Antimicrobial peptides in oyster hemolymph: the bacterial connection.

Diane Defer, Florie Desriac, Joël Henry, Nathalie Bourgougnon, Michèle Baudy-Floc'H, Benjamin Brillet, Patrick Le Chevalier, Yannick Fleury

Antimicrobial Peptides in oyster hemolymph: the bacterial connection

Version auteur de l'article publié dans : *Fish & Shellfish Immunology*, Volume 34, Issue 6, June 2013, Pages 1439–1447

Diane Defer[1,2], Florie Desriac[1], Joël Henry[3], Nathalie Bourgougnon[2], Michèle Baudy-Floc'h[4] Benjamin Brillet[1], Patrick Le Chevalier[1], and Yannick Fleury[1*]

[1] Université de Brest, Institut Universitaire de Technologie, Laboratoire Universitaire de Biodiversité et d'Ecologie Microbienne EA3882, Université Européenne de Bretagne,
6 rue de l'université, 29334 Quimper Cedex (France)

[2] Université de Bretagne Sud, Centre d'Enseignement et de Recherche Yves Coppens, Laboratoire de Biotechnologie et Chimie Marines EA3884, Université Européenne de Bretagne, Campus de Tohannic , BP573, 56017 Vannes Cedex (France)

[3] Université de Caen Basse Normandie,
CNRS INEE - FRE3484 BioMEA – IBFA, 14032 Caen Cedex (France)

[4] Université de Rennes 1, Sciences Chimiques de Rennes U.M.R. 6226, Campus de Beaulieu, Bat. 10A, 10C, Avenue du Général Leclerc, 35042 RENNES Cedex (France)

[*] correspondence :

Yannick Fleury, Institut Universitaire de Technologie,

Laboratoire Universitaire de Biodiversité et d'Ecologie Microbienne EA3882,

Université de Brest, Université Européenne de Bretagne,

6 rue de l'université, 29334 Quimper Cedex

Tel, (33) 2 98 64 19 30; Fax, (33) 2 98 64 19 69;

email : fleury@univ-brest.fr

Antimicrobial Peptides in oyster hemolymph : the bacterial connection

Abstract

We have explored antimicrobial compounds in oyster hemolymph and purified four active peptides with molecular masses of 4464, 3158, 655 and 636 Da. While no exploitable structural elements were obtained for the former three, a partial amino acid sequence (X-P-P-X-X-I-V) was obtained for the latter, named *Cg*-636. Due to both its low MM and the presence of exotic amino acid residue (X), we suspected a bacterial origin and tracked cultivable hemolymph-resident bacteria of oyster for their antimicrobial abilities. Supernatants of 224 hemolymph resident bacteria coming from 60 oysters were screened against 10 target bacteria including aquaculture pathogens. Around 2 % (5 strains) revealed antimicrobial activities. They belong to *Pseudoalteromonas* and *Vibrio genera.* Two closely related strains named h*Cg*-6 and h*Cg*-42 have been shown to produce Bacteriocin-Like Inhibitory Substances (BLIS) even in oyster hemolymph. We report herein first BLIS-producing bacteria isolated from

bivalve hemolymph. These results strongly suggest that hemolymph resident bacteria may prevent pathogen establishment and pave the way for considering a role of resident bacteria into bivalve defense.

keywords:

antimicrobial peptide, Bacteriocin-like inhibitory substance, hemolymph, bivalve, resident bacteria, probiotic

1.Introduction

Marine organisms live under the highest microbial pressure and threat on earth due to microbial concentrations in seawater, estimated at 10^4 CFU.mL^{-1} for bacteria, 10^3 CFU.mL^{-1} for fungi and around 3.10^6 viruses. mL^{-1} [1][2]. So, to fight against microbial infection, marine organisms have successfully spelled out and implemented efficient and potent strategies and the first of them are antimicrobial peptides (AMPs)[3]. It is now universally accepted in the scientific community that AMPs are ubiquitous in the living kingdom (for reviews the reader is referred to [4][5][6][7]). All these antimicrobial peptides have been gathered in various generalist databases such as APD2 [8], cAMP [9] or DAMPD [10] or specialized ones such as Defensin knowledgebase [11] or Bactibase [12]. And yet, in spite of a higher biodiversity in marine environment, AMPs are far less-described from marine sources[3][7][13][14]. Among marine organisms, filter feeders such as mollusc bivalves are particularly exposed to microbial challenge due to their way of feeding. Therefore, it is not surprising that AMPs were described from mussels, one of the most efficient filter feeder bivalves. Indeed, since 1996, no fewer than 6 cystein-rich AMP families have been described in mussels *eg* defensin, myticin, mytilin and mytimycin, mytimacins and big defensins [15][16][17][18][19], displaying a real chemical arsenal. It was

demonstrated that mussel AMP families *eg* myticins, mytilins and defensins were differentially distributed throughout the organism and released in hemolymph plasma under bacterial challenge for a systemic response [18][20]. On the other hand, oyster AMPs are more recent, dating back to 2005 [21]. American oyster defensin (AOD) and *Cg*-Defm were respectively purified from gill and mantle [21][22]. The latter was shown to be constitutively produced in mantle while two isoforms named *Cg*-Defh1 and *Cg*-Defh2 were shown to be expressed in hemocytes [22][23]. As for mussels, 3 members of big defensin family were also identified in oyster hemocytes [24]. These defensins have been shown to exert their antibacterial activity by targeting lipid II [25]. No AMPs have ever been described to be released into oyster hemolymph to provide a systemic response to infection although antibacterial activity has been described in hemolymph plasma in oysters [26] [27] [28].

Furthermore, the natural presence of bacteria in hemolymph of healthy bivalves is now well-accepted but not very documented although this resident microflora should play a role in oyster development and health [29]. In this study, we have investigated this paradox. We have first analyzed oyster hemolymph for antimicrobial peptides using a functional approach. We report herein the purification and partial characterization of antimicrobial peptides from oyster hemolymph. In a second step, we examined cultivable resident bacteria in oyster hemolymph for their antibacterial abilities. We report the isolation of hemolymph-resident bacterial strains exhibiting antibacterial potency and their abilities to produce antimicrobial peptides in hemolymph *in vitro* suggesting a potential role in bivalve defense.

2. Materiel and Methods

2.1 Biological material

2.1.1 Hemolymph sampling and conditioning

Oysters, *Crassostrea gigas*, were collected in the Rhuys peninsula, Morbihan gulf, France (47°30'50 North, 2° 37' 50 West, WGS84 system). They were off-size for commercial markets, about 12 cm long and 5 cm wide. After careful opening, oyster hemolymph (1-3 mL) was collected in the pericardic cavity using disposable sterile needle.

For bacterial isolation, each individual hemolymph sample (1.5 mL) was directly laid onto marine agar (Difco™ Marine Agar 2216) using automated spiral plater (WASP, AES Chemunex, France) and incubated 72h at 18°C. For antimicrobial studies and bacterial growth assay, hemolymph samples (about 500 mL) were pooled, centrifugated (6000*g* for 10 min at 4°C) and then sterilized, using disposable filter (0.22 µm, SFCA serum Filter Unit, Nalgene).

2.1.2 Culture hemolymph-associated bacteria and identification

After 72h incubation at 18°C, hemolymph-inoculated marine agar plates were observed and numbered. Using morphological criteria, about five colonies *per* plate, that is to say *per* oyster were selected and sub-cultured in marine broth for 48H at 18°C. Culture supernatants were then collected by centrifugation and sterilized using 0.22 µm filters. Hemolymph-associated bacteria were identified using 16S rDNA gene sequencing. Bacteria were collected by centrifugation (6000*g* for 5 min at 4°C) and chemically lysed (SDS 3% at pH 12). DNA was extracted

with isoamyl phenol chlorophorm (1:24:25, v/v/v), washed twice in cold ethanol 70% and dried under *vacuum* before storage in Tris EDTA (TE) buffer. Using two couples of universal primers (W18:9^{F}, W20:1462^{R}) or (27^{F}, 1492^{R}) and PCR masterMix (Promega®), 16S rDNA was amplified to generate 1500pb PCR products. They were controlled using 1% agarose gel electrophoresis before sequencing (GATC Biotech, Germany). Partial 16S rDNA sequences were compared with GeneBank entries using BlastN to identify bacterial genus. Phylogenic trees were built using MEGA 5 program package. Nucleotide sequences inferior to 1000 nucleotides were excluded. The 16S rDNA gene sequences obtained were deposited in the GenBank database.

2.1.3 Target strains and growth conditions

Four Gram-positive and six Gram-negative bacteria were used as target bacteria. Culture conditions are presented Table 1. *Pseudoalteromonas prydzensis* ACAM 620T and all strains isolated from oyster hemolymph were grown at 18°C onto Marine Broth or Marine Agar (Marine Agar 2216, DIFCO^{TM}).

Table 1 : Culture conditions of target bacteria

Bacteria	Strain	Medium	Temperature
Bacillus megaterium	ATCC 10778	LB	30°C
Lactococcus garviae	ATCC 43921	TSB	30°C
Micrococcus luteus	ATCC 10240	TSB	37°C
Vagococcus salmoninarum	18–96	TSB	30°C
Aeromonas hydrophila	CIP 7614	TSB	30°C
Escherichia coli	ATCC 25922	TSB	37°C
Listonella anguillarum	NCBIM 829	TSB+NaCl(1.5%, w/v)	25°C
Salmonella enterica	CIP 8297	TSB	37°C
Vibrio alginolyticus	CIP 103360	MB	18°C
Yersinia ruckeri	ATCC 29473	TSB	30°C

2.1.4 Antimicrobial assay

Antimicrobial activity was assayed in liquid medium. Minimal inhibitory concentrations were determined in standard 96-well microtiter plates against the bacterial panel as previously described by Defer et al, 2009 [28] and adapted from [30]. Chromatographic fractions were assayed

against target bacteria at 10^5 CFU.mL^{-1} coming from an exponential growing phase culture in a final volume of 100µl. The plates were incubated for 48h at the optimal growth temperature. Bacterial growth was measured at 600 nm for optical density. Evaluation was carried out in triplicate. MIC was defined as the lowest protein concentration displaying 100% growth inhibition.

Culture supernatants coming from bacteria isolated from oyster hemolymph were collected after centrifugation (6000*g* for 10 min at 4°C) and filtration (0.22 µm, SFCA serum Filter Unit, Nalgene). Antibacterial activity was investigated using the well-diffusion method. Buffered with phosphate 100 mM pH 7 (in order to avoid organic acid inhibition) medium agar was inoculated with target bacteria at 1×10^6 CFU.mL^{-1} and plated in a sterile Petri dish. Wells (diameter, 5 mm) were punched in the agar plate and 50 µl of culture supernatants to be assayed were added. The plate cultures were incubated at optimal growth temperature for 18h. Negative control (marine broth) and positive controls were used (lysozyme or Nisaplin® (1 mg.mL^{-1}) for Gram-positive bacteria and polymyxine B (1 mg.mL^{-1}) for Gram-negative bacteria). Growth inhibition of the indicator bacterium was evaluated by the inhibition zone size surrounding the wells after 18 H of incubation. Assays were carried out in duplicate. For activity quantification, a serial two-fold dilution of supernatant in sterile water was assayed against target bacteria. The reciprocal of the highest dilution showing a 1-mm zone of inhibition around the well was arbitrarily defined as the number of units of antibacterial activity [31]. Each unit of activity was determined from two independent experiments performed in duplicate.

