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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 biofilm grown in tryptic soy broth supplemented by 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, rifampicin, ciprofloxacin, tobramycin). Rheological studies showed that ASM was more elastic than viscous, as also observed for sputa from CF patients, with elastic and viscous moduli respectively similar and slightly lower than those of CF sputa. Biofilms formed by MSSA ATCC 25923 and MRSA 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 (bacterial counts) being similar in both media. Full concentration-response curves of antibiotics obtained after 24 h incubation of biofilms showed that all antibiotics were drastically less potent and less efficient in ASM than in TGN towards 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. Use of ASM may help to determine effective drug concentrations or to evaluate new therapeutic options against biofilms in CF patients.
INTRODUCTION

Cystic fibrosis (CF) is a recessive genetic disease affecting mainly Caucasian individuals, with around 70,000 cases around the world. The disease results from a malfunction of a chloride channel (cystic fibrosis transmembrane conductance regulator) affecting multiples organs like 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 bacterial response to antibiotics (4,5).

*Staphylococcus aureus* is the most prevalent microorganism detected in infants and 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 a slow growth and generating therefore small colonies on agar plates. Among them, the best characterized are electron transport-deficient SCVs (auxotroph 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). Up till now, many studies have mainly concentrated on the activity of antibiotics against *Pseudomonas aeruginosa* biofilms, because it is 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 biofilm 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, rifampicin), 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, 6 sputa were studied, which were previously categorized as purulent (uniformly green or yellow) or mucopurulent (mixture of purulent and mucoid parts) by visual inspection (17). Their deformation and flow under an applied stress was studied by rheology (Figure 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 viscous moduli between 0.0001% and 0.1% of shear strain. For ASM, stability was observed at shear strain between 0.0001% and 0.1% for the elastic modulus but only at shear strains between 0.0001% -0.001% and 0.05% for the viscous modulus. In this range of non-destructive shear strains, purulent samples showed higher elastic and viscous moduli than mucopurulent samples, while ASM had an elastic modulus very similar, and a viscous modulus about 3 times lower as compared to 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 was then studied for two S. aureus strains, namely the methicillin-sensitive (MSSA) strain ATCC 25923 and the methicillin-resistant (MRSA) strain ATCC 33591. Two different media were compared, namely, TGN (tryptic soy broth supplemented with glucose and NaCl), previously used in our laboratory for growing staphylococcal biofilms (14,18), and ASM. Figure 2 shows the evolution over time of viability (assessed by cfu counting), 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 approx. 10^7 cfu/mL that remained
stable over three days (left panels). Metabolic activity (middle panels) also increased rapidly
over the first 12 h and reached a plateau after approx. 24 h in TGN, with fluorescence signals
higher for ATCC 33591 than ATCC 25923. In ASM, metabolic activity was lower 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 over the first 24 h in both media and for both strains (right panels), with no major
change thereafter except for ATCC 25923 in TGN, for which biomass decreased from day 1
to day 3. Again, absorbance value were higher in TGN than in ASM. Based on these results,
we opted to use 24 h biofilms for further experiments in which antibiotics were added for 24
additional hours.

Antibiotic activity against planktonic cultures

Table 1 shows the MICs of the selected antibiotics against the two bacterial strains in the
media used above for biofilms studies as compared to Mueller Hinton Broth cation-adjusted
(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 (± 1 dilution), slightly higher (2 dilutions for vancomycin against
ATCC 25923 and azithromycin against ATCC 33591) or higher (3 or more dilutions for
vancomycin and azithromycin against ATCC 33591 and tobramycin against ATCC 25923) as
compared to MHB-ca. In ASM, MICs were similar (± 1 dilution), slightly higher (2 dilutions for
vancomycin against ATCC 25923 or azithromycin and tobramycin against ATCC 33591) or
lower (for linezolid against ATCC 25923) as compared to MHB-ca.

