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Susceptibility of *Staphylococcus aureus* Growing on Fibronectin-Coated Surfaces to Bactericidal Antibiotics

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Several recent studies have shown that bacteria either grown in vitro as adherent biofilms or recovered from infected prosthetic devices have decreased susceptibilities to antimicrobial killing. To further study the microbial and environmental factors responsible for this decreased antibiotic susceptibility, we developed an in vitro model of surface-adherent *Staphylococcus aureus* growing on polymethylmethacrylate coverslips coated with pure fibronectin. After exponential growth for 4 h, the population of fibronectin-attached *S. aureus* remained constant for a further 48-h period, as evaluated by CFU counts of organisms quantitatively removed from the coverslips. At selected time points, surface-bound organisms were exposed to bactericidal concentrations of either oxacillin, vancomycin, fleroxacin, or gentamicin in short-term (0.5 to 2 h) or long-term (24 h) killing assays. Whereas at 2 h surface-growing organisms were still optimally killed by all antimicrobial agents, at 4 and 24 h attached bacteria expressed markedly altered susceptibilities to these agents. The decrease in susceptibility was moderate for fleroxacin, more important for oxacillin and vancomycin, and extensive for gentamicin. When surface-attached *S. aureus* was compared with bacteria grown in a fluid phase, both populations showed a parallel time-dependent decrease in their susceptibilities to either oxacillin, vancomycin, or fleroxacin. In contrast, attached organisms became considerably more resistant to gentamicin than suspended bacteria did. Subpopulations of organisms spontaneously released from coverslips during antibiotic exposure also showed markedly reduced susceptibilities to antimicrobial killing. This simple model of *S. aureus* colonization of in vitro fibronectin-coated surfaces might represent a useful approach to the study of the physiological and biochemical changes that underlie the decreased antibiotic susceptibilities of biomaterial-attached organisms.

Infection is a major complication in patients with implanted devices. Several clinical (3, 4, 23) and experimental (11, 21) studies have documented the frequent failure of antimicrobial agents to eradicate staphylococcal infections associated with orthopedic prostheses, central nervous system shunts, or artificial valves. The reasons for this lack of effectiveness are still poorly understood and may involve alterations in the properties of the bacteria that colonize implants, impairment in the host defense mechanisms (41, 43), or both.

Tissue cage models of foreign body infection, whether subcutaneously implanted in guinea pigs (38, 39) or rats (8, 25), have further documented the lack of in vivo efficacy of several antistaphylococcal agents that are highly active in vitro. Tissue cage models, which reproduce characteristics typical of those of clinical foreign body infections (42, 43), have also demonstrated that the impaired in vivo efficacies of antimicrobial agents occurred despite the presence of adequate antibiotic concentrations at the true site of infection, namely, in the inflammatory exudative fluid produced by tissue cages (8, 25, 38, 39).

In a recent study (9), we have found that suspensions of *Staphylococcus aureus* recovered from the foreign body surface and tissue cage fluid showed markedly decreased susceptibilities to the in vitro killing effects of antistaphylococcal antibiotics when compared with the susceptibilities of bacteria of the same strains grown in batch cultures under conventional conditions.

Various in vitro systems have recently been developed (2, 16, 24, 32) for reproducing the conditions of decreased antibiotic killing observed in vivo. Those studies have generally shown that the bacteria that grow on surfaces, in particular, those that form a biofilm by bacterial production of extracellular polymers (slime), were not eradicated by bactericidal concentrations of antimicrobial agents. It has been suggested that biofilm-enclosed organisms escape antibiotic killing because the extracellular material prevents diffusion and bacterial uptake of antimicrobial agents (1, 10, 19, 22, 34, 40), but some investigators have reported that resistance is independent of bacterial slime-producing characteristics (20, 31). Other factors may be responsible for the decreased susceptibilities of attached bacteria, e.g., reduced oxygen tension and pH, nutrient deprivation (1, 2, 5, 17), or a slow growth rate (1, 5, 6, 12, 13, 17, 18, 39). The individual contribution of each of these factors, in particular, the contribution of growth rate, is not yet clearly defined. One concern (5, 6, 18) has been the lack of a direct comparison between freshly established (within hours) and older populations of surface-bound bacteria and the lack of a fluid-phase standard with growth rates comparable to those of solid-phase organisms.

This report describes a procedure that allows easy monitoring of time-dependent changes in the growth rate and antibiotic susceptibility of *S. aureus* attached to fibronectin-coated polymer surfaces. The responses of surface-bound bacteria to antimicrobial agents were compared with those of fluid-phase organisms with similar growth rates. Growth of staphylococci on solid-phase fibronectin produced selective and significant changes in their susceptibilities to different antibiotics.

