

Identification and functional characterization of the astacidin family of proline-rich host defence peptides (PcAst) from the red swamp crayfish (*Procambarus clarkii*, Girard 1852)

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ABSTRACT

This study reports the identification of four novel proline-rich antimicrobial peptides (PR-AMP) from the transcriptome of the red swamp crayfish *Procambarus clarkii*. The newly identified putative peptides (PcAst-1b, -1c, -2 and -3), which are related with the previously identified hemocyte-specific PR-AMP astacidin-1, are encoded by the multi-genic astacidin gene family. The screening of available and proprietary transcriptomes allowed to define the taxonomical range of distribution of this gene family to Astacoidea and Parastacoidea. The antimicrobial properties of three synthetic PcAst peptides (PcAst-1a, -1b/c and -2), were characterized against reference bacteria or multidrug resistant clinical isolates, and their cytotoxicity was evaluated towards human transformed cell lines. The antimicrobial activity ranged from potent and broad-spectrum, in low-salt medium, to poor, whereas it was generally low in full nutrient broth. No significant toxic effects were observed on cultured human cells. RNA-seq data from 12 different tissues indicated a strong specificity for haemocytes under naïve physiological condition, with moderate expression (5-fold lower) in gills. Quantitative real time PCR revealed a rapid (within 2 h) and significant up-regulation of PcAst-1a (Astacidin 1) and PcAst-2 expression in response to LPS injection. Due to the variation in antimicrobial potency and inducibility, the roles of the other astacidins (PcAst-1b, -1c and -3) need to be further investigated to determine their significance to the immune responses of the red swamp crayfish.

1. Introduction

Phagocytosis, encapsulation and melanisation are key innate immune responses that invertebrates enact against pathogen infections (Jiravanichpaisal et al., 2010; Liu et al., 2007). Crustaceans are one of the most abundant and diverse group of metazoans and, due to their colonization of widely different environments, they are considered an important source of bioactive molecules (Zhu et al., 2019). In particular, the lack of an adaptive immune system has led to the evolution of effective antibiotic molecules, including different types of antimicrobial peptides (AMPs). These molecules can be highly diverse in their primary structure, but they generally share a cationic nature and common

structural features such as an amphipathic residue arrangement, as well as functional characteristics such as inducibility following exposure of the organism to pathogen associated molecular patterns (PAMPs) (Ganz and Lehrer, 1994; Hancock and Diamond, 2000). In fact, AMPs are recognised as important components of the innate immune system, nearly universally present in multicellular organisms (Hoffmann et al., 1999; Jiravanichpaisal et al., 2010; Zasloff, 2002).

Invertebrate AMPs are commonly grouped into three families, based on some common structural features: *i*) cysteine-free, linear peptides that adopt an amphipathic, alpha-helical structure and generally have a membranolytic mode-of-action (well-known examples are insect cecropins) (Steiner et al., 1981), *ii*) peptides with two or more cysteine

Abbreviations: DiOC6, 3,3'-dihexyloxycarbocyanine iodide; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine DOPC; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PI, propidium iodide; PG, phosphatidylglycerol; LPS, Lipopolysaccharide; cDNA, complementary DNA; PAMP, pathogen-associated molecular patterns

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residues forming disulfide bridged, β -sheet structures (e.g. defensins) (Kagan et al., 1994), and *iii*) linear peptides with an overrepresentation of proline and/or glycine residues, which may or may not have disulphide-stabilized structures (Otvos, 2002).

The first AMP to be partially characterized in crustaceans was a peptide pertaining to the third type, isolated from the haemolymph of the shore crab *Carcinus maenas*; a 6.5 kDa proline-rich peptide (Schnapp et al., 1996) and other similar peptides have been recently reported from three species of mud crabs (Imjongjirak et al., 2017). This original report was shortly followed by the identification of penaeidins, a family of proline-rich AMPs that also contain a C-terminal, Cys-rich domain (Cuthbertson et al., 2008; Destoumieux et al., 1997; Wu et al., 2019). These AMPs, found in shrimps and prawns of the *Penaeus* genus, are active against both Gram-negative and Gram-positive bacteria. The first evidence of proline-rich AMPs in crayfish dates to 2007 (Jiravanichpaisal et al., 2007), when astacidin 2 was characterized from the plasma of *Pacifastacus leniusculus*. This AMP is relatively short (14–16 aa in length) and shows a broad range of antibacterial activity, inhibiting the growth of both Gram-positive and -negative bacteria.

The red swamp crayfish, *Procambarus clarkii*, is widely distributed in natural environments (Lodge et al., 2012), and it is also a cultured species worldwide (Gong et al., 2012; Jin et al., 2019). Due to its economic importance for aquaculture, its immune system has been extensively studied (Dai et al., 2017; Du et al., 2016; Wu et al., 2017; Zhou et al., 2017). However, only a single 20 aa-long astacidin, highly similar to *P. leniusculus* astacidin 2 and mainly expressed in haemocytes and gills, has been identified so far (Shi et al., 2014) in this species.

Here we report the identification of four potential transcripts pertaining to the proline-rich astacidin family in *P. clarkii*, thanks to a whole-transcriptome sequencing approach. The functional characterisation of these peptides, together with the elucidation of their molecular diversity, evolutionary history, constitutive expression in different tissues and inducibility after LPS-challenge, allowed to shed some light on their possible role in crayfish immunity.

