



HAL
open science

Streamlined copper defenses make *Bordetella pertussis* reliant on custom-made operon

Alex Rivera-Millot, Stéphanie Slupek, Jonathan Chatagnon, Gauthier Roy, Jean-Michel Saliou, Gabriel Billon, Véronique Alaimo, David Hot, Sophie Salomé-Desnoullez, Camille Locht, et al.

► To cite this version:

Alex Rivera-Millot, Stéphanie Slupek, Jonathan Chatagnon, Gauthier Roy, Jean-Michel Saliou, et al.. Streamlined copper defenses make *Bordetella pertussis* reliant on custom-made operon. *Communications Biology*, 2021, 4, pp.46. 10.1038/s42003-020-01580-2 . hal-03097387v1

HAL Id: hal-03097387

<https://cnrs.hal.science/hal-03097387v1>

Submitted on 5 Jan 2021 (v1), last revised 24 Aug 2021 (v2)

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License

1

2

3

Streamlined copper defenses make *Bordetella pertussis* reliant

4

on custom-made operon

5

6 Alex Rivera-Millot¹, Stéphanie Slupek¹, Jonathan Chatagnon¹, Gauthier Roy¹, Jean-Michel

7 Saliou², Gabriel Billon³, Véronique Alaimo³, David Hot², Sophie Salomé- Desnoulez^{1,4},

8 Camille Locht¹, Rudy Antoine^{1*}, Françoise Jacob-Dubuisson^{1*}

9

10 ¹ Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019- UMR 9017-

11 CIIL-Center of Infection and Immunity of Lille, Lille, France

12 ² Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, US 41 - UMS 2014 -

13 PLBS, F-59000 Lille, France

14 ³ Univ. Lille, CNRS, UMR 8516 – LASIRE – Laboratoire de Spectroscopie pour les

15 Interactions, la Réactivité et l'Environnement, F-59000 Lille, France

16 ⁴ Bio Imaging Center Lille platform (BICeL), Univ. Lille, Lille, France

17

18 **Keywords:** *Bordetella pertussis*, Copper homeostasis, Phagocytosis, Oxidative stress,

19 Evolution, Metallochaperone, Host-restricted pathogen, CopZ

20 For correspondence: rudy.antoine@inserm.fr; francoise.jacob@ibl.cnrs.fr

21 **Abstract**

22 Copper is both essential and toxic to living beings, which tightly control its intracellular
23 concentration. At the host-pathogen interface, copper is used by phagocytic cells to kill
24 invading microorganisms. We investigated copper homeostasis in *Bordetella pertussis*, which
25 lives in the human respiratory mucosa and has no environmental reservoir. *B. pertussis* has
26 considerably streamlined copper homeostasis mechanisms relative to other Gram-negative
27 bacteria. Its single remaining defense line consists in a metallochaperone diverted for copper
28 passivation, CopZ, and two peroxide detoxification enzymes, PrxGrx and GorB, which
29 together fight stresses encountered in phagocytic cells. Those proteins are encoded by an
30 original, composite operon assembled in an environmental ancestor and which is under
31 sensitive control by copper. This system appears to contribute to persistent infection in the
32 nasal cavity of *B. pertussis*-infected mice. Combining responses to co-occurring stresses in a
33 tailored operon reveals a strategy adopted by a host-restricted pathogen to optimize survival at
34 minimal energy expenditure.

35

36 **Introduction**

37 Copper is an essential trace element bioavailable in its soluble Cu^{2+} form in oxygen-rich
38 environments ¹. As the redox potential of the $\text{Cu}^{1+}/\text{Cu}^{2+}$ pair is exquisitely tuned for
39 enzymatic reactions, most living organisms use copper for oxidation reactions and in electron
40 transfer chains. However, copper excess is highly toxic. In the reducing environment of the
41 cytoplasm, Cu^{1+} displaces iron and other transition metals from their sites in metalloenzymes,
42 causing loss of function and deregulating metal import systems ^{2,3}. Cu^{1+} also indirectly
43 generates intracellular oxidative stress ^{4,5}. Because of its toxicity, living organisms have
44 evolved a variety of systems to fend off excess of this metal. As such, ubiquitous copper-
45 specific P_{IB} -type ATPases remove copper from the cytoplasm, in partnership with
46 cytoplasmic copper chaperones in both eukaryotes and prokaryotes ^{1,6,7}.

47 Eukaryotic organisms take advantage of copper toxicity to kill invading microorganisms
48 ^{8,9}. Predatory protists, such as amoebae, and phagocytic cells of higher eukaryotes import
49 copper for bactericidal purposes ^{10,11}. In turn, bacteria have developed various resistance
50 mechanisms against copper excess ^{12,13}. Copper extrusion systems include CopA-type
51 ATPases, as well as CusABCF-type Resistance-Nodulation-Cell Division (RND) transporters
52 in Gram-negative bacteria, and other less well characterized proteins ^{1,14}. Oxidation of Cu^{1+} in
53 the periplasm is catalyzed by PcoA-type multi-copper oxidases (MCO) ¹⁵. Specific
54 metallochaperones also contribute to copper homeostasis as part of export systems or by
55 copper sequestration ^{16,17}.

56 Environmental bacteria are likely to encounter occasional copper excess or predation by
57 protists. Therefore, they have developed large resistance arsenals against copper intoxication.
58 Conversely, bacteria with restricted ecological niches, such as pathogens or commensals,
59 appear to have fewer copper homeostasis genes ¹⁸. Here, we investigated copper homeostasis

60 in the whooping cough agent *Bordetella pertussis*, a predominantly extracellular pathogen
61 whose sole natural niche is the human upper respiratory tract ¹⁹. Unlike its relative *Bordetella*
62 *bronchiseptica*, whose lifestyles alternate between the environment and a mammalian host, *B.*
63 *pertussis* is host-restricted and was thus used here as a model to study how genomic reduction
64 by niche specialization affects copper homeostasis at the host-pathogen interface. We
65 discovered that *B. pertussis* has eliminated most resistance systems typically found in Gram-
66 negative bacteria. Its only line of defense consists of an original operon to withstand copper
67 excess and peroxides, both encountered in phagocytic cells.

68

69 **Results**

70 **Loss of copper homeostasis systems in *B. pertussis*.** Comparative genomic analyses
71 revealed that the exclusively human pathogen *B. pertussis* and its close relative *B.*
72 *bronchiseptica* share common sets of copper-homeostasis genes, including genes coding for a
73 CopA ATPase, a PcoA-PcoB-type MCO system, and CopZ, CopI and CusF copper
74 chaperones, but both lack *cusABC* genes ¹⁸. A copper storage protein (*csp*) gene is present in
75 the *B. bronchiseptica* but not in the *B. pertussis* genome ¹⁸. Both species also have genes
76 coding for CueR-type regulators and CopRS-type two-component systems that control copper
77 homeostasis genes in other bacteria ^{20,21}.

78 Nevertheless, growth of the two species in the presence of copper revealed distinct
79 phenotypes (Fig. 1). After a prolonged lag, Cu-treated *B. pertussis* started growing at the same
80 rate as the untreated culture, suggesting that copper has a bacteriostatic effect, whereas growth
81 of Cu-treated *B. bronchiseptica* started at the same time as the control but at a slower pace,
82 indicating a rapid adaptation to copper-rich conditions. The apparent resistance of both
83 species to high concentrations of copper is misleading and due to growth medium compounds

84 chelating the metal ion, in particular Tris, amino acids and glutathione, which markedly
85 reduces its effective concentration. We hypothesized that *B. pertussis* started growing after
86 the toxic Cu¹⁺ ion resulting from the reduction of Cu²⁺ by ascorbate, another component of the
87 growth medium, was re-oxidized thanks to strong oxygenation of the cultures. This was
88 confirmed by the observation that the addition of fresh ascorbate after a few hours to *B.*
89 *pertussis* cultured in the presence of 5 mM copper prevented bacterial growth (Supplementary
90 Figure S1).

91 RNA sequencing (RNA-seq) analyses of *B. pertussis* grown in the presence of 2 mM
92 CuSO₄ showed altered expression of a number of stress response and metabolic genes and of
93 a few Cu-specific genes (Fig. 2a and b and Supplementary Data S1). They include *bp2860*
94 and *bp2722* that code for P_{IB}-type ATPases, the latter being Zn-specific²², and *bp1727*,
95 *bp1728* and *bp1729* that form an operon (Supplementary Figure S2) and code for a CopZ-like
96 copper chaperone, a putative glutaredoxin and a putative oxidoreductase, respectively.
97 Copper-repressed *bp2921* and *bp2922* were not further investigated here. A 30-min Cu
98 treatment of *B. pertussis* confirmed up-regulation of *bp2860* and *bp1727-bp1728-bp1729*
99 (Fig. 2c and Supplementary Data S2). In contrast, *bp3314-bp3315-bp3316* coding for CopI,
100 PcoA, and PcoB homologues were hardly expressed and not regulated by copper in *B.*
101 *pertussis*, in contrast to their orthologues in *B. bronchiseptica* (Figs. 2b and 2d and
102 Supplementary Data S3). Proteomic analyses of the two organisms in the presence of copper
103 showed increased production of the proteins coded by the upregulated genes in both bacteria,
104 except for the membrane protein CopA and the small protein CopZ (Fig. 2b, Supplementary
105 Data S4 and S5). Because the size of CopZ might have hampered its detection in global
106 proteomic analyses, we analyzed extracts of copper-treated *B. pertussis* by denaturing
107 electrophoresis and mass fingerprinting of the putative CopZ protein band (Fig. 3). This
108 confirmed that CopZ is strongly overproduced by *B. pertussis* in the presence of copper.

109 To investigate the role of copper homeostasis genes, we inactivated *bp3314-bp3315-*
110 *bp3316* (*pcoA* operon), *bp0157-bp0158* (*copRS*), *bp2860* (*copA*) and *bp1727* (*copZ*) in *B.*
111 *pertussis*. Except for the latter, growth of those mutants was affected by the addition of copper
112 to the medium to the same extent as that of the parental strain (Supplementary Figure S3). In
113 all available *B. pertussis* genomes, three IS481 sequences are present upstream of *bp3314* and
114 one is located within the 5' end of *bp2860* (Supplementary Figures S4 and S5), which most
115 likely inactivated the *pcoA* and *copA* operons, as evidenced by their low expression levels
116 both in the presence and in the absence of copper (Fig. 2b). In contrast, the *copA* and *pcoA*
117 operons are strongly upregulated by copper and probably functional in *B. bronchiseptica*.
118 Altogether thus, *B. pertussis* has considerably streamlined its response to copper excess,
119 maintaining only one system functional.

