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Comparison of the Antibiotic Activities of Daptomycin, Vancomycin, and the Investigational Fluoroquinolone Delafloxacin against Biofilms from *Staphylococcus aureus* Clinical Isolates

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Biofilm-related infections remain a scourge. In an *in vitro* model of biofilms using *Staphylococcus aureus* reference strains, delafloxacin and daptomycin were found to be the most active among the antibiotics from 8 different pharmacological classes (J. Bauer, W. Siala, P. M. Tulkens, and F. Van Bambeke, Antimicrob. Agents Chemother. 57:2726–2737, 2013, doi:10.1128/AAC.00181-13). In this study, we compared delafloxacin to daptomycin and vancomycin using biofilms produced by 7 clinical strains (*S. aureus* epidemic clones CC5 and CC8) in order to rationalize the differences observed between the antibiotics and strains. The effects of the antibiotics on bacterial viability (resazurin reduction assay) and biomass (crystal violet staining) were measured and correlated with the proportion of polysaccharides in the matrix, the local microenvironmental pH (micro-pH), and the antibiotic penetration in the biofilm. At clinically meaningful concentrations, delafloxacin, daptomycin, and vancomycin caused a ≥25% reduction in viability against the biofilms formed by 5, 4, and 3 strains, respectively. The antibiotic penetration within the biofilms ranged from 0.6 to 52% for delafloxacin, 0.2 to 10% for daptomycin, and 0.2 to 1% for vancomycin; for delafloxacin, this was inversely related to the polysaccharide proportion in the matrix. Six biofilms were acidic, explaining the high potency of delafloxacin (lower MICs at acidic pH). Norspermidine and norspermine (disassembling the biofilm matrix) drastically increased delafloxacin potency and efficacy (50% reduction in viability for 6 biofilms at clinically meaningful concentrations) in direct correlation with its increased penetration within the biofilm, while they only modestly improved daptomycin efficacy (50% reduction in viability for 2 biofilms) and penetration, and they showed marginal effects with vancomycin. Delafloxacin potency and efficacy against biofilms are benefited by its penetration into the matrix and the local acidic micro-pH.

Biofilms consist of communities of bacteria encased in a matrix made of polymeric substances, including DNA, proteins, polysaccharides, and teichoic acids in Gram-positive bacteria. About 80% of human bacterial infections are associated with these structures that can develop on the surface of tissues or foreign bodies (1). *Staphylococcus aureus* is one of the pathogens associated most frequently with these biofilm-related infections, and more specifically those presenting a persistent character, like osteomyelitis, rhinosinusitis, otitis media, endocarditis, or orthopedic implant infections (2, 3).

Studies performed *in vitro* and in animal models have shown that antibiotics are less active against bacteria growing in biofilms than against their planktonic counterparts (4–9). This apparent resistance has been attributed to a conjunction of factors related to the metabolic state of bacteria (a slow growth rate, emergence of small colony variants, and the presence of a significant population of persisters [cells in a dormant state]), the heterogeneity of the environment (gradients of pH, nutrients, or oxygen within the biofilm), or the obstacle to antibiotic access imposed by the matrix (binding, inactivation, or poor diffusion). Yet, direct experimental evidence of a cause-and-effect relationship between these factors and the lack of efficacy of antibiotics is not always well documented (1).

We recently developed an *in vitro* pharmacodynamic model allowing for a comparison of antibiotic relative potencies and maximal efficacies against biofilms of *S. aureus* which used quantitative and qualitative approaches in parallel (10). This model showed that among the antibiotics from 8 pharmacological classes, the investigational fluoroquinolone delafloxacin (11, 12) and daptomycin performed better than all others when tested against biofilms formed by both methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) reference strains (ATCC 25923 and ATCC 33591). In the present study, we extended the analysis to biofilms produced by 7 clinical isolates belonging to multilocus sequence typing clonal complexes (CC) CC5 and CC8, two of the most pandemic human lineages that have acquired mobile genetic elements carrying drug resistance or virulence genes, making then well adapted for colonization, pathogenicity, and a poor response to antibiotic treatment (13). Delafloxacin was compared to daptomycin and vancomycin as typical examples of highly and poorly active antibiotics, respectively, when tested in biofilms formed from the reference strains (10). We found that the biofilms produced from the clinical strains tested were generally much more refractory to antibiotics than those produced by reference strains, with marked differences

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TABLE 1 Description of clinical strains included in the study

Strain	Molecular characterization[f]							Clinical origin
	16S[a]	*nuc*[a]	*mecA*[b]	*spa* type[c]	MLST[d]	TSST-1[e]	PVL[e]	
2011S027	+	+	−	t002	CC5	+	−	Cellulitis and bacteremia
Surv 2003/1083	+	+	−	t002	CC5	+	−	Chirurgical wound
Surv 2005/104	+	+	+	t002	CC5	−	−	Skin
2009S028	+	+	+	t002	CC5	+	−	Nasal carriage
2009S025	+	+	+	t002	CC5	+	−	Ear
Surv 2005/179	+	+	+	t008	CC8	−	−	Skin
Surv 2003/651	+	+	+	051	CC8	−	−	Respiratory infection

[a] PCR amplification of *nuc* (*S. aureus*-specific region of the thermonuclease gene) and of a genus-specific 16S rRNA sequence (14).
[b] PCR amplification of *mecA* (detection of methicillin resistance) (14).
[c] *S. aureus* protein A gene (14).
[d] Multilocus sequence typing allelic profile (clonal complex) (14).
[e] PCR amplification of TSST-1 and PVL genes encoding the corresponding toxins (15).
[f] +, gene present; −, gene absent.

among the strains, but delafloxacin and daptomycin were more active than vancomycin. For delafloxacin, activity was dependent on biofilm pH and on the antibiotic penetration within the biofilm, which was itself related to the proportion of carbohydrates present in the matrix. Our data therefore suggest that examining biofilm properties may help predict antibiotic activity. We also show that destructuring the matrix by means of polycationic compounds, such as norspermine or norspermidine, greatly increases delafloxacin activity by improving its diffusion, underlining the interest in developing adjunctive therapies for the treatment of biofilm-related infections.

MATERIALS AND METHODS

Materials. Delafloxacin (95.7% potency) was procured from Melinta Therapeutics (New Haven, CT), and daptomycin (100% potency) was from Novartis Pharma AG (Basel, Switzerland). Vancomycin was used as a powder for injection (chlorhydrate form, without excipients) approved for human use in Belgium and in compliance with the provisions of the European Pharmacopoeia (Vancomycine Mylan; Mylan, Inc., Canonsburg, PA). Norspermidine and norspermine were from Sigma-Aldrich (St. Louis, MO). Fluorescent products (including Bodipy-FL-labeled vancomycin) were obtained from Molecular Probes (Eugene, OR), except Bodipy-FL-daptomycin, which was a kind gift from Cubist Pharmaceuticals (Lexington, MA). The media for bacterial culture were from Becton Dickinson Company (Franklin Lakes, NJ).

Bacterial strains, culture conditions, and biofilm model. ATCC 33591 (MRSA) was used as a reference strain. Seven clinical strains isolated from various sites but all belonging to the pandemic clonal complexes CC5 or CC8 were selected from the collection of the Belgian Reference Centre for *S. aureus* (Hôpital Erasme, Université Libre de Bruxelles, Brussels, Belgium). They were characterized as previously described (14, 15) with respect to toxin expression and molecular typing (Table 1).

The biofilms were obtained using as a starting inoculum bacteria transferred from frozen stocks onto Trypticase soy agar plates and incubated overnight at 37°C, after which 10 colonies were inoculated in Trypticase soy broth (TSB) supplemented with 2% NaCl and 1% glucose, and the bacterial density was adjusted to an optical density at 620 nm (OD_{620}) of 0.005. For quantitative analysis, 200 μl of bacterial suspension was cultivated in 96-well plates (European catalog no. 734-2327; VWR [Radnor, PA] tissue culture plates) at 37°C for 24 h so as to generate a mature biofilm (10). For confocal microscopy studies, the biofilms were grown on glass coverslips (20 mm in diameter; VWR) placed in 12-well plates, covered with 4 ml of bacterial suspension, and incubated at 37°C for 24 h.

Antibiotic susceptibility testing and activity against bacteria growing in biofilms. Unless stated otherwise, the MICs were determined by microdilution in cation-adjusted Mueller-Hinton broth (CA-MHB), according to the recommendations of the CLSI for delafloxacin and vancomycin, and with addition of $CaCl_2$ (so as to reach a final concentration of 50 mg/liter) for daptomycin (16). Antibiotic activity was determined against 24-h biofilms, as previously described (10). In brief, the culture medium was removed and replaced by the same medium (control) or medium containing antibiotics at increasing concentrations (1- to 256-fold their MIC in broth). The biofilms were reincubated for 48 h at 37°C and then treated as previously described (10) for the quantitative determination of biomass (crystal violet staining) or bacterial viability (reduction of resazurin in fluorescent resorufin). Crystal violet absorbance was measured at 570 nm, and resorufin fluorescence was measured at an excitation wavelength of 560 nm and an emission wavelength of 590 nm ($\lambda_{exc\ 560}/\lambda_{em\ 590}$) using a SPECTRAmax Gemini XS microplate spectrofluorometer (Molecular Devices LLC, Sunnyvale, CA).

Assay of polysaccharides. In a first assay, calcofluor white (CFW), which binds to exopolysaccharides containing beta1-3 and/or beta1-4 linkages (17, 18), was used to compare the exopolysaccharide contents of the biofilms from different strains. To this effect, 24-h-old biofilms were washed with 1 ml phosphate-buffered saline (PBS), added with 100 μl of a CFW solution at 0.5 mg/ml, and left for 5 min in the dark at room temperature. CFW fluorescence was then measured at $\lambda_{exc\ 365}/\lambda_{em\ 440}$ using a microplate spectrofluorometer. In a second stage, the exopolysaccharides were purified exactly as previously described (19), after which their concentrations were determined according to a method described for quantifying carbohydrates, including polysaccharides (20), using glucan as a standard. In brief, 500 μl of purified exopolysaccharides was mixed with 1.5 ml of concentrated sulfuric acid, vortexed for 30 s, and cooled in ice for 2 min, after which UV light absorption was read at 315 nm. The diameter of exopolysaccharide supramolecular particles was measured by dynamic light scattering (Zetasizer Nano ZS; Malvern, France) using 20 μl of purified exopolysaccharides dissolved in 980 μl of distilled water (19).

Confocal laser scanning microscopy visualization of biofilms. The biofilm samples were imaged by confocal laser scanning microscopy in a Cell Observer SD microscope (Carl Zeiss AG, Oberkochen, Germany) using spinning disc technology (Yokogawa, Tokyo, Japan) and controlled by the AxioVision software (AxioVs40 version 4.8.2.0). The biofilms were stained using either the LIVE/DEAD bacterial viability kit (L-7007), as described previously (10), or 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (RedoxSensor vitality kit; Invitrogen, Carlsbad, CA). CTC is a colorless, nonfluorescent, and membrane-permeable compound that is readily reduced via electron transport activity to a fluorescent insoluble CTC-formazan that accumulates inside bacteria (21). The biofilm-over-

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grown coverslips were washed with 1 ml PBS buffer, labeled with 0.5 mM CTC for 30 min, and observed via confocal laser scanning microscopy (excitation laser line, 488 nm; emission filter, monomeric red fluorescent protein [mRFP], 598 to 660 nm). The optimal confocal settings (camera exposure time and confocal scanner unit [CSU] disk speed) were determined in preliminary experiments. Image stacks of each sample were acquired at a resolution of 700 by 500 pixels and recorded using a z-stack module for the acquisition of image series from different focus planes and used to construct three-dimensional images with the AxioVision software.

Spatial distribution of biofilm pH microenvironments. Seminaphthorhodafluor-4F 5-(and-6) carboxylic acid (C-SNARF-4) is a reliable pH indicator of the bacterial biofilm microenvironment (22). This dye exists as a mixture of fluorescent forms (monoanionic [naphthol] and dianionic [naphtholate]) that are predominant at a pH of >5 but are nonfluorescent (neutral and cationic) at lower pH values. The relative concentrations of the protonated and unprotonated forms, and therefore the local pH, can be estimated by measuring the 580 nm/640 nm fluorescence ratio using confocal laser scanning microscopy (23). Twenty-four-hour biofilms cultivated on glass coverslips were incubated for 2 h in water, after which C-SNARF-4 was added at a final concentration of 10 μM in a volume of 1 ml. The coverslips were then incubated in the dark for 30 min. The solution was then carefully removed with a pipette. The C-SNARF-4-treated biofilms were examined by confocal microscopy (excitation at 488 nm and emission detected in two channels at 580 nm and 640 nm). The fluorescence intensity was determined by calculating the densitometric mean for each channel using the outline tool of the AxioVision software. The ratio of the emission intensity between the two channels was then calculated, and the microenvironment pH was determined from the ratio $(Fluo_{em580\ sample} - Fluo_{em580\ background})/(Fluo_{em640\ sample} - Fluo_{em640\ background})$ (Fluo, fluorescence), using calibration standards (solutions of 50 mM HEPES buffer adjusted at pHs ranging from 5.2 to 8, with a 0.2-pH unit interval) imaged using settings identical to those used for the biofilm sample (see Fig. S1 in the supplemental material).

