Calprotectin-Mediated Zinc Chelation Inhibits Pseudomonas aeruginosa Protease Activity in Cystic Fibrosis Sputum

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ABSTRACT

Pseudomonas aeruginosa induces pathways indicative of low zinc availability in the cystic fibrosis (CF) lung environment. To learn more about P. aeruginosa zinc access in CF, we grew P. aeruginosa strain PAO1 directly in expectorated CF sputum. The P. aeruginosa Zur transcriptional repressor controls the response to low intracellular zinc, and we used the NanoString methodology to monitor levels of Zur-regulated transcripts, including those encoding a zincophore system, a zinc importer, and paralogs of zinc containing proteins that do not require zinc for activity. Zur-controlled transcripts were induced in sputum-grown P. aeruginosa compared to those grown in control cultures but not if the sputum was amended with zinc. Amendment of sputum with ferrous iron did not reduce expression of Zur-regulated genes. A reporter fusion to a Zur-regulated promoter had variable activity in P. aeruginosa grown in sputum from different donors, and this variation inversely correlated with sputum zinc concentrations. Recombinant human calprotectin (CP), a divalent-metal binding protein released by neutrophils, was sufficient to induce a zinc starvation response in P. aeruginosa grown in laboratory medium or zinc-amended CF sputum, indicating that CP is functional in the sputum environment. Zinc metalloproteases comprise a large fraction of secreted zinc-binding P. aeruginosa proteins. Here, we show that recombinant CP inhibited both LasB-mediated casein degradation and LasA-mediated lysis of Staphylococcus aureus, which was reversible with added zinc. These studies reveal the potential for CP-mediated zinc chelation to posttranslationally inhibit zinc metalloprotease activity and thereby affect the protease-dependent physiology and/or virulence of P. aeruginosa in the CF lung environment.

IMPORTANCE

The factors that contribute to worse outcomes in individuals with cystic fibrosis (CF) with chronic Pseudomonas aeruginosa infections are not well understood. Therefore, there is a need to understand environmental factors within the CF airway that contribute to P. aeruginosa colonization and infection. We demonstrate that growing bacteria in CF sputum induces a zinc starvation response that inversely correlates with sputum zinc levels. Additionally, both calprotectin and a chemical zinc chelator inhibit the proteolytic activities of LasA and LasB proteases, suggesting that extracellular zinc chelators can influence proteolytic activity and thus P. aeruginosa virulence and nutrient acquisition in vivo.

KEYWORDS

Pseudomonas aeruginosa, calprotectin, cystic fibrosis, proteases, zinc
However, studies investigating the transcriptional response of *P. aeruginosa* in CF sputum show that a common gene expression pattern is the increased expression of zinc uptake and transport genes (4–9), which are normally expressed when zinc is limited. The *P. aeruginosa* zinc starvation response is regulated by the zinc uptake regulator (Zur), which is a transcriptional repressor (10). When intracellular zinc is high, Zur monomers bind zinc, dimerize, and bind DNA to repress gene expression of zinc uptake pathways. When intracellular zinc becomes low, the dimeric, zinc-bound fraction of Zur decreases, which leads to derepression of genes involved in zinc uptake and the expression of zinc-free paralogs of essential proteins (zinc-sparing response). The *P. aeruginosa* Zur regulon (11, 12) includes genes involved in zinc uptake, i.e., the zinc transporter-encoding operon *znuABCD* (10, 13, 14) and the zincophore-encoding operon *cntILMO* (15, 16). In addition, Zur regulates expression of zinc-free paralogs of ribosomal proteins (PA3600 and PA3601) (13, 17) and a transcriptional regulator (*dksA2*) (18) so as to reduce the requirement for zinc or to make the zinc stored in these proteins available for other cellular processes (19).

The host, on the other hand, utilizes nutritional immunity to sequester metal ions away from pathogens to reduce bacterial growth and control infection (20). One of the most abundant zinc-binding host proteins in CF is calprotectin (CP), which was previously named “the cystic fibrosis antigen” because of its abundance in the serum, sputum, and bronchoalveolar lavage fluid (BALF) of individuals with CF (2, 21–24). Neutrophils recruited to sites of inflammation release CP as S100A8/A9 heterodimers (25, 26), which then form tetramers in environments with sufficient levels of calcium (27, 28). Each heterodimer has two divalent-metal binding sites: the His3-Asp site has high affinity for zinc, while the His6 site is capable of binding divalent zinc, iron, manganese, or nickel (29). CP is thought to induce zinc limitation as a means to control infections caused by *Staphylococcus aureus*, *Acinetobacter baumannii* in tissues, and *Salmonella enterica* serovar Typhimurium in the gastrointestinal tract (30–32). However, little is known about the effect of CP-mediated zinc sequestration on *P. aeruginosa* growth and physiology.

Additionally, CP has been shown to inhibit the activity of metalloproteases such as host matrix metalloproteinases via zinc chelation (33). *P. aeruginosa* regulates expression of several metalloenzymes, including zinc metalloproteases, by quorum sensing (QS), which is a mechanism that regulates gene expression in accordance with cell density through the secretion of signal molecules. The secretion of zinc metalloproteases LasB (PA3724), LasA (PA1871), AprA (PA1249), ImpA (PA0572), and PepB (PA2939) (Table 1) are regulated by transcriptional regulators LasR and RhlR involved in QS (34, 35). This coordinated expression may be of particular importance for optimal protease activity given recent findings showing that zinc metalloproteases LasB and LasA in addition to the serine protease protease IV are activated, after being secreted, by a QS-induced proteolytic cascade, in which LasB activates protease IV and then protease IV, in turn, activates LasA (36, 37). Expression of these zinc metalloproteases is important for *P. aeruginosa* colonization and virulence because they play key roles in processes such as degrading host proteins (e.g., elastin) (38), invading host cells (39), evading host immune responses (40–42), and lysing other bacteria (e.g., *S. aureus*) (43, 44). While incubation of *P. aeruginosa* zinc metalloproteases with chemical zinc chelators

### TABLE 1 Zinc metalloproteases secreted by *P. aeruginosa*

| Gene no. | PDB entry | Protein name and description |
|----------|------------|-----------------------------|
| PA0572   | 5KDW      | ImpA, immunomodulating metalloprotease of *P. aeruginosa* |
| PA1249   | 1KAP      | AprA, alkaline metalloprotease or aeruginolysin |
| PA1871   | 3IT5      | LasA, staphylolytic protease |
| PA2939   | NA        | PepB or PaAP, aminopeptidase |
| PA3724   | 1EZM      | LasB, elastase or pseudolysin |

*From *P. aeruginosa* genome website, https://www.pseudomonas.com/.*

*From Protein Data Bank (PDB) website, https://www.rcsb.org/.*
inhibits their activity (45, 46), the effect of physiologically relevant zinc chelators such as CP on the activity of P. aeruginosa zinc metalloproteases remains unclear.