120

121 **Function of the *copZ* operon.** A strong effect of copper excess on growth was observed with
122 the Δ *bp1727* (*copZ*) mutant, and therefore we focused on that operon. We generated two
123 additional knock-out mutants (Δ *bp1727-bp1728-bp1729* and Δ *bp1728-bp1729*) and measured
124 their growth in the presence or absence of copper. Copper excess caused a major growth
125 defect to two of the mutants, Δ *bp1727* and Δ *bp1727-bp1728-bp1729*, and the phenotype of
126 the latter was complemented by ectopic expression of the operon (Fig. 4a). Glutathione is a
127 major player in Cu homeostasis²³. *B. pertussis* cannot synthesize glutathione but actively
128 imports it, as indicated by the high expression levels of the glutathione-specific ABC
129 transporter genes *bp3828-3831* in all conditions (Supplementary Data S1 and S2). The growth
130 defect of *B. pertussis* in the presence of copper was exacerbated in the absence of glutathione
131 in the culture medium, supporting its role for copper tolerance in *B. pertussis* (Fig. 4b).
132 Altogether the growth data show that CopZ, encoded by the first gene of the operon, plays a
133 major role in copper resistance, unlike the other two proteins.

134 In other bacteria, CopZ binds Cu^{1+} and transfers it to an ATPase partner for export ²⁴.
135 Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) analyses were
136 performed with purified CopZ incubated with Cu or with Fe as a control. Three Cu ions and
137 one Fe ion were bound per CopZ monomer, respectively (Fig. 5a). We reasoned that in both
138 cases one ion was chelated by the 6-His tag used for recombinant CopZ purification.
139 Therefore, subtraction of a Cu ion yields a ratio of 2 Cu per CopZ monomer. This value is
140 consistent with crystal structures of homologues (pdb accession numbers 6FF2 and 2QIF)
141 showing CopZ dimerization mediated by 4 Cu ions bound by conserved Cys and His residues
142 ²⁵ (Supplementary Figure S6). Thus, CopZ is a copper binding protein, which in the absence
143 of a functional CopA in *B. pertussis* most likely sequesters cytosolic copper to counter its
144 toxicity.

145 Homology searches indicated that *bp1728* and *bp1729* code for a chimeric peroxiredoxin-
146 glutaredoxin protein ²⁶ and a glutathione reductase, respectively. Orthologues of these
147 enzymes were shown to reduce peroxides at the expense of glutathione and to regenerate
148 glutathione using NAD(P)H, respectively ^{27,28}. Using purified recombinant BP1728 and
149 BP1729 proteins in *in vitro* enzymatic assays ^{27,28}, we confirmed that these proteins are a
150 glutathione-dependent peroxidase and a glutathione reductase, respectively (Fig. 5b-d). We
151 thereafter called them PrxGrx (<http://peroxibase.toulouse.inra.fr/>) and GorB.

152 To test whether the PrxGrx-GorB system protects bacteria against peroxides, we
153 determined mutant survival after oxidative stress ²⁹. A 30-min exposure killed each of the
154 three mutant strains to a greater extent than their parental strain, and the phenotype of the
155 three-gene mutant was complemented by ectopic expression of the operon (Fig. 4c). The
156 phenotype of the ΔcopZ mutant indicated that, in addition to PrxGrx-GorB, CopZ also
157 protects *B. pertussis* against reactive oxygen species (ROS). Together, the three proteins

158 might endow *B. pertussis* with some capability to fend off both copper and peroxides (Fig.
159 5e).

160 As a human-restricted pathogen, *B. pertussis* may simultaneously encounter oxidative and
161 copper stresses in phagosomes of phagocytic cells. We thus tested the role of the operon in
162 intracellular survival within human macrophages and in a murine model of lung and nasal
163 colonization. The deletion mutant was killed faster by macrophages than its parental strain
164 (Fig. 4d). We also subjected the *copZ* and *prxgrx-gorB* mutants to this assay. Both were killed
165 faster than the wild type (wt) strain in macrophages, especially at the 1-hour time point
166 (Supplementary Figure S7). This shows that both CopZ and the Prxgrx-GorB system
167 contribute to survival early after phagocytosis. Finally, we tested the effect of deleting the
168 entire operon in an animal model of colonization. The profiles of colonization in the lungs
169 over time by the mutant and the wt strains were not significantly different, although at the late
170 time point (21 days) more animals appeared to have cleared the mutant bacteria. Furthermore,
171 the deletion mutant was cleared more quickly from nasopharynxes than the parental strain
172 (Figs. 4e and f).

173

174 **Copper regulation of the *copZ-prxgrx-gorB* operon.** Quantitative RT-PCR (qRT-PCR) on
175 *prxgrx* showed a 30-fold up-regulation of the operon by copper, even at micromolar
176 concentrations (Fig. 6a and Supplementary Figure S8). qRT-PCR experiments on the very
177 small *copZ* gene were unsuccessful, as no qRT-PCR primer pairs were found to work.
178 Inactivation of *bp1726*, in antisense orientation of the *copZ-prxgrx-gorB* operon and coding
179 for a putative CueR regulator ²⁰, resulted in a markedly decreased but not abolished
180 transcriptional response of *prxgrx* to copper (Fig. 6a). The three genes are co-transcribed
181 (Supplementary Figure S2) and up-regulated by copper (Fig. 2 and Supplementary Data S1
182 and S2), and we showed that the second is controlled by CueR. We can thus reasonably

183 assume that the entire operon is under the control of CueR. Interestingly, though, the
184 remaining copper-induced up-regulation of *prxgrx* in the *cueR* KO mutant indicates that the
185 operon may also be controlled by a second system directly or indirectly activated by copper.

186 We determined the transcriptional start site of the operon by 5' rapid amplification of
187 cDNA ends (5' RACE). The 5' UTR is 61-nucleotides long, and putative CueR binding sites
188 were identified within the promoter, as described for *Escherichia coli*³⁰ (Supplementary
189 Figure S5). Attempts to produce a recombinant CueR protein in *E. coli* to be used for
190 electrophoretic migration shift assays (EMSA) were unsuccessful, suggesting that its over-
191 production may be toxic to *E. coli*.

192 We then turned to human macrophages to determine if the operon is up-regulated upon
193 phagocytosis. It was shown earlier that the ATP7A Cu-transporting ATPase is overexpressed
194 and localizes to phagosomes in activated murine macrophages⁹. We thus reasoned that
195 copper might trigger expression of the *copZ-prxgrx-gorB* operon in *B. pertussis* early after
196 phagocytosis. By using epifluorescence microscopy, we showed that differentiation and
197 activation of THP1 cells cause overproduction of ATP7A. In addition, the Cu transporter
198 partially redistributes from a mainly ER/Golgi localization in resting cells to a more
199 punctuated localization corresponding to cytosolic vesicles (Supplementary Figure S9).
200 Endosomes are thus likely to be loaded with copper. Then, to visualize the activation of the
201 operon in intracellular bacteria, we introduced a transcriptional fusion between the promoter
202 of the operon and an mRFP1 reporter gene in *B. pertussis* and put the bacteria in contact with
203 differentiated THP1 cells deprived of copper or supplemented with copper. After one hour of
204 contact, fluorescence of bacteria engulfed by copper-replete macrophages was significantly
205 more intense than in copper-deprived macrophages (Fig. 7a and b). The rapid induction of the
206 *copZ-prxgrx-gorB* promoter indicates that copper is an early signal for intracellular bacteria,
207 and that CopZ-PrxGrx-GorB serves as a defense system in early phagosomes. This is

208 supported by the role of that system for bacterial survival at early time points after
209 phagocytosis (Fig. 4d).

210

211 **Regulation by peroxides.** As shown above, residual regulation of *prxgrx* by copper was
212 observed in the *cueR* KO strain (Fig. 6a). Since copper excess generates oxidative stress⁴, we
213 hypothesized that a ROS-sensing regulator might be involved in this residual control of
214 *prxgrx*. We examined all known prokaryotic PrxGrx homologues and searched for putative
215 regulatory genes in the corresponding loci. An *oxyR* gene was found directly adjacent and in
216 antisense orientation in 19.4 % of cases (Supplementary Data S6). OxyR regulators have been
217 shown to control bacterial responses to peroxides^{31,32}.

218 Treatment of *B. pertussis* with H₂O₂ resulted in a 4-fold upregulation of *prxgrx*, which was
219 abolished by inactivation of the putative *oxyR* gene, *bp1613* (Fig. 6b). Using EMSA, we
220 detected a protein-DNA complex between recombinant OxyR and the *copZ-prxgrx* intergenic
221 region but not the *cueR-copZ* intergenic region (Fig. 6c and Supplementary Figure S10).
222 Putative OxyR binding boxes can be found in the *copZ-prxgrx* intergenic region
223 (Supplementary Figure S11). Thus, two transcriptional activators control the expression of
224 this operon in response to distinct signals (Fig. 6d). The three genes are strongly up-regulated
225 by copper through CueR, and in addition, the last two genes are more modestly up-regulated
226 by peroxides through OxyR.

227 Finally, we searched β proteobacterial genomes for similar composite operons to determine
228 the phylogenetic context in which they arose. Out of 86 bacterial genera, 16 harbor a *prxgrx*
229 gene, of which 11 have *prxgrx-gorB* operons. Only *Bordetella* and *Achromobacter* have
230 complete *copZ-prxgrx-gorB* operons. The latter two genera encompass pathogenic or
231 opportunistic species. Occurrence of this operon thus appears to be linked to eukaryotic cell-
232 associated lifestyles.

233

234 **Discussion**

235 Copper is both an essential and toxic metal for living organisms, including bacteria, yet
236 copper homeostasis has been studied only in a few model organisms. We describe here that *B.*
237 *pertussis*, a host-restricted, mostly extracellular bacterium that lives on human mucosal
238 surfaces, has considerably streamlined its resistance against copper intoxication. *B. pertussis*
239 mainly relies on a composite, three-protein system strongly upregulated by copper to counter
240 both copper excess and peroxide stress. This system evolved from an environmental bacterial
241 ancestor by diverting a ubiquitous cytoplasmic metallochaperone for copper sequestration and
242 recruiting genes involved in protection against oxidative stress in an operon regulated by
243 copper. This assembly enables the bacterium to mount a strong response against two stresses
244 simultaneously encountered in the endosomal compartment. Molecular traces of this evolution
245 can be found in the OxyR-mediated regulation of the last two genes of the operon in response
246 to peroxide stress. Persistence of this ancestral control may be useful to a strictly aerobic
247 bacterium when it resides outside of the phagosomal environment, as respiration also
248 generates ROS³³.