Penetration of antibiotics within the biofilms. To study the delafloxacin penetration within the biofilms, we took advantage of its intrinsic fluorescence ($\lambda_{exc\ 395}/\lambda_{em\ 450}$). Twenty-four-hour biofilm-overgrown coverslips were incubated for 1 h with 50 mg/liter delafloxacin dissolved in distilled water and thereafter stained with the LIVE/DEAD bacterial viability kit, as described above. The biofilms were washed twice with 1 ml PBS and imaged using confocal microscopy, with the following excitation/emission wavelengths: 488 nm/500 to 550 nm for Syto 9, 561 nm/570 to 620 nm propidium iodide (for LIVE/DEAD staining), and 400 nm/420 to 450 nm for delafloxacin. To study daptomycin and vancomycin penetration within the biofilms, 24-h biofilms were exposed for 1 h to 20 mg/liter Bodipy-FL-daptomycin or Bodipy-FL-vancomycin and then to 0.5 mM CTC for 30 min in the dark, and they were washed twice with 1 ml PBS buffer. The confocal images were obtained using an λ_{exc} of 488 nm and recording emitted fluorescence at 570 to 620 nm for Bodipy-FL-labeled antibiotics and 500 to 550 nm for CTC-formazan. The antibiotic concentrations within the biofilms were then calculated using calibration curves of the antibiotic solutions examined under the microscope and using the same settings (concentrations of 5 to 100 mg/liter for delafloxacin and 0.5 to 10 mg/liter for Bodipy-FL-daptomycin and -vancomycin).

Data analyses and statistical analyses. Curve-fitting analyses of the concentration-effect relationships were made with GraphPad Prism version 4.03 (GraphPad Software, San Diego, CA, USA). The data were used to fit a sigmoid function (Hill equation, variable slope) by nonlinear regression, as previously done with this model [10]) and to calculate the maximal relative efficacies (E_{max}) (maximum reduction in biofilm mass production or in viable bacteria extrapolated for an infinitely large antibiotic concentration) and relative potencies (C_{25}, C_{50}, or C_{75}, concentrations allowing 25, 50, or 75% reduction of the parameter investigated, respectively). Statistical analyses were made with GraphPad InStat version 3.06 (GraphPad Software) and correlations with JMP version 10.02 (SAS Institute, Inc., Cary, NC).

RESULTS

Susceptibility testing. Table 2 shows the activities of the antibiotics against all strains included in this study when tested using standard susceptibility testing methods (broth microdilution). The MICs of daptomycin and vancomycin were equivalent against all strains (0.5 and 1 mg/liter, respectively), while those of delafloxacin were lower, ranging from 0.004 to 0.125 mg/liter.

Biofilm characterization. Figure 1 (left and middle panels) compares the viability and the biomass obtained after 24-h culture of biofilms for the 7 clinical isolates investigated in the present study in comparison with the *S. aureus* reference strain ATCC 33591. With respect to viability, three *S. aureus* clinical strains (2003/1083, 2009S025, and 2011S027) gave a signal similar to that of ATCC 33591, while the signal generated by the four other strains was only 2/3 of this value. With respect to biomass, crystal violet staining was lower than for the reference strain, with Surv2003/1083, 2009S025, and Surv2003/651 being the highest biofilm producers, Surv2005/104 and 2009S28 the lowest producers, and 2011S027 and Surv2005/179 showing intermediate behavior. The content of the biofilms in β-polysaccharides was also estimated using calcofluor white. As illustrated in Fig. 1 (right panel), biofilm produced by strain Surv2003/651 contained more polysaccharides than did the biofilms obtained with the other strains. Yet, when the results were normalized for the amount of biomass produced, the biofilms obtained from strains Surv2005/104 and 2009S28 were those containing the highest proportion of polysaccharides, followed by biofilms from strains 2011S027, Surv2005/179, and Surv2003/651, and then from strains ATCC 33591, Surv2003/1083, and 2009S025.

Activities of antibiotics against biofilms. Antibiotic activity was then evaluated against 24-h biofilms incubated for 48 h at a broad range of antibiotic concentrations in order to obtain full concentration-effect responses and to calculate the corresponding parameters of the Hill equation fitted to the data. Typical results obtained with the biofilms formed from 2 strains are shown in Fig. 2, with the results for the biofilms obtained from the other strains presented in Fig. S2 in the supplemental material. The relative potencies concerning viability are presented in Table 2. They were presented as C_{25} values (concentrations reducing the resorufin fluorescence signal by 25%) because, due to low activity, further reduction was not obtained for all strains. Against strain 2011S027, all drugs displayed concentration-dependent activity, with delafloxacin being by far the most potent and the most efficient among the three antibiotics tested, being able to reduce the biomass by approximately 50% at the lowest concentration tested (0.1 mg/liter) and to almost sterilize the biofilm at a concentration close to 1 mg/liter. Daptomycin was less potent and less efficient, while vancomycin was globally poorly active with respect to both viability and biomass. In sharp contrast, the biofilm produced by strain Surv2003/651 was totally refractory to all 3 antibiotics, despite the low MICs observed for this strain. Similar to strain Surv2003/651, strain Surv2005/179 was not sensitive to antibiotics in the biofilms. The biofilms produced by the 4 other clinical isolates showed intermediate behavior, with delafloxacin being much more effective and potent than the other drugs against strain 2009S025 and with daptomycin being more potent against strains 2009S028 and Surv2005/104. Comparing the relative potencies

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TABLE 2 Antibiotic properties and biofilm pHs for the reference strain and the clinical isolates used in the study

Strain	Antibiotic[e]	MIC (mg/liter)	Concn (mg/liter) causing 25% reduction in resorufin fluorescence in biofilms[a]	Antibiotic penetration in biofilm (%)[b]	Biofilm pH[c]
MRSA ATCC 33591	DFX	0.004	**0.002**[d]	60.05	6.12, 5.63
	DAP	0.5	**1.3**	5.99	
	VAN	1	**1.6**	0.44	
MSSA 2011S027	DFX	0.004	**0.2**	51.8	5.28, 5.92
	DAP	0.5	**7.1**	9.98	
	VAN	1	**19.5**	0.4	
MRSA Surv 2003/1083	DFX	0.004	**9.2**	15.6	6.41, 6.35
	DAP	0.5	**6.0**	4.28	
	VAN	1	36.0	0.96	
MRSA Surv 2005/104	DFX	0.125	**13.2**	5.72	6.15, 5.46
	DAP	0.5	**1.2**	8.72	
	VAN	1	**15.0**	0.24	
MRSA 2009S028	DFX	0.016	**7.1**	16.84	5.94, 5.95
	DAP	0.5	**0.4**	7.32	
	VAN	1	**12.3**	0.41	
MSSA 2009S025	DFX	0.004	**1.5**	23.9	5.87, 5.57
	DAP	0.5	>250	2.64	
	VAN	1	>250	0.26	
MRSA Surv 2005/179	DFX	0.016	>250	13.02	6.67, 5.71
	DAP	0.5	>250	0.26	
	VAN	1	>250	0.32	
MRSA Surv 2003/651	DFX	0.125	>250	0.62	6.19, 5.55
	DAP	0.5	>250	0.20	
	VAN	1	>250	0.22	

[a] Calculated using the Hill equation of the concentration-response curve (see Fig. 2; see also Fig. S2 in the supplemental material).
[b] Percentage of the concentration added in the culture medium; see Fig. 4 and also Fig. S5 in the supplemental material.
[c] Values at 2- and 30-μm depths, respectively (Fig. 3; see also Fig. S4 in the supplemental material for values measured at different depths in the biofilm).
[d] Boldface indicates values that are lower than the free C_{max} reached in patients receiving projected doses (DFX C_{max}, 10 to 16 mg/liter for intravenous [i.v.] doses of 300 to 450 mg; free fraction, 84% [28, 29]) or conventional doses (DAP C_{max}, 94 mg/liter for i.v. dose of 6 mg/kg of body weight; free fraction, 8.5 % [Cubicin SPC]; VAN C_{max}, 63 mg/liter for i.v. dose of 15 mg/kg; mean free fraction, 45% [vancomycin SPC]).
[e] DFX, delafloxacin; DAP, daptomycin; VAN, vancomycin.

from a clinical perspective, delafloxacin achieved a 25% reduction in the resorufin fluorescence signal at a range of clinically achievable concentrations (≤free human maximum concentration of drug in serum [C_{max}]) against 5 out of the 7 clinical isolates versus 4/7 for daptomycin and 3/7 for vancomycin (Table 2).

We then looked for a possible correlation between the relative potency of each antibiotic (measured as the C_{25}) and the characteristics of the biofilms presented in Fig. 1. As illustrated in Fig. S3 in the supplemental material, there was no significant correlation between relative potency and biomass (middle panel; $P > 0.2$), but a trend (P, ~0.1) to a higher potency for delafloxacin (i.e., lower C_{25} values) against biofilms containing more metabolically active bacteria (higher resorufin fluorescence [RF] signal; left panel) or those presenting a smaller proportion of polysaccharides in their matrix (lower CFW-to-crystal violet [CV] ratio; right panel).

Biofilm microenvironmental pH. An acidic environment is known to modulate antibiotic intrinsic activity (24) and, more specifically, to markedly improve that of delafloxacin (11). We therefore measured the microenvironmental pH (micro-pH) in the depth of the biofilm using the pH-sensitive probe C-SNARF-4 (22). Table 2 shows the pH values measured at the top and bottom of each biofilm, with full data sets illustrated for 3 selected strains in Fig. 3 (upper panels) and for the other strains in Fig. S4 in the supplemental material. Biofilms from 6 out of the 7 clinical strains showed a pH profile similar to that of the reference strain ATCC 33591, with acidic pH recorded at the biofilm surface (~5.3 at 0 μm to ~6.0 at 2 μm) and slightly higher values (0.5 to 1 pH units) in the deepest plane of the biofilm. Only the biofilm of Surv2005/179 was almost neutral at the surface (pH 6.8), but the pH deep in the biofilm was similar to that observed with the other strains. The middle panel of Fig. 3 illustrates for the same strains the influence of pH on delafloxacin MIC (planktonic bacteria) in a range covering the pH measured in the biofilms (see Fig. S4 for the other strains). The MICs were 3 to 9 dilutions lower at the pH of the biofilm than those at a neutral pH (pH 7.4). In contrast, the vancomycin and daptomycin MICs were not affected by pH (maximum 1 dilution change over a pH range from 5.6 to 7.4; data not shown). The lower panel of Fig. 3 shows the correlation between the pH measured 2 μm below the biofilm surface and the relative

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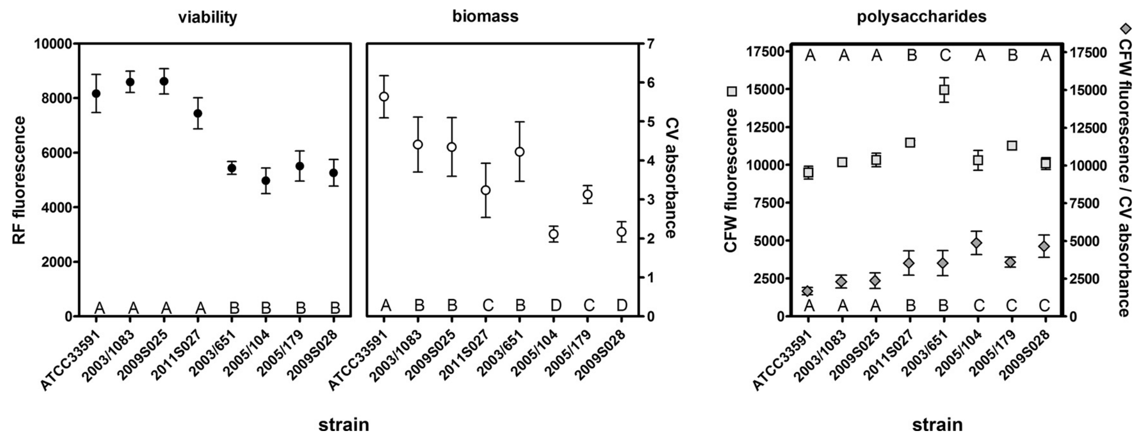

FIG 1 Biofilm characterization. Resorufin fluorescence signal (RF) (left), crystal violet absorbance (CV) (middle), and calcofluor white fluorescence (CFW) signal (right, squares) measured in 24-h biofilm. The right axis in the right graph shows CFW fluorescence values normalized with respect to biomass, as evaluated by CV absorbance (diamonds). The data are the means ± standard deviations (SD) of 8 wells. Statistical analysis was performed using an analysis of variance (ANOVA) with Dunnett's *post hoc* test; the strains with different letters for each data set denote statistically significant differences among them (*, $P < 0.05$).

potency of delafloxacin within the biofilms. The correlation was only partial (strains with similar MICs showing different potencies), suggesting that parameters other than pH influence delafloxacin activity within the biofilm.

Antibiotic penetration within the biofilm. The lack of penetration has been reported as a key factor limiting antibiotic activity against bacteria developing in the depth of biofilms (25–27). We therefore examined using confocal laser scanning microscopy the

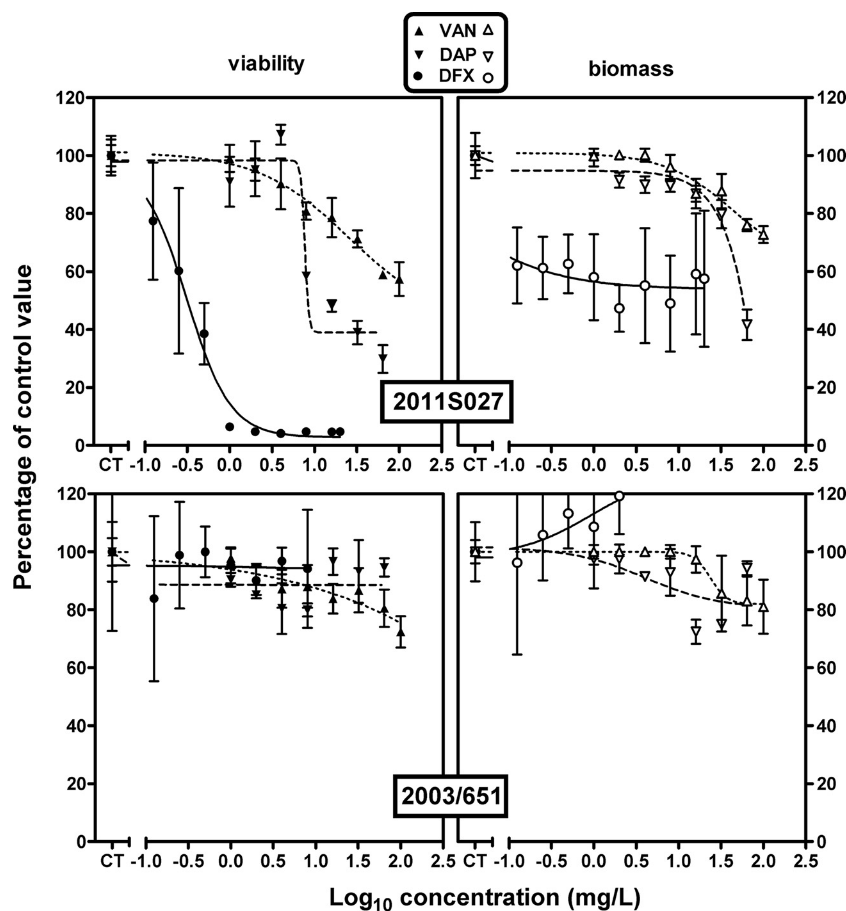

FIG 2 Activities of antibiotics against biofilms. Concentration-response activities of antibiotics against 24-h biofilms of strain 2011S027 (top) or 2003/651 (bottom). Twenty-four-hour biofilms were incubated with increasing concentrations of antibiotics for 48 h (DFX, delafloxacin; DAP, daptomycin; VAN, vancomycin). The ordinate shows the change in viability (assessed by resorufin fluorescence; left) or in biomass (assessed by crystal violet absorbance; right) as the percentage of the control (CT) value (no antibiotic present). All values are the means ± standard deviations (SD) of 8 wells (when not visible, the SD bars are smaller than the size of the symbols).

