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Gallium Induces the Production of Virulence Factors in *Pseudomonas aeruginosa*

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Summary

Gallium, a novel antimicrobial, interferes with iron homeostasis in *Pseudomonas aeruginosa*. However, iron deficiency increases virulence, and we found gallium also increases the production of virulence factors in laboratory and clinical strains.

Abstract

The novel antimicrobial gallium is a non-redox iron III analogue with bacteriostatic and bactericidal properties, effective for the treatment of *Pseudomonas aeruginosa in vitro* and *in vivo* in mouse and rabbit infection models. It interferes with iron metabolism, transport and presumably its homeostasis. Since gallium exerts its antimicrobial effects by competing with iron, we hypothesized that it ultimately will lead cells to an iron deficiency status. Since iron deficiency promotes the expression of virulence factors *in vitro* and promotes the pathogenicity of *Pseudomonas aeruginosa* in animal models, it is anticipated that treatment with gallium will also promote the production of virulence factors. To test this hypothesis, the reference strain PA14 and two clinical isolates from cystic fibrosis patients were exposed to gallium and their production of pyocyanin, rhamnolipids, elastase, alkaline protease, alginate, pyoverdine and biofilm was determined. Gallium treatment induced the production of all the virulence factors tested in the three strains except for pyoverdine. In addition, since the Ga-induced virulence factors are quorum sensing controlled, co-administration of Ga and the quorum quencher brominated furanone C-30 was assayed, and it was found that C-30 alleviated growth inhibition from gallium. Hence, adding both C-30 and gallium may be more effective in the treatment of *P. aeruginosa* infections.

Introduction

The antipseudomonal properties of $Ga(NO_3)_3$ were described recently (Kaneko*, et al.*, 2007). This formulation is used in the clinic for the treatment of hypercalcemia of malignancy; gallium has bacteriostatic/bactericidal properties *in vitro*, depending in on the doses and iron availability, while *in vivo* it promotes healing of pulmonary mice infections (Kaneko*, et al.*, 2007), and bacterial keratitis in rabbits, when coupled with desferrioxamine (Banin*, et al.*, 2008). In addition other formulations like Ga-maltolate are also effective in treating murine infections (DeLeon*, et al.*, 2009). The psychochemical properties of Ga (III) are very similar to those of Fe (III) but unlike iron, gallium cannot be reduced, so it was proposed that Ga (III) may interfere with several processes requiring iron redox cycling, like (i) electron transport by the respiratory chain via iron mediated redox reactions of the respiratory complexes (Anraku, 1988), (ii) DNA synthesis via the iron dependency of ribonucleotide reductase (Fontecave*, et al.*, 1990), (iii) protection against oxidative stress via iron dependent catalase and superoxide dismutase (Hassett*, et al.*, 1993, Frederick*, et al.*, 2001), and (iv) transport of Fe(III) by the siderophore pyoverdine, because, in order to be internalized Fe(III) complexed with pyoverdine must be first reduced to Fe(II) (Yeats*, et al.*, 2004). Since gallium exerts its antimicrobial effects by competing with iron, it ultimately causes iron deficiency and since growth at low iron levels dramatically increases *P. aeruginosa* virulence, we hypothesized that Ga treatment may also increase virulence.

Several studies demonstrated that iron deficiency promotes *P. aeruginosa* virulence; for example, Sokol and Woods (Sokol & Woods, 1984) found that iron limitation increases the production of elastase and exotoxin A in cultures and creases tissue damage/inflammation in rat lung infections.; Also, low iron levels induce proteases, elastase, phospholipase C, alginate, pyoverdine, pyochelin, hemolysin and adherence and increases renal bacterial load and tissue pathology in mice (Mittal*, et al.*, 2008). Several other studies reported similar findings (Lamont*, et al.*, 2002, Ochsner*, et al.*, 2002, Kim*, et al.*, 2003). Indeed Kaneko et al. (Kaneko*, et al.*, 2007) that first proposed Ga as an antipseudomonal were aware of the effect of iron deficiency and the induction of virulence factors and concluded based in DNA microarrays that Ga had no effect on the expression of virulence genes. Nevertheless, five pyochelin synthesis genes *pchABCGH* were among the most induced in that study. Moreover Ga was used at very low concentration that was innocuous to growth. However to combat *P. aeruginosa* infections, Ga must be used at a concentration able to decrease growth and/or kill bacteria.

