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Fragments of β-thymosin from the sea urchin *Paracentrotus lividus* as potential antimicrobial peptides against staphylococcal biofilms

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The immune mediators in echinoderms can be a potential source of novel antimicrobial peptides (AMPs) applied toward controlling pathogenic staphylococcal biofilms that are intrinsically resistant to conventional antibiotics. The peptide fraction <5 kDa from the cytosol of coelomocytes of the sea urchin *Paracentrotus lividus* (5-CC) was tested against a group of Gram-positive and Gram-negative pathogen reference strains. The 5-CC of *P. lividus* was active against all planktonic-tested strains but also showed antibiofilm properties against staphylococcal strains. Additionally, we demonstrated the presence of three small peptides in the 5-CC belonging to segment 9-41 of a *P. lividus* β-thymosin. The smallest of these peptides in particular, showed the common chemical–physical characteristics of AMPs. This novel AMP from β-thymosin has high potential activity as an antibiofilm agent, acting on slow-growing bacterial cells that exhibit a reduced susceptibility to conventional antibiotics and represent a reservoir for recurrent biofilm-associated infections.

Keywords: antibiofilm agents; antimicrobial peptides (AMPs); staphylococcal biofilms

Introduction

Staphylococci can induce a wide spectrum of infectious diseases associated with remarkable morbidity and mortality.[1] Pathogenic staphylococci have an extraordinary ability to acquire several antibiotic resistance traits, and the rise of community and hospital-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) is a major health problem worldwide. This scenario has created an urgent need for novel therapeutic approaches[2] to control drug-resistant bacterial strains, not only in their free-living planktonic form, but also when encountered as biofilms—bacterial communities able to grow on surfaces and surrounded by an extracellular polymeric substance (EPS) matrix.

The ability to form biofilms, is probably the most important virulence factor of staphylococci in the development of the chronic and persistent form of several infectious diseases in humans such as otitis media, osteomyelitis, endophtalmitis, urinary tract infections, acute septic arthritis, native valve endocarditis, burn or wound infections, and cystic fibrosis-associated pulmonary infections.[3–9]

Furthermore, staphylococcal biofilms are commonly isolated from medical device-related infections with *S. aureus* mainly involved in metal-biomaterial infections, while *Staphylococcus epidermidis* is more often observed in polymer-associated infections.[10] The Gram-positive pathogens *S. aureus*, *S. epidermidis,* and *Enterococcus faecalis* represent more than 50% of the species isolated from patients with medical device–associated infections,[11] and catheter-related bloodstream infections (CRBSIs) during intensive care unit (ICU) stays in four European countries (France, Germany, Italy,

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UK) have an estimated cost of € 163.9 million in health care.[12]

Staphylococcal biofilm resistance to antibiotics: a multifactorial mechanism

Bacterial biofilms are more resistant to host immune defense systems and display a significantly high degree of antibiotic tolerance.[13] Antibiotic resistance in biofilms is multifactorial, because biofilm structured bacteria develop different mechanisms of resistance. The bacteria in the external layers of the community, for example, are more active in cell division and energetic metabolism compared to the internal layers, due to oxygen and nutrient gradients from the top to the bottom of a biofilm. A metabolically heterogeneous bacterial population differs markedly from a free-living (planktonic) population[14] and nutrient-depleted zones can result in stationary phase-like cells (dormant metabolic state) with reduced susceptibility to antibiotics.[15]

The EPS matrix may retard the rate of penetration of antibiotics enough to induce the expression of genes that mediate resistance within the biofilm.[3] Other well-known mechanisms such as the production of enzymes that degrade antibiotics, alteration of targets, or overexpression of efflux pumps that have a broad range of substrates, are associated with the planktonic cells, but bacterial cells growing in biofilm increase horizontal gene transmission so they can easily spread antibiotic resistance traits.[16] Furthermore, specialized populations of persister cells in the *S. aureus* biofilms, remain in a dormant state in the presence of an antibiotic, with no growth and no death.[17] This mechanism is believed to be responsible for recurrent infections in hospital settings because the persister cells give rise to a normal bacterial colony after drug removal.

