

New Insight into Daptomycin Bioavailability and Localization in Staphylococcus aureus Biofilms by Dynamic Fluorescence Imaging

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New insight into daptomycin bioavailability and localization in S. aureus biofilms by dynamic

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AAC

18 Staphylococcus aureus (S. aureus) is one of the most frequent pathogens responsible for biofilm-19 associated infections (BAI) and the choice of antibiotics to treat these infections remains a challenge 20 for the medical community. In particular, daptomycin has been reported to fail against implant-21 associated S. aureus infections in clinical practice while its association with rifampicin remains a good 22 candidate for BAI treatment. To improve our understanding of such resistance/tolerance toward 23 daptomycin, we took advantage of the dynamic fluorescence imaging tools (time-lapse imaging and 24 FRAP) to locally and accurately assess the antibiotic diffusion-reaction in methicillin-susceptible and 25 methicillin-resistant S. aureus biofilms. To provide a realistic representation of daptomycin action, we optimized an in vitro model built on the basis of our recently published in vivo mouse model of 26 27 prosthetic vascular graft infections. We demonstrated that at therapeutic concentrations, daptomycin 28 was inefficient to eradicate biofilms while the matrix was not a shield to the antibiotic diffusion and to 29 its interaction with its bacterial target. In the presence of rifampicin, daptomycin was still present in the 30 vicinity of the bacterial cells allowing the prevention of the emergence of rifampicin-resistant mutants. 31 Conclusions derived from this study strongly suggest that S. aureus biofilms resistance/tolerance 32 towards daptomycin may be more likely related to a physiological change involving structural 33 modifications of the membrane, which is a strain-dependent process.

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35 INTRODUCTION

Staphylococcus aureus (S. aureus) is a Gram-positive bacteria shown to be the most frequent cause of biofilm-associated infections (BAI) (1) and one of the major cause of morbidity and mortality in hospitals and communities (2). Unlike planktonic cells, biofilms exhibit specific phenotypic traits allowing them to resist host defenses and antibiotic treatments (3), which frequently leads to chronic infections such as endocarditis, sinusitis, and osteomyelitis but also to implant-associated infections (4).

42 Among the most recent clinically-used antibiotics, daptomycin is a cyclic lipopeptide approved 43 for the treatment of serious staphylococcal infections such as bacteremia and implant-related infections 44 (5). Daptomycin is a calcium-dependent antibiotic, acting by insertion into the Gram-positive cytoplasmic membranes where it forms oligomeric pores, causing potassium ion leakage and 45 46 subsequent membrane depolarization, leading ultimately to cell death (6). As is the case for many 47 antibiotics, daptomycin has been shown to exhibit a significant bactericidal activity against planktonic cells (7–9). However, the eradication of adherent bacteria is rarely achieved despite the large number of 48 in vitro and animal studies in which daptomycin activity was evaluated (10-16). Besides the results of 49 50 the literature that appear controversial (17), direct comparison between studies is not directly possible 51 since biofilm growth and treatment protocols used greatly differ.

To get a realistic representation of daptomycin action against *S. aureus* biofilms, we developed an *in vitro* model built on the basis of our recently published *in vivo* study on *S. aureus* prosthetic vascular graft infections using the same strains (18). The interest of this approach was the possibility to use fluorescence imaging techniques that cannot be employed *in vivo* (confocal laser scanning microscopy, time-lapse and fluorescence recovery after photobleaching) to examine the penetration, diffusion, bioavailability and localization of the fluorescently-labelled antibiotic inside the biofilms. To validate this approach, we monitored for 72 h the activity of daptomycin against biofilms formed by Page **3** of **30** 59 methicillin-susceptible and methicillin-resistant clinical and collection strains. In addition, we enriched 60 the culture medium with proteins and calcium to mimic the *in vivo* physiological conditions of our 61 mouse model (18). The same experiments were performed in the presence of rifampicin, an antibiotic 62 that can be combined with daptomycin for recalcitrant *S. aureus* BAI.