Antibiotic activity against biofilms

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

Results are presented as concentration-response curves in Figure 3A for drugs known as
slowly bactericidal (meropenem, vancomycin), or bacteriostatic (linezolid, azithromycin), and
Pharmacodynamic parameters, calculated based on the sigmoidal regression of the concentration-response curves, are summarized in Figure 4 for maximal efficacy ($E_{\text{max}}$; corresponding to the maximal decrease in metabolic activity or cfu as extrapolated for an infinitively large concentration) and in Figure 5 for relative potency (estimated by the concentration needed to reduce cfus of $1 \log_{10}$ [$C_{-1\log}$]) and metabolic activity or biomass, of 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 metabolic activity decreasing in parallel with cfus in most of the cases (33% reduction in metabolic activity corresponding to approx. $1 \log_{10}$ cfu decrease). The largest discrepancy between concentration-response curves towards 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 details, 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 antibiotics to which ATCC 33591 is resistant was low towards viability, metabolic activity and biofilm. Third, when comparing efficacy among antibiotics, the bacteriostatic drug azithromycin was globally the less effective (less negative $E_{\text{max}}$). The other bacteriostatic drug linezolid was as effective as rapidly bactericidal drugs (rifampicin, ciprofloxacin, tobramycin) towards viability and as effective as slowly bactericidal drugs (meropenem, vancomycin), rifampicin, and ciprofloxacin towards metabolic activity causing a higher reduction in crystal violet staining than other antibiotics against both bacterial strains in both media. Rifampicin was the most effective antibiotic towards biomass, causing a more marked reduction in crystal violet staining than other antibiotics against both bacterial strains in in both media. Fourth, the potency of drugs was low in biofilms, since $C_{-1\log}$ (viability) and $C_{33}$ (metabolic activity and biomass) were higher
than their MIC (except for azithromycin [towards viability and metabolic activity] and meropenem [against ATCC 25923] in TGN). If we now consider these results in a clinical perspective, a 1 log₁₀ reduction in cfu or a 33% reduction in metabolic activity was obtained at concentrations that can be reached in human serum (see Table 1 for values) in TGN for all drugs except (i) ciprofloxacin and tobramycin against both strains and (ii) meropenem and azithromycin against ATCC 33591, to which this strain is resistant. In contrast, only modest effects were observed in ASM at concentrations found in human serum (see green vertical dotted lines in Figures 3A-B or green horizontal lines in Figure 5 corresponding to the human Cₘₐₓ after administration of a conventional dose).

**SCV analysis and resazurin metabolism**

The large difference in the activity of tobramycin when assessed by cfu counting vs. 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, we observed, specifically for these samples, the presence of large hemolytic colonies and small non-hemolytic colonies (Figure 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 TSA and TSA supplemented by hemin (TSAH), menadione (TSAM), or both (TSAHM). Large and small colonies were observed on plates exposed to tobramycin (tested at 5, 10 and 100 mg/L), with no significant differences in the total number of colonies counted on the various types of agar plates.

To further explore the reasons for discrepancies between fluorescence signal and cfu counts in biofilms exposed to high concentrations of tobramycin, we compared the capacity to metabolize resazurin of planktonic and biofilm cultures of isogenic strains, namely COL (K7) and its two stable SCVs variants COL-\(\text{menD}\) (K8; menadione-dependent) and COL-\(\text{hemB}\) (K9; hemin-dependent). Planktonic cultures at increasing OD₆₂₀nm were incubated during 30 minutes with resazurin (Figure 7 left panel). 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 to a lower extent for COL (K7) than for SCVs (Figure 7B; right panel).
DISCUSSION

Many studies investigating the activity of antibiotics against biofilms are considering only parameters like the minimum biofilm inhibitory concentration (MBIC) and the minimum biofilm eradication concentration (MBEC) to establish their potential interest (19-21). Yet, these methods lack standardization (19) and do not allow to examine in details antibiotic pharmacodynamics.

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 it combines a series of advantages for high throughput experiments, like the possibility to test many conditions in parallel and the low amount of material and drug needed (21).

As 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 activity of antibiotics. The first one consisted in 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 the ASM. This has indeed not been examined in previous papers using ASM media 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 sputa 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 from those measured in mucopurulent sputa and approx. 10-fold lower than those of purulent sputa. Yet, only a small number of sputum samples could be tested here but we know from larger series that there is a broad dispersion in mucus
viscoelastic properties for infected sputa (30). ASM may thus reasonably represent mucus characteristics 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, a lower metabolic activity for equivalent bacterial counts and a slightly lower biomass in ASM as compared to TGN. This low metabolic activity in ASM vs. TGN seems specific for the biofilm mode of life, as it is observed (i) for both 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 cfu and 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 of 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 % in metabolic activity or of 1 log$_{10}$ cfu was rarely reached in ASM, and at drug concentrations that are higher than 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 planktonic cultures (14,18,22-24,31-35). 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 ($3 \log_{10}$ cfu reduction) was rarely obtained, with however the limitation that a plateau value was not reached in many cases. Maximal efficacy is also markedly lower in ASM vs. 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 tolerant to antibiotics relying on bacterial multiplication to exert their effect (4). We cannot totally exclude 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 (39-41).

