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Pyoverdine and Proteases Affect the Response of *Pseudomonas aeruginosa* to Gallium in Human Serum

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Gallium is an iron mimetic which has recently been repurposed as an antibacterial agent due to its capability to disrupt bacterial iron metabolism. In this study, the antibacterial activity of gallium nitrate [$Ga(NO_3)_3$] was investigated in complement-free human serum (HS) on 55 *Pseudomonas aeruginosa* clinical isolates from cystic fibrosis and non-cystic fibrosis patients. The susceptibility of *P. aeruginosa* to $Ga(NO_3)_3$ in HS was dependent on the bacterial ability to acquire iron from serum binding proteins (i.e., transferrin). The extent of serum protein degradation correlated well with *P. aeruginosa* growth in HS, while pyoverdine production did not. However, pyoverdine-deficient *P. aeruginosa* strains were unable to grow in HS and overcome iron restriction, albeit capable of releasing proteases. Predigestion of HS with proteinase K promoted the growth of all strains, irrespective of their ability to produce proteases and/or pyoverdine. The MICs of $Ga(NO_3)_3$ were higher in HS than in an iron-poor Casamino Acids medium, where proteolysis does not affect iron availability. Coherently, strains displaying high proteolytic activity were less susceptible to $Ga(NO_3)_3$ in HS. Our data support a model in which both pyoverdine and proteases affect the response of *P. aeruginosa* to $Ga(NO_3)_3$ in HS. The relatively high $Ga(NO_3)_3$ concentration required to inhibit the growth of highly proteolytic *P. aeruginosa* isolates in HS poses a limitation to the potential of $Ga(NO_3)_3$ in the treatment of *P. aeruginosa* bloodstream infections.

Pseudomonas aeruginosa is a major nosocomial pathogen and the leading cause of chronic lung infection in cystic fibrosis (CF) patients. As for other pathogens, *P. aeruginosa* is faced with severe iron limitation in the mammalian host, since iron is withheld by the host's iron-binding proteins, such as transferrin (Tf) in serum and lactoferrin in mucosal secretions (1). Iron acquisition is crucial for *P. aeruginosa* pathogenicity, as inferred by the presence of multiple iron uptake systems, including the production of siderophores (i.e., pyoverdine [PVD] and pyochelin) (2, 3) and several proteases that cleave the host's iron binding proteins, thereby increasing iron availability *in vivo* (4).

The loss of efficacy of conventional antibiotic therapies for *P. aeruginosa* infection calls for the development of novel therapeutic options aimed at inhibiting both acute and chronic infections. In recent years, attention has been directed to antimicrobial approaches targeting bacterial iron metabolism, since iron is essential for bacteria to cause infection (5, 6). Due to its chemical similarity with iron, the metal gallium (Ga^{3+}) perturbs several iron-dependent biological processes (7), thereby inhibiting the growth of many bacterial species, including *P. aeruginosa* (8–12). Therefore, the repurposing of gallium-based drugs for antibacterial therapy has attracted recent interest, as testified by the initiation of a clinical trial aimed at monitoring the effect of Ganite, the FDA-approved $Ga(NO_3)_3$ formulation, administered to CF patients chronically infected by *P. aeruginosa* (13, 14). Since sepsis represents a frequent complication of *P. aeruginosa* primary infection, with life-threatening consequences and a heavy impact on health care (15), drugs that counteract the bloodstream dissemination of *P. aeruginosa* are needed.

The aim of the present study was to test the efficacy of $Ga(NO_3)_3$ in inhibiting *P. aeruginosa* growth in complement-free human serum (HS), which provides a vehicle for systemic dissemination of infection. We demonstrate that the growth of *P. aeruginosa* in HS is dependent on the ability of individual strains to release proteases that cleave serum proteins, including Tf, as well as on the production of the PVD siderophore, ultimately causing iron release from serum proteins and stimulation of bacterial growth. This phenomenon should be taken into account when assessing the antibacterial efficacy of gallium, since the ability of bacteria to retrieve iron from the host would counteract growth inhibition based on iron mimetism (8–11).