It has been observed that *S. aureus* in biofilms is 100–1000 times less susceptible to antibiotics than equivalent populations of planktonic bacteria.[18] Conventional antibiotics can be effective against metabolically active bacterial cells but currently no effective therapies for staphylococcal biofilms exist. Early removal of the device or surgical intervention, remain the most effective means to treat biofilm-associated infections, to date.[4] Therefore, there is an urgent need for novel treatments, strategies, and antistaphylococcal biofilm agents.

Discovery of novel antistaphylococcal biofilm agents

In the biofilm preclinical research field, three different approaches are primarily followed: screening of novel compounds (synthetic or natural) that inhibit staphylococcal biofilms through direct effects on bacterial growth and viability; target-based strategy for discovering agents that show antibiofilm properties by targeting specific pathways essential for staphylococcal biofilm formation; and enzymes that target staphylococcal biofilm matrix.[19,20] This paper will focus on discovery of novel antimicrobial peptides (AMPs) derived from the beta-thymosin peptide of *Paracentrotus lividus* as new antistaphylococcal biofilm agents.

Activity-based screening of antibiofilm agents

We focused on the immune system of marine invertebrates as a relatively underexplored source of new antimicrobial agents. Echinoderms are intertidal benthic organisms that are constantly exposed to a persistent threat of infection by high concentrations of bacteria and viruses from the marine environment. The survival of these organisms depends on efficient antimicrobial mechanisms that protect them against pathogens. We focused on the coelomocytes, the immune mediators in echinoderms. In particular, our study focused on the sea urchin, *Paracentrotus lividus,* which is a common species in the Mediterranean sea. The coelomocytes of echinoderms are responsible of a wide repertoire of cellular and humoral immunologic functions, including cellular recognition, phagocytosis, cytotoxicity, and the production of antimicrobial peptides (AMPs).[21,22] In addition to AMPs, a 60-kDa protein, which showed antibacterial activity, has been isolated from lysates of coelomocytes from *P. lividus*.[23] The survival and fitness of *P. lividus* in marine environments suggest that its innate immune system is potent and effective, since this species is a long-living organism. Moreover, it lives in an infralitoral environment where it is exposed to pathogenic attacks from invading microorganisms also of anthropic origin, and it is not fouled, so it clearly has developed strategies to prevent bacterial colonization on its surface. All these biological and ecological aspects render the sea urchin *P. lividus* a good source

for AMPs with high potential as novel antimicrobial molecules.

Antimicrobial peptides (AMPs) are characterized by a small molecular size (<10 kDa, or ~10–50 amino acids) and broad antibacterial activity. A large variety of AMPs have been described in marine invertebrates and represent the major humoral defense system against pathogens: defensin, mytticin, and mytilin in mussels; penaeidin in shrimp; tachyplesin; and polyphemusin in horseshoe crab; clavanin; and styelin in ascidians, and Ci-PAP-A22 in *Ciona intestinalis*.[24–32]

AMPs in *P. lividus* have not been previously evaluated. We studied the antimicrobial and antistaphylococcal biofilm activity of a 5-kDa peptide fraction from coelomocytes cytosol (5-CC) against reference strains and isolates of human and animal origin.

