Acinetobacter baumannii Coordinates Urea Metabolism with Metal Import To Resist Host-Mediated Metal Limitation

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ABSTRACT During infection, bacterial pathogens must adapt to a nutrient metal-limited environment that is imposed by the host. The innate immune protein calprotectin inhibits bacterial growth in vitro by chelating the divalent metal ions zinc (Zn^{2+}, Zn) and manganese (Mn^{2+}, Mn), but pathogenic bacteria are able to cause disease in the presence of this antimicrobial protein in vivo. One such pathogen is Acinetobacter baumannii, a Gram-negative bacterium that causes pneumonia and bloodstream infections that can be complicated by resistance to multiple antibiotics. A. baumannii inhibition by calprotectin is dependent on calprotectin Mn binding, but the mechanisms employed by A. baumannii to overcome Mn limitation have not been identified. This work demonstrates that A. baumannii coordinates transcription of an NRAMP family Mn transporter and a urea carboxylase to resist the antimicrobial activities of calprotectin. This NRAMP family transporter facilitates Mn accumulation and growth of A. baumannii in the presence of calprotectin. A. baumannii is found to utilize urea as a sole nitrogen source, and urea utilization requires the urea carboxylase encoded in an operon with the NRAMP family transporter. Moreover, urea carboxylase activity is essential for calprotectin resistance in A. baumannii. Finally, evidence is provided that this system combats calprotectin in vivo, as deletion of the transporter impairs A. baumannii fitness in a mouse model of pneumonia, and this fitness defect is modulated by the presence of calprotectin. These findings reveal that A. baumannii has evolved mechanisms to subvert host-mediated metal sequestration and they uncover a connection between metal starvation and metabolic stress.

IMPORTANCE Acinetobacter baumannii is a bacterium that causes bloodstream, wound, urinary tract, and pneumonia infections, with a high disease burden in intensive care units. Treatment of A. baumannii infection is complicated by resistance to most antibiotics in use today, and resistance to last-resort therapies has become commonplace. New treatments for A. baumannii infection are desperately needed, but our current understanding of the bacterial factors required to cause infection is limited. We previously found that the abundant innate immune protein calprotectin inhibits the growth of A. baumannii by withholding essential metals. Despite this, A. baumannii is still able to infect wild-type mice, which produce calprotectin during infection. Here, we identify factors employed by A. baumannii during infection to overcome calprotectin-mediated metal sequestration. Moreover, we expose a connection between metal starvation and metabolism that may be a “chink in the armor” of A. baumannii and lead to new treatment options.
Calprotectin is important for host defense against *A. baumannii* in the lung. Calprotectin comprises 45% of the cytoplasmic protein in neutrophils (10), and *A. baumannii* infection of the murine lung leads to robust recruitment of neutrophils, which are necessary for bacterial clearance (11). Neutrophil recruitment causes a dramatic accumulation of calprotectin that colocalizes with sites of lobar inflammation and *A. baumannii* colonization (12). Calprotectin-deficient mice have increased bacterial burdens and mortality from *A. baumannii* pneumonia (13). Finally, recombinant calprotectin inhibits *A. baumannii* growth *in vitro*, and this is dependent on an intact hexahistidine Mn binding site within calprotectin; this finding suggests that *A. baumannii* requires Mn for full fitness (8, 13).

Mn is an essential cofactor for life and is predominantly utilized as a redox-active cofactor for enzymes, including superoxide dismutase and ribonucleotide reductase (14). Several families of Mn transporters have been identified in bacteria. The most widely conserved of these are Mn ATP binding cassette (ABC) transporters and the natural resistance-associated macrophage protein (NRAMP) family of Mn transporters, which are important for the virulence of many bacterial pathogens (14). NRAMP family transporters are transmembrane proteins that utilize the proton motive force as an energy source for transport (14). For the pathogen *Staphylococcus aureus*, both an NRAMP family transporter and an ABC family Mn transporter are important for bacterial resistance to calprotectin (15). To date, no Mn transporters have been characterized in *A. baumannii*.

