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The diversification of the antimicrobial peptides from marine worms is driven by environmental conditions

Bruno Renato [1], Boidin-Wichlacz Céline [1], Melnyk Oleg [1], Zeppilli Daniela [2], Landon Céline [3], Thomas Frédéric [4], Cambon Marie-Anne [2], Lafond Mickael [5], Mabrouk Kamel [6], Massol François [1], Hourdez Stéphane [7], Maresca Marc [5], Jollivet Didier [8], Tasiemski Aurélie [1, *]

[1] Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 - UMR 9017 - CIIL - Center for Infection and Immunity of Lille, F-59000 Lille, France
[2] Univ. Brest, CNRS, Ifremer, UMR6197 Biologie et Ecologie des Ecosystèmes marins Profonds, F-29280 Plouzané, France
[3] Center for Molecular Biophysics, CNRS, UPR 4301, Orleans, France
[4] CREEC/(CREES), MIVEGEC, Unité Mixte de Recherches, IRD 224–CNRS 5290–Université de Montpellier, Montpellier, France
[5] Aix-Marseille Univ, CNRS, Centrale Marseille, iSm2, Marseille F-13013, France
[6] Aix-Marseille Univ, CNRS, UMR 7273, ICR, Marseille F-13013, France
[7] Sorbonne Université, LECOB, UMR 8222, Observatoire Océanologique de Banyuls, 1 Avenue Pierre Fabre, 66650, Banyuls-sur-Mer, France
[8] Sorbonne Université, CNRS, UMR 7144 AD2M, Station Biologique de Roscoff, Place Georges Teissier CS90074, Roscoff F-29688, France

*Corresponding author : Aurélie Tasiemski, email address : aurelie.tasiemski@univ-lille.fr

Abstract :

Antimicrobial peptides (AMPs) play a key role in the external immunity of animals, offering an interesting model for studying the influence of the environment on the diversification and evolution of immune effectors., alvinellacin (ALV), arenicin (ARE) and polaricin (POL, a novel AMP identified here), characterized from three marine worms inhabiting contrasted habitats (‘hot’ vents, temperate and polar respectively), possess a well conserved BRICHOS domain in their precursor molecule despite a profound amino acid and structural diversification of the C-terminal part containing the core peptide. Data not only showed that ARE, ALV and POL display an optimal bactericidal activity against the bacteria typical of the habitat where each worm species lives but also that this killing efficacy is optimal under the thermochemical conditions encountered by their producers in their environment. Moreover, the correlation between species habitat and the cysteine contents of POL, ARE and ALV led us to investigate the importance of disulfide bridges in their biological efficacy as a function of abiotic pressures (pH and temperature). The construction of variants using non-proteinogenic residues instead of cysteines (α-aminobutyric acid variants) leading to AMPs devoid of disulfide bridges, provided evidence that the disulfide pattern of the three AMPs allows for a better bactericidal activity and suggests an adaptive way to sustain the fluctuations of the worm's environment. This work shows that the external immune effectors exemplified here by BRICHOS AMPs are evolving under strong diversifying environmental pressures to be structurally shaped and more efficient/specific under the ecological niche of their producer.

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Highlights

► A novel antimicrobial component named polaricin belonging to the BRICHOS-AMP family was identified from a polar marine annelid ► Comparison of BRICHOS-AMPs from annelids inhabiting contrasted habitats shows a profound diversification of the AMP sequence ► AMPs are structurally shaped and more efficient/specific under the ecological niche of their producer ► AMPs display an optimal killing efficacy against the bacteria and under the environmental conditions typical of each worm's habitat ► This is the first evidence than external immune effectors are evolving to fit environmental pressures

Keywords : Environment, Annelids, Polar, Hydrothermal, External immunity

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1. INTRODUCTION

External immunity constitutes the first defense of metazoans to face pathogens by handling the surrounding microbial communities to both avoid infection and establish symbiosis (Otti et al., 2014). As such, it can be viewed as an extended weapon of the immune system to prevent infection (Otti et al., 2014). Many organisms including plants, invertebrates, vertebrates and, even bacteria, secrete antimicrobial peptides (AMPs) as an extrinsic protective shield against the surrounding microbiome that is usually rich in potentially pathogenic agents (Bosch et al., 2021; Rakers et al., 2013; Wang, 2014; Zasloff, 2002). AMPs are predominantly short cationic peptides (up to 100 amino acids), with a broad-spectrum of activities against diverse microbes (bacteria, virus or fungi) with a rapid and selective killing processing mode (Zasloff, 2002). Lacking antibodies, invertebrates nevertheless benefit from a powerful innate immune system, characterized, among other processes, by a great variety of AMPs (Hancock et al., 2006).

Marine organisms constitute almost half of the biodiversity of the Earth, and after a long-term diversification (Cambrian explosion: 541 to 485 million years ago) and co-evolution with fungi, bacteria and archaea, acquired the capacity to produce many unique antimicrobial substances (Bruno et al., 2020, 2019; Kang et al., 2015). Marine AMPs were found to be structurally different from their counterparts produced by terrestrial species, usually displaying novel structures, taxa-specific or even species-specific (Bruno et al., 2020, 2019; Cheung et al., 2015). A long evolutionary story of marine organisms under diversifying environmental pressures, such as the microbial community composition and/or highly fluctuating abiotic factors (temperature, pH, salinity…) likely have driven the evolution of the physiological adaptation of most marine invertebrates, including on their immune functions (Nalini et al., 2018).

In particular, worms have colonized and dominated in biomass most of marine and freshwater habitats, copying with a wide number of environmental conditions and bacterial assemblages, especially in extreme environments such as polar regions, deep-sea hydrothermal vents, or highly anthropized shallow areas (Bulgheresi, 2011; Heip et al., 1985). In the case of annelids (ringed worms), the majority of AMPs is species-specific, probably as the result of a long parallel evolution of their defense arsenal to a wide variety of habitats on Earth (aquatic and terrestrial), mimicking their highly diverse life styles and

ecology (A.Tasiemski, 2008). In this context, the BRICHOS-domain AMP family of marine annelids living under extreme conditions, which is specifically involved in the worms' external immune defense constitutes a unique and attractive model to study the adaptive evolution of the immune system to environmental changes.

BRICHOS (initially found in Bri2, chondromodulin, and prosurfactant protein C in human) is a 100 amino acids domain, present in several protein precursors, which act as an intramolecular chaperone (Hedlund et al., 2009; Johansson et al., 2006; Kim et al., 2010; Sánchez-Pulido et al., 2002; Willander et al., 2011). Despite their very old phylogenetic history (at the end of the Cambrian diversification), BRICHOS precursors all display the same structure consisting of: (i) a highly hydrophobic N-terminal signal sequence, (ii) a pro-region containing a BRICHOS domain, and (iii) a C-terminal region with β-sheet propensities. In the unique case of marine worms, this latter corresponds to an AMP, which exerts its antimicrobial activities once cleaved from the precursor (Bruno et al., 2019; Ovchinnikova et al., 2004; Panteleev et al., 2018; Tasiemski et al., 2014). Among the BRICHOS-domain AMPs, arenicin (ARE) was the first identified from *Arenicola marina*, an annelid inhabiting the intertidal zone of the European temperate shore (Ovchinnikova et al., 2004). Later, the AMP alvinellacin (ALV) was purified from the deep-sea hydrothermal vent annelid *Alvinella pompejana*, that inhabits the hottest part of this peculiar environment (Tasiemski et al., 2014). Finally, another annelid BRICHOS-domain AMP, the nicomicin, was identified from *Nicomache minor,* a polychaete colonizing both temperate and arctic habitats (Panteleev et al., 2018).