Biological activity of a 5-kDa peptide fraction from coelomocytes cytosol

The 5-CC of *P. lividus* was tested against a Gram-positive (*S. aureus* and *S. epidermidis*, including drug-resistant strains) group and a Gram-negative (*Pseudomonas aeruginosa*, *E. coli*) group, and planktonic reference yeast (*C. albicans*, *C. tropicalis*) strains by using a microdilution method and determining the minimum inhibitory concentration (MIC). The 5-CC showed a broad antimicrobial activity against all tested strains (Table 1). Moreover, 5-CC showed antibiofilm properties against staphylococcal biofilms of reference strains *S. epidermidis* DSM 3269 and *S. aureus* ATCC 29213. The antimicrobial efficacy of 5-CC against biofilms of the clinical strain *S. 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) of 5-CC the formation of young (six hours old) and mature (24-hours old) staphylococcal biofilms was inhibited. We observed an interesting inhibitory effect of 5-CC at a sub-MIC concentration, either on the formation of a young biofilm (six hours old) of *S. epidermidis* 1457 or on the formation of a mature biofilm (24 hours old) of the same clinical strain (Fig. 1).[36] 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 was no sign of dead cells, the reduction in bacterial adhesion could be due to an interference of 5-CC peptides with microbial surface proteins (adhesins, autolysins) that facilitate attachment to surfaces in the first step of staphylococcal biofilm formation.[33,34]

Table 1. Antimicrobial activity of a 5 kDa peptide fraction from celomocytes (5-CC)[a]

	5-CC MIC values in mg/mL
S. aureus ATCC 29213	126.8
S. aureus ATCC 25923	63.4
S. aureus ATCC 43866	63.4
S. epidermidis 1457	126.8
S. epidermidis DSM 3269	253.7
E. coli ATCC 25922	126.8
P. aeruginosa ATCC 9027	253.7
C. albicans ATCC 10231	31.7
C. tropicalis ATCC 13813	15.8

[a]Values *in vitro* expressed in mg/mL for all strains tested.

Considering that biofilms can be found in virtually all natural ecosystems that support microbial growth, they also have enormous impact in veterinary medicine because they can be responsible for the failure in antimicrobial therapy in bacterial infections or in a failure of properly sanitizing of food processing plants. Many aspects of the farm management, in animal health and welfare and in food processing premises should be reconsidered in the light of these very common bacterial communities. *S. aureus* is a major pathogen of mastitis, which is one of the most common diseases in dairy cattle, and we are currently testing the 5-CC against some *S. aureus* isolates of animal origin. From preliminary experiments, we determined good antistaphylococcal activity against planktonic *S. aureus* isolates (MIC values ranging from 0.62 to 0.31 μg/mL). We