2.2 Enzymatic digestion

To define the chemical nature of the antimicrobial compounds detected, both chromatographic fractions and culture supernatants were subjected to proteolytic digestion. Samples in 50 mM phosphate buffer, pH 8 were incubated either with proteinase K (Sigma, P-6556) or trypsin (Sigma, T-1426) or α-chymotrypsin (Sigma C-4129) at an enzyme to substrate ratio of 1 to 20 (w/w). After a one-hour incubation at 37°C, samples were assayed for antibacterial activity against *M. luteus* for hemolymph fractions and *Y. ruckeri* or *L. anguillarum* for supernatants of hemolymph-associated bacteria. Samples in 50 mM phosphate buffer pH 8 without enzyme incubated one hour at 37°C were used as positive control.

2.3 SDS-PAGE and overlay assays

Active fractions and supernatants were examined using 16.5% polyacrylamide gel Tris-Tricine, pH 8.8 to allow suitable resolution of small peptides [32]. Sample solutions (1-5 µg) were dissolved (v/v) in sample buffer (2X) containing 5% SDS, 12% glycerol, 2% β-mercaptoethanol, 10% Coomassie Brilliant Blue G, and 5% 1 M Tris-HCl, pH 6.8, and heated at 100 °C for 5 min. Electrophoresis was done at constant voltage of 100 V for 2 h. Gels were fixed in 50% (v/v) methanol and 10% (v/v) acetic acid for 20 min and stained with Coomassie Brilliant Blue R-250 (Bio-Rad). To test for antibacterial activity, unstained polyacrylamide gels were washed with sterile water for 30 min, placed into sterile Petri dishes, and overlaid with adapted broth agar (8 $g.L^{-1}$) inoculated at 10^6 $UFC.mL^{-1}$ with the target bacteria. Petri dishes were incubated for 18h at optimal temperature of target bacteria and examined for growth inhibition zones (adapted from [33]).

2.4 AMPs purification from oyster hemolymph

2.4.1 C-18 Solid phase extraction

Filtrated hemolymph was directly loaded and fractionated onto C-18 cartridges (SPE/C18 UPTI-clean, Interchim, France) equilibrated with 10% Acetonitrile (ACN), 0.1% trifluoro-acetic acid (TFA). Elution was performed sequentially with 10%, 40% and 80% ACN, 0.1%TFA. Lyophilized fractions were reconstituted in sterile ultrapure water (1% (v/v) of initial hemolymph pool volume) and named H_{10}, H_{40} and H_{80}. Protein concentration was determined using the microBCA protein assay kit (Interchim, France). The H_{10}, H_{40} and H_{80} fractions were kept frozen at −20 °C until antimicrobial assays were performed.

2.4.2 Purification of antimicrobial peptides

H_{40} fractions were loaded onto a calibrated size-exclusion column (TSK G2000 SWXL, 5µm, 300X7.8 mm, Tosoh Bioscience, Japan) equilibrated in ultra pure water, 45% ACN, 0.1% TFA. Fractions (0.5mL) were collected at a flow rate of 0.5mL.min^{-1}, freeze-dried, dissolved in sterile ultrapure water and finally assayed for antibacterial activity as described above. Pooled active fractions were lyophilized and dissolved in H_2O, 0.1%TFA and further fractionated onto Uptisphere C18 column (C18 5HSC 25QS, 5µm, 250X4.6 mm, Interchim, France). After an initial 5 min washing step in 20%ACN in 0.1%TFA/water, elution was achieved in 60 min at a flow rate of 0.8 mL.min^{-1} with a 20 to 50% linear gradient of ACN, 0.07% TFA. Fractions were monitored for antibacterial activity. The active fraction was further analyzed by mass spectrometry.

2.5 Peptide characterization

2.5.1 Mass spectrometry

Analyses were performed with a HPLC Surveyor chain connected on-line to an orthogonal electrospray source (Deca XP MS-n Thermo Fisher Scientific) operated in the positive electrospray ionization mode (ESI+). The ions were focused into an ion trap, suitable for MS and MS/MS analyses. The capillary exit of the electrospray ion source was set at 70 V, the octapole at 3 V. A counter flow of nitrogen was used as nebulizing gas. Xcalibur data system was used to acquire the data, which were further processed with Sequest data system. The Chromafix C18 fraction of each extract was concentrated on Zip Tip C18 solid phase extraction microcolumn (Millipore), eluted with 5 ml of acetonitrile/0.1% formic acid and dried. The pellet was resuspended in 10 ml of 0.1% formic acid in water to be injected onto a C18 Thermo Hypersil column (0.5 mm X 50 mm, 3 µm) with an acetonitrile linear gradient of 1% per minute in 0.1% formic acid, from 2 to 60%. The MS data were acquired in the scan mode considering the positive ion signal.

2.5.2 Edman microsequencing

Purified antimicrobial peptides were blotted onto Prosorb (Applied Biosystems) before subjected to Edman degradation in an Applied Biosystems 492 automated protein sequencer.

3. Results

To explore the potential of hemolymph-associated bacteria to produce antimicrobial compounds in hemolymph, a dual approach was adopted. Indeed hemolymph was investigated in parallel for antimicrobial activity and for bacteria producing antimicrobial compounds. The adopted strategy is presented Figure 1.

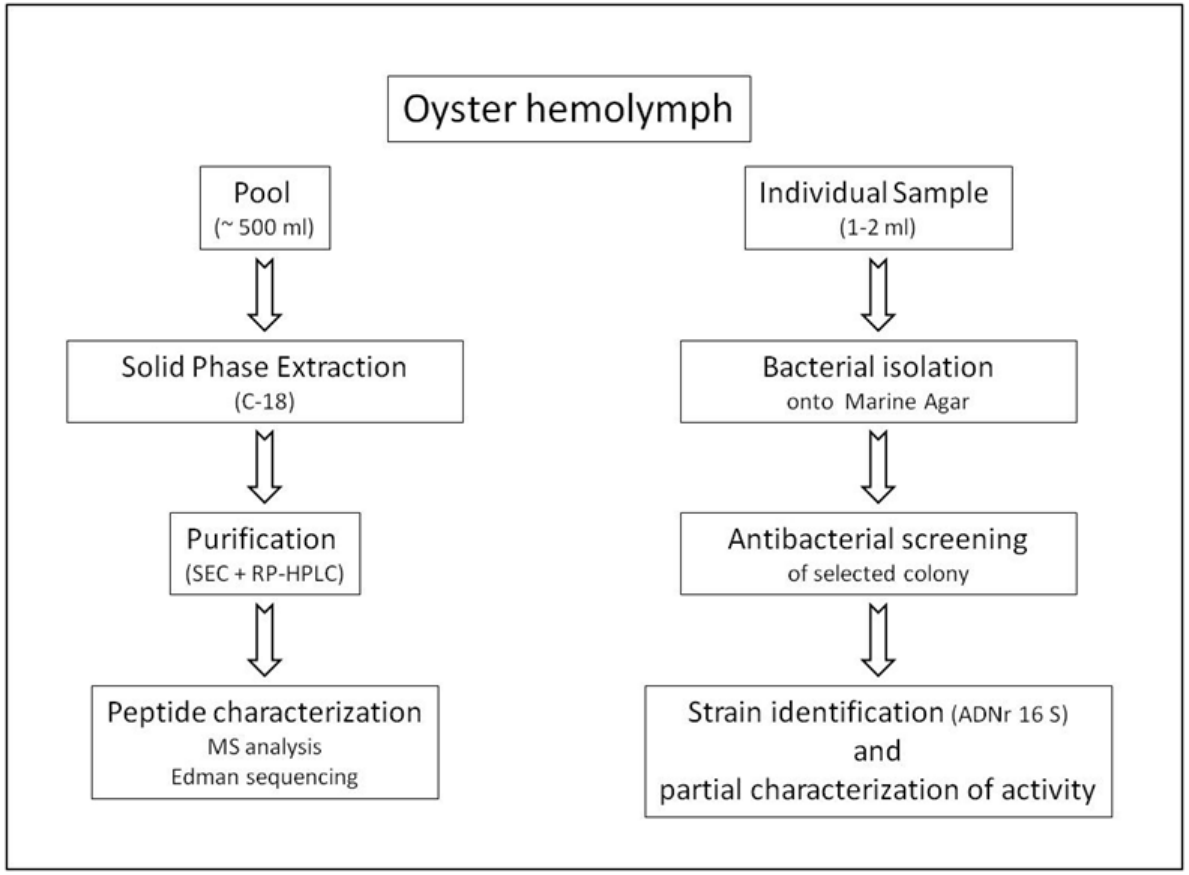


Fig. 1. Global strategy used to track AMPs and BLIS-producing bacteria in oyster hemolymph.

3.1 Antimicrobial peptides in hemolymph

3.1.1 Antibacterial activity in hemolymph fractions

Filtrated hemolymph exhibited a partial antibacterial activity since only a significant growth delay of target cells was observed (data not shown). In order to concentrate, hemolymph pool (around 500mL collected from about 200 oysters) was extracted onto C-18 cartridges, eluted in a three-step protocol increasing ACN proportion (10, 40 and 80%) and finally freeze-dried. Resulting fractions named respectively H_{10}, H_{40} and H_{80} were assayed against bacterial target cells. While the H_{10} fraction did not show any antibacterial activity, a potent one was found in the H_{40} and H_{80} fractions (Table 2). Both of them present very low MICs, around 20 $\mu g.mL^{-1}$, against two Gram-positive bacteria, *B. megaterium* and *M. luteus*. Only the H_{40} fraction exhibited an anti Gram-negative activity, limited to *Y. ruckeri* and with MIC being up to 160 $\mu g.mL^{-1}$. Moreover, the fact that antibacterial activity was recovered into H_{40} and H_{80} fractions demonstrates the hydrophobic character of the active compound(s).

Table 2. Antibacterial spectrum of activity of the hemolymph fractions expressed as MICs (µg.mL^{-1})

		Hemolymph fractions			
		H_{10}	**H_{40}**	**H_{80}**	**Positive Control**
	[Prot] µg.mL^{-1}	1000	630	150	µg.mL^{-1}
Target bacteria		**MIC (µg.mL^{-1})**			
Bacillus megaterium	ATCC 10778	-	20	37	1
Micrococcus luteus	ATCC 10240	-	20	9	4
Vagococcoccus salmoninarum	18-96	-	-	-	64
Aeromonas hydrophyla	CIP 7614	-	-	-	1
Escheriachia coli	ATCC 25922	-	-	-	4
Listonella anguillarum	NCBIM 829	-	-	-	1
Vibrio alginolyticus	CIP 103360	-	-	-	16
Yersinia ruckeri	ATCC 29473	-	160	-	1

(-) means that no inhibitory effect was observed. Lyzozyme and Polymyxin B were respectively used as positive control for Gram-positive and Gram-negative bacteria.

3.1.2 Partial characterization of the active(s) compound(s)

In order to investigate the chemical nature of the active compound(s), the H_{40} fraction was subjected to various basic assays such as protease treatments. We first used proteinase K, a broad-specificity serine protease, in order to display the proteinic nature. Incubation at 37°C for 1h with

proteinase K resulted in a total loss of antibacterial activity, MIC being higher than 630 $\mu g.mL^{-1}$ (Table 2). We can deduce that the active compound(s) are at least partially of proteinic nature. To confirm and get structural insights onto amino acids composition, H_{40} fraction was subjected to specific peptidases, trypsin and α-chymotrypsin. Trypsin treatment resulted in a total loss of antibacterial activity while only a residual activity (MIC = 630 $\mu g.mL^{-1}$) was detected when treated with α-chymotrypsin, (Table 3). So it appears that endopeptidase treatments cause at least a dramatic reduction of antibacterial activity.

