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 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 post-translationally inhibit zinc metalloprotease activity and thereby impact the protease-dependent physiology and/or virulence of *P. aeruginosa* in the CF lung environment.

21 **Importance**

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

30 **Introduction**

31 In cystic fibrosis (CF), microbes such as *Pseudomonas aeruginosa* colonize airway mucus
32 where they then compete with host cells and other microbes for nutrients, including metals.
33 Divalent metal ions (e.g., Zn^{2+}, Fe^{2+}, Mn^{2+}, etc.) are essential micronutrients for host and microbe
34 alike, in part, because they act as cofactors in enzymes important for a variety of cellular functions.
35 While the concentration of metals, such as zinc, in CF sputum can vary, the concentration of zinc
36 in expectorated sputum from CF patients is elevated, on average, compared to levels in samples
37 from healthy controls (1-3). However, studies investigating the transcriptional response of *P.*
38 *aeruginosa* in CF sputum show that a common gene expression pattern is the increased
39 expression of zinc uptake and transport genes (4-9), which are normally expressed when zinc is
40 limited. The *P. aeruginosa* zinc-starvation response is regulated by the zinc uptake regulator
41 (Zur), which is a transcriptional repressor (10). When intracellular zinc is high, Zur monomers bind
42 zinc, dimerize, and bind DNA to repress gene expression of zinc uptake pathways. When
43 intracellular zinc becomes low, the dimeric, zinc-bound fraction of Zur decreases, which leads to
44 derepression of genes involved in zinc uptake and the expression of zinc-free paralogs of
45 essential proteins (zinc-sparing response). The *P. aeruginosa* Zur regulon (11, 12) includes the
46 zinc transporter-encoding operon *znuABCD* (10, 13, 14), the zincophore-encoding operon
47 *cntILMO* (15, 16), and zinc-free paralogs of ribosomal proteins (*PA3600* and *PA3601*) (13, 17)
48 and transcription factors (*dksA2*) (18). These responses not only reduce the requirement for zinc
49 but liberate the zinc that was stored in the zinc-dependent forms of these proteins (19).

50 The host, on the other hand, utilizes nutritional immunity to sequester metal ions away
51 from pathogens to reduce bacterial growth and control infection (20). One of the most abundant
52 zinc-binding host proteins in CF is calprotectin (CP), which was previously named "the cystic
53 fibrosis antigen" because of its abundance in the serum, sputum, and bronchoalveolar lavage
54 fluid (BALF) of individuals with CF (2, 21-24). Neutrophils recruited to sites of inflammation release
55 CP as S100A8/A9 heterodimers (25, 26), which then form tetramers in environments with

56 sufficient levels of calcium (27, 28). Each heterodimer has two divalent-metal binding sites: one
57 site has high affinity for zinc and low affinity for manganese while the other site is capable of
58 binding divalent manganese, iron, zinc, or nickel (29). CP is thought to induce zinc limitation as a
59 means to control infections caused by *Staphylococcus aureus*, *Acinetobacter baumannii* in
60 tissues, and *Salmonella enterica* serovar Typhimurium in the gastrointestinal tract (30-32).
61 However, little is known about the effect of CP-mediated zinc sequestration on *P. aeruginosa*
62 growth and physiology.

63 Additionally, CP has been shown to inhibit the activity of metalloproteases such as host
64 matrix metalloproteinases via zinc chelation (33). *P. aeruginosa* regulates expression of several
65 metalloenzymes, including zinc metalloproteases, by quorum sensing (QS), which is a
66 mechanism that regulates gene expression in accordance with cell density through the secretion
67 of signal molecules. The secretion of zinc metalloproteases LasB (PA3724), LasA (PA1871), AprA
68 (PA1249), ImpA (PA0572), PepB (PA2939), and Protease IV (PA4175) (**Table 1**) are regulated
69 by transcriptional regulators LasR and RhlR involved in QS (34, 35). This coordinated expression
70 may be of particular importance for optimal protease activity given recent findings showing that
71 LasB, Protease IV, and LasA are activated after being secreted by a QS-induced proteolytic
72 cascade in which LasB activates Protease IV and then Protease IV, in turn, activates LasA (36,
73 37). Expression of these zinc metalloproteases is important for *P. aeruginosa* colonization and
74 virulence because they play key roles in processes such as degrading host proteins (e.g., elastin)
75 (38), invading host cells (39), evading host immune responses (40-42), and lysing other bacteria
76 (e.g., *S. aureus*) (43, 44). While incubation of *P. aeruginosa* zinc metalloproteases with chemical
77 zinc chelators inhibits their activity (45, 46), the effect of physiologically relevant zinc chelators
78 such as CP on the activity of *P. aeruginosa* zinc metalloproteases remains unclear.

79 To test these hypotheses, we used a novel method in which *P. aeruginosa* strain PAO1
80 was grown directly in unamended expectorated CF sputum and matched sputum samples treated
81 with divalent metals (e.g., Zn^{2+} and Fe^{2+}) and zinc chelators (e.g., TPEN and CP). The effect of

82 zinc chelators on *P. aeruginosa* zinc metalloprotease activity was further assessed using
83 protease-specific assays. Overall, our findings support a model in which zinc chelation by CP in
84 the mucus of the CF lung may impact the ecology of colonizing *P. aeruginosa* by inhibiting the
85 activity of proteases involved in processes such as nutrient acquisition and interspecies
86 competition.
87
88 **Results**
89 ***P. aeruginosa* exhibits a Zur-regulated zinc-starvation response when grown in CF sputum**
90 **samples from different donors**
91 Given that recent studies show that *P. aeruginosa* increases expression of Zur-regulated
92 genes in CF sputum (4-6), we first constructed a *lacZ* fusion to the promoter of *PA3600* on the
93 chromosome of *P. aeruginosa* strain PAO1 (PAO1 *att*::P_{PA3600}-*lacZ*) to act as a tool to explore
94 factors that influence the activation of the Zur regulon. *PA3600* encodes the Zur-regulated zinc-
95 independent isoform of the 50s ribosomal protein L36 (11-13, 17). Activation of the *PA3600*
96 promoter was first confirmed by measuring activity by *P. aeruginosa* grown in culture medium
97 (LB), medium containing TPEN (*N,N,N′,N′*-tetrakis-2-pyridylmethyl-ethylenediamine), or medium
98 containing both TPEN and zinc (**Fig. 1a**). TPEN is a membrane permeable metal ion chelator with
99 a high affinity for zinc (47) and was therefore used to induce a zinc-starvation response in *P.*
100 *aeruginosa*. *P. aeruginosa* grown for 3 h in LB had little promoter activity (~23 Miller Units [MU]),
101 while growth in medium containing TPEN resulted in a seven-fold increase in promoter activity
102 (~150 MU) (**Fig. 1a**). The addition of TPEN and an excess of zinc (1 mM) did not stimulate
103 promoter activity (**Fig. 1a**). The ability of sputum to activate the *PA3600* promoter was then
104 determined by growing *P. aeruginosa* in M63 minimal medium containing 0.2% glucose (M63),
105 culture medium plus TPEN (positive control), or expectorated CF sputum from 10 different donors
106 (**Fig. 1b**). While *P. aeruginosa* grown for 3 h in M63 exhibited greater promoter activity (~85 MU)
107 than when grown in LB (~23 MU), growth in CF sputum resulted in a three-fold increase in

108 promoter activity (~281 MU). Average promoter activation in CF sputum was statistically the same
109 as promoter activity induced by TPEN (**Fig. 1b**).
110 To further assess the activity of Zur in CF sputum, we used a multiplex method to assess
111 expression of *PA3600* and three additional Zur-regulated genes. To do so, we used NanoString
112 technology, which is a hybridization-based method that is quantitative, not hindered by
113 contaminating DNA in sputum, and requires only a small amount of RNA. Consequently,
114 NanoString works well for the analysis of small clinical sample aliquots (e.g., sputum) as
115 previously demonstrated (48, 49). In this study, NanoString technology allowed for the analysis
116 of subset of Zur-regulated genes: *PA3600*, *cntO*, *znuA*, and *dksA2*. Analysis showed an induction
117 of these Zur-regulated genes in *P. aeruginosa* grown in sputum compared to M63 (**Fig. 1c**).
118 Amending samples with excess zinc (1 mM) was sufficient to reduce the expression of Zur-
119 regulated genes (**Fig. 1c**). Studies have shown regulatory crosstalk between iron and zinc as iron
120 starvation was previously shown to increase expression of Zur-regulated genes *cntO*, *cntM*, and
121 *amiA*, but not *znuA* (50). However, amending sputum samples with excess ferrous iron (1 mM)
122 did not reduce expression of Zur-regulated genes (**Fig. 1c**). Together these data support the
123 model that *P. aeruginosa* has limited access to zinc in sputum and that zinc and iron limitation are
124 separate signals.
125
126 **Activation of the Zur-regulated *PA3600* promoter in CF sputum is inversely correlated with**
127 **concentration of zinc in sputum samples**
128 While promoter activity of *P. aeruginosa* grown in CF sputum samples was overall higher
129 than medium controls, there was a range of promoter activity across sputum samples from
130 different subjects (**Fig. 1b**). We hypothesized that differences in promoter activities between
131 sputum samples from different CF patients were due to differences in sputum zinc concentrations.
132 To test this, inductively coupled plasma mass spectrometry (ICP-MS) was performed on
133 homogenized CF sputum samples to measure total metals (i.e., zinc, iron, and manganese)