To test these hypotheses, we used a novel method in which P. aeruginosa strain PA01 was grown directly in unamended expectorated CF sputum and matched sputum samples treated with divalent metals (e.g., Zn²⁺ and Fe²⁺) and zinc chelators (e.g., TPEN [N,N,N',N'-tetrakis-2-pyridylmethyl-ethylenediamine] and CP). The effect of zinc chelators on P. aeruginosa zinc metalloprotease activity was further assessed using protease-specific assays. Overall, our findings support a model in which zinc chelation by CP in the mucus of the CF lung may affect the ecology of colonizing P. aeruginosa by inhibiting the activity of proteases involved in processes such as nutrient acquisition and interspecies competition.

RESULTS

P. aeruginosa exhibits a Zur-regulated zinc starvation response when grown in CF sputum samples from different donors. Given that recent studies have shown that P. aeruginosa increases expression of Zur-regulated genes in CF sputum (4–6), we first aimed to construct a lacZ fusion to the promoter of a Zur-regulated gene on the chromosome of P. aeruginosa to act as a tool to explore factors that influence the activation of the Zur regulon. While the P. aeruginosa Zur regulon contains several candidates, we selected ribosomal protein PA3600 given the abundance of ribosomes in bacterial cells (10, 47) and the high Zur responsiveness of zinc-independent ribosomal proteins relative to that of other Zur-regulated genes (48). P. aeruginosa encodes two forms of the 50s ribosomal protein L36. PA4242/RpmJ is the zinc-dependent isoform that contains the zinc ribbon motif CXXC...CXXXXXH, while Zur-regulated PA3600 is the zinc-independent isoform that lacks the zinc ribbon motif (see Fig. S1 in the supplemental material) (11–13, 17). PA4242 is highly similar (87% identity) to RpmJ, the zinc-dependent isofom of L36 encoded by Escherichia coli strain K-12 (Fig. S1), and as such the gene is commonly annotated as rpmJ (48). PA3600 is highly similar (80% identity) to RpmJ2, the zinc-independent isoform of L36 encoded by E. coli strain K-12 (Fig. S1), and thus we will refer to PA3600 as RpmJ2 for the remainder of this study.

To further assess the activity of Zur in CF sputum, we used a multiplex method to assess expression of rpmJ2 and three additional Zur-regulated genes. To do so, we used NanoString technology, which is a hybridization-based method that is quantitative, is not hindered by contaminating DNA in sputum, and requires only a small amount of RNA. Consequently, NanoString works well for the analysis of small clinical sample aliquots (e.g., sputum) as previously demonstrated (50, 51). In this study, NanoString technology allowed for the analysis of a subset of Zur-regulated genes: rpmJ2, cntO, znuA, and dksA2. Analysis showed an induction of these Zur-regulated genes in P. aeruginosa grown in sputum compared to those grown in M63 (Fig. 1C).
Amending samples with excess (1 mM) zinc was sufficient to reduce the expression of Zur-regulated genes (Fig. 1C). Studies have shown regulatory cross talk between iron and zinc, as iron starvation was previously shown to increase expression of Zur-regulated genes cntO, cntM, and amiA but not znuA (52). However, amending sputum samples with excess (1 mM) ferrous iron did not reduce expression of Zur-regulated genes (Fig. 1C). Together, these data support the model that *P. aeruginosa* has limited access to zinc in sputum and that zinc and iron limitation are separate signals.

Activation of the Zur-regulated rpmJ2 promoter in CF sputum is inversely correlated with concentration of zinc in sputum samples. While promoter activity of *P. aeruginosa* grown in CF sputum samples was overall higher than that of *P. aeruginosa* grown in medium controls, there was a range of promoter activity across sputum samples from different subjects (Fig. 1B). We hypothesized that differences in promoter activities between sputum samples from different CF patients were due to differences in sputum zinc concentrations. To test this, inductively coupled plasma-mass spectrometry (ICP-MS) was performed on homogenized CF sputum samples to measure total metal (i.e., zinc, iron, and manganese) concentrations. The ability of these same sputum samples to activate the rpmJ2 promoter in reporter strain PAO1 att::PrpmJ2-lacZ was tested in parallel. The data showed a significant inverse correlation between sputum zinc concentration and induction of the rpmJ2 promoter across tested sputum samples (Fig. 2). There was no significant correlation between sputum iron or manganese concentrations and induction of the rpmJ2 promoter (Fig. 2B; Fig. S2A and B). Induction of the rpmJ2 promoter was also compared to clinical information, primarily lung function (percent of forced expiratory volume in 1 s, \( \text{FEV}_1 \)).
FEV1%) at the time of sputum collection, but there was no correlation found between FEV1% and rpmJ2 promoter activity (Fig. S2C). Therefore, the derepression of Zur-regulated genes in P. aeruginosa grown in CF sputum inversely correlates with the total zinc concentration in sputum samples.