2. Materials and methods

2.1. Peptide identification

The identification of novel astacidin peptides followed a robust procedure based on the use of conserved signal peptides, similar to that previously reported for identifying AMPs in placozoa and teleost fish (Simunić et al., 2014; Tessera et al., 2012), since these are known to be generally well conserved to allow the efficient secretion of defence peptides. Briefly, the signal sequence (MRLLYLLGVVLVALMAAVPSQA) from *Pacifastacus leniusculus* astacidin (UniProt entry Q0PJU7) (Jiravanichpaisal et al., 2007) was identified using SignalP 4.1 (Petersen et al., 2011), and used to query available *Procambarus clarkii* transcriptomic resources for transcripts encoding sequences related to AMPs with TBLASTN. These resources included the dbEST database (Boguski et al., 1993) as well as a comprehensive transcriptome assembly (hereafter defined ATLAS) deriving from 12 different *P. clarkii* tissues (Unpublished results). Similarity searches resulted in numerous hits which were manually inspected upon translation, using EMBOSS transeq (Rice et al., 2000), to identify those with features compatible with putative proline-rich AMPs (e.g. cationicity, proline and arginine residue distribution).

2.2. Phylogenetic analyses

The available draft genome of the marbled crayfish *Procambarus f. f. virginalis* (Gutekunst et al., 2018) was screened for the presence of astacidin using the four sequences identified in *P. clarkii* as queries. Significant hits (based on an e-value cutoff of 1 E^{-5}) were further inspected, fragmented contigs were re-assembled using the CLC Genomics Workbench v.11 (Qiagen, Hilden, Germany) and annotated upon alignment with genomic and cDNA sequences from congeneric species. Donor and

acceptor splicing sites were refined using Genie (Reese et al., 1997).

Astacidin-like sequences were also searched for in other available transcriptomes from Astacidea, which were either downloaded from the NCBI TSA database (when available), or *de novo* assembled with the CLC genomics Workbench v.11, including those from: *Astacus astacus* (Theissinger et al., 2016), various *Cambarus* species (*C. cryptodytes*, *C. dubius*, *C. graysoni*, *C. hamulatus*, *C. nerterius*, *C. rusticiformis*, *C. setosus*) (unpublished), *Cherax cainii*, *C. destructor* (Ali et al., 2015), *C. quadricarinatus* (Tan et al., 2016), *Enoplozetopus cf. daumi* MCZ IZ 74073 (unpublished), *Homarus americanus* (McGrath et al., 2016), *Nephrops norvegicus* (Rotllant et al., 2017), *Pacifastacus leniusculus* (unpublished), *Pontastacus leptodactylus* (Tom et al., 2014), various species of *Procambarus* (*P. fallax*, *P. horsti*, *P. lucifugus*, *P. pallidus*) (unpublished), *Orconectes (Faxonius) australis* and *O. (F.) incomptus* (unpublished). Positive hits were detected based on a minimum e-value threshold of 1 E^{-3} and further refined based on detection by a custom HMM profile obtained from the multiple sequences alignment of the conserved signal peptide of all astacidins generated, using HMMER v.3.2.1 (Finn et al., 2011).

The phylogeny of astacidins was then inferred using MrBayes v.3.2.6 (Huelsenbeck and Ronquist, 2001), from the multiple sequence alignment of all available full-length astacidin sequences. The phylogenetic analysis was performed by running two independent Markov chain Monte Carlo (MCMC) analyses in parallel for 200,000 generations, until all estimated parameters of the model of molecular evolution (LG + G + I) reached an ESS value > 200 .

2.3. Peptide synthesis

Three novel astacidin peptide sequences (PcAst-1a, 1b/c and -2) from *P. clarkii* were selected for chemical synthesis, based on suitable physico-chemical characteristics with respect to potential antimicrobial activity. The peptides were obtained from GenicBio (Shanghai, China) and SynPeptide (Shanghai, China), all at $> 95\%$ purity as confirmed by RP-HPLC and mass-spectrometry (PcAst-1a and -1b/c were synthesized as C-terminally amidated minus the terminal GK residues). Chromatographic separation was achieved on a reversed-phase column (C18, 5 μm , $4.6 \times 250 \text{ mm}$) using a 1–70% acetonitrile/0.1% TFA gradient in 25 min at a 1 ml/min flow rate. Peptide stock concentrations were determined by dissolving accurately weighed aliquots of peptide in doubly distilled water, and further verified by using the extinction coefficients at 214 nm, calculated as described by Kuipers and Gruppen (2007).

