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GENETICS AND MOLECULAR BIOLOGY

The Molecular Basis of *Acinetobacter baumannii* Cadmium Toxicity and Resistance

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ABSTRACT *Acinetobacter* species are ubiquitous Gram-negative bacteria that can be found in water, in soil, and as commensals of the human skin. The successful inhabitation of *Acinetobacter* species in diverse environments is primarily attributable to the expression of an arsenal of stress resistance determinants, which includes an extensive repertoire of metal ion efflux systems. Metal ion homeostasis in the hospital pathogen *Acinetobacter baumannii* contributes to pathogenesis; however, insights into its metal ion transporters for environmental persistence are lacking. Here, we studied the impact of cadmium stress on *A. baumannii*. Our functional genomics and independent mutant analyses revealed a primary role for CzcE, a member of the cation diffusion facilitator (CDF) superfamily, in resisting cadmium stress. We also show that the CzcCBA heavy metal efflux system contributes to cadmium efflux. Collectively, these systems provide *A. baumannii* with a comprehensive cadmium translocation pathway from the cytoplasm to the periplasm and subsequently the extracellular space. Furthermore, analysis of the *A. baumannii* metallome under cadmium stress showed zinc depletion, as well as copper enrichment, both of which are likely to influence cellular fitness. Overall, this work provides new knowledge on the role of a broad arsenal of membrane transporters in *A. baumannii* metal ion homeostasis.

IMPORTANCE Cadmium toxicity is a widespread problem, yet the interaction of this heavy metal with biological systems is poorly understood. Some microbes have evolved traits to proactively counteract cadmium toxicity, including *Acinetobacter baumannii*, which is notorious for persisting in harsh environments. Here, we show that *A. baumannii* utilizes a dedicated cadmium efflux protein in concert with a system that is primarily attuned to zinc efflux to efficiently overcome cadmium stress. The molecular characterization of *A. baumannii* under cadmium stress revealed how active cadmium efflux plays a key role in preventing the dysregulation of bacterial metal ion homeostasis, which appeared to be a primary means by which cadmium exerts toxicity upon the bacterium.

KEYWORDS *Acinetobacter*, cadmium, heavy metal, cell membranes, efflux

The heavy metal cadmium is enriched in industrial settings and mining sites through anthropogenic activity. Although various metalloproteins retain their functions when bound to cadmium (1), cadmium is primarily associated with toxicity in biological systems (2). Despite cadmium being redox inactive, cadmium stress is commonly connected to the induction of oxidative stress in both humans and microbes (3). Recent advances

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in the field have revealed cadmium-metalloprotein interactions as a primary feature of cadmium toxicity (4, 5). In addition to dysregulation of central carbon metabolism and lipid homeostasis (6), cadmium stress in *Streptococcus pneumoniae* causes manganese and zinc starvation via interaction with noncognate metalloproteins, these being a manganese-recruiting protein and a zinc-responsive transcriptional regulator (5). Manganese is the primary cofactor for superoxide dismutase in *S. pneumoniae*; hence, cadmium-induced manganese depletion leads to oxidative stress susceptibility (5, 7). In *Salmonella enterica*, excess cadmium was found to affect zinc and manganese, but not iron, accumulation (4). Conversely, iron supplementation was shown to improve the growth of *Bacillus subtilis* when exposed to high levels of cadmium (8). These studies show that dysregulation of metal ion homeostasis and protein mismetallation by cadmium is central to its toxicity in cells.

Acinetobacter baumannii is rapidly emerging as one of the world's most antimicrobial-resistant bacterial pathogens (9–11). Its highly effective intrinsic and acquired stress resistance features have allowed this human pathogen to survive in the hospital environment and thrive when colonizing immunocompromised individuals (12, 13). Various members of the genus *Acinetobacter*, including some strains of *A. baumannii*, have a strong environmental presence. This has resulted in the *A. baumannii* genome harboring diverse metabolic and stress resistance features that are typically not linked to pathogenicity (14, 15). Its environmental roots are also reflected in its extensive arsenal of putative heavy metal export mechanisms that may enable persistence under diverse metal ion stresses (16, 17). *A. baumannii* metal ion biology is a rapidly expanding field of research. In particular, its diverse array of metal ion acquisition systems has been studied intently (18–24). In contrast, our understanding of heavy metal resistance is limited, with only the roles of zinc and copper toxicity being studied in recent years (16, 17, 25–27). Metal ion toxicity analyses in *A. baumannii* have revealed widespread implications for cellular physiology, including dysregulation of metal ion homeostasis, oxidative stress susceptibility, and alterations in membrane biology. Similar to the impact of cadmium on the dysregulation of *S. pneumoniae* and *S. enterica* metal ion homeostasis, zinc stress in *A. baumannii* has been shown to result in copper depletion (16).

The *A. baumannii* genome harbors a broad arsenal of both highly conserved and recently acquired efflux systems that have putative functions in metal resistance (16). The P-type ATPase CopA is the primary mediator of cytoplasmic copper efflux in *A. baumannii* and plays a role in its virulence potential (26). The complexity of copper homeostasis in the periplasm has also been studied (27), but strain-to-strain variation complicates defining its significance across the species (16, 26). Zinc resistance is mediated by an efficient efflux pathway that consists of the cation diffusion facilitator (CDF) family member CzcD, a periplasmic zinc chaperone named CzcI, and the heavy metal efflux (HME) system CzcCBA (25).

Current models propose that bacterial resistance to cadmium toxicity is mediated via various mechanisms. Bacterial cell wall components such as polysaccharides, proteins, and lipids can prevent cadmium from entering the cell (28). Once cadmium passes the cell wall layer, intercellular metal sequestration and efflux pumps play a significant role in detoxifying the cell from cadmium toxicity. Metallothioneins are widely known metal chelators involved in copper and cadmium resistance, especially in eukaryotes (29), while their role in bacterial microorganisms has been less explored until recently (30). Bacterial cadmium efflux can be mediated by HME, CDF, or P-type ATPase family members. Examples include CadA, a P-type ATPase from *Streptococcus thermophilus* (31), the CDF member YiiP from *Escherichia coli* (32, 33), and the CznABC HME system from *Helicobacter pylori* (34). These systems may display overlapping substrate profiles within a bacterial species, which requires experimental verification. Although a recent study has provided insights into the role of the C-terminal domain in CDF metal specificity (35), overall, the specificity for transition metals varies greatly between members within the CDF, HME, and P-type ATPase families and is difficult to predict based on protein sequence analyses.

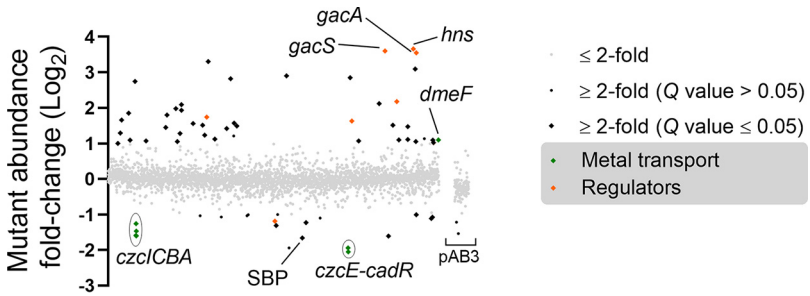

FIG 1 The *A. baumannii* cadmium resistome. The effect of cadmium treatment (60 μM for 16 h) on the abundance of gene mutations (Tn*5* insertions) mapping to the *A. baumannii* ATCC 17978 chromosome and plasmid pAB3 was determined by TraDIS analysis. The data are means from biological triplicates (also presented in Table S3). The *Q* value was determined using a previously described method (53).