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MATERIALS AND METHODS

Chemicals and materials. Iso-Sensitest (IST) broth, a semidefined medium, and phosphate-buffered saline (PBS) solutions were purchased from Oxoid (Basingstoke, United Kingdom) and GIBCO (Paisley, United Kingdom), respectively. Fibronectin purified from pooled human plasma by affinity chromatography on gelatin-Sepharose 4B (35, 36) was a generous gift of J. J. Morgenthaler (Blood Transfusion Service, Swiss Red Cross, Bern, Switzerland). [^{3}H]thymidine was purchased from Amersham (Amersham, United Kingdom).

Bacterial strain. *S. aureus* I20, an isolate from the bloodstream of a patient with an intravenous catheter infection, was used for the study. The strain showed decreased susceptibility to killing by antibiotics when recovered from an experimental foreign body infection in rats (9). No significant slime production was found when *S. aureus* I20 was tested qualitatively by the tube method described by Christensen et al. (7).

Antimicrobial agents and standard susceptibility tests. Standard reference preparations of drugs used in the present investigation were obtained from their respective distributors, as follows: oxacillin from Sigma (St. Louis, Mo.), vancomycin from Lilly (Giessen, Germany), fleroxacin from Hoffmann-La Roche (Basel, Switzerland), and gentamicin from Schering (Kenilworth, N.J.). The MIC of each agent for *S. aureus* I20 was determined in Mueller-Hinton broth (Difco, Detroit, Mich.) by the tube macrodilution method with a standard inoculum of 5×10^5 to 2×10^6 CFU/ml (30). To screen for possible carryover of each antibiotic during MBC determinations, 100-μl portions were taken from all tubes with no growth and were subcultured, either undiluted or 10-fold diluted, on Mueller-Hinton agar (Difco) for 24 h at 37°C. The MBC was defined as the lowest concentration that killed 99.9% of the original inoculum (29).

Attachment and growth of *S. aureus* I20 on fibronectin-coated surfaces. Procedures for adsorption of fibronectin on coverslips and adhesion of *S. aureus* I20 in suspension have previously been described in detail (35, 36). Briefly, ethanol-cleaned and heat-sterilized polymethylmethacrylate coverslips (8 by 8 mm) were coated individually with a monolayer of fibronectin (300 ng/cm^2) by incubation for 1 h at 37°C in a 1-ml volume of PBS containing 10 μg of purified fibronectin. After being rinsed, each fibronectin-coated coverslip was incubated for 60 min in a shaking water bath at 37°C with a 1-ml suspension containing 10^8 CFU of washed log-phase *S. aureus* I20 in PBS with 1 mM Ca^{2+} and Mg^{2+}. These organisms came from an overnight culture which was diluted to 2×10^7 CFU/ml in IST broth, and the organisms were grown for 3 h. When indicated, [^{3}H]thymidine was added to the 3-h culture. At the end of the attachment period, the fluids containing unbound bacteria were drained. Coverslips were rinsed twice in 1 ml of PBS and were transferred to new tubes containing 1 ml of IST broth. They were then incubated at 37°C without agitation to allow growth of attached organisms for various periods of time ranging from 2 to 48 h. The number of surface-bound *S. aureus* I20 organisms resulting from growth on fibronectin-coated coverslips in IST broth was estimated by measuring the CFU counts of the bacteria removed from the solid phase by the following methods. At the end of the incubation, the fluids containing spontaneously released bacteria were drained; after gentle rinsing in PBS, the coverslips were transferred to tubes containing 1 ml of PBS with 100 μg of bovine trypsin (Serva, New York, N.Y.) at 37°C, vortexed for 30 s, and sonicated for 3 min at 60 W in a low-output cleaning sonicator (model 2200; Brandson Ultrasonics, Branbury, Conn.). Duplicate 100-μl portions of appropriate dilutions were plated onto Mueller-Hinton agar, and the plates were incubated for 24 to 48 h at 37°C. This method was shown microscopically to detach efficiently surface-bound *S. aureus* I20 and to disrupt bacterial clumps. Further control experiments performed with [^{3}H]thymidine-labeled bacteria indicated that more than 90% of attached *S. aureus* I20 organisms were removed from the coverslips. Controls proved that this procedure did not affect bacterial viability.

Killing assays of surface-bound *S. aureus* I20. At selected time points during growth on coverslips, namely, at either 2, 4, or 24 h, bactericidal assays on attached *S. aureus* I20 were performed. For each condition, duplicate or triplicate coverslips were gently rinsed and transferred to 1 ml of fresh IST medium containing bactericidal concentrations (four to eight times greater than the MBC) of antibiotics, either 4 μg of oxacillin per ml or 8 μg of vancomycin, fleroxacin, or gentamicin per ml. Short-term killing assays were performed at 37°C for 2 h with oxacillin or vancomycin, for 1 h with fleroxacin, and for 30 min with gentamicin. In addition, long-term killing assays were performed at 37°C for 24 h with the same agents at the same concentrations. At the end of the incubation period, organisms were detached from coverslips as described above. Killing of surface-bound *S. aureus* I20 was defined as the average decrease (expressed in $\log_{10}$ CFU per coverslip) in colony counts of antibiotic-exposed organisms compared with those measured on parallel coverslips before antibiotic exposure.