249 Genomic, transcriptomic and proteomic analyses demonstrated the loss in *B. pertussis* of
250 most well-known resistance mechanisms against copper excess. This contrasts with other
251 respiratory tract pathogens such as *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*
252 and *B. bronchiseptica*^{34,35}. Unlike *B. pertussis*, *M. tuberculosis* is a genuine intracellular
253 bacterium that resides within macrophages and other cell types, *P. aeruginosa* can occupy
254 various niches including environmental milieus, and *B. bronchiseptica* alternates between
255 pathogenic and environmental life cycles³⁶. In humans, copper is not free but complexed with
256 proteins such as ceruloplasmin or metallothionein. Therefore, *B. pertussis* is unlikely to

257 experience copper excess, except when it is engulfed by phagocytic cells, a fate it actively
258 tries to avoid^{37,38}. *B. pertussis* could thus afford the loss of most defense mechanisms against
259 copper intoxication, because of its specialization to a restricted niche.

260 *B. pertussis* is mainly an extracellular pathogen, with limited survival rates upon
261 phagocytosis³⁹. Therefore, it may be surprising that it has nonetheless conserved an active-
262 defense system. Nevertheless, *B. pertussis* has been shown to occasionally reside within
263 phagocytic cells³⁹⁻⁴¹. We propose thus that *B. pertussis* adopts a two-pronged strategy with
264 respect to phagocytic cells while limiting energy expenditure. The major arm of this strategy
265 uses immunomodulatory virulence factors to target neutrophils and macrophages by disabling,
266 delaying or dysregulating their responses to infection^{37,38,42-45}. The second arm is to
267 overproduce specific proteins, including those identified here, that enable bacteria to survive
268 to some extent within specific compartments in macrophages, probably with early endosomal
269 characteristics³⁹, while those phagocytes face the virulence factors produced by extracellular
270 bacteria. Absence of the *copZ-prxgrx-gorB* operon had little effect on *B. pertussis*
271 colonization of the lungs but shortened colonization of the nasal cavity of mice. Interestingly,
272 in murine nasal tissues, a large influx of immune cells occurs after one to two weeks of
273 infection, and a sizeable proportion of bacteria appear to reside within immune cells in that
274 organ (V. Dubois, personal communication). This might contribute to explain the detrimental
275 effect of inactivating the operon on nasal cavity colonization.

276 Copper and peroxide stresses are interlinked, as copper excess indirectly causes oxidative
277 stress⁴. Placing the *copZ*, *prxgrx* and *gorB* genes under copper control is thus an efficient way
278 of fighting co-occurring threats, especially considering that CopZ also protects the bacterium
279 from peroxide stress. This activity most likely stems from CopZ's ability to form redox-
280 sensitive Cys-mediated dimers⁴⁶. This property is reminiscent of the eukaryotic CopZ
281 homologue, Atox1, both an anti-oxidant protein and the copper chaperone partner of the

282 CopA homologues ATP7A/B^{47,48}. In *B. pertussis*, CopZ sequesters copper but no longer
283 participates in its export. In some bacteria, these two functions are performed by distinct
284 CopZ paralogues¹⁷.

285 Pathogenic *Bordetella* species are thought to have derived from environmental ancestors⁴⁹.
286 The *copZ-prxgrx-gorB* operon was only found in two closely related genera, *Bordetella* and
287 *Achromobacter*, arguing that it originated in a common environmental ancestor. Known
288 *Achromobacter* species are environmental bacteria or opportunistic pathogens. The need to
289 survive predation by protists, which employ killing mechanisms similar to those of
290 macrophages, including the use of copper¹⁰, exerts a strong selective pressure on
291 environmental bacteria. This selective pressure most likely accounts for the panoply of
292 resistance systems found in *Achromobacter*¹⁸, which most likely were already present in the
293 environmental ancestor of *Achromobacter* and *Bordetella*. Persistence in amoeba could
294 constitute one of the evolutionary steps allowing *Bordetellae* to infect mammals. *B.*
295 *bronchiseptica* exemplifies an intermediate in this evolution, as this respiratory pathogen of
296 mammals which is able to multiply in amoebae and potentially uses them as transmission
297 vectors has conserved ancestral genes for intracellular persistence^{36,50}. As a host-restricted
298 pathogen, *B. pertussis* has traded most of this ability for a lighter coding potential. *B. pertussis*
299 has a number of cuproproteins¹⁸ and therefore requires copper for growth. It might thus also
300 use CopZ for nutritional passivation⁵¹, i.e. as a copper store for future needs.

301

302 **Methods**

303 **Culture conditions.** Modified Stainer-Scholte (SS) liquid medium and Bordet-Gengou blood-
304 agar (BGA) medium were used with the appropriate antibiotics to grow *B. pertussis* BPSM
305 and its derivatives. As copper ions are Lewis acids and *B. pertussis* requires buffered medium

306 close to pH 7, a stock solution (62 mM of CuSO₄, 100 mM Tris, pH 5.5) in the fraction A of
307 SS medium was used to supplement the medium with copper. The control cultures were
308 prepared by adding the same volumes of 100 mM Tris-HCl (pH 5.5) to SS medium. To record
309 growth curves, the optical density at 630 nm was continuously measured using an Elocheck
310 device (Biotronix). For growth yields, the *B. pertussis* cultures were started at OD₆₀₀ of 0.1,
311 and after 24 h of growth optical density values at 600 nm (OD₆₀₀) were determined using an
312 Ultrospec 10 spectrophotometer (Biochrom) with five biological replicates for each condition
313 and strain. Glutathione was omitted from the culture medium where indicated. The turbidities
314 measured using the Elocheck and Ultrospec apparatuses, which use different wavelengths and
315 pathlengths, cannot be directly compared. For transcriptomic, proteomic and qRT-PCR
316 experiments, the *B. pertussis* cultures were started at OD₆₀₀ of 0.1 to reach OD₆₀₀ values of 1.5
317 after 16-20 h, and *B. bronchiseptica* cultures were started at OD₆₀₀ of 0.08 to reach OD₆₀₀
318 values of 1.5-1.8 after 10-12 h. To subject *B. pertussis* to oxidative stress for qRT-PCR
319 analysis, the bacteria were grown to OD₆₀₀ of 1.5, and 10 mM H₂O₂ was added to the cultures
320 for 30 minutes. To measure survival to oxidative shock, the bacteria were grown for 36 h on
321 BGA from standardized stocks, resuspended in PBS (pH 7.2) containing 500 μM of
322 hypoxanthine, and then diluted in the same solution to obtain ~ 50,000 bacteria in 300 μl in
323 15-ml tubes. 0.05 U.ml⁻¹ xanthine oxidase was added to produce H₂O₂ and O₂^{-•}²⁹, and the
324 bacteria were incubated for 30 minutes at 37 °C with shaking. Serial dilutions were plated
325 onto BGA to count CFUs, using control suspensions incubated as above. Three biological
326 replicates and three technical replicates were made. CFU counts of the initial suspensions
327 were used as references.

328 **Construction of strains and plasmids.** Deletion mutants were obtained by allelic exchange
329 ⁵². Complementation was performed at the *ure* chromosomal locus ⁵². The inactivation
330 mutants were constructed using the suicide vector pFUS2 ⁵³. For pProm1727-mRFP1, the

331 *cueR-copZ* intergenic region including the first 7 codons of *copZ*, and the *mRFPI* gene
332 without its initiation codon were amplified by PCR and cloned as BamHI-XbaI and XbaI-
333 HindIII restriction fragments, respectively, to generate a translational fusion in pBBR1-MCS5
334 ⁵⁴. All primers are listed in the Supplementary Data S7.

335 **RNA sequencing and qRT-PCR.** For RNA extractions, the cultures were stopped by adding
336 2 mL of a mixture of phenol:ethanol (5:95) to 8 mL of bacterial suspensions. Bacteria were
337 pelleted, and total RNA was extracted with TriReagent (InVitrogen) ⁵⁵. Genomic DNA was
338 removed by a DNase I treatment (Sigma Aldrich). DNA-depleted total RNA was treated with
339 the RiboZero rRNA Removal kit (Illumina). The rRNA-depleted RNA was then used to
340 generate the Illumina libraries using the TrueSeq RNA library, followed with sequencing on
341 an Illumina NextSeq 500 benchtop sequencer on SR150 high output run mode. The RNA-seq
342 data were analyzed using Rockhopper v2.0.3 with the default parameters to calculate the reads
343 per kilobase per million base pairs (RPKM) values for each coding sequence using the *B.*
344 *pertussis* Tohama I BX470248 genome annotation. All RNA-seq experiments were performed
345 on biological duplicates. The results fully supported preliminary microarray analyses for *B.*
346 *pertussis*. qRT-PCR were performed on 3 or 4 separate cultures with at least 3 technical
347 replicates for each condition. To map the transcriptional initiation site by rapid amplification
348 of cDNA 5' ends (RACE), total RNA obtained as described above was treated with the
349 GeneRacer kit (Invitrogen) using appropriate RACE primers (Supplementary Data S7).

350 **Mass spectrometry proteomic analyses.** *B. bronchiseptica* and *B. pertussis* were grown in
351 SS medium supplemented or not with 2 mM CuSO₄ for 10 h and 16 h, respectively, and the
352 cultures were stopped at OD₆₀₀ of 1.6-1.8. The bacteria were collected by centrifugation,
353 resuspended in 50 mM Tris-HCl (pH 7.2), 100 mM NaCl, with Complete protease inhibitor
354 (Roche) and lysed with a French Press. Clarified lysates were ultracentrifuged for 1 hour at
355 100,000 g at 4 °C to separate soluble and insoluble fractions. Both fractions were heated at

356 100°C in 5% SDS, 5% β-mercaptoethanol, 1 mM EDTA, 10% glycerol, 10 mM Tris (pH 8)
357 for 3 min and loaded on a 10% acrylamide SDS-PAGE gel. The migration was stopped soon
358 after the samples entered the separating gel. The gel was briefly stained with Coomassie Blue,
359 and one gel slice was taken for each sample. The gel plugs were washed twice in 25 mM
360 NH₄HCO₃ (50 μL) and acetonitrile (50 μL), the Cys residues were reduced and alkylated by
361 adding 50 μL dithiothreitol (10 mM solution) and incubating at 57°C before the addition of 50
362 μL iodoacetamide (55 mM solution). The plugs were dehydrated with acetonitrile, and the
363 proteins were digested overnight at room temperature using porcine trypsin (12.5 ng/50 μL) in
364 25 mM NH₄HCO₃. Tryptic peptides were extracted first with 60% acetonitrile and 5% formic
365 acid for 1 h, and then with 100% acetonitrile until dehydration of the gel plugs. The extracts
366 were pooled and excess acetonitrile was evaporated before analysis. Peptide fractionation was
367 performed using a 500-mm reversed-phase column at 55 °C and with a gradient separation of
368 170 min. Eluted peptides were analyzed using Q-Extractive instruments (Fisher Scientific)⁵⁶.
369 Raw data collected during nanoLC-MS/MS analyses were processed and converted into *.mgf
370 peak list format with Proteome Discoverer 1.4 (Thermo Fisher Scientific). MS/MS data were
371 interpreted using search engine Mascot (version 2.4.0, Matrix Science, London, UK) installed
372 on a local server. Searches were performed with a tolerance on mass measurement of 10 ppm
373 for precursor ions and 0.02 Da for fragment ions, against two target decoy databases
374 composed of all potential open reading frames of at least 30 residues between stop codons of
375 *B. pertussis* or *B. bronchiseptica* (54890 and 74418 entries, respectively), plus sequences of
376 recombinant trypsin and classical contaminants (118 entries). Cys carbamidomethylation or
377 propionamidation, Met oxidation, and protein N-terminal acetylation were searched as
378 variable modifications. Up to one trypsin missed cleavage was allowed. Peptides were filtered
379 out with Proline 2.0 according to the cutoff set for proteins hits with 1 or more peptides larger
380 than 9 residues, ion score > 10 and 2% protein false positive rates⁵⁶. Spectral counting

381 analyses were performed with Proline 2.0. The p-values were calculated with a Student's T-
382 test (confidence level: 95%). To obtain q-values, the p-values were adjusted for multiple
383 testing correction to control the false discovery rate by following the Benjamini-Hochberg
384 procedure (Q= 5%)⁵⁷.

