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ORIGINAL ARTICLE

Antimicrobial and antistaphylococcal biofilm activity from the sea urchin *Paracentrotus lividus*

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Abstract

Aims: Staphylococcal biofilm-associated infections are resistant to conventional antibiotics. Consequently, new agents are needed to treat them. With this aim, we focused on the effector cells (coelomocytes) of the sea urchin *Paracentrotus lividus* immune system.

Methods and Results: We tested the activity of the 5-kDa peptide fraction of the cytosol from coelomocytes (5-CC) against a group of Gram-positive, Gram-negative bacteria and fungi. We determined minimal inhibitory concentrations (MICs) ranging from 253·7 to 15·8 mg ml^{-1}. We observed an inhibitory activity and antibiofilm properties of 5-CC against staphylococcal biofilms of reference strains *Staphylococcus epidermidis* DSM 3269 and *Staphylococcus aureus* ATCC 29213. The antimicrobial efficacy of 5-CC against the biofilms of clinical strain *Staph. epidermidis* 1457 was also tested using live/dead staining in combination with confocal laser scanning microscopy. At a sub-MIC concentration (31·7 mg ml^{-1}) of 5-CC the formation of young (6-h old) and mature (24-h old) staphylococcal biofilms was inhibited.

Conclusions: The biological activity of 5-CC could be attributed to three peptides belonging to the sequence segment 9–41 of a beta-thymosin of *P. lividus*.

Significance and Impact of the Study: The effector cells of *P. lividus* represent an interesting source of marine invertebrates-derived antimicrobial agents in the development of new strategies to treat staphylococcal biofilms.

Introduction

Staphylococcal biofilms are the leading cause of device-related infections of medical relevance. *Staphylococcus aureus* is an important cause of metal biomaterial, bone joint and soft tissue infections, while *Staphylococcus epidermidis* is seen more often in polymer-associated infections (Götz 2004). Together, the Gram-positive pathogens *Staph. aureus*, *Staph. epidermidis* and *Enterococcus faecalis* represent more than 50% of the species isolated from patients with medical device-associated infections (Donelli *et al.* 2007).

Many infections such as otitis media, sinusitis, cholesteatoma, tonsillitis and adenoiditis are caused by biofilm-forming mucosal pathogens (*Pseudomonas aeruginosa*, *Staph. aureus*, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*). The role of biofilms in the persistence of chronic infection of otolaryngology interest was first recognized in otitis media. Further studies have confirmed the presence of biofilms in chronic tonsillitis and in chronic rhinosinusitis (Post *et al.* 2007).

The treatment of these kinds of infections is complicated as microbial biofilms are typically highly resistant to conventional antibiotics (Gilbert *et al.* 1997). The discovery of anti-infective agents active not only against planktonic micro-organisms but also against biofilms represents an important goal (Projan and Youngman

2002). The immune system of marine invertebrates is a poorly explored source of new antimicrobial agents. In particular, we focused on the coelomocytes effector cells of the sea urchin *Paracentrotus lividus* immune system. The coelomocytes of *P. lividus* show a wide repertoire of immunological functions, including cellular recognition, phagocytosis and cytotoxicity (Arizza *et al.* 2007). A 60-kDa protein, which showed antibacterial activity, has been isolated from lysates of coelomocytes from *P. lividus* (Stabili *et al.* 1996). Furthermore, from biological and ecological points of view, there are two good reasons to justify the choice of this species: (i) it is a long-living organism and (ii) it lives in an infralitoral environment where it is exposed to pathogenic attacks from invading micro-organisms. The survival and fitness of *P. lividus* in marine environments suggest that its innate immune system is potent and effective.

In this study, we focused on antimicrobial peptides (AMPs) characterized by a small molecular size (<10 kDa) and a wide antibacterial activity (Boman 1995; Cellura *et al.* 2007). A large variety of AMPs have been described in marine invertebrates: defensin (Hubert *et al.* 1996), myticin (Mitta *et al.* 1999) and mytilin (Mitta *et al.* 2000) in mussels; penaeidin (Destoumieux *et al.* 2000) in shrimp; tachyplesin and polyphemusin in horseshoe crab (Miyata *et al.* 1989), clavanin and styelin in ascidians (Lee *et al.* 1997), and Ci-PAP-A22 in *Ciona intestinalis* (Fedders and Leippe 2008). As far as we know, there are no studies about AMPs in *P. lividus*. Thus, we turned our attention to the evaluation of the antimicrobial activity of a 5-kDa peptide fraction from coelomocytes cytosol (5-CC) of the above-mentioned species against a group of important human pathogens. We also showed the *in vitro* efficacy of 5-CC in inhibiting the formation of *Staph. epidermidis* 1457 biofilm, a clinical strain isolated from an infected central venous catheter, as well as the antibiofilm activity of 5-CC against reference staphylococcal biofilms.