Recombinant CP induces a P. aeruginosa zinc starvation response in vitro and in expectorated CF sputum. Studies report elevated levels of zinc in CF sputum (1, 2). Our ICP-MS data show that the sputum sample in our study that elicited the strongest zinc starvation response had a zinc concentration of \( \approx 2 \mu g/g \) (\( \approx 2,000 \mu g/\text{liter}, \approx 31 \mu M \)) (Fig. 2A). Given the concomitant high zinc concentration in our CF sputum samples and the elevated zinc starvation response in P. aeruginosa grown in these CF sputum samples, it is likely that the zinc in our CF sputum samples is bound by zinc-sequestering proteins. CP is one such host zinc-sequestering protein that is found in high concentrations in the sputum of CF patients (2, 22, 23). CP has also been shown to induce expression of Zur-regulated genes in P. aeruginosa (53).

Therefore, we hypothesized that CP binds zinc to induce a zinc starvation response in P. aeruginosa grown in CF sputum. To test this, we first expressed and purified recombinant human CP as previously described (54) and as illustrated in Fig. S3. The ability of our recombinant CP to induce a zinc starvation response was tested by growing P. aeruginosa strain PAO1 att::P_{rpmJ2}-lacZ in culture medium (LB), medium containing CP, or medium containing CP and divalent zinc, iron, or manganese (Fig. 3A). CP concentrations in the sputum of CF patients have been reported to reach up to 3 mg/ml with an interquartile range of 0.5 mg/ml to 1 mg/ml (2, 23); we used 1 mg/ml (\( \approx 40 \mu M \)) CP, which is at the highest end of the interquartile range, for all CP-based experiments. Growing P. aeruginosa in medium containing 1 mg/ml CP resulted in a 4-fold increase in promoter activation (\( \approx 92 \) MU) compared to that of the control (\( \approx 25 \) MU) (Fig. 3A). The addition of excess (1 mM) zinc in the presence of CP completely prevented promoter activation (\( \approx 17 \) MU) (Fig. 3A). The addition of 1 mM iron had an intermediate effect (\( \approx 44 \) MU), while the addition of 1 mM manganese had a small though statistically significant effect (\( \approx 77 \) MU) (Fig. 3A). These results suggest that CP primarily binds zinc and iron, but not manganese, under the conditions tested. Furthermore, given the intermediate effect of iron, these results suggest that excess iron displaces zinc from the His6 binding site but not from the His3Asp site, consistent with reported findings (55), effectively causing CP to bind half the amount of zinc. These results confirm that our purified recombinant human CP can induce a zinc starvation response in P. aeruginosa which is quenched with the addition of exogenous zinc.

Despite the reportedly high concentrations of CP in the serum, sputum, and BALF of CF patients (2, 21–24), P. aeruginosa appears to be able to access enough zinc to...
Various environmental factors may influence CP zinc binding, such as calcium concentrations (56), pH (57), or the presence of oxidants (58, 59). Additionally, while CP in its tetrameric state is resistant to proteolytic degradation, CP is susceptible to oxidation, which in turn makes it susceptible to proteolytic degradation by both host and bacterial proteases (58, 59). Because it was unclear if CP in sputum would remain intact and/or active to bind zinc, we tested the ability of recombinant human CP to bind zinc and thereby induce a zinc starvation response in 

P. aeruginosa

strains. 

P. aeruginosa

strains were grown in culture medium (Control), medium with 40 μM CP (CP), or medium with 40 μM CP and 1 mM ZnSO₄·7 H₂O (CP+Zn (II)), (NH₄)₂Fe(SO₄)₂·6 H₂O (CP+Fe(III)), or MnCl₂·4 H₂O (CP+Mn(III)) for 3 h. The data shown represent the mean ± SD from three independent experiments. (B) 

P. aeruginosa

strain PAO1 PₚrpmJ²-lacZ was inoculated into CF sputum from 11 different donors. The sputum was divided and left untreated (Control), treated with 100 μM ZnSO₄·7 H₂O (Zn), or treated with 40 μM CP and 100 μM ZnSO₄·7 H₂O (CP+Zn) for 3 h. Different-color dots represent samples from different donors. The same-color dots connected by a line are from the same CF sputum donor. Data were analyzed by RM one-way ANOVA with Tukey’s multiple-comparison test. (C) Representative growth curves of 

P. aeruginosa

strain PAO1 PₚrpmJ²-lacZ grown in LB, LB containing 50 μM TPEN, or LB containing 40 μM CP. Data shown represent the mean ± SD from three technical replicates and are representative of three independent experiments. (D) OD₆₀₀ at 16 h of 

P. aeruginosa

strain PAO1 PₚrpmJ²-lacZ grown in LB, LB containing 50 μM TPEN, or LB containing 40 μM CP. Data shown represent the mean ± SD from three independent experiments. The data in panel A and panel D were analyzed by one-way ANOVA with Tukey’s multiple-comparison test. Samples marked with the same letter are not significantly different; samples marked with different letters are significantly different \( (P < 0.05) \).
lower concentrations of zinc than those of samples that induce low promoter activity, comparatively (Fig. 2). The high promoter activity by *P. aeruginosa* was readily quenched by the addition of zinc but remained high when CP was also added (Fig. 3B; green, lavender, and lilac). Conversely, the low promoter activity by *P. aeruginosa* grown in sputum samples with presumably high zinc was not affected greatly by the addition of zinc or CP (Fig. 3B; pink, light pink, and gray). Overall, these data show that addition of recombinant CP to zinc-amended sputum can induce a zinc starvation response dependent on sputum zinc concentration.

Previous studies have shown that CP (60) or a biologically equivalent CP derivative (55, 61, 62) affects *P. aeruginosa* growth rate and yield in culture to different extents depending on culture conditions. The concentrations of TPEN and CP that we found to induce a zinc starvation response in *P. aeruginosa* in LB medium (Fig. 1A; Fig. 3A) had minor effects on growth. In this study, we found that both TPEN and CP did not affect growth rate (Fig. 3C) but caused entry into stationary phase at a lower optical density (OD) (Fig. 3D).