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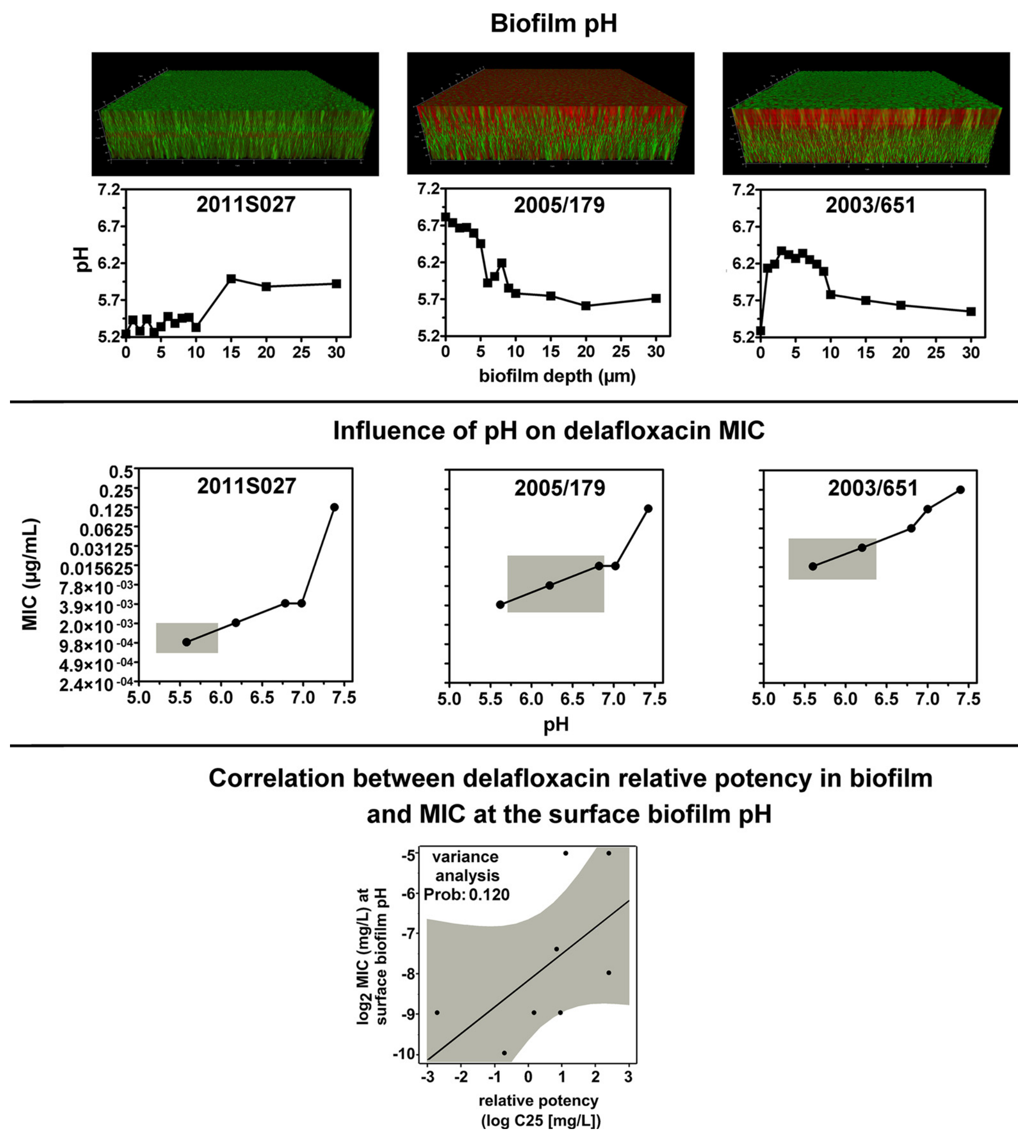

FIG 3 Micro-pH in biofilms and influence on delafloxacin activity. Top panels, evaluation of micro-pH within biofilms of three selected strains, as evaluated by confocal microscopy using C-SNARF-4 as a pH-sensitive probe (fluorescence emitted shifting from red to green upon acidification). The graph below each image shows the corresponding micro-pH in the depth of the biofilm. Middle, influence of pH on the MIC of delafloxacin in MHB adjusted to different pH values. The gray squares highlight the range of pH measured in the corresponding biofilm. The strain numbers are at the top of each graph. Bottom, correlation between the relative potency of delafloxacin (C_{25} [concentration reducing viability by 25%], as determined based on the concentration-response curves presented in Fig. 2; see also Fig. S2 in the supplemental material) and the MIC at a pH corresponding to the one measured at 2 μm below the surface of the corresponding biofilm.

capacities of the three drugs to diffuse within the biofilm thickness, taking advantage of the fluorescent character of delafloxacin (blue signal) and using daptomycin or vancomycin coupled to Bodipy-FL (green signal) as a control. Biofilms were labeled using LIVE/DEAD staining (green/red staining) for those treated by delafloxacin and CTC (red staining) for those treated by Bodipy-FL-labeled antibiotics. Table 2 shows the percentage of penetration of each antibiotic at the surface or in the depth of the biofilm compared to the antibiotic concentration added in the medium bathing the biofilm. Confocal images and fluorescence signal analysis are shown in Fig. 4 for the three same selected strains and in Fig. S5 in the supplemental material for the other strains. Although variable, the penetration of delafloxacin in the biofilm thickness was much better than that for the other antibiotics, reaching up to 52% of the whole structure for the biofilms produced by the clinical isolates, whereas it did not exceed 10% for Bodipy-FL-daptomycin, and it was <1% for Bodipy-FL-vancomycin. Interestingly, the penetration of all antibiotics was of the same order of magnitude in the biofilms produced by the reference strain and by the MSSA clinical strain 2011S027, against which antibiotics were the most active. In order to better delineate a potential relationship between the penetration of the drugs through the biofilm and their activity on viability within the biofilm, we plotted the relative potency of each drug (as determined by the concentration needed to reduce viability within the biofilm by 25% [C_{25}]) versus its relative penetration within the biofilm (using values measured close to the

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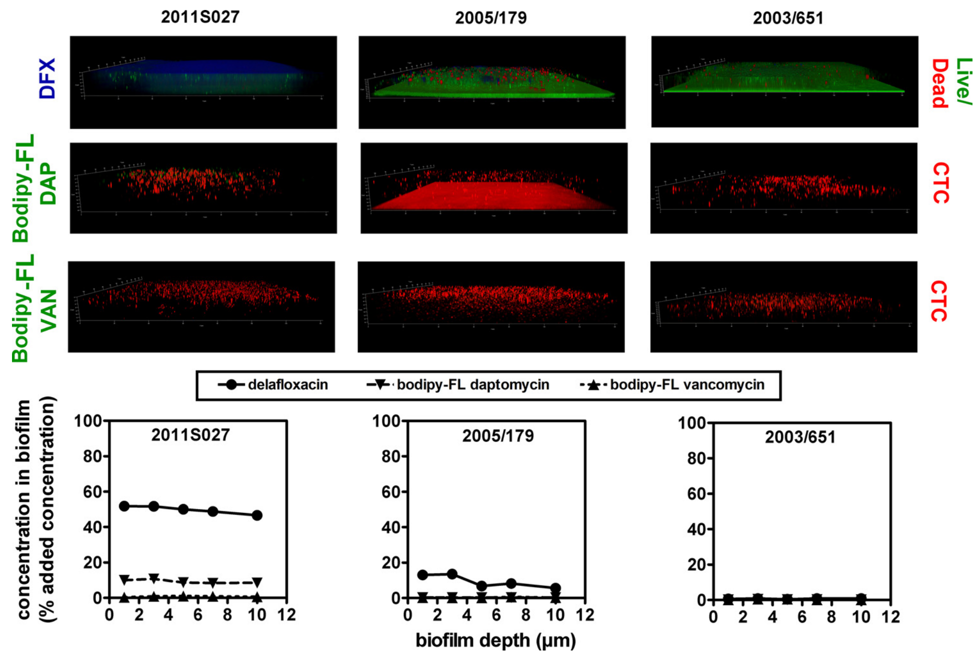

FIG 4 Penetration of antibiotics within biofilms. Confocal images of biofilms incubated for 1 h with 50 mg/liter delafloxacin (top [blue]), 20 mg/liter Bodipy-FL-daptomycin (middle [green]), or 20 mg/liter Bodipy-FL-vancomycin (bottom [green]) and labeled with LIVE/DEAD stain (top: red, dead; green, live) or CTC (middle and bottom: red). The graphs below each column compare the relative penetration of the drugs within the depth of the corresponding biofilm, expressed as the percentage of the added concentration (DFX, delafloxacin; DAP, daptomycin; VAN, vancomycin).

surface). Strain MRSA Surv2005/179 was excluded from the analysis, because the neutral character of its biofilm may constitute a confounding factor. As shown in Fig. 5, there was a statistically significant correlation between the relative potency and relative penetration within the biofilm for delafloxacin and daptomycin, while vancomycin relative potency was always low, due to poor penetration of the drug. Figure 5 also shows that a penetration of 5% was sufficient for delafloxacin or daptomycin to achieve a 25% reduction in viability within the biofilms at a clinically meaningful range of concentrations (MIC free C_{max} range; see footnotes to Table 2 for values). This effect was achieved for 6/7 of the tested strains for delafloxacin versus 5/7 for daptomycin.

Influence of polyamines on antibiotic activities against biofilms. Norspermidine and related polyamines have been shown to trigger the disassembly of *S. aureus* biofilms by interacting with matrix exopolysaccharides (19). Because a large diffusion into the matrix and a low proportion of polysaccharides in the matrix seemed to critically determine the activities of the antibiotics in our model, we explored whether the addition of polyamines would improve this activity by increasing antibiotic penetration. We first checked that norspermine and norspermidine had no detectable activity on the biofilms when used alone and did not change the MICs of the bacteria when tested in broth (planktonic forms) (data not shown). We then exposed 24-h biofilms to antibiotics in the presence of 200 μM norspermidine or norspermine. The data are illustrated in Fig. 6 for the biofilms produced by strain Surv2003/651, which were fully refractory to any of the antibiotics tested when used alone (Fig. 2). Quite strikingly, norspermine and norspermidine markedly improved the activities of both delafloxacin and daptomycin, so as to reach a 40% reduction in both resorufin and crystal violet signals at 20 mg/liter (the highest concentration tested for delafloxacin). This effect was related to a marked increase in antibiotic relative penetration within the biofilm (0 to 50% for delafloxacin and 30% for daptomycin). In contrast, the polyamines added to vancomycin did not improve its penetration within the biofilm and only modestly improved its efficacy, with no change in potency for viability and no detectable effect on biomass. To further document the effect of norspermidine on biofilms, we compared the concentrations and sizes of the exopolysaccharides purified from Surv2003/651 biofilms that were exposed to 200 μM norspermidine for 24 h or incubated for the same time under control conditions. Norspermidine drastically reduced both the concentrations and the diameters of exopolysaccharide supramolecular particles (27.9 ± 3.0 μg/ml and 0.95 ± 1.2 nm in norspermidine-exposed samples versus 157.9 ± 5.2 μg/ml and 280.4 ± 4.3 nm under control conditions).

