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1 **Cross-regulation and cross-talk of conserved and accessory two-component**
2 **regulatory systems orchestrate *Pseudomonas* copper resistance**

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Short title

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***Pseudomonas* copper-responsive TCSs: cross-regulation and cross-talk**

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12

13 **Abstract**

14

Bacteria use diverse strategies and molecular machinery to maintain copper

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homeostasis and to cope with its toxic effects. Some genetic elements providing copper

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resistance are acquired by horizontal gene transfer; however, little is known about how they

17

are controlled and integrated into the central regulatory network. Here, we studied two

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copper-responsive systems in a clinical isolate of *Pseudomonas paraeruginosa* and deciphered

19

the regulatory and cross-regulation mechanisms. To do so, we combined mutagenesis,

20 transcriptional fusion analyses and copper sensitivity phenotypes. Our results showed that the
21 accessory CusRS two-component system (TCS) responds to copper and activates both its own
22 expression and that of the adjacent nine-gene operon to provide resistance to elevated levels
23 of extracellular copper. The same locus was also found to be regulated by two core-genome-
24 encoded TCSs - the copper-responsive CopRS and the zinc-responsive CzcRS. Although the
25 target palindromic sequence – ATTCATnnATGTAAT – is the same for the three response
26 regulators, transcriptional outcomes differ. Thus, depending on the operon/regulator pair,
27 binding can result in different activation levels (from none to high), with the systems
28 demonstrating considerable plasticity. Unexpectedly, although the classical CusRS and the
29 noncanonical CopRS TCSs rely on distinct signaling mechanisms (kinase-based vs.
30 phosphatase-based), we discovered cross-talk in the absence of the cognate sensory kinases.
31 This cross-talk occurred between the proteins of these two otherwise independent systems.
32 The entire locus is part of an Integrative and Conjugative Element, and was found in other
33 *Pseudomonas* strains where its expression could provide copper resistance under appropriate
34 conditions. The results presented here illustrate how acquired genetic elements can become
35 part of endogenous regulatory networks, providing a physiological advantage. They also
36 highlight the potential for broader effects of accessory regulatory proteins through
37 interference with core regulatory proteins.

38 **Author Summary**

39 Two-component regulatory systems play a key role in bacterial life by detecting and
40 integrating a wide range of signals, allowing bacteria to continuously monitor and adapt to a
41 changing environment. They weave a complex network with a few highly interconnected
42 phosphorelays and numerous cross-regulations. Our study reveals connections between zinc

43 and copper homeostasis in a pathogenic bacterium, with cross-regulation observed for three
44 independent, closely related transcriptional regulators, CzcR, CopR and CusR. Zinc and copper
45 play a major role in host-pathogen interactions, and bacteria that synergize their responses to
46 the two elements can harness a growth advantage and enhanced fitness in specific conditions.
47 We also observed unexpected cross-talk between the core genome-encoded CopRS system
48 and the horizontally acquired CusRS system, although the molecular control exerted by a
49 histidine kinase on its cognate regulator differs. This plasticity observed within the two
50 signaling systems ensures a normal regulatory response to the copper signal required to
51 maintain copper homeostasis.

52

53 **Introduction**

54 Copper can be considered to be a heavy metal “Dr. Jekyll and Mr. Hyde”, as it is an
55 essential trace element in biological systems but, in excess, generates free radicals with
56 deleterious consequences [1,2]. As an antimicrobial, copper is extensively used to treat crops,
57 to enhance breeding, and as part of medicine, with the first reports of use dating back to
58 ancient civilizations. Human physiology exploits its properties, notably by accumulating
59 copper in phagocytic cells and at infection sites where it contributes to protection against
60 viruses, fungi and bacteria [3]. However, bacteria also use copper and have developed
61 mechanisms to deal with its excess.

62 To maintain copper homeostasis, Gram-negative bacteria use various molecular
63 mechanisms and machinery [1,2,4,5]. Cuproproteins are essentially extracellular or
64 periplasmic, participating in important biological processes such as respiration, while
65 chaperones and storage proteins capture intracellular copper cations. Copper transits from

66 the extracellular compartment to the cytosol, through porins or other membrane
67 transporters. Enzymes or chemical chelators, such as glutathione, also manipulate the redox
68 state of copper to allow chaperone loading and extrudation in case of excess. Periplasmic
69 chaperones provide copper for cuproprotein biogenesis or deliver it to export systems. These
70 export systems involve P1B-type ATPases or Resistance-Nodulation-Division (RND)-family
71 multidrug efflux pumps. Periplasmic multi-copper oxidases also contribute significantly to
72 periplasmic detoxification, by oxidizing Cu(I) to Cu(II) they reduce the metal's toxicity [2,5].
73 Synthesis of these molecular machines to ensure an equilibrium between cellular uptake and
74 detoxification is mainly orchestrated by the actions of two types of regulators, MerR-like
75 activators such as CueR that respond to cytoplasmic Cu(I), and two-component regulatory
76 systems (TCSs) that sense periplasmic Cu(I), like the CusRS system [1,2,5]. TCSs are widespread
77 in bacteria and play key roles in monitoring and triggering an adaptive response to
78 environmental cues [6–8]. They comprise sensory histidine kinase (HK) proteins and cognate
79 response regulators (RRs) communicating by phosphotransfer. Phosphorylation of RRs often
80 results in modulation of gene expression, as most RRs are transcription factors. The copper
81 resistance of bacterial species is also highly dependent on their genetic diversity. Indeed,
82 additional determinants can be acquired through horizontal gene transfer (HGT), and these
83 acquisitions tend to increase bacterial survival under copper stress [9,10]. For example, the
84 13-gene 37-kb GI-7 islet found in some *Pseudomonas aeruginosa* isolates was found to provide
85 them a copper-resistant phenotype that plays a key role in bacterial colonization and
86 persistence in hospital environments [10,11]. The accessory genes acquired are often
87 associated with metal and/or antibiotic resistance, such as the *pco/sil* genes carried on the
88 widely distributed Tn7-like structures or on the CHASRI (Copper Homeostasis and Silver
89 Resistance Island) [9,10,12–15].

90 The focus of our study, *P. aeruginosa*, is a metabolically versatile Gram-negative
91 bacterium that exhibits high resistance to copper [5]. This opportunistic animal and plant
92 pathogen is ubiquitously present in the environment, in soil and aquatic habitats. It is one of
93 the major causes of nosocomial infections, establishing acute and chronic infections in
94 immunocompromized patients [16,17]. Importantly, the ability of this bacterium to cope with
95 copper stress was shown to be essential for its pathogenicity in a mouse model [18]. Like other
96 bacteria, *P. aeruginosa* uses several strategies and systems to resist copper poisoning and
97 cope with varying concentrations encountered in its environment [5,19]. Expression of the
98 copper-resistance genes is mainly orchestrated by the cytoplasmic CueR regulator and a TCS,
99 CopRS [19,20]. The cytoplasmic Cu(I)-sensing CueR is the “first responder”, triggered after a
100 copper shock, mainly to control genes whose products are involved in cytoplasmic copper
101 tolerance, notably including an RND pump, the cytoplasmic chaperones CopZ1 and CopZ2, and
102 CopA1. When high periplasmic Cu(I)/Cu(II) levels persist, the “second responder” CopR
103 activates genes involved in periplasmic detoxification, such as *pcoAB* and *ptrA* [5]. The
104 physiological importance of CopRS and its targets in terms of bacterial persistence and
105 virulence has been underlined in chronic and acute wound infections [21]. Recently, the TCS
106 DbsRS was shown to be an additional contributor to the copper response and to copper
107 resistance [22]. CopRS responds only to copper, whereas DbsRS also responds to silver,
108 upregulating the expression of genes involved in protein disulfide bond formation, among
109 others. Interestingly, DbsRS requires more than 4-fold higher levels of copper for activation
110 compared to the other two regulatory TCSs [22]. A short pulse of high copper causes a general
111 stress response in addition to specific copper responses [20]. This general response is probably
112 due to the generation of reactive oxygen species. In contrast, when bacteria grow in a high-
113 copper medium, passive transport functions are downregulated to reduce membrane

114 permeability. Nevertheless, both acute and chronic high-copper conditions trigger the
115 synthesis of targets such as the tripartite RND pump CzcCBA, copper-related proteins PcoAB
116 and P-type ATPase CopA1, MexPQ-OpmE, and the three TCSs – CopRS, DbsRS, and CzcRS [20].
117 The urinary isolate IHMA87 [23], related to *Pseudomonas paraeruginosa* PA7 [24,25],
118 expresses additional, ICE-encoded TCS and copper-resistance proteins. This article reports
119 their molecular and phenotypic characterization.

120 Our results indicated that the accessory TCS is integrated into the core regulatory
121 network for copper homeostasis at *i*) the signal level, by detecting and responding to copper,
122 *ii*) the transcriptional level, as expression of its genes is controlled by several core regulators
123 responding to distinct cues, and *iii*) the molecular level, as we found cross-talk to occur with
124 non-cognate partners. Therefore, regulatory genes that accompany physiologically relevant
125 genes on mobile elements appear as an important part of the bacteria's conserved regulatory
126 network.

127

128 **Results**

129 **CusRS is a copper-responsive and strain-specific TCS**

130 The two divergently transcribed operons (*IHMA87_02165-66* and *IHMA87_02164-57*)
131 present in the urinary isolate IHMA87 are reminiscent of the *copSR-copABGOFCDK* locus
132 identified in the soil-based heavy metal-tolerant β -proteobacterium *Achromobacter* sp. AO22
133 [26] (Fig 1A). These predicted operons are absent from reference strains of *P. aeruginosa*
134 (PAO1, PA14) and from taxonomic outlier PA7, the reference strain of a *P. aeruginosa* clade
135 that has recently been proposed as a new species called *P. paraeruginosa* [25]. The locus is

136 present in a genomic island described hereafter. The regulatory genes *IHMA87_02165-66*
137 encode a putative copper-responsive TCS that shares homology with *P. aeruginosa* CopRS. We
138 named this TCS CusRS due to the strong identity of the predicted proteins with the *E. coli* CusR
139 and CusS (70.7% and 41.5% amino acid identity, respectively). For the *IHMA87_02164-57*
140 genes, we used the “*pcoA2B2GOFCDK*” nomenclature, with “*A2 and B2*” as copies of *pcoA* and
141 *pcoB* genes are already present in the core genome of *P. aeruginosa* and *P. paraaeruginosa*
142 (www.pseudomonas.com; [27]). The predicted operon codes for several proteins required for
143 metal detoxification, including the multi-copper oxidase PcoA2, a putative P-type ATPase
144 (PcoF) and copper-binding periplasmic proteins likely to play a role as chaperones (PcoC, PcoG
145 and PcoK).