Materials and methods

Microbial strains

The strains used were *Staph. aureus* ATCC 25923, *Staph. aureus* ATCC 29213, *Staph. aureus* ATCC 43866, *Staph. epidermidis* DSM 3269, *Staph. epidermidis* 1457, *Escherichia coli* ATCC 25922, *Ps. aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231 and *Candida tropicalis* ATCC 13803.

Animals and bleeding procedure

Sea urchins collected from the Gulf of Palermo, were maintained at 15°C in marine aquaria equipped with biological and physical filters and fed with commercial invertebrate food (Azoo; Taikong Corp., Taipei, Taiwan). The coelomic fluid (CF) was withdrawn by inserting the needle of a syringe, preloaded with isosmotic anticoagulant solution (20 mmol l^{-1} Tris, 0·5 mol l^{-1} NaCl, 70 mmol l^{-1} EDTA pH 7·5) (ISO–EDTA) into the peristomal membrane. After centrifugation (900 ***g*** for 10 min at 4°C), the coelomocytes were washed two times in ISO–EDTA and resuspended at 5×10^6 cells ml^{-1} in ISO–EDTA. Coelomocyte number was calculated with a haemocytometer chamber, and dead cells were evaluated by using the eosin-Y exclusion test (0·5% in ISO–EDTA).

Preparation of coelomocyte lysate supernatant

Acid-soluble protein extracts were prepared according to the previously described method (Mercado *et al.* 2005) with some slight modifications. Coelomocytes (10×10^6) were suspended in a solution of 10% acetic acid in ISO without EDTA, sonicated (SonifierBranson, model B-15; Danbury, CT, USA) for 1 min at 0°C (1 pulse s^{-1}, 70% duty cycle) and centrifuged at 27 000 ***g*** for 30 min at 4°C to remove any precipitate. After centrifugation, the supernatants were filtered (0·45 μm, Millex™; Millipore Corp.) and freeze-dried to be later redissolved in H_2O.

A 5-kDa peptide fraction of the cytosol from celomocytes (5-CC) was obtained by using a filter through a membrane with a nominal size of 5 kDa (Ultrafree-0.5 PBCC Centrifugal filter Unit; Amicon Millipore, MA, USA).

Minimum inhibitory concentrations (MIC)

MICs were determined by a micromethod as previously described (Schillaci *et al.* 2005).

Biofilms susceptibility testing: static chamber system

Biofilms of the clinical strain *Staph. epidermidis* 1457 were cultivated in coverglass cell culture chambers (Nunc, Roskilde, Denmark) (Jager *et al.* 2005). Overnight cultures of *Staph. epidermidis* in Tryptic Soy Broth (TSB, Oxoid) medium supplemented with 0·25% glucose were diluted to OD_{600} = 0·001 then inoculated into wells of chambers (1·5 ml per well) and incubated at 37°C for 6 h (young biofilm) and 24 h (mature biofilm). To evaluate the inhibitory activity of biofilm formation, a sub-MIC concentration of 5-CC equal to 31·7 mg ml^{-1} was directly added to the bacterial suspension at time zero.

After incubation, the chambers were washed gently four times with sterile phosphate-buffered saline (PBS), then

stained with 1 μmol l^{-1} SYTO9 (green fluorescent stain for living cells) and 1 μmol l^{-1} Propidium Iodide (PI, red fluorescent stain for dead cells; Molecular Probes Inc., Eugene, OR, USA) for 15 min and observed with a confocal laser scanning microscope (CLSM).

Microscopy and image acquisition

The microscopic observations and image acquisition were performed with a Zeiss LSM 510 CLMS (Carl Zeiss, Jena, Germany) equipped with detectors and filter sets for monitoring staining with LIVE/DEAD kit. Biofilm images were obtained using a 63×/1·4 objective or a 40×/1·3i objective. Simulated section images were generated using the IMARIS software package (Bitplane AG, Zurich, Switzerland).