Killing assays of fluid-phase *S. aureus* I20. Bactericidal assays were also performed on suspensions of *S. aureus* I20 derived either from liquid cultures in IST broth or from prior solid-phase cultures. Liquid cultures of *S. aureus* I20 were performed in IST broth at 37°C without agitation with an initial inoculum of 2×10^5 to 5×10^5 CFU. Test tubes did not contain coverslips in these experiments. When freshly detached organisms were exposed to antibiotics in the fluid phase, they came from a prior culture on fibronectin-coated coverslips as described above and were detached from the surface by sonication and trypsin treatment. Conditions for short- and long-term killing assays were identical to those used for surface-bound organisms. At 2 and 4 h, antibiotics were directly added to duplicate suspensions of *S. aureus* I20. At 24 h, however, the organisms that reached the stationary phase (10^9 CFU/ml) were washed by centrifugation and were resuspended in fresh medium at concentrations of 10^8 and 10^6 CFU per ml of IST broth before exposure to bactericidal antibiotics. Controls performed at serial 10-fold dilutions with plating of 100-μl portions on Mueller-Hinton agar indicated the absence of significant carryover effects with *S. aureus* I20 in suspension. Experiments were performed in duplicate or triplicate.

Quantification of bacteria released from the surface. Solid-phase cultures of *S. aureus* I20 radiolabeled with [^{3}H]thymidine were used to quantify bacterial release from coverslips by radioactive counts (in counts per minute), because CFU counts are affected by bacterial growth and killing in addition to bacterial release. To validate the assumption that radioactive counts represented cell-associated radioactivity, sedimentation of the counts by low-speed sedimentation or retention by 0.22-μm-pore-size filters (Millipore, Bedford, Mass.) was assessed. The percentage of radioactivity incorporated into bacterial DNA was evaluated from tritium counts selectively released from the radiolabeled bacteria by a 30-min treatment with 10 μg of lysostaphin (Applied