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. The 55 *P. aeruginosa* clinical isolates used in this study are listed in Table S1 in the supplemental material. The collection included 27 isolates from respiratory secretions of CF patients and 28 from other pathological samples from non-CF patients, also including the reference strains PAO1 (ATCC 15692) and PA14 (16). *P. aeruginosa* was grown in iron-free Casamino Acids medium (DCAA) (17), Mueller-Hinton broth (MH; Difco), or HS supplemented or not with the appropriate $FeCl_3$ concentration. Control experiments were conducted in HS that was predigested for 18 h at 37°C with 100 μg/ml proteinase K (Promega) and then inactivated for 30 min at 65°C. Complete HS hydrolysis has been qualitatively verified by SDS-PAGE (see Fig. S1 in the supplemental material).

HS was obtained from 125 healthy donors, following illustration, approval, and subscription of informed consent. Complement was inactivated by incubation at 56°C for 30 min, and the bulk of HS was sterilized by filtration as previously described (18) and then stored at +4°C until used. Bulk HS chemistry was as follows: total serum proteins, 80 mg/ml;

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total iron, 0.70 μg/ml; ferritin, 0.243 μg/ml; Tf, 2.63 mg/ml (64 μM total iron-binding sites); total iron binding capacity, 4.27 mg/ml (20% Tf saturation, equivalent to 51.5 μM unsaturated iron binding sites). $Ga(NO_3)_3$ was purchased from Sigma-Aldrich.

Bacterial growth and PVD determinations. Bacterial growth (optical density at 600 nm [OD_{600}]) was monitored in 96-well microtiter plates using a Wallac 1420 Victor3V multilabel plate reader (PerkinElmer). PVD levels in culture supernatants were measured as the OD_{405} upon dilution in 100 mM Tris-HCl (pH 8) (19).

PVD purification. For PVD purification, the methods of Meyer et al. (20) were used with minor modifications. *P. aeruginosa* Δ*pchD* was grown in DCAA for 18 h. The culture supernatant was purified by filtration through a Sep-Pak C_{18} Vac-Cartridge 3cc (Waters). After the polymer packing was solvated with 10 hold-up volumes of 50% (vol/vol) methanol, the cartridge was flushed with 10 hold-up volumes of double-distilled water. The filtered culture supernatant containing PVD was loaded, and the unwanted components were eluted with double-distilled water. PVD was then eluted with a small quantity of 50% (vol/vol) methanol, evaporated to dryness in a desiccator, and dissolved in a small volume of double-distilled water. The PVD concentration was determined by spectrophotometric measurement of the apo form at OD_{405} ($\varepsilon = 1.4 \times 10^4\ M^{-1}\ cm^{-1}$) (21).

$Ga(NO_3)_3$ susceptibility tests. $Ga(NO_3)_3$ activity was tested in 96-well microtiter plates. Briefly, *P. aeruginosa* clinical isolates were grown overnight at 37°C in DCAA supplemented with 40 μM $FeCl_3$ and then diluted to an OD_{600} of 0.01 in 200 μl (final volume) of HS or DCAA containing increasing $Ga(NO_3)_3$ concentrations (0 to 128 μM). Microtiter plates were incubated for up to 48 h at 37°C with shaking (120 rpm). Susceptibility assays in MH were performed using a $Ga(NO_3)_3$ concentration range of 0 to 512 μM and a standard broth microdilution experimental procedure (22). MICs were defined as the lowest $Ga(NO_3)_3$ concentrations that completely inhibited *P. aeruginosa* growth, as detected by visual inspection of the plates. Each strain was tested in at least two independent experiments.

Quantification of hydrolyzed HS proteins. Supernatants from *P. aeruginosa* cultures in HS were analyzed both quantitatively and qualitatively to determine HS protein hydrolysis. For quantitative measurements, supernatants were supplemented with 13% trichloroacetic acid (TCA) in order to precipitate nonhydrolyzed proteins (23). Subsequently, the hydrolyzed (i.e., TCA-nonprecipitable) protein fraction was quantified by the Lowry assay (24). For qualitative measurements, supernatants were diluted 1:16 in gel loading buffer (0.25 M Tris-HCl, 2% SDS, 10% 2-mercaptoethanol, 20% glycerol), heated at 100°C for 10 min, and subjected to electrophoresis in 0.1% SDS–12% polyacrylamide gels (SDS-PAGE). Gels were stained with Coomassie brilliant blue.