Staphylococcal biofilm susceptibility testing: crystal violet method

Staphylococcus epidermidis DSM 3269 and *Staph. aureus* ATCC 29213 were grown on TSB (Sigma) containing 2% glucose, for 24 h at 37°C in a shaking bath and then diluted 1 : 200 as to obtain a suspension whose optical density (OD) at 570 nm was about 0·015. The diluted suspension was added to the wells (100 μl per well) of a polystyrene microtiter plate and incubated for 24 h at 37°C; then the wells were washed three times with 200 μl of sterile PBS and finally air-dried in inverted position at 37°C. Each of the wells was then filled with 100 μl of Mueller–Hinton broth. With the exception of the positive (growth) control wells, the Mueller–Hinton broth was supplemented with 5-CC at concentrations, obtained by dilution, ranging from 126·8 to 7·9 mg ml^{-1} and incubated at 37°C for 24 h. Following this incubation period, the medium was removed, the plates were air-dried in inverted position, and then each well was stained with a crystal violet solution as described (Pitts *et al.* 2003). The optical density (OD) of each well was read by a microplate reader (ELX 800; Bio-Tek instruments) at 570 nm. Comparing the average of optical density of the growth control (not treated) wells with that of sample wells, we calculated the inhibition percentages for each concentration of the 5-CC by the following formula:

$$\text{OD growth control} - \text{OD sample}/\text{OD growth control} \times 100$$

The ability to prevent formation of staphylococcal biofilms was assessed by directly adding 5-CC at concentrations ranging from 126·8 to 7·9 mg ml^{-1} in 100 μl of the bacterial suspensions inoculated in the wells of a 96-well plate at 37°C at time zero. After 24 h, the wells were washed, stained and read as already described.

Identification of 5-kDa peptide fraction (5-CC) components

SDS-polyacrylamide gel electrophoresis

SDS-PAGE in slab gel was performed according to a described method (Laemmli 1970); proteins were stained with Coomassie Blue. Gels were calibrated with molecular weight standard proteins (Sigma), and the molecular weights of the resulting bands were calculated by image analysis software (AlphaEasyFC; Alpha Innotech Corp.). Gels were satined with silver stain (Bio-Rad). The average of three independent experiments was evaluated.

RP-HPLC-nESI-MSMS

HPLC-grade water and CH_3CN were provided by Carlo Erba (Milan, Italy). Capillary RP-HPLC/nESI-MSMS was performed using an Ultimate 3000 LC system (Dionex Corporation, Sunnyvale, CA, USA) coupled online with a linear ion trap nano-electrospray mass spectrometer (LTQ; Thermo Fischer Scientific, San Jose, CA, USA). Ionization was performed with liquid junction and a non-coated capillary probe (20 mm i.d.; New Objective).

An amount of 2 mg of 5-CC was freeze-dried and redissolved in H_2O–0·1% formic acid (FA) (1 mg ml^{-1}). Ten microlitres of this solution was directly loaded onto a C18 μ-precolumn cartridge (0·3 μm × 5 mm, 100 Å, 5 μm, PepMap; Dionex) equilibrated with 0·1% aqueous FA at a flow rate of 20 μl min^{-1} for 4 min. Subsequently, the extract was applied onto a C18 capillary column (150 × 0·18 mm, 300 Å, 5 μm; Thermo Electron) and eluted at room temperature with a linear gradient of CH_3CN-0·1% FA/H_2O–0·1% FA from 5% to 50% in 50 min at a flow rate of 2 μl min^{-1}.

Repetitive mass spectra were acquired from *m/z* 350 to 2000 mass range in positive ion mode using the following electrospray ion source parameters: capillary temperature, 220°C; spray voltage, 1·8 kV.

Peptide ions were analysed by the *N*th-dependent method as follows: (i) full MS scan (mass to charge ratio 350 : 2000); (ii) ZoomScan (scan two major ions with higher resolution; isolation width 2 Da); and (iii) MS/MS of these two ions (Q 0·250, activation time 5 50 of the ms, collision energy 23 a.u.).

Database search and protein identification

MSMS data were used to perform protein identifications by searching in nonredundant protein sequence database (NCBInr) using the MOWSE algorithm as implemented in the MS search engine MASCOT (Matrix Science) (http://www.matrixscience.com). The MSMS data were extracted as DTA files by means of Bioworks 3.2 (Thermo Fischer Scientific) and converted into Mascot generic

format. The following parameters were used for database searches: taxonomy: Eukaryota; no enzyme as cleavage specificity; mass tolerance of (1·2 Da for the precursor ions and a tolerance of 0·6 Da for the fragment ions; allowed modifications: oxidation of Met (variable), transformation of N-terminal Gln and N-terminal Glu residue in the pyroglutamic acid form (variable).

