



Activity of Antibiotics against *Staphylococcus aureus* in an *In Vitro* Model of Biofilms in the Context of Cystic Fibrosis: Influence of the Culture Medium

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ABSTRACT *Staphylococcus aureus* is a highly prevalent pathogen in the respiratory tract of young patients with cystic fibrosis (CF) and causes biofilm-related infections. Here, we set up an *in vitro* model of a biofilm grown in Trypticase soy broth supplemented with glucose and NaCl (TGN) or in artificial sputum medium (ASM) and used it to evaluate on a pharmacodynamic basis the activity of antibiotics used in CF patients and active on staphylococci (meropenem, vancomycin, azithromycin, linezolid, rifampin, ciprofloxacin, tobramycin). Rheological studies showed that ASM was more elastic than viscous, as was also observed for sputa from CF patients, with elastic and viscous moduli being, respectively, similar to and slightly lower than those of CF sputa. Biofilms formed by methicillin-sensitive *S. aureus* strain ATCC 25923 and methicillin-resistant *S. aureus* strain ATCC 33591 reached maturity after 24 h, with biomass (measured by crystal violet staining) and metabolic activity (assessed by following resazurin metabolization) being lower in ASM than in TGN and viability (assessed by bacterial counts) being similar in both media. Full concentration-response curves of antibiotics obtained after 24 h of incubation of biofilms showed that all antibiotics were drastically less potent and less efficient in ASM than in TGN toward viability, metabolic activity, and biomass. Tobramycin selected for small-colony variants, specifically in biofilms grown in ASM; the auxotrophism of these variants could not be established. These data highlight the major influence exerted by the culture medium on *S. aureus* responsiveness to antibiotics in biofilms. The use of ASM may help to determine effective drug concentrations or to evaluate new therapeutic options against biofilms in CF patients.

KEYWORDS *Staphylococcus aureus*, antibiotics, biofilms, cystic fibrosis, small-colony variants

Cystic fibrosis (CF) is a recessive genetic disease affecting mainly Caucasian individuals, with about 70,000 cases around the world. The disease results from a malfunction of a chloride channel (the cystic fibrosis transmembrane conductance regulator) affecting multiple organs, like the airways, pancreas, intestines, liver, reproductive tract, and sweat glands, and causing in these tissues the accumulation of a viscous, difficult-to-evacuate mucus. This stagnation is a propitious field for bacterial colonization and biofilm-related infections (1, 2). Biofilms are defined as microbial communities embedded in a self-produced matrix of extracellular polymeric substances (EPS), mainly extracellular DNA, polysaccharides, and proteins (3, 4). This lifestyle protects bacteria against host defense and antibiotics not only through the barrier effect exerted by the biofilm matrix but also because of metabolic changes affecting the bacterial response to antibiotics (4, 5).

Staphylococcus aureus is the most prevalent microorganism detected in infants and

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children suffering from CF, being detected in about 80% of patients aged 10 to 17 years (6). In chronic infections, *S. aureus* can adopt specific phenotypes, like small-colony variants (SCVs), characterized by slow growth and, therefore, the generation of small colonies on agar plates. Among them, the best characterized are electron transport-deficient SCVs (auxotrophs for hemin, menadione, or thiamine) that can be induced by aminoglycoside treatment and thymidine-dependent SCVs, mainly recovered after long-term treatment by trimethoprim-sulfamethoxazole in cystic fibrosis patients (7). Fatty acid-dependent and CO₂-dependent SCVs have also been described (8, 9).

S. aureus is well-known to form biofilms that contribute to its capacity to cause chronic infections, including in CF patients (4, 10–12). Until now, many studies have mainly concentrated on the activity of antibiotics against *Pseudomonas aeruginosa* biofilms, because they are the most prevalent in the adult CF population (6). Data are thus critically lacking regarding staphylococcal biofilms.

The aim of the present study was to set up an *in vitro* model of biofilms by *S. aureus* using a medium mimicking the viscoelastic properties of the sputum found in CF patients and referred to as artificial sputum medium (ASM) (13). This model was then used to study the activity of antibiotics on a pharmacodynamic basis in order to evaluate their relative potency and maximal efficacy (14). As antibiotics, we selected representative molecules among antistaphylococcal agents (vancomycin, linezolid, rifampin) but also among those that are frequently used in CF patients, based on their broad spectrum of activity (carbapenems, fluoroquinolones, aminoglycosides) or on their anti-inflammatory effect (azithromycin) (5, 15, 16).

RESULTS

Rheological properties of ASM. As a preliminary step to this work, we compared the viscoelasticity of artificial sputum medium (ASM) with that of sputa collected from CF patients in order to evaluate the capacity of this medium to mimic, in this respect, the environment faced by microbes in the lungs of CF patients. To this effect, six sputum specimens which were previously categorized as purulent (uniformly green or yellow) or mucopurulent (mixture of purulent and mucoid parts) by visual inspection were studied (17). Their deformation and flow under an applied stress were studied by rheology (Fig. 1). Upon application of an increasing shear strain to the samples, we observed that the linear viscoelastic region of CF samples remained steady for both the elastic and the viscous moduli between 0.0001% and 0.1% of shear strain. For ASM, stability was observed at a shear strain of between 0.0001% and 0.1% for the elastic modulus but only at shear strains of between 0.0001% and 0.05% for the viscous modulus. In this range of nondestructive shear strains, purulent samples showed higher elastic and viscous moduli than mucopurulent samples, while ASM had an elastic modulus very similar to the values measured for mucopurulent samples and a viscous modulus about 3 times lower than the values measured for mucopurulent samples. Both CF sputa and ASM were more elastic than viscous.

Kinetics of biofilm development in different media. The kinetics of biofilm development were then studied for two *S. aureus* strains, namely, methicillin-sensitive *S. aureus* (MSSA) strain ATCC 25923 and *S. aureus* methicillin-resistant (MRSA) strain ATCC 33591. Two different media were compared, namely, Trypticase soy broth supplemented with glucose and NaCl (TGN), previously used for growing staphylococcal biofilms (14, 18), and ASM. Figure 2 shows the evolution over time of viability (assessed by CFU counts), metabolic activity (metabolization of resazurin in fluorescent resorufin), and biomass (absorbance of crystal violet) in these biofilms. In both media, both strains grew quickly during the first 12 h to reach a plateau at approximately 10⁷ CFU/ml that remained stable over 3 days (Fig. 2, left). Metabolic activity (Fig. 2, middle) also increased rapidly over the first 12 h and reached a plateau after approximately 24 h in TGN, with the fluorescence signals being higher for ATCC 33591 than for ATCC 25923. In ASM, metabolic activity was lower than that in TGN and similar for both bacterial strains, reaching a maximum after 12 h and decreasing thereafter to reach a plateau at fluorescence values 1/3 of those observed in TGN at 12 h. Biomass also grew quickly

