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Efficient zinc uptake is critical for the ability of *Pseudomonas aeruginosa* to express virulence traits and colonize the human lung

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ABSTRACT

We have recently shown that *Pseudomonas aeruginosa*, an opportunistic pathogen that chronically infects the lungs of patients with cystic fibrosis (CF) and other forms of lung disease, is extremely efficient in recruiting zinc from the environment and that this capability is required for its ability to cause acute lung infections in mice. To verify that *P. aeruginosa* faces zinc shortage when colonizing the lungs of human patients, we analyzed the expression of three genes that are highly induced under conditions of zinc deficiency (*zrmA*, *dksA2* and *rpmE2*), in bacteria in the sputum of patients with inflammatory lung disease. All three genes were expressed in all the analyzed sputum samples to a level much higher than that of bacteria grown in zinc-containing laboratory medium, supporting the hypothesis that *P. aeruginosa* is under zinc starvation during lung infections. We also found that the expression of several virulence traits that play a central role in the ability of *P. aeruginosa* to colonize the lung is affected by disruption of the most important zinc importing systems. Virulence features dependent on zinc intake include swarming and swimming motility and the ability to form biofilms. Furthermore, alterations in zinc assimilation interfere with the synthesis of the siderophore pyoverdine, suggesting that zinc recruitment could modulate iron uptake and affect siderophore-mediated cell signaling. Our results reveal that zinc uptake is likely to play a key role in the ability of *P. aeruginosa* to cause chronic lung infections and strongly modulates critical virulence traits of the pathogen. Taking into account the recent discovery that zinc uptake in *P. aeruginosa* is promoted by the release of a small molecular weight molecule showing high affinity for zinc, our data suggest novel and effective possibilities to control lung infections by these bacteria.

1. Introduction

P. aeruginosa is able to cause chronic lung infections in patients with cystic fibrosis (CF), non-CF bronchiectasis and chronic obstructive pulmonary disease, promoting the progressive and irreversible loss of the respiratory function. The ability of pathogens to evade the host immune responses and thrive during infection is significantly affected by their ability to import transition metals, which are used as cofactors by a large number of proteins [1,2]. Several lines of evidence show that *P. aeruginosa*'s capacity to assimilate iron though a series of diversified and efficient strategies is important for its ability to colonize successfully the CF lung [3–5]. Recently, we have shown that this microorganism is also equipped with different high-affinity zinc import systems that are needed to establish lethal lung infections in mice and for the expression of some virulence factors [6,7]. These observations suggest that zinc availability is limited in the lung mucosa, in agreement with the observation that the zinc-sequestering neutrophilic protein Calprotectin is extremely abundant in sputum from CF patients [8,9]. However, this hypothesis is an apparent contradiction with reports that sputum from patients with CF and non-CF bronchiectasis contain much higher levels of zinc than control healthy patients [10]. In the case of CF patients, zinc levels were found to decrease after antibiotic therapies to treat exacerbations, correlating the zinc concentration to the presence of infecting bacteria [9]. Moreover, it has been recently suggested that resistance to highly levels of zinc could contribute to the infectivity of *P. aeruginosa* clinical isolates [11]. The goal of this study was to investigate the bioavailability of zinc for bacteria that colonize the lung and to assess whether the zinc import

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Table 1
Bacterial strains.

Strains	*Genotype*	*Source*
P. aeruginosa		
PAO1	Wild type strain	Lab collection
PA14	Wild type strain	Lab collection
MDO101	PA14 *znuA:gm*	[5]
MDO111	PA14 *zrmA:gm*	[6]
MDO113	PA14 *znuA:scar zrmA:gm*	[6]
MDO115	PA14 *zrmB:gm*	[6]
MDO117	PA14 *znuA:scar zrmB:gm*	[6]
DUN008-9	isolated from patient with non-CF bronchiectasis	This study
DUN013-3	isolated from patient with non-CF bronchiectasis	This study
DUN013-4	isolated from patient with non-CF bronchiectasis	This study
DUN016-3	isolated from patient with non-CF bronchiectasis	This study
DUN036-1	Isolated from patient with cystic fibrosis	This study
DUN038-1	Isolated from patient with chronic obstructive pulmonary disease	This study
E. coli		
DH5α	φ80 Δ*lac*Z 15Δ (*lac-arg*F) U169 *deo*R*rec*A1 *end*A1 *hsd*R17 (rk^-, mk^+) *pho*A*sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1	Lab collection
HB101	F^- *mcrBmrr hsdS20*($r_B^-m_B^-$) *rec*A13 *leu*B6 *ara*-14 *pro*A2 *lacY1 gal*K2 xyl-5 mtl-1 *rps*L20(Sm^R) glnV44 λ^-	Lab collection

mechanisms may promote the ability of *P. aeruginosa* to establish pulmonary infections. To this goal, we analyzed the expression of three genes, *rpmE2, dskA2* and *zrmA,* used by *P. aeruginosa* to adapt to conditions of zinc deficiency in bacteria in the sputum of patients with chronic lung disease. *rpmE2* and *dksA2*, encode for the zinc-independent paralogs of the ribosomal protein L31 and of the transcriptional regulator DksA, respectively, whereas *zrmA* encodes the TonB-dependent outer membrane receptor of a zinc binding metallophore [7]. In addition, we examined the role of zinc homeostasis on the expression of additional virulence-related traits that contribute to the establishment of pulmonary infections.