385 To identify CopZ, *B. pertussis* extracts were separated by electrophoresis using
386 commercial Tricine 16% gels (InVitrogen) and stained with colloidal Coomassie blue dye.
387 The fast-migrating protein band was digested in the gel with trypsin, and the peptide
388 fingerprints were obtained by matrix assisted laser desorption/ ionization time-of-flight mass
389 spectrometry.

390 **Protein production.** For the production of CopZ, PrxGrx and GorB, recombinant pQE30
391 derivatives (Qiagen) were introduced into *E. coli* M15(pREp4). The cells were grown in LB
392 medium at 37°C under orbital shaking, and expression was induced at OD₆₀₀ of 0.8 with 1
393 mM IPTG for 3 hours. Production of recombinant OxyR was done in *E. coli*
394 SG13009(pREp4) in M9 medium with 2% casaminoacids and 250 μM ascorbate. At OD₆₀₀ of
395 0.4, 100 μM IPTG was added, and the culture was continued for 16 hours at 14 °C. The
396 bacteria were collected, and the pellets were resuspended in 50 mM Tris-HCl (pH 7.5), 100
397 mM NaCl, 10 mM imidazole, plus 1 mM TCEP for CopZ and OxyR. The bacteria were lysed
398 with a French press, and the lysates were clarified by centrifugation. The proteins were
399 purified by chromatography on Ni⁺⁺ columns. Concentrations were measured using the BCA
400 assay kit (ThermoFisher Scientific) for PrxGrx and GorB, and with the Qbit protein assay kit
401 for CopZ and OxyR (ThermoFisher Scientific).

402 **Metal binding to CopZ.** Purified CopZ (30 μM) was incubated in 50 mM Tris-HCl (pH 7.5),
403 100 mM NaCl, 1 mM TCEP and 100 mM EDTA for 30 minutes with stirring at room
404 temperature to remove bound metal ions and then dialyzed against the same buffer without

405 EDTA, using 3.5-kD cutoff membranes. The apo form of the protein precipitated in the
406 absence of TCEP. Iron sulfate or copper chloride solutions were prepared in the same buffer
407 containing 1 mM TCEP and 25 mM ascorbate. CopZ was mixed with 125 μ M of either metal
408 for 5 minutes at room temperature, the samples were then dialyzed against the same buffer
409 and finally acidified with 6% nitric acid. Elemental Cu and Fe analyses were performed by
410 ICP-AES (Agilent, model 5110) in axial mode. The wavelengths selected for Cu and Fe
411 analyses were 327.395 nm and 238.204 nm, respectively. ^{63}Cu and ^{65}Cu concentrations were
412 both analysed to evidence potential interferences. To remove polyatomic interferences, a 100
413 mL min^{-1} He flow was added at the collision reaction interface. Impurity of ^{129}Xe in the Ar
414 gas used for generating the plasma was chosen as internal standard to detect any instrumental
415 drift. External calibrations were performed from standard solution at 1g L^{-1} (Astasol),
416 adequately diluted and acidified to be in the range of the unknown concentrations.

417 **Enzymatic assays.** Buffer exchange was performed to obtain the purified recombinant
418 proteins in 0.1 M sodium/potassium phosphate (pH 7.6). Enzymatic activity of GorB was
419 followed at 25 °C in the same buffer containing 0.3 mM NADPH, 8 mM GSH, and H_2O_2 at
420 concentrations ranging from 100 μ M to 2 mM. The latter was used to generate the
421 corresponding concentrations of oxidized glutathione, before starting the reaction by adding
422 the enzyme at a concentration of 0.5 nM ²⁷. The decrease of NADPH concentration was
423 followed by measuring the absorbance of the solution at 340 nm. Activity of PrxGrx was
424 detected in a coupled assay performed at 25°C in the same buffer with 0.3 mM NADPH, 6
425 mM GSH, 0.25 nM GorB and 1 μ M PrxGrx ²⁸. 4 mM H_2O_2 was added last as the substrate of
426 the first reaction to generate the substrate of the second reaction, oxidized glutathione.
427 However, as H_2O_2 also directly generates oxidized glutathione, the activity of PrxGrx was
428 detected by the increased rate at which NADPH was consumed when both enzymes were

429 present in the reaction relative to the reaction rate with GorB only. No enzymatic constants
430 were determined because of the coupled reaction set up.

431 **EMSA.** Amplicons of 127 bp and 139 bp, corresponding to the *cueR-copZ* (IGR 1) and *copZ-*
432 *prxgrx* (IGR 2) intergenic regions, respectively, were mixed at 1 μ M with 0, 2 or 20 μ M
433 recombinant OxyR, in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM MgCl₂, 1 mM
434 TCEP. After 30 min at 37°C, the samples were loaded onto a 6% acrylamide gel containing
435 25 mM Tris-HCl (pH 8.8), 200 mM glycine, 10 mM MgCl₂. Electrophoresis was performed
436 on ice for 90 min at 25 mA in the same buffer. DNA was detected by ethidium bromide
437 staining.

438 **Phagocytosis assays.** THP1 cells were cultured in RPMI medium with 10% fetal bovine
439 serum (RPMI/FBS). They were then treated with 50 ng/mL phorbol 12-myristate 13-acetate
440 (PMA, Sigma) for 24 h to induce differentiation into macrophages and washed in PBS before
441 adding fresh medium. The bacteria were grown at 37°C on BGA for 48 hours, resuspended in
442 PBS and incubated with the macrophages at a multiplicity of infection (MOI) of 100 for 30
443 min at 37°C with 5% CO₂. The macrophages were then washed with PBS containing 100
444 μ g/ml polymyxin B to eliminate extracellular bacteria. For the following incubation the
445 concentration of polymyxin was lowered to 5 μ g/ml in RPMI/FBS, and one and three hours
446 later macrophages were washed with PBS and lysed with 0.1% saponine. Serial dilutions were
447 plated onto BGA for CFU counting. The experiments were performed at least in biological
448 triplicates.

449 **Fluorescence microscopy.** To detect intracellular bacteria, the THP1 cells were cultured as
450 described above. RPMI/FBS was supplemented with 50 μ M bathocuproine disulfonate (BCS)
451 or 20 μ M CuCl₂ during THP1 differentiation and for the following 12 hours, and after
452 washing the cells in RPMI, fresh RPMI/FBS was added 1 hour before contact. Bacteria

453 harboring the reporter plasmid pProm1727-mRFP1 were grown for 12-13 hours in SS
454 medium containing 50 μ M BCS. They were collected by centrifugation, resuspended in PBS
455 to an OD₆₀₀ of 1 and incubated with macrophages at a MOI of 100. After one hour of contact,
456 extracellular bacteria were eliminated, RPMI/FBS supplemented with copper or BCS was
457 added, and the incubation was continued for one hour at 37°C. Finally, the cells were fixed
458 with Formalin (Sigma) for 15 minutes at room temperature and washed three times with PBS.
459 The cell nuclei and the plasma membranes were labeled with DAPI at 30 μ g/ml (Sigma) and
460 Wheat Germ Agglutinin Alexa Fluor™ 488 Conjugate (Invitrogen) at 5 μ g/ml for 10 minutes
461 at room temperature. Cells were then washed twice with PBS. Images were captured by
462 confocal microscopy with a spinning disk microscope Nikon/Gataca system csu-w1. The
463 images were analyzed with the Fiji software⁵⁹. All parameters were set to the same values on
464 all images. The bacteria were identified and counted with the 3D Object Counter module, and
465 the fluorescence intensity was calculated by evaluating the average gray levels of the red
466 objects. Three images containing each between 200 and 266 detectable bacteria were analyzed
467 for each condition.

468 For ATP7A labeling, THP1 cells were cultured as above. One million cells were placed
469 on top of coverslips in each well of a 24-well plate. The cells were then treated for 24 h with
470 PMA in RPMI/FBS as above, with PMA + LPS (500 ng/mL), or with PMA for 24 h and then
471 placed in contact with bacteria at a MOI of 100 for 2 h. The cells were then centrifuged for 3
472 minutes at 300 g, and the supernatant was removed. The centrifugation step was necessary to
473 pellet the non-differentiated, non-adherent control cells. Fixation was performed for 45 min at
474 room temperature with the eBioscience Fixation buffer (ThermoFisher Scientific). The cells
475 were then washed three times by centrifugation and addition of eBioscience Permeabilization
476 buffer (ThermoFisher Scientific). They were then incubated for 35 min with 5% goat serum in
477 the permeabilization buffer, followed by an incubation of 2 h with a monoclonal antibody

478 against ATP7A (Invitrogen MA5-27720) diluted 600 folds. The cells were washed three times
479 as above, before adding Alexa Fluor 488-AffiniPure Donkey Anti-Mouse IgG (Jackson: 715-
480 545-151) diluted 1/500 in permeabilization buffer with 5% goat serum also containing DAPI
481 as described above. After 1 h incubation the cells were washed three times with PBS. Images
482 were obtained using an Evos M5000 microscope with an apochromatic X60 Olympus
483 objective. The final image is a projection of 100 Z stacks with 0.2- μ m steps. All images were
484 captured with the same exposure parameters. The fluorescence intensity of the cells was
485 calculated with the Fiji software (ImageJ) in two dimensions.

