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The CpxAR signaling system confers a fitness advantage for flea gut colonization by the plague bacillus

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ABSTRACT The adaptation of *Yersinia pestis*, the flea-borne plague agent, to fluctuating environmental conditions is essential for the successful colonization of the flea vector. A previous comparative transcriptomic analysis showed that the Cpx pathway of *Y. pestis* is up-regulated in infected fleas. The CpxAR two-component system is a component of the envelope stress response and is critical for maintaining the integrity of the cell. Here, a phenotypic screening revealed a survival defect of the *cpxAR* mutant to oxidative stress and copper. The measured copper concentration in the digestive tract contents of fed fleas increased fourfold during the digestive process. By direct analysis of phosphorylation of CpxR by a Phos-Tag gel approach, we demonstrated that biologically relevant concentrations of copper triggered the system. Then, a competitive challenge highlighted the role of the CpxAR system in bacterial fitness during flea infection. Lastly, an *in vitro* sequential exposure to copper and then H_2O_2 to mimic the flea suggests a model in which, within the insect digestive tract, the CpxAR system would be triggered by copper, establishing an oxidative stress response.

IMPORTANCE The bacterium *Yersinia pestis* is the agent of flea-borne plague. Our knowledge of the mechanisms used by the plague bacillus to infect the flea vector is limited. The up-regulation of the envelope stress response under the control of the Cpx signaling pathway was previously shown in a transcriptomic study. Here, our *in vivo* and *in vitro* approaches suggest a model in which *Y. pestis* uses the CpxAR phosphorelay system to sense and respond to the copper present in the flea gut, thereby optimizing the flea gut colonization. In other words, the system is essential for bacterial fitness in the flea.

KEYWORDS *Yersinia pestis*, plague, flea, *cpxAR*, fitness

Plague, caused by the bacterium *Yersinia pestis*, is a devasting infectious disease that has ravaged human populations for centuries (1). Despite the availability of effective antibiotics and improved public health measures, plague remains a serious threat in many parts of the world, particularly in Africa, Asia, and the Americas, where it continues to cause sporadic outbreaks and pose a significant public health challenge (2). Understanding the biology, transmission, and pathology of this fatal disease is critical to prevent and control it.

Y. pestis is transmitted to mammals by flea bites. During infection of the insect, the bacterium must colonize and obstruct the flea's foregut to increase the likelihood of transmission to a new host (3–7). Shortly after ingestion, *Y. pestis* may elicit a flea response in the proventriculus, leading to the formation of a soft bactericidal mass to which it resists. The plague bacillus then consolidates this mass by secreting poly-β-1,6-N-acetyl-D-glucosamine, blocking the passage of food and leading to the regurgitation of infected blood during feeding (3, 4, 6, 7). However, the exact mechanism of how

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Y. pestis colonizes the flea digestive tract and forms the bacterial mass is still not fully understood (7, 8).

The flea presents an inconstant environment with physicochemical parameters that are gradually or abruptly changing before, during, and after feeding (7, 9–12). Adaptation to these fluctuating conditions is mainly achieved by two-component systems or phosphorelays. The CpxAR two-component regulatory system is part of the envelope stress response and is critical for maintaining the integrity of the cell (13). The system consists of the inner membrane histidine sensor kinase CpxA and its cognate cytoplasmic response regulator CpxR (14–16). Upon specific stress, the CpxA autophosphorylates and transfers its phosphate group to CpxR. In *Escherichia coli*, the phosphorylated regulator modulates dozens of genes involved in protein folding and degradation, chemotaxis, motility, multidrug efflux, and DNA repair (17–19). The system is negatively regulated by the periplasmic protein CpxP, which interacts with CpxA and inhibits its autophosphorylation (20). Previous studies have reported that the CpxAR system enables bacteria to respond to several stresses such as oxidative (21, 22) and osmotic stresses (23) and is involved in virulence (24–27).

In *Yersinia* species, the system is associated with virulence in *Yersinia enterocolitica* (28, 29) and *Yersinia pseudotuberculosis* (30, 31). Despite the system's involvement in *Y. pestis*' resistance to human neutrophils' bactericidal activity (32), no role in bubonic or pulmonary plague has been found (33). An upregulation of *cpxA* and *cpxP* genes was described in fleas compared with a planktonic culture in a comparative transcriptomic analysis (34), suggesting that the CpxAR system may be activated during insect colonization. However, the role of the CpxAR system in the flea has not yet been investigated. It should be highlighted that the *cpxP* gene is not essential for biofilm formation and flea colonization (34).

Here, we investigated the role of the CpxAR system in flea colonization. We identified that the CpxAR system was involved in the survival of H_2O_2 and copper, two stresses found in the flea. However, the phosphorylation of CpxR is only enhanced upon exposure to copper. Lastly, by co-infecting fleas with the *cpxAR* mutant and the wild-type strain, we revealed a role for CpxAR in bacterial fitness during flea infection.

