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Pyochelin Potentiates the Inhibitory Activity of Gallium on *Pseudomonas aeruginosa*

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Gallium (Ga) is an iron mimetic that has successfully been repurposed for antibacterial chemotherapy. To improve the antibacterial potency of Ga on *Pseudomonas aeruginosa*, the effect of complexation with a variety of siderophores and synthetic chelators was tested. Ga complexed with the pyochelin siderophore (at a 1:2 ratio) was more efficient than $Ga(NO_3)_3$ in inhibiting *P. aeruginosa* growth, and its activity was dependent on increased Ga entrance into the cell through the pyochelin translocon.

Iron (Fe) is an essential nutrient for nearly all forms of life, being the cofactor of many vital enzymes involved in DNA synthesis, metabolism, and the oxidative stress response (1). Pathogenic bacteria must counteract an Fe-poor environment during infection, since Fe is unavailable to invading pathogens due to sequestration by the Fe carrier and storage proteins of the host (2). Bacteria have evolved multiple strategies to acquire Fe from the host, the most common being through the production of siderophores. These compounds are secreted in the extracellular milieu, where they form stable complexes with Fe and other transition metals (depending on their coordination chemistry) and convey the metal to the bacterial cell via specific active transport systems (3). Given the importance of Fe in bacterial metabolism and the paucity of effective antibiotics for multidrug-resistant bacteria, Fe uptake and metabolism have recently been assessed as targets for the development of new antibacterials (4–7).

Gallium (Ga^{III}) is a semimetal that shares a number of chemical similarities with the oxidized Fe form (Fe^{III}). The most prominent Fe^{III} mimetic features of Ga^{III} are the nucleus radius and coordination chemistry, which enable Ga^{III} to replace Fe^{III} in Fe-containing enzymes. Being that Ga^{III} is redox inactive, its incorporation in Fe^{III}-containing enzymes results in the overall disruption of Fe metabolism (8). The antimicrobial properties of $Ga(NO_3)_3$, the active component of the FDA-approved formulation Ganite (Genta), have been investigated in a number of species (recently reviewed in references 9–12). In particular, Ga^{III} inhibits both planktonic and biofilm growth of the opportunistic pathogen *Pseudomonas aeruginosa* and causes significant protection from *P. aeruginosa* infection in animal models (13, 14).

In the present study, we attempted to improve the antibacterial activity of Ga^{III} on *P. aeruginosa* by complexation with suitable carriers (either synthetic chelators or siderophores) that are actively taken up by the bacterium and that stimulate, to a variable extent, its growth under conditions of extreme Fe deficiency (see Fig. S1 in the supplemental material). Both the *P. aeruginosa* reference strain PAO1 and the cystic fibrosis isolate TR1 (15) were used for growth promotion/inhibition assays. Ga^{III}-chelator complexes were generated by mixing, in the appropriate ratios (Fig. 1), aqueous solutions of $Ga(NO_3)_3$ with ferrichrome (FER) (Sigma), sodium dicitrate (CIT) (Sigma), desferrioxamine (DFO) (Novartis), sodium salicylate (SAL) (Sigma), and the autologous siderophores pyoverdine (PVD) and pyochelin (PCH). PVD and PCH were purified from culture supernatants of a PCH-defective *P. aeruginosa* mutant (PAO1Δ*pchD*; see Table S1 and Supplemental Experimental Procedures in the supplemental material) and a PVD-defective *P. aeruginosa* mutant (PAO1Δ*pvdA*) (16), respectively, according to previously published procedures (17–19). The growth inhibitory activity of each Ga^{III} complex was then assayed in the Fe-poor medium DCAA (18) and compared to the activity of each chelator or $Ga(NO_3)_3$ alone (Fig. 1; see also Fig. S2 in the supplemental material). In line with previous results (13), $Ga(NO_3)_3$ inhibited *P. aeruginosa* growth in a dose-dependent manner at concentrations of >3.13 μM. Consistent with previous findings (13), $Ga(NO_3)_3$ showed bacteriostatic activity at a growth inhibitory concentration (12.5 μM; data not shown).

The PCH-Ga^{III} complex was the only combination endowed with higher inhibitory activity than that of $Ga(NO_3)_3$ alone. PVD-Ga^{III}, SAL-Ga^{III}, and CIT-Ga^{III} complexes showed a moderate protective effect on *P. aeruginosa*, since they decreased Ga^{III}-mediated growth inhibition in a dose-dependent manner. Notably, FER-Ga^{III} and DFO-Ga^{III} abrogated growth inhibition by Ga^{III}, as one would expect for a compound endowed with strong Ga^{III}-scavenging activity (Fig. 1; see also Fig. S2 in the supplemental material). A similar response to Ga^{III} and its complexes was observed for both the *P. aeruginosa* PAO1 and TR1 strains (Fig. 1; see also Fig. S2 in the supplemental material).