486 **Animal experiments.** The bacteria were grown on BGA for 48 h and resuspended in sterile
487 PBS to 10^6 bacteria per 20 μ L. Female, 6-weeks-old JAX TM BALB/ cByJ mice (Charles
488 River) were anesthetized intraperitoneally with a mixture of ketamine, atropine and valium
489 and infected by intranasal inoculation with 10^6 bacteria. Groups of 3-5 animals per bacterial
490 strain were sacrificed 3 h, and 4, 7, 14, or 21 days post-inoculation. Their lungs and naso-
491 pharynxes were removed in a sterile manner and homogenized using an Ultra Thurax
492 apparatus ⁶⁰. The suspensions were serially diluted in PBS and plated onto BGA for CFU
493 counting. All the experiments were carried out in accordance with the guidelines of the
494 French Ministry of Research regarding animal experiments, and the protocols were approved
495 by the Ethical Committees of the Region Nord Pas de Calais and the Ministry of Research
496 (agreement number APAFIS#9107±201603311654342 V3).

497 **Bioinformatic analyses.** The regions upstream of CueR-regulated genes in *E. coli* and
498 *Rubrivivax gelatinosus* (*copA* and *cueO*) were aligned, and Glam2 from the MEME 5.1.0
499 suite ⁶¹ was used to define a pattern. This motif was sought on the genome of *B. pertussis*
500 using Glam2scan. To identify homologues of PrxGrx, the NCBI nr database was searched
501 with the HMMER software ⁶² for proteins carrying both Redoxin (Pfam: PF08534) and
502 Glutaredoxin (Pfam: PF00462) domain signatures. The corresponding genes and the five

503 upstream and downstream genes were retrieved from databases of prokaryotic DNA
504 sequences found in the NCBI site (GenBank, RefSeq and WGS).

505 **Statistics and Reproducibility.** Three to six independent biological samples were used to
506 determine growth yields and survival to oxidative stress or macrophages. For the animal
507 colonization experiments, 4 and 5 mice per time point were used for the wt and mutant
508 bacteria, respectively. To determine the Cu/CopZ ratios, three individual samples were
509 processed. For experiments using samples of small sizes, statistical tests were performed with
510 the GraphPad Prism software using non-parametric two-tailed Mann-Whitney tests
511 (confidence level 95%). For qRT-PCR 3 independent biological samples were used. Given
512 that the *cueR* and *oxyR* KO bacteria grew poorly in the presence of copper or hydrogen
513 peroxide, respectively, the error bars were large. No statistical analyses were performed in
514 those cases. For quantification of the fluorescence of intracellular bacteria, large numbers of
515 bacteria were used (>600). In that case, the sample sizes allowed to use a parametric two-
516 tailed Student T-test (confidence level 95%; 1381 degrees of freedom) of the GraphPad Prism
517 software.

518 **Data availability statement.** The transcriptomic data have been deposited in the GEO
519 repository of NCBI (GEO accession: GSE145049). The mass spectrometry proteomics data
520 have been deposited in the ProteomeXchange Consortium via the PRIDE⁵⁸ partner repository
521 with the dataset identifier PXD020900 and 10.6019/PXD020900. The data used to generate
522 the graphs and charts can be found in Supplementary Data S8.

523 **Code Availability statement.** The code generated in this work to retrieve the genetic
524 environment of genes of interest is available to readers without restriction. It can be accessed
525 using the following link: <https://github.com/rudantoine/GeneEnv.git>

526

527 **Competing interests.** The authors declare no competing interests.

528 **Authors contributions.** A.R.-M., R.A and F.J.-D. designed the study. A.R.-M., J.C., S.S.
529 G.R., J.-M.S., G.B., V.A., D.H., S.S.-D. performed the experiments. A.R.-M., R.A and F.J.-D.
530 analyzed the data. A.R.-M. and F.J.-D. wrote the manuscript. C.L. provided advise. All
531 authors read and made corrections to the manuscript.

532 **Acknowledgments**

533 We thank Violaine Dubois for sharing unpublished data, Hervé Drobecq for mass
534 fingerprinting analyses and Blanche Daunou for preliminary work with recombinant proteins.
535 A. R.-M. and G. R. acknowledge the support of doctoral fellowships from the University of
536 Lille- Region Hauts-de-France and from the University of Lille, respectively. A. R.-M. also
537 thanks the Fondation de la Recherche Médicale (FRM) for their support. This work was
538 funded by the Institut National de la Santé et de la Recherche Médicale (INSERM) and the
539 University of Lille. ICP-AES measurements were performed on the Chevreul Institute
540 Platform (U-Lille / CNRS). The Region Hauts de France and the French government are
541 acknowledged for co-funding this equipment.

542

543 **References**

- 544 1. Solioz, M. *Copper and Bacteria. Evolution, homeostasis and toxicity*, Springer Nature
545 Switzerland AG, Cham, Switzerland (2018).
- 546 2. Chillappagari, S. *et al.* Copper stress affects iron homeostasis by destabilizing iron-sulfur
547 cluster formation in *Bacillus subtilis*. *J Bacteriol* **192**, 2512-24 (2010).
- 548 3. Macomber, L. & Imlay, J.A. The iron-sulfur clusters of dehydratases are primary intracellular
549 targets of copper toxicity. *Proc Natl Acad Sci USA* **106**, 8344-9 (2009).
- 550 4. Dalecki, A.G., Crawford, C.L. & Wolschendorf, F. Copper and Antibiotics: Discovery, Modes of
551 Action, and Opportunities for Medicinal Applications. *Adv Microb Physiol* **70**, 193-260 (2017).
- 552 5. Hodgkinson, V. & Petris, M.J. Copper homeostasis at the host-pathogen interface. *J Biol Chem*
553 **287**, 13549-55 (2012).
- 554 6. Migocka, M. Copper-transporting ATPases: The evolutionarily conserved machineries for
555 balancing copper in living systems. *IUBMB Life* **67**, 737-45 (2015).

- 556 7. O'Halloran, T.V. & Culotta, V.C. Metallochaperones, an intracellular shuttle service for metal
557 ions. *J Biol Chem* **275**, 25057-60 (2000).
- 558 8. Stafford, S.L. *et al.* Metal ions in macrophage antimicrobial pathways: emerging roles for zinc
559 and copper. *Biosci Rep* **33** (2013).
- 560 9. White, C., Lee, J., Kambe, T., Fritsche, K. & Petris, M.J. A role for the ATP7A copper-
561 transporting ATPase in macrophage bactericidal activity. *J Biol Chem* **284**, 33949-56 (2009).
- 562 10. Hao, X. *et al.* A role for copper in protozoan grazing - two billion years selecting for bacterial
563 copper resistance. *Mol Microbiol* **102**, 628-641 (2016).
- 564 11. Sheldon, J.R. & Skaar, E.P. Metals as phagocyte antimicrobial effectors. *Curr Opin Immunol*
565 **60**, 1-9 (2019).
- 566 12. Chandrangsu, P., Rensing, C. & Helmann, J.D. Metal homeostasis and resistance in bacteria.
567 *Nat Rev Microbiol* **15**, 338-350 (2017).
- 568 13. Ladomersky, E. & Petris, M.J. Copper tolerance and virulence in bacteria. *Metallomics* **7**, 957-
569 64 (2015).
- 570 14. Arguello, J.M., Raimunda, D. & Padilla-Benavides, T. Mechanisms of copper homeostasis in
571 bacteria. *Front Cell Infect Microbiol* **3**, 73 (2013).
- 572 15. Singh, S.K., Grass, G., Rensing, C. & Montfort, W.R. Cuprous oxidase activity of CueO from
573 *Escherichia coli*. *J Bacteriol* **186**, 7815-7 (2004).
- 574 16. Durand, A. *et al.* c-Type Cytochrome Assembly Is a Key Target of Copper Toxicity within the
575 Bacterial Periplasm. *mBio* **6**, e01007-15 (2015).
- 576 17. Novoa-Aponte, L., Ramirez, D. & Arguello, J.M. The interplay of the metallosensor CueR with
577 two distinct CopZ chaperones defines copper homeostasis in *Pseudomonas aeruginosa*. *J Biol Chem*
578 **294**, 4934-4945 (2019).
- 579 18. Antoine, R., Rivera-Millot, A., Roy, G. & Jacob-Dubuisson, F. Relationships Between Copper-
580 Related Proteomes and Lifestyles in beta Proteobacteria. *Front Microbiol* **10**, 2217 (2019).
- 581 19. Melvin, J.A., Scheller, E.V., Miller, J.F. & Cotter, P.A. *Bordetella pertussis* pathogenesis:
582 current and future challenges. *Nat Rev Microbiol* **12**, 274-88 (2014).
- 583 20. Capdevila, D.A., Edmonds, K.A. & Giedroc, D.P. Metallochaperones and metalloregulation in
584 bacteria. *Essays Biochem* **61**, 177-200 (2017).
- 585 21. Rademacher, C. & Masepohl, B. Copper-responsive gene regulation in bacteria. *Microbiology*
586 **158**, 2451-2464 (2012).
- 587 22. Kidd, S.P. & Brown, N.L. ZccR--a MerR-like regulator from *Bordetella pertussis* which responds
588 to zinc, cadmium, and cobalt. *Biochem Biophys Res Commun* **302**, 697-702 (2003).
- 589 23. Helbig, K., Bleuel, C., Krauss, G.J. & Nies, D.H. Glutathione and transition-metal homeostasis
590 in *Escherichia coli*. *J Bacteriol* **190**, 5431-8 (2008).
- 591 24. Radford, D.S. *et al.* CopZ from *Bacillus subtilis* interacts in vivo with a copper exporting CPx-
592 type ATPase CopA. *FEMS Microbiol Lett* **220**, 105-12 (2003).
- 593 25. Hearnshaw, S. *et al.* A tetranuclear Cu(I) cluster in the metallochaperone protein CopZ.
594 *Biochemistry* **48**, 9324-6 (2009).
- 595 26. Rouhier, N. & Jacquot, J.P. Molecular and catalytic properties of a peroxiredoxin-glutaredoxin
596 hybrid from *Neisseria meningitidis*. *FEBS Lett* **554**, 149-53 (2003).
- 597 27. Vergauwen, B. *et al.* Characterization of glutathione amide reductase from *Chromatium*
598 *gracile*. Identification of a novel thiol peroxidase (Prx/Grx) fueled by glutathione amide redox cycling.
599 *J Biol Chem* **276**, 20890-7 (2001).
- 600 28. Pauwels, F., Vergauwen, B., Vanrobaeys, F., Devreese, B. & Van Beeumen, J.J. Purification and
601 characterization of a chimeric enzyme from *Haemophilus influenzae* Rd that exhibits glutathione-
602 dependent peroxidase activity. *J Biol Chem* **278**, 16658-66 (2003).
- 603 29. Omsland, A., Miranda, K.M., Friedman, R.L. & Boitano, S. *Bordetella bronchiseptica* responses
604 to physiological reactive nitrogen and oxygen stresses. *FEMS Microbiol Lett* **284**, 92-101 (2008).
- 605 30. Yamamoto, K. & Ishihama, A. Transcriptional response of *Escherichia coli* to external copper.
606 *Mol Microbiol* **56**, 215-27 (2005).
- 607 31. Green, J. & Paget, M.S. Bacterial redox sensors. *Nat Rev Microbiol* **2**, 954-66 (2004).