Zinc metalloproteases are enriched among *P. aeruginosa*-secreted zinc-binding proteins. CP, which is not membrane permeable, may have effects on pathogens beyond limiting zinc availability for growth by binding or competing for metals in the extracellular environment. We performed a UniProt Knowledgebase (UniProtKB) analysis of the *P. aeruginosa* strain PAO1 proteome, which identified at least 72 zinc-binding proteins (Table 2). Of those 72, 64 were described by gene ontology (GO) molecular function as having catalytic activity (Table 2), which is consistent with the role of zinc as a cofactor. Of those 64 zinc-binding enzymes, 12 were further described as proteases and 5 of those were secreted zinc metalloproteases LasB, LasA, AprA, ImpA, and PepB (Table 2). We performed a second UniProtKB analysis of the *P. aeruginosa* strain PAO1 proteome that identified at least 34 secreted proteins, of which 6 were proteases and included the 5 aforementioned zinc metalloproteases in addition to protease IV (PA4175). While protease IV is not a zinc metalloprotease, its enzymatic activity is dependent on zinc. Protease IV enzymatic activity is reduced under zinc-limited conditions and in a *P. aeruginosa* mutant lacking the zinc importer-encoding gene *znuA* (14), which may be due to lower LasB activity, which is required to activate protease IV (36, 37). These analyses suggest that while 83% of secreted proteases, important virulence factors, are zinc metalloproteases, 100% of secreted proteases are either directly or indirectly zinc dependent. Overall, previously published studies and curated databases suggest that CP (2, 22, 23) and *P. aeruginosa*-secreted zinc metalloproteases (63) are abundant in the extracellular milieu of the CF mucus environment.

**Zinc chelation inhibits LasB-mediated proteolysis.** Given the importance of zinc to the activity of extracellular zinc metalloenzymes, we hypothesized that zinc

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**TABLE 2 Characteristics of zinc-binding proteins in *P. aeruginosa* as annotated by UniProtKB**

| GO molecular function | No. of proteins total | No. of proteins with indicated subcellular localization | | |
| --- | --- | --- | --- | --- |
| Zinc binding | 72 | 8 | 5 | 21 | 38 |
| Catalytic activity: nonpeptidase | 52 | 3 | 16 | 33 |
| Catalytic activity: peptidase | 12 | 5 | 3 | 1 | 3 |
| Structural binding activity | 2 |  |  | 2 |
| Molecular function regulator | 2 |  | 1 | 1 |
| ATPase-coupled protein transmembrane transporter activity | 1 |  |  | 1 |

*From protein knowledgebase (UniProtKB) website, [https://www.uniprot.org/uniprot/](https://www.uniprot.org/uniprot/).

*Gene ontology (GO).*
chelation by TPEN and CP would inhibit the activity of secreted zinc metalloproteases. Our initial studies suggested that LasB and LasA accounted for the majority of proteolytic activity by *P. aeruginosa* strain PAO1 (wild type [WT]) because filtered supernatants from ΔlasAB cultures spotted onto milk plates cleared the milk plates substantially less than did filtered WT supernatants (Fig. 4A, insets i and ii). As a result, this study focuses on the effect of zinc chelation on LasB activity.

To test the above hypothesis, LasB activity was determined quantitatively using azocasein as a substrate. The azocasein degradation assay was previously described to measure total proteolytic activity (14). However, by comparing the ability of *P. aeruginosa* WT, ΔlasA, and ΔlasAB supernatants to degrade azocasein, we found that azocasein degradation was LasB-dependent under the conditions tested (Fig. 4A). As a result, we tested the effect of TPEN and CP on LasB activity using the azocasein degradation assay. *P. aeruginosa* supernatants were filtered and then left untreated, treated with TPEN or CP, or treated with both TPEN or CP and divalent zinc, iron, or manganese. Treatment with TPEN or CP inhibited LasB enzymatic activity, while addition of 1 mM zinc, but not of iron or manganese, in the presence of TPEN or CP restored LasB activity (Fig. 4B and C). Furthermore, treatment of ΔlasAB supernatants with TPEN (Fig. S4A) or CP (Fig. S4B) without or with the addition of excess zinc did not alter azocasein degradation. Therefore, treatment of *P. aeruginosa* cell-free supernatants with zinc chelators TPEN and CP inhibits LasB-mediated caseinolytic activity.

**Zinc chelation inhibits LasA-mediated lysis of S. aureus.** LasA activity was determined by monitoring the decrease in absorbance at 595 nm of a heat-killed *S. aureus* suspension as previously described (14). Use of *P. aeruginosa* strain PAO1 (WT), ΔlasA, and ΔlasA+lasA (complemented mutant) supernatants confirmed that LasA is necessary for the lysis of *S. aureus* and that this assay measures LasA-mediated lysis of *S. aureus* under the conditions tested (Fig. 5A and B). This assay was then used to measure LasA activity in *P. aeruginosa* cell-free supernatants left untreated, treated with TPEN or CP, or treated with both TPEN or CP and divalent zinc, iron, or manganese. Treatment of supernatants with TPEN or CP inhibited LasA activity, while treatment with TPEN or CP in the presence of excess zinc (500 μM and 160 μM, respectively), but not iron or manganese, restored LasA activity (Fig. 5C to F). Furthermore, treatment of ΔlasA supernatants with zinc, TPEN, or CP had no effect on lysis of *S. aureus*, confirming that treatment of supernatants did not have LasA-independent cytotoxic effects on *S. aureus* (Fig. S6). Therefore,
treatment of \textit{P. aeruginosa} cell-free supernatants with zinc chelators TPEN and CP inhibits LasA-mediated lysis of \textit{S. aureus}.