In this study, we defined the genome-wide cadmium stress adaptation mechanisms in *A. baumannii*. This work identified both CDF and HME systems that play key roles in cadmium export in *A. baumannii*, which were confirmed by single-gene mutant analyses. We also show that the bacterium encodes a highly efficient cadmium-sensing regulator for the transcriptional activation of the primary cadmium resistance mechanism. By using global metallomic analyses under cadmium stress, this study provides novel insights into cadmium toxicity.

RESULTS AND DISCUSSION

***A. baumannii* employs multiple transport systems to resist cadmium stress.** To delineate the molecular basis of cadmium toxicity and the mechanisms that aid in cadmium stress resistance, we created a Tn*5*-based random transposon mutant library in the laboratory reference strain *A. baumannii* ATCC 17978. Transposon-directed insertion site sequencing (TraDIS) sequencing of the base library in rich medium revealed a density of at least 113,000 unique mutants. We subjected 10^9 mutant cells to a subinhibitory concentration (60 μM) of cadmium for 16 h. This concentration was found to present a minor but significant impact on proliferation of this strain (Fig. S1) and was thus deemed appropriate for TraDIS analysis. Cadmium exposure resulted in 67 genes displaying significantly differential numbers of insertions (fold change of $\geq$2; *Q* value, $\leq$0.05) (Table S3): decreased mutant fitness (decreased transposon insertions) in 23 genes and enhanced fitness (increased transposon insertions) in 44 genes.

The TraDIS analyses identified ACX60_13165 (CadR), which encodes a putative MerR-like transcriptional regulator, as most affected by cadmium treatment ($-$4.1-fold change) (Fig. 1). The regulatory gene is transcribed divergently from the CDF family transport gene *czcE* (ACX60_13160), which our analyses defined as the gene with the second greatest loss of represented transposon mutants under cadmium stress ($-$3.8-fold change). The previously characterized HME-RND zinc efflux system CzcCBA (ACX60_01405-15) and its metal chaperone, encoded by *czcI* (ACX60_01400) (25), were also shown to be important in conferring cadmium resistance, where mutant abundance decreased between $-$2.4- and $-$3.0-fold. The CDF member *czcD*, previously revealed to play a role in zinc efflux, was not found to be significantly impacted by cadmium treatment. We identified that transposon mutants of a putative DmeF-like cobalt-exporting CDF (ACX60_18165) were enriched under cadmium stress (2.1-fold change). Interestingly, independent mutant analyses revealed a marginal role for the *A. baumannii dmeF* member during cadmium stress but a primary role in cobalt resistance (Fig. S2).

A solute-binding protein (SBP; ACX60_10710) with similarity to methionine and metal ion binding SBPs such as NlpA (COG1464.1) was impacted by cadmium stress ($-$3.2-fold change). The gene encoding this periplasmic protein is located in an operon

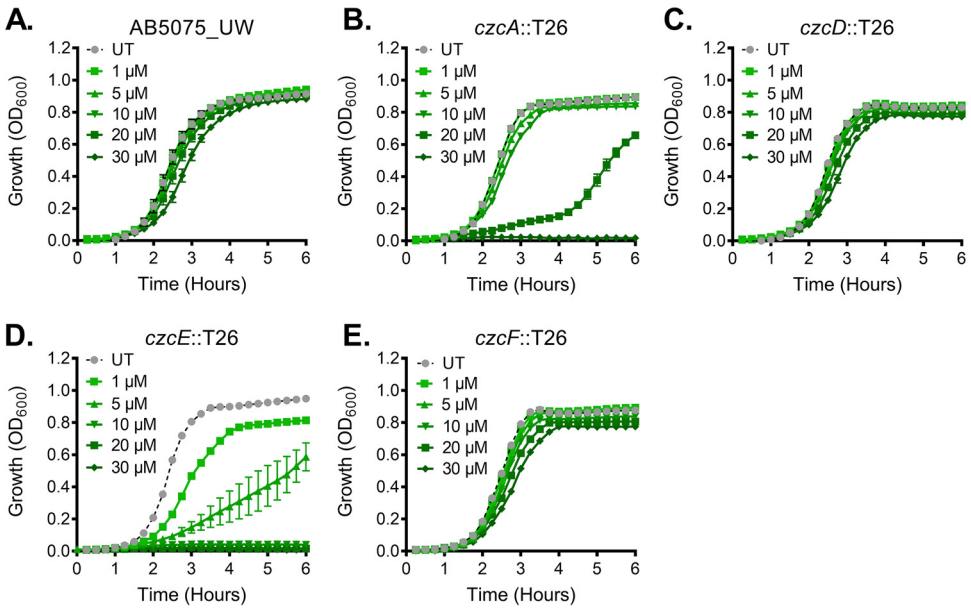

FIG 2 Cadmium resistance in *A. baumannii*. The effects of cadmium (1, 5, 10, 20, or 30 μM $CdCl_2$) on growth of (A) wild-type, (B) *czcA*::T26, (C) *czcD*::T26, (D) *czcE*::T26, and (E) *czcF*::T26 cells were compared to that of untreated (UT) cells. The growth was examined by measuring the OD_{600}. The data are the means from at least biological triplicates (with standard errors of the means [SEM]). The visibility of error bars may be occluded by the symbols.

that also encodes its putative ATP-binding cassette (ABC) transporter for cargo delivery and subsequent translocation across the inner membrane, but none of these genes were affected by cadmium stress. Hence, the SBP may play a role in periplasmic metal buffering instead of facilitating transport.

The relatively large number of genes in which insertional disruption proved to impart a positive effect included various transcriptional regulators. H-NS (12.6-fold change) is one of *A. baumannii*'s most prominent global transcriptional regulators (36). Although no role in metal ion stress has been indicated in previous transcriptomic analyses of an ATCC 17978 *hns* mutant, in *Salmonella*, the ferric uptake regulator (Fur) regulates the expression of *hns*, and H-NS competes with Fur for some gene targets in *Salmonella* (37, 38). The GacAS regulatory system, mutation of which is beneficial to survival during cadmium stress (11.7- and 12.1-fold changes, respectively), plays a key role during infection and in lifestyle changes, including biofilm formation (39). Other regulators include those with putative roles in sensing saturated fatty acids, including ACX60_05275 (3.3-fold change) and ACX60_17890 (2.0-fold change). This may indicate that *A. baumannii* adapts to cadmium stress by changing the structure and subsequently the biophysical properties of its membrane. Analysis of the total fatty acid profile of *A. baumannii* under cadmium stress revealed minor but significant changes in 16:1 fatty acids (1.5% decrease) and 18:0 fatty acids (0.9% increase) (Fig. S3). These changes may indicate a slight increase in the membrane rigidity following cadmium exposure (40).