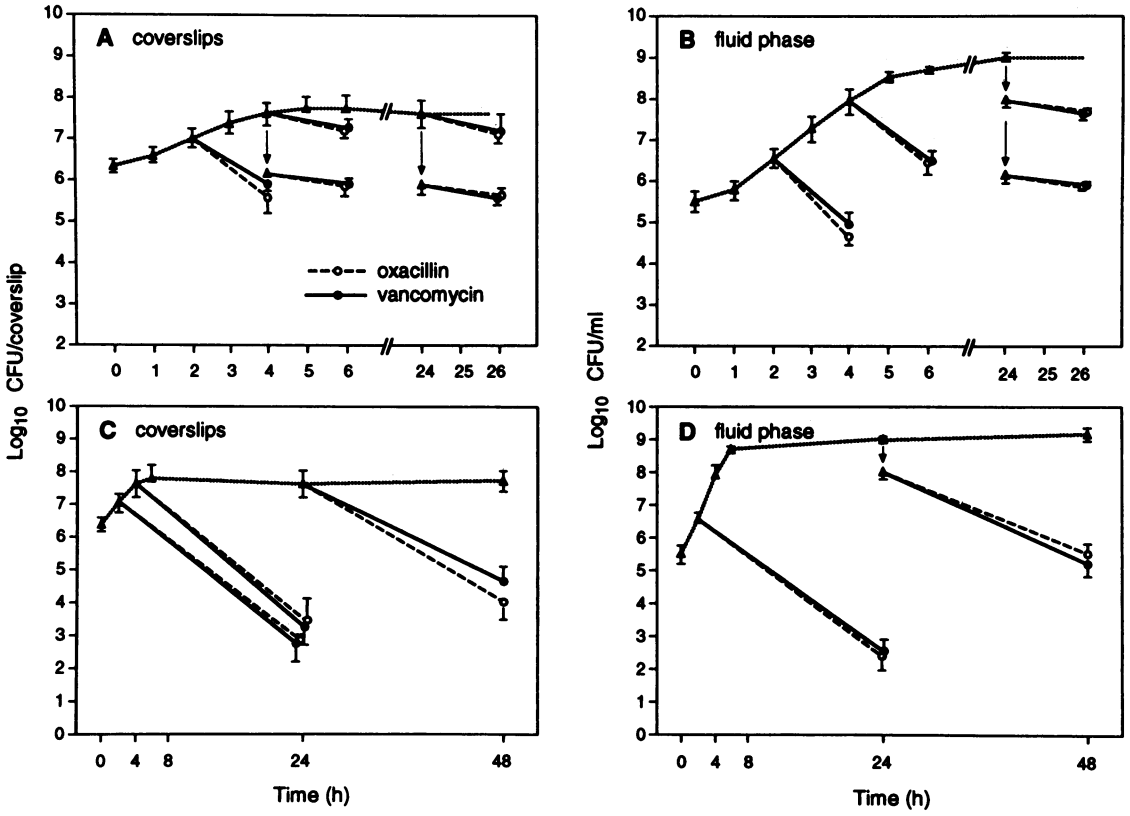

FIG. 1. Growth curve (▲) and rate of in vitro killing of *S. aureus* I20 by either oxacillin (4 μg/ml) or vancomycin (8 μg/ml). Short-term (A and B) or long-term (C and D) assays were performed with organisms growing either on fibronectin-coated coverslips (A and C) or in the fluid phase (B and D). In panel A, arrows indicate organisms detached from coverslips and diluted; in panels B and D, arrows indicate fluid-phase organisms incubated at different dilutions. Results are expressed as means ± SDs of three to five experiments.

Microbiology, Brooklyn, N.Y.) per ml and DNase (Sigma) in PBS supplemented with 1 mM Ca^{2+} and Mg^{2+}.

Data analysis. Each experiment that evaluated growth and killing of either solid- or fluid-phase organisms was performed at least three times. Results were expressed as means ± standard deviations (SDs). Differences between means were estimated by Student's *t* test with two-tailed significance levels. *P* values of <0.05 were considered statistically significant.

RESULTS

Standard susceptibility tests. The MICs and MBCs of oxacillin, vancomycin, fleroxacin, and gentamicin for *S. aureus* I20 were 0.5 and 1.0, 1.0 and 2.0, 1.0 and 1.0, and 1.0 and 2.0 μg/ml, respectively. Similar MBCs were obtained by plating either undiluted or 10-fold-diluted samples, which indicated the absence of any significant carryover of antibiotics.

Characteristics of the solid-phase assay. During the initial 6-h incubation period, the average viable counts of *S. aureus* I20 growing on fibronectin-coated coverslips were assayed every hour and increased 20-fold, from 6.34 $\log_{10}$ CFU per coverslip at zero time to 7.62 $\log_{10}$ CFU per coverslip at 4 h (Fig. 1). Thereafter, viable counts of surface-bound bacteria remained stable through the further 48-h period at an average value of 7.76 $\log_{10}$ CFU per coverslip (Fig. 1).

For selected time points, the reproducibility of the number of attached organisms in different sets of coverslips before exposure to antibiotics was tested in control experiments. Directly after attachment or after 4 h of growth, surface-bound organisms showed minimal deviations from one set to another: the average $\log_{10}$ counts of six sets of duplicate coverslips ranged from 6.24 to 6.44 (mean ± SD, 6.32 ± 0.08) at zero time and from 7.80 to 8.05 (7.92 ± 0.10) at 4 h of growth.

Susceptibilities of surface-bound and fluid-phase organisms to bactericidal antibiotics. The susceptibilities of surface-bound *S. aureus* I20 to bactericidal concentrations of either oxacillin (4 μg/ml) or vancomycin (8 μg/ml) at selected time points are shown in Fig. 1 and were compared with those of organisms tested separately in the fluid phase. During the initial 4-h incubation period, fluid-phase bacteria (without coverslips) grew at a faster rate than solid-phase organisms, with average generation times of 26 and 45 min, respectively. In short-term killing assays performed for 2 h, exponentially growing 2-h cultures of surface-attached and fluid-phase *S. aureus* I20 showed average decreases in CFU with oxacillin of 1.42 and 1.86 $\log_{10}$ and with vancomycin of 1.10 and 1.59 $\log_{10}$, respectively. These differences in the killing rate of solid- and fluid-phase organisms were not statistically significant. At 4 h, the killing of attached bacteria was significantly ($P < 0.02$) lower than that of organisms in the liquid phase incubated with either oxacillin (0.42 versus 1.49 $\log_{10}$ CFU) or vancomycin (0.34 versus 1.37 $\log_{10}$ CFU). In contrast, both solid- and fluid-phase *S. aureus* I20 at steady state at 24 h showed decreased susceptibilities to killing by cell wall-active agents in short-term assays, since neither oxacillin nor