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64 MATERIALS AND METHODS

65 Bacterial strains

Four *S. aureus* strains were tested in the present study: two of them were collection strains (methicillinsusceptible *S. aureus* (MSSA) ATCC 27217 and methicillin-resistant *S. aureus* (MRSA) ATCC 33591) and two others were isolated from patients with *S. aureus* bloodstream infections (MSSA 176 and MRSA BCB8). All the strains were kept at -80°C in Tryptic Soy Broth (TSB; Biomérieux, France) containing 20% (vol/vol) glycerol. The frozen cells were subcultured twice in TSB (one 8-hour culture followed by an overnight culture) to constitute the stock cultures from which aliquots were kept at -20°C. Bacterial growth and experiments were both conducted at 37°C.

73 Antimicrobial agents and medium

74 Daptomycin and rifampicin were both purchased from Sigma (France). The fluorescently-labelled 75 antibiotic BODIPY-FL®-daptomycin was a kind gift from Cubist Pharmaceuticals (MA, USA) and 76 BODIPY-FL® was purchased from Invitrogen (France). According to the manufacturer's instructions, 77 the stock solutions were prepared by diluting daptomycin and BODIPY®-FL-daptomycin in dimethylsulfoxide (1 mg/mL), and rifampicin in sterile water (2 mg/mL), further kept at -20°C. Before 78 79 use, the solutions were diluted in TSB enriched with proteins (BSA, Bovine Serum Albumin, 36 g/L; Sigma, France) and calcium (CaCl₂, 2H₂O, 50 mg/L; Sigma, France) to mimic in vivo physiological 80 81 levels. It has been checked that, in these conditions, the final concentration of dimethylsulfoxide was

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non-cytotoxic for the bacteria. Clinically-meaningful concentrations were used in this study: 20 µg/mL
for both daptomycin and rifampicin. When combined, the antibiotics were mixed together before
application above the biofilm surface.

85 Susceptibility testing

The MICs of daptomycin and rifampicin were determined by the broth microdilution method in cationadjusted Mueller-Hinton broth (CAMHB), according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Media were supplemented with 50 mg/L Ca²⁺ for daptomycin.

89 Characterization of molecular interactions between daptomycin and rifampicin by absorption 90 and fluorescence spectroscopies

Absorption spectra of antibiotics alone and in combination were measured with a Varian's Cary® 300 spectrophotometer (Agilent Technologies, France). The corresponding fluorescence emission spectra were recorded using a Fluorolog-3 (Jobin Yvon, Inc., France) fluorescence spectrophotometer mounted with front-face detection geometry by exciting daptomycin at 360 nm. The measurements were made in quintuplicate on each sample.

96 In vitro biofilm preparation and antibiotics activities

97 Biofilms were studied in a polystyrene microtiter plate-based assay since it has been shown that this 98 material has physicochemical properties close to that of biomaterial surfaces such as polyethylene 99 terephthalate that is used in vascular grafts (19, 20). For the preparation of S. aureus biofilms, 250-µL 100 portions of an overnight subculture adjusted to an optical density of 0.02 at 600 nm (corresponding to ${\sim}10^8$ CFU/mL) were added to 96-well microplates (µClear; Greiner Bio-One, France). After a 1.5-h 101 102 adhesion period at 37°C, the wells were rinsed with sterile physiological water (150 mM NaCl) in order 103 to eliminate non-adherent cells, refilled with sterile TSB enriched with proteins and calcium (TSBpc) 104 and then incubated for 24 h at 37°C to allow biofilm growth.

105 To assess antibiotics activities, the 24 h-biofilms were rinsed with a 150 mM NaCl aqueous solution 106 before adding the antibiotics solutions diluted in TSBpc as described previously. Viable culturable 107 bacteria were then counted at regular interval times: 0 h (when antibiotics are added), 24, 48 and 72 h 108 after antibiotics injection. For each time, bacterial cultures were centrifuged 10 min at 7000g in order to 109 eliminate the excess of antibiotic. The bacterial pellet was dispersed in NaCl 150 mM sterile saline 110 solution, centrifuged again and dispersed in the same conditions. Successive decimal dilutions were 111 then realized. For each dilution, six drops (10 μ L) were deposited on Tryptic Soy Agar (TSA) plates (Biomérieux, France) and incubated at 37°C during 24 h. CFUs were counted and averaged for each 112

113 dilution at each time. The detection limit of viable culturable cells was 100 CFU/mL.