Interestingly, precursors of BRICHOS-AMPs show a well-conserved BRICHOS domain but highly divergent primary AMP sequences, suggesting either a strong diversifying selection at the interspecific level of the antibiotic peptides to face the diverse microbial communities, and/or abiotic conditions, or some exon shuffling between the well-conserved BRICHOS domain and AMPs of different genomic origins (Papot et al., 2017). To enlarge our panel of BRICHOS-AMPs from extreme habitats, we report here a novel AMP, named polaricin (POL), from an undescribed terebellid polychaete belonging to the genus *Amphitritides* and endemic to subtidal polar waters of the Antarctic habitat. POL together with ALV and ARE, represent relevant molecules to study AMP adaptation to environmental stressors as they have evolved under highly contrasted (polar, temperate and hot vents respectively)

environmental conditions from a shared but distant ancestor (Ovchinnikova et al., 2004; Tasiemski et al., 2014) (Fig. 1).

In this work, we focused our attention on how selective pressures associated with biotic and abiotic factors have affected the structure and bioactivity of the three POL, ALV and ARE peptides. Through combined analyses of their bioactivities and structural characteristics under various set of environmental and bacterial conditions, we deciphered whether these immune peptides better suited to the environmental conditions that characterize the worms' habitats.

2. MATERIAL AND METHODS

2.1. AMPs and habitat specificities of the worms from which they were identified

Alvinellacin (ALV) was isolated from *Alvinella pompejana* (Annelid, Polychaeta, Terebellida), a polychaete that inhabits parchment-like tubes along the outer walls of active hydrothermal vents chimneys of East Pacific Rise (Desbruyères and Laubier, 1980). Its habitat is characterized by frequent and unpredictable mixing of hot and anoxic fluids with cold seawater (2-3 °C), which exposes the worm to extreme temperatures (from 2 °C to up to 105 °C) that greatly vary inside the tube (21 °C to 80 °C with a thermal optimum around 42 °C) and acidic gradients (pH from 5.33 to 6.4) due to the mixing of bottom seawater with the acidic vent fluid (Desbruyères et al., 1998; Di Meo-Savoie et al., 2004; Gaill and Le Bris, 2007; Le Bris et al., 2005; Ravaux et al., 2013; Von Damm, 2000).

Arenicin (ARE) was purified from the lugworm *Arenicola marina* (Annelid, Polychaeta, Scolecida), a polychaete that inhabits temperate near-shore (intertidal) sediments, digging tunnels in the sand (HOBSON, 1967; Riisgard and Banta, 1998). The burrows of these worms are exposed to periodic tidal emersion, which imposes severe thermal variations (-5 °C to 25 °C) and temporal hypoxia with mild acidic conditions (pH from 5.6 to 8.2) (Alyakrinskaya, 2003; Bat and Raffaelli, 1998; Juretschke and Kamp, 1990; Shumway and Davenport, 1977; Sommer et al., 1997; Sommer and Pörtner, 1999, 2002; Toulmond, 1975; Weber, 1979).

Polaricin (POL) was identified by homology search in the transcriptome of *Amphitritides* sp. (Annelid, Polychaeta, Terebellida), a marine annelid inhabiting marine sediments at the base of sponges or bryozoans of the Antarctic plateau at depth of 100-150 m. This "spaghetti worm" is sedentary and lives inside a slightly consolidated tube made with sand and debris

on the Antarctic continental plateau where it faces extremely cold but stable coastal waters (about -1.8 °C), with very small fluctuations of temperature and pH (Aoki et al., 2005; Matson et al., 2011; Mcneil et al., 2010; Thiel et al., 1996).

2.2. Molecular identification of the AMP precursors

The molecular characterization of the preproarenicin and preproalvinellacin has been described in previous studies done by Ovchinnikova, T. V. *et al* (Ovchinnikova et al., 2004) and by Tasiemski A. *et al* (Tasiemski et al., 2014).

The complete cDNA sequence of prepropolaricin (GenBank accession number OQ474903) was obtained by homology with the preproalvinellacin gene (Tasiemski et al. 2014, GenBank accession number KJ489380) from a tblastx query on the transcriptome assembly previously obtained for the Antarctic *Amphitritides* sp. under scrutiny (blast database) using the software Geneious (blast cut-off p-value = 10^{-15}). The *Amphitritides* RNAseq dataset used for the assembly was obtained from an Illumina 150 bp paired-ends sequencing of a stranded mRNA library on a HiSeq 2500 machine at Genome Québec (half a line: 150 M PE reads). Transcripts (Ngenes=99970, N50=1440 bp, median contig length=488 bp, GC%=36.2) were then obtained with the Trinity 2.4.0 software from R1 and R2 pairs after trimming adapters and regions of low PHRED scores (trimmomatic 0.36: ILLUMINACLIP: illumina. fa: 2:30:10, LEADING:5, TRAILING:5, SLIDINGWINDOW:4:15 MINLEN:36) and a normalization of the reads before assembly.

2.3. Alignments of the precursor amino acid sequences and identification of the BRICHOS and AMP domains

The BRICHOS domain sequence was identified by using MyHits Motif Scan (https://myhits.isb-sib.ch/cgi-bin/motif_scan). The SignalP 5.0 software (https://www.cbs.dtu.dk/services/SignalP/) was used to detect the presence of signal peptide.

The amino acid sequence alignments of peptides precursors of ALV (GenBank accession number KJ489380), ARE (GenBank accession number AY684856), nicomicin (GenBank accession number MH898866) and POL, was performed with CLC Sequence Viewer software (version 8.0).The computation of homology/identity percentage was performed at the SIB (ExPASy software, https://www.expasy.org/) using the BLAST network service (BLASTP,

version 2.2.31+). The amino acid sequences of the three AMPs presented in our alignment were used for the chemical synthesis of native and ABU peptides as described further on.

2.4. Chemical synthesis of AMPs with (ALV, ARE, POL) and without (ABU ALV, ABU ARE, ABU POL) disulfide bridges

The amino acid sequences used for the peptide syntheses are presented in tables S1 and S2. The chemically synthesized peptides (purity of 98%), *i.e.,* ALV, ARE and POL, were produced by GenScript (Leiden, Netherlands) with the cysteine (Cys) pattern involved in disulfide bridges accordingly to their native forms:

-ALV: Four cysteine residues engaged in two intramolecular disulfide bridges (Cys3-Cys21; Cys7-Cys17);

-ARE: Two cysteine residues engaged in one intramolecular disulfide bridge (Cys3-Cys20);

-POL: One cysteine (Cys10) engaged in one intermolecular disulfide bridge (Cys10-Cys10).

The disulfide bridges were removed by replacing all the cysteine residues by ABU residues (hydrophobic non-proteinogenic alpha amino acid, also known as homoalanine) which do not associate covalently and thus do not form disulfide bridges (Fajloun et al., 2000; Sabatier et al., 2009). The α-aminobutyric acid (ABU) modified peptides were chemically produced using the solid-phase peptide synthesis method using an automated peptide synthesizer (Model 433A, Applied Biosystems Inc.) in the Radical Chemistry Institute by the Radical Organic Chemistry and Specialty Polymers Team (CROPS) laboratory (France, Marseilles) (Mabrouk et al., 2007; Merrifield, 1986).. The identity and degree of homogeneity of the peptides were verified by: (1) analytical C18 reversed- phase HPLC (NUCLEODUR 100-5 C18, 250 × 10 mm, MACHERY NAGEL) using a 60 min linear gradient from 0 to 60% of buffer B [0.08% (v/v) TFA/acetonitrile] in buffer A [0.1% (v/v) TFA/H_2O], at a flow rate of 1 mL/min; (2) amino acid analysis after acidolysis [6 M HCl/2% (w/v) phenol, 20 h, 118 °C, N_2 atmosphere] and (3) molecular mass analysis by MALDI-TOF mass spectrometry. The physicochemical properties of all the synthesized peptides (molecular weight, isoelectric point, net charge at neutral pH) were calculated with the Innovagen Pepcalc.com server (Innovagen AB, SE-22370 Lund, SWEDEN).