The second improvement brought to the model consists in following bacterial viability by measuring in parallel metabolic activity and bacterial counts. The detection of fluorescent metabolites as surrogate for viability is essentially justified by easiness and rapidity in high throughput assays (42). We complemented it here with cfu counting because we were suspecting that the metabolization of molecules used as probes could be altered in dormant but viable bacteria that are present in biofilms (43,44).

While, in most of the cases, cfu counts and metabolic activity were affected to a similar extent by antibiotics, a noticeable exception concerns tobramycin: it caused a marked reduction in cfus 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* (7,45-48). 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 SCV phenotype when induced by aminoglycosides. This result is coherent with a recent study describing the existence of SCVs for which no known auxotrophism could be evidenced (49). SCVs are thought to play a pathological role in patients with cystic fibrosis (46). 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 only used reference strains and not clinical isolates collected from patients with cystic fibrosis. This study should therefore be considered as a first step to establish a strong pharmacodynamic model that could 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 test them thereafter in a dynamic model reproducing pharmacokinetic fluctuations overtime (38). In the meantime, however, the broad range of concentrations we tested covers human peak serum levels ($C_{\text{max}}$). Our data therefore leads 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 (methicillin-sensitive strain) and ATCC 33591 (methicillin-resistant strain) were used for all experiments. COL (K7; wild-type, hospital-acquired methicillin-resistant *Staphylococcus aureus*), and its *menD* and *hemB* SCV stable mutants (K8 and K9, constructed by allelic replacement with an *ermC* cassette-inactivated *menD* gene and an *ermB* cassette-inactivated *hemB* gene, respectively [50,51]) were used for experiments focusing on the small colony variant (SCV) phenotype. All strains, including SCVs, were routinely grown on TSA. SCVs remained stable in these conditions (no revertants observed).

Antibiotics were obtained as microbiological standards as follows: azithromycin (potency 100%), from Teva (Petach Tikva, Israel); ciprofloxacin HCl (potency 93.9%), from Bayer (Leverkusen, Germany); tobramycin (potency 100%), from Galephar (Marche-en-Famenne, Belgium); linezolid (potency 100%), from RibX Pharmaceuticals (presently Melinta Therapeutics, New Haven, CT); rifampicin (potency 97%), from Sigma-Aldrich. 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 [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), Tris 1.81g (Calbiochem; San Diego, CA), and 5 mL egg yolk emulsion (Sigma-Aldrich). All
compounds were autoclaved except egg yolk emulsion which 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. These 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 sputa, 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 were loaded on a MCR102 rheometer (Anton Paar, Graz, Austria) set up at 37°C. Elastic and viscous moduli were recorded during application of strains between 0.0001% and 10% at a constant frequency of 10 Hz, using the 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$_{620}$ nm adjusted to 0.005) and then incubated at 37°C for 24h 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 were inoculated in the 96-well plates and incubated during 2h at 37°C to favour attachment, after which 180 µL of ASM were added to also reach an inoculum of $10^7$ cfu/mL. Plates were then incubated at 37°C for 24h.

**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) (52), and compared to those measured in TGN and ASM following the same protocol. Yet, direct visual reading of MICs was not possible in ASM due to the natural turbidity of this medium. Aliquots were therefore spread on TSA and incubated overnight; MICs were defined as the lower concentration for which there was no change in cfu as compared to initial values.

**Activity of antibiotics against biofilms**

After 24h of incubation, the culture medium of biofilms was removed and replaced with fresh medium (control) or medium supplemented with antibiotics at concentrations ranging from $10^{-3}$ to $10^3$ mg/L in order to obtain full concentration-response curves. Biofilms were reincubated for 24h at 37°C. At the end of the incubation period, the medium was removed and the biofilm was washed once with 200 µL of phosphate buffered saline (PBS). Biofilm biomass was quantified using crystal violet, a cationic dye that non-specifically stains negatively-charged constituents in biofilms (18). Washed biofilms were fixed by heat at 60°C for about 1 hour and incubated during 10 minutes at room temperature with 200 µL of crystal violet (VWR) (final concentration 0.2 g/L). After removing the excess of crystal violet, plates were washed under running water and dried. The dye fixed to the biofilm was resolubilized in 200 µL of 66% acetic acid and incubated 1h at room temperature. Absorbance was measured at 570 nm using a SPECTRAmax Gemini XS microplate spectrophotometer (Molecular Devices LLC, Sunnyvale, CA). Metabolic activity in biofilms was quantified using the resazurin assay, which is based on the reduction by living bacteria of the weakly fluorescent blue-coloured dye resazurin in the pink-colored highly fluorescent resorufin (18). Washed biofilms were incubated with 10 mg/L
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 1mL of sterile PBS in microcentrifugation tubes. The tubes were vortexed and placed during 5 minutes in a sonication bath (Branson® Ultrasonic cleaner 3510E-MT, frequency 40kHz; Danbury, CT) to disrupt the biofilm, and vortexed again, after which aliquots were taken and diluted before spreading on TSA plates. Unless stated otherwise, colonies were counted after 24h of incubation at 37°C.