114 Percentage of rifampicin-resistant mutants in biofilms

115 To measure percentages of rifampicin-resistant mutants, the antibiotics solutions (rifampicin combined or not with daptomycin) were added to 24 h-biofilms. CFUs were counted on TSA plates containing or 116 not rifampicin (20 µg/mL) at 24 and 48 h after antibiotics injections. The percentages were obtained by 117 118 calculating the ratio between the number of CFUs grown on rifampicin-containing TSA plates and the 119 number of CFUs grown on rifampicin-free TSA plates.

120 **Statistical analysis**

The mean \log_{10} CFU/mL and biovolumes for each therapy were compared with each other using 121 122 ANOVA variance analyses. They were performed using the Statgraphics software (Manugistics, 123 Rockville, USA). Significance was defined as a P-value associated with a Fisher test value lower than 124 0.05.

125 Confocal Laser Scanning Microscopy (CLSM)

126 Visualization of antibiotics activities against biofilms using Live/Dead® staining

127 24 h-biofilms prepared as described previously were observed 24, 48 and 72 h after antibiotics addition using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, France) implemented 128 Page 6 of 30 Antimicrobial Agents and

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129 at the Centre de Photonique Biomédicale (CPBM, Orsay, France). Prior to each observation, bacteria 130 were stained with 2.5 µM Syto9[®] (Life Technologies, France), a green cell-permeant nucleic acid dye, 131 and 30 µM propidium iodide (PI) (Life Technologies, France), a red nucleic acid dye that can penetrate 132 cells with compromised membranes (dead cells) only. Syto9® and PI were sequentially excited at 488 133 nm and 543 nm respectively and their fluorescence emissions were collected between 500 and 600 nm 134 for Syto9® and between 640 and 750 nm for PI. Images were acquired using a 63x oil immersion 135 objective with a 1.4 numerical aperture. The size of the confocal images was 512 x 512 pixels (82 x 82 μ m²), recorded with a z-step of 1 μ m. For each biofilm, four typical regions were analyzed. Images 136

137 were reconstructed in 3D using ICY® Software.

Quantification of biovolumes and maximum thickness

Maximum thickness (µm) was measured directly from xyz stacks. Biovolumes (µm³) were calculated by binarizing images with a java script executed by ICY as described previously (21). The biovolume was then defined as the overall volume of cells in the observation field. The percentage of dead cells corresponds to the ratio between biovolumes of PI-stained bacteria and Syto9®-stained bacteria.

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Antibiotics penetration and localization inside biofilms

To study the penetration of BODIPY®-FL-daptomycin and its combination with rifampicin within 24 h-biofilms, we employed time-lapse microscopy, as described before (22), using the same Leica TCS SP5 confocal microscope. Briefly, the fluorescence intensity evolution over time was observed in a defined focal plane (5 μm above the substratum surface). As soon as the TSBpc-diluted solutions of BODIPY-FL®-daptomycin combined or not with rifampicin were added above the biofilm, fluorescence intensity images were acquired every second during 15 min. Simultaneously, transmission images were acquired to ensure that no structural alteration of the biofilm occurred during this process.

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The labelled antibiotic was excited with a continuous argon laser line at 488 nm through a 63x oil immersion objective and the emitted fluorescence was recorded within 500 and 600 nm.

The corresponding diffusive penetration coefficients (D_p) through the biofilms were determined according to the relationship previously described by Stewart (23):

$$D_{\rm p} = 1.03 \text{ x } L^2 / t_{90} \tag{Eq. 1}$$

where L is the biofilm thickness and t_{90} is the time required to attain 90% of the equilibrium staining intensity at the deeper layers of the biofilm.

