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Stylicins, a new family of antimicrobial peptides from the Pacific blue shrimp *Litopenaeus stylirostris*

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Abstract:

The present study reports the characterization of Ls-Stylicin1, a novel antimicrobial peptide from the penaeid shrimp, *Litopenaeus stylirostris*. The predicted mature peptide of 82 residues is negatively charged (theoretical p*I* = 5.0) and characterized by a proline-rich N-terminal region and a C-terminal region containing 13 cysteine residues. The recombinant Ls-Stylicin1 has been isolated in both monomeric and dimeric forms. Both display strong antifungal activity against *Fusarium oxysporum* (1.25 μM < MIC < 2.5 μM), a pathogenic fungus of shrimp, but lower antimicrobial activity against Gram (−) bacteria, *Vibrio* sp. (40 μM < MIC < 80 μM). However, rLs-Stylicin1 is able to agglutinate *Vibrio penaeicidae in vitro* in agreement with its potent LPS-binding activity on immobilized LPS of *V. penaeicidae* (dissociation constant (K_d) of 9.6×10^{-8} M). This molecule with no evident homology to other hitherto described antimicrobial peptides but identified herein several species of penaeid shrimp is thought to be the first member of a shrimp antimicrobial peptide family, which we termed stylicins.

Keywords: Ls-Stylicin1; Peptide; Shrimp; Antifungal; Agglutination; *Vibrio*

1. Introduction

Infectious diseases are one of the main limitations to the sustainable development of shrimp aquaculture worldwide. Over the past 20 years, progresses have been made in the understanding of shrimp immunity, through the characterization in particular of antimicrobials and other defense molecules (Aguirre-Guzman *et al*., 2009).

Several families of antimicrobial peptides have been isolated. Those include penaeidins, isolated the first time from the Pacific white shrimp *Litopenaeus vannamei* (Destoumieux *et al.*, 1997) and which primary structure are characterized by the presence of a N-terminal domain rich in proline residues and a C-terminal domain containing 6 cysteine residues (Destoumieux *et al.*, 2000). Penaeidins display antifungal and antibacterial activity with a predominant activity against Gram-positive bacteria (Bachère *et al.*, 2000) and agglutinating activity against *Vibrio* strains (Munoz, 2002). Cysteine-rich antimicrobial peptides from shrimp also include crustins, possessing 12 cysteine residues and a whey-acidic-protein domain (WAP) and exclusively active against Gram-positive bacteria (Bartlett *et al*, 2002; Zhang *et al*; 2007; Amparyup *et al.*, 2008). Also antilipopolysaccharide factor (ALF) such as recombinant form of ALFpm3 (Amparyup *et al.*, 2008) exhibited a broad spectrum of activity against various strains of bacteria and fungi and was able to reduce WSSV propagation and prolong the survival of shrimps. Also lysozyme such as lysozyme C (Mai *et al*., 2009) from the blue shrimp (*Litopenaeus stylirostris*) demonstrated to be not only active against Gram-positive and Gram-negative bacteria but also against shrimp pathogens Vibrio sp.

Besides antimicrobial peptides, several effectors of shrimp immunity have been characterized that are involved in the recognition of microorganisms by binding molecules on the surface of invading bacteria. To date, those Pattern Recognition Proteins (PRPs) (Medzhitov *et al.*, 2002; Janeway *et al.*, 2002) include C-type lectins as PmLec from *Penaeus*

monodon, a lipopolysaccharide binding protein which has strong hemagglutinating and bacterial-agglutinating activity as well as opsonic effect (Luo *et al.*, 2006).

In spite of such progresses, the mechanisms by which the shrimp can circumvent infections remain largely unknown. Thus, the capacity of the shrimp *Litopenaeus stylirostris* to survive an infection with the highly pathogenic bacteria *Vibrio penaeicidae* have been investigated (de Lorgeril *et al*., 2005) by genomic approach. Thus, a relationship has been evidenced between the successful anti-infectious response of the shrimp and the abundance of transcripts for genes involved in immunity. Among them, genes encoding antimicrobials have been evidenced such as penaeidin 3 and lysozyme. Interestingly, a new transcript encoding a putative peptide rich in cysteines (13 Cys) and prolines was also identified. This transcript presented sequence homologies with a mouse cryptdin-related mRNA (Ouellette *et al*., 1990). Abundantly expressed in the haemocytes of shrimp able to survive the infection, the peptide could be involved in the immune response against the pathogenic bacteria.

In this report, we have further characterized this new cysteine and proline-rich peptide and investigated its biological properties in terms of antimicrobial, LPS-binding and agglutinating properties. For that, a recombinant peptide was produced in *Escherichia coli* system and purified to homogeneity. The “cryptin-like” peptide from the initial study (de Lorgeril *et al*., 2005) was renamed here Ls-Stylicin1 after the name of the shrimp *Litopenaeus stylirostris.*

2. Materials and methods

2.1. Nucleic sequences analysis

Total genomic DNA was extracted from the haemocytes of juvenile *L. stylirostris* according to the *Current Protocols in Molecular Biology* (Wiley *et al.*, 2003) as well as total mRNA using Trizol (Invitrogen) for cDNA synthesis (Invitrogen). Based on Litsty 004-A-01

L, the cDNA sequence identified by de Lorgeril, GenBank/EMBL Data Bank with the accession number **CV699274** (de Lorgeril et *al.,* 2005), homologous primers, R1F (5'-ATGAAGACCTACAGTC-3') and R1R (5'-GACTGTAGGTCTTCAT-3') were designed and used for PCR amplification. The reaction was performed by incubating the reaction mixtures at 94 °C for 10 min, followed by successive cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min for 30 cycles using *Taq* DNA polymerase (Qbiogene). Amplification products were then cloned into TopoTA cloning vector (Invitrogen) for sequencing. The subsequent sequences (DNA and cDNA) were compared with those available in Genbank data bases at the National Center for Biotechnology Information (NCBI) using the BLAST network service. Sequence alignments were made using Clustal W (Higgins *et al*., 1992) and Multalin programs (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html). Peptide sequences were analysed by the Blast and ScanProsite Tools.

2.2. Recombinant expression

Recombinant *L. stylirostris* Stylicin1 (rLs-Stylicin1) was expressed in *E. coli* as an N-terminal His6-tagged fusion protein using the pET-28b (+) system (Novagen, Madison, WI). A Met-coding trideoxynucleotide was incorporated 5' of the Ls-Stylicin1 cDNA and cloned in-frame with the N-terminal His6 in the *Bam*H1/*Sac*1 sites of pET-28a (+) by PCR amplification, using forward primer R2F (5'GTCTTGCACACGGGATCCATGTCTTCCTTTTCACC-3') paired with reverse primer R2R (5'-GTGGCTTCGTTCTCGAGCTCTTATCCTTTATAACC-3'). The reaction was performed by incubating the reaction mixtures at 94 °C for 10 min, followed by successive cycles at 94 °C for 1min, 55 °C for 1min, and 72 °C for 1 min for 30 cycles using *Taq* DNA polymerase (Qbiogene). The underlined codon in the forward primer denotes a Met codon to

incorporate a CNBr cleavage site immediately upstream of the N-terminus of the designed peptide. The construction was sequenced using an ABI Prism 377 DNA sequencer. The rLs-Stylicin1 was expressed in *E. coli* BL21 (DE3) pLysS cells (Novagen) transformed with the pET-28a (+)/rLs-Stylicin1 construct. The cells were grown at 37 °C to A_{600} 0.8 in Luria-Bertani (LB) medium (10 g of bacto-Tryptone, 5 g of bacto yeast extract, and 10 g of NaCl) supplemented with 50 µg/ml kanamycin. Expression of fusion proteins was induced with 0.5 mM isopropyl-α-D-1-thiogalactopyranoside. After growth at 37 °C for 12 h, bacterial cells were harvested by centrifugation and stored at -20 °C. The cells were lysed by resuspension in 6 M guanidine HCl in 100 mM Tris-HCl, pH 8.1, followed by sonication at 40% amplitude for 2 min using a Vibra cell Sonifier 450 (Branson Ultrasons, Annemasse, France). The lysate was clarified by centrifugation in a Sorvall SA-600 rotor at 10,000 *g* for 30 min at 4 °C prior to protein purification.