134 concentrations. The ability of these same sputum samples to activate the *PA3600* promoter in
135 reporter strain PAO1 *att*::P_{PA3600}-*lacZ* was tested in parallel. The data showed a significant inverse
136 correlation between sputum zinc concentration and induction of the *PA3600* promoter across
137 tested sputum samples (**Fig. 2**). There was no significant correlation between sputum iron or
138 manganese concentrations and induction of the *PA3600* promoter (**Fig. 2b**; **Fig. S1a**; **Fig. S1b**).
139 Induction of the *PA3600* promoter was also compared to clinical information, primarily lung
140 function (FEV1%) at the time of sputum collection, but there was no correlation found between
141 FEV1% and *PA3600* promoter activity (**Fig. S1c**). Therefore, the derepression of Zur-regulated
142 genes in *P. aeruginosa* grown in CF sputum inversely correlates with the total zinc concentration
143 in sputum samples.
144

145 **Recombinant CP induces a *P. aeruginosa* zinc-starvation response *in vitro* and in**
146 **expectorated CF sputum**

147 Studies report elevated levels of zinc in CF sputum (1, 2). Our ICP-MS data show that the
148 sputum sample in our study that elicited the strongest zinc-starvation response had a zinc
149 concentration of ~2 μg/g (~2000 μg/L, ~31 μM) (**Fig. 2a**). Given the concomitant high zinc
150 concentration in our CF sputum samples and the elevated zinc starvation response in *P.*
151 *aeruginosa* grown in these CF sputum samples, it is likely that the zinc in our CF sputum samples
152 is bound by zinc-sequestering proteins. CP is one such host zinc-sequestering protein that is
153 found in high concentrations in the sputum of CF patients (2, 22). CP has also been shown to
154 induce expression of Zur-regulated genes in *P. aeruginosa* strain PA14 (51). Therefore, we
155 hypothesized that CP binds zinc to induce a zinc starvation response in *P. aeruginosa* grown in
156 CF sputum. To test this, we first expressed and purified recombinant human CP as previously
157 described (52) and as illustrated in **Fig. S2**. The ability of our recombinant CP to induce a zinc-
158 starvation response was tested by growing *P. aeruginosa* strain PAO1 *att*::P_{PA3600}-*lacZ* in culture
159 medium (LB), medium containing CP, or medium containing CP and zinc (**Fig. 3a**). CP

160 concentrations in the lung can reach 1 mg/ml (~40 μM) (29), therefore, 1 mg/ml CP was used for
161 all CP-based experiments. Growing *P. aeruginosa* in medium containing 1 mg/ml CP resulted in
162 a four-fold increase in promoter activation (~92 MU) compared to the control (~25 MU) (**Fig. 3a**).
163 The addition of excess zinc (1 mM) in the presence of CP prevented promoter activation (**Fig.**
164 **3a**). These results confirm that our purified recombinant human CP can induce a zinc-starvation
165 response in *P. aeruginosa* which is quenched with the addition of exogenous zinc.

166 Despite the reportedly high concentrations of CP in the serum, sputum, and BALF of CF
167 patients (2, 21-24), *P. aeruginosa* appears to be able to access enough zinc to persist. Various
168 environmental factors may influence CP zinc binding such as calcium concentrations (53), pH
169 (54), or the presence of oxidants (55, 56). Additionally, while CP in its tetrameric state is resistant
170 to proteolytic degradation, CP is susceptible to oxidation which in turn makes it susceptible to
171 proteolytic degradation by both host and bacterial proteases (55, 56). Because it was unclear if
172 CP in sputum would remain intact and/or active to bind zinc, we tested the ability of recombinant
173 human CP to bind zinc and thereby induce a zinc-starvation response in *P. aeruginosa* grown in
174 CF sputum. *P. aeruginosa* strain PAO1 *att*::P_{PA3600}-*lacZ* was grown in unamended CF sputum,
175 sputum supplemented with 1 mM zinc, and sputum supplemented with both 1 mM zinc and 1
176 mg/ml (~40 μM) CP (**Fig. 3b**). The addition of zinc lowered *PA3600* promoter activity in sputum
177 (**Fig. 3b**), supporting our NanoString data (**Fig. 1c**), while addition of CP to zinc-amended sputum
178 significantly prevented reduction of promoter activity (**Fig. 3b**). These data confirm that
179 recombinant CP added to CF sputum remains intact to bind zinc, which induces a zinc starvation
180 response in colonizing *P. aeruginosa*.

181 While recombinant CP added to zinc-amended sputum increased *P. aeruginosa PA3600*
182 promoter activity on average compared to zinc-amended sputum controls, the CF sputum
183 samples tested varied in their responses (**Fig. 3b, Fig. 1b**). The inverse correlation between
184 sputum zinc concentrations and induction of the *PA3600* promoter suggests that sputum samples
185 that result in high promoter activity have lower concentrations of zinc than samples that induce

186 low promoter activity, comparatively (**Fig. 2**). The high promoter activity by *P. aeruginosa* was
187 readily quenched by the addition of zinc but remained high when CP was also added (**Fig. 3b**;
188 green, lavender, lilac). Conversely, the low promoter activity by *P. aeruginosa* grown in sputum
189 samples with presumably high zinc is not affected greatly by the addition of zinc nor CP (**Fig. 3b**;
190 pink, light pink, gray). Overall, these data show that addition of recombinant CP to zinc-amended
191 sputum can induce a zinc-starvation response dependent on sputum zinc concentration.
192

193 **Zinc metalloproteases are enriched amongst *P. aeruginosa*-secreted zinc-binding proteins**

194 Since both TPEN and CP were confirmed to bind zinc and induce a zinc-starvation
195 response in *P. aeruginosa* in culture medium (**Fig. 1a**, **Fig. 3a**), we wanted to further measure the
196 effects of TPEN- and CP-mediated zinc sequestration on *P. aeruginosa* growth. Addition of TPEN
197 or CP to cultures grown in LB decreased the final OD_{600} of *P. aeruginosa* compared to control
198 conditions (**Fig. 3d**), but neither inhibited earlier growth stages (**Fig. 3c**). These data show that *P.*
199 *aeruginosa* grows in the presence of CP under the conditions tested.

200 While CP does not prevent the growth of *P. aeruginosa*, little is known about how CP-
201 mediated zinc starvation affects *P. aeruginosa* physiology. Unlike the chemical chelator TPEN,
202 CP is not membrane permeable and instead exerts its effects on pathogens by binding metals in
203 the extracellular environment. CF sputum has been reported to contain high concentrations of
204 both CP (2, 22, 23) and secreted *P. aeruginosa* proteases including zinc metalloproteases (57).
205 We performed a UniProt Knowledgebase (UniProtKB) analysis of the *P. aeruginosa* strain PAO1
206 proteome, which identified at least 72 zinc-binding proteins (**Table 2**). Of those 72, 64 were
207 described by Gene Ontology (GO) molecular function as having catalytic activity (**Table 2**), which
208 is consistent with the role of zinc as a cofactor. Of those 64 zinc-binding enzymes, 12 were further
209 described as proteases and 5 of those were secreted zinc metalloproteases LasB, LasA, AprA,
210 ImpA, and PepB (**Table 2**). We performed a second UniProtKB analysis of the *P. aeruginosa*
211 strain PAO1 proteome that identified at least 34 secreted proteins, of which 6 were proteases and

212 included the 5 aforementioned zinc metalloproteases in addition to Protease IV (PA4175).
213 UniProtKB does not show Protease IV as binding zinc, but Protease IV has been described as a
214 zinc metalloprotease and its enzymatic activity is reduced in a *P. aeruginosa* mutant lacking the
215 zinc importer-encoding gene *znuA* (14). These analyses suggest that 83-100% of secreted
216 proteases, important virulence factors, are zinc metalloproteases. Overall, previously published
217 studies and curated databases suggest that CP and *P. aeruginosa*-secreted zinc
218 metalloproteases are abundant in the extracellular milieu of the CF mucus environment.
219

220 **Zinc chelation inhibits LasB-mediated proteolysis**

221 Given the importance of zinc to the activity of zinc metalloenzymes, we hypothesized that
222 zinc chelation by TPEN and CP would inhibit the activity of secreted zinc metalloproteases. Our
223 initial studies suggested that LasB and LasA accounted for the majority of proteolytic activity by
224 *P. aeruginosa* strain PAO1 (WT) because filtered supernatants from Δ*lasAB* cultures spotted onto
225 milk plates cleared the milk plates substantially less than filtered WT supernatants (**Fig. 4a**, inset
226 i-ii). As a result, this study focuses on the effect of zinc chelation on LasB and LasA activity.

