

ANTIBIOFILM ACTIVITY OF *COBETIA MARINA* FILTRATE UPON *STAPHYLOCOCCUS EPIDERMIDIS* CATHETER-RELATED ISOLATES

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ABSTRACT

We report the antibiofilm activity by the sponge-associated bacterium *Cobetia marina* upon *Staphylococcus epidermidis* clinical isolates obtained from central venous catheters. Antibiofilm activity/antimicrobial susceptibility correlation might predict the action of the metabolite(s) upon *Staphylococcus epidermidis* in the clinic, making it a possible adjuvant in therapies against biofilm-associated infections.

Key words: biofilms, antibiofilm activity, *Staphylococcus epidermidis*, *Cobetia marina*, *Darwinella* sp.

Medical device-related infections represent a serious clinical problem, since the majority of hospital patients undergo procedures for the insertion of foreign devices. Indeed, staphylococcal implant-related infections are the prototype of a biofilm infection and coagulase-negative staphylococci, mainly *Staphylococcus epidermidis*, predominate as the agent of these biomedical infections (11, 12). Once the biofilm is established, therapeutic options become limited due to difficulties with the perfusion of antimicrobial agents into biofilm and to the metabolic latent state of bacteria in the biofilm lifestyle. Thus, interference with cell-cell signaling by modulating *quorum sensing* (QS) to prevent biofilm formation might be an attractive strategy to treat these infections. In this alternative approach, which does not interfere with bacterial growth, the switching off of virulence

expression and attenuation of the pathogen make microorganisms more susceptible to antimicrobials and to the immune system (9). The potential advantages of antivirulence therapies include the large number of bacterial functions that can be inhibited, the preservation of normal human microbiota and the reduction in selective pressure that may result in decreased resistance (4).

Marine sponges are reservoirs of microorganisms and their survival in this particular environment resulted in the ability to produce unique compounds as a manner of combating enemies (16), an ability which we wanted to exploit in order to target pathogenic biofilms. To our knowledge this is the first report on the production of culture filtrate obtained from the marine Gram-negative bacterium *Cobetia marina*, which interferes significantly in *S. epidermidis* biofilm formation.

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Marine sponge sampling, metabolite(s) production and bacterial strains

Marine sponges were collected in the Ilha do Arvoredo nature reserve, Santa Catarina, Brazil (27°S 16' 42.4"/48°W 22' 30.8") in November 2007. A sponge fragment was deposited in the Porifera Collection of the Museu de Ciências Naturais, Fundação Zoobotânica do Rio Grande do Sul, Brazil, referenced as MCNPOR 7831. The bacterium strain 224 (FN677754), identified as *Cobetia* sp. by 16S rRNA gene analysis as described elsewhere (1), presents 99.6% sequence similarity to *Cobetia marina* and was associated with the marine sponge *Darwinella* sp. Up to now, the only activity related with the genus *Cobetia* is its biosurfactant effect upon hydrocarbons (13). It was isolated on agar plates (NaCl 24 g L⁻¹, MgCl₂.6H₂O 11 g L⁻¹, yeast extract 1 g L⁻¹, peptone 1 g L⁻¹ and agar 20 g L⁻¹) and selected to provide an antibiofilm culture filtrate. The culture medium for metabolites production consisted of yeast extract 4.5 g L⁻¹, dextrin 50 g L⁻¹, peptone 2 g L⁻¹, NaCl 24 g L⁻¹ and MgCl₂.6H₂O 11 g L⁻¹, resulting in a pH of 5.6, and was incubated in flask at 150 rpm and 28 °C for 96 h. After centrifugation, the supernatant was filtered, giving a culture filtrate named 224CF (84.0 mg mL⁻¹) that was used in our assays.

Staphylococcus epidermidis ATCC 35984, *Pseudomonas aeruginosa* ATCC 27853 and thirteen *S. epidermidis* isolates from central venous catheters (CVC) at the Hospital de Clínicas de Porto Alegre during 2008 were used in this study. They were grown in Luria Bertani (LB) medium and a bacterial suspension equivalent to 10⁸ CFU mL⁻¹ was used in the assays.