Proteolytic activity. For qualitative determination of proteolytic activity, *P. aeruginosa* strains were grown in LB, diluted to an OD_{600} of ~1 in saline, and spotted (5 μl) onto Luria-Bertani agar (LA) plates containing 2% skim milk (Difco). Proteolytic activity was detected as a clear halo around the bacterial patch after 18 h of growth at 37°C.

Western blot detection of Tf in HS and the PVD biosynthetic enzyme PvdA in *P. aeruginosa* cells. HS proteins and *P. aeruginosa* whole-cell lysates were separated by SDS-PAGE and electrotransferred onto nitrocellulose filters (Hybond C extra; Amersham) using a semidry transfer unit (Hoefer Scientific Instruments) for 1 h at 150 mA. Filters were blocked with 2× TBST (100 mM Tris-HCl [pH 8.0], 1.0 M NaCl, 0.1% Tween 20) containing 2% bovine serum albumin, washed with 2× TBST, incubated with a monoclonal anti-PvdA antibody (25) or an anti-human Tf rabbit polyclonal antibody (Epitomics) diluted 1:100 or 1:3,000 in 2× TBST, respectively. Proteins were detected with a secondary anti-mouse (Promega) or anti-rabbit (Calbiochem) alkaline phosphatase-conjugated antibody. Blots were developed with the 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) and nitroblue tetrazolium chloride (NBT) reagents for colorimetric detection (Promega).

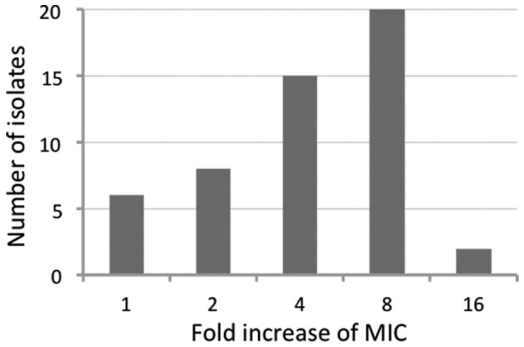

FIG 1 Fold increase of the $Ga(NO_3)_3$ MICs in HS, relative to that in DCAA, for 51 *P. aeruginosa* clinical isolates.

Detection of quorum sensing (QS) signal molecules. To determine the production of *N*-3-oxododecanoyl homoserine lactone ($3OC_{12}$-HSL) and *N*-butanoyl-L-homoserine lactone (C_4-HSL), 100-μl volumes of overnight cultures of each clinical isolate diluted in LB at OD_{600} 0.01 were cocultured with 100-μl volumes of overnight cultures of reporter strains PA14-R3 for $3OC_{12}$-HSL and PAO-JP2 (pKD-*rhlA*) for C_4-HSL (26, 27) diluted in LB at an OD_{600} of 0.045. Microtiter plates (final volume, 200 μl) were incubated at 37°C with shaking, and cell density (OD_{600}) and bioluminescence (light counts per second) were simultaneously measured after 6 h in a Wallac 1420 Victor3V multilabel plate reader (PerkinElmer). Luminescence values were normalized by the cell density (OD_{600}).

Statistical analyses. Spearman correlation coefficients (r_s) were used as a measure of the relative validity between *P. aeruginosa* growth in HS and protein hydrolysis or PVD production. Student's *t* test was used to determine significant differences in $Ga(NO_3)_3$ MICs, using the GraphPad Prism software.

RESULTS

***P. aeruginosa* susceptibility to gallium is lower in HS than in an iron-poor laboratory medium.** The anti-*P. aeruginosa* activity of $Ga(NO_3)_3$ was preliminarily tested in complement-free HS on 55 *P. aeruginosa* clinical isolates collected from respiratory secretions of CF patients (27 isolates) and from a variety of pathological samples from non-CF patients (28 isolates) (see Table S1 in the supplemental material). All but four isolates, namely, SP18, SP20, SP21, and FM1, grew in HS. *P. aeruginosa* growth in HS was invariably characterized by a long lag phase of 10 to 18 h and attained stationary phase after 36 to 54 h (data not shown). The MIC of $Ga(NO_3)_3$ was determined both in HS and in the iron-free Casamino Acids medium DCAA (17), for comparison. The great majority of isolates (72%) displayed a ≥4-fold increase in $Ga(NO_3)_3$ MIC in HS compared with that in DCAA (Fig. 1; see also Table S1 in the supplemental material), indicating a reduced susceptibility of *P. aeruginosa* to $Ga(NO_3)_3$ in HS.