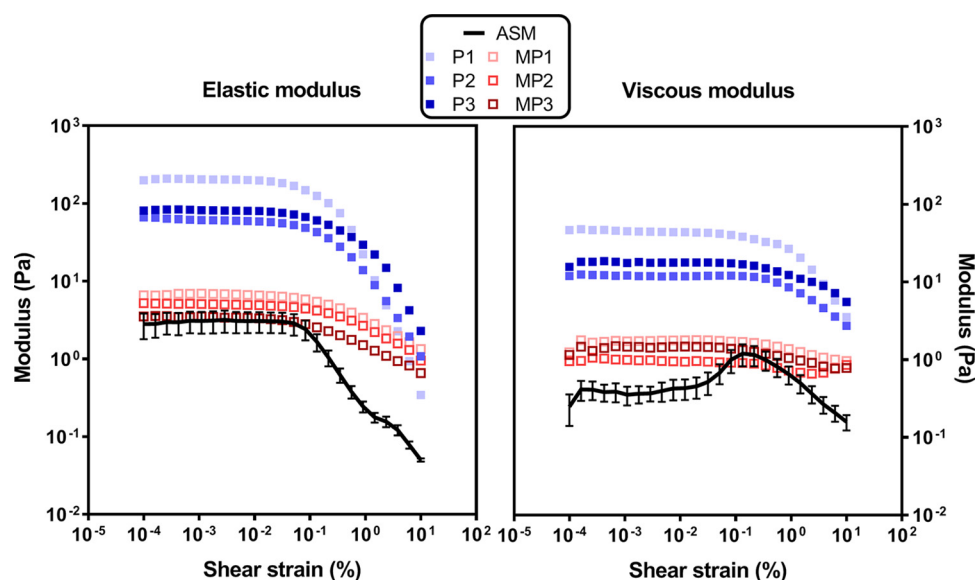


FIG 1 Viscoelasticity of artificial sputum medium (ASM; black curve) compared to that of six sputum specimens that were collected from CF patients and that showed a purulent (samples P1, P2, and P3 [blue closed squares]) or mucopurulent (samples MP1, MP2, and MP3 [red open squares]) appearance, shown as individual data. The graphs show the elastic modulus (left) and the viscous modulus (right), both of which are expressed in pascals (Pa; kilograms · meter⁻¹ · second⁻²). A linear viscoelastic region is observed at between 0.0001% and 0.1% of shear strain.

over the first 24 h in both media and for both strains (Fig. 2, right), with no major change being seen thereafter, except for ATCC 25923 in TGN, for which biomass decreased from day 1 to day 3. Again, absorbance values were higher in TGN than in ASM. Based on these results, we opted to use 24-h-old biofilms for further experiments, in which antibiotics were added for an additional 24 hours.

Antibiotic activity against planktonic cultures. Table 1 shows the MICs of the selected antibiotics against the two bacterial strains in the above-described media used for the biofilm studies compared to those in cation-adjusted Mueller-Hinton broth (MHB-ca). ATCC 25923 was fully susceptible to all antibiotics tested, while ATCC 33591 was resistant to meropenem, azithromycin, and tobramycin.

In TGN, MICs were similar to (± 1 dilution), slightly higher than (2 dilutions for vancomycin against ATCC 25923 and azithromycin against ATCC 33591), or higher than (3 or more dilutions for vancomycin and azithromycin against ATCC 33591 and tobramycin against ATCC 25923) those in MHB-ca. In ASM, MICs were similar to (± 1 dilution), slightly higher than (2 dilutions for vancomycin against ATCC 25923 or azithromycin and tobramycin against ATCC 33591), or lower than (for linezolid against ATCC 25923) those in MHB-ca.

Antibiotic activity against biofilms. We then evaluated the activity of the same antibiotics against bacterial viability, metabolic activity, and biofilm biomass after 24 h of incubation in TGN or in ASM.

Results are presented as concentration-response curves in Fig. 3A for drugs known to be slowly bactericidal (meropenem, vancomycin) or bacteriostatic (linezolid, azithromycin) and in Fig. 3B for drugs that are rapidly bactericidal (rifampin, ciprofloxacin, and tobramycin). Pharmacodynamic parameters, calculated based on the sigmoidal regression of the concentration-response curves, are summarized in Fig. 4 for maximal efficacy (E_{\max} ; corresponding to the maximal decrease in metabolic activity or number of CFU extrapolated for an infinitely large concentration) and in Fig. 5 for relative potency (estimated by the concentration needed to reduce the number of CFU by 1 log₁₀ [$C_{-1 \log}$] and metabolic activity or biomass by 33% [C_{33}]).

Considering first the results as a whole, all antibiotics showed concentration-dependent effects on both bacterial viability within the biofilm and biomass, with

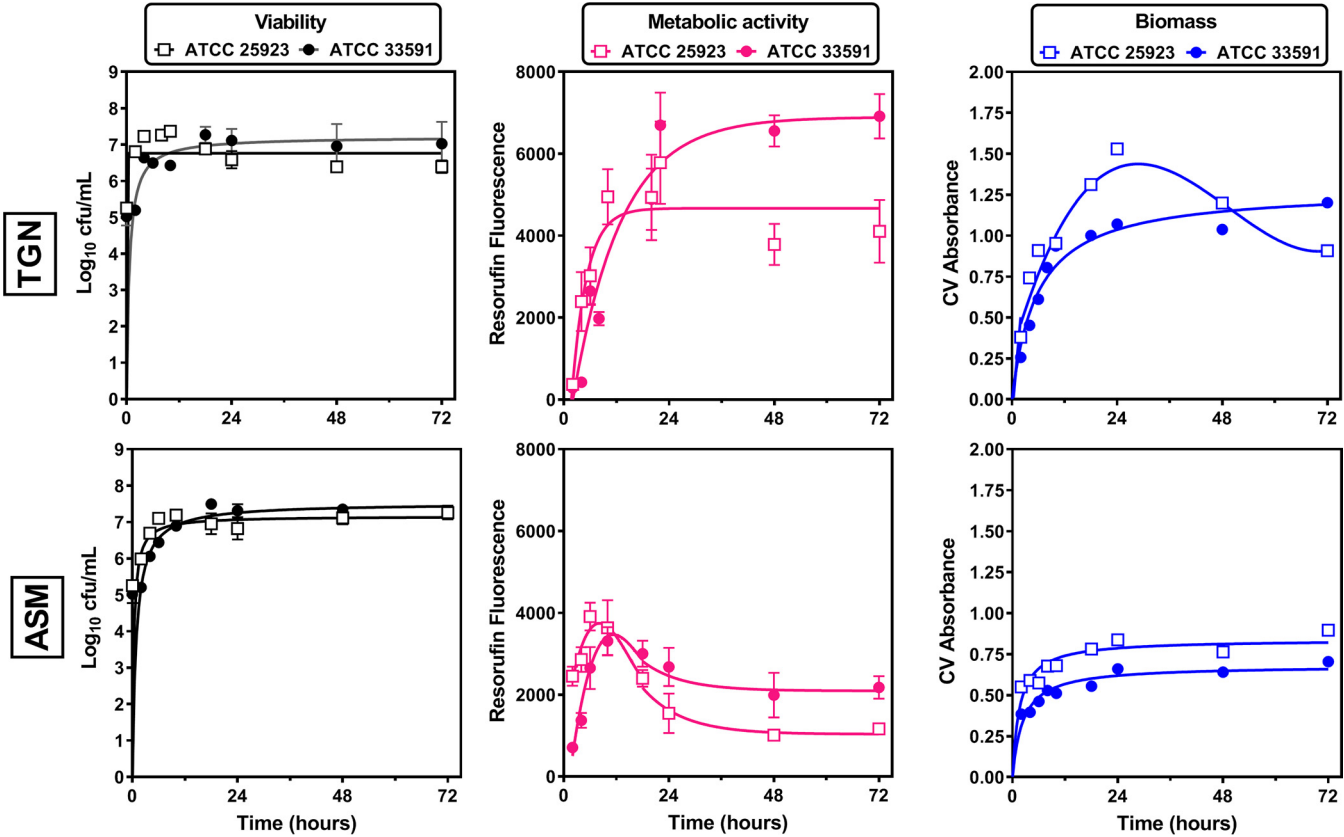


FIG 2 Characterization of the biofilm model in two different media. Evolution over time in Trypticase soy broth supplemented with 1% glucose and 2% NaCl (TGN; top) and in artificial sputum medium (ASM; bottom) of viability (assessed by CFU counts), metabolic activity (metabolization of resazurin in fluorescent resorufin), and biomass (absorbance of crystal violet [CV]). Data are means \pm standard deviations (SD) from at least 3 independent experiments. When the error bars are not visible, the SD were smaller than the size of the symbols.

metabolic activity decreasing in parallel with the number of CFU in most of the cases (a 33% reduction in metabolic activity, corresponding to an approximately 1- \log_{10} -CFU decrease). The largest discrepancy between concentration-response curves for viability and metabolic activity was observed with tobramycin in ASM: the decrease in CFU counts was indeed much more marked than the reduction in metabolic activity at high tobramycin concentrations.