RESULTS

The CpxAR system is required for survival to H_2O_2 and copper oxide stresses

During the flea colonization process, *Y. pestis* forms a biofilm to increase the likelihood of its transmission by the insect (7, 35). However, no evidence was found for a role for CpxAR in biofilm formation (Fig. 1A). We therefore investigated whether the system might be involved in adaptation to fluctuating environmental conditions. Indeed, once ingested by the hematophagous insect, *Y. pestis* must adapt to increased osmolarity [300 to 600 mOsM (11)], to a highly oxidizing environment [approximately 10 to 17 mM (36)] and fluctuating metal concentrations (12). Hence, we sought to investigate the system's contribution to coping with these different environmental stresses. Deletion of the *cpxRA* locus did not result in any significant growth defect at various osmolarities (Fig. 1B) relevant to those found in the flea gut (11). To assay the susceptibility to ROS, we analyzed the survival of each strain after treatment with hydrogen peroxide (Fig. 1C). The Δ*cpxAR* mutant was more susceptible than the wild-type strain. After 1 h of exposure, only 44% of the population was still viable when plated on lysogeny broth (LB). The complemented strain displayed a similar level of susceptibility as the wild-type strain (Fig. 1C). To extend our phenotypic analysis of the stresses that *Y. pestis* might encounter within the arthropod's gut, we analyzed whether the CpxAR system was involved in cupric oxide resistance. Indeed, copper is known to be involved in the insect's innate immune system (37–39). No differences in growth could be observed when Cu^{2+} was added up to 0.5 mM (Fig. 1D). With the addition of 5 mM Cu^{2+} into the plate, the wild-type strain or the complemented strain showed an increased sensitivity to the presence of copper, while the Δ*cpxAR* mutant was unable to grow (Fig. 1D). These

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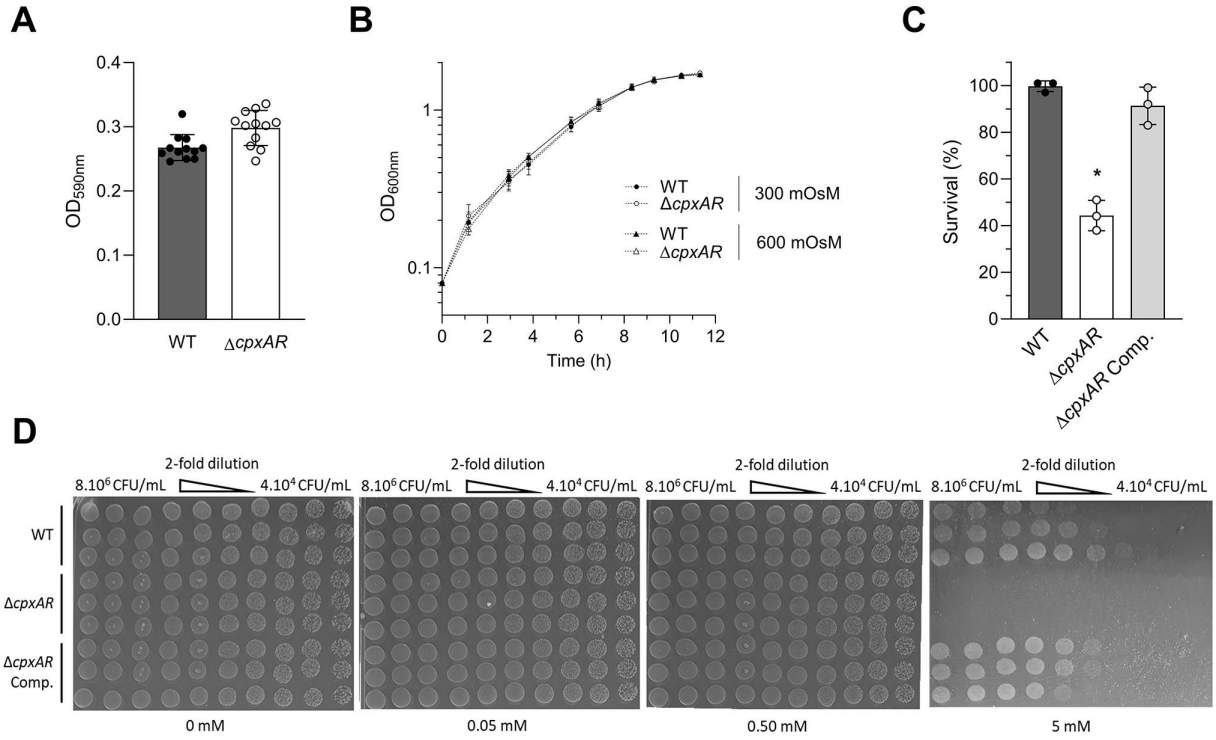


FIG 1 Phenotypic characterization of the Δ*cpxAR* mutant in flea-mimicking conditions. (A) Cristal violet assay showing biofilm formation of the wild-type (WT) and the *ΔcpxAR* strain. Data are quoted as the mean ± SD from at least three independent experiments. (B) Assessment of the WT and the Δ*cpxAR* strain growth in LB at 300 mOsM and 600 mOsM by measuring optical density at 600 nm (OD600nm) over time. (C) Survival of *Y. pestis* WT, the Δ*cpxAR*, and the complemented strain after exposure to H_2O_2 (20 mM) for 1 h. The mean percentage of survival ± SD values from three independent experiments is shown. Statistical analyses were performed with GraphPad Prism V10.0.2 using the Kruskal-Wallis test with Dunn's correction for multiple comparisons (*$P < 0.05$). (D) Photos showing the growth of the WT, Δ*cpxAR*, and complemented strain on LB agar plates containing different concentrations of $CuSO_4$. For each strain, different quantities of bacteria (8×10^6 CFU/mL to 4×10^5 CFU/mL) from three different cultures were spotted.

findings suggest that the CpxAR system is involved in oxidative stress and Cu^{2+} resistance *in vitro*.