Results and Discussion

In order to test our hypothesis, it was first confirmed that decreasing iron levels increased the production of virulence factors., For these experiments, *P. aeruginosa* PA14 was grown in minimal succinate medium supplemented with $FeCl_3$ 2.5 μM (Ren et al., 2005) at 37ºC with 200 rpm shaking for 15 h, in the absence and presence of the iron chelating protein lactoferrin, purchased from SIGMA (St Louis, MO, USA) at 0.1, 0.25, and 0.5 mg/mL and the virulence factors were determined as reported previously: pyoverdine (Ren*, et al.*, 2005), pyocyanin (Essar*, et al.*, 1990) and elastase (Ohman*, et al.*, 1980) were determined as reported previously **(supplementary methods).** As expected, lactoferrin treatment increased the production of virulence factors in a dose response manner, with the maximal induction occurring at 0.5 mg/mL: 1.6, 3.0, and 5.4-fold for pyoverdine, pyocyanin and elastase, respectively. Also as expected, adding **a higher concentration of $FeCl_3$ (12.5 μM)** to the medium reverted the effects of lactoferrin (Supporting Information, Fig. S1).

With confirmation that decreasing iron availability via lactoferrin increases virulence factor production, the effect of $Ga(NO_3)_3$ in the production of pyocyanin, elastase, rhamnolipids, alginate, alkaline protease and biofilm formation of the reference strain PA14 was tested, under the same culture conditions used to evaluate lactoferrin. Alginate, alkaline protease, biofilm formation and rhamnolipids were determined as described previously (Knutson & Jeanes, 1968, Howe & Iglewski, 1984, O'Toole & Kolter, 1998, Wilhelm*, et al.*, 2007) **(supplementary methods).** The effect of Ga on PA14 was assayed at two concentrations. Ga 10 μM that decreased the growth rate (μ) ~ 10%, without a significant effect in maximal cell density at 15 h, and Ga 25 μM that decreased ~ 30% both the growth rate and a the maximal cell density. Since the decrease in cell density was significant, cultures with Ga 25 μM were allowed to grow until they reached a similar final density than the cultures without Ga (O.D. 600 nm~ 2.5). For biofilm formation, the 96-well/crystal violet staining assay was performed **(supplementary methods)** As shown in Figure 1, **in general**, the virulence factors increased with both Ga concentrations, except for rhamnolipids when Ga 10 μM was used (0.48-fold decrease respect to the control without Ga), and pyoverdine that decreased 0.5-fold with Ga 25 μM **and biofilm that remained unchanged with Ga 10 μM.** The maximal inductions, at Ga 25 μM, were **all significant ($p < 0.05$)** of: 1.5, 2.0, 2.0, 2.1, 3.5, and 5.0-fold for rhamnolipids, biofilm, pyocyanin, alkaline protease, elastase, and alginate, respectively.

Furthermore the effect of Ga in the production of virulence factors in two clinical strains isolated from infants with cystic fibrosis was also evaluated (descriptions of the clinical strains in Supporting Information); those isolates were selected after a screening using Ga 25 μM to select strains with similar Ga tolerance profiles compared to PA14. Similar to the case of PA14, the production of virulence factors increased with Ga 25 μM treatment: 5.8, 3.6, 9.1, 8.6, 2.0, and 1.7-fold for pyocyanin, elastase, biofilm, alkaline protease, rhamnolipids and alginate for the INP-37 strain and 3.7, 1.7, 16.6, 2.0, 2.2, and 1.7 for the INP-58M strain, while pyoverdine decreased 0.56 fold for INP-37 and increased 1.32 fold for INP-58M (Figure 1). **All inductions at Ga 25 μM were significant ($p < 0.05$), except for pyoverdine and alginate of the strain INP-58M.** Hence, Ga induces the expression of virulence factors in **the selected two** clinical strains as it does with PA14. **Further research is needed to address the frequency of this phenomenon in larger collections of clinical strains from CF patients and other kind of infections.**