2.3. Purification and refolding

His-tagged rLs-Stylicin1 fusion proteins were purified by affinity chromatography by incubating cell lysates with nickel-nitrilotriacetic acid resin (Novagen) at a ratio of 25:1 (v/v) in 6 M guanidine HCl, 20mM Tris-HCl (pH 8.0) for 4 h at 4 °C. Fusion proteins were eluted with two volumes of 6 M guanidine HCl, 1 M imidazole, 20mM Tris-HCl (pH 6.4), dialyzed against 5% acetic acid (HOAc) in SpectraPor dialysis membranes (Spectrum Laboratories Inc., Rancho Dominguez), and lyophilized. His-tagged rLs-Stylicin1 was then loaded on a gel filtration Superdex 200 (Pharmacia) column equilibrated in 5% acetic acid solution (flow rate of 1ml/min) and eluted fractions were pooled, frizzed and lyophilised. The methionine residue introduced at the rLs-Stylicin1 N terminus was subjected to CNBr cleavage by dissolving the lyophilized His-tagged fusion proteins in 50% formic acid, addition of solid CNBr to 10 mg/ml (final concentration), and incubation of the mixtures for 8 h in the darkness at 25°C.

The cleavage reaction was terminated by adding 10 volumes of water, followed by freezing and lyophilization. The cleaved fusion peptide mixture was folded at pH 8.1 in a buffer solution containing 0.1 M NaHCO3 and 3mM reduced and 0.3mM oxidized glutathione in the presence of 2 M urea and 25% *N*,*N*-dimethylformamide (Wu *et al.*,2003). Then, the folded peptide mixture, rLs-Stylicin1, was resuspended in 1mL of acetic acid 5% buffer then loaded on a gel filtration Superdex 100 (Pharmacia) column equilibrated with the same buffer. Monomeric and dimeric form of rLs-Stylicin1 fractions were isolated, lyophilised and stored at room temperature. Peptide purity was analysed by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) using the Laemmli's method (Laemmli., 1970). Samples were prepared by heating for 10 min at 100°C in an equal volume of sample buffer (0.1 M citrate-phosphate buffer, 5% SDS, 0.9% 2-mercaptoethanol, 20% glycerol, pH 6.8).

2.4. Mass spectrometry analysis

To determine the number of cysteine residues that may be involved in the intramolecular and intermolecular disulfide bridges, mass spectrometry analysis was performed on monomeric and dimeric forms of rLs-Stylicin1. Matrix-assisted Laser Desorption/Ionisation-Time Of-Flight Mass Spectrometry (MALDI-TOF-MS) was carried out on a Bruker ULTRAFLEX mass spectrometer, set on a positive linear mode.

2.5. Immunolocalization

Antibodies against Ls-Stylicin1 were raised in mice (Balb/c). Briefly, 1mg of rLs-Stylicin1 were coupled to ovalbumin (Sigma) in a 1: 1 mass ratio after activation with 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (Pierce). Mice (4 weeks old) received three subcutaneous injections (50µg each) of ovalbumin-coupled rLs-Stylicin1 at day 0 with

complete Freund's adjuvant, and at days 15 and 30 with incomplete Freund's adjuvant. Mouse tumor cells ($5x10^6$ cells in 500µl RPMI 1640, (Gibco) were injected in the mouse peritoneal cavity at day 30. Then days after cells injection, ascitic fluid was withdrawn and clarified by centrifugation (800 X g, 15 min and 4°C). Immunoglobulins G (IgG) for each sera were purified on a Hitrap protein G sepharose column using Gradifrac (Pharmacia). Reactivity and specificity of purified IgG were tested by ELISA (indirect) against rLs-Stylicin1.

L. stylirostris shrimp (20g) were obtained from French Polynesia Island. Haemolymph was taken from the ventral sinus located at the first abdominal segment, under an equal volume of anticoagulant Modified Alsever Solution (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7) and centrifuged at 800g for 15 minutes (4°C) to separate the blood cells from plasma. Immunofluorescence analyses were carried out using polyclonal antibodies specific for Ls-Stylicin1, according to the method of Munoz (Munoz *et al.*, 2004). Briefly, haemocytes, fixed with paraformaldehyde, were cytocentrifuged (900 rpm, 10 min) on L-polylysine slides. After permeabilization with 0.1% Triton X-100 (10 minutes), the haemocytes were incubated overnight (4°C) with anti-Ls-Stylicin1 (2.3µg/ml) in PBS/Skimmed-Milk (PBS/SM) at 0.5% or irrelevant mouse IgG2a anti IgM (3µg/ml) of *Dicentrarchus labrax* (Fish Teleostean) (control 1) or anti-Ls-Stylicin1(2.3µg/ml) pre-incubated with the recombinant peptide (10µg/ml) (control 2). After three washes in phosphate buffer saline (PBS) a second incubation was performed for 1h at room temperature with anti-mouse FITC-conjugated (Pierce) diluted at 1: 200 (0.5µg/ml) in PBS/SM 0.5%. Slides were washed three times in PBS, fixed with Moviol then observed by confocal microscopy (Leica TCS 4D).

2.6. Antimicrobial assays

The antibacterial and the antifungal activity spectrum of purified rLs-Stylicin1 was investigated against a panel of Gram-positive, Gram-negative bacteria strains, fungi and yeast

171 strains including pathogenic strains for shrimp and molluscs. Briefly, four Gram-positive
172 bacteria (*Micrococcus lysodeikticus* ATCC 4698; *Staphylococcus aureus* ATCC 25293;
173 *Bacillus subtilis* and *Aerococcus viridans*, IBMC Strasbourg collection) and seven Gram-
174 negative bacteria (*Escherichia coli* 363 ATCC 11775; *Salmonella typhimurium,* IBMC
175 Strasbourg collection; *Vibrio penaeicidae* AM101; *V. splendidus* LGP32 and *V.*
176 *nigripulchritudo* SFn1,IFREMER collection; *Pseudomonas aeruginosa and Enterococcus*
177 *faecalis*, LIBRAGEN collection, Toulouse), were used in a liquid growth inhibition assay
178 (Destoumieux *et al*., 1997). In brief, 10µL of rLs-Stylicin1 dilutions were incubated in 96-
179 well microtiter plates with 100µl of bacteria suspension, starting at OD 600nm = 0.001, in
180 Poor Broth nutrient medium (1% bactotryptone, 0.5% (w/v) NaCl, pH 7.5). Bacterial growth
181 was assayed by measuring OD_{600nm} after 24 h incubation from 20°C to 37°C according to the
182 optimal grow temperature of each bacteria. The minimal inhibitory concentration (MIC) was
183 evaluated by testing serial replica dilutions, defined as the lowest peptide concentration that
184 prevents any growth (Hancock *et al*., 1995). The Minimal Bactericidal Concentration (MBC)
185 was determined as the lowest concentration that kills 99.9 % of bacteria.