The concentration of copper increases during digestion of the blood meals in the flea's gut

While the H_2O_2 concentration in the flea has been reported previously (36), the concentration of copper in the insect has never been investigated. Therefore, we measured by inductively coupled plasma mass spectrometry (ICP-MS) the concentration of copper in the flea gut at different time points after feeding on mouse blood (12 h, 24 h, and 6 days) and in the mouse blood used to feed fleas. In the flea gut, the average Cu^{2+} concentration increased to 18.0 ± 2.0 µM at 12 h, to 20.1 ± 5.4 µM at 24 h, and reached 34.8 ± 4.6 µM 6 days after the blood meal. An average concentration of 7.3 ± 7.0 µM was measured in mouse blood. The 4.7-fold difference in Cu^{2+} concentration between mouse blood and flea strengthens the idea that copper could be a potential signal for the CpxAR system, while *Y. pestis* passes through the insect.

Phosphorylation of CpxR increases in the presence of copper

CpxAR is needed for resistance to H_2O_2 and Cu^{2+} *in vitro*, two fluctuating parameters in the flea compared with the mammalian host. Therefore, we next wondered whether H_2O_2 and Cu^{2+} are a stimulus triggering the *Y. pestis'* CpxAR system. To test this hypothesis, we assayed the ratio of CpxR-phosphorylated form (CpxR~*P*)/CpxR total in the presence of H_2O_2 or Cu^{2+} using a polyacrylamide Phos-Tag approach (40). The Phos-tag molecule reversibly binds the phosphate group ($-PO_3^{2-}$) attached to CpxR and

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consequently slows the migration of the CpxR-phosphorylated form compared with the non-phosphorylated form (Fig. 2A). The (CpxR~*P*)/CpxR ratio in *Y. pestis* exposed or not to 20 mM H_2O_2 was about 2.5% (Fig. 2B). In contrast, the ratio was ~34,5% (14-fold increase) when the bacterium was exposed to 50 µM Cu^{2+}. This result supports the idea that copper could be a triggering signal for the CpxAR system within the flea.

Loss of the CpxAR system led to a competitive fitness defect in the flea

Given that the CpxAR system is required *in vitro* for survival under two stresses encountered by the bacterium in the flea gut (Fig. 1C and D) and that copper triggers the system (Fig. 2), we sought to determine the role in the production of a transmissible infection in flea. Noting that the system is not required for biofilm formation *in vitro* (Fig. 1A) and that the *cpxP* mutant (which is unable to inhibit CpxAR) has no impact on flea blockage (34), we wondered whether the CpxAR phosphorelay might play a role in bacterial fitness within the insect gut. To investigate this hypothesis, a competitive co-infection of the Δ*cpxAR* mutant with the wild-type strain was performed. Cohorts of fleas were co-infected in a 1:1 ratio of either Δ*cpxAR*/wild-type or Δ*cpxAR*/Δ*cpxAR* complemented (Fig. 3). During the co-infection with the wild-type and the Δ*cpxAR* strain, the number of Δ*cpxAR* bacteria significantly decreased at 13 days post-infection. A similar observation was obtained when the fleas were co-infected with the Δ*cpxAR* mutant and its complemented strain (Fig. 3).

The CpxAR system promoted survival to coexposure to H_2O_2 and copper

To shed light on the role of the CpxAR system during flea infection, we tested whether the competitive defect in flea of the *cpxAR* mutant compared with the wild-type strain was due to a slight growth defect of the mutant *in vitro*. To this, culture media were inoculated in a 1:1 ratio of the Δ*cpxAR* mutant and the wild-type strain (Fig. 4A). No significant difference was observed between both strains after 3 h and 8 h of culture (Fig. 4A). Therefore, the role of *cpxAR* in the flea does not appear to be the result of a general growth defect. In the flea, the bacterium is co-exposed to oxidative stress (36) and copper (the present study), two stresses sensed and inducing a bacterial response via CpxAR (41). It is therefore possible that the exposure to each stress or both stresses simultaneously could explain the growth defect of the *cpxAR* mutant in the flea compared with the wild-type strain. To test this hypothesis, we measured the bacterial survival after pre-incubation of the bacteria with (or without) copper for 1 h (to trigger

