The zinc transporter ZnuABC is critical for the virulence of *Chromobacterium violaceum* and contributes to diverse zinc-dependent physiological processes

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ABSTRACT

Chromobacterium violaceum is a ubiquitous environmental bacterium that causes sporadic life-threatening infections in humans. How *C. violaceum* acquires zinc to colonize environmental and host niches is unknown. In this work, we demonstrated that *C. violaceum* employs the zinc uptake system ZnuABC to overcome zinc limitation in the host, ensuring the zinc supply for several physiological demands. Our data indicated that the *C. violaceum* ZnuABC transporter is encoded in a *zur*-CV_RS15045-CV_RS15040-*znuCBA* operon. This operon was repressed by the zinc uptake regulator Zur and derepressed in the presence of the host protein calprotectin (CP) and the synthetic metal chelator EDTA. A $\Delta znuCBA$ mutant strain showed impaired growth under these zinc-chelated conditions. Moreover, the deletion of *znuCBA* provoked a reduction in violacein production, swimming motility, biofilm formation, and bacterial competition. Remarkably, the $\Delta znuCBA$ mutant strain was highly attenuated for virulence in an *in vivo* mouse infection model and showed a low capacity to colonize the liver, grow in the presence of CP, and resist neutrophil killing. Overall, our findings demonstrate that ZnuABC is essential for *C. violaceum* virulence, contributing to subvert the zinc-based host nutritional immunity.

KEYWORDS: metalloregulator, Zinc Uptake Regulator, *Chromobacterium violaceum*, zinc homeostasis, bacterial virulence, bacterial zinc transporter, ZnuABC

INTRODUCTION

The transition metal zinc plays an essential role in both eukaryotic and prokaryotic cells (1, 2). Zinc functions as a structural and catalytic cofactor of several classes of proteins such as proteases (3), endopeptidases (4, 5), metallo- β -lactamases (6), and transcription factors (2). Large fractions of the proteome of bacteria (about 5-6%) and vertebrates (about 8-10%) are zinc-binding proteins (2, 7). Maintenance of bacterial zinc homeostasis requires the coordinated expression of transporters for zinc acquisition and

efflux (8). In most Gram-negative bacteria, the Zinc Uptake Regulator Zur is the main metal-sensing regulator that represses genes encoding zinc acquisition systems, such as the ATP-binding cassette (ABC) transporter ZnuABC (8-10). The ZnuABC is a high-affinity tripartite zinc importer composed of a soluble periplasmic zinc-binding protein (ZnuA), a transmembrane protein (ZnuB), and an ATPase (ZnuC) (8, 10). In some cases, Zur also acts as an activator of zinc efflux systems under zinc excess, such as in *Streptomyces coelicolor* (9), *Xanthomonas campestris* (11), and *Caulobacter crescentus* (12).

Zinc also plays a central role during bacterial infection (1, 13). Vertebrate hosts use distinct strategies to make zinc and other metals unavailable to bacterial pathogens, a process termed nutritional immunity (13). During infection, neutrophils express a large amount of calprotectin (CP), a S100A8/S100A9 heterodimer of the S100 family of EF-hand calcium-binding proteins (14, 15). CP binds zinc, manganese, and ferrous iron with high affinity (16, 17) and causes growth inhibition of a large variety of bacteria (18-22). On the other hand, bacteria overcome CP-mediated zinc chelation by expressing high affinity zinc transporters, such as the ZnuABC, as demonstrated in *Salmonella enterica* serovar Typhimurium (19) and *Acinetobacter baumannii* (23, 24). The battle between hosts and bacteria also involves zinc intoxication. For instance, macrophages deliver zinc-containing vesicles to promote microbial clearance, while bacteria express zinc efflux systems to alleviate zinc toxicity (25, 26).

Metal homeostasis is critical for environmental pathogenic bacteria, which need to deal with metal fluctuations in free-living and host-associated conditions. In previous works, we demonstrated the importance of iron for the virulence and the physiology of *Chromobacterium violaceum* (27, 28), a Gram-negative environmental opportunistic pathogen. *C. violaceum* causes human infections with mortality rates ranging from 35 to 53% of the cases (29, 30). Several traits contribute to *C. violaceum* pathogenicity, such as

the high intrinsic antibiotic resistance (29, 30), the secretion of effectors by a Cpi1 type III secretion system (31-33), and the production of catecholate-type siderophores regulated by the Ferric Uptake Regulator Fur (27, 28). However, the relevance of zinc homeostasis for the physiology and virulence of *C. violaceum* and how this bacterium acquires zinc are largely unknown.

In this work, we characterized the high affinity ABC-type transporter ZnuABC of *C. violaceum*. We demonstrated that ZnuABC is encoded in a Zur-repressed *zur*-CV_RS15045-CV_RS15040-*znuCBA* operon. A $\Delta znuCBA$ mutant strain showed impaired growth under zinc-chelated conditions and reduced violacein production, swimming motility, biofilm formation, and bacterial competition. Using growth curves under CP-treatment, neutrophil killing assays, and an *in vivo* mouse infection model, we demonstrated that ZnuABC exerts a key role in *C. violaceum* virulence by overcoming the host-imposed and CP-mediated zinc limitation.

