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A Maturing and Mature Staphylococcal Biofilm Model for Screening of Antibiotic Activity

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Abstract

Background: *S. aureus* causes biofilm-related infections that are difficult to treat due, in part, to phenotypical differences between adherent and planktonic cells. We have developed a model of young (6 h) and mature (24 h) biofilms and have set up methods allowing for the quantitative evaluation of AB activity on both bacterial survival and matrix production. This model was used here to screen the activity of antistaphylococcal AB.

Methods: Biofilms of S. aureus ATCC25923 (MSSA) were cultivated in 96-well plates for 6 or 24 h and then exposed to AB. Total biofilm mass (matrix + cells) was measured using crystal violet staining. The amount of viable cells within the matrix was measured using the redox indicator resazurin (reduced to resorufin [Lett. Appl. Microbiol. 2008, 49:249-54]).

Results: During maturation, resorufin fluorescence increased up to 5-fold after 24 h and decreased then slightly to 3-fold after 72 h (compared to a 6 h-old biofilm). The table shows drug effects on viable cells and matrix. All AB were active on young biofilms, but most of them were less (MXF/RIF > VAN/QD) or not (FUS/LZD/AZM) active on bacteria in mature biofilms and did only poorly act on total biofilm mass. Yet, OXA and DAP (high conc.) showed marked effects on both viable cells and matrix.

antibiotic	MIC[a] (mg/L)	6 h-old biofilm[b] [% of control after 24h of incubation]			24 h-old biofilm[c] [% of control after 48h of incubation]		
		Resorufin Fluorescence (% of AB-free control)			Resorufin Fluorescence (% of AB-free control)		Crystal violet Abs. (% of AB-free control)
		1 x MIC	8 x MIC	32 x MIC	8 x MIC	32 x MIC	32 x MIC
OXA	**0.125**	0.9 ± 0.7	1.3 ± 0.2	0.3 ± 0.2	2.0 ± 0.2	0.5 ± 0.02	9.7 ± 1.3
DAP	**1**	27.9 ± 8.9	0.9 ± 0.4	-*	96.6 ± 5.9	1.1 ± 0.1	23.9 ± 4.9
RIF	**0.031**	3.21 ± 0.16	2.6 ± 0.1	1.9 ± 0.1	29.0± 9.8	37.4 ± 14.6	43.3 ± 9.0
MXF	**0.031**	67.4 ± 11.9	4.5 ± 5.3	12.6 ± 1.1	54.8 ± 6.3	42.7 ± 4.3	69.5 ± 3.2
VAN	**1**	113.0 ± 9.6	10.2 ± 0.3	8.8 ± 1.7	98.3 ± 7.0	48.3 ± 6.1	66.0 ± 3.3
QD	**1**	6.2 ± 0.6	6.7 ± 0.4	9.1 ± 0.7	68.5 ± 2.3	66.3 ± 3.6	85.0 ± 6.4
FUS[d]	**0.25**	10.5 ± 4.7	4.3 ± 0.5	4.0 ± 0.01	104.3 ± 12.2	97.9 ± 5.9	119.7 ± 6.7
LZD	**1**	49.1 ± 8.0	6.2 ± 0.4	5.7 ± 0.2	115.0 ± 3.1	105.9 ± 4.6	97.2 ± 8.09
AZM	**1**	124.4 ± 4.8	43.7 ± 1.7	10.3 ± 0.1	141.0 ± 4.6	131.7 ± 6.5	147.2 ± 7.8

[a]determined by microdilution in MH broth
[b]cultivated in 96-well plates for 6 h before medium was replaced by medium + AB for 24 h
[c]cultivated in 96-well plates for 24 h before medium was replaced by medium + AB for 48 h
[d] fusidic acid
*no detectable signal

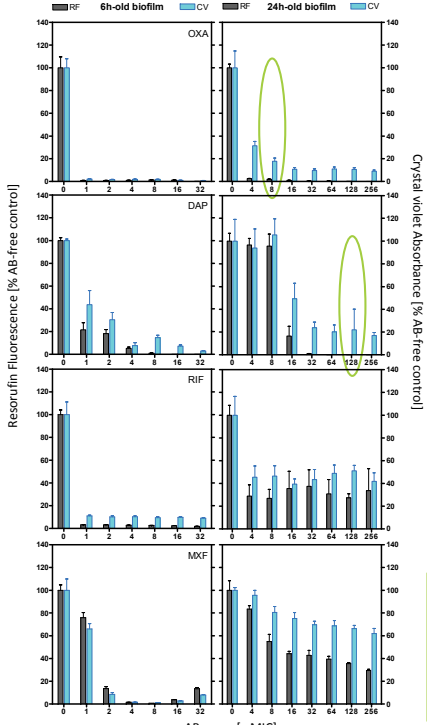
Conclusion: Even when bactericidal in broth, AB are poorly active against bacteria in mature biofilms, with the exception of OXA and DAP. This may be due to poor penetration and/or activity on matrix, suggesting the need of searching for agents able to destroy it.