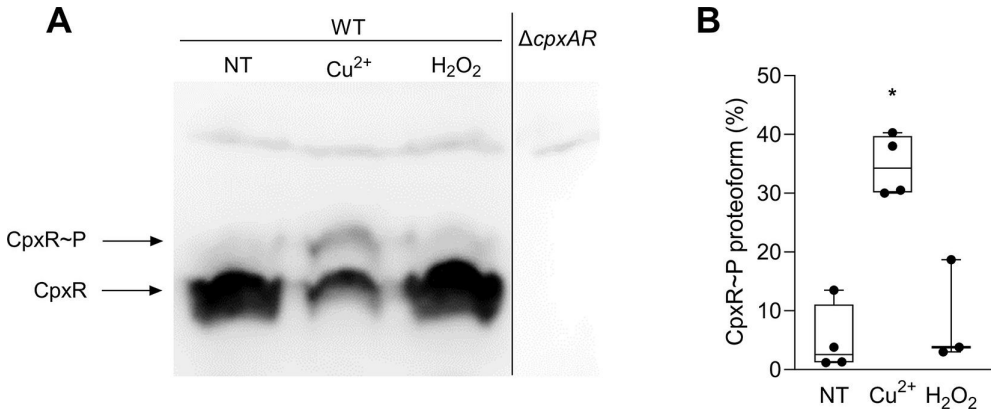


FIG 2 The copper is a cue for CpxR activation. Assessment of CpxR phosphorylation with or without exposure to oxidant stresses. (A) CpxR and CpxR~*P* proteoforms were separated by Phos-Tag acrylamide gel after extraction from the bacterial pellet and were revealed by western blot. This result is representative of three independent experiments. (B) Box-and-whisker plots (Tukey) show the percentage of the CpxR~*P* proteoform/CpxR total in the WT strain. Data are the results from at least three independent experiments. Statistical analysis was performed with GraphPad Prism V10.0.2 using the Mann-Whitney test (*$P < 0.01$).

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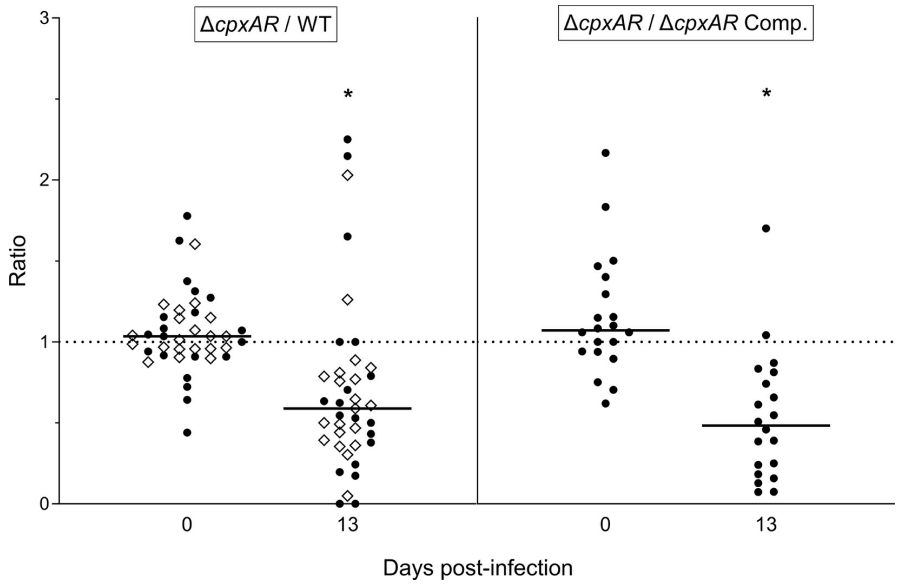


FIG 3 The CpxAR system provides a fitness advantage in the flea gut. Bacterial loads immediately after and 13 days after the infectious meal. Experiments were performed with *Y. pestis* Δ*cpxAR* mutant and *Y. pestis* WT Kim6+ or *Y. pestis*-complemented strain. The data correspond to samples from two independent experiments (white diamond: first experiment, black circles: second experiment). Each symbol indicates the ratio within an individual flea. The horizontal lines indicate the median CFU per flea. Statistical analyses were performed using the Kruskal-Wallis test and a Conover post-hoc test with a Benjamini-Hochberg adjustment for multiple testing. Any test with a *P* value < 0.05 is declared as significant.

the CpxAR system) and then exposure to 20 mM H_2O_2 for 1 h and 3 h, as control bacteria were also exposed to copper for 3 h (Fig. 4B). Regardless of the genetic background and time of exposure, copper had no deleterious effect. In contrast, H_2O_2 killed the Δ*cpxAR* mutant, as expected. Interestingly, the incubation of the bacteria with H_2O_2 after exposure to copper decreased the viability of both the wild-type and Δ*cpxAR* mutant. However, 3 h after treatment, the wild-type strain had recovered, while the Δ*cpxAR* mutant no longer appeared viable. Lastly, regardless of the treatment used and the time of exposure to the stress, the complemented strain showed a similar profile as the wild-type strain. These results suggest that triggering the CpxAR system can improve *Y. pestis* survival when the bacterium is co-exposed to biologically relevant concentrations (i.e., found in the flea) of copper and H_2O_2.

