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***Pseudomonas aeruginosa* capability to recruit zinc under conditions of limited metal availability is affected by inactivation of the ZnuABC transporter**

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Abstract

The ability of a large number of bacterial pathogens to multiply in the infected host and cause disease is dependent on their ability to express high affinity zinc importers. In many bacteria ZnuABC, a transporter of the ABC family, plays a central role in the process of zinc uptake in zinc poor environments, including the tissues of the infected host. To initiate an investigation into the relevance of the zinc uptake apparatus for *Pseudomonas aeruginosa* pathogenicity, we have generated a *znuA* mutant in the PA14 strain. We have found that this mutant strain displays a limited growth defect in zinc depleted media. The *znuA* mutant strain is more sensitive than the wild type strain to calprotectin-mediated growth inhibition, but both the strains are highly resistant to this zinc sequestering antimicrobial protein. Moreover, intracellular zinc content is not evidently affected by inactivation of the ZnuABC transporter. These findings suggest that *P. aeruginosa* is equipped with redundant mechanisms for the acquisition of zinc that might favor *P. aeruginosa* colonization of environments containing low levels of this metal. Nonetheless, deletion of *znuA* affects alginate production, reduces the activity of extracellular zinc-containing proteases, including LasA, LasB and Protease IV, and decreases the ability of *P. aeruginosa* to disseminate during systemic infections. These results indicate that efficient zinc acquisition is

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critical for the expression of various virulence features typical of *P. aeruginosa* and that ZnuABC also plays an important role in zinc homeostasis in this microorganism.

Introduction

A key feature of the host response to bacterial infections is the activation of strategies aimed at the sequestration of transition metals, such as iron, zinc and manganese, to starve microorganisms from nutrients which are essential for their growth¹. In fact, metals are structural or catalytic cofactors in a wide number of proteins and a reduction in their intake may severely affect the ability of bacteria to multiply and produce virulence factors. The importance of these metals for bacterial physiology has been highlighted by bioinformatics studies which have indicated that about 4% of all bacterial proteins contain non-heme iron and 5-6% of these proteins contain zinc^{2, 3}. Zinc, in particular, is present in enzymes from all six functional classes, including several enzymes participating to central metabolic pathways and a vast assortment of distinct cellular functions.

Strategies to hamper metal recruitment by infectious bacteria, include the release of metal sequestering proteins at mucosal sites of infection, and the activation of metal transport systems which removes metal ions not tightly bound to proteins from extracellular and specific intracellular compartments. To overcome such metal sequestration mechanisms, pathogens produce high affinity metal uptake systems which favor the recruitment of metals in environments where these elements are very scarce and enhance their proliferation in inflamed tissues^{1, 4}. In most Gram-negative bacteria a central factor in the response to zinc deficiency is played the high affinity zinc uptake system ZnuABC⁵. ZnuABC is a member of the ATP-binding cassette-type class of transporters, whose expression is under the transcriptional control of Zur, a metalloregulatory DNA-binding protein with high sensitivity to free intracellular zinc^{6, 7}. In several bacteria, including *Brucella abortus*⁸, *Salmonella enterica*^{9, 10} and *Campylobacter jejuni*¹¹, deletion of the *znuABC* operon or of the gene encoding for ZnuA, the soluble periplasmic component of the transporter, is associated with major defects in growth in zinc poor media and to a dramatic loss of virulence. In other bacteria, additional zinc import systems contribute to metal import under zinc deficiency and disruption of *znuABC* is not associated to clear-cut phenotypes. For example, in response to zinc deficiency *Neisseria meningitidis*¹², *Yersinia pestis*^{13, 14} and *Acinetobacter baumannii*^{15, 16} produce membrane channels which specifically favor the entrance of zinc ions within cells.

Historically *Pseudomonas aeruginosa*, the leading cause of morbidity and mortality in Cystic Fibrosis (CF) patients, has been the focus of intensive research to understand how this organism acquires iron in the tissues of the host¹⁷. These studies have revealed that the sputum from most CF patients chronically infected by *P. aeruginosa* contains high levels of the siderophores pyoverdine and pyochelin, suggesting that these iron-binding molecules have a crucial role in the recruitment of iron during infections. Moreover, the pathways for heme-iron and ferrous ions uptake are active during lung infections¹⁸, underscoring the relevance of an adequate iron supply for the ability of *P. aeruginosa* to colonize this tissue. Quite surprisingly very few studies have explored the relevance of zinc import systems for

this microorganism. This is a significant gap in knowledge if we consider that zinc-dependent enzymes are known to contribute to antibiotic resistance and to different pathways modulating *P. aeruginosa* pathogenicity. For example, the arsenal of *P. aeruginosa* secreted virulence factors include several proteases which use zinc as cofactor¹⁹⁻²² and evidence has been provided for a critical role of zinc in their maturation and activation²⁰.

Nevertheless, a few sparse literature observations support the hypothesis that the mechanisms of zinc uptake play an important role also in *P. aeruginosa*. For example, a study aimed at a large scale isolation of *P. aeruginosa* virulence factors identified as a major candidate a gene whose disruption caused a more than 100-fold reduction in *P. aeruginosa* lethal dose in neutropenic mice²³. This gene, denominated *np20*, encodes Zur, the general regulator of zinc uptake under zinc deficiency²⁴. Subsequent studies have revealed that *np20* is strongly induced by the respiratory mucus isolated by CF patients and, together the whole *znuABC* operon, is significantly up-regulated in bacteria recovered from the sputum of patients²⁵. Moreover, CF lung disease is characterized by a massive and persistent recruitment of polymorphonuclear neutrophils in the airway lumen, leading to the release of large amounts of calprotectin (CP)^{26, 27}. Recent studies have established that CP controls microbial growth by sequestering zinc and manganese ions at sites of infection and that the ability of several pathogens to withstand the antimicrobial activity of CP relies on their ability to produce metal transporters characterized by high affinity for zinc or manganese^{15, 28-30}. The observation that CP is one of the most abundant components of the sputum proteome from CF patients, suggest that the extraordinary ability of *P. aeruginosa* to colonize the inflamed CF lung must be supported by effective strategies to counteract CP-induced metal starvation.