**DISCUSSION**

Here, we show that \textit{P. aeruginosa} strain PAO1 grown in aliquots of expectorated CF sputum exhibits a zinc starvation response despite relatively high concentrations of zinc in the sputum samples. Treatment with recombinant host CP was sufficient to induce a zinc starvation response in \textit{P. aeruginosa} grown in zinc-amended CF sputum samples from different subjects, demonstrating that CP retains its function in sputum. Furthermore, treatment of \textit{P. aeruginosa} supernatants with CP inhibited the activity of secreted, extracellular zinc metalloproteases LasB and LasA. The data presented in this study support a model in which CP released from recruited neutrophils sequesters zinc from the environment to induce a zinc starvation response in \textit{P. aeruginosa} and...
sequesters zinc from secreted virulence factors, including zinc-dependent metalloproteases such as LasB and LasA. LasB is a protease that can degrade host proteins, such as elastin, as well as peptides. These degraded proteins/peptides can then be taken up and utilized as nutrients by *P. aeruginosa*. LasA is a protease that lyses *S. aureus* by cleaving pentaglycine bridges of peptidoglycan. LasA-mediated lysis of *S. aureus* allows *P. aeruginosa* to take up nutrients released from lysed *S. aureus* as well as to outcompete *S. aureus* in the CF lung. During infection, neutrophils are recruited to sites of infection/inflammation. Neutrophils may then release cellular contents such as CP. CP can then bind bioavailable zinc away from *P. aeruginosa*, thus reducing the overall abundance of *P. aeruginosa*, while also inducing a zinc starvation response by *P. aeruginosa*. Additionally, CP can bind zinc away from both LasB and LasA, thereby inhibiting their proteolytic activity. Furthermore, LasB and LasA activity have been shown to induce neutrophil extracellular traps (NETs). Therefore, CP-mediated inhibition of LasB and LasA activity may lead to less NET formation and, subsequently, less CP release. Arrows indicate a positive interaction.

A variety of strategies have been used to learn about the environment that *P. aeruginosa* encounters in the CF lung, including analysis of bacteria grown in buffered media supplemented with CF sputum compared to bacteria grown in laboratory media (8, 9) and direct analysis of gene expression by bacteria in expectorated CF sputum (4, 5, 64). While studies have varied in their techniques, transcriptomic analyses have found that genes induced by low intracellular zinc are elevated in sputum samples relative to those in controls (4–9). Our model differs from previous models, as it measures the transcriptional response of *P. aeruginosa* grown directly in expectorated sputum from a variety of CF patients. Our study also found that *P. aeruginosa* activates its zinc starvation response in CF sputum on average but revealed differences across samples from different CF donors (Fig. 1B and C; Fig. 3B). These findings taken together underscore the fact that *P. aeruginosa* growth in laboratory media would not recapitulate the effect of low-zinc conditions in the context of CF. To this end, our CF sputum model is one way to provide a low-zinc environment and allows for investigation of the response of *P. aeruginosa* across sputum samples from different donors which vary in levels of host factors like CP. This same approach would also enable the investigation of different *P. aeruginosa* strains in sputum aliquots from a single donor.

CP concentrations in the sputum of CF patients vary but have been reported to reach up to 3 mg/ml (~120 μM), with the majority of sputum samples measuring between 0.5 mg/ml (~20 μM) and 1 mg/ml (~40 μM) (2, 23). Zinc concentrations in CF sputum similarly vary but are generally high relative to those in sputum from non-CF patients.
individuals and other biological compartments. Smith et al. (1) found that the zinc concentration of 45 CF sputum samples ranged from 678 \( \mu \text{g/liter} \) (\((-10 \mu \text{M})\) to 1,181 \( \mu \text{g/liter} \) (\((-18 \mu \text{M})\)) compared to 103 \( \mu \text{g/liter} \) (\((-2 \mu \text{M})\)) to 597 \( \mu \text{g/liter} \) (\((-9 \mu \text{M})\)) in 8 non-CF sputum samples. Li et al. (3) reported that the zinc concentration of 118 CF sputum samples ranged from \(-5 \mu \text{M}\) to \(-145 \mu \text{M}\). Gray et al. reported that among 23 CF sputum samples, the median concentration was 135 \( \mu \text{g/liter} \) (\((-2 \mu \text{M})\)) with an interquartile range of 54 to 210 \( \mu \text{g/liter} \) (\((-0.8 \text{ to } 3 \mu \text{M})\)) (84). In this study, the zinc concentration of 8 CF sputum samples ranged from 1 \( \mu \text{g/g} \) (\((-15 \mu \text{M})\)) to 8 \( \mu \text{g/g} \) (\((-116 \mu \text{M})\)) (Fig. 2A). The persistence of \( P. \text{aeruginosa} \) infections in the CF lung is thus likely influenced by levels of zinc, CP, other host and microbial zinc-binding factors, and its own cellular zinc requirements in this specific environment.

There is mounting evidence that divalent-metal sequestration by CP affects \( P. \text{aeruginosa} \). Wakeman et al. (53) demonstrated that CP-mediated genetic responses in \( P. \text{aeruginosa} \) were reversed upon treatment with zinc \( \text{in vitro} \) and that \( P. \text{aeruginosa} \) and CP colocalized at sites of inflammation within a CF lung explant. D’Orazio et al. showed that CP-mediated growth inhibition was enhanced in \( P. \text{aeruginosa} \) strain \( \Delta \text{znuA} \), which is a mutant lacking the gene encoding the small zinc-binding protein of the ZnuABC zinc importer resulting in reduced intracellular zinc accumulation (13, 14). Zygiel et al. (61) showed that treatment with CP significantly reduced intracellular iron and manganese in \( P. \text{aeruginosa} \) but did not significantly affect intracellular zinc, though intracellular zinc trended downward (61). Our data show that CP induces a Zur-regulated zinc starvation response \( \text{in vitro} \) and in expectorated CF sputum which is repressed upon the addition of excess zinc (Fig. 3A and B). We also observed CP-mediated growth defects \( \text{in vitro} \) (Fig. 3C) similar to those reported by Zygiel et al. (61) which were previously attributed to ferrous iron chelation by CP. Taken together, the data show that \( P. \text{aeruginosa} \) and CP colocalize at sites of inflammation in the CF lung and that CP is capable of inducing zinc and/or iron starvation responses depending on test conditions.