DISCUSSION

Over the last 30 years, the role of the CpxAR system in virulence has been investigated in various pathogens. In *Y. pestis*, this system has been studied for its contribution to the bubonic and pulmonary plague forms in rodents (33), but its role in the arthropod part has not been explored. While no role in rodents could be observed (33), our study identified a fitness-competitiveness defect against the wild-type strain (Fig. 3). Furthermore, neither *in vitro* growth nor biofilm was affected by the deletion of the *cpxRA* operon (Fig. 1A and B). These data are comparable to those observed with *Vibrio cholerae*, the pathogen responsible for cholera. In *V. cholerae*, *cpxP* expression is increased in the mouse small intestine, but deletion of the *cpxAR* system does not lead to a growth defect or loss of virulence (42). With a few exceptions where the system is crucial for virulence [*Dickeya dadantii* (27), *Citrobacter rodentium* (24), and *E. coli* UPEC (43)], our data, combined with the literature, confirm that *cpxAR* is more a virulence lifestyle gene than a true virulence gene *per se* (44), as has been observed in *Legionella pneumophila* (25), *Salmonella enterica* (26), and *Y. enterocolitica* (28). In other words, the CpxAR system is involved in host colonization, rather than being required for direct interaction with the host.

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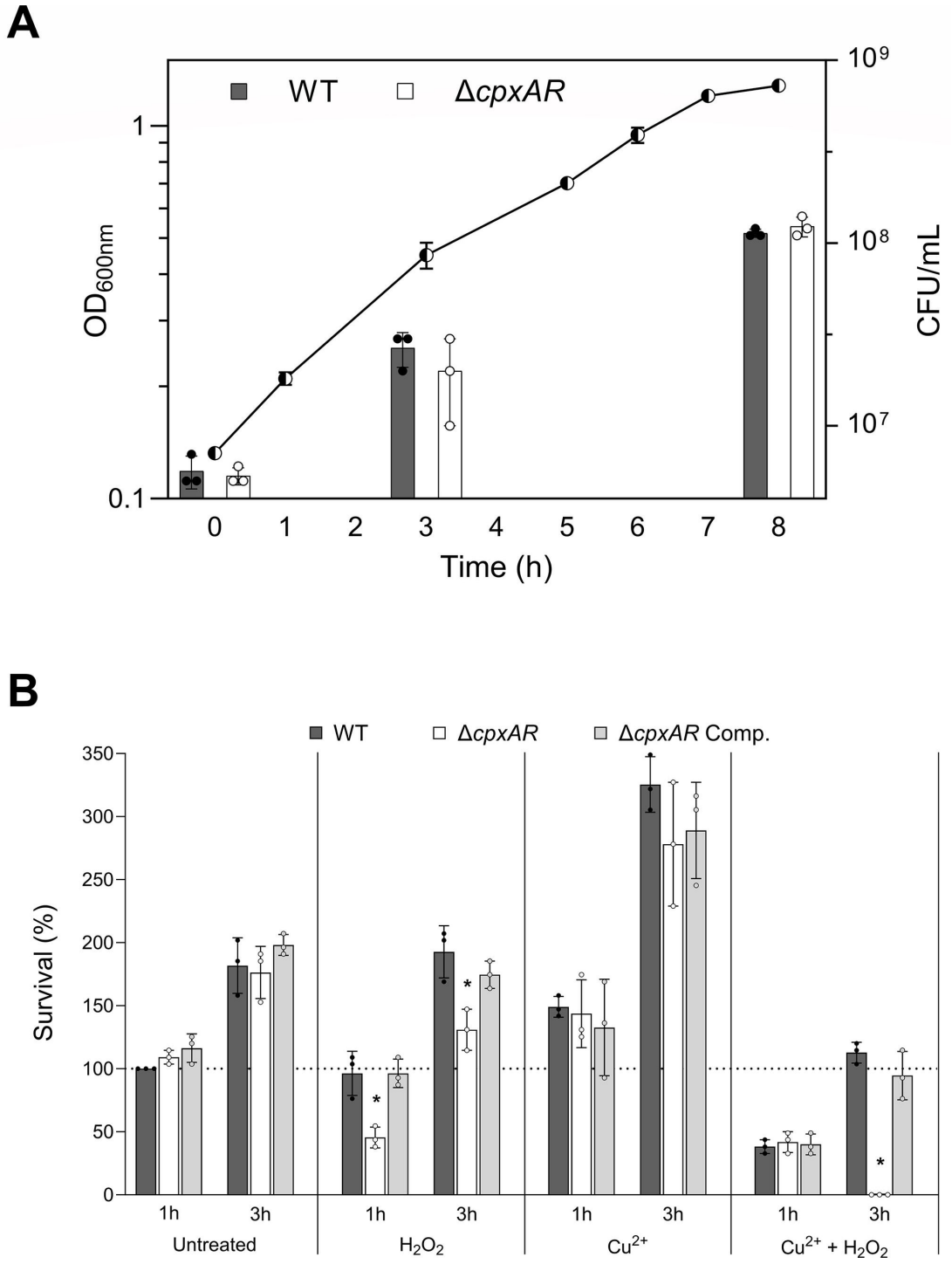


FIG 4 Copper-activated CpxAR protects *Y. pestis* against oxidative stress. (A) *In vitro* LB co-cultures did not display any bacterial fitness defect of the ΔcpxAR compared with the WT strain. The co-culture growth curve is represented by linked half-black and half-white dots. The number of CFU/mL of the total population and the mutant was determined by enumeration on LB plates and LB plates, respectively, containing trimethoprim at 0, 3, and 8 h of culture. (B) *In vitro* assessment of the copper pre-exposed *Y. pestis* survival to 1 h and 3 h of H_2O_2 stress treatment in HPLM and the control conditions (untreated, H_2O_2, or copper). Statistical analysis was performed with GraphPad Prism V10.0.2 using a two-way ANOVA multiple comparison test (*$P < 0.05$).