***A. baumannii* employs a dedicated inner membrane cadmium exporter.** To confirm the roles of the putative cadmium transporters in cadmium stress resistance as observed by TraDIS (Fig. S4), we examined mutants with mutations in *czcA* (ABUW_0268), *czcD* (ABUW_0269), *czcE* (ABUW_2851), and *czcF* (ABUW_3664). *A. baumannii* AB5075_UW and its mutant derivatives were grown with increasing concentrations of cadmium (1, 5, 10, 20, or 30 μM $CdCl_2$) (Fig. 2A to E). We found that inactivation of *czcE* rendered the cells hypersusceptible to cadmium stress, with significant growth perturbation seen at concentrations as low as 1 μM cadmium, which is approximately 30-fold lower than the AB5075_UW

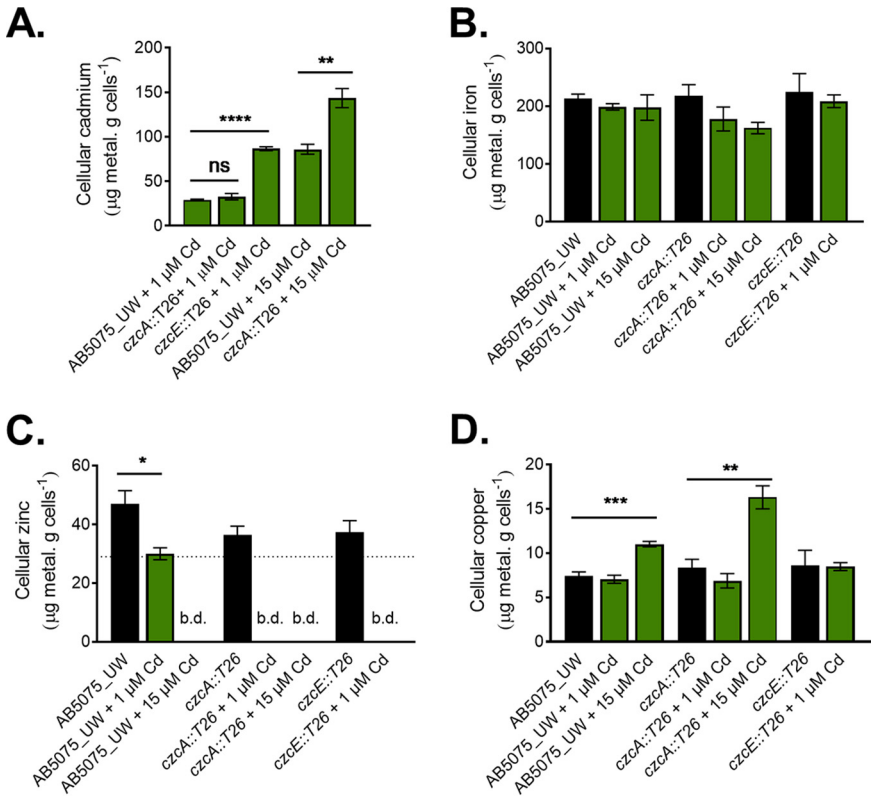

FIG 3 Impact of cadmium exposure on *A. baumannii* metal ion homeostasis. The accumulation of (A) cadmium, (B) iron, (C) zinc, and (D) copper in wild-type, *czcA*::T26, or *czcE*::T26 cells with or without cadmium supplementation (1 μM or 15 μM $CdCl_2$), as determined by inductively coupled plasma mass spectrometry. Metal ion levels were quantified as the weight of the metal (μg) per the weight of the dry cell pellet (g). For all panels, the data are the mean of at least biological triplicates (with SEM). Statistical analyses were performed using a two-tailed Student's *t* test; n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. The detection limit of zinc is indicated by the dotted line (b.d., below detection).

parental strain. In addition to a primary role in zinc resistance (25), CzcCBA contributes to cadmium resistance, with the *czcA*::T26 mutant showing increased sensitivity at cadmium concentrations of 20 μM or greater. Consistent with the examination of these efflux systems in strain AB50875_UW, the ATCC 17978 Δ*czcA* and Δ*czcE* mutants displayed increased susceptibility to cadmium stress (Fig. S5). CzcD and CzcF were found not to play a major role in cadmium resistance. The concerted efforts from this study and previous work (25) have identified significant roles for CzcA, CzcD, and CzcE in zinc and/or cadmium resistance, but no major role has been identified for CzcF. Hence, we examined the response of CzcF to stress induced by other transition metals (zinc, copper, nickel, cobalt, and iron), which revealed a minor role in zinc and nickel resistance (Fig. S6).

The impact of cadmium on the *A. baumannii* metallome. Bacterial cells tightly balance metal ion abundances, as hyperaccumulation or deficit beyond the cellular set points can have detrimental impacts on metalloprotein function and homeostasis of other metals. TraDIS analyses provided some insight into the impact of cadmium stress on cobalt homeostasis (Fig. 1 and Fig. S2). First, we examined cadmium accumulation in *A. baumannii* strain AB5075_UW and the *czcA*::T26 and *czcE*::T26 mutant strains. These analyses revealed that *czcA* inactivation did not impact accumulation following supplementation with 1 μM cadmium. In contrast, the *czcE*::T26 mutant accumulated cadmium at nearly 8-fold-higher levels than the wild type (Fig. 3A). Since growth of the *czcE*::T26 mutant was dramatically perturbed at cadmium concentrations greater than 1 μM, only the wild type and *czcA*::T26 mutant were examined in 15 μM cadmium. The