Additionally, while Filkins et al. (65) showed that \( \text{in vitro} \) coculture of \( P. \text{aeruginosa} \) and \( S. \text{aureus} \) on CF bronchial epithelial cells reduced the viability of \( S. \text{aureus} \), Wakeman et al. (53) showed that zinc chelation by CP promotes \( P. \text{aeruginosa} \) and \( S. \text{aureus} \) coculture \( \text{in vitro} \), \( \text{in vivo} \), and \( \text{ex vivo} \) models, in part by downregulating genes encoding anti-staphylococcal factors such as pyocyanin, hydrogen cyanide, and \( \text{Pseudomonas quinolone signal/2-heptyl-4-hydroxyquinoline N-oxide (PQS/HQNO)} \). Interestingly, treatment of \( P. \text{aeruginosa} \) with CP did not reduce the expression of \( \text{lasA} \), though the functionality of LasA was not tested (53). In this study, we show that CP-mediated zinc chelation inhibits LasA-mediated lysis of \( S. \text{aureus} \) by \( P. \text{aeruginosa} \text{ in vitro} \) (Fig. 5E and F). Therefore, while LasA may be expressed and secreted by \( P. \text{aeruginosa} \) in the presence of CP, CP may posttranslationally inhibit LasA activity via zinc sequestration. Furthermore, colonization of the CF airways is usually described as a pattern of succession where \( S. \text{aureus} \) is the predominant colonizer early on in younger patients before being outcompeted by \( P. \text{aeruginosa} \) in older patients (65). However, Fischer et al. (66) recently showed that \( P. \text{aeruginosa} \) and \( S. \text{aureus} \) chronically cocolonize the CF lung. Wakeman et al. also showed that \( P. \text{aeruginosa} \), \( S. \text{aureus} \), and CP colocalize in CF lung explants (53). Further studies are required to determine if CP modulates protease-dependent and/or protease-independent cocolonization of \( P. \text{aeruginosa} \) and \( S. \text{aureus} \) in the CF lung.

Notably, \( P. \text{aeruginosa} \) strains chronically adapted to the CF lung, including \( \text{lasR}^{-} \text{loss-of-function (LasR}^{-}\text{)} \) mutants, have a reduced capacity to outcompete \( S. \text{aureus} \) (67). LasR is a QS regulator that positively regulates the expression and secretion of several virulence factors, including zinc metalloproteases LasB, LasA, AprA, ImpA, PepB, and protease IV (34, 35). However, LasR\(^{-}\) strains commonly arise during chronic CF infection and are associated with worse lung function (68–73). While LasR\(^{-}\) strains are common in CF infections, virulence factors regulated by LasR such as zinc metalloproteases are still reported to be abundant in CF sputum (63). Recent work by Mould et al. showed that when LasR\(^{+}\) and LasR\(^{-}\) strains were cocultured, the LasR\(^{+}\) strain increased...
production of RhlR-controlled virulence factors by the LasR– strain (74). Interestingly, LasB and LasA are reportedly regulated by both the LasR and RhlR QS regulators (35). Therefore, further investigation is needed to understand how intra- and interspecies interactions within populations colonizing the CF airway affect the secretion and function of virulence factors such as zinc metalloproteases LasB and LasA.

LasB is an abundant protease with broad substrate specificity that is implicated in amino acid liberation and consumption (75). In addition to nutrient acquisition, LasB also plays a role in the ability of P. aeruginosa to invade host epithelial cells (39) and to evade host immune responses via processes such as degrading cytokines (40). Interestingly, degradation of proinflammatory cytokines interleukin 8 (IL-8) and IL-6 by LasB reduces neutrophil recruitment and the overall IL-8 and IL-6 response (40). While LasB-mediated cytokine degradation has been reported to reduce neutrophil recruitment, LasB can also induce neutrophil extracellular traps (NETs) (76, 77). Neutrophils recruited to sites of inflammation can release CP through processes such as NET formation (78), and in this study we show that CP-mediated zinc chelation inhibits the activity of secreted LasB (Fig. 4C). Taken together, there appears to be a complex interplay between LasB, neutrophils, and CP during the course of infection which may contribute to exacerbations in CF. Furthermore, recent work suggests that secreted LasB activates protease IV which then predominantly processes and activates LasA (36, 37). Therefore, CP-mediated inhibition of secreted LasB activity may have downstream effects on the processing and activity of other secreted zinc metalloproteases.

In conclusion, the results of our study show that CP can induce a zinc starvation response in P. aeruginosa in CF sputum as well as chelate zinc to inhibit the activity of virulence-associated zinc metalloproteases. Other zinc-binding host proteins (79) or zincophores from other microbes (31) may also modulate the activity of P. aeruginosa zinc metalloproteases. Future studies will focus on how competition for zinc in a zinc-limited or zinc-chelating environment such as CF mucus shapes polymicrobial infections and patient outcomes, particularly considering the observed variability in zinc concentration and availability across CF patients.

MATERIALS AND METHODS

Strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. P. aeruginosa and Escherichia coli strains were maintained on tryptic soy broth (LB) (1% tryptone, 0.5% yeast extract, 0.5% NaCl) with 1.5% agar and routinely grown in LB on a roller drum at 37°C. P. aeruginosa plasmid strains were maintained by supplementing media with 300 μg/ml carbencillin or 60 μg/ml gentamicin. E. coli plasmid strains were maintained by supplementing media with 100 μg/ml carbenicillin. S. aureus SH1000 was maintained on Trypticase soy with 1.5% agar (TSA) or grown in Trypticase soy broth (TSB) on a roller drum at 37°C. Saccharomyces cerevisiae strains for cloning were maintained on yeast-peptone-dextrose (YPD) medium with 2% agar.

Construction of plasmids. Primers used for plasmid construction are listed in Table S2. All plasmids were sequenced at the Molecular Biology Core at the Geisel School of Medicine at Dartmouth. Plasmid strain (DH3229) was constructed using a S. cerevisiae recombination technique as previously described (80). Plasmid strain (DH3229) served as the vector backbone for this construct. GH121_P

Saccharomyces cerevisiae strains for cloning were maintained on yeast-peptone-dextrose (YPD) medium with 2% agar.

