Cloning of mRNA Sequences for Two Antibacterial Peptides in a Hemipteran Insect, Riptortus clavatus

Authors: Miura, Ken, Ueno, Satoshi, Kamiya, Katsumi, Kobayashi, Jun, Matsuoka, Hiroyuki, et al.

Source: Zoological Science, 13(1) : 111-117

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.13.111

ZOOLOGICAL SCIENCE **13**: 111–117 (1996)

Cloning of mRNA Sequences for Two Antibacterial Peptides in a Hemipteran Insect, *Riptortus clavatus*

Ken Miura[1], Satoshi Ueno[1], Katsumi Kamiya[1], Jun Kobayashi[2], Hiroyuki Matsuoka[1], Katsuhiko Ando[1] and Yasuo Chinzei[1]

[1]*Department of Medical Zoology, School of Medicine and* [2]*Laboratory of Molecular Bioengineering, Faculty of Engineering, Mie University, Tsu 514, Japan*

ABSTRACT—*Escherichia coli* injection rapidly induced bactericidal activity in the hemolymph of a hemipteran insect, *Riptortus clavatus*. This activity reached its maximum at 9 hr after injection and thereafter declined slowly. Two types of cDNA clones involved in this response were isolated by differential screening. The predominant type encoded for an open reading frame of 678 amino acids, which consisted of fourteen tandem repeats. Each repeat was rich in charged residues and had a proline-rich region which had striking sequence similarities to proline-rich antibacterial peptides from other insect species, indicating these clones encode a multipeptide precursor of antibacterial peptides. The other type encoded for a glycine-rich peptide similar to a known antibacterial peptide as well. Northern blot analyses revealed rapid induction of mRNAs corresponding to these clones after the injection. To our knowledge, this is the first report on the mRNA sequences of antibacterial peptides of hemimetabolous insects, and the second report on the occurrence of multipeptide precursor structure in insect antibacterial peptides.

INTRODUCTION

Insects have both cellular and humoral self-defense systems to protect themselves against bacterial challenges. A well-characterized humoral defense is a rapid and transient synthesis of a battery of antibacterial peptides upon infection. Usually, these are small sized cationic peptides having bactericidal activity against a wide spectrum of bacteria, and their expressions are thought to be regulated at the transcriptional level (for review, see Boman *et al.*, 1991; Hultmark, 1993). Up to now several antibacterial peptides have been purified, and in some cases, characterized at the nucleic acid sequence level. These studies have been done almost exclusively in higher, holometabolous (showing complete metamorphosis) insects including Lepidoptera, Diptera, Hymenoptera and Coleoptera. Antibacterial peptides so far isolated from the higher insects can be classified as follows. i) The cecropins are about 4-kDa peptides and active against both gram-negative and gram-positive bacteria (Hultmark *et al.*, 1982; Qu *et al.*, 1982; Okuda and Natori, 1985; Dickinson *et al.*, 1988; Taniai *et al.*, 1992). ii) The insect defensins are 4-kDa in size, active against gram-positive bacteria and possess six cysteine residues engaged in three intramolecular disulfide bridges (Matsuyama and Natori, 1988; Lambert *et al.*, 1989; Fujiwara *et al.*, 1990; Bulet *et al.*, 1992). This class of peptides are absent in Lepidoptera. iii) Glycine-rich peptides such as attacins (Hultmark *et al.*, 1983), sarcotoxin II (Ando and Natori, 1988), diptericin (Dimarcq *et al.*, 1988) and coleoptericin (Bulet *et al.*, 1991) are active against a limited number of gram-negative bacteria. iv) Small sized proline-rich peptides, apidaecin (Casteels *et al.*, 1989) and abaecin (Casteels *et al.*, 1990) from honeybee and drosocin (Bulet *et al.*, 1993) from fruitfly are active against gram-negative bacteria. On the other hand, our knowledge about antibacterial peptides of hemimetabolous (showing incomplete metamorphosis) insects is quite limited. Recently Cociancich *et al.* (1994) have isolated and characterized three antibacterial peptides from a hemipteran insect, *Pyrrochoris apterus*: namely, the insect defensin of this species, a proline-rich peptide carrying an *O*-glycosylated substitution and a glycine-rich peptide. Experimentally, antibacterial peptides are induced rapidly in hemolymph following bacterial component injection or body wall injury. Increasing evidence suggests that this insect "immune" reaction is analogous to innate immunity in vertebrates, especially to the acute phase response. Recently, we have noticed that in the hemipteran insects, the bean bug, *Riptortus clavatus*, bactericidal activity can also be induced in the hemolymph by injection of formaldehyde-fixed *E. coli*. The present study was conducted in an attempt to directly identify genes involved in acute phase response of this species by a new approach, differential screening. In this paper, we describe cDNA cloning, sequence analysis and expression of two antibacterial peptide genes.

