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In Vitro Activity of Gallium Maltolate against Staphylococci in Logarithmic, Stationary, and Biofilm Growth Phases: Comparison of Conventional and Calorimetric Susceptibility Testing Methods▽

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Ga^{3+} is a semimetal element that competes for the iron-binding sites of transporters and enzymes. We investigated the activity of gallium maltolate (GaM), an organic gallium salt with high solubility, against laboratory and clinical strains of methicillin-susceptible *Staphylococcus aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), methicillin-susceptible *Staphylococcus epidermidis* (MSSE), and methicillin-resistant *S. epidermidis* (MRSE) in logarithmic or stationary phase and in biofilms. The MICs of GaM were higher for *S. aureus* (375 to 2000 μg/ml) than *S. epidermidis* (94 to 200 μg/ml). Minimal biofilm inhibitory concentrations were 3,000 to ≥6,000 μg/ml (*S. aureus*) and 94 to 3,000 μg/ml (*S. epidermidis*). In time-kill studies, GaM exhibited a slow and dose-dependent killing, with maximal action at 24 h against *S. aureus* of 1.9 $\log_{10}$ CFU/ml (MSSA) and 3.3 $\log_{10}$ CFU/ml (MRSA) at 3× MIC and 2.9 $\log_{10}$ CFU/ml (MSSE) and 4.0 $\log_{10}$ CFU/ml (MRSE) against *S. epidermidis* at 10× MIC. In calorimetric studies, growth-related heat production was inhibited by GaM at subinhibitory concentrations; and the minimal heat inhibition concentrations were 188 to 4,500 μg/ml (MSSA), 94 to 1,500 μg/ml (MRSA), and 94 to 375 μg/ml (MSSE and MRSE), which correlated well with the MICs. Thus, calorimetry was a fast, accurate, and simple method useful for investigation of antimicrobial activity at subinhibitory concentrations. In conclusion, GaM exhibited activity against staphylococci in different growth phases, including in stationary phase and biofilms, but high concentrations were required. These data support the potential topical use of GaM, including its use for the treatment of wound infections, MRSA decolonization, and coating of implants.

Staphylococcus aureus continues to represent the major cause of infection in the outpatient and health care settings. The therapeutic options for multiresistant strains, including methicillin-resistant *S. aureus* (MRSA) strains, are limited (7, 16). In addition, coagulase-negative staphylococci often cause chronic low-grade infections associated with implanted devices, on which they can grow embedded in a protective extracellular matrix known as a biofilm (11). In the biofilm, bacteria can persist in a low metabolic, stationary growth phase, in which they resist killing by the host immune system and antimicrobials (14, 28). Both the spread of multiresistant staphylococci and the increased use of temporary implants (vascular catheters, pins from external fixation devices) and permanent implants (e.g., joint prosthesis, breast implants, and cardiac or brain pacemakers), drive the need for new antimicrobial agents for innovative therapeutic strategies (1, 7, 23, 29).

The antimicrobial activities of different inorganic salts, such as sodium metabisulfite and copper silicate, against planktonic and adherent staphylococci in vitro have already been investigated (6, 15). Gallium (Ga^{3+}) is a semimetal element which competes with Fe^{3+} for binding to proteins and chelators. Ga^{3+} is virtually irreducible under physiological conditions, while Fe^{3+} participates in the redox reaction, in which it is readily reduced to Fe^{2+}. Thus, by replacing Fe^{3+}, Ga^{3+} interferes with bacterial DNA and protein synthesis and blocks the redox reactions that depend on iron electron acquisition (3).

Staphylococci are known to have an avidity for iron. Several mechanisms of iron recruitment have previously been studied and have been described to be essential virulence factors (12, 19, 20, 24, 25). As a consequence, iron uptake and metabolism may constitute a potential target in the effort to develop agents that may be used to combat staphylococcal infections.

In the form of the nitrate salt, gallium demonstrated bactericidal activity against planktonic and adherent *Pseudomonas aeruginosa* and *Burkholderia cepacia* in vitro (17, 22). Gallium maltolate (GaM), a highly soluble gallium formulation (4), was effective in vivo against *P. aeruginosa* and *S. aureus* infection after topical subcutaneous injection in thermally injured mice (13). However, to our knowledge, the in vitro activity of gallium against staphylococci has not yet been characterized. Gallium was chosen from among the elements with antibacterial properties due to its theoretical principle of activity (i.e., competition for Fe^{3+}), its selective mechanism of action against bacteria (with low predicted tissue toxicity), in vitro data from studies with other gram-negative bacilli, and previous experience with its use for diagnostic purposes in human nuclear medicine (3).

