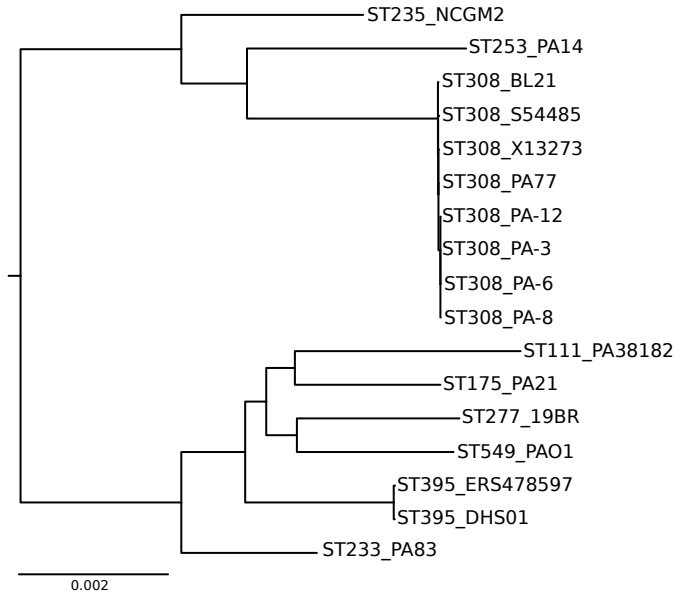
L

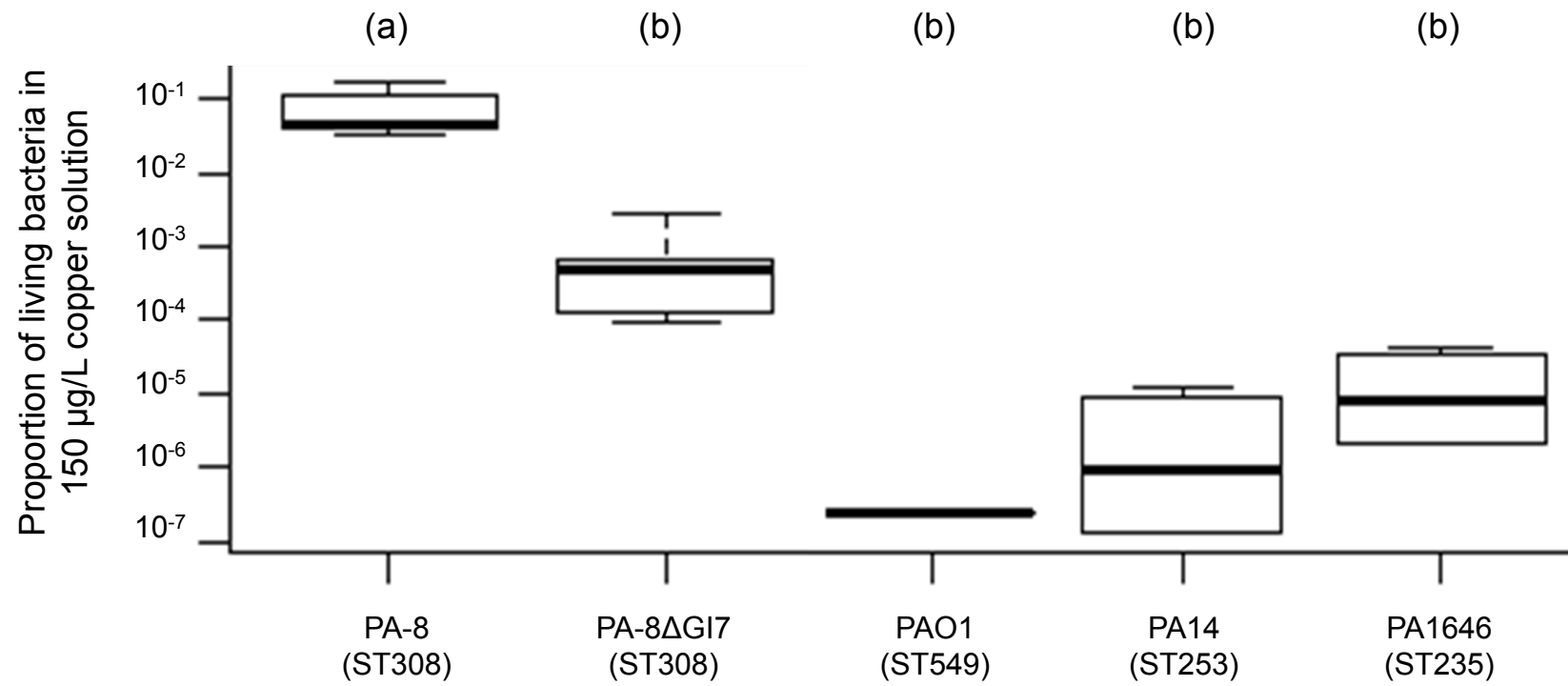
Green building

4	
3	
2	
1	
0	H <u>Unused shower</u>
-1	
-2	I <u>Technical facility</u>
-3	

K







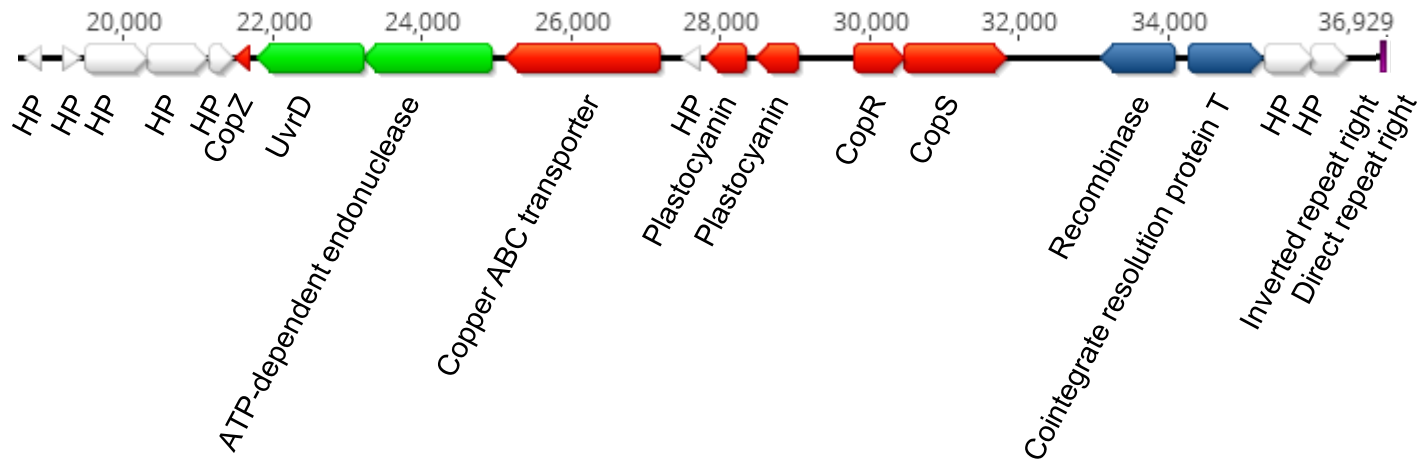
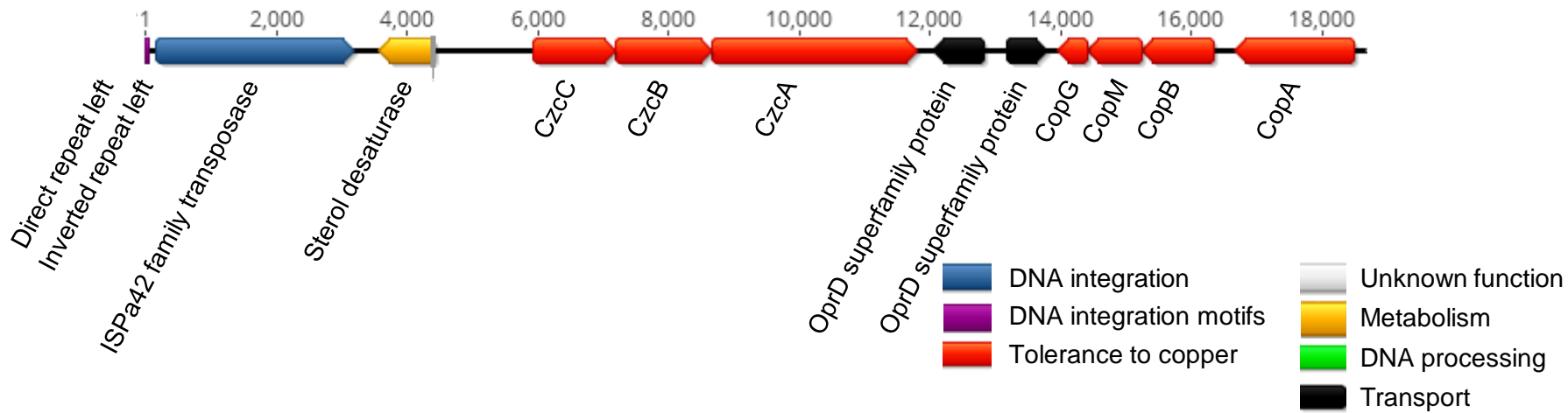


Table 1. Isolation sites of 14 *P. aeruginosa* isolates collected from the hospital plumbing system (Besançon, France).

Location	Site sampled	Cu ²⁺ concentration (µg/L) ^a	Isolation of <i>P. aeruginosa</i>		<i>P. aeruginosa</i> isolates ^b
			With the recommended method	With the adapted method	
Orange building	Unused faucet (A)	NA	No	No	
	Technical facility (B)	NA	No	No	
	Unused shower (C)	13.4 ± 1.4	No	Yes	PA-1, PA-2, PA-7
Grey building	Unused faucet (E)	7.3 ± 0.2	No	Yes	PA-5
	Crawl space (D)	2.7 ± 0.1	No	Yes	PA-10, PA-12^b
	Storage area (F)	NA	No	No	
	Storage area (G)	NA	No	No	
Green building	Unused shower (H)	30.3 ± 25.4	No	Yes	PA-4
	Technical facility (I)	3.3 ± 2.3	No	Yes	PA-11
Blue building	Storage area (J)	143.9 ± 1.9	No	Yes	PA-8^b
Water inlets	Blue building (K)	6.0 ± 0.9	No	Yes	PA-6^b , PA-13, PA-14
	Orange, grey, green buildings (L)	0.6 ± 0.0	No	Yes	PA-3^b , PA-9

^aGeometric mean of three values ± SD.

^bIsolates for which the genome was fully sequenced are shown in bold.

NA: not available



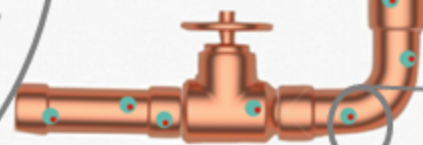
Copper-resistant environmental or phytopathogenic *Pseudomonas* sp.



HGT



Copper-resistant *Pseudomonas aeruginosa*



?



Proportion of living bacteria in 150 µg/L copper solution