Looking, then, to the data in more detail, four main observations can be made. First, the activity of antibiotics was globally lower in ASM than in TGN with respect to both their maximal efficacy and their relative potency. Second, as expected, the efficacy of

TABLE 1 Antibiotic susceptibility of bacterial strains in different media

Antibiotic	MIC (mg/liter) ^a						EUCAST ^c susceptibility breakpoint (mg/liter)	Human C _{max} (mg/liter) ^d
	ATCC 25923			ATCC 33591				
	MHB-ca	TGN	ASM ^b	MHB-ca	TGN	ASM ^b		
Meropenem	0.06	0.125	0.125	8	16	8	4	50
Vancomycin	1	4	4	1	8	2	2	60
Linezolid	2	2	0.5	1	1	0.5	4	15
Azithromycin	1	1	1	128	512	512	2	0.5
Rifampin	0.015–0.03	0.0039	0.031	0.03–0.06	0.001	0.015	0.5	18
Ciprofloxacin	0.25	0.25	0.5	0.25	0.25	0.5	1	3.7
Tobramycin	0.25	4	0.5	64	1,024	256	1	15

^aMHB-ca, cation-adjusted Mueller-Hinton broth; TGN, Trypticase soy broth supplemented with 1% glucose and 2% NaCl; ASM, artificial sputum medium.

^bThe MIC was determined by CFU counting after plating due to the turbidity of the medium.

^cEUCAST, European Committee on Antimicrobial Susceptibility Testing.

^dBased on the Belgian summary of the product characteristics for each drug.

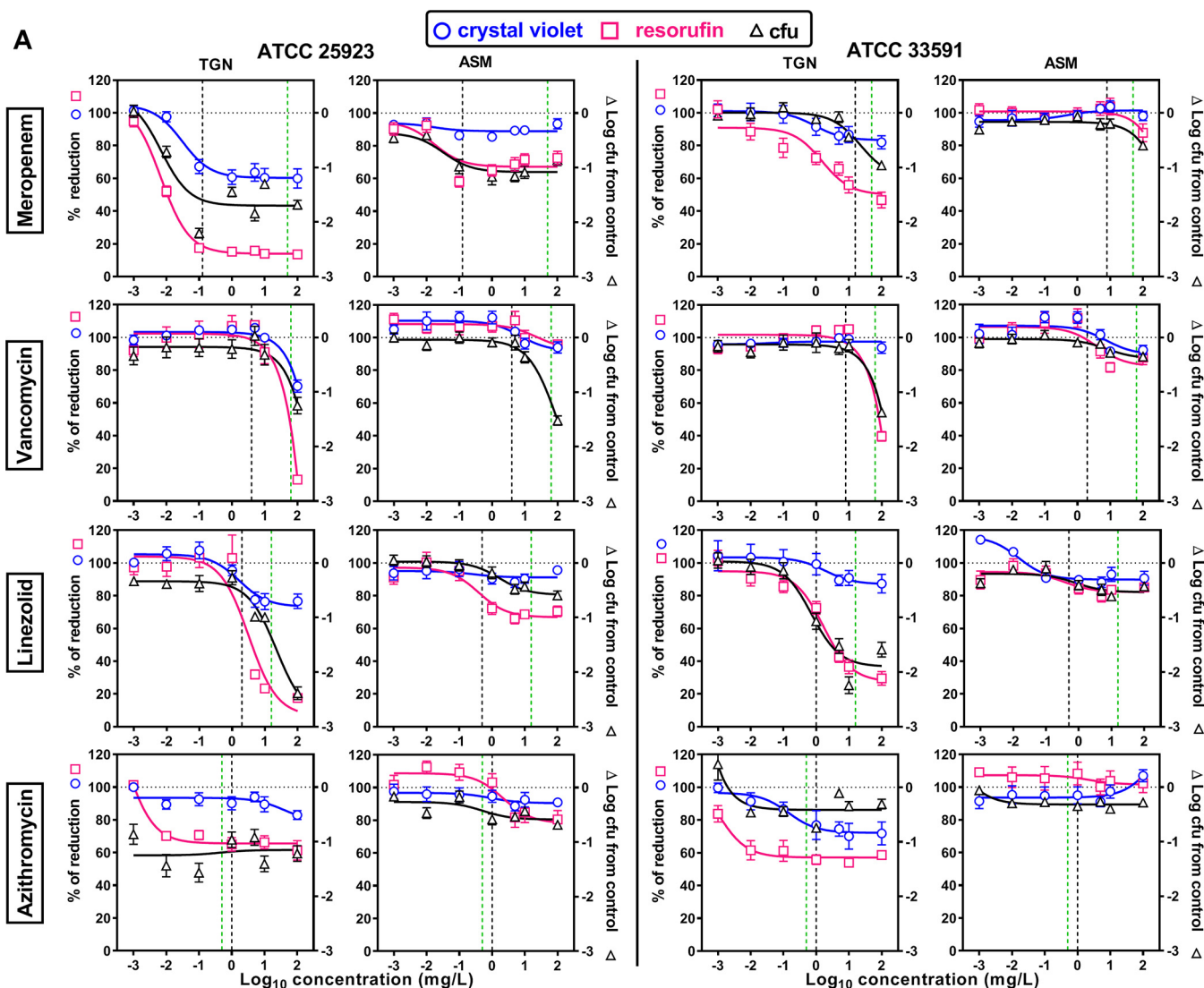


FIG 3 Activities of antibiotics against biofilms. Concentration-response activity of antibiotics against 24-h-old biofilms of strains ATCC 25923 (left) and ATCC 33591 (right) grown in Trypticase soy broth supplemented with 1% glucose and 2% NaCl (TGN) or in artificial sputum medium (ASM). Biofilms were incubated with increasing concentration of antibiotics for 24 h (panel A: meropenem, vancomycin, linezolid, azithromycin; panel B: rifampin, ciprofloxacin, tobramycin). The left ordinate shows the decrease in metabolic activity (resazurin assay; pink open squares) or biofilm mass (crystal violet assay [CV]; blue open circles) as a percentage of the control value (no antibiotic present). The right ordinate shows the change in viability (CFU counts; black open triangles) as the reduction (in log scale) from the control value (no antibiotic present). The vertical black dotted lines are the MIC of the antibiotic in the corresponding medium, and the green dotted lines are the human C_{max} after conventional dosing (Table 1). Note that the MIC of tobramycin in TGN against ATCC 33591 is higher than 1,000 mg/liter and, therefore, not visible on the graph. All values are means \pm SEM from 3 to 7 independent experiments performed in triplicate (when not visible, the error bars are smaller than the size of the symbols).

