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Characterization and Function of the First Antibiotic Isolated from a Vent Organism: The Extremophile Metazoan *Alvinella pompejana*

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**Abstract**

The emblematic hydrothermal worm *Alvinella pompejana* is one of the most thermo tolerant animal known on Earth. It relies on a symbiotic association offering a unique opportunity to discover biochemical adaptations that allow animals to thrive in such a hostile habitat. Here, by studying the Pompeii worm, we report on the discovery of the first antibiotic peptide from a deep-sea organism, namely alvinellacin. After purification and peptide sequencing, both the gene and the peptide tertiary structures were elucidated. As epibionts are not cultivated so far and because of lethal decompression effects upon Alvinella sampling, we developed shipboard biological assays to demonstrate that in addition to act in the first line of defense against microbial invasion, alvinellacin shapes and controls the worm’s epibiotic microflora. Our results provide insights into the nature of an abyssal antimicrobial peptide (AMP) and into the manner in which an extremophile eukaryote uses it to interact with the particular microbial community of the hydrothermal vent ecosystem. Unlike earlier studies done on hydrothermal vents that all focused on the microbial side of the symbiosis, our work gives a view of this interaction from the host side.

**Introduction**

*Alvinella pompejana* is a polychaetous annelid that inhabits active deep-sea hydrothermal vents along the East Pacific Rise, where it colonizes the walls of actively venting high-temperature chimneys [1]. The environment of the worm is characterized by extreme physicochemical gradients, high pressure and bursts of elevated temperatures which can be as high as 105°C [2]. To date, the Pompeii worm is considered as one of the most eurythermal and thermotolerant metazoa known on Earth [3–6].

One of the striking features of this annelid is its association with a unique epibiotic bacterial community that forms cohesive hair-like projections from mucous glands lining the dorsal intersegmental spaces [3]. Numerous studies, including metagenomic analyses, evidenced that the microflora is composed of a multispecies complex of 12 to 15 phylogenotypes of which >98% are Epsilonproteobacteria, a dominating taxonomic group in hydrothermal vents [7]. These bacteria have been suggested to provide *Alvinella* with a stable source of nutrients and may detoxify the environment of the worm from reactive heavy metals and free hydrogen sulfide [1].

Central theme in beneficial bacterial-host interaction is that hosts must protect themselves against inappropriate colonization and replication of the symbiotic flora [8]. Various mechanisms are employed to control the symbionts without compromising host vitality. Amongst them, beneficial partnership between symbiotic bacteria and the immune reactions of the host has been widely invoked in mammals and insects [8]. The molecular interactions between the two partners of the association seem to modulate host immunity, and in turn the immune system shapes the composition of the microbiota.

Antimicrobial peptides (AMPs) are small sized molecules naturally produced by bacteria, protists, fungi, plants and animals. Their large distribution in nature within both unicellular and multicellular organisms suggests that they are crucial immune effectors which presumably have evolved under positive selection.
for a long period of time [9]. Recently, Login et al demonstrated that coelopteracin A, an AMP produced by the beetle belonging to the Staphylococcus genus, keeps endosymbionts under control within the bacteriocytes [10]. By comparison with the number of AMPS isolated from terrestrial invertebrates (~1500), relatively few AMPS (≈40) have been characterized from marine organisms [11]. Yet, marine animals are permanently in close contact with very high densities of microbes (10^9 to 10^7 per ml) suggesting that their immune effectors are effective in microbial growth inhibition and killing [12]. Although AMPS have been found in numerous marine invertebrate taxa such as Cnidarians, Annelids, Mollusks, Arthropods, Tunicates and Echinoderms [11], there is no evidence of active AMPS in organisms living in the deep-sea. To date, the aspect of AMP coevolution under selective pressures associated with the abyssal environment has never been investigated, whereas many life forms, in such an extreme habitat, rely on a symbiotic association.

Here, we describe the nature of the first abyssal AMP found in a symbiotic animal, its common origin with AMPS of coastal annelids as well as the manner in which an extremophile eukaryote uses it to interact with the particular microbial community of the hydrothermal vent ecosystem.

Material and Methods

Bacterial strains. The bacterial strains used in this study are listed in S.1 in Material and Methods S1. Epibionts were scraped with a thin razor from 1 cm^2 of the tegument of freshly harvested Alvinella pompejana. Epibionts were then minced and cultured in Leibovitz L-15 medium under sterile conditions on board. Incubations without bacteria were performed under the same conditions as controls.