Table 3. Protease sensitivity of the H_{40} fraction

Hemolymph fraction H_{40}	MIC $\mu g.mL^{-1}$
- proteinase K-treated	> 630
- tryspsin-treated	> 630
- α-Chymotrypsin-treated	630
- control	20

control means H_{40} fraction incubated for 1H at 37°C in 50 mM phosphate buffer, pH 8.

To assess the molecular size of the active compound(s) in the H_{40} fraction unambiguously, we used a method developed for bacteriocin studies. It consists in a combination of electrophoretic analysis (SDS-PAGE) and antibacterial bioassay. After electrophoretic migration, washed SDS-

PAGE gel was overlaid with agar medium inoculated with target cells. After incubation overnight, a single inhibition zone was observed in the 3.5 kDa size zone (Figure 2). Results from solid phase extraction, enzymatic treatments and molecular size evaluation showed that antibacterial activity in oyster hemolymph was arising from hydrophobic, proteinaceous and low MM compounds which are structural characteristics of antimicrobial peptides.

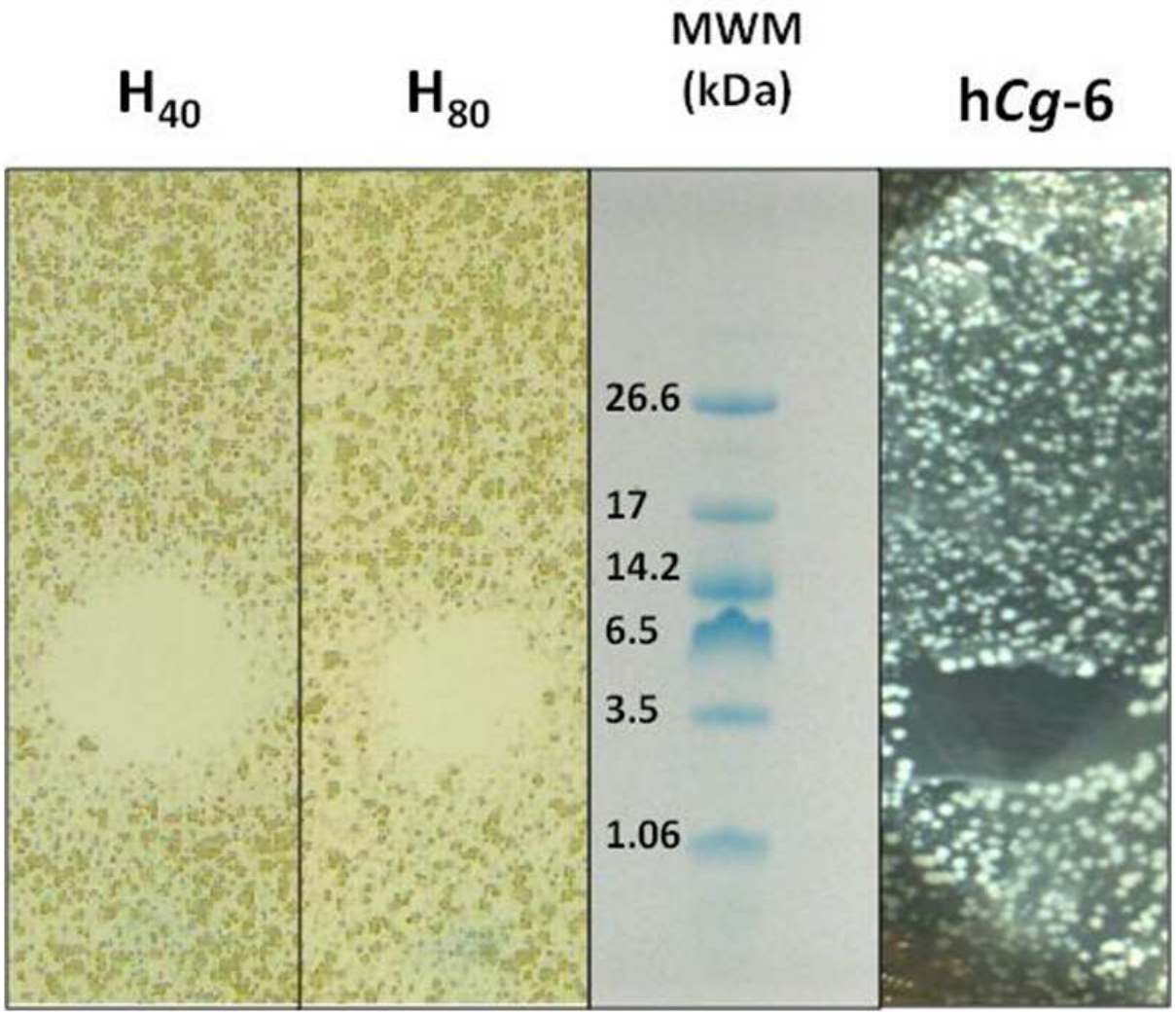


Fig. 2. SDS-polyacrylamide gel electrophoresis of hemolymph fractions (H40 and H80) and culture supernatant of strain HCg-6 overlaid respectively with culture broth agar containing target bacteria *M. luteus* and *Y. ruckeri*.

3.1.3 Purifications of antibacterial peptide(s)

Based on molecular size and hydrophobic character determined above, we planned a two-step protocol to purify the active peptide(s) detected in the H_{40} fraction. Antibacterial activity against the most sensitive strain, *M. luteus*, was used as a functional assay. The H_{40} fraction was first loaded onto a size-exclusion chromatography. Active fractions were further purified by reverse phase HPLC. Finally, the purified and active peptide was directly subjected to mass spectrometry analysis. With this strategy, we isolated a 4464 Da active peptide (Figure 3). Unfortunately, no structural elements were obtained using automated Edman degradation. Three new purifications were successively attempted using the same protocol arising from different hemolymph pools. Each of them resulted in different antibacterial peptides each exhibiting different MM namely 3158 Da, 655 Da and finally 636 Da (Figure 3).

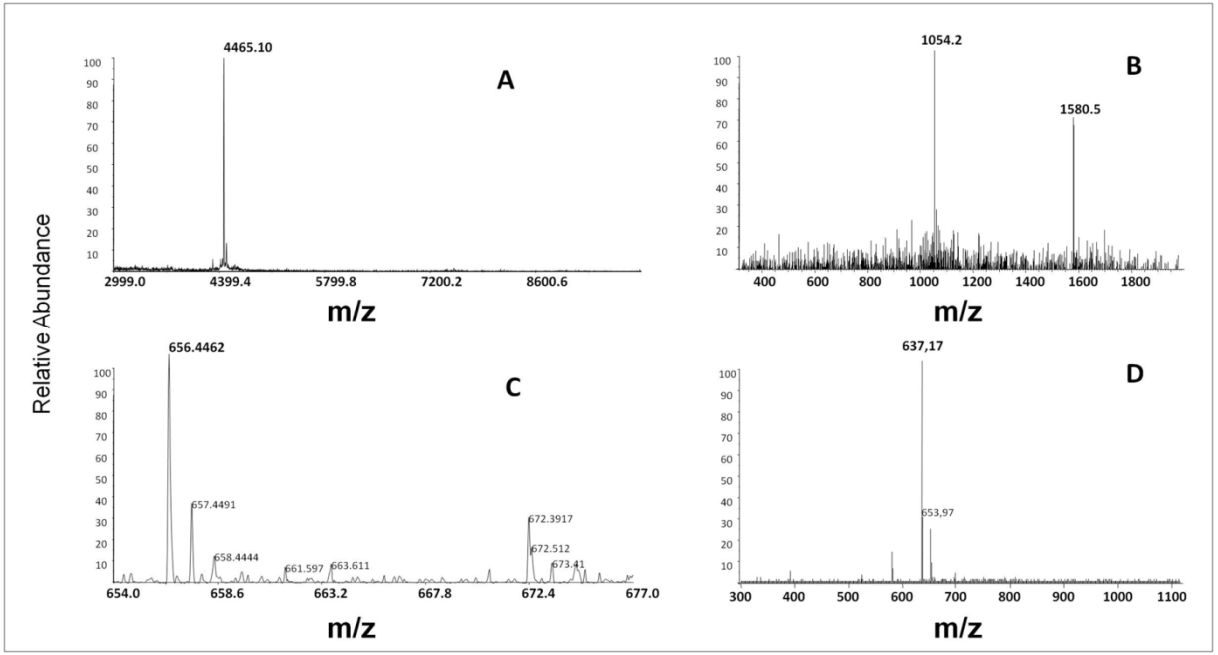


Fig. 3. Molecular weight of the antimicrobial peptides purified from four hemolymph pools (A to D) using an electrospray ionisation mass spectrometry.

There is no denying that to accept that purified peptides were hemolymph-pool dependent. No structural elements were obtained using Edman degradation except for the 636 Da peptide. The primary structure was partially determined as X-P-P-X-X-I-V, where X defines non-standard amino acid. It was named *Cg*-636 due to both its origin, *Crassostrea gigas,* and its MM. In the light of sequence and mass elements, we speculate that the *Cg*-636 peptide is composed of small exotic amino acid residues. In the face of such results, we suspected a bacterial origin of these peptides. Such an hypothesis is particularly attractive since (i) it would explain, at least partially, the four peptides purified from four hemolymph

pools and also since (ii) it has never been explored in bivalves, the bacterial presence in bivalve hemolymph being generally assessed for their potential pathogenicity.

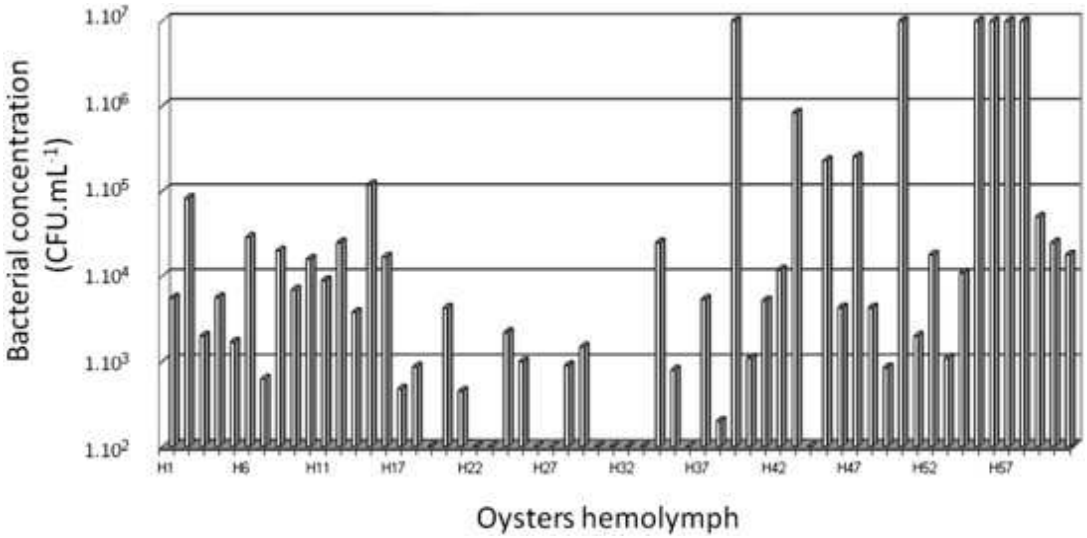


Fig. 4. Bacterial concentrations in oyster hemolymph. The symbol (&ssdiam) indicates that a strain exhibiting antibacterial activity was detected in hemolymph sample.

