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Sputum containing zinc enhances carbapenem resistance, biofilm formation and virulence of *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa chronic lung infections are the leading cause of mortality in cystic fibrosis patients, a serious problem which is notably due to the numerous *P. aeruginosa* virulence factors, to its ability to form biofilms and to resist the effects of most antibiotics. Production of virulence factors and biofilm formation by *P. aeruginosa* is highly coordinated through complex regulatory systems. We recently found that CzcRS, the zinc and cadmium-specific two-component system is not only involved in metal resistance, but also in virulence and carbapenem antibiotic resistance in *P. aeruginosa*. Interestingly, zinc has been shown to be enriched in the lung secretions of cystic fibrosis patients. In this study, we investigated whether zinc might favor *P. aeruginosa* pathogenicity using an artificial sputum medium to mimic the cystic fibrosis lung environment. Our results show that zinc supplementation triggers a dual *P. aeruginosa* response: (i) it exacerbates pathogenicity by a CzcRS two-component system-dependent mechanism and (ii) it stimulates biofilm formation by a CzcRS-independent mechanism. Furthermore, *P. aeruginosa* cells embedded in these biofilms exhibited increased resistance to carbapenems. We identified a novel Zn-sensitive regulatory circuit controlling the expression of the OprD porin and modifying the carbapenem resistance profile. Altogether our data demonstrated that zinc levels in the sputum of cystic fibrosis patients might aggravate *P. aeruginosa* infection. Targeting zinc levels in sputum would be a valuable strategy to curb the increasing burden of *P. aeruginosa* infections in cystic fibrosis patients.

1. Introduction

Trace metals such as zinc, copper, manganese and iron are crucial for numerous processes in mammalian cells. They are mainly involved as cofactors for various enzymes or biochemical reactions. An excess of these elements is, however, deleterious, since trace metals may interfere with proteins, interact with DNA and induce the formation of reactive oxygen species that oxidize and alter cellular components [1]. Metal homeostasis is therefore a tightly regulated process to avoid cellular damage and metal balance disorders which are responsible for several human diseases [2,3]. It is known that under certain circumstances, trace metal homeostasis is strongly disturbed. For instance, burn patients suffer from trace metal depletion as a result of extensive exudative losses in the injured areas. This leads to a significant increase in Zn and Cu concentrations within the burn wounds and to serum depletion of these trace metals [4]. In cystic fibrosis patients, the viscous mucus that accumulates in the lungs is enriched in Cu, Zn and Fe [5,6]. Since those special environments enriched in trace elements are also often colonized with bacterial pathogens, it is of prime importance to understand whether such trace metal levels affect the metabolism and virulence factors expression of bacterial pathogens. A link between iron availability in the host and bacterial virulence has long been established (for reviews [7,8]). There is an increasing body of evidence, however, that several other metals such as Zn, Cu or Ni might also play a role in bacterial virulence [9–12], and might function as a selective pressure for antibiotic resistance, affecting the treatment of infectious diseases [13]. We previously characterized one such co-regulation mechanism linking metal response to antibiotic resistance and pathogenicity of the Gram-negative bacterium *Pseudomonas aeruginosa* [14–16]. This

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major opportunistic pathogen causes chronic infections in cystic fibrosis patients and sepsis in burn victims. It is the main cause of morbidity and mortality among these patient populations [17]. *P. aeruginosa* expresses virulence factors in a coordinated, cell density-dependent manner, by the way of interconnected quorum sensing systems (reviewed in [18]).

In this bacterium, the presence of Zn, Cd or Cu, induces the expression of the metal-inducible CzcRS two-component system (TCS) that activates the expression of a metal efflux pump CzcCBA and down-regulates the expression of the OprD porin [14,15]. Since this porin is the route of entry of carbapenem antibiotics [19,20], the cells become resistant to this major family of anti-Pseudomonas compounds often used as the last line of treatment. At the same time, the CzcRS TCS modulates the expression of virulence factors by controlling the *las* quorum sensing system [16].

Considering these data and the fact that the sputum of cystic fibrosis patients is enriched in Zn [5,6], we decided to test the effects of this metal on *P. aeruginosa* physiology, virulence and carbapenem resistance using an artificial sputum medium. Our data revealed that Zn is a key factor involved in the two phases of the *P. aeruginosa* infection process. First, Zn facilitates the initial phase by promoting the induction of virulence factor expression, then it stimulates biofilm formation during the chronic phase. Additionally, the presence of Zn induces carbapenem resistance in both planktonic cells and biofilm cells.

2. Materials and methods

2.1. Strains and growth conditions

P. aeruginosa PAO1 (laboratory strain) and the *ΔczcRS* mutant [14] deleted for the *czcRS* TCS were used in this work. Bacterial strains were grown at 37 °C in artificial sputum (AS) medium [21] with modified proportions of mucin and salmon sperm DNA (invitrogen) as previously described [22]. The AS medium was supplemented or not with 0.2 mM $ZnCl_2$. This concentration corresponds to the value obtained by atomic absorption spectrometry in the lung tissue [23] and was also used recently for the analysis of the *Enterococcus faecalis* transcriptome, revealing strong changes in the expression profile of transporters and genes involved in metabolism and cell envelope synthesis [24]. Cultures were grown on a rotary shaker (260 rpm) for the planktonic cell analysis presented in Fig. 1. Static cultures were used for biofilm quantification and planktonic versus biofilm RT-qPCR analysis.