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We investigated the in vitro activity of GaM against staphylococci in the logarithmic and stationary phases and in biofilms. Standard in vitro tests for determination of its MIC, its minimal bactericidal concentrations (MBC) in the logarithmic and stationary growth phases (10), and its activity against biofilms were used. To better investigate the activity of GaM at subinhibitory concentrations, we studied its effect on bacterial heat production in cultures exposed to GaM using a newly developed calorimetric assay. Antimicrobial assays were performed in a synthetic iron-limited medium. The aim of the study was to investigate the activity of GaM against staphylococci in order to assess the potential systemic or topical applications, including for the treatment of wound infections, MRSA decolonization, and the coating of implants.

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MATERIALS AND METHODS

Laboratory bacterial strains. *S. aureus* ATCC 29213 (a methicillin-susceptible *S. aureus* [MSSA] strain), *S. aureus* ATCC 43300 (a methicillin-resistant *S. aureus* [MRSA] strain), *Staphylococcus epidermidis* 1457 (a methicillin-susceptible *S. epidermidis* [MSSE] strain), and *S. epidermidis* B3972 (a methicillin-resistant *S. epidermidis* [MRSE] strain) were used. The bacteria were stored at −70°C by using a cryovial bead preservation system (Microbank; Pro-Lab Diagnostics, Richmond Hill, ON, Canada). Single cryovial beads were cultured overnight on Columbia sheep blood agar plates (Becton Dickinson, Heidelberg, Germany). Inocula were prepared from the subcultures of two to three colonies resuspended in Trypticase soy broth and incubated overnight at 37°C without shaking. The overnight cultures were diluted with the appropriate medium to an inoculum of $\approx 1 \times 10^6$ CFU/ml.

Clinical isolates. For susceptibility screening, 20 genotypically distinct clinical isolates, 5 per bacterial susceptibility group, were used. The clinical strains were collected from nonrelated patients admitted to University Hospital in Basel, Switzerland, between January 2005 and December 2008, and were isolated from intraoperative tissue specimens ($n = 3$), blood ($n = 6$), urine ($n = 8$), or synovial fluid ($n = 3$). The *S. aureus* isolates were characterized by pulsed-field gel electrophoresis pattern analysis with a Chef DR III system (Bio-Rad) for the separation of SmaI-digested genomic DNA and by use of the Pearson correlation, as described previously (27). The isolates displayed a Pearson correlation of <75% (2). The clinical isolates were screened for their susceptibility to methicillin by a broth microdilution procedure (Merlin Diagnostika, Bornheim-Hersel, Germany), and the results were interpreted in accordance with the guidelines of the Clinical and Laboratory Standards Institute (formerly the NCCLS). The inocula of the clinical isolates were prepared from bacterial overnight cultures on Columbia sheep blood agar plates resuspended to a McFarland standard of 0.5 and further diluted in the appropriate medium to an inoculum of $\approx 1 \times 10^6$ CFU/ml.

Chemicals. Susceptibility assays were performed in iron-limited media, including RPMI 1640 (Invitrogen, Basel, Switzerland) supplemented with 5% pyruvate and 5% glutamate (RPMI) or 0.01 M phosphate-buffered saline (PBS), pH 7.4. GaM was kindly provided by Titan Pharmaceuticals (South San Francisco, CA). Stock solutions were freshly prepared and sterile filtered on the day of the assays.

Doubling time. The doubling times of the laboratory bacterial strains were investigated in Mueller-Hinton broth (MHB) and RPMI. Overnight cultures diluted 1:100 were further grown in MHB and in RPMI until mid-logarithmic phase. From the mid-logarithmic phase, each culture was diluted 1:100 (*S. aureus*) or 1:10 (*S. epidermidis*) in either MHB or RPMI and incubated at 37°C with shaking at 180 rpm (for MSSA, MRSA, and MRSE) or without shaking (for MSSE, to avoid the clot formation induced by shaking). The optical density at 600 nm (OD_{600}) was measured every 30 min for 10 h (for MSSA, MRSA, and MRSE) or every 60 min for 12 h (for MSSE). The $\log_2 OD_{600}$ values were plotted versus time, and a linear regression equation was determined for the logarithmic growth phase. The inverse of the slope was defined as the bacterial doubling time (in minutes). Experiments were performed in triplicate, and the results are expressed as means ± standard deviations (SDs). Comparisons of the doubling times were performed by the use of Student's *t* test. Differences were considered significant when P values were <0.05.