3.2 Antibacterial bacteria in hemolymph

Hemolymph, 1.5 mL *per* oyster, was collected sterilely from the pericardic cavity. It was immediately laid down onto Marine Agar using automated spiral plater. After incubation 72h at 18°C, colony-forming units were counted. Bacterial counting revealed a great disparity in bacterial concentration in oyster hemolymph (Figure 4) since about 20% of the oysters analyzed exhibited less than 10^2 CFU.mL^{-1}, while a

bacterial concentration higher than 10^7 CFU.mL^{-1} was determined for around 10% of oysters. Excluding these extremes, most of the oysters collected (70%) displayed an average bacterial concentration in hemolymph of $1.2.10^4$ CFU.mL^{-1}.

Starting from each hemolymph sample plated, macroscopically different colonies were sub-cultured in Marine broth for 48H. From hemolymph samples coming from 60 oysters, 224 strains were cultivated. Their supernatants were assayed using the well-diffusion method against a panel of 10 bacterial targets including *M. luteus* and *Y. ruckeri* as well as significant pathogenic bacteria in aquaculture (Table 4). Antibacterial activity was detected in the supernatant of five strains, that is to say around 2.2% of the isolated strains. These strains were named h*Cg*-xx referring to their origin, hemolymph of *C. gigas* number xx. The active strains were mainly active against Gram-negative bacteria. Only supernatants from strains h*Cg*-11/2 and h*Cg*-42 exhibited activity against both Gram-positive and -negative bacteria. The *E. coli* strain tested was not inhibited by the h*Cg*-strains supernatants while the *A. hydrophyla*, *L. anguillarum* and *Y. ruckeri* strains, pathogenic in aquaculture, were the most sensitive strains.

Table 4. Antibacterial activity and protease sensitivity of the culture supernatant of hemolymph-associated strains

Supernatant from strain	*hCg-6*	*hCg-10*	*hCg-11/2*	*hCg-11/3*	*hCg-42*	Reference
isolated from hemolymph of oyster n°	6	10	11	11	42	
Target bacteria						
Bacillus megaterium ATCC 10778	-	-	-	-	+	+++
Lactococcus garviae ATCC 43921	-	-	+++	-	+	+++
Micrococcus luteus ATCC 10240	-	-	+++	-	-	+++
Vagococcoccus salmoninarum 18-96	-	-	-	-	-	+++
Aeromonas hydrophila *CIP 7614*	+	+++	+	+++	++	+++
Escherichia coli ATCC 25922	-	-	-	-	-	+++
Listonella anguillarum NCBIM 829	+++	+++	++	+++	+	+++
Salmonella enterica CIP 8297	+	-	++	-	-	+++
Vibrio alginolyticus CIP 103360	-	+	-	+	ND	ND
Yersinia ruckeri ATCC 29473	++	++	+++	+++	+++	+++
Antibacterial activity (%) after protease treatments						
Proteinase K	0	0	0	65	0	
Trypsin	100	79	71	88	50	
α-Chymotrypsin	ND	83	65	88	ND	
Control	100	100	100	100	100	

The symbol (-) means that no inhibition was detected using the well-diffusion assay while (+) indicates that an inhibition halo was observed. (+), (++) and (+++) were used to quantify the size of the inhibition zone : + < 1 mm large, 1mm < ++ < 2 mm and +++ > 3 mm. ND : not determined Lyzozyme and Polymyxin B were respectively used as positive reference for Gram-positive and Gram-negative bacteria. Nisaplin® was used as reference for *L.garviae*. Control means h*Cg*-strain supernatant incubated in 50 mM phosphate buffer, pH 8 for 1H at 37°C.

The 16S rDNA partial sequences of the strains were deposited in the NCBI nucleotide sequence database, Gene bank. Accession numbers are JX912482, JX912480, JX912478, JX912479 and JX912481 respectively for strains h*Cg*-6, h*Cg*-10, h*Cg*-11/2, h*Cg*-11/3 and h*Cg*-42. Identification of active h*Cg*-strains from oyster hemolymph was determined by BLAST analysis of 16S rDNA gene sequence. All the h*Cg*-strains belong to the *Gammaproteobacteria* class, strains h*Cg*-6, -10 and -42 being affiliated to *Pseudoalteromonas* genus while strains h*Cg*-11/2 and h*Cg*-11/3 were identified as *Vibrio* genera (Figure 5). The 16S rRNA gene sequences from *Pseudoalteromonas* published type strains compilated from NCBI taxonomy browser and those determined in this study permitted to build phylogenetic trees using MEGA5 software. The phylogenetic tree of the *Pseudoalteromas* strains revealed that the h*Cg*-6 and h*Cg*-42 strains are very closely related although they were coming from two different oysters. Although their 16S rDNA nucleotide sequences exhibited 100% identity, they were considered as different strains since their plasmid profiles were different (data not shown). They form a cluster close to *Pseudoalteromonas prydzensis* and *Pseudoalteromonas mariniglutinosa* exhibiting 99% identity respectively to strain MB8-11 and KMM3635. The strain h*Cg*-10, more distant from h*Cg*-6 and -42 (Figure 5), is related to *Pseudoalteromonas paragorgicola* (97 % identity to strain KMM3548) and *Pseudoalteromonas elyakovii* (97% identity to strain KMM162T). The phylotype h*Cg*-10 may represent new *Pseudoalteromonas* specie [34].

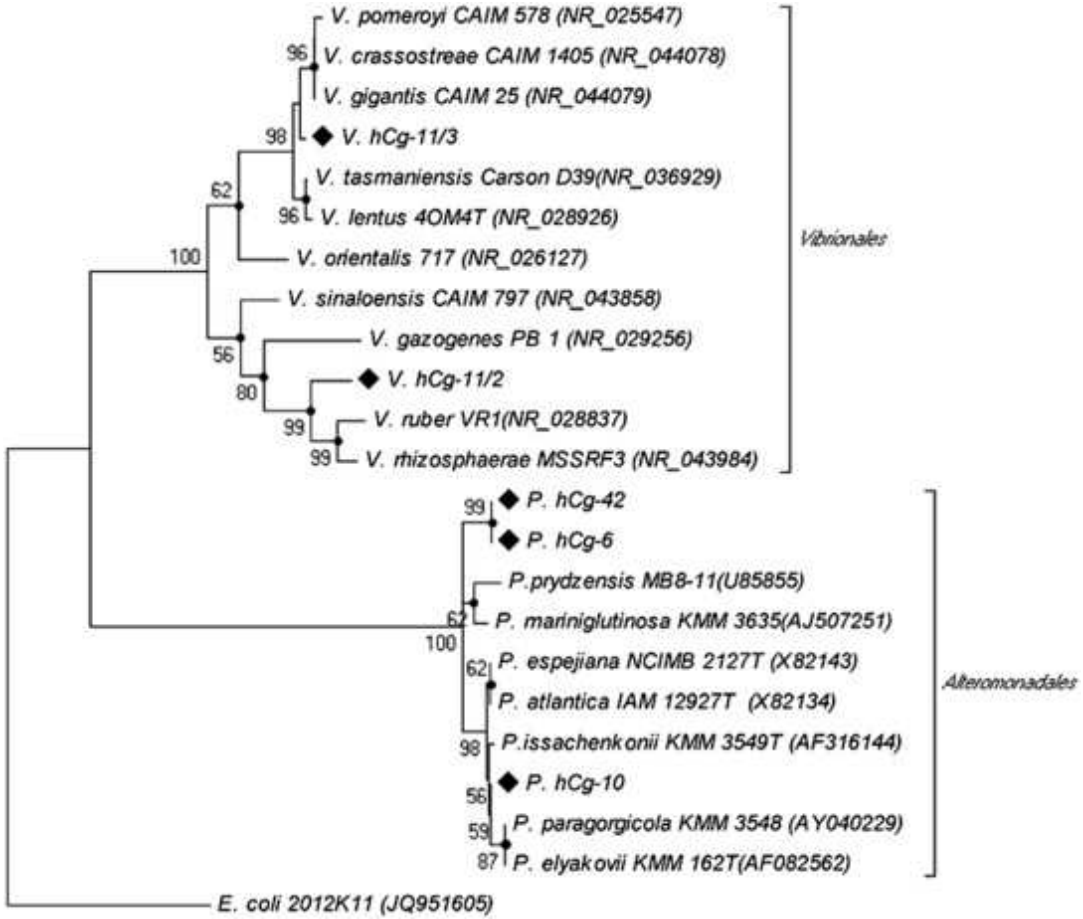


Fig. 5. Neighbor-joining tree indicating the phylogenetic relationships inferred from partial 16S rDNA gene sequences of strains h*Cg* within the two order of the *ã Proteobacteria* phylum*: Alteromonadales* and *Vibrionales*. Bootstrap values (expressed as percentage of 1000 replications) > 50% are shown at branching point. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum parsimony and the maximum-likelihood algorithms. The *Enterobacteriales* member *Escherichia coli* 2012K11 (position 208–1220) was used as outgroup. Empty circles indicate sequences determined in this study. Bar, 0.01 substitutions *per* nucleotide.

In contrast to h*Cg*-6 and -42, the strains h*Cg*-11/2 to h*Cg*-11/3 were isolated from the same hemolymph sample (oyster 11). Regarding the phylogenetic tree based on 16S rDNA gene sequences of the *Vibrio*, strains h*Cg*-11/2 and h*Cg*-11/3 are respectively affiliated to the cluster *V. gigantis/crassostreae/tasmaniensis* and *V.rhizospherae/ruber* (Figure 5).

3.3 BLIS production by hemolymph-resident bacteria

Supernatants from strains h*Cg*-6, -10, 11/2, -11/3 and -42 were subjected to protease treatments in exactly the same way as H40 fraction i.e. using proteinase K, trypsin and α-chymotrypsin. Resulting antibacterial activity was estimated using a serial two-fold dilution method. All protease treatments resulted in a more or less drastic reduction of antibacterial activity according to the producing strains suggesting at least a proteinic part of the active compounds (Table 4). When analyzed using the SDS-PAGE overlaid with target bacteria, only supernatants from *Pseudoalteromonas* h*Cg*-6 and h*Cg*-42 exhibited an inhibition halo in the 3.5 kDa migration zone (Figure 2). We assumed that the active compounds in supernatants h*Cg*-10, -11/2 and -11/3 did not withstand denaturating treatment prior to electrophoresis. However that may be, it emerges that the *Pseudoalteromonas* h*Cg*-6 and h*Cg*-42 strains produce low MM, antibacterial and proteinaceous compounds. Such compounds present all the characteristics BLIS [35].

To get new insight into the BLIS-production abilities of the h*Cg*-6 and h*Cg*-42 strains, they were grown in various media. Marine broth was used as a positive control. After a 48 H incubation at 18°C, the biomass yielded was similar in each medium, *e.g.* around 10^9 CFU.mL^{-1}. Supernatants were collected in order to quantify antibacterial activity. When cultivated in Sea Salt peptone or Sea Salt LB, these strains have exhibited a

BLIS-production level equivalent to production in marine broth but no activity was detectable after cultivation in TSB with or without Sea Salt (Table 5). Amazingly, the antibacterial activity recovered undergoes a all or nothing rule. To mimic *in vivo* conditions, sterile hemolymph was also assayed as a culture medium. The closest phylogenic strain, *Pseudoalteromonas prydzensis* ACAM 620^{T}, was used as a negative control. *Pseudoalteromonas* h*Cg*-6 and -42 were shown to be able to grow in hemolymph (data not shown). Moreover, antibacterial activity was detected in supernatant at a level as high as the positive control one (Table 5). These results indicate that *Pseudoalteromonas* hCg-6 and -42 strains are able to produce BLIS in oyster hemolymph *in vitro*.