To observe the localization of the fluorescently-labelled daptomycin within biofilms, bacteria were counterstained with the FM4-64 dye (Life Technologies, France): this dye was also excited at 488 nm but its fluorescence emission was collected within the range 640-750 nm. The images (512 x 512 pixels) of both fluorophores were simultaneously recorded with a z-step of 1 μ m.

162 Antibiotic diffusion and bioavailability inside biofilms using FRAP experiments

163 Image-based Fluorescence Recovery after Photobleaching (FRAP) measurements was used to assess 164 local diffusion and bioavailability of the fluorescently-labelled daptomycin. Briefly, FRAP is based on a brief excitation of fluorescent molecules by a very intense light source in a user-defined region to 165 166 irreversibly photobleach their fluorescence. Fluorescence recovery is then probed over time at a low 167 light power in the same photobleached region (22, 24). All time-resolved measurements were obtained using the same confocal microscope. The time course of fluorescence intensity recovery was analyzed 168 169 with mathematical models, giving access to the quantitative mobility of the fluorescent molecules and 170 allowing to determine the diffusion coefficients. For all FRAP experiments, the fluorescence intensity 171 image size was fixed to 512 x 128 pixels with an 80-nm pixel size and recorded using a 12-bit resolution. The line scan rate was fixed to 1400 Hz, corresponding to a total time between frames of \sim 172 173 265 ms. As determined previously, the full widths at half maximum in xy and z (along the optical axis)

of the bleached profile were 0.8 μ m and 14 μ m, respectively, allowing to neglect diffusion along the axial/vertical axis and thus to consider only two-dimensional diffusion. Each FRAP experiment started with the acquisition of 50 images at 7% of laser maximum intensity (7 μ W) followed by a 200-ms single bleached spot at 100% laser intensity. A series of 450 single section images was then collected with the laser power attenuated to its initial value (7% of the bleach intensity). The first image was recorded 365 ms after the beginning of bleaching.

180

181 RESULTS

182 Susceptibility testing

The MICs obtained for daptomycin and rifampicin against the four *S. aureus* strains are presented in Table 1. All isolates in planktonic conditions were susceptible to daptomycin and rifampicin. Breakpoint values of drugs according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2016 are: 2 mg/L for vancomycin; 1 mg/L for daptomycin; for rifampicin $S \le 0.06$ and R > 0.5 mg/L.

188 Spectroscopic characterization of the interaction between rifampicin and daptomycin

Neither the photonic absorption properties nor the fluorescence emission spectrum of daptomycin were
significantly influenced by the addition of rifampicin (Fig. 1), revealing the absence of cross-reaction
between the two antibiotics.

BODIPY-FL®-daptomycin penetration, diffusion, bioavailability and localization inside S.
 aureus biofilms

194 *Time-lapse imaging:* time-lapse experiments were performed to visualize *in situ* the penetration of the 195 fluorescently-labelled daptomycin and its combination with rifampicin throughout the deepest layers of 196 *S. aureus* biofilms. By setting the focal plane 5 µm above the substratum surface, we demonstrated that BODIPY-FL®-daptomycin penetrated the biofilms (~30 μ m thickness) within few minutes: fluorescence intensity was measured a few seconds after antibiotic addition and increased rapidly to reach 90% of the maximal intensity in 9 min (Fig. S1 and Movie S4). The penetration coefficients obtained from Eq. 1 ranged from 2.5 ± 0.7 μ m²/s for *S. aureus* 27217, 176 and 33591 to 4.9 μ m²/s ± 0.7 μ m²/s for *S. aureus* BCB8. These values are both of the same order by comparison with BODIPY-FL® alone for which the penetration coefficient is superior to 140 μ m²/s (22). The coefficients were not statistically different in comparison with those of daptomycin in the presence of rifampicin.