Antimicrobial susceptibility of planktonic bacteria. The MIC and the MBC in the logarithmic growth phase (MBC_{log}) were evaluated in RPMI by a broth macrodilution method according to the guidelines of the Clinical and Laboratory Standards Institute (10). The MBC in the stationary growth phase (MBC_{stat}) was assayed in PBS, as described previously (31). The bacterial counts in this medium remained within ±15% of the initial inoculum within 24 h. Ten twofold serial dilutions were prepared from a GaM stock solution of 6,000 μg/ml. The MIC was defined as the lowest GaM concentration that prevented visible bacterial growth, and the MBC was defined as the lowest GaM concentration that reduced the numbers of CFU/ml by ≥99.9% of the initial inoculum. The MICs and MBCs for the laboratory strains were determined three times, and the results are expressed as the medians. The MICs of GaM for the clinical isolates were then determined.

Antimicrobial susceptibility of biofilm bacteria. The activity of GaM against staphylococcal biofilms was tested by a broth microdilution assay, as described previously (8, 21) and as modified by Sandoe et al. (26). Briefly, 200 μl of overnight cultures diluted in 1% glucose-supplemented RPMI (inoculum, $\approx 1 \times 10^6$ CFU/ml) were distributed into 96-well microtiter plates (Nuclon Delta; Nunc, Roskilde, Denmark). The frame wells were filled with medium only and served as a negative control for growth. Biofilms were formed on pegs of modified polystyrene microtiter lids (TSP system; Nunc), immersed into the wells, and cultured for 20 h at 37°C. The pegged lids were then rinsed with PBS and transferred to a second microtiter plate (antimicrobial susceptibility plate) containing twofold serial dilutions of GaM at concentrations between 6,000 μg/ml and 10 μg/ml; six wells were filled with medium only and served as positive growth controls. The plates were incubated for an additional 20 h at 37°C. On the third day, the peg lids were again rinsed with PBS and transferred to recovery plates, into each well of which 200 μl of RPMI had been distributed. The plates were shaken for 5 min at 250 rpm to enhance bacterial regrowth from the biofilms and were then incubated at 37°C without shaking. After 48 h of incubation, the bacterial growth occurring on the recovery plates was measured as the turbidity at OD_{600} by using an enzyme-linked immunosorbent assay reader. The lowest concentration of GaM which prevented biofilm regrowth in the recovery plate was defined as the minimal biofilm inhibitory concentration (MBIC). The experiments were performed four times on different days in triplicate, and the results are reported as the median values. The screening of clinical isolates was performed in triplicate, and the median MBICs are reported.

As a control, strain-dependent biofilm formation was evaluated as described previously (9, 30). Briefly, 200 μl of bacterial inoculum, prepared as described above, was distributed in six replicates in polystyrene, 96-well, flat-bottomed tissue culture plates (Falcon; Becton Dickinson, France). The plates were incubated for 20 h at 37°C. The medium was then gently aspirated from each well. The bacteria adhering to the plate bottoms were washed with 200 μl PBS and air dried for about 2 h. The biofilms were stained with a solution of 0.5% crystal violet in 70% methanol for 15 min. The stain was then removed and the wells were rinsed twice with 300 μl of PBS. The stained adherent bacteria were resuspended from each well with 200 μl of 70% methanol and transferred to a new plate. The OD_{490} of the latter was measured, each value was subtracted from the mean absorbance for the blank wells (negative control), and the mean for six wells for each strain was calculated. The value derived from 3 SDs above the mean OD_{490} for the blank was used as the breakpoint for classifying the strain as able or not able to form biofilms under the in vitro conditions used (9).

Time-kill studies. The killing profiles of four GaM concentrations against laboratory strains were assessed in parallel to growth controls. Samples were incubated at 37°C without shaking for 24 h (for the *S. aureus* strains) and 48 h (for the *S. epidermidis* strains). The bacterial concentrations (CFU/ml) were determined by plating aliquots of appropriate dilutions on Muller-Hinton agar plates at 0 h, 2 h, 4 h, 6 h, 8 h, 24 h, and 48 h (the last time point was used for *S. epidermidis* only). The bacterial colonies were enumerated after 24 h of incubation at 37°C. The results are plotted as the reduction in the $\log_{10}$ CFU/ml over time, defined as $\log_{10}(\text{CFU/ml})_t - \log_{10}(\text{CFU/ml})_0$, where t is time and the 0 indicates time zero. Each strain was tested three times on different days. The figures were plotted by using Prism (version 4.0) software (GraphPad Software, La Jolla, CA).