To shed some light on the possible contribution of the Zn import apparatus to the ability of *P. aeruginosa* to colonize the lung of CF patients, we have undertaken a characterization of a mutant strain unable to express a functional ZnuABC importer. Our results reveal that ZnuABC is critical for the expression of different virulence features of this pathogen and suggest that other high affinity zinc import systems contribute to zinc homeostasis in *P. aeruginosa*.

Experimental

Reagents

Antibiotics, bovine serum albumin, 2-nitrophenyl β -D-galactopyranoside (ONPG), ethylenediaminetetraacetic acid disodium salt (EDTA), FeSO₄, ZnSO₄, CuSO₄, MnCl₂ trichloroacetic acid, azocasein, elastin congo-red, hide-remazol brilliant blue R, N-p-Tosyl-Gly-Pro-Lys 4-nitroanilide acetate salt, alginic acid from brown algae and carbazole were purchased from Sigma Aldrich. Ammonium metavanadate was purchased from Acros Organics™. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. High fidelity DNA polymerase Expand was obtained from Roche Diagnostics, GmbH (Mannheim, Germany). All other chemicals were purchased from VWR International. The oligonucleotides were synthesized by Primm (Milan, Italy).

Bacterial strains, plasmids and growth media

The strains, plasmids and primers used in this study are listed in Table 1. *Escherichia coli* and *P. aeruginosa* were routinely grown at 37°C in Luria-Bertani (LB) broth (10 g Bacto tryptone per liter, 5 g yeast extract per liter, 10 g NaCl per liter) eventually solidified by the addition of 1.5% (w/v) agar. *Staphylococcus aureus* SH1000³¹ was grown at 37°C in brain heart infusion (BHI) broth and agar (Becton Dickinson). *P. aeruginosa* was grown under zinc limiting conditions in Vogel-Bonner minimal-medium E (VB-MM: 0.04 g anhydrous MgSO₄ per liter, 2 g citric acid per liter, 10 g anhydrous K₂HPO₄ per liter, 3.5 g NaNH₄HPO₄·4H₂O per liter, 2 g glucose per liter)³². To avoid zinc contamination from glassware, minimal media were prepared in disposable plastic containers and sterilized by filtration in Vacuum Filter-Storage Bottle Systems, 0.22 µm (Corning). Before use, the quality of each minimal medium batch was verified by monitoring the accumulation of ZnuA in *Salmonella* Typhimurium SA140 strain, which is abolished by zinc concentrations below 1 µM⁹.

Pseudomonas isolation agar (PIA; Difco) plates were used to select exconjugants when pEX18Tc²⁹ was used as a suicide vector.

Media were supplemented with antibiotics for marker selection or plasmid maintenance as follows. For *E. coli*, we used 50 µg/ml kanamycin, 10 µg/ml gentamicin, 10 µg/ml tetracycline and 100 µg/ml carbenicillin. For *P. aeruginosa* we used 100 µg/ml gentamicin, 100 µg/ml tetracycline and 500 µg/ml carbenicillin.

Mutant strains construction

Deletion of the *znuA* gene was obtained by the gene replacement method described by Hoang *et al.*³³. Two DNA fragments, flanking and entering into the *znuA* gene, were PCR-amplified using PA14 strain chromosomal DNA as a template and subsequently ligated to generate a DNA region encompassing the *znuA* gene, but lacking a 517 bp fragment of the coding sequence. The primers used to amplify the upstream fragment were PAO4(A), containing an *EcoRI* restriction site at its 5' end, and PAO5, containing a *BamHI* restriction site at its 5' end. This 800 bp fragment (denominated NT) includes at its 3' 264 nucleotides encoding the C-terminus of ZnuA. The primers used to amplify the second DNA fragment were PAO6, containing a *BamHI* site at its 5' end, and PAO7(B), containing a *HindIII* site at its 5' end. This 800 bp DNA fragment (denominated CT) starts 641 nucleotides upstream the start codon of the *znuA* gene.

The NT and CT DNA fragments was digested with the appropriate restriction enzymes, ligated and inserted into the *EcoRI* and *HindIII* sites of plasmid pUC18. Then, the 830 bp FRT-*aacC1*-FRT cassette from plasmid pPS856 (Hoang *et al.*, 1998), which contains the gene that confers resistance to gentamicin (*aacC1*, gentamicin acetyltransferase 3-1 gene) flanked by the Flp recombinase target (FRT) sequences, was inserted in the *BamHI* site between the NT and CT cloned fragments. The 2.43 Kb DNA fragment comprising the NT fragment, the *aacC1* gene and the CT fragment was amplified using primers PAO4(B) and PAO7(B), digested with *HindIII* and inserted into the compatible sites of the gene replacement vector pEX18Tc³³. The final construct, denominated *PaKO_{znuA}pEX18Tc*, was

mobilized from *E. coli* DH5- α to *P. aeruginosa* strains via pRK2013-mediated triparental mating^{34, 35}. Transconjugants were plated onto *Pseudomonas* isolation agar plates containing 100 μ g/ml of gentamicin to select for single-crossover events in *P. aeruginosa*. Gm^R-transconjugants were streaked for isolated colonies on LB-agar plates containing 100 μ g/ml of gentamicin and 5% sucrose to select for the loss of the vector sequence³³.