Only proteins that met the following criteria were accepted as unambiguously identified: MASCOT score > 76 [probability-based MOWSE score: $-10 \times \log(P)$, where P is the probability that the observed match is a random event. Score >76 indicates identity or extensive homology ($P < 0{\cdot}05$)]. Additionally, every peptide used for protein identification was checked for (i) y-ion series: ≥80% of the y-ions should be available; (ii) presence of the b-ions; and (iii) e value $<1 \times 10^{-10}$ (probability that the observed match is a random peptide).

Results

Antimicrobial activity of 5-CC

The antimicrobial activity of the 5-kDa peptide fraction from coelomocytes cytosol (5-CC) of *P. lividus* expressed as MICs against microbial reference strains (planktonic cells) is listed in Table 1. We tested 5-CC at concentrations ranging from 507·5 to 7·9 mg ml^{-1} against a group of Gram-positive and Gram-negative bacterial reference strains and against two human pathogen fungi. The 5-CC resulted active against all tested microbial strains with MIC values ranging from 15·8 mg ml^{-1} against *C. tropicalis* ATCC 13803 to 253·7 mg ml^{-1} against *Ps. aeruginosa* ATCC 9027.

5-CC resulted effective, with MIC values ranging from 253·7 to 63·4 mg ml^{-1}, against planktonic staphylococcal strains *Staph. epidermidis* DSM 3269, *Staph. epidermidis* 1457, *Staph. aureus* ATCC 29213, *Staph. aureus* ATCC 25923 and methicillin-resistant *Staph. aureus* ATCC 43866.

Table 1 Antimicrobial activity of 5-kDa peptide fraction from celomocytes (5-CC). Values *in vitro* expressed in mg ml^{-1} for all strains tested

	5-CC (5 kDa total cytosol) Minimum inhibitory concentrations (MIC) values in mg ml^{-1}
Staphylococcus aureus ATCC 29213	126·8
Staph. aureus ATCC 25923	63·4
Staph. aureus ATCC 43866	63·4
Staphylococcus epidermidis 1457	126·8
Staph. epidermidis DSM 3269	253·7
Escherichia coli ATCC 25922	126·8
Pseudomonas aeruginosa ATCC 9027	253·7
Candida albicans ATCC 10231	31·7
Candida tropicalis ATCC 13813	15·8

Antibacterial activity of 5-CC against staphylococcal biofilms

The biofilm of clinical strain *Staph. epidermidis* 1457 was tested for its susceptibility to 5-CC by CLSM combined with viability staining. A treatment with a sub-MIC concentration of 5-CC equal to 31·7 mg ml^{-1} (the MIC value against planktonic form of the same strain was 126·8 mg ml^{-1}) was found to inhibit adhesion, and biofilm formation after 6 h (young biofilm) and 24 h (mature biofilm) with a significant reduction of viable cells was evident (Fig. 1).

The activity of 5-CC on biofilm formation and against preformed 24-h old biofilms of reference strains *Staph. epidermidis* DSM 3269 and *Staph. aureus* ATCC 29213 was investigated by crystal violet method. At concentrations of 5-CC between 126·8 and 7·9 mg ml^{-1}, interesting inhibition percentages ranging from 85% to 50% were found for preventative activity of *Staph. epidermidis* biofilm formation. For *Staph. aureus*, we obtained lower inhibition percentages ranging from 61% to 28·4% at concentrations of 5-CC between 126·8 and 15·8 mg ml^{-1} (Fig. 2).

The antibiofilm activity of 5-CC against preformed 24-h old *Staph. aureus* ATCC 29213 biofilms at concentrations ranging from 126·8 and 15·8 mg ml^{-1} was estimated, and inhibition percentages from 61·8 to 37·4 were obtained. With similar concentrations of 5-CC, weaker antibiofilm effects were found against preformed *Staph. epidermidis* DSM 3269 biofilms (Fig. 3).