Inhibition of growth-related heat production by GaM. Viable and growing microorganisms produce heat, which can be measured with a calorimeter (5, 18). This property allows the potency of an antimicrobial against bacterial growth to be measured (18). We evaluated the inhibition of bacterial heat production in the presence of different concentrations of GaM over 24 h. From a GaM stock solution of 6,000 μg/liter, nine twofold serial dilutions were prepared in RPMI and 2 ml was transferred into sterile 4-ml glass ampoules. The adjusted bacterial cultures were then inoculated under the meniscus in the ampoules, and the

TABLE 1. In vitro susceptibilities of four laboratory strains of staphylococci in logarithmic, stationary, and biofilm growth phases to GaM and corresponding calorimetry parameters

Organism (strain)	MIC (μg/ml)	MBC_{log} (μg/ml)	MBC_{stat} (μg/ml)	MBIC (μg/ml)	MHIC (μg/ml)
MSSA (ATCC 29213)	2,000	>6,000	>6,000	6,000	4,500
MRSA (ATCC 43300)	1,000	6,000	>6,000	6,000	1,500
MSSE (1457)	100	2,000	6,000	280	375
MRSE (B3972)	200	1,500	1,500	560	375

ampoules were sealed so that they were air tight and were sequentially introduced into the calorimetry channels.

A 48-channel batch calorimeter (model 3102 TAM III thermal activity monitor; TA Instruments, New Castle, DE) was used to measure the heat flow at 37°C, controlled at ±0.0001°C, and at an analytical sensitivity of ±0.2 μW. The heat flow (in microwatts) was measured at 10-s intervals for 24 h. The curve of total heat (in Joules) versus time was determined by integration of the area below the heat flow-time curve. After measurement of the heat flow was completed, the content of each ampoule was assessed for visual growth. The minimal heat inhibitory concentration (MHIC) was defined as the lowest GaM concentration that led to growth-related heat production of <0.25 J after 24 h. Bacterial cultures without GaM were used as positive (growth) controls. Experiments were performed two times on different days in duplicate for the laboratory strains. The clinical isolates were then screened and the MHICs were collected.

RESULTS

Doubling time in iron-limited medium. The replication of the *S. epidermidis* laboratory strains was slower than that of the *S. aureus* laboratory strains, with the doubling times in RPMI (mean ± SD) being 46 ± 10 min (MSSA) and 55 ± 8 min (MRSA) for the *S. aureus* strains and 114 ± 27 min (MSSE) and 108 ± 17 min (MRSE) for the *S. epidermidis* strains. No significant differences in doubling times were observed between strains cultured in RPMI or MHB ($P > 0.05$).

Antimicrobial susceptibilities of planktonic bacteria. Table 1 summarizes the in vitro susceptibilities of the laboratory strains to GaM. The MICs were about 10-fold higher for the *S. aureus* laboratory strains than for the *S. epidermidis* laboratory strains. GaM at concentrations up to 6,000 μg/ml was not bactericidal against the MSSA strains, whereas a 3-$\log_{10}$-CFU reduction against the other strains was reached. MBC_{log} values were between 6× and 20× the respective MICs. In the stationary phase, GaM exhibited bactericidal activity only against *S. epidermidis*; the MBC_{stat} was 60× the MIC for the MSSE strain and 7.5× the MIC for the MRSE strain. Table 2 shows the results for the clinical isolates. GaM inhibited the growth of all strains tested. Clinical isolates of MSSA and *S. epidermidis* exhibited MICs similar to those for the corresponding laboratory strains, whereas MRSA isolates had median MICs about four times lower than those for the corresponding laboratory strains.

Antimicrobial susceptibilities of biofilm bacteria. In biofilm susceptibility studies, the GaM MBICs for the laboratory strains were 3× the MIC for the MSSA strains, 6× the MIC for the MRSA strains, and 2.8× the MIC for the MSSE and MRSE strains (Table 1). The formation of biofilms on the pegged lids was confirmed by the growth of positive control biofilm cultures in the recovery plates. Three of the five MSSA clinical isolates screened for their MBICs (Table 2) had MBICs of >6,000 μg/ml. The MRSA isolates displayed better susceptibility, with only two strains having MBICs of >6,000 μg/ml. The median MBICs for the MSSE and MRSE isolates were 375 and 750 μg/ml, respectively, with the biofilm forms of all isolates displaying susceptibility to GaM. Strain DJ192 did not grow in the recovery plate in wells with and without GaM during the 48 h of incubation. Therefore, the MBIC for this strain could not be measured.