antibiotics to which ATCC 33591 is resistant was low for viability, metabolic activity, and biomass. Third, when comparing efficacy among the antibiotics, the bacteriostatic drug azithromycin was globally less effective (it had a less negative E_{max}). The other bacteriostatic drug, linezolid, was as effective as the rapidly bactericidal drugs (rifampin, ciprofloxacin, tobramycin) towards viability and as effective as slowly bactericidal drugs (meropenem, vancomycin), rifampin, and ciprofloxacin towards metabolic activity, causing a higher reduction in crystal violet staining than the other antibiotics of both bacterial strains in both media. Rifampin was the most effective antibiotic towards biomass, causing a more marked reduction in crystal violet staining than the other antibiotics of both bacterial strains in both media. Fourth, the potency of the drugs was low in biofilms, since the $C_{-1 \log}$ (viability) and C_{33} (metabolic activity and biomass) were higher than their MIC (except for azithromycin [towards viability and metabolic

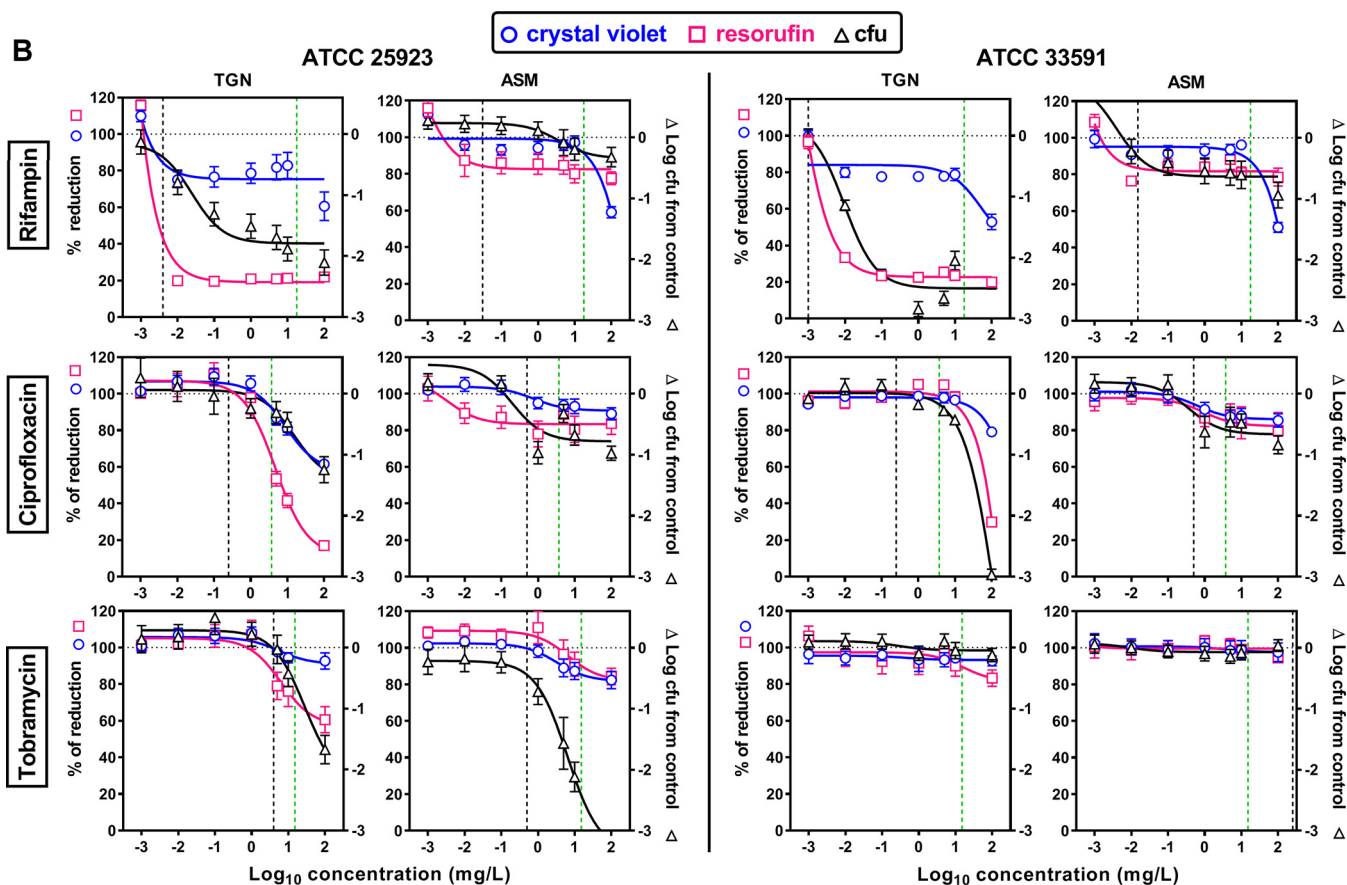


FIG 3 (Continued)

activity] and meropenem [against ATCC 25923] in TGN). If we now consider these results in a clinical perspective, a 1-log₁₀ reduction in the number of CFU or a 33% reduction in metabolic activity was obtained at concentrations that can be reached in human serum (see Table 1 for the values) in TGN for all drugs except (i) ciprofloxacin and tobramycin against both strains and (ii) meropenem and azithromycin against ATCC 33591 (ATCC 33591 is resistant to both meropenem and azithromycin). In contrast, only modest effects were observed in ASM at concentrations found in human serum (see the green vertical dotted lines in Fig. 3 and the green horizontal lines in Fig. 5, corresponding to the human maximum concentration in serum [C_{max}] after administration of a conventional dose).

SCV analysis and resazurin metabolism. The large difference in the activity of tobramycin, when assessed by the CFU count versus the resorufin fluorescence against ATCC 25923 biofilms grown in ASM, suggests the presence of weak metabolizer phenotypes. When carefully examining the morphology of colonies grown on Columbia blood agar (CBA), we observed, specifically for these samples, the presence of large hemolytic colonies and small nonhemolytic colonies (Fig. 6). Aminoglycosides are known to induce the formation of hemin- or menadione-dependent small-colony variants (SCVs) (7). We therefore also plated samples collected from tobramycin-treated biofilms on Trypticase soy agar (TSA) and TSA supplemented with hemin (TSAH), menadione (TSAM), or both hemin and menadione (TSAHM). Large and small colonies were observed on plates exposed to tobramycin (tested at 5, 10, and 100 mg/liter), with no significant differences in the total number of colonies counted on the various types of agar plates being seen.