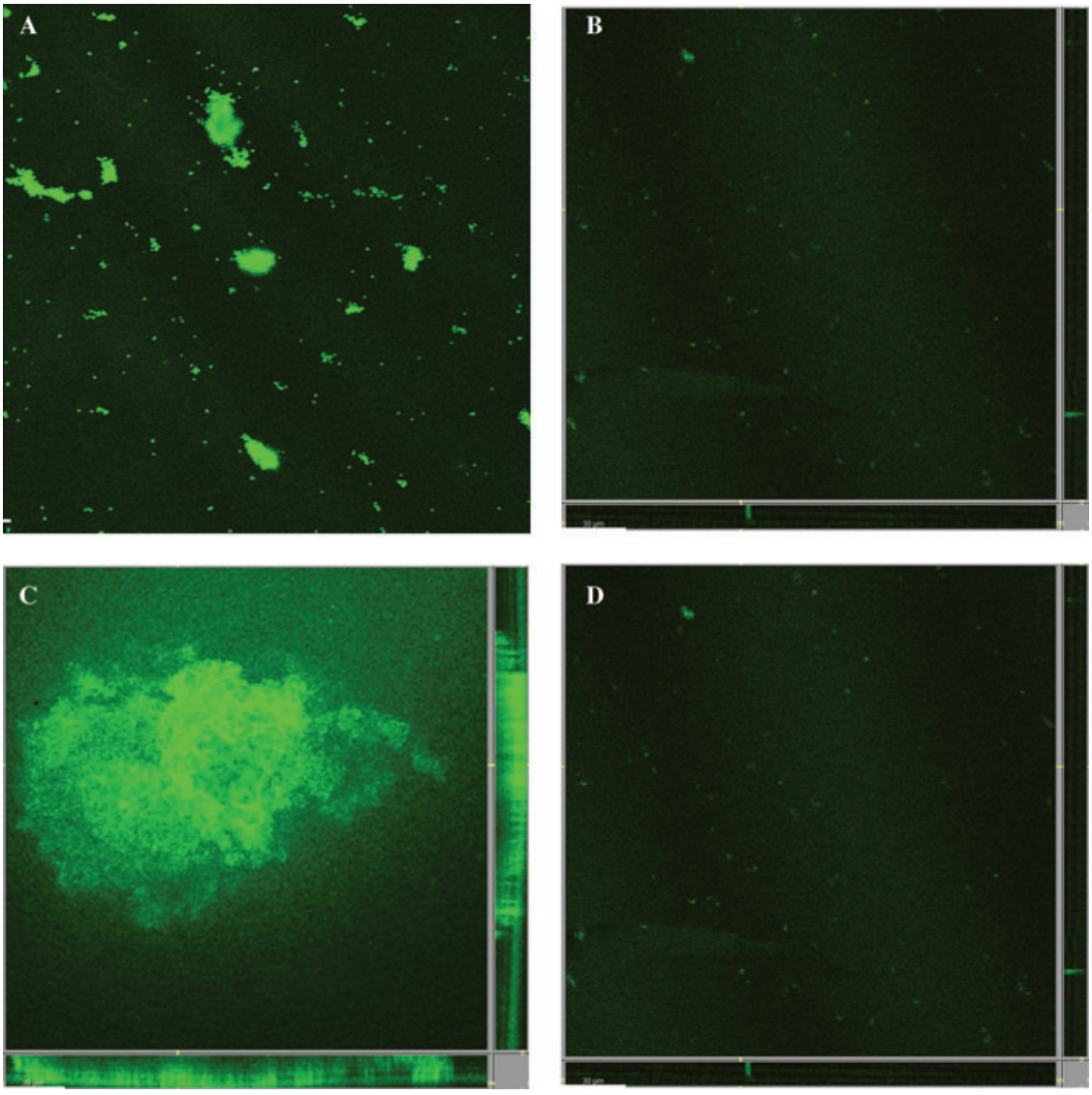

Figure 1. Preventative inhibitory activity of 5-CC. (Panel A) *Staphylococcus epidermidis* 1457 growth control (six hours old); (panel B) treated with a concentration of 31.7 mg/mL after six hours; (panel C) *S. epidermidis* 1457 growth control (24 hours old); (panel D) treated with a concentration of 31.7 mg/mL after 24 hours. 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.[36]

also plan to evaluate the activity of 5-CC against preformed 24-h-old biofilms of staphylococcal isolates of veterinary importance.

AMPs from β-thymosin of *P. lividus*

The antimicrobial defense system of marine invertebrates is based solely on an innate immune system that includes both humoral and cellular responses. AMPs constitute a major component of their humoral immunity. They are short cationic, amphipathic sequences of amino acids ranging around 10–50 amino acids in length. Marine invertebrate AMPs display broad antimicrobial spectra, even against human pathogens.[35]

In our experimental work, we observed that 5-CC possessed a broad antimicrobial activity against all tested pathogens. Small-sized molecules with a 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: the (9–19), (12–31), and (24–41) fragments of a β-thymosin of *P. lividus* (NCBInr acc.no/gi/22474470) whose molecular mass is 4592 Da.[36] We found by BLAST analysis that β-thymosin of *P. lividus* has an identity of 87% with human β-thymosin 10.

The β-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 and have several biological functions due to direct and indirect effects on the actin cytoskeleton. There is little information about the function of thymosins in invertebrates, but their presence has been reported in marine invertebrates[37,38] and in insects where they are upregulated by microbial infections.[39]

By analyzing some important chemical–physical properties, such as hydrophobicity, charge, and presence of hydrophobic residues on the same not polar face, we found that the smallest fragment, fragment 1, (9–19 of β-thymosin), 11 amino acids in length, has a good chance of being an antimicrobial peptide:[40] it has a net positive charge because of an excess number of lysine residues, and it has three hydrophobic residues on the same face and a total hydrophobic ratio of 36%. Hydrophobic and charged residues may permit interaction with bacterial membranes.[41] Moreover fragment 1 of β-thymosin has an alpha-helix structure, the most common structure of AMPs in nature, and has a similarity with already described AMPs produced by a variety of organisms, for instance, a similarity of 35% with the Jelleine III found in royal jelly of honeybees (*Apis mellifera)*.[42]

