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Innate Immunity

ISOLATION OF SEVERAL CYSTEINE-RICH ANTIMICROBIAL PEPTIDES FROM THE BLOOD OF A MOLLUSC, *MYTILUS EDULIS**

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We have isolated from the blood of immune-challenged and untreated mussels (*Mytilus edulis*) antibacterial and antifungal peptides. We have characterized two isoforms of a novel 34-residue, cysteine-rich, peptide with potent bactericidal activity and partially characterized a novel 6.2-kDa antifungal peptide containing 12 cysteines. We report the presence of two members of the insect defensin family of antibacterial peptides and provide a phylogenetic analysis that indicates that mollusc and arthropod defensins have a common ancestry. Our data argue that circulating antimicrobial peptides represent an ancient host defense mechanism that predated the separation between molluscs and arthropods at the root of the Cambrian, about 545 million years ago.

The antimicrobial defense of molluscs has attracted surprisingly little attention in view of the economic interest of many representatives of this phylum. Although generally considered to be capable of mounting an efficient host defense, molluscs are obviously susceptible to several microbial pathogens, as illustrated by the frequent waves of lethal diseases affecting populations of oysters and mussels, for instance. The current view holds that molluscs rely predominantly on cellular defense reactions in which invading microorganisms are either phagocytosed or encapsulated by blood cells or lymph cells. The presence of macromolecules with antimicrobial activity has been reported from the mucus of the giant snail *Achatina fulica* (the 150-kDa achacin; Refs. 1–3) and from egg mass and purple fluid of the sea hare, *Aplysia kurodai* (4, 5). Remarkably though, molluscs had not been reported, to date, to contain in their blood antimicrobial peptides to fight off bacteria and fungi. Such molecules are one of the hallmarks of the antimicrobial host defense of arthropods (for review, see Refs. 6 and 7), and they play a significant role in the survival against invading bacteria and fungi in higher insects, as illustrated recently for *Drosophila* (8). It was unclear whether the absence of any report on such molecules in molluscs reflected the lack of, or inaccurate, research in this field, or whether indeed molluscs had developed basically different strategies for their antimicrobial defense.

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We were intrigued by this situation, and in the course of a broad range phylogenetic study on the antimicrobial peptides in invertebrates, one of us (S. C.) collected specimens of the lamellibranch *Mytilus edulis* at the Zoological Outstation of the St. Petersburg University on the White Sea. Applying the same methodologies that we have used for isolating antimicrobial peptides from insects, we were able to detect in the blood of control and bacteria-challenged mussels the presence of several antibacterial and antifungal peptides. We report here the characterization of two types of small sized, cationic, cysteine-rich peptides; one of these, which we name mytilin, is a 34-residue, novel peptide with potent bactericidal activity. The other is clearly a member of the large family of 35–43-residue, cationic, cysteine-rich peptides which we had isolated initially from immune-reactive larvae of the fleshfly *Phormia terranovae* and named insect defensin (9; for sapecin, a synonym for insect defensin, see also Ref. 10). These peptides are widespread among insects and scorpions. We also report the partial characterization of a novel cysteine-rich antifungal peptide from the blood of *M. edulis*. Our data strongly argue that circulating antimicrobial peptides represent an ancient host defense mechanism that predated the separation between the phyla of arthropods and molluscs, believed to have occurred at the root of the Cambrian, *i.e.* 545 million years ago.

MATERIALS AND METHODS

Animals and Blood Collection

Adult mussels (*M. edulis*) were collected from a White Sea natural population in the North Karelia region (Russia). Molluscs were immune challenged as follows. The valves were opened gently, and a needle, dipped previously into a suspension of live *Escherichia coli* D31, served for repeated pricking (five to seven times) into the anterior adductor muscle, the mantle, and the gonads. After challenge, the mussels were returned to the sea. After 4 days, blood was collected (approximately 1 ml/animal) with a syringe from the hemal sinus. Blood was supplemented with aprotinin (5 μg/ml) and frozen until use.

In a control experiment, a blood sample from unchallenged *M. edulis* was prepared in the same conditions.

Microrganisms

Bacteria—The bacterial strains were those used in previous studies (11, 12) plus the following marine bacteria: *Alteromonas carrageenovora* (ATCC 43555), *Pseudomonas alginovora*, and *Cytophaga drobachiensis* (gifts from T. Barbeyron, Station Biologique, Roscoff, France).