To remove the gentamicin resistance marker, plasmid pFLP2³³, which contains the Flp recombinase, was transferred from *E. coli* to *P. aeruginosa*. Colonies were grown on *Pseudomonas* isolation agar plates containing carbenicillin (500 μ g/ml) to select for pFLP2-containing *P. aeruginosa*. Then gentamicin-sensitive colonies were isolated and plated on LB agar containing 5% sucrose to select strains lacking the pFLP2 plasmid. The deletion of the gene of interest and the loss of FRT-*aacCI*-FRT cassette was confirmed by PCR and Southern blot analyses. The *znuA* deletion mutants with or without the gentamicin resistance cassette were referred to as MDO101 and MDO102, respectively. Most of the experiments described in this paper were carried out either with MDO101 or MDO102, obtaining essentially identical results.

To carry out complementation assays, the *znuA* gene was amplified by PCR using the oligonucleotides 5'-*znuA* and 3'-*znuA* (see Table 1) and the so obtained DNA fragment was inserted into the *Bam*HI and *Sac*I restriction sites of pTZ110. The resulting plasmid, ZnuApTZ110, was introduced into MDO101 and MDO102.

Analyses of bacterial growth under low zinc availability

Bacterial cultures grown overnight in VB-MM at 37°C with shaking were diluted 1:1000 in the same medium supplemented or not with 5 μ M ZnSO₄. Aliquots of 200 μ l of these dilutions were inoculated in a 96-well plate (Becton-Dickinson) and incubated at 37°C with shaking. Bacterial growth was monitored at 595 nm every hour for 12 hours by a microtiter-plate reader (Sunrise™ Tecan). Assays were performed in triplicate and each strain was tested in at least three independent experiments. Bacterial growth was analyzed also on VB-MM-agar plates. Overnight cultures grown in Luria-Bertani broth (LB) were normalized to an optical density at 600 nm of 1.0. Bacterial suspensions were serially diluted 1:10 and 5 μ l of each dilution were plates on VB-MM-agar plate, supplemented or not with appropriate concentration of metals and/or chelating agent EDTA.

Construction of *znuA-lacZ* fusion and β -galactosidase activity assay

A 290 bp DNA fragment located upstream of the *znuA* coding sequence was amplified by PCR using the oligonucleotides 5'-PromPAO1 and 3'-PromPAO1(A) (Table 1) and PA14 chromosomal DNA as template. The obtained DNA fragment was digested with the restriction enzymes *Bam*HI and *Hind*III and inserted upstream of a promoterless *lacZ* gene into the promoter probe plasmid pTZ110³⁶ obtaining PROM*znuA*pTZ110. The fusion was subsequently transferred to the wild-type and mutant strains by triparental mating³⁴. The positive clones for the acquisition of the fusion were selected on selective medium for *Pseudomonas* (PIA) containing the antibiotic carbenicillin and β -galactosidase activity was measured by standard procedures³⁷.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis

Wild type PA14 and MD101 strains were grown at 37°C in standard Vogel-Bonner minimal medium or in the same medium supplemented with 3 µM ZnSO₄ for 18 hours at 37°C in 50 ml polypropylene tubes. Aliquots of 2 mL of cell cultures were collected, centrifuged at 18000×g for 5 minutes and then the pellet was washed with 2 ml of phosphate buffer saline (PBS) containing 1 mM EDTA to remove excess zinc.

The periplasmic fractions were obtained by resuspending bacterial pellets in 2 ml of 20% sucrose, 30 mM Tris-HCl pH 8.0, 1 mM EDTA and 1 mg/ml lysozyme. After a 10 minutes incubation on ice, cells were centrifuged for 5 min at 18000xg and the supernatants, containing the periplasmic fractions, were removed. Pellets corresponding to the whole cells were freeze dried by using a Modulyo freeze-dryer (ThermoSavant, USA) and weighted by an AX26 DeltaRange™ balance (Mettler-Toledo, Greifensee, Switzerland). Acid digestion of samples and ICP-MS analysis was carried out essentially as previously described^{38, 39}. Method sensitivity, repeatability, and trueness were assessed by calculating the limits of detection, limits of quantification, coefficients of variation, and the recovery functions, respectively, for each analysed elements³⁸. The periplasmic fractions were diluted tenfold with 1% HNO₃ and analyzed by applying the standard addition method as described in a previous study³⁸.

The significance of the differences between the measured element concentrations was evaluated by a two factor ANOVA performed with cell strain and zinc concentration in cell media as the independent factors. Fisher post hoc test was used to perform pairwise multiple comparisons. *p* values less than 0.05 were considered statistically significant. Statistical analysis was performed with Statistica 6.0 (StatSoft Inc., Tulsa, OK) software.

Calprotectin resistance assay

Overnight cultures of *P. aeruginosa* were diluted 1:50 in LB added back and incubated with shaking for an additional 1 hour at 37 °C. For the CP inhibition assay, growth conditions consisted of 60 percent LB and 40 percent CP buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM β-mercaptoethanol, 3 mM CaCl₂) ratio plus increasing concentrations of CP (0-1000 µg/mL). PA14 wild type or the PA14 *znuA* mutant strains were diluted 1:100 and incubated at 37 °C with shaking. Growth was monitored at 2 hour intervals throughout the time course. Bacterial growth in the presence of CP was normalized to growth without CP from data obtained after 8 hours. Statistical significance was determined by Student's *t* test with a *p* < 0.05 being considered significant.

Protease assays

To obtain culture supernatants, 6.0 ml of culture medium (LB or VB-MM) were inoculated with bacteria and incubated overnight at 37 °C with shaking. Then, samples were centrifuged twice at 13000 × g for 15 minutes at 4 °C, and the supernatants were stored at -20 °C.