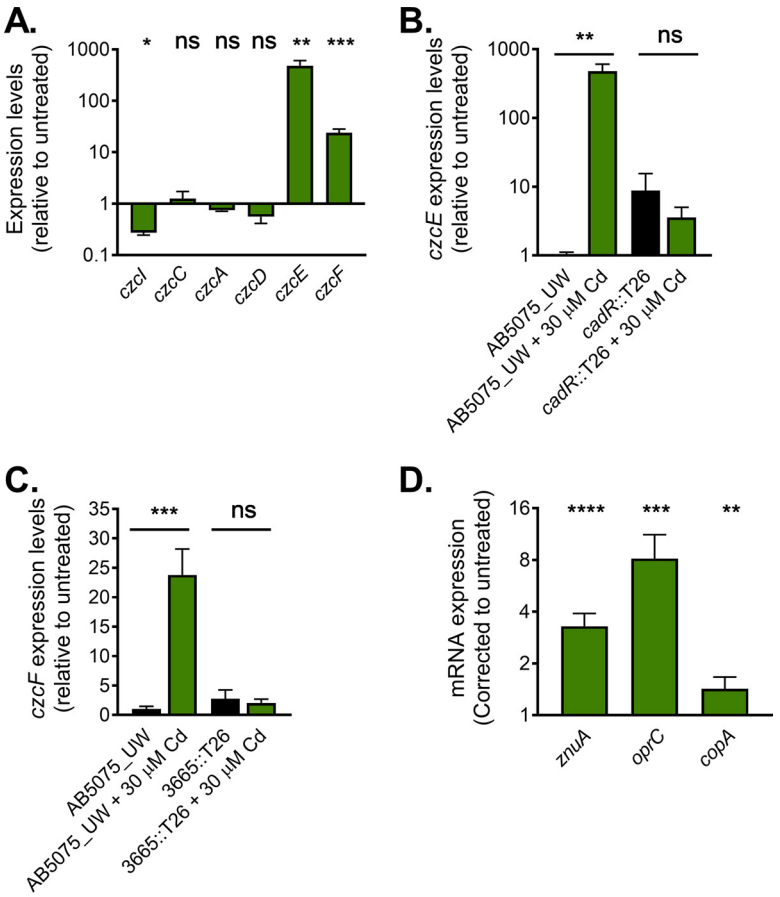

FIG 4 Regulation of cadmium resistance. (A) The transcriptional responses of *czcI*, *czcC*, *czcA*, *czcD*, *czcE*, and *czcF* in wild-type AB5075_UW cells treated with 30 μM $CdCl_2$ were examined by qRT-PCR. The transcription levels of (B) *czcE* and (C) *czcF* were examined in regulator mutant and AB5075_UW wild-type cells by qRT-PCR following treatment with 30 μM $CdCl_2$. (D) The transcription levels of *znuA*, *oprC*, and *copA* in wild-type cells were examined by qRT-PCR following treatment with 30 μM $CdCl_2$ for 30 min. For all panels, the data are means (SEM) for at least biological triplicates. The visibility of error bars may be occluded by the symbols. Statistical analyses were performed using a two-tailed Student's *t* test. n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

czcA::T26 mutant accumulated cadmium at greater levels than those observed for the wild type (144 versus 86 μg cadmium·g cells^{-1}) (Fig. 3A).

Examination of the impact of cadmium on the most abundant *A. baumannii* metal ions revealed that zinc and copper levels were affected, but not iron (Fig. 3B to D). Hence, an association between the enrichment of transposon mutants in the global regulator HNS and cadmium-induced changes in cellular iron could not be made. Therefore, the role of HNS during cadmium stress requires further study in *A. baumannii*. Zinc levels in the wild type were significantly lower upon exposure to 1 μM cadmium and below the limit of detection upon exposure to 15 μM cadmium. Consistent with these findings, zinc levels in the *czcA*::T26 and *czcA*::T26 mutants decreased to a greater extent upon exposure to 1 μM cadmium than those in the wild type (Fig. 3B). Interestingly, the wild type and *czcA*::T26 mutant hyperaccumulated copper when exposed to 15 μM cadmium (Fig. 3D). Despite the similar cellular cadmium levels observed in the *czcE*::T26 mutant following treatment with 1 μM cadmium and the wild type with 15 μM cadmium (Fig. 2A), the accumulation of copper was not affected in the *czcE*::T26 mutant (Fig. 2D).

Cadmium stress influences the *A. baumannii* metalloregulator responses. Transcriptional analyses revealed that *czcE* is upregulated 480-fold by cadmium stress (Fig. 4A), while *czcI* was significantly downregulated by cadmium stress. Analysis of

CadR (ABUW_2852), a MerR-type regulator divergently transcribed from *czcE*, suggested that it was responsible for activation of *czcE*, which is consistent with the TraDIS analysis (Fig. 4B). We found that *czcF* was significantly upregulated upon treatment with cadmium, despite it not playing a major role in cadmium resistance (Fig. 4C and Fig. S6). Similar to *czcE*, we found that *czcF* is regulated by a divergently transcribed MerR-type regulator, ABUW_3665 (Fig. 4C).

Our metallomic analyses determined that cadmium toxicity impacts zinc and copper homeostasis (Fig. 2). To ascertain the impact of cadmium stress on the expression of metal ion homeostasis contributors, we analyzed transcription of *znuA*, encoding a component of the major zinc acquisition system ZnuABC (18), *oprC*, encoding a putative copper acquisition system (41), and *copA*, encoding the primary copper efflux system (25). Under cadmium stress, *A. baumannii* increases expression of *znuA* (3.3 $\log_2$-fold) (Fig. 4D), which may suggest a role in attempting to compensate for zinc starvation. Interestingly, cadmium-induced copper overaccumulation may be a result of the significant upregulation of *oprC* (8.2 $\log_2$-fold) (Fig. 4D), although further experiments are required to conclusively define this mechanism.

Conclusions. Cadmium resistance in *A. baumannii* was found to be primarily mediated by the CDF member CzcE. However, mutation of *czcA*, the critical inner membrane component of an HME system, resulted in *A. baumannii* growth perturbation in the presence of cadmium, which suggests that cytoplasmic cadmium is translocated into the periplasm by CzcE and then exported into the extracellular space by CzcCBA. The combination of a highly attuned cadmium sensing regulator (CadR) and dedicated inner-membrane cadmium transporter illustrates the significance of cadmium efflux in the organism. The seemingly opportunistic role of CzcCBA in cadmium resistance indicates that cadmium exerts greater toxicity in the cytoplasm than in the periplasm, which is consistent with previous reports (42, 43). However, the less significant role of CzcCBA than CzcE in cadmium resistance may also be due to the lack of its upregulation, rather than an inability to carry out export from either the cytoplasm or periplasm. To date, the transcriptional regulator(s) of *czcCBA* has not been identified.

Cadmium stress resulted in copper overaccumulation with transcriptional analyses revealing *oprC* as a possible copper import system gene. Although *oprC* has been shown to be regulated by OxyR in *Acinetobacter oleivorans* (44), recent analyses of OxyR in *A. baumannii* did not identify such an association (45). Hence, the physiological role of OprC warrants further investigation. Zinc homeostasis was also affected by cadmium stress, resulting in a reduction in cellular zinc. However, the molecular basis behind cadmium-induced zinc depletion in *A. baumannii* remains unclear. These results show that cadmium stress results in an inverse dysregulation of copper and zinc homeostasis, which is at least in part consistent with our previous analysis of these metals (16).

The impact of toxic heavy metals on cell membranes is well established (46), but how its modulation aids in resistance is poorly understood. Cadmium stress induced a change in the composition of the *A. baumannii* fatty acids via an overall reduction in double bonds and by extension membrane fluidity. Although regulation of fatty acids biosynthesis is vastly distinct in *S. pneumoniae*, a change in the biophysical properties of the membrane similar to that in *A. baumannii* under cadmium stress was observed (6). The unique means of membrane-mediated resistance to heavy metal stress was also evidenced in the analysis of *Cupriavidus metallidurans* and *Pseudomonas putida*, which identified a role for a fatty acid *cis-trans* isomerase and a cyclopropane synthase (47). These types of enzymes allow rapid biochemical change to facilitate a major biophysical shift but are lacking in most *A. baumannii* strains, including ATCC 17978, and were therefore not identified by TraDIS. However, international clone I isolates, which include AB5075_UW, harbor a genetic locus that encodes a cyclopropane synthase as well as a putative two-component regulatory system for heavy metal sensing. Here, we found that *A. baumannii* ATCC 17978 is likely to employ fatty acid desaturases to combat membrane-mediated cadmium stress, but the link between heavy metal resistance

and membrane hemostasis may be stronger in some clonal lineages, such as those of the international clone I group.