2. Material and methods

2.1. Bacterial strains and growth media

The bacterial strains used in this study are listed in Table 1. All *P. aeruginosa* mutant strains derive from the PA14 strain. Bacteria were routinely cultured at 37 °C in Luria-Bertani (LB) broth (10 g Bactotryptone per liter, 5 g yeast extract per liter, 10 g NaCl per liter). Growth under zinc-limiting conditions was obtained using the Vogel-Bonner minimal-medium E (VB-MM: 0.04 g anhydrous $MgSO_4$ per liter, 2 g citric acid per liter, 10 g anhydrous K_2HPO_4 per liter, 3.5 g $NaNH_4HPO_4$-$4H_2O$ per liter, 2 g glucose per liter)[12] or by adding EDTA to solid, semi-solid or liquid media (either LB, Difco nutrient broth or VB-MM). Zinc contaminations of VB-MM were minimized as previously described [13]. For RT-qPCR analysis bacteria were grown in Kings B medium as described previously [5]. Antibiotics for *P. aeruginosa* were used at the following concentrations: 100 μg ml^{-1} gentamicin and 300–500 μg ml^{-1} carbenicillin.

2.2. Construction of lacZ fusions and β-galactosidase activity assay

All the plasmids used for the β-galactosidase activity assay are listed in Table 2. Promoter-*lacZ* reporter plasmids were obtained by fusing a promoterless *lacZ* coding sequence to either the *fliE* or the *fleQ* promoter in plasmid pTZ110, as previously described [6]. The *fliE* promoter was amplified with primers *fliE*_1 and *fliE*_2, whereas the *fleQ* promoter was amplified with the primers *fleQ_1 and fleQ_2* (Table 2). The resulting plasmids p*fliE*pTZ110 and p*fleQ*pTZ110 were subsequently transformed in *E. coli* DH5α and transferred to *P. aeruginosa* PA14 wild-type and mutant strains by triparental mating [14]. The positive clones carrying the construct were isolated on a *Pseudomonas* selective medium (PIA) containing the antibiotic carbenicillin (500 μg ml^{-1}) and β-galactosidase activity was measured by standard procedures [15].

2.3. Bacterial motility assays

Swarming and swimming motility assays were performed as previously described with minor modifications [16]. Swarm plates consisted of Difco nutrient broth with 0.5% Difco Bacto agar and 0.5% glucose. Swim plates consisted of LB broth containing 0.3% Difco Bacto agar. Both plates were further supplemented with appropriate concentration of the chelating agent EDTA and/or zinc and iron. Overnight cultures of *P. aeruginosa* grown in LB were suspended and diluted to an OD_{600} of 0.2, and 5 μl was spotted onto the swarm and swim plates, which were subsequently incubated at 37 °C for 18 h.

2.4. Rhamnolipids quantification

P. aeruginosa PA14 strains were grown in nutrient broth, in VB-MM or in VB-MM supplemented with 3 μM $ZnSO_4$ at 37 °C until mid-exponential or stationary phase. Rhamnolipids concentration in bacterial culture supernatant was detected as previously described [17]. The amount of rhamnolipid was measured by orcinol assay using rhamnose as a standard. The absorbance at 415 nm was normalized to initial cell density.

2.5. Biofilm formation

Biofilm formation assay was performed in 96-well polypropylene plates (Greiner bio-one) as previously described with some modifications [18]. Overnight cultures of *P. aeruginosa* grown in LB were diluted in VB-MM to get 10^7cfu/200 μl and then inoculated at 37 °C without shaking for 48 h. After the incubation, the wells of the plates were washed three times with distilled water to remove planktonic cells and media. Subsequently, the wells were stained with 0.1% crystal violet for 30 min and then washed three times with distilled water. Crystal violet was solubilised with 95% ethanol. Adherent biofilm was quantified by measuring the Optical Density (OD) at 550 nm and normalizing to initial cell density, using a microplate reader (SunriseTMTecan).

2.6. Pyoverdine quantification assay

P. aeruginosa PA14 strains were grown in standard Vogel-Bonner minimal medium at 37 °C with shaking for 22 h. Pyoverdine was quantitated as previously described with minor modifications [19]. Bacterial cultures were centrifuged at 4800*g* for 20 min, and culture supernatants were collected and filter sterilized (0.22 μm GE healthcare). Cell-free supernatants were diluted in 100 mM Tris-HCl (pH 8.0). Pyoverdine was measured in supernatants by recording absorbance at 405 nm as previously described and normalized to initial cell density.

2.7. Measurement of gene expression by RT-qPCR

Measurement of *P. aeruginosa* gene expression in freshly-expectorated sputum and in laboratory-grown *P. aeruginosa* bacteria was carried out as described previously [5,20]. Briefly, sputum samples were collected from patients attending Dunedin Hospital under the approval of the New Zealand Health and Disability Ethics Committees (NYTY/10/12/106). Individuals with cystic fibrosis (CF) and non-CF bronchiectasis were recruited, and written informed consent was provided by all participants. Sputum was expectorated directly into 20 ml of RNAlater (Qiagen). RNA was then extracted and cDNA synthesized. *P. aeruginosa* strain PAO1 was grown in Kings B medium to late-log phase, RNA extracted from the bacteria present in 0.5 ml of culture, and cDNA prepared. Gene-specific qPCR primers (Table 2) with high (> 1.8) amplification efficiency were developed for *rpmE*2, *zrmA* and