RESULTS

The *C. violaceum* ZnuABC transporter is encoded in a Zur-repressed *zur*-CV_RS15045-CV_RS15040-*znuCBA* operon. *C. violaceum* possesses two members of the Ferric Uptake Regulator (Fur) family of transcription factors, the previously characterized Fur (27) and the undescribed Zinc Uptake Regulator Zur (CV_RS15050). Genome inspection revealed that *zur* composes a putative operon with other five genes (Fig. 1a): CV_RS15045, encoding an uncharacterized protein belonging to the Cluster of Orthologous Groups (COG) COG0523, CV_RS15040, encoding a small accessory hypothetical protein, and the *znuC*, *znuB*, and *znuA* genes, which encode a putative ZnuABC transport system (Fig. 1a). Zur represses Zur-regulated genes by binding in a palindromic regulatory sequence, known as Zur-box (9-12). To identify a putative Zur binding site in *C. violaceum*, we searched for conserved DNA motifs upstream of *zur* in several genomes of

Chromobacterium, using the motif discovery tool MEME (34). This analysis returned a possible Zur-binding motif (Fig. 1b) located upstream of *zur* that resembles the previously characterized Zur-boxes of *Pseudomonas protegens* (35) and *Neisseria meningitidis* (36). We designed primers in three genes of the possible operon (Fig. 1a) to verify by reverse transcriptase-polymerase chain reaction (RT-PCR) if they were co-transcribed. Firstly, we confirmed the absence of DNA contamination in the isolated total RNA by PCR (Fig. 1c, upper panel). Using the same combination of primers, we detected co-transcription by RT-PCR (Fig. 1c, down panel), indicating the occurrence of a *zur*-CV_RS15045-CV_RS15040-*znuCBA* operon in *C. violaceum*. To investigate Zur-mediated regulation of this operon in *C. violaceum*, we generated a fusion of the promoter region of *zur* (*pzur*) with a promoterless *lacZ* gene. The β -galactosidase activity assays revealed that in the wild-type (WT) strain, the expression of this operon doubled from zinc-replete (LB and LB plus zinc) to zinc-chelated conditions (EDTA and TPEN treatments) (Fig. 1d). As a control, the *pzur-lacZ* fusion was unresponsive to iron-replete (FeSO_4) or iron-chelated conditions (2,2'-DP treatment) (Fig. 1d). In the Δzur strain, regardless of the treatment, the *pzur* expression was derepressed, demonstrating that Zur acts as a repressor of this operon (Fig. 1d). Altogether, these results indicated that *C. violaceum* possesses a zinc-responsive, Zur-regulated operon containing six genes potentially involved in zinc homeostasis. Hereafter, we will focus on the characterization of the *C. violaceum* ZnuABC.

The absence of ZnuABC impairs the growth of *C. violaceum* in zinc limitation. To

study how *C. violaceum* copes with zinc availability *in vitro*, we determined the minimal inhibitory concentration (MIC) of the zinc-chelating agent EDTA. The WT and $\Delta znuCBA$ strains showed the same MIC for EDTA (128 mM) (Fig. 2a). Considering that EDTA can chelate other divalent cations, we tested different combinations of metals (zinc or iron) and metal-chelators (EDTA or 2,2'-DP) to assess the growth after 24 h cultivation (Fig. 2b) and

the growth inhibition by disk diffusion assays (Fig. 2c, d). Under a condition of EDTA plus zinc (LB 1 mM EDTA 250 μ M ZnCl₂), the $\Delta znuCBA$ mutant had an impaired growth compared to that of the WT and complement strains (Fig. 2b). In agreement, in the disk diffusion assays, the $\Delta znuCBA$ mutant was more sensitive to EDTA treatment (inhibition halos from 62.5 mM EDTA) than the WT or the complemented strain (inhibition halos from 250 mM EDTA) (Fig. 2c). As a control, all strains showed the same profile of growth (Fig. 2b) or growth inhibition (Fig. 2d) under iron chelated (2,2'-DP) or iron supplemented conditions (FeSO₄). These data demonstrate that ZnuABC is required for growth under zinc, but not iron, limitation. To further characterize the effects of zinc deficiency in a $\Delta znuCBA$ mutant strain, we performed growth curves in LB medium with different zinc conditions. The Δzur and $\Delta znuCBA$ mutant strains showed the same growth profile as that of the WT strain in LB (Fig. 3a) or LB plus 1 mM EDTA (Fig. 3b), raising the possibility that the intracellular zinc content would still be sufficient to maintain proper cell physiology. To investigate this hypothesis, we treated all pre-inocula with 1 mM EDTA and measured growth curves in LB (Fig. 3c) and LB plus 1 mM EDTA (Fig. 3d). In these experiments, the growth of $\Delta znuCBA$ was mildly compromised in LB (Fig. 3c) and severely compromised in LB plus 1 mM EDTA (Fig. 3d) compared to that of the WT strain. In both cases, these phenotypes were reverted in the complemented strain. Zinc supplementation in the growth curves using EDTA-treated pre-inocula was not able to fully restore the growth defect of the $\Delta znuCBA$ mutant strain (Fig. 3e). Overall, these experiments demonstrate that the ZnuABC transporter plays an important role in zinc acquisition in *C. violaceum*, especially under severe zinc scarcity, when the intracellular zinc pool was previously depleted.