Fungi—The filamentous fungi *Neurospora crassa* (CBS 327-54) and *Fusarium culmorum* (IMI 180420) were a gift from W. F. Broekaert (Janssens Laboratory of Genetics, Catholic University of Leuven, Heverlee, Belgium). They were grown on a five-cereal medium, and spores were harvested as described previously (13).

Antimicrobial Assays

Antibacterial Assay and Determination of the Minimal Inhibitory Concentration (MIC)[1]—During the various steps of peptide purification, antibacterial activity was monitored by a liquid growth inhibition assay on the Gram-positive strain *M. luteus* and on the Gram-negative strain *E. coli* D31 in the conditions described previously (14).

The MIC values are expressed according to Ref. 15 as an interval (a–b), where (a) represents the highest peptide concentration tested at which bacteria are still growing and (b) the lowest concentration that causes 100% growth inhibition.

Control antibiotic peptides, MSI-94 (a broad spectrum linear amphipathic peptide derived from magainin; Ref. 16) and PGLa (a naturally occurring antibiotic from frog; Ref. 16) were gifts from M. A. Zasloff (Magainin Pharmaceuticals, Plymouth Meeting, PA).

Bactericidal Assay—10 μl of purified peptide, at a concentration of 10 times the MIC, was mixed with 90 μl of an exponential phase culture of *M. luteus* at a starting OD_{600} = 0.01. 10-μl fraction aliquots were incubated separately for different time intervals at 25 °C and plated on nutrient agar. The number of colony-forming units was counted after an overnight incubation at 37 °C. As control, 10 μl of water was incubated with the bacterial culture.

All antibacterial assays were performed in Poor Broth nutrient medium (PB: 1% BactoTrypton, 0.5% NaCl, w/v; pH 7.5) except for marine bacteria assays, which were performed in Zobell's medium (0.5% BactoTrypton, 0.1% yeast extract, w/v, in water containing 450 mM NaCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, and 5 mM KCl).

Antifungal Assay—Antifungal activity against *N. crassa* was detected by a liquid growth inhibition assay as described by Fehlbaum and associates (11).

Peptide Extraction

The blood of *M. edulis* (40 ml from bacteria-challenged mussels, 10 ml from controls) was diluted (v/v) with a solution of 0.1% trifluoroacetic acid in the presence of aprotinin (1.5 μM, final concentration) and phenylthiourea (20 μM, final concentration). Acidic extraction was performed for 30 min in an ice-cold water bath under gentle stirring, and the extract was centrifuged at 10,000 × *g* for 20 min at 4 °C.

Peptide Purification

Step I: Solid Phase Extraction—After centrifugation, the acidic supernatant was loaded onto a 12-ml Sep-Pak Vac C_{18} (Waters Associates) equilibrated with acidified water (0.05% trifluoroacetic acid). Stepwise elutions were performed with solutions of 5, 50, and 80% acetonitrile in 0.05% trifluoroacetic acid. The eluted fractions were concentrated under vacuum (Speed Vac concentrator) and reconstituted in MilliQ water.

Step II: Reversed Phase HPLC—As the bulk of the antimicrobial activity was detected in the 50% solid phase extraction fraction, only this fraction was submitted to reversed phase HPLC on an aquapore OD 300 column (4.6 × 220 mm, Brownlee). Elution was achieved with a linear gradient of 5–55% acetonitrile in 0.05% trifluoroacetic acid over 90 min at a flow rate of 1 ml/min. The absorbance peaks were hand collected, dried under vacuum, and reconstituted in MilliQ water. The presence of antibacterial and antifungal activities was detected by the liquid growth inhibition assays described above.

Step III: Gel Permeation HPLC—The active fractions were analyzed by gel permeation HPLC using two serially linked columns (Ultraspherogel SEC 3000 and SEC 2000 columns, 7.5 × 300 mm, Beckman). Elution was performed under isocratic conditions with 30% acetonitrile in 0.05% trifluoroacetic acid at a flow rate of 0.5 ml/min. All the fractions recovered were treated as in step II.

Final Purification Steps—For the final purification of the active compounds, the selected fractions were analyzed on the same reversed phase column as in step II, using adapted diphasic linear gradients of acetonitrile in 0.05% trifluoroacetic acid at a flow rate of 1 ml/min. The gradient used for *Mytilus* defensins was from 2 to 14% over 10 min and from 14 to 24% over 40 min. Mytilins were purified by a gradient from 2 to 15% over 10 min and from 15 to 25% over 40 min. For the *Mytilus* antifungal peptide, the acetonitrile gradient was from 5 to 19% over 10 min followed by a 40-min gradient of acetonitrile from 19 to 29%.