Calprotectin-mediated Mn deprivation restricts *A. baumannii* growth, presumably because Mn-dependent bacterial processes are rendered inactive without their cognate cofactor. However, exactly which bacterial processes are inhibited and how the bacterium responds to these alterations in physiology remain unknown. For instance, multiple metabolic enzymes involved in carbon metabolism, including phosphoglyceromutase (16) and pyruvate carboxylase (17), require Mn or are activated by Mn, but whether central metabolic processes are altered by calprotectin-mediated Mn sequestration is unclear. We hypothesized that understanding the effects of calprotectin exposure on *A. baumannii* physiology *in vitro* may uncover bacterial processes essential for infection in niches where calprotectin is abundant.

The overall goal of this study was to identify mechanisms by which *A. baumannii* overcomes calprotectin-based nutritional immunity. An operon that contains a putative Mn transporter and urea catabolism enzymes from the urea amidolyase family was identified. Based on transcriptional regulation, we hypothesized that urea amidolyase is a component of the *A. baumannii* response to calprotectin-mediated Mn sequestration. Mn transport was demonstrated to be important for growth in the presence of calprotectin and colonization of the murine lung. Urea catabolism was found to be vital for growth in the presence of calprotectin and functionally linked to Mn acquisition in *A. baumannii*. Taken together, these results uncover that host-mediated metal sequestration restricts metabolism in bacterial pathogens, and they broaden the understanding of the bacterial factors required to survive this restriction.

**RESULTS**

*A. baumannii* encodes an NRAMP family transporter that mediates resistance to calprotectin. We hypothesized that *A. baumannii* is able to overcome calprotectin-mediated Mn chelation by utilizing a metal transporter system that has high affinity for Mn. To identify predicted Mn transporters in *A. baumannii*, the KEGG database (18, 19) was searched for Mn transporter orthologs in the *A. baumannii* ATCC 17978 genome. This search identified only one gene encoding a protein with similarity to NRAMP or ABC family Mn transporters, the gene *A1S_1266*. *A1S_1266* encodes a potential NRAMP family member. NRAMP family members are integral membrane proteins that transport divalent cations, often having specificity for Mn(II) (14). *A1S_1266* is in a predicted operon containing genes that catabolize urea to ammonia, which we named the manganese and urea metabolism (*mum*) operon (Fig. 1A). As *A1S_1266* is predicted to encode a transporter, this gene was named *mumT* (Fig. 1A).

We hypothesized that *mumT* encodes an Mn importer that is important for growth under Mn-restricted conditions, such as upon exposure to calprotectin. Consistent with this, normalized *mumT* transcript abundance increased upon calprotectin treatment (Fig. 1B). Genetic deletion of *mumT* delayed *A. baumannii* growth in the presence of recombinant calprotectin, as did deletion of the Zn import gene *znuB* (Fig. 1C and D). This growth lag could be complemented by the expression of *mumT* from a plasmid or by the addition of excess Mn (see Fig. S1A to C in the supplemental material). Importantly, growth inhibition of a Δ*znuB* mutant in the presence of calprotectin was only fully rescued by the addition of excess Zn and was not rescued by excess Mn alone (20). We hypothesized that if MumT preferentially imports Mn, loss of *mumT* would increase resistance to toxic levels of Mn but not other divalent cations. In support of this, the Δ*mumT* mutant was able to grow in 3 mM Mn, which is highly toxic to wild-type *A. baumannii* (Fig. 1E and F), whereas the Δ*mumT* mutant was more sensitive than wild-type *A. baumannii* to Fe toxicity and had similar sensitivity to Zn toxicity (see Fig. S1D and E). Finally, to determine whether *mumT* is required for Mn acquisition, cellular Mn concentrations were measured by inductively coupled plasma mass spectrometry (ICP-MS) (Fig. 1G). The Δ*mumT* strain had lower Mn levels in cell pellets than did wild-type *A. baumannii*, and this defect was complemented by expression of *mumT* in trans. In contrast, cellular Zn and Fe levels in the Δ*mumT* mutant were not significantly different than the wild-type *A. baumannii* levels (see Fig. S1F). Together, these results demonstrate that *A. baumannii* *mumT* is important for growth under Mn-restricted conditions and for accumulation of cellular Mn.