In addition to a link with lipid homeostasis, heavy metals are known to influence antibiotic resistance, including in *Acinetobacter* species (48, 49), but this is not well understood from a genetic perspective. Genomic analyses did not identify any putative antibiotic resistance markers in close proximity to the primary cadmium efflux system, *czcE*, or the cadmium/zinc resistance locus (*czcICBAD*). However, *czcF* was found to be part of a mobile genetic element that also harbors a putative arsenate resistance mechanism (ABUW_3667 to ABUW_3671), which supports the coexistence of distinct antimicrobial resistance mechanisms.

In summary, this study has identified the previously unknown cadmium efflux pathways of *A. baumannii*. These systems are highly conserved across this species and genus; hence, this work is broadly applicable to *Acinetobacter* biology, exemplified by our parallel analysis of two distinct strains. We have garnered new knowledge of environmental stress adaptation strategies, with systems that have unique and shared metal ion specificities. The analyses of cadmium stress across a range of distinct metal ion export systems also provided novel insights into the alignment of metal sensors and efflux systems, which is of major significance to bacterial fitness in a broad range of environments.

MATERIALS AND METHODS

Bacterial strains, chemicals, media, and growth conditions. *Acinetobacter baumannii* AB5075_UW mutant derivatives were purchased from the Manoil laboratory (50), and T26 transposon insertions were confirmed by PCR. The *A. baumannii* ATCC 17978 *czcA* and *czcE* mutants were generated previously in our laboratory (25) (see Tables S1 and S2 for strains and oligonucleotides used in the study, respectively). All chemicals were purchased from Sigma-Aldrich (Australia) unless otherwise indicated.

A. baumannii strains were routinely grown in Luria-Bertani broth (LB) containing 1% tryptone (BD Bacto), 0.5% yeast extract (BD Bacto), and 0.5% sodium chloride. For overnight culturing, a single colony from LB agar (1.5%) was used to inoculate 4 ml of LB medium. Overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.01 in 200 μl LB for growth assays or 20 ml LB for all other analyses. For growth assays, cultures in LB medium were incubated at 37°C with shaking in a FLUOstar Omega spectrophotometer (BMG Labtech), with the OD_{600} values presented. The 20-ml cultures used for all other analyses were incubated at 37°C in an Innova 40R shaking incubator (Eppendorf) at 230 rpm until they reached an OD_{600} of 0.7.

Construction of transposon mutant library. *A. baumannii* ATCC 17978 was used to generate a dense transposon library. The library was constructed using the protocol previously described (51). Briefly, transposomes were prepared using an EZ-Tn*5* transposase (Epicentre Biotechnology) and a custom transposon carrying a kanamycin resistance cassette amplified from the pUT_Km plasmid (52) using the primer sets listed in Table S2. The transposomes (0.25 μl) were electroporated into 60 μl of freshly prepared electrocompetent *A. baumannii* cells using a Bio-Rad GenePulser II (1.8 kV, 25 μF, and 200 Ω) in a 1-mm electrode gap cuvette (Bio-Rad). Cells were recovered by resuspension in 1 ml of SOC (super optimal broth with catabolite repression) medium and incubated at 37°C with shaking (200 rpm) for 2 h. Tn*5* insertion recombinants were selected on Mueller-Hinton (MH) agar (BD Bacto) supplemented with 7 $\mu g \cdot ml^{-1}$ kanamycin. Approximately 12 to 16 transformations were performed and pooled for each batch, with 10,000 to 50,000 resulting transformants. Approximately 250,000 mutants were collected and pooled from a total of 10 batches, snap-frozen, and stored in 20% glycerol at −80°C.

Time-kill assay for the selection of cadmium concentration for mutant library treatment. Approximately 10^9 CFU from an overnight ATCC 17978 culture was subcultured into 10 ml fresh MH broth spiked with different $CdCl_2$ concentrations (0, 3, 60, 80 160, and 320 μM) and incubated at 37°C with shaking. At 0, 1, 2, 4, 5 and 24 h, 100-μl samples were taken and 10-fold serially diluted in sterile phosphate-buffered saline (PBS), and 10 μl of each dilution was spotted on MH agar. Colonies were enumerated to determine the number of surviving cells after overnight incubation at 37°C.

TraDIS of mutant library and data analysis. Approximately 10^9 viable Tn*5* mutant cells were inoculated into 10 ml of MH broth (BD Bacto) and grown at 37°C for 8 h with shaking (200 rpm). A total of 500 μl containing approximately 10^9 cells was seeded into 10 ml fresh MH broth with or without 60 μM $CdCl_2$ in duplicate and grown for 16 h at 37°C with shaking (200 rpm). Genomic DNA was then extracted from approximately 10^{10} cells using the DNeasy UltraClean microbial kit (Qiagen) according to the manufacture's protocol. Sequencing and analysis of the transposon mutant library were performed using the transposon-directed insertion site sequencing (TraDIS) protocol as described previously (53). The primer sets used for PCR amplification of TraDIS fragments (Pf5_PCR) and sequencing (Pf5_Seq) are listed in Table S2. Samples were sequenced on a HiSeq2500 Illumina sequencing platform, generating approximately 2 million 50-bp single-end reads per sample, as previously described (54). TraDIS sequence reads were analyzed using the BioTraDIS pipeline with default parameters (as described in reference 53).

Mutant genome analyses. Genomic DNA of *czcA*, *czcE*, and *cadR* mutants was analyzed to confirm that no mutations other than the target gene were present, using previously published methods (40).

Briefly, DNA was extracted on a QIASymphony SP system with a QIASymphony DSP virus/pathogen kit (Qiagen, Hilden, Germany). DNA concentrations were quantified using a Quant-IT double-stranded-DNA (dsDNA) high-sensitivity kit (Thermo Fisher Scientific, MA, USA). Sequencing libraries were generated using the Illumina Nextera XT library preparation kit (Illumina Inc., CA, USA) following the manufacturer's instructions with minor modifications. Half volume was used for tagmentation reagents, amplification reagents, and input DNA. Library cleanup was performed using an AxyPrep MAG PCR cleanup kit (Corning Inc., NY, USA), and libraries were pooled manually and sequenced on a NextSeq 550 platform with a NextSeq 500/550 midoutput kit v2.5 (300 cycles) (Illumina Inc.). The Snippy pipeline version 4.3.6 was employed for single nucleotide polymorphism (SNP) identification (https://github.com/tseemann/snippy).

