Quantitative and qualitative assessment of antibiotic activity against *Staphylococcus aureus* Biofilm.

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Abstract

BACKGROUND ***Staphylococcus aureus*** **causes severe and chronic diseases (osteomyelitis, periodontitis, chronic wound infection, endocarditis…) that are associated to its capacity to grow in biofilms. Understanding how antimicrobial agents act upon these biofilms is essential in order to select appropriate treatments. We have tested the activity of four anti-infective agents (vancomycin, fusidic acid, daptomycin, and the ceragenin CSA-13) and one detergent (cetrimide) against MSSA and MRSA mature biofilms, using a combined qualitative and quantitative approach.**

METHODS Strains used were MSSA ATCC25923 and MRSA ATCC33591. Biofilms were grown in 96-well plates (quantitative analysis) or on coverslips in 12-well plates (qualitative analysis) in TSB added by 1 % glucose and 2 % NaCl for 24h and then exposed to the selected agents at 32 times their MIC during 48h. Quantitative analysis: biofilm mass was evaluated by measuring the OD of crystal violet and viability of bacteria, using the redox indicator resazurin (reduced to fluorescent resorufin by viable bacteria). Qualitative analysis in confocal laser scanning microscopy (CLSM): bacteria integrity was evaluated with the BacLight Live/Dead staining, and respiratory activity of living bacteria, using 5-cyano-2,3-ditolyl tetrazolium chloride CTC (reduced to fluorescent CTC-formazan).

RESULTS CSA-13, daptomycin and cetrimide (respective MICs: 1, 0.5, and 5-10 mg/L) were the most effective in reducing metabolic and respiratory activities (70-100 % reduction) and in disrupting bacterial membrane integrity (75-97 % damaged bacteria) of both MSSA and MRSA biofilms. Vancomycin and fusidic acid were poorly effective against biofilms (5-20 % effects) although they display low MICs (1 and 0.25 mg/L) against planktonic bacteria.

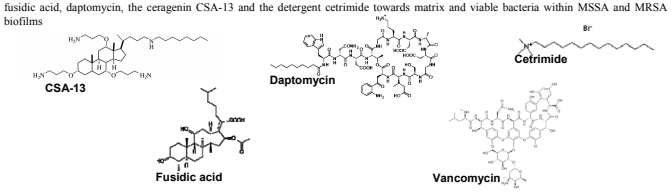
Pharmacological Agent [a]	MIC (mg/L)		% of cells with damaged membrane [b]		CTC-formazan fluorescence [c]		Crystal violet absorbance [c]		Resorufin fluorescence [c]	
	MSSA	MRSA	MSSA	MRSA	MSSA	MRSA	MSSA	MRSA	MSSA	MRSA
CSA-13	1	1	92.29	86.77	5.9±2.8	8.9±3.2	43.65±15	0±0.5	5.7±2.6	6.3±9
Daptomycin	0.5	0.5	97.01	75.11	7.8±2.7	31.6±7.6	110±59	98±6.1	4.7±1.33	31.15±1.7
Cetrimide	5	10	78.75	87.18	1.6±0.9	10.1±1.6	205±58	95±26	-0.16±0.1	-0.7±0.1
Vancomycin	1	1	5.47	4.74	84.2±2.2	82.1±4.2	171±31	93.6±18	79 ±4.1	77.2±9.2
Fusidic acid	0.25	0.25	0	0	91.9±6.9	95.4±9.3	91±29.1	99.8±8.4	84.5±9	99.4±5.2

qualitative analysis (blue), quantitative analysis (red)
a: Biofilm grown in the same medium for 24 h and then exposed during 48 h to antibiotics at concentration of 32 X their MIC
b: Intensities of the green (500-550nm) and red (620-650nm) emission were acquired with CSLM, and the green/red fluorescence ratio were calculated for Known live/dead proportion of *S.aureus*. We determine the relationship between %live bacteria (intact membrane) (x) and green/red fluorescence ratio (y): [y=8.3845 xs+0.1463].Percentage of damaged cells is calculated between 1-5µm of biofilm depth.
c: Calculated based on data expressed in percentage of the value recorded in the absence of antibiotic (control).