To further explore the reasons for the discrepancies between the fluorescence signal and the CFU counts in biofilms exposed to high concentrations of tobramycin, we

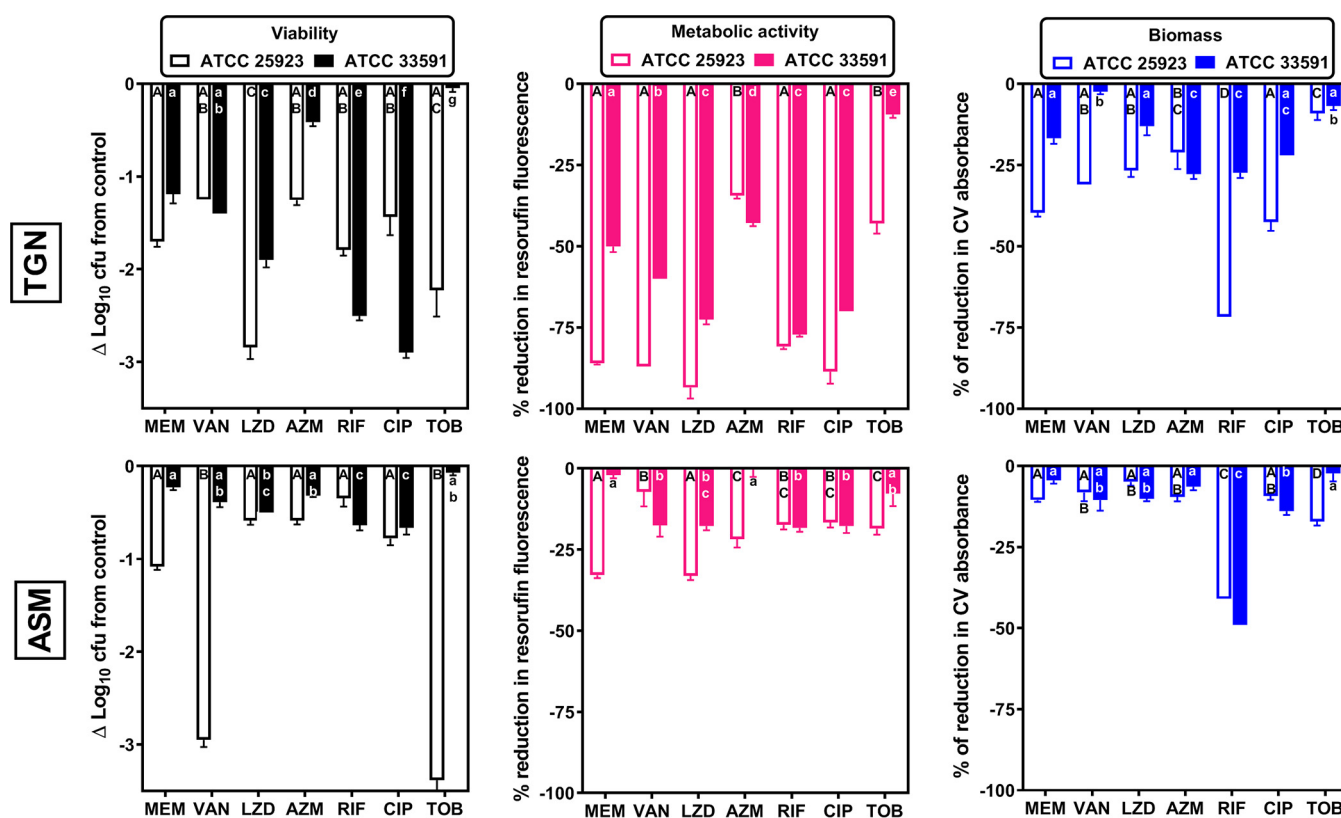


FIG 4 Comparison of antibiotic maximal efficacies (E_{\max}) expressed as the reduction in the number of CFU from that for the control (left) or as a percentage of the reduction in metabolic activity (resorufin fluorescence; middle) or biofilm mass (crystal violet [CV] absorbance; right) compared to that for an untreated biofilm for strain ATCC 25923 (open bar) or ATCC 33591 (closed bars) grown in Trypticase soy broth supplemented with 1% glucose and 2% NaCl (TGN) or in artificial sputum medium (ASM). MEM, meropenem; VAN, vancomycin; LZD, linezolid; AZM, azithromycin; RIF, rifampin; CIP, ciprofloxacin; TOB, tobramycin. Values are means \pm SEM. Statistical analyses were by one-way analysis of variance (ANOVA) with Tukey's posttest for multiple comparisons; values with different letters are significantly different from each other ($P < 0.05$). Capital letters compare the results for the antibiotics for strain ATCC 25923; lowercase letters compare the results for the antibiotics for strain ATCC 33591.

compared the capacity to metabolize resazurin of planktonic and biofilm cultures of isogenic strains, namely COL (K7) and its two stable SCVs, COL *menD* (K8; menadione dependent) and COL *hemB* (K9; hemin dependent). Planktonic cultures at increasing optical densities at 620 nm (OD_{620}) were incubated for 30 min with resazurin (Fig. 7, left). We observed for all strains that resazurin metabolization increased linearly with the optical density of the bacterial suspension and was not lower for SCVs than for the normal-phenotype parental strain. In biofilms grown in TGN, the fluorescence signal was not lower for both SCVs than for COL (K7). In ASM, the metabolic capacity of all strains was markedly reduced, but it was reduced to a lower extent for COL (K7) than for SCVs (Fig. 7B, right).

DISCUSSION

Many studies investigating the activity of antibiotics against biofilms consider only parameters like the minimum biofilm inhibitory concentration (MBIC) and the minimum biofilm eradication concentration (MBEC) to establish potential interest in them (19–21), yet these methods lack standardization (19) and do not allow examination of antibiotic pharmacodynamics in detail.

In this work, we set up an *in vitro* model to study the pharmacodynamics of antibiotics against staphylococcal biofilms in the context of cystic fibrosis. This model allows for a quick quantification of biofilm biomass and bacterial metabolic activity and viability. Biofilms were grown in microtiter plates, a support that is widely used (14, 18, 22–24) because they combine a series of advantages for high-throughput experiments,