Table 5. BLIS-production in various media

Antibacterial activity (%)	Marine Broth	LB +Sea Salts	Peptone +Sea Salts	TSB	TSB +Sea Salts	Hemolymph
Pseudoalt. hCg-6	100	100	100	0	0	100
Pseudoalt. hCg-42	100	100	100	0	0	100
Pseudoalt prydzensis	0	0	0	0	0	0

LB and TSB respectively mean Luria Broth and Tryptic Soy Broth.

4. Discussion

The present study report the purification and partial characterization of antimicrobial peptides and for the first time isolation of BLIS-producing bacteria from oyster hemolymph. Antimicrobial compounds detected in a concentrated fraction of hemolymph, named H_{40}, were shown to be low MW, amphipathic and proteinaceous compounds. All these characters designate them as antimicrobial peptide [36]. Four purifications conducted from different hemolymph pools led to as many bioactive peptides exhibiting different MW (*eg* 4464,1 Da, 3158.4 Da, 655 Da and finally 636,1 Da). The 4464 Da peptide exhibited a MM similar to that of AOD [21], *Cg*-Defm [22] and *Cg*-Defh1 and *Cg*-Defh2 [23]. As recombinant oyster defensins, antibacterial activity of the 4464 Da peptide was much more potent against Gram positive bacteria even though the main oyster pathogens belong to Gram-negative bacteria [25]. It seems that oysters have developed a strategy based on synergy to complete its set of AMPs. Proline rich peptides (*Cg*-Prps) expressed in hemocyte have exhibited potent synergistic antibacterial activity with *Cg*-Def [37]. Moreover, a member of the LPS-binding protein and bactericidal/permeability-increasing protein (BPI) family has recently been identified in *Crassostrea gigas* oyster (*Cg*-BPI). *Cg*-BPI production was shown to be constitutive in tissues in contact with the environment and triggered by bacterial challenge in hemocytes [38]. A synergistic effect has also been emphasized between the *Cg*-Defs themselves [39]. For most of these defense compounds, production and/or release have been shown to result from a bacterial challenge suggesting pathogen-associated molecular pattern implication [24].

Regarding the active peptide purified herein, the only structural elements obtained were a partial amino-acids sequence for the latter one (636 Da): X-P-P-X-X-I-V, where X refers to non-standard residues. It was therefore called *Cg*-636. The exotic amino-acid residues account for 212.64

Da that is to say for each of them an average MM minus H_2O around 71 Da for each of them. This simple calculation orientates towards small unusual amino acid residues such as Dehydro-alanine (Dha) whose MM minus H_2O is 69.06 Da. The only Dha-containing antibacterial peptides known to date are lantibiotics [40], ribosomally-synthetized and highly post-translationally modified peptides produced by Gram-positive bacteria.

Querying *Crassostrea gigas* genome or expressed sequence tags databases such as GigaDB [41] and GigasBase [42] were fruitless.

Antimicrobial peptide databases, cAMP [9], APD2 [8], DAMPD [10], Defensins knowledgebase [11], were requested for peptide length inferior to 10 amino acids residues coming from invertebrates. Only jelleines met these criteria [43] but did not exhibit any homology at the structural level. Research was broadened to microbial peptides such as bacteriocin and nonribosomal peptides by querying specialized databases such as Norine [44] or Bactibase [12], also without anysuccess. A microbial origin of the *Cg*-636 peptide was hypothesized and investigated.

Bacterial presence in bivalve hemolymph is known for years [45] but to the best of our knowledge, hemolymph-resident bacteria have never been explored for their antimicrobial activities. Indeed, the bacterial presence in oyster hemolymph is generally assessed for their potential pathogenicity. No information is available about the role of resident bacteria in hemolymph, if any, in bivalve health.

Hemolymph plating onto marine agar and bacterial counting has revealed the great disparity into bacterial concentration ranging from less than 10^2 to more than 10^7 $CFU.mL^{-1}$. Five strains exhibiting antibacterial activity were identified as *Vibrio and Pseudoalteromonas* species. These two genera are classically found in bivalve hemolymph [45] and some of them have been shown to be non pathogenic for oyster [46].

Vibrio and especially *Pseudoalteromonas* antimicrobial activities have been already documented [35][47][48][49][50]. However, to the best of our knowledge, this is the first report of antimicrobial strains isolation within mollusc hemolymph.

We attempted to identify the chemical property of the active compounds produced by the hemolymph-resident bacteria isolated and named h*Cg*-. We demonstrated unambiguously that the h*Cg*-6 and h*Cg*-42 are BLIS producing bacteria. *Pseudoalteromonas* sp. are well-known for producing antimicrobial low-molecular weight metabolites such as the alkaloids Tambjamines [51], thiomarinol [52], methylbutanoic acids [53], isatin [54]. But very few proteinic antimicrobial compounds have been characterized from *Pseudoalteromonas*. To date, three proteins named P-153 (MM 87 kDa), a L-amino acids oxydase (MM 110 kDa) and recently PfaP (MM 77 kDa) have been purified and characterized from respectively *Pseudoalteromonas piscicida* [55], *luteoviolacea* [56] and *flavipulchra*[57].

The BLIS-production ability of the h*Cg*-6 and h*Cg*-42 strains was shown to be culture-conditions dependent, another BLIS producer trait. The biosynthesis regulation ways are under the control of stress *stimuli* for most microcins and lactic acid bacteria bacteriocins (for review see [58][59]). Finally, and most significantly, a BLIS-production was detected in hemolymph *in vitro.* It appears therefore that h*Cg*-6 and h*Cg*-42 strains may directly inhibit the invasion of pathogens and/or modulate the composition of the microbiota. Such a function has been proposed for resident microflora in corals [60][61]. Therefore hemolymph-resident microflora may play a role in the oyster defense and so constitute a pertinent source of new probiotics in aquaculture. Our results throw a new light on hemolymph-resident microbiota in oyster and raise the questions of its role in bivalve health.

Acknowledgments

Thanks are given to Dr JP Bowman from Tasmanian Institute of Agriculture, Australia, for the generous gift of *Pseudoalteromonas prydzensis* ACAM 620^T, to Estelle Bellanger-Thuillier for her technical assistance and to Hervé Bourdon for critical reading. This work was partly funded by the region Bretagne (Biprobio project, ARED 6227). Florie Desriac was supported by a “Quimper-communauté” grant for pHD thesis.

Bibliography

[1] Zweifel UL, Hagstrom A. Total Counts of Marine Bacteria Include a Large Fraction of Non-Nucleoid-Containing Bacteria (Ghosts). Appl Environ Microbiol 1995;61:2180–2185.

[2] Suttle CA. Viruses in the sea. Nature 2005;437:356–361. doi:10.1038/nature04160

[3] Otero-González AJ, Magalhães BS, Garcia-Villarino M, López-Abarrategui C, Sousa DA, Dias SC, et al. Antimicrobial peptides from marine invertebrates as a new frontier for microbial infection control. FASEB J 2010;24:1320–1334. doi:10.1096/fj.09-143388

[4] Afacan NJ, Yeung ATY, Pena OM, Hancock REW. Therapeutic potential of host defense peptides in antibiotic-resistant infections. Curr. Pharm. Des. 2012;18:807–819.

[5] Nakatsuji T, Gallo RL. Antimicrobial peptides: old molecules with new ideas. J. Invest. Dermatol. 2012;132:887–895. doi:10.1038/jid.2011.387

[6] Pasupuleti M, Schmidtchen A, Malmsten M. Antimicrobial peptides: key components of the innate immune system. Crit. Rev. Biotechnol. 2012;32:143–171. doi:10.3109/07388551.2011.594423

[7] Sperstad SV, Haug T, Blencke H-M, Styrvold OB, Li C, Stensvåg K. Antimicrobial peptides from marine invertebrates: challenges and perspectives in marine antimicrobial peptide discovery. Biotechnol. Adv. 2011;29:519–530. doi:10.1016/j.biotechadv.2011.05.021

[8] Wang G, Li X, Wang Z. APD2: the updated antimicrobial peptide database and its application in peptide design. Nucleic Acids Res 2009;37:D933–D937. doi:10.1093/nar/gkn823

[9] Thomas S, Karnik S, Barai RS, Jayaraman VK, Idicula-Thomas S. CAMP: a useful resource for research on antimicrobial peptides. Nucleic Acids Res. 2010;38:D774–780. doi:10.1093/nar/gkp1021

[10] Seshadri Sundararajan V, Gabere MN, Pretorius A, Adam S, Christoffels A, Lehväslaiho M, et al. DAMPD: a manually curated antimicrobial peptide database. Nucleic Acids Research 2011. doi:10.1093/nar/gkr1063

[11] Seebah S, Suresh A, Zhuo S, Choong YH, Chua H, Chuon D, et al. Defensins knowledgebase: a manually curated database and information source focused on the defensins family of antimicrobial peptides. Nucleic Acids Res 2007;35:D265–D268. doi:10.1093/nar/gkl866

[12] Hammami R, Zouhir A, Le Lay C, Ben Hamida J, Fliss I. BACTIBASE second release: a database and tool platform for bacteriocin characterization. BMC Microbiology 2010;10:22. doi:10.1186/1471-2180-10-22

[13] Tincu JA, Taylor SW. Antimicrobial Peptides from Marine Invertebrates. Antimicrob Agents Chemother 2004;48:3645–3654. doi:10.1128/AAC.48.10.3645-3654.2004

[14] Rajanbabu V, Chen J-Y. Applications of antimicrobial peptides from fish and perspectives for the future. Peptides 2011;32:415–420. doi:10.1016/j.peptides.2010.11.005

[15] Hubert F, Noël T, Roch P. A Member of the Arthropod Defensin Family from Edible Mediterranean Mussels (Mytilus galloprovincialis). European Journal of Biochemistry 1996;240:302–306. doi:10.1111/j.1432-1033.1996.0302h.x

[16] Charlet M, Chernysh S, Philippe H, Hetru C, Hoffmann JA, Bulet P. Innate Immunity. Journal of Biological Chemistry 1996;271:21808 – 21813. doi:10.1074/jbc.271.36.21808

[17] Mitta G, Hubert F, Dyrynda EA, Boudry P, Roch P. Mytilin B and MGD2, two antimicrobial peptides of marine mussels: gene structure and expression analysis. Dev. Comp. Immunol. 2000;24:381–393.

[18] Mitta G, Vandenbulcke F, Noël T, Romestand B, Beauvillain JC, Salzet M, et al. Differential distribution and defence involvement of antimicrobial peptides in mussel. J. Cell. Sci. 2000;113 (Pt 15):2759–2769.

[19] Gerdol M, De Moro G, Manfrin C, Venier P, Pallavicini A. Big defensins and mytimacins, new AMP families of the Mediterranean mussel Mytilus galloprovincialis. Developmental & Comparative Immunology 2012;36:390–399. doi:10.1016/j.dci.2011.08.003

[20] Mitta G, Vandenbulcke F, Hubert F, Roch P. Mussel defensins are synthesised and processed in granulocytes then released into the plasma after bacterial challenge. J. Cell. Sci. 1999;112 (Pt 23):4233–4242.