146 Previous DAPseq experiments showed that, *in vitro*, recombinant CusR mainly binds to
147 the 254-bp intergenic sequence of the *cusR* and *pcoA2* genes of the *IHMA87* genome [28]. The
148 summit of the enriched DAPseq peak pointed to a perfect palindromic sequence
149 “ATTCATnnATGTAAT” found upstream of a putative *cusR* promoter identified by BPROM
150 (www.softberry.com) (Fig 1B). This palindromic sequence has similarities to the CopR
151 consensus sequence in *P. aeruginosa* (CTGACAn₅GTAAT) (PRODORIC, www.prodoric.de). CusR
152 binding to the intergenic sequence was confirmed by Electrophoretic Mobility Shift Assays
153 (EMSA), and its specificity was demonstrated by competition with an unlabeled probe (Fig 1C),
154 suggesting that CusR might regulate its own expression as well as that of the genes transcribed
155 in the opposite orientation.

156 To assess the copper-responsiveness of the two operons and the involvement of the
157 CusRS system in this response, we analyzed the expression of transcriptional *lacZ* reporter
158 fusions with 500-bp sequences upstream of the *cusR* and *pcoA2* coding sequences in the wild-

159 type strain and the *cusR*-deleted mutant. For copper stress conditions, we exposed the
160 bacteria to 0.5 mM CuSO₄ for 2.5 h. This concentration and duration do not affect bacterial
161 viability [19]. Exposure of the wild-type strain to copper led to significant stimulation of
162 expression of both *cusR* and *pcoA2* (Fig 1D,E). In contrast, no induction of *pcoA2* was observed
163 following exposure of the *cusR* mutant to copper (Fig 1E), and activation of the *cusR* promoter,
164 while still detected, was reduced about two-fold (Fig 1D). CusR therefore appears to control
165 expression of both operons in a copper-dependent manner, but *cusR* expression must also be
166 controlled by another copper-responsive regulatory factor.

167 To assess the importance of promoter elements, we constructed and analyzed a set of
168 *lacZ* fusions associated with *cusR* upstream sequences of various lengths. Deletions affecting
169 the palindromic sequence identified or the predicted -10/-35 boxes abolished expression,
170 whereas a short fragment containing the palindrome maintained a copper response, but
171 reduced both basal and activated expression levels (Fig 1D). Interestingly, mutation of a 4-bp
172 sequence within the palindrome (ATTACATnnATAGCCT) led to complete loss of the copper-
173 responsiveness of the promoter. When examining the *pcoA2* promoter region (Fig 1E),
174 shortening the sequence reduced the fold-change in expression following exposure to copper,
175 whereas mutations in the palindrome led to the complete loss of copper-mediated *PpcoA2*
176 activation. As expression of the *pcoA2* promoter is CusR-dependent, we concluded that CusR
177 controls *pcoA2* expression by binding to the palindromic sequence identified.

178 To assess the extent of the *pco* operon, the mRNA levels of several genes within the
179 locus (*pcoB2*, *pcoF*, and *pcoK*) and that of a downstream gene encoding a putative LysR-type
180 transcription factor were measured by RTqPCR. Transcription was increased for all targets
181 following copper stimulation, although the level of induction measured decreased with the

182 distance between the gene and the *pcoA2* promoter (S1 Fig). In all cases, interruption of *pcoA2*
183 transcription by introducing an omega interposon abolished the copper-inducing effect down
184 to *lysR*, indicating that the last gene in the locus, *lysR*, is part of the same transcriptional unit.

185 Overall, these data show that the CusRS system in IHMA87 is functional and responds
186 to copper. Following binding to a single site, CusR regulates its own expression and controls
187 the expression of the nine-gene “*pcoA2*” operon, which contains genes that may play a role
188 in copper resistance.

189 **CusR and the *pcoA2* operon contribute to copper resistance**

190 Copper concentrations in urine are known to increase during UTIs, as part of an innate
191 antimicrobial strategy [3,29]. Given the origin of IHMA87, we first questioned whether it had
192 higher copper resistance compared to three reference strains. For these reference strains, we
193 found *P. aeruginosa* PAO1 and PA14 to be more sensitive to 20 mM CuSO₄ than the *P.*
194 *paraeruginosa* strain PA7 (S2 Fig). In these tests, IHMA87 had the highest resistance capacity,
195 which could be provided by the additional, putative copper-resistance machinery, Pco,
196 acquired through HGT. To verify whether this was a common feature of UTI isolates, we also
197 studied two others – JT87 and TA19, which like IHMA87 are members of the PA7-like group.
198 These other strains showed variable resistance profiles, with TA19 being the most sensitive of
199 all the strains tested. Thus, high copper resistance does not appear to be a common feature
200 of UTI isolates.

201 Therefore, we investigated the role of the CusRS system and the *pcoA2* operon-
202 encoded proteins in the high copper resistance of IHMA87. First, CusR was compared to the
203 two well-described copper regulators, CopR and CueR. To do so, survival of individual, double,
204 and triple mutants was analyzed on minimal medium in the presence of high copper

205 concentrations (5 mM or 20 mM) and compared to that of the wild-type IHMA87 strain. As
206 shown in Fig 2A, mutation of the *cueR* gene did not affect copper resistance whatever the
207 genetic background, whereas the absence of CopR considerably reduced survival of the
208 bacteria, revealing a key role of CopR and its regulon in the response to copper stress. When
209 CopR was absent, the additional loss of CusR made the bacteria highly sensitive to copper
210 toxicity. This sensitivity was observable at 5 mM CuSO₄ and accentuated at the higher
211 concentration (Fig 2A). To determine whether this phenotype was caused by the absence of
212 the CusR regulator *per se*, by the lack of *pcoA2-lysR* expression, or by a combined effect, we
213 introduced in several mutants an omega interposon into the *pcoA2* gene, stopping
214 transcription of the operon from the first gene. This interposon strongly affected the copper
215 resistance of the $\Delta copR$ *pcoA2::Ω* strain compared to that of the $\Delta copR$ mutant, with an effect
216 observable from 5 mM CuSO₄ (Fig 2B). Thus, the proteins encoded by the CusR-regulated
217 *pcoA2* operon are involved in copper detoxification and resistance.

218 We further investigated the contribution to copper resistance of the different copper
219 regulatory systems and the proteins encoded by the *pcoA2* operon. To do so, we followed the
220 growth of appropriate mutant strains in lysogeny broth (LB) and compared it to IHMA87 wild-
221 type growth. Firstly, increasing the copper concentration from 1 to 4 mM led to an extended
222 lag phase for wild-type growth (S3A Fig). Significant cell death has been reported in the
223 bacterial population after exposure to this high copper concentration for 2 h [19]. As the lag
224 phase is a dynamic and adaptive period preparing bacteria for cell division, its extension
225 indicated that more time is required both to repair altered cells and for physiological
226 recuperation by stressed cells before exponential growth can occur [30,31]. Secondly, we
227 observed that *cueR* and *copR* mutant growth was even more dramatically affected than wild-
228 type growth in the presence of 4 mM CuSO₄. For these strains, especially $\Delta copR$, the lag phase

229 was considerably extended (S3A Fig). This increased delay before division indicated that the
230 copper-resistance mechanisms controlled by the two regulators are important for survival in
231 and adaptation to the stress. Interestingly, growth of $\Delta cusR$ and $pcoA2::\Omega$ mutants was
232 unaffected, and was similar to IHMA87 growth, indicating that these mutations had no effect
233 on the copper resistance. In contrast, the lack of both CopR and Pco proteins completely
234 abolished bacterial growth (S2A Fig). This result further underlined the important role in
235 bacterial copper resistance played by the proteins encoded by the $pcoA2$ operon. In the
236 conditions used here, this role can be masked by proteins from the CopR regulon. Finally, we
237 introduced an arabinose-inducible promoter (*PBAD*) into the chromosome, upstream of the
238 $pcoA2$ operon, and compared bacterial growth of the wild-type and modified strains over a
239 range of copper concentrations. Increased expression of the operon conferred a clear growth
240 advantage relative to wild-type bacteria, allowing faster adaptation to exposure to 5 mM
241 $CuSO_4$, as indicated by the shorter lag phase (S3B Fig).

242 In conclusion, IHMA87 has a high intrinsic capacity to resist copper stress that can be
243 further increased by overexpressing the $pcoA2$ operon.

244 **Three TCSs cross-regulate the *cusR-pcoA2* intergenic region**

245 As shown in Fig 1D, copper activates *cusR* expression even in the absence of CusR. As
246 CopR binds *in vitro* to the *cusR-pcoA2* intergenic region [28], we assessed its role as well as
247 that of the copper-responsive regulator CueR in the residual activity of *PcusR* and that of the
248 divergent promoter. To do so, we used transcriptional *lacZ* fusions in several genetic
249 backgrounds lacking one, two, or all three regulators. We found that CueR was dispensable in
250 all the conditions tested (Fig 3A, B). However, induction of *cusRS* expression was reduced in
251 the absence of CopR compared to the wild-type background, and was lost in the $\Delta cusR\Delta copR$

252 mutant. This result confirmed CopR as the additional element responsible for the complete
253 copper induction of *cusRS* in combination with CusR. Activation of the *cusRS* promoter by both
254 CusR and CopR was abrogated by mutating the palindrome (Fig 1D), demonstrating that the
255 same intergenic binding site is required *in vivo* for activation by both regulators.

256 For the expression of the *pcoA2* operon, as mentioned above, no copper induction was
257 observed in the $\Delta cusR$ strain (Fig 1E, 3B). Interestingly, *pcoA2* expression was higher in $\Delta copR$
258 than in the parental strain upon exposure to copper, suggesting a negative regulatory role of
259 CopR. In contrast, expression of *pcoA2* genes was abolished in the $\Delta cusR\Delta copR$ strain,
260 indicating that the increased activation observed in the presence of copper in $\Delta copR$ was the
261 result of CusR activity (Fig 3B). This observation showed that CopR binding does not lead to
262 transcriptional activation of the *pcoA2* promoter and, by competing for binding on the same
263 palindrome, CopR limits access for CusR, therefore reducing *pcoA2* activation.

264 To further explore cross-regulation between the different pathways, we tested
265 whether CusR affects the expression of *copA1* and *pcoA*, which are respectively regulated by
266 CueR and CopR [19]. Expression of the *copA1* promoter was abolished in the absence of CueR,
267 but was unaffected by deletion of either *cusR* or *copR* (Fig 3C) as expected based on *in vitro*
268 binding data [28]. *pcoA* expression had almost the opposite profile, with no effect observed
269 following *cueR* deletion, but a complete lack of copper-induced expression in the *copR* mutant
270 and a slight reduction in the copper-responsiveness in the *cusR* mutant (Fig 3D). This latter
271 result was unexpected as *in vitro* results indicate that CusR does not bind to this promoter
272 [28]. Suspecting an indirect effect, we measured expression of *PpcoA-lacZ* and *PpcoA2-lacZ*
273 fusions in the *pcoA2::Ω* strain. Levels of both transcriptional fusions were decreased in the
274 presence of copper compared to levels in the wild-type strain, whereas the level of CueR-

275 regulated *copA* expression was similar for the two strains (Fig 3E). Based on these
276 observations, the proteins encoded by the *pcoA2* operon may be required for the CopRS and
277 CusRS systems to fully develop their copper-dependent activity. The effect is probably due to
278 altered copper compartmentalization in the bacteria. These data also revealed an additional
279 indirect layer of regulation, since the primary target of CusR, the *pcoA2* operon, seems
280 important for both TCSs to mount a copper response.