Introduction & Objectives

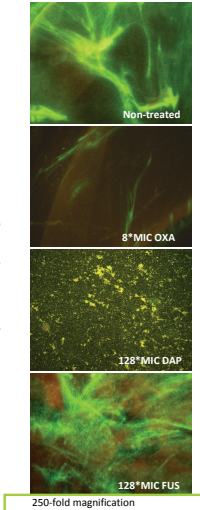
Infections by *S. aureus* related to biofilm formation represent a major problem in the hospital. Little is known however about the treatment of such infections. Appropriate biofilm models are therefore needed to evaluate the activity of available or novel antibiotics. Young and mature biofilms have rarely been compared, even though this might have important implications in a clinical context. Our aims were (i) to develop a suitable model of both maturing and mature biofilm, (ii) to set up methods allowing for the quantitative assessment of antibiotic activity and (iii) to examine the effect of different antibiotic classes on matrix and viable bacteria within young and mature biofilms.

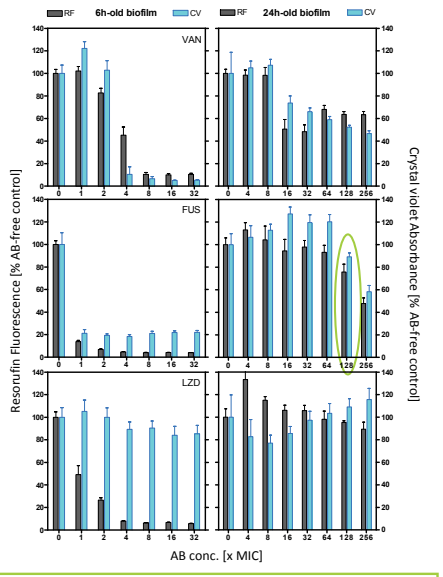
Results

The graphs show the effect of selected antibiotics on the viability of embedded cells (resorufin fluorescence, RF) and quantity of the surrounding matrix (crystal violet absorbance, CV). Direct comparison of 6h-old (young) biofilms and 24h-old (matured) biofilms demonstrate significant differences in response to antibiotic treatment. While in case of young biofilms, most antibiotics (except LZD and AZM [not shown]) were able to markeldy decrease the amount of viable cells as well as total biofilm mass, most antibiotics failed against mature biofilms. Only OXA and DAP were highly effective in matrix-desintegration of matured biofilms and killed the majority of bacteria.



LIVE/DEAD-staining of 24h-old biofilms incubated in the presence of AB for 48h (conditions are corresponding to the green oval on the graphs)





250-fold magnification
ALIVE — DEAD

The consequences of AB-treatment were further investigated by fluorescent microscopy using the LIVE/DEAD Kit based on the DNA - binding dyes Syto9 (membrane permeable, green- fluorescent) and propidium iodide (impermeable, red-fluorescent). Using a combining Filter, intact cells are green-fluorescent (ALIVE), while bacteria with damaged membranes (DEAD) appear orange since they contain both dyes. Comparing non-treated biofilms with treated ones, differences in structure, total volume the biofilm, cell integrity and cell-clustering could be observed. While FUS causes no clear effect, a strong decrease of the biofilm was detected for DAP and OXA (already at low concentration).

Methods

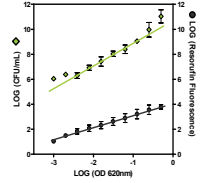
S. aureus ATCC25923 (MSSA) was cultivated in TSB complemented with 1% glucose and 2% NaCl. Static biofilms of different ages were obtained in 96-well plates and exposed to bacteriostatic (AZM, LZD, fusidic acid) or bactericidal (DAP, MXF, OXA, Q-D, RIF, VAN) antibiotics. Before and after 1 - 2 days of antibiotic treatment, biofilms were characterized. Total biofilm mass (matrix + cells) was measured by classical crystal violet staining (modified method after Christensen et al. 1985, J Clin Microbiol. Vol.22, No. 6).

The amount of viable cells within the matrix was determined using the redox indicator resazurin (blue, non-fluorescent) which is metabolized to fluorescent resorufin (pink) by viable bacteria only (Toté et al. 2010, Appl Environ Microbiol. Vol.76, No.10).

The AB-effect on 24h-old biofilms was also observed by Fluorescence Microscopy in combination with the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes).

Resazurin-Assay

To identify the best conditions for evaluating biofilms by using the resazurin-assay, the influence of resazurin concentration, solvents and incubation times was examined on planktonic cells and/or biofilms. After 30 min of incubation, an initial resazurin concentration of 10µg/mL allows clear differentiation between cell-suspensions of optical densities (OD) between 0.016 and 1. By counting CFUs and measuring resorufin fluorescence (RF) of planktonic cells simultaneously, we could establish a correlation between CFUs and RF as a function of OD.



Conclusion

Appropriate antibiotics for biofilm treatment should not only effectively act on bacteria but also show a strong matrix-degradable effect. Only those antibiotics that are capable of causing a significant reduction in the biofilm matrix seem also able to act upon viable bacteria. This suggests that the killing of the formerly embedded bacteria is required to prevent a re-growth starting from single persisting cells.

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