2.2. MIC determination

The minimum inhibitory concentration (MIC) was determined, in AS medium supplemented or not with 0.2 mM $ZnCl_2$, by using the broth dilution method in 96-well microtiter plates as previously described [25]. Tienam (imipenem) powder was freshly resuspended and diluted in AS medium. 100 µl of a doubling dilution series (final concentration from 64 µg/mL to 0.125 µl/mL) were dispensed into a 96-well microplate, 100 µl of bacterial inoculum was then added to each well and incubated at 37 °C under static conditions for 12 h. Growth was detected by the addition of 20 µl p-iodonitrotetrazolium violet (INT, Sigma–Aldrich) solution at 2 mg/ml, followed by incubation for several hours [26]. The highest dilution of imipenem in which no red-purple color appears corresponds to its MIC.

2.3. CzcR antiserum production and western blot analysis

The full *czcR* gene was amplified by PCR (primers czcr5: CCACATATGCGCATCCTTATTATCGAAGATGAA and czcR3: CCCGGATCCCGGCGCGCTTCCAGGACGTAGCCG), digested with *Nde*I and *BamH*I and cloned into the *Nde*I-*BamH*I restriction sites of the pET15b vector (Novagen). The plasmid was introduced into *Escherichia coli* BL21(DE3) [27]. Overexpression and purification were performed under denaturing conditions using a nickel affinity column (Ni-NTA superflow, Qiagen) according to the Qiagen protocol. Fractions containing the recombinant protein were pooled and purified by SDS–PAGE in a 12% gel. Polyclonal antiserum was raised in rabbits by four three-weekly injections of 100 µg of purified recombinant protein. Western blot analysis was performed as previously described [16] using AS medium with or without the addition of 0.2 mM $ZnCl_2$.

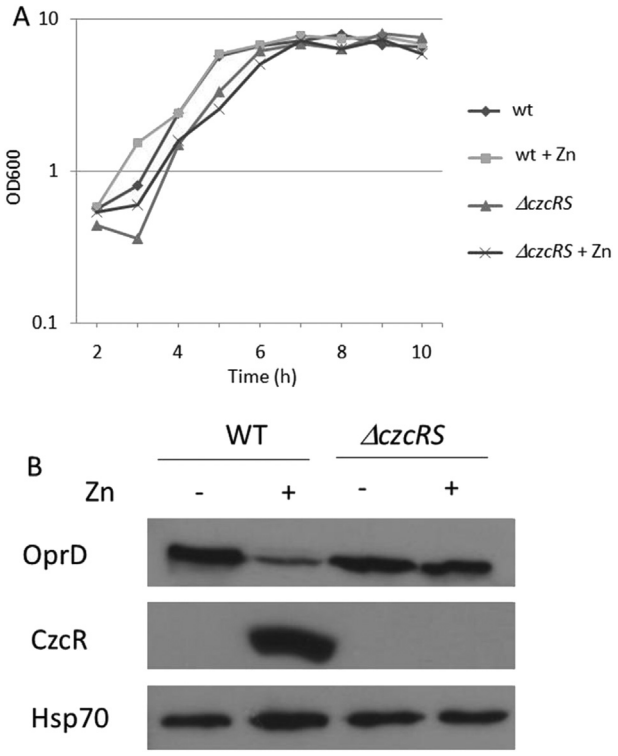

Fig. 1. Effect of Zn on growth and on OprD expression in AS medium. A) Growth curves of *Pseudomonas aeruginosa* wild type PAO1 and *ΔczcRS* mutant in AS medium in the presence or absence of 0.2 mM $ZnCl_2$ (+Zn). B) Western blot analysis of OprD porin and CzcR regulator in the wild type and the *ΔczcRS* mutant strains. Total protein was extracted from strains cultivated in AS medium in the presence or absence of 0.2 mM $ZnCl_2$. Blots were exposed to anti-OprD, anti-CzcR or anti-Hsp70 (loading control) antibody.

2.4. Biofilm quantification

Overnight precultures in AS medium were diluted to an OD_{600} of 0.05 in AS medium, supplemented or not with 0.2 mM $ZnCl_2$. 200 µL were dispensed to a 96-well plate and incubated for 15 h at 37 °C without shaking. The culture, containing planktonic cells, was removed and adherent biofilm was quantified as previously described [16]. Measurements were performed three times in three independent experiments.

2.5. RNA extraction and qRT-PCR

RNA extraction was performed on cultures grown in AS medium as described for biofilm quantification. After incubation, the planktonic cells were harvested by gently pipetting each well, without disturbing the biofilm formed around the wall. Before collection, the biofilm cells (attached to the walls) were released by