[21] Seo J-K, Crawford JM, Stone KL, Noga EJ. Purification of a novel arthropod defensin from the American oyster, Crassostrea virginica. Biochemical and Biophysical Research Communications 2005;338:1998–2004. doi:10.1016/j.bbrc.2005.11.013

[22] Gueguen Y, Herpin A, Aumelas A, Garnier J, Fievet J, Escoubas J-M, et al. Characterization of a defensin from the oyster Crassostrea gigas. Recombinant production, folding, solution structure, antimicrobial activities, and gene expression. J. Biol. Chem. 2006;281:313–323. doi:10.1074/jbc.M510850200

[23] Gonzalez M, Gueguen Y, Desserre G, De Lorgeril J, Romestand B, Bachère E. Molecular characterization of two isoforms of defensin from hemocytes of the oyster Crassostrea gigas. Developmental & Comparative Immunology 2007;31:332–339. doi:10.1016/j.dci.2006.07.006

[24] Rosa RD, Santini A, Fievet J, Bulet P, Destoumieux-Garzón D, Bachère E. Big Defensins, a Diverse Family of Antimicrobial Peptides That Follows Different Patterns of Expression in Hemocytes of the Oyster Crassostrea gigas. PLoS One 2011;6. doi:10.1371/journal.pone.0025594

[25] Schmitt P, Wilmes M, Pugnière M, Aumelas A, Bachère E, Sahl H-G, et al. Insight into Invertebrate Defensin Mechanism of Action. J Biol Chem 2010;285:29208–29216. doi:10.1074/jbc.M110.143388

[26] Hubert F, Van der Knaap W, Noël T, Roch P. Cytotoxic and antibacterial properties of Mytilus galloprovincialis, Ostrea edulis and Crassostrea gigas (bivalve molluscs) hemolymph. Aquatic Living Resources 1996;9:115–124. doi:10.1051/alr:1996015

[27] Anderson RS, Beaven AE. Antibacterial Activities of Oyster (Crassostrea Virginica) and Mussel (Mytilus Edulis and Geukensia Demissa) Plasma. Aquatic Living Resources 2001;14:343–349. doi:10.1016/S0990-7440(01)01143-3

[28] Defer D, Bourgougnon N, Fleury Y. Screening for antibacterial and antiviral activities in three bivalve and two gastropod marine molluscs. Aquaculture 2009;293:1–7. doi:10.1016/j.aquaculture.2009.03.047

[29] Schmitt P, Rosa RD, Duperthuy M, De Lorgeril J, Bachère E, Destoumieux-Garzón D. The Antimicrobial Defense of the Pacific Oyster, Crassostrea gigas. How Diversity may Compensate for Scarcity in the Regulation of Resident/Pathogenic Microflora. Front Microbiol 2012;3:160. doi:10.3389/fmicb.2012.00160

[30] Wiegand I, Hilpert K, Hancock REW. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nature Protocols 2008;3:163–175. doi:10.1038/nprot.2007.521

[31] Fleury Y, Dayem MA, Montagne JJ, Chaboisseau E, Le Caer JP, Nicolas P, et al. Covalent structure, synthesis, and structure-function studies of mesentericin Y 105(37), a defensive peptide from gram-positive bacteria Leuconostoc mesenteroides. J. Biol. Chem. 1996;271:14421–14429.

[32] Schägger H, Von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. 1987;166:368–379.

[33] Bhunia AK, Johnson MG. A modified method to directly detect in SDS-PAGE the bacteriocin of Pediococcus acidilactici. Letters in

Applied Microbiology 1992;15:5–7. doi:10.1111/j.1472-765X.1992.tb00709.x

[34] Stackebrandt E, Ebers J. Taxonomic parameters revisited : tarnished gold standards. 2006;6:152–156.

[35] Desriac F, Defer D, Bourgougnon N, Brillet B, Le Chevalier P, Fleury Y. Bacteriocin as Weapons in the Marine Animal-Associated Bacteria Warfare: Inventory and Potential Applications as an Aquaculture Probiotic. Mar Drugs 2010;8:1153–1177. doi:10.3390/md8041153

[36] Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. Pharmacol. Rev. 2003;55:27–55. doi:10.1124/pr.55.1.2

[37] Gueguen Y, Romestand B, Fievet J, Schmitt P, Destoumieux-Garzón D, Vandenbulcke F, et al. Oyster hemocytes express a proline-rich peptide displaying synergistic antimicrobial activity with a defensin. Mol. Immunol. 2009;46:516–522. doi:10.1016/j.molimm.2008.07.021

[38] Gonzalez M, Gueguen Y, Destoumieux-Garzón D, Romestand B, Fievet J, Pugnière M, et al. Evidence of a bactericidal permeability increasing protein in an invertebrate, the Crassostrea gigas Cg-BPI. Proceedings of the National Academy of Sciences 2007;104:17759 – 17764. doi:10.1073/pnas.0702281104

[39] Schmitt P, Lorgeril J de, Gueguen Y, Destoumieux-Garzón D, Bachère E. Expression, tissue localization and synergy of antimicrobial peptides and proteins in the immune response of the oyster Crassostrea gigas. Developmental & Comparative Immunology 2012;37:363–370. doi:10.1016/j.dci.2012.01.004

[40] Field D, Hill C, Cotter PD, Ross RP. The dawning of a “Golden era” in lantibiotic bioengineering. Mol. Microbiol. 2010;78:1077–1087. doi:10.1111/j.1365-2958.2010.07406.x

[41] Zhang G, Fang X, Guo X, Li L, Luo R, Xu F, et al. The oyster genome reveals stress adaptation and complexity of shell formation. Nature 2012;490:49–54. doi:10.1038/nature11413

[42] Gueguen Y, Cadoret JP, Flament D, Barreau-Roumiguière C, Girardot AL, Garnier J, et al. Immune gene discovery by expressed sequence tags generated from hemocytes of the bacteria-challenged oyster, Crassostrea gigas. Gene 2003;303:139–145.

[43] Fontana R, Mendes MA, Souza BM de, Konno K, César LMM, Malaspina O, et al. Jelleines: a family of antimicrobial peptides from the Royal Jelly of honeybees (Apis mellifera). Peptides 2004;25:919–928. doi:10.1016/j.peptides.2004.03.016

[44] Caboche S, Pupin M, Leclère V, Fontaine A, Jacques P, Kucherov G. NORINE: a database of nonribosomal peptides. Nucleic Acids Res 2008;36:D326–D331. doi:10.1093/nar/gkm792

[45] Olafsen JA, Mikkelsen HV, Giæver HM, Høvik Hansen G. Indigenous Bacteria in Hemolymph and Tissues of Marine Bivalves at Low Temperatures. Appl Environ Microbiol 1993;59:1848–1854.

[46] Garnier M, Labreuche Y, Garcia C, Robert M, Nicolas J-L. Evidence for the Involvement of Pathogenic Bacteria in Summer Mortalities of the Pacific Oyster Crassostrea gigas. Microbial Ecology 2007;53:187–196. doi:10.1007/s00248-006-9061-9

[47] Bowman JP. Bioactive compound synthetic capacity and ecological significance of marine bacterial genus pseudoalteromonas. Mar Drugs 2007;5:220–241.

[48] Flemer B, Kennedy J, Margassery LM, Morrissey JP, O'Gara F, Dobson ADW. Diversity and antimicrobial activities of microbes from two Irish marine sponges, Suberites carnosus and Leucosolenia sp. J. Appl. Microbiol. 2012;112:289–301. doi:10.1111/j.1365-2672.2011.05211.x

[49] Gram L, Melchiorsen J, Bruhn JB. Antibacterial activity of marine culturable bacteria collected from a global sampling of ocean surface waters and surface swabs of marine organisms. Mar. Biotechnol. 2010;12:439–451. doi:10.1007/s10126-009-9233-y

[50] Romanenko LA, Uchino M, Kalinovskaya NI, Mikhailov VV. Isolation, phylogenetic analysis and screening of marine mollusc-associated bacteria for antimicrobial, hemolytic and surface activities. Microbiol. Res. 2008;163:633–644.

[51] Pinkerton DM, Banwell MG, Garson MJ, Kumar N, De Moraes MO, Cavalcanti BC, et al. Antimicrobial and cytotoxic activities of synthetically derived tambjamines C and E - J, BE-18591, and a related alkaloid from the marine bacterium Pseudoalteromonas tunicata. Chem. Biodivers. 2010;7:1311–1324. doi:10.1002/cbdv.201000030

[52] Murphy AC, Fukuda D, Song Z, Hothersall J, Cox RJ, Willis CL, et al. Engineered thiomarinol antibiotics active against MRSA are generated by mutagenesis and mutasynthesis of Pseudoalteromonas SANK73390. Angew. Chem. Int. Ed. Engl. 2011;50:3271–3274. doi:10.1002/anie.201007029

[53] Hayashida-Soiza G, Uchida A, Mori N, Kuwahara Y, Ishida Y. Purification and characterization of antibacterial substances produced by a marine bacterium Pseudoalteromonas haloplanktis strain. J. Appl. Microbiol. 2008;105:1672–1677. doi:10.1111/j.1365-2672.2008.03878.x

[54] Kalinovskaya NI, Ivanova EP, Alexeeva YV, Gorshkova NM, Kuznetsova TA, Dmitrenok AS, et al. Low-molecular-weight, biologically active compounds from marine Pseudoalteromonas species. Curr. Microbiol. 2004;48:441–446. doi:10.1007/s00284-003-4240-0

[55] Longeon A, Peduzzi J, Barthélemy M, Corre S, Nicolas J-L, Guyot M. Purification and partial identification of novel antimicrobial protein from marine bacterium Pseudoalteromonas species strain X153. Mar. Biotechnol. 2004;6:633–641. doi:10.1007/s10126-004-3009-1

[56] Gómez D, Espinosa E, Bertazzo M, Lucas-Elío P, Solano F, Sanchez-Amat A. The macromolecule with antimicrobial activity synthesized by Pseudoalteromonas luteoviolacea strains is an L-amino acid oxidase. Appl. Microbiol. Biotechnol. 2008;79:925–930. doi:10.1007/s00253-008-1499-x

[57] Yu M, Wang J, Tang K, Shi X, Wang S, Zhu W-M, et al. Purification and characterization of antibacterial compounds of Pseudoalteromonas flavipulchra JG1. Microbiology 2011;158:835–842. doi:10.1099/mic.0.055970-0

[58] Duquesne S, Destoumieux-Garzón D, Peduzzi J, Rebuffat S. Microcins, gene-encoded antibacterial peptides from enterobacteria. Nat Prod Rep 2007;24:708–734. doi:10.1039/b516237h

[59] De Vuyst L, Leroy F. Bacteriocins from lactic acid bacteria: production, purification, and food applications. J. Mol. Microbiol. Biotechnol. 2007;13:194–199. doi:10.1159/000104752

[60] Shnit-Orland M, Kushmaro A. Coral mucus-associated bacteria: a possible first line of defense. FEMS Microbiol. Ecol. 2009;67:371–380. doi:10.1111/j.1574-6941.2008.00644.x

[61] Kvennefors ECE, Sampayo E, Kerr C, Vieira G, Roff G, Barnes AC. Regulation of bacterial communities through antimicrobial activity by the coral holobiont. Microb. Ecol. 2012;63:605–618. doi:10.1007/s00248-011-9946-0

FIGURES AND CAPTIONS

Fig. 1. Global strategy used to track AMPs and BLIS-producing bacteria in oyster hemolymph