***C. violaceum* ZnuABC is required for growth in zinc limitation imposed by calprotectin.** Calprotectin (CP) is a zinc, manganese, and iron chelating protein released from neutrophils that plays a central role in host defense against bacterial pathogens (17-

19, 21). To investigate the antimicrobial role of CP against *C. violaceum*, we used a wild-type CP (CP WT) and a selectively inactivated CP with mutations in both the noncanonical (SI) and the canonical (SII) transition metal-binding sites (CP Δ SI/SII) (17). Determination of the 50% growth inhibitory concentration (IC₅₀) of CP WT indicated that *C. violaceum* is more resistant to CP than an *Escherichia coli* DH5 α strain, used as a control (IC₅₀ of 534.6 $\pm$ 13.8 and 287.5 $\pm$ 22.3, respectively). We measured growth curves in LB supplemented with 500 μ g/ml of CP WT or CP Δ SI/SII, using CP treated or untreated pre-inocula (Fig. 4). When bacteria were treated with CP WT and the growth curve was performed in LB with CP WT, the Δ znuCBA mutant strain showed impaired growth compared to that of the WT and complement strains (Fig. 4a). This effect was not detected when CP Δ SI/SII was present in both the pre-inocula and inocula (Fig. 4b), indicating a metal binding-dependent CP effect. When CP WT was only present in the growth curve, without pre-treatment, all strains presented the same growth profile (Fig. 4c), confirming the requirement of ZnuABC for growth when the intracellular zinc pool was depleted. We also measured the β -galactosidase activity of the *pzur-lacZ* fusion in the WT strain in the presence of CP (Fig. 4d). The expression of *pzur* increased two-fold with the CP WT or EDTA treatments compared to the untreated control. Treatment with the CP Δ SI/SII had no significant effect (Fig. 4d). These data indicate that CP imposes zinc limitation to *C. violaceum*, which in turn responds by expressing ZnuABC to overcome CP-mediated zinc chelation.

ZnuABC ensures the supply of zinc required for violacein production, swimming motility, biofilm formation, and bacterial competition. During the MIC assays (Fig. 2a), we observed that EDTA had an inhibitory effect on the production of the pigment violacein. Further characterization indicated that the production of violacein was inhibited at 1 mM EDTA in the WT strain, whereas in the Δ znuCBA mutant, inhibition was achieved from 0.031 mM EDTA. This phenotype was reverted with the addition of zinc and after genetic

complementation (Fig. 5a). These data suggest that zinc is required for violacein production in *C. violaceum*. We tested whether other phenotypic traits, such as swimming motility and biofilm, could be affected in zinc limitation on the dependence of ZnuABC. Motility tests in semi-solid M9CH agar plates indicated that the flagellar swimming motility of *C. violaceum* WT decreased upon the addition of EDTA. Still, when zinc was added together with EDTA, the motility matched that of the untreated M9CH condition (Fig. 5b). The $\Delta znuCBA$ mutant strain was less motile than the WT, Δzur , and $\Delta znuCBA[pMR20znuCBA]$ strains (Fig. 5c). These results indicate that *C. violaceum* requires zinc acquisition via ZnuABC for proper swimming motility. We quantified biofilm formation on static conditions of cultures grown in glass tubes in LB or LB plus 100 μ M EDTA (Fig. 5d). The biofilm formation in LB was not affected in the Δzur or $\Delta znuCBA$ mutant strains compared to that of the WT strain. However, in zinc depleted conditions, the $\Delta znuCBA$ mutant strain produced less biofilm than the other strains. Zinc depletion caused an overall drop in biofilm formation (Fig. 5d). These data indicated that ZnuABC is important for biofilm formation under zinc deficiency in *C. violaceum*. We wondered if zinc could be important for *C. violaceum* in the context of interspecies competition. In liquid co-culture assays with or without zinc supplementation, the $\Delta znuCBA$ mutant strain was outcompeted by *Burkholderia cepacia* and *S. Typhimurium* (Fig. 5e), indicating that ZnuABC is important for *C. violaceum* to compete with other bacteria.

ZnuABC contributes to *C. violaceum* virulence and resistance to neutrophils. To verify whether zinc-related genes are involved in the pathogenesis of *C. violaceum*, we performed virulence assays in BALB/c mice, as previously determined (27, 28, 37). The Δzur mutant strain was as virulent as the WT strain (most animals died 2 days post infection) (Fig. 6a). On the other hand, when infected with the $\Delta znuCBA$ mutant strain, all animals remained alive 10 days post infection, demonstrating that the absence of *znuCBA*

renders *C. violaceum* highly attenuated for virulence. This phenotype was partially rescued in the complemented strain (Fig. 6a). We also evaluated the *C. violaceum* ability to colonize the host by assessing the bacterial CFU in mice 16 hours post infection. The livers of mice infected with the $\Delta znuCBA$ mutant strain had lower bacterial burdens than that of the WT or complemented strain (Fig. 6b). Additionally, we tested the survival of *C. violaceum* when incubated with neutrophils isolated from the peritoneal cavity of BALB/c mice. The CFU of the $\Delta znuCBA$ mutant strain decreased in the presence of neutrophils, while the CFU of the WT and complemented strains remained similar to that of these strains maintained in the control situation, without neutrophils (Fig. 6c). Altogether, these experiments showed that ZnuABC plays a key role in the virulence of *C. violaceum*, most likely by contributing to zinc acquisition in the context of infection, where neutrophils release CP to impose zinc limitation.