2.4. Preparation of liposomes

Anionic and neutral LUVs (large unilamellar vesicles) were prepared by dissolving dry phosphatidylglycerol (PG) (Avanti Polar Lipids, Alabaster, Alabama, USA) or 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Avanti Polar Lipids), respectively, in chloroform/methanol (2:1) solution. The solution was evaporated in a small round-bottom flask under constant rotation, using a dry nitrogen stream and then vacuum-dried for 24 h. The liposome cake was resuspended in 1 ml of 10 mM sodium phosphate buffer (SPB) to a final concentration of 5 mM phospholipid and spun for 1 h at a temperature above the T_c (lipid critical temperature). The vesicle suspensions were then disrupted by several freeze–thaw cycles, from liquid N_2 , prior to passage through successive polycarbonate filters with 1 μm , 0.4 μm and 0.1 μm pores, using a mini-extruder (Avanti Polar Lipids), and resuspended to a final phospholipid concentration of 0.4 mM. Considering liposome membrane surface area of a $\sim 100 \text{ nm}$ diameter liposome, and area of a phospholipid head group ($\sim 0.7\text{--}1 \text{ nm}^2$) (Tossi et al., 2000), the concentration of liposomes was estimated from the average number of phospholipids/liposomes to be about 5 nM.

2.5. Circular dichroism

CD spectra were obtained on a J-710 spectropolarimeter (Jasco, Tokyo, Japan), as the accumulation of three scans, and were measured

in a) SPB solution, b) 50% TFE in SPB, c) sodium dodecyl sulphate micelles (10 mM SDS in SPB), d) anionic LUVs (PG) in SPB or e) neutral LUVs (DOPC) in SPB.

2.6. Antimicrobial activity

The *in vitro* testing was carried by determining the minimal inhibitory concentration (MIC) against either reference (ATCC) laboratory strains (*Escherichia coli* ATCC 25922, *Acinetobacter baumannii* ATCC 19606 and *Staphylococcus aureus* ATCC 29213) or clinical isolates of these bacteria, some multi-drug resistant, as previously described in detail in Rončević et al. (2017). The MIC was assessed using the serial two-fold microdilution method according to the CLSI protocol (Clinical and Laboratory Standards Institute (CLSI), 2017). Bacteria were cultured in either full Mueller Hinton (MHB or 20% Mueller Hinton broth in SPB) to the mid exponential phase, and then added to serial dilutions of astacidin peptides to a final load of 5×10^5 CFU/ml in 100 μ l per well, and incubated at 37 °C for 18 h. The MIC was visually determined as the lowest concentration of the peptide showing no detectable bacterial growth, and accepted if it was the consensus value of an experiment performed in triplicate.

For determination of minimal bactericidal concentration (MBC), 4 μ l of bacterial suspensions were taken from the wells corresponding to MIC, 2 \times MIC, and 4 \times MIC and then plated on MH agar plates. Plates were incubated for 18 h at 37 °C to allow the viable colony counts and the MBC determined as the peptide concentration causing no visible growth.

2.7. Cytotoxicity assay

The cytotoxic effect of astacidin peptides was determined on both lymphoid and epithelial cell line models. For *in vitro* maintenance, the human MEC-1 lymphoid tumour cell line (Stacchini et al., 1999) was cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 10% fetal bovine serum (FBS) (complete medium) and sub-cultured two-three times a week for not more than 20 passages; the epithelial cell lines (human bladder cancer cell lines T24 and TCCSUP) purchased from ATCC (LGC Standards, Teddington, Middlesex, UK), were sub-cultured in a Dulbecco's modified Eagle's medium (DMEM Euroclone, Milano, Italy) containing 4.5 g/L glucose, 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin, 100 μ g/mL streptomycin).

Cytotoxicity assays were carried out by seeding cells into 96-well culture plates; MEC-1 were diluted to 10^6 cells/ml and treated with astacidin peptides for 24 h, after which 20 μ l of MTT dye (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. Formazan crystals were solubilized with acidic isopropanol (0.04 N HCl in absolute isopropanol) and the absorbance measured at 540 nm and 630 nm with microplate reader (Tecan Sunrise, Männedorf, Switzerland). T24 and TCCSUP epithelial cell lines were sown at a density of 3×10^5 cells/ml and treated with astacidin peptides for 4, 24, 48 and 72 h, after which cells were incubated with MTT dye (0.5 g/L) at 37 °C for 2 h. Formazan crystals were solubilized with 10% dimethylsulphoxide (DMSO) and formazan formation was measured at 570 nm with ELx808 Ultra Microplate Reader (BioTek, Inc., Winooski, Vermont, USA).

All measurements were done at least in triplicate and the data were calculated in relation to the untreated control (100%).

2.8. Animal maintenance

Twenty adult *P. clarkii* females, at intermolt stage (body weight 37 ± 8 g), were collected from an artificial river inside the "Bonifica del Branco" (45°46' N, 13°30' E, GO, Italy) and kept in two separate 120 L tanks provided with closed circuit filtered and thoroughly aerated tap water at ~18 °C. They were fed fish pellets (Sera granular, Heisenberg, Germany) three times a week, for a week, before the start of the experiment.

The experiments on these animals complied with the current laws of Italy, the country where these were performed. No specific permits were required for studies that did not involve endangered or protected species. Individuals were maintained in appropriate laboratory conditions to guarantee their welfare and responsiveness. After the experiments were completed, the crayfish were sacrificed by hypothermia.

2.9. LPS-challenge in *P. clarkii*

Two groups of three crayfish each were injected with 0.025 μ g/g body weight of lipopolysaccharide from *E. coli* O111:B4 (Sigma) solubilized in PBS 1X in the abdominal cavity and sacrificed at 2 and 4 h post-injection. In parallel, two control groups, consisting of 3 crayfish each, for the two experimental points considered, were injected with crustacean saline solution (NaCl 14.5 g, CaCl₂ 0.72 g, MgSO₄ 3.18 g, KCl 0.35 g, HEPES 5 mM, NaHCO₃ 0.5 g at pH 7.4 in a final volume of 1.2 L), and also sacrificed at 2 and 4 h post-injection.