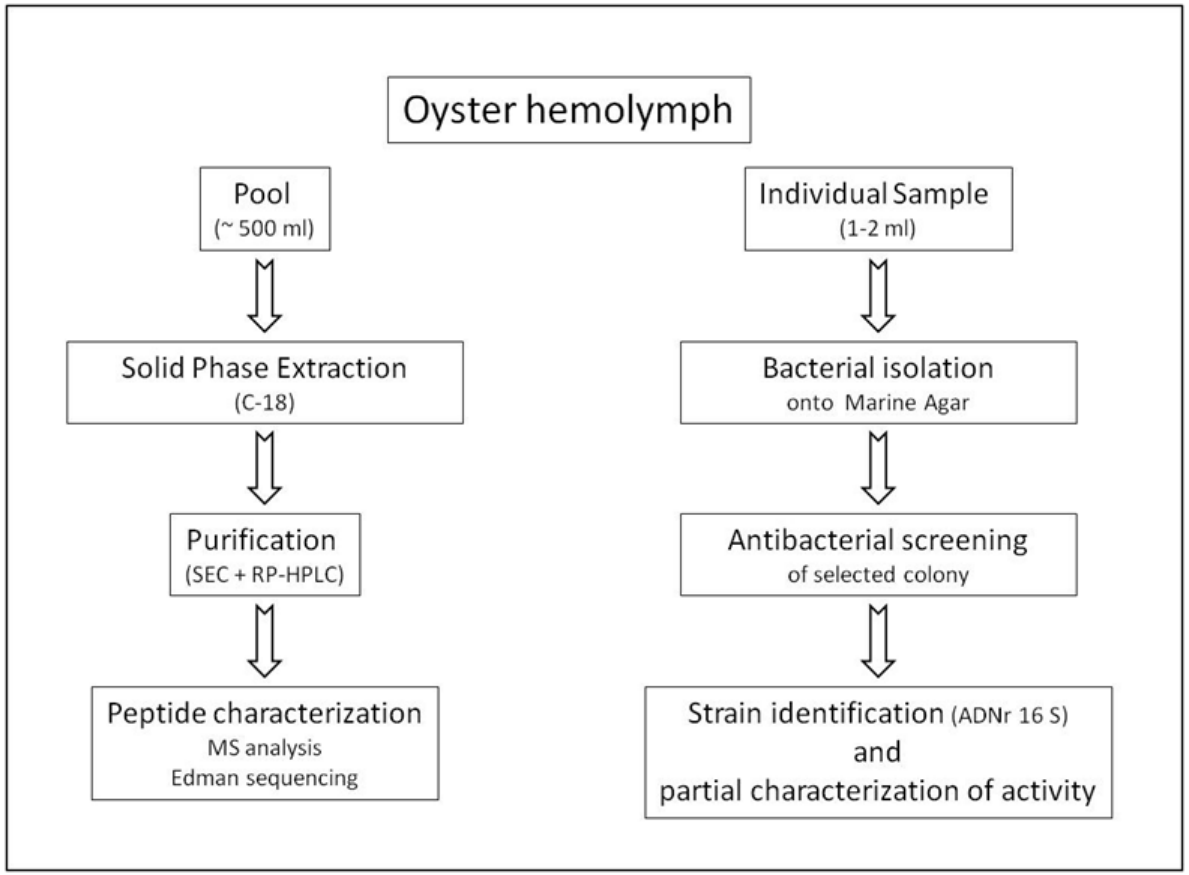
Fig. 2. SDS-Polyacrylamide gel electrophoresis of hemolymph fractions (H40 and H80) and culture supernatant of strain HCg-6 overlaid respectively with culture broth agar containing target bacteria *M. luteus* and *Y. ruckeri*.

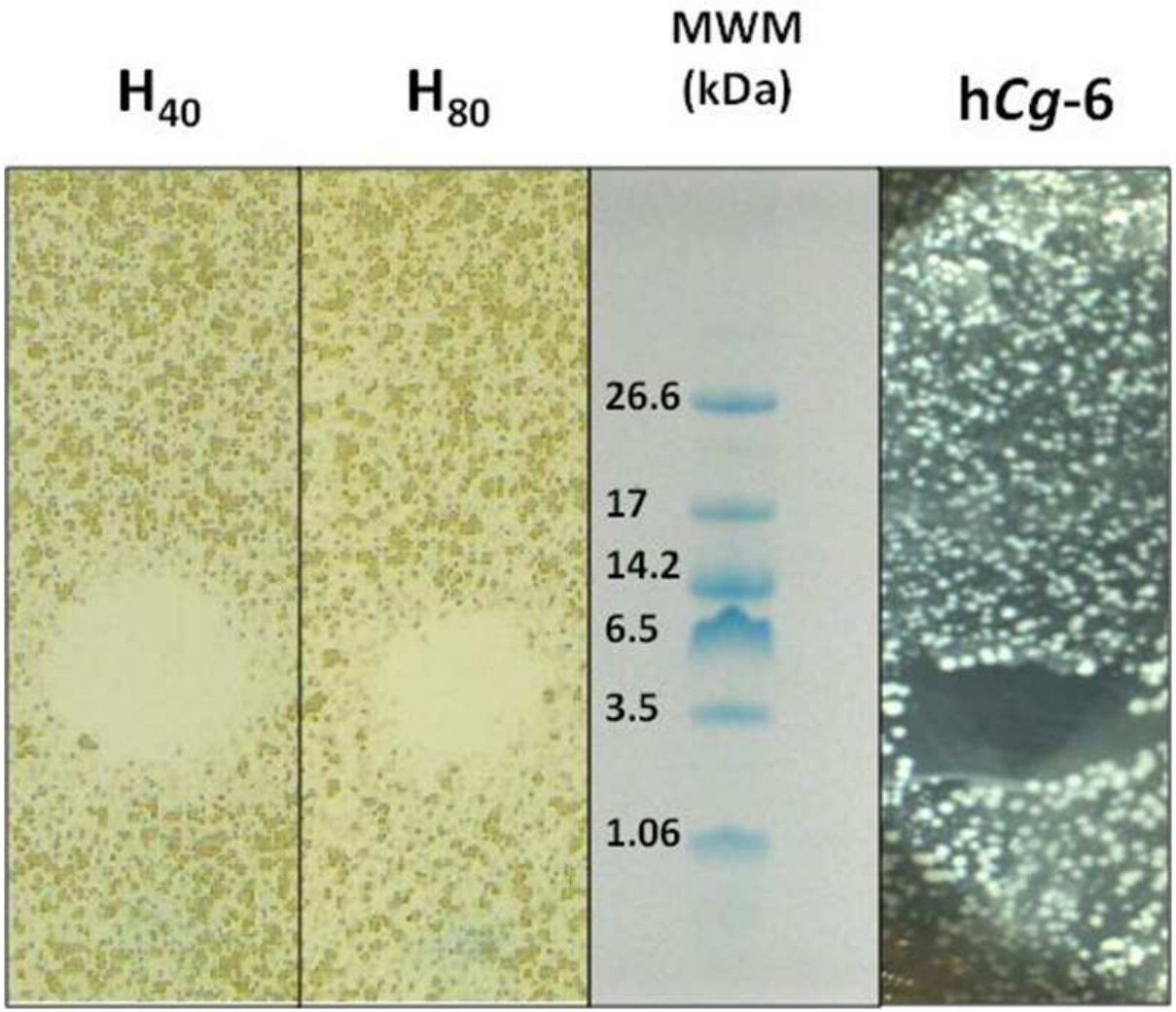
Fig. 3. Molecular Weight of the antimicrobial peptides purified from four hemolymph pools (A to D) using an electrospray ionisation mass spectrometry.

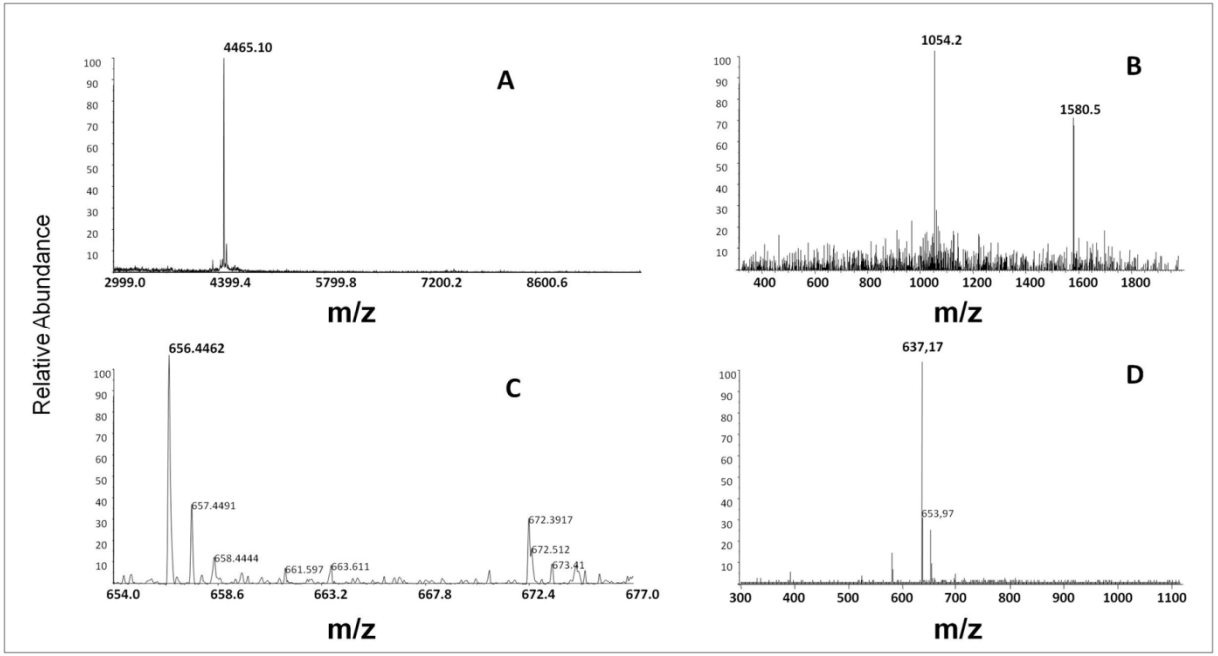
Fig. 4. Bacterial concentrations in oyster hemolymph.

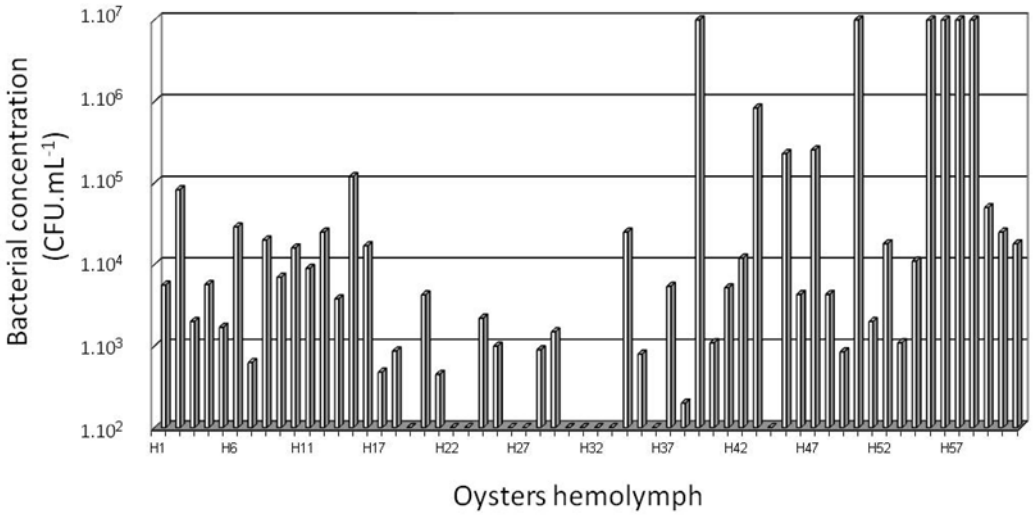
The symbol (◆) indicates that a strain exhibiting antibacterial activity was detected in hemolymph sample.