2.5. Comparison of the three-dimensional (3D) structures of ALV, ARE and POL

The 3D structure of ARE and ALV was recovered from previous studies (Ovchinnikova et al., 2004; Tasiemski et al., 2014). 3D peptide models of the non-covalent homodimer 1:1 of POL

and of the monomer were predicted using Alpha Fold (https://alphafold.ebi.ac.uk/). The confidence in the accuracy of the predicted structures is indicated by the predicted local distance difference test (pLDDT): score 0 to 100. The peptides 3D illustrations were generated using ChimeraX (https://www.cgl.ucsf.edu/chimerax/).

2.6. Mass spectrometry of POL

A sample of POL (about 2 µL, at concentration of 2 mg/mL) was used for characterization by analytical UPLC–MS using a System Ultimate 3000 UPLC (Thermofisher) equipped with an Acquity peptide BEH300 C18 column (1.7 µm, 2.1 × 100 mm, 300 Å), a diode array detector and a mass spectrometer (Ion trap LCQfleet). Analysis was performed at 70 °C using a linear gradient of 0-70% of eluent B in eluent A over 20 min at a flow rate of 0.4 mL min^{-1} (eluent A = 0.1% TFA in H_2O; eluent B = 0.1% TFA in acetonitrile). The column eluate was monitored by UV at 215 nm. The peptide masses were measured by on-line UPLC–MS (LCQ Fleet Ion Trap Mass Spectrometer, ThermoFisherScientific): heat temperature 450 °C, spray voltage 2.8 kV, capillary temperature 400 °C, capillary voltage 10 V, tube lens voltage 75 V.

2.7. Bacterial strains used for antimicrobial assays

A series of bacterial strains was used to estimate the killing efficiency of the different AMPs and test whether they are more efficient against bacteria living in the same environment as the worms. The bacterial strains used in this study are all Gram-negative from different marine environments:

--*Pseudomonas sp.* AT1288 and *Vibrio diabolicus* HE800 provided by Ifremer LMEE culture collection (Mission HERO 1991) were isolated from a mix of tubes of *A. pompejana* and small pieces of the surrounding polymetallic sulfides of deep-sea hydrothermal chimneys (East Pacific Rise) (Raguénès et al., 1997).

--*Shewanella algae*, *Oceanisphaera donghaensis*, and *Vibrio fluvialis* were isolated from intertidal mud sediment near to the burrow of *Arenicola marina.* Raw sediment samples were incubated overnight in a liquid Marine Broth media at 28 °C to stimulate bacterial growth. Well-growing colonies were isolated by multiple pricking out onto agar plates containing marine seawater to identify different bacterial strains. Pure colonies were identified with the Universally applicable bacterial primer set 16S-F and 16S-R (forward primer: 5'-TCCTACGGGAGGCAGCAGT -3'; reverse primer: 5'-

GGACTACCAGGGTATCTAATCCTGTT -3') as described in *Cuvillier et al* (Cuvillier-hot et al., 2017) . The sequences were compared against nucleotide sequences from the Eztaxon database using BLASTn UPGMA trees were constructed using the Mega-X software package (http://www.megasoftware.net) (Fig. S4).

--*Vibrio alginolyticus* provided by the Ifremer is worldwide distributed in coastal waters of temperate and also polar areas (Holt and Bruun, 2005; Urakawa and Rivera, 2006). *V. alginolyticus* is genetically closely related to *V. diabolicus* (Fig. S4).

2.8. Determination of the spectrum of antimicrobial activities of ALV, ARE and POL

Peptides Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC), were determined by the broth microdilution method against bacterial growth in microtiter plates as previously described by Bulet (Bulet et al., 1991). The AMPs were resuspended in sterile pure water. Briefly, 100 µL of bacterial dilutions (10^6 Colony Forming Unit/mL) were added to each well into sterile 96 well flat bottom plates (CELLSTAR, Greiner bio-one) containing serial dilutions (from 40 to 0.00195 µg/mL) of peptides (10 µL per well). The plates were incubated overnight at 28 °C on a rotary shaker (140 rpm). The MIC was determined as the lowest peptide concentration at which observable microbial growth was inhibited, the absorbance (600 nm) measurements were realized with a microtiter plate reader (Tecan Sunrise Microplate Reader). MBC was measured by streaking on proper agar (MHB with 15 g/L bacteriological agar, Euromedex) Petri dishes, the whole volume of each well (110 µL) with no bacterial growth in the previously obtained MIC plates. After incubation for about 24 hours at 28°C, the MBC was defined as the peptide concentration where no colony growth was observed. All tests were conducted in triplicate.

A 'home *versus* away' graph was constructed from antimicrobial assays against the whole set of environmental marine bacteria with log-transformed MIC data organized into two groups, depending on the habitat (polar/temperate intertidal *vs* deep-sea hot vents) to highlight patterns of AMP efficiency in both native and transplanted habitats.

2.9. Kinetics of bactericidal activities of ALV, ARE and POL against *Vibrio* strains depending on the thermal range

Kinetics of bactericidal activity of the tree AMPs were assessed on two bacteria belonging to the same genus but colonizing two contrasted habitats: *V. alginolyticus* (polar and

temperate) and *V. diabolicus* ("hot" hydrothermal vent) according to the protocol described in Tasiemski et al (Tasiemski et al., 2004).

Cultures were incubated with peptides (at a concentration of 5-fold MICs) for 5, 10, 15, 30, 60, 120 min and 24 h at two temperatures (10 °C and 42 °C) to mimic the worms' thermal environment. Viabilities of *Vibrio* strains were assessed by plating the bacterial suspensions on MHB agar plates (sterile Petri dish, 90 mm). The plates were then incubated overnight at 28 °C and the resulting bacterial colonies were counted (CFU/mL). Three independent experiments were run in parallel and data were log-transformed (log CFU/mL).

2.10. Thermostability assays on ALV, ARE, POL and their ABU forms against *Vibrio* strains

The biological activities of AMPs were evaluated after their exposition to a thermal stress (high and low temperatures). Two *Vibrio* species genetically closely related, *V. alginolyticus* and *V. diabolicus* were chosen for this assay, as representatives of the two marine environments. The 10^6 CFU/mL diluted culture was added (100 µL per well) into sterile 96-well flat bottom plates (CELLSTAR, Greiner bio-one) containing a volume of 10 µL/well of peptides (at a concentration of 5-fold MIC values) previously incubated for 30 minutes and 1 hour at 4, 10, 42 and 90 °C. The plates were then incubated overnight at 28°C at 140 rpm. The antimicrobial activity was evaluated by measuring bacteria growth on a microtiter plate reader (Tecan Sunrise Microplate Reader) at 600 nm. The results were expressed as percentage of bacterial growth in comparison with the control (no AMP added to the MHB medium). The tests were conducted in triplicates.