227 To test the above hypothesis, LasB activity was determined quantitatively using azocasein
228 as a substrate. The azocasein degradation assay was previously described to measure total
229 proteolytic activity (14). However, by comparing the ability of *P. aeruginosa* WT, Δ*lasA*, and
230 Δ*lasAB* supernatants to degrade azocasein, we found that azocasein degradation was LasB-
231 dependent under the conditions tested (**Fig. 4a**). As a result, we tested the effect of TPEN and
232 CP on LasB activity using the azocasein degradation assay. *P. aeruginosa* supernatants were
233 filtered and then left untreated, treated with TPEN or CP, or treated with both TPEN or CP and
234 zinc. Treatment with TPEN or CP inhibited LasB enzymatic activity while addition of excess zinc
235 (1 mM) in the presence of TPEN or CP restored LasB activity (**Fig. 4b**, **Fig. 4c**). Furthermore,
236 treatment of Δ*lasAB* supernatants with TPEN (**Fig. S3a**) or CP (**Fig. S3b**) without or with the
237 addition of excess zinc did not alter azocasein degradation. Therefore, treatment of *P. aeruginosa*

238 cell-free supernatants with zinc chelators TPEN and CP inhibits LasB-mediated caseinolytic
239 activity.
240
241 **Zinc chelation inhibits LasA-mediated lysis of *S. aureus***
242 LasA activity was determined by monitoring the decrease in absorbance at 595 nm of a
243 heat-killed *S. aureus* suspension as previously described (14). Use of *P. aeruginosa* strain PAO1
244 (WT), Δ*lasA*, and Δ*lasA*+*lasA* (complemented mutant) supernatants confirmed that LasA is
245 necessary for the lysis of *S. aureus* and that this assay measures LasA-mediated lysis of *S.*
246 *aureus* under the conditions tested (**Fig. 5a-b**). This assay was then used to measure LasA
247 activity in *P. aeruginosa* cell-free supernatants left untreated, treated with TPEN or CP, or treated
248 with both TPEN or CP and zinc. Treatment of supernatants with TPEN or CP inhibited LasA
249 activity while treatment with TPEN or CP in the presence of excess zinc (500 μM and 160 μM,
250 respectively) restored LasA activity (**Fig. 5c-f**). Furthermore, treatment of Δ*lasA* supernatants with
251 zinc, TPEN, or CP had no effect on lysis of *S. aureus*, confirming that treatment of supernatants
252 did not have LasA-independent cytotoxic effects on *S. aureus* (**Fig. S5**). Therefore, treatment of
253 *P. aeruginosa* cell-free supernatants with zinc chelators TPEN and CP inhibits LasA-mediated
254 lysis of *S. aureus*.
255
256 **Discussion**
257 Here we show that *P. aeruginosa* strain PAO1 grown in aliquots of expectorated CF
258 sputum exhibits a zinc-starvation response despite relatively high concentrations of zinc in the
259 sputum samples. Treatment with recombinant host CP was sufficient to induce a zinc-starvation
260 response in *P. aeruginosa* grown in zinc-amended CF sputum samples from different subjects,
261 demonstrating that CP retains its function in sputum. Furthermore, treatment of *P. aeruginosa*
262 supernatants with CP inhibited the activity of secreted, extracellular zinc metalloproteases LasB
263 and LasA. The data presented in this study support a model in which CP released from recruited

264 neutrophils sequesters zinc from the environment to induce a zinc-starvation response in *P.*
265 *aeruginosa* and sequesters zinc from secreted virulence factors including zinc-dependent
266 metalloproteases LasA and LasB inhibiting *S. aureus* lysis, degradation of peptides, and/or
267 nutrient acquisition (**Fig. 6**).

268 A variety of strategies have been used to learn about the environment that *P. aeruginosa*
269 encounters in the CF lung including analysis of bacteria grown in buffered media supplemented
270 with CF sputum compared to bacteria grown in laboratory media (8, 9), and direct analysis of
271 gene expression by bacteria in expectorated CF sputum (4, 5, 58). While studies have varied in
272 their techniques, transcriptomic analyses have found that genes induced by low intracellular zinc
273 are elevated in sputum samples relative to controls (4-9). Our model differs from previous models
274 as it measures the transcriptional response of *P. aeruginosa* grown directly in expectorated
275 sputum from a variety of CF patients. Our study also found that *P. aeruginosa* activates its zinc-
276 starvation response in CF sputum on average but revealed differences across samples from
277 different CF donors (**Fig. 1b-c, Fig. 3b**). These findings taken together underscore the fact that
278 *P. aeruginosa* growth in laboratory media would not recapitulate the effect of low-zinc conditions
279 in the context of CF. To this end, our CF sputum model is one way to provide a low-zinc
280 environment and allows for investigation of the response of *P. aeruginosa* across sputum samples
281 from different donors which vary in levels of host factors like CP. This same approach would also
282 enable the investigation of different *P. aeruginosa* strains in sputum aliquots from a single donor.

283 CP concentrations during infections can reach 1 mg/ml or ~40 μM which is often posited
284 to be higher than or in excess of the bioavailable zinc concentration in most environments (29).
285 However, zinc concentrations in CF sputum are high relative to sputum from non-CF individuals
286 and other biological compartments. Smith et al. (1) found that the zinc concentration of 45 CF
287 sputum samples ranged from 678 μg/L (~10 μM) to 1181 μg/L (~18 μM) compared to 103 μg/L
288 (~2 μM) to 597 μg/L (~9 μM) in 8 non-CF sputum samples. Li et al. (3) reported that the zinc
289 concentration of 118 CF sputum samples ranged from ~5 μM to ~145 μM. In this study, the zinc

290 concentration of 8 CF sputum samples ranged from 1.002 μg/g (~15 μM) to 7.562 μg/g (~116 μM)
291 (**Fig. 2a**). Therefore, under certain conditions or in some microenvironments, CP may not be in
292 excess of environmental zinc.

293 There is mounting evidence that divalent-metal sequestration by CP affects *P. aeruginosa*.
294 Wakeman et al. (51) demonstrated that CP-mediated genetic responses in *P. aeruginosa* were
295 reversed upon treatment with zinc *in vitro* and that *P. aeruginosa* and CP colocalized at sites of
296 inflammation within a CF lung explant. D'Orazio et al. showed that CP-mediated growth inhibition
297 was enhanced in *P. aeruginosa* strain Δ*znuA*, which is a mutant lacking the gene encoding the
298 small zinc-binding protein of the ZnuABC zinc importer resulting in reduced intracellular zinc
299 accumulation (13, 14). Zygiel et al. (59) showed that treatment with CP significantly reduced
300 intracellular iron and manganese in *P. aeruginosa*, but did not significantly affect intracellular zinc,
301 though intracellular zinc trended downward (59). Our data show that CP induces a Zur-regulated
302 zinc-starvation response *in vitro* and in expectorated CF sputum which is repressed upon the
303 addition of excess zinc (**Fig. 3a-b**). We also observed CP-mediated growth defects *in vitro* (**Fig.
304 3c**) similar to those reported by Zygiel et al. (59) which were previously attributed to ferrous iron
305 chelation by CP. Taken together, the data show that *P. aeruginosa* and CP colocalize at sites of
306 inflammation in the CF lung and that CP is capable of inducing zinc- and/or iron-starvation
307 responses depending on test conditions.

308 Additionally, while Filkins et al. (60) showed that *in vitro* co-culture of *P. aeruginosa* and
309 *S. aureus* on CF bronchial epithelial cells reduced the viability of *S. aureus*, Wakeman et al. (51)
310 showed that zinc chelation by CP promotes *P. aeruginosa* and *S. aureus* co-culture in *in vitro*, *in
311 vivo*, and *ex vivo* models, in part, by downregulating genes encoding anti-staphylococcal factors
312 such as pyocyanin, hydrogen cyanide, and PQS/HQNO. Interestingly, treatment of *P. aeruginosa*
313 with CP did not reduce the expression of *lasA* though the functionality of LasA was not tested
314 (51). In this study, we show that CP-mediated zinc chelation inhibits LasA-mediated lysis of *S.
315 aureus* by *P. aeruginosa in vitro* (**Fig. 5e-f**). Therefore, while LasA may be expressed and

316 secreted by *P. aeruginosa* in the presence of CP, CP may post-translationally inhibit LasA activity
317 via zinc sequestration. Furthermore, colonization of the CF airways is usually described as a
318 pattern of succession where *S. aureus* is the predominant colonizer early on in younger patients
319 before being outcompeted by *P. aeruginosa* in older patients (60). However, Fischer et al. (61)
320 recently showed that *P. aeruginosa* and *S. aureus* chronically co-colonize the CF lung. Wakeman
321 et al. also showed that *P. aeruginosa*, *S. aureus*, and CP colocalize in CF lung explants (51).
322 Further studies are required to determine if CP modulates protease-dependent and/or protease-
323 independent co-colonization of *P. aeruginosa* and *S. aureus* in the CF lung.