Fig. 5. Neighbour-joining tree indicating the Phylogenetic relationships inferred from partial 16S rDNA gene sequences of strains h*Cg* within the two order of the γ *Proteobacteria* phylum*: Alteromonadales* and *Vibrionales*. Bootstrap values (expressed as percentage of 1000 replications) > 50% are shown at branching point. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum parsimony and the maximum-likelihood algorithms. The *Enterobacteriales* member *Escherichia coli* 2012K11 (position 208-1220) was used as outgroup. Empty circles indicate sequences determined in this study. Bar, 0.01 substitutions per nucleotide.


Oyster hemolymph
Pool
(~ 500 ml)
Solid Phase Extraction
(C-18)
Purification
(SEC + RP-HPLC)
Peptide characterization
MS analysis
Edman sequencing
Individual Sample
(1-2 ml)
Bacterial isolation
onto Marine Agar
Antibacterial screening
of selected colony
Strain identification (ADNr 16 S)
and
partial characterization of activity


H40
H80
MWM
(kDa)
hCg-6
26.6
17
14.2
6.5
3.5
1.06


Relative Abundance
A
4465.10
2999.0
4399.4
5799.8
7200.2
8600.6
m/z
B
1054.2
1580.5
400
600
800
1000
1200
1400
1600
1800
m/z
C
656.4462
657.4491
658.4444
661.597
663.611
672.3917
672.512
673.41
654.0
658.6
663.2
667.8
672.4
677.0
m/z
D
637,17
653,97
300
400
500
600
700
800
900
1000
1100
m/z


Bacterial concentration (CFU.mL-1)
1.10^7
1.10^6
1.10^5
1.10^4
1.10^3
1.10^2
H1
H6
H11
H17
H22
H27
H32
H37
H42
H47
H52
H57
Oysters hemolymph

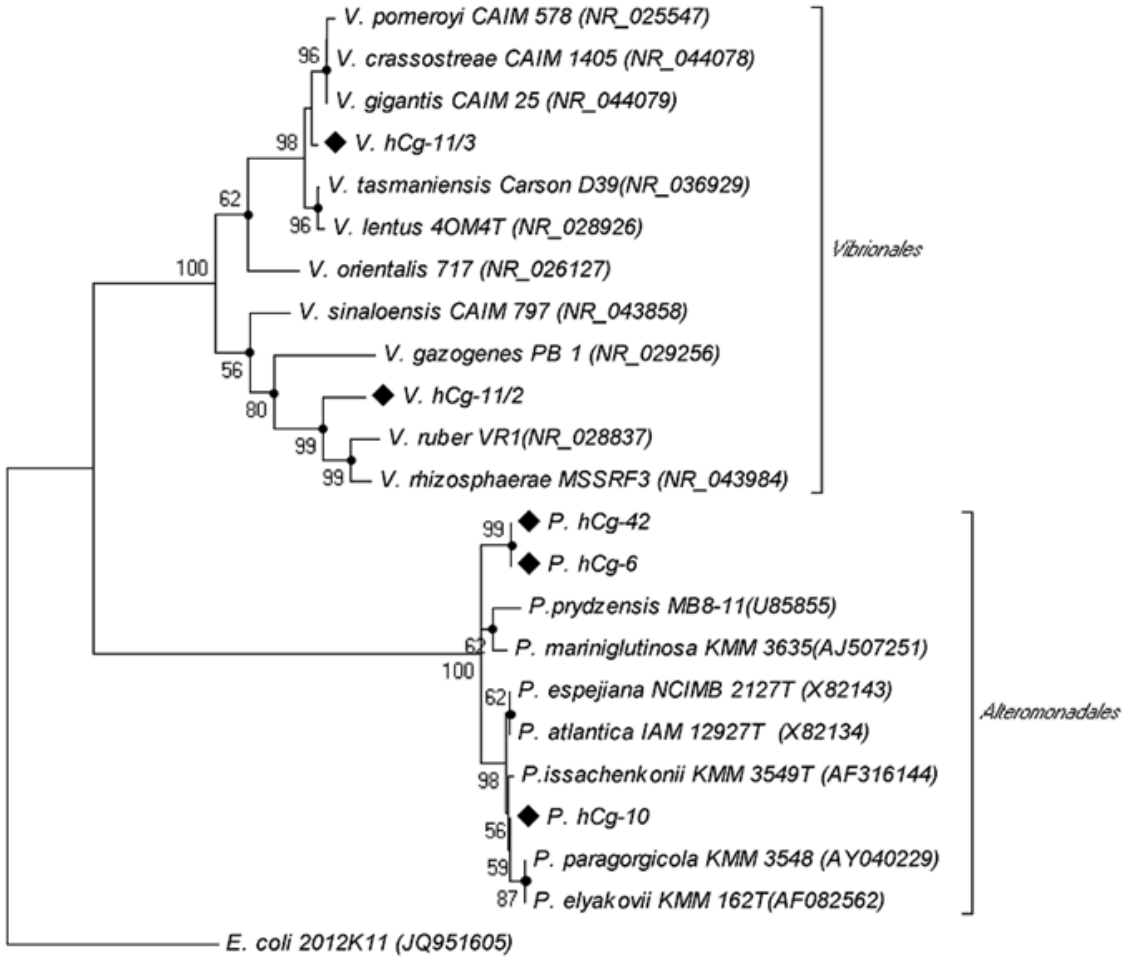

V. pomeroyi CAIM 578 (NR_025547)
96
V. crassostreae CAIM 1405 (NR_044078)
V. gigantis CAIM 25 (NR_044079)
98
V. hCg-11/3
V. tasmaniensis Carson D39(NR_036929)
62
96
V. lentus 4OM4T (NR_028926)
Vibrionales
100
V. orientalis 717 (NR_026127)
V. sinaloensis CAIM 797 (NR_043858)
V. gazogenes PB 1 (NR_029256)
56
80
V. hCg-11/2
99
V. ruber VR1(NR_028837)
99
V. rhizosphaerae MSSRF3 (NR_043984)
99
P. hCg-42
P. hCg-6
P.prydzensis MB8-11(U85855)
62
P. mariniglutinosa KMM 3635(AJ507251)
100
62
P. espejiana NCIMB 2127T (X82143)
Alteromonadales
P. atlantica IAM 12927T (X82134)
98
P.issachenkonii KMM 3549T (AF316144)
56
P. hCg-10
59
P. paragorgicola KMM 3548 (AY040229)
87
P. elyakovii KMM 162T(AF082562)
E. coli 2012K11 (JQ951605)

Table 1 : Culture conditions of target bacteria

Bacteria	Strain	Medium	Temperature
Bacillus megaterium	ATCC 10778	LB	30°C
Lactococcus garviae	ATCC 43921	TSB	30°C
Micrococcus luteus	ATCC 10240	TSB	37°C
Vagococcus salmoninarum	18–96	TSB	30°C
Aeromonas hydrophila	CIP 7614	TSB	30°C
Escherichia coli	ATCC 25922	TSB	37°C
Listonella anguillarum	NCBIM 829	TSB+NaCl(1.5%, w/v)	25°C
Salmonella enterica	CIP 8297	TSB	37°C
Vibrio alginolyticus	CIP 103360	MB	18°C
Yersinia ruckeri	ATCC 29473	TSB	30°C

Table 2. Antibacterial spectrum of activity of the hemolymph fractions expressed as MICs (µg.mL^{-1})

		Hemolymph fractions			
		H_{10}	**H_{40}**	**H_{80}**	**Positive Control**
	[Prot] µg.mL^{-1}	1000	630	150	µg.mL^{-1}
Target bacteria		**MIC (µg.mL^{-1})**			
Bacillus megaterium	ATCC 10778	-	20	37	1
Micrococcus luteus	ATCC 10240	-	20	9	4
Vagococcus salmoninarum	18-96	-	-	-	64
Aeromonas hydrophyla	CIP 7614	-	-	-	1
Escheriachia coli	ATCC 25922	-	-	-	4
Listonella anguillarum	NCBIM 829	-	-	-	1
Vibrio alginolyticus	CIP 103360	-	-	-	16
Yersinia ruckeri	ATCC 29473	-	160	-	1

(-) means that no inhibitory effect was observed. Lyzozyme and Polymyxin B were respectively used as positive control for Gram-positive and Gram-negative bacteria.

Table 3. Protease sensitivity of the H_{40} fraction

Hemolymph fraction H_{40}	MIC $\mu g.mL^{-1}$
- proteinase K-treated	> 630
- tryspsin-treated	> 630
- α-Chymotrypsin-treated	630
- control	20

control means H_{40} fraction incubated for 1H at 37°C in 50 mM phosphate buffer, pH 8.

Table 4. Antibacterial activity and protease sensitivity of the culture supernatant of hemolymph-associated strains

Supernatant from strain	*hCg-6*	*hCg-10*	*hCg-11/2*	*hCg-11/3*	*hCg-42*	Reference
isolated from hemolymph of oyster n°	6	10	11	11	42	
Target bacteria						
Bacillus megaterium ATCC 10778	-	-	-	-	+	+++
Lactococcus garviae ATCC 43921	-	-	+++	-	+	+++
Micrococcus luteus ATCC 10240	-	-	+++	-	-	+++
Vagococcus salmoninarum 18-96	-	-	-	-	-	+++
Aeromonas hydrophila *CIP 7614*	+	+++	+	+++	++	+++
Escherichia coli ATCC 25922	-	-	-	-	-	+++
Listonella anguillarum NCBIM 829	+++	+++	++	+++	+	+++
Salmonella enterica CIP 8297	+	-	++	-	-	+++
Vibrio alginolyticus CIP 103360	-	+	-	+	ND	ND
Yersinia ruckeri ATCC 29473	++	++	+++	+++	+++	+++
Antibacterial activity (%) after protease treatments						
Proteinase K	0	0	0	65	0	
Trypsin	100	79	71	88	50	
α-Chymotrypsin	ND	83	65	88	ND	
Control	100	100	100	100	100	

The symbol (-) means that no inhibition was detected using the well-diffusion assay while (+) indicates that an inhibition halo was observed. (+), (++) and (+++) were used to quantify the size of the inhibition zone : + < 1 mm large, 1mm < ++ < 2 mm and +++ > 3 mm. ND : not determined

Lyzozyme and Polymyxin B were respectively used as positive reference for Gram-positive and Gram-negative bacteria. Nisaplin® was used as reference for *L.garviae*. Control means h*Cg*-strain supernatant incubated in 50 mM phosphate buffer, pH 8 for 1H at 37°C.

Table 5. BLIS-production in various media

Antibacterial activity (%)	Marine Broth	LB +Sea Salts	Peptone +Sea Salts	TSB	TSB +Sea Salts	Hemolymph
Pseudoalt. hCg-6	100	100	100	0	0	100
Pseudoalt. hCg-42	100	100	100	0	0	100
Pseudoalt prydzensis	0	0	0	0	0	0

LB and TSB respectively mean Luria Broth and Tryptic Soy Broth.