*mumT* upregulation in the presence of calprotectin requires the LysR family transcriptional regulator MumR. *mumT* is present immediately downstream from a gene that encodes a predicted LysR family transcriptional regulator, *mumR* (Fig. 1A). We hypothesized that *mumR* is required for transcriptional control of *mumT*. To understand the contributions of *mumR* to *mumT* regulation, a strain containing an in-frame deletion of *mumR* was generated. The abundance of *mumT* transcript was significantly decreased in the Δ*mumR* strain relative to that with wild-type *A. baumannii* (Fig. 2A). This suggested that MumR activates *mumT* expression. To confirm this finding, activity of the *mumT* promoter was investigated using a reporter system. The *mumT* promoter was cloned into a plasmid harboring the Photobacterium luminescens luciferase operon, *luxABCDE*, such that activity of the *mumT* promoter resulted in luminescence. This vector was transformed into wild-type *A. baumannii* and Δ*mumR* strain cells, and luminescence was measured under various growth conditions. In
rich medium, the mumT promoter was active in wild-type A.baumannii but not in the ΔmumR strain (Fig. 2B). When incubated with 125 μg/ml calprotectin, the activity of the mumT promoter was significantly enhanced in wild-type A. baumannii, and this was dependent on mumR. This result demonstrated that calprotectin exposure induces expression from the mumT promoter.

mumT is an operon with mumC, which encodes a urea carboxylase that contributes to A. baumannii urea utilization. Based on the small intergenic distances between the open reading frames (ORFs) for mumT (A1S_1266) and mumC (A1S_1270), we predicted that these genes constitute an operon. This was confirmed by performing PCR on cDNA prepared using RNA isolated from wild-type A. baumannii (see Fig. S2A in the supplemental material). PCR products were amplified across adjacent ORFs for mumT, mumL, mumU, mumH, and mumC, and no products were amplified for primers designed to amplify the mumR-mumT or mumC-A1S_1271 junctions, demonstrating that mumTLUHC form an operon.

Next, we sought to identify the functions of members of the mum operon. MumH is homologous to allophanate hydrolase, and MumC is a putative member of the biotin carboxylase family. In fungi, the enzyme urea amidolyase contains urea carboxylase and allophanate hydrolase domains. Urea amidolyase converts urea first to allophanate via biotin-mediated carboxylation (urea carboxylase domain) and then converts allophanate to carbon dioxide and ammonia via hydrolysis (allophanate hydrolase domain) (21). Because mumH and mumC are adjacent ORFs, we posited that MumC is a biotin-dependent urea carboxylase and
MumH and MumC mediate urea degradation (Fig. 3A). Urea amidolyase enzymatic activity has been characterized in enzymes cloned from *Oleomonas sagaranensis* (22, 23), but the roles of urea amidolyase in bacterial physiology and pathogenesis remain unknown.

To evaluate the importance of each *mum* gene in urea degradation, in-frame deletions of *mumL*, *mumU*, *mumH*, and *mumC* were generated. Inactivation of *mumC*, but not of *mumH*, impaired growth in the presence of toxic levels of urea (Fig. 3B and C) but not in LB alone (see Fig. S2B in the supplemental material). While *A. baumannii* cannot grow using urea as a sole carbon source (data not shown), *A. baumannii* can utilize urea as a sole nitrogen source in a manner dependent on the presence of *mumC* (Fig. 3D and E). Importantly, the *ΔmumH* mutant was capable of utilizing urea as a sole nitrogen source (Fig. 3E), which indicated that the single ammonia molecule produced by MumC-mediated urea carboxylation (Fig. 3A) is sufficient to support *A. baumannii* growth as a sole nitrogen source.

Urea carboxylase activity in *Oleomonas sagaranensis* and *Candida utilis* requires either Mg$^{2+}$, Mn$^{2+}$, or Co$^{2+}$ (23, 24), suggesting that *A. baumannii* utilization of urea by MumC may require Mn$^{2+}$. To address this, we chelated Mn by the addition of calprotectin to medium containing urea as the sole nitrogen source. The addition of calprotectin was sufficient to substantially impair growth in urea (Fig. 3F and G). Importantly, growth was not inhibited when a variant of calprotectin (∆S1) that is unable to bind Mn (8) was added to the medium. Consistent with these findings, *mumT* inactivation decreased growth in high concentrations of urea, which was complemented by the expression of *mumT* in *trans*, demonstrating that the MumT Mn transporter is important to enable urea degradation (see Fig. S2C and D in the supplemental material). Together, these results demonstrated that *mumC* is vital for catabolism of urea in *A. baumannii* and suggest that urea catabolism may be altered by Mn availability.