TABLE 2. In vitro susceptibilities of 20 clinical isolates of staphylococci in the logarithmic and biofilm growth phases to GaM and the corresponding calorimetry parameters

Organism and strain	MIC (μg/ml)	MBIC (μg/ml)	MHIC (μg/ml)
MSSA			
B14097/07	750	>6,000	375
T6477/05	1,500	6,000	750
S2626/07	1,500	>6,000	375
T642/05	750	>6,000	188
B10984/07	750	3,000	188
MRSA			
T3011	375	>6,000	94
U8927-1/07	375	3,000	94
M1082/07	375	3,000	94
S1771.07	375	6,000	375
B12570/07	375	>6,000	375
MSSE			
DJ67	188	1,500	188
DJ134	188	3,000	1,500
DJ169	188	750	94
DJ198	94	3,000	94
DJ254	94	3,000	188
MRSE			
DJ146	94	3,000	188
DJ192	94	—[a]	375
B102285	94	94	375
B102534	188	750	375
B103110	94	750	188

[a] —, biofilm susceptibility was not available due to the absence of growth for the growth control.

Both laboratory strains and clinical isolates were positive for biofilm formation after crystal violet staining of the bacteria adhering to the bottoms of polystyrene 96-well tissue culture plates. The OD_{490} breakpoint was 0.08, calculated as 3 SDs (3 × 0.009) above the mean OD_{490} (0.053) for the blank wells. The mean OD_{490}s ± SDs (for both the laboratory strains and the clinical isolates) were 0.104 ± 0.044 (MSSA), 0.184 ± 0.074 (MRSA), 0.244 ± 0.077 (MSSE), and 0.187 ± 0.070 (MRSE).

Time-kill studies. Figure 1 shows the reduction in the $\log_{10}$ CFU/ml of the laboratory strains in the presence of GaM over 24 h (*S. aureus*) or 48 h (*S. epidermidis*) of incubation. For all strains tested, GaM inhibited bacterial growth in a time-dependent and dose-dependent manner. At subinhibitory concentrations, only a slight reduction and a delay in the net bacterial counts were detected compared to those for the growth controls. Against MSSA and MRSA isolates, the highest levels of killing after 24 h, achieved with GaM at 3× the MIC, were 1.9 and 3.3 $\log_{10}$ CFU/ml, respectively. Against the MSSE isolates, GaM at 10× the MIC reduced bacterial viability by 2.9 and 4.4 $\log_{10}$ CFU/ml after 24 h and 48 h, respec-

