SHORT COMMUNICATION

Gallium induces the production of virulence factors in *Pseudomonas aeruginosa*

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In this study the authors show that gallium, which has antimicrobial properties, interferes with iron homeostasis in *Pseudomonas aeruginosa* and thereby increases expression of virulence factors. This is an important finding highly relevant for treatment of *Pseudomonas* infections.

Keywords
*Pseudomonas aeruginosa*; gallium nitrate; iron; virulence factors; biofilms.

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The antipseudomonal properties of Ga(NO₃)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, such as (1) electron transport by the respiratory chain via iron-mediated redox reactions of the respiratory complexes (Anraku, 1988), (2) DNA synthesis via the iron dependency of ribonucleotide reductase (Fontecave et al., 1990), (3) protection against oxidative stress via iron-dependent catalase and superoxide dismutase (Hassett et al., 1993; Frederick et al., 2001), and (4) 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). As gallium exerts its antimicrobial effects by competing with iron, we hypothesized that it ultimately will lead cells to an iron deficiency status. As iron deficiency promotes the expression of virulence factors *in vitro* and promotes the pathogenicity of *P. 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 patients with cystic fibrosis 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, as 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.

Abstract
The novel antimicrobial gallium is a nonredox 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. As gallium exerts its antimicrobial effects by competing with iron, we hypothesized that it ultimately will lead cells to an iron deficiency status. As iron deficiency promotes the expression of virulence factors *in vitro* and promotes the pathogenicity of *P. 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 patients with cystic fibrosis 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, as 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.
Woods (1984) found that iron limitation increases the production of elastase and exotoxin A in cultures and increases 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. (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 on 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.

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 FeCl₃ 2.5 μM (Ren et al., 2005) at 37°C with 200 r.p.m. shaking for 15 h, in the absence and presence of the iron-chelating protein lactoferrin, purchased from Sigma (St Louis, MO) at 0.1, 0.25, and 0.5 mg mL⁻¹, 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 (Supporting Information, Data S1). 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: 1.6-, 3.0-, and 5.4-fold for pyoverdine, pyocyanin, and elastase, respectively. Also as expected, adding a higher concentration of FeCl₃ (12.5 μM) to the medium reverted the effects of lactoferrin (Fig. S1).

With confirmation that decreasing iron availability via lactoferrin increases virulence factor production, the effect of Ga(NO₃)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 & Jeans, 1968; Howe & Iglewski, 1984; O'Toole & Kolter, 1998; Wilhelm et al., 2007; Data S1). The effect of Ga on PA14 was assayed at two concentrations. Ga 10 μM that decreased the growth rate (μ) c. 10%, without a significant effect in maximal cell density at 15 h, and Ga 25 μM that decreased c. 30% both the growth rate and the maximal cell density. As the decrease in cell density was significant, cultures with Ga 25 μM were allowed to grow until they reached a similar final density as the cultures without Ga (OD₆₀₀ nm c. 2.5). For biofilm formation, the 96-well/crystal violet staining assay was performed (Data S1) As shown in Fig. 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-, 2.1-, 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 (see Table S1); those isolates were selected after a screening using Ga 25 μM to select strains with similar Ga tolerance profiles compared with 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-fold for the INP-58M strain, while pyoverdine decreased 0.56-fold for INP-37 and increased 1.32-fold for INP-58M (Fig. 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 patients with CF 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, as 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 twofold 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 as Ga does not bind to Fe(II)-binding molecules (Logan et al., 1981), it may not affect Fe (II) uptake.

As 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) (Fig. S2a). The clinical isolates showed similar behavior (Fig. S2b and c). These results are relevant as recently it was demonstrated that Fe (II) is available for *P. aeruginosa* in vivo and has an important role in promoting pulmonary infections in patients with CF (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 twofold 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 dependent 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 (Data S1), and biofilm formation was induced 2.0-, 1.72-, and 2.5-fold for PA14, INP-37, and INP-58M, 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). As 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 (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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References


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Lactoferrin induces the production of the virulence factors: pyocyanin, elastase, and pyoverdine in Pseudomonas aeruginosa PA14.
Fig. S2. Protection of Fe (II) and Fe (III) against Ga growth inhibition.
Fig. S3. Growth inhibition of Ga is potentiated by the addition of furanone C-30.
Table S1. Description of the clinical strains.
Data S1. Materials and Methods.

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