**Identification and metabolic activity of small colony variants**

When appropriate, the presence of SCV was determined by spreading bacteria recovered from biofilms on 5 types of agar plates, namely TSA, TSA supplemented with 1 mg/L hemin (TSAH) or menadione (TSAM), TSA supplemented with both 1 mg/L hemin and menadione (TSAMH) and Columbia blood agar plates (CBA) (45,53). Colonies were counted after 24h of incubation at 37°C. In parallel, we compared the capability of stable SCV and of normal phenotype strains to metabolize resazurin into resorufin. Bacterial suspensions at different OD_{620nm} were prepared in PBS starting from overnight cultures on TSA. 96-well plates were inoculated with 200 µL of bacterial suspension mixed with resazurin (final concentration: 10 mg/L) 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 Graph-Pad Prism version 8.02 (GraphPad Software, San Diego, CA). Data were used to fit a sigmoid function, which allowed us to calculate maximal efficacy (\(E_{\text{max}}\); maximal reduction in viability or biomass for an infinitely large concentration of antibiotic) and relative potencies (\(C_{33}\); antibiotic concentration needed to reach 33% of viability or biomass reduction within the biofilm). Statistical analyses were
performed with Graph Pad Instat version 3.06 (GraphPad Software) or Graph-Pad Prism version 8.02.
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Figure 1. Viscoelasticity of ASM (Artificial Sputum Medium; black curve) as compared to six sputa collected from CF patients and showing a purulent (P1, P2, P3 [blue closed squares]) or mucopurulent (MP1, MP2, MP3 [red open squares]) appearance, shown as individual data. The graphs show the elastic modulus (left) and the viscous modulus (right), both expressed in Pascal (Pa; kg.m^{-1}.s^{-2}). A linear viscoelastic region is observed between 0.0001% and 0.1% of shear strain.

Figure 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; upper panels) and in Artificial Sputum Medium (ASM; lower panels) of viability (assessed by cfu counting), metabolic activity (metabolization of resazurin in fluorescent resorufin), and biomass (absorbance of crystal violet [CV]). Data are means ± standard deviations (SD) of at least 3 independent experiments. When not visible, the SD are smaller than the size of symbols.

Figure 3A. Activities of antibiotics against biofilms. Concentration-responses activity of antibiotics against 24h-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 during 24 h (meropenem, vancomycin, linezolid, azithromycin). Left ordinate shows the decrease in metabolic activity (resazurin assay, pink open square) or biofilm mass (crystal violet assay [CV], blue open circle) as a percentage of the control value (no antibiotic present). Right ordinate shows the change in viability (cfu counting, black open triangle) as the reduction in log scale from the control value (no antibiotic present). The vertical black dotted line is the MIC of the antibiotic in the corresponding medium and the green dotted line, the human C_{max} after conventional dosing (see Table 1). All values are means ± SEM of 3 to 7 independent experiments performed in triplicates (when not visible the error bars are smaller than the size of symbols).

Figure 3B. Activities of antibiotics against biofilms. Concentration-responses activity of antibiotics against 24h-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 during 24 h (rifampicin, ciprofloxacin and tobramycin). Left ordinate shows the decrease in metabolic activity (resazurin assay, pink open square) or biofilm mass (crystal violet assay [CV], blue open circle) as a percentage of the control value (no antibiotics present). Right ordinate shows the change in viability (cfu counting, black open triangle) as the reduction in log scale from the control value (no antibiotics present). The vertical black dotted line is the MIC of the antibiotic in the corresponding medium and the green dotted line, the human C_{max}
after conventional dosing (see Table 1). Note that the MIC of tobramycin in TGN against ATCC 33591 is higher than 1000 mg/L and therefore not visible on the graph. All values are means ± SEM of 3 to 7 independent experiments performed in triplicates (when not visible the error bars are smaller than the size of symbols).