*mumC* is important for *A. baumannii* growth in calprotectin. The entire *mum* operon is upregulated following exposure to calprotectin (Fig. 4A). Based on this observation, we hypothesized that additional *mum* genes may be important for growth in calprotectin. The sensitivities of *A. baumannii* *ΔmumL*, *ΔmumU*, *ΔmumH*, and *ΔmumC* mutant strains to calprotectin growth inhibition were evaluated (Fig. 4B). The strains lacking *mumL* and *mumU* exhibited sensitivity to calprotectin similar to that of wild-type *A. baumannii* (Fig. 4C; see also Fig. S3A in the supplemental material). This demonstrated that *mumL* and *mumU* are not important for growth in the presence of calprotectin. In contrast, the *ΔmumH* and *ΔmumC* mutants, strains harboring in-frame deletions of allophosphate hydrolase and urea carboxylase, respectively, had increased sensitivity to calprotectin (Fig. 4B and C). The *ΔmumC* mutant did not exhibit decreased transcription of *mumT* (see Fig. S3B). The growth deficit of the *ΔmumC* mutant in calprotectin could be complemented by the addition of exogenous Mn to the medium (see Fig. S1C in the supplemental material) or by providing a copy of *mumC* in *trans* (see Fig. S3C and D). Importantly, the *ΔmumC* strain did not exhibit resistance to Mn toxicity (see Fig. S3E), suggesting that its role in calprotectin resistance is not related to Mn transport. These data indicate that urea catabolism via *mumH* and *mumC* is important for *A. baumannii* resistance to calprotectin.

*mumT* contributes to the fitness of *A. baumannii* in a murine pneumonia model. To investigate the contribution of *mumT* to *A. baumannii* fitness in the murine lung, C57BL/6 mice were inoculated intranasally with a 1:1 mixture of wild-type *A. baumannii* and the *ΔmumT* mutant. After 36 h, bacterial burdens were quantified from the lungs and the liver, a site of systemic dissemination (Fig. 5A and B). Strain *ΔmumT* burdens were significantly lower than wild-type burdens in both the lungs and the livers of C57BL/6 mice, indicating that *mumT* contributes to fitness in this infection model. To define the role of calprotectin in the fitness defect of the *ΔmumT* strain, calprotectin-deficient mice (*S100A9−/−*) were also coinfected with wild-type *A. baumannii* and the *ΔmumT* strain. As in C57BL/6 mice, *ΔmumT* strain burdens were significantly lower than those in the wild type in the lungs of calprotectin-deficient mice. However, the fitness defect of the *ΔmumT* strain in the liver was completely rescued in the absence of calprotectin. These findings indicate that calprotectin is vital for limiting *A. baumannii* *ΔmumT* strain dissemination to the liver. To elucidate whether the differential rescue of the *ΔmumT* mutant in the liver and the lung of calprotectin-deficient mice correlates with Mn concentrations, *A. baumannii*-infected livers and lungs were subjected to ICP-MS analysis. Mn abundance in the liver was over 25-fold greater than Mn abundance in the lung (Fig. 5C). The high level of Mn in the liver correlated with increased fitness of the *ΔmumT* strain in the liver relative to that in the lung in calprotectin-deficient mice; this indicated that the liver of a calprotectin-deficient mouse is Mn replete. The result indicating that the *ΔmumT* strain is attenuated in the livers of wild-type mice suggests that calprotectin is sufficient to Mn starve *A. baumannii* even in the Mn-abundant liver. Taken as a whole, these data reveal that *mumT* is important for *A. baumannii* fitness during infection and demonstrate that calprotectin is important for preventing *ΔmumT* strain dissemination to the murine liver.