Total proteolytic activity was determined using azocasein as substrate, using a procedure previously described^{20, 40} with minor modifications. Briefly, 0.1 ml of each culture

supernatant was added to 0.9 ml of 10 mM Tris-HCl, pH 8.0 containing 5 mg of azocasein, and the reaction mixture was incubated at 37 °C for 30 minutes. The undigested substrate was precipitated by adding trichloroacetic acid (3.3%) to each reaction tube and removed by centrifugation (13000 × g, 20 minutes). The absorbance at 415 nm was measured by a microtiter-plate reader (Sunrise™ Tecan).

Elastolytic activity in culture supernatants was quantified by using insoluble elastin Congo red as substrate, as described^{20, 41}. 0.1 ml of each supernatant was added to 0.4 ml of 10 mM Tris-HCl, pH 8.0 containing 5 mg of substrate. The mixture of reaction was incubated at 37 °C under shaking. After 24 hours, undigested elastin was removed by centrifugation at 13000 × g for 10 minutes and the absorbance at 492 nm measured using a microtiter-plate reader (Sunrise™ Tecan).

Staphylolytic activity of supernatant samples of *P. aeruginosa* strains was determined by monitoring the decrease in absorbance at 595 nm of a heat-killed *Staphylococcus aureus* suspension^{42, 43}. *S. aureus* strain SH1000³¹ was cultured in BHI broth overnight at 37 °C with shaking. Bacteria were pelleted by centrifugation, resuspended in 20 mM Tris-HCl, pH 8.8 to a final optical density at 595 nm of 1.0, and killed at 100 °C for 30 minutes. Aliquots (0.1 ml) of supernatants were added to 0.9 ml of heat-killed bacteria suspension. Staphylolytic activity was determined by measuring the change in absorbance at 595 nm every 15 minutes for 3 hours by using a microtiter-plate reader (Sunrise™ Tecan).

Protease IV activity was measured using the chromogenic peptide Chromozym PL (tosyl-Gly-Pro.Lys-p-nitroanilide) as substrate^{43, 44}. The supernatant (0.1 ml) was added to 0.4 ml of reaction mixture (50 mM Tris-HCl, pH 8.0, 150 mM NaCl mixed with 40 µg of Chromozym PL). The reactions were incubated at 37 °C for 30 minutes and then read in a microtiter plate at 415 nm using a Sunrise™ Tecan plate reader.

The activity of alkaline protease was determined using Hide powder azur. The supernatant (0.1 ml) was added to 0.4 ml of 20 mM Tris-HCl, pH 8.0 containing 5 mg of substrate. Samples were incubated at 37 °C for 1 hour in a shaking incubator, centrifuged at 13000 × g for 10 minutes, and the absorbance at 595 nm was then determined.

Student's *t*-test was used to determine the significance of differences.

Alginate production determination

P. aeruginosa strains were grown at 30 °C for 48 hours on PIA plates supplemented or not with ammonium metavanadate (PIA-AMV) plates⁴⁵. All biological material was recovered from the surface of each PIA-AMV plate and suspended in 25 ml of PBS. The optical density at 600 nm of the bacterial suspension in PBS was measured and adjusted. The bacterial suspensions were spun at 1300 rpm to pellet bacterial cells and the supernatant fractions were recovered. Levels of alginate were assayed using the carbazole assay described by Knutson and Jeanes⁴⁶ with minor modifications. The supernatant (0.1 ml) was mixed with 2.5 ml of borate-sulfuric acid reagent (10 mM H₃BO₃ in concentrated H₂SO₄) and 75 µl of carbazole reagent (0.1% in ethanol). The reaction was then incubated at 55 °C for 30 minutes, and the absorbance at 530 nm was measured spectrophotometrically. The

alginate concentration was quantified using a standard curve of *Macrocystis pyrifera* alginate (0 to 60 µg/ml). Statistical significance was determined by Student's *t* test.

Mouse infection studies

For animal studies we used C57Bl/6 or CD1 male mice (Charles River). Mice were acclimatized for a minimum of 1 week before use. Mice were housed in sterile ventilated cages in a humidity (40-60%) and temperature (20-22°C) controlled environment, with a 12 hours light/dark cycle (lights on at 7 AM). *P. aeruginosa* strains (wild type PA14 and MDO101) were cultured in VB-MM to exponential phase (optical density at 600 nm approx. 0.5) and diluted in sterile PBS at the appropriate concentration.

For mouse competition assays, we carried out a first experiment with animals of 8 weeks of age and a second one with animals of 12 weeks of age, obtaining essentially the same results. Groups of 6 animals were inoculated intraperitoneally with 0.2 ml of PBS containing 2×10^6 colony forming units (cfu) and sacrificed after 18 hours. Bacteria were recovered from spleen and lung, plated for single colonies, and 200 colonies were picked on selective PIA plates containing 100 µg/ml of gentamicin. The competitive index (CI) was calculated using the formula $CI = \text{output (strain A/strain B)} / \text{input (strain A/strain B)}$.

For acute lung infections, PA14 and MDO101 strains were grown to exponential phase in VB-MM, pelleted by centrifugation ($2700 \times g$, 15 minutes), washed twice with sterile PBS, and appropriate dilutions in sterile PBS were prepared. Mice were anesthetized with 2.5% avertin (2,2,2-tribromethanol, 97%; Sigma Aldrich) in 0.9% NaCl, intubated with a 22-gauge venous catheter and inoculated with 5×10^6 cfu into both lung lobes. After infection, mortality and body weight were monitored for one week. Statistical analysis was performed using Fisher's exact test (two tailed) for categorical variables.

Ethics Statement

Animal studies performed either at the Italian Istituto Superiore di Sanità (ISS) or at the San Raffaele Scientific Institute (Milan, Italy) were carried out according to the Italian Ministry of Health guidelines for the use and care of experimental animals. All experiments were previously approved by the local Ethical Committee and carried out under the supervision of certified veterinarians.