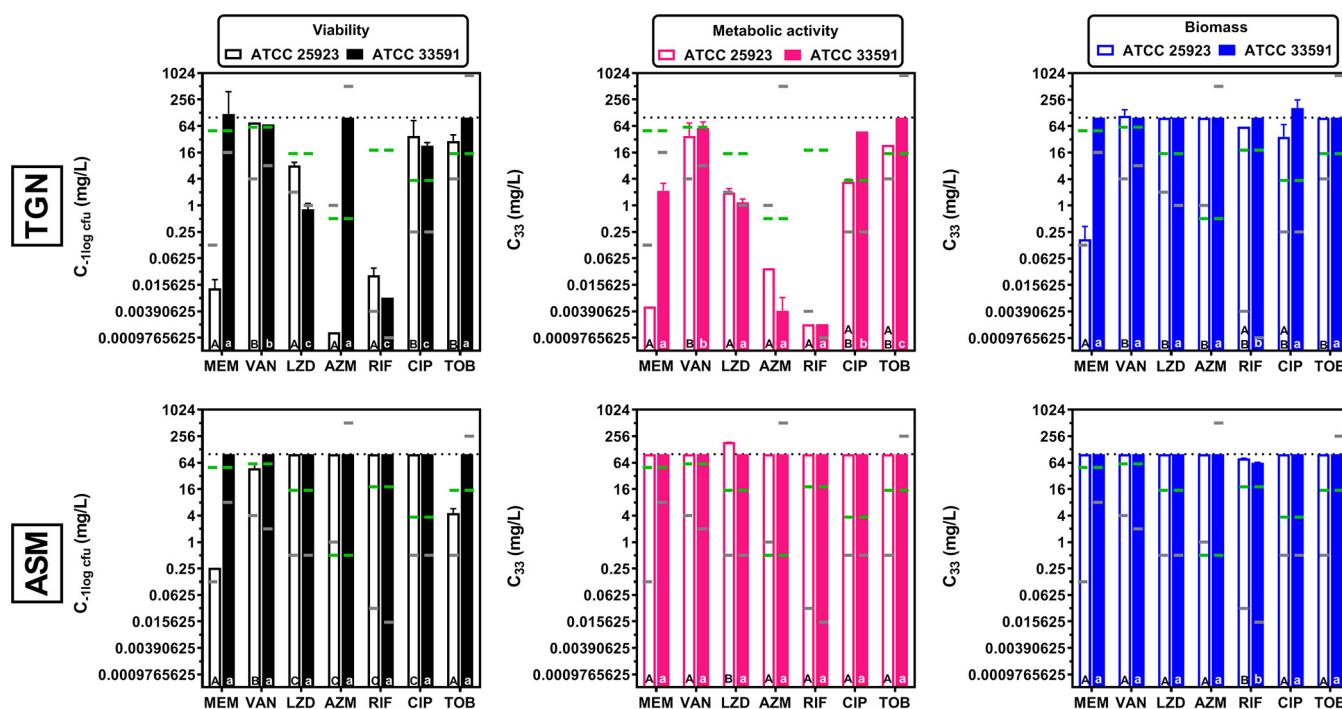


FIG 5 Comparison of antibiotic relative potencies ($C_{-1 \log}$ or C_{33} , expressed in milligrams per liter) for viability (left), metabolic activity (resorufin fluorescence; middle), or biofilm mass (crystal violet [CV] absorbance) for strain ATCC 25923 (open bars) or ATCC 33591 (closed bars) grown in Trypticase soy broth supplemented with 1% glucose and 2% NaCl (TGN) or in artificial sputum medium (ASM). Green horizontal lines correspond to the human C_{max} after administration of a conventional dose. Gray horizontal lines correspond to the MIC shown in Table 1. MEM, meropenem; VAN, vancomycin; LZD, linezolid; AZM, azithromycin; RIF, rifampin; CIP, ciprofloxacin; TOB, tobramycin. Values are means \pm SEM. Statistical analyses were by one-way analysis of variance (ANOVA) with Tukey's posttest for multiple comparisons; values with different letters are significantly different from each other ($P < 0.05$). Capital letters compare the results for the antibiotics for strain ATCC 25923; lowercase letters compare the results for the antibiotics for strain ATCC 33591. The gray horizontal lines are the MIC of the antibiotic in the corresponding medium, and the green horizontal lines are the human C_{max} after conventional dosing (Table 1).

like the possibility to test many conditions in parallel and the need for small amounts of material and drug (21).

Compared to our previous work (14), we introduce here two major improvements to our *in vitro* model to study biofilm formation by *S. aureus* and the activity of antibiotics.

The first one consisted of comparing TGN, a broth medium optimized for *S. aureus* biofilm growth, with ASM, a medium designed to mimic the viscoelastic properties of the mucus produced by patients with cystic fibrosis. To the best of our knowledge, the present work is the first to experimentally study the viscoelastic properties of ASM. This has indeed not been examined in previous papers using ASM of similar composition (13, 25–27). The general shape of the viscous modulus measured with the agar-containing ASM used here differs from that of sputum but is reminiscent of that described for solid agar preparations subjected to the same amplitude sweep oscillatory test (28). More importantly, we show that agar-containing ASM shares with CF sputa a higher elasticity than viscosity (17, 29, 30) and that the values of these parameters in ASM are close to those measured in mucopurulent sputa and approximately 10-fold lower than those measured in purulent sputa. Although only a small number of sputum samples could be tested here, we know from a larger series that there is a broad dispersion in mucus viscoelastic properties for infected sputa (30). ASM may thus reasonably represent the characteristics of mucus from part of the CF population.

By comparing the influence of culture conditions on biofilm growth, we demonstrate that a stable biofilm can be obtained after 24 h of culture in both media, with, however, lower metabolic activity for equivalent bacterial counts and a slightly lower biomass being seen in ASM than in TGN. This lower metabolic activity in ASM than in TGN seems to be specific for the biofilm mode of life, as it was observed (i) for both

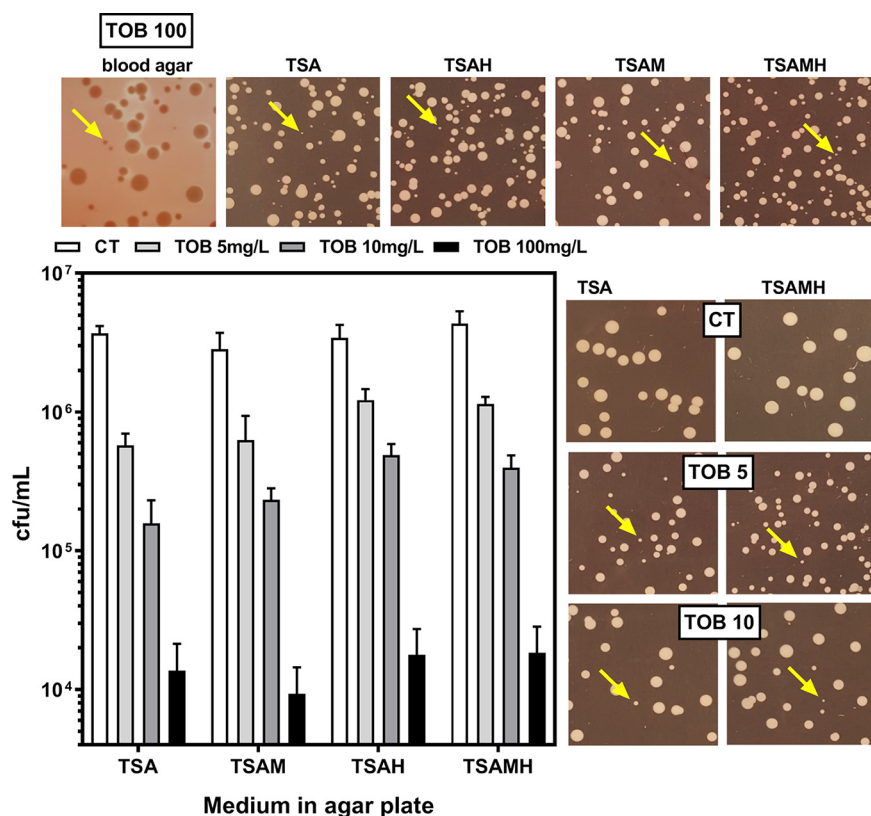


FIG 6 Morphology and counts of colonies from biofilms of ATCC 25923 cultivated in artificial sputum medium (ASM) and exposed for 24 h to tobramycin (TOB) at 5 (TOB 5), 10 (TOB 10), or 100 (TOB 100) mg/liter or under control conditions (no antibiotic added as a control [CT]). Samples were plated on Columbia blood agar, TSA, or TSA supplemented with either 1 mg/liter hemin (TSAH), menadione (TSAM), or both hemin and menadione (TSAMH). Yellow arrows indicate a typical small colony.

bacterial strains as well as for K7 or its SCVs in biofilms but not in planktonic cultures and (ii) for incubation times longer than 12 h (when both the number of CFU and the biomass have reached a plateau value). When examining antibiotic activity in these media, all data from concentration-effect curves could be fitted to sigmoidal regressions, the equation for which allowed us to compare two pharmacodynamic parameters, namely, the relative potency and maximal efficacy (14).