324 Notably, *P. aeruginosa* strains chronically adapted to the CF lung, including *lasR* loss-of-
325 function (LasR-) mutants, have a reduced capacity to outcompete *S. aureus* (62). LasR is a QS
326 regulator that positively regulates the expression and secretion of several virulence factors
327 including zinc metalloproteases LasB, LasA, AprA, ImpA, PepB, and Protease IV (34, 35).
328 However, LasR- strains commonly arise during chronic CF infection and are associated with
329 worse lung function (63-68). While LasR- strains are common in CF infections, virulence factors
330 regulated by LasR such as zinc metalloproteases are still reported to be abundant in CF sputum
331 (57). Recent work by Mould et al. showed that when LasR+ and LasR- strains were cocultured,
332 the LasR+ strain increased production of RhlR-controlled virulence factors by the LasR- strain
333 (69). Interestingly, LasB and LasA are reportedly regulated by both the LasR and RhlR QS
334 regulators (35). Therefore, further investigation is needed to understand how intra- and
335 interspecies interactions within populations colonizing the CF airway impact the secretion and
336 function of virulence factors such as zinc metalloproteases LasB and LasA.

337 LasB is an abundant protease with broad substrate specificity that is implicated in amino
338 acid liberation and consumption (70). In addition to nutrient acquisition, LasB also plays a role in
339 the ability of *P. aeruginosa* to invade host epithelial cells (39) and to evade host immune
340 responses via processes such as degrading cytokines (40). Interestingly, degradation of pro-
341 inflammatory cytokines IL-8 and IL-6 by LasB reduces neutrophil recruitment and the overall IL-8

342 and IL-6 response (40). While LasB-mediated cytokine degradation has been reported to reduce
343 neutrophil recruitment, LasB can also induce neutrophil extracellular traps (NETs) (71, 72).
344 Neutrophils recruited to sites of inflammation can release CP through processes such as NET
345 formation (73) and in this study we show that CP-mediated zinc chelation inhibits the activity of
346 secreted LasB (**Fig. 4c**). Taken together, there appears to be a complex interplay between LasB,
347 neutrophils, and CP during the course of infection which may contribute to exacerbations in CF.
348 Furthermore, recent work suggests that secreted LasB activates Protease IV which then
349 predominantly processes and activates LasA (36, 37). Therefore, CP-mediated inhibition of
350 secreted LasB activity may have downstream effects on the processing and activity of other
351 secreted zinc metalloproteases.

352 In conclusion, the results of our study show that CP can induce a zinc-starvation response
353 in *P. aeruginosa* in CF sputum as well as chelate zinc to inhibit the activity of virulence-associated
354 zinc metalloproteases. Future studies will focus on how competition for zinc in a zinc-limited or
355 zinc-chelating environment such as CF mucus shapes polymicrobial infections and patient
356 outcomes, particularly considering the observed variability in zinc concentration and availability
357 across CF patients.

358 **Materials and Methods**

359 **Strains and growth conditions**

360 Bacterial strains and plasmids used in this study are listed in **Table S1**. *P. aeruginosa* and
361 *Escherichia coli* strains were maintained on lysogeny broth (LB) (1% tryptone, 0.5% yeast extract,
362 0.5% NaCl) with 1.5% agar and routinely grown in LB on a roller drum at 37°C LB. *P. aeruginosa*
363 plasmid strains were maintained by supplementing media with 300 µg/ml carbenicillin or 60 µg/ml
364 gentamicin. *E. coli* plasmid strains were maintained by supplementing media with 100 µg/ml
365 carbenicillin. *S. aureus* SH1000 was maintained on trypticase soy with 1.5% agar (TSA) or grown
366 in trypticase soy broth (TSB) on a roller drum at 37°C. *Saccharomyces cerevisiae* strains for
367 cloning were maintained on yeast-peptone-dextrose (YPD) medium with 2% agar.

368

369 **Construction of plasmids**

370 Primers used for plasmid construction are listed in **Table S2**. All plasmids were sequenced at the
371 Molecular Biology Core at the Geisel School of Medicine at Dartmouth. Plasmid GH121_P_{PA3600}-
372 *lacZ* (DH3229) was constructed using a *S. cerevisiae* recombination technique as previously
373 described (74). Plasmid GH121_P_{pqsA}-*lacZ* served as the vector backbone for this construct.
374 GH121_P_{PA3600}-*lacZ* was purified from yeast using Zymoprep™ Yeast Plasmid Miniprep II
375 according to manufacturer's protocol and transformed into electrocompetent *E. coli* strain S17 by
376 electroporation. The plasmid was introduced into *P. aeruginosa* by conjugation and recombinants
377 were obtained using sucrose counter-selection and genotype screening by PCR.

378

379 Complementation plasmid pMQ70_*lasA* was generated using the NEBuilder HiFi DNA assembly
380 cloning kit (New England BioLabs). *P. aeruginosa* strain PAO1V Δ*lasA* was complemented *in*
381 *trans* by inserting a functional copy of *lasA* amplified from PAO1V genomic DNA into plasmid
382 pMQ70 under the control of the arabinose-inducible *BAD* promoter generating plasmid
383 pMQ70_*lasA*. Plasmid pMQ70_*lasA* was transformed into Δ*lasA* by electroporation.

384

385 **Cystic Fibrosis (CF) sputum collection**

386 Sputum samples were collected in accordance with protocols approved by the Committee for the
387 Protection of Human Subjects at Dartmouth. Expectorated sputum samples used in this study
388 were collected from adult subjects with CF during a routine office visit or upon admission for
389 treatment of a disease exacerbation. Sputum samples were frozen upon collection and stored at
390 −80°C until use.

391

392 **Beta-galactosidase (β-Gal) assay**

393 *P. aeruginosa* cells with a promoter fusion to *lacZ* integrated at the *att* locus were grown in 5 mL
394 cultures of LB at 37°C for 16 h. Overnight cultures were diluted 1:50 in 50 ml culture medium (LB
395 or M63) and then grown to an OD_{600} of 0.5. The cells were then centrifuged at 4,500 x g for 10
396 min, resuspended in culture medium, centrifuged at 10,000 x g for 2 min, and then resuspended
397 in 500 µl culture medium. Ten µl of cell suspension were added per 100 µl culture medium or
398 sputum sample in a 2 ml microcentrifuge tube. Samples were incubated at 37°C with shaking for
399 3 h. β-Gal activity was measured as described by Miller (75) using 50 µl of sample.

400

401 **RNA isolation and NanoString analysis**

402 Unamended sputum or sputum amended with 1 mM $ZnSO_4 \cdot 7\ H_2O$ or $(NH_4)_2Fe(SO_4)_2 \cdot 6\ H_2O$
403 (100 µL) was added to 2 ml microcentrifuge tubes. *P. aeruginosa* strain PAO1 was grown in 5 mL
404 cultures of LB at 37°C for 16 h. Overnight cultures were diluted 1:50 in 50 ml M63 minimal medium
405 with 0.2% glucose and then grown to an OD_{600} of 0.5. The cells were then centrifuged at 4,500 x
406 g for 10 min, washed with water, centrifuged, and then resuspended in 500 µl water. Ten µl of cell
407 suspension were added per 100 µl M63 minimal medium with 0.2% glucose (control) or sputum
408 sample in a 2 ml microcentrifuge tube. Samples were then incubated at 37°C with shaking for 3
409 h. TriZol (900 µl) was added to 100 µl sputum containing 10 µl of PAO1 cell suspension. Samples

410 were stored overnight. RNA was prepared following DirectZol kit instructions and eluted in 50 µl
411 water.
412
413 For NanoString, 5 µl of a 1:10 dilution of RNA was used. Diluted RNA was applied to the codeset
414 PaV4 and processed as previously reported (49). Counts were normalized to the geometric mean
415 of spiked-in technical controls. Normalized counts were used for Z-score calculations and
416 heatmap construction.
417
418 **Measurement of zinc in sputum samples**
419 Sputum samples for zinc analysis were stored at −80°C until processed. Sputum zinc was
420 quantified by inductively coupled plasma-mass spectrometry (ICP-MS) following nitric acid
421 digestion of organic material according to the method of Heck et al. and is expressed as µg zinc
422 per g of sputum (76). ICP-MS was performed by the Dartmouth Trace Element Analysis (TEA)
423 Core.
424
425 **Expression and Purification of recombinant calprotectin (CP)**
426 Plasmid S100A8/A9 was obtained from Futami et al. (52) and recombinant CP was expressed
427 and purified as previously described with minor modification. Plasmid S100A8/A9 was first
428 confirmed by Sanger sequencing and then transformed into *E. coli* T7 Express cells. Transformed
429 T7 Express cells were then grown in LB containing 100 µg/ml carbenicillin at 37°C with shaking
430 and induced at about an OD_{600} of 0.5 with 0.5 mM β-D-1-thiogalactopyranoside (IPTG) for 3 h.
431 Cultures were centrifuged at 13,260 x g for 10 min at 4°C. Supernatant was discarded. Cell pellets
432 were resuspended in 30 ml wash solution (150 mM NaCl), transferred to a 50 ml conical tube,
433 and then centrifuged at 3,210 x g for 10 min at 4°C. Supernatant was discarded. Pellets were
434 weighed and then stored at -20°C.
435