281 Mining and reanalyzing the DAPseq data [28] indicated that three other RRs strongly
282 targeted the *pcoA2-cusR* intergenic sequence centered at the same position (S4A Fig). These
283 RRs are the two poorly characterized regulators IrlR and MmnR, and the well-known zinc-
284 responsive protein CzcR that binds to the sequence “GAAAC₆GTAAT” [32]. Additional target
285 genes were found to be shared by CopR, CusR and CzcR (S4B,C Fig), presumably due to the
286 extensive similarity of their consensus sequences, especially the second half-site “GTAAT”.
287 Therefore, we analyzed the roles of these additional RRs on the expression of both the *cusRS*
288 and *pcoA2* operons.

289 The TCS CzcRS is known to respond to several signals, including zinc stress [33]. We
290 therefore assessed its effect on *pcoA2* and *cusRS* expression in the presence of copper and/or
291 zinc. As a control of CzcR activity, we introduced a *PczcC-lacZ* fusion into the wild-type and
292 $\Delta czcR$ strains, as a known CzcR target. Fig 4 shows that, as expected, the *czcC* promoter was
293 upregulated after the addition of zinc, with or without the addition of copper, and that this
294 upregulation was CzcR-dependent. Furthermore, the expression of *pcoA2* was activated by
295 zinc to almost the same level as by copper. Exposure to both stimuli was slightly, but
296 significantly, more efficient. Zinc activation was lost in the absence of CzcR, and the *pcoA2*
297 expression level dropped to that observed in the wild-type in the presence of copper alone

298 (Fig 4). Therefore, both CusR and CzcR control and activate expression of the *pcoA2* operon to
299 similar extents. A distinct expression profile was observed for the *cusR* promoter: while copper
300 strongly activated its expression, the induction following exposure to zinc was lower. In
301 addition, zinc bypassed the copper effect when bacteria were simultaneously exposed to both
302 metals. In the absence of CzcR, this “restraining” effect was lost, and the high-copper-
303 inducible effect was restored, suggesting that CzcR must conceal the CusR binding site.

304 We then assessed a potential regulatory role of MmnR and IrlR and observed no
305 *mmnR* or *irlR* deletion phenotypes in terms of *pcoA2* operon and *cusR* expression, even in the
306 presence of copper (S6 Fig). However, we cannot exclude that this was due to an absence of
307 signal and activation of the sensor in our experimental conditions.

308 Overall, the data indicate that *cusRS* and *pcoA2* operons are controlled *in vivo* by at
309 least three TCSs: CusRS, CopRS, and CzcRS. The three RRs bind to the same palindromic site
310 but modulate expression of the divergent operons differently. Indeed, while CusR and CzcR
311 activate both operons, even if the latter is less effective on the *pcoA2* promoter, CopR only
312 activates *cusRS*, repressing the *pcoA2* operon by hindering the positive action of CusR (S7 Fig).

313 **Molecular cross-talk between the CopRS and CusRS systems**

314 The *P. aeruginosa* CopRS system works in an unusual way [34]. Instead of relying on a
315 conventional “phosphorylation-based mechanism” where the sensor allows the
316 phosphorylation of the response regulator upon detecting a signal, CopS displays phosphatase
317 activity toward CopR. Exposure to copper thus relieves the inhibition by allowing CopR to
318 activate its target genes. The mechanism leading to phosphorylation of CopR remains
319 unidentified. We confirmed the “phosphatase-based mechanism” of the CopRS system in the
320 *P. paraeruginosa* strain IHMA87, as demonstrated in Fig 5A. Indeed, in a *cusRS* background,

321 the absence of CopS led to a high-copper-blind expression of *pcoA*, whereas constitutive
322 phosphatase activity of the sensor CopS_{His235A} mutation abolished *pcoA* expression. The same
323 pattern was observed for *cusR* expression (Fig 5A). This result also confirmed that CopR can
324 activate expression of this promoter in a $\Delta cusRS$ background, as previously suggested by the
325 data presented in Fig 3A.

326 We then analyzed the functioning of the CusRS system in a genetic background lacking
327 the CopR and CopS proteins to avoid any protein interference. As shown in Fig 5B, neither
328 *pcoA2* nor *cusRS* operons were activated in the strain lacking the sensor protein CusS. This
329 result indicates both that this HK is required for CusR activation by phosphorylation, and that
330 no other protein can phosphorylate CusR. We also confirmed that the phosphorylated form
331 of CusR is the active form, because no gene expression was observed when its conserved
332 phosphorylation site (Asp₅₄) was mutated to an alanine (Fig 5B). In addition, mutation of the
333 autophosphorylation site of CusS (His₂₆₆) abolished the expression of the two operons,
334 indicating that it is required for the system to function properly (autophosphorylation prior to
335 phosphotransfer). Altogether, these data show that the CusRS system is a canonical TCS,
336 functioning like the *E. coli* CusRS system, with the detection of a signal triggering a
337 phosphorylation cascade ultimately leading to target gene activation.

338 The two TCSs were then studied in different combinations of mutants of their HK gene
339 to obtain information on possible cross-talk between non-cognate HKs and RRs. The results
340 are summarized schematically in S7 Fig. Focusing on a CopR-only-activated promoter, we
341 found that deletion of *copS* led to an identical copper response pattern of *PpcoA-lacZ*
342 expression to that observed in the wild-type strain or in a strain lacking the *cusS* gene (Fig 5C).
343 However, deletion of both *copS* and *cusS* led to the strong constitutive activation of *PpcoA*

344 (copper-blind expression) that was observed in the $\Delta copS$ mutant in the absence of CusRS (Fig
345 5A). This result indicates that the HK CusS can compensate for an absence of the HK CopS,
346 controlling CopR activity in the absence of the cognate partner. Thus, like CopS, CusS could act
347 as a CopR phosphatase to prevent gene expression in the absence of copper when its cognate
348 partner is absent. Alternatively, interaction between CusS and CopR in the absence of CopS
349 might prevent phosphorylation of CopR in the absence of signal. We further observed that
350 control of CopR by CusS does not require its cognate HK to be absent. Indeed, normal
351 expression of *PpcoA* was observed when the constitutive phosphatase CopS_{H235A} was
352 produced (Fig 5C), indicating that CusS permits CopR phosphorylation following detection of
353 copper.

354 To determine whether CopS could also interact with CusRS, we used *PcusR-lacZ*
355 (Fig 5D) and *PpcoA2-lacZ* (Fig 5E) transcriptional fusions in several mutant genetic
356 backgrounds. As expected, absence of *copS* had little effect on the two promoters: indeed
357 CusS could control the phosphorylation status of CusR and of CopR, as observed above, and
358 both these proteins activated transcription only in response to the copper signal. However,
359 when *cusS* was deleted, in the absence or presence of copper, a slight increase in *PcusR*
360 expression levels was observed (Fig 5D), and the effect on *PpcoA2* expression was even more
361 pronounced (Fig 5E). Transcriptional activation could be attributed to activity of
362 phosphorylated CusR as the signal was lost when CusR_{D51A} was synthesized in the $\Delta cusS$
363 background mutant. Thus, CopS appears to permit CusR phosphorylation in the presence of
364 copper, but also in its absence, and to control its phosphorylation status, although not as
365 tightly as the cognate HK CusS. *PcusR* activation was not observed in the CopS_{H235A} $\Delta cusS$ strain,
366 supporting the need for CopS for phosphorylation of CusR (Fig 5D). Copper-blind expression
367 of both *PcusR* and *PpcoA2* was then observed in the $\Delta cusS\Delta copS$ mutant. As CusR cannot be

368 phosphorylated in bacteria lacking both sensors (CusS and CopS), the *PcusR* expression
369 pattern observed (Fig 5D) can be attributed mainly to dysregulated CopR activity (Fig 5C).
370 However, high levels of unphosphorylated CusR also contributed to these activities as the
371 copper-blind expression of *PcusR* noticed in *CusR_{D514A}ΔcopSΔcusS* was still observed, albeit to
372 a lesser extent (Fig 5D). This unphosphorylated CusR activity was clearly observed on CusR-
373 only-activated *pcoA2* promoter in *ΔcusSΔcopS* that displayed copper-blind expression (Fig 5E):
374 as expected, similar activity levels were then observed when the CusR phosphorylation site is
375 mutated in the double mutant background (*CusR_{D514A}ΔcopSΔcusS*) (Fig 5E). Furthermore,
376 *PpcoA2* expression levels were lower in *ΔcusSΔcopS* compared to in *ΔcusS*, which underscores
377 the negative effect of phosphorylated CopR binding to the promoter (Fig 5E). In the
378 *CopS_{H235A}ΔcusS* background, the *pcoA2* promoter was not expressed, as no CusR is synthesized
379 (Fig 5D).

380 Finally, we wondered whether the CopRS and CusRS systems respond to and modulate
381 their regulon within the same range of copper concentrations. We compared their individual
382 sensitivities to copper and their sensitivity relative to that of the cytoplasmic detector CueR.
383 To do so, we used *lacZ* fusions to monitor the expression of a specific target of each
384 transcriptional regulator over time in response to different copper levels. We found CopRS
385 and CusRS to display a similar sensitivity and response to copper, inducing their target in the
386 presence of 0.5 mM CuSO₄. In contrast, CueR activates its target at lower copper
387 concentrations (from 0.01 mM) (S8 Fig).

388 To conclude on cross-talk, not only do the CopRS and CusRS systems share similar DNA-
389 binding sites and a target sequence, their HK can compensate for each other, with higher
390 efficiency observed for CusS.