The *mum* system is broadly conserved across bacteria. Calprotectin has antimicrobial activity against numerous pathogens (8). Because of the importance of the *mum* operon in *A. bauman-
**FIG 3** The mum operon contributes to *A. baumannii* urea utilization. (A) Predicted reactions catalyzed by urea carboxylase MumC and allophanate hydrolase MumH. Urea is converted to allophanate via biotin-mediated carboxylation of urea by urea carboxylase MumC in a reaction that requires Mg or Mn. Allophanate is hydrolyzed by allophanate hydrolase MumH to carbon dioxide and ammonia. (B) Growth of wild-type *A. baumannii* (WT) and ΔmumT, ΔmumL, ΔmumU, ΔmumH, and ΔmumC mutant strains in 700 mM urea over time. (C) Growth of wild-type *A. baumannii* and ΔmumT, ΔmumL, ΔmumU, ΔmumH, and ΔmumC mutant strains in 700 mM urea, relative to growth in LB alone at 24 h. For experiments shown in panels B and C, data are combined from 3 or more independent experiments performed in technical duplicate. Significance was calculated using one-way analysis of variance for each condition, comparing each strain to the wild-type control with Dunnett’s multiple-comparisons test. (D) Growth of wild-type *A. baumannii* and the ΔmumC mutant with 8 mM urea as the sole nitrogen source. Wild-type cells grown with no nitrogen source were included as a negative control. (E) Utilization of 8 mM urea as a nitrogen source by wild-type *A. baumannii* and the ΔmumC mutant strains. The OD₆₀₀ at 9 h is shown. Data in panels D and E are from a single experiment performed in biological duplicate. Data are representative of 5 independent experiments. Significance was calculated using one-way analysis of variance for each condition, comparing each strain to the wild-type control with Dunnett’s multiple-comparisons test. (F) Wild-type *A. baumannii* growth with urea as primary nitrogen source in the presence of 780 μg/ml calprotectin or calprotectin ΔS1, a variant of calprotectin unable to bind Mn. For experiments F and G, data are from a single experiment performed in biological triplicate and are representative of 4 independent experiments. Significance was calculated using one-way analysis of variance for each condition, comparing each strain to the wild-type control with Dunnett’s multiple-comparisons test. ***, *P* < 0.001; ****, *P* < 0.0001.

The conservation of this system was investigated (Fig. 6). The mum operon was present in all *A. baumannii* strains queried and other *Acinetobacter* species. A mumR homolog was present adjacent to the mum operon in all *Acinetobacter* species interrogated but was not present outside the *Acinetobacter* genus, suggesting that *Acinetobacter* has evolved with a unique regulatory mechanism for this operon. The mum operon is present with at least four of the five genes retained in some other *Gammaproteobacteria*, including the urinary pathogen *Proteus mirabilis*, and more distantly related *Proteobacteria*, including *Agrobacterium tumefaciens*. Portions of the mum operon, including NRAMP family transporters and allophanate hydrolase genes, are also present in diverse bacterial phyla, including *Actinobacteria* and *Firmicutes*. Importantly, portions of the mum operon are present in diverse bacterial pathogens, including *S. aureus* and *Neisseria meningitidis*. These observations indicate that the mum
operon is broadly conserved across bacteria but not in other domains of life.

**DISCUSSION**

*A. baumannii* colonization of the murine lung generates a robust immune response, which ultimately results in copious amounts of calprotectin being present at the host-pathogen interface (12). Here, we demonstrated that the *mum* operon responds to calprotectin and contributes to *A. baumannii* calprotectin resistance (Fig. 7). MumT was established as a Mn transporter, the unique transcriptional regulation of *mumT* by *mumR* was determined, and a link between *mumT* and urea catabolism via *mumC* was identified. Additionally, urea catabolism was identified as the first metabolic pathway linked to calprotectin resistance, an important step in identifying the mechanisms by which calprotectin disrupts bacterial physiology and inhibits bacterial growth during infection. Finally, the *mum* system was demonstrated to be important for *A. baumannii* fitness in the murine lung and liver, and calprotectin was found to be required by Mn-starved *A. baumannii* in the liver.

*mumT* encodes an NRAMP family homolog. *mumT* is unique compared to previously identified NRAMPs, as MumT shares low sequence homology with reported NRAMPs (<25%), and NRAMPs are typically monocistronic (25). Inactivation of the *S. aureus* NRAMP family member MntH increases sensitivity to calprotectin, although a second Mn transporter (MntABC) must be deleted to see a dramatic growth difference in the presence of calprotectin (15). Similar to this finding, deletion of *mumT* delays *A. baumannii* growth in the presence of calprotectin, and this growth difference is reversed by the addition of excess Mn to the medium. In contrast to results obtained with *S. aureus*, *A. baumannii* growth is significantly decreased by inactivation of *mumT* alone, suggesting that *A. baumannii* may not encode another high-affinity Mn import system. Previously reported NRAMP family transporters have varied specificities for Mn, Fe, and other divalent cations (26–31). The metal specificity of MumT was evalu-
uated by determining sensitivity to toxic levels of Mn, Fe, and Zn. The \( \Delta \text{mum}T \) strain was less sensitive than wild-type \( A. \text{baumannii} \) to Mn toxicity. This is consistent with the decreased ability of this strain to accumulate Mn, as measured by ICP-MS. Together, the increased sensitivity of the \( \Delta \text{mum}T \) mutant to calprotectin and decreased sensitivity of the \( \Delta \text{mum}T \) mutant to toxicity of Mn strongly suggest that \( \text{mum}T \) encodes a transporter that imports Mn. Interestingly, the \( \Delta \text{mum}T \) strain had increased sensitivity to Mn.