Haemolymph was withdrawn with a 10 ml needle-less syringe and haemocytes were pelleted through centrifugation at 4°C, 2000 xg for 1 min.

2.10. Gene expression analysis

The basal expression levels of astacidins in naïve animals were evaluated through the analysis of RNA-seq data from 12 tissues (brain, eyestalk, green glands, ventral ganglia, heart, hepatopancreas, gills, haemocytes, muscle, Y-organ and epidermis, ovary and testis) Illumina sequenced (depth 2x100 bp), derived from a transcriptome study (Unpublished results).

The CLC Genomics Workbench v.12 (Qiagen, Hilden, Germany) was used to map the reads from each tissue to the assembly, using the RNA-seq analysis tool with the following parameter settings: mismatch cost 2, gap insertion and deletion costs 3, end gaps 0, length fraction 0.95, similarity fraction 0.98.

The total RNA from LPS-challenged crayfish was extracted from haemocytes by following the TRI Reagent® (Sigma-Aldrich) manufacturer's protocol. The concentration and quality of extracted RNAs were checked using an Agilent 2100 Bioanalyzer and the RNA 6000 Nano kit (Agilent Technologies). cDNAs were produced using the GoTaq 2 Step RT qPCR kit (Promega), starting from 1 μ g of total RNA and following the manufacturer's instructions. A dilution of 1/5th of the initial cDNA was used for quantitative-Real Time PCR (qRT-PCR) validations.

Specific primers were designed through Primer3Web version 4.0.0 (Untergasser et al., 2012) and Oligo Calculator version 3.26 (Kibbe, 2007) to predict possible secondary structures and hairpin formations, as reported in Table 1.

The qRT-PCR reactions were performed in triplicate on experimental samples by using the PerfeCTa SYBR® Green supermix (Quanta bio), and following the thermal cycle: initial denaturation 95 °C for 2'; 35 cycles composed of two-steps reaction 95 °C for 1', annealing and elongation 60 °C for 30"; followed by a melting curve from 65 °C to 95° with a temperature increment of 0.5 °C every 5". Each PCR reaction was performed in a final volume of 15 μ l, with a final concentration of 1 X supermix, 0.3 μ M of each primer and 1 μ l of cDNA.

The Elongation Factor 1-alpha (EF1 α), previously validated as stable reference gene for qPCR analyses in *P. clarkii* (Manfrin et al., 2015), was used for internal normalization across samples. The CFX Maestro software (Bio-Rad) was used to calculate the relative expression of different astacidins among the experimental samples, following the $\Delta\Delta$ Ct method. Differentially expressed genes were identified using the Shapiro-Wilk test, based on a p-value threshold of 0.05.

3. Results and discussion

3.1. Astacidin identification

The putative AMP cDNA sequences identified in *P. clarkii* thanks to their similarity with the signal peptide of the previously reported *P.*

Table 1Primers used for qRT PCR validation. EF1 α : Elongation factor 1 alpha served as reference gene. bp: base pairs.

Primer ID	Forward	Reverse	Amplicon size (bp)
EF1 α	AGATCTGAAACGTGGTTTTGTT	TCAATCTTTTCAGAAAGTTCGT	186
Pc-Ast-1 a	TCCCGCCTACCGTCCT	CAAGAGGGAAGGGAAGCC	115
Pc-Ast-1 b/c	ACTTCGCCGCTCCTGGCTA	TGTGGCTGGTGATGGTAGAG	103
Pc-Ast-2	TGACTGGAGGATCACAACTCT	GTAAGGCGGGGATAAAAAG	122
Pc-Ast-3	TGACTGGAGGATCACAACTCT	CCTCAGTGGACGGTCAGG	134

leniusculus astacidin (Jiravanichpaisal et al., 2007) were translated and analysed, looking for typical features of PR-AMPs (cationic charge, a pattern of alternating proline and arginine residues). The query sequence had been originally assigned the suffix “-2” by Jiravanichpaisal et al. due to the previous discovery of the unrelated hemocyanin-derived AMP Astacidin 1 from the same species (Lee et al., 2003). However, to avoid nomenclature confusion in future assignments and to maintain consistency with phylogenetic evidence (see section 3.2), we will hereafter refer to this peptide as *PleAstacidin-1* (see Table 2), and consider its orthologous AMPs as members to the astacidin-1 clade.

The first AMP identified in *P. clarkii* was named PcAst-1a, and its 43 aa long precursor displays a well supported signal peptide, which would result, upon proteolytic cleavage, in a mature PR-AMP of 20 aa, with a C-terminal amidation signal. Another slightly longer mature peptide (25 residues) was assigned the name PcAst-1b, with an additional variant (His₅ → Tyr₅ within the signal peptide region) named PcAst-1c.