Results

Analysis of the contribution of *ZnuA* to *P. aeruginosa* growth in zinc poor media

We have analyzed bacterial growth in a chemically defined medium (VB-MM) containing only contaminant traces of zinc³⁸. Unlike other bacterial strains tested under similar conditions^{9, 47} the *P. aeruginosa znuA* mutant strain showed only a marginal growth defect with respect to the wild type strain (Figure 1A). A more marked zinc-dependent growth defect was observed in VB-MM agar plates in the presence of 10 µM EDTA (Figure 1B). All these growth defects were complemented by a plasmid containing the *znuA* gene (data not shown). We have also tested the ability of CP to inhibit *P. aeruginosa* growth. Figure 2 shows that incubation of bacteria with 1000 µg/ml of purified CP caused a 75% growth

inhibition of the wild type strain and a 85% growth inhibition of the *znuA* strain. Although the difference between the two strains is statistically significant, it should be noted that the concentration of CP used in this assay is able to completely inhibit growth of other bacteria such *A. baumannii*⁴⁸ and *S. Typhimurium*³⁰.

ZnuA expression as a function of zinc availability

To gain information on the involvement of ZnuABC in the response of *P. aeruginosa* to zinc paucity, we have analyzed the expression of *znuA* using a transcriptional fusion between the *znuA* promoter and the *lacZ* reporter gene. The results reported in Figure 3A and 3C show that *znuA* expression is higher in VB-MM than in LB medium and is induced by chelating agents and repressed by zinc. Moreover, β -galactosidase expression from the reporter plasmid in response to EDTA is higher when measured in the *znuA* mutant strain than in wild type PA14 (Figure 3B and 3D), indicating that the absence of ZnuA subtly modifies intracellular zinc availability. Although this picture is coherent with the idea that the ZnuABC is under control of Zur and is induced under conditions of zinc deficiency, it should be noted that the *znuA* mutant strain shows a significant basal level of expression either in LB medium or in VB-MM supplemented with zinc. This finding contrasts with previous studies on other bacteria showing that micromolar zinc concentrations nearly abolish *znuA* expression in other bacteria^{9, 10, 47}. We hypothesize that a constitutive basal level of *znuABC* expression could be the consequence of the specific gene arrangement of the *znuABC* operon in *P. aeruginosa*. In fact, whereas in other bacteria the *zur* gene and *znuABC* are located in separated chromosomal locations, *P. aeruginosa* is characterized by the insertion of *np20* (*zur*) between the divergently transcribed *znuA* and *znuC* genes (see Supplementary Figure 1).

Elemental profile of wild type and mutant *P. aeruginosa* strains

Previous studies have shown that inactivation of *znuA* in *Salmonella* causes marked modifications in the intracellular concentration of various metal ions³⁸. To gain further insight into the role of ZnuA in metal homeostasis in *P. aeruginosa*, we have measured the intracellular content of several metal ions in wild type and *znuA* mutant strains grown in VB-MM, in the presence or absence of zinc supplementation, by ICP-MS. We found that wild type and *znuA* mutant strains show remarkable differences in their ionic profiles either when cultivated in zinc-free VB-MM or in the zinc-replete medium (supplementary Figure 2 and supplementary Table 1). The mutant strain accumulated higher levels of Cu, Co, Cr and Rb and a lower amount of Mn in the Zn-depleted medium. Most of these differences were abrogated in the zinc-containing medium, where, however, the mutant strain exhibited a higher content of Mg and K. Both the wild type and *znuA* strains showed a marked decrease in Mn and Ni content when cultivated in presence of zinc, a behavior which is suggestive of zinc-mediated inhibition of metal specific importers^{49, 50}. In contrast, Sr uptake was enhanced in presence of zinc. Quite unexpectedly, the *znuA* mutant strain contained less Mn than the wild type strain when cultivated in standard VB-MM. This observation, which deserves further investigations, suggests that ZnuABC could have an important role in manganese uptake in *P. aeruginosa*. No significant differences were found for Fe and other metals such as Ca, Na, and Al (not shown). As far as intracellular zinc content is concerned, the *znuA* mutant contained the same amount of zinc as the wild type

strain when cultivated in minimal medium (Figure 4A), further supporting the hypothesis that other uptake systems may compensate for the absence of a functional ZnuABC transporter. For both strains there was a statistically significant increase in zinc content in the zinc rich medium. The analysis of the metal content of cytoplasmic (spheroplasts) and periplasmic fractions (Figure 4B and C) suggests that the intracellular zinc content is modestly affected by the presence or absence of ZnuA and by the extracellular concentration of the metal, whereas the periplasmic zinc concentration varies as a function of zinc concentration in the medium. This observation suggests that *P. aeruginosa* is able to control the cytoplasmic concentration of zinc in environmental conditions characterized by an excess of the metal, while the periplasmic environment reflects the concentration of the metal present in the medium.

ZnuA is required for efficient alginate production

Recent studies have shown that PA14 develops a mucoid phenotype when cultivated in PIA plates supplemented with ammonium metavanadate^{45, 51}, a condition that likely induces cell surface stress and activation of the alginate synthetic pathway⁵². In line with the above mentioned studies, we have observed that wild type PA14 does not show a mucoid phenotype when plated on standard PIA agar plates, but overproduces alginate in the presence of ammonium metavanadate (Supplementary Figure 3). In contrast, we observed that the *znuA* mutant strain is unable to produce high levels of alginate under the same conditions. A quantitative evaluation of alginate production through the carbazole assay, revealed that the *znuA* mutation halves the ability of *P. aeruginosa* PA14 to produce this exopolysaccharide in response to ammonium metavanadate (Figure 5).

Disruption of the *znuA* gene affects extracellular proteases activity

To evaluate the possibility that alterations in zinc homeostasis due to *znuA* inactivation might affect the release of *P. aeruginosa* virulence factors, we measured total extracellular protease activity as well as the specific activity of four distinct zinc-containing proteases, i.e. LasA, LasB, protease IV and alkaline protease.