Primary cell culture. Freshly harvested coelomic cells were cultured in Leibovitz L-15 medium under sterile conditions on board. For microbial treatment, cells were separately incubated in 500 µL of medium containing 10 µL of killed bacteria, for 12 h. Incubations without bacteria were performed under the same conditions as controls.

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Gene characterization and gene expression

The nucleotidic sequence coding the preproalvinellacin precursor was obtained by blasting the amino acid sequence of Alvinellacin to the Alvinella EST database (TERA 00513) [21].

Gene structure. The complete gene sequence of the preproalvinellacin (Genbank accession number KJ489380) was obtained from the cloning of PCR-products coming from the nested amplification of a series of A. pompejana gDNA using specific primers targeted on the 5' and 3' ends of the cDNA. Used primers are as followed: AP_alvinellacinF starting from the first methionine codon: 5'-ATG ACG TAT TCT GTA GTT GTG ACG-3'; AP_alvinellacinR1 (in the 3'UTR region): 5'-TAG GCA GGA CGG AGG CGC CAG ATC A-3'; and AP_alvinellacinR2 (starting on codon stop): 5'-CTC AGT GAA ATG AAG CAG GTG AGT GT-3'. PCR amplifications were obtained following 40 cycles of 96°C for 45 s, 60°C for 45 s and 72°C for 4 min after a first denaturation of gDNA at 96°C for 4 min and a final elongation of 10 min. Putative splicing sites (ACEs and ISEs) and both mobile and regulatory elements were detected using ACESCAN2 web server (http://genes.mit.edu/
acescan2/index.html) and modules of the geneinfinity (http://www.geneinfinity.org/sp/sp_coding.html), webgene (http://www.itb.cnr.it/webgene/) and the TE tools (ergmanlab.smith.man.ac.uk/?page_id = 295) platforms. The complete gene sequence of preprocapitellacin was obtained by blasting the preproalvinellacin in the Capitella teleta genome database (http://genome.jgi-psf.org/Capca1/Capca1.home.html).

Quantitative Reverse Transcription PCR. RNA from cells were extracted (Qiazol, Qiagen) and used for cDNA synthesis with an oligoT according to the protocol of the manufacturer (SuperScript II; Invitrogen). The primers used for quantification were designed with the Primer3 Input software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 www.cgi).

-Alvinellacin primers: forward: 5'-TGACATCGTGAAG-GAACTCG-3'; reverse: 5'-CCGTTCCTACCAACTTTCCA-3'.
-Ribosomal Protein 26S primers (Alvinella database [21]: TERA01523): forward: 5'-CCGGCTAGTTCAAGATGACC-3'; reverse: 5'-AGCTGCTGCCTCCACTATGT-3'.

The RP26S was used as the reference gene. Real Time reactions were conducted on a CFX96 qPCR system (BioRad) using a hot start, then 40 cycles at 94°C, 15 s; 56°C, 30 s; 72°C, 30 s., and a final extension step at 72°C for 3 min. Analysis of relative gene expression data was performed using the ΔΔCt method. For each couple of primers, a plot of the log cDNA dilution versus ΔCt was generated to validate the qPCR experiments (data not shown). Reference and target were amplified in separated wells.

Alvinellacin production sites

Polyclonal antiserum. The alvinellacin antiserum was raised in two New Zealand White rabbits (Saprophyte pathogen-free). The chemically synthesized peptide was coupled to OVA and used for the immunization procedure according to the protocol described previously [22]. The reactivity of the antibody was tested by Dot Immunobinding Assay (DIA) using 1 μL of the RP HPLC fractions [23].

Immunocytochemistry and immunohistochemistry. Cells or tissues were fixed on board in 4% paraformaldehyde. Later, the SHANDON Cytospin 3 was used to spin cell suspension onto polylysine slides (8 min, 2,000 rpm). Immunocytochemistry and immunohistochemistry were performed with the rabbit anti-alvinellacin (1:100) and the FITC-conjugated anti-rabbit secondary antibody (1:100; Jackson Immunoresearch Laboratories) according to a protocol already described by our group [21]. Samples were examined using a confocal microscope (Zeiss LSM 510).

Coelomocyte structure. The coelomocytes were collected, and immediately fixed in 3% glutaraldehyde according to the protocol previously described [24]. Coelomocytes were observed on a Hitachi H 600 electron microscope.