Gahlot and collaborators have reported that in *Y. pseudotuberculosis*, the species from which *Y. pestis* emerged, the CpxAR system is involved in biofilm formation (30). Considering that the CpxAR system is identical between *Y. pseudotuberculosis* and *Y.*

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pestis, they assume that CpxAR would play a similar role in *Y. pestis* (30). However, our data do not support this idea (Fig. 1A). There are at least two issues that need to be raised. Firstly, Gahlot and colleagues used the highly biofilm-producing *Y. pseudotuberculosis* strain YPIII (serotype O3), whereas *Y. pestis* appears to be a clonal emergence from the mild biofilm-producing *Y. pseudotuberculosis* serotype O:1b (45, 46). Secondly, the example of the OmpR-EnvZ or RcsCDB two-component systems highlights the fact that, during its emergence, *Y. pestis* rewrote its regulatory networks to support its new skills (11, 47, 48). Since the CpxA-CpxR protein sequences are identical between *Y. pseudotuberculosis* and *Y. pestis* (30), only the environmental component in the equation "Phenotypic traits = Genetic + Environment" can explain the phenotypic difference. In other words, it is the selection pressure of the environment (i.e., different envelope composition and stresses encountered) that has led to a shift in the output of the CpxAR system and so its role. Therefore, our study confirms that even if the protein sequences of CpxA-CpxR are conserved between *Y. pestis* and *Y. pseudotuberculosis*, their implications in physiology and regulatory networks are tailored to the life cycle of each species. This observation reinforces the idea that it is not possible to extrapolate from studies carried out on *Y. pseudotuberculosis* to *Y. pestis*.

The role of the CpxAR system of *Y. pestis* in the bacterial fitness during the flea colonization suggests a role in bacterial population dynamics to cope with changing environmental conditions. This idea is reinforced by the system's involvement in survival under oxidative stress and Cu^{2+} *in vitro*. The relationship between these two stresses and the CpxAR system had already been observed previously in other species such as *S. enterica* serovar Typhimurium (41, 49). In *Y. pestis*, while the Δ*cpxAR* mutant survival was impacted *in vitro* by H_2O_2 oxidative stress (Fig. 1C) at a concentration similar to that measured in the flea (36), this stress does not serve as a signal for the CpxAR system (Fig. 2). Interestingly, Cu^{2+} at biologically relevant concentrations does not cause growth defects (Fig. 1D and 4B) but can trigger the CpxAR system (Fig. 2) and could be a physiological cue in *Y. pestis* as it is the case for *Salmonella enterica* serovar Typhimurium (41). In addition, when *Y. pestis* is pre-incubated *in vitro* with copper and then exposed to oxidative stress, the absence of the CpxAR system was deleterious for bacterial survival (Fig. 4B). Yet, it should be borne in the mind that the Δ*cpxAR* mutant was still fully able to form a biofilm (Fig. 1A) and therefore to cluster in a matrix. Biofilms have been described as a protective environment against stress (50). This could explain why the Δ*cpxAR* mutant is not cleared in the flea. Our results suggest a model in which copper triggers the CpxAR system, thereby contributing to the oxidative stress resistance of *Y. pestis* during flea colonization.

How copper can activate the CpxAR system and what bacterial determinants are deployed to protect the cell from oxidative stress remain to be elucidated. In *Salmonella enterica*, the CpxAR system protects against high copper concentrations (1 mM) and oxidative stress (5/6 mM) (41). Pre-incubation with copper also confers a protective effect against oxidative stress. The CpxAR system regulates the scsABCD locus encoding proteins with homologies to thioredoxin-type proteins. Lopez et al. propose that regulation of the ScsABCD system by the CpxAR system restores the redox balance of the *Salmonella* envelope (41). Yet, it remains unclear how copper triggers the system. A recent study by Andrieu et al. suggests that the CpxAR system can detect specific oxidative stress (49). In their study, they reported that *N*-chlorotaurine oxidative stress directly triggers the activation of the CpxAR system. Interestingly, other oxidative stress such as H_2O_2 did not elicit the system (49). Here, the presence of copper could lead to a type of oxidative stress different (51) from what is encountered when using H_2O_2.

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MATERIALS AND METHODS

Strains, mutants, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The mutant strain was generated using the lambda red recombinase-based method (52) with some modifications (6, 53). The coding sequence of the *cpxRA* locus was replaced by a trimethoprim resistance cassette flanked by flippase recognition target (FRT) sites that had been amplified from the pEP1087 (Table 1). The mutant strain was complemented with the mini-Tn7 (54) containing a WT copy of the *cpxRA* locus under the control of its own promoter (Table 1). Mutations were confirmed by PCR with the primer sets given in Table 1 and sequencing following the method described previously (11).

The strains were grown in lysogeny broth (LB, 240230, BD), brain heart infusion (BHI, 211059, BD), or human plasma-like medium (HPLM, A4899101, Gibco) at 21°C or 28°C. When needed, agar (15 $g.L^{-1}$), kanamycin (50 $\mu g.mL^{-1}$), and trimethoprim (25 $\mu g.mL^{-1}$) were added to obtain solid and/or selective media. The LB osmolality is 345 mOsM (56). To obtain a high-osmolarity medium, NaCl was added. The HPLM concentration of copper was determined on three different lots by ICP-MS and was $0.9 \pm 3.3*10^{-2}$ µM. To compare the growth rate of different strains, bacteria were cultured at 21°C. The growth in LB was monitored by measuring the OD_{600nm}.