436 Cell pellets were resuspended in 85 mL lysis buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM
437 $MgCl_2$) supplemented with Benzonase-HC to control viscosity of the sample. Cells were then
438 lysed using the microfluidizer with 3 passages at 18,000 psi. Final volume was about 100 ml. 15%
439 polyethylenimine (PEI) was added dropwise to a final concentration of 0.7% to precipitate nucleic
440 acids (about 5 ml). Samples were then centrifuged at 23,280 x g for 10 min at 4°C. Pellet
441 containing intact cells and precipitated nucleic acids was discarded. NH_4SO_4 (61.27 g) was added
442 slowly to clarified supernatant (about 115 ml) while stirring at 4°C until a saturation of 80%. The
443 sample become gradually turbid. Sample was stirred for an additional 30 min after complete
444 saturation. Sample was then centrifuged at 23,280 x g for 10 min at 4°C. Supernatant was
445 discarded and the pellet was dissolved in about 30 ml solubilization buffer (50 mM Tris-HCl pH
446 7.5, 30 mM dithiothreitol [DTT]) and incubated for 1 h at 37°C. Dissolved pellet was transferred to
447 dialysis cassettes and dialyzed overnight in 50 mM sodium phosphate pH 6.0 at 4°C using 3.5
448 kDa cut-off dialysis cassettes to change buffer. Sample was then centrifuged at 23,280 x g for 10
449 min at 4°C to remove any pellet.
450
451 CP was then purified using a HiTrap SP column (stored in 20% ethanol). The column was washed
452 with 5 column volumes (CV) of H_2O at about 5 ml/min. The column was then washed with 5 CV
453 of 100% SP Sepharose HP buffer B (50 mM sodium phosphate pH 6.0, 1 mM DTT, 1 M NaCl;
454 filtered/degassed) at about 5 ml/min. The column was equilibrated with 10 CV of SP Sepharose
455 HP buffer A (50 mM sodium phosphate pH 6.0, 1 mM DTT; filtered/degassed) at about 5 ml/min.
456 A superloop was assembled with the appropriate volume for sample application. Sample was then
457 loaded in the column using the superloop at 2.5 ml/min. The column was then washed with 10
458 CV of SP Sepharose HP buffer A at about 5 ml/min. The column was then washed with a step
459 gradient of SP Sepharose HP buffer B: 5 CV of 5% SP Sepharose HP buffer B, 10 CV at 30% SP
460 Sepharose HP buffer B and 5 CV at 100% SP Sepharose HP buffer B at about 5 ml/min.

461 Fractions were analyzed using SDS-PAGE (15% gel) and the appropriate fractions were then
462 pooled.
463
464 CP was then purified using a HiLoad 26/600 Sephadex S75 and CP buffer (50 mM Tris-HCl pH
465 7.5, 150 mM NaCl, 1 mM DTT; filtered/degassed). Sample (about 13 ml) was loaded in a 50 ml
466 superloop. Sample was then run on the HiLoad 26/600 Superdex 75p, a program composed of 2
467 CV equilibration, injection of 12 ml sample and elution with 1.2 CV at 2.6 ml/min. Flow rate is 2.6
468 ml/min and collection of 7 ml/tube. Tubes corresponding to three different fractions were pooled
469 to make fractions F1_I, F2_I, and F3_I. All other tubes containing calprotectin from both HiTrap
470 runs were concentrated using YM-10 Amicon centrifugal filters and re-loaded in the HiLoad
471 26/600 superdex 75 as before. Tubes corresponding to three different fractions were pooled to
472 make fractions F1_II, F2_II, and F3_II. Samples from all six fractions were analyzed using SDS-
473 PAGE (4-12% gel). Fractions F1_I and F1_II, F2_I and F2_II, and F3_I and F3_II were combined
474 to make fractions F1, F2, and F3, respectively. Fractions were concentrated with YM-10 Amicon
475 centrifugal filters. The final concentrations of the fractions were determined using a Bradford
476 protein assay.
477
478 **Protease assays**
479 *P. aeruginosa* culture supernatants were used for protease assays. 5 ml overnight cultures in LB
480 were centrifuged at 4,500 x g for 10 min. Supernatants were then filter sterilized using a 0.22 µm
481 syringe filter. For TPEN experiments, undiluted supernatants were used. For CP experiments,
482 stored aliquots of CP were first diluted to 3 mg/ml in CP buffer without DTT (50 mM Tris-HCl pH
483 7.5, 150 mM NaCl). Then 1 part 3 mg/ml CP was added to 2 parts supernatant for a final
484 concentration of 1 mg/ml.
485

486 Caseinolytic activity was determined qualitatively by spotting *P. aeruginosa* supernatants onto 1%
487 milk plates or quantitatively using azocasein as a substrate as previously described (14) with
488 modification. In brief, *P. aeruginosa* culture supernatants were treated overnight (16 h) with 50
489 μM TPEN or an equivalent volume of 100% EtOH, 1 mg/ml (~40 μM) CP or an equivalent volume
490 of CP buffer without DTT, and/or 1 mM $ZnSO_4 \cdot 7\ H_2O$ or an equivalent volume of diH_2O.
491 Treatment of WT supernatants with 50 μM to 2 mM $ZnSO_4 \cdot 7\ H_2O$ was found not to affect LasB
492 activity (**Fig. S3c**). The supernatants were then incubated at 37°C overnight (16 h). Supernatants
493 (25 μl) were mixed with 150 μl 2% azocasein in 10 mM Tris-HCl, 8 mM $CaCl_2$, pH 7.4. Samples
494 were incubated at 37°C for 15 min. 228 μl of 10% TCA were added to each sample, vortexed,
495 then incubated at room temperature for 15 min. Samples were then centrifuged for 10 min at
496 10,000 x g. Cleared supernatants (100 μl) were added to wells of a 96-well flat-bottom polystyrene
497 plate containing 200 μl 1 M NaOH. Absorbance was read at 440 nm.
498
499 Staphylolytic activity was determined by monitoring the decrease in absorbance at 595 nm of a
500 heat-killed *S. aureus* suspension as previously described (14) with modification. *S. aureus* strain
501 SH1000 (77) was cultured in TSB overnight (16 h) at 37°C with rolling. Cultures were centrifuged
502 at 4,500 x g for 10 min, resuspended in 20 mM Tris-HCl, pH 8.8 to a final OD_{600} of 1.0, and then
503 killed by heating at 100°C for 30 min. Heat-killed *S. aureus* suspensions were cooled to room
504 temperature before use. *P. aeruginosa* culture supernatants were treated overnight (16 h) with 50
505 μM TPEN or an equivalent volume of 100% EtOH, 1 mg/ml (~40 μM) CP or an equivalent volume
506 of CP buffer without DTT, and/or 160-500 μM $ZnSO_4 \cdot 7\ H_2O$ or an equivalent volume of diH_2O.
507 Because increasing concentrations of zinc were previously reported to inhibit LasA activity (46),
508 an appropriate concentration of zinc to use in add-back experiments was determined
509 experimentally. For undiluted WT supernatants, the addition of 500 μM zinc had no effect on LasA
510 activity, while increasing concentrations of zinc inhibited LasA-mediated lysis of *S. aureus* (**Fig.
511 S4a-b**). Therefore, we used 500 μM zinc for TPEN-based experiments. For CP-buffer diluted WT

512 supernatants, the addition of 50 μM zinc had no effect on LasA activity, while increasing
513 concentrations of zinc inhibited LasA-mediated lysis of *S. aureus* (**Fig. S4c-d**). However, a
514 tetramer of CP can potentially bind up to four zinc ions. Therefore, to ensure that zinc would be
515 in excess in CP-based experiments, we used 160 μM zinc which was four times the concentration
516 of CP but still less than 250 μM zinc which was the concentration tested that started to inhibit
517 LasA activity independent of CP. *P. aeruginosa* supernatants (20 μl) were added to 180 μl of heat-
518 killed *S. aureus* in wells of a 96-well flat-bottom polystyrene plate. Staphylolytic activity was
519 determined by monitoring the change in absorbance at 595 nm every 15 min for 3 h using a plate
520 reader. The plate was shaken before each read.
521

522 **Statistical analysis**

523 Statistical analysis was performed using GraphPad Prism 8 and results were expressed as the
524 mean values plus or minus standard deviations. Unless otherwise noted, one-way analysis of
525 variance (ANOVA) followed by Tukey's multiple-comparison test was performed to determine
526 statistical significance of the data. See the figure legends for other specific statistical tests used.
527

