Changes in CoNS biofilm formation, composition, structure and antimicrobial resistance due to growth in sub-inhibitory concentrations of dicloxacillin

N.Cerca[1], S. Martins[1], S. Sillankorva[1], G. Pier[2], R. Oliveira[1], J. Azeredo[1]

[1] Centro de Engenharia Biologica, Universidade do Minho, Campus de Gualtar, 4710 Braga, Portugal
(E-mail: ncerca@deb.uminho.pt; silviamartins@zmail.pt; s.sillankorva@deb.uminho.pt; roliveira@deb.uminho.pt; *jazeredo@deb.uminho.pt)*
[2] Channing Laboratory, Brigham Women's Hospital, Harvard Medical School, Boston, USA
(E-mail; gpier@rics.bwh.harvard.edu)

Abstract
Many studies have demonstrated that low concentrations of antibiotics can inhibit microbial adherence to medical-device surfaces. However, little is known about the changes that might occur in bacterial physiology of biofilms formed under sub-inhibitory (subMIC) concentrations of antibiotics. In this study, biofilms of two coagulase negative staphylococci (CoNS) species were formed with and without the presence of subMIC concentrations of dicloxacillin and changes in the ability to produce biofilms as well as in the composition of biofilm matrix were evaluated. Biofilms formed in the presence of dicloxacillin developed less amount of biomass and exhibited a different composition of the biofilm matrix. Bacterial physiological alterations triggered by biofilm formation under subMIC concentrations of antibiotics were also evaluated. The results showed that bacteria surface characteristics, like hydrophobicity and elemental composition as well as the expression of PNAG molecules were affected. Additionally, an increase in resistance to several antibiotics was observed in biofilm cells formed in the presence of dicloxacillin.

Keywords
Biofilm formation; Dicloxacillin; *Staphylococcus epidermidis*; *Staphylococcus haemolyticus*; Sub-MIC inhibition;

INTRODUCTION

Coagulase-negative staphylococci (CoNS), like *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*, are now well established as major nosocomial pathogens associated with infections of indwelling medical devices (Voung *et al.*, 2002). Many studies have demonstrated that low concentrations of antibiotics can inhibit microbial adherence to medical-device surfaces (Pagano *et al.*, 2004). Besides the reduced capability of adhesion of the bacteria, little is known about other changes implicated in the virulence of CoNS that might occur due to biofilm formation in the presence of low concentrations of antibiotics. It has been suggested that growth in the presence of sub-inhibitory antibiotic concentration may lead to an increase in antibiotic resistance (Arciola *et al.*, 2002).

The aim of this work was to evaluate the ability of biofilm formation and changes in the physiology of bacterial CoNS cells triggered by biofilm formation under low concentrations of antibiotics. Factors such as biofilm formation ability, cell surface properties, production of specific molecules responsible for biofilm formation (intercellular adhesin PNAG) and resistance to antibiotics are the virulence factors of CoNS that will be addressed in this work

METHODS

Strains

In this work 2 CoNS clinical strains were used: *S. haemolyticus* M176 and *S. epidermidis* M187 were both isolated from patients with peritonitis associated with renal dialysis patients.

Biofilm formation

S. haemolyticus M176 and *S. epidermidis* M187 biofilms were formed in a 96 well microtiter plate in TSB supplemented with 0.25% glucose for 24 hours, at 37°C and 150 rpm. These biofilms were considered the controls (CT). To evaluate the effect of subMIC concentrations of dicloxacilin in biofilm formation, biofilms were formed in culture media supplemented with 8μg/ml (=1/2 MIC) of dicloxacillin (DIC).

Biofilm quantification and observation

Biofilm quantification was determined as described previously (Heilmann *et al.*,1996) using a rapid colorimetric assay. For SEM observations, biofilms were formed on acrylic plates and dried at 80°C for 48 hours.

Bacterial cells characterization

Biofilms were scraped from the substrate and sonicated for 10 s at 20W. Bacterial cells were then harvested by centrifugation (10500×g, 6 min, 4°C) and washed twice in culture medium. Contact angle determinations were performed using water as a reference liquid on air-dried bacterial cell lawns deposited on a nitrocellulose filter (Bos *et al.*, 2004). Hemagglutination assays were performed according to Rupp *et al.* (1992) using horse erythrocytes. Cells were prepared for XPS analysis as described by Rouxhet *et al.* (1994).

Biofilm matrix composition

The biofilm matrix was extracted as briefly described: biofilms were scraped from the substratum surfaces, sonicated for 30 s, 20W, then vortexed for 2 min, centrifuged at 10500g, 6 min, 4°C, and the supernatants were filtered and stored at –20°C before being used in the quantification assays. Proteins and polysaccharides of the biofilm matrix were determined by Lowry and phenol- sulfuric acid methods, respectively. SDS-PAGE of the extracted matrix was performed as described by Smith *et al.*(1991).