To ascertain the purity of the peptides, an additional step was performed on a narrow bore reversed phase column (Delta Pak HPI C_{18} PEEK, 2 × 150 mm, Waters Associates) developed in the same diphasic gradients as above but at a temperature of 40 °C instead of 25 °C and at a flow rate of 0.25 ml/min.

All HPLC purifications were performed using Waters HPLC systems (model 510) equipped with tunable absorbance detectors (model 481). The column effluent was monitored by absorbance at 225 nm. For the purification step at 40 °C, a Waters 626 pump system was used.

Capillary Zone Electrophoresis

The purity of the peptides was ascertained further by capillary zone electrophoresis. Analysis was conducted on a model 270A-HT capillary electrophoresis system (Applied Biosystems) equipped with a fused silica capillary as described previously (17).

Structure Determination

Matrix-assisted Laser Desorption/Ionization-Time of Flight-Mass Spectrometry—Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry was performed on a Bruker Biflex (Bremen, Germany) mass spectrometer operating in a positive linear mode as described previously (17).

Reduction and S-Pyridylethylation—1–2 nmol of purified peptide was submitted to reduction and alkylation. The procedure was that used in a previous paper from this laboratory (18).

Microsequence Analysis—Automated Edman degradation of the native and pyridylethylated peptides and detection of phenylthiohydantoin derivatives were performed on a pulse liquid automatic sequenator (Applied Biosystems, model 473A).

RESULTS

Isolation of Several Antimicrobial Peptides from the Blood of M. edulis

Blood was collected from bacteria-challenged and untreated *M. edulis*. Cells and debris were removed by centrifugation, and the supernatant was acidified and applied on a Sep-Pak Vac C_{18} column. Elution was performed sequentially with 5, 50, and 80% acetonitrile in 0.05% trifluoroacetic acid. Antibacterial (anti-*M. luteus* and anti-*E. coli*) and antifungal (anti-*N. crassa*) activities were only found in the 50% acetonitrile fractions (data not shown) from both challenged and untreated mussels. The active molecules of the 50% eluate were submitted to reversed phase HPLC and eluted with a gradient of 5–55% acetonitrile, yielding the chromatogram shown in Fig. 1. No clear-cut differences were observed between the chromatograms from bacteria-challenged and control mussels (data not shown). Moreover, the same fractions from both samples were found to contain the antibacterial and antifungal activities, indicating that a bacterial challenge did not induce any new antimicrobial substances in the blood of mussels. Antibacterial activity, both against *M. luteus* and *E. coli*, was present in six distinct zones in the chromatogram (AB1 to AB6), and antifungal activity was recorded in two peaks (AF1, AF2) (Fig. 1), indicating that mussel blood contains a complex spectrum of antimicrobial molecules. The present study focuses on the substances eluted between 17 and 23% acetonitrile in the gradient. The active compounds present in the fractions AB1, AB2, AB3, and AF1 were subjected to gel permeation HPLC followed by two successive purification steps by reversed phase HPLC. Antimicrobial activity was monitored from aliquots of the fractions, as above. Five molecules (two in fraction AB1) were purified to apparent homogeneity as judged by capillary zone electrophoresis (data not shown) and subjected to chemical characterization.

Characterization of Isolated Antimicrobial Peptides

Cysteine-rich Peptides with Sequence Similarity to Arthropod Defensins—From the large fraction AB1 (Fig. 1), two antibacterial peptides (approximately 10 μg of each) were purified to homogeneity. Mass measurements of the two active molecules yielded two closely related masses, 4314.3 and 4392.4 Da. The fact that only one mass was obtained for each of these com-

[1] The abbreviations used are: MIC, minimal inhibitory concentration; HPLC, high performance liquid chromatography; AB, antibacterial; AF, antifungal.