Antibiofilm activity, antibiotic activity and filtrate culture characterization

Two protocols adapted from Antunes *et al.* (2) in 96-well polystyrene flat-bottom microtiter plates (Costar 3595 Corning Inc., NY, USA) were used. In the (i) antibiofilm formation assay, 100 µL of bacterial suspension, 100 µL of 224CF and 50 µL of LB were incubated at 37 °C for 24 h to

S. epidermidis, and for 8 h to *P. aeruginosa*, while in the (ii) biofilm eradication assay we added 100 µL of 224CF and 100 µL of LB after the period of biofilm formation. Afterwards, in both protocols, the wells were washed and heat-fixed by exposing them to hot air at 60 °C for 1 h. The adherent biofilm layer formed was stained with 0.4% crystal violet for 15 min. The stain was rinsed off, resuspended with DMSO (Sigma, St. Louis, MO, USA) and absorbance was measured at 570 nm (Spectramax M2e, Molecular Devices, CA, USA). The biofilm formation control was considered to represent 100% of biofilm formation, and the 224CF was replaced by 100 µL of LB.

Antibacterial assays were performed by measuring the bacterial growth (OD₆₀₀). As control, the culture filtrate was replaced by 100 µL of LB and was considered 100% of planktonic bacterial growth.

The strains were classified in the biofilm assay as described earlier (15), as weak (W) biofilm producer (average OD value ≤ 2 × OD_c) and moderate (M) biofilm producer (average OD value ≤ 4 × OD_c), where OD_c = average OD of negative control × (3 × standard deviation of negative control). Susceptibility profiles were obtained by microdilution using a MicroScan Walk-Away 96 SI automated system (Dade Behring Inc., IL, USA), using breakpoint values defined by CLSI 2008 (Table 1).

The culture filtrate inhibited up to 84.7 ± 3.4 % (p<0.001) and 30.0 ± 14.4 % (p<0.05) of biofilm formation by *S. epidermidis* ATCC 35984 and *P. aeruginosa* ATCC 27853, respectively. However, 224CF had no capacity to destroy preformed biofilms and no antibacterial activity either against these microorganisms. The effect against *S. epidermidis* biofilm formation was dose dependent, presenting values of 41.1 ± 8.8 %, 54.5 ± 4.9 %, 84.7 ± 3.4 % and 79.3 ± 1.8 % with 224CF concentrations in the wells of 8.4 mg mL⁻¹, 16.8 mg mL⁻¹, 25.2 mg mL⁻¹ and 33.6 mg mL⁻¹, respectively. The 224CF antibiofilm activity against *S. epidermidis* was thermo-resistant when autoclaved (15 min at 121 °C), but the it was completely eliminated when 224CF was treated with

100 µg mL⁻¹ of proteinase K (Sigma, St. Louis, MO, USA) for 24 h at 37 °C and inactivated by boiling for 15 min. According to these results, a heat-resistant peptide might be present in 224CF, acting as a modulator of the *S. epidermidis* QS system and inhibiting biofilm formation. The QS-inhibitors commonly known to interfere in QS of *Staphylococcus* spp. are cyclic and linear peptide molecules (9). Heat-resistant peptides and proteins with biological activities have already been described, such as bacteriocin and coagulant molecules (3, 7).

Due to the higher activity observed against *S. epidermidis* compared to *P. aeruginosa*, we decided to test the culture filtrate against 13 samples of CVC *S. epidermidis* isolates. Interestingly, 224CF showed statistically significant inhibition of the biofilm formation in 10 isolates (Table 1, p value < 0.05). The correlation of 224CF activity with their

antimicrobial susceptibility might predict the action of the metabolite(s) present in the filtrate upon *S. epidermidis* in the clinic, making it a possible adjuvant in therapies directed against biofilm-associated infections. After statistical analysis, 224CF was found to be more active against the isolates simultaneously susceptible to clindamycin, gentamicin and rifampicin, while there was no relationship with the susceptibility profile to penicillins, cephalosporins and quinolones. The range of 224CF antibiofilm activity is not associated with the level of biofilm production of the isolates, since the isolate 117 (weak biofilm producer) had its biofilm formation inhibited in same order of isolate 113 (moderate biofilm producer), 83.4 ± 8.5 % and 90.2 ± 1.6 %, respectively. Similar observations were found by the isolates 92 and 74b (Table 1, p value < 0.05).