The same experiment was also conducted in triplicate with the ABU forms to determine the role of the disulfide bridges in the thermal stability of the three native AMPS

2.11. pH-stability assay of ALV, ARE, POL and their ABU forms against *V. alginolyticus*

The biological activities of the three AMPs were measured after their exposition to an acid/basic stress following the method developed by Yang *et al.* 2017 (Na Yang et al., 2017). For comparative purposes, only a strain sensitive to the three AMPs was used: *V diabolicus* was excluded and *V. alginolyticus* was chosen because of its lowest MICs to all three AMPs. One colony of *V. alginolyticus* was grown in MHB medium overnight at 28 °C (140 rpm). Diluted bacterial cultures (1×10^6 CFU/mL) were added (100 µL per well) into sterile 96-well

flat bottom plates (CELLSTAR, Greiner bio-one) containing serial dilutions of peptides (from 20 to 0.00195 µg/mL), previously incubated for 3 hours at 10 °C in a pH buffered solution. Four acid-to-basic 100 mM solutions (sodium acetate buffer (pH 4.0), sodium phosphate buffer (pH 6.0), Tris-HCL buffer (pH 8.0), and glycine-NaOH buffer (pH 10.0)) were tested against control samples incubated in MHB medium (pH 7.4) at 10 °C. The plates were then incubated overnight at 28°C and 140 rpm. The antimicrobial activity of peptides was determined by the broth microdilution method (Wiegand et al., 2008). All tests were conducted in triplicates and the results were expressed as MIC values (in µM).

The same experiment was then conducted in triplicates with the ABU forms to determine the role of the disulfide bridges in the pH stability of the three native AMPs.

3. RESULTS

3.1. The newly identified Polaricin (POL) supports a wider sequence and structural diversification of AMPs from the BRICHOS-AMP family

The molecular identification of prepropolaricin was performed following a tblastx on the transcriptome assembly of the Antarctic terebellid worm *Amphitritides* sp. using the sequence of the preproalvinellacin as query (Fig. 2 and Fig. S1). Pfam analysis revealed the presence of a conserved BRICHOS domain, in line with the previously described members of BRICHOS-AMP family (Ovchinnikova et al., 2004; Panteleev et al., 2018; Tasiemski et al., 2014). Data highlighted a higher percentage of sequence identity of the BRICHOS domain compared to the AMP itself which seems to have evolved at a much faster rate (Table S1). As opposed to the AMP itself, the BRICHOS domain displays a clear phylogenetic signal in our set of polychaetes, *Alvinella* and *Amphitritides* being more closely related to each other than to other annelid species even if they inhabit contrasted habitats.

POL is processed from the C-terminal part of prepropolaricin (Fig. 2). It is a cationic 19 amino acids peptide (Table S2) containing a single cysteine residue by contrast with ARE and ALV, which respectively possess two and four cysteine residues involved in the formation of disulfide bridges that stabilize a β-hairpin conformation (Fig. 3).

MALDI-TOF mass spectrometry analyses of POL revealed that 3/4 of the polar AMP molecules dimerize presumably through an intermolecular bond engaging the cysteine residue of POL (Fig. S2). Depending on the dimerization state, the predicted 3D structure for

POL, based on an *in silico* approach (see Methods) is different: The monomer adopts an alpha-helix conformation while the non-covalent homodimer forms two parallel β-strands exposing face to face each cysteine residue. The best predicted models are presented in Figure 3.

3.2. BRICHOS-AMPs co-evolved with the microbial community of the worms from which they are identified

Crossed antimicrobial assays (Table 1) were performed between bacterial strains (cultivable under MIC assay conditions) typical of the polar/temperate shallow water or hot vents, and the three BRICHOS-AMPs (*i.e.*, POL, ARE and ALV). AMPs specificities for microbial communities characterizing thermal habitats encountered by the worms were then analyzed *via* a "home *versus* away" diagram, a method commonly used in ecology to identify local adaptation (see Material and methods) (Savolainen et al., 2013). To do so, MIC values of the three AMPs against the different strains were plotted to build the diagram (Fig. 4). The peptides which are the best locally adapted to the home site of their producers display the lowest values of MIC (higher fitness level) and *vice versa*. Analysis showed that ALV displayed a strong antimicrobial activity against all the bacteria tested with a better fitness against hydrothermal vent bacteria than against temperate/polar bacteria. ARE was also active against all the strains tested including *S. algae*, showing unlike ALV, a better fitness against temperate-coastal bacteria than against hydrothermal vent bacteria. Among the three peptides, POL was not active against the hot vent *V. diabolicus* and has the lowest efficacy against all the other bacterial strains tested. However, its fitness was the best against *V. alginolyticus* a strain that can also be found in polar habitat. Consequently, BRICHOS-AMPs are locally adapted to (*i.e.* specifically target) the microbial community associated with the worm habitat from which they are identified.

3.3. BRICHOS-AMPs are able to handle the thermal and pH ranges sustained by the worms

In a first step, the bactericidal activity, which reflects the requested time for an AMP to kill bacteria, was investigated through killing-time kinetics assays against the two *Vibrio* strains typical of the deep-sea 'hot' vents (*V. diabolicus*) and of the intertidal temperate/polar environment (*V. alginolyticus*) (Fig. 5). The antimicrobial efficacy was tested either at 10 °C, which corresponds to the optimal temperature of *Arenicola marina* (A. Sommer, 1999;

Alyakrinskaya, 2003; Bat and Raffaelli, 1998; Juretschke and Kamp, 1990; Shumway and Davenport, 1977; Sommer et al., 1997; Sommer and Pörtner, 2002; Toulmond, 1975; Weber, 1979) or at 42 °C, which corresponds to the optimal temperature of *A. pompejana* (Desbruyères et al., 1998; Di Meo-Savoie et al., 2004; Gaill and Le Bris, 2007; Le Bris et al., 2005; Ravaux et al., 2013; Von Damm, 2000). The assay was first adjusted according to the growth curve of the bacteria previously determined at the different temperatures (Fig. S3). The observed effects are consequently not reflecting a direct impact of these temperatures on the growth of these bacterial strains but rather differential AMP activities on the bacteria. Figure 5 shows that POL was slightly active against *V. alginolyticus* at 10 °C and inactive against *V. diabolicus* at both 10 °C and 42 °C. Figure 5 also shows that ARE and ALV are bactericidal against both strains with a better efficacy against their respective surrounding *Vibrio* communities. When mimicking local temperatures, the efficiency of ARE and ALV is even more pronounced against the local strain (i.e., 15 min to kill 100% of *V. diabolicus* at 42 °C versus >24 h at 10 °C for ALV and 30 min to kill 100% of *V. alginolyticus* at 10 °C versus 24 h at 42 °C for ARE).

In a second step, the thermal stabilities of the three AMPs were tested by comparing their bactericidal properties after being incubated for 30 min to 1 h at 4 °C, 10 °C, 42 °C and even 90 °C (Fig. 6A). The "polar" AMP (POL) exerted its antimicrobial activity against *V. alginolyticus* at 4 °C and 10 °C only and lost all its activity following incubations at 42 °C and 90 °C. By contrast, the "vent" AMP, ALV remained active against both bacteria following incubations at 42 °C and 90 °C with a 50% decrease of its activity after 1 h of incubation for the highest temperature tested. Data also confirmed the higher efficacy of ALV toward *V. diabolicus*. The "temperate" ARE displayed an intermediate pattern in which the activity started to decrease by 80% at 42 °C and almost disappeared after 1 h at 90 °C with only 20% of residual activity.

The effect of the environmental pH on the three AMPs was evaluated by comparing their antimicrobial efficacies at various pH (4, 6, 7.4, 8 and 10) corresponding to the pH range of the habitat of each worm (Fig. 3B), the narrowest range being the polar environment (7.8-8.6 *versus* 3.9-8.5 for the hot vent). Because POL is not active against *V. diabolicus*, only *V. alginolyticus* was used for the assays (Fig. 7A). The peptides remained soluble at all the pH values. Antibacterial activities of ARE and ALV were not affected by their incubation under

acidic-to-basic pH conditions although POL exhibited a reduced efficiency (4-fold under the most extreme conditions pH 4 and 10) as soon as the pH is not neutral.