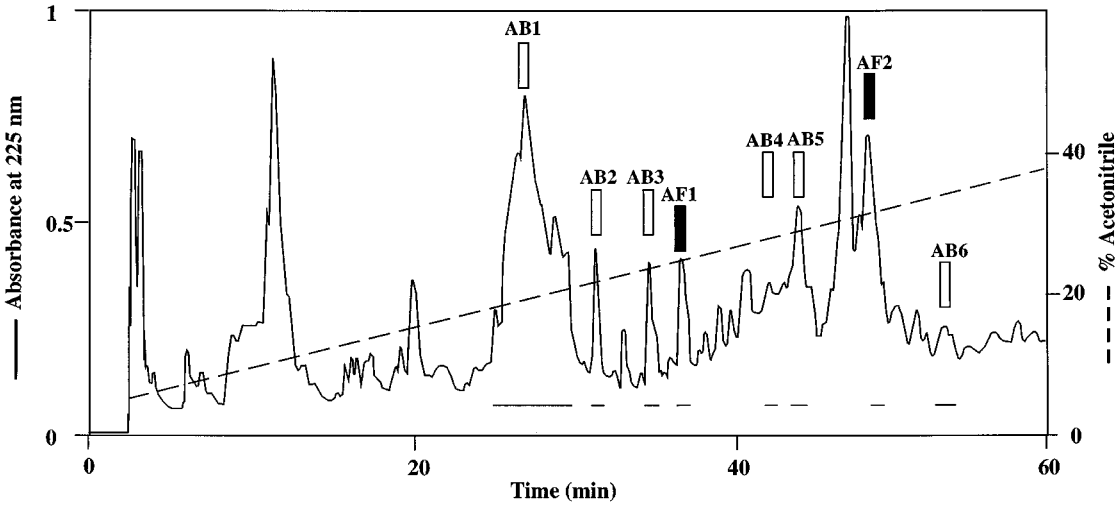

FIG. 1. **Reversed phase HPLC separation of immune blood of *M. edulis* (step II).** After prepurification by solid phase extraction, the active material present in the fraction eluted with 50% acetonitrile was analyzed on an Aquapore OD 300 column. Elution was performed with a linear gradient (*dotted line*) from 5 to 55% acetonitrile in acidified water (0.05% trifluoroacetic acid) over 90 min at a flow rate of 1 ml/min. Absorbance peaks were monitored at 225 nm (*full line*). Antimicrobial activities were detected by liquid growth inhibition assays; antibactericidal activities (*white column*) against *M. luteus* and *E. coli* D31; antifungal activity (*black column*) against *N. crassa*. To facilitate subsequent purifications, adjacent active peaks were combined into larger fractions; the *lines* under the chromatogram represent the fraction size.

pounds provides an additional demonstration for their purity. Next, we submitted each peptide (1 nmol) to sequencing by Edman degradation. The 4314.3-Da peptide, referred to as peptide A, consists of 37 residues including six cysteines, the identity of which was ascertained by pyridylethylation (Fig. 2*a*). No phenylthiohydantoin amino acids were observed at positions 21 and 28 on both native and pyridylethylated peptides. For the 4392.4-Da peptide (peptide B), a 35-residue NH_2-terminal sequence was obtained including also six pyridylethylated cysteines (Fig. 2*a*), whereas no signal was observed at position 28. Peptides A and B were very close in sequence and show a high degree of similarity with arthropod defensins, a large family of cysteine-rich cationic peptides. The positions of the cysteines in this family are highly conserved, and their array is identical with that of peptides A and B (Fig. 2*a*). This strengthens the hypothesis that the mussel peptides are true evolutionary homologs of arthropod defensins. Peptides A and B are therefore bonafide members of this family, and we will refer to them as *Mytilus* defensins A and B in Fig. 2*a*.

In peptide A, neither residue 21 nor 28 corresponds to a cysteine. Assuming that the six cysteines are engaged in three intramolecular disulfide bridges, the mass difference (391.5 Da) between tha measured mass (4314.3 Da) and the calculated mass (3922.8 Da) could not account for any combination between two conventional amino acids (highest mass for two tryptophan residues, 372 Da). The most probable explanation is that the amino acids in these positions are modified or carry a substitution. Such a situation would be unique among the defensins isolated so far. The elucidation of this problem will call for large scale preparation of native material and the use of additional physicochemical methods. A similar situation is observed for residue 28 in peptide B. For this peptide, the sequence determination by Edman degradation after pyridylethylation gave no signal after residue 35, and in view of the sequences of the other members of the defensin family (including peptide A) we assume that peptide B has at its COOH terminus several additional residues, which would account for the relatively higher mass of this peptide.

In the purification assays, *Mytilus* defensins A and B were consistently more active against *M. luteus* than *E. coli*.