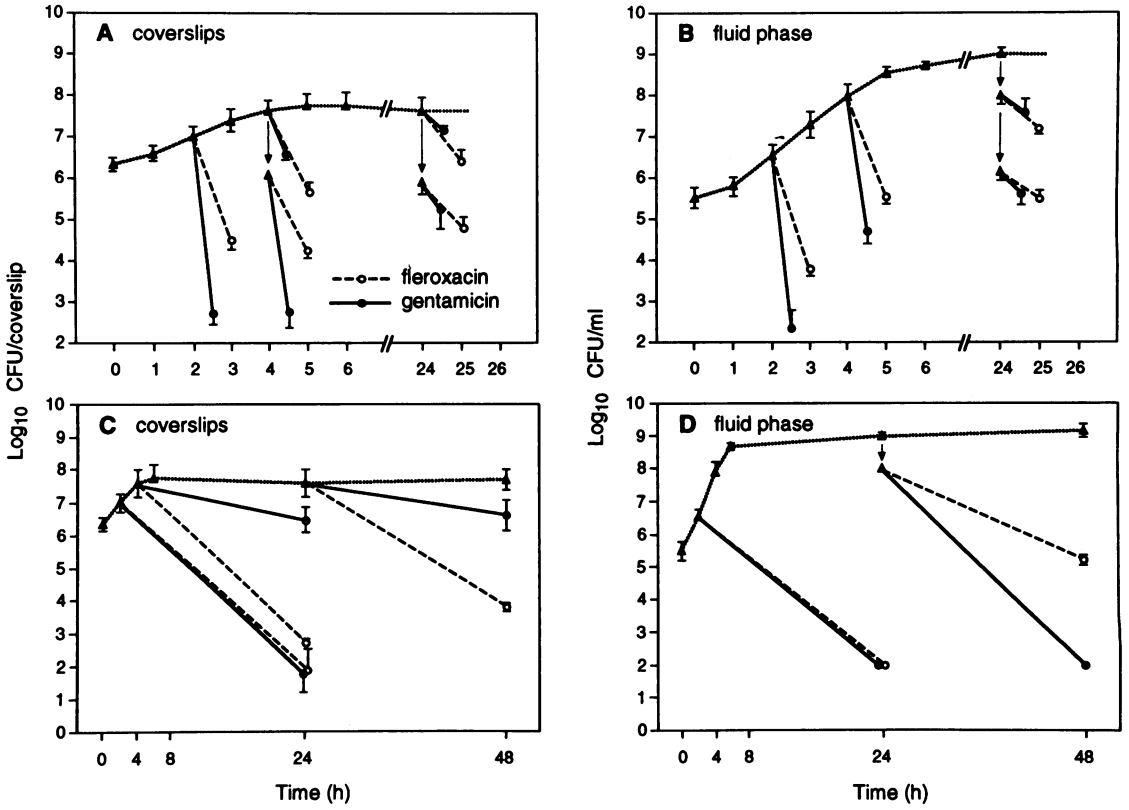

FIG. 2. Growth curve (▲) and rate of in vitro killing of *S. aureus* I20 by either fleroxacin or gentamicin (8 μg/ml). Short-term (A and B) or long-term (C and D) assays were performed with organisms growing either on fibronectin-coated coverslips (A and C) or in the fluid phase (B and D). In panel A, arrows indicate organisms detached from coverslips and diluted; in panels B and D, arrows indicate fluid-phase organisms incubated at different dilutions. Results are expressed as means ± SDs of three to five experiments.

vancomycin could reduce the colony counts of stationary-phase organisms by even 0.5 $\log_{10}$ CFU (Fig. 1A and B).

Surface-bound and fluid-phase *S. aureus* I20 were also exposed to cell wall-active antibiotics in long-term (24-h) killing assays. Both actively growing and stationary-phase organisms tested at 2, 4, or 24 h were significantly killed (>3 $\log_{10}$ CFU) by both oxacillin and vancomycin (Fig. 1C and D).

The susceptibility of either surface-bound or fluid-phase *S. aureus* I20 to a bactericidal (8 μg/ml) concentration of either fleroxacin or gentamicin was also tested at selected time points (Fig. 2). In short-term (1-h) killing assays performed with fleroxacin, the average reduction in CFU was not significantly different for solid- and fluid-phase organisms, reaching 2.53 and 2.80 $\log_{10}$ at 2 h, 1.95 and 2.38 $\log_{10}$ at 4 h, and 1.20 and 0.84 $\log_{10}$ at 24 h, respectively (Fig. 2A and B). Long-term killing assays confirmed the similar susceptibilities to fleroxacin of attached and fluid-phase organisms, which were eradicated by more than 3.5 $\log_{10}$ CFU when tested at either 2 or 24 h (Fig. 2C and D).

In contrast to its susceptibilities to other antibiotics, *S. aureus* I20 showed a progressive decrease in susceptibility to gentamicin which was selectively promoted by a surface location. Exponentially growing 2-h cultures in the solid phase were still susceptible to the bactericidal effect of gentamicin in short-term (30-min) killing assays, with an average decrease in CFU counts of 4.30 $\log_{10}$ (Fig. 2A). At 4 h, the susceptibility of attached bacteria to gentamicin was sharply reduced (killing of 1.06 $\log_{10}$ CFU), while fluid-phase *S. aureus* I20 tested in short-term assays was killed by >3 $\log_{10}$ CFU (Fig. 2A and B). At 24 h, both attached and fluid-phase bacteria were initially (30-min incubation) resistant to the killing effect of gentamicin, but a longer incubation period with the drug resulted in a selective resistance of the surface-bound organisms versus the fluid-phase ones, with a decrease in CFU of 1.05 $\log_{10}$ for the former and more than 6 $\log_{10}$ CFU for the latter (Fig. 2C and D).