3.4. Importance of disulfide bridges for the antibacterial activities of BRICHOS-AMPs under different thermal and pH conditions

The more the marine worms are exposed to high temperatures and extreme pH values, the more their BRICHOS-AMP exhibits a high content of cysteine residues engaged in disulfide bridges (Fig. 3). Cysteine replacement by alpha amino butyric acid (ABU) in the three AMPs allowed us to remove the intermolecular (ABU-POL) or intramolecular disulfide bridges (ABU-ALV, ABU-ARE) without modifying their main biochemical features (summarized in Table S3).

When comparing both the MIC and MBC values (Table 2) of the three AMPs and their ABU forms, ABU ALV and ABU ARE were slightly less efficient against *V. alginolyticus* while ABU POL has become slightly more active against this bacterium. Against *V. diabolicus,* the replacement of cysteines had a strong negative effect on the killing efficiency of the three AMPs. The disulfide bridges are likely to play a crucial role in the mode of action against *V. diabolicus*.

The importance of the cysteines in the thermostability of the BRICHOS-AMPs was also investigated. The ABU forms of the three BRICHOS-AMPs were exposed for 30 min to 1 h to the three temperatures (4 °C, 10 °C and 42 °C) considered as optimal for their respective worms (Fig. 6B) before being used in antibacterial assays against *V. alginolyticus* and *V. diabolicus*. ABU-POL kept the same activity as POL (Fig. 6A) against *V. alginolyticus* at 4 °C and 10 °C. However, when incubated at higher temperatures (42 °C and 90 °C), ABU-POL still inhibited partly the growth of *V. alginolyticus* unlike the native peptide POL, which is inactive under such conditions. ABU-POL even showed a reduced but still existing activity against this bacterium after being incubated 30 min at 90 °C. Both POL and its ABU form were inactive against *V. diabolicus* whatever the AMP thermal incubations tested. ABU-ARE was as active as ARE against *V. alginolyticus* and *V. diabolicus* at 4 °C and 10 °C. At greater temperatures than 42 °C, ABU-ARE lost almost all its activity against the two tested strains. Without disulfide bridges, ALV was only active against *V. alginolyticus* at 4 °C and 10 °C. No activity was observed at 42 °C and above.

Because POL, ABU-POL and ABU-ALV were not active against *V. diabolicus*, the importance of the disulfide bridges in the pH stability of the ABU-AMPs *versus* the AMPs was only investigated against *V. alginolyticus* (Fig. 7). Under acidic/basic conditions, ABU-variants kept their antibacterial activity against *V. alginolyticus* (Fig. 7B), displaying a slight decrease of activity (2-4-fold) especially under the most extreme pH conditions (4 and 10). ABU-POL was more resistant to pH changes than the native form, with an antimicrobial activity that slightly increased compared to POL.

4. DISCUSSION

The members of the BRICHOS AMP family constitute a remarkably attractive model to study the evolution of AMPs, as actors of worm's immune defense in extreme and fluctuating environmental conditions. In a previous study, genetic analyses of the *ALV precursor* encoding gene in two sister and syntopic species of alvinellid worms (*A. pompejana* and *A. caudata*) sharing the same hydrothermal vent conditions, showed no differences of the AMP sequence over a geographic range spanning the 6.000 km of the East Pacific Rise (Bulle et al., 2022)(Papot et al., 2017). Despite multiple genetic duplications the *ALV precursor* gene in both sister species (Papot et al., 2017), sharing the same biotic and abiotic environmental conditions constitutes a strong genetic purifying mean of maintaining exactly the same AMP to interact with common microbiome during the evolution race (Bulmer and Crozier, 2004; Fuller et al., 2011).

Here, an "*in silico* approach" consisting in blasting the sequence of preproalvinellacin on the Antarctic *Amphitritides sp.* transcriptome (Tasiemski et al., 2014) allowed us to identify a novel member of the BRICHOS-AMP family, named polaricin (POL). POL does not show any similarities with other known molecules. Focusing on BRICHOS-AMP precursors (ARE, POL and ALV) from annelids living in opposite habitats (temperate, polar, hot vent), we provided evidence for very small similarities in AMP primary sequences and conformations, while exhibiting a nearly conserved BRICHOS domain.

According to the mass spectrometry analysis, the Antarctic AMP seems to adopt a dimerized form presumably through a disulfide bridge engaging each unique cysteine residue of POL, what is unusual for an AMP. The difference between the predicted structural conformations of the homodimer (two parallel beta strands) *versus* the monomeric form (alpha helix) of

POL underlines puzzling mechanisms that require further investigations. As observed for the hemoglobin of a deep sea polychaetes, the cysteinylation of the free cysteine of POL is also possible. Forming a disulfide bond between the POL cysteine and a free cysteine would either prevent POL intermolecular dimerization or protect the free cysteine from oxidative stress (Hourdez et al., 2002).

As shown by Nuclear Magnetic Resonance (NMR) (Ovchinnikova et al., 2004; Tasiemski et al., 2014), ALV and ARE share the same monomeric organization despite great differences in primary structure: Two twisted antiparallel β-strands, forming a β-hairpin conformation stabilized by two or one disulfide bond(s) respectively. The best predicted 3D structures of POL are totally different. Interestingly the predicted alpha helix structure is strongly reminiscent of nicomicin, the monomeric BRICHOS AMP from a cold temperate and arctic annelid (Panteleev et al., 2018). These data support a common origin of the precursor molecule with a clear divergent evolution of the AMP itself, which seems to be driven by strong disruptive ecological constrains associated with the worm's lifestyle.

According to common-garden approaches, the manipulation of environmental factors (microbial community, temperature and pH) is a powerful method to test for the role of the species *preferenda* in the evolution of their external immune defenses (Otti et al., 2014). Using the "home *versus* away" approach, we showed that ARE and ALV are locally adapted to the bacterial communities of the worm's environment they belong to, while POL is maladapted to interact with both the shallow temperate and the deep hydrothermal-vent microbiota. According to the MIC data, ALV and ARE indeed kill faster and more efficiently the bacterial strains encountered in the habitat of their own worm. Interestingly, POL was inefficient against the hydrothermal vent *V. diabolicus* but displayed the best efficacy against *V. alginolyticus,* a bacterium that colonizes both polar and cold temperate habitats, in support a local adaptation of this AMP. The demonstration would have been even more convincing by observing a strong activity of POL against a strictly polar marine bacterium but this requires the use of a strain cultivable under the conditions of a MIC assay. Such a strain was not available so far. Unlike *Vibrio*, *Pseudomonas sp.* isolated from hydrothermal vents was easily killed by the three AMPs regardless of their habitat of origin. The genus *Pseudomonas* is cosmopolitan to marine waters and terrestrial habitats (e.g., freshwater, soil…) and can be considered as one of the most successful bacterial groups on Earth. Worms studied here likely have encountered species from the genus *Pseudomonas* close to the

species used here. Such AMP specificities support the role of AMPs in invertebrate adaptation to environmental changes through selective constraints associated with the surrounding bacterial communities as previously shown in studies demonstrating their involvement in the immune defense and in the control of the vital ectosymbiosis of the worm *A. pompejana* and of the shrimp *Rimicaris exoculata* (Bloa et al., 2020; Tasiemski et al., 2014). Data confirmed not only that ARE, ALV and POL display an optimal killing activity against the species of *Vibrio* typical of the habitat where each worm species lives, but also that this killing efficacy is optimal/kept under the thermo-chemical conditions under which the worms live.