Results and Discussion

Nature of Alvinella AMP and evolutionary link with AMPs from coastal species

To date, only one AMP isolated from marine species belongs to an AMP family already characterized in terrestrial species [11].
alvinellacin is processed from a larger precursor molecule and S2). As for most AMPs from all invertebrate phyla, mature named alvinellacin, from the coelomocytes of as antimicrobial peptides (AMPs) [26]. We purified and identified that sterilize the coelomic fluid by releasing humoral factors such a compartment that includes mobile cells, named coelomocytes an anatomy of annelids is characterized by the presence of a coelom, combined with the analysis of the activity from the Pompeii worm, a biochemical approach was proceeded to identify peptides with antibiotic confined to certain taxa or even species, as observed for AMPs of The majority of marine AMPs presents novel structures and is containing a signal peptide and an anionic proregion [27] (Figure 1A).

Following a BLAST search, the mature peptide did not display any similarity in its primary structure with other known proteins. However, the proregion had ~33% identity to the proregion of AMPs from coastal annelids: arenicin from Arenicola marina and capitellacin, a putative peptide inferred from the genome sequence of Capitella teleta (Figure S3). Pfam analysis of the proregions revealed the presence of a conserved BRICHOS domain. So far, this 100 amino acids domain has never been reported in other AMP precursors than preproarenicin [29].

Despite the lack of an obvious similarity between the primary structures of alvinellacin, arenicin and capitellacin, we analyzed and compared their three-dimensional structures. Using NMR spectroscopy and mass spectrometry, we determined the tertiary structure of alvinellacin (Figures S4 and S5, Tables S1 and S2) and compared it to the solved and predicted structures of arenicin and capitellacin, respectively (Figure 1B). Alvinellacin and capitellacin are stabilized by two disulfide bonds, whereas arenicin possesses only one cystine. Like capitellacin, alvinellacin folds into a double-stranded antiparallel beta-sheet resembling the structure of arenicin [29]. Consequently, the three AMP precursors i.e. preproalvinellacin, preprocapitellacin and preproarenicin harbor the conserved pattern of almost all the BRICHOS containing proteins: a hydrophobic domain (here, the signal peptide), a linker region, the BRICHOS domain itself and a C terminal region with beta-sheet propensities (here, the AMP) [28].

To date, prorregions of AMP precursor are essentially known to be implicated in cell chemotaxy and/or protection against the cytotoxic activities of certain AMPs [30]. The BRICHOS domain has been found as a constituent of proteins associated with a wide variety of human diseases such as dementia, respiratory distress and cancer [31].

Recent data evidence that BRICHOS participates in the complex post-translational processing of proteins, and functions as an intramolecular chaperone domain that can bind beta hairpin motifs and prevents them from beta sheet aggregation and amyloid fibril formation [28]. Because of their strand-loop-strand structure, it seems reasonable that alvinellacin like the two other AMPs interacts with BRICHOS. Coastal and, even more, hydrothermal annelids are naturally submitted to strong hypoxic and thermal stresses. We hypothesize that the presence of the BRICHOS domain might be an evolution-driven adaptation of the worms to warrant the correct folding of their AMP under extreme conditions such as hypoxia and/or eurythermality. All these suggestions should be experimentally tested: BRICHOS might also have a novel function in A. pompejana that remains undiscovered.

As a conserved gene structure constitutes a convincing evidence for evolutionary relatedness between protein families, we also characterized the complete gene sequence of alvinellacin and compared it to the capitellacin gene [32] (Figure S6). Both genes display a 5 introns/6 exons structure with nearly all conserved intron-splicing positions. Given the taxonomic position of Capitella and Alvinella [33], their gene structure along with the prorregion sequence identity and the three-dimensional peptide structure, strongly indicate that alvinellacin and capitellacin presumably together with arenicin, share an ancient origin and are evolutionary correlated since hundred millions of years. Further detailed comparisons of the prorregions showed a high level of amino acid changes in the first part of the propieces; that may be also attributable to an adaptive ‘hot spot’ of mutations (functional change) in the face of the very long period of time since divergence between the two polychaeta species. The low amino acid
investigation because of lethal decompression effects upon environmental microbes. 
Alvinellacin in the first line of defense towards environmental microbes:

In general, microbial invasion into the host causes bacterial infection which prompts an immune response such as the release of AMPs to eliminate invaders. Since alvinellacin was isolated from the coelomocytes, these cells are likely to produce and secrete the AMP into the coelomic fluid where it exerts its antibacterial activities. The presence of a signal peptide in the alvinellacin precursor (Figure 1A) together with the results obtained by immunocytochemistry (see below) corroborates this assumption. The antimicrobial activity of alvinellacin was then evaluated (Figure 2 and Table 1). As the worm’s coelomic fluid composition is not very different from seawater, assays were performed at salt concentrations mimicking this environment [35]. Under these conditions, alvinellacin’s activity was constant primarily against the Gram-negative bacteria. This may represent an adaptation of the worm to its associated microorganisms, which have been shown to be predominantly Gram-negative bacteria [3,36]. We then wondered whether an exposure to various microorganisms might have differential impacts on the synthesis of alvinellacin. Usually, to investigate the immune response of an organism, animals are submitted to experimental infections and variations of immune markers are quantified. Since Alvinella precludes in vivo investigation because of lethal decompression effects upon conservation in the AMP sequences compared to the proregions suggests that they might have evolved independently. To the best of our knowledge, AMP proregions are not known to interfere with components of the external environment as AMPs do by interacting with microbes. Thus, the mature AMP presumably evolved to respond to the specific microbial communities (hydrothermal or coastal habitats) as well as to the specific lifestyle (symbiotic or not) of the worms while the proregion did not. This is consistent with the observation that the C-terminal propeptides of (symbiotic or not) of the worms while the proregion did not. This is consistent with the observation that the C-terminal propeptides of the interstitial collagen of Alvinella and Arenicola are similar, while the helical domain of the mature protein, which is located in the extracellular matrix and is presumably more exposed to environmental conditions, is not. [34].

Alvinellacin controls and shapes the epibiotic flora:

While a key role of AMPs in fighting infections is well described, very recent studies also evidenced that these effector molecules can be employed to regulate/control the symbiotic microflora [10,30].
The strong and vital relationship between *Alvinella* and its epibionts prompted us to investigate such an alternative function of alvinellacin. Immunohistochemistry experiments showed that alvinellacin is expressed constitutively by epithelial cells of the tegument associated with the epibiotic microflora, i.e. the dorsal but not the ventral epidermis (Figures 4A vs 4B). This observation supports the idea that alvinellacin may prevent bacterial entrance and/or keep epibionts under control. Accordingly, we determined the antimicrobial potency of alvinellacin against epibionts (Figure 5). As epibionts have not been cultivated so far, we carried out a shipboard antimicrobial assay aimed at detecting epibiont-cell damage in response to exposure to alvinellacin. Interestingly, alvinellacin significantly targeted epibionts that correspond to filamentous bacteria (epibiont types 5 to 9). In particular, alvinellacin killed 100% of the two most abundant morphotypes within this group (types 6 and 7). In contrast, the presence/absence of alvinellacin on epibionts types 1 and 4 did not have distinguishable effects and other epibionts (types 2 and 3) are not affected by alvinellacin. Altogether, the data suggest that alvinellacin controls epibiosis by selectively killing the most dominant part of the filamentous bacteria found on the dorsal part of the worm.

That is reminiscent of the role of the defensin HD5 in shaping the composition of the symbiotic microflora of the digestive tract in...
mammals [41]. We hypothesize that high production of alvinellacin by epidermal cells selects and shapes the epibiotic microflora and prevents microbiota from over proliferating and subsequently penetrating the underlying tissue. To test this hypothesis, an accidental invasion was simulated by incubating coelomocytes with epibionts (Figure 4C). The distribution of alvinellacin-immune reactivity was compared by immunofluorescence in unchallenged versus challenged cells. Under basal conditions (t = 0), the AMP was strongly detectable inside the cells, suggesting that this active compound is stored after synthesis. One hour after the bacterial infestation, the immune staining inside the cells faded, evidencing that alvinellacin is secreted rapidly when the cells are challenged by microorganisms. The induction of transcription observed by RT-qPCR in the cells incubated for 12 h with epibionts probably contributes to the renewal of the alvinellacin peptide stock (Figure 3B). Overall, these

Figure 4. Alvinellacin is produced by tissues or cells in contact with epibionts. (A) Picture of Alvinella showing the distribution of epibionts. (B) Immunohistochemistry data evidence that alvinellacin peptide accumulates in tissue hosting epibionts i.e. the dorsal but not the ventral tegument. (C) Accidental entrance of epibionts stimulates the secretion of alvinellacin by circulating cells. Images of immunodetection of alvinellacin in coelomocytes incubated with epibionts. After one hour of exposure, the signal was reduced evidencing an extracellular secretion of the peptide. Control is performed with preimmune serum 1: FITC fluorescence, 2: transmission, 1+2: overlay.