391 **Horizontal acquisition of the *cusRS* and *pcoA2* operons**

392 As mentioned above, the reference *P. paraeruginosa* strain PA7 lacks the *cusRS* and *pcoA2*
393 operons. Analysis of the genomic environment surrounding this copper-related locus showed
394 that it is present on a genomic island that presents the features of an Integrative and
395 Conjugative Element (ICE) (Fig 6A). ICEs are large mobile genetic elements that are crucial for
396 the genetic plasticity of bacteria [35,36]. Consequently, the copper-related locus was probably
397 acquired by HGT. We named this island ICE1_IHMA, and it is one of three predicted ICEs in the
398 IHMA87 genome. It is 96,269 bp long, inserted at a tRNA-Gly site, and has an average GC
399 content of 64%. Among the 100 predicted coding sequences present on ICE1_IHMA, some
400 correspond to core ICE genes, required for the element's life cycle. These include signature
401 genes coding for an integrase of phage origin (*int*) and a MOB(H)-type relaxase [37]. In
402 addition, the element possesses a set of mating-pore formation (MPF) genes encoding a T4SS
403 (*tfc* genes) including an ATPase (*tfc16/virB4*) and a Type IV coupling protein (*tfc6/t4cp2*).
404 Among the eight types of MPF system – classed based on diverse features – ICE1_IHMA is a
405 type G (MPF_G). These systems are frequently found in Proteobacteria [38]. ICEs also carry
406 specific cargo genes, which usually provide the host with benefits and diverse phenotypic
407 properties [35]. Based on sequence homology and the organization of its core genes,
408 ICE1_IHMA is highly similar to PAGI-2, a prevalent *clc*-like family ICE in *P. aeruginosa* [16] (Fig
409 6A). By comparison with PAGI-2, we defined two specific cargo modules in ICE1_IHMA, named
410 A and B. Module A comprises four genes, of which three (*IHMA87_02154-52*) are predicted to
411 encode a CzcCBA-type heavy metal RND efflux pump. To assess whether this pump could be
412 involved in copper efflux, we deleted *IHMA87_02154-52*. No effect was observed in terms of
413 survival of the mutant obtained upon exposure to a high copper concentration (S2 Fig). In line

414 with this result, the promoter region of *IHMA87_02154-52* was not targeted *in vitro* by CusR
415 or CopR [28]. The other module, Module B is composed of 21 cargo genes, among them the
416 *cusRS* and *pcoA2* operons, but also several genes predicted to be involved in resistance to
417 chromate and arsenic compounds. Copper, arsenic, and chromium resistance genes
418 frequently co-occur in mobile genetic elements, favoring the spread of both metal resistance
419 genes and antibiotic resistance. The spread of such resistance is of concern because co-
420 selection promotes the acquisition of antibiotic resistance [10,39]. Although the gene
421 encoding the LysR-type regulator is present on PAGI-2, it was considered to be part of Module
422 B, since we demonstrated that it was part of the *pcoA2* operon.

423 As ICEs are modular and cargo genes can be exchanged, we assessed how the two cargo
424 modules are distributed in bacterial genomes by performing blast searches against the NCBI
425 database (Fig 6B). Modules A and B from ICE1_IHMA are both present in several bacteria from
426 various genera. The most frequently represented genus was *Pseudomonas*, and the
427 corresponding predominant species are *aeruginosa* and *paraeruginosa*. The two modules
428 were also identified in other genera with distinct distributions, for example in a few
429 *Achromobacter* and *Stenotrophomonas* genomes, whereas only Module A was identified in
430 some *Comamonas* genomes. Interestingly, if the *lysR* gene located at the 3' extremity of the
431 *pcoA2* operon is excluded from the search for Module B, identical results were obtained. This
432 further emphasizes its co-occurrence with the *cusRS* and *pcoA2* operons, and suggests a
433 possible role in their regulation.

434 We next focused our analysis on the distribution of the region from *lysR* to *cusS*, which
435 comprises the entire sequence of the two operons (Fig 6B). Initially, the search provided more
436 hits than obtained for searches with the whole of Module B, suggesting that this region can
437 be acquired or maintained independently of the rest of the module. The genera harboring the

438 locus were similar to those with the module B, but the distribution varied with a higher ratio
439 of *Pseudomonas*. Among species, the region was mostly represented in *P. aeruginosa* and
440 *P. paraeruginosa*, with only two strains of *Pseudomonas* not belonging to these species. We
441 then examined the distribution and conservation of the *lysR-cusS* region over a database of
442 494 complete *P. aeruginosa* and *P. paraeruginosa* genomes. The clades were named based on
443 the approach described by Freschi *et al.* [40] from 1 to 5, and, as proposed by Rudra *et al.* [25],
444 outlier group 3 was (re)classified as *P. paraeruginosa* (*P.p*). Bioinformatics analysis revealed
445 the locus to be present in several strains of *P. aeruginosa* clades 1 and 2, and (as expected) in
446 the IHMA87 strain, which is classed in clade 3 (Fig 6C). The locus was not detected in the few
447 clade 4 and 5 strains for which genomes are available (www.pseudomonas.com; [27]). The
448 locus was present as part of a putative ICE in over 80% of genomes. In the remaining 20% of
449 genomes, it was part of regions bearing some similarity to an ICE, as determined based on
450 neighboring genes and their predicted functions. The reason these regions were not
451 immediately predicted to be ICEs can be attributed either to ICE degeneracy, or to a detection
452 failure. In all the loci identified, the motif recognized by CusR in the *pcoA2-cusRS* intergenic
453 sequence was conserved, indicating that the copper-resistance genes can be integrated into
454 the bacterial regulatory network, allowing them to be readily expressed in their host under
455 appropriate conditions.

456 Taken together, these data show that the *lysR-cusS* locus involved in copper resistance
457 is mainly found in the *Pseudomonas* genus where it can be acquired by HGT as part of an ICE.
458 As demonstrated by our research, the integration of this locus into the regulatory network
459 involves cross-regulation and cross-talk with endogenous regulatory actors. These constraints
460 must limit the acquisition of the locus by other genera.

461 Discussion

462 In this study, we examined the integration of an acquired copper-resistance locus into
463 a housekeeping system, both at the level of regulatory pathways and in terms of the
464 protection it affords against toxic copper concentrations. The ICE-encoded copper-responsive
465 TCS CusRS was shown to control expression of its own gene and a divergently transcribed nine-
466 gene operon, *pcoA2B2GOFCDK-lysR*. The copper-related elements described here were
467 initially acquired by HGT, and are not the only example of accessory copper genes found in *P.*
468 *aeruginosa* species [11]. In bacteria, acquired systems can be even more efficient in coping
469 with copper stress than the core mechanisms, as observed for the *copXL* locus in
470 *Staphylococcus aureus* [41]. Although capable of conferring bacterial copper resistance, the
471 results presented here showed that the positive effects of the *P. paraaeruginosa* Pco proteins
472 are masked by those of the core copper homeostasis proteins, controlled by the CopRS system
473 [19]. However, mutation of the *pcoA2* operon reduced the metal response of both the CopRS
474 and the CusRS regulatory systems (Fig 2B): this indicates that Pco proteins also play a role in
475 maintaining copper compartmentalization that affects signal detection by both acquired and
476 conserved sensory HKs. Our results showed that the *pcoA2* operon contributes to overall
477 copper homeostasis as it is under the control of the main core TCS CopRS. The control of
478 accessory genes by housekeeping TCSs has previously been documented for copper
479 homeostasis regulation. For example, in *E. coli*, although chromosome-encoded CusRS and
480 plasmid-borne PcoRS systems are independent TCSs, CusR activates expression of the
481 plasmid-encoded *pcoE* gene [42]. Similarly, in emerging pathogenic *S. aureus* strains, the core-
482 encoded regulator CsoR controls expression of genes present in several mobile elements,
483 conferring a phenotype that is hyper tolerant to copper [41,43]. In the *P. paraaeruginosa* strain

484 studied here, we found CopR to downregulate expression of the *pcoA2* operon, probably by
485 competing with CusR for DNA binding (Fig 3B). This finding was unexpected as the *pcoA2*
486 operon does not appear to be a poisoned gift, given that it can improve the bacteria's
487 resistance to copper stress when artificially overexpressed.

488 Although the homologous CopRS and CusRS systems respond to the same molecule
489 over the same concentration range, their downstream signaling mechanisms differ. The HKs
490 are often bifunctional enzymes with both phosphatase and autokinase activities, differentially
491 triggered depending on the signal detection [44]. We confirmed that the CopRS system relies
492 on a “phosphatase mechanism” in *P. paraeruginosa* like that observed in *P. aeruginosa* [34],
493 where CopS dephosphorylates CopR. The copper signal shuts down the phosphatase activity
494 of CopS and, consequently, CopR becomes active, triggering gene expression. With this *modus*
495 *operandi*, absence of HK leads to a copper-blind activation of the CopR regulon [34]. In the
496 strain studied here, the CusRS system acted like a canonical TCS, functioning more like the
497 recently discovered copper-responsive DbsRS TCS in *P. aeruginosa* [22]. DsbS can
498 dephosphorylate its partner DsbR and, upon direct binding of copper, it can
499 autophosphorylate. The phosphate is subsequently transferred to DsbR, which in turn
500 activates target genes, notably those responsible for copper resistance [22]. Although no CusR
501 dephosphorylation was observed, the data indicated that CusS HK could dephosphorylate the
502 non-cognate CopR in the absence of CopS.

503 The *P. paraeruginosa* IHMA87 proteins CusR and CusS share 70.7% and 41.5%
504 sequence identity, respectively, with their *E. coli* homologs [27]. In addition, both CusR
505 regulators have a perfectly palindromic DNA-binding site and a very restrictive regulon [42].
506 Only one significant target sequence was identified for IHMA87 CusR by DAPseq [28]. This

507 sequence is the palindrome “ATTCATnnATGTAAT”, located in the intergenic sequence *pcoA2-*
508 *cusR*. It is perfectly conserved in the equivalent locus in *Achromobacter* sp. AO22 [26]. *E. coli*
509 CusR preferentially binds to the palindrome “AAAATGACAAnnTTGTCATTTT” [42], located
510 between the two divergent operons *cusRS* and *cusC(F)BA*. Although no other chromosomal
511 binding site could be identified [45], *E. coli* CusR also directly controls expression of the
512 plasmid-borne gene *pcoE*, which has palindromic sequence (TGACAAnnTTGTCAT) in its
513 promoter. Another feature of the *E. coli* CusRS system is its ability to confer resistance to silver
514 toxicity. Thus, the HK CusS was shown to bind to both copper and silver [46,47]. As observed
515 for chromosomally-encoded CusRS and plasmid-encoded PcoRS systems in *E. coli* [42], CopRS
516 and CusRS are independent systems. However, in addition to the known cross-regulation (at
517 the level of both gene target and signal) [44], we demonstrated *in vivo* molecular
518 communication between the unrelated HK-RR pairs. Variable levels of cross-talk within TCS
519 signaling pathways have also been reported in other bacteria, ranging from absent (or not
520 observed) to highly represented as in the case of *Mycobacterium tuberculosis* [48,49]. As TCSs
521 often arose during evolution through gene duplication, the high levels of sequence identity
522 shared by the paralogs may favor interactions. However, distinct mechanisms normally evolve
523 rapidly after duplication to insulate the systems, preventing inappropriate interactions and
524 favoring the specificity of the molecular interactions within each system. Although generally
525 considered to be a disadvantage for the bacteria, as it decreases the specific response and
526 may trigger inappropriate responses, cross-talk can also be beneficial. Indeed, it can provide
527 some evolutionary benefits, such as complex signal-processing mechanisms or priming to
528 respond to future stimuli [44,49,50]. In the IHMA87 strain, our results indicate that CusS could
529 compensate for the absence of CopS by maintaining the relevant CopR phosphorylation status
530 thanks to its copper-responsive function. CusS could also control CopR activity in the presence

531 of the constitutive phosphatase CopS_{H235A} (Fig 5C). The reciprocal reaction was less efficient,
532 as CopS was only partially capable of replacing CusS in its interaction with CusR. Whether the
533 system also controls CusR phosphorylation is less clear, as this reaction requires a
534 phosphotransfer step not reported for the CopS-CopR pair. In addition, results were blurred
535 by cross-regulation exerted by the two TCSs on target genes (Fig 5D,E). We nevertheless
536 produced schematic models that fitted the *in vivo* results indicating the cross-talk between
537 CusRS and CopRS (S7 Fig). These models will need to be consolidated by further experiments,
538 in particular to determine the extent of the protein interactions and the HK activities toward
539 the cognate/non-cognate RRs.