Table 1. Inhibition of *S. epidermidis* clinical isolates biofilm formation by *C. marina* 224CF and profiles of susceptibility to antimicrobials.

<i>S. epidermidis</i> isolate	Biofilm producer	<i>C. marina</i> 224CF inhibition	CL	GN	RF	A/C	OX	A/S	CZ	CX	CP	LV	NO
92	W	31.3 ± 19.9 %*	R	R	R	R	R	R	R	R	R	R	R
146	M	73.7 ± 7.0 %*	R	R	R	R	R	R	R	R	R	R	R
181	M	4.3 ± 5.4 %	R	R	R	R	R	R	R	R	S	S	S
183b	M	34.9 ± 24.0 %	R	S	R	R	R	S	R	R	S	S	S
74b	M	33.5 ± 15.1 %*	R	R	S	R	R	R	R	R	R	R	R
199	M	0 %	R	R	S	R	R	R	R	R	R	R	R
196	M	46.9 ± 3.1 %*	R	R	S	R	R	R	R	R	R	R	R
50	M	72.5 ± 5.2 %*	R	S	S	R	R	R	R	R	R	R	R
73	M	75.5 ± 5.1 %*	R	S	S	R	R	R	R	R	R	R	R
117	W	83.4 ± 8.5 %*	S	S	S	R	R	R	R	R	R	R	R
172	M	80.2 ± 15.8 %*	S	S	S	R	S	S	S	S	R	R	R
113	M	90.2 ± 1.6 %*	S	S	S	R	R	R	R	R	S	S	S
45	W	75.4 ± 2.8 %*	S	S	S	S	S	S	S	S	S	S	S
ATCC 35984	W	84.7 ± 3.4 %*	R	R	S	R	R	R	R	R	S	S	S

Results represent mean ± standard deviation of 3 experiments. * represents significant difference in relation to control (p < 0.05). M and W represent moderate and weak biofilm producers, respectively. R represents resistant and S represents sensitive to the respective antibiotic; CL= clindamycin; GN= gentamicin; RF= rifampicin; A/C= amoxicillin/clavulanate; OX= oxacillin; A/S= ampicillin/sulbactam; CZ= cefazolin; CX= ceftriaxone; CP= ciprofloxacin; LV= levofloxacin and NO= norfloxacin.

Statistical analysis

All experiments were performed at least in triplicate and analyzed by Student's *t*-Test using SPSS 12.0 software. Analysis and correlations involving clinical isolates were done using Statistica 9.0.

Scanning electron microscopy (SEM)

S. epidermidis ATCC 35984 was grown in sterile 96-well microtiter plates holding pieces of glass coverslips, using LB medium in the untreated biofilm (control) and 224CF in the treated group for 24 h at 37 °C. Samples were prepared as

described elsewhere (5) and examined in a JEOL JSM-6060 scanning electron microscope. A dense and uniform staphylococcal biofilm covered the glass surface, visualized by the self-produced extracellular polymeric substances (EPS) in the control (Figure 1 panels A and C), while the 224CF-treated biofilm displayed a significant reduction in the number of adherent bacteria and in the size of aggregates, which were reduced to small clusters or even single cells. Moreover, very little EPS appeared to have been formed (Figure 1 panels B and D). These results corroborate with the inhibition observed in the biofilm assay.