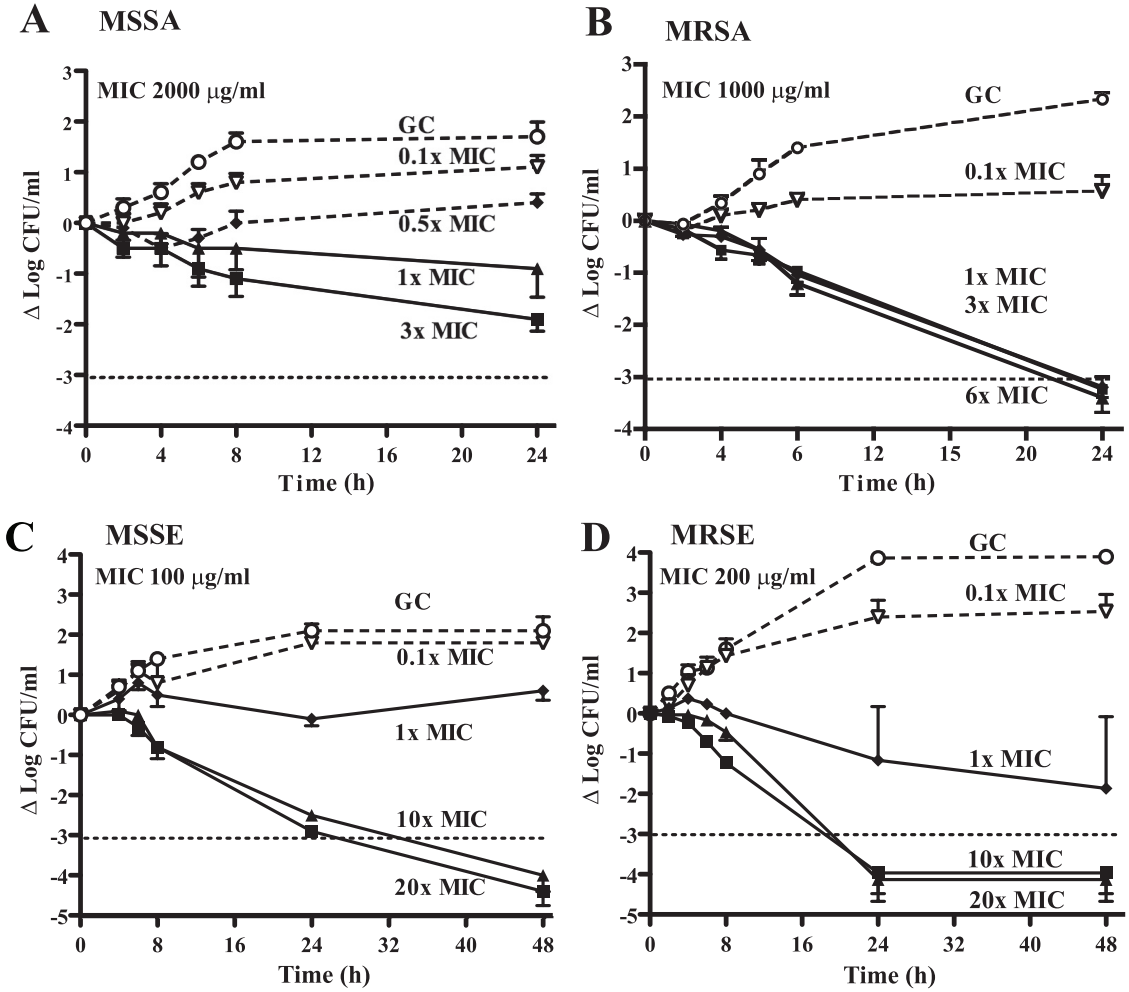

FIG. 1. Time-kill curves of the activity of GaM in RPMI against MSSA (A), MRSA (B), MSSE (C), and MRSE (D). Values are the mean numbers of CFU/ml ± SDs of three measurements. Continuous lines represent kill curves (at inhibitory concentrations) and dashed lines represent growth curves (at subinhibitory concentrations) over 24 h (*S. aureus*) or 48 h (*S. epidermidis*) of incubation. GaM concentrations are reported as multiples of the MIC for the corresponding test strain (MICs are listed in Table 1). GC, growth controls tested in RPMI without GaM; horizontal dotted line, 3-$\log_{10}$-CFU/ml reduction. The scales on the *x* and *y* axes are adapted for *S. aureus* and *S. epidermidis*.

tively, while the counts for the MRSE isolates were already reduced by 4.0 $\log_{10}$ CFU/ml at 24 h. Bactericidal activity was demonstrated against the MRSA, MSSE, and MRSE isolates and was time dependent.

Inhibition of growth-related heat production. Figure 2 shows representative calorimetry curves of the total heat generated by each of the laboratory strains cultured in RPMI at 37°C over 24 h. The inhibition of the growth-related total heat produced by the strains in the presence and the absence of GaM was measured. The total heat generated over 24 h without GaM was similar for all strains and ranged from 4 to 5 J. The curves for the laboratory strains were analyzed and were found to follow a two-phase course: an initial rapid increase in the amount of heat, corresponding to the logarithmic growth phase, occurred in the MSSA strains at between 0 and 4 h of incubation, in the MRSA and MSSE strains at between 3 and 7 h, and in the MRSE strains at between 4 and 9 h. In the second phase, the rate of increase in the amount of heat produced was lower but was continuous up to 24 h for all isolates except the MSSA isolates, which reached a heat production plateau at ≈16 h of incubation. At subinhibitory concentrations, GaM reduced the amount of heat produced in a dose-dependent manner. Cultures with total heat of <0.25 J at 24 h did not display any visual turbidity, and thus, 0.25 J was used as the total heat breakpoint for the evaluation of MHICs. The MHICs for the laboratory and clinical isolates (Table 1 and 2) correlated with the MICs for all strains, with values being ±4× the respective MICs for all except one MSSE isolate (isolate DJ134), for which the MHIC was 6× higher than the MIC.