Cystic fibrosis (CF) sputum collection. Sputum samples were collected in accordance with protocols approved by the Committee for the Protection of Human Subjects at Dartmouth. Expectorated sputum samples used in this study were collected from adult subjects with CF during a routine office visit or upon admission for treatment of a disease exacerbation. Sputum samples were frozen upon collection and stored at −80°C until use.

Beta-galactosidase assay. P. aeruginosa cells with a promoter fusion to lacZ integrated at the att locus were grown in 5 ml cultures of LB at 37°C for 16 h. Overnight cultures were diluted 1:50 in 50 ml of culture medium (LB or M63) and then grown to an optical density at 600 nm (OD600) of 0.5. The cells were then centrifuged and resuspended in culture medium, centrifuged at 10,000 × g for 2 min, and then resuspended in 500 μl of culture medium. Ten μl of cell suspension were
added per 100 μl of culture medium or sputum sample in a 2-ml microcentrifuge tube. Samples were incubated at 37°C with shaking for 3 h. Beta-galactosidase (β-Gal) activity was measured as described by Miller (81) using 50 μl of sample.

**RNA isolation and NanoString analysis.** Unamended sputum or sputum amended with 1 mM ZnSO4, 7 H2O or (NH4)2Fe(SO4)2·6 H2O (100 μl) was added to 2-ml microcentrifuge tubes. *P. aeruginosa* strain PA01 was grown in 5 ml cultures of LB at 37°C for 16 h. Overnight cultures were diluted 1:50 in 50 ml M63 minimal medium with 0.2% glucose and then grown to an O600 of 0.5. The cells were then centrifuged at 4,500 × g for 10 min with water, centrifuged, and then resuspended in 500 μl of water. Ten μl of cell suspension were added per 100 μl M63 minimal medium with 0.2% glucose (control) or sputum sample in a 2-ml microcentrifuge tube. Samples were then incubated at 37°C with shaking for 3 h. TRIzol (900 μl) was added to 100 μl of sputum containing 10 μl of PA01 cell suspension. Samples were stored overnight. RNA was prepared following DirectZol kit instructions and eluted in 50 μl of water.

For NanoString, 5 μl of a 1:10 dilution of RNA was used. Diluted RNA was applied to the codeset PaV4 and processed as previously reported (51). Counts were normalized to the geometric mean of spiked-in technical controls. Normalized counts were used for Z-score calculations and heatmap construction.

**Measurement of zinc in sputum samples.** Sputum samples for zinc analysis were stored at −80°C until processed. Sputum zinc was quantified by inductively coupled plasma-mass spectrometry (ICP-MS) following nitric acid digestion of organic material according to the method of Heck et al. and is expressed as micrograms of zinc per gram of sputum (82). ICP-MS was performed by the Dartmouth Trace Element Analysis (TEA) Core.

**Expression and purification of recombinant calprotectin.** Plasmid S100A8/A9 was obtained from Futami et al. (54), and recombinant calprotectin (CP) was expressed and purified as previously described with minor modifications. Plasmid S100A8/A9 was first confirmed by Sanger sequencing and then transformed into *E. coli* T7 Express cells. Transformed T7 Express cells were then grown in LB containing 100 μg/ml carbenicillin. Supplemented with Benzonase-HC to control viscosity of the sample. Cells were then lysed using the microfluidizer with 3 passages at 18,000 lb/in². Final volume was about 100 ml. Fifteen percent polyethylenimine (PEI) was added dropwise to a final concentration of 0.7% to precipitate nucleic acids (about 5 ml). Samples were then centrifuged at 23,280 × g for 10 min at 4°C. Pellet containing intact cells and precipitated nucleic acids was discarded. NH4SO4 (61.27 g) was added slowly to clarify supernatant (about 115 ml) while stirring at 4°C until a saturation of 80%. The sample became gradually turbid. Sample was stirred for an additional 30 min after complete saturation. Sample was then centrifuged at 23,280 × g for 10 min at 4°C. Supernatant was discarded and the pellet was dissolved in about 30 ml of solubilization buffer (50 mM Tris-HCl [pH 7.5], 30 mM dithiothreitol [DTT]) and incubated for 1 h at 37°C. Dissolved pellet was then dialyzed against 50 mM sodium phosphate (pH 6.0) at 4°C using 3.5 kDa cutoff dialysis cassettes to change buffer. Sample was then centrifuged at 23,280 × g for 10 min at 4°C to remove any pellet.

CP was then purified using a HiTrap SP column (stored in 20% ethanol). The column was washed with 5 column volumes (CV) of H2O at about 5 ml/min. The column was then washed with 5 CV of 100 mM NaCl, 5 mM MgCl2 supplemented with Benzonase-HC to control viscosity of the sample. Cells were then lysed using the microfluidizer with 3 passages at 18,000 lb/in². Final volume was about 100 ml. Fifteen percent polyethylenimine (PEI) was added dropwise to a final concentration of 0.7% to precipitate nucleic acids (about 5 ml). Samples were then centrifuged at 23,280 × g for 10 min at 4°C. Pellet containing intact cells and precipitated nucleic acids was discarded. NH4SO4 (61.27 g) was added slowly to clarify supernatant (about 115 ml) while stirring at 4°C until a saturation of 80%. The sample became gradually turbid. Sample was stirred for an additional 30 min after complete saturation. Sample was then centrifuged at 23,280 × g for 10 min at 4°C. Supernatant was discarded and the pellet was dissolved in about 30 ml of solubilization buffer (50 mM Tris-HCl [pH 7.5], 30 mM dithiothreitol [DTT]) and incubated at 37°C. Dissolved pellet was then dialyzed against 50 mM sodium phosphate (pH 6.0) at 4°C using 3.5 kDa cutoff dialysis cassettes to change buffer. Sample was then centrifuged at 23,280 × g for 10 min at 4°C to remove any pellet.