To further analyze whether the antibiotic susceptibility changes observed on *S. aureus* I20 attached to fibronectin-coated surfaces were specific to the solid-phase location at the time of the assay or reflected more permanent metabolic changes, control experiments were performed. In those experiments, the organisms were removed from coverslips and tested in the liquid phase. As shown in Fig. 1A and 2A, bacteria freshly removed from the coverslips at either 4 or 24 h and tested in dilute suspensions of 10^6 CFU/ml were killed by either oxacillin, vancomycin, or fleroxacin at exactly the same rate as the more densely packed surface-bound organisms were. The only notable exception was gentamicin, which, at 4 h, but not at 24 h, was more active on dilute, freshly suspended bacteria than on surface-bound organisms (Fig. 2A). Additional experiments testing the influence of various cell densities of *S. aureus* I20 on the bactericidal activity of each antibiotic were performed on bacteria in suspension. Liquid-phase cultures of *S. aureus* I20 in the stationary phase (24 h) were diluted to either 10^6 or 10^8 CFU/ml and were exposed to short-term bactericidal assays. Since organisms at both inocula were killed similarly by each

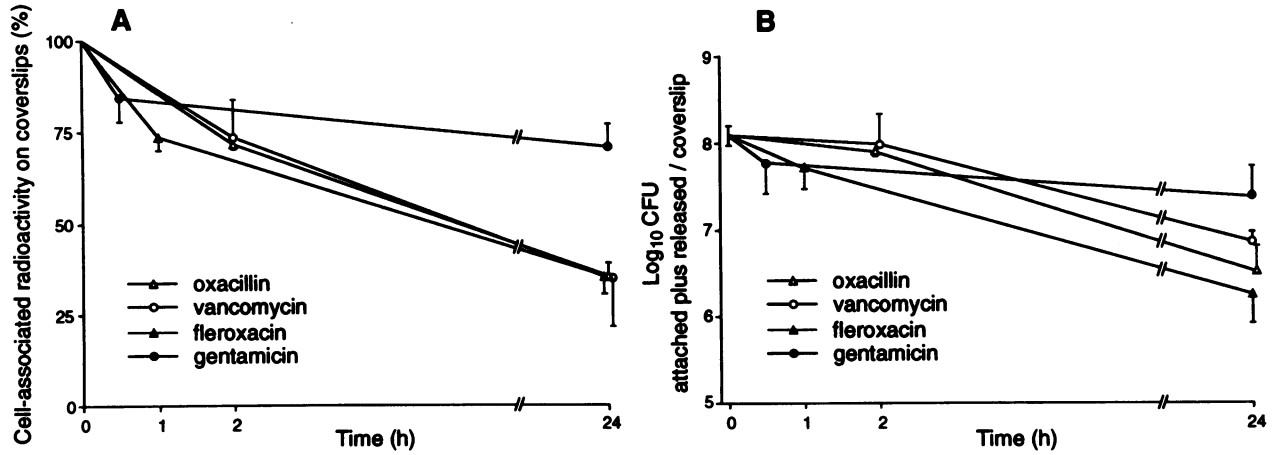

FIG. 3. (A) Percentage of *S. aureus* I20 (cell-associated radioactivity) remaining attached to fibronectin-coated coverslips during long-term incubation with antibiotics. (B) Cumulative survival of attached and spontaneously released bacteria during long-term incubation with antibiotics. In panels A and B, zero time indicates the time of addition of 4 μg of oxacillin per ml or 8 μg of either vancomycin, fleroxacin, or gentamicin per ml to a 24-h solid-phase bacterial culture. Results are expressed as means ± SDs of three to five experiments.

antibiotic (Fig. 1B and 2B), this indicated that cell density itself is not a critical factor that interferes with the bactericidal assays.

A further important environmental parameter known to influence bactericidal activity is limitation of essential nutrients. This possibility was excluded by performing control experiments which showed equivalent growth rates of solid- and liquid-phase cultures of *S. aureus* I20 in either undiluted or 10-fold-diluted IST broth (data not shown).