FRAP imaging: FRAP was used to measure the local diffusion of BODIPY-FL®-daptomycin and its interaction with bacteria within biofilms. According to the FRAP principle (22, 24, 25), if the fluorescently-labelled daptomycin molecules are allowed to move freely in the sample, a total fluorescence recovery is observed, meaning that the fluorescence is redistributed in the defined region. Conversely, if the fluorescence recovery is not total after the photobleaching, it means that a fraction of molecules is not diffusing freely and thus interacts with its local environment. The other fraction diffuses and is thus bioavailable.

211 First, we checked that no bacterial movement occurred during image acquisition by representing 212 kymograms, two-dimensional graphs of fluorescence intensity measured along a line (here a straight line drawn on the full width of the images) for each image of the FRAP series (Fig. 2a). A typical 213 214 FRAP curve of BODIPY-FL®-daptomycin in S. aureus biofilms is presented in Fig. 2b. Whatever the 215 bacterial strains or the treatments used (daptomycin alone and in combination with rifampicin), we 216 demonstrated here that the fluorescence recovery was not total after photobleaching: only 20% of 217 BODIPY-FL-®-daptomycin molecules interacted with the environment, meaning that a large excess of 218 molecules (80%) were diffusing freely in the defined-regions and thus bioavailable (mean local 219 diffusion coefficient, 7.1 +/- 0.6 μ m²/s).

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220 Localization of the fluorescently-labelled daptomycin combined or not with rifampicin depending on

the surrounding environment: As daptomycin is known to be highly bound to serum proteins (90-93%) (26), we addressed the question of whether there could be a different localization of the fluorescently-labelled daptomycin in a protein-enriched medium. As shown in Fig. 3, fluorescence intensity images were significantly different depending on the surrounding medium: regardless of the strain tested, the antibiotic appeared to be colocalized with the FM4-64 dye at the bacterial site when the surrounding medium was a NaCl aqueous solution supplemented in calcium while the antibiotic localization appeared to be mainly extracellular when the medium was enriched with proteins (TSBpc).

The addition of rifampicin did not affect daptomycin localization in the biofilm.

229 Combining 3D-fluorescence imaging and time-kill studies to assess S. aureus biofilms inactivation

230 in the presence of daptomycin alone and in association with rifampicin

We used confocal microscopy to describe the three-dimensional structures of biofilms and the temporaldistribution of both live and dead cells throughout the biofilm thickness.

Fluorescence intensity images showed that the biofilms formed by the four strains yielded similar compact structures (Fig. 4a and Fig. S2 controls). Their thicknesses were neither significantly variable from one strain to another (25-29 μ m, *P* > 0.05), nor between 24 h and 72 h. This is a reasonable result given that the culture medium (TSBpc) was renewed before the first observation but not over time: thus biofilm development mainly occurred during the first 24 h.

When treated with daptomycin, biofilms exhibited more free-of-cells areas compared with controls (Fig. 4a and Fig. S2). This can be related to significant decreases in biofilms thicknesses (19-21 μ m in the presence of daptomycin, P < 0.05) by comparison with the control biofilms. Moreover, in the presence of daptomycin, no statistically significant change in the proportion of cell death over time was quantified (10-30 %, P > 0.05) (Fig. 4b). However, the MRSA clinical isolate (BCB8) was more

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susceptible to daptomycin: ~ 60 % of cell death was quantified at 24 h, a value that decreased beyond 243 (40% at 72 h, P < 0.05) due to cell regrowth (see below). 244

245 By comparison with the monotherapy treatment, the biofilms structures and thicknesses were not affected when they were treated with daptomycin in combination with rifampicin. However, in these 246 247 conditions, a significantly higher proportion of cell death was observed, achieving 85% at 72 h (P <0.01) (Fig. 4b). For the two MSSA strains (27217, 176) and the MRSA collection strain (33591), the 248 249 antibiotics association activity gradually increased over time (Fig. 4) while the maximum activity 250 against the MRSA clinical strain (BCB8) was reached within 24 h.