A fourth sequence, named PcAst-2, perfectly matched a transcript upregulated in the haemocytes of white spot syndrome virus-infected *P. clarkii* (Shi et al., 2010). This 31 aa long PR-AMP, produced by the proteolytic cleavage of a 53aa long precursor, was found to be significantly different and longer than the three Astacidin-1 isoforms, and lacked the GK amidation signal (see Table 2). An additional 50 aa long precursor sequence, named PcAst-3, shared close similarity with PcAst-2 in its N-terminal region, but had a shorter mature peptide (28 aa), which contained the GK amidation signal at the end of an anionic Tyr-rich C-terminal section (see Fig. 1a).

All *P. clarkii* astacidins, as well as the orthologous sequences from other crustaceans (see Table 2 and section 3.2), have a content and distribution of proline and arginine residues consistent with their classification within the class of PR-AMPs (Scocchi et al., 2011). Besides crustaceans (e.g. mud crab PR-AMPs, crab arasins and shrimp penaeidins), this type of AMPs is also found in insects (e.g. apidaecins, pyrrhocoricins, drosocins, oncocyns) (Bulet et al., 1999; Otvos, 2000) and artiodactyl mammalian species (e.g. bactenecins) (Scocchi et al., 2011). Among marine invertebrates, PR-AMPs have been also occasionally described in molluscs, such as in the case of mussel myticalins (Leoni et al., 2017) and oyster Cg-Prp (Gueguen et al., 2009).

However, no significant sequence similarity was observed between astacidins and other PR-AMP families, with the possible exception of a central motif in the PcAst-2 sequence (RPPYL PxPxPRPxPxP) (see Fig. 1b), which is present in cetartiodactyl cathelicidin PR-AMPs as well as in insect oncocin (Knappe et al., 2010; Mardirossian et al., 2018; Scocchi et al., 2011). This may represent an interesting case of convergent evolution pointing to a conserved sequence motif that may be functionally relevant for PR-AMPs, regardless of their origin. In this respect, it should be pointed out that some PR-AMPs, including those listed in Fig. 1b, have a mode-of-action which involves their translocation into susceptible bacteria via a membrane transporter, and the subsequent inactivation of bacterial ribosome subunits (Graf et al., 2017).

3.2. Gene architecture and taxonomical distribution of astacidins

We investigated the gene structure of astacidins in *P. virginialis*, the only species of this genus with a genome currently available. In spite of its highly fragmented nature (nearly 2 million scaffolds with a N50

close to 1 kb), we could retrieve one full-length astacidin gene (orthologous to PcAst-2, i.e. sharing 98% sequence identity at the nucleotide level within the CDS) and an additional partial hit for a gene orthologous to PcAst-3 (i.e. sharing 95% sequence identity at the nucleotide level). No sequences orthologous to PcAst-1a or -1b/c could be detected.

PviAstacidin-2 displays a genomic architecture comprising two exons and a phase 2 intron splitting the open reading frame close to its 3' end (Fig. 2). Although the 5' and 3' boundaries of the gene could not be defined with certainty, due to the absence of mRNA data for this species, the alignment with the *P. clarkii* cDNA permitted to define a first exon comprising at least 367 nucleotides, a 685 nucleotides-long intron and a second exon comprising at least 866 nucleotides, of which the 3' end was well supported by the presence of a potential AATAAA mRNA polyadenylation site. The *PviAstacidin-3* ortholog is only a partial sequence that however includes the entire sequence of exon 1, which contains the complete ORF, implying that, in this case, exon 2 only includes the 3'UTR sequence.

Numerous astacidin orthologous sequences were identified in the transcriptomes of several crayfish species of the superfamily Astacoidea (see Table 2), in spite of the absence of RNA-seq data specifically obtained from circulating haemocytes, the main tissue of production of these AMPs (see section 3.5). Phylogenetic inference suggests that all astacidins derive from two ancestral lineages, of which members can now be found in most (if not all) crayfish species (see Fig. 3). These two lineages correspond to PcAst-1a and PcAst-2/3 orthologs, respectively. In particular, sequences orthologous to PcAst-2 and -3 could be found, with minor variations, in multiple *Procambarus* and *Cambarus* species, as well as in *A. astacus*, *P. leptodactylus* and *P. leniusculus* (Fig. 3). Members of the Astacidin-1 lineage were detected in different *Procambarus*, *Cambarus* and *Orconectes* (*Faxonius*) species, as well as in *P. leniusculus*. *P. clarkii* was the only species where a gene duplication event, originating PcAst-1a and -1b/c, was apparent.

Although transcriptomic screening suggests that no astacidin-like sequences are present in two of the four Astacoidea superfamilies (Enoplometopoidea and Nephropoidea), astacidin-like sequences were found in *Cherax* spp., indicating a taxonomical distribution covering both Astacoidea and Parastacoidea. While one of the sequences identified in *C. quadricarnatus* (*CquAstacidin-X1*) belonged to the main clade including all Astacoidea astacidins (and may be more closely related to the Astacidin-1 group, see Table 2), two orthologous sequences from *C. cainii* and *C. quadricarnatus* (*CquAstacidin-X2* and *CquAstacidin-X3*) clustered in a divergent, basal lineage (Fig. 3).

Overall, in spite of the remarkable sequence divergence in the antimicrobial peptide region, all astacidins were defined as being characterized by a highly conserved, 22 aa long signal peptide, which might be effectively used for classification purposes, and for which the 50% majority rule consensus was MRLLYLLXLVLVALMAAVPSQA. With respect to the AMP domain, astacidin-2 orthologs and the two divergent *Cherax* spp. sequences both lacked the characteristic GK C-terminal amidation motif present in all other astacidins.