- 608 32. Hillion, M. & Antelmann, H. Thiol-based redox switches in prokaryotes. *Biol Chem* **396**, 415-
609 44 (2015).
- 610 33. Imlay, J.A. Where in the world do bacteria experience oxidative stress? *Environ Microbiol* **21**,
611 521-530 (2019).
- 612 34. Samanovic, M.I., Ding, C., Thiele, D.J. & Darwin, K.H. Copper in microbial pathogenesis:
613 meddling with the metal. *Cell Host Microbe* **11**, 106-15 (2012).
- 614 35. Quintana, J., Novoa-Aponte, L. & Arguello, J.M. Copper homeostasis networks in the
615 bacterium *Pseudomonas aeruginosa*. *J Biol Chem* **292**, 15691-15704 (2017).
- 616 36. Taylor-Mulneix, D.L. *et al.* *Bordetella bronchiseptica* exploits the complex life cycle of
617 *Dictyostelium discoideum* as an amplifying transmission vector. *PLoS Biol* **15**, e2000420 (2017).
- 618 37. Andreasen, C. & Carbonetti, N.H. Pertussis toxin inhibits early chemokine production to delay
619 neutrophil recruitment in response to *Bordetella pertussis* respiratory tract infection in mice. *Infect*
620 *Immun* **76**, 5139-48 (2008).
- 621 38. Ahmad, J.N. *et al.* *Bordetella* Adenylate Cyclase Toxin Inhibits Monocyte-to-Macrophage
622 Transition and Dedifferentiates Human Alveolar Macrophages into Monocyte-like Cells. *mBio* **10**,
623 01743-19 (2019).
- 624 39. Lamberti, Y.A., Hayes, J.A., Perez Vidakovics, M.L., Harvill, E.T. & Rodriguez, M.E. Intracellular
625 trafficking of *Bordetella pertussis* in human macrophages. *Infect Immun* **78**, 907-13 (2010).
- 626 40. Cafiero, J.H., Lamberti, Y.A., Surmann, K., Vecerek, B. & Rodriguez, M.E. A *Bordetella pertussis*
627 MgtC homolog plays a role in the intracellular survival. *PLoS One* **13**, e0203204 (2018).
- 628 41. Lamberti, Y., Perez Vidakovics, M.L., van der Pol, L.W. & Rodriguez, M.E. Cholesterol-rich
629 domains are involved in *Bordetella pertussis* phagocytosis and intracellular survival in neutrophils.
630 *Microb Pathog* **44**, 501-11 (2008).
- 631 42. Boyd, A.P. *et al.* *Bordetella pertussis* adenylate cyclase toxin modulates innate and adaptive
632 immune responses: distinct roles for acylation and enzymatic activity in immunomodulation and cell
633 death. *J Immunol* **175**, 730-8 (2005).
- 634 43. Carbonetti, N.H. Immunomodulation in the pathogenesis of *Bordetella pertussis* infection and
635 disease. *Curr Opin Pharmacol* **7**, 272-8 (2007).
- 636 44. Fedele, G., Bianco, M. & Ausiello, C.M. The virulence factors of *Bordetella pertussis*: talented
637 modulators of host immune response. *Arch Immunol Ther Exp (Warsz)* **61**, 445-57 (2013).
- 638 45. de Gouw, D., Diavatopoulos, D.A., Bootsma, H.J., Hermans, P.W. & Mooi, F.R. Pertussis: a
639 matter of immune modulation. *FEMS Microbiol Rev* **35**, 441-74 (2011).
- 640 46. Utz, M. *et al.* The Cu chaperone CopZ is required for Cu homeostasis in *Rhodobacter*
641 *capsulatus* and influences cytochrome cbb3 oxidase assembly. *Mol Microbiol* **111**, 764-783 (2019).
- 642 47. Brose, J., La Fontaine, S., Wedd, A.G. & Xiao, Z. Redox sulfur chemistry of the copper
643 chaperone Atox1 is regulated by the enzyme glutaredoxin 1, the reduction potential of the
644 glutathione couple GSSG/2GSH and the availability of Cu(I). *Metallomics* **6**, 793-808 (2014).
- 645 48. Hatori, Y. & Lutsenko, S. An expanding range of functions for the copper
646 chaperone/antioxidant protein Atox1. *Antioxid Redox Signal* **19**, 945-57 (2013).
- 647 49. Hamidou Soumana, I., Linz, B. & Harvill, E.T. Environmental Origin of the Genus *Bordetella*.
648 *Front Microbiol* **8**, 28 (2017).
- 649 50. Rivera, I. *et al.* Conservation of Ancient Genetic Pathways for Intracellular Persistence Among
650 Animal Pathogenic *Bordetellae*. *Front Microbiol* **10**, 2839 (2019).
- 651 51. Koh, E.I., Robinson, A.E., Bandara, N., Rogers, B.E. & Henderson, J.P. Copper import in
652 *Escherichia coli* by the yersiniabactin metallophore system. *Nat Chem Biol* **13**, 1016-1021 (2017).
- 653 52. Rivera-Millot, A. *et al.* Characterization of a Bvg-regulated fatty acid methyl-transferase in
654 *Bordetella pertussis*. *PLoS ONE* **12**, e0176396 (2017).
- 655 53. Antoine, R. *et al.* New virulence-activated and virulence-repressed genes identified by
656 systematic gene inactivation and generation of transcriptional fusions in *Bordetella pertussis*. *J*
657 *Bacteriol* **182**, 5902-5905 (2000).
- 658 54. Kovach, M.E. *et al.* Four new derivatives of the broad-host-range cloning vector pBBR1MCS,
659 carrying different antibiotic-resistance cassettes. *Gene* **166**, 175-6 (1995).

- 660 55. Lesne, E. *et al.* Distinct virulence ranges for infection of mice by *Bordetella pertussis* revealed
661 by engineering of the sensor-kinase BvgS. *PLoS One* **13**, e0204861 (2018).
- 662 56. Veyron-Churlet, R. *et al.* Rv0613c/MSMEG_1285 Interacts with HBHA and Mediates Its
663 Proper Cell-Surface Exposure in Mycobacteria. *Int J Mol Sci* **19** (2018).
- 664 57. Benjamini, Y., Drai, D., Elmer, G., Kafkafi, N. & Golani, I. Controlling the false discovery rate in
665 behavior genetics research. *Behav Brain Res* **125**, 279-84 (2001).
- 666 58. Perez-Riverol, Y. *et al.* The PRIDE database and related tools and resources in 2019:
667 improving support for quantification data. *Nucleic Acids Res* **47**(D1):D442-D450 (2019).
- 668 59. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**,
669 676-82 (2012).
- 670 60. Solans, L. *et al.* IL-17-dependent SIgA-mediated protection against nasal *Bordetella pertussis*
671 infection by live attenuated BPZE1 vaccine. *Mucosal Immunol* **11**, 1753-1762 (2018).
- 672 61. Frith, M.C., Saunders, N.F.W., Kobe, B. & Bailey, T.L. Discovering sequence motifs with
673 arbitrary insertions and deletions. *PLoS Comput Biol*, **5** (2008).
- 674 62. Johnson, L.S., Eddy, S.R. & Portugaly, E. Hidden Markov model speed heuristic and iterative
675 HMM search procedure. *BMC Bioinformatics* **11**, 431 (2010).

676

677

678 **Figure 1. Effect of copper on *B. pertussis* and *B. bronchiseptica* growth.** Growth curves of
679 *B. pertussis* BPSM and *B. bronchiseptica* RB50 in SS medium supplemented (blue curves) or
680 not (orange curves) with 5 mM CuSO₄ are shown. The turbidity of the cultures was measured
681 using an Elocheck device. The values shown in ordinate (absorbency units) do not correspond
682 to optical density measurements classically obtained with a spectrometer, since the Elocheck
683 instrument uses distinct pathlength and wavelength.

684

685 **Figure 2. Copper regulation of homeostasis systems in *B. pertussis* and *B. bronchiseptica*.**
686 **(a, c and d)** RNAseq analyses of *B. pertussis* grown for 16 hours in the presence of 2 mM
687 CuSO₄ in SS medium **(a)**, *B. pertussis* grown in SS medium and treated for 30 minutes with 2
688 mM of CuSO₄ **(c)**, and *B. bronchiseptica* grown for 12 hours in the presence of 2 mM CuSO₄
689 **(d)**. Comparisons were made with bacteria grown in standard conditions. Each gene is
690 represented by a dot. The x and y axes show absolute levels of gene expression in reads per
691 kilobase per million base pairs (RPKM) in standard and copper conditions, respectively. The
692 genes indicated in blue indicate genes of interest with the strongest regulation factors. The full
693 sets of data are shown in Supplementary Tables S1, S2 and S3. **(b)** Summary of the
694 transcriptomic and proteomic analyses performed after growing bacteria as in **(a)** and **(c)** in
695 medium supplemented with 2 mM CuSO₄. Standard culture conditions were used for
696 comparisons. * indicates small proteins difficult to detect by global proteomic approaches.

697

698 **Figure 3. Identification of CopZ in *B. pertussis*.** **(a)** Lysates of wild type *B. pertussis* (wt) or
699 the deletion mutant ($\Delta bp1727$) grown in SS medium supplemented (+ Cu) or not with 2 mM
700 CuSO₄ were subjected to SDS-PAGE electrophoresis in Tris-tricine gels for the detection of
701 CopZ. The gel was stained with colloidal Coomassie blue dye. CopZ migrates below the 10
702 kDa band of the markers. **(b and c)** The protein band was cut from the gel, and CopZ was
703 identified by mass fingerprinting analyses. The m/z ratios of the peptides identified by
704 MALDI-TOF are shown in panel b, and the sequences of the peptides are in panel c. This
705 experiment complements the proteomic analyses, because the small size of CopZ hampered
706 its detection in global proteomic approaches (see Fig. 2b and Supplementary Data S4).