Figure 7 illustrates the influence of polyamines on the relative potencies of the antibiotics against the 7 clinical isolates by showing the concentration of antibiotic needed to cause a 25, 50, or 75% reduction in resorufin fluorescence within a biofilm under control conditions or in the presence of polyamines (using the Hill equations describing the concentration-effect relationships, as illustrated in Fig. 6 for strain Surv2003/651). Thus, at a concentration mimicking the anticipated free C_{max} of delafloxacin in patients (13 mg/liter [28, 29]), delafloxacin in combination with norspermine caused a 25% reduction in resorufin fluorescence against all 7 strains, 50% against 6 strains, and 75% against 3 strains. Delafloxacin in combination with norspermidine was slightly less efficient. Norspermidine, and to a lesser extent norspermine, were also highly synergistic with daptomycin, but for most of the strains, the concentrations needed to reach a 50 or 75% reduction in fluorescence remained higher than the free hu-

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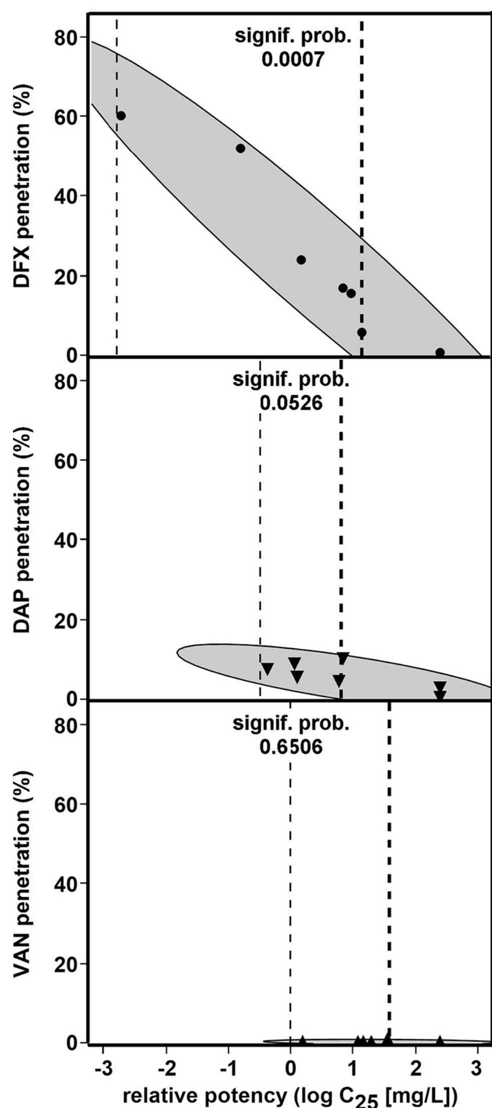

FIG 5 Correlation between activity against biofilm and antibiotic penetration within biofilms. Correlation between the relative potency of antibiotics (C_{25} [concentration reducing viability by 25%], as determined based on concentration-response curves presented in Fig. 2; see also Fig. S2 in the supplemental material) and the penetration of antibiotics within biofilms for all strains, except strain 2005/179, for which neutral pH may constitute a confounding factor. The thin dotted lines correspond to the lower MICs of the drug among the strains tested, and the thick dotted lines correspond to the free human C_{max} for each antibiotic (as observed upon treatment with a conventional dose projected from clinical trials for delafloxacin; see footnote *a* in Table 2 for values). DFX, delafloxacin; DAP, daptomycin; VAN, vancomycin.

man C_{max} (8 mg/liter [Cubicin SPC]). Both polyamines only slightly improved vancomycin activity.

DISCUSSION

To the best of our knowledge, this study is one of the first to examine in a systematic fashion the link between the activities of antibiotics, their penetration within biofilms, the biofilm micro-pH, and the biofilm polysaccharide content using a series of clinical *S. aureus* strains from two major contemporary epidemic clones. Two critical observations are that (i) antibiotic activity is lower against biofilms produced by these clinical isolates than against those produced by the reference strains (10), and (ii) there is huge variability in the susceptibilities of the biofilms produced by different isolates to the same antibiotic. These differences might stem from variations in the chemical composition and in the biophysical properties of the biofilms.

With respect to matrix composition, *S. aureus* biofilms consist mainly of polysaccharides (poly-*N*-acetylglucosamine and teichoic acid), extracellular matrix proteins, and DNA released from bacteria (30–33), but the relative amounts of these components may differ among the biofilms formed from different strains due to differences in the expression of the genes regulating biofilm formation (32, 34). The matrix composition of the biofilms formed by the strains used in our study is not known, but we observed major differences in the capacities of the strains to form biofilms. Yet, differences in biomass did not correlate with differences in antibiotic activity. Actually, there is even a trend toward a higher potency for delafloxacin against biofilms with higher biomass or viability. This may be due to the fact that fluoroquinolones are more reliant on bacterial growth than is daptomycin in order to exert their bactericidal effect (35). Interestingly, delafloxacin potency was inversely proportional to the biofilm content in polysaccharides relative to the whole biomass. Although the exact nature of these polysaccharides needs to be determined, the data are consistent with the generally accepted concept that exopolysaccharides in the matrix represent an initial barrier that delays the penetration of antimicrobials (36). Of note also, strains Surv2005/179 and Surv2003/651, which are the least susceptible to antibiotics when tested in biofilms, are associated with multilocus sequence type (MLST) CC8, which is claimed to be a stronger biofilm producer than CC5 (37). In our hands, CC8 rather appeared as producing a matrix rich in polysaccharides, but the number of strains belonging to this lineage was too low to draw meaningful conclusions.

With respect to the biophysical properties of the biofilms, we explored the potential importance of two of them, namely, (i) the local pH along the thickness of the biofilm and its variation and (ii) the ability of the biofilms to let antibiotics diffuse into them.

Acidic pH considerably increases the potency of delafloxacin when tested against planktonic forms of *S. aureus* by increasing its intrabacterial accumulation (11), but it does not substantially affect the potency of vancomycin or daptomycin (38 and this study). Except for one strain, the biofilms were globally slightly acidic, reaching a pH value at which delafloxacin MICs are typically 1/100 of those observed at a neutral pH. This may contribute to its superior relative potency compared to that of daptomycin and vancomycin. But antibiotic access to its bacterial target is also a clear prerequisite for activity, and biofilms are known to present a barrier to the free diffusion of antibiotics (25, 26, 39, 40). We found here that the penetration of Bodipy-FL-vancomycin within the biofilm was very limited for all strains, which may explain the poor activity of vancomycin. These observations confirm those of Jefferson et al. (41) but are in contradiction to those of Daddi Oubekka et al. (42), who showed that Bodipy-FL-vancomycin appreciably diffuses within biofilms. The data from Daddi Oubekka et al., however, were obtained using another experimental approach that follows the dynamics of the process (correlative time-resolved fluorescence microscopy) rather than measuring actual concentrations. Of note, Daddi Oubekka et al. did not observe any antibacterial activity for vancomycin within the biofilms, and therefore, they concluded that poor penetration was not a critical

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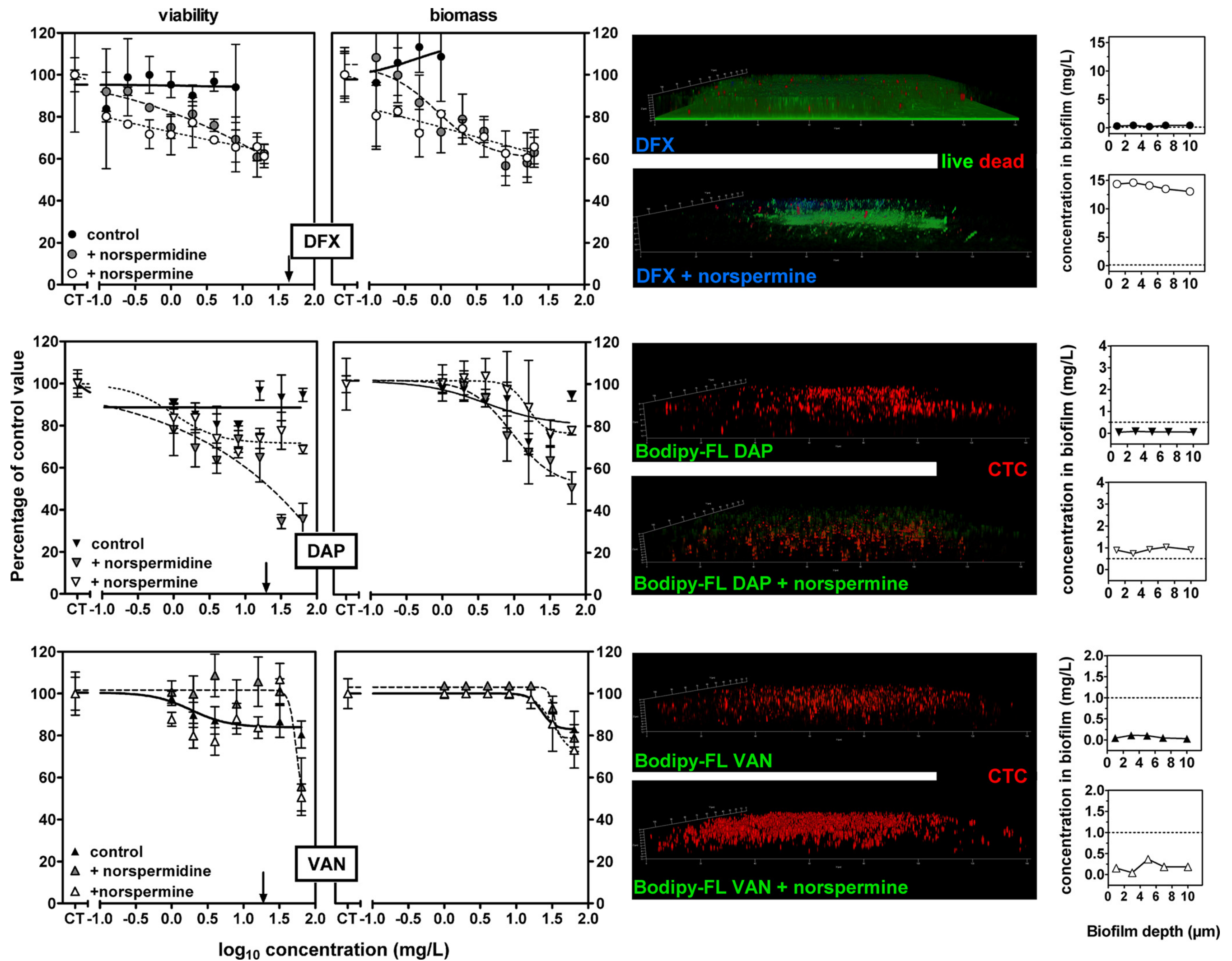

FIG 6 Influence of polyamines on antibiotic activity against biofilm of strain 2003/651. Left, concentration-response activities of antibiotics against 24-h biofilms incubated with increasing concentrations of antibiotics for 48 h (DFX, delafloxacin; DAP, daptomycin; VAN, vancomycin) in the absence or presence of 200 μM norspermine or norspermidine. The ordinate shows the change in viability (assessed by resorufin fluorescence; left) or in biomass (assessed by crystal violet absorbance; right) as the percentage of the control value (no antibiotic present). All values are the means ± standard deviations (SD) of 8 wells (when not visible, the SD bars are smaller than the size of the symbols). The arrows point to the concentration of antibiotic used in confocal microscopy. Middle, confocal images of biofilms incubated for 1 h with 50 mg/liter delafloxacin (blue), 20 mg/liter Bodipy-FL-daptomycin (green), or 20 mg/liter Bodipy-FL-vancomycin (green) in the absence or presence of 200 μM norspermine and labeled with LIVE/DEAD staining (top: red, dead; green, live) or CTC (middle and bottom: red). Right, relative penetration (in mg/liter) of the drugs within the depth of the corresponding biofilms, under control conditions, or in the presence of 200 μM norspermidine. The horizontal dotted line corresponds to the MIC of each antibiotic.

factor in preventing antibiotics from exerting their action within biofilms. Our data clearly suggest the opposite. Yet, Daddi Oubekka et al. also propose that the nature of the biofilm matrix may critically determine the diffusibility of drugs, which is consistent with our own observations. For Bodipy-FL-daptomycin, we noticed low penetration rates that are in line with the low diffusibility (28%) observed for this molecule in a biofilm formed by a reference strain of *Staphylococcus epidermidis* (43). We cannot, however, exclude that Bodipy itself modifies the diffusion capacities of the drug. While coupling with Bodipy does not change the MIC of vancomycin, it reduces that of daptomycin by about 2-fold (41, 44). For delafloxacin, which was followed with fluorescence microscopy without chemical modification, we observed that its penetration, although highly variable among the biofilms, was, generally speaking, higher than that of both daptomycin and vancomycin and clearly correlated with antibiotic activity. This higher capacity of delafloxacin to diffuse in biofilms compared to that of daptomycin and vancomycin may be at least partly due to its smaller overall size (molecular mass, 441 g/mol) (45) than that of vancomycin (1,449 g/mol) and daptomycin (1,620 g/mol), especially if one considers that coupling with Bodipy increases their molecular mass by about 275 g/mol. The better penetration of delafloxacin may also be favorably influenced by the acidic pH of the biofilm. Delafloxacin, indeed, is a weak acid, with a pK_a of ~5.6 (calculated with Reaxys; see also reference 11), and its uncharged species represent, therefore, about half of its total concentration in the depth of the biofilms formed with strains 2005/179 and 2003/651. Putting these parameters together, Fig. 8 illustrates