Identification of 5-kDa peptide fraction (5-CC) components

Three principal peptides were detected by SDS-PAGE of the 5-CC fraction. The size of the peptides was estimated by comparing them to molecular weight standard proteins (Fig. 4). In order to confirm the presence of peptides and determine their sequence, the 5-CC fraction was subjected to RP-HPLC/nESI-MSMS. The MSMS data obtained were used to perform database searches and

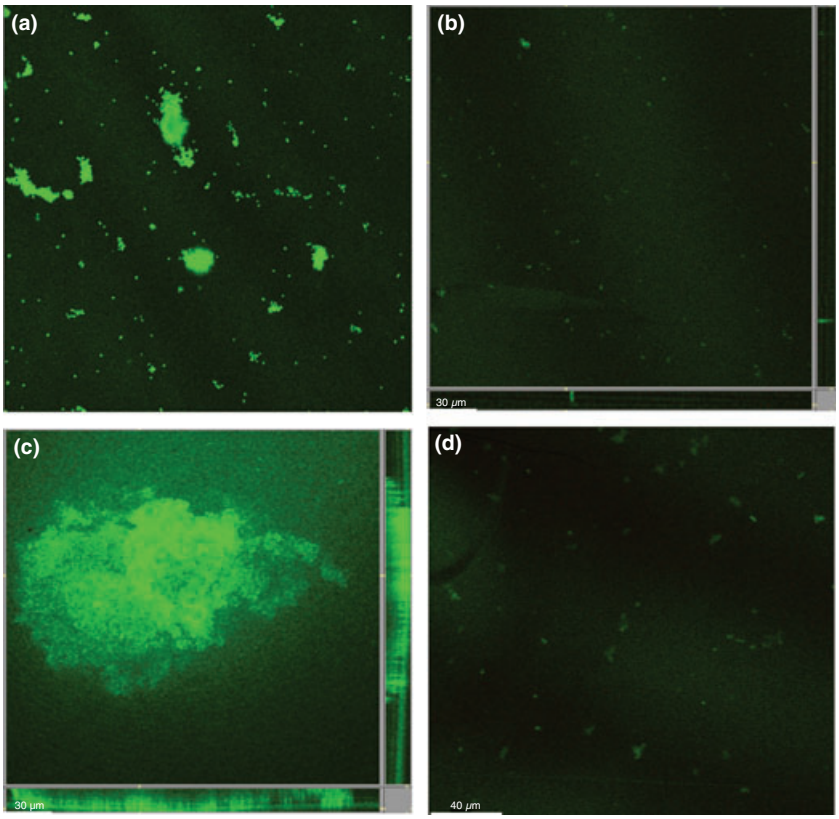

Figure 1 Preventative inhibitory activity of 5-CC. (a) *Staphylococcus epidermidis 1457* growth control 6-h old; (b) treated with a concentration of 31·7 mg ml^{-1} after 6 h; (c) *Staph. epidermidis 1457* growth control 24-h old; (d) treated with a concentration of 31·7 mg ml^{-1} after 24 h. After 6- or 24-h treatment, the biofilms were stained with LIVE/DEAD materials (SYTO9, green; PI, red) and observed using CLMS. The assays were repeated at least twice, and similar results were obtained.

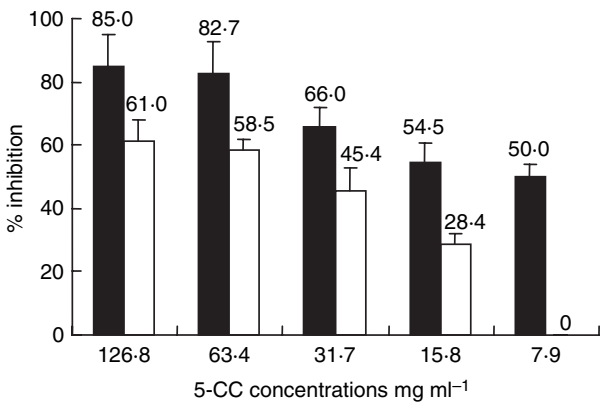

Figure 2 Preventative inhibitory activity of 5-CC on *Staphylococcus epidermidis* DSM 3269 and *Staphylococcus aureus* 29213 biofilm formation. Activity expressed as inhibition percentages; Values are the mean of at least three independent determinations. Error bars represent standard deviations. (■) *Staphylococcus epidermidis* and (□) *Staph. aureus*.