No orthologous sequences were detected in the transcriptomes of other, non-Astacoidea decapods, indicating that astacidins represent a taxonomically restricted gene family (Khalturin et al., 2009) or that any orthologous sequences in other crustaceans are too divergent from those of Astacoidea to enable detection by homology-based methods.

Table 2
Sequence and properties of astacin peptides identified in different Astacidae species.

Peptide	Species	Family	Sequence ^{1,2}
<i>Astacin 1</i>			
PcAst-1a (*)	<i>Procambarus clarkii</i>	Cambaridae	SNGYRP-----AYRPA YRPSYRPGK
PleAstacin-1 [§]	<i>Pacifastacus leniusculus</i>	Astacidae	SLGYRP-----RPNYRP--RPIYRPGK
PcAst-1 b/c (*)	<i>P. clarkii</i>	Cambaridae	SNVYRPPY--RPVYRPLRFPYRPGK
PluAstacin-1	<i>P. lucifugus</i>	“	SHGYRPPYLP-----RPRRPPIYRPGK
CgrAstacin-1	<i>Cambarus graysoni</i>	“	SSGHRPIYRPPRPPIYRPLRPIYRPGK
ChuAstacin-1	<i>C. humulatus</i>	“	SNGHRPIYRPPRPPIYRPLRPFVYRPGK
CduAstacin-1	<i>C. dubius</i>	“	SNGHRPIYRPPRPPIYRPPQRPPIYRPGK
CneAstacin-1	<i>C. nerterius</i>	“	SNGHRPIYRPPRPPIYRPPQRPPIYRPGK
CruAstacin1	<i>C. rusticiformis</i>	“	SNGHRPIYRPPRPPIYRPLRPIYRPGK
OauAstacin-1	<i>Orconectes (Faxonius) australis</i>	“	SNGHRPIYRPPRPPIYRPLRPFVYRPGK
OinAstacin-1	<i>O. (F.) incomptus</i>	“	SNGHRPIYRPPRPPIYRPLRPFVYRPGK
CquAstacin-X1	<i>Cherax quadraticus</i>	Parastacidae	GRHQRPYLPRFRYRPGK
<i>Astacin 2</i>			
PcAst-2 (*)	<i>P. clarkii</i>	Cambaridae	FYPRPYRPPYLPDPRPFPRPLPAFGHEFRRH
PpaAstacin-2	<i>P. pallidus</i>	“	FYPRPYRPPYLPDPRPFPRPLPAFGNEFRRH
PluAstacin-2	<i>P. lucifugus</i>	“	FYPRPYRPPYLPDPRPFPRPLPAFGNEFRRH
PviAstacin-2	<i>P. virginalis</i>	“	FYPRPYRPPYLPDPRPFPRPLPAFGNEFRRH
PfaAstacin-2	<i>P. fallax</i>	“	FYPRPYRPPYLPDPRPFPRPLPAFGNEFRRH
PhoAstacin-2	<i>P. horsti</i>	“	FYPRPYRPPYLPDPRPFPRPLPAFGNEFRRH
CgrAstacin-2	<i>C. graysoni</i>	“	FYPRPYRPPYLPDPRPFPRPLPAFGDGYRPR
CneAstacin-2	<i>C. nerterius</i>	“	FYPRPYRPPYLPDPRPFPRPLPAFGDGYRPR
CruAstacin-2	<i>C. rusticiformis</i>	“	FYPRPYRPPYLPDPRPFPRPLPAFGDGYRPR
CteAstacin-2	<i>C. tenebrosus</i>	“	FYPRPYRPPYLPDPRPFPRPLPAFGDGYRPR
AasAstacin-2	<i>A. astacus</i>	Astacidae	FYPQFRPPYVPDPRPFPRFPAYDAI
PleAstacin-2 [§]	<i>P. leniusculus</i>	“	FYPRPYRPPYLPDPRPFPRPLPAYDAI
<i>Astacin 3</i>			
PcAst-3	<i>P. clarkii</i>	Cambaridae	FIPRPDRPLRPYPRPFVDYGDYGIYGGK
PviAstacin-3	<i>P. virginalis</i>	“	YIPRPDRLLRTYPRPFVDYGDYGIYGGK
CteAstacin-3	<i>C. tenebrosus</i>	“	YIPRPYRPLR-YPRPFVDYGDYGIYGGK
AasAstacin-3	<i>A. astacus</i>	Astacidae	FIPRPYRPPRPYPRPFVDYGDYGIYGGK
PleAstacin-3	<i>P. leniusculus</i>	“	FIPNPYRQPRPYPKPFVDYGDYGIYGGK
<i>Others</i>			
CquAstacin-X2	<i>C. quadraticus</i>	Parastacidae	LRFDPPYVWPEYRPHYFPPVYYK
CcaAstacin-X2	<i>C. cainii</i>	“	LRFDPPYVSWPEYRPHYFPPVYYK

1) Residues that are conserved in $\geq 50\%$ of peptides in any astacin peptide family are shaded.

2) Astacin peptides with GK (italic, underlined) as C-terminal residues are likely amidated at the third last residue.