251 Another observation of interest (Fig. 4a) is that upon daptomycin exposure, dead cells were observed 252 over the whole biofilm depth, including the basal layer of cells in contact with the substratum, 253 providing further evidence of the antibiotic penetration throughout the deepest layers of the biofilms. 254 This result is even more observable for the BCB8 strain because of the greater proportion of dead cells 255 involved by the action of daptomycin. This process was also observed when daptomycin was used in 256 association with rifampicin.

257 These data from fluorescence imaging were supported by CFU counts of suspended biofilms (Fig. 5). 258 The results confirm that daptomycin alone was ineffective against biofilms formed by the two MSSA 259 strains (27217, 176) and the MRSA collection strain (33591). For the MRSA clinical isolate (BCB8), 260 only \sim 1-2-log reduction in bacterial counts was measured at 24 h before regrowth was observed to 261 reach the same values as for the MSSA biofilms.

262 In accordance with fluorescence imaging data, the activity of the antibiotics combination was much 263 greater than the monotherapy. The sensitivity of the microbiological method allowed determining that the cell population decreased by ~ 4 log after 72 h of treatment (P < 0.05). The emergence of 264

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rifampicin-resistant mutants was verified. As presented in Fig. 6, daptomycin dramatically prevented
the emergence of rifampicin-resistant mutants when used in combination.

267

268 DISCUSSION

The choice of antibiotics to treat *S. aureus* BAI remains a challenge for the medical community. In this context, the ambivalence of the published results on daptomycin activity is a relevant example. Despite increasing data about daptomycin as an option to treat implant-associated *S. aureus* infections, as many failures (18, 27) as successes (7–9, 12) have been reported both in clinical practice and in laboratory models. This highlights that *S. aureus* BAI resistance/tolerance mechanisms to antimicrobials deserve more attention.

275 The biofilm-associated exopolymeric matrix may be considered to act as a shield to the 276 antimicrobial diffusion-reaction (28–32) by delaying its penetration and/or reducing its bioavailability. 277 To verify this hypothesis non-invasively, we took advantage of dynamic fluorescence imaging 278 methods: confocal microscopy, time-lapse imaging and FRAP. For our biofilm model and whatever the 279 bacterial strain, no failure of daptomycin penetrability or bioavailability was observed. The opposite finding described by Siala et al. (31) may be related to the conditions of fluorescence acquisition that 280 were not well-adapted to BODIPY-FL® fluorescence. In this study, time-lapse fluorescence imaging 281 282 experiments demonstrated that daptomycin rapidly reached the biofilms deepest layers while section 283 views of fluorescence intensity images presented in Fig. 3 ascertain the presence of the fluorescentlylabelled antibiotic through the whole biofilm structure. Furthermore, FRAP results ascertained that only 284 285 $\sim 20\%$ of the antibiotic molecules were immobilized. Thus, the majority of the antibiotic molecules 286 were in free movement and could be bioavailable through the biomass (~80% of non-immobilized 287 molecules).

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We further addressed the question of whether or not daptomycin reached its bacterial target. 288 289 Fluorescence intensity images provided interesting information, showing that the fluorescently-labelled 290 antibiotic was distributed majorly in the extracellular matrix rather than in the bacterial cell membranes 291 (Fig. 3). This is in agreement with the well-known property of daptomycin to have a very high degree 292 of protein-binding, especially with serum albumin (90-93%) (26, 33) which is naturally present in 293 physiological conditions. Nevertheless, the fluorescence recovery curves obtained by FRAP 294 experiments in free medium and in the biofilms strongly suggested the reversibility of daptomycin 295 protein-binding (33, 34): the equilibrium between the bound and unbound states may conserve the 296 apparent mobility of the antibiotic. Additional experiments were performed in a protein-free medium (a 297 saline solution supplemented with calcium ions). In this case, bacterial cell membranes appeared as hot 298 spots on fluorescence images, consistently with the described antibiotic interaction with its target (6). 299 Surprisingly, whether the medium was protein-free or not, daptomycin exhibited the same lack of 300 effectiveness, as revealed by time-kill studies performed by fluorescent live-dead staining and 301 conventional plating on agar (data not shown in the absence of proteins). Thus, the interaction with the 302 matrix components cannot explain biofilm tolerance to the antibiotic.