Quantification of bacterial release during antibiotic exposure. To validate the long-term antibiotic killing assays performed on 24-h solid-phase cultures, it was important to exclude an excessive contribution of bacterial release to the observed decrease in viable counts (Fig. 1 and 2). Since CFU counts were an unreliable parameter in the presence of antibiotics, we determined in a further set of experiments the proportion of cell-associated radioactivity which was released during antibiotic exposure. At 24 h, 20 to 30% of the initially attached radioactivity was still present on coverslips. The cellular location of this radioactivity was validated by control experiments which showed that >90% of the tritium counts of the 24-h bacterial culture were sedimentable by low-speed centrifugation and were retained by 0.22-μm-pore-size filters. These counts were quantitatively solubilized (>90%) by treatment of the bacterial culture with lysostaphin and DNase.

During the initial 30-min to 2-h incubation period, the radioactivity released from 24-h solid-phase cultures was less than 30%, regardless of the antibiotic tested. After 24 h of incubation with antibiotics, the proportion of released radioactivity averaged 65% for oxacillin-, vancomycin-, and fleroxacin-treated cultures and averaged 29% for gentamicin-treated cultures (Fig. 3A).

Despite the fact that the counts per minute released from the coverslips probably reflected those from a mixture of viable and killed organisms whose proportion was unknown, it was possible to predict how bacterial release could affect the accuracy of killing estimates obtained by CFU counts. In the hypothetical situation in which radioactivity release from the coverslips would exclusively involve viable bacteria, the reduction in CFU counts, as presented in Fig. 1 and 2, would overestimate the killing of surface-bound organisms. For this situation, bacterial killing on coverslips can be estimated as follows: $\log_{10}(CFU_{init} \times cpm_{fin}/cpm_{init}) - \log_{10} CFU_{fin}$, where CFU_{init} and cpm_{init} represent the initial (preantibiotic) viable and radioactivity counts per coverslip, respectively, and CFU_{fin} and cpm_{fin} represent the final (postantibiotic) viable and radioactivity counts per coverslip, respectively.

Comparisons of corrected killing estimates with uncorrected ones revealed marginal differences. The decrease in $\log_{10}$ CFU per coverslip for surface-bound organisms was shifted from 3.16 to 2.71 for a 24-h exposure to oxacillin, from 2.84 to 2.39 for vancomycin, from 4.04 to 3.59 for fleroxacin, and from 1.38 to 1.23 for gentamicin. Therefore, release of viable organisms during antibiotic exposure was not an important reason for the decrease in CFU counts of antibiotic-exposed organisms on coverslips.

The unknown proportion of bacteria leaving coverslips as viable versus killed units did not allow a precise measurement of bacterial killing in the fluid phase; however, we could make an estimation of this killing by recording the cumulative survival of attached and released bacteria during antibiotic exposure. Viable counts of released organisms added to those of attached ones (Fig. 3B) showed a high cumulative survival in the presence of oxacillin, vancomycin, fleroxacin, and gentamicin, with reductions in CFU counts of 1.61 $\log_{10}$ CFU (versus 3.16 $\log_{10}$ CFU for surface-bound organisms alone), 1.27 $\log_{10}$ CFU (versus 2.84 $\log_{10}$ CFU), 1.88 $\log_{10}$ CFU (versus 4.04 $\log_{10}$ CFU), and 0.65 $\log_{10}$ CFU (versus 1.38 $\log_{10}$ CFU), respectively. In other terms, the great majority of the viable counts of the total population after antibiotic exposure were contributed by organisms released in the fluid phase (>97% with oxacillin, vancomycin, and fleroxacin and 81% with gentamicin), indicating that the bacteria were poorly susceptible to the antimicrobial agents in this compartment.

DISCUSSION

A novel method for studying the antibiotic susceptibilities of surface-adherent staphylococci was developed. The model described here is an extension of a previously described procedure for studying *S. aureus* adhesion to polymethylmethacrylate coverslips either coated with pure fi-

bronectin in vitro (36) or explanted from animal models of foreign body infections (35). Fibronectin is considered an important host protein in promoting *S. aureus* attachment to biomaterial surfaces. In vitro coating of polymeric surfaces by fibronectin can reproducibly promote *S. aureus* adhesion. Fibronectin-mediated attachment results in a homogeneous distribution of bacteria on the coverslips. This distribution contributed to the precise monitoring of subsequent growth of the surface-bound organisms.

The present study showed that *S. aureus* growing on surfaces is initially as susceptible to bactericidal antibiotics as fluid-phase bacteria. Longer incubation of the surface-bound organisms, up to the stationary phase, produced a significant decrease in their susceptibilities to antimicrobial killing. These susceptibility changes were not expressed to the same extent for all antibiotics, being moderate for fleroxacin, more important for oxacillin and vancomycin, and major for gentamicin. The susceptibilities of surface-bound and fluid-phase *S. aureus* decreased in a nearly parallel way for oxacillin, vancomycin, and fleroxacin. With gentamicin, bacterial susceptibility was far more affected when the bacteria were grown on the solid surface than when they were grown in the fluid phase. Not only surface-bound organisms but also those released from fibronectin-coated coverslips during antibiotic exposure were found to be significantly more resistant to antibiotic killing than bacteria grown and tested exclusively in the fluid phase.