Both proteases and PVD contribute to *P. aeruginosa* growth in HS. The antibacterial activity of gallium depends on iron availability, being evident only under iron-limiting conditions (8, 11). It was therefore surprising to observe a poor antibacterial activity for $Ga(NO_3)_3$ in HS, where the free iron concentration is known to be limiting for bacterial growth (1). This effect could be due to the ability of *P. aeruginosa* to acquire iron from Tf via siderophores, or proteases, or both (4, 28). Increased iron availability would thus promote growth and ultimately counteract the antibacterial activity of an iron-mimetic inhibitor, like $Ga(NO_3)_3$ (8–11). Therefore, the ability of clinical isolates to hydrolyze serum proteins and/or produce PVD was investigated. The amount of

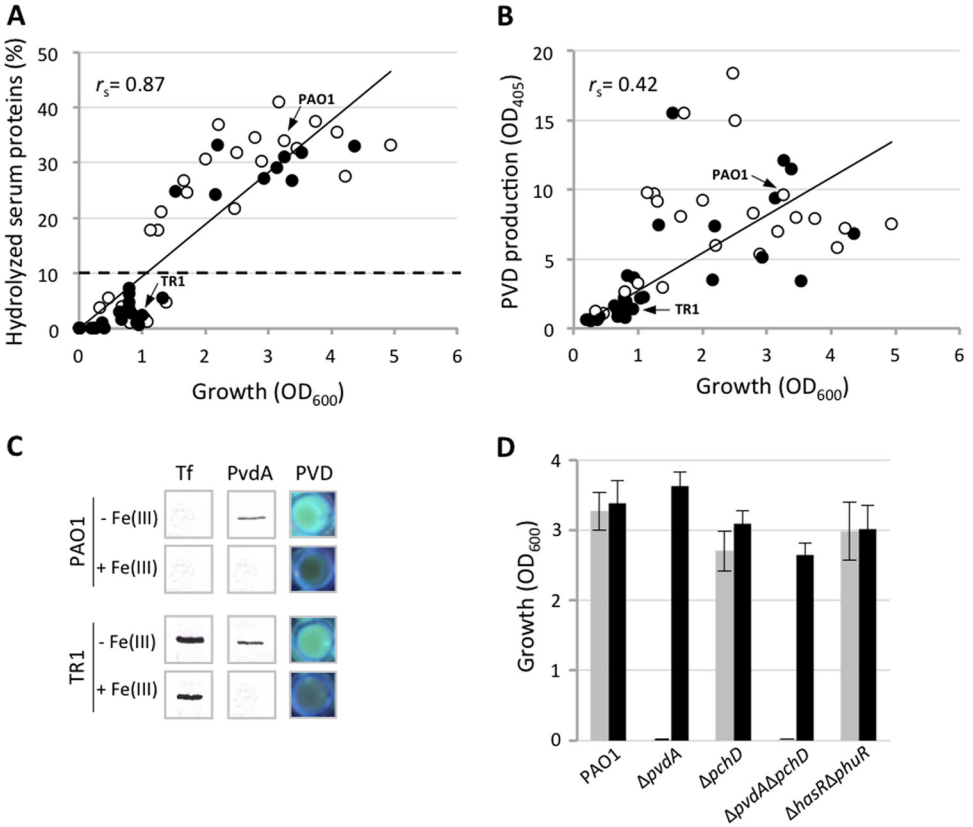

FIG 2 (A) Correlation analysis between hydrolysis of HS proteins and *P. aeruginosa* growth in HS. Hydrolyzed HS proteins were quantified as TCA-nonprecipitable, Lowry-reactive material and are expressed as percentages relative to total HS proteins. An arbitrary cutoff value of 10% of hydrolyzed serum proteins was set to discriminate between weak and strong HS-hydrolyzing isolates (dotted line). (B) Correlation analysis between pyoverdine (PVD) production (OD_{405}) and *P. aeruginosa* growth in HS. Black and white circles represent CF and non-CF isolates, respectively. Arrows indicate strains PAO1 and TR1, representative of HS hydrolyzing and nonhydrolyzing isolates, respectively. The Spearman rank correlation coefficients (r_s) are indicated. (C) Western blot analysis of Tf and PVD biosynthetic enzyme (PvdA) levels in HS culture supernatants and bacterial cell lysates, respectively. *P. aeruginosa* strains PAO1 and TR1 were grown in HS supplemented or not with 100 μM $FeCl_3$ [+Fe(III) and −Fe(III), respectively]. PVD production was qualitatively assayed by fluorescence emission under UV light. (D) Growth of *P. aeruginosa* PAO1 mutants impaired in different iron uptake systems in HS (gray bars) and in HS supplemented with 100 μM $FeCl_3$ (black bars). In all experiments, the mean value of results for at least two independent microtiter plate assays was considered.