Assessment of biofilm formation *in vitro*

Biofilms were assayed as previously described (11). Briefly, bacteria were incubated in LB supplemented with 4 mM $MgCl_2$ and 4 mM $CaCl_2$ for 48 h at 21°C with 180 rpm shaking in a 24-well plate (1×10^7 bacteria in 1 mL). Then, the medium was removed, and the attached bacterial biofilm was stained with a crystal violet dye solution (0.01%) for 15 min at room temperature. Next, the wells were rinsed three times with ddH_2O. The dye attached to the biofilm was released with the addition of an ethanol-acetone (80:20) solution. Lastly, the solution was transferred to a 96-well plate, and the absorbance of each well was measured at 542 nm (ELx800 microplate reader, BioTek).

TABLE 1 Strains, vectors, and primers used in this study

Strain, plasmid, or primer	Description or sequence (5′–3′)[a]	Reference
Y. pestis strains		
KIM6+ (WT)	Wild-type strain pYV- negative strain	(55)
Δ*cpxAR*	Δ*cpxAR*::*dfrB,* Tmp^R	This study
Δ*cpxAR* Comp.	Δ*cpxAR*::*dfrB*, miniTn7::*cpxRA-aphA3′*, Kan^R	This study
Vectors		
pKD4	ri6K vector, Kan^R cassette flanked by FRT sites	(52)
pEP1087	derived from pKD4, Tmp^R cassette flanked by FRT sites	(53)
pEP1013	vector encoding the Red recombinase, Amp^R	(53)
pEP1436	pEP1013 encoding the endonuclease I-*Sce* I	(6)
pUC18R6K-mini-Tn7	riR6K vector bearing empty R. mini-Tn7T, Amp^R	(54)
pUC18R6K-mini-Tn7::*cpxRA-aphA3′*	pUC18R6K-mini-Tn7 encoding the fusion *cpxRA-aphA3′*	This study
Primers		
CpxAR-F	ACGGTATCGAAACCTTGAAAGAATTACGCCAACACCATCAGACCCCGGTGGTGTAGGCTGGAGCTGCTTC	This study
CpxAR-R	GACGCCCGATCTCGGGCTTCATCTGTACGATAAAATGGCCGGAATATTTGCATATGAATATCCTCCTTAG	This study
CpxAR-FC-PstI	TATCTGCAGGTGCATTGCCTCGCGATCTT	This study
CpxAR-RC-EcoRV	TATGATATCGCATTATCGGGTTACCGTTG	This study
CpxAR-check-F	CTATTAGTTGATGATGACCG	This study
CpxAR-check-R	AAGATTTAAGCGGATGCAGC	This study
CpxARi-CF	AGTTCACCCTGCTCTATCTG	This study
CpxARi-CR	GCGATAACACCAGCAAGTCA	This study

[a]Tmp^R (dfrB), Kan^R (aphA3′), and Amp^R (bla) indicate resistance to trimethoprim, kanamycin, and ampicillin, respectively.

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Survival assays

To evaluate bacterial survival to H_2O_2, *Y. pestis* KIM6+ WT, the Δ*cpxAR*, and the complemented strains were grown to the mid-log phase and were harvested by centrifugation (4,000 × *g*, 5 min). Bacteria were resuspended and incubated in one volume of 1× phosphate-buffered saline (PBS) supplemented with 20 mM H_2O_2 for 1 h at 21°C under 180 rpm. After incubation, bacteria were plated on LB agar and enumerated after 48 h incubation at 28°C.

To evaluate bacterial survival to copper, overnight cultures of *Y. pestis* KIM6+ WT, the Δ*cpxAR*, and the complemented strains were diluted in fresh LB to 8×10^6 CFU/mL. Then, bacteria were twofold serial diluted to 4×10^5 CFU/mL and spotted on LB agar plates supplemented with 50 µM, 500 µM ,or 5 mM $CuSO_4$. Bacterial survival was determined after 48 h incubation at 28°C.

To evaluate bacterial survival to H_2O_2 after copper exposition, overnight cultures of *Y. pestis* KIM6+ WT, the Δ*cpxAR* and the complemented strains were thricely washed in HPLM medium and diluted in fresh HPLM medium to 1×10^8 bacteria/mL supplemented with 50 µM of $CuSO_4$. After 1 h pre-incubation at 21°C, bacteria were exposed to 20 mM H_2O_2 for up to 3 h. After 1 h and 3 h incubation to oxidative stress, bacteria were plated on LB agar and enumerated after 48 h incubation at 28°C.