Accepted December 4, 1995
Received October 16, 1995

MATERIALS AND METHODS

Insects

The bean bugs, *Riptortus clavatus* were collected in Ishigaki Island, Okinawa pref. and reared at 25°C under a 16 hr light/8 hr

dark cycle. F_1 and F_2 adult females were used in all experiments. Bugs were injected with 1 μl of a suspension of formaldehyde-fixed *E.coli* LE392 (1.6×10^9 cells/ml in 0.9% NaCl) into the hemocoel while under CO_2 anesthetization. Bugs were kept at 25°C until dissection. Hereafter, insects treated as described above are designated as "immunized" insects. Hemolymph was collected and diluted 5-fold with 0.9% NaCl. Hemolymph samples were centrifuged at 12,000 rpm for 5 min, and the supernatant stored at −80°C until use.

Growth inhibition assay

Bactericidal activity in the hemolymph sample was estimated as follows. Five milliliters of 0.6% melted LB agarose (52°C) were mixed with 100 μl of viable *E. coli* LE392 suspension (1.6×10^9 cells/ml), and poured into a 9 cm plastic dish. Wells of 4 mm in diameter were punched after the agarose layer was solidified. Five microliters each of hemolymph sample was applied into the wells, and incubated at 37°C overnight. The samples containing bactericidal activity produced clear zones where the bacterial growth was not seen (growth inhibition zone). The area of the growth inhibition zone was measured. Since a liner relationship was observed between areas of growth inhibition zone and relative quantity of immunized hemolymph (data not shown), we concluded that this assay was able to be used to estimate bactericidal activity.

Extraction of RNA and construction of cDNA library

Total RNA was extracted from the fat body of naive or immunized female adults by AGPC method (Chomczynski and Sacchi, 1987). RNA preparations were measured colorimetrically. A260/A280 and A260/A230 of RNA preparations were always above 1.7 and 2.0, respectively. In some cases, the total RNA fraction was further purified to poly(A)$^+$ RNA by using oligo(dT)-latex (Japan Synthetic Rubber Co.). The cDNA library was constructed from poly(A)$^+$ RNA isolated from the fat body of the female adults 9 hr after immunization using a cDNA Synthesis Kit (Pharmacia) according to the instructions of the manufacturer. Oligo(dT)-primed synthesis of the first cDNA strand was done using Molony murine leukemia virus reverse transcriptase. RNase H and DNA polymerase I were used for second strand cDNA synthesis. The cDNA preparation was ligated to *Eco*RI-digested λgt10 arms (Stratagene) after the addition of *Eco*RI/*Not*I linkers and packaged *in vitro* using a commercial kit (Nippon Gene). *E. coli* NM514 cells were infected with recombinant phages, giving a titer of 1.72×10^7 pfu/μg RNA.

Differential screening

Single-stranded cDNA probes were prepared as described in Sambrook *et al.* (1989). One microgram each of fat body poly(A)$^+$ RNA from naive or immunized (9 hr) female adults was reverse-transcribed by random priming. The reaction mixture had a total volume of 25 μl of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 4 mM dithiothreitol, 0.8 mM each of dGTP, dATP and dTTP, 4.8 μM dCTP, containing [α-^{32}P] dCTP (100 μCi, ICN), RNAase inhibitor (40 U, TOYOBO), random hexamer (9 μg, BRL) and SuperScript reverse transcriptase (200 U, BRL). After 1 hr incubation at 37°C, the reaction was stopped by addition of EDTA (final conc. 20 mM) and sodium dodecyl sulfate (SDS, final conc. 0.8% w/v). The mixture was then incubated at 68°C for 30 min after the addition of 3 μl of 3N NaOH. The solution was neutralized and extracted with phenol : chloroform (1:1). The single-stranded cDNA preparation was purified by gel filtration (Nick column, Pharmacia). *E. coli* NM514 cells were infected with phages at 37°C for 20 min. The infected cells were mixed with melted LB top agarose, plated on LB plates and incubated at 37°C for 9 hr. A set of duplicate plaque lifts was taken onto nylon membrane (Hybond N+, Amersham) for each plate. The lifts were prehybridized for 1 hr at 68°C in a solution containing 5× Denhardt's solution, 5× SSPE, 10 μg/ml of denatured salmon sperm DNA, and 0.5% (w/v) SDS, and the subsequent hybridization step was done in the same solution supplemented with radioactive probes prepared either from naive or immunized insects at 68°C overnight. The lifts were washed sequentially : twice in 2×SSPE at 68°C for 20 min; once in 1×SSPE at 68°C for 20 min. After washing the lifts were autoradiographed with Fuji HR-S X-ray films using intensifying screens at −80°C overnight. Plaques giving differentially expressed signals were picked up, plated and screened again in the same way. Plaques giving positive signals were purified through the two cycles of screening. Phage DNA was purified by using a commercial kit (QIAGEN), and the insert cut out by *Eco*RI. The size of the cDNA insert was determined by agarose gel electrophoresis.