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778

779 **Figure Legends**

780 **Fig. 1** *P. aeruginosa* inoculated into expectorated CF sputum from different donors exhibits a
781 zinc-starvation response. (**a**) *P. aeruginosa* strain PAO1 P_{PA3600}-*lacZ* was grown in LB (Control),
782 LB with 50 μM TPEN (TPEN), or LB with 50 μM TPEN and 1 mM $ZnSO_4 \cdot 7\ H_2O$ (TPEN+Zn) for
783 3 h. The data shown represent the mean ± SD from three independent experiments. (**b**) *P.*
784 *aeruginosa* strain PAO1 P_{PA3600}-*lacZ* was grown in M63 (Control), M63 with 50 μM TPEN (TPEN),
785 or expectorated CF sputum (sputum) for 3 h. Each point in the sputum set indicates a separate
786 sample from a different donor. The data were analyzed by Brown-Forsythe and Welch ANOVA
787 with Dunnett's T3 multiple comparisons test. (**c**) *P. aeruginosa* strain PAO1 was inoculated into
788 M63 (M63) or into sputum from two different donors (Sputum 1 and Sputum 2). The sputum was
789 divided and left untreated (Sputum), treated with 1 mM $ZnSO_4 \cdot 7\ H_2O$ (Sputum+Zn), or treated
790 with 1 mM $(NH_4)_2Fe(SO_4)_2 \cdot 6\ H_2O$ (Sputum+Fe). Each condition was analyzed in triplicate. The
791 same lowercase letters indicate samples that are not significantly different and different lowercase
792 letters indicate significant differences ($p<0.05$). *$p<0.05$, **$p<0.01$

793

794 **Fig. 2** Activation of the *PA3600* promoter in CF sputum by *P. aeruginosa* is inversely correlated
795 with total sputum zinc concentration. (**a**) *P. aeruginosa* strain PAO1 P_{PA3600}-*lacZ* was inoculated
796 into 8 different CF sputum samples. Zinc concentration of the same 8 CF sputum samples was
797 determined by ICP-MS. B-Gal activity on the left y-axis (Miller Units; gray bars) was then
798 compared to sputum zinc concentration on the right y-axis (μg/g; red dots), (**b**) Pearson correlation
799 matrix comparing B-Gal activity (Miller units), sputum zinc concentration, sputum iron
800 concentration, and sputum manganese concentration. *$p<0.05$, **$p<0.01$

801

802 **Fig. 3** Recombinant human CP added to CF sputum and culture medium induces a zinc-starvation
803 response by *P. aeruginosa*. (**a**) *P. aeruginosa* strain PAO1 P_{PA3600}-*lacZ* was grown in culture
804 medium (Control), medium with 40 μM CP (CP), or medium with 40 μM CP and 1 mM $ZnSO_4 \cdot 7$

805 H_2O (CP+Zn) for 3 h. The data shown represent the mean ± SD from three independent
806 experiments. (**b**) *P. aeruginosa* strain PAO1 P_{PA3600}-*lacZ* was inoculated into CF sputum from 11
807 different donors. The sputum was divided and left untreated (Control), treated with 100 μM $ZnSO_4$
808 • 7 H_2O (Zn), or treated with 40 μM CP and 100 μM $ZnSO_4$ • 7 H_2O (CP+Zn) for 3 h. Different color
809 dots represent samples from different donors. The same color dots connected by a line are from
810 the same CF sputum donor. Data were analyzed by RM one-way ANOVA with Tukey's multiple
811 comparisons test. (**c**) Representative growth curves of *P. aeruginosa* strain PAO1 P_{PA3600}-*lacZ*
812 grown in LB, LB containing 50 μM TPEN, or LB containing 40 μM CP. Data shown represent the
813 mean ± SD of three technical replicates and are representative of three independent experiments.
814 (**d**) OD_{600} at 16 h of *P. aeruginosa* strain PAO1 P_{PA3600}-*lacZ* grown in LB, LB containing 50 μM
815 TPEN, or LB containing 40 μM CP. Data shown represent the mean ± SD of three independent
816 experiments. The same lowercase letters indicate samples that are not significantly different and
817 different lowercase letters indicate significant differences (p<0.05).
818
819 **Fig. 4** Zinc chelation inhibits LasB enzymatic activity. (**a**) Filtered supernatants from 16 h cultures
820 of WT, Δ*lasA*, and Δ*lasAB* were incubated with 2% azocasein for 15 min. Inset are images
821 showing the ability of (**i**) WT and (**ii**) Δ*lasAB* cell-free supernatants to clear milk plates after 16 h.
822 (**b**) Filtered supernatants from WT 16 h cultures were left untreated (Control), treated with 50 μM
823 TPEN (TPEN), or treated with 50 μM TPEN and 1 mM $ZnSO_4$ • 7 H_2O (TPEN+Zn) for an additional
824 16 h. Supernatants were then incubated with 2% azocasein for 15 min. The data shown represent
825 the mean ± SD from three independent experiments. (**c**) Filtered supernatants from WT 16 h
826 cultures were left untreated (Control), treated with 40 μM CP (CP), or treated with 40 μM CP and
827 1 mM $ZnSO_4$ • 7 H_2O (CP+Zn) for an additional 16 h. Supernatants were then incubated with 2%
828 azocasein for 15 min. The same lowercase letters indicate samples that are not significantly
829 different and different lowercase letters indicate significant differences (p<0.05). An enzyme unit
830 (U) is defined as 1 μmol min^{-1}.

831

832 **Fig. 5** Zinc chelation inhibits LasA enzymatic activity. (**a**-**b**) Lysis of heat-killed *S. aureus* strain
833 SH1000 by cell-free supernatants from WT, Δ*lasA*, and Δ*lasA*+*lasA* (Δ*lasA* complemented *in*
834 *trans* under the Control of arabinose-inducible P_{BAD}) 16 h cultures. (**c**-**d**) Lysis of heat-killed *S.*
835 *aureus* strain SH1000 by WT and Δ*lasA* cell-free supernatants. WT supernatant was divided and
836 left untreated (WT), treated with 50 μM TPEN (WT+TPEN), or treated with 50 μM TPEN and 500
837 μM $ZnSO_4 \cdot 7\ H_2O$ (WT+TPEN+Zn). (**e**-**f**) Lysis of heat-killed *S. aureus* strain SH1000 by WT and
838 Δ*lasA* cell-free supernatants. WT supernatant was divided and left untreated (WT), treated with
839 40 μM CP (WT+CP), or treated with 40 μM CP and 160 μM $ZnSO_4 \cdot 7\ H_2O$ (WT+CP+Zn). (**a**), (**c**),
840 (**e**) The data represent the mean from three independent experiments. Error bars have been
841 omitted for clarity. (**b**), (**d**), (**f**) Quantification of data in (**a**), (**c**), and (**e**), respectively, using area
842 under the curve (AUC). Data are the mean ± SD from three independent experiments. The same
843 lowercase letters indicate samples that are not significantly different and different lowercase
844 letters indicate significant differences (p<0.05).

845

846 **Fig. 6** Model of the effects of CP-mediated zinc chelation in the CF lung on *P. aeruginosa*. *P.*
847 *aeruginosa* colonizes the mucus in the airways of CF patients to high densities, which in part
848 requires the uptake and utilization of zinc. At high densities, *P. aeruginosa* secretes a variety of
849 quorum sensing-dependent virulence factors including zinc metalloproteases such as LasB and
850 LasA. LasB is a protease that can degrade host proteins, such as elastin, as well as peptides.
851 These degraded proteins/peptides can then be taken up and utilized as nutrients by *P.*
852 *aeruginosa*. LasA is a protease that lyses *S. aureus* by cleaving pentaglycine bridges of
853 peptidoglycan. LasA-mediated lysis of *S. aureus* allows *P. aeruginosa* to take up nutrients
854 released from lysed *S. aureus* as well as to outcompete *S. aureus* in the CF lung. During infection,
855 neutrophils are recruited to sites of infection/inflammation. Neutrophils may then release cellular
856 contents such as CP. CP can then bind bioavailable zinc away from *P. aeruginosa* thus reducing

857 the overall abundance of *P. aeruginosa*, while also inducing a zinc-starvation response by *P.*
858 *aeruginosa*. Additionally, CP can bind zinc away from both LasB and LasA thereby inhibiting their
859 proteolytic activity. Furthermore, LasB and LasA activity have been shown to induce neutrophil
860 extracellular traps (NETs). Therefore, CP-mediated inhibition of LasB and LasA activity may lead
861 to less NET formation and, subsequently, less CP release. Black arrows indicate a positive
862 interaction. Red arrows indicate an inhibitory interaction.

863 **Tables**

864 **Table 1** Zinc metalloproteases secreted by *P. aeruginosa*

Gene Number[a]	PDB Entry[b]	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	N/A	PepB or PaAP, aminopeptidase
PA3724	1EZM	LasB, elastase or pseudolysin
PA4175	N/A	Protease IV, endoprotease

865 [a] From P. aeruginosa genome website, https://www.pseudomonas.com/.