Deletion of *znuA* caused a noteworthy decrease of total extracellular protease activity either in bacteria cultivated in LB medium or in VB-MM (Figure 6, panels A and B). The addition of zinc to VB-MM led to a 4 fold increase of total proteolytic activity, highlighting the importance of this metal for the production of active proteases. A comparable behavior was also observed for LasA, LasB and protease IV, whose activity was decreased in the *znuA* strain cultivated in VB-MM and, in the case of protease IV, also in LB medium (Supplementary Figure 4). Although the activity of all these proteases increased in bacteria cultivated in VB-MM supplemented with zinc, it should be noted that in most cases the addition of zinc is not sufficient to equalize the activity in the two strains. This observation suggests that ZnuA is also necessary for correct zinc homeostasis in zinc replete environments.

An exception to this general model is represented by alkaline protease, whose activity is not affected in the *znuA* mutant strain (Figure 6 and Supplementary Figure 4)

Mice infections

To analyze the role of ZnuA in zinc uptake *in vivo*, we have initially carried out competition assays in C57Bl/6 mice infected intraperitoneally. The *znuA* mutant proved to be significantly disadvantaged with respect to the wild type strain (Figure 7). However, it should be noted that high numbers of *znuA* cells were recovered from tissues, indicating that both the wild type and the *znuA* mutant strains have high ability to proliferate in the infected host. This observation is in contrast to studies carried out with other bacteria that have shown that the wild-type strain largely outcompetes the *znuA* mutant strain during infections¹⁰. Similar results were obtained in CD1 mice (data not shown).

In line with the idea that *znuA* disruption has a minor effect on *P. aeruginosa* virulence, we were not able to observe significant differences in the mortality induced by the wild type and the *znuA* PA14 strains in lung infected C57Bl/6 mice (Supplementary Figure 4)

Discussion

The pathways enabling bacteria to recruit transition metals within the infected host are key to bacterial survival and attractive candidates for the development of novel anti-infective treatments. For example, based on the observation that gallium interferes with iron metabolism in *P. aeruginosa* it has been proposed that gallium-based treatments could be used to eradicate this pathogen from CF patients⁵³. Whereas some studies have clearly indicated that zinc promotes *P. aeruginosa* virulence^{20, 54}, only a handful of studies have directly addressed the importance of zinc uptake for the ability of this pathogen to cause disease. As a first step to evaluate the role of zinc importers in *P. aeruginosa* pathogenicity we have carried out a characterization of a PA14 strain lacking a functional ZnuABC transporter.

It has been recently shown that PAO1 mutant strain lacking the genes encoding for ZnuA, ZnuC or ZnuB have a slightly reduced ability to grow in LB medium supplemented with 0.5 mM EDTA⁵⁵. As bacterial responses to chelating agents are sometimes ambiguous⁵⁶, we analyzed growth of a PA14 mutant strain lacking *znuA* in a chemically defined minimal medium containing only contaminant traces of zinc. In line with the results of Ellison et al⁵⁵, the mutant strain exhibited a modest growth defect (Figure 1A) and a more marked phenotype could be observed only in minimal medium agar plates supplemented with EDTA (Figure 1b), a result possibly favored by the ability of the solid agar support to limit zinc availability to bacteria⁵⁷. The small impact of *znuA* deletion on bacterial growth is accompanied by the substantial equality of the intracellular zinc content between the wild-type and the mutant strains grown in minimal medium (Figure 4). However, it should be noted that ICP-MS analyses provide information on the total amount of zinc present in cells, but cannot distinguish between the zinc tightly bound to macromolecules and the small quota of labile metal which regulates transcription from Zur-regulated genes. Instead, an analysis of *znuA* transcription from a reporter plasmid (Figure 3, panels B and D) suggests that the strain lacking ZnuA contains a reduced pool of free zinc. At the same time, significant differences were detected in the global ionic profiles of wild type and *znuA* mutant PA14 strains (supplementary Figure 2 and Supplementary Table 1), suggesting that

small alterations in zinc homeostasis may significantly affect the metal content of the bacterial cell.

These observations suggest that *P. aeruginosa* continues to import the zinc necessary for its growth also in the absence of a functional ZnuABC importer. This conclusion is also supported by the remarkable ability of *P. aeruginosa* to grow in the presence of high concentrations of CP (Figure 2). CP concentration in the sputum of CF patients correlates with zinc levels⁵⁸. Both CP and zinc levels decrease following treatment of CF exacerbations, suggesting that most of the zinc present in lung secretion could be tightly bound to CP. These observations support the hypothesis that CP is abundantly released in the airway secretions in an effort to contain bacterial proliferation, but that the ability of *P. aeruginosa* to colonize the CF lung is based on the ability to withstand the host mechanisms of zinc sequestration.

The capability of *P. aeruginosa* to grow efficiently without a functional *znuA* gene or in the presence of high amounts of CP is suggestive of the existence of additional high affinity zinc importers. Recent studies have in fact revealed that some pathogens possess more than one mechanism to acquire zinc from the environment. For example, *Listeria monocytogenes* expresses two ABC-type zinc importers (ZnuABC and ZurAM), both contributing to virulence, but individually able to ensure efficient growth in a chemically defined media⁵⁹. Redundant zinc import systems have been identified in other bacteria able to colonize the airways, such as *N. meningitidis*, *A. baumannii* and *Y. pestis*. Under conditions of zinc deficiency, all these bacteria produce membrane proteins that facilitate zinc import. In particular, it has been recently shown that the siderophore yersiniabactin may function as a zincophore in *Y. pestis*, thus mediating zinc uptake through a inner membrane receptor specifically recognizing the zinc-yersinabactin complex¹³. Although previous studies have suggested the existence of other zinc importers in *P. aeruginosa*^{55, 60}, the identity of the other transporter(s) ensuring efficient zinc uptake in the absence of a functional ZnuABC importer is still to be determined.