Sequencing and analysis of sequence data

The cDNA inserts having *Eco*RI overhangs on both ends were subcloned into Bluescript II SK+(Stratagene). Serial deletion mutants were made by exonuclease III followed by Mung bean nuclease using a commercial kit (Nippon Gene). Deletion mutants were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using a *Taq* dye-deoxy terminator cycle sequencing kit and an Applied Biosystem Model 373A sequencer (ABI Inc.). Editing and analysis of cDNA sequence data were performed with a commercial program (GENETYX Ver. 8.0, SDC Software Development Co.). DNA and Amino acid sequence homology searches were done by a fasta program through an e-mail server of DNA Data Bank of Japan.

Northern blot hybridization

Radioactive cDNA probes were prepared from cDNA inserts by using a cDNA multi-primer labeling kit (Nippon Gene) and [α-^{32}P]-dCTP (ICN). Five micrograms per lane of total RNA preparation were run on a 1.2% agarose-formaldehyde gel, then transferred onto nylon membrane (Hybond N+, Amersham) under alkaline conditions. The membrane was hybridized with a cDNA probe at 42°C in a solution of 50% formamide (v/v), 5 × SSPE, 5 × Denhardt's solution, and 10 μg/ml of denatured salmon sperm DNA (Sigma) following prehybridization. The membrane was washed twice at 68°C in 2 × SSPE for 20 min, once in 1 × SSPE for 20 min and finally in 0.1 × SSPE for 20 min, then autoradiographed with Fuji HR-S X-ray films using intensifying screens at −80°C overnight.

RESULTS AND DISCUSSION

Figure 1 shows the time course of bactericidal activity induction in the hemolymph up to the first 48 hr after immunization. In the hemolymph of naive insects (0 hr), bactericidal activity was negligible while it was first recognized in hemolymph at about 1 hr after injection. Growth inhibition zone increased in size sharply up to 9 hr, reached its maximum, then decreased slowly. The bactericidal activity was thermo-stable up to 90°C and destroyed completely by proteinase K treatment (data not shown), suggesting that the activity could be attributed to rather small, proteineous factors. This immediate induction represents an acute phase response in this insect. We attempted to isolate cDNA clones associated with this response using differential screening.

The time course of bactericidal activity induction in immune hemolymph suggested that the acute phase-related mRNA would accumulate in relatively large quantities in insects around 9 hr after injection. A cDNA library was constructed from fat body poly(A)$^+$ RNA of the insects 9 hr after injection. The library was screened differentially by using two sorts of single-stranded cDNA probes prepared

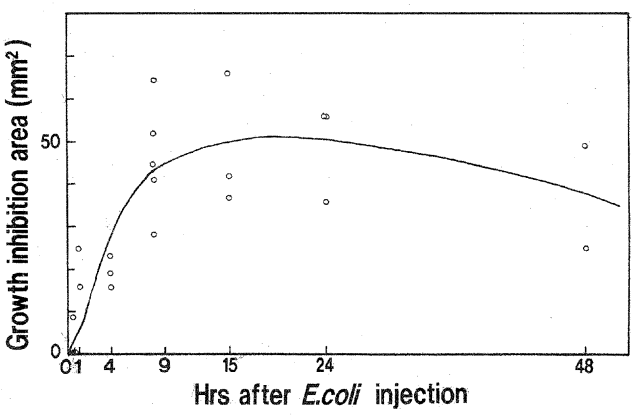

Fig .1. Time course of induction of bactericidal activity following *E. coli* injection. The activity was determined by growth inhibition assay. The hemolymph sample equivalent to 1 μl of original hemolymph was applied, and the areas of growth inhibition zones measured after incubation at 37°C. Open circles represent the results of individual insects.

from insects 9 hr after immunization and naive insects ((+) and (−) probes, respectively). Finally, 8 (+)-specific cDNA clones were isolated by screening approximately 1.44×10^4 clones of independent recombinant phage. These (+)-specific clones were categorized into two groups by restriction mapping and cross-hybridization. Group A consisted of seven clones which carried a cDNA insert of around 2000 bp. Restriction mapping of group A clones with *Kpn*I implied the existence of repeated sequences (data not shown). Group B consisted of only one clone carrying a cDNA insert of 600 bp in size. One representative clone each was selected from both groups : λdiff2 from group A ; λdiff16 from group B. The cDNA inserts of the representatives were gel-purified and subcloned into Bluescript II SK+ for further characterization.