Novel Cysteine-rich Peptides: Mytilins—The active compound present in fraction AB2 (Fig. 1) was purified to homogeneity (approximately 55 μg) using a four-step procedure (see "Materials and Methods"), and 500 pmol was sequenced by Edman degradation. The following sequence of 33 residues was obtained: G*X*ASR*X*KAK*X*AGRR*X*KGWASASFRGR*XYXKX*FR. A mass of 3773.7 Da was determined by mass spectrometry. Assuming that the seven blanks in the sequence corresponded to cysteines, we have submitted 2 nmol of the native peptide to reduction and alkylation with 4-vinylpyridine. The product of the reaction was analyzed both by mass spectrometry and sequencing. The mass of the pyridylethylated peptide (4623.5 Da) exceeded that of the native peptide by 849.8 Da, which corresponds approximately to the mass of eight pyridylethyl groups added to reduced cysteines. Sequencing by Edman degradation of the pyridylethylated peptide yielded a sequence of 34 residues: the 33 NH_2-terminal amino acids were identical to those identified in the previous sequencing experiment on the native molecule (see above) and included seven cysteines. In addition, a COOH-terminal cysteine was detected in the pyridylethylated peptide, giving a total of eight cysteines (Fig. 2*b*). The mass calculated from the primary structure data is 3781.5 Da, in excess of 8 Da to that obtained by mass measurement of the native peptide (3773.7 Da). This indicates that the eight cysteines are engaged in the formation of four intramolecular disulfide bridges. A search in the peptide sequence data bases did not yield any homology with known peptides. We propose the name of mytilin for this novel 34-residue, cationic (calculated pI: 10.4), cysteine-rich antibacterial peptide.

Using the same strategy, we have isolated and fully characterized from fraction AB3 (Fig. 1) an isoform of mytilin with a mass of 3974.3 Da. This isoform consists also of 34 residues and has eight cysteines engaged in four intramolecular disulfide bridges. Thirteen residues are distinct between the two isoforms, which we will refer as mytilin A (3773.7 Da) and B (3974.3 Da) (Fig. 2*b*).

Partial Characterization of an Antifungal Peptide: Mytimycin—We have next analyzed the more hydrophobic compound present in fraction AF1 (Fig. 1). Approximately 25 μg of pure material was obtained by a four-step purification procedure (see "Materials and Methods"). Mass spectrometry measurements yielded a single mass of 6233.5 Da. After reduction and alkylation of 2 nmol of peptide, the mass increased to 7533.4 Da, indicating the possible presence of 12 cysteines. The pyri-

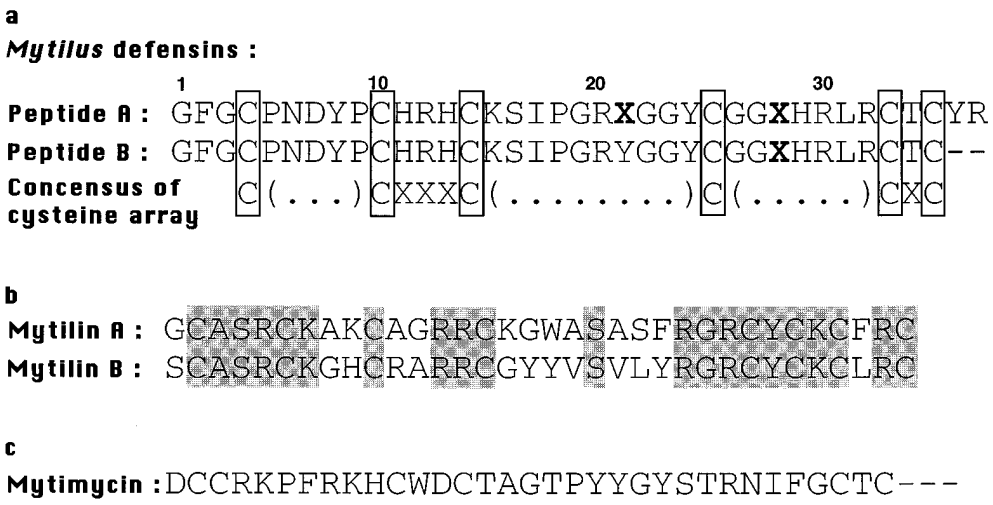

FIG. 2. **Amino acid sequences (in one-letter code) of the antimicrobial peptides isolated from *M. edulis*.** *Panel a*, *Mytilus* defensins A and B (cysteines are *boxed* with the consensus cysteine array deduced from arthropod defensins; unidentified residues after derivatization are in *bold* characters). *Panel b*, mytilins A and B (identical residues are *boxed*). *Panel c*, *Mytilus* antifungal peptide referred to as mytimycin (partial NH_2-terminal sequence).

dylethylated peptide was sequenced by Edman degradation, and a 32-residue NH_2-terminal partial sequence was obtained (Fig. 2*c*), corresponding to roughly half of the sequence. We did not detect any homology with reported peptide sequences in protein data bases. This peptide delays the growth of *N. crassa* and *F. culmorum*. We will refer to it as mytimycin.