540 The *in vivo* cross-talk detected between the CusRS and CopRS systems in
541 *P. paraeruginosa* was unexpected. First, CopRS is encoded by the core genome of the
542 bacterium whereas CusRS is coded by genes present in the HGT-acquired ICE. Second, while
543 the RRs of the two systems share 69% sequence identity, the HKs CopS and CusS only share
544 36.4% identity. These two HKs also act differently on their cognate RRs. Third, cross-talk
545 usually involves the absence of both the reciprocal RR and the cognate HK of two distinct TCSs,
546 as observed for CpxA–CpxR and EnvZ–OmpR in *E. coli* and mentioned for cross-talk from CusS
547 to OmpR and CpxR [44,50]. The reason is that the RRs usually compete with other RRs for
548 interaction with their cognate partner(s) to prevent misregulation. However, here, in the
549 absence of CopS, CusS controlled the activity of CusR and CopR normally. Moreover, it did so
550 even in the presence of the constitutive phosphatase CopS_{H235A}. Although it originated from a
551 lateral transfer, our bioinformatics analyses revealed that the locus is mainly present in
552 *P. aeruginosa* and *P. paraeruginosa* strains, where the genes are probably readily expressed.
553 The expression advantage of copper resistance on bacterial fitness might explain this
554 distribution. In addition, the cross-regulation and cross-talk with endogenous regulatory TCSs

555 may be required for appropriate integration of the locus, making it possible to tightly control
556 expression without creating a burden. This would naturally limit spread of this ICE to other
557 species.

558 Despite having been acquired by HGT, we also observed that the *cusRS-pcoA2* locus
559 was a site of fierce competition for regulation, completely integrated into the conserved TCS
560 regulatory network. Indeed, four core genome-encoded RRs – IrlR, MmnR, CzcR and CopR – in
561 addition to the accessory CusR can bind the *cusRS-pcoA2* intergenic region *in vitro* [28]. All
562 these RRs belong to the OmpR family with winged-HTH effector domains. The corresponding
563 genes are organized in a two-gene operon with downstream, overlapping genes encoding the
564 cognate HK (www.pseudomonas.com; [27]). Phylogenetic analysis clustered them together,
565 inferring that they emerged by RR-HK pair duplication [51]. A genome-wide binding assay
566 revealed these closely related regulators to share similar DNA-binding motifs, and extensively
567 overlapping gene targets (Fig S4) [28]. The physiological relevance of IrlR and MmnR binding
568 could not be assessed here, as the signals detected by their cognate HK are as yet unknown.
569 Although the *mmnRS* locus was reported to be upregulated in response to copper shock [52],
570 we observed no effect of *mmnR* inactivation on the expression of *cusRS* and *pcoA2* in the
571 presence of copper (S5 Fig).

572 Even if the five RRs bind to similar targets *in vitro*, their physiological role is more
573 delimited, as CopR, CusR and CzcR are known to have their own regulon with only a few shared
574 and cross-regulated targets. Similarly, in *Pseudomonas stutzeri*, comparison of the *in vitro*
575 binding sites and regulons of CzcR and the paralogs CopR1/CopR2 revealed limited target
576 sharing and cross-regulation [53]. In addition, their binding was shown to induce either
577 activation or repression with response amplitudes varying depending on the promoter bound

578 [53]. Along the same lines, while CusR, CopR and CzcR could bind to the same DNA region, we
579 observed that their effects on the expression of *cusRS-pcoA2* ranged from activation (to
580 various extents) to no activation at all (illustrated in S6 Fig). The molecular dynamics of the
581 TCSs, signal perception by HKs, and phosphorylated RR levels are among other important *in*
582 *vivo* parameters that will need to be investigated. Nevertheless, based on the results
583 presented here, these variable outcomes may also be due to differences in RR-DNA and/or
584 RR-RNA polymerase interaction, as demonstrated for the *E. coli* paralogs KdpR and OmpR [54].
585 Indeed, a new set of target genes for duplicated RRs can evolve through changes in the DNA-
586 binding sites recognized and their capacity to interact with the RNA polymerase [55].
587 However, sometimes the second mechanism is not without consequences, as shown here,
588 where CopR not only fails to activate *pcoA2* promoter expression, but actually inhibits it by
589 competing with CusR for binding. Furthermore, as proposed for CopRS and CzcRS in *P. stutzeri*
590 [53], we cannot exclude the possibility that cross-talk between these two homologous TCSs
591 exists, adding a level of complexity to the regulatory networks. Overall, our findings
592 emphasized that the effects of a regulator binding to a promoter cannot always be predicted
593 and must be determined on a case-by-case basis.

594 This study also illustrated the connection between zinc and copper homeostasis, with
595 cross-regulation observed with CzcR, CopR and CusR. The two metals play a major role in host-
596 pathogen interactions and, during infection, higher copper and zinc concentrations can be
597 encountered by bacteria under the same physiological conditions. For example, in
598 phagolysosomes [56], these two metal ions contribute to bacterial killing. Synergized
599 responses to the two elements relying on cross-regulation have been described in *P.*
600 *aeruginosa* [57,58]. For instance, high copper concentrations lead to both increased zinc and
601 copper resistance, and overproduced CopR can activate expression of *czcRS*, which in turn

602 activates CzcCAB synthesis [57]. The regulator CzcR also controls the expression of the CopR-
603 regulated *ptrA-czcE-queF* operon [33,58]. In a previous study, we found that PtrA is a
604 periplasmic protein involved in copper tolerance [59]. In addition, CzcE was recently found to
605 be a novel element required for zinc resistance [33]. Therefore, this operon harbors genes
606 involved in tolerance to both metals. The data presented here further demonstrated that CzcR
607 activates expression of the *pcoA2* operon, which is integrated in the RR regulon. As CzcRS is
608 known to respond to cadmium in addition to zinc [33], this broadens the range of signals
609 capable of triggering expression of the *pcoA2* and *cusRS* operons.

610 In conclusion, some *P. aeruginosa* strains harbor ICE carrying genes for copper
611 resistance, as well as cognate regulatory elements that are completely integrated into the
612 bacterial regulatory network. Synthesis of this accessory copper system is tightly controlled in
613 the intergenic regulatory region, which is a hot spot for binding of five RRs. The RRs compete
614 for DNA binding and their docking stimulates or silences gene expression. The reason for the
615 negative effect exerted by the regulator CopR is not known, but expression of the *pcoA2*
616 operon can be modulated by signals other than copper. Therefore, the accessory copper-
617 resistant mechanisms might be physiologically relevant in (as yet unidentified) conditions
618 encountered in the host or in the environment.

619

620 **Material and Methods**

621 **Bacterial strains and growth conditions**

622 The *E. coli*, *P. aeruginosa* and *P. paraaeruginosa* strains used in this study are described
623 in S1 Table. Bacteria were grown aerobically in LB at 37 °C with agitation. To assess the effect

624 of copper on gene expression, overnight cultures were diluted to an OD₆₀₀ of 0.1 in LB
625 supplemented with the indicated CuSO₄ concentrations, and growth was continued until the
626 OD₆₀₀ reached around 1.0 (2.5 h). Growth in 96-well plates at 37 °C with agitation was
627 assessed by following the OD₅₉₅ of 200-μL cultures in LB with the CuSO₄ concentrations
628 indicated. Plates were read by a microplate reader at the indicated times. For copper
629 sensitivity plate assays, 15 g/L agar was added to M9 minimal medium (48 mM Na₂HPO₄,
630 22 mM KH₂PO₄, 9 mM NaCl, 19 mM NH₄Cl, 2 mM MgSO₄, 0.1 mM CaCl₂, 0.2% Glucose)
631 supplemented with CuSO₄. When LB cultures reached OD₆₀₀ of 1.0, 10 μL of a 10-fold serial
632 dilution of the cultures were spotted on the M9 media and the plates were incubated for 1 to
633 2 days at 37 °C. Antibiotics were added at the following concentrations (in μg/mL): 200
634 spectinomycin (Sm), 100 ampicillin (Ap), 50 gentamicin (Gm), 10 tetracycline (Tc), and 10
635 chloramphenicol (Cm) for *E. coli*; 75 Gm and 75 Tc in presence of 5 irgasan (Irg) in LB for *P.*
636 *aeruginosa*.

637 **Plasmids and genetic manipulation**

638 The plasmids used and the primers for PCR are listed in S1 and S2 Tables, respectively.
639 All constructions were verified by sequencing.

640 To generate *P. paraeruginosa* deletion mutants, upstream (sF1/sR1) and downstream
641 (sF2/sR2) flanking regions of the different genes were fused and cloned into an *Sma*I-cut
642 pEXG2 plasmid by sequence- and ligation-independent cloning (SLIC) using the appropriate
643 primer pairs [60].

644 For chromosomal insertion of the *PBAD* upstream *pcoA2* operon, a pEXG2 plasmid was
645 created by SLIC with upstream and downstream sequence of the *pcoA2* promoter sequence,
646 creating a *Spe*I site 52 bp upstream of the start codon. Then, the 1580-bp “*araC-PBAD*”

647 fragment was excised from pSW196 using *Xba*I and cloned into the *Spe*I site created, to
648 produce pEXG2-PBAD-*pcoA2*-Sp.

649 For mutagenesis by insertion of the Ω interposon, the MCS-contained *Bam*HI site was
650 first deleted from pEXG2 (pEXG2 Δ B). Then a *de novo Bam*HI site was created while using SLIC
651 to fuse two fragments of *pcoA2* in *Sma*I-cut pEXG2 Δ B. Finally, the Ω interposon from pHP45 Ω
652 was introduced into *Bam*HI-cut pEXG2 Δ B-IHMA_02164.

653 To replace residue His by Ala in CusS and CopS, we used SLIC to generate a fragment
654 with the mutated codon and create a *Sac*II site to allow subsequent restriction analysis
655 screening of PCR-amplified fragments.

656 To create the transcriptional *lacZ* fusions, the miniCTX-*lacZ* plasmid was first modified
657 to prevent transcriptional interference by integrating the strong *rrnB* terminator sequence
658 into its *Xho*I-*Kpn*I sites, as described in Trouillon *et al.* [61], to produce miniCTX-*TrrnB-lacZ*.
659 Then, fragments comprising the ATG and around 500 bp upstream of each target analyzed
660 were amplified using the appropriate pairs (sF/sR) for insertion into *Sma*I-cut miniCTX-*TrrnB-*
661 *lacZ* by SLIC. To create mutations in the regulator binding sites, another pair of overlapping
662 mutated primers (sR1/sF2) were used to create two overlapping fragments (sF/sR1 et sF2/sR),
663 also inserted into *Sma*I-cut miniCTX-*TrrnB-lacZ* by SLIC.