186 Susceptibility of *Fusarium oxysporum* (a gift from A. Vey, INRA Saint Christol-lez-
187 Alès, France), *Penicillium* sp and *Botrytis cinerae* (fungi collection, IUT Department Génie
188 Biologique, Montpellier), *Aspergillus fumigatus, Neurospora crassa* and *Candida albicans*
189 (LIBRAGEN collection, Toulouse) were tested by a Fehlbaum modified liquid growth
190 inhibition assay (Fehlbaum *et al*., 1994). In brief, fungal spores 100 µl final concentration 10^4
191 spores mL^{-1} were suspended in a Potatoes Dextrose Broth (Difco) complemented with
192 tetracycline (10 µg/ml final concentration) and 10µL of rLs-Stylicin1 dilutions in microtiter
193 plates. Peptide was replaced by 10 µl of sterile water in controls. Growth inhibition was
194 observed under the microscope after 24 or 48h incubation at 30°C and quantified by OD 600

nm measurement after 48 h. The Minimal Inhibitory Concentration (MIC) was defined as described above.

2.7. Binding and agglutination assays

The binding of rLs-Stylicin1 to *V. penaeicidae* AM101 was examined. Briefly, an overnight culture of fresh cells was prepared. Cells were washed twice with PBS and centrifuged (1000 rpm, 30 min at room temperature) to eliminate the culture media. Cells were suspended in 1 ml PBS ($1x10^7$ cells/ml) and rLs-Stylicin1 added at a concentration of 10µg/ml in PBS. Peptides were substituted with PBS for the control. The mix was incubated for two hours at 4°C then washed 3 times with PBS. Mouse anti rLs-Stylicin1 IgG (laboratory product) was added (10µg/ml in PBS/SM 0.5%) for 1 h at 37°C then washed 3 times in PBS. FITC-conjugated anti-mousse IgG (Pierce Interchim) diluted 500 fold (2µg/ml) in PBS/SM 0.5%, was added for 1 h at 37°C. Cells were washed three times in PBS and, after fitting with glycerine buffer, were observed by fluorescence microscopy.

The quantitative binding assay of rLs-Stylicin1 to LPS was determined by an enzyme-linked immunosorbent assay (ELISA) using the anti-rLs-Stylicin1 antibody prepared as described previously. A microtiter plate was coated with 50 µl of 5 µg/ml *E. coli* 0127:B8 LPS (SIGMA) and *V. penaeicidae* LPS (Lab purification using the Bligh and Dyer method; Blight & Dyer, 1959) in 0.1 M Na_2CO_3, 20 mM EDTA, pH 9.6, for 3 h at 50 C. The plate was washed twice with PBS and then blocked with 5% (w/v) BSA in PBS buffer for 1h at room temperature and subsequently incubated with various amounts of purified rLs-Stylicin1 (0 – 16.8µM) for 1 h at room temperature. Control was the wells without rLs-Stylicin1 incubation. The plate was washed as above and then incubated with 50µl of 10µg/ml mouse anti rLs-Stylicin1 IgG in 0.1% (w/v) BSA/PBS for 2 h at 37 C. After washing as above the plate was incubated with an horseradish peroxidase-conjugated anti-mouse IgG (Pierce Interchim)

(1:5000 dilution, 0.2µg/ml) in 0.1% (w/v) BSA/PBS for 1 h at room temperature, washed again and developed with orthophenylenediamine in substrate buffer (0.1m citric acid, 0.1M sodium acetate, pH 5.4 containing 0.33% H_2O_2). The reaction was stopped after 15 min by adding 50µl of 4N H_2SO_4 and the plate was analyzed in a microtiter plate reader Multiskan Ex Labsystems at 490 nm. Experiments were performed in one independent repeat in triplicate for statistical analyses. The apparent dissociation constants (K_d) and the maximum binding (A_{max}) parameters were calculated with KaleidaGraph for Windows, Software, using nonlinearly fitting as $A=A_{max}[L]/(Kd+[L])$, where A is the absorbance at 490 nm and [L] is the ligand concentration.

The agglutination of *V. penaeicidae* bacteria by rLs-Stylicin1 was considered. Briefly, 20 µl of *V. penaeicidae* fresh cells ($1x10^8$cells/ml) were mixed with 10 µL of 160 µM peptide solution in PBS. The mixture was then incubated for 1 hour at 37°C and the agglutination observed by optical microscopy. Peptides were substituted with PBS for the control.

3. Results

3.1. Sequence analysis

Nucleic sequences of Ls-stylicin1 were cloned from the haemocytes of *L. stylirostris*. The Ls-Stylicin1 cDNA sequence (GenBank accession no. **EU177435)** was 325 bp-long (Fig. 1). The corresponding genomic sequence (GenBank accession no. **EU177436**) obtained by PCR-amplification was 525 bp-long. Sequence analysis of the Ls-Stylicin1 gene showed that it is composed of two exons and one intron (213 bp) inserted between nucleotide 77 and nucleotide 78 of the cloned cDNA sequence (Fig. 1). The coding sequence was compared with sequences available in Genbank databases (NCBI) using the BLASTn and BLASTx network service. It revealed more than 80% sequence similarity with several sequences from penaeid shrimp: these include cDNA sequences from *L. stylirostris* (GenBank accession nos.

CV758538 and **CV699274**), *L. vannamei* (GenBank accession nos. **BE188497**, **BE188619**, **BE188495**, **CK591498**, **CK591516**, **CK591492**, and **CV468241**), *Penaeus monodon* (GenBank accession nos. **DW042940** and **DW678039**) and two genomic sequences from *L. stylirostris* (GenBank accession no. **EU177436** and **EU177437**).

The amino acid sequence deduced from Ls-stylicin1 cDNA is 104 residues long (Fig. 1). It has a predicted 22 amino acid signal peptide from Met^{1} to Gly^{22}, as determined by Van Heijne's rules (Nielsen *et al.*, 1997), and a putative Lys^{98}-Lys^{99} prohormone convertase site in its C-terminal sequence. The predicted mature Ls-stylicin1 is therefore an 82 amino acid peptide, starting from Ser^{23} and ending at Gly^{104}, which is determined to be anionic with a calculated pI of 5.00. Sequence analysis of the mature Ls-Stylicin1 revealed the presence of 16 proline residues representing 19.5% of the whole sequence. Those proline residues are concentrated in the N-terminal. Besides, Ls-Stylicin1 contains 13 cysteine residues representing 15.9% of the whole sequence, which are present in the C-terminal part of the molecule (Fig. 1). No whey acidic protein (WAP) domain, lectin amino-acid motifs (CTLD), ligand-binding (QPD) motifs or potential glycosylation site were identified in the Ls-Stylicin1 amino acid sequence.