A recent literature survey of studies of surface-bound microbial populations, frequently referred to as biofilms, revealed that in most cases bacteria tested for antibiotic susceptibility were grown for long periods, frequently exceeding 24 h (2, 14, 19, 20, 24, 32, 37–39). A majority of investigators using either static experimental systems (20, 24, 38, 39) or dynamic experimental systems like the Robbins device or chemostats (2, 14, 19, 32, 37) considered biofilm populations to be in a slow mode of growth or in a dormant state, although no direct estimates of either the growth or the turnover rate of adherent bacteria were provided. A frequent characteristic of in vitro bacterial biofilm studies was the presence of extended polysaccharide glycocalices, especially in the case of *Pseudomonas aeruginosa* (2, 32) and *Staphylococcus epidermidis* (14, 19). In these complex experimental conditions, it was difficult to sort out the individual contribution of several important parameters such as the thickness and composition of the exopolysaccharide layer, the high densities of adherent bacteria, the limitation of essential nutrients, altered metabolism, or slow growth rate. In our study with *S. aureus* I20, we found no evidence by microscopy for a significant exopolysaccharide layer surrounding fibronectin-attached organisms, thus excluding a possible influence of this parameter. Furthermore, we could minimize the potential effects of nutrient limitation by incubating surface-bound organisms in a rich liquid medium containing an excess of essential nutrients during growth and antibiotic testing.

Solid- and fluid-phase bacteria at equivalent growth phases showed parallel changes in their susceptibilities to oxacillin, vancomycin, and fleroxacin. This suggested that for these antibiotics growth rate and/or growth-related metabolic events play a major role in the susceptibility decrease of surface-bound organisms. In contrast, the rapid and substantial loss of susceptibility to gentamicin observed for solid-phase *S. aureus* was not related only to decreased growth rate, because fluid-phase organisms growing at similar rates remained more susceptible than adherent bacteria. Previous studies also described a drastic loss of aminoglycoside activity toward adherent populations of either *S. epidermidis*, *P. aeruginosa*, or *Escherichia coli*, which was explained either by a restricted drug penetration through thick bacterial biofilms (2, 19, 32) or by growth rate-related effects (13). Because neither of these mechanisms could explain the loss of susceptibility to gentamicin of *S. aureus* I20 in our experimental system, we looked for the possible emergence of aminoglycoside-resistant variants during exposure to concentrations of gentamicin exceeding the MBC. Several studies have previously described the in vitro and in vivo production of small, nonhemolytic colonies showing increased resistance to aminoglycoside antibiotics. These so-called small-colony variants, which developed during therapy with aminoglycosides or in vitro exposure, were shown to have a defective energy-dependent transport system of aminoglycosides, which led to reduced intracellular accumulations and bactericidal activities of these antimicrobial agents (26–28, 33). A careful screening of colony morphology during growth and antibiotic exposure of solid-phase *S. aureus* did not show emergence of small-colony variants. Further experiments are planned to study the physiological parameters of gentamicin-tolerant populations of *S. aureus* during adhesion on fibronectin-coated surfaces.

Very few studies have estimated the antibiotic susceptibilities of bacteria spontaneously released from adherent biofilms. In one report (37), the bacteria shed from biofilms of *S. epidermidis* and *P. aeruginosa* were described as having an intermediate response between those of the more resistant adherent organisms and the more susceptible fluid-phase ones. In another experimental system of growth rate-controlled biofilms (16), the susceptibilities of the released organisms varied widely, depending on the bacterial species; gram-negative bacilli were highly susceptible to antibiotics (12, 13, 15), whereas *S. epidermidis* released from surfaces was more resistant to killing than surface-bound organisms were (15). Our results are a further indication of the decreased susceptibility to antibiotic killing of *S. aureus* released from surfaces. These results might have some in vivo relevance. We have recently reported that *S. aureus* recovered from an experimental model of a chronically infected foreign body was resistant to antimicrobial agents when tested in vitro (9). Not only surface-bound organisms but also those recovered from the fluid surrounding the foreign body—which might be shed bacteria—were killed slowly and incompletely by all the antibiotics tested. We hope that the simplified in vitro model reported here will help investigators to study the factors responsible for the resistance of in vivo-growing microorganisms to bactericidal agents.

In conclusion, the development of an in vitro model that made use of fibronectin-coated coverslip surfaces for promoting *S. aureus* attachment and colonization allowed us to study in detail how the susceptibilities of surface-bound organisms to different antibiotics were affected by growth rate, surface density, and other environmental parameters. The bacteria that colonize surfaces or that are spontaneously released from them represent an interesting subpopulation of *S. aureus* whose physiological and biochemical properties deserve further study.

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