664 The pEXG2- and miniCTX-*TrrnB-lacZ*-derived vectors were transferred into *P.*
665 *paraeruginosa* IHMA87 strains by triparental mating using the helper plasmid pRK600. Allelic
666 exchanges for mutagenesis were selected as previously described [62]. To create the mutant
667 encoding the CusR_{D51A} protein, the mutated sequence was introduced into the Δ *cusR* mutants
668 to replace the deleted gene.

669 For *E. coli* overproduction of CusR protein with an N-terminal 6xHis-Tag, the *cusR* gene
670 was first amplified by PCR using IHMA87 genomic DNA as template, and SLIC was used to
671 insert the fragment into the pET15b plasmid cut with *NdeI* and *BamHI*.

672 **β -galactosidase activity assay**

673 β -galactosidase activity was assessed at least in triplicate as previously described
674 [63,64], after 2.5 h of growth in the indicated media. On the bar graphs, activities were
675 expressed in Miller Units (MU), and error bars correspond to the standard error of the mean
676 (SEM). When relevant, statistical significance was assessed using ANOVA test with Tukey's
677 method.

678 **Reverse Transcription-quantitative Polymerase Chain Reaction (RTqPCR)**

679 Extraction of total RNA, cDNA synthesis and qPCR were carried out as previously
680 described [61] with the following modifications: the cDNA was synthesized using 3 μ g of RNA
681 and the SuperScript IV first-strand synthesis system (Invitrogen). For each strain and condition,
682 mRNA expression was measured for three biological replicates. Data were analyzed with the
683 CFX Manager software (Bio-Rad), and expression levels were normalized relative to *rpoD*
684 reference Cq values using the Pfaffl method. The sequences of the primers used can be found
685 in S2 Table. Statistical analyses were carried out with *T*-test.

686 **Production and purification of CusR**

687 His₆CusR (H₆CusR) was produced in *E. coli* BL21 Star (DE3) strains harboring pET15b-
688 CusR, grown in LB at 37 °C. Expression was induced by adding 1 mM isopropyl β -D-1-
689 thiogalactopyranoside when cultures had reached an OD₆₀₀ of 0.6. Cells were grown for a
690 further 2 h at 37 °C and 150 rpm, and then harvested by centrifugation. They were

691 resuspended in 20 mL of IMAC buffer (25 mM Tris-HCl, 500 mM NaCl, pH 8) containing 10 mM
692 Imidazole and supplemented with Protease Inhibitor Mixture (Complete, Roche Applied
693 Science) for lysis at 4 °C by sonication. After centrifugation at 200,000 × g for 30 min at 4 °C,
694 the soluble fraction was loaded onto a 1-mL anion exchange column (HiTrap 1 mL HP, GE
695 Healthcare) and purified on an Akta purifier system. The protein was eluted by applying a 30-
696 mL gradient ranging from 10 to 200 mM Imidazole in IMAC buffer at a flow rate of 1 mL/min.
697 Eluted protein was diluted 1:20 in Tris-NaCl buffer (50 mM Tris- HCl, 150 mM NaCl, pH 8) for
698 conservation before use.

699 **Electrophoretic Mobility Shift Assays (EMSA)**

700 Probes were generated by annealing complementary primer pairs, one of which was
701 Cy5-labeled, to form the fluorescent probe. 0.5 nM of the resulting 60-bp DNA fragments were
702 incubated for 15 min at 25 °C in EMSA Buffer (20 mM HEPES, 80 mM KCl, 20 mM MgCl₂,
703 0.8 mM EDTA, glycerol 7.5%, 0.1 mM dithiothreitol, pH 7.9). For competition assays,
704 unlabeled DNA probes were added to the reaction at either 0.5 nM (equal amount) or 200 nM
705 (400-fold excess). The proteins were then added at the indicated concentrations in a final
706 reaction volume of 20 µL and incubated for a further 20 min at 25 °C. Samples were then
707 loaded onto a native 5% Tris-Borate-EDTA (TBE) polyacrylamide gel and run at 100 V and 4 °C
708 in cold 0.5X TBE Buffer. Fluorescence imaging was performed using an ImageQuant 800
709 imager.

710 **Bioinformatics analyses**

711 ICEs were identified and delineated using the web-based tool ICEfinder [65]. ICEs were
712 typed using ConjScan [66] on the Pasteur Galaxy platform [67]. Sequences were aligned and
713 visualized with clinker [68]. Blast analyses were performed with BLASTn [69]. Complete

714 genome sequences of *P. aeruginosa* (n= 487) and *P. paraeruginosa* (n=7) were downloaded
715 from the Pseudomonas Genome Database [27]. Genomes were annotated with Prokka [70],
716 and core genomes were determined with Roary [71] before alignment with MAFFT using the
717 default parameters [72] on the Galaxy server [73]. The resulting alignment was used to build
718 a maximum-likelihood phylogenetic tree with FASTTREE [74] on the Galaxy server. The
719 phylogenetic tree was finally visualized and annotated using iTOL [75].

720

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950

951 **Supporting Information**

952

953 **S1 Fig. Delineation of the *pcoA2* operon.**

954 **S2 Fig. Copper resistance of different UTI isolates.**

955 **S3 Fig. Copper resistance in rich media.**

956 **S4 Fig. CusR, CopR and CzcR targets.**

957 **S5 Fig. Effect of lack of IrlR and MmnR on the copper-related locus.**

958 **S6 Fig. CusR, CopR and CzcR : three regulators, three different outcomes.**

959 **S7 Fig. Models of CopRS and CusRS mechanisms and cross-talk.**

960 **S8 Fig. Similar copper sensitivity of CusRS and CopRS.**

961 **S1 Table. Bacterial strains and plasmids used in this study**

962 **S2 Table. Primers used in this study**

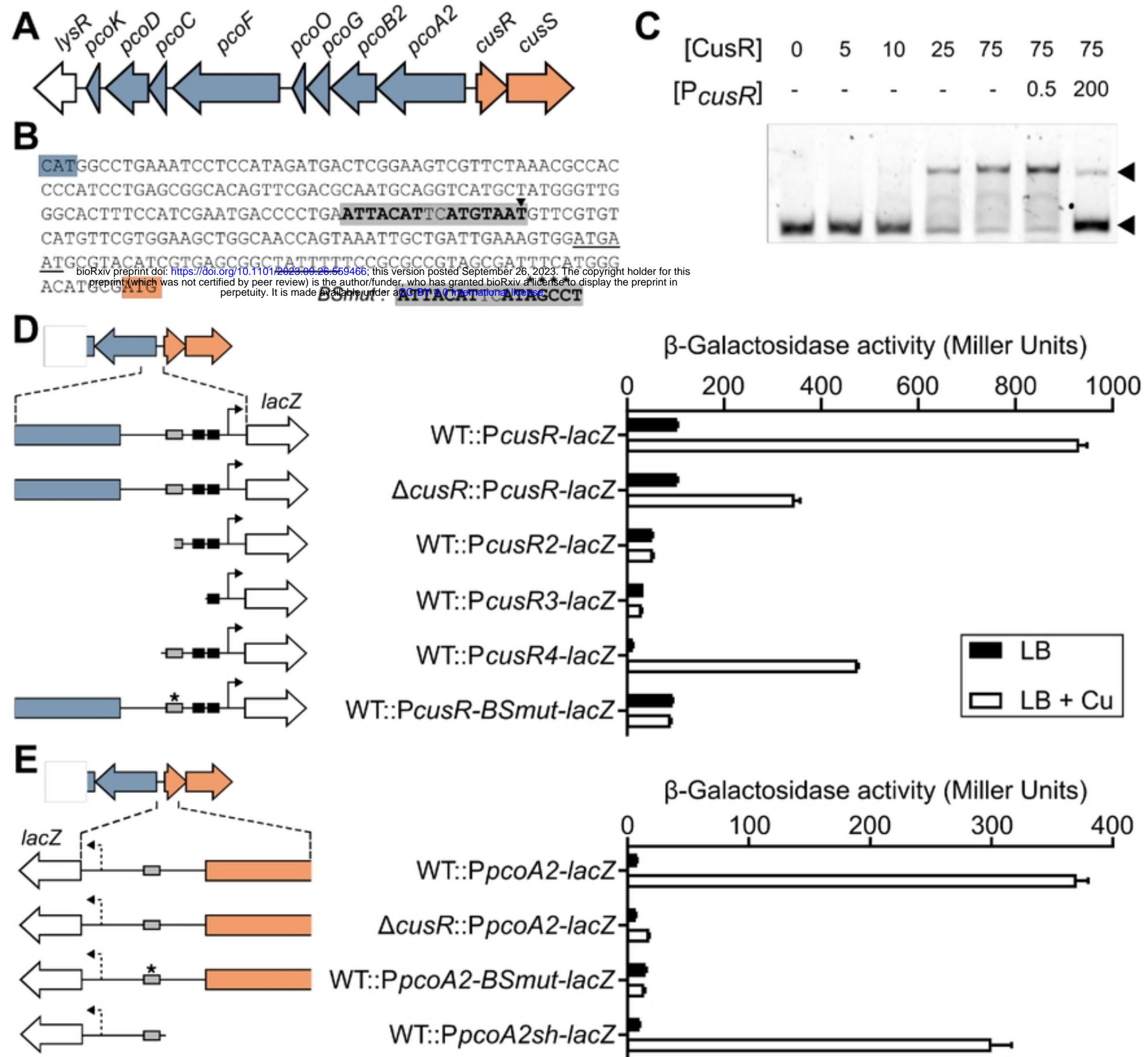


Fig 1. CusRS is a copper responsive and self-regulated TCS. (A) Genetic organization of the copper-related gene locus identified in strain IHMA87. Blue: copper-resistance genes; Orange: regulatory genes. (B) The *pcoA2-cusR* intergenic sequence, framed by the start codon (colored highlights) of each divergent gene. The -10/-35 sequences of the *cusR* promoter (underlined) were predicted using BPR0M. The putative CusR

binding site, the palindromic sequence, is in bold and highlighted in gray, with the summit of the peak identified by previous DAPseq [28] identified by an arrowhead. The mutated sequence (*B_Smut*) used in the study is indicated in the inset, with the 4 mutated bases indicated by asterisks. (C) Electrophoretic mobility shift assay of CusR on the *pcoA2-cusR* intergenic region. Up to 75 nM of recombinant His6-CusR protein were incubated with 0.5 nM Cy5-labeled 60-mer probe for 15 min before electrophoresis. For competition assays, excess unlabeled *PcusR* probe (200 nM, 400-fold) was added to the reaction. Arrowheads indicate the positions of unbound free probes (bottom) and CusR/probe complexes (top). (D, E) β -galactosidase activities of the wild-type IHMA87 strain (WT) and the $\Delta cusR$ strain harboring the indicated *PcusR-lacZ* (D) and *PpcoA2-lacZ* (E) transcriptional fusions. The different regions studied are illustrated in the left panels. Black rectangles: putative -10/-35 boxes of *cusR* promoter; Gray rectangle: palindromic sequence, with the *B_Smut* sequence indicated by an asterisk. Arrow: putative transcription start sites, the arrow is dotted for *pcoA2* as it was not predicted by BPR0M. Experiments were performed in triplicate and error bars indicate the SEM.