Fragment 2 (12–31 of β-thymosin), 20 amino acids in length, has a positive charge and a similarity of 40.9% with maculatin, a peptide obtained from skin glands of the tree frog *Litoria genimaculate*.[43] Fragment 3 (24–41 of β-thymosin), and β-thymosin itself, are negatively charged and have little chance to be AMPs.

Interestingly, the entire sequence from amino acid 9 to amino acid 41, may form alpha helices and have at least five residues on the same hydrophobic face. This region may interact with membranes and has also good chance to be an AMP; moreover it has a similarity of 39% with latarcins, antimicrobial and cytolytic peptides from the venom of the spider *Lachesana tarabaevi* (Table 2).[44]

AMPs from β-thymosin of *P. lividus* as potential antibiofilm agents

The tolerance of biofilms to antibiotics is mainly due to the slow growth and low metabolic activity

Table 2. Chemical–physical properties of *P. lividus* β-thymosin fragments and similarity with already described AMPs

Peptide	MW	Total net charge	Percentage hydrophobic residues	Sequence and hydrophobic residues (underlined) on the same face	Similarity (> 35%)
Fragment 1 (9–19)	1251.7	+1	36%	E V A S F D K S K L K	Jelleine III
Fragment 2 (12–31)	2293.2	+2	20%	S F D K S K L K K A E T Q E K N T L P T	Maculatin
Fragment 3 (24–41)	2088.1	−1	16%	Q E KN T L P T K ET I EQE K T A	
Entire sequence (9–41)	3745.1	0	24%	E V A S F D K S K L K K A E T Q E K N T L P T K E T I E Q E K T A	Latarcin
β-thymosin (1–41)	4583.1	−1	26%	MADKPDVSEVASFDKSKLKKAE TQEKNTLPTKETIEQEKTA	

of bacteria in such communities, so they are intrinsically resistant to antibiotics, such as β-lactams, which target dividing and metabolically active cells. On the contrary, the prevalent mechanism of action of AMPs is due to their ability to permeabilize and/or to form pores within the cytoplasmic membranes, so they have a high potential to act also on slow-growing or even nongrowing bacteria that exhibit a reduced susceptibility to many antibiotics and represent a reservoir for recurrent biofilm infections. The AMPs also have a high potential for inhibiting biofilm formation, in fact, they can act at several stages of biofilm formation and with different mechanisms of action: they may minimize initial adhesion of microbial cells to abiotic surfaces by altering the adhesive features of plastic surfaces, or by binding to microbial surfaces via electrostatic interactions, or may prevent biofilm maturation by killing the early surface colonizers, or by inhibiting quorum sensing (QS), that is, the communication system used by many bacteria to build a biofilm.[45]

Staphylococcal biofilms are responsible for many biomaterial associated infections (BAI), including persistent forms of some infectious diseases in humans. The continual increase in the use of medical devices is associated with a significant risk of infectious complications, including blood stream infections, septic thrombophlebitis, endocarditis, metastatic infections, and sepsis.[46,47] Biofilm associated infections of indwelling medical devices are usually resolved after replacement of the device but involve a prolonged hospital stay and increased healthcare costs. Considering also that increasing numbers of elderly patients require indwelling medical devices like artificial knees and hips, a new generation of antiinfective agents effective in the prevention or eradication of biofilms is needed.[48]

AMPs derived from β-thymosin of *P. lividus* for their chemical–physical characteristics and predicted activity are attractive candidates for potential therapeutic development in medical and veterinary field. Our current experimental work is aimed to confirm the predicted activity of the fragments of β-thymosin and to improve their potential as novel effective chemical countermeasures against staphylococcal biofims.

Conflicts of interest

The authors declare no conflicts of interest.

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