Cellular metal ion content analysis. Untreated and metal-stressed bacteria (LB supplemented with 1 μM $CdCl_2$ or 15 μM $CdCl_2$) were harvested at mid-log phase (OD_{600}, 0.7) and washed, by resuspension and centrifugation at 7,000 $\times$ *g* for 8 min, first with PBS containing 5 mM EDTA and then with PBS as described previously (25, 26, 55, 56). Bacterial pellets were desiccated at 95°C overnight, followed by determination of dry cell weight, resuspension of pellets in 35% HNO_3, and boiling at 95°C for 1 h. Samples were diluted to a final concentration of 3.5% HNO_3 and analyzed by inductively coupled plasma mass spectrometry (ICP-MS) on an Agilent Solution 8900 QQQ ICP-MS (Adelaide Microscopy, University of Adelaide).

qRT-PCR. For RNA extraction and qRT-PCR analysis, untreated or metal-stressed bacteria (LB supplemented with 30 μM $CdCl_2$) were harvested when they reached mid-log phase (OD_{600}, 0.7), and cells were lysed in QiaZol (Qiagen) as described previously (57, 58). RNA was extracted and purified using a PureLink RNA minikit (Thermo Fisher Scientific), with on-column DNase I treatment, according to the manufacturer's instructions. qRT-PCR was performed using the SuperScript III one-step RT-PCR kit (Thermo Fisher Scientific) on a QuantStudio 7 Flex System (Thermo Fisher Scientific). Transcription levels were corrected to those obtained for *GAPDH* prior to normalization to the transcription levels observed for untreated *A. baumannii* cultures. Oligonucleotide sequences are listed in Table S2.

Bacterial lipid extraction and gas chromatography-mass spectrometry analysis. Overnight cultures of *A. baumannii* AB5075_UW were diluted to an OD_{600} of 0.05 in fresh LB medium (20 ml) and grown to mid-log phase (OD_{600}, 0.7), with treated cultures supplemented with 15 μM $CdCl_2$, as per the metal accumulation analyses. Cells were harvested by centrifugation at 7,000 $\times$ *g* for 10 min and washed once with PBS; processed pellets were then resuspended in 50 μl of 1.5% NaCl buffer. For lipid extraction, 1 ml of chloroform-methanol (2:1 [vol/vol]) was added to the cell suspension, mixed vigorously for 2 min, and then incubated at room temperature for 10 min. Following the addition of 200 μl 1.5% NaCl, the cell suspension was mixed vigorously for 1 min and centrifuged at 6,000 $\times$ *g* for phase separation. The lower phase was recovered and concentrated via nitrogen evaporation. All samples were stored at −20°C prior to gas chromatography-mass spectrometry (GC-MS) analysis.

To generate fatty acid methyl esters (FAMEs), concentrated lipid samples were resuspended in 1:1 chloroform–trimethylsulfonium hydroxide (TMSOH) and subsequently analyzed using an Agilent 7890A GC system with a 30-m Agilent DB-FastFAME column (Agilent Technologies). Mass spectrometry was completed using a coupled Agilent 5975C MSD system (Agilent Technologies). FAME species were differentiated and determined by comparing to the FAME mix c4-24 standard (Sigma-Aldrich). Data analysis was completed using the Agilent MassHunter Qualitative Navigator software (Agilent Technologies).

Data availability. TraDIS sequence reads were deposited in the European Nucleotide Archive under accession number ERP118051. The data are accessible via BioProject accession number PRJNA746061.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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B.A.E., K.A.H., A.K.C., I.T.P., J.C.P., and C.A.M. designed the study. S.F.A., F.G.A., R.M., K.A.H., N.N.D., K.G., and B.A.E. performed the experiments. B.A.E., F.G.A., S.F.A., K.A.H., A.K.C., R.M., I.T.P., C.A.M., and J.C.P. contributed to the drafting of the manuscript.

REFERENCES

1. Malgieri G, Palmieri M, Esposito S, Maione V, Russo L, Baglivo I, de Paola I, Milardi D, Diana D, Zaccaro L, Pedone PV, Fattorusso R, Isernia C. 2014. Zinc to cadmium replacement in the prokaryotic zinc-finger domain. Metallomics 6:96–104. https://doi.org/10.1039/c3mt00208j.
2. Dietrich N, Tan CH, Cubillas C, Earley BJ, Kornfeld K. 2016. Insights into zinc and cadmium biology in the nematode *Caenorhabditis elegans*. Arch Biochem Biophys 611:120–133. https://doi.org/10.1016/j.abb.2016.05.021.
3. Cuypers A, Plusquin M, Remans T, Jozefczak M, Keunen E, Gielen H, Opdenakker K, Nair AR, Munters E, Artois TJ, Nawrot T, Vangronsveld J, Smeets K. 2010. Cadmium stress: an oxidative challenge. Biometals 23:927–940. https://doi.org/10.1007/s10534-010-9329-x.
4. Ammendola S, Cerasi M, Battistoni A. 2014. Deregulation of transition metals homeostasis is a key feature of cadmium toxicity in Salmonella. Biometals 27:703–714. https://doi.org/10.1007/s10534-014-9763-2.