DISCUSSION

Gallium exerts its antimicrobial action by targeting iron metabolism and thereby interfering with an important virulence factor for infection and the persistence of staphylococci (24, 25). We evaluated the antimicrobial activity of gallium in the form of GaM against four staphylococcal laboratory strains

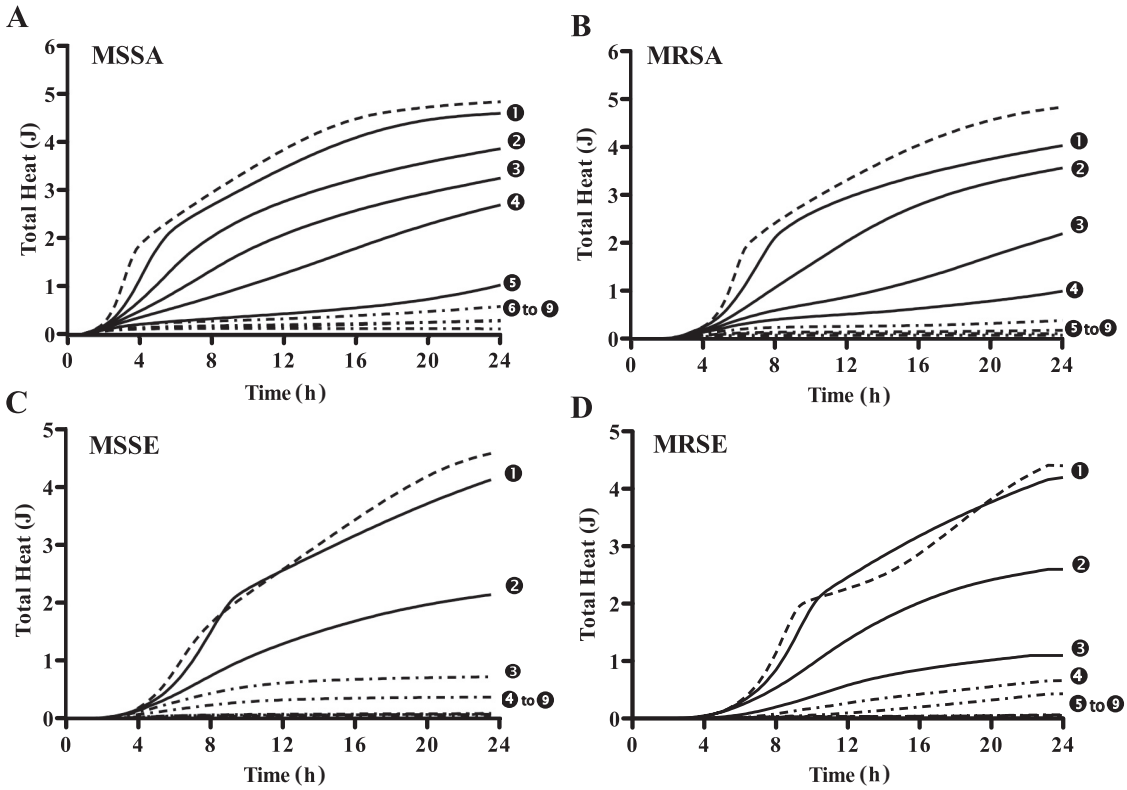

FIG. 2. Calorimetry curves representing the total heat generated by MSSA (A), MRSA (B), MSSE (C), and MRSE (D) at 37°C for 24 h in the presence of twofold dilutions of GaM in RPMI. Dashed lines, growth controls without GaM; continuous lines, GaM concentrations below the MIC; dashed-dot lines, GaM concentrations above the MIC for the corresponding test strain. The scales on the *y* axes are adapted for *S. aureus* and *S. epidermidis*. GC, growth control; curve 1, 23 μg/ml GaM; curve 2, 46 μg/ml GaM; curve 3, 94 μg/ml GaM; curve 4, 188 μg/ml GaM; curve 5, 375 μg/ml GaM; curve 6, 750 μg/ml GaM; curve 7, 1,500 μg/ml GaM; curve 8, 3,000 μg/ml GaM; curve 9, 6,000 μg/ml GaM.

and 20 distinct clinical isolates. Assays were performed in a defined synthetic iron-limited medium (RPMI). By determination of the bacterial doubling time, we found that the RPMI iron-restricted medium was appropriate for use instead of the standard medium (MHB) for susceptibility testing. Indeed, in our assay, RPMI supported staphylococcal growth as well as MHB did.