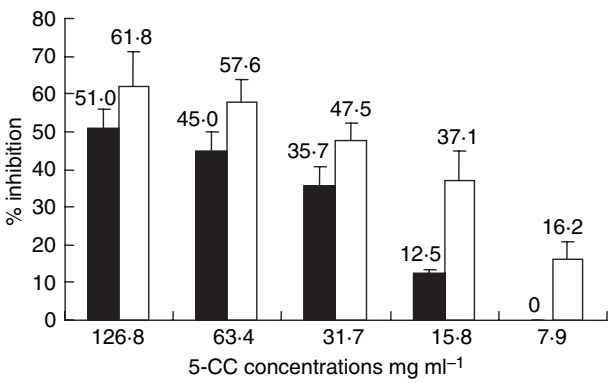

Figure 3 Antibiofilm activity of 5-CC against preformed 24-h-old biofilm of *Staphylococcus epidermidis DSM 3269* and *Staphylococcus aureus* 29213. Activity expressed as inhibition percentages; Values are the mean of at least three independent determinations. Error bars represent standard deviations. (■) *Staphylococcus epidermidis* and (□) *Staph. aureus*.

allowed the identification of three peptides belonging to the sequence segment 9–41 of a beta-thymosin of *P. lividus* (NCBInr acc. no gi|22474470) whose molecular mass is 4592 Da. The sequences of identified peptides are reported in Table 2.

Discussion

The antimicrobial defence system of marine invertebrates is based solely on an innate immune system that includes both humoral and cellular responses. AMPs constitute

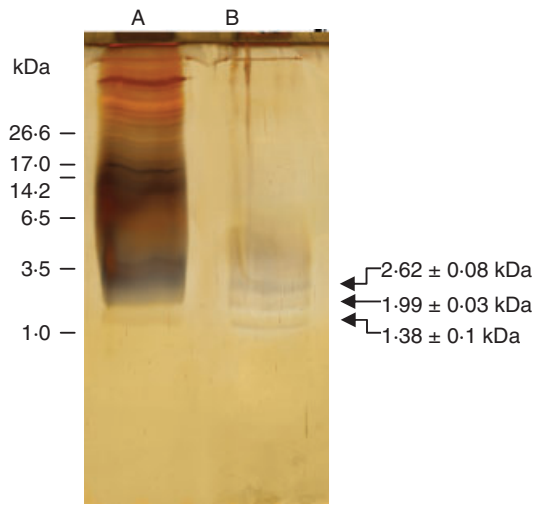

Figure 4 Silver stain of Tris/glycine SDS-PAGE of *Paracentrotus lividus* cytosol. The samples were running in a 20% gel, stained with. A: 30 μl of unfractionated cytosol. B: 30 μl of 5-CC. Molecular weight markers are on the left.

Table 2 Identification of principal peptides of 5-CC by ESI-MS. The three peptides belonging to the sequence segment 9–41 of a beta-thymosin of *Paracentrotus lividus*.

Position	ESI-MS calculated MH+	Sequence
9–19	1251·7	EVASFDKSKLK
12–31	2293·2	SFDKSKLKKAETQEKNTLPT
24–41	2088·1	QEKNTLPTKETIEQEKTA

a major component of their humoral immunity. They comprise short sequences of amino acids ranging around 10–50 amino acids in length and have a net positive charge because of their excess number of cationic residues. Marine invertebrates AMPs display broad antimicrobial spectra, even against human pathogens (Tincu and Taylor 2004).

In this work, we observed that 5-CC possesses a broad antimicrobial activity against all tested human pathogens. Small-sized molecules and broad antimicrobial spectrum are two common characteristics of AMPs, hence we employed RP-HPLC/nESI-MSMS to confirm the presence of AMPs in the 5-CC content. Three principal peptides in 5-CC, whose molecular weights were respectively 1251·7, 2088·1 and 2292·2 Da, were identified: they were the (9–19), (12–31), (24–41) fragments of a beta-thymosin of *P. lividus*.

The beta-thymosins are a family of highly conserved polar 5-kDa peptides originally thought to be thymic hormones. They are present at high concentrations in almost every cell from vertebrate phyla, but their presence has also been reported in marine invertebrates (Safer and Chowrashi 1997). Thymosin β4, as well as other members of this ubiquitous peptide family, were identified as the main intracellular G-actin sequestering peptides (Safer *et al.* 1990). Furthermore, several biological effects are attributed to beta-thymosins: induction of metalloproteinases, chemotaxis, angiogenesis and inhibition of inflammation as well as the inhibition of bone marrow stem cell proliferation in vertebrates (Huff *et al.* 2001). Beta-thymosin is also described as one of the AMP of platelets from animals and human beings (Tang *et al.* 2002).

It is possible that the antimicrobial and antistaphylococcal biofilm inhibitory activity of 5-CC is associated with the three identified peptides that matched with beta-thymosin of *P. lividus*. In particular, the smallest peptide, 11 amino acids in length, has a good chance of being an antimicrobial peptide (Wang and Wang 2004): it has a net positive charge because of an excess number of lysine residues, and it has three hydrophobic residues and a total hydrophobic ratio of 36%. Hydrophobic and charged residues may permit interaction with bacterial membranes (Hancock *et al.* 2006).