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**Figure 5.** Comparison of antibiotics relative potencies (C\text{-log} or C\text{33}) expressed in mg/L for viability (left panels), metabolic activity (resorufin fluorescence; middle panels) or biofilm mass (crystal violet [CV] absorbance) for strains 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). Green horizontal lines correspond to the human C\text{max} after administration of a conventional dose. Grey horizontal lines correspond to MIC shown in table 1. MEM, meropenem; VAN, vancomycin; LZD, linezolid; AZM, azithromycin; RIF, rifampicin; CIP, ciprofloxacin; TOB, tobramycin. Values are means ± SEM. Statistical analyses: one-way analysis of variance (ANOVA) with Tukey’s post-test for multiple comparisons; values with different letters are significantly different from each other (p<0.05). Capital letters: comparison between antibiotics for strain ATCC 25923; lowercase letters: comparison between antibiotics for strain ATCC 33591. The grey horizontal lines are the MIC of the antibiotic in the corresponding medium and the green horizontal line, the human C\text{max} after conventional dosing (see Table 1).

**Figure 6.** Morphology and counts of colonies from biofilms of ATCC 25923 cultivated in artificial sputum medium (ASM) and exposed during 24 h to tobramycin (TOB) at 5, 10, or 100 mg/L or in control conditions (no antibiotic added; CT). Samples were plated on Columbia blood agar, TSA, or TSA supplemented with either 1 mg/L hemin (TSAH), menadione (TSAM) or both (TSAMH). Yellow arrows indicate a typical small colony.
**Figure 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 panel: Resorufin fluorescence signal recorded after 30 minutes of incubation of planktonic bacteria at increasing inocula with 10 mg/L resazurin. Right panel: Resorufin fluorescence signal recorded after 30 minutes of incubation of 24h-old biofilm with 10 mg/L resazurin. Data are means ± SD of triplicates in a single experiment or means ± SEM of at least 3 independent experiments performed in triplicate. Statistical analyses comparing strains in each individual medium: one-way analysis of variance (ANOVA) with Tukey’s post-test for multiple comparisons; values with different letters are significantly different from each other (p<0.05).
Table 1: Antibiotic susceptibility of bacteria strains in different media

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>MIC (mg/L)</th>
<th>ATCC 25923</th>
<th>ATCC 33591</th>
<th>EUCAST&lt;sup&gt;c&lt;/sup&gt; Susceptibility breakpoint (mg/L)</th>
<th>Human C&lt;sub&gt;max&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt; (mg/L)&lt;sup&gt;d&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MHB-ca&lt;sup&gt;a&lt;/sup&gt;</td>
<td>TGN&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ASM&lt;sub&gt;a,b&lt;/sub&gt;</td>
<td>MHB-ca&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Meropenem</td>
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<td>0.125</td>
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<td>16</td>
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<td>4</td>
<td>1</td>
<td>8</td>
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<tr>
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<td>2</td>
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<td>1</td>
<td>1</td>
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<tr>
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<td>1</td>
<td>1</td>
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<td>0.0039</td>
<td>0.031</td>
<td>0.03-0.06</td>
<td>0.001</td>
</tr>
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<td>0.25</td>
<td>0.5</td>
<td>0.25</td>
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<td>4</td>
<td>0.5</td>
<td>64</td>
<td>1024</td>
</tr>
</tbody>
</table>

<sup>a</sup> MHB-ca: Muller-Hinton broth cation-adjusted; TGN: Trypticase Soy Broth supplemented with 1% glucose and 2% NaCl; ASM: Artificial Sputum Medium

<sup>b</sup>MIC determined by cfu counting after plating due to the turbidity of the medium

<sup>c</sup> EUCAST: European Committee on Antimicrobial Susceptibility Testing

<sup>d</sup> based on the Belgian summary of product characteristics for each drug
Figure 1. Viscoelasticity of ASM (Artificial Sputum Medium; black curve) as compared to six sputa collected from CF patients and showing a purulent (P1, P2, P3 [blue closed squares]) or mucopurulent (MP1, MP2, MP3 [red open squares]) appearance, shown as individual data. The graphs show the elastic modulus (left) and the viscous modulus (right), both expressed in Pascal (Pa; kg.m\(^{-1}\).s\(^{-2}\)). A linear viscoelastic region is observed between 0.0001% and 0.1% of shear strain.
Figure 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; upper panels) and in Artificial Sputum Medium (ASM; lower panels) of viability (assessed by cfu counting), metabolic activity (metabolization of resazurin in fluorescent resorufin), and biomass (absorbance of crystal violet [CV]). Data are means ± standard deviations (SD) of at least 3 independent experiments. When not visible, the SD are smaller than the size of symbols.
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