Regarding the relative potency, we noticed that a reduction of 33% of metabolic activity or of 1 log₁₀ CFU was rarely reached in ASM and at drug concentrations that were higher than those in TGN or than the MIC measured for planktonic cultures. Although these effects do not strictly correspond to bacteriostasis, our results confirm that higher concentrations of antibiotics are needed to act against biofilms than against planktonic cultures (14, 18, 22–24, 31–35). The low penetration and/or bioavailability of antibiotics in the biofilm matrix is probably a main reason for this loss in potency (3, 4, 18) and may become even more critical in ASM. Supporting this assumption, it has been shown that the permeability coefficients of antimycobacterial antibiotics were low in mucus from cystic fibrosis patients or artificial mucus, which has been attributed to the viscoelastic properties of this medium (36). More globally, the viscoelasticity of biofilms, which critically depends on their structure and composition, plays a pivotal role in their protective effect against mechanical and chemical challenges (37).

Regarding the maximal efficacy, we noticed that a bactericidal effect (3-log₁₀-CFU reduction) was rarely obtained, with, however, the limitation that a plateau value was not reached in many cases. Maximal efficacy was also markedly lower in ASM than in TGN for all drugs. This might be ascribed to a reduced metabolic rate in ASM (documented here by measuring resazurin reduction), which may render bacteria more

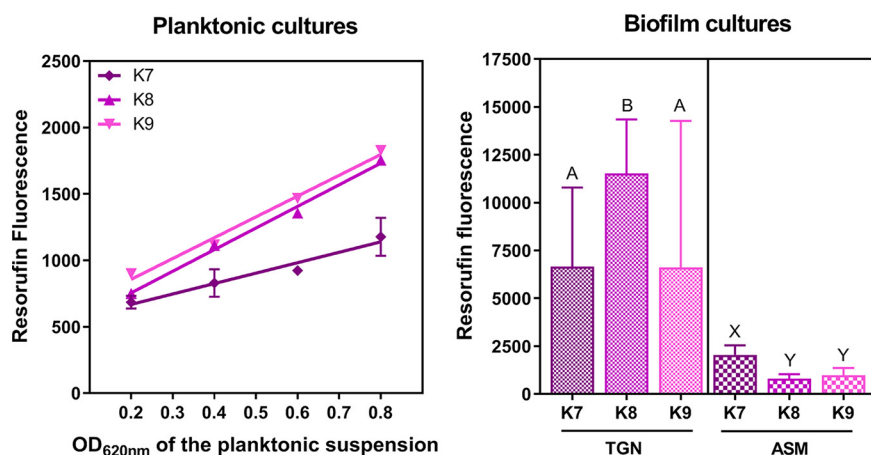


FIG 7 Metabolic activity in planktonic cultures or biofilm cultures grown in Trypticase soy broth supplemented with 1% glucose and 2% NaCl (TGN) or in artificial sputum medium (ASM). (Left) Resorufin fluorescence signal recorded after 30 min of incubation of planktonic bacteria at increasing inocula with 10-mg/liter resazurin. (Right) Resorufin fluorescence signal recorded after 30 min of incubation of a 24-h-old biofilm with 10-mg/liter resazurin. Data are means \pm SD for triplicates in a single experiment or means \pm SEM from at least 3 independent experiments performed in triplicate. Statistical analyses comparing strains in each individual medium were performed by one-way analysis of variance (ANOVA) with Tukey's posttest for multiple comparisons; values with different letters are significantly different from each other ($P < 0.05$).

tolerant to antibiotics relying on bacterial multiplication to exert their effect (4). We cannot totally exclude the possibility, however, that slow metabolism also indirectly affects relative potency, by impairing the uptake inside bacteria of antibiotics requiring active transport, like aminoglycosides. Intriguingly, linezolid is more effective than other bacteriostatic or slowly bactericidal drugs in TGN, in accordance with our previous data with this molecule (14, 38). A direct comparison with other literature data is difficult because most of the papers report MBEC values only (39, 40).

The second improvement brought to the model consists of following bacterial viability by measuring in parallel metabolic activity and bacterial counts. The detection of fluorescent metabolites as a surrogate for viability is essentially justified by the ease and rapidity of their use in high-throughput assays (41). We complemented it here with CFU counting because we suspected that the metabolism of molecules used as probes could be altered in the dormant but viable bacteria that are present in biofilms (42, 43).

While, in most of the cases, CFU counts and metabolic activity were affected to a similar extent by antibiotics, a noticeable exception concerned tobramycin: it caused a marked reduction in the number of CFU for ATCC 25923 in biofilms grown in ASM but only a marginal reduction in metabolic activity. Aminoglycosides are known to induce the formation of small-colony variants of *S. aureus* both *in vivo* and *in vitro* (7, 44–47). We demonstrate here the presence of small colonies, which strongly suggests that tobramycin selects for SCVs specifically in biofilms cultivated in ASM. At this stage, we could not determine a single metabolic defect associated with this phenotypic switch, since small colonies were still observed on media enriched in hemin and/or in menadione, i.e., the two factors that usually revert the SCV phenotype when induced by aminoglycosides. This result is coherent with that of a recent study describing the existence of SCVs for which no known auxotrophism could be evidenced (48). SCVs are thought to play a pathological role in patients with cystic fibrosis (45). Our data suggest that viscous sputum may contribute to facilitate this selection upon antibiotic treatment.

From a clinical point of view our study suffers from three major limitations. First, we used only reference strains and not clinical isolates collected from patients with cystic fibrosis. This study should therefore be considered a first step to establish a strong

pharmacodynamic model that can be now applied to other strains or other drugs. Second, we did not consider the possible role of the microaerophilic environment that may exist in the sputum of patients with cystic fibrosis and may affect antibiotic activity (27). Third, bacteria were exposed to constant concentrations of antibiotics for a fixed period of time. These conditions do not mimic the pharmacokinetic profile of the drugs in the lungs. Our high-throughput system may be helpful to select the most effective drugs to be tested thereafter in a dynamic model reproducing pharmacokinetic fluctuations over time (38). In the meantime, however, the broad range of concentrations that we tested covers human peak serum levels (C_{\max}). Our data therefore lead to the conclusion that serum concentrations are probably too low to be active on biofilms developing in the respiratory tract. Delivery of drugs directly to the lungs via inhalation may help to overcome this issue.

MATERIALS AND METHODS

Bacterial strains and antibiotics. *S. aureus* ATCC 25923 (a methicillin-sensitive strain) and ATCC 33591 (a methicillin-resistant strain) were used for all experiments. COL (K7; a wild-type, hospital-acquired methicillin-resistant *Staphylococcus aureus* strain) and its *menD* and *hemB* small-colony variant (SCV)-stable mutants (K8 and K9, respectively, constructed by allelic replacement with an *ermC* cassette-inactivated *menD* gene and an *ermB* cassette-inactivated *hemB* gene, respectively [49, 50]) were used for experiments focusing on the SCV phenotype. All strains, including SCVs, were routinely grown on TSA. SCVs remained stable under these conditions (no revertants were observed).

Antibiotics were obtained as microbiological standards, as follows: azithromycin (potency, 100%) was from Teva (Petach Tikva, Israel), ciprofloxacin HCl (potency, 93.9%) was from Bayer (Leverkusen, Germany), tobramycin (potency, 100%) was from Galephar (Marche-en-Famenne, Belgium), linezolid (potency, 100%) was from Rib-X Pharmaceuticals (presently Melinta Therapeutics, New Haven, CT), and rifampin (potency, 97%) was from Sigma-Aldrich (St. Louis, MO). Meropenem (potency, 74%) and vancomycin (potency 97.5%) were procured as generic drug branded products for human parenteral use distributed for clinical use in Belgium by Sandoz (Holzkirchen, Germany) and Mylan (Cannonsburg, PA), respectively.