Chronic staphylococcal infections and device-related infections can be attributed to the growth of bacteria as biofilms. Staphylococcal biofilms are intrinsically resistant to conventional antibiotics, and currently no effective therapies that target microbial biofilms exist. Early removal of the device or surgical interventions remains the most effective means to treat biofilm-associated infections (Brady *et al.* 2008). Therefore, novel antibiofilm agents, treatments and strategies are needed. The inhibition of formation of staphylococcal biofilms on surfaces of medical devices is one of the possible strategies to combat them.

In our study, we observed an interesting inhibitory effect of 5-CC at a sub-MIC concentration, either on the formation of a young biofilm (6-h old) of *Staph. epidermidis* 1457 or on the formation of a mature biofilm (24-h old) of the same clinical strain. The susceptibility to antimicrobial treatment of a biofilm can depend on the stage of development (age) of the biofilm itself. A mature biofilm can be more tolerant to antimicrobial treatment than a young biofilm. Live/dead staining was used to assay bacterial viability with or without treatment. As there is no sign of dead cells, the reduction in bacterial adhesion could be because of an interference of 5-CC peptides with microbial surface proteins (adhesins, autolysins) that facilitate attachment to plastic surfaces in the first step of staphylococcal biofilm formation (Patti and Hook 1994; Heilmann *et al.* 1997). Of course, further studies are needed to explain the mechanism of action of 5-CC in the activity of prevention of adhesion and biofilm formation.

5-CC also showed a preventative activity and a good antibiofilm activity against preformed staphylococcal reference strains.

We believe that 5-CC is an interesting source of antimicrobial compounds with pharmaceutical potential (drugs or drug leads) as antistaphylococcal biofilms agents. Nevertheless, new studies are needed to correlate the activity of 5-CC with its principal constituents, and we are currently evaluating and comparing the antimicrobial properties of each single peptide and of different combinations of the three peptides.

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References

Arizza, V., Giaramita, F.T., Parrinello, D., Cammarata, M. and Parrinello, N. (2007) Cell cooperation in coelomocyte cytotoxic activity of *Paracentrotus lividus* coelomocytes. *Comp Biochem Physiol A Mol Integr Physiol* **147**, 389–394.

Boman, H. (1995) Peptide antibiotics and their role in innate immunity. *Annu Rev Immunol* **13**, 61–92.

Brady, R.A., Leid, J.G., Calhoun, J.H., Costerton, J.W. and Shirtliff, M.E. (2008) Osteomyelitis and the role of biofilm in chronic infection. *FEMS Immunol Med Microbiol* **52**, 13–22.

Cellura, C., Toubiana, M., Parrinello, N. and Roch, P. (2007) Specific expression of antimicrobial peptide and HSP70 genes in response to heat-shock and several bacterial challenges in mussels. *Fish Shellfish Immunol* **22**, 340–350.

Destoumieux, D., Munoz, M., Bulet, P. and Bachère, E. (2000) Penaeidins, a family of antimicrobial peptides from penaeid shrimp (Crustacea, Decapoda). *Cell Mol Life Sci* **57**, 1260–1271.

Donelli, G., Francolini, I., Romoli, D., Guaglianone, E., Piozzi, A., Ragunath, C. and Kaplan, J.B. (2007) Synergistic activity of dispersin B and cefamandole nafate in inhibition of staphylococcal biofilm growth on polyurethanes. *Antimicrob Agents Chemother* **51**, 2733–2740.

Fedders, H. and Leippe, M.A. (2008) Reverse search for antimicrobial peptides in *Ciona intestinalis*: identification of a gene family expressed in hemocytes and evaluation of activity. *Dev Comp Immunol* **32**, 286–298.

Gilbert, P., Das, J. and Foley, I. (1997) Biofilm susceptibility to antimicrobials. *Adv Dent Res* **11**, 160–167.

Götz, F. (2004) Staphylococci in colonization and disease: prospective targets for drugs and vaccine . *Curr Opin Microbiol* **7**, 477–487.

Hancock, R.E.W., Brown, K.L. and Mookherjee, N. (2006) Host defence peptides from invertebrates-emerging antimicrobial strategies. *Immunobiology* **211**, 315–322.