EPS can vary greatly between biofilms, depending on the microorganisms present, the shear forces experienced, the temperature and the availability of nutrients (6). Poly-N-acetylglucosamine is considered the main component

of staphylococcal matrix, but proteins and extracellular teichoic acid also are present (8). Nevertheless, *P. aeruginosa* EPS may contain alginate, Pel (from pellicles) - a glucose-rich matrix polysaccharide polymer and Psl (from polysaccharide synthesis locus) - a mannose-rich and galactose-rich polysaccharide (14). The presence of two distinct molecules in the filtrate affecting *S. epidermidis* and *P. aeruginosa* separately or the marked difference in the EPS composition could explain the 224CF activity upon each bacterium. It is known that there is a connection existing between cell density and the EPS production, which is QS-regulated (10). Thus, it might be possible that 224CF is able to modulate the QS of *S. epidermidis* and thereby prevent EPS production, blocking the irreversible adhesion to a surface and, consequently, the formation of biofilm.

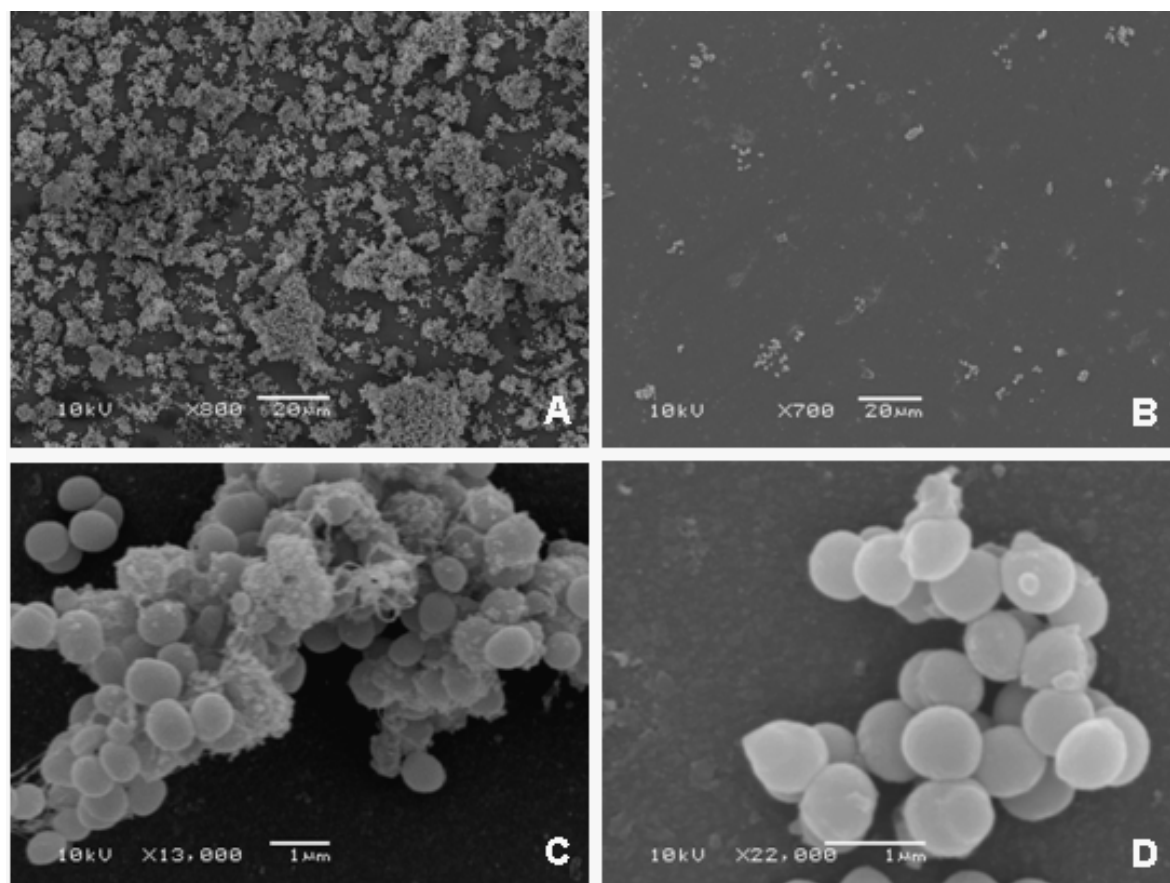


Figure 1. SEM images of the *S. epidermidis* ATCC 35984 biofilms. Panels A and C are untreated, while panels B and D are 224CF-treated biofilms. Scale bars are shown in the figures.

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