Against laboratory strains and by using the criteria applied to conventional antimicrobial substances, *S. epidermidis* was more susceptible to GaM than *S. aureus.* Moreover, bactericidal activity was detected against all staphylococci except MSSA in logarithmic phase and also against *S. epidermidis* in the stationary growth phase. The time-kill studies demonstrated that the killing mechanism of GaM occurs in a time-dependent and dose-dependent fashion. Since the *S. epidermidis* strains had longer doubling times than the *S. aureus* strains (in both RPMI and MHB), the time-kill studies were prolonged to 48 h for the MSSE and MRSE strains. Indeed, the killing of MSSE was improved at 48 h compared to that at 24 h. Better killing was achieved with GaM in time-kill studies than in the MIC/MBC macrodilution assay. This discrepancy was mainly observed with the MRSA strain, for which a 3-log reduction was already achieved at the MIC in the time-kill study. The reason for the better killing action may be correlated to the different conditions under which bacteria are cultured for the MBC test and for the kill-curve studies. The MBC assay requires incubation under static conditions, and kill-curve studies are performed with repetitive shaking at each sampling time point. By shaking, the bacteria get access to more oxygen in the growth medium, and they may thus also maintain better metabolic activity at late times of incubation. Since GaM displayed better efficacy against replicating bacteria, in the time-kill curve studies, the 3-log reduction may already occur at concentrations lower than the MBC.

The activity of GaM against biofilm-embedded bacteria was measured by determining the MBICs for laboratory strains and 20 clinical isolates. All isolates were classified as being able to form biofilms by the crystal violet staining assay. However, one of the MRSE isolates could not be further evaluated for biofilm susceptibility to GaM because of a lack of regrowth from the subcultured pegged lids. GaM exhibited inhibitory activity against biofilms of the remaining strains, with the MBICs being between 3,000 and 6,000 μg/ml (for the *S. aureus* strains) and between 280 and 3,000 μg/ml (for the *S. epidermidis* strains). For 5 of 10 clinical *S. aureus* isolates tested, no inhibition of the biofilms was observed at concentrations up to 6,000 μg/ml.

By measurement of bacterial heat production, we compared this novel calorimetric method with conventional susceptibility tests and we evaluated the effect of GaM at subinhibitory concentrations on bacterial metabolism and growth. The concentrations of GaM required for heat inhibition correlated well with the MICs, confirming the direct link between heat mea-

surements and bacterial growth. The discrepancies in the absolute values of MHIC and MIC that eventually appeared could possibly have occurred because of the culture conditions. Indeed, for heat measurement, the bacteria were cultured in sealed ampoules with limited oxygen availability, while the broth macrodilution assays were performed in snap-lid tubes with sufficient oxygen availability. However, differences of two- to fourfold may still represent a good correlation of the two parameters. Thus, we suggest the potential use of calorimetry as a fast, accurate, and simple method for investigation of the antimicrobial activities of new substances at subinhibitory concentrations. The major finding was that, in accordance with the results of the time-kill studies performed, the GaM mechanism of inhibition of heat production was also dose dependent and time dependent, and a reduction in the level of heat production compared to that for the controls was measured at GaM concentrations of up to 23 μg/ml.

The in vitro activity of GaM against laboratory and clinical strains of staphylococci occurred at MICs ranging from 100 to 2,000 μg/ml. After the administration of a single oral dose of 500 mg of GaM to three healthy volunteers, the median peak concentration of gallium in serum was 1 μg/ml, which is 100- to 2,000-fold lower than the MICs that were measured (4). These data suggest that the systemic application of GaM will probably not achieve therapeutic concentrations in humans. However, a subcutaneous injection of GaM (25 mg/kg of body weight) was highly protective against a wound infection induced by *P. aeruginosa* and *S. aureus* in a thermally injured mouse infection model (13). The findings of the latter study, together with the results of our in vitro study, would support the potential use of GaM for local administration for the prevention and treatment of wound infections. Moreover, due to its activity at subinhibitory concentrations, the use of low doses of GaM in combination with standard antimicrobials may be investigated to elucidate their potential synergistic effects or ability to prevent the development of resistance. GaM is also a promising candidate for topical use, by which high local concentrations can be achieved. In view of the increasing rates of resistance of *S. aureus* to oxacillin and mupirocin, GaM may become an option (alone or in combination with other agents) for treatment for skin and mucosal decolonization. Finally, the antibiofilm activity displayed in this study may suggest that gallium may be used for implant coating as a strategy for the prevention of staphylococcal adherence. Animal and clinical studies are needed to further characterize the therapeutic and preventive potentials of gallium, and these would then need to be followed by toxicity studies. A safe and efficacious gallium-containing formulation for the treatment of a broad spectrum of biofilm-forming or multiresistant microbes would be highly desirable.

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