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results reinforce the role of alvinellacin in keeping symbionts under control.

**Conclusion**

Altogether, the data indicate the production of an original AMP from a deep-sea animal that endorses a durable relationship with Epsilonproteobacteria and possibly archaea in the face of the hostile vent habitat. Alvinellacin appears to act as a first line of defence against microbial invasion. The specificity of the gene induction along with the selective anti epibiotic activity and the expression in tissues exposed to the environment suggest that alvinellacin is actively participating in the surveillance of the epibiotic community. The conservation of the proregion and the gene structure of alvinellacin with AMPs of coastal annelids, suggest a common origin of the molecules. To draw a decisive conclusion regarding the gene evolution of alvinellacin, we plan to search for related genes in more than 30 annelid species living in various habitats. Such phylogenetic analysis will aim at determining whether the amino acid sequences of the antimicrobial part of the precursor diverged between species in order to face (i) contrasted temperatures, (ii) different microbial environments and/or (iii) to allow the establishment of epibioses.

**Supporting Information**

**Figure S1** Alvinellacin purification and molecular identification. Material eluting at 60% acetonitrile (ACN) upon solid phase extraction was loaded onto a C18 column (250×4 mm, Vydac). Elution was performed with a linear gradient of acetonitrile in acidified water (dotted line), and absorbance was monitored at 225 nm. Each individually collected fraction was tested for its antimicrobial activity (white bar) and its immunoreactivity to the alvinellacin Ab by DIA (grey bar). Fractions containing antimicrobially active alvinellacin were further purified by two additional RP-HPLC purification steps. Asterisk shows the active final fraction containing alvinellacin.

**Figure S2** MS spectrum of native alvinellacin. Analysis of purified alvinellacin by MALDI TOF-MS shows a m/z value of 2,600.35 MH+ which perfectly matches the theoretical mass of the peptide including two disulfide bonds.

**Figure S3** Sequence alignments of the precursors of alvinellacin, capitellacin, and two arenicin isoforms.

**Figure S4** Intact protein MS spectrum of alvinellacin measured by nanoESI-Orbitrap MS. (A) Full range MS survey spectrum. (B) Zoom-in of the [M+5H]5+ charge state species in a. A small species (indicated as asterisk) found next to the major component was identified as the methionine oxidation product of alvinellacin. The experimentally determined monoisotopic MW of alvinellacin was 2,599.2221 Da. (C) Display of theoretical MW (2,599.2067 Da) of alvinellacin and its isotope distribution at charge state 5. The results indicated that all four cysteines are involved in the formation of disulfide bonds.

**Figure S5** Time-course analysis of the proteolytic cleavage of alvinellacin. The products of alvinellacin digestion were analyzed by nanoESI-Orbitrap MS. (A) Peptide MS survey spectra of alvinellacin digested with Lys-C at 35°C (overnight). (B) Subsequent digestion of the Lys-C-digest with trypsin after
Table S1 Structural statistics for the 10 best structures of alvinellacin showing the lowest target functions. None of the distance constraints was violated by more than 0.5 Å in any structure.

Table S2 Disulfide-connected peptide fragments of alvinellacin observed after proteolytic cleavage. Peptides with oxidized cysteines were successively digested using the proteases Lys-C and trypsin. The resulting peptides were analyzed by offline nanoESI-Orbitrap MS/MS as shown in Figure S2. The results unambiguously indicated two disulfide linkages between C1-C4 and C2-C3.

References


Acknowledgments

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Author Contributions

Conceived and designed the experiments: A. Tasiemski ML, JG SJ FG. Performed the experiments: A. Tasiemski CBW DJ VCH SJ CV CWH A. Tholey CG ML, JG FDS OH. Analyzed the data: A. Tasiemski SJ CV DJ ML. Contributed reagents/materials/analysis tools: FP. Wrote the paper: A. Tasiemski SJ CV DJ ML.

Material and Methods S1

(PDOCX)

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