The active molecules were all found in the blood of unchallenged mussels at similar concentrations (see "Discussion"). They were purified as above, and their identities were confirmed by mass spectrometry measurements (data not shown).

Activity Spectrum and Mode of Action of Mytilin A on Bacteria

Activity Spectrum—The quantity of mytilin A (14 nmol) extracted in this study was sufficient to investigate the activity spectrum of this peptide against a variety of bacterial strains (Table I). In the liquid growth inhibition assay, mytilin A showed a marked activity against the following Gram-positive strains: *Aerococcus viridans*, *Bacillus megaterium*, and *Micrococcus luteus* (MIC: 0.6–1.2 μM); and *Enterococcus faecalis* and *Staphylococcus aureus*, (MIC: 2.5–5 and 5–10 μM, respectively). Mytilin A had a similar effect on the two Gram-negative strains *E. coli* D31 (MIC: 2.5–5 μM) and *E. coli* D22 (MIC: 5–10 μM) and also affected the marine species *A. carrageenovora*, *P. alginovora*, and *Cytophaga drobachiensis* (MIC: 2.5–5 μM). The other strains tested (*E. coli* 1106 and *Salmonella typhimurium*) were not affected, even at a concentration of 10 μM.

Bactericidal Assay—*M. luteus* was incubated at a starting OD_{600} = 0.01 in the presence of mytilin A at a concentration 10 times higher than the MIC value. Incubations of serial aliquots were performed for 1, 3, and 10 min and 1, 3, and 6 h, after which the bacteria were plated on nutrient agar and the colony-forming units counted after 24 h. The results (data not shown) were clear-cut in that a 1-min period of incubation in the presence of mytilin A was sufficient to kill all bacteria. Mytilin A appears thus as a potent and rapid bactericidal compound.

Phylogenetic Analysis of Defensins

The unexpected discovery of defensin homologs in the blood of a mollusc prompted us to undertake a phylogenetic analysis of the defensin family. For this, we have hand-aligned a large array of defensins using the six cysteines as landmarks. The aligned sequences were submitted to analysis using MUST and PAUP software (19, 20). Only a subsample of the sequences including the new mussel defensins was used in the present work. Both distance (Fig. 3) and parsimony trees (not shown) displayed three salient features.

TABLE I
Activity spectrum of mytilin A compared with two control antibiotic peptides (PGLa and MSI-94)

The minimal inhibitory concentration is expressed in μM.

Bacteria	MIC		
	Mytilin A	PGLa	MSI-94
		μM	
Gram-positive bacteria			
M. luteus	0.6–1.2	0.3–0.6	0.5–1
A. viridans	0.6–1.2	0.6–1.2	0.5–1
B. megaterium	0.6–1.2	0.6–1.2	0.5–1
E. faecalis	2.5–5	>52	4–40
S. aureus	5–10	0.6–1.2	1–2
Gram-negative bacteria			
E. coli D31	2.5–5	1.2–2.4	1–2
E. coli D22	5–10	1.2–2.4	1–2
E. coli 1106	>10	0.6–1.2	1–2
S. typhimurium	>10	5–52	2–4
A. carrageenovora	2.5–5	>52	5–10
P. alginovora	2.5–5	>52	10–20
C. drobachiensis	2.5–5	>52	5–10

On an unrooted tree, the sequences were split into two clearly distinct sets, the split being supported by very high bootstrap values (22). First, the two mussel sequences and those of the scorpion and the paleopteran insect *Aeschna* (dragonfly) (for sequence information, see Fig. 4) grouped in one set, while all defensins from neopteran insects grouped in the other (Hemiptera, Coleoptera, Diptera and Hymenoptera). Second, the branch length of the first set of species was short compared with that of the second, indicating strong differences in evolutionary rates. It can be seen on the tree, for example, that the distance separating the dragonfly defensin sequence from that of the mollusc *Mytilus*, or the scorpion, *i.e.* organisms belonging to different phyla, is smaller than that separating *Drosophila* from *Aedes*, *i.e.* species belonging to two orders of dipteran insects. Third, there was no universal pattern of grouping *versus* dispersal of the various sequences belonging to a single species. Thus, the *Aedes* sequences for example were tightly grouped, suggesting either recent diversification or repeated homogenization, whereas the *Sarcophaga* sequences were spread all over the tree. It is impossible to clearly identify orthologous genes, *i.e.* to locate the various duplication events

over the branches of the tree, because the gene sampling (both within genome and within organism) and the resolving power of molecular phylogeny (see below) are poor.