It appears, therefore, that some Ga^{III} complexes alleviate Ga^{III} inhibition rather than potentiate it, suggesting that they behave as Ga^{III} scavengers rather than vehicles of Ga^{III} to the cell. To gain further insight into the effect of the different chelators on Ga^{III} activity, bacteria were grown in DCAA containing $Ga(NO_3)_3$ at a fixed inhibitory concentration (12.5 μM) and increasing concentrations of the different chelators in order to obtain different chelator-to-Ga^{III} ratios (Fig. 2). DFO and FER protected *P. aeruginosa* from the growth inhibitory activity of $Ga(NO_3)_3$, even at 1:2 and

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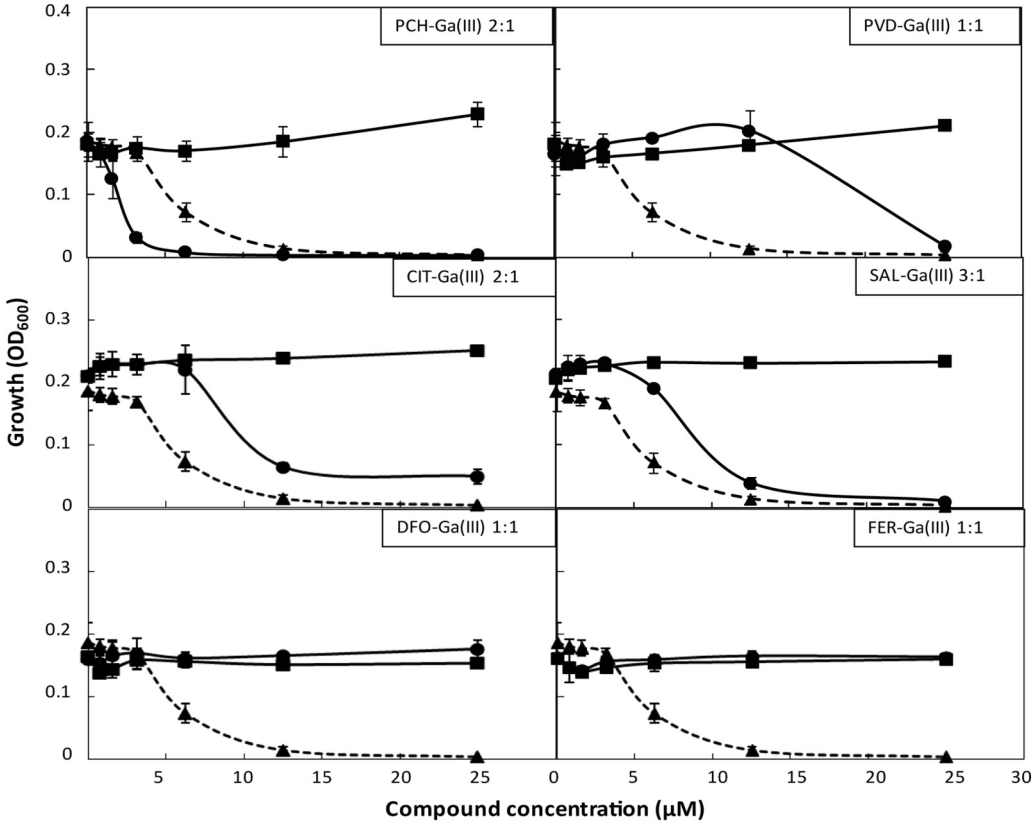

FIG 1 Effect of $Ga(NO_3)_3$ and Ga^{III} complexes on *P. aeruginosa* PAO1 growth. Growth (optical density at 600 nm [OD_{600}]) of *P. aeruginosa* PAO1 in microtiter plates containing (per well) 200 μl DCAA supplemented with different concentrations of $Ga(NO_3)_3$ (triangles and dashed lines), the indicated Ga^{III}-chelator complex (circles and solid lines), or the chelator alone as a control (squares and solid lines), after 24 h at 37°C. The stoichiometry (binding ratio) of each Ga^{III}-chelator complex is indicated in the inset of each panel. The data are the mean ± standard deviation from at least two independent experiments.