Phos-Tag and SDS-PAGE immunoblot analysis

Gel retardation of the phosphorylated form of CpxR was performed as described previously (27, 40) with some modifications. Briefly, 2×10^8 bacteria harvested by centrifugation were suspended and lysed with 20 µL of 1 M formic acid and vortexed thoroughly. Acid was neutralized by 5 µL of 5 N NaOH, and then 6 µL of 5× SDS-PAGE loading buffer [composition: 250 mM Tris-HCl pH 6.8, 4% (wt/vol) SDS, 30% (wt/vol) glycerol, 20% (vol/vol) β-Mercaptoethanol, 1 mM $MnCl_2$, and 0.02% (wt/vol) bromophenol blue] was added. The sample was loaded onto Phos-tag acrylamide gels composed of a 12.5% resolving solution [12.5% (wt/vol) 37.5:1 acrylamide/N,N′methylenebisacrylamide, 375 mM Tris (pH 8.8) and 0.1% (wt/vol) SDS, 35 µM Phos-tag acrylamide and 70 µM $MnCl_2$] and a 4% stacking solution [4% (wt/vol) 37.5:1 acrylamide/N,N′methylenebisacrylamide, 125 mM Tris (pH 6.8) and 0.1% (wt/vol) SDS]. The gels were run at 4°C under constant voltage (130 V) with fresh running buffer [0.1% (wt/vol) SDS, 25 mM Tris, and 192 mM glycine pH 8.8].

Then, a 15-minute wash with transfer buffer (25 mM Tris and 192 mM glycine) supplied with 2 mM EDTA, followed by two 15-minute washes without EDTA, was performed before transferring the gel to 0.22-µm PVDF membrane using a Trans-Blot Turbo Blotting system (Bio-Rad) with a pre-programmed protocol (2.5 A, up to 25 V, 7 min). Western blotting against CpxR was performed using standard protocols with the rabbit anti-CpxR polyclonal antibodies (27) at a dilution of 1:1,000 and anti-rabbit secondary antibody coupled to horseradish peroxidase at a dilution of 1:5,000. Blots were imaged by chemiluminescent detection (SuperSignal West Pico PLUS from Thermo Scientific and Amersham ImageQuant 800 imaging system from Cytiva).

Flea infection

Flea infection was assayed as previously described (57, 58). Starved *Xenopsylla cheopis* rat fleas were allowed to feed on heparinized mouse blood containing 5×10^8 bacteria per mL (grown at 37°C overnight in BHI), using an artificial apparatus (feeder) for 1 h. For co-infection assay, equivalent amounts of each strain were added to the blood and then placed into the feeder. Then, cohorts of 100 female fleas were collected. Fleas were allowed to feed mouse blood at 2, 6, 9, and 13 days post-infection. To determine *Y. pestis* competitive fitness in fleas, groups of 20 females were collected at different times post-infection to individually plate them (after trituration) on LB agar plates containing 1 µg/mL irgasan, 10 µg/mL hemin, and either kanamycin or trimethoprim to select for the mutant strains. The CFUs were counted after 48 h at 28°C.

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ICP-MS analysis

The midgut content from fed *X. cheopis* was collected using the following methods. Intact digestive tracts lacking their Malpighian tubule system were collected from fleas under a dissecting microscope. The midgut epithelium was pierced, and the midgut contents were carefully expressed. Six to nine midgut contents were pooled and diluted fivefold in H_2O to provide sufficient samples for subsequent analyses. Mouse blood was collected in a heparinized tube (Becton Dickinson). Fifty microliters of mice blood or 45 µL of fleas gut was treated as follows: in the first step, the samples were attacked with a mixture of 100 µL of H_2O_2 (Promochem, 30% high purity) and 200 µL of HNO_3 (Fisher Scientific, 67%–69%, Optima, and trace metal grade) at room temperature during 24 h to extract Cu from the matrixes. The samples were then diluted with Milli-Q water (18.2 MΩ.cm) to get 10 mL of solution. Before ICP-MS analysis, these solutions were systematically filtrated at 0.45 µm (Sartorius, acetate cellulose membrane). Copper analyses (^{63}Cu isotope chosen) were performed with an inductively coupled plasma-single quadrupole mass spectrometer (ICP-MS 7850, Agilent Technologies) using He as collision gas and Kinetic Energy Discrimination to eliminate spectral interferences. To take into account the potential drift of the spectrometer, an internal standard (^{72}Ge) was added to the samples. To control the quality of the results, the water SLRS-6-certified standard was also analyzed. The certified values were 24.0 ± 1.8 µg/L and our experimental result was 25 ± 1 µg/L. Finally, the limit of detection was estimated to be 0.02 µg/L in this run of analysis, and the average blank values (including all the steps of sample preparation) did not exceed 10% of the sample's values. Blank values were therefore neglected for the calculations.

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S.B.G. conceived and supervised the project. B.R., A.D., V.A., G.B., F.S., and S.B.G. contributed to the study design. B.R., A.D., V.A., and S.B.G. performed the experiments. B.R., A.D., V.A., G.B., F.S., and S.B.G analyzed the data. C.L. performed statistical analysis. F.P. reared the mice and the fleas. S.B.G. wrote the manuscript with input from all authors.

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DATA AVAILABILITY

All experimental data pertinent are accessible for review in the paper.

ETHICS APPROVAL

The animal experiments were carried out following the European directive 2010/63/EU and the French governmental decree 2013-118, and all protocols had been approved by the French Ministry of Higher Education, Research and Innovation, and the Ethics Committee in Animal Experimentation. All animal husbandry adhered to the current European and French regulations.

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