hydrolyzed proteins was measured in HS after bacterial growth, as TCA-nonprecipitable material (Fig. 2A), and qualitatively verified by SDS-PAGE analysis of serum proteins relative to the uninoculated control (see Fig. S1 in the supplemental material). The growth of *P. aeruginosa* clinical isolates in HS correlated well with their ability to hydrolyze serum proteins (r_s = 0.87) (Fig. 2A), while poor correlation was observed between PVD production and growth in HS (r_s = 0.42) (Fig. 2B). These data suggest that increased availability of nutrients (e.g., free iron and amino acids) and the consequent *P. aeruginosa* growth promotion in HS primarily depend on protein degradation rather than on PVD production, at least during late growth, when protease activity and PVD production were measured. Accordingly, Tf was completely degraded in spent HS obtained from HS-hydrolyzing strains such as the type strain PAO1, as opposed to weak HS hydrolyzers, such as the CF isolate TR1, regardless of the presence or absence of exogenously added $FeCl_3$ (Fig. 2C and see Fig. S1 in the supplemental material). Interestingly, both PAO1 and TR1 produced PVD, as documented by their fluorescent phenotype in HS and expression of the iron-repressible PvdA enzyme, implicated in an early step of PVD biogenesis (Fig. 2C) (29). This observation confirms that HS is perceived by *P. aeruginosa* as an iron-poor environment, inductive of PVD production.

Of note, significantly lower HS proteolysis was observed for CF than for non-CF isolates ($P < 0.05$, chi-square test; data not shown), consistent with the notion that *P. aeruginosa* isolates from chronic CF infections generally display low protease activity (30).

To gain insight into the contribution of individual iron acquisition systems to *P. aeruginosa* growth in HS, the reference strain PAO1 and isogenic deletion mutants impaired in heme uptake (Δ*hasR* Δ*phuR* [31]) and siderophore-dependent iron uptake (Δ*pvdA*, impaired in PVD biosynthesis [32]; Δ*pchD*, impaired in pyochelin biosynthesis [33], or Δ*pvdA* Δ*pchD*, defective in production of both siderophores [34]) were cultured in HS supplemented or not with an excess of iron (Fig. 2D). Only the PVD-defective mutants (Δ*pvdA* and Δ*pvdA* Δ*pchD*) showed impaired growth in HS compared with the wild type, whereas addition of $FeCl_3$ (100 μM) rescued the growth of the mutants to wild-type levels (Fig. 2D). Conversely, mutations in heme uptake or pyochelin biosynthesis genes did not affect bacterial growth in HS (Fig. 2D).

PVD is essential for *P. aeruginosa* growth initiation in HS. The above-described results prompted us to investigate whether the lack of growth in HS of the clinical isolates SP18, SP20, SP21, and FM1 was due to a defect in PVD production. These strains displayed a PVD-defective phenotype when grown in the iron-