Translated sequences identified previously revealed five peptides sequences from penaeid shrimps, two from *L. stylirostris*, two partial sequences from *L. vannamei* and one partial sequence from *P. monodon* (Fig. 2). Moreover, compared with peptidic sequences available in Genbank data bases (NCBI) using the BLASTp network service, no significant sequence homology was found with antimicrobials, cysteine-rich, proline–rich peptides or any other well characterized peptides. Altogether, this suggests that Ls-Stylicin1 is the first member of a novel family of peptides from penaeid shrimp.

3.2. Recombinant expression of Ls-Stylicin1 in *E. coli* and purification

The 82 amino-acid recombinant peptide, rLs-Stylicin1, corresponds to the deduced mature peptide sequence, Ser^{23}–Gly^{104} of the Ls-Stylicin1 studied here (Fig. 1). The rLs-Stylicin1 produced in the heterologous expression system (pET-28b (+) vector) and folded, appeared as a monomer form with a molecular mass of 8.9 kDa (Fig 3A) and multimeric forms (Fig. 3B) as revealed by non reducing gel electrophoresis. The two major forms, monomer and dimer, were purified by gel filtration chromatography. The homogenous preparation of monomer (Fig. 3A) was mass measured at 8920.40 Daltons by MALDI-TOF MS (Fig. 3C). In comparison to the calculated mass of 8932.20 Daltons, the 11.80 Dalton losses may correspond to the mass of 12 hydrogen elements coming from 12 of the 13 cysteine residues present in the molecule and implicated in disulfide bridge formation.

The second homogenous preparation of homodimer was mass measured at 17838.81 Daltons by MALDI-TOF MS (Fig. 3D). In comparison to the calculated mass of 17864.40 Daltons (dimer with 1 disulfide bridge cross-linkage), the 25.59 Daltons loss in mass corresponds to the mass of 26 hydrogen elements coming from 24 residues of cysteine implicated in monomeric disulfide bridges formation and from 2 residues may implicated in cross link formation of homodimer.

3.3. Ls-Stylicin1 localization in haemocytes

The peptide localization within haemocytes was investigated by indirect immunofluorescence using specific antibodies. Immunofluorescence assays showed a strong immunolabelling for all the haemocyte populations (Fig. 4A, right and left panel) including hyaline and granular cells, as evidenced on the same view observed by light microscopy. Moreover, Ls-Stylicin1 appears to be localized in small cytoplasmic granules of the haemocytes. No fluorescence was observed on haemocytes incubated with irrelevant mouse

IgG2a anti IgM of *Dicentrarchus labrax*. (Fig. 4B) or anti-rLs-Stylicin1 pre-incubated with rLs-Stylicin1 peptide (Fig. 4C).

3.4. Antimicrobial activities

The monomer form displays weak bacteriostatic activity (up to 4 times the MIC) against *Vibrio* species, particularly the shrimp pathogenic strains *V. penaeicidae* and *V. nigripulchritudo,* with MIC values from 40 µM to 80 µM (Tab. 1)**.** Otherwise, no activity against other Gram-negative or Gram-positive bacteria tested was detected up to concentrations of 160 µM. Among the six fungal and yeast species tested, antifungal activity was observed against the filamentous shrimp pathogen, *F. oxysporum* with both monomeric and dimeric forms (Tab. 1) (Souheil *et al*., 1999). In addition, we noted that, at concentrations of 2.5 µM (the MIC) *F. oxysporum* spore germination was inhibited but with no abnormal morphology. Otherwise, no activity against *Penicillium* sp, *B. cinerae, A. fumigatus, N. crassa,* or *C. albicans* was detected up to peptide concentrations of 100 µM.

Interestingly, similar antimicrobial effects were observed with the dimer, a bacteriostatic effect against *Vibrio* sp and an antifungal effect against *F. oxysporum* at the peptide concentrations of 20 µM and 1.25 µM respectively.

3.5. *V. penaeicidae* binding assays

To assay the binding of rLs-Stylicin1 to *V. penaeicidae*, bacteria were incubated with rLs-Stylicin1 and stained with anti-Ls-Stylicin1 IgG followed by FITC-conjugated anti-mouse IgG immunostaining. Immunofluorescence assays showed a strong labelling of bacteria was observed (Fig. 5A). The ability of rLs-Stylicin1 to bind *V. penaeicidae* cells was therefore investigated. To determine the potential agglutination properties of rLs-Stylicin1, fresh cells of *V. penaeicidae* were incubated with increasing concentration of rLs-Stylicin1. Bacteria

agglutination was observed by light microscopy after 1 hour incubation at 37°C with highly concentrated rLs-Stylicin1 (160 µM) (Fig. 5C).

Because Ls-Stylicin1 may bind to the cell surface components of Gram-negative bacteria, we tested LPS as a candidate ligand for rLs-Stylicin1. To measure binding of rLs-Stylicin1 to LPS, we performed an enzyme-linked immunosorbent assay in which the LPS from *V. penaeicidae* or *E. coli* (O127:B8) was coated onto a microplate. We showed that rLs-Stylicin1 binds to both LPSs in a concentration-dependent manner (Fig. 6). Nonlinear regression analysis with a one site binding model ($0.93 < R^2 < 0.96$) revealed that rLs-Stylicin1 bound to *V. penaeicidae* and *E. coli* LPS with an apparent dissociation constant (K_d) of 0.096 and 0.102 µM, respectively.

4. Discussion

In this study we report the characterization of Ls-Stylicin1, a novel antimicrobial peptide from the penaeid shrimp, *L. stylirostris*. The nucleotide and deduced amino-acid sequences of the Ls-Stylicin1 showed high sequence similarities (80%) to unknown peptides from penaeid shrimp previously deposited in Genbank. All sequences contain a highly similar proline-rich region at the N-terminus and similar C-terminal cysteine-rich region. Such structural are also characterized of penaeidins, a family of antimicrobial peptides from penaeid shrimp (Bachere *et al*., 2000; Gueguen *et al.*, 2006). However, no sequence similarity, no conserved WAP domain, lectin amino-acid motifs (CTLD), ligand-binding (QPD) motifs, or known PRP motifs was found between Ls-Stylicin1 and other antimicrobial peptides well characterized up to now. Therefore Ls-Stylicin1 was proposed to be the first member of a new family of peptides present in penaeid shrimp.

The recombinant Ls-Stylicin1 was first investigated for its potential antimicrobial activities. The peptide proved to be poorly antibacterial with MICs in the range of 40 to 80

µM. Because those MICs are rather high, we speculated that a direct antibacterial activity such as the typical membrane disruption was unlikely. Moreover, the peptide displays a negative net charge at a physiological pH and is rather anionic in contrast with most of the antimicrobial peptides identified so far.