Due the properties of these virulence factors, they may eventually help bacteria to counteract gallium toxicity. For example, *P. aeruginosa* rhamnolipids bind heavy metals (Ochoa-Loza*, et al.*, 2001) and decrease the toxicity of Cd (II) (Sandrin*, et al.*, 2000). Moreover Al (III), which belongs to the same chemical group as Ga (III), has the highest reported affinity for rhamnolipids, while Fe (III) affinity is much lower (Ochoa-Loza*, et al.*, 2001). In consequence, the secretion of rhamnolipids may confer resistance to Ga (III) by sequestering it. Similarly the exopolysaccharide alginate binds heavy metals including Al (III) (Gregor*, et al.*, 1996). In addition since elastase cleaves transferrin releasing the bound iron (Britigan*, et al.*, 1993), its activity could contribute to cope with gallium by increasing iron availability. Similarly, if alkaline protease cleaves iron bound proteins it may release iron. Finally, for pyocyanin, we recently demonstrated that its production increased 2-fold in a PA14 spontaneous Ga (III) resistant mutant, that treatment with Ga induces its production, that pyocyanin(-) mutants are more sensitive to Ga than the wild-type strain, and that its addition to the medium protects the wild-type strain but not a mutant unable to transport Fe (II) against the bacteriostatic effects of Ga (García-Contreras*, et al.*, 2013). A possible explanation for this protective effect is that pyocyanin catalyzes the reduction of Fe (III) to Fe (II) (Cox, 1986), and that since Ga does not bind to Fe(II) binding molecules (Logan, et al., 1981), it may not affect Fe (II) uptake.

Since our proposed mechanism implies that Fe (III) is reduced to Fe (II), Fe (II) should have stronger protective effects against Ga than Fe (III). Consistent with

this mechanism, we found that for the PA14 strain, adding 5 μM of Fe(II) to the succinate medium protects the cells against up to 100 μM Ga; in contrast, growth is severely affected with lower Ga (inhibited 82% with 50 μM Ga) when adding the same amount of Fe (III) (Supporting Information, Fig. S2A).The clinical isolates showed similar behavior (Fig. S2B **and S2C**). These results are relevant since recently it was demonstrated that Fe (II) is available for *P. aeruginosa in vivo* and has an important role in promoting pulmonary infections in CF patients (Hunter*, et al.*, 2013). Interestingly, the biofilm inhibition properties of Ga that had been described previously (Kaneko*, et al.*, 2007) were seen here for initial PA14 biofilms with 10 and 25 μM of Ga when cultured during 15 or 24 h(García-Contreras*, et al.*, 2013); however, we show here that for longer times (48 h), the biofilm formation of PA14 increased 2-fold in the presence of Ga 25 μM and that biofilm formation of the clinical strains increased significantly (up to 17-fold). **In addition, Ga 25 μM inhibited PA14 biofilm formation by 30 % but had no effect on the biofilms of both clinical strains after 18 h of treatment, confirming that the effect of Ga on biofilm is time dependant and not the same for all strains. Furthermore, biofilms of the three strains were developed in the presence and absence of Ga 25 μM for 48 h, replacing the medium every 12 h to avoid nutrient limitation (supplementary methods), and biofilm formation was induced 2.0, 1.72, and 2.5 fold for PA14, INP-37 and INP-58 M, respectively (P <0.05). Currently we do not know the mechanisms leading to biofilm stimulation by Ga, but it is important to note that the mechanism by which Ga inhibits biofilm is not well understood (Rzhepishevska*, et al.*, 2011)**.Since all the virulence factors tested are controlled by quorum sensing (Winzer & Williams, 2001), the effect of adding Ga concomitantly with the quorum quenching compound furanone C-30 (Hentzer*, et al.*, 2003) was evaluated in the hope of reducing production of the virulence factors. In PA14, C-30 potentiates the growth inhibitory effects of Ga (Supporting Information, Fig. S3) by blocking the induction of QS controlled virulence factors. Also, Ga did not exhibit any supra-additive effects in killing when combined with gentamycin, ceftazidime, imipenem, or ciprofloxacin (not shown).

Overall, our results suggest that treatment of *P. aeruginosa* infections with Ga may eventually enhance virulence, promoting the damage associated with the infection instead of alleviating it.; However, a combination therapy with Ga and quorum quenchers may be more effective to treat *P. aeruginosa* infections than Ga monotherapy. Our observations require further study such as the evaluation of the effect of Ga (alone or combined with quorum quenchers) in a broader collection of clinical isolates and in the virulence of laboratory/clinical strains in animal models.

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