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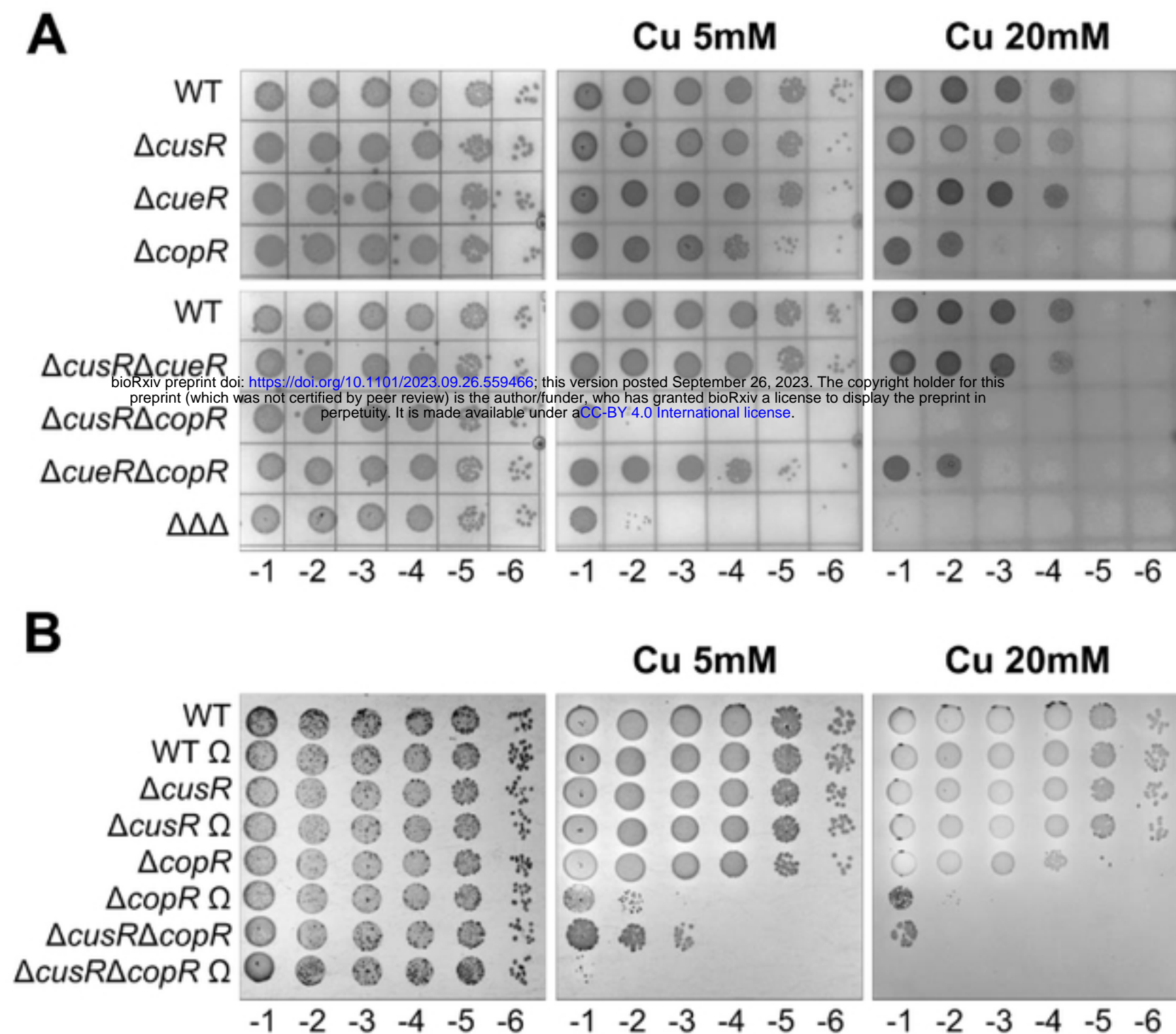


Fig 2. The CusR regulon plays a role in bacterial copper resistance. (A, B) Copper sensitivity plate assays of the different strains used in the study. $\Delta\Delta\Delta$ corresponds to the $\Delta cusR\Delta cueR\Delta copR$ triple mutant and Ω to the $pcoA2::\Omega$ background. 10 μ l of 10-fold serial dilutions, as indicated under images (10^{-1} to 10^{-6}), were deposited on M9 plates containing the $CuSO_4$ concentration indicated. Plates were observed after 24 h (M9) or 48 h (Cu) of growth at 37°C.

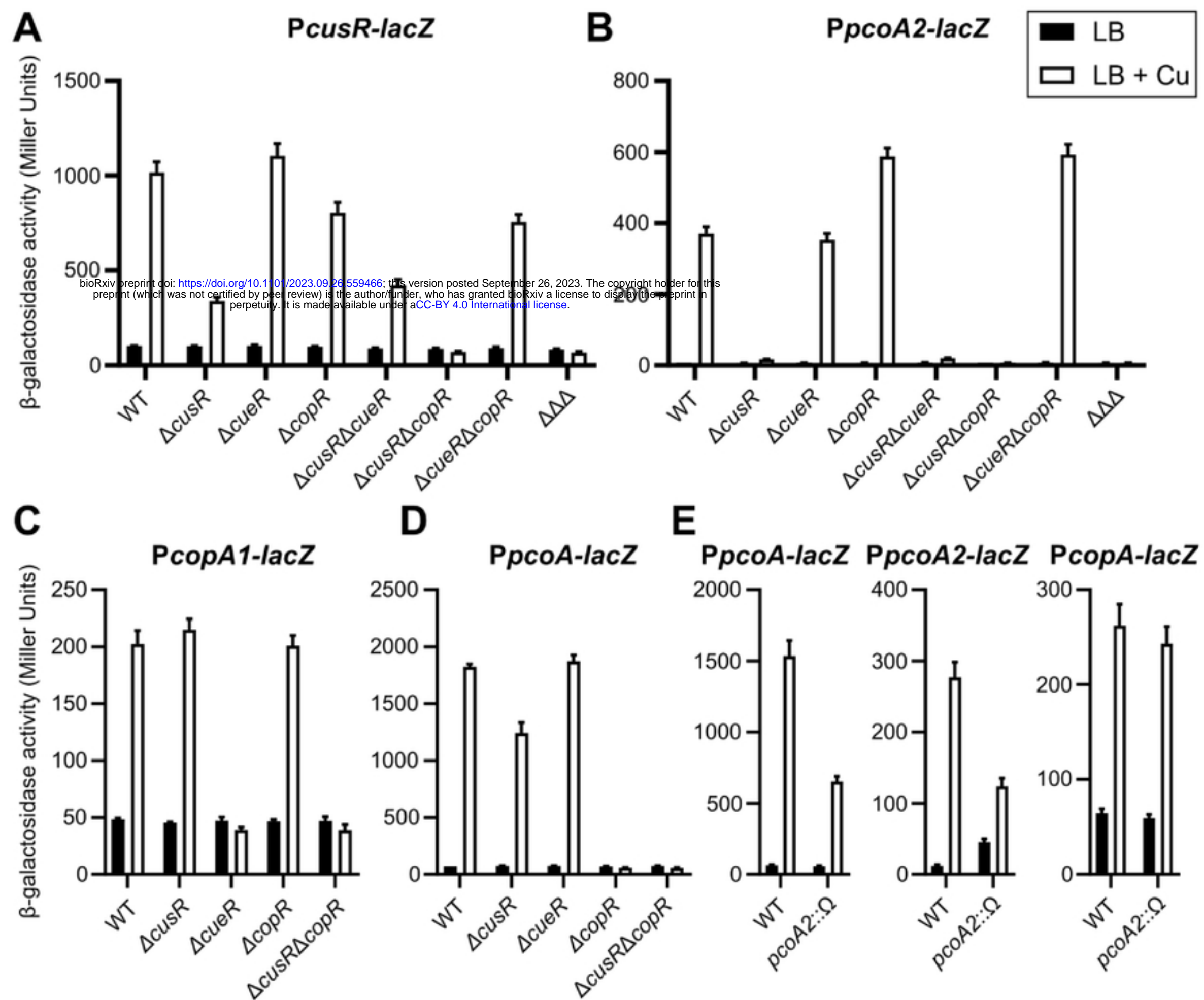


Fig 3. Cross-regulation by CusRS and CopRS. β -galactosidase activities are shown for strains harboring the *PcusR-lacZ* (A), *PpcoA2-lacZ* (B), *PcopA1-lacZ* (C), or *PpcoA-lacZ* (D) transcriptional fusions. $\Delta\Delta\Delta$ corresponds to the $\Delta cusR\Delta cueR\Delta copR$ triple mutant. (E) β -galactosidase activities of the wild-type and *pcoA2:: Ω* strains containing the indicated transcriptional fusions. Experiments were performed in triplicate; error bars correspond to the SEM.