Besides, rLs-Stylicin1 is active against *Vibrio* sp with bacteriostatic activity. We therefore hypothesized that the mechanism of bacterial growth inhibition could rely on the ability of rLs-Stylicin1 to bind Gram-negative bacteria and their cell walls. From those binding assays, rLs-Stylicin1 appeared to function as a pattern recognition protein, especially through its interaction with LPS present in cell wall of Gram-negative bacteria, and more particularly LPS from V. *penaeicidae*. Indeed, the rLs-Stylicin1 strongly bound to immobilized LPS of both *V. penaeicidae* and *E. coli* with a dissociation constant (Kd) of $0;96x10^{-7}$ and $1.02x10^{-7}$ M, respectively. The binding constants of rLs-Stylicin1 for LPS are in agreement with the values obtained with other known binding proteins, such as polymyxine B, human CD14 (Kirkland *et al.*, 1993), the limulus factor C (Tan *et al.*, 2000), the *Crassostreas gigas* BPI (Gonzalez *et* al., 2007) or *Penaeus monodon* rALFPm3 (Somboonwiwat *et al*., 2008), which range from 3.3×10^{-7} M to 2.3×10^{-10} M. This strongly suggests that LPS is one of the target molecules for Ls-Stylicin1. It is tempting to speculate that the proline-rich region of rLs-Stylicin1 could be implicated in the LPS binding as reported for other antibacterial peptides (Gennaro *et al*., 2002; Li *et al*., 2006; Cuthbertson *et al*., 2004) and, like peneidins, in bacterial aggregation, rather than in direct bacterial killing (Munoz, 2002). On that basis, we hypothesize that the antibacterial activities are the result of a combination of rLs-Stylicin1 LPS-binding and rLs-Stylicin1 multimerization processing. This is supported by our experimental data showing a bacteriostatic activity observed at low peptide concentration as well as *in vitro* agglutination at high peptide concentration, when rLs-Stylicin1 multimerization is likely to occur. Such agglutination may also occur *in vivo*

thereby facilitating the haemocyte-mediated bacterial clearance. Beyond that, the whole process may help to protect shrimp from *V. penaeicidae* infections.

While poorly antimicrobial, Ls-Stylicin1 proved to be strongly active against the filamentous fungus pathogenic for shrimp *F. oxysporum* (MIC = 2.5 µM). Other cysteine-rich peptides that exhibit antifungal activity have been previously characterized. All these peptides display a conserved pairing of six cysteine residues such as the shrimp penaeidins (Bachere *et al*., 2000), the potatoes Snakin1 (Segura *et al*., 1999) or the horse shoe crab Tachycitin (Kawabata *et al*., 1996; Suetake *et al*., 2002). Moreover they are cationic and display chitin-binding activity. The molecule described here has no conserved pairing of six cysteine residues; it is anionic and does not bind chitin (data not show). So it can hardly be compared with earlier described antifungal peptides. Based on its antifungal activity, Ls-Stylicin1 could bind molecules of *F. oxysporum* cell wall and affect the fungi grow. Proteins that bind both LPS (as Ls-Stylicin1) and fungi cell wall molecules such as the β1-3 glucan (schoffelmeer *et al.*, 1999) have been previously described in penaeid shrimps. They are referred to as the lipopolysaccharides / β1-3-glucan binding proteins (LGBP) from *P. stylirostris* (Roux *et al.*, 2002), from *P. monodon* (Sritunyalucksana *et al.*, 2002) and from *P. vannamei* (Romo-Figueroa *et al.*, 2004). Moreover, the antifungal activity of this peptide was comparable to the C-terminal fragment of Hemocyanin PvHCt, an anionic peptide (theorical pI= 6.04) strictly anti-fungal, with MIC value from 3 to 50 µM (Destoumieux *et al* 2001). Given that no structure homology is found between rLs-Stylicin1 and PvHCt. The negative charge of both peptides at physiological pH may play an important role in their anti-fungal activities.

Monomer and dimer of Ls-Stylicin1 exhibits similar antimicrobial activity spectrum, suggesting that the antimicrobial activity of rLs-stylicin1 is independent of its tertiary structure. Those results are consistent with the previously described antimicrobial peptide, the

bactenecin. Indeed, both form of this peptide are demonstrated to completely kill both *Staphylococcus aureus* and *E. coli* at concentration of 8 µM. However, unlike the monomer, the homodimer of bactenecin displayed membrane-lytic activity and consequently has more potent antibacterial activity than the monomer at physiological conditions (Lee *et al.*, 2009). The same probably does not apply to Ls-Stylicin1, since Ls-Stylicin1 homodimer have a bacteriostatic effect on bacteria. Future studies will help understanding the role of stylicin multimerization in shrimp defense.

Tissue-specific expression analysis of Ls-Stylicin1 in adult *L. stylirostris* shrimp revealed that this peptide is expressed and localized in haemocytes. Moreover, it has been previously demonstrated that the corresponding mRNA was over-expressed in the haemocyte of *L. stylirostris* shrimp surviving an experimental infection with *V. penaeicidae* (de Lorgeril *et al.*, 2005). This suggests that Ls-Stylicin1 is abundantly produced in hemocytes of shrimp surviving a *V. penaeicidae* challenge. The shrimp immune response to infection involves massive hemocyte recruitment to infection sites where penaeidins are released (Munoz *et al*., 2004). There, like penaeidins, Ls-stylicins could participate to the isolation and elimination of the bacteria. The presence of a signal peptide support the hypothesis of Ls-Stylicin1 release. Alternatively, Ls-Stylicin1 facilitates the intracellular elimination of pathogens by phagocytes through a potential opsonic effect, base on the LPS-binding activity of stylicins. This potential involvement in defense reaction through binding to pathogens deserves further investigation.

In conclusion, as presented in the results section, several stylicin-like peptides are present in the public EST databases, all of them being found in penaeid shrimp. Moreover, we have identified a second Stylicin coding cDNA sequence from *L. stylirostris* (data not shown). Together with the experimental data from this article, this indicates that Ls-Stylicin1 is the

first member of a new family of antimicrobial peptides from penaeid shrimp, and primarily active against pathogenic filamentous fungi.

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Figures

Fig. 1. Ls-Stylicin1 cDNA sequence (**EU177435**, 312 bp) and deduced peptide (104 amino acids). The start and stop codons are shown in bold. The 22 amino acid signal peptide sequence is indicated by a solid underline. Putative proline rich region is indicated in tight region. Conserved cysteine residues are marked with ▲. Lysine–lysine prohormone convertases site is marked with ■. Sequence and position of the intron(213 bp) from the corresponding genomic sequence (**EU177436**) are indicated by a box.

Fig. 2. Alignment of consensus translated sequences find in GeneBank Database. Ls-Stylicin1 (**EU177435** and **CV758538**) and Ls-2 (**EU177437**, **EU177437**) from *L. stylirostris* shrimp. Pm-1 (**DW042940** and **DW678039**) from *P. Monodon.* Lv-1 (**BE188497**, **BE188495**) and Lv-2 (**CK591498**, **CV468241**, **CK591492**) partial sequences from *L. vannamei* shrimp. Variant amino acids are indicated in black regions.

Fig. 3. SDS–PAGE Tris-Tricine analysis of the purified rLs-Stylicin1. Purified monomer (**A**, lane 1) and multimer (**B**, lane1). Lane M is the molecular weight marker. MALDI-TOF MS spectra of the purified monomer (**C**) and dimer (**D**).

Fig. 4. Immunolocalization of rLs-Stylicin1 peptide in hemocytes. *L. stylirostris* hemocytes + anti-rLs-Stylicin1(**A**); *L. stylirostris* hemocytes + irrelevant mouse IgG2A anti IgM of *Dicentrarchus labrax* (**B**). *L. stylirostris* hemocytes+ anti-rLs-Stylicin1 pre-incubated with rLs-Stylicin1 (**C**). Granular cell (G), Hyaline cell (H).