Interestingly, although inactivation of *znuA* is accompanied by limited growth defects, we found that different virulence-related features are altered in the absence of a functional ZnuABC transporter. We have shown that the absence of *znuA* affects the capability of *P. aeruginosa* to produce the exopolysaccharide alginate in response to stress induced by ammonium metavanadate in PIA agar plates (Figure 5 and supplementary Figure 3). Alginate forms a capsule that protects *P. aeruginosa* from phagocytosis, antibiotics and a variety of host antimicrobial factors⁶¹⁻⁶³. Moreover, alginate production is favored by anaerobic conditions and this contributes to persistence and to chronic *P. aeruginosa* infections in the lung of CF patients^{64, 65}. Alginate production is negatively regulated by the anti-sigma factor MucA⁶¹. Alginate-overproducing mucoid strains of *P. aeruginosa* are generally characterized by mutations in *mucA*, whereas non-mucoid strains can induce production of the exopolysaccharide through a finely regulated pathway leading to the proteolytic inactivation of MucA. This pathway involves zinc-containing proteases, such as MucP and ClpX⁶¹. Based on this, our data suggest that the decreased ability of the *znuA* mutant to secrete alginate could be explained by alterations in the expression and/or maturation of zinc-depending enzymes.

This possibility is also supported by the data showing that zinc availability promotes secretion of active extracellular proteases and that the supernatants from cultures of the *znuA* mutant strain contain lower protease activity than those from the wild type strain (Figure 6). In particular, our data demonstrate that the activity of three distinct zinc-containing extracellular proteases, LasA, LasB and protease IV, is decreased in the mutant strain. An exception to this trend is represented by alkaline protease, whose activity is significantly induced by zinc addition to the minimal medium (Figure 6E), but is very similar in the wild type and mutant strains. Whereas, in *P. aeruginosa* most proteases are secreted through the general secretion pathway and mature in the periplasmic compartment²¹, alkaline protease is exported through an independent secretory pathway resembling the type I secretion system^{66, 67}. Little is known about the mechanisms of zinc incorporation in *P. aeruginosa* proteases, but our data suggest that alkaline protease may acquire zinc in a distinct cellular compartment with respect to the other proteases. Proteins secreted via the general secretory pathways usually incorporate the metal in the periplasmic compartment⁶⁸, whereas proteins secreted by the type I secretion system bypass the periplasm and mature in the extracellular milieu⁶⁹. It is worth noting that, although the difference does not reach statistical significance, the periplasmic fraction from the *znuA* strain contains a lower amount of zinc compared to that from the wild type strain (figure 4C). This observation supports the hypothesis that the absence of ZnuA subtly modulates zinc distribution in the cell. Another possibility consistent with such observations is that ZnuA, in addition to its well-known role in the transport of zinc, could play a role as a chaperone facilitating zinc acquisition by periplasmic zinc proteins. This possibility could explain the observation that *znuA* is significantly expressed in bacteria growing in LB or in media supplemented with zinc (Figure 3) and that the total extracellular protease activity (Figure 6A) and the activity of protease IV is significantly decreased in LB. We propose that the constitutive level of expression of *znuA* is a consequence of the peculiar organization of the *znuABC* operon in *P. aeruginosa* (supplementary Figure 1). The arrangement of the operon is suggestive of a leaky regulation of transcription of the *znuA* gene to ensure the constant production of the Zur regulator that is needed to control the expression of several other genes⁷⁰⁻⁷². We actually observed that two other Zur-regulated genes (*rpm2* and *dksA2*) are more tightly controlled than *znuA* under zinc replete conditions (data not shown). We suggest that the specific arrangement of the *znuABC* operon is indicative of a constitutive role of ZnuA which may be related to the need for maintaining a constant influx of zinc or for a role in the control of zinc distribution in the periplasmic space.

We have also carried out experiments to evaluate the consequence of *znuA* deletion on *P. aeruginosa* virulence in mice. Competition experiments in intraperitoneally infected C57Bl/6 and CD1 mice revealed that wild type PA14 colonize the spleen and the lungs more efficiently than the *znuA* mutant strain. However, unlike the case with other bacteria where the *znuA* mutant shows a dramatic loss of capability to disseminate within the host compared to wild type strain^{4, 10}, the competitive index between the two strains is rather low and both the strains induce a comparable mortality in C57Bl/6 mice infected with a 2×10^6 CFU dose (data not shown). At the same time, preliminary acute lung infection experiments confirm that wild type and *znuA* PA14 strains induce comparable mortality and loss of weight (Supplementary Figure 5).

In conclusion this study reveals that inactivation of *znuA* affects the ability of *P. aeruginosa* to produce alginate and secrete extracellular proteases, thus supporting the idea that zinc acquisition is critical for full virulence of this pathogen. At the same time, however, we observed that *P. aeruginosa* has a remarkable resistance to the antimicrobial activity of CP and that the *znuA* mutant strain shows modest growth defects in Zn-limited environments, elevated intracellular Zn content and a limited reduction of virulence in mice. These observations suggest that *P. aeruginosa* is equipped with redundant mechanisms for the acquisition of Zn which may compensate for the absence of a functional ZnuABC transporter. We propose that the high resistance to zinc restriction may significantly contribute to colonization of the CF lung.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Growth of *P. aeruginosa* in chemically defined VB-MM

A) Wild type (squares) and *znuA* (circles) *P. aeruginosa* PA14 strains were grown in standard VB-MM (filled symbols) or in the same medium supplemented with 5 μ M ZnSO₄ (open symbols). **B)** Wild type and *znuA* *P. aeruginosa* PA14 strains were grown in VB-MM agar plates, in VB-MM agar plates supplemented with 10 μ M EDTA or in VB-MM agar plates supplemented with 10 μ M EDTA and 10 μ M ZnSO₄.