(*) These sequences were selected for peptide synthesis and functional characterization.

([§]) As explained in the text, *PleAstacin-1* had been previously designed as astacin-2 by [Jiravanichpaisal et al. \(2007\)](#); to avoid nomenclature confusion and maintain consistency with phylogenetic evidence (see section 3.2), we renamed this sequence *PleAstacin-1* and re-assigned the name *PleAstacin-2* to a newly identified AMPs supported by transcriptomic evidence.

3.3. Peptide structure

The CD spectra for PcAst-1a, -1b/c and -2 in aqueous phosphate buffer were compatible with an extended conformation, which was not surprising given the high content of proline (incompatible with a helical conformation) and relatively short size of the peptides (unlikely to lead to β -hairpin formation) (Fig. 4). In the presence of SDS micelles, all spectra became more positive in the region about 230 nm, suggesting a possible contribution from a polyproline type-II conformation under these conditions. There is little change in spectra in the presence of

LUVs formed by DOPC, suggesting that the peptides do not interact with neutral biological membranes. There was instead a significant change in the spectra of all the peptides in the presence of anionic LUVs formed by PG. Furthermore, these spectra resembled those in the presence of the organic solvent TFE. This suggests that the peptides interact with the surface of anionic biological membranes, likely via electrostatic interactions, and that they then are susceptible to the anisotropic environment at the membrane surface. In any case, results would point to a preferred interaction with bacterial rather than animal cells, which might result in a more significant effect on the former.

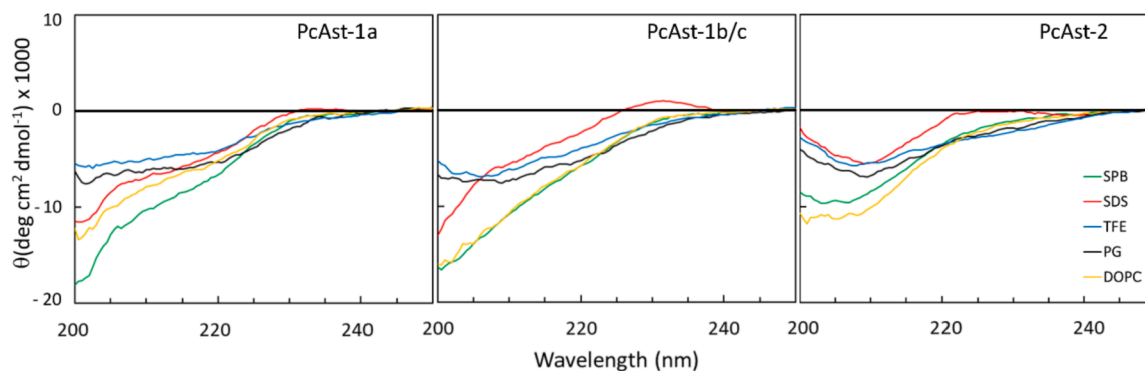


Fig. 4. CD spectra for *P. clarkii* astacidins under different conditions. Spectra are the accumulation of three scans carried out with 20 μM peptide in aqueous buffer, in the presence of TFE and SDS micelles or anionic (phosphatidylglycerol, PG) or neutral (dioctanoyl phosphatidylcholine, DOPC) LUVs, representing model biological membranes.

Table 3

Antimicrobial activity of astacidin peptides in 20% MH expressed as Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) both expressed in μM .

Bacterial strains	PcAst-1a		PcAst-1b/c		PcAst-2	
	MIC	MBC	MIC	MBC	MIC	MBC
<i>S. aureus</i> ATCC 29213	32	> 32	2	4	32	32
<i>S. aureus</i> c.i.	> 32	/	32	> 32	> 32	/
<i>E. coli</i> ATCC 25922	32	> 32	1	1	32	> 32
<i>E. coli</i> c.i.	> 32	/	2	2	> 32	/
<i>A. baumannii</i> ATCC 19606	> 32	/	2	4	8	8
<i>A. baumannii</i> c.i.	> 32	/	2	4	8	8

Table 4

Astacidins and EF1 α gene expression in 12 different tissues. Expression levels are shown as TPMs (Transcripts Per Million) values and quantified using a coloured scale. Black > 2000, dark grey (2000-500), mid grey (499-100), light grey (99-10), white < 10 TPMs.

Astacidin	Brain	Eyestalk	Haemocytes	Green glands	Ventral ganglia	Y organ	Hepatopancreas	Heart	Testis	Ovary	Gills	Muscle
PcAst-1a	494	128	7729	109	369	187	10	169	19	48	1420	21
PcAst-1b/c	612	71	4458	45	183	103	4	74	14	24	1048	12
PcAst-2	13	7	309	5	15	6	0	5	2	3	24	2
PcAst-3	11	5	183	3	17	7	1	7	3	1	22	1
EF1a	1340	955	1614	1600	1594	1076	740	1110	807	1423	1185	649

concentrations, although some specific types of cells may be more susceptible than others. This is in line with the prediction made based on CD spectra (see above).