303 The particular physiology of embedded bacteria should be thus considered and more 304 specifically cells with a low metabolic activity. Previous studies using a BrdU immunofluorescent 305 labelling technique demonstrated that the large majority of staphylococci cells in a biofilm were actually in a low-metabolic state (35, 36). Additionally, in the present study, the comparison of cell 306 viability results obtained by CFU counts and fluorescence imaging highlighted a significant proportion 307 308 of viable cells detected by Live-Dead® staining but not by CFU measurements. This subpopulation 309 may be considered as viable but non-culturable (VBNC), a subpopulation known to have a slow 310 metabolism (37–39). Moreover, it has been demonstrated that daptomycin is poorly effective against bacteria in stationary stage (7, 27). One can thus reasonably suggest that for bacteria with low-311

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metabolic activity, the cell membrane dysregulation induced by daptomycin may be slower and/or 312 313 more difficult to attain due to structural modifications of the cell membrane. This assumption is 314 supported by the reported data revealing that daptomycin displays a concentration-dependent bactericidal activity against dormant cells (7, 9, 12). In the present study, we tested a double 315 316 concentration of daptomycin (40 µg/mL) on the different S. aureus biofilms, almost leading to 317 bactericidal effects after 24 h of drug exposure (Fig. S3) and showing no cell-regrowth over time. 318 However, biofilm clearance was not reached. This achievement was reported to occur in a daptomycin concentration equal to or greater than 100 μ g/mL but may not be relevant in clinical practice (7, 9, 12). 319

320 One particularity in this study concerns the BCB8 clinical isolate, which discriminated itself by 321 a twice higher penetration coefficient by comparison with the other strains tested and a greater 322 susceptibility as revealed by the observation of a larger proportion of dead cells over the whole biofilm 323 thickness, including the basal layer in contact with the substratum. These results are in line with those 324 obtained in vivo (18), which demonstrated a strain-dependent activity of daptomycin against S. aureus 325 biofilms. In view of the antibiotic mechanism of action which is supposed to target the plasma 326 membrane, the observed variable response depending on the bacterial strain may be due to a change in 327 membrane composition or conformation from a strain to another.

328 Facing the lack of daptomycin efficiency in treating recalcitrant S. aureus BAI, the addition of 329 rifampicin has raised great interest (10–13, 15, 40–42). Through this study, we demonstrated that the 330 combined therapy was indeed highly efficient against S. aureus biofilms, but did not allow total 331 bacterial clearance. Both antibiotics have been shown not to cross-react with each other, as evidenced 332 by steady-state fluorescence spectroscopy. Moreover, the penetration, diffusion and localization of the 333 fluorescently-labelled daptomycin were not affected by the presence of rifampicin. We also proved here that rifampicin-resistant mutants emerged when biofilms were treated with rifampicin alone, but 334 335 not when treated with the antibiotic combination. Altogether, presented data confirm that daptomycin Page 15 of 30

prevents the emergence of rifampicin resistant mutants, allowing the bactericidal activity of rifampicinto quickly occur in time, regardless of the cell physiological state.

In conclusion, consistently with the previous *in vivo* study aiming at evaluating the antibiotic efficacy in *S. aureus* prosthetic vascular graft infections (18), we demonstrated in the present *in vitro* model a strain-dependent lack of daptomycin activity toward biofilms. Dynamic fluorescence microscopy allowed discarding a lack of antibiotic availability and interaction with bacteria. Given the mode of action of daptomycin, these observations suggest a membrane-dependent factor of tolerance in such biofilms. Therefore, to provide a better understanding of daptomycin reduced activity against biofilms, a further step should concern the analysis of the membranes composition.

