Establishment of a maturing and mature staphylococcal biofilm model suitable for screening of antibiotic activity

Julia. Bauer, P.M. Tulkens, F. Van Bambeke

Louvain Drug Research Institute (Cellular and Molecular Pharmacology), Université catholique de Louvain, Brussels, Belgium,
avenue E. Mounier, Bâtiment Van Helmont, FACM 7370, email: julia.bauer@uclouvain.be

Introduction

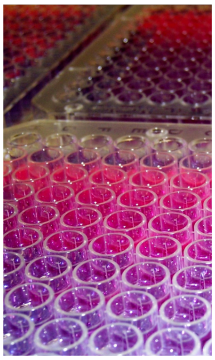
Staphylococcus aureus causes biofilm-related infections that are difficult to treat, partially due to phenotypical differences between adherent and planktonic cells. Appropriate models are needed to test for the activity of available and novel antibiotics against biofilms. However, young (< 1 day) and mature biofilms have rarely been compared, even though this might have important implications in a clinical context.

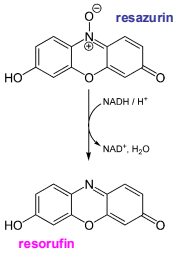
Objectives

Our aims were (i) to develop a suitable model of both maturing and mature biofilms and (ii) to set up methods allowing for the quantitative assessment of antibiotic activity on both bacterial survival and matrix production.

Methods

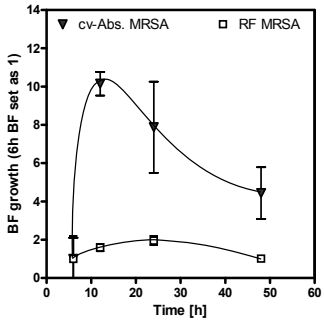
S. aureus ATCC25923 (MSSA) and ATCC33591 (MRSA) were used. Static biofilms of different ages were obtained in 96-well plates and exposed to bacteriostatic (AZM, LZD, fusidic acid) or bactericidal (DAP, MXF, OXA, Q-D, RIF, VAN) antibiotics. Before and after 1 - 2 days of antibiotic-incubation biofilms 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).





Biofilm Development

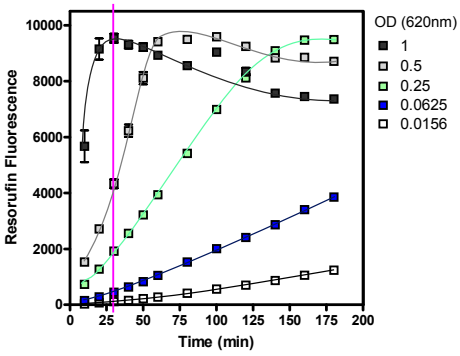
Within the first 48 h of growth, crystal violet absorbance changes significantly. In contrast, resorufin fluorescence is relatively constant, indicating that production of extracellular substances varies strongly during biofilm maturation whereas the number of viable cells remains stable.



Validation of the resazurin-assay

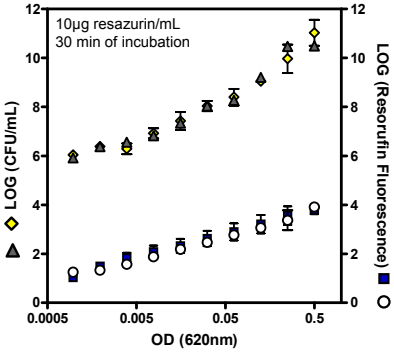
To identify the best conditions for evaluating biofilms by using the resazurin-assay, the influence of resazurin concentration, solvents (TSB and MHB enable high fluorescence-detection showing low background background emission) and incubation times was examined on planktonic cells and/or biofilms. After 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 (**A**). Since resazurin is a non-toxic agent, cells continue, or, in case of antibiotic-replacement, start growing during the incubation time. For this reason short incubation times are preferable. Higher resazurin concentrations make it difficult to distinguish between low cell numbers, while lower concentrations do not suffice at high cell densities: When resazurin is completely reduced to resorufin, a time-dependent decrease of fluorescence can be observed which is due to the subsequent conversion of resorufin into colourless and non-fluorescent hydroresorufin.
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 (**B**).

A Time-dependency of resazurin-incubation



10µg resazurin/mL
Planktonic cells; MSSA

B Correlation CFU-resorufin fluorescence

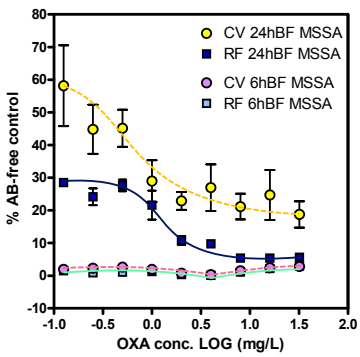


Results

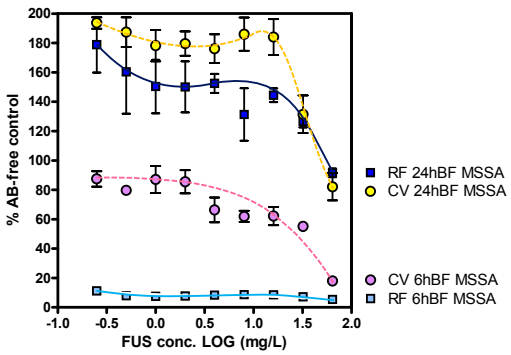
While most antibiotics were active on young biofilms they were less active (OXA > DAP >MXF / RIF > QD) or inactive (VAN / FUS / LZD / AZM) on bacteria in mature biofilms.
As an illustration, Panel **C** and **D** show the effect of Oxacillin and fusidic acid on a young (6h) and a matured (24h) biofilms of MSSA. OXA significantly reduces the number of viable cells within the 24h old biofilm (to 30-10% of the control, using AB concentrations between 1*MIC and 256*MIC), as well as the total biofilm mass (to at least 60-20%). In contrast, in the presence of FUS, the number of viable cells and the biofilm mass do not drop below 80% of control values for a 24 h biofilm.

CV: cristal violet absorbance
RF: resorufin fluorescence

C Effect of OXA



D Effect of FUS



Antibiotic-studies: Interpretation of the results
Studies of antibiotic-caused effects on biofilms require the exchange of medium after distinct times of growth. Depending on strain and age of the biofilm an increase or decrease in crystal violet absorbance and resorufin fluorescence is observed. For this reason, in all experiments an AB-free control is performed under identical conditions and set as 100%.

Summary

Resazurin is a promising indicator to determine the viable burden of biofilm. In combination with crystal violet staining the resazurin method allows an evaluation of drug effects on viable cells and matrix regarding young and matured biofilms of *Staphylococcus aureus*.