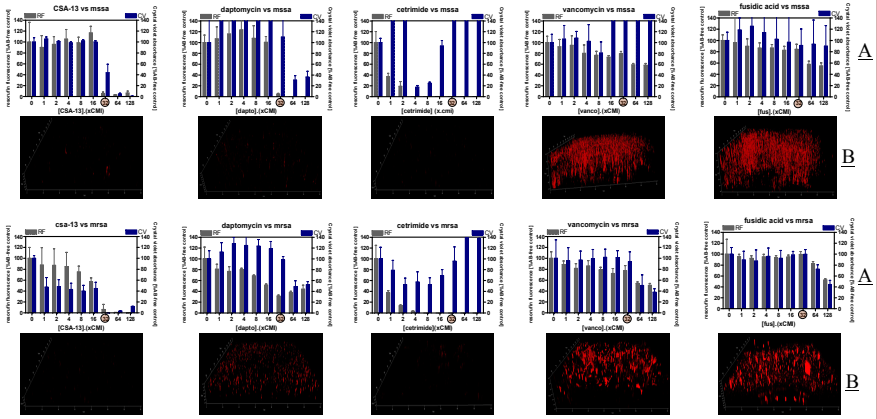
CONCLUSIONS. Among the agents investigated those that present an amphipathic structure with an hydrophilic head and a lipoophilic tail are the most active on biofilms, suggesting that detergent-like properties are needed to act upon both bacteria and matrix in biofilm.

Introduction and Objectives

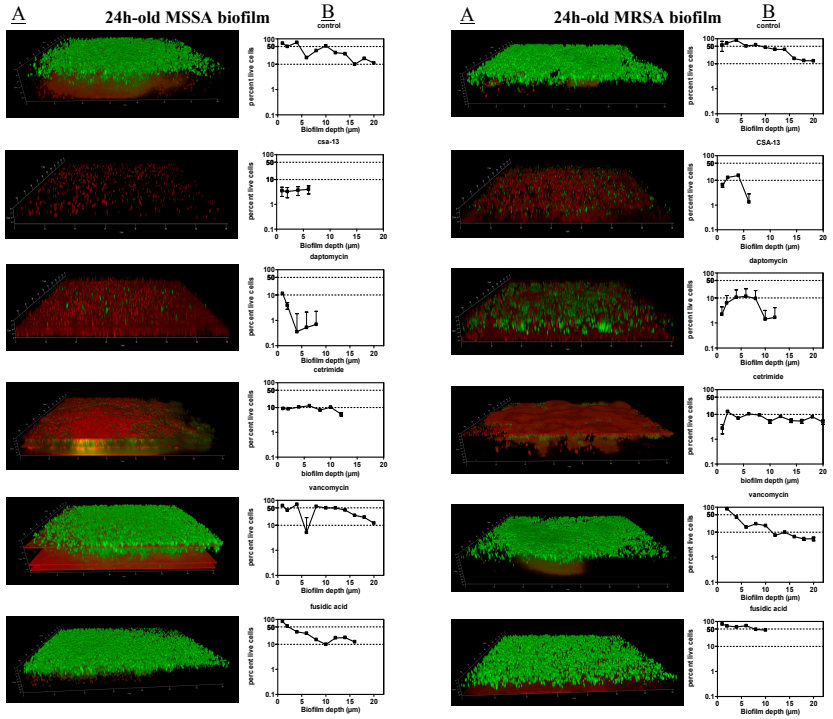
A biofilm can be defined as a microbially-derived sessile community, typified by cells that are attached to a substratum, interface, or to each other,and are embedded in a matrix of extracellular polymeric substance. By adopting this sessile mode of life, biofilm-embedded microorganisms benefit from a number of advantages over their planktonic counterparts. A reduced susceptibility to many antimicrobial agents is an important advantage of this mode of life, and ahas been associated with severe and persistent infections. We developed a model to evaluate the activity of available and novel pharmacological agents against *S. aureus* biofilms and used it to assess the activity of vancomycin, fusidic acid, daptomycin, the ceragenin CSA-13 and the detergent cetrimide towards matrix and viable bacteria within MSSA and MRSA biofilms



Results



A: Response to increasing concentrations of antimicrobial agents of 24h-old biofilms of MSSA (up) or MRSA (down). The graphs show the effect of selected agents on the viability of embedded cells (resorufin fluorescence, RF) and quantity of surrounding matrix (crystal violet, CV). 24-h biofilms were incubated with increasing concentrations (x fold MIC) of antimicrobial agents for 48 h. The ordinate shows the change in resorufin fluorescence (RF) or in crystal violet absorbance (CV) in percentage of the control value (no antibacterial agent present). All values are means ± standard deviations (SD) of 4 wells. **B: 3D images in confocal laser scanning microscopy of 24h-biofilm of MSSA (ATCC25923) or MRSA (ATCC33591) after CTC staining:** the respiratory activity of the embedded cells was determined by staining with 0.5mM of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) after exposure to selected agents at 32 X their MIC during 48 h. Viable cells were identified in CSLM by the presence of intracellular , red CTC-Formazan.