866 [b] From Protein Data Bank (PDB) website, https://www.rcsb.org/.

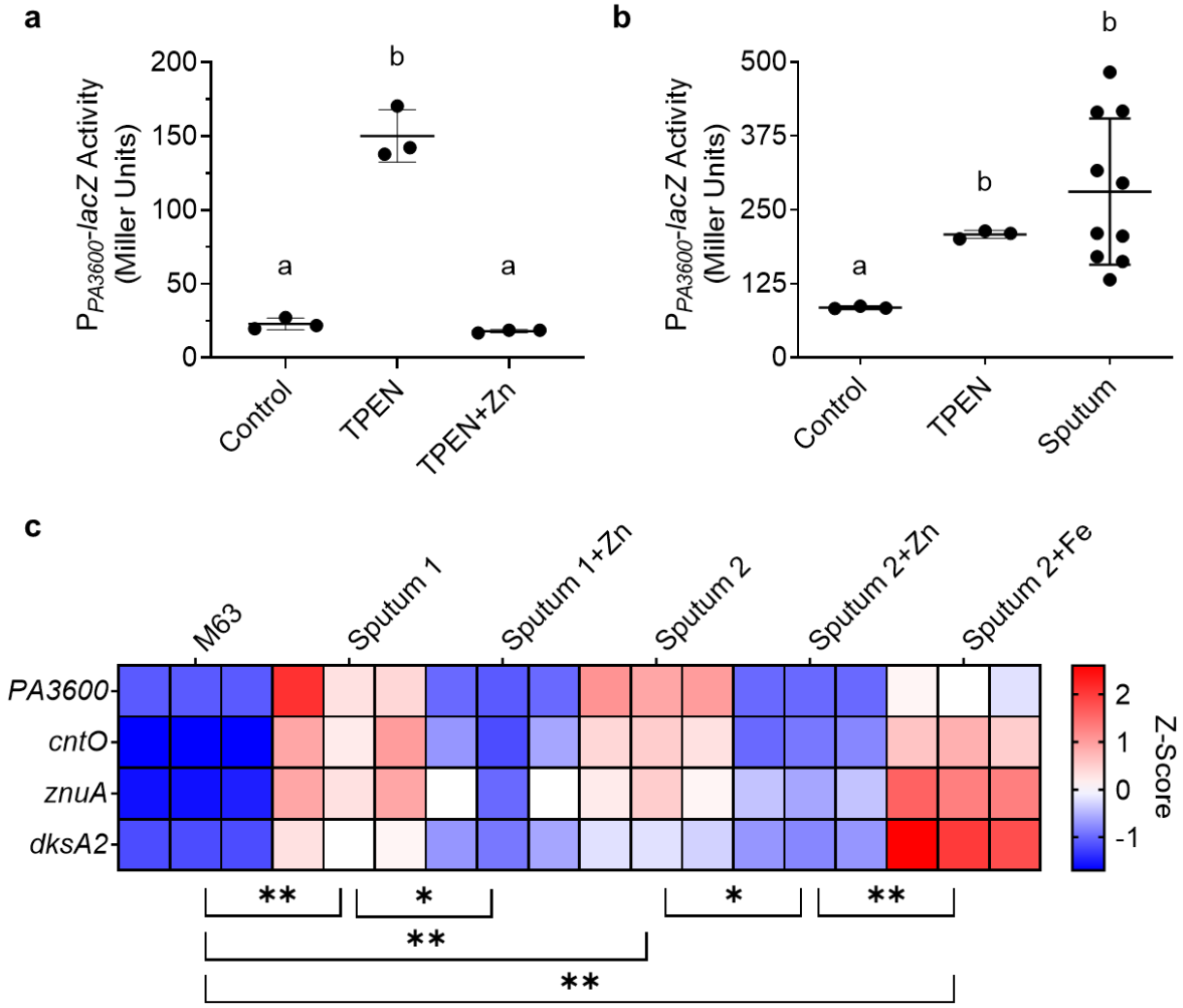
867

868 **Table 2** Characteristics of zinc-binding proteins in *P. aeruginosa* as annotated by UniProtKB[a]

		Subcellular Localization			
GO Molecular Function[b]	**Number of Proteins**	**Secreted**	**Inner Membrane**	**Cytoplasm**	**Not Listed**
Zinc-Binding	72	8	5	21	38
Catalytic Activity: Non-peptidase	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	-	-

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

870 [b] Gene Ontology (GO)



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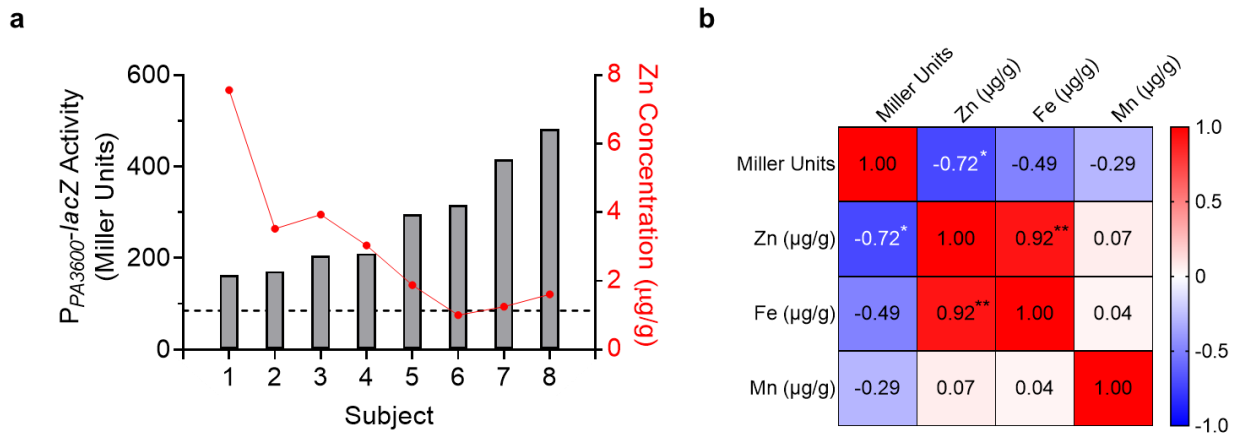

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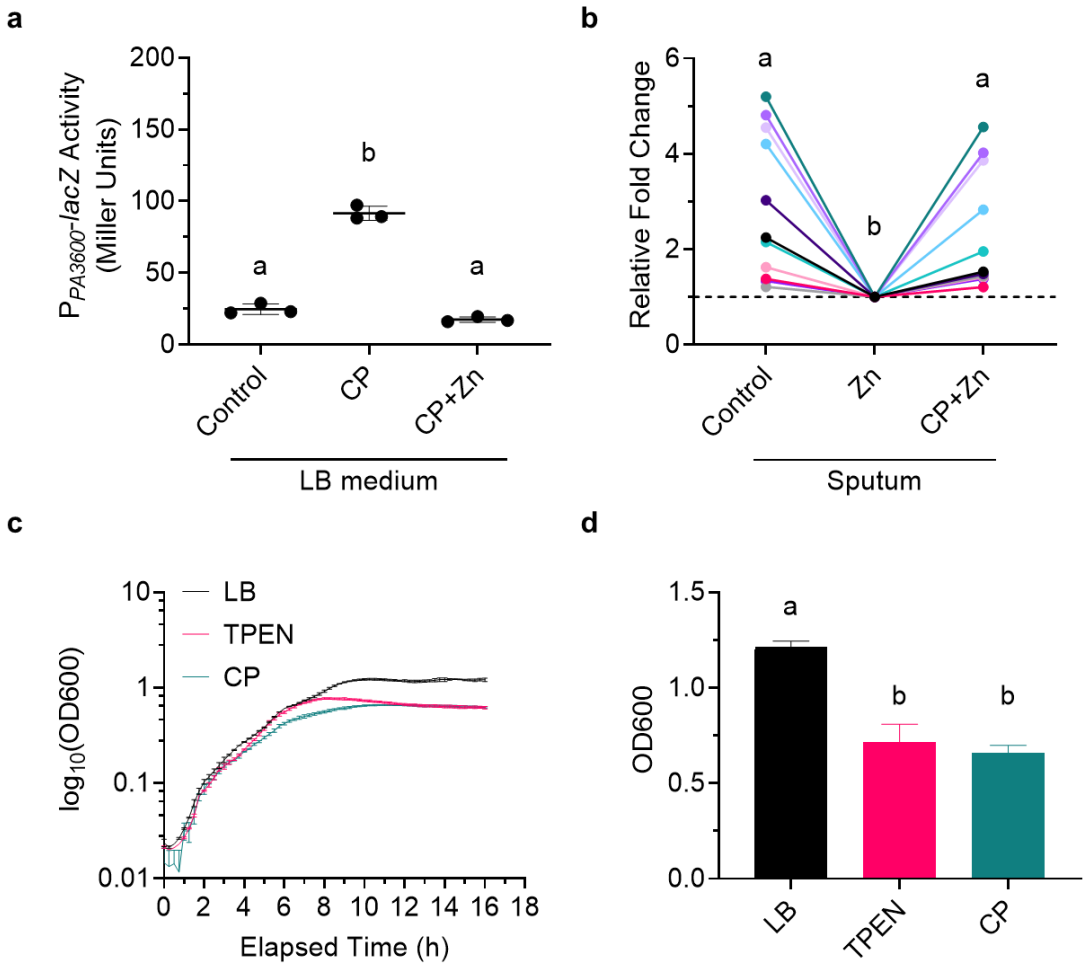