Fig. 5. Immunofixation of rLs-Stylicin1 peptide on *V. penaeicidae* (Vp) membrane observed under a fluorescence microscope (40x). *V. penaeicidae* + rLs-Stylicin1 + anti- rLs-Stylicin1

IgG (**A**); *V. penaeicidae* + anti- rLs-Stylicin1 IgG (negative control) (**B**). Agglutination of *V. penaeicidae* observed under optical microscope in presence of highly concentrate rLs-Stylicin1 (**C**); *V. penaeicidae* negative control (**D**).

Fig. 6. Quantitative binding of rLs-Stylicin1 to immobilized LPS. The microtiter plates were coated with 5 µg of *E. coli* LPS (-○-) or *V. penaeicidae* LPS (-●-) and incubated with increasing concentration of purified rLs-Stylicin1 in Tris buffer containing 0.1 mg/ml BSA. Bond rLs-Stylicin1 was detected using purified mouse anti rLs-Stylicin1 antibodies as described under "Experimental Procedures." Each point represents the mean of three individual measurements ± S.E. In the second graph, the lines represents the corresponding curves calculated for one-site binding (R^2 = 0.97).

Tab. 1.

		MIC (µM)
Gram(+) bacteria	*Micrococcus lysodeikticus*	>160
	Staphylococcus aureus	>160
	Bacillus subtilis	>160
	Aerococcus viridans	>160
Gram (-) bacteria	*Enterococcus faecalis*	>160
	Pseudomonas aeruginosa	>160
	Escherichia coli 363	>160
	Salmonella typhimurium	>160
	***Vibrio splendidus* LGP32**	**80**
	Vibrio penaecidae*	**40**
	Vibrio nigripulchritudo*	**80**
Fungi and yeast	***Fusarium oxysporum****	**2.5**
	Penicillium spp	>100
	Botrytis cinerae	>100
	Aspergillus fumigatus	>100
	Neurospora crassa	>100
	Candida albicans	>100

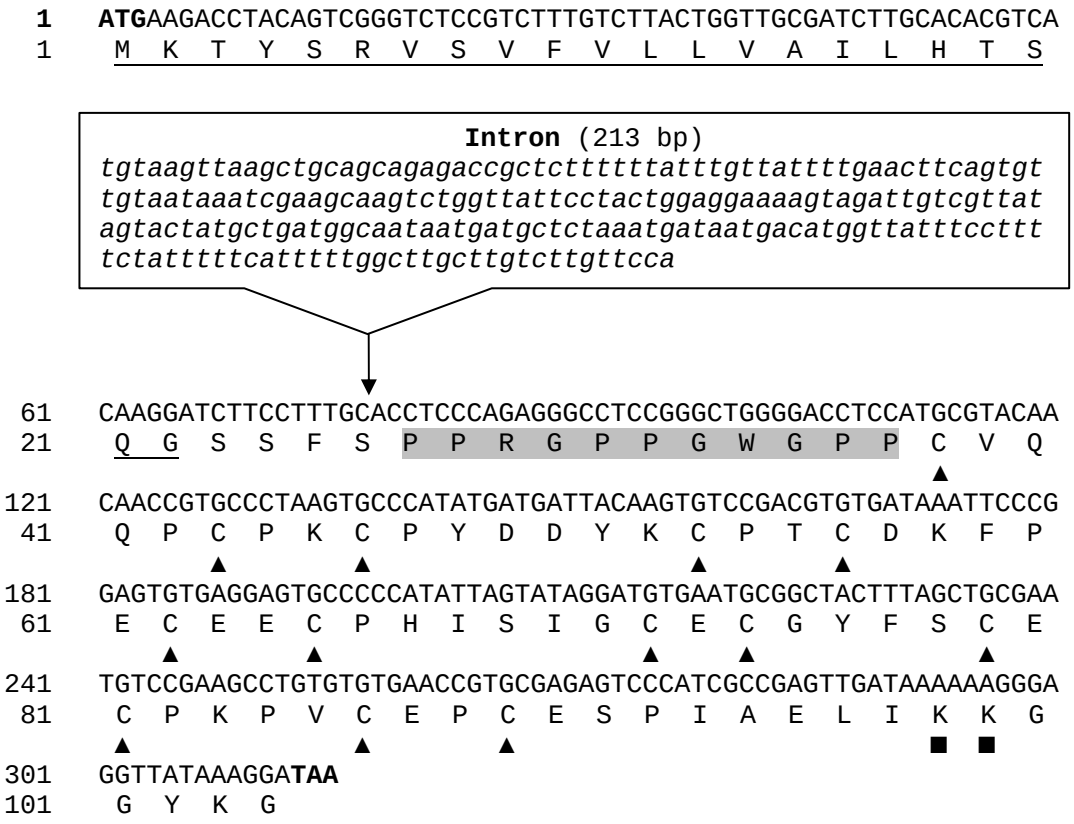
1 ATGAAGACCTACAGTCGGGTCTCCGTCTTTGTCTTACTGGTTGCGATCTTGCACACGTCA
1 M K T Y S R V S V F V L L V A I L H T S
Intron (213 bp)
tgtaagttaagctgcagcagagaccgctctttttatttgttattttgaacttcagtgt
tgtaataaatcgaagcaagtctggttattcctactggaggaaaagtagattgtcgttat
agtactatgctgatggcaataatgatgctctaaatgataatgacatggttatttccttt
tctatttttcatttttggcttgcttgtcttgttcca
61 CAAGGATCTTCCTTTGCACCTCCCAGAGGGCCTCCGGGCTGGGGACCTCCATGCGTACAA
21 Q G S S F S P P R G P P G W G P P C V Q
121 CAACCGTGCCCTAAGTGCCCATATGATGATTACAAGTGTCCGACGTGTGATAAATTCCCG
41 Q P C P K C P Y D D Y K C P T C D K F P
181 GAGTGTGAGGAGTGCCCCCATATTAGTATAGGATGTGAATGCGGCTACTTTAGCTGCGAA
61 E C E E C P H I S I G C E C G Y F S C E
241 TGTCCGAAGCCTGTGTGTGAACCGTGCGAGAGTCCCATCGCCGAGTTGATAAAAAAGGGA
81 C P K P V C E P C E S P I A E L I K K G
301 GGTTATAAAGGATAA
101 G Y K G