The correlation between species habitat and the cysteine content of the BRICHOS-AMPs led us to investigate the importance of disulfide bridges in the biological efficacy of the three molecules as a function of abiotic pressures (pH and temperature). The use of ABU-chemical variants, provided evidence of the strong involvement of internal disulfide bridges in the stability of BRICHOS-AMPs when exposed to thermal and/or pH stresses. This allowed us to conclude that the disulfide bond patterns of ALV and ARE are not only designed for a better bacterial killing ability but also a way to sustain the high fluctuations of the worm's environment (tidal emersion and seasonality for *A. marina* and chaotic mixing of hot fluid with cold seawater for *A. pompejana*) (Alyakrinskaya, 2003; Desbruyères et al., 1998; Pradillon et al., 2005; Shumway and Davenport, 1977). These results are consistent with earlier results on chemically synthesized fragments (*i.e.* not naturally existing) designed from an ARE isoform (N Yang et al. 2017): the synthetic fragment containing disulfide bridges were not affected by pH variations (4.0 to 10.0) and more thermostable (range 20-80 °C) than their respective linear fragments (devoid of disulfide bridges) which also exhibited lowest antibacterial activities (Na Yang et al., 2017). By ensuring the β-hairpin structure, the disulfide bridges confer both a higher stability and a much more efficient bactericidal activity to ALV and ARE (Andrä et al., 2009; Ju-Un Lee Dong-Il Kang, Wan Long Zhu, Song Yub Shin, Kyung-Soo Hahm, 2007; Lai et al., 2002; Nan et al., 2012; Wu et al., 1999).

Unexpectedly, ABU-POL was more effective against both mesophilic and thermophilic bacteria, displaying a more stable structure at higher temperatures and more acid or basic waters than the native dimeric form. This could lend support for the prevention of disulfide bridge formation through cysteinylation *in vivo* for this species, and will need to be investigated further. To date, the dimerization of AMPs was mainly performed with the aim

of improving the performance of synthetic peptides for therapeutic use (reviewed in Lorenzon et al., 2019 (Lorenzon et al., 2019)). Chemical dimerization *via* the creation of disulfide bridges of some AMPs such as magainin and histatin conducted to an enhanced antimicrobial potency and a reinforced resistance to proteases while the dimerization of others such as the alpha helical aurein, reduced the antimicrobial activities by changing the mechanism of action of the peptide (Lorenzon et al., 2013). In the specific case of POL, the formation of homodimers might have been selected during the evolution race, probably as a response to the progressive cooling of the Antarctic continent, about 25 Mya (Cowart et al., 2022; Rogers et al., 2007). Although less efficient against temperate to thermophilic bacterial strains, peptide dimerization might be advantageous against polar psychrophilic microbes and such hypothesis as well as the structural conformation of POL still need to be investigated.

Conclusion and perspectives

Based on POL, a novel BRICHOS-AMP sequence discovered in a new terebellid polychaete strictly inhabiting Antarctica, we have performed a comparative analysis between different BRICHOS-domain AMPs, specific of marine polychaetes inhabiting contrasted thermal habitats. Due to its unusual single cysteine, further investigations are however required to elucidate both the structure and the mode of action of this novel polar AMP. The presence of a BRICHOS domain linked to a potent AMP and its successful evolution in polychaetes raised the matter of fully investigating on their specific molecular interaction in particular by investigating the role of the BRICHOS domain as a molecular chaperone to the folding/unfolding of the host defense factors of these worms.

For the first time, we provided evidence that BRICHOS-domain AMPs evolved under strong diversifying environmental pressures to be more efficient under the ecological preferences of the worm they belong to. They are structurally shaped and adapted to exert their activities under specific conditions and against specific microbial targets. There is ample evidence of the importance of bacterial pathogens in the evolution of AMPs providing protection against bacteria that have rapidly evolved to escape the antibacterial response. The present data bring to light for the first time an adaptation of AMPs, not in an infectious context but rather in an environmental and evolutionary context. This work highlights the importance of considering thermal and pH conditions of the habitat in the evolution and

efficacy of the antimicrobial immune response involved in host interactions with the local microbiome. These surveys could provide guidelines of the evolutionary response of marine organisms to future scenarios, like ocean warming and acidification (Mcculloch et al., 2012; Sternberg and Thomas, 2014; Wilkins et al., 2019). The present data also support the importance of considering the optimal/natural environment of active substances when investigating and optimizing their use in therapeutic treatment of microbial diseases known to modify/alter the cellular microenvironment (Crabbé et al., 2014).

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FIGURE LEGENDS

Figure 1: Geographical distribution of the three polychaetes studied and description of their marine habitats.

Figure 2: Alignment of the precursors of the BRICHOS-AMP family members: polaricin (POL), nicomicin, alvinellacin (ALV) and arenicin (ARE). BRICHOS and AMP regions are respectively in black and dotted black rectangles. In the AMP region, cysteine residues involved in disulfide bridges are highlighted in black circles. The number of residues relative to the N-terminus segment of the precursor is shown on the right side of each sequence.
The color of amino-acids background underlines the higher level of conservation in the BRICHOS region as compared to the AMP region itself. The alignment was generated using CLC Sequence Viewer software (version 8.0).

Figure 3: Three-dimensional structures of ALV, ARE and the structures of POL generated by Alpha Fold software. AlphaFold produces a per-residue confidence score (pLDDT) between 0 and 100: Very high confidence (pLDDT > 90); Confident (90 > pLDDT > 70); Low (70 > pLDDT > 50); Very low (pLDDT < 50).
Number of cysteines in the three AMPs as a function of thermal and pH encountered in the habitat of each worm.

Figure 4: A representation of BRICHOS-AMPs local adaptation (home *vs.* away diagram) to temperate-coastal and hydrothermal vents-deep sea habitats, using their MIC values against environmental bacterial strains. Y-axis, representative of fitness factor, stands for peptides MIC log-transformed data: higher MIC values correspond to lower fitness levels. The colored areas gather the values belonging to the same peptide. Dashed lines, connecting the areas across the two habitats, show the molecular adaptation of ARE and ALV to their respective habitats, displaying lower MIC values against “home strains” and *vice versa*. Lacking strictly polar bacterial strains, POL displays the highest MIC values against the investigated “away strains”. Peptides and bacteria sharing a common environment are shown by the same color: red for hot vent, blue for temperate and purple for polar. The cases of not-active peptide are represented with – on the top of the diagram, as leaning to infinity.

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Figure 5: Kinetics profiles of the bactericidal activities of ALV, ARE and POL against *V. alginolyticus* and *V. diabolicus*, at 10 °C and 42 °C. The results are shown as time-plot of *Vibrio spp.* viability (log_{10} of CFU/mL), without (control) and with peptides. Temperatures, peptides and strains belonging to the temperate, hydrothermal vent and polar environments are in blue, red and purple respectively. The dotted grey lines are the negative controls with no peptides.

Figure 6: Effect of temperature on biological activity of (A) native peptides and (B) ABU-peptides, against *V. alginolyticus* and *V. diabolicus*, after 30 minutes to 1 hour of peptide incubation at different temperatures (4, 10, 42 and 90 °C). Each bar is expressed as the average with error bars, representing ±SD from 3 independent experiments (n = 3). The Y-axis (in A) displays a break in the range of values to improve its readability.

Figure 7: Effect of pH on the biological activity of (A) native peptides and (B) ABU-peptides activities, against *V. alginolyticus*. The tests were performed after peptide incubation (3 hours) under acid/basic conditions (at pH 4, 6, 8 and 10). The control is represented by peptide MIC values in MHB medium (pH 7.4) for each peptide.