Culture media for biofilm cultures. Two media were used in parallel, namely, Trypticase soy broth (VWR, Radnor, PA) supplemented with 1% glucose and 2% NaCl (TGN) (14, 18) or artificial sputum medium (ASM) (adapted from previous work [13, 25, 26]). This medium aims at mimicking the composition and viscoelastic properties of the mucus found in the respiratory tract of patients with cystic fibrosis. It contains, per liter, 10 g mucin (Sigma-Aldrich, St. Louis, MO), 4 g DNA (Sigma-Aldrich), 5.9 mg DTPA (diethylenetriaminepentaacetic acid; Sigma-Aldrich), 5 g NaCl (VWR), 2.2 g KCl (Sigma-Aldrich), 3 g agar (Becton, Dickinson, Franklin Lakes, NJ), 5 g amino acids (Becton, Dickinson), 1.81 g Tris (Calbiochem, San Diego, CA), and 5 ml egg yolk emulsion (Sigma-Aldrich). All compounds except egg yolk emulsion were autoclaved; the egg yolk emulsion was added aseptically to the autoclaved medium, after which the pH was adjusted to 7 with NaOH.

Rheology. Rheology was used to evaluate the viscoelastic properties of artificial sputum medium in comparison with CF sputa. The latter sputa were previously collected from six CF patients by expectoration during physiotherapy and immediately frozen at -80°C (17). The experimental protocol was approved by the Ethics Committee of the Université catholique de Louvain (UCL; registration number B403201422928). Among the six sputum specimen, two were characterized at the time of collection for the presence of microorganisms and found to be contaminated by *S. aureus* (sample P3) and by *Pseudomonas aeruginosa* and *Aspergillus fumigatus* (sample MP3), respectively. Upon application of an increasing shear strain to the samples, we followed, during an amplitude sweep oscillatory test, their storage/elastic modulus, representing the tendency to recover the original shape after a stress-induced deformation, and their loss/viscous modulus, corresponding to the resistance to flow (29). We also determined the linear viscoelastic region (LVER) of each sample, i.e., the region of small deformations in which the viscoelastic parameters remain constant (17). As previously described (17), 600 mg of sample was loaded on an MCR102 rheometer (Anton Paar, Graz, Austria) set up at 37°C . During application of the strains, elastic and viscous moduli between 0.0001% and 10% were recorded at a constant frequency of 10 Hz, using RheoCompass software.

Development of the biofilm model. Biofilms were grown in 96-well plates (European catalog number 734–2327; VWR), as previously described (18), using TGN or ASM. In brief, a bacterial suspension was prepared in cation-adjusted MHB starting from overnight cultures on Trypticase soy agar. When using TGN for biofilm growth, 96-well plates were inoculated (200 μl /well) at approximately 10^7 CFU/ml (OD_{620} adjusted to 0.005) and then incubated at 37°C for 24 h so as to obtain a mature biofilm. When ASM was used for biofilm growth, 20 μl of a suspension at 10^8 CFU/ml in cation-adjusted MHB was inoculated in the 96-well plates and the plates were incubated for 2 h at 37°C to favor attachment, after which 180 μl of ASM was added to also reach an inoculum of 10^7 CFU/ml. The plates were then incubated at 37°C for 24 h.

Susceptibility testing. MICs were determined by microdilution following the guidelines of the Clinical and Laboratory Standards Institute, using cation-adjusted Mueller-Hinton broth (MHB-ca) (51), and compared to those measured in TGN and ASM following the same protocol. Direct visual reading of the MICs was not possible in ASM, however, due to the natural turbidity of this medium. Aliquots were

therefore spread on TSA and incubated overnight; MICs were defined as the lowest concentration at which there was no change in the number of CFU compared to the initial values.

Activity of antibiotics against biofilms. After 24 h of incubation, the culture medium of the biofilms was removed and replaced with fresh medium (control) or medium supplemented with antibiotics at concentrations ranging from 10^{-3} to 10^3 mg/liter in order to obtain full concentration-response curves. Biofilms were reincubated for 24 h at 37°C. At the end of the incubation period, the medium was removed and the biofilm was washed once with 200 μ l of phosphate-buffered saline (PBS). Biofilm biomass was quantified using crystal violet, a cationic dye that nonspecifically stains negatively charged constituents in biofilms (18). Washed biofilms were fixed by heat at 60°C for about 1 h and incubated for 10 min at room temperature with 200 μ l of crystal violet (final concentration, 0.2 g/liter; VWR). After removing the excess crystal violet, the plates were washed under running water and dried. The dye fixed to the biofilm was resolubilized in 200 μ l of 66% acetic acid and incubated for 1 h at room temperature. The absorbance was measured at 570 nm using a SpectraMax Gemini XS microplate spectrophotometer (Molecular Devices LLC, Sunnyvale, CA). The metabolic activity in the biofilms was quantified using the resazurin assay, which is based on the reduction by living bacteria of the weakly fluorescent blue dye resazurin in pink highly fluorescent resorufin (18). Washed biofilms were incubated with 10 mg/liter resazurin (Sigma-Aldrich) for 30 min at room temperature in the dark. Resorufin fluorescence was measured at a wavelength of 590 nm with an excitation wavelength of 550 nm using a SpectraMax Gemini XS microplate spectrofluorometer. Bacterial counts were also determined in washed biofilms resuspended in 1 ml of sterile PBS in microcentrifugation tubes. The tubes were vortexed and placed for 5 min in a sonication bath (Bransonic Ultrasonic cleaner 3510E-MT; frequency, 40 kHz; Danbury, CT) to disrupt the biofilm and vortexed again, after which aliquots were taken and diluted before they were spread on TSA plates. Unless stated otherwise, the colonies were counted after 24 h of incubation at 37°C.

Identification and metabolic activity of small-colony variants. When appropriate, the presence of SCVs was determined by spreading the bacteria recovered from biofilms on 5 types of agar plates, namely, TSA, TSA supplemented with 1 mg/liter hemin (TSAH) or menadione (TSAM), TSA supplemented with both 1 mg/liter hemin and menadione (TSAMH), and Columbia blood agar (CBA) plates (44, 52). The colonies were counted after 24 h of incubation at 37°C. In parallel, we compared the capability of stable SCVs and of normal-phenotype strains to metabolize resazurin into resorufin. Bacterial suspensions at different OD_{620} values were prepared in PBS starting from overnight cultures on TSA. Ninety-six-well plates were inoculated with 200 μ l of a bacterial suspension mixed with resazurin (final concentration, 10 mg/liter) and then incubated for 30 min at room temperature in the dark, after which resorufin fluorescence was measured as described above.

Curve-fitting and statistical analyses. Curve-fitting analyses were made using GraphPad Prism (version 8.02) software (GraphPad Software, San Diego, CA). Data were used to fit a sigmoid function, which allowed us to calculate the maximal efficacy (E_{max} ; the maximal reduction in viability or biomass for an infinitely large concentration of antibiotic) and relative potencies (the antibiotic concentration needed to reach 33% viability or a 33% biomass reduction within the biofilm [C_{33}]). Statistical analyses were performed with GraphPad Instat (version 3.06; GraphPad Software) or GraphPad Prism (version 8.02) software.

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