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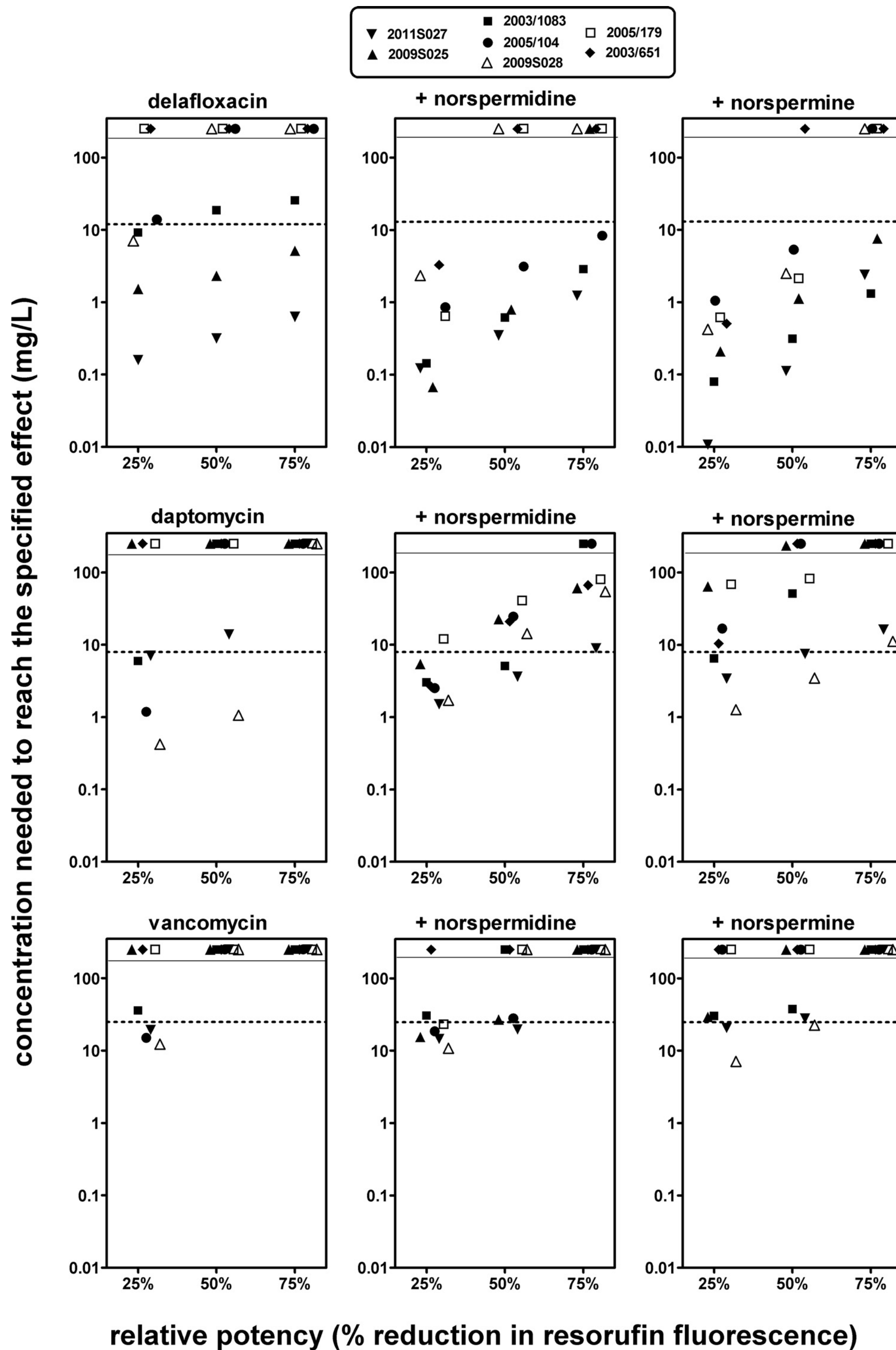

FIG 7 Influence of polycations on the activities of antibiotics against biofilms from clinical stains. The graphs show the concentrations of each antibiotic needed to reach the reductions in viability indicated on the abscissa under control conditions (left) or in the presence of 200 μM norspermidine (middle) or norspermine (right), calculated based on the equation of sigmoid concentration-response curves obtained for each strain in experiments similar to the one illustrated in Fig. 6. Top, delafloxacin; middle, daptomycin; bottom, vancomycin. The horizontal dotted lines highlight the free serum C_{max} values reached (or projected for delafloxacin) in the sera of patients receiving conventional doses. The thin line separates values that were higher than the highest concentration tested (and were set arbitrarily at 250 mg/liter).

the respective roles of delafloxacin MIC and penetration in the biofilm on its activity. Thus, strains can be separated into two clusters with respect to MICs, but their susceptibility to this antibiotic within the biofilm rather depends on the capacity of the drug to reach the bacteria within the structure, which itself is influenced by the proportion of polysaccharides present in the matrix. This can be explained by the fact that for those biofilms that are permeable to delafloxacin, the local concentrations are far

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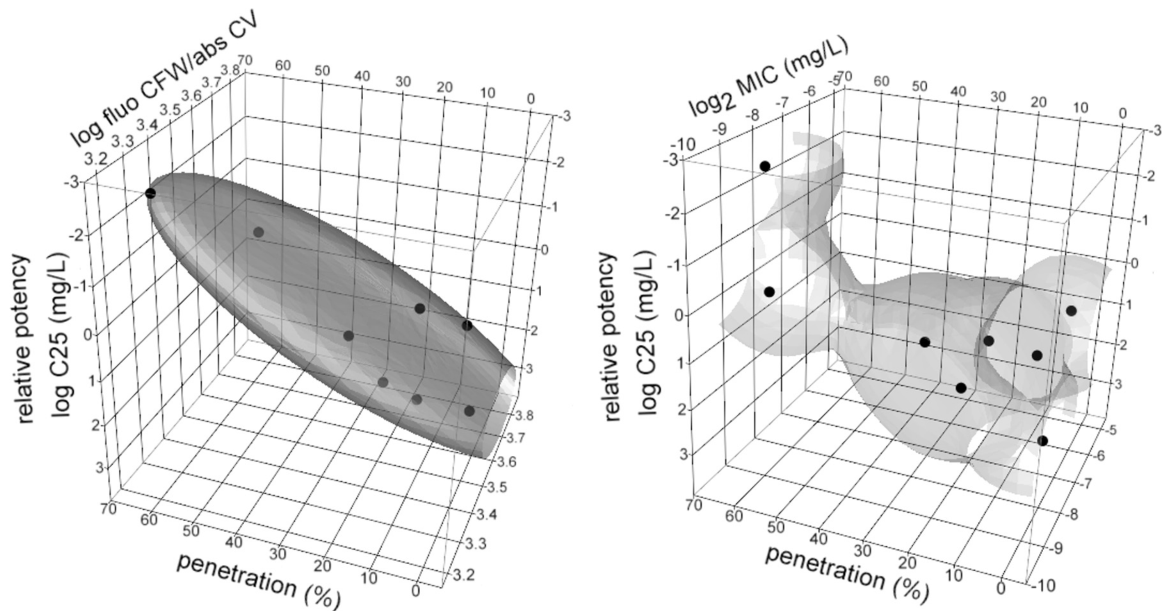

FIG 8 Correlation between relative potency of delafloxacin, its penetration within biofilms, and the proportion of polysaccharides in biofilms (left) or the MIC of the antibiotic at the biofilm pH (right). Shown are the data from the 7 clinical isolates and ATCC 33591, with relative potency estimated as the C_{25} (concentration needed to reduced the viability within the biofilms by 25%), penetration within biofilms determined with confocal microscopy (see Table 2 for values), and the ratio of calcofluor white fluorescence to crystal violet absorbance shown in Fig. 1 or the MIC at the biofilm pH shown in Fig. 3; see also Fig. S4 in the supplemental material. The shaded areas show the normal contour (left panel) or nonparametric density contours (right panel).

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above the MIC, whatever its value. Taken together, these data highlight a potential advantage of delafloxacin over vancomycin or even daptomycin in this respect. This advantage is further documented by the drastic gain in relative potency observed for this drug, and for daptomycin to a lesser extent, when tested in the presence of norspermidine and norspermine. We show here that norspermidine and norspermine markedly improve the diffusibility of antibiotics, especially those with a low molecular weight, like delafloxacin, and cause a commensurate improvement in activity, which is consistent with a weakening of the matrix meshwork. Compounds possessing a common motif consisting of three methylene groups flanked by two amino groups have been shown to collapse the exopolysaccharide network in *Bacillus subtilis* biofilms by interacting with negative charges and favoring biofilm disassembly (19). Other authors propose instead that norspermidine, added during culture at the concentration used in our experiments, can inhibit biofilm formation in an exopolysaccharide-independent manner by inhibiting *B. subtilis* growth (46). None of these authors examined the effect of norspermidine on preformed biofilms of *S. aureus*, but the fact that norspermidine drastically reduces both the content and size of the polysaccharides in the Surv2003/651 biofilm suggests that the first mechanism took place in our model. Yet, whatever the molecular reason for this synergy, our observations clearly open perspectives in the search of drug candidates with similar properties to be used as an adjuvant in biofilm treatments.

A third critical observation made in this study is that delafloxacin proved to be at least as potent and effective as daptomycin against these difficult-to-treat biofilms.

Daptomycin is nowadays considered to be an antibiotic of choice for the treatment of biofilm-related infections (47–51), and it showed activity against 5 of the 7 isolates tested in our study, if one considers clinically achievable concentrations. In the range of its foreseeable clinically relevant concentrations (28, 29), delafloxacin displayed activity against 6 of the 7 clinical isolates. Delafloxacin is tremendously more potent than other fluoroquinolones against *S. aureus*, with MICs as low as 0.001 mg/liter against susceptible strains and ranging from 0.015 to 1 mg/liter for strains with high-level resistance to both moxifloxacin and levofloxacin (MICs up to 8 and 64 mg/liter, respectively) (11, 52, 53). Therefore, delafloxacin may warrant further evaluation in *in vivo* biofilm models with Gram-positive organisms as a potential alternative to the currently available options. Our data also point to the interest in developing strategies aimed at restructuring the matrix to be combined with antibiotics in order to improve antibacterial activity.

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REFERENCES

1. **Römling U, Balsalobre C.** 2012. Biofilm infections, their resilience to therapy and innovative treatment strategies. J. Intern. Med. **272:**541–561. http://dx.doi.org/10.1111/joim.12004.
2. **Costerton JW, Stewart PS, Greenberg EP.** 1999. Bacterial biofilms: a common cause of persistent infections. Science **284:**1318–1322. http://dx.doi.org/10.1126/science.284.5418.1318.
3. **Lynch AS, Robertson GT.** 2008. Bacterial and fungal biofilm infections.

Annu. Rev. Med. **59:**415–428. http://dx.doi.org/10.1146/annurev.med.59.110106.132000.