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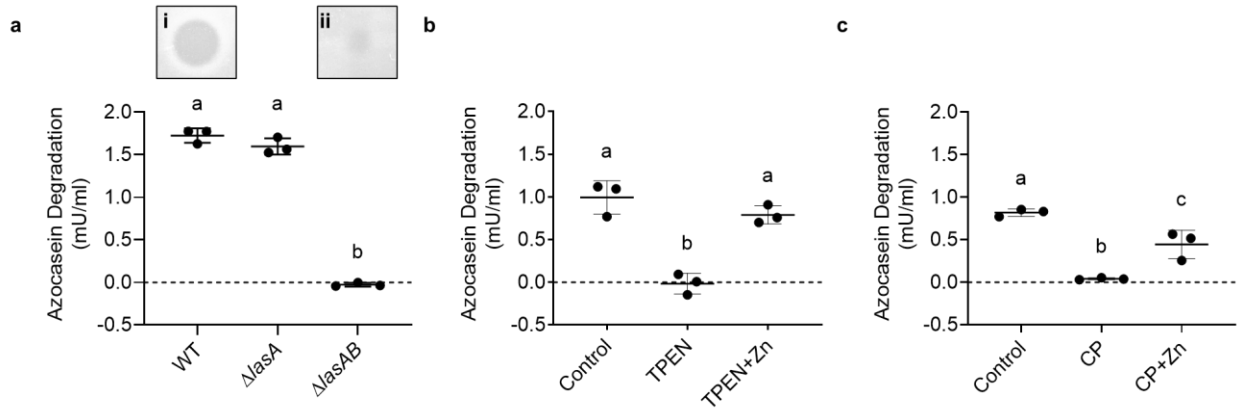

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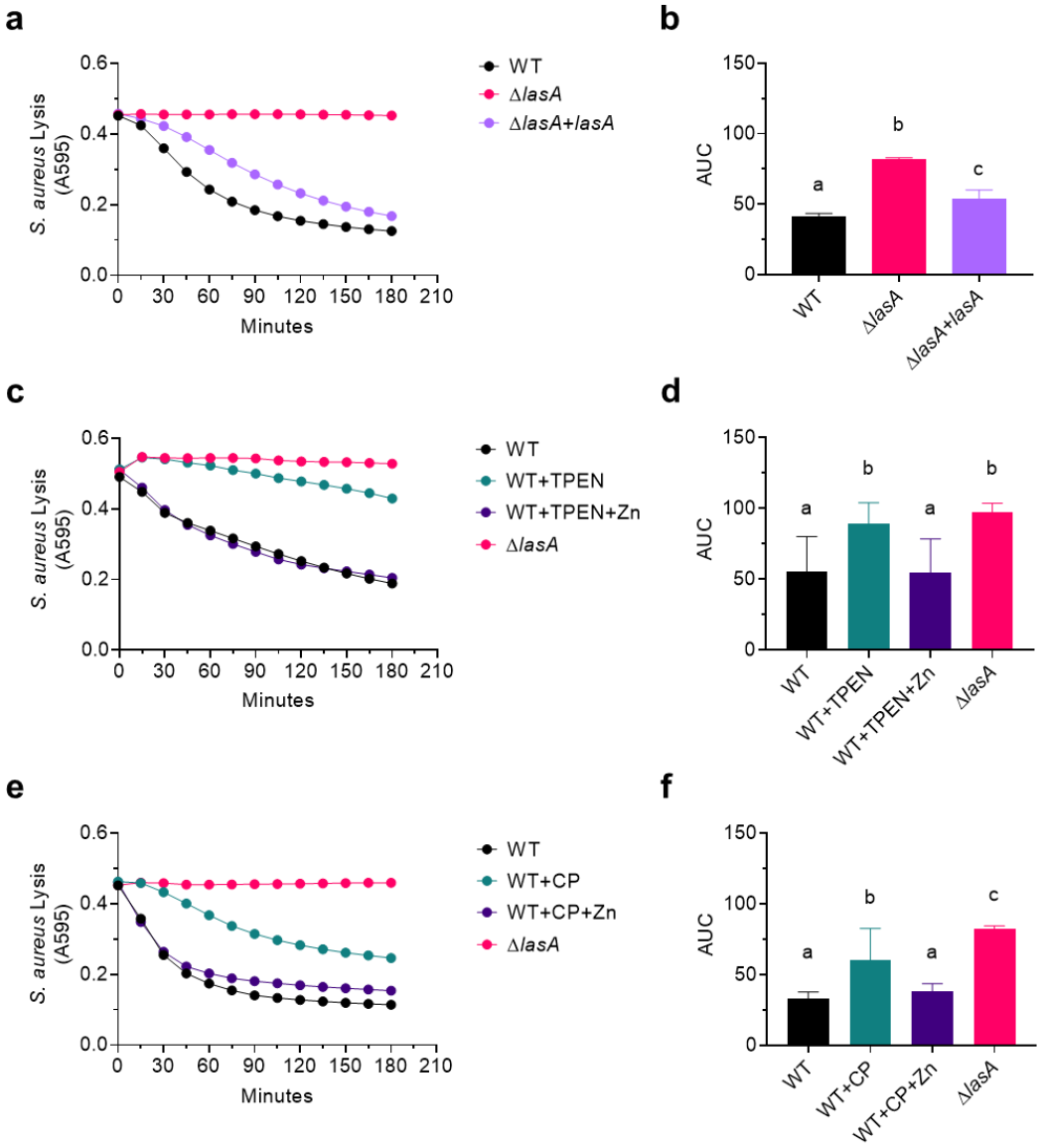

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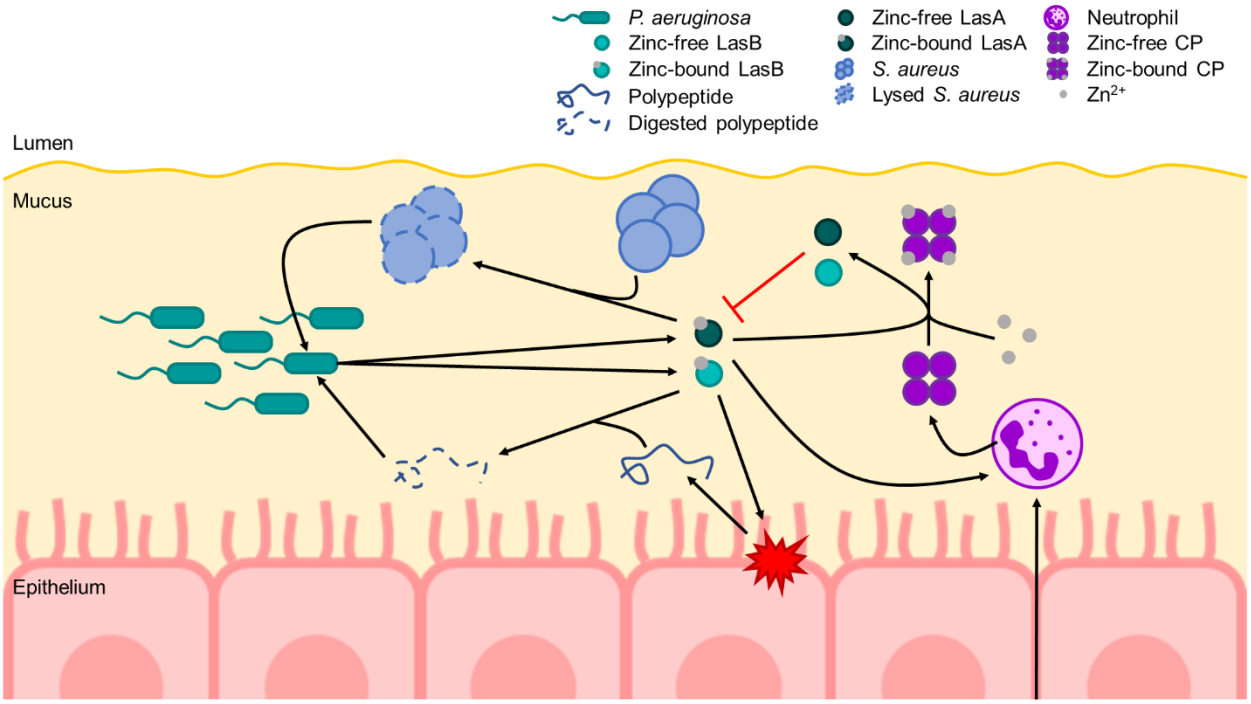

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