DISCUSSION

In this study, we characterized the zinc uptake transporter ZnuABC in the environmental pathogen *C. violaceum*. We described the role of ZnuABC in several zinc-dependent processes, including overcoming host nutritional immunity during infection. The *znuCBA* genes are transcribed in an operon with *zur*, and two genes encoding hypothetical proteins. This operon is repressed by Zur and derepressed in the presence of the host protein CP and the synthetic zinc chelator EDTA. We showed that the deletion of *znuCBA* renders *C. violaceum* more sensitive to zinc-depleted conditions. The compromised zinc supply affected violacein production, swimming motility, biofilm formation, and bacterial competition. Furthermore, we demonstrated, using a murine model of infection, that ZnuABC is required for *C. violaceum* virulence, liver colonization, and resistance to neutrophil.

The ZnuABC transporters have been characterized as high affinity zinc importers in several Gram-negative bacteria (3, 10, 19, 23, 24). Although our study lacks a biochemical assay to demonstrate the zinc uptake activity of ZnuABC directly, we provide evidence that ZnuABC may act as a high affinity zinc transporter in *C. violaceum*: (i) the *znuCBA* genes are part of a Zur-repressed operon that respond to zinc limitation; (ii) the absence of *znuCBA* impairs the growth of *C. violaceum* only under severe zinc scarcity, when the intracellular zinc pool was previously depleted with EDTA or CP. In several bacteria, such as *A. baumannii*, *Agrobacterium tumefaciens*, and *Pseudomonas aeruginosa*, the genes *znuA*, *znuB*, *znuC*, and *zur* are located near in the genome; commonly, *zur* is transcribed together with *znuCB* and divergently from *znuA* (24, 38, 39). In *C. violaceum*, *znuCBA* compose an operon with *zur*, CV_RS15040, and CV_RS15045. The gene CV_RS15040 encodes a small hypothetical protein (63 aa) with a predicted helical transmembrane segment. It would be interesting in future studies to address whether CV_RS15040 could be an accessory protein of the ZnuABC system. The gene CV_RS15045 encodes a putative GTPase of the COG0523 family of metallochaperones (40). Studied members of this family involved with the response to zinc limitation include YeiR of *E. coli* (41), YciC of *Agrobacterium tumefaciens* (38), ZagA of *Bacillus subtilis* (42), and ZigA of *A. baumannii* (43, 44).

Our data indicated that *C. violaceum* requires the ZnuABC system to supply zinc to several physiological processes, such as violacein production, swimming motility, biofilm formation, and bacterial competition. It has been described the requirement of zinc for enzymes of the violacein biosynthetic pathway in *C. violaceum* (45), for flagellar motility in *Proteus mirabilis*, *S. Typhimurium*, and *P. aeruginosa* (46-48), for biofilm formation via surface adhesion proteins in *Staphylococcus epidermidis* and *Staphylococcus aureus* (49), and for bacterial competition in a context of polymicrobial infections (19, 50). Although

further studies are needed to understand how these processes were impaired under zinc deficiency in *C. violaceum*, our data suggest an adaptive response to ensure the zinc supply for key metabolic pathways. A global investigation of the Zur regulon could elucidate these aspects of the zinc homeostasis in *C. violaceum*.

Zinc acquisition is crucial for bacterial pathogenesis, but the contribution of the ZnuABC transporters varies according to the bacterial pathogen. The involvement of ZnuABC in virulence has been described in *S. Typhimurium* (51), *A. baumannii* (23, 24), and *E. coli* (52). Previous work from our group has indicated that *C. violaceum* relies on siderophores to overcome the iron limitation imposed by the host during infection (27, 28), but the role of zinc for the *C. violaceum* virulence remained unknown. In this study, we determined that the ABC-type transporter ZnuABC is essential for the pathogenesis of *C. violaceum* in mice, given that a $\Delta znuCBA$ mutant strain was highly attenuated for virulence. The low capacity of $\Delta znuCBA$ to colonize the liver, grow in the presence of CP, and resist neutrophil killing, indicate that ZnuABC is a primary mechanism for *C. violaceum* to overcome zinc limitation in the host.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this work are described in Table 1. *E. coli* strains were cultivated in Luria-Bertani (LB) medium. *C. violaceum* strains were cultivated at 37°C in LB or M9 minimal medium supplemented with 0.1% casein hydrolysate (M9CH) (27, 28). Kanamycin (50 µg/ml), ampicillin (100 µg/ml) or tetracycline (10 µg/ml) were used in the cultures, when required. Conditions of zinc sufficiency were achieved by supplementation with ZnCl₂. Zinc deficient conditions were determined by minimal inhibitory concentration (MIC) experiments, using the chelators ethylenediamine tetraacetic acid (EDTA) and N,N,N',N'-tetrakis (2-

pyridylmethyl) ethylenediamine (TPEN). For iron, we used FeSO₄ (iron sufficiency) or 2,2'-dipyridyl (2,2'-DP) (iron deficiency), as previously defined for *C. violaceum* (27, 28).