**FIG 6** The \( \text{mum} \) system is broadly conserved across bacteria. Genetic alignments of representative organisms that are predicted to contain orthologs of the \( \text{mum} \) system in adjacent loci. Eleven organisms are depicted of the 88 total organisms identified in the SEED database. The numbers underneath each gene correspond to amino acid similarity, based on Clustal W2 alignment to the representative \( A. \text{baumannii} \) genes. Genes shown in light grey with no outline are not part of the \( A. \text{baumannii} \) \( \text{mum} \) operon.

**FIG 7** Model for the \( \text{mum} \) system response to calprotectin-mediated manganese sequestration. Calprotectin induces \( A. \text{baumannii} \) Mn starvation and an adaptive response. \( \text{mum} \) activation is orchestrated by the transcriptional regulator, MumR, which enhances \( \text{mum} \) expression. \( \text{mum}T \) encodes an NRAMP family transporter, MumT, that increases cellular Mn content by facilitating Mn import into the cytoplasm. MumT increases \( A. \text{baumannii} \) fitness in the lung, presumably by providing Mn for important intracellular processes. \( \text{mum}C \) encodes a urea carboxylase enzyme, MumC, that likely utilizes Mn as cofactor. MumC catabolizes urea to ammonia and carbon dioxide, enables the use of urea as a sole nitrogen source, and provides resistance to calprotectin.
Fe toxicity. We hypothesize that the enhanced sensitivity of the \( \Delta \text{mumT} \) strain to Fe toxicity stems from disruption of the Mn:Fe ratio. This is consistent with the finding that the Mn:Fe ratio is important for Neisseria meningitidis to survive metal toxicity (32).

The \( \Delta \text{mumT} \) mutant was less fit than wild-type \( A. \text{baumannii} \) for colonizing the lung and dissemination to the liver. Of note, the \( \Delta \text{mumT} \) strain was not attenuated in dissemination to the livers of mice lacking calprotectin, consistent with the model showing that calprotectin is required to starve \( A. \text{baumannii} \) of Mn and prevents colonization of the liver. \( S. \text{aureus} \) inactivated for Mn transporters \( \text{mntH} \) and \( \text{mntT} \) is also attenuated in the livers of wild-type but not calprotectin-deficient mice (15). Similarly, \( A. \text{baumannii} \) inactivated for the Zn transporter \( \text{znuABC} \) is significantly attenuated in the livers of wild-type mice but not significantly attenuated in calprotectin-deficient mice (13). Together, these findings implicate calprotectin metal sequestration as particularly important in host defense of the liver relative to other organs. The heightened efficacy of calprotectin in the liver may be because the liver, the site of Mn absorption into the systemic circulation and Mn excretion into bile, is the most Mn-replete organ in the body (33). The concentrations of Mn in the \( A. \text{baumannii} \)-infected liver are approximately 25-fold higher than Mn concentrations in the \( A. \text{baumannii} \)-infected lung. In this setting of excess Mn, the \( \Delta \text{mumT} \) mutant appeared to be capable of importing sufficient Mn through other transport systems, unless calprotectin was present to sequester Mn. The \( \Delta \text{mumT} \) strain was less fit than wild-type \( A. \text{baumannii} \) in the lungs of mice lacking calprotectin, suggesting that additional stresses beyond calprotectin exist in the lung that decrease the fitness of this mutant strain.

Unlike other reported NRAMP family transporters, \( \text{mumT} \) is in an operon. The other genes in this operon were not previously described in \( A. \text{baumannii} \) and lacked an obvious link to Mn homeostasis. A predicted function was not identified for \( \text{mumU} \) or \( \text{mnt} \), but based on sequence homology, \( \text{mumH} \) and \( \text{mumC} \) are predicted to encode allophanate hydrolase and urea carboxylase, enzymes that catalyze the biotin- and ATP-dependent two-step catabolism of urea to ammonia and carbon dioxide. Homologs of these genes in \( O. \text{sagaranensis} \) have been cloned and their enzymatic activities verified (22, 23); however, allophanate hydrolase in \( P. \text{fluorescens} \) functions in cyanuric acid metabolism, not urea metabolism (34). Therefore, the physiological role of these enzymes in bacteria can vary. \( A. \text{baumannii} \) utilized urea as a nitrogen source but not a carbon source, and urea nitrogen utilization depends on \( \text{mumC} \). These results demonstrate that urea carboxylase has a physiological role in urea catabolism in this bacterium.