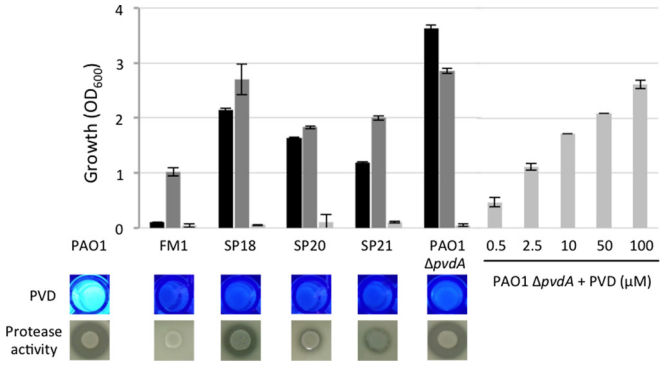

FIG 3 Growth (OD_{600}) of PVD-defective *P. aeruginosa* strains in HS supplemented with 100 μM $FeCl_3$ (black bars), predigested HS (dark gray bars), and HS (light gray bars). Growth of the PAO1 Δ*pvdA* mutant in HS was rescued by addition of increasing concentrations of PVD. Growth values (OD_{600}) for the wild-type strain PAO1 were 3.39 ± 0.32, 4.56 ± 0.01, and 3.25 ± 0.27 in HS supplemented with 100 μM $FeCl_3$, predigested HS, and HS, respectively. Qualitative data on PVD production in DCAA and proteolytic activity on skim milk agar plates are shown for each strain.

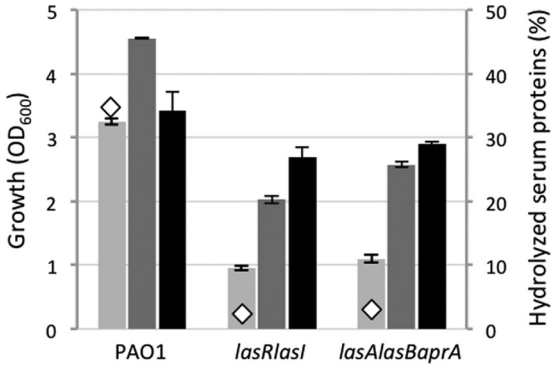

FIG 4 Growth (OD_{600}) and hydrolysis of HS proteins (relative to total HS proteins; diamonds) by *P. aeruginosa* PAO1 and *lasR lasI* and *lasA lasB aprA* mutants, grown for 48 h at 37°C in HS (light gray bars), predigested HS (dark gray bars), and HS supplemented with 100 μM $FeCl_3$ (black bars). Values represent the mean (±standard deviation) of results for three independent assays. Standard deviations are not visible when the bars representing them are smaller than the symbol of the corresponding data point.

poor minimal medium DCAA (Fig. 3) and were able to grow in predigested HS (Fig. 3), where iron is released from Tf and available to bacteria (Fig. 3). Moreover, all isolates except FM1 grew in HS supplemented with 100 μM $FeCl_3$. Notably, FM1 was also the only strain that was impaired in protease production on milk agar plates (Fig. 3). Exogenously added PVD stimulated the growth of the PAO1 Δ*pvdA* mutant in HS in a dose-dependent manner, even at concentrations far below the maximum PVD levels achievable by *P. aeruginosa* PAO1 in iron-depleted laboratory cultures (>200 μM) (19). These results support the essential role of PVD for *P. aeruginosa* growth in HS.

QS-controlled proteases increase *P. aeruginosa* growth in HS. The inability of protease-proficient *P. aeruginosa* isolates SP18, SP20, and SP21 to grow in HS is in apparent contrast with the good correlation observed between protease production and growth in HS (Fig. 2A). Since the expression of protease in *P. aeruginosa* is a growth phase-dependent phenomenon regulated by quorum sensing (QS) (35), we hypothesized that bacteria need to reach a certain cell density to allow the protease-dependent growth increment. In this view, PVD might play a prominent role at early growth stages, when protease production is scarce. Then, in response to increased cell density and QS, protease-mediated iron mobilization from Tf would further support growth. To address this point, we determined the growth in HS of a *P. aeruginosa* PAO1 QS-defective mutant (*lasR lasI*) (36) and a protease-defective mutant (*lasA lasB aprA*) (37), both proficient in PVD production (data not shown). Both mutants produced barely detectable levels of proteases and showed a ca. 3-fold reduction in growth yields in HS, relative to that of the wild-type PAO1 (Fig. 4). Notably, both predigestion of HS and supplementation with 100 μM $FeCl_3$ strongly stimulated the growth of QS- and protease-defective mutants (Fig. 4). These findings strongly suggest that QS-controlled protease production provides *P. aeruginosa* with incremental growth capabilities in HS, likely through proteolytic mobilization of Tf-bound iron. Moreover, a transposon mutant deficient in the iron- and PVD-regulated protease PrpL (38, 39) showed significant growth reduction in HS and decreased hydrolysis of HS proteins (20% and 35%, respectively, relative to the parental strain PAO1; data not shown), corroborating the importance of protease production for *P. aeruginosa* growth in HS.