The nucleotide and deduced amino acid sequence for entire insert of λdiff2 clone is shown in Figure 2. λdiff2 had an insert of 2242 bp. The start codon (ATG) was located 24 bp downstream from the 5' end. First terminal codon (TAA) was at position 2058–2060. A recognition sequence for the addition of poly(A)$^+$ tail (AATAAA) was located at position 2225–2230. Thus, an open reading frame extending 679 codons was deduced. This corresponds to a peptide with a molecular mass of 76367 daltons. As expected from the results of restriction mapping, the nucleotide sequence consisted of fifteen tandem repeats of a highly conserved short sequence, one of which, repeat #15 was located in the 3'-untranslated region. A typical repeat contained 141 bp encoding for 47 codons. The alignment of the fourteen-times repeated amino acid sequences is shown in Figure 3. The sequences were rich in charged residues and had a proline-rich core sequence. Nucleotide and deduced amino acid sequence of insert of λdiff16 is shown in Figure 4. The first start codon (ATG) was seen at position 32–34. The sequence consisted of 574 nucleotides and an open reading frame extended for 151 codons (m =16367.17) including the first terminal codon (TAA). The deduced amino acid sequence was rich in glycine residues (15.33%). The 3'-untranslated region contained polyadenylation signal (AATAAA) at position 553–555.

The time course of gene transcript accumulation was investigated for λdiff2 and λdiff16 clones by Northern blotting (Fig. 5). The estimated sizes of the transcripts (2.4 kb and 0.7 kb) corresponded well to the length of the λdiff2 and λdiff16 inserts. The bands of signals probed by the λdiff2 cDNA insert were rather broad and obscured by the less dense bands of rRNA. This may suggest the occurrence of λdiff2 variants having different numbers of repeats as reported in apidaecin (Casteels-Josson *et al.*, 1993). We did not investigate further this point. The λdiff2 transcript was detectable by 1 hr after immunization, increased sharply to its maximum at 9 hr, thereafter decreased gradually and returned to trace amounts at 48 hr. Although the level of the expression of λdiff16 was much lower than that of λdiff2 and its signal was very week at 1 hr by overnight exposure, the induction profile of λdiff16 was appreciably similar to that of λdiff2: first detected at 1hr, peaked at 9hr, then slowly declined. This suggests that the regulation of the two genes shares a common mechanism. These profiles are well-coincident with that of bactericidal activity in immunized hemolymph (Fig. 1). Therefore, when the bactericidal activity is taken as an index of the final output of the acute phase response in this species, the two genes are apparently early genes involved in the acute phase response. From these results, these genes are thought to encode either antibacterial molecules themselves or molecules involved in transduction of bacterial infection signals. In order to elucidate the relationship between these genes and genes whose sequence information is available, DNA and protein data bases were searched by a fasta program of DNA Data Bank of Japan. The DNA data base search failed to establish similarity among published DNA sequences. On the other hand, the protein data base search revealed that λdiff16 shares appreciable homology to hemiptericin, a glycine-rich, attacin-like antibacterial peptide purified from other hemipteran species, *Pyrrhocoris apterus* (Cociancich *et al.*, 1994) : 42.9% in 91 residues in the C-terminal half. This indicates that λdiff16 encodes a counterpart of hemiptericin in *R. clavatus*. Then, the sequence of the λdiff16 was aligned to published amino acid sequences of attacin-like antibacterial peptides. The amino acid sequence of λdiff16 showed homology to those of diptericin (24.6% in C-terminal 65 residues (Dimarcq *et al.*, 1988)) and attacin E (21.3% in C-terminal 89 residues (Hultmark *et al.*, 1983)).

As for λdiff2, a striking similarity was found between a proline-rich region of each repeat and pyrrhocoricin, a proline-rich antibacterial peptide isolated also from *Pyrrhocoris apterus* (Cociancich *et al.*, 1994): over 80% similarity for typical repeats #2 to #13. The core sequences of the λdiff2 repeat #1 and #2 were aligned to published amino acid sequences of proline-rich antibacterial peptides, drosocin from *Drosophila melanogaster* (Bulet *et al.*, 1993)