707

708 **Figure 4. Role of the operon in *B. pertussis* and in host-pathogen interactions.** **(a and b)**
709 Growth yields of *B. pertussis* after 24 h in SS medium **(a)** or in SS medium devoid of
710 glutathione **(b)**, supplemented or not with 2 mM CuSO₄. The cultures were inoculated at

711 initial OD₆₀₀ values of 0.1, and after 24 h the OD₆₀₀ were determined. Note the different y axis
712 scales between (a) and (b). wt, wild type (parental) strain; Δ27, Δ28-29 and Δ27-29, KO
713 mutants for *bp1727 (copZ)*, *bp1728-bp1729 (prxgrx-gorB)* and the three genes, respectively.
714 The various strains are represented using different symbols and shades of grey. Δ27-29/+27-
715 29 represents the latter mutant complemented by expression of the operon at another
716 chromosomal locus. The horizontal orange lines represent mean values. (c) Survival of the
717 same strains to an oxidative shock of 30 minutes. (d) Intracellular survival of *B. pertussis* in
718 THP1 macrophages. The horizontal orange lines represent mean values. See Supplementary
719 Figure S6 for the data of the individual *copZ* and *prxgrx-gorB* KO mutants. (e and f)
720 Colonization of mice lungs (e) and nasopharynxes (f) after nasal infections with the parental
721 strain and the *copZ-prxgrx-gorB* KO mutant. The numbers of bacteria are indicated for each
722 mouse and organ (black circles: parental bacteria, red squares: mutant). The lines connect the
723 geometric means of the counts at each time point, and the dotted lines indicate the thresholds
724 of detection. For all the assays, statistical analyses were performed using two-tailed Mann-
725 Whitney tests (*, $p < 0.05$; **, $p < 0.005$). For panels a and b, 5 biologically independent
726 samples were used. For panels c and d, 4 and 6 biological samples were used, respectively.
727 For panels e and f, 5 and 4 animals per time point were used for the KO and wt strains,
728 respectively.

729

730 **Figure 5. Activities of the proteins coded by the *bp1727-1728-1729 (copZ-prxgrx-gorB)***
731 **operon.** (a) The metal/protein ratios of recombinant CopZ were determined by ICP-AES. The
732 apo form of CopZ was incubated or not with iron (gray) or copper (black), and both ions were
733 measured. Three independent samples were processed. (b) Oxidation of NADPH by
734 recombinant GorB over time. H₂O₂ was added at the indicated concentrations to generate the
735 glutathione disulfide (GSSG) substrate of GorB, before adding the enzyme. The reaction was
736 followed by the decrease of absorbency at 340 nm. (c) Plot of the reaction rate as a function of
737 substrate concentration. A k_{cat}/K_m value of $323,000 \pm 46,000 \text{ M}^{-1} \text{ s}^{-1}$ for GSSG was estimated
738 based on Michaelis-Menten kinetics. One or two measurements were performed at each
739 substrate concentration. (d) Effect of recombinant PrxGrx on the rate of oxidation of NADPH
740 by GorB. The red and blue curves show reaction rates when GorB was present alone or when
741 both enzymes were present, respectively. H₂O₂ was added last as a substrate of the first
742 reaction. However, as H₂O₂ also generates GSSG, which initiates the second reaction, PrxGrx
743 activity was detected by the increased rate of the reaction when both enzymes are present, and

744 no enzymatic constants could be determined. (e) Schematic representation of the functions of
745 the three proteins. By chelating Cu^{1+} , CopZ prevents the ion from generating hydroxyl
746 radicals through the Fenton reaction. H_2O_2 is reduced to H_2O through the activity of PrxGrx,
747 at the expense of reduced glutathione (GSH). The product of that reaction, glutathione
748 disulfide, is reduced through the activity of GorB at the expense of NADPH.

749

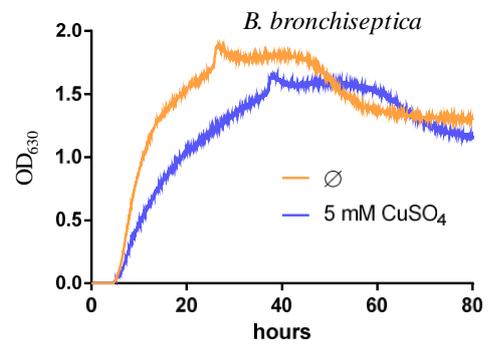
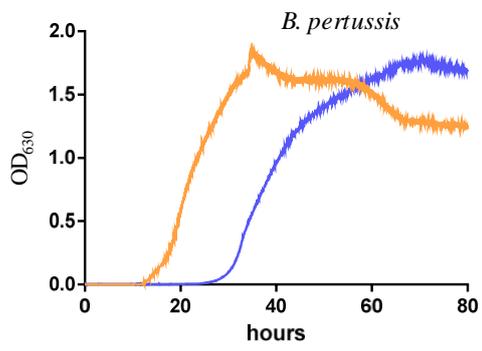
750 **Figure 6. Regulation of the operon.** (a and b) qRT-PCR analyses of the parental and the
751 *cueR* KO strains treated for 30 min with 2 mM CuSO_4 (a) or 10 mM H_2O_2 (b), showing the
752 expression levels of *prxgrx* relative to untreated controls. Data were normalized with the
753 housekeeping gene *bp3416*. Three biological replicates were performed. (c) EMSA with
754 recombinant OxyR and DNA fragments of the *cueR-copZ* and *copZ-prxgrx* intergenic regions,
755 IGR 1 and IGR 2, respectively. The uncropped gel is shown in Supplementary Figure S10. (d)
756 Schematic representation of the locus, with sequences of the putative CueR and OxyR boxes.
757 The site of transcription initiation was determined by 5'RACE (Supplementary Figure S5).
758 The putative OxyR binding sites were identified by their similarity with the *E. coli* consensus
759 sequences, and alignments of the *Bordetella* and *Achromobacter* sequences were used to build
760 the consensus motif (Supplementary Figure S11).

761

762 **Figure 7. Upregulation of the operon in macrophages.** (a) Representative images of THP1
763 macrophages having engulfed *B. pertussis* harboring the *mRPF1* gene under the control of the
764 *copZ-prxgrx-gorB* operon promoter. Macrophages were either starved of copper using a
765 chelator or treated with copper chloride prior to contact with bacteria. The bacteria are red,
766 cell membranes are green, and nuclei are blue. In the two fields shown at the left, the bacteria
767 were projected as objects, showing that similar numbers are present in both conditions. All
768 image panels are shown at the same magnification. (b) Levels of fluorescence (arbitrary units)
769 of intracellular bacteria in the two conditions. Statistical analyses were performed using a
770 two-tailed Student T-test (****, $p < 0.0001$). The horizontal orange lines represent median
771 values ($n > 500$ in both cases).