Fig.1.

```
                1                                                  50
Ls-Stylicin1    MKTYSRVSVFVLLVAVLHTSQGSSFSPPRGPPGWGPPCVQQPCPKCPYDD
Ls-2            MKTYSRVSVFVLLVAIVHTSQGSSFAPPSRLPGWEPPCVPQQCPPCPYDD
Pm-1            MRTFSQVSGFVLLVAIVRMSQGSSFYAPSGPPGERHPCPPQLCPVCPG--
Lv-1                                                  PQECPPCPYDD
Lv-2            MKTYSQVSVFVLLVAIAHTSQGSSFSPPRGPPGWKLPCVPQECPPCPYDD


                51                                                 106
Ls-Stylicin1    YKCPTCDKFPECEE-CPHISIGCECGYF-SCECPKPVCEPCESPIAELIKKGGYKG
Ls-2            -ECPKCGGFPACEE-CPDIHIGCDCPFYHSCLCRQPVCEPCESPIAELIKKGGYKG
Pm-1            -ECPKCESFPVCHEVCPDIAIGCDCPFYHSCECRHRVCPPCENPIAELIKKGGYNG
Lv-1            -ECPKCEELPACEE-CPDIHIGCDCPFYHSCLCRQPVCEPCESPIAELIKKGGYKG
Lv-2            -ECPKCGGFPVCHEVCTDI.................................
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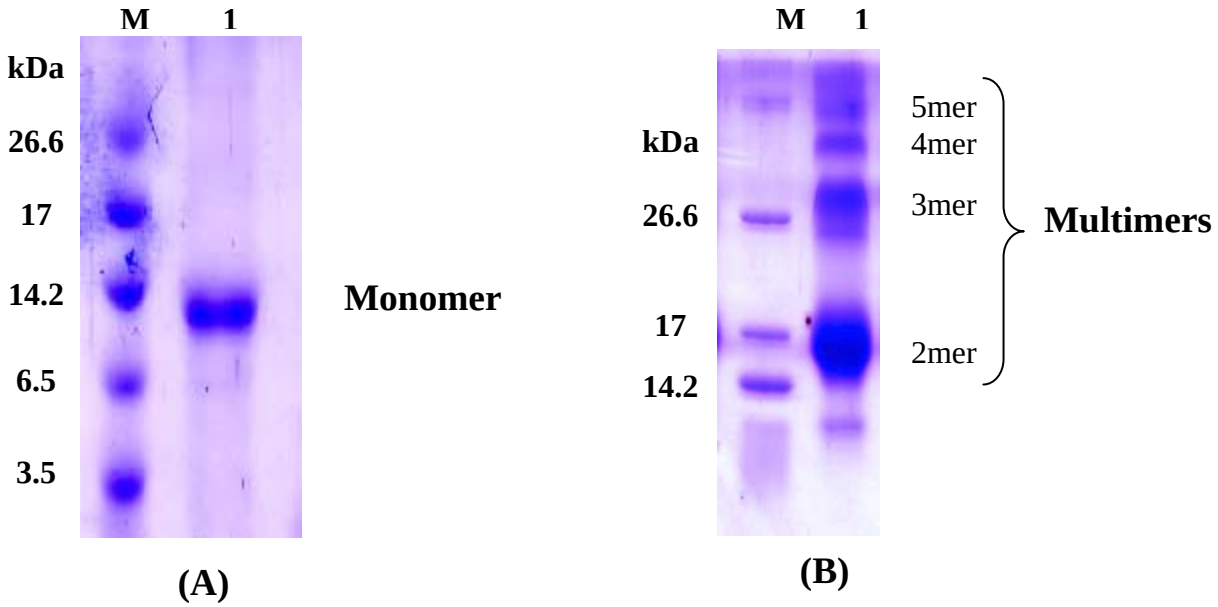