**Fractionation of the fractions.** Fractions were analyzed using SDS-PAGE (15% gel) and the appropriate fractions were then pooled. CP was then purified using a HiLoad 26/600 Sephadex 575 and CP buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM DTT; filtered/degassed). Sample (about 13 ml) was loaded in a 50 ml superloop. Sample was then run on the HiLoad 26/600 Superdex 75p, a program composed of 2 CV equilibration, injection of 12 ml of sample, and elution with 1.2 CV at 3 ml/min. Flow rate is 3 ml/min and collection of 7 ml/tube. Tubes corresponding to three different fractions were pooled to make fractions F1_I, F2_I, and F3_I. All other tubes containing calprotectin from both HiTrap runs were concentrated using YM-10 Amicon centrifugal filters and reloaded in the HiLoad 26/600 superdex 75 as before. Tubes corresponding to three different fractions were pooled to make fractions F1_II, F2_II, and F3_II. Samples from all six fractions were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4 to 12% gel). Fractions F1_I and F1_II, F2_I and F2_II, and F3_I and F3_II were combined to make fractions F1, F2, and F3, respectively. Fractions were concentrated with YM-10 Amicon centrifugal filters. The final concentrations of the fractions were determined using a Bradford protein assay.

**Growth assays.** Growth of *P. aeruginosa* in the presence of TPEN or CP was measured as previously described (61) with modification. *P. aeruginosa* was grown for 16 h in 5 ml LB with rolling at 37°C. Samples were then centrifuged and washed once in LB before resuspending the pellet in LB. Washed
samples were adjusted to an OD_{600} of 1.0 in LB. Samples were then diluted 1:10 in LB, LB plus 50 \mu M TPEN, or LB plus 40 \mu M CP to an OD_{600} of 0.1. In triplicate, 100-\mu l aliquots of samples or media alone (negative controls) were added to wells of a 96-well flat-bottom plate containing 100 \mu l of LB, LB plus 50 \mu M TPEN, or LB plus 40 \mu M CP. Plates were incubated statically at 37°C in a plate reader for 16 h, during which time the OD_{600} was measured every 15 min following a brief shake.

Protease assays. *P. aeruginosa* culture supernatants were used for protease assays. Five milliliters of overnight cultures in LB was centrifuged at 4,500 \times g for 10 min. Supernatants were then filter sterilized using a 0.22-\mu m syringe filter. For TPEN experiments, undiluted supernatants were used. For CP experiments, stored aliquots of CP were first diluted to 3 mg/ml in CP buffer without DTT (50 mM Tris-HCl (pH 7.5), 150 mM NaCl). Then, 1 part 3 mg/ml CP was added to 2 parts supernatant for a final concentration of 1 mg/ml.

Caseinolytic activity was determined qualitatively by spotting *P. aeruginosa* supernatants onto 1% milk plates or quantitatively using azocasein as a substrate as previously described (14) with modification. In brief, *P. aeruginosa* culture supernatants were treated overnight (16 h) with 50 \mu M TPEN or an equivalent volume of 100% ethanol (EtOH), 1 mg/ml (~40 \mu M) CP or an equivalent volume of CP buffer without DTT, and/or 1 mM ZnSO_{4}·7 H_{2}O or an equivalent volume of diH_{2}O. Treatment of WT supernatants with 50 \mu M to 2 mM ZnSO_{4}·7 H_{2}O was found not to affect LasB activity (Fig. S4C). The supernatants were then incubated at 37°C overnight (16 h). Supernatants (25 \mu l) were mixed with 150 \mu l 2% azocasein in 10 mM Tris-HCl, 8 mM CaCl_{2} (pH 7.4). Samples were incubated at 37°C for 15 min. To each sample, 228 \mu l of 10% TCA was added and the sample was vortexed and then incubated at room temperature for 15 min. Samples were then centrifuged for 10 min at 10,000 \times g. Cleared supernatants (100 \mu l) were added to wells of a 96-well flat-bottom polystyrene plate containing 200 \mu l of 1 M NaOH. Absorbance was read at 440 nm.

Staphylolytic activity was determined by monitoring the decrease in absorbance at 595 nm of a heat-killed *S. aureus* suspension as previously described (14) with modification. *S. aureus* strain SH1000 (83) was cultured in TSB overnight (16 h) at 37°C with rolling. Cultures were centrifuged at 4,500 \times g for 10 min, resuspended in 20 mM Tris-HCl (pH 8.8) to a final OD_{600} of 1.0, and then killed by heating at 100°C for 30 min. Heat-killed *S. aureus* suspensions were cooled to room temperature before use. *P. aeruginosa* culture supernatants were treated overnight (16 h) with 50 \mu M TPEN or an equivalent volume of 100% EtOH, 1 mg/ml (~40 \mu M) CP or an equivalent volume of CP buffer without DTT, and/or 160 to 500 \mu M ZnSO_{4}·7 H_{2}O or an equivalent volume of diH_{2}O. Because increasing concentrations of zinc were previously reported to inhibit LasA activity (46), an appropriate concentration of zinc to use in add-back experiments was determined experimentally. For undiluted WT supernatants, the addition of 500 \mu M zinc had no effect on LasA activity, while increasing concentrations of zinc inhibited LasA-mediated lysis of *S. aureus* (Fig. S5C and D). Therefore, we used 500 \mu M zinc for TPEN-based experiments. For CP buffer-diluted WT supernatants, the addition of 50 \mu M zinc had no effect on LasA activity, while increasing concentrations of zinc inhibited LasA-mediated lysis of *S. aureus* (Fig. S5C and D). To ensure that zinc would be in excess in TPEN-based experiments, we used 160 \mu M zinc, which was four times the concentration of CP but still less than 250 \mu M zinc, which was the concentration tested that started to inhibit LasA activity independent of CP. *P. aeruginosa* supernatants (20 \mu l) were added to 180 \mu l of heat-killed *S. aureus* in wells of a 96-well flat-bottom polystyrene plate. Staphylolytic activity was determined by monitoring the change in absorbance at 595 nm every 15 min for 3 h using a plate reader. The plate was shaken before each read.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 8 and results were expressed as the mean values plus or minus standard deviations. Unless otherwise noted, one-way analysis of variance (ANOVA) followed by Tukey’s multiple-comparison test was performed to determine statistical significance of the data. See the figure legends for other specific statistical tests used.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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