A: 3D images in confocal laser scanning microscopy of 24h-biofilm of MSSA (ATCC25923) or MRSA (ATCC33591) after live/dead staining.. Images of control conditions or after exposure to selected agents at 32 X their MIC during 48 h. Biofilm were stained with SYTO9 (green; viable cells) and propidium iodide (red; dead cells). **B: quantitative analysis of images** percentage of living cells is determined through the depth of the biofilm, as calculated from the SYTO9 / propidium iodide fluorescence ratio. The relationship between fluorescence ratio(y) and percentage of living bacteria (intact membrane) green/red (x) is calculated : [y=8.3845 xs+0.1463].

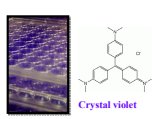
Methods

Bacterial strains, culture conditions and Biofilm development
Two *S.aureus* strains were used in this study; ATCC25923 (MSSA) and ATCC33591 (MRSA). Bacteria were transferred from the stock culture onto TSA plates (Trypticase Soy Agar) and incubated overnight at 37°C. For the quanytitative analysis, biofilms were cultivated in polystyrene 96-well plates. For qualitative analysis, biofilms were cultivated on glass cover slips placed in 12-well plates. Biofilms were grown in TGN medium (Trypticase Soy Broth (TSB); 2% NaCl; 1% glucose) at 37°C with an initial inoculum adjusted to an OD620nm of 0.005.

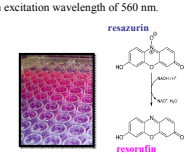
Quantitative analysis

Crystal violet staining: Biofilm production was evaluated by measuring the absorbance of crystal violet, a cationic dye which stains non-specifically all (negatively-charged) biofilm constituents based on ionic interactions. Biofilms were fixed by heat at 60 °C for about 1 hour and stained by a 15 minutes incubation at room temperature with 150 µL of a 2.3% crystal violet solution (Sigma-Aldrich, St. Louis, MO). After elimination of the excess of crystal violet under running water, the dye fixed to the biofilm was resolubilized by addition of 200 µL of 33% glacial acetic acid and incubation at room temperature for 1 hour without shaking. Crystal violet absorbance was measured at 570 nm .

Redox indicator rezasurin assay: Viability was determined using the blue-colored phenoxazin dye resazurin, which is reduced by viable bacteria in the pink, fluorescent compound resorufin. Biofilms were incubated with 10µg/mL resazurin (Sigma-Aldrich) in TSB during 30 minutes at room temperature in the dark. Resorufin fluorescence was measured at a wavelength of 590 nm using an excitation wavelength of 560 nm.



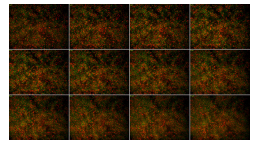
Detection of **matrix and cells** by non-specific staining of all biofilm constituents



Detection of **viable bacteria** by measuring the *in situ* local redox potential

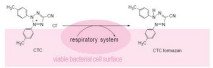
Qualitative analysis

LIVE/DEAD staining: In order to evaluate cell membrane integrity after antibiotic treatment, the BacLight Live/Dead bacterial viability kit (L-7007; Molecular Probes, Oregon, USA) was used. The kit contains Syto9 and propidium iodide to differentiate between cells with intact membranes (live: green) and membrane damaged cells (dead: red), respectively. When both dyes are present, propidium iodide causes reduced fluorescence of Syto9 resulting in a red fluorescence signal for damaged cells and green fluorescence for intact cells when a dual emission filter (Emgreen~500nm; Emred~620nm) is used. Biofilms were incubated with the dyes in the dark for 30min at room temperature in the dark, and. The staining result was directly observed by confocal laser scanning microscopy (CSLM), with capture of image stacks of each sample using Z-Stack module for acquisition of image series from different focus planes. Fluorescence ratio of each focus plane was determined (z-axis separation of 1µm). Image stacks are used also to render three-dimensional images by AxioVision software.



Example of an image stacks after biofilm staining with Live/Dead bacterial viability kit

5-cyano-2,3-ditolyl tetrazolium chloride (CTC) staining: To detect respiratory activity of metabolically active bacteria within the biofilm, we used CTC, a colourless, non-fluorescent and membrane permeable compound which is readily reduced via electron transport activity to fluorescent, insoluble CTC-formazan that accumulates inside bacteria. Biofilm-overgrown cover slips were washed, labeled with CTC and observed in CSLM.



conclusion

In spite of their low and similar MICs (0.25-1mg/L), antibiotics display markedly different effects against biofilms, suggesting that intrinsic activity is not predictive of activity in this model.
Daptomycin, CSA-13 and cetremide are the most effective molecules against mature biofilms, considering both matrix thickness and bacterial viability, suggesting that detergent-like properties are critical to act in the deepness of the matrix.

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