Resistance to antibiotics

The MIC of bacterial cells of biofilms formed with and without the presence of sub-inhibitory concentrations of dicloxacillin was determined as follows: biofilms were scraped from the substratum surface, sonicated for 10 s, 20W, adjusted to a standard cell inoculum, and incubated in 96-well microtitter plates with several two-fold dilutions of dicloxacillin, tetracycline and rifampicin.

RESULTS AND DISCUSSION

The amount of biofilm formed by *S. haemolyticus* M176 in TSB supplemented with glucose was significantly lower ($p<0.05$) than that of biofilm of *S. epidermidis* M187, formed in the same conditions, demonstrating a strain specificity on the ability to form biofilm. The use of dicloxacillin in a low concentration (8μg/ml=1/2 MIC) was effective in reducing the amount of biofilm formed by both strains (Fig.1), being more effective on *S. haemolyticus* than on *S. epidermidis*.

Besides the reduction in biofilm formation, other physiological changes were observed. Table 1 summarizes the effect of dicloxacillin in some bacterial cell properties. Cells entrapped in biofilms formed in sub-inhibitory concentrations of dicloxacillin have significantly lower ($p<0.05$) water contact angles and hemagglutination titters. This last parameter can be used as an indirect measure of the production of PNAG (Mack *et al.*,1999), the molecule responsible for biofilm formation in CoNS (Mack *et al.*,1994). The reduction in PNAG production can explain the reduction in biofilm formation.

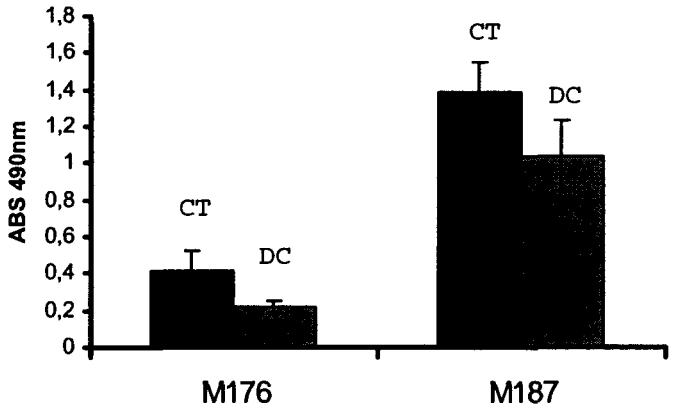

Figure 1. Amount of biofilm formed by *S. haemolyticus* M176 and *S. epidermidis* M187 in the presence of subMIC concentration of dicloxacillin (DC) and in the absence (CT), evaluated by the violet crystal colorimetric assay.

XPS analysis also revealed differences in the surface elemental composition of the biofilm cells. The high O/C ratio observed is a common CoNS characteristic and can be related to the presence of a slime layer surrounding the cell wall (van der Mei *et al.*, 2000).

Table 1. Bacterial cell surface properties of the biofilms of *S. haemolyticus* M176 and *S. epidermidis* M187 formed in the presence (DIC) and absence (CT) of subMIC concentration of dicloxacillin.

Strain	Contact angle	Hemagglutination titer	Surface elemental composition		
			N/C	O/C	P/C
M176 CT	36.4 (± 2.2)	1 : 4	0.152	0.397	0.031
M176 DIC	31.8 (± 1.7)	1 : 2	0.191	0.441	0.027
M187 CT	28.0 (± 1.1)	1 : 8	0.192	0.480	0.032
M187 DIC	22.9 (± 1.6)	1 : 4	0.145	0.399	0.038

The biofilm matrices of both CoNS species presented a lower protein content per cell when formed in dicloxacillin, with *S. haemolyticus* biofilms having a significantly ($p<0.05$) higher content in polysaccharides per cell (Table 2).

Table 2. Composition of the biofilm matrix, expressed in $\mu g/10^8$ cell, of biofilms of *S. haemolyticus* M176 and *S. epidermidis* M187 formed in the presence (DIC) and absence (CT) of subMIC concentration of dicloxacillin

Strain	Proteins	Polysaccharides
M176 CT	3.26(± 0.35)	0.98(± 0.48)
M176 DIC	1.37(± 0.38)	1.62(± 0.46)
M187 CT	3.39(± 0.20)	1.35(± 0.35)
M187 DIC	2.81(± 0.74)	1.18(± 0.15)

The biofilm matrices of both Staphylococcal species exhibited a 26 Da band when formed in dicloxacillin, which is absent in the controls (Fig. 2). However, in the case of *S. epidermidis* a 126 Da band present in the control is missing in cells from biofilms formed under subMIC conditions.