***In silico* prediction of a Zur binding site.** To find a putative Zur box in *C. violaceum*, we searched for conserved DNA binding motifs upstream of *zur*, using the MEME (Multiple Em for Motif Elicitation) tool (34). The DNA sequences (300 bp upstream of the *zur* start codon) used in the MEME analysis were obtained from 23 draft genomes of species belonging to the *Chromobacterium* genus, using the Integrated Microbial Genomes and Microbiomes (IMG/M) website (53). The DNA binding motifs predicted by MEME were searched against databases of known DNA binding motifs, using the Tomtom motif comparison tool (54).

Construction of *C. violaceum* mutant and complemented strains. To generate the Δzur and $\Delta znuCBA$ mutant strains, we used a mutagenesis protocol based on two-step homologous recombination and sucrose counter-selection, as previously established for *C. violaceum* (27, 28). The primers used for cloning, sequencing, and mutant confirmation are listed in Table 2. For complementation of the $\Delta znuCBA$ mutant strain, the three genes were PCR-amplified from the WT strain and cloned into the replicative plasmid pMR20. The primers used for cloning are listed in Table 2. The construct was mobilized into the $\Delta znuCBA$ mutant strain by conjugation (27, 28).

RNA isolation and reverse-transcriptase polymerase chain reaction (RT-PCR). Wild-type *C. violaceum* was grown at 37°C in LB medium until the mid-exponential growth phase, and the cells were harvested by centrifugation. The total RNA was extracted using TRIzol reagent (Invitrogen) and purified with Direct-zol RNA Miniprep Plus (Zymo Research), following the manufacturer's instructions. Purified RNA was tested in PCR reactions with the same set of primers used in RT-PCR (Table 2) to check for genomic DNA contamination. The co-transcription was analyzed by RT-PCR using SuperScript III One-

298 Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen), following the
299 manufacturer's instructions.

300 **Calprotectin IC₅₀ assays.** CP was purified as previously described (21) and stored in a CP
301 buffer (100 mM NaCl, 3 mM CaCl₂, 20 mM Tris pH 7.5, and 5 mM 2-mercaptoethanol). The
302 integrity of the WT and Δ SI/SII versions of purified CP was confirmed by SDS-PAGE
303 analysis. Two bands, one of 8 kDa (S100A8) and the other of 14 kDa (S100A9), were
304 observed (data not shown). To assess the CP antimicrobial activity against *C. violaceum*,
305 the 50% growth inhibitory concentration (IC₅₀) of CP was calculated as previously described
306 (17) with minor modifications. Cultures of *E. coli* DH5 α and *C. violaceum* were diluted to an
307 OD₆₀₀ of 0.1 in LB and CP buffer (38% LB, 62% CB buffer), and WT or Δ SI/SII CP were
308 added at several quantities (serial dilution from 960 μ g/ml to 120 μ g/ml). After 8 h
309 incubation in a 96-well plate with agitation of 120 rpm, the OD₆₀₀ was measured, and the
310 IC₅₀ was calculated in GraphPad Prism version 8 (GraphPad, San Diego, CA). The
311 experiments were performed in three independent biological replicates.

312 **Construction of transcriptional *lacZ* fusions and β -galactosidase activity assay.**

313 The upstream region of *zur* (CV_RS15050) was PCR-amplified using proper primers (Table
314 2). The product was cloned directly into the pGEM-T easy plasmid (Promega), and
315 subcloned into the pRK*lacZ*290 vector, as an *Eco*RI/*Hind*III fragment (Table 1). The
316 construct containing the *zur-lacZ* transcriptional fusion was introduced into the *C. violaceum*
317 strains. The strains harboring this construct were grown until the mid-exponential phase in
318 LB medium, and were either untreated or treated with the indicated concentrations of ZnCl₂,
319 TPEN, EDTA, 2,2'-DP, or FeSO₄ for 2 h. For the CP-treated cells, overnight cultures were
320 diluted 1/6 in LB medium and CP buffer (38% LB, 62% CB buffer). Then, 500 μ g/ml CP or 1
321 mM EDTA were added, and after 4 h the promoter activity was measured. The β -

galactosidase activity assay was performed as previously described (55), using cultures from three independent biological replicates.

Minimal Inhibitory Concentration (MIC) assay. To assess the effects of the metal chelator EDTA, MIC assays were performed with the WT and $\Delta znuCBA$ strains. Overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.01 in LB medium in the presence of serially-diluted concentrations of EDTA. After 24 h cultivation at 37°C with agitation (250 rpm), the OD_{600} of all samples was measured. The experiments were performed in three independent biological replicates.

Disk diffusion assays. To test the sensitivity to EDTA or 2,2'-DP, overnight cultures were diluted to an OD_{600} of 0.1, and 500 μ l were spread on LB agar plates. Sterile paper disks were placed on the cultures. The indicated concentrations of EDTA or 2,2'-DP were dropped in each paper disk (10 μ l). After 24 h incubation at 37°C, the zone of growth inhibition was measured. The experiments were performed in three independent biological replicates.

Growth curves. Overnight cultures of *C. violaceum* were diluted in 10-15 ml of LB medium to an OD_{600} of 0.01. When necessary, the pre-inocula were treated with 1 mM EDTA, 500 μ M CP WT or 500 μ M CP $\Delta SI/SII$. For the growth curves, in LB or LB plus the indicated treatments, the diluted cultures were grown at 37°C under agitation (250 rpm), and aliquots were withdrawn for OD_{600} measurement in an Eppendorf BioPhotometer. Growth curves with CP were performed in diluted LB (38% LB, 62% CB buffer). All experiments were performed in three independent biological replicates.