3.5. Astacidins expression in *P. clarkii*

Following the transcriptomic characterisation of PcAst-1a, PcAst-1b, PcAst-1c, PcAst-2 and PcAst-3, their expression has been quantified

using available RNAseq data collected from 12 different tissues of naïve (unexposed to bacteria or PAMPs) *P. clarkii*. Table 4 shows the Transcripts per Million (TPM) obtained from the RNA-seq analysis on these 12 different tissues. PcAst-1b and -1c have been clustered together since they differ only in a single amino acid within the signal peptide region.

Despite significant differences among the five isoforms, haemocytes clearly represent the specific compartment for expression of all five astacidins under naïve conditions. PcAst-1a, and -1b/c are the most

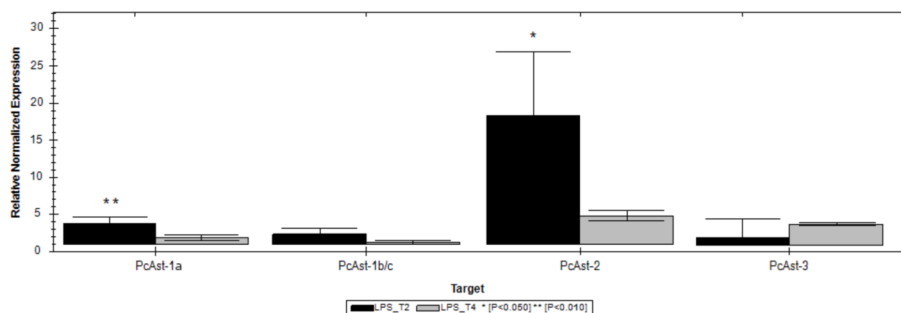


Fig. 5. Relative expression of *P. clarkii* astacidins in haemocytes following LPS-challenge at 2 and 4 h post injection (LPS_T2 and LPS_T4, respectively). The $\Delta\Delta\text{Ct}$ method was applied by using control groups and EF1- α as calibrator samples. Statistically significant p-values are indicated by asterisks.

expressed in haemocytes (~7700-4400 TPMs), and were also found in gills (~1400-1000 TPMs, about 5-fold less expressed than haemocytes). PcAst-2 was also expressed at a significant level in haemocytes (~300 TPMs, about 24-fold less than PcAst-1a), but its expression fell in barely detectable in all the other tissues. PcAst-3 reached a level of ~180 TPMs in haemocytes, being the least expressed astacidin isoform (42-fold less than PcAst-1a). In general, astacidins were poorly expressed in all the other tissues, including hepatopancreas, which suggests that astacidins are strictly involved in haemocyte-mediated immunity.

Although the presence of PcAst1a in the gills of naïve animals supports the findings of Shi and colleagues that PcAst expression could be induced in the gills as well (Shi et al., 2014), this observation could also be explained by the presence of the haemolymph in the gills, since this tissue is in direct contact with the open circulatory system (Burggren et al., 1974). The pattern of inducibility of the newly characterized PcAsts was experimentally investigated by the withdrawal of haemolymph from healthy *P. clarkii* female individuals in intermolt, 2 and 4 h post- LPS-injection.

The relative expression of astacidins was then estimated using qRT-PCR, using the elongation factor 1 alpha (EF1 α) as a reference gene. The $\Delta\Delta C_t$ method was applied to detect statistically significant differences with a non-challenged (buffer injected) control group, at two selected time points (Fig. 5). The results indicated a significant up-regulation of PcAst-1a and PcAst-2 at 2 h post injection (p-value < 0.0095 and < 0.0201, respectively), whereas none of the four astacidins tested were differentially regulated 4 h after the injection. The early inducibility of PcAst-1a and PcAst-2 suggests their possible involvement in an early-defence against Gram-negative bacteria infection. On the other hand, the lack of modulation of PcAst-1b, -1c and PcAst-3 in response to LPS-challenge suggests that the expression of these AMPs may be induced by other PAMPs, not necessarily associated with the Gram-negative bacterial cell wall.

4. Conclusions

Three different types of astacidin proline rich AMPs (PR-AMPs) were identified in several crayfish species from the superfamily Astacoidea. Phylogenetic inference, together with the screening of available sequencing data, allowed the identification of orthologous sequences also in members of the Parastacoidea superfamily. RNAseq analyses pointed out that all members of the PcAst-1 class are highly expressed in haemocytes of naïve crayfish, and to a lesser extent also in gills, showing a high basal expression level and a strong tissue-specificity. The inducibility of PcAst-1a and PcAst-2 on challenge with LPS, suggests that PcAsts are involved in the immune response, while the triggering elements remain to be identified for PcAst-1b/c and -3. Primary structural features (high number of Pro residues) and circular dichroism studies suggest that these peptides have a simple linear structure and might interact selectively with the surface of anionic biological membranes. It still remains to be determined whether the activity of astacidins on Gram-negative bacteria depends on their internalization and subsequent inactivation of ribosomes, as reported for other mammalian and insect PR-AMPs that share a conserved sequence motif.

Data deposition

PcAst nucleotide sequences have been deposited at GenBank under the following accession numbers: PcAst-1a_astacidin MN848347; PcAst-1b_astacidin MN848348; PcAst-1c_astacidin MN848349; PcAst-2_astacidin MN848350; PcAst-3_astacidin MN848351.

Declaration of competing interest

All authors listed on the paper declare to have no conflict of interest of any nature both financial, personal and related to other people or organizations.

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