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Credit author statement:

Renato Bruno: Writing the initial draft, performing the experiments, data presentation, investigation. Céline Boidin-Wichlacz: Design of methodology, performing the experiments, data presentation. Daniela Zeppilli: Critical review and co PI of the Pioneer Project. Oleg Melnyk: Mass spectrometry experiments and analyses. Céline Landon, Frederic Thomas and Mickael Lafond: Critical review, commentary. Marie-Anne Cambon: Provision of biological materials. Kamel Mabrouk: Provision and design of the ABU peptides. François Massol: Statistical analyses, creation of models, conceptualization. Stéphane Hourdez: Design and creation of the polar annelid databank, critical review. Marc Maresca: Design of methodology, performing the experiments, critical review. Didier Jollivet: Design and creation of the polar annelid databank, identification of POL from transcriptomic analyses, conceptualization, critical review. Aurélie Tasiemski: Data presentation, writing the initial draft, review and final editing of the published work, conceptualization, supervision and project administration.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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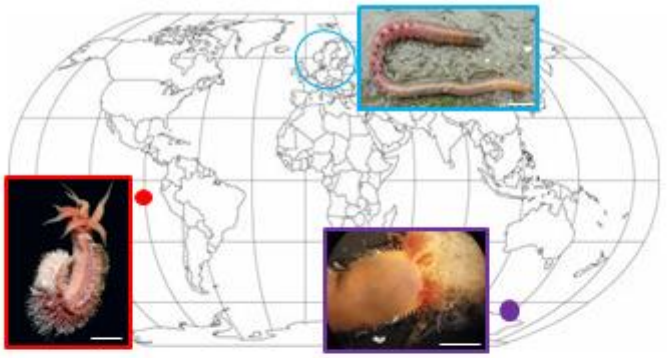
Figure 1



Annelid species	*Amphitritides sp.*	*Arenicola marina*	*Alvinella pompejana*
AMP name	Polaricin (POL)	Arenicin (ARE)	Alvinellacin (ALV)
Environment	Polar, coastal	Temperate, coastal	Hot chimneys of hydrothermal vents, deep sea
Distribution	Antarctica (Adelie Land)	North Sea	East Pacific Rise 9 North

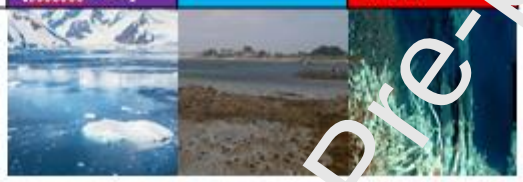


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Figure 2

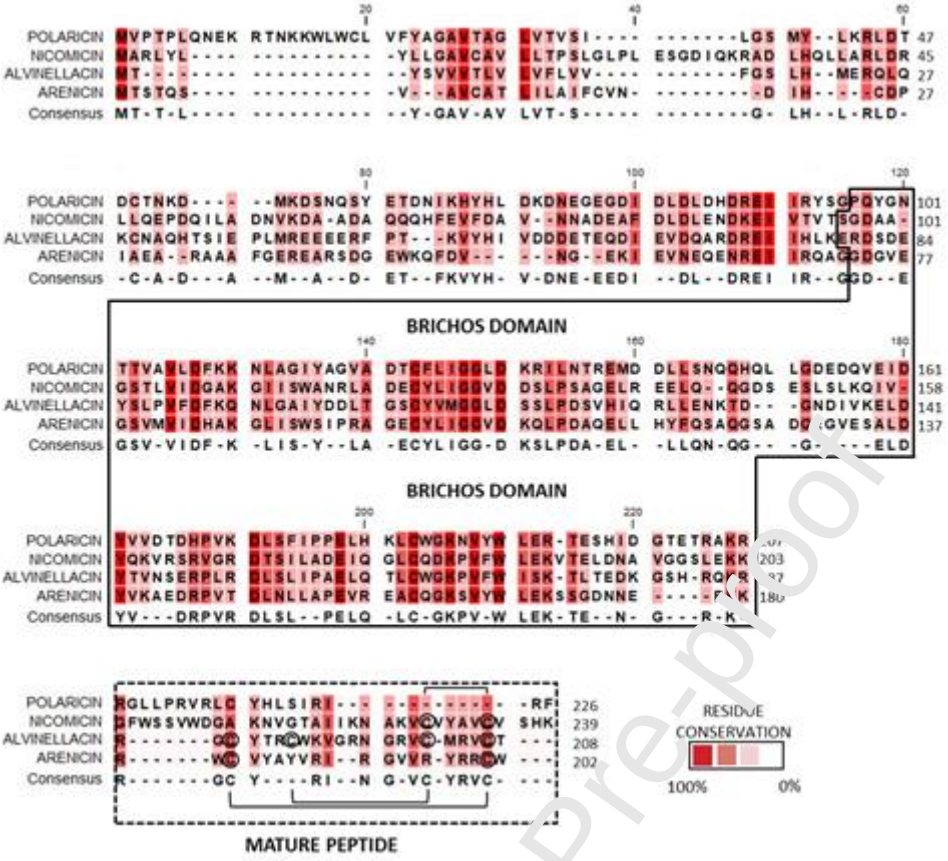

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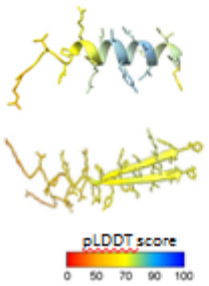
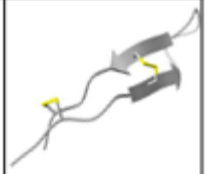
Figure 3

	POL	ARE	ALV
Structure		 PDB : 2JSB	 PDB : 2LLR
Number of Cys	1	2	4
Number of intramolecular disulfide bridges	0	1	2

Thermal range of the worms' habitat

			Amphitritides sp.	*Arenicola marina*	*Alvinella pompejana*
HABITAT	T°	Range	-1.8 °C – 1 °C	5 °C – 25 °C	2 °C - >100 °C
		Optimum	~0 °C	~ °C	~42 °C