1:4 chelator-to-Ga^{III} ratios, which is suggestive of a strong Ga^{III} scavenging effect (Fig. 2). Similar results were obtained with PVD, though at higher PVD-to-Ga^{III} ratios. SAL and CIT showed a poor scavenging effect, being unable to counteract the $Ga(NO_3)_3$ inhibitory effect even in the presence of an excess of chelator (chelator-to-Ga^{III} ratio, 4:1). PCH never rescued *P. aeruginosa* growth in the presence of $Ga(NO_3)_3$ at all ratios tested (Fig. 2). Again, comparable results were obtained for the *P. aeruginosa* clinical strain TR1 (see Fig. S3 in the supplemental material). Taken together, these results indicate that DFO, FER, and PVD are strong inhibitors of Ga^{III} activity, while PCH exerts an opposite effect, suggesting that PCH acts as a "Trojan horse" that conveys Ga^{III} into the cell.

To shed more light on the mechanism by which PCH potentiates Ga^{III} activity, we generated *P. aeruginosa* PAO1 mutants impaired in PCH biosynthesis (Δ*pchD*) or uptake (Δ*fptAX* and Δ*fptX*, with deletions of the whole PCH translocon or the inner membrane transporter only, respectively) (for details, see reference 20; see also Table S1 and Supplemental Experimental Procedures in the supplemental material). We reasoned that if the Ga^{III} inhibitory effects were enhanced by PCH production and internalization, these PCH synthesis and uptake mutants would be more resistant to $Ga(NO_3)_3$. The effect of $Ga(NO_3)_3$ on these strains was thus assessed in DCAA supplemented with increasing $Ga(NO_3)_3$ concentrations (0 to 100 μM), and the data were expressed as the MICs at 24 h and 48 h (Fig. 3A). While all strains showed comparable growth levels in DCAA without $Ga(NO_3)_3$ (data not shown), the Δ*pchD*, Δ*fptAX*, and Δ*fptX* strains were more resistant to $Ga(NO_3)_3$ than the wild-type PAO1 (Fig. 3A), suggesting that PCH production and transport into the cell contribute to Ga^{III} inhibitory activity. To confirm that the reduction of Ga^{III} susceptibility in PCH-defective mutants was due to impaired internalization of Ga^{III} into the cell, intracellular Ga^{III} levels were measured in each strain grown in DCAA supplemented with a subinhibitory $Ga(NO_3)_3$ concentration (3 μM) by means of inductively coupled plasma optical emission spectrometry (ICP-OES) using an ICP-OES Varian 710 spectrometer (Agilent Technologies) (Fig. 3B). In line with the higher resistance shown by the Δ*pchD*, Δ*fptAX*, and Δ*fptX* mutants, the intracellular levels of Ga^{III} were significantly lower in these mutants than those in wild-type PAO1 (Fig. 3B), although not nil, suggesting that Ga^{III} may enter the cell via an alternative route(s). This is consistent with the recent finding that the HitAB Fe transport system is also involved in Ga^{III} uptake by *P. aeruginosa* (21).

In conclusion, our data demonstrate that siderophores and synthetic chelators affect in different manners the *in vitro* activity of Ga^{III} on *P. aeruginosa*. Among many Ga^{III} complexes tested, only the endogenous siderophore PCH facilitates Ga^{III} entrance into the cell through its specific uptake machinery, thereby potentiating the antipseudomonal activity of Ga^{III}. These findings might be of guidance for the future design of more effective Ga^{III} delivery systems to *P. aeruginosa*.