Fig.2.


M
1
kDa
26.6
17
14.2
6.5
3.5
Monomer
(A)
M
1
kDa
26.6
17
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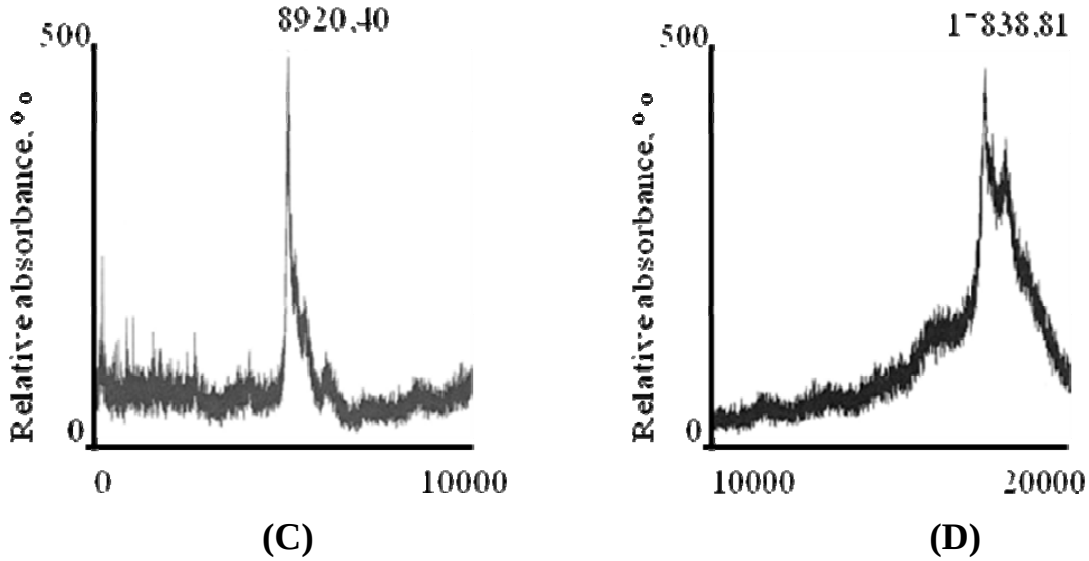
5mer
4mer
3mer
2mer
Multimers
(B)


500
8920.40
Relative absorbance, %
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0
10000
(C)
500
17838.81
Relative absorbance, %
0
10000
20000
(D)

Fig.3.

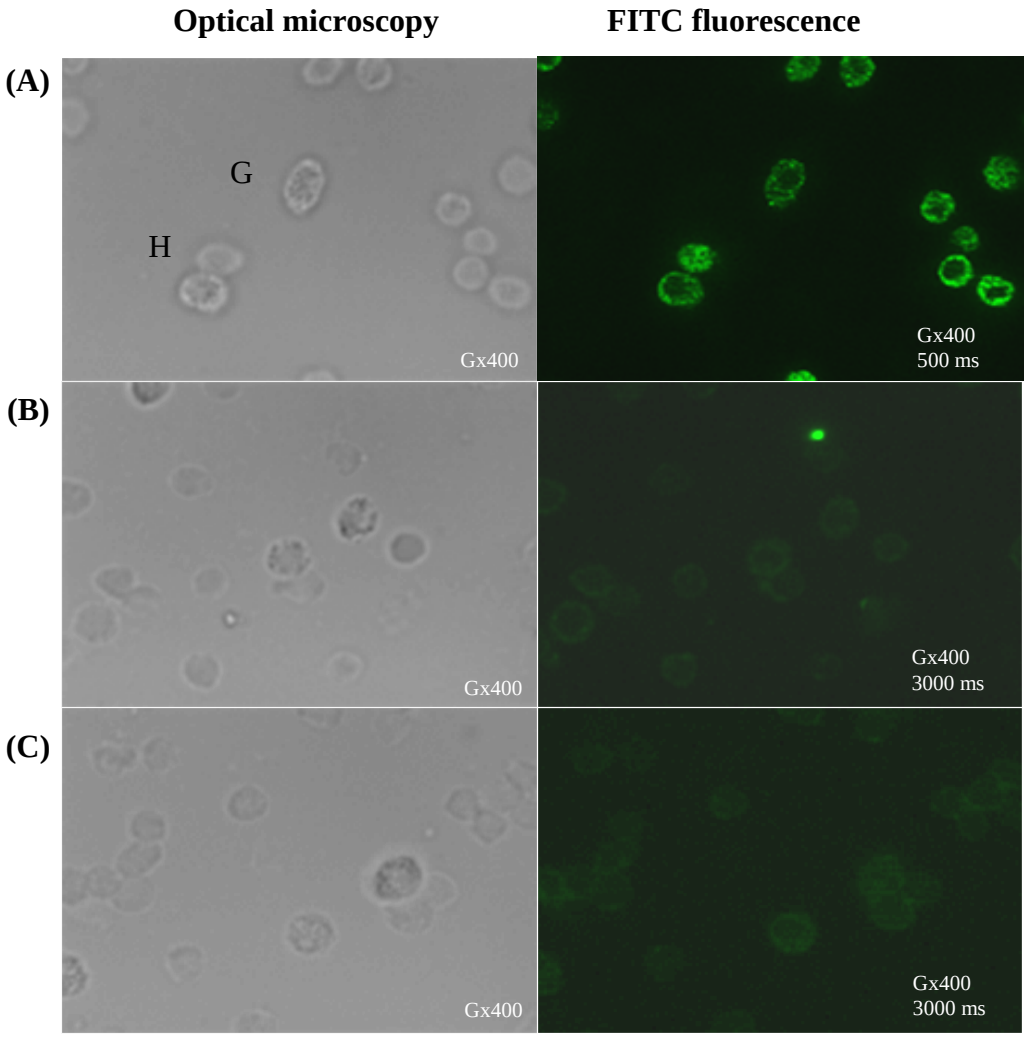
Optical microscopy
FITC fluorescence
(A)
G
H
Gx400
Gx400
500 ms
(B)
Gx400
Gx400
3000 ms
(C)
Gx400
Gx400
3000 ms

Fig.4.

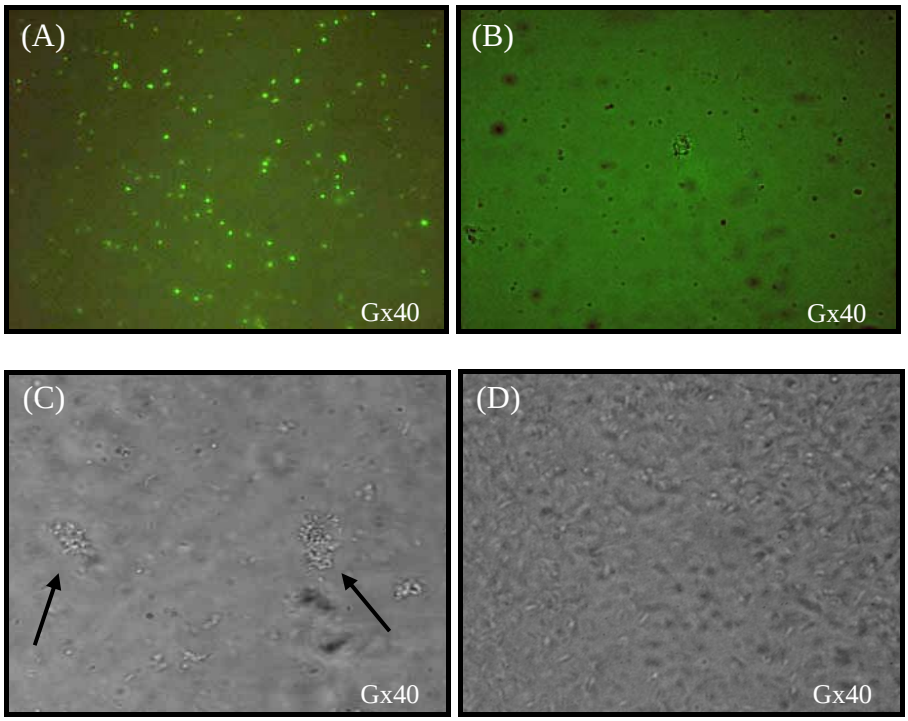

Fig.5.

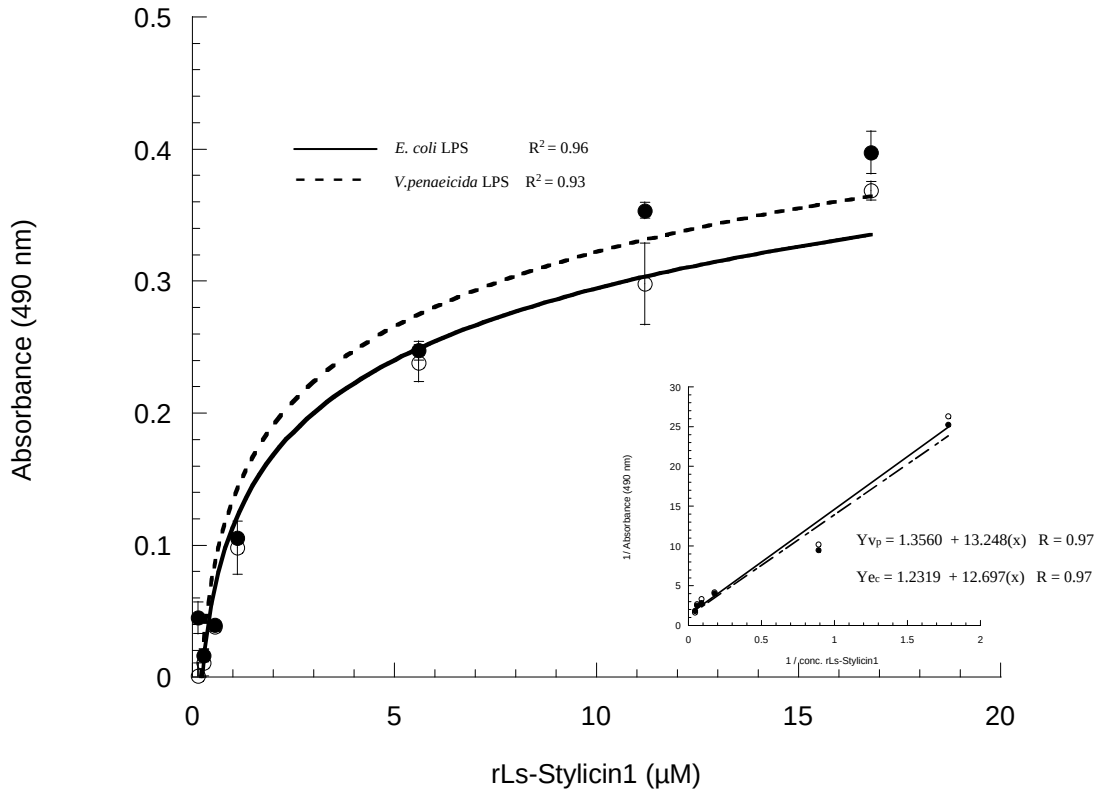
0.5
0.4
0.3
0.2
0.1
0
Absorbance (490 nm)
E. coli LPS R2 = 0.96
V.penaeicida LPS R2 = 0.93
0
5
10
15
20
rLs-Stylicin1 (μM)
1/ Absorbance (490 nm)
1 / conc. rLs-Stylicin1
Yvp = 1.3560 + 13.248(x) R = 0.97
Yec = 1.2319 + 12.697(x) R = 0.97

Fig. 6.