The detailed topology within the Neoptera should be taken with reservation for two reasons. First, the sequences are quite short, and second, they are substantially saturated (*i.e.* they have accumulated multiple substitutions at the same site during the course of evolution), a process that reduces the resolving power of molecular data (see Ref. 23). Nonetheless, it is worth noting that the order of emergence of taxa within that set of species is broadly in agreement with traditional insect phylogeny, Hemiptera emerging at the basis of this set, followed by Coleoptera and subsequently by the dipteran/hymenopteran clade.

DISCUSSION

The data presented in this report establish for the first time that the blood of mussels contains marked antimicrobial activity that is related to the presence of several antibacterial and antifungal molecules. Remarkably, these molecules appear to be constitutively present. Although in this regard, our study has to be considered as preliminary, we have no indication that pricking the mussels with a bacteria-soaked needle has modified the spectrum or the concentration of the antimicrobial molecules. This situation contrasts with that observed in the higher insect orders, where the synthesis of antimicrobial peptides is rapidly and transiently induced by an immune challenge. Interestingly though, the constitutive presence of antimicrobial peptides in the blood is also observed in scorpions, which represent a group of arthropods which has appeared about 150 million years before the higher insects. It is tempting to propose that the constitutive presence of antimicrobial peptides in the blood is an ancient situation in the invertebrate host defense. It certainly necessitates the continuous synthesis of these peptides, which are presumably degraded in the blood by proteases with a short half-life time, and is thus energy-consuming. However, the constitutive presence of these peptides does not require the existence of a complex recognition system of microorganisms nor regulatory cascades leading to the rapid transcription of antimicrobial peptides genes upon septic injury. We may speculate that such a sophisticated system has evolved later in evolution and provided the advantage that antimicrobial molecules are produced only at times of septic injury. A broader analysis of the presence, whether constitutive or inducible, of antimicrobial peptides in representative species of other invertebrate classes and phyla will certainly be rewarding in this respect.

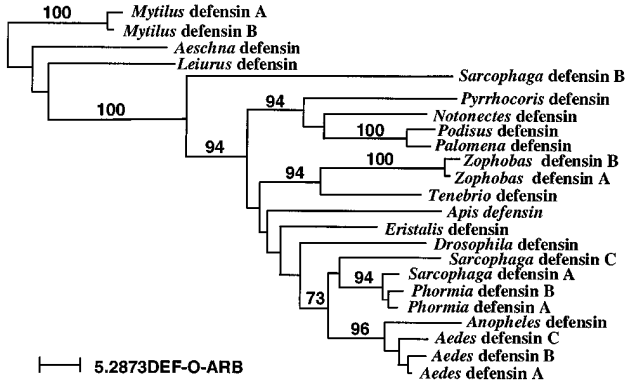

FIG. 3. **Distance tree of defensin sequences using the neighbor joining method.** The tree was rooted on the mussel sequences. The *numbers* above the branches indicate the percentage of times the species, which are to the *right*, grouped together in the bootstrap trees. The *scale bar* indicates the frequency of amino acid differences.

Although our inventory of the antimicrobial peptides in the blood of *M. edulis* is as yet incomplete, it shows that mussels, like insects, produce both antibacterial and antifungal peptides. Whereas the first antibacterial peptides were characterized in insects 15 years ago, it has only recently been appreciated that insects also produce antifungal peptides. Only three inducible antifungal peptides have been characterized so far in this class, namely drosomycin and metchnikowin, both from *Drosophila* (11, 24) and thanatin from *Podisus* (25). They show no sequence similarity with the antifungal peptide isolated in this study, at least in the 34 NH_2-terminal residues that were identified. The antifungal peptide mytimycin is rich in cysteine residues (12 for a total molecular mass of 6.2 kDa) and certainly adopts a very compact structure. It exhibits a marked activity against the two filamentous fungi tested; we have not yet had the opportunity to test this molecule against other fungi for lack of biological material.

