Pyochelin Potentiates the Inhibitory Activity of Gallium on Pseudomonas aeruginosa

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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 (GaIII) is a semimetal that shares a number of chemical similarities with the oxidized Fe form (FeIII). The most prominent FeIII mimetic features of GaIII are the nucleus radius and coordination chemistry, which enable GaIII to replace FeIII in Fe-containing enzymes. Being that GaIII is redox inactive, its incorporation in FeIII-containing enzymes results in the overall disruption of Fe metabolism (8). The antimicrobial properties of Ga(NO3)3, the active component of the FDA-approved formulation Ganite (Genta), have been investigated in a number of species (9–12). In particular, GaIII 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 GaIII 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. GaIII-chelator complexes were generated by mixing, in the appropriate ratios (Fig. 1), aqueous solutions of Ga(NO3)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 PCH were purified from culture supernatants of a PCH-defective P. aeruginosa mutant (PAO1ΔpchD; see Table S1 and Supplemental Experimental Procedures in the supplemental material) and a PVD-defective P. aeruginosa mutant (PAO1ΔpvdA) (16), respectively, according to previously published procedures (17–19). The growth inhibitory activity of each GaIII complex was then assayed in the Fe-poor medium DCAA (18) and compared to the activity of each chelator or Ga(NO3)3 alone (Fig. 1; see also Fig. S2 in the supplemental material). In line with previous results (13), Ga(NO3)3 inhibited P. aeruginosa growth in a dose-dependent manner at concentrations of >3.13 μM. Consistent with previous findings (13), Ga(NO3)3 showed bacteriostatic activity at a growth inhibitory concentration (12.5 μM; data not shown).

The PCH-GaIII complex was the only combination endowed with higher inhibitory activity than that of Ga(NO3)3 alone. PVD-GaIII, SAL-GaIII, and CIT-GaIII complexes showed a moderate protective effect on P. aeruginosa, since they decreased GaIII-mediated growth inhibition in a dose-dependent manner. Notably, FER-GaIII and DFO-GaIII abrogated growth inhibition by GaIII, as one would expect for a compound endowed with strong GaIII-scavenging activity (Fig. 1; see also Fig. S2 in the supplemental material). A similar response to GaIII 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 GaIII complexes alleviate GaIII inhibition rather than potentiate it, suggesting that they behave as GaIII scavengers rather than vehicles of GaIII to the cell. To gain further insight into the effect of the different chelators on GaIII activity, bacteria were grown in DCAA containing Ga(NO3)3 at a fixed inhibitory concentration (12.5 μM) and increasing concentrations of the different chelators in order to obtain different chelator-to-GaIII ratios (Fig. 2). DFO and FER protected P. aeruginosa from the growth inhibitory activity of Ga(NO3)3, even at 1:2 and...
1:4 chelator-to-Ga\textsuperscript{III} ratios, which is suggestive of a strong Ga\textsuperscript{III} scavenging effect (Fig. 2). Similar results were obtained with PVD, though at higher PVD-to-Ga\textsuperscript{III} ratios. SAL and CIT showed a poor scavenging effect, being unable to counteract the Ga(NO\textsubscript{3})\textsubscript{3} inhibitory effect even in the presence of an excess of chelator (chelator-to-Ga\textsuperscript{III} ratio, 4:1). PCH never rescued \textit{P. aeruginosa} growth in the presence of Ga(NO\textsubscript{3})\textsubscript{3} at all ratios tested (Fig. 2). Again, comparable results were obtained for the \textit{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\textsuperscript{III} activity, while PCH exerts an opposite effect, suggesting that PCH acts as a "Trojan horse" that conveys Ga\textsuperscript{III} into the cell.

To shed more light on the mechanism by which PCH potentiates Ga\textsuperscript{III} activity, we generated \textit{P. aeruginosa} PAO1 mutants impaired in PCH biosynthesis (\textit{pchD}) or uptake (\textit{fptAX} and \textit{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). Taken together, these results indicate that DFO, FER, and PVD are strong inhibitors of Ga\textsuperscript{III} activity, while PCH exerts an opposite effect, suggesting that PCH acts as a "Trojan horse" that conveys Ga\textsuperscript{III} into the cell.

![FIG 1 Effect of Ga(NO\textsubscript{3})\textsubscript{3} and Ga\textsuperscript{III} complexes on \textit{P. aeruginosa} PAO1 growth. Growth (optical density at 600 nm [OD\textsubscript{600}]) of \textit{P. aeruginosa} PAO1 in microtiter plates containing (per well) 200 \textmu{l} DCAA supplemented with different concentrations of Ga(NO\textsubscript{3})\textsubscript{3} (triangles and dashed lines), the indicated Ga\textsuperscript{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\textsuperscript{III}-chelator complex is indicated in the inset of each panel. The data are the mean ± standard deviation from at least two independent experiments.](http://aac.asm.org/)
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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REFERENCES