HS proteolysis impairs gallium activity. As anticipated, the growth of different *P. aeruginosa* clinical isolates in HS correlates well with their ability to produce proteases. Consequently, we hypothesized that proteolytic release of iron from HS proteins could be responsible for the loss of the antibacterial activity of gallium (8–11). After an arbitrary cutoff value of 10% hydrolyzed HS proteins was set (Fig. 2A, dotted line), isolates were split into two groups, defined as strong HS hydrolyzers (27 isolates composed of 9 CF and 18 non-CF isolates) and weak HS hydrolyzers (24 isolates composed of 17 CF and 7 non-CF isolates). Notably, the median $Ga(NO_3)_3$ MIC for weak HS hydrolyzers was 16 μM, while strong HS hydrolyzers displayed a median MIC of 64 μM (Fig. 5A). In line with this observation, the $Ga(NO_3)_3$ MICs of the protease-

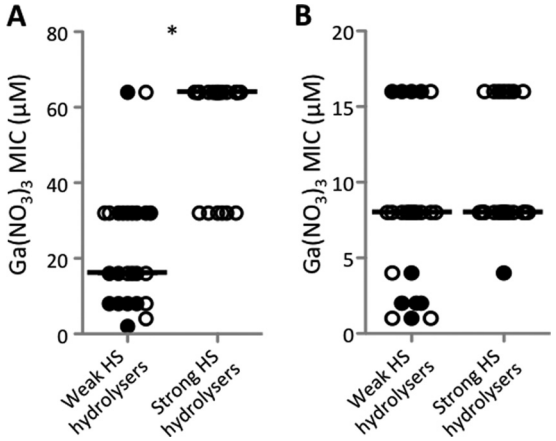

FIG 5 MICs of $Ga(NO_3)_3$ for 51 *P. aeruginosa* clinical isolates grown for 48 h in HS (A) and for 24 h in DCAA (B). PVD-defective isolates were not included. For each isolate, the mean value of results for two independent assays was considered. Isolates were grouped according to their ability to hydrolyze serum proteins (see Fig. 1A). The horizontal line is the median for each group. Black and white circles represent CF and non-CF isolates, respectively. An asterisk indicates a statistically significant difference ($P < 0.001$; Student's *t* test) between the two groups.

defective PAO1 *lasR lasI* and *lasA lasB aprA* mutants in HS were reproducibly 2-fold lower than that of their parental strain (32 μM versus 64 μM). The lower inhibitory effect of $Ga(NO_3)_3$ on strong HS hydrolyzers than on weak HS hydrolyzers (Fig. 5A) confirms that proteolytic activity, most plausibly Tf degradation, has a major impact on the anti-*P. aeruginosa* activity of $Ga(NO_3)_3$ in HS. This was corroborated by the finding that in predigested HS, the MIC of $Ga(NO_3)_3$ was >256 μM for all strains tested (data not shown), and stimulation of bacterial growth was much greater for weak HS hydrolyzers than strong HS hydrolyzers (see Fig. S2 in the supplemental material). Notably, weak and strong HS hydrolyzers displayed similar growth yields and $Ga(NO_3)_3$ susceptibilities when grown in the Casamino Acids-based iron-poor medium DCAA, where amino acids are readily available and proteolytic activity is dispensable for bacterial growth (no complexes with iron-binding proteins are present in this medium [17]) (Fig. 5B; see Fig. S3A in the supplemental material). As expected, the effect of $Ga(NO_3)_3$ in HS was completely abrogated by the addition of an excess of $FeCl_3$ [Fe(III):Ga(III) molar ratio, 5:1]) (see Fig. S3B in the supplemental material), and MICs of >512 μM were determined for all strains in MH, where the iron concentration is high (14 μM [40]).

DISCUSSION

Interfering with *P. aeruginosa* iron metabolism represents a promising strategy to develop novel therapeutic options, and the repurposing of gallium-based drugs has recently been discussed as an alternative strategy for antibacterial chemotherapy (14).