4. **Parra-Ruiz J, Bravo-Molina A, Peña-Monje A, Hernández-Quero J.** 2012. Activity of linezolid and high-dose daptomycin, alone or in combination, in an *in vitro* model of *Staphylococcus aureus* biofilm. J. Antimicrob. Chemother. **67:**2682–2685. http://dx.doi.org/10.1093/jac/dks272.
5. **Mataraci E, Dosler S.** 2012. *In vitro* activities of antibiotics and antimicrobial cationic peptides alone and in combination against methicillin-resistant *Staphylococcus aureus* biofilms. Antimicrob. Agents Chemother. **56:**6366–6371. http://dx.doi.org/10.1128/AAC.01180-12.
6. **Kirby AE, Garner K, Levin BR.** 2012. The relative contributions of physical structure and cell density to the antibiotic susceptibility of bacteria in biofilms. Antimicrob. Agents Chemother. **56:**2967–2975. http://dx.doi.org/10.1128/AAC.06480-11.
7. **Parra-Ruiz J, Vidaillac C, Rose WE, Rybak MJ.** 2010. Activities of high-dose daptomycin, vancomycin, and moxifloxacin alone or in combination with clarithromycin or rifampin in a novel *in vitro* model of *Staphylococcus aureus* biofilm. Antimicrob. Agents Chemother. **54:**4329–4334. http://dx.doi.org/10.1128/AAC.00455-10.
8. **John AK, Schmaler M, Khanna N, Landmann R.** 2011. Reversible daptomycin tolerance of adherent staphylococci in an implant infection model. Antimicrob. Agents Chemother. **55:**3510–3516. http://dx.doi.org/10.1128/AAC.00172-11.
9. **Murillo O, Doménech A, Garcia A, Tubau F, Cabellos C, Gudiol F, Ariza J.** 2006. Efficacy of high doses of levofloxacin in experimental foreign-body infection by methicillin-susceptible *Staphylococcus aureus*. Antimicrob. Agents Chemother. **50:**4011–4017. http://dx.doi.org/10.1128/AAC.00523-06.
10. **Bauer J, Siala W, Tulkens PM, Van Bambeke F.** 2013. A combined pharmacodynamic quantitative and qualitative model reveals the potent activity of daptomycin and delafloxacin against *Staphylococcus aureus* biofilms. Antimicrob. Agents Chemother. **57:**2726–2737. http://dx.doi.org/10.1128/AAC.00181-13.
11. **Lemaire S, Tulkens PM, Van Bambeke F.** 2011. Contrasting effects of acidic pH on the extracellular and intracellular activities of the anti-Gram-positive fluoroquinolones moxifloxacin and delafloxacin against *Staphylococcus aureus*. Antimicrob. Agents Chemother. **55:**649–658. http://dx.doi.org/10.1128/AAC.01201-10.
12. **Ohshita Y, Yazaki A.** 1997. *In vitro* studies with WQ-3034, a newly synthesized acidic fluoroquinolone, abstr. F-164. Abstr. 37th Intersci. Conf. Antimicrob. Agents Chemother., Toronto, Canada. American Society for Microbiology, Washington, DC.
13. **Que YA, Moreillon P.** 2014. *Staphylococcus aureus* (including staphylococcal toxic shock), p 195. *In* Mandell GL, Bennett JE, Dolin R (ed), Mandell, Douglas, and Bennett's principles and practice of infectious diseases. Elsevier Churchill Livingstone, Philadelphia, PA.
14. **Denis O, Deplano A, Nonhoff C, De Ryck R, de Mendonça R, Rottiers S, Vanhoof R, Struelens MJ.** 2004. National surveillance of methicillin-resistant *Staphylococcus aureus* in Belgian hospitals indicates rapid diversification of epidemic clones. Antimicrob. Agents Chemother. **48:**3625–3629http://dx.doi.org/10.1128/AAC.48.9.3625-3629.2004.
15. **Denis O, Deplano A, De Beenhouwer H, Hallin M, Huysmans G, Garrino MG, Glupczynski Y, Malaviolle X, Vergison A, Struelens MJ.** 2005. Polyclonal emergence and importation of community-acquired methicillin-resistant *Staphylococcus aureus* strains harbouring Panton-Valentine leucocidin genes in Belgium. J. Antimicrob. Chemother. **56:**1103–1106. http://dx.doi.org/10.1093/jac/dki379.
16. **Clinical and Laboratory Standards Institute.** 2013. Performance standards for antimicrobial susceptibility testing. 23th informational supplement. CLSI MS100-S23. Clinical and Laboratory Standards Institute, Wayne, PA.
17. **Ramaswamy S, Dworkin M, Downard J.** 1997. Identification and characterization of *Myxococcus xanthus* mutants deficient in calcofluor white binding. J. Bacteriol. **179:**2863–2871.
18. **McLennan MK, Ringoir DD, Frirdich E, Svensson SL, Wells DH, Jarrell H, Szymanski CM, Gaynor EC.** 2008. *Campylobacter jejuni* biofilms up-regulated in the absence of the stringent response utilize a calcofluor white-reactive polysaccharide. J. Bacteriol. **190:**1097–1107. http://dx.doi.org/10.1128/JB.00516-07.
19. **Kolodkin-Gal I, Cao S, Chai L, Böttcher T, Kolter R, Clardy J, Losick R.** 2012. A self-produced trigger for biofilm disassembly that targets exopolysaccharide. Cell **149:**684–692. http://dx.doi.org/10.1016/j.cell.2012.02.055.
20. **Albalasmeh AA, Berhe AA, Ghezzehei TA.** 2013. A new method for rapid determination of carbohydrate and total carbon concentrations using UV spectrophotometry. Carbohydr. Polym. **97:**253–261. http://dx.doi.org/10.1016/j.carbpol.2013.04.072.
21. **Kim J, Pitts B, Stewart PS, Camper A, Yoon J.** 2008. Comparison of the antimicrobial effects of chlorine, silver ion, and tobramycin on biofilm. Antimicrob. Agents Chemother. **52:**1446–1453. http://dx.doi.org/10.1128/AAC.00054-07.
22. **Hunter RC, Beveridge TJ.** 2005. Application of a pH-sensitive fluoroprobe (C-SNARF-4) for pH microenvironment analysis in *Pseudomonas aeruginosa* biofilms. Appl. Environ. Microbiol. **71:**2501–2510. http://dx.doi.org/10.1128/AEM.71.5.2501-2510.2005.
23. **Muller-Borer BJ, Yang H, Marzouk SA, Lemasters JJ, Cascio WE.** 1998. pHi and pHo at different depths in perfused myocardium measured by confocal fluorescence microscopy. Am. J. Physiol. **275:**H1937–H1947.
24. **Baudoux P, Bles N, Lemaire S, Mingeot-Leclercq MP, Tulkens PM, Van Bambeke F.** 2007. Combined effect of pH and concentration on the activities of gentamicin and oxacillin against *Staphylococcus aureus* in pharmacodynamic models of extracellular and intracellular infections. J. Antimicrob. Chemother. **59:**246–253. http://dx.doi.org/10.1093/jac/dkl489.
25. **Pibalpakdee P, Wongratanacheewin S, Taweechaisupapong S, Niumsup PR.** 2012. Diffusion and activity of antibiotics against *Burkholderia pseudomallei* biofilms. Int. J. Antimicrob. Agents **39:**356–359. http://dx.doi.org/10.1016/j.ijantimicag.2011.12.010.
26. **Singh R, Ray P, Das A, Sharma M.** 2010. Penetration of antibiotics through *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. J. Antimicrob. Chemother. **65:**1955–1958. http://dx.doi.org/10.1093/jac/dkq257.
27. **Anderl JN, Franklin MJ, Stewart PS.** 2000. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. Antimicrob. Agents Chemother. **44:**1818–1824. http://dx.doi.org/10.1128/AAC.44.7.1818-1824.2000.
28. **Lawrence L, Benedict M, Hart J, Hawkins A, Danping L, Medlock M, Hopkins S, Burak E.** 2011. Pharmacokinetics (PK) and safety of single doses of delafloxacin administered intravenously in healthy human subjects, poster A2-045a. 51th Intersci. Conf. Antimicrob. Agents Chemother., Chicago, IL. American Society for Microbiology, Washington, DC.
29. **Rubino CM, Bhavnani SM, Burak E, Ambrose PG.** 2010. Pharmacokinetic-pharmacodynamic target attainment analyses supporting delafloxacin phase 3 dose regimen decisions, poster A1-681. Abstr. 50th Intersci. Conf. Antimicrob. Agents Chemother., Boston, MA. American Society for Microbiology, Washington, DC.
30. **Gil C, Solano C, Burgui S, Latasa C, García B, Toledo-Arana A, Lasa I, Valle J.** 2014. Biofilm matrix exoproteins induce a protective immune response against *Staphylococcus aureus* biofilm infection. Infect. Immun. **82:**1017–1029. http://dx.doi.org/10.1128/IAI.01419-13.
31. **Kogan G, Sadovskaya I, Chaignon P, Chokr A, Jabbouri S.** 2006. Biofilms of clinical strains of *Staphylococcus* that do not contain polysaccharide intercellular adhesin. FEMS Microbiol. Lett. **255:**11–16. http://dx.doi.org/10.1111/j.1574-6968.2005.00043.x.
32. **Sadovskaya I, Chaignon P, Kogan G, Chokr A, Vinogradov E, Jabbouri S.** 2006. Carbohydrate-containing components of biofilms produced *in vitro* by some staphylococcal strains related to orthopaedic prosthesis infections. FEMS Immunol. Med. Microbiol. **47:**75–82. http://dx.doi.org/10.1111/j.1574-695X.2006.00068.x.
33. **Mann EE, Rice KC, Boles BR, Endres JL, Ranjit D, Chandramohan L, Tsang LH, Smeltzer MS, Horswill AR, Bayles KW.** 2009. Modulation of eDNA release and degradation affects *Staphylococcus aureus* biofilm maturation. PLoS One **4:**e5822. http://dx.doi.org/10.1371/journal.pone.0005822.
34. **Atshan SS, Shamsudin MN, Sekawi Z, Lung LTT, Hamat RA, Karunanidhi A, Ali AM, Ghaznavi-Rad E, Ghasemzadeh-Moghaddam H, Seng JSC, Nathan JJ, Pei CP.** 2012. Prevalence of adhesion and regulation of biofilm-related genes in different clones of *Staphylococcus aureus*. J. Biomed. Biotechnol. **2012:**976972. http://dx.doi.org/10.1155/2012/976972.
35. **Mascio CT, Alder JD, Silverman JA.** 2007. Bactericidal action of daptomycin against stationary-phase and nondividing *Staphylococcus aureus* cells. Antimicrob. Agents Chemother. **51:**4255–4260. http://dx.doi.org/10.1128/AAC.00824-07.
36. **Mah TF, O'Toole GA.** 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. **9:**34–39. http://dx.doi.org/10.1016/S0966-842X(00)01913-2.

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37. **Croes S, Deurenberg RH, Boumans MLL, Beisser PS, Neef C, Stobberingh EE.** 2009. *Staphylococcus aureus* biofilm formation at the physiologic glucose concentration depends on the *S. aureus* lineage. BMC Microbiol. **9:**229. http://dx.doi.org/10.1186/1471-2180-9-229.
38. **Barcia-Macay M, Seral C, Mingeot-Leclercq MP, Tulkens PM, Van Bambeke F.** 2006. Pharmacodynamic evaluation of the intracellular activities of antibiotics against *Staphylococcus aureus* in a model of THP-1 macrophages. Antimicrob. Agents Chemother. **50:**841–851. http://dx.doi.org/10.1128/AAC.50.3.841-851.2006.
39. **Rodríguez-Martínez JM, Ballesta S, Pascual A.** 2007. Activity and penetration of fosfomycin, ciprofloxacin, amoxicillin/clavulanic acid and co-trimoxazole in *Escherichia coli* and *Pseudomonas aeruginosa* biofilms. Int. J. Antimicrob. Agents **30:**366–368. http://dx.doi.org/10.1016/j.ijantimicag.2007.05.005.
40. **Stewart PS.** 2002. Mechanisms of antibiotic resistance in bacterial biofilms. Int. J. Med. Microbiol. **292:**107–113. http://dx.doi.org/10.1078/1438-4221-00196.
41. **Jefferson KK, Goldmann DA, Pier GB.** 2005. Use of confocal microscopy to analyze the rate of vancomycin penetration through *Staphylococcus aureus* biofilms. Antimicrob. Agents Chemother. **49:**2467–2473. http://dx.doi.org/10.1128/AAC.49.6.2467-2473.2005.
42. **Daddi Oubekka S, Briandet R, Fontaine-Aupart MP, Steenkeste K.** 2012. Correlative time-resolved fluorescence microscopy to assess antibiotic diffusion-reaction in biofilms. Antimicrob. Agents Chemother. **56:**3349–3358. http://dx.doi.org/10.1128/AAC.00216-12.
43. **Stewart PS, Davison WM, Steenbergen JN.** 2009. Daptomycin rapidly penetrates a *Staphylococcus epidermidis* biofilm. Antimicrob. Agents Chemother. **53:**3505–3507. http://dx.doi.org/10.1128/AAC.01728-08.
44. **Pogliano J, Pogliano N, Silverman JA.** 2012. Daptomycin-mediated reorganization of membrane architecture causes mislocalization of essential cell division proteins. J. Bacteriol. **194:**4494–4504. http://dx.doi.org/10.1128/JB.00011-12.
45. **Stewart PS.** 2003. Diffusion in biofilms. J. Bacteriol. **185:**1485–1491. http://dx.doi.org/10.1128/JB.185.5.1485-1491.2003.
46. **Hobley L, Kim SH, Maezato Y, Wyllie S, Fairlamb AH, Stanley-Wall NR, Michael AJ.** 2014. Norspermidine is not a self-produced trigger for biofilm disassembly. Cell **156:**844–854. http://dx.doi.org/10.1016/j.cell.2014.01.012.
47. **Meije Y, Almirante B, Del Pozo JL, Martín MT, Fernández-Hidalgo N, Shan A, Basas J, Pahissa A, Gavaldà J.** 2014. Daptomycin is effective as antibiotic-lock therapy in a model of *Staphylococcus aureus* catheter-related infection. J. Infect. http://dx.doi.org/10.1016/j.jinf.2014.01.001.
48. **Seaton RA, Malizos KN, Viale P, Gargalianos-Kakolyris P, Santantonio T, Petrelli E, Pathan R, Heep M, Chaves RL.** 2013. Daptomycin use in patients with osteomyelitis: a preliminary report from the EU-CORE^{SM} database. J. Antimicrob. Chemother. **68:**1642–1649. http://dx.doi.org/10.1093/jac/dkt067.
49. **Domínguez-Herrera J, Docobo-Pérez F, López-Rojas R, Pichardo C, Ruiz-Valderas R, Lepe JA, Pachón J.** 2012. Efficacy of daptomycin versus vancomycin in an experimental model of foreign-body and systemic infection caused by biofilm producers and methicillin-resistant *Staphylococcus epidermidis*. Antimicrob. Agents Chemother. **56:**613–617. http://dx.doi.org/10.1128/AAC.05606-11.
50. **Van Praagh AD, Li T, Zhang S, Arya A, Chen L, Zhang XX, Bertolami S, Mortin LI.** 2011. Daptomycin antibiotic lock therapy in a rat model of staphylococcal central venous catheter biofilm infections. Antimicrob. Agents Chemother. **55:**4081–4089. http://dx.doi.org/10.1128/AAC.00147-11.
51. **LaPlante KL, Woodmansee S.** 2009. Activities of daptomycin and vancomycin alone and in combination with rifampin and gentamicin against biofilm-forming methicillin-resistant *Staphylococcus aureus* isolates in an experimental model of endocarditis. Antimicrob. Agents Chemother. **53:**3880–3886. http://dx.doi.org/10.1128/AAC.00134-09.
52. **Remy JM, Tow-Keogh CA, McConnell TS, Dalton JM, Devito JA.** 2012. Activity of delafloxacin against methicillin-resistant *Staphylococcus aureus*: resistance selection and characterization. J. Antimicrob. Chemother. **67:**2814–2820. http://dx.doi.org/10.1093/jac/dks307.
53. **Almer LS, Hoffrage JB, Keller EL, Flamm RK, Shortridge VD.** 2004. *In vitro* and bactericidal activities of ABT-492, a novel fluoroquinolone, against Gram-positive and Gram-negative organisms. Antimicrob. Agents Chemother. **48:**2771–2777. http://dx.doi.org/10.1128/AAC.48.7.2771-2777.2004.

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Supplemental material

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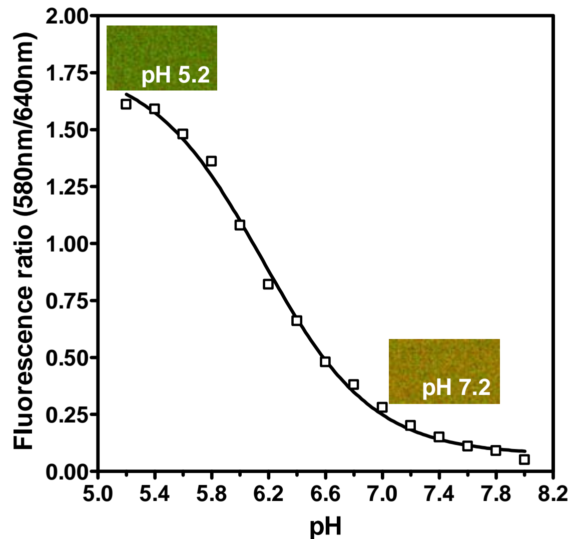
Figure S1

Calibration curve used for pH determination within biofilm. C-SNARF-4 solutions at different pH were examined in confocal scanning laser microscopy and fluorescence was recoded at 580 and 640 nm upon excitation at 488 nm. The built equation [calculated from the equation of the sigmoidal regression : Fluorescence ratio =BOTTOM + (TOP-BOTTOM)/(1+10^((LogEC50-pH)*HILLSLOPE))] was used to estimate pH within biofilms stained with C-SNARF-4.