5. Begg SL, Eijkelkamp BA, Luo Z, Counago RM, Morey JR, Maher MJ, Ong CL, McEwan AG, Kobe B, O'Mara ML, Paton JC, McDevitt CA. 2015. Dysregulation of transition metal ion homeostasis is the molecular basis for cadmium toxicity in *Streptococcus pneumoniae*. Nat Commun 6:6418. https://doi.org/10.1038/ncomms7418.
6. Neville SL, Eijkelkamp BA, Lothian A, Paton JC, Roberts BR, Rosch JW, McDevitt CA. 2020. Cadmium stress dictates central carbon flux and alters membrane composition in *Streptococcus pneumoniae*. Commun Biol 3:694. https://doi.org/10.1038/s42003-020-01417-y.
7. Eijkelkamp BA, Morey JR, Ween MP, Ong CL, McEwan AG, Paton JC, McDevitt CA. 2014. Extracellular zinc competitively inhibits manganese uptake and compromises oxidative stress management in *Streptococcus pneumoniae*. PLoS One 9:e89427. https://doi.org/10.1371/journal.pone.0089427.
8. Wu Z, Zheng R, Liu G, Liu R, Wu S, Sun C. 2021. Calcium protects bacteria against cadmium stress via reducing nitric oxide production and increasing iron acquisition. Environ Microbiol 23:3541–3553. https://doi.org/10.1111/1462-2920.15237.
9. WHO. 2017. WHO publishes list of bacteria for which new antibiotics are urgently needed. WHO Media Centre.
10. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, Pulcini C, Kahlmeter G, Kluytmans J, Carmeli Y, Ouellette M, Outterson K, Patel J, Cavaleri M, Cox EM, Houchens CR, Grayson ML, Hansen P, Singh N, Theuretzbacher U, Magrini N, WHO Pathogens Priority List Working Group. 2018. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis 18:318–327. https://doi.org/10.1016/S1473-3099(17)30753-3.
11. CDC. 2019. 2019 antibiotic resistance threats report. CDC.
12. Cerqueira GM, Peleg AY. 2011. Insights into *Acinetobacter baumannii* pathogenicity. IUBMB Life 63:1055–1060. https://doi.org/10.1002/iub.533.
13. Peleg AY, de Breij A, Adams MD, Cerqueira GM, Mocali S, Galardini M, Nibbering PH, Earl AM, Ward DV, Paterson DL, Seifert H, Dijkshoorn L. 2012. The success of acinetobacter species; genetic, metabolic and virulence attributes. PLoS One 7:e46984. https://doi.org/10.1371/journal.pone.0046984.
14. Adams MD, Goglin K, Molyneaux N, Hujer KM, Lavender H, Jamison JJ, MacDonald IJ, Martin KM, Russo T, Campagnari AA, Hujer AM, Bonomo RA, Gill SR. 2008. Comparative genome sequence analysis of multidrug-resistant *Acinetobacter baumannii*. J Bacteriol 190:8053–8064. https://doi.org/10.1128/JB.00834-08.
15. Farrugia DN, Elbourne LD, Hassan KA, Eijkelkamp BA, Tetu SG, Brown MH, Shah BS, Peleg AY, Mabbutt BC, Paulsen IT. 2013. The complete genome and phenome of a community-acquired *Acinetobacter baumannii*. PLoS One 8:e58628. https://doi.org/10.1371/journal.pone.0058628.
16. Hassan KA, Pederick VG, Elbourne LD, Paulsen IT, Paton JC, McDevitt CA, Eijkelkamp BA. 2017. Zinc stress induces copper depletion in *Acinetobacter baumannii*. BMC Microbiol 17:59. https://doi.org/10.1186/s12866-017-0965-y.
17. Williams CL, Neu HM, Gilbreath JJ, Michel SL, Zurawski DV, Merrell DS. 2016. Copper resistance of the emerging pathogen *Acinetobacter baumannii*. Appl Environ Microbiol 82:6174–6188. https://doi.org/10.1128/AEM.01813-16.
18. Hood MI, Mortensen BL, Moore JL, Zhang Y, Kehl-Fie TE, Sugitani N, Chazin WJ, Caprioli RM, Skaar EP. 2012. Identification of an *Acinetobacter baumannii* zinc acquisition system that facilitates resistance to calprotectin-mediated zinc sequestration. PLoS Pathog 8:e1003068. https://doi.org/10.1371/journal.ppat.1003068.
19. Mortensen BL, Rathi S, Chazin WJ, Skaar EP. 2014. *Acinetobacter baumannii* response to host-mediated zinc limitation requires the transcriptional regulator Zur. J Bacteriol 196:2616–2626. https://doi.org/10.1128/JB.01650-14.
20. Nairn BL, Lonergan ZR, Wang J, Braymer JJ, Zhang Y, Calcutt MW, Lisher JP, Gilston BA, Chazin WJ, de Crecy-Lagard V, Giedroc DP, Skaar EP. 2016. The response of *Acinetobacter baumannii* to zinc starvation. Cell Host Microbe 19:826–836. https://doi.org/10.1016/j.chom.2016.05.007.
21. Gaddy JA, Arivett BA, McConnell MJ, Lopez-Rojas R, Pachon J, Actis LA. 2012. Role of acinetobactin-mediated iron acquisition functions in the interaction of *Acinetobacter baumannii* strain ATCC 19606T with human lung epithelial cells, *Galleria mellonella* caterpillars, and mice. Infect Immun 80:1015–1024. https://doi.org/10.1128/IAI.06279-11.
22. Nwugo CC, Gaddy JA, Zimbler DL, Actis LA. 2011. Deciphering the iron response in *Acinetobacter baumannii*: a proteomics approach. J Proteomics 74:44–58. https://doi.org/10.1016/j.jprot.2010.07.010.
23. Zimbler DL, Penwell WF, Gaddy JA, Menke SM, Tomaras AP, Connerly PL, Actis LA. 2009. Iron acquisition functions expressed by the human pathogen *Acinetobacter baumannii*. Biometals 22:23–32. https://doi.org/10.1007/s10534-008-9202-3.
24. Eijkelkamp BA, Hassan KA, Paulsen IT, Brown MH. 2011. Investigation of the human pathogen *Acinetobacter baumannii* under iron limiting conditions. BMC Genomics 12:126. https://doi.org/10.1186/1471-2164-12-126.
25. Alquethamy SF, Adams FG, Naidu V, Khorvash M, Pederick VG, Zang M, Paton JC, Paulsen IT, Hassan KA, Cain AK, McDevitt CA, Eijkelkamp BA. 2020. The role of zinc efflux during *Acinetobacter baumannii* infection. ACS Infect Dis 6:150–158. https://doi.org/10.1021/acsinfecdis.9b00351.
26. Alquethamy SF, Khorvash M, Pederick VG, Whittall JJ, Paton JC, Paulsen IT, Hassan KA, McDevitt CA, Eijkelkamp BA. 2019. The role of the CopA copper efflux system in *Acinetobacter baumannii* virulence. Int J Mol Sci 20:575. https://doi.org/10.3390/ijms20030575.
27. Williams CL, Neu HM, Alamneh YA, Reddinger RM, Jacobs AC, Singh S, Abu-Taleb R, Michel SLJ, Zurawski DV, Merrell DS. 2020. Characterization of *Acinetobacter baumannii* copper resistance reveals a role in virulence. Front Microbiol 11:16. https://doi.org/10.3389/fmicb.2020.00016.
28. Deb S, Ahmed SF, Basu M. 2013. Metal accumulation in cell wall: a possible mechanism of cadmium resistance by *Pseudomonas stutzeri*. Bull Environ Contam Toxicol 90:323–328. https://doi.org/10.1007/s00128-012-0933-z.
29. Klaassen CD, Liu J, Diwan BA. 2009. Metallothionein protection of cadmium toxicity. Toxicol Appl Pharmacol 238:215–220. https://doi.org/10.1016/j.taap.2009.03.026.
30. Li X, Islam MM, Chen L, Wang L, Zheng X. 2020. Metagenomics-guided discovery of potential bacterial metallothionein genes from the soil microbiome that confer Cu and/or Cd resistance. Appl Environ Microbiol 86:e02907-19. https://doi.org/10.1128/AEM.02907-19.
31. Schirawski J, Hagens W, Fitzgerald GF, Van Sinderen D. 2002. Molecular characterization of cadmium resistance in *Streptococcus thermophilus* strain 4134: an example of lateral gene transfer. Appl Environ Microbiol 68:5508–5516. https://doi.org/10.1128/AEM.68.11.5508-5516.2002.
32. Lu M, Fu D. 2007. Structure of the zinc transporter YiiP. Science 317:1746–1748. https://doi.org/10.1126/science.1143748.
33. Wei Y, Fu D. 2006. Binding and transport of metal ions at the dimer interface of the *Escherichia coli* metal transporter YiiP. J Biol Chem 281:23492–23502. https://doi.org/10.1074/jbc.M602254200.
34. Stahler FN, Odenbreit S, Haas R, Wilrich J, Van Vliet AH, Kusters JG, Kist M, Bereswill S. 2006. The novel *Helicobacter pylori* CznABC metal efflux pump is required for cadmium, zinc, and nickel resistance, urease modulation, and gastric colonization. Infect Immun 74:3845–3852. https://doi.org/10.1128/IAI.02025-05.
35. Udagedara SR, La Porta DM, Spehar C, Purohit G, Hein MJA, Fatmous ME, Casas Garcia GP, Ganio K, McDevitt CA, Maher MJ. 2020. Structural and functional characterizations of the C-terminal domains of CzcD proteins. J Inorg Biochem 208:111087. https://doi.org/10.1016/j.jinorgbio.2020.111087.
36. Eijkelkamp BA, Stroeher UH, Hassan KA, Elbourne LD, Paulsen IT, Brown MH. 2013. H-NS plays a role in expression of *Acinetobacter baumannii* virulence features. Infect Immun 81:2574–2583. https://doi.org/10.1128/IAI.00065-13.
37. Prajapat MK, Saini S. 2012. Interplay between Fur and HNS in controlling virulence gene expression in *Salmonella typhimurium*. Comput Biol Med 42:1133–1140. https://doi.org/10.1016/j.compbiomed.2012.09.005.
38. Troxell B, Sikes ML, Fink RC, Vazquez-Torres A, Jones-Carson J, Hassan HM. 2011. Fur negatively regulates hns and is required for the expression of HilA and virulence in *Salmonella enterica* serovar Typhimurium. J Bacteriol 193:497–505. https://doi.org/10.1128/JB.00942-10.
39. Cerqueira GM, Kostoulias X, Khoo C, Aibinu I, Qu Y, Traven A, Peleg AY. 2014. A global virulence regulator in *Acinetobacter baumannii* and its control of the phenylacetic acid catabolic pathway. J Infect Dis 210:46–55. https://doi.org/10.1093/infdis/jiu024.
40. Adams FG, Pokhrel A, Brazel EB, Semenec L, Li L, Trappetti C, Paton JC, Cain AK, Paulsen IT, Eijkelkamp BA. 2021. *Acinetobacter baumannii* fatty acid desaturases facilitate survival in distinct environments. ACS Infect Dis 7:2221–2228. https://doi.org/10.1021/acsinfecdis.1c00192.
41. Abdollahi S, Rasooli I, Mousavi Gargari SL. 2018. The role of TonB-dependent copper receptor in virulence of *Acinetobacter baumannii*. Infect Genet Evol 60:181–190. https://doi.org/10.1016/j.meegid.2018.03.001.
42. Helbig K, Bleuel C, Krauss GJ, Nies DH. 2008. Glutathione and transition-metal homeostasis in *Escherichia coli*. J Bacteriol 190:5431–5438. https://doi.org/10.1128/JB.00271-08.