Since \( \text{mumC} \) is in an operon with \( \text{mumT} \), we investigated whether \( \text{mumC} \) requires \( \text{mumT} \)-delivered Mn for activity. Previous reports suggested that urea carboxylase activity requires divalent cations (23, 24). A strain inactivated for \( \text{mumT} \) was impaired for growth in urea as a sole nitrogen source and in the presence of toxic levels of urea. Furthermore, this effect was specific to inactivation of \( \text{mumT} \) and does not occur when other genes in the operon are inactivated. Therefore, this finding suggests that \( \text{mumC} \)-mediated urea catabolism is Mn dependent.

It is well established that calprotectin inhibits bacterial growth in vitro and hampers the growth of some bacteria during infection (35). Ostensibly, calprotectin inhibits growth by suppressing metal-dependent bacterial processes. However, it is unclear what specific bacterial processes are inhibited and how this affects bacterial physiology; currently, the only bacterial process known to be inhibited by calprotectin is \( S. \text{aureus} \) superoxide dismutase activity (36). Because evolutionary conservation of genomic organization can suggest similar function, we hypothesized that other genes in the \( \text{mum} \) operon may be important for resistance to calprotectin. In keeping with this, inactivation of \( \text{mumC} \) significantly decreased growth in the presence of calprotectin. This result demonstrated that urea degradation increases the ability of \( A. \text{baumannii} \) to combat calprotectin metal limitation. Furthermore, calprotectin, but not calprotectin lacking the ability to tightly bind Mn, completely inhibited growth of \( A. \text{baumannii} \) utilizing urea as a sole nitrogen source. One interpretation of these results is that urea degradation is a Mn-, Zn-, or Fe-dependent process that is inhibited by calprotectin.

The question remains: why is urea degradation important for growth in the presence of calprotectin? Urea is generated as a by-product of metabolism in rich medium, and calprotectin-mediated metal starvation may cause a metabolic strain by inhibiting metal-dependent metabolic processes. This could lead to a buildup of urea that requires \( \text{mumC} \)-mediated breakdown. Future work to query this hypothesis will also help define metabolic pathways in \( A. \text{baumannii} \). Alternatively, urea and/or ammonia could serve as a signaling molecule within the bacterial cell. Our findings emphasize the importance of improving the understanding of \( A. \text{baumannii} \) metabolism and the role of metabolism in \( A. \text{baumannii} \) virulence. In this regard, we report that calprotectin-mediated metal starvation and urea catabolism are linked in \( A. \text{baumannii} \).

The finding that urea carboxylase is important for defense against the antimicrobial protein calprotectin in vitro extends the known role of urea in microbial pathogenesis. There are two described pathways for catabolizing urea in bacteria: urease and urea amidolyase (37). Urease is a key virulence factor for Helicobacter pylori, as it is required for local alkalinization and chemotaxis in the stomach (38–40). \( P. \text{mirabilis} \) also utilizes urea as a virulence factor in the bladder, the site of host urea excretion; urease activity of \( P. \text{mirabilis} \) alters the pH and causes calculus formation in urine (41). Urease is also required for virulence of the fungal pathogens Cryptococcus neoformans (42) and Coccioidoides posadasii (43). The only reported virulence role for urea amidolyase systems is for \( C. \text{albicans} \), which uses urea-produced ammonia to regulate pH and induce the yeast-to-hypha transition; this system is important for escape from macrophages and colonization of the kidney (44, 45). The present study indicates that urea amidolyase systems are also important for defense against the human antimicrobial protein calprotectin.

The \( \text{mum} \) operon is conserved across many, but not all, bacteria. The \( \text{mum} \) operon is present in many nonpathogenic organisms, including Acinetobacter baylyi, suggesting this operon did not evolve exclusively as a virulence factor. However, it is present in many pathogens, including \( S. \text{aureus} \). Therefore, a better understanding of the genes within this operon may reveal drug targets for the treatment of multidrug-resistant infections.