Swimming motility. The swimming motility assay was performed in M9CH 0.3% agar plates as previously described (27). When indicated, the M9CH plates were supplemented with 1 mM EDTA and/or 250 μ M $ZnCl_2$. Swimming motility was determined as the halo

diameter of bacterial spreading at 24 h and 48 h. The experiments were performed in three independent biological replicates.

Static biofilm measurement. All strains were cultured in glass tubes without agitation in LB medium with or without 100 μ M EDTA at 37°C for 24 h, before staining with 0.1% (w/v) crystal violet. Stained biofilms were resuspended with 100% ethanol (1 ml), followed by measurement of OD₆₀₀ (27). The experiments were performed in six biological replicates. Data are plotted as the ratio of biofilm measurement by bacterial growth.

Interspecies competition. The *C. violaceum* WT and $\Delta znuCBA$ strains were co-cultivated with other bacteria. The *C. violaceum* strains (resistant to ampicillin) and the ampicillin-sensitive bacteria *B. cepacia* and *S. Typhimurium* were grown overnight in LB. Bacterial cultures were diluted 1:20 in LB or LB plus 250 μ M ZnCl₂ and mixed 1:1. After incubation for 2 h at 37°C and 250 rpm, the co-cultures were serially diluted and plated in selective LB ampicillin plates for CFU enumeration. For comparison, the CFU of each bacterium grown in the same conditions, but without co-cultivation, was also determined in LB plates.

Mouse virulence assays. 6-week-old female BALB/c mice were used to inject intraperitoneally 10⁶ CFU from the *C. violaceum* strains, as previously determined (27, 28, 31, 37). Animal survival was followed up for 10 days post infection. To determine the bacterial burdens in the liver, mice were infected as previously stated and euthanized after 16 h. Livers were aseptically collected and blended in PBS. Serial dilutions were plated on LB for CFU quantification. For the *in vitro* neutrophil killing assays, we used a previously described protocol of thioglycolate-induced peritonitis to obtain a neutrophil enriched fraction from the peritoneal cavity of BALB/c mice (28). The *C. violaceum* strains were added to the neutrophils in a Hank's Balanced Salt Solution (HBSS) supplemented with 2% fetal bovine serum at a multiplicity of infection (MOI) of 10:1. After 2 h incubation at 37°C with gentle agitation, the neutrophils were lysed by adding an equal volume of water. The

vortexed suspensions were serially diluted and plated in LB for CFU quantification. The CFU of the strains grown in HBSS was determined, as a control. The procedures with mice were performed under the Ethical Principles in Animal Research adopted by the National Council for the Control of Animal Experimentation (CONCEA). The protocol (181/2017) was approved by the Local Ethical Animal Committee (CEUA) of FMRP-USP.

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 8 (GraphPad, San Diego, CA). Data distribution was assessed using a Shapiro-Wilk test of normality, assuming statistical significance at 0.05. Multiple comparison tests were performed and are indicated in each figure.

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FIGURE LEGENDS

Fig 1. The *C. violaceum* ZnuABC is encoded in a Zur-repressed, zinc responsive operon. (A) Genomic context of the genes *zur*, CV_RS15045, CV_RS15040, and *znuCBA*. The red box upstream of *zur* indicates a predicted Zur-box. The arrows indicate the

annealing positions of the primers used in the RT-PCR reactions. (B) The putative Zur binding site predicted upstream of *zur* from *Chromobacterium* spp. This DNA binding motif matches the known Zur-boxes of *Pseudomonas protegens* Pf-5 and *Neisseria meningitidis* MC58. (C) Co-transcription of genes of the *zur*-CV_RS15045-CV_RS15040-*znuCBA* operon. Control PCR reactions (upper panel) using genomic DNA or RNA as templates. RT-PCR reactions (down panel) using the indicated primers (“a” and “b”, 1552 bp; “c” and “d”, 1613 bp) confirmed co-transcription of the genes of the operon. (D) Expression of the promoter region of *zur* (*pzur-lacZ*) determined by β -galactosidase activity assay. Strains harboring the *pzur-lacZ* fusion were grown in LB medium, and the cultures were treated for 2 h with the indicated concentrations of ZnCl_2 , EDTA, TPEN, FeSO_4 , and 2,2'-DP. These experiments were performed in three independent biological replicates. Statistical analysis performed by One-way ANOVA followed by Dunnett's multiple comparison test. Samples were compared to the untreated LB culture of WT[*pzur-lacZ*] or Δzur [*pzur-lacZ*]. * $P=0.0358$, *** $P=0.0008$, **** $P<0.0001$, where not indicated = not significant.