43. Helbig K, Grosse C, Nies DH. 2008. Cadmium toxicity in glutathione mutants of *Escherichia coli*. J Bacteriol 190:5439–5454. https://doi.org/10.1128/JB.00272-08.
44. Kim J, Cho Y, Jang IA, Park W. 2015. Molecular mechanism involved in the response to hydrogen peroxide stress in *Acinetobacter oleivorans* DR1. Appl Microbiol Biotechnol 99:10611–10626. https://doi.org/10.1007/s00253-015-6914-5.
45. Juttukonda LJ, Green ER, Lonergan ZR, Heffern MC, Chang CJ, Skaar EP. 2019. *Acinetobacter baumannii* OxyR regulates the transcriptional response to hydrogen peroxide. Infect Immun 87:e00413-18. https://doi.org/10.1128/IAI.00413-18.
46. Garcia JJ, Martinez-Ballarin E, Millan-Plano S, Allue JL, Albendea C, Fuentes L, Escanero JF. 2005. Effects of trace elements on membrane fluidity. J Trace Elem Med Biol 19:19–22. https://doi.org/10.1016/j.jtemb.2005.07.007.
47. Shamim S, Rehman A, Qazi MH. 2014. Cadmium-resistance mechanism in the bacteria *Cupriavidus metallidurans* CH34 and Pseudomonas putida mt2. Arch Environ Contam Toxicol 67:149–157. https://doi.org/10.1007/s00244-014-0009-7.
48. Din G, Farooqi A, Sajjad W, Irfan M, Gul S, Ali Shah A. 2021. Cadmium and antibiotic-resistant *Acinetobacter calcoaceticus* strain STP14 reported from sewage treatment plant. J Basic Microbiol 61:230–240. https://doi.org/10.1002/jobm.202000538.
49. Bazzi W, Abou Fayad AG, Nasser A, Haraoui LP, Dewachi O, Abou-Sitta G, Nguyen VK, Abara A, Karah N, Landecker H, Knapp C, McEvoy MM, Zaman MH, Higgins PG, Matar GM. 2020. Heavy metal toxicity in armed conflicts potentiates AMR in *A. baumannii* by selecting for antibiotic and heavy metal co-resistance mechanisms. Front Microbiol 11:68. https://doi.org/10.3389/fmicb.2020.00068.
50. Gallagher LA, Ramage E, Weiss EJ, Radey M, Hayden HS, Held KG, Huse HK, Zurawski DV, Brittnacher MJ, Manoil C. 2015. Resources for genetic and genomic analysis of emerging pathogen *Acinetobacter baumannii*. J Bacteriol 197:2027–2035. https://doi.org/10.1128/JB.00131-15.
51. Langridge GC, Phan MD, Turner DJ, Perkins TT, Parts L, Haase J, Charles I, Maskell DJ, Peters SE, Dougan G, Wain J, Parkhill J, Turner AK. 2009. Simultaneous assay of every *Salmonella* Typhi gene using one million transposon mutants. Genome Res 19:2308–2316. https://doi.org/10.1101/gr.097097.109.
52. Herrero M, de Lorenzo V, Timmis KN. 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J Bacteriol 172:6557–6567. https://doi.org/10.1128/jb.172.11.6557-6567.1990.
53. Barquist L, Mayho M, Cummins C, Cain AK, Boinett CJ, Page AJ, Langridge GC, Quail MA, Keane JA, Parkhill J. 2016. The TraDIS toolkit: sequencing and analysis for dense transposon mutant libraries. Bioinformatics 32:1109–1111. https://doi.org/10.1093/bioinformatics/btw022.
54. Hassan KA, Cain AK, Huang TTao, Liu Q, Elbourne LDH, Boinett CJ, Brzoska AJ, Li L, Ostrowski M, Nhu NTK, Nhu TDH, Baker S, Parkhill J, Paulsen IT. 2016. Fluorescence-based flow sorting in parallel with transposon insertion site sequencing identifies multidrug efflux systems in *Acinetobacter baumannii*. mBio 7:e01200-16. https://doi.org/10.1128/mBio.01200-16.
55. Pederick VG, Eijkelkamp BA, Begg SL, Ween MP, McAllister LJ, Paton JC, McDevitt CA. 2015. ZnuA and zinc homeostasis in *Pseudomonas aeruginosa*. Sci Rep 5:13139. https://doi.org/10.1038/srep13139.
56. Eijkelkamp BA, Begg SL, Pederick VG, Trapetti C, Gregory MK, Whittall JJ, Paton JC, McDevitt CA. 2018. Arachidonic acid stress impacts Pneumococcal fatty acid homeostasis. Front Microbiol 9:813. https://doi.org/10.3389/fmicb.2018.00813.
57. Hassan KA, Jackson SM, Penesyan A, Patching SG, Tetu SG, Eijkelkamp BA, Brown MH, Henderson PJ, Paulsen IT. 2013. Transcriptomic and biochemical analyses identify a family of chlorhexidine efflux proteins. Proc Natl Acad Sci U S A 110:20254–20259. https://doi.org/10.1073/pnas.1317052110.
58. Giles SK, Stroeher UH, Eijkelkamp BA, Brown MH. 2015. Identification of genes essential for pellicle formation in *Acinetobacter baumannii*. BMC Microbiol 15:116. https://doi.org/10.1186/s12866-015-0440-6.