**MATERIALS AND METHODS**

**Bacterial strains and reagents.** The strains used in this study are described in Table S1 in the supplemental material. All strains are derivatives of the human clinical isolate \( A. \text{baumannii} \) ATCC 17978. Cloning was performed in Escherichia coli DH5α. Bacteria were routinely grown in lysogenic broth (LB) at 37°C unless otherwise noted. Solid medium contained 1.5% agar. Antibiotics were added at the following concentrations for
A. baumannii and E. coli, respectively; 500 μg ml⁻¹ and 100 μg ml⁻¹ ampicillin, 40 μg ml⁻¹ kanamycin, 10 μg ml⁻¹, and 5 μg ml⁻¹ tetracycline. All antibiotics were purchased from Sigma (St. Louis, MO). In-frame deletion strains (ΔmumR, ΔmumT, ΔmumL, ΔmumU, ΔmumH, and ΔmumC mutant strains) were generated via homologous recombination utilizing the suicide plasmid pFLP2 and screened by PCR and Southern blotting as previously described (46). Some constructs were generated by ligating the stitched PCR product directly into pFLP2 (for the ΔmumR and ΔmumT mutants) or by using Gibson recombineering (for the ΔmumL, ΔmumU, ΔmumH, and ΔmumC mutants) (New England BioLabs, Ipswich, MA). Primers used to generate in-frame deletion strains, complementation plasmids, and reporter plasmids are listed in Table S2 in the supplemental material. Complementation vectors for the ΔmumT and ΔmumC strains were constructed in pWH1266 under control of the 16S promoter (rpo1) as previously described (46), except that complementation vectors did not include a c-Myc tag and the mumC complementation vector was cloned between EcoRV and BamHI sites. p.

All antibiotics were purchased from Sigma (St. Louis, MO). Inoculation with 1-tetracycline. Recombinant bacterial cultures were then transferred to preweighed metal-free 15-ml conical tubes (VWR, Radnor, PA). Pellets were harvested by centrifugation, washed twice with Milli-Q deionized water, and dried. The pellet weight was then recorded using an analytical balance (Mettler-Toledo, Columbus, OH). Pellets were digested with 1 ml 50% HNO₃ (optima-grade metal-free; Fisher, Waltham, MA) at 50°C overnight, diluted with 9 ml Milli-Q deionized water, and subcultured 1:50 in LB containing 500 μg ml⁻¹ ampicillin. Bacteria were subcultured 1:50 for 1 h, and then cultures were diluted 1:100 to 10 ml of 60% LB–40% calprotectin buffer containing 500 μg ml⁻¹ ampicillin and grown for 8 h. Bacterial cultures were then transferred to preweighed metal-free 15-ml conical tubes (VWR, Radnor, PA). Pellets were harvested by centrifugation, washed twice with Milli-Q deionized water, and dried. The pellet weight was then recorded using an analytical balance (Mettler-Toledo, Columbus, OH). Pellets were digested with 1 ml 50% HNO₃ (optima-grade metal-free; Fisher, Waltham, MA) at 50°C overnight, diluted with 9 ml Milli-Q deionized water, weighed using an analytical balance, and subjected to mass spectrometry. Whole organs from A. baumannii-infected mice were homogenized in 1 ml PBS and digested in 2 ml HNO₃ and 500 μl H₂O₂ (optima-grade metal-free; Fisher, Waltham, MA) at 90°C overnight in metal-free Teflon jars for digestion. Digested samples were then diluted with 9 ml Milli-Q deionized water and submitted for ICP-MS analysis at the Vanderbilt Mass Spectrometry Research Center. Levels of 65Zn, 55Mn, and 56Fe were measured, concentrations were determined by utilizing a standard curve for each metal, and results were normalized by dilution factor.

Determining the conservation of the mum operon in silico. The “compare region” feature of the SEED viewer (51) was used to identify genomic regions similar to the mum operon, with mumT set as the focus gene. From the 58 Archaea, 962 Bacteria, and 562 Eukarya genomes in the SEED database at the time of query, 88 were found to include sets of genes with similar sequences. Genomic regions from 11 organisms were selected for protein alignments. Protein sequences were downloaded from the SEED database and aligned by using ClustalW2 (52, 53) for comparison to the 17978 homolog.
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