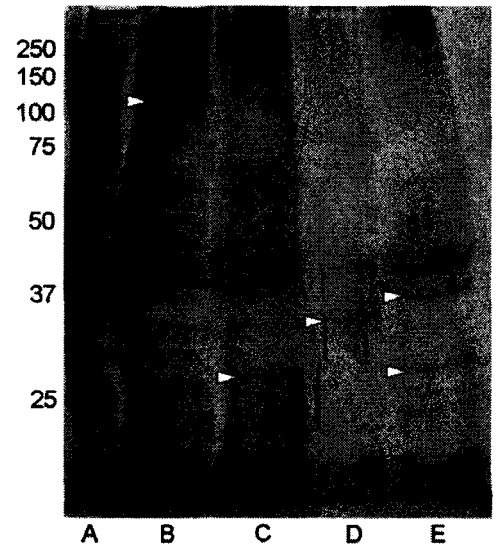

Figure 2. SDS-Page of biofilm matrices. standard sample (A), M187 CT (B), M187 DIC (C), M176 DIC (D); M176 CT (E).

The SEM observations clearly demonstrate differences in biofilm morphology due to the effect of dicloxacillin (Fig. 3). Two major differences can be found in SEM observations: (i) biofilms formed in the presence of subMIC concentrations of dicloxacillin have a lower cell density; (ii) more extracellular material is observed in control biofilms, comparing with the biofilms formed under subMIC conditions. .

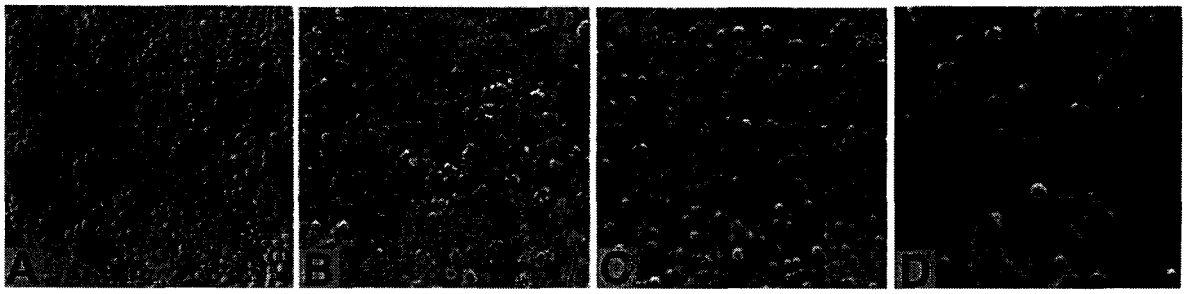

Figure 3. SEM observations of biofilm surface. A) M187 CT; B): M176 CT; C): M876 DIC; D) M176 DIC.

The growth in subMIC concentrations of dicloxacillin also induced an increase in the resistance of the biofilm cells to agents with different mechanisms of action. This resistance is especially accentuated for dicloxacillin (Fig.4).

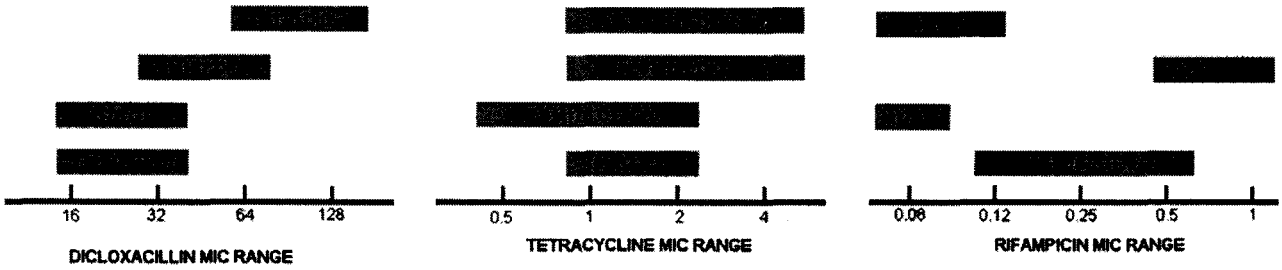

Figure 4. Shift in the MIC range of dicloxacillin, tetracycline and rifampicin, of the biofilm cells formed in the presence (DIC) and absence (CT) of subMIC concentrations of dicloxacillin.

CONCLUSIONS

Although the use of sub-inhibitory concentrations of antibiotics is effective in reducing the amount of biofilms formed, which may have some clinical interest, other changes that occur in the physiology of biofilm cells, like the increase in antibiotic resistance, might compromise the general

use of this strategy to prevent biofilm formation. It has been suggested that the matrix of biofilms can be responsible for the increasing resistance to antibiotics (Voung *et al.*, 2002). In this study, both changes in the biofilm matrix and also in antimicrobial resistance were observed. Other studies have also reported an increase in antibiotic resistance due to the growth in subMIC concentrations of antibiotics (Tambe *et al.*, 2001). Even so, the prophylaxis use of antibiotics to prevent CoNS infections is widely used (Haessler *et al*, 2003). However, new approaches are being developed, based on the same principle of subMIC growth, but using other molecules, like nonsteroidal anti-inflamatory drugs (Arciola *et al.*, 1998), which do not carry the problem of developing antimicrobial resistance.

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