772

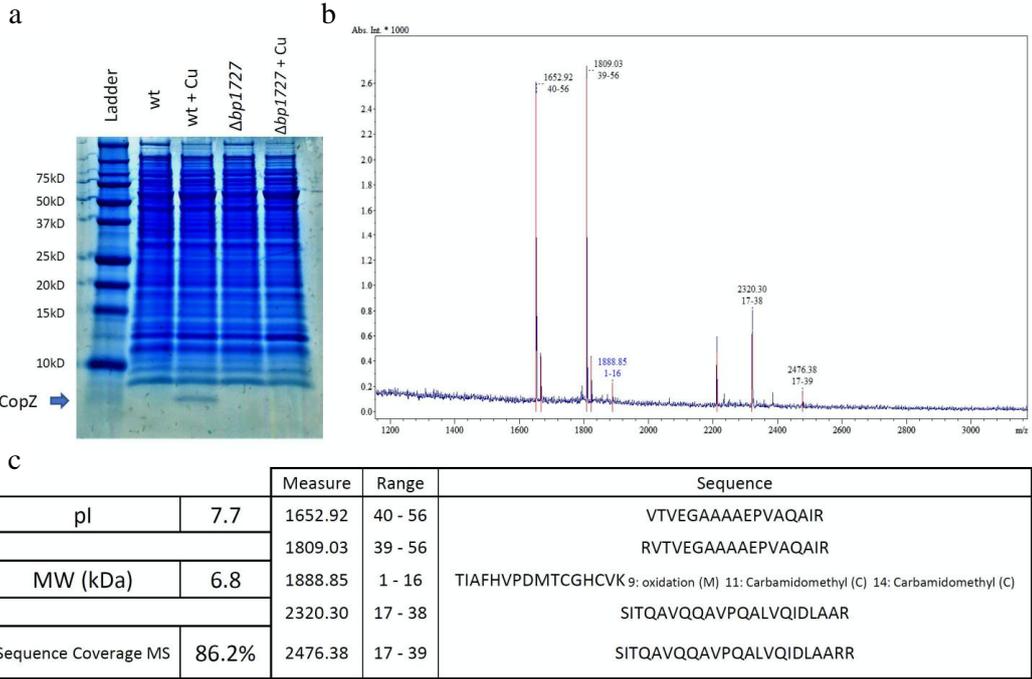
773



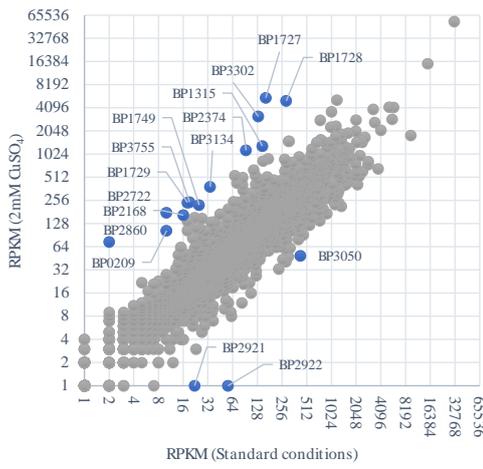
774

775

776



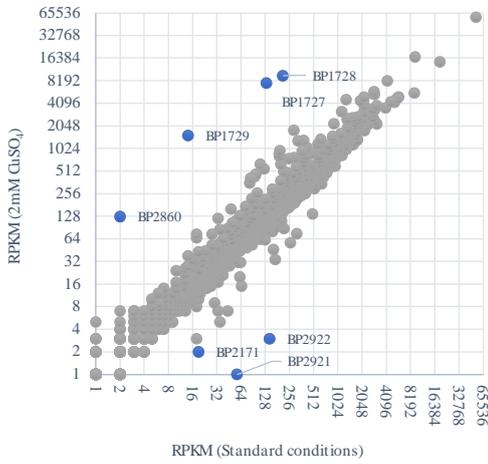
a



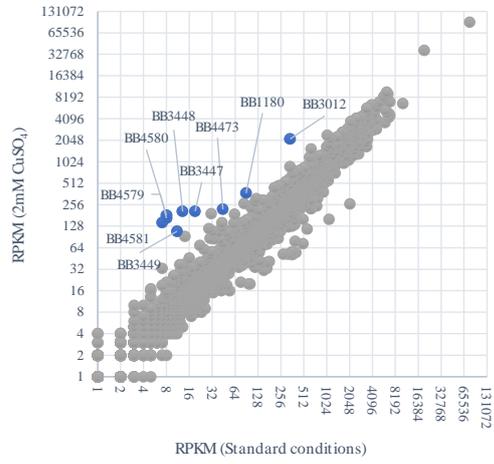
b

Protein	Gene	<i>B. pertussis</i>				<i>B. bronchiseptica</i>				
		Transcriptomics (RPKM)		Proteomics (Spectral count)		Transcriptomics (RPKM)		Proteomics (Spectral count)		
		standard	Copper	standard	Copper	standard	Copper	standard	Copper	
CopZ [*]	<i>bp1727</i>	162	5550	0	2	<i>bb3012</i>	338	2145	0	0
PrxGrx	<i>bp1728</i>	288	5059	1	6	<i>bb3011</i>	478	664	10	22
GorB	<i>bp1729</i>	18	241	0	57	<i>bb3010</i>	42	23	1	8
CopA	<i>bp2860</i>	2	74	0	0	<i>bb1180</i>	89	373	0	0
CopI [*]	<i>bp3314</i>	1	1	0	0	<i>bb4581</i>	7	145	0	17
PcoA	<i>bp3315</i>	2	2	0	0	<i>bb4580</i>	8	183	0	52
PcoB	<i>bp3316</i>	1	2	0	0	<i>bb4579</i>	8	166	0	18
Blue copper protein [*]	<i>bp0156</i>	34	26	0	4	<i>bb4473</i>	44	222	0	17
CopS	<i>bp0157</i>	4	5	0	0	<i>bb4472</i>	12	12	1	0
CopR	<i>bp0158</i>	13	12	0	0	<i>bb4471</i>	25	27	1	1
CueR	<i>bp1726</i>	78	181	0	2	<i>bb3013</i>	138	208	0	0
CusF [*]	<i>bp3088</i>	820	989	0	0	<i>bb0177</i>	160	144	0	0
Csp [*]						<i>bb2842</i>	7	8	0	0
CusABC										

c



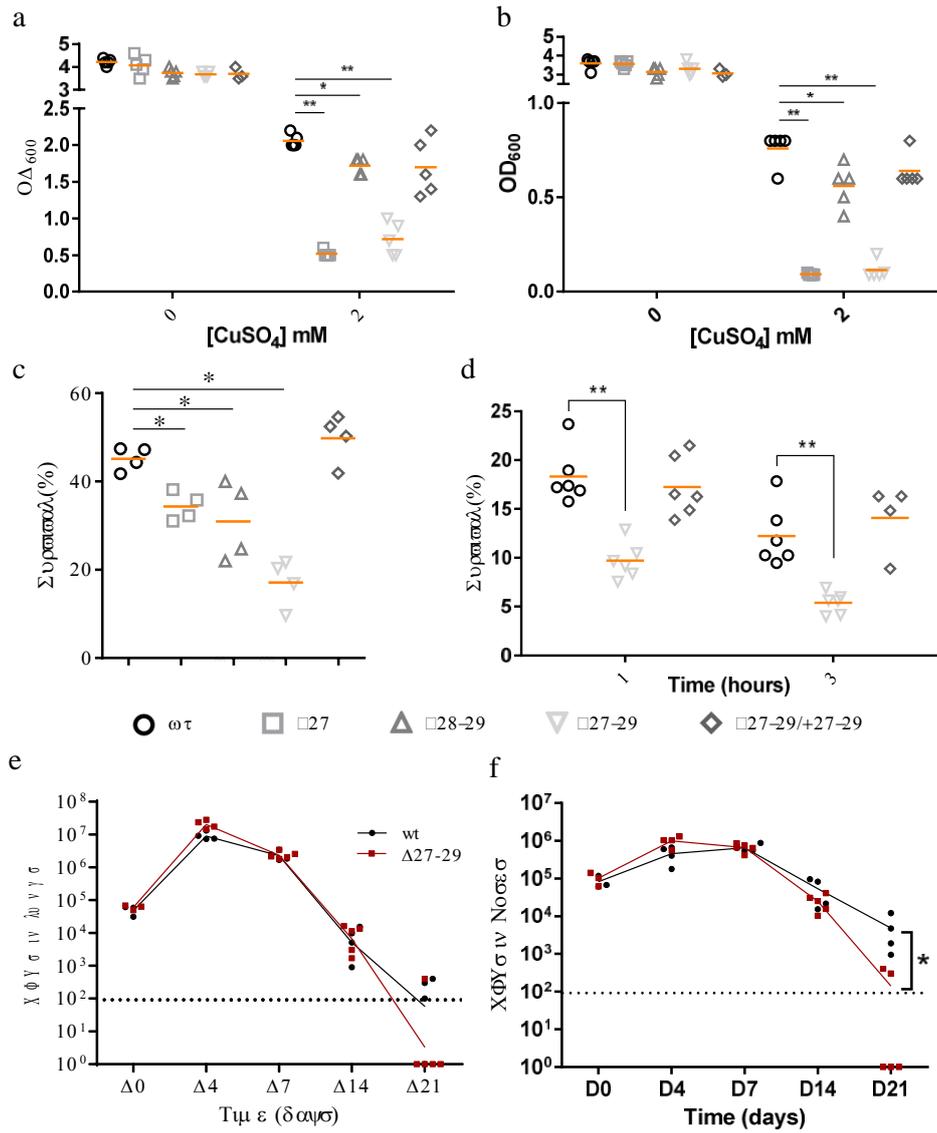
d



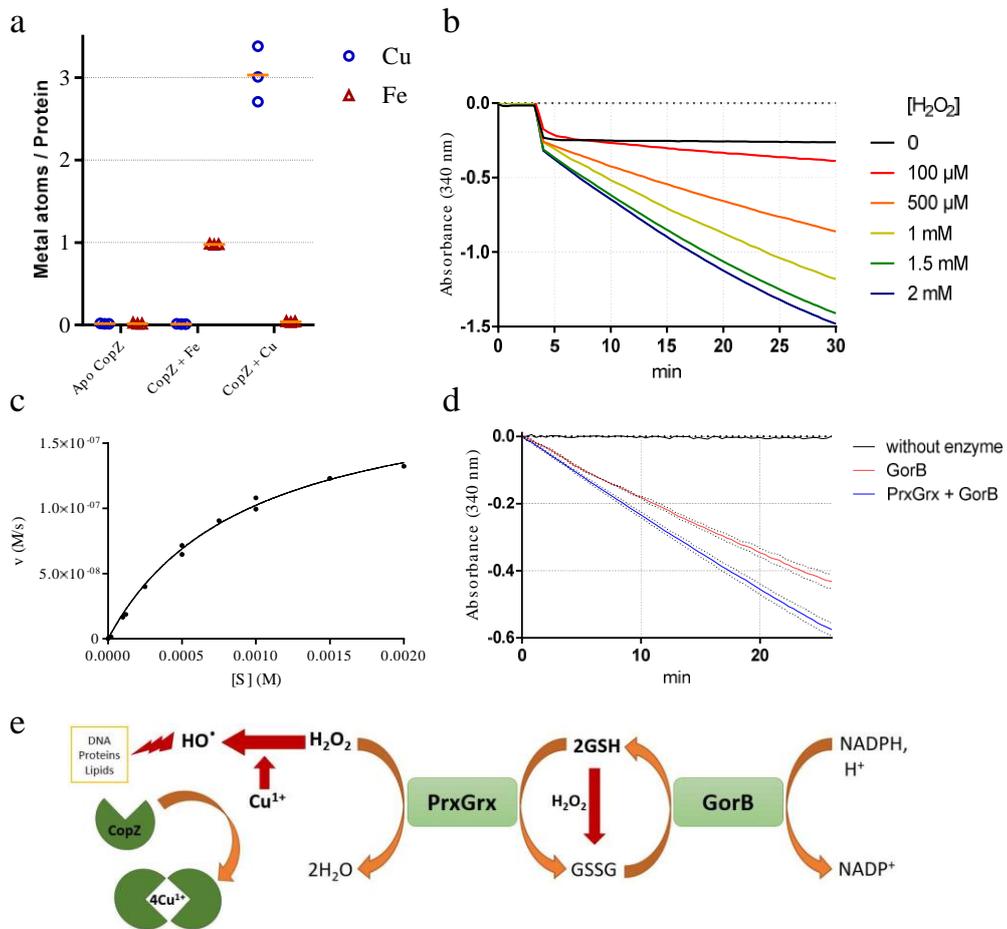
778

779

780

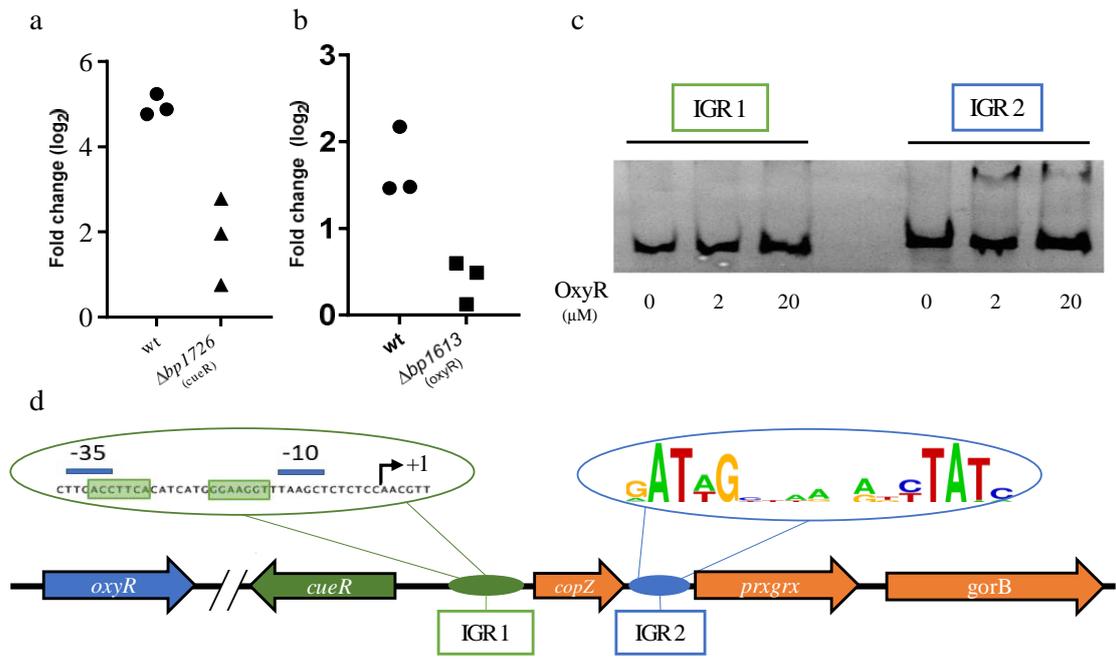


781
782
783



784

785



786

787