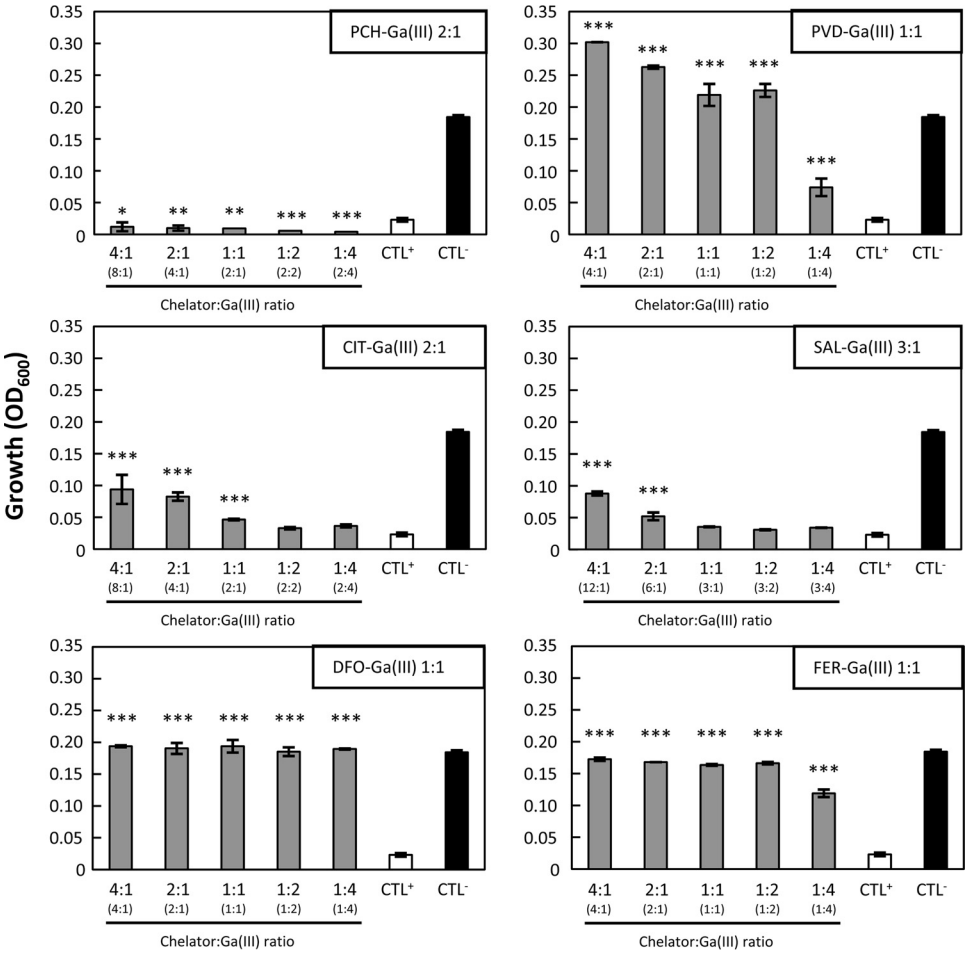

FIG 2 Effect of different chelator-to-Ga^{III} ratios on *P. aeruginosa* PAO1 growth. *P. aeruginosa* PAO1 was grown for 24 h at 37°C in microtiter plates containing (per well) 200 μl DCAA supplemented with 12.5 μM $Ga(NO_3)_3$ and various concentrations of each chelator to obtain different chelator-to-Ga^{III} ratios. The chelator-to-Ga^{III} ratio (*x* axis) takes into account the binding stoichiometry, as indicated in the inset of each panel. The actual chelator-to-Ga^{III} ratio is given in parentheses on the *x* axis. CTL^+, growth in the presence of 12.5 μM $Ga(NO_3)_3$; CTL^-, growth without chelators or $Ga(NO_3)_3$. Growth was measured as the OD_{600} (*y* axis). Each value is the mean ± standard deviation from the results of three independent experiments. Statistically significant differences compared to CTL^+ are indicated (analysis of variance [ANOVA]): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

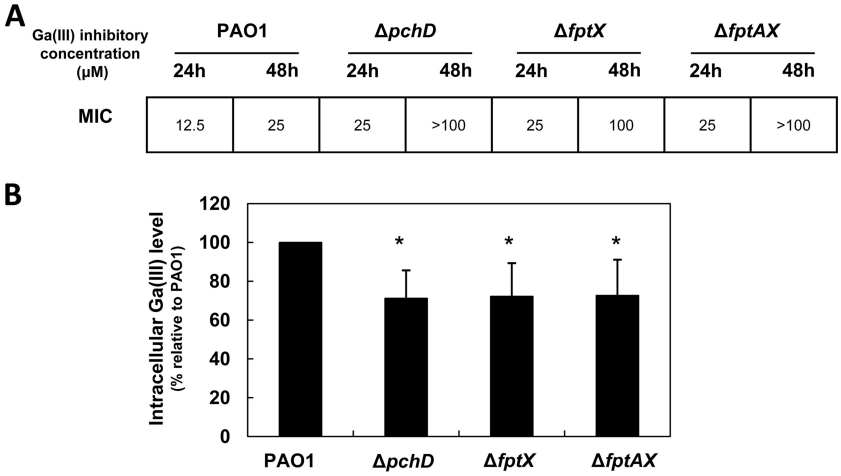

FIG 3 Ga^{III} inhibitory activity and intracellular Ga^{III} levels in *P. aeruginosa* PAO1 and isogenic PCH-defective mutants. (A) MICs of $Ga(NO_3)_3$ (μM) for *P. aeruginosa* PAO1 and isogenic PCH-transport mutants grown for 24 h and 48 h in 200 μl DCAA supplemented with increasing $Ga(NO_3)_3$ concentrations (0 to 100 μM). (B) Intracellular concentrations of Ga^{III} in the same *P. aeruginosa* strains grown in DCAA supplemented with 3 μM $Ga(NO_3)_3$ for 14 h, measured by the means of ICP-OES. The values are expressed as the percentage relative to PAO1 and represent the mean ± standard deviation from three independent experiments. Intracellular Ga^{III} levels in wild-type PAO1 varied from 0.47 to 1.41 ng Ga^{III}/mg total cell proteins, depending on the experiment. *, statistically significant differences relative to PAO1 ($P < 0.05$, ANOVA).

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