Figure 2. CP-mediated inhibition of *P. aeruginosa* growth *in vitro*

Growth of wild type and *znuA* PA14 strains in the presence of 1 mg/ml CP with respect to untreated controls. Data represent the average $\pm$ SD of nine replicates. * $p < 0.05$.

Figure 3. *znuA* expression in *P. aeruginosa* PA14

A) Expression of *znuA* in LB medium. Wild type PA14 carrying plasmid PROM $znuA$ pTZ110 was grown overnight in LB medium, LB containing 0.5 mM EDTA or LB containing 0.5 mM EDTA and 0.5 mM ZnSO₄ and β -galactosidase activity was determined as described in the Experimental section. B) Differences in *znuA* expression in wild type and *znuA* mutant (MD101) strains in LB supplemented with 0.5mM EDTA. C) Expression of *znuA* in VB-MM medium. Wild type PA14 carrying plasmid PROM $znuA$ pTZ110 was grown overnight in VB-MM in the presence or absence of 10 μ M zinc and EDTA supplementation. D) Differences in *znuA* expression in wild type and *znuA* mutant (MD101) strains in VB-MM supplemented with 10 μ M EDTA. * $p < 0.05$, ** $p < 0.01$.

Figure 4. Zinc content of wild type and *znuA* PA14 strains grown in VB-MM and VB-MM supplemented with 3 μ M ZnSO_4

A) Total zinc content determined on bacterial pellets as recovered from overnight cultures in VB-MM. **B)** Zinc content in bacterial spheroplasts, obtained after removal of the periplasmic fractions. **C)** Zinc concentration in periplasmic fractions. Data are mean concentrations obtained from 4 independent bacterial cultures and bars indicate mean standard deviations. * $p < 0.05$.

Figure 5. Alginate production in wild type and *znuA* PA14 strains. The level of alginate produced by wild type and *znuA* PA14 strains grown in PIA agar plates supplemented with ammonium vanadate was measured by the carbazole assay

Data are means $\pm$ SD of four independent experiments. * $p < 0.05$.

Figure 6. Effect of *znuA* mutation on extracellular protease activity

A) Total protease activity in the supernatants from wild type and *znuA* PA14 strains grown in LB medium. **B)** Total protease activity in the supernatants from bacteria grown in VB-MM or in VB-MM supplemented with 10 μ M zinc. **C)** LasA, **D)** LasB, **E)** Protease IV, **F)** Alkaline protease activity in the supernatants from bacteria grown overnight in VB-MM or in VB-MM supplemented with 10 μ M zinc. Data are means $\pm$ SD of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$.

Figure 7. Competition assay in intraperitoneally infected mice

Bacteria were recovered from the spleens and lungs of C57Bl/6 mice infected with mixed inocula (wild type vs *znuA*) and enumerated in agar plates. * $p < 0.05$, ** $p < 0.01$

TABLE 1

Bacterial strains, plasmids and primers used

Strain, plasmid or primer	Genotype or description	Reference(s) or source
<i>E. coli</i>		
DH5 α	ϕ 80 <i>lacZ</i> 15 (<i>lac-argF</i>) U169 <i>deoR recA1 endA1 hsdR17</i> (rk ⁻ , mk ⁺) <i>phoA supE44</i> λ^- <i>thi-1 gyrA96 relA1</i>	Lab collection
HB101	F ⁻ <i>mcrB mrr hsdS20</i> (r _B ⁻ m _B ⁻) <i>recA13 leuB6</i> ara-14 <i>proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20</i> (Sm ^R) <i>glnV44</i> λ^-	Lab collection
<i>P. aeruginosa</i>		
PA14	Wild type strain	He et al. ⁷³
MDO101	PA14 <i>znuA::gm</i>	This study
MDO102	MDO101 <i>znuA::scar</i>	This study
<i>S. aureus</i>		
SH1000	Functional <i>rsbU</i> derivative of 8325-4 <i>rsbU</i> ⁺	Horsburgh et al. ³¹
Plasmids		
pUC18		Lab collection
pEX18Tc	Broad-host-range gene replacement vector with MCS from pUC18; <i>sacB</i> ⁺ ;Tc ^R ,oriT ⁺	Hoang et al. ³³
pRK2013	Broad-host-range helper vector; ColE1-Tra(RK2) ⁺ ,kan ^R	Figurski, & Helinski, ³⁴
<i>Pa</i> KO _{<i>znuA</i>} pEX18Tc		This study
pTZ110		Schweizer & Chuanchue, ³⁶
ZnuApTZ110		This study
PROM _{<i>znuA</i>} pTZ110		This study
pPS856	Ap ^R , Gm ^R	Hoang et al. ³³
pFLP2	Bhr Flp recombinase-producing plasmid	Hoang et al. ³³
Primers^a		
PAO4(A)	CGGAATTCCTCGTGGACTTTCTCGAGG	
PAO4(B)	CATAAGCTTCCTCGTGGACTTTCTCGAGG	
PAO5	CGGGATCCGTGTTTCGCGGTATCCGCGGT	
PAO6	CGGGATCCGCAGCAGTACGTCCGGCT	
PAO7(B)	CATAAGCTTTTCGCTCAGCACTGCCTGGC	
5'-PROMPAO1	TTTAAGCTTGC GCGGCGGCCGTCGTTT	
3'-PROMPAO1 (A)	TTTGGATCCGCGGCACTCACATGAAAGGT	
5'ZnuA	CCCGGATCCCAGCGGCTTGTGGCTCTG	
3'ZnuA	CCCGAGCTCGCGTCTGTCTTGGCCATCC	

^a Restriction sites are underlined