Fig 2. The absence of ZnuABC affects the sensitivity of *C. violaceum* to zinc, but not to iron, limitation. (A) The MIC of EDTA in LB medium was the same for the WT and ΔznuCBA strains. Data from three biological replicates are shown as mean and standard deviation. Statistical analysis performed by unpaired *t*-test for each EDTA concentration indicated the absence of statistical significance. (B) Growth of the indicated strains in LB in different conditions of zinc (EDTA, ZnCl_2) and iron (2,2'-DP and FeSO_4) availability. The growth was evaluated after 24 h cultivation. Statistical analysis performed by One-way ANOVA followed by Dunnett's multiple comparison test. Samples were compared to the WT strain in each condition. **** $P<0.0001$, where not indicated = not significant. (C and D) Disk diffusion assays for the chelating agents EDTA (C) and 2,2'-DP (D) with the indicated strains. The diameter of the zone of inhibition was measured in three independent biological

replicates. Statistical analysis performed by One-way ANOVA followed by Dunnett's multiple comparison test. Samples were compared to the WT strain in each concentration of EDTA or 2,2'-DP. **** $P < 0.0001$, where not indicated = not significant.

Fig 3. Intracellular zinc depletion by EDTA treatment impairs the growth of $\Delta znuCBA$.

Growth curves of the indicated strains in LB (A and C) and LB supplemented with 1 mM EDTA (B and D), with pre-inocula untreated (A and B) or treated with 1 mM EDTA (C and D). (E) Growth curves of EDTA-treated pre-inocula in LB supplemented with 1 mM EDTA and 200 μ M $ZnCl_2$. Growth curves were determined in three independent biological replicates and are represented as the mean with standard deviation.

Fig 4. Calprotectin decreases the growth of $\Delta znuCBA$. (A-C) Growth curves of the indicated strains determined in the presence of 500 μ M CP WT (A and C) or 500 μ M CP $\Delta SI/SII$ (B). (A) Pre-inocula treated with 500 μ g/ml CP WT and growth curves performed without (continuous lines) or with (dashed lines) 500 μ g/ml CP WT. (B) Pre-inocula treated with 500 μ g/ml CP $\Delta SI/SII$ and growth curves performed without (continuous lines) or with (dashed lines) 500 μ g/ml CP $\Delta SI/SII$. (C) Untreated pre-inocula used in growth curves performed without (continuous lines) or with (dashed lines) 500 μ g/ml CP WT. (D) Effect of CP on the expression of the promoter region of *zur* (*pzur-lacZ*). The β -galactosidase activity assays were performed 4 h after the treatment with 500 μ M CP WT, 500 μ M CP $\Delta SI/SII$, or 1 mM EDTA. Experiments were performed in three independent biological replicates and are represented as the mean with standard deviation. Statistical analysis performed by One-way ANOVA followed by Dunnett's multiple comparison test. Samples of each treated condition were compared with the samples in LB. *** $P = 0.0004$, **** $P < 0.0001$.

Fig 5. Zinc limitation or mutation of *znuCBA* decrease violacein production, swimming motility, biofilm formation, and bacterial competition. (A) Effect of EDTA and zinc on violacein production in the indicated strains. Photos acquired after 24 h

cultivation. (B and C) Effect of EDTA and zinc on swimming motility. (B) WT strain in M9CH or M9CH plus 1 mM EDTA, 200 μ M ZnCl₂, or both EDTA and ZnCl₂. (C) The indicated strains in M9CH. The halo diameter of bacterial spreading was measured at 24 h and 48 h. Experiments were performed in three independent biological replicates and are represented as the mean with standard deviation. Statistical analysis performed by One-way ANOVA followed by Dunnett's multiple comparison test. Comparisons were halo diameter of each treated condition with untreated M9CH (A) or mutant strains with WT strain (B). * $P=0.039$, **** $P<0.0001$. (D) Effect of EDTA on biofilm in the indicated strains. Static biofilm was determined by crystal violet staining after 24 h of cultivation in LB or LB plus 100 μ M EDTA. Data were plotted as the ratio of biofilm quantification by cell density of the cultures (OD₆₀₀). Statistical analysis performed by One-way ANOVA followed by Dunnett's multiple comparison test. ** $P=0.003$. (E) The absence of ZnuABC affects *C. violaceum* competition. Interspecies competition of the indicated *C. violaceum* strains co-cultured with *Burkholderia cepacia* ATCC 17759 or *Salmonella* Typhimurium ATCC 14028. Experiments were performed with or without 250 μ M ZnCl₂ in two biological replicates.

Fig 6. The Δ znuCBA mutant strain is highly attenuated for virulence in mice and susceptible to neutrophil killing. (A) Survival plot of infected BALB/c mice. Animals were i.p. injected with 10⁶ CFU of the indicated strains. Animal survival was monitored for up to 10 days. Statistical analysis was performed by the log rank (Mantel-Cox) test; ** $P=0.0074$, **** $P<0.0001$. (B) Bacterial burden in the liver. BALB/c mice (n=5 for WT; n=6 for Δ znuCBA and Δ znuCBA[pMR20znuCBA]) were infected with 10⁶ CFU. After 16 h of infection, livers were collected, homogenized in PBS, diluted, and plated for CFU quantification. Statistical analyses were performed by One-way ANOVA followed by Dunnett's multiple-comparison test, comparing with the WT strain. ** $P=0.0047$. (C) Neutrophil microbicidal activity. Neutrophils were infected *in vitro* with the *C. violaceum* strains at a MOI of 10 and

664 incubated at 37°C for 2 h. The neutrophils were lysed, and diluted samples were plated on
665 LB for bacterial CFU quantification. As a control, bacteria incubated with HBSS and
666 maintained in the same conditions were also quantified. Statistical analyses were
667 performed by One-way ANOVA followed by Dunnett's multiple-comparison test, comparing
668 with the WT strain. ** $P=0.0053$.
669