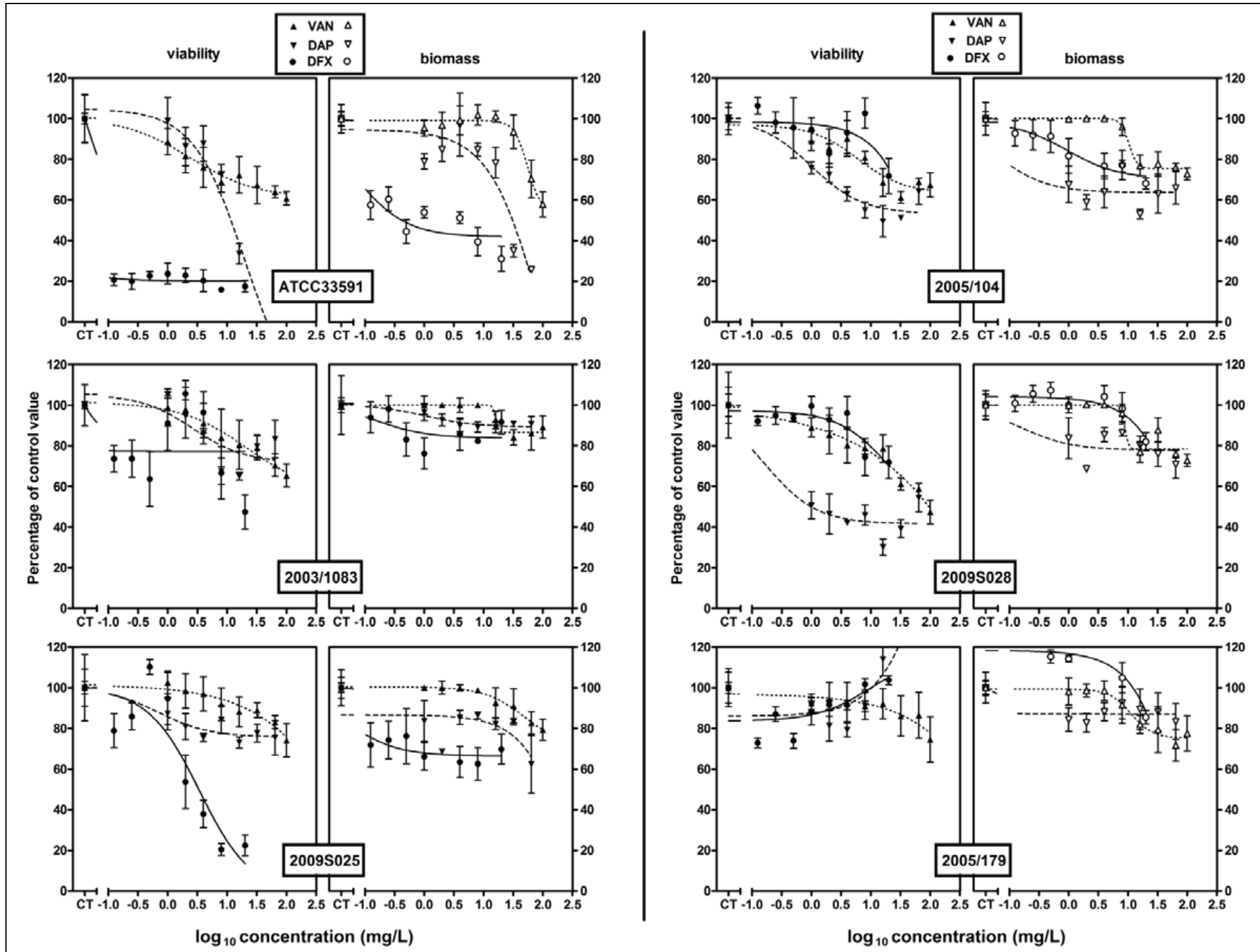


Figure S2

Concentration-response activity of antibiotics against 24 h biofilms of clinical isolates and of ATCC33591 (reference strain). 24h biofilms were incubated with increasing concentrations of antibiotics for 48 h (DFX: delafloxacin, DAP: daptomycin; VAN: vancomycin). The ordinate shows the change in viability (assessed by the resorufin fluorescence; left) or in biomass (assessed by crystal violet absorbance; right) in percentage of the control value (no antibiotic present). All values are means ± standard deviations (SD) of 8 wells (when not visible, the SD bars are smaller than the size of the symbols).

Figure S3

Correlation between capacity of forming biofilms in control conditions (left: viability, as assessed by resorufin [RF] fluorescence; middle, biomass, as assessed by crystal violet [CV] absorbance) or the relative proportion of polysaccharide in each biofilm matrix (as assessed by the calcofluor white [CFW] fluorescence-to-crystal violet [CV] absorbance ratio) and the relative potency of antibiotics (C_{25} [concentration reducing viability of 25%] as determined based on concentration-response curves presented in Figures 2 and S2). DFX: delafloxacin, DAP: daptomycin; VAN: vancomycin). Data from MRSA ATCC33591 and all clinical isolates. Signification probability values from the analysis of variance.

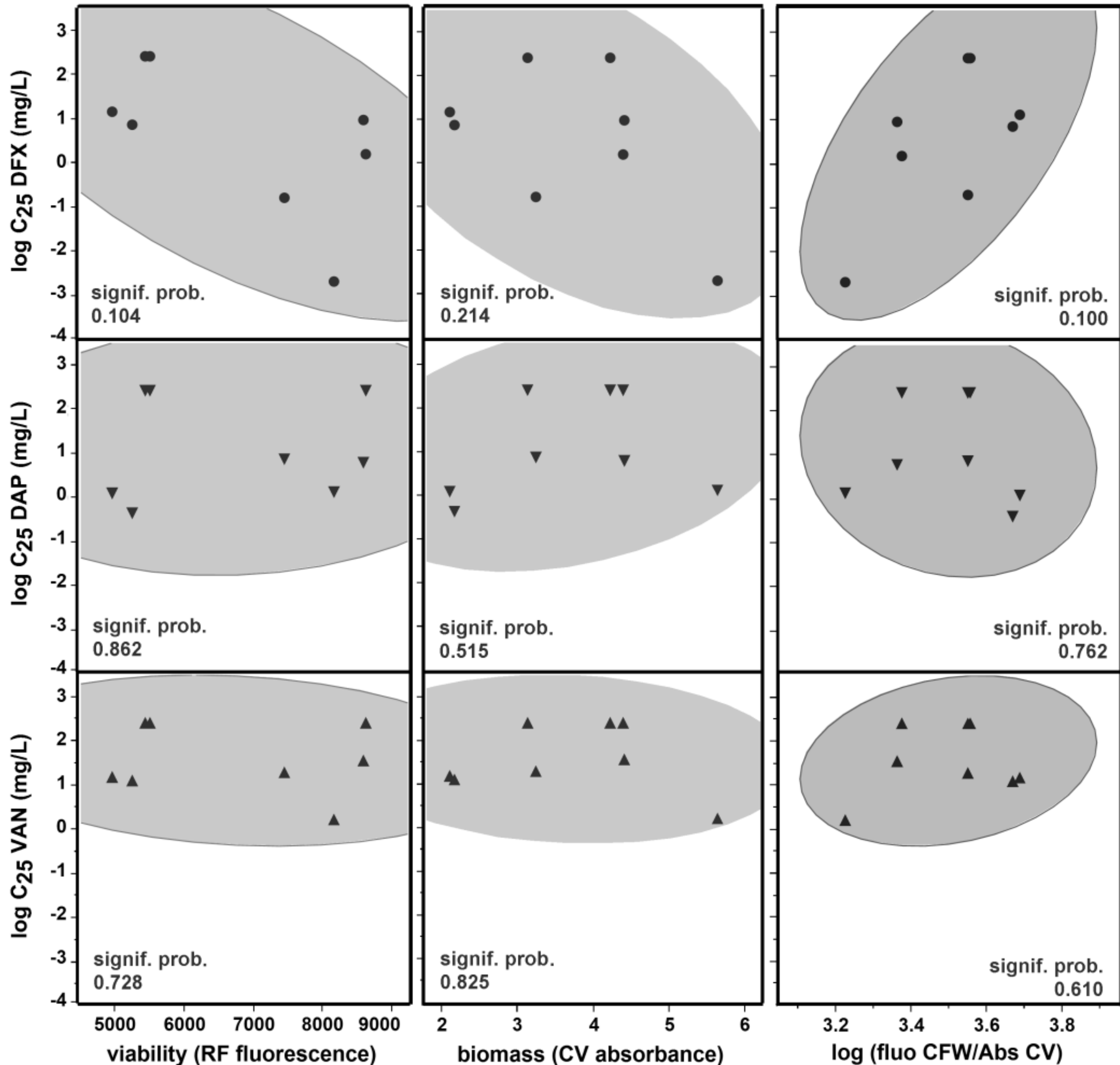


Figure S4

<u>Top panel</u>: Evaluation of micropH within biofilm of clincial isolates as evaluated in confocal microscopy using C-SNARF-4 as a pH sensitive probe (fluorescence emitted shifting from red to green upon acidification). The graph below each image shows the corresponding micropH in the deepness of the biofilm.
<u>Middle panel:</u> influence of pH on the MIC of delafloxacin in MHB adjusted to different pH values. Gray squares highlight the range of pH measured in the corresponding biofilm.

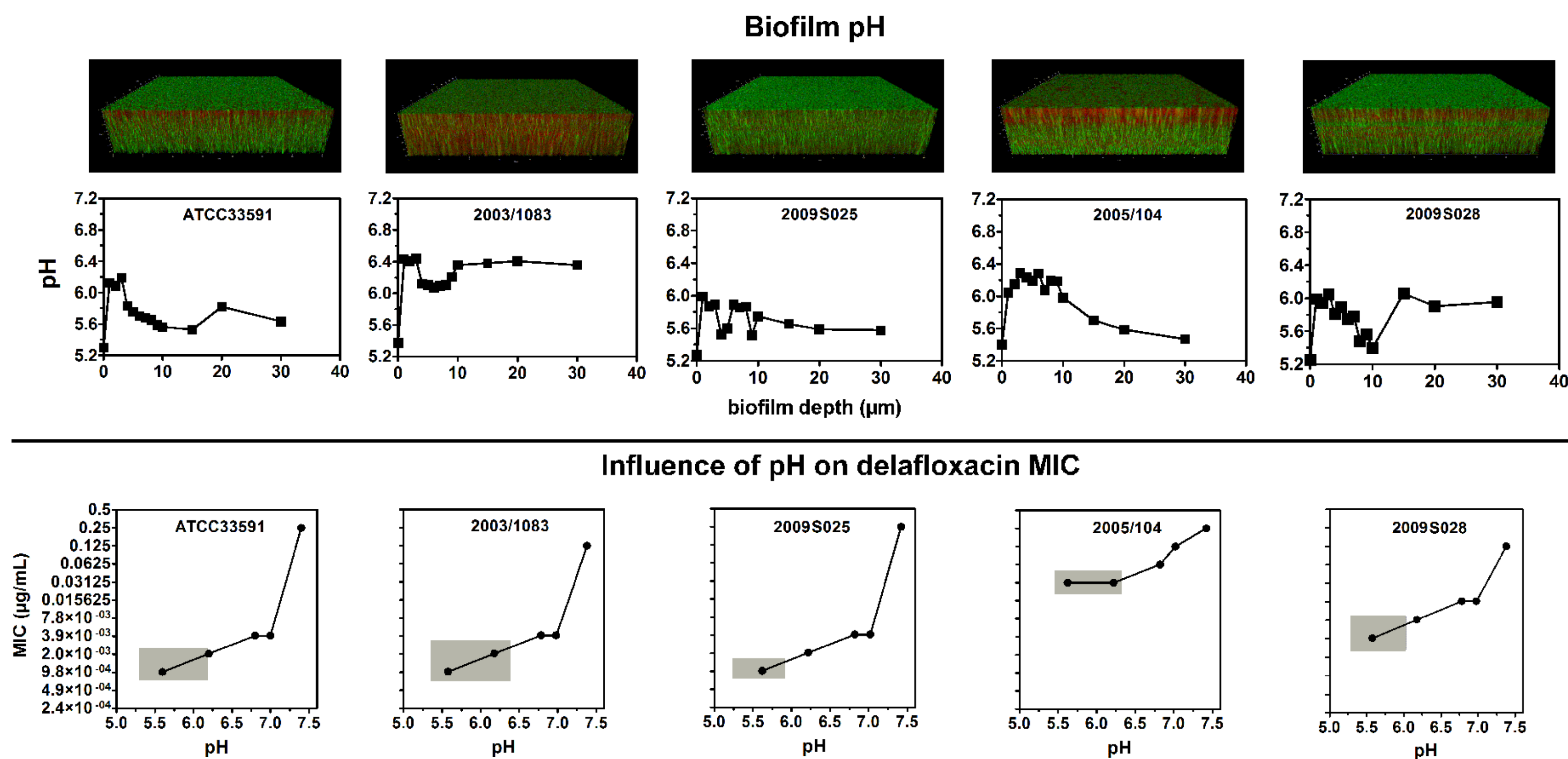


Figure S5 :

Confocal images of biofilm incubated during 1 h with 50 mg/L delafloxacin (top [blue]), 20 mg/L Bodipy-FL daptomycin (middle [green]) or 20 mg/L Bodipy-FL vancomycin (bottom [green]) and labeled with live/dead staining (top [red: dead; green: live]) or CTC (bottom [red]).
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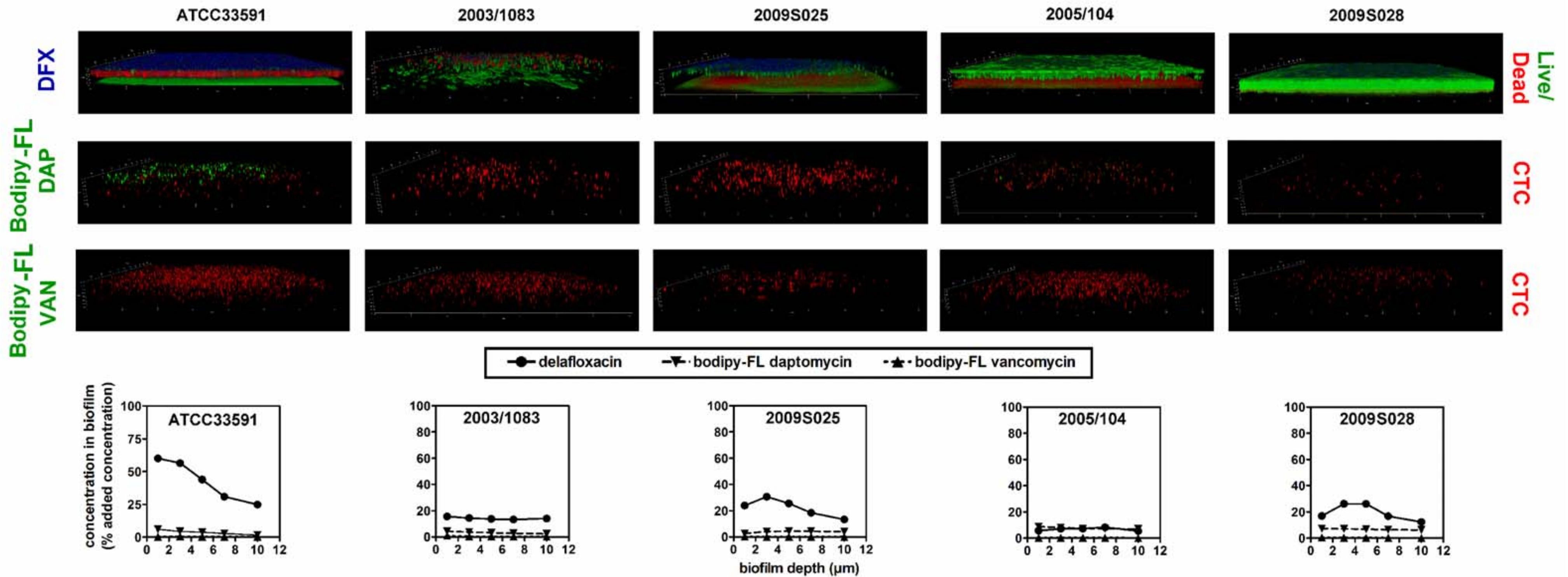