345

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Antibiotic	MSSA ATCC 27217	MSSA 176	MRSA ATCC 33591	MRSA BCB8
Daptomycin	0.25	0.5	0.25	0.125
Rifampicin	< 0.06	0.015	0.0075	< 0.06

Table 1: MICs (mg/L) of daptomycin and rifampicin against the four S. aureus strains (± 5%). 481

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484 Fig. 1: Absorption (straight lines) and fluorescence spectra (dashed lines) of daptomycin (20 µg/mL)

alone and combined to rifampicin (20 µg/mL). Excitation wavelength: 360 nm. Daptomycin alone is 485

486 represented in black and its combination with rifampicin in grey.

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Fig. 2: FRAP acquisitions for BODIPY-FL®-daptomycin inside S. aureus biofilms. (a) Kymogram 488 489 representation (x,t) of FRAP acquisitions. The line along which the kymogram was done is 38 μ m. (b) 490 Typical fluorescence recovery curves representative of six different zones for each condition: 491 BODIPY-FL®-daptomycin inside biofilms (black) and inside TSBpc without biofilm (grey). The 492 kymogram and fluorescence recovery curve presented here are the ones obtained for MSSA 27217 493 biofilms since they were representative of the data obtained for the other strains only in the presence or 494 not of rifampicin.

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BODIPY-FL®daptomycin in TSBpc

BODIPY-FL®-

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25 . 30 vein in biofilm

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497 Fig. 3: Fluorescence imaging of BODIPY-FL®-daptomycin (green channel) and FM4-64 (red channel) 498 in S. aureus biofilms. Merged images are also shown. On the top, the surrounding medium is a NaCl 499 (150 mM) aqueous solution supplemented with calcium ions (50 mg/L). On the bottom, the 500 surrounding medium is TSB enriched with proteins (36 g/L) and calcium ions (50 mg/L). Only images 501 of MSSA 27217 biofilms are represented since they were representative of all biofilms visualized for 502 other strains in the presence or not of rifampicin.

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505 Fig. 4: (a) Visualization of MSSA 27217 and MRSA BCB8 biofilms using 3D reconstruction to 506 observe biofilm thickness. Images were collected without any drug exposure (control) and after 72 h 507 exposure to unlabeled daptomycin (20 µg/mL) alone and in association with rifampicin (20 µg/mL). 508 Dead cells were stained red with propidium iodide and all bacteria were stained green with Syto9[®]. 509 The acquisition was performed on the whole biofilm thickness with an axial displacement of 1 µm. 510 Images dimension is $82x82\mu m^2$. The scale bar corresponds to 20 μm and mean thickness values of the 511 biofilms over time (from 24 to 72 h) are written in white on each image. (b) Percentage of dead cells 512 evolution over time calculated from three series of biofilms images treated or not with daptomycin (20 513 μ g/mL) alone or in combination with rifampicin (20 μ g/mL). Black bars, controls; grey bars, 514 daptomycin alone; open bars, daptomycin-rifampicin combination. Error bars represent the standard 515 deviation.

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517

518 Fig. 5: Time-kill curves of daptomycin (20 μ g/mL) activities combined or not with rifampicin (20

519 µg/mL) against MSSA 27217 and MRSA BCB8 biofilms. Filled circles: controls; filled squares:

520 daptomycin; filled triangles: daptomycin + rifampicin. Error bars represent the standard deviation.

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Fig. 6: Number of rifampicin-resistant mutants determined in MSSA 27217 biofilms counted on rifampicin-containing TSA plates. Above each bar is shown the percentage of rifampicin-resistant mutants in *S. aureus* biofilms among the total bacterial population. Error bars represent the standard deviation. White bars, rifampicin alone (20 μ g/mL); black bars, daptomycin-rifampicin combination (20 μ g/mL for both antibiotics).

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(a)



х





FM4-64

Merge

AAC

(a)

MSSA 27217

Control

28 µm

Daptomycin

19 um

22 µm



(b)







Time (hours)

AAC



10

94%

Ι

Time (hours)

0,05% T

24

100%

I

0,04 % T

48

□Rifampicin

■ Daptomycin + Rifampicin

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