The four antibacterial peptides characterized in this study are cysteine-rich, cationic, small sized peptides. Two are isoforms of defensins, and two are isoforms of a novel type of antibacterial peptides, which we refer to as mytilins.

Mytilus Defensins—Nearly 30 defensins have now been isolated and fully or partially characterized from various arthropod sources (for review, see Ref. 6). These molecules are present in scorpions (Chelicerata), in the ancient insect orders of the Paleoptera, and in the recent insect orders of the Hemiptera, Hymenoptera, Coleoptera, and Diptera. They all have six cysteines positioned in a highly conserved array which allows for a complex three-dimensional structure worked out in detail for the defensin of *P. terranovae* (26, 27). *Phormia* defensin consists of a central amphipathic α-helix with an extended NH_2-terminal loop and a COOH-terminal antiparallel β-sheet. The helix is stabilized via two disulfide bridges to the β-sheet, and the NH_2-terminal loop is linked to one of the strands of this sheet via the third disulfide bridge. The cysteine consensus motif is present in all hitherto characterized arthropod defensins and, as illustrated in Fig. 4, is identical to that found in *Mytilus* defensins. Within the large family of arthropod defensins, the mussel defensin A is strikingly close to that of the dragonfly *Aeschna cyanea* and the scorpion *Leiurus quinquestriatus*. The sequences of the three peptides are identical to 75%, using the Clustal method with the PAM250 residue weight table. When the defensins of *Mytilus*, *Aeschna*, and *Leiurus* are aligned against that of *Phormia*, for which the three-dimensional structure is known, a gap has to be introduced in the NH_2-terminal part, which corresponds to the extended loop of *Phormia* defensin. Assuming that the three-dimensional structure of *Mytilus* defensin is basically similar to that of *Phormia*, we can propose that the NH_2-terminal loop is shorter than in the case of *Phormia* defensin.

FIG. 4. **Comparison of *Mytilus* defensin A (in one-letter code) with *Aeschna* defensin (18), *Leiurus* defensin (28), and *Phormia* defensin (9).** The sequences are aligned for similarities. Identical and conservative residues are *boxed*, and *gaps* are introduced for optimal alignment. The unidentified residues are in *bold* characters.

The phylogenetic analysis of a subset of defensin sequences (Fig. 3) revealed one striking fact: the sequences group into two sets, one slowly evolving contains the mussel, scorpion, and dragonfly as representatives; and the other, fast evolving, contains all of the neopteran insects. It is interesting to note that the slow evolving set corresponds to species that have predominantly constitutively expressed defensins (as shown here for the mussel), whereas all of the fast evolving sequences of the second set are of the inducible type. The defensins of neopteran insects thus have functional constraints that are different from those of other protostomians, and it may be speculated that the regulated gene expression has allowed these specializations.

Mytilins—These molecules are remarkably rich in cysteine residues with respect to their relatively small size (eight residues for a mass of 3.7 kDa), which leads to the assumption that their three-dimensional structure is highly compact. The connectivity of the disulfide bridges has still to be worked out, but given the cysteine array in the primary sequence, it is clearly different from those of the other hitherto characterized cysteine-rich antimicrobial peptides from arthropods (insect defensins, tachyplesin, drosomycin, thanatin) (6, 25), frogs (brevinins) (29), mammals (mammalian defensins, protegrins) (30), or plants (plant defensins, γ-thionins) (31). It is of interest to note that mytilin A has a rapid bactericidal activity, which is evocative of the action of insect defensins from the fleshfly *P. terranovae*. The latter molecules disrupt within seconds the permeability barrier of the cytoplasmic membrane of Gram-positive bacteria, resulting in a partial depolarization, a decrease in cytoplasmic ATP, an inhibition of respiration, and a rapidly ensuing death (32).

The concentration of mytilins (isoform A plus isoform B) in the blood of mussels can be estimated at approximately 2 μM, assuming a 50% recovery in our purification procedures. This is in the range of the MIC determined for most of the bacteria that we tested. As is the case for defensins, mytilins appear somewhat more active against Gram-positive bacteria than against Gram-negative cells.

In conclusion, our study indicates that the host defense of molluscs, like that of arthropods, involves the synthesis of both antibacterial and antifungal, small sized, cationic peptides. Interestingly, some of these, the defensins, appear to have a common ancestry, and we speculate that they have evolved from a protodefensin existing already at the root of the Cambrian explosion.

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