Heilmann, C., Hussain, M., Peters, G. and Götz, F. (1997) Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystirene surface. *Mol Microbiol* **20**, 1013–1024.

Hubert, F., Noël, T. and Roch, Ph. (1996) A new member of the arthropod defensin family from edible Mediterranean mussels (*Mytilus galloprovincialis*). *Eur J Biochem* **240**, 302–306.

Huff, T., Müller, C.G.S., Otto, A.M., Netzker, R. and Hannappel, E. (2001) β-Thymosins, small acidic peptides with multiple functions. *Int J Biochem Cell Biol* **33**, 205–220.

Jager, S., Mack, D., Rohde, H., Horstkotte, M.A. and Knoblock, J.K. (2005) Disintegration of *Staphylococcus epidermidis* biofilms under glucose-limiting conditions depends on the activity of the alternative sigma factor sigmaB. *Appl Environ Microbiol* **71**, 5577–5581.

Laemmli, U.K. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lee, I.H., Cho, Y. and Lehrer, R.I. (1997) Styelins, broad-spectrum antimicrobial peptides from the solitary tunicate, *Styela clava*. *Comp Biochem Physiol B Biochem Mol Biol* **118**, 515–521.

Mercado, L., Schmitt, P., Marshall, S.H. and Arenas, G. (2005) Tissues of the mussel *Mytilus edulis chilensis*: a new source for antimicrobial peptides. *J Biochem* **8**, 284–290.

Mitta, G.F., Hubert, T., Noël, B. and Roch, P. (1999) Myticin, a novel cysteine-rich antimicrobial peptide isolated from hemocytes and plasma of the mussel *Mytilus galloprovincialis*. *Eur J Biochem* **265**, 71–78.

Mitta, G.F., Vandenbulcke, F., Hubert, M., Salzet, M. and Roch, P. (2000) Involvement of mytilins in mussel antimicrobial defense. *J Biol Chem* **275**, 12954–12962.

Miyata, T., Tokunaga, F., Yoneya, T., Yoshikawa, K., Iwanaga, S., Niwa, M., Takao, T. and Shimonishi, Y. (1989) Antimicrobial peptides, isolated from horseshoe crab hemocytes, tachyplesin II, and polyphemusins I and II: chemical structures and biological activity. *J Biochem* **106**, 663–668.

Patti, J.M. and Hook, M. (1994) Microbial adhesins recognizing extracellular matrix macromolecules. *Curr Opin Cell Biol* **6**, 752–758.

Pitts, B., Hamilton, B.A., Zelver, N. and Stewart, P.S. (2003) A microtiter-plate screening methodfor biofilm disinfection and removal. *J Microbiol Methods* **54**, 269–276.

Post, J.C., Hiller, N.L., Nistico, L., Stoodley, P. and Ehrlich, G.D. (2007) The role of biofilms in otolaryngologic infections: update 2007. *Curr Opin Otolaryngol Head Neck Surg* **15**, 347–351.

Projan, S.J. and Youngman, P.J. (2002) Antimicrobials: new solutions badly needed. *Curr Opin Microbiol* **5**, 463–465.

Safer, D. and Chowrashi, P.K. (1997) Beta-thymosin from marine invertebrates: primary structure and interaction with actin. *Cell Motil Cytoskeleton* **38**, 163–171.

Safer, D., Golla, R. and Nachmias, V.T. (1990) Isolation of a 5-kilodalton actin sequestering peptide from human blood platelets. *Proc Natl Acad Sci USA* **87**, 2536–2540.

Schillaci, D., Petruso, S. and Sciortino, V. (2005) 3,4,5,3′,5′-Pentabromo-2(2′-hydroxybenzoyl) pyrrole: a potential lead compound as anti-Gram-positive and anti-biofilm agent. *Int J Antimicrob Agents* **25**, 338–340.

Stabili, L., Pagliara, P. and Roch, P. (1996) Antibacterial activity in the coelomocytes of the sea urchin *Paracentrotus lividus*. *Comp Biochem Physiol B Biochem Mol Biol* **113**, 639–644.

Tang, Y.Q., Yeaman, M.R. and Selsted, M.E. (2002) Antimicrobial peptides from human platelets. *Infect Immun* **70**, 6524–6533.

Tincu, J.A. and Taylor, S.W. (2004) Antimicrobial peptides from marine invertebrates. *Antimicrob Agents Chemother* **48**, 3645–3654.

Wang, Z. and Wang, G. (2004) ADP: The antimicrobial peptide database. *Nucleic Acids Res* **32**, D590–D592.