In this work, we investigated the ability of $Ga(NO_3)_3$ to inhibit the growth of *P. aeruginosa* in HS. We report that *P. aeruginosa* is less susceptible to gallium-mediated growth inhibition in HS than in an iron-poor laboratory medium (Fig. 1), as opposed to what was recently reported for *Acinetobacter baumannii*, which is a scarcely proteolytic species (18, 41, 42). This different behavior depends on the ability of *P. aeruginosa* to acquire iron from Tf, by means of the PVD siderophore and hydrolysis of HS proteins, which ultimately provides sufficient iron to counteract gallium inhibition. Upon testing a representative collection of diverse *P. aeruginosa* clinical isolates, we report a very good correlation between HS proteolysis and the ability to grow in HS (Fig. 2A), indicating that iron availability in HS no longer limits bacterial growth when proteases are produced.

Interestingly, we found that clinical isolates and laboratory strains defective in PVD production (namely SP18, SP20, SP21, FM1, PAO1 Δ*pvdA* Δ*pchD*, and PAO1 Δ*pvdA*) cannot grow in HS (Fig. 2 and 3), in line with previous findings (28). Since protease production by *P. aeruginosa* is regulated by QS (35), we also observed that QS- and protease-defective mutants were inhibited by HS, although growth yields were restored to the wild-type levels by predigestion of HS proteins or supplementation of HS with an excess of $FeCl_3$ (Fig. 4). Altogether, these data depict a scenario in which PVD production is essential to ensure initial growth in HS, while protease production, which likely starts during late growth being regulated by QS, allows *P. aeruginosa* to reach higher cell densities by making essential nutrients (e.g., iron and amino acids) readily available. Therefore, it appears that production of proteases is necessary but *per se* insufficient to allow *P. aeruginosa* growth in HS, since protease-proficient strains that are unable to produce PVD do not grow in HS (Fig. 3). In contrast, protease-deficient strains able to produce PVD grow in HS, albeit with reduced yields (Fig. 4). Accordingly, we found that QS-deficient clinical isolates impaired in protease production (about 15% in our strain collection) could grow in HS when they were proficient in PVD production (see Table S1 in the supplemental material).

Given the importance of protease production and iron release from Tf for *P. aeruginosa* growth in HS, we observed that weak HS hydrolyzers were more susceptible to gallium-mediated growth inhibition in HS than strong HS hydrolyzers (Fig. 5A) and that this difference was abolished when $Ga(NO_3)_3$ MICs were measured in DCAA (Fig. 5B) or in the presence of an excess of iron, e.g., in MH and predigested HS.

Overall, our findings highlight the need for appropriate *in vitro* conditions to assess growth inhibition by an iron mimetic. Compared with conventional laboratory media (e.g., DCAA and MH), HS provides a more realistic environment to assess the antibacterial activity of gallium (11). From a practical viewpoint, the $Ga(NO_3)_3$ concentrations required to inhibit the growth of the majority of *P. aeruginosa* isolates in HS are very close to or even higher than those achievable in plasma for cancer therapy. In fact, the recommended dosing regimens for the treatment of cancer-related hypercalcemia attain a peak serum concentration of $Ga(NO_3)_3$ of ca. 28 μM (7, 43). This gallium concentration is insufficient to suppress the growth in HS of highly proteolytic *P. aeruginosa* strains (Fig. 5A), which predominate among non-CF isolates, thus raising concern about the potential of $Ga(NO_3)_3$ in the treatment of systemic *P. aeruginosa* infection. It should also be considered that because of the high hemolytic activity of *P. aeruginosa* (44), some iron sources such as heme could be more readily available in whole blood. Thus, it cannot be excluded that iron acquisition strategies other than pyoverdine (e.g., heme uptake) could counteract the iron-mimetic activity of gallium *in vivo*. On the other hand, the antibacterial activity of the complement and the host's immune system are likely to synergize with gallium *in vivo*. Indeed, $Ga(NO_3)_3$ provided effective protection from *P. aeruginosa* lung infection in mice (8), and preliminary results point to its efficacy in reducing bacterial load during CF lung infection (13). However, the strain-dependent ability of *P. aeruginosa* to mobilize iron from the host iron-containing proteins and grow in serum (or other body fluids) should be taken into account in the future development of gallium-based antibacterial therapies.

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