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Figure 4

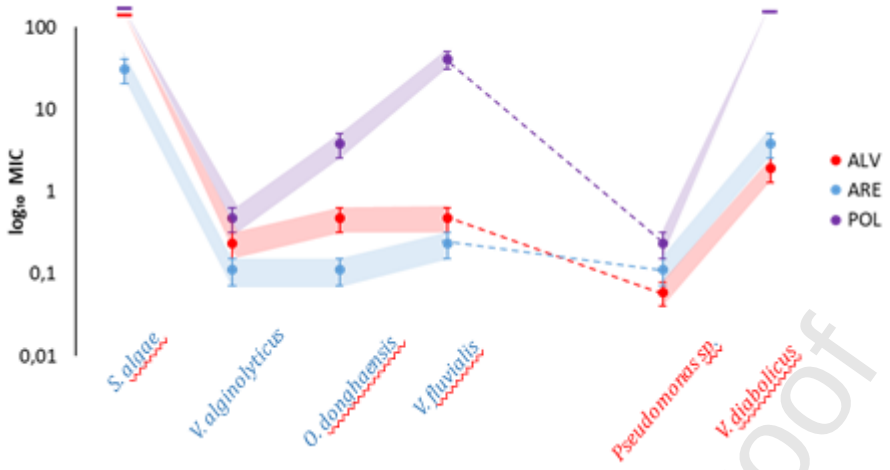

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Figure 5

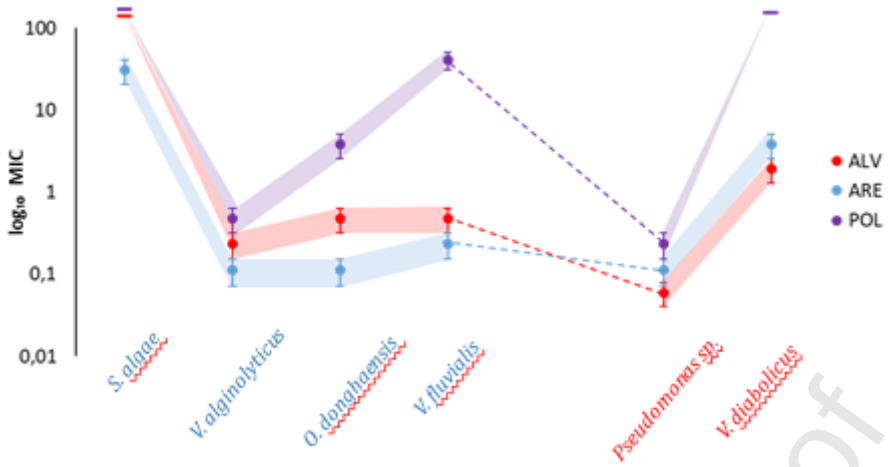

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Figure 6

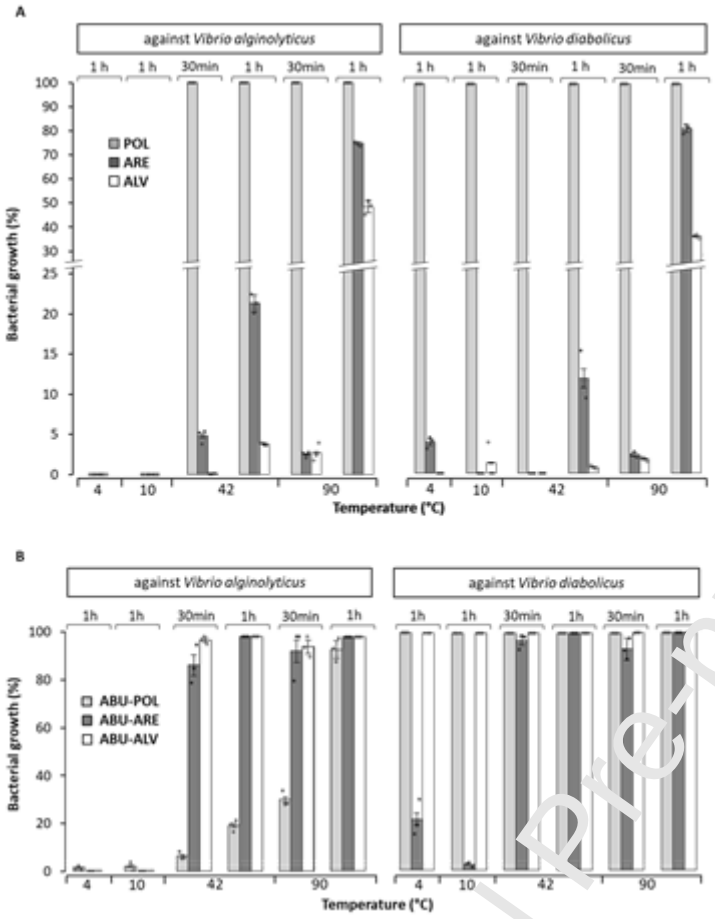

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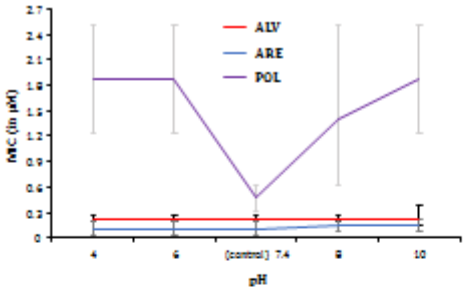
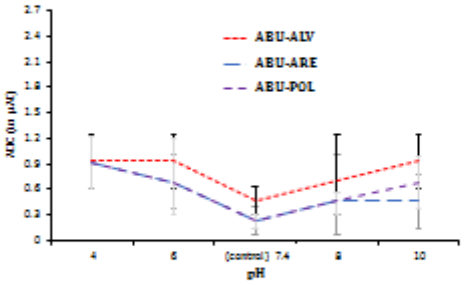
Figure 7





pH range of the worms' habitat

RABUM			*Amphiritidae* sp.	*Arenicola marina*	*Alvinella pompejana*
	pH	range	7.8 – 8.6	2.4 – 8.4	2.9 – 8.5
		optimum	7.5 - 8	6.5 - 8	6.7 - 8

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TABLES

Table 1: Antibacterial activities against environmental bacteria of the three BRICHOS-AMPs: the "cold" POL, "temperate" ARE and "hot" ALV. MIC: Minimal Inhibitory Concentration; MBC: Minimal Bactericidal Concentration; > not active at this concentration and beyond

		MIC (µM)			MBC (µM)		
HABITATS	BACTERIA	ALV	ARE	POL	ALV	ARE	POL
Temperate, polar Intertidal	*Vibrio alginolyticus*	0.31	0.15	0.625	0.625	0.31	5
Temperate Intertidal	*Vibrio fluvialis*	0.625	0.31	40	10	1.25	>40
Temperate Intertidal	*Shewanella algae*	>40	40	>40	>40	>40	>40
Temperate Intertidal	*Oceanisphaera donghaensis*	0.625	0.15	5	1.25	0.625	20
Hot vent Deep sea	*Vibrio diabolicus*	2.5	5	>40	20	20	>20
Hot vent Deep sea	*Pseudomonas sp.*	0.07	0.15	0.31	0.31	1.25	5

Table 2: Comparison of the antibacterial activities against the hot vent *Vibrio diabolicus* and the polar/temperate *Vibrio alginolyticus* of the three BRICHOS-AMPs (the "cold" POL, "temperate" ARE and "hot" ALV) *versus* their respective ABU variants devoid of disulfide bridges. MIC: Minimal Inhibitory Concentration; MBC: Minimal Bactericidal Concentration; > not active at this concentration and beyond

	MIC (µM)						MBC (µM)					
BACTERIA	ALV	ABU-ALV	ARE	ABU-ARE	POL	ABU-POL	ALV	ABU-ALV	ARE	ABU-ARE	POL	ABU-POL
V. alginolyticus	0.31	0.62	0.15	0.31	0.625	0.31	0.625	1.25	0.31	1.25	5	2.5
V. diabolicus	2.5	>40	5	>40	>40	>40	20	>40	20	>40	>20	>40

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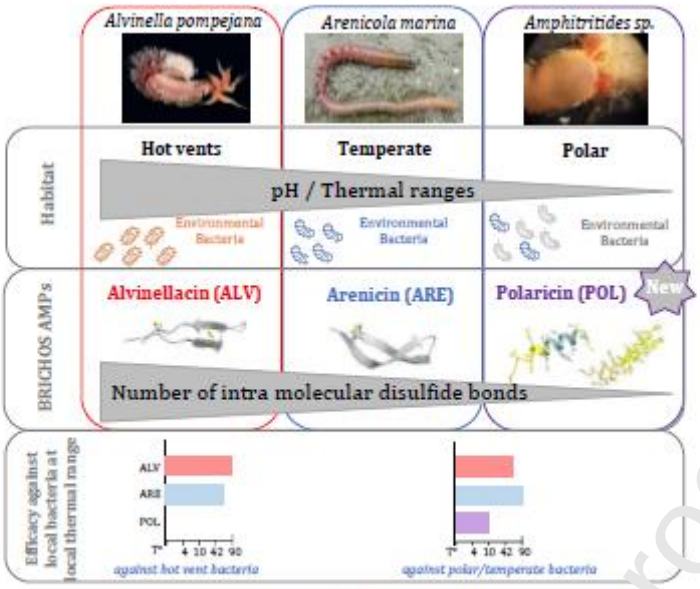
Graphical abstract



➢ Local adaptation of AMPs to the ecological niche of the producers

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Highlights:

• A novel antimicrobial component named polaricin belonging to the BRICHOS-AMP family was identified from a polar marine annelid

• Comparison of BRICHOS-AMPs from annelids inhabiting contrasted habitats shows a profound diversification of the AMP sequence

• AMPs are structurally shaped and more efficient/specific under the ecological niche of their producer

• AMPs display an optimal killing efficacy against the bacteria and under the environmental conditions typical of each worm's habitat

• This is the first evidence than external immune effectors are evolving to fit environmental pressures

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