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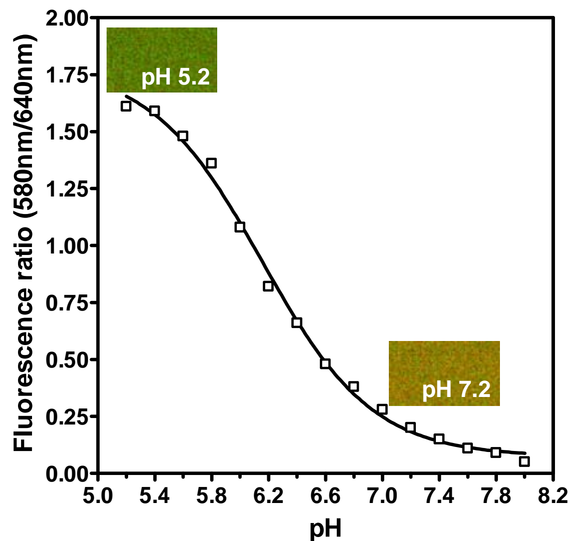

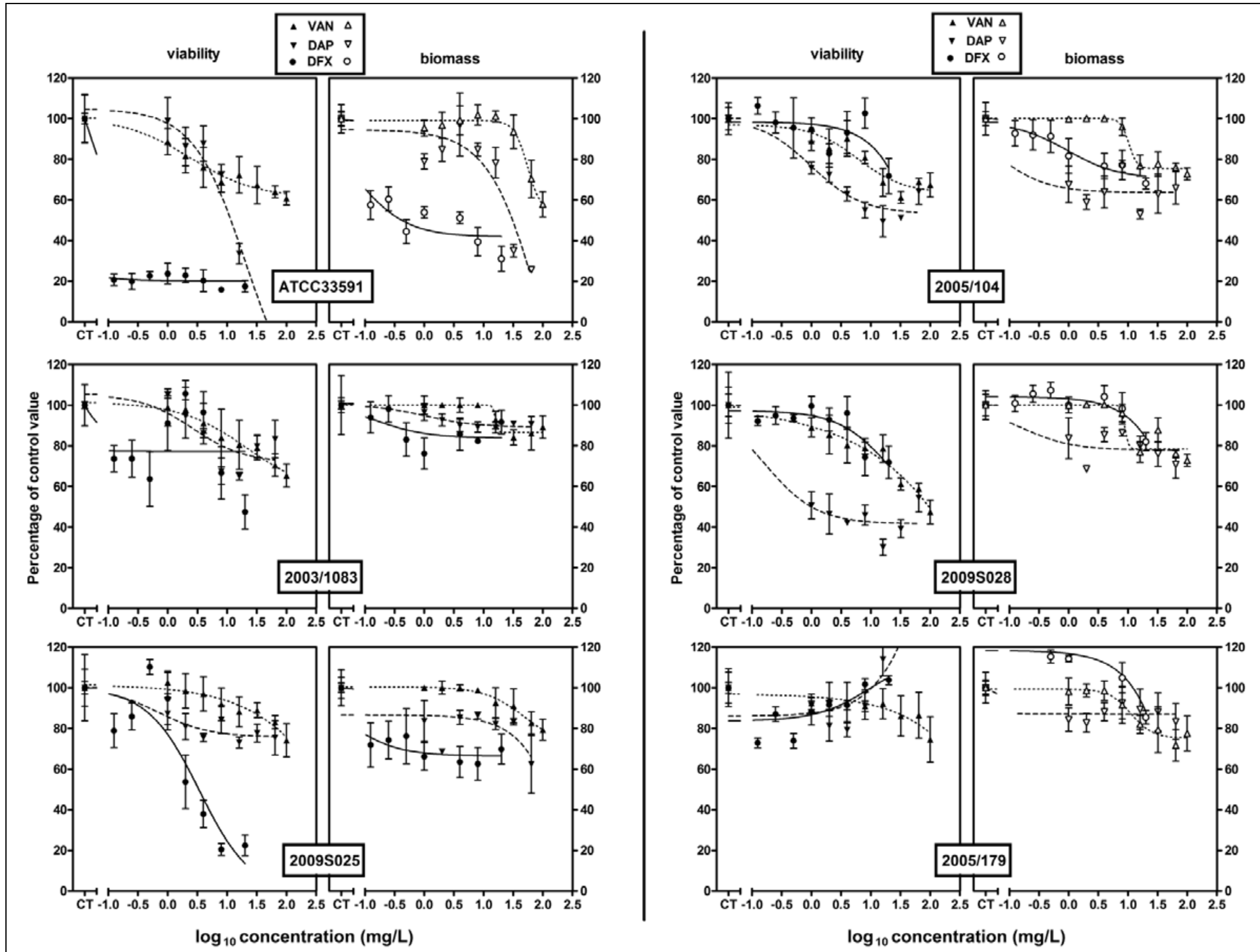

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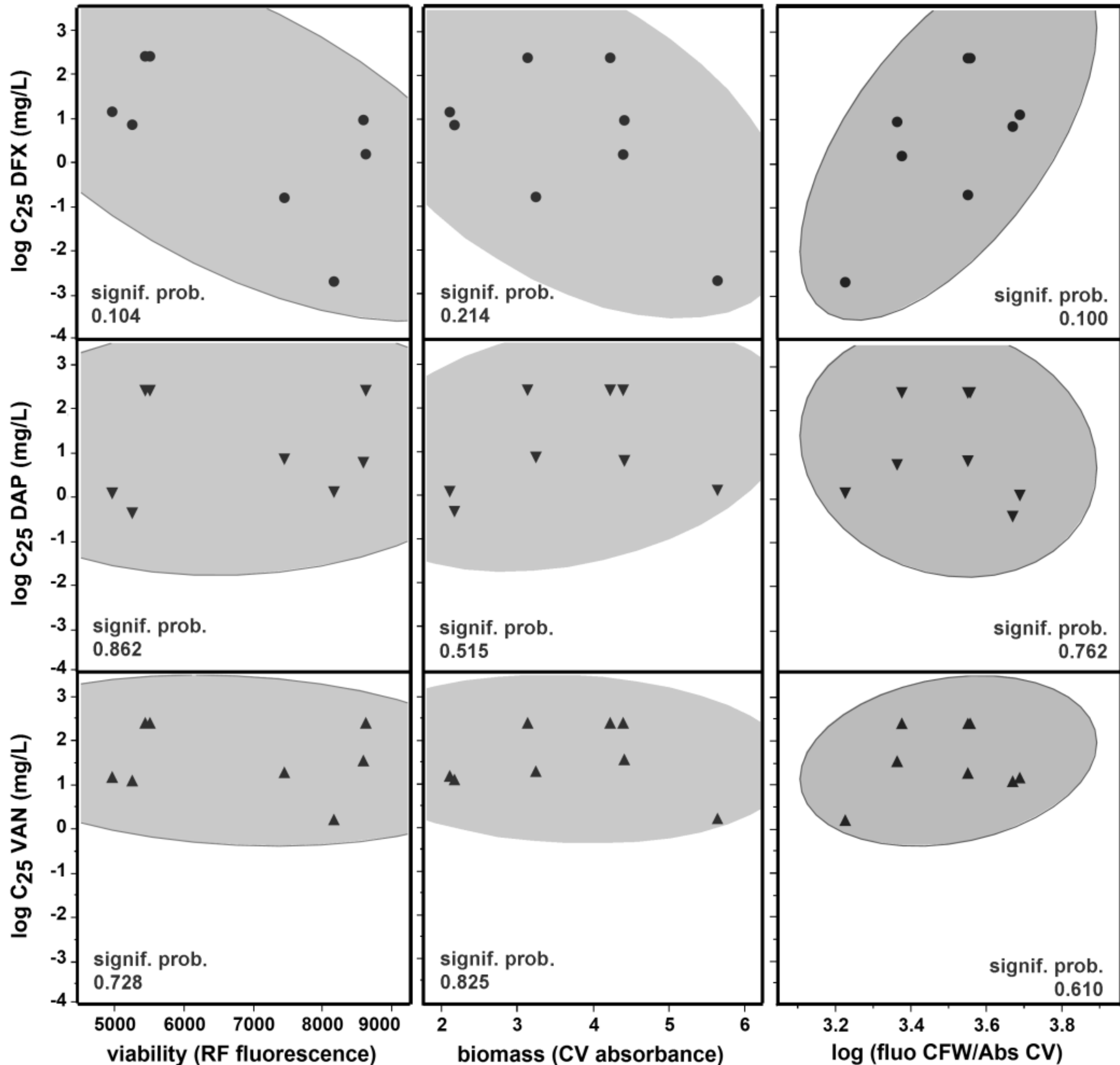

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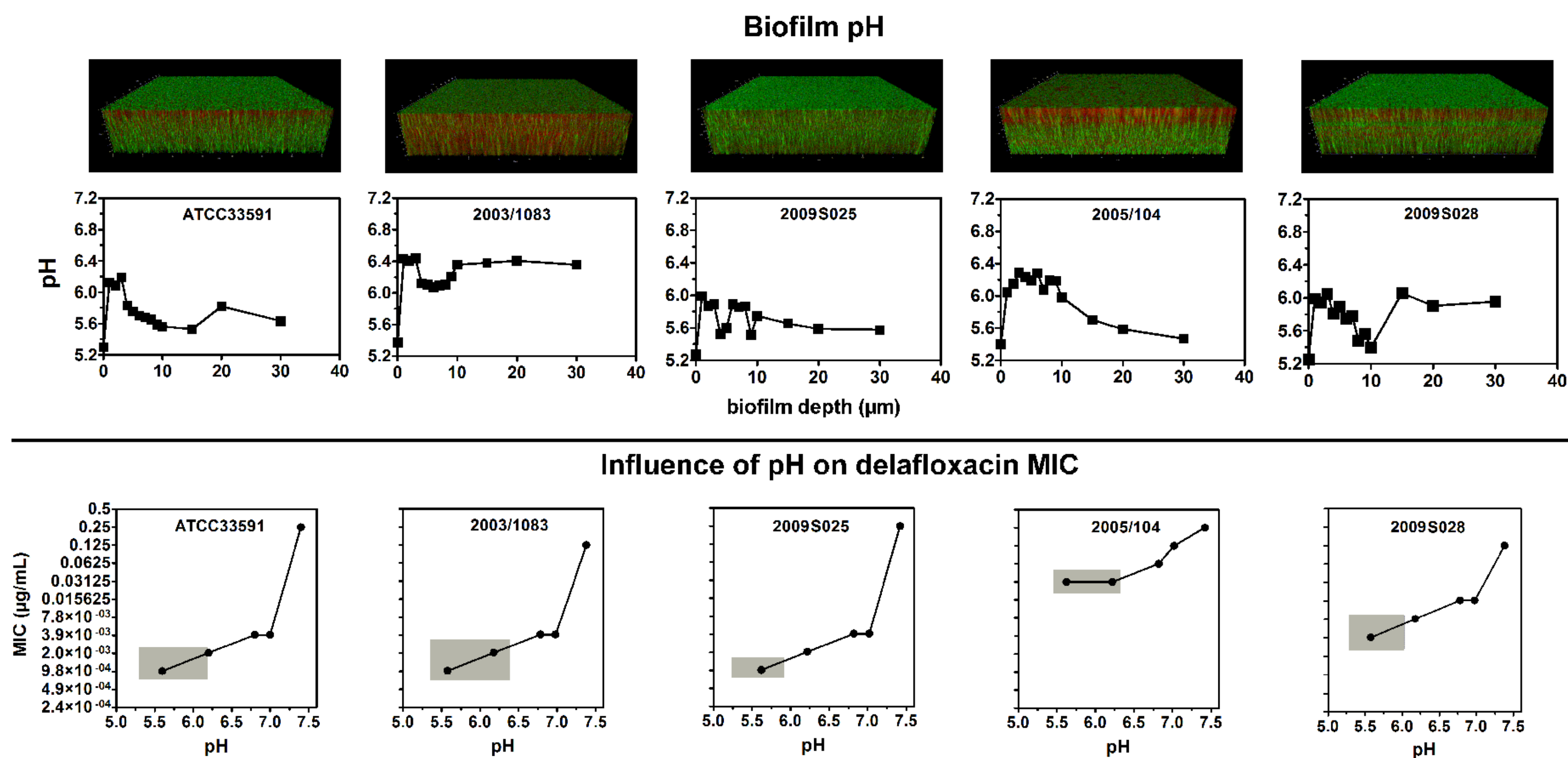

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