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description ^a	Reference or source
<i>Escherichia coli</i>		
DH5α	<i>E. coli</i> strain for cloning purposes	(56)
S17-1	<i>E. coli</i> strain for plasmid mobilization	(57)
<i>Chromobacterium violaceum</i>		
WT	<i>C. violaceum</i> ATCC 12472 WT strain with sequenced reference genome	(58)
WT[pMR20]	WT strain harboring the empty pMR20 plasmid	This work
Δ <i>zur</i>	WT strain with <i>zur</i> (CV_RS15050) deleted	This work
Δ <i>znuCBA</i>	WT strain with <i>znuC</i> , <i>znuB</i> , and <i>znuA</i> (CV_RS15035, CV_RS15030, and CV_RS15025) deleted	This work
Δ <i>znuCBA</i> [pMR20]	Δ <i>znuCBA</i> strain harboring the empty pMR20 plasmid	This work
Δ <i>znuCBA</i> [pMR20 <i>znuCBA</i>]	Δ <i>znuCBA</i> mutant complemented with the genes <i>znuCBA</i>	This work
WT[p <i>zur-lacZ</i>]	WT strain with fusion of the promoter region of <i>zur</i> to <i>lacZ</i>	This work
Δ <i>zur</i> [p <i>zur-lacZ</i>]	Δ <i>zur</i> mutant with fusion of the promoter region of <i>zur</i> to <i>lacZ</i>	This work
<i>Burkholderia cepacia</i>	<i>B. cepacia</i> ATCC 17759 WT strain with sequenced reference genome	(59)
<i>Salmonella</i> Typhimurium	<i>S. Typhimurium</i> ATCC 14028 WT strain	(60)
Plasmids		
pNPTS138	Suicide vector containing <i>oriT</i> , <i>sacB</i> , Kan ^r	M. R. K. Alley
pMR20	Broad-host-range low-copy vector containing <i>oriT</i> , Tet ^r	(61)
pGEM-T easy	Cloning plasmid, Amp ^r	Promega
pRK <i>lacZ</i> 290	pRK2-derived vector with promoterless <i>lacZ</i> gene, Tet ^r	(62)

^aKan, kanamycin; Tet, tetracycline; Amp, ampicillin; r, resistance.

Table 2. Primers used in this work

Purpose and primer name	Sequence (5'→3') ^a	Description
Construction of mutant strain		
Zur_del1	ATTATT <u>GGGCCCC</u> GGCCCCGGCGGATCAATCC	<i>Apal/HindIII</i> upstream flanking fragment with 626 bp
Zur_del2	ATTATTA <u>AAGCTT</u> CTCGGCGGCGGCGAGATAGG	<i>HindIII/BamHI</i> downstream flanking fragment with 629 bp
Zur_del3	ATTATTA <u>AAGCTT</u> CTGGCCGGACGCTGCCAGT	<i>HindIII/BamHI</i> downstream flanking fragment with 634 bp
Zur_del4	ATTATT <u>GGATCC</u> GCCAGGCGACATCCAGCTGG	<i>Apal/HindIII</i> upstream flanking fragment with 647 bp
ZnuCBA_del1	ATTATT <u>GGGCCCC</u> GCTGTTCCCGCCCAAGTC	<i>HindIII/BamHI</i> downstream flanking fragment with 634 bp
ZnuCBA_del2	ATTATTA <u>AAGCTT</u> GATGTGGTGCACCGCCGG	<i>HindIII/BamHI</i> downstream flanking fragment with 634 bp
ZnuCBA_del3	ATTATTA <u>AAGCTT</u> GAGTCCGAGGCCTCGGCC	<i>HindIII/BamHI</i> downstream flanking fragment with 634 bp
ZnuCBA_del4	ATTATT <u>GGATCC</u> CTGATCGCGATGGGCCTCT	<i>HindIII/BamHI</i> downstream flanking fragment with 634 bp
M13Fw	GTAAAACGACGGCCAGT	Sequencing of DNA fragments into plasmids
M13Rv	AGCGGATAACAATTTTCAC	
Construction of complemented strains		
ZnuCBA_comp_Fw	ATTATTA <u>AAGCTT</u> GCTCTTGGTGTGGTGGGCAT	<i>HindIII/XhoI</i> 2671 bp product with <i>znuCBA</i> genes
ZnuCBA_comp_Rv	ATTATT <u>CTCGAG</u> CGCCGACAGCCTGCGCCG	
RT-PCR		
a	CGGCCGCGTCAATGAAGTGG	1552 bp product encompassing <i>zur</i> and <i>znuC</i> genes
b	CGAACGGCCCGCTCTGGT	1613 bp product encompassing <i>znuC</i> and <i>znuA</i> genes
c	CGGCATGGCCCGCGCGAT	
d	ACCACGGTCACGCCGCGG	
β-galactosidase assays		
LacZ_zur_Fw	ATTATTGAATTCGCTTGTACTGCTTGAACAC	<i>EcoRI/HindIII</i> 347 bp product for pRK/lacZ290 cloning
LacZ_zur_Rv	ATTATTA <u>AAGCTT</u> CAGCTTCAGCACCTGGC	

^aUnderlined sequences indicate the restriction enzyme recognition sites used for cloning purposes

a)

b)

c)

d)

e)