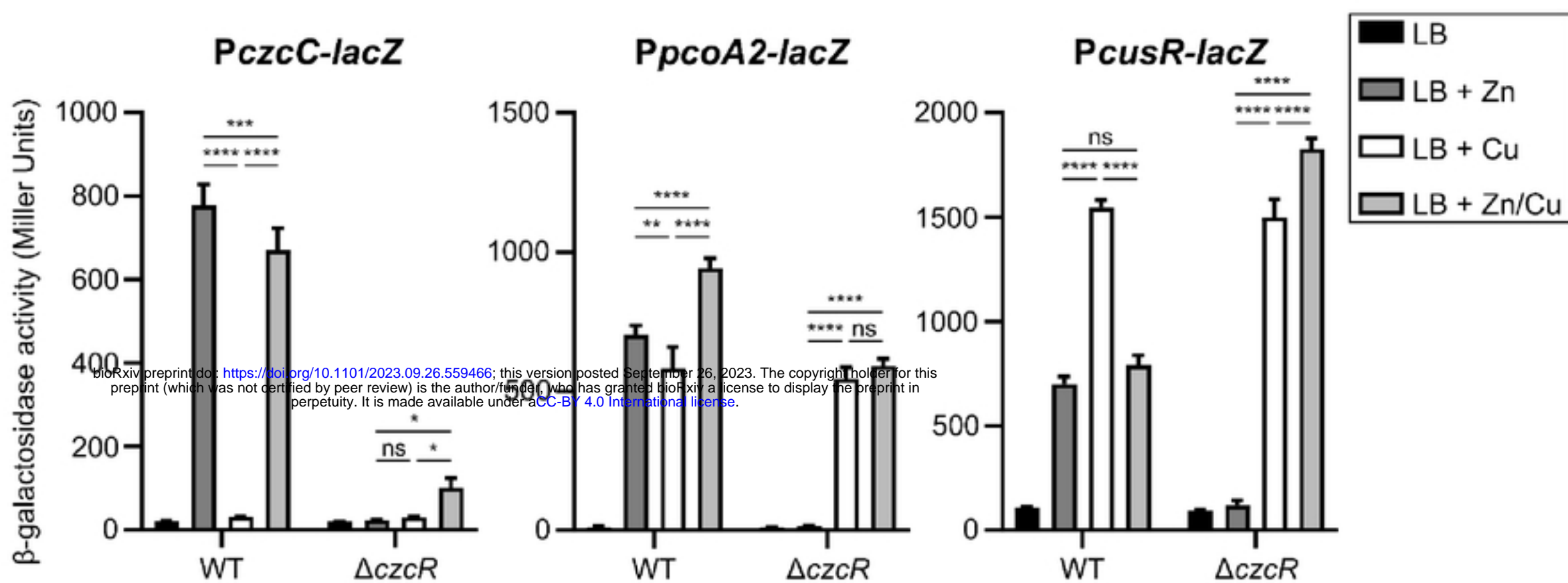


Fig 4. CusRS regulon is also controlled by CzcRS. β -galactosidase activities of the IHMA87 strain (WT) and its isogenic *czcR* mutant harboring the three transcriptional fusions indicated. Activities were measured after 2.5 h of growth in LB or LB supplemented with 0.5 mM $ZnCl_2$, 0.5 mM $CuSO_4$, or 0.5 mM $ZnCl_2/0.5$ mM $CuSO_4$. Error bars indicate the SEM. Statistical significance was assessed by ANOVA, applying Tukey's method (p -value, 0.05 [*], 0.01 [**], 0.001 [***], or 0.0001 [****]).

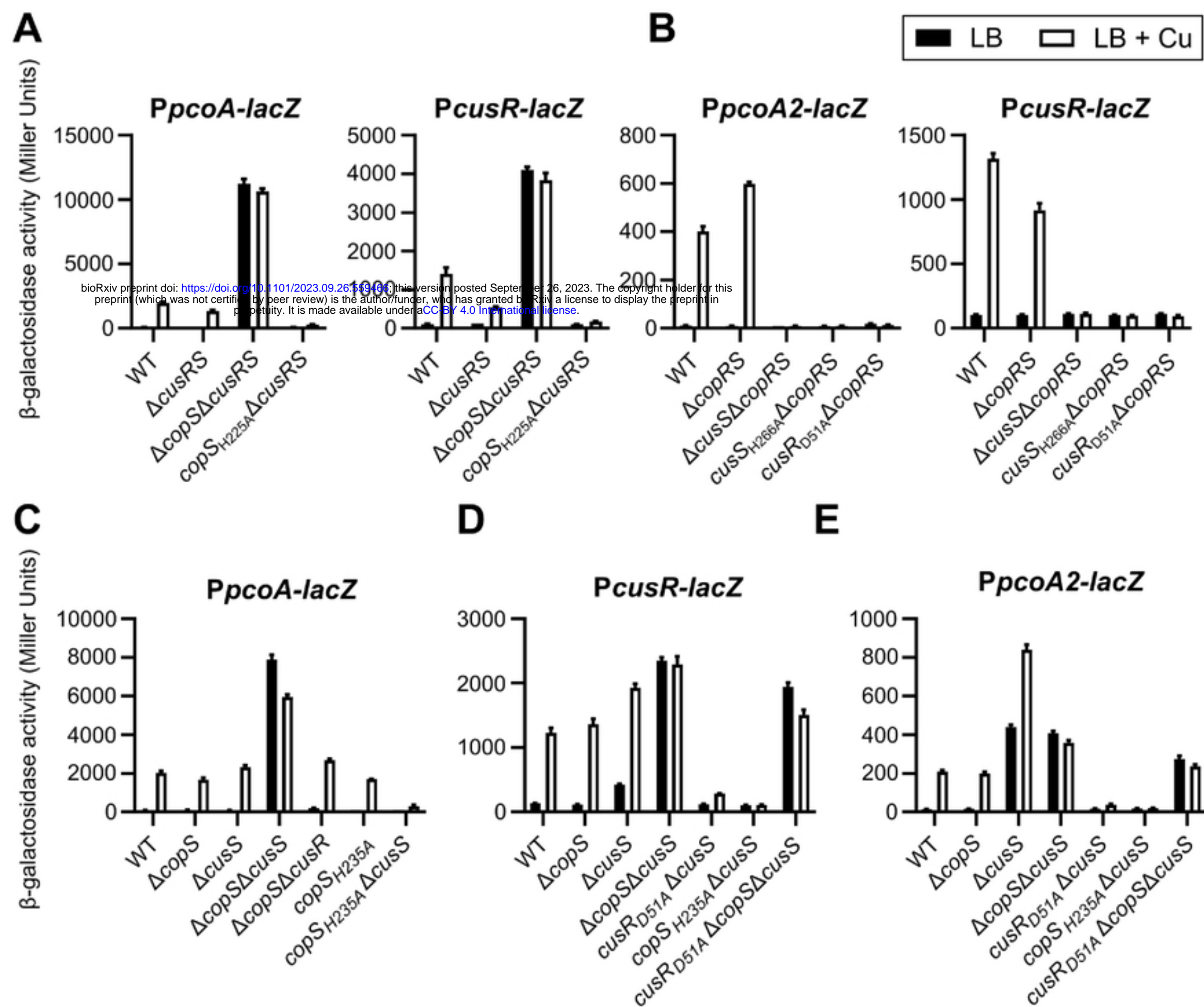


Fig 5. TCS mechanisms and cross-talk. β -galactosidase activities of the strains harboring the transcriptional fusions indicated, after 2.5 h in LB or LB supplemented with 0.5 mM $CuSO_4$. Error bars indicate the SEM.

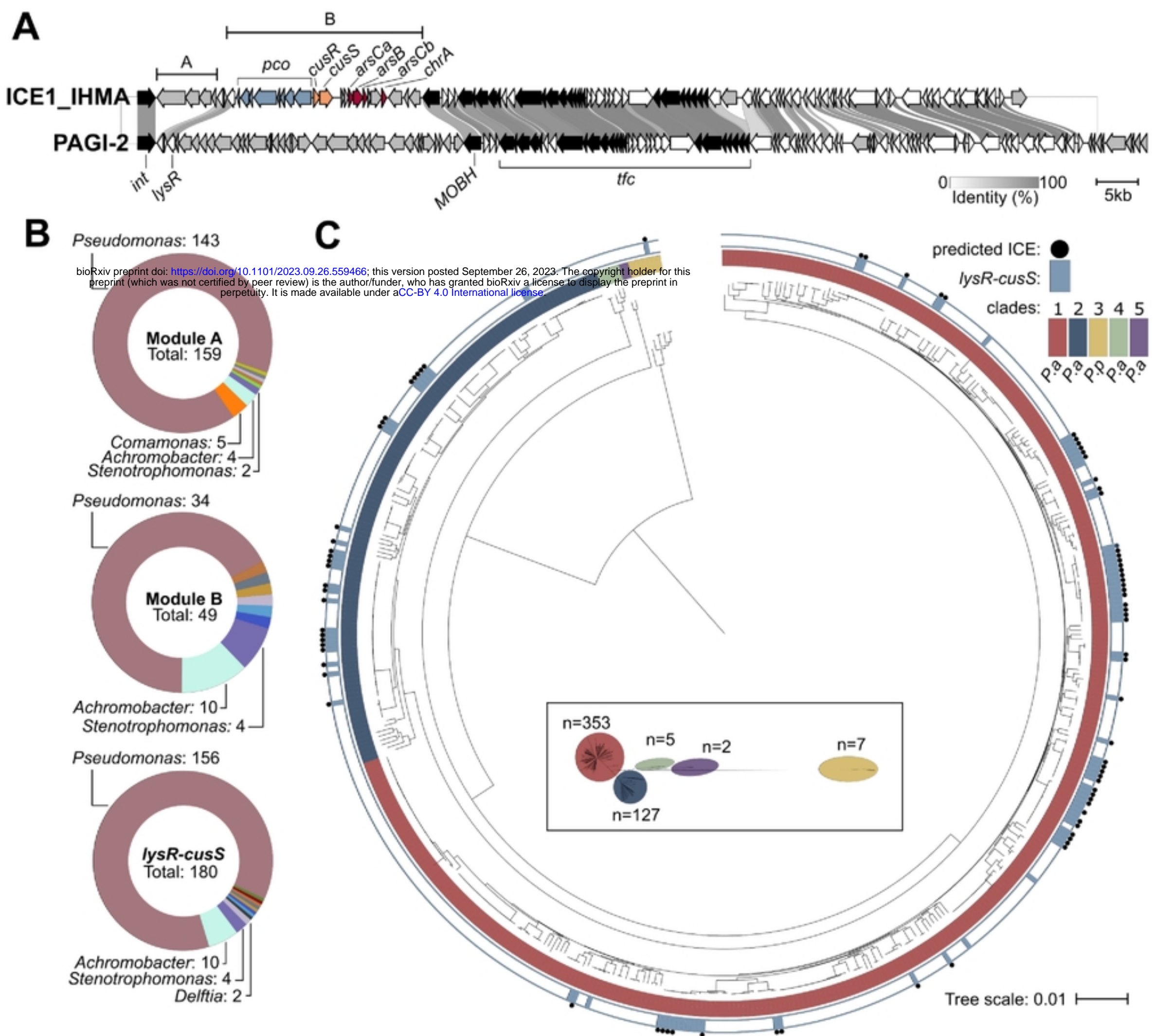


Fig 6. Conservation of *lysR-cusS* locus in *P. aeruginosa* and *P. paraeruginosa* species. (A) Comparison of the sequences of ICE1_IHMA and PAGI-2. ORFs are color-coded as follows: *pcoA2B2GOFCDK*, blue; *cusRS*, orange; genes involved in conjugation, transfer and integration of ICE, black; homologous genes, white; genes without any corresponding counterparts in ICE1_IHMA and PAGI-2, gray. Intensity of the link

between ORFs was based on % identity between the orthologous proteins. (B) Distribution of genera across genomes harboring modules carried on ICE1_IHMA, as defined in (A). Genera with a single representative are not shown. Module A: *Delftia*, *Diaphorobacter*, *Ralstonia* and *Variovorax*; Module B: *Aerosticca*, *Delftia*, *Tistrella* and *Variovorax*; *lysR-cusS* locus: *Aerosticca*, *Bordetella*, *Delftia*, *Raoultella*,

Ralstonia, *Tistrella*, *Variovorax* and *Moraxellaceae*. (C) Phylogeny of *P. aeruginosa* and *P. paraaeruginosa* species and distribution of *lysR-cusS*. Inset shows unrooted tree at scale, with the number of genomes for each clade. The presence of the locus is indicated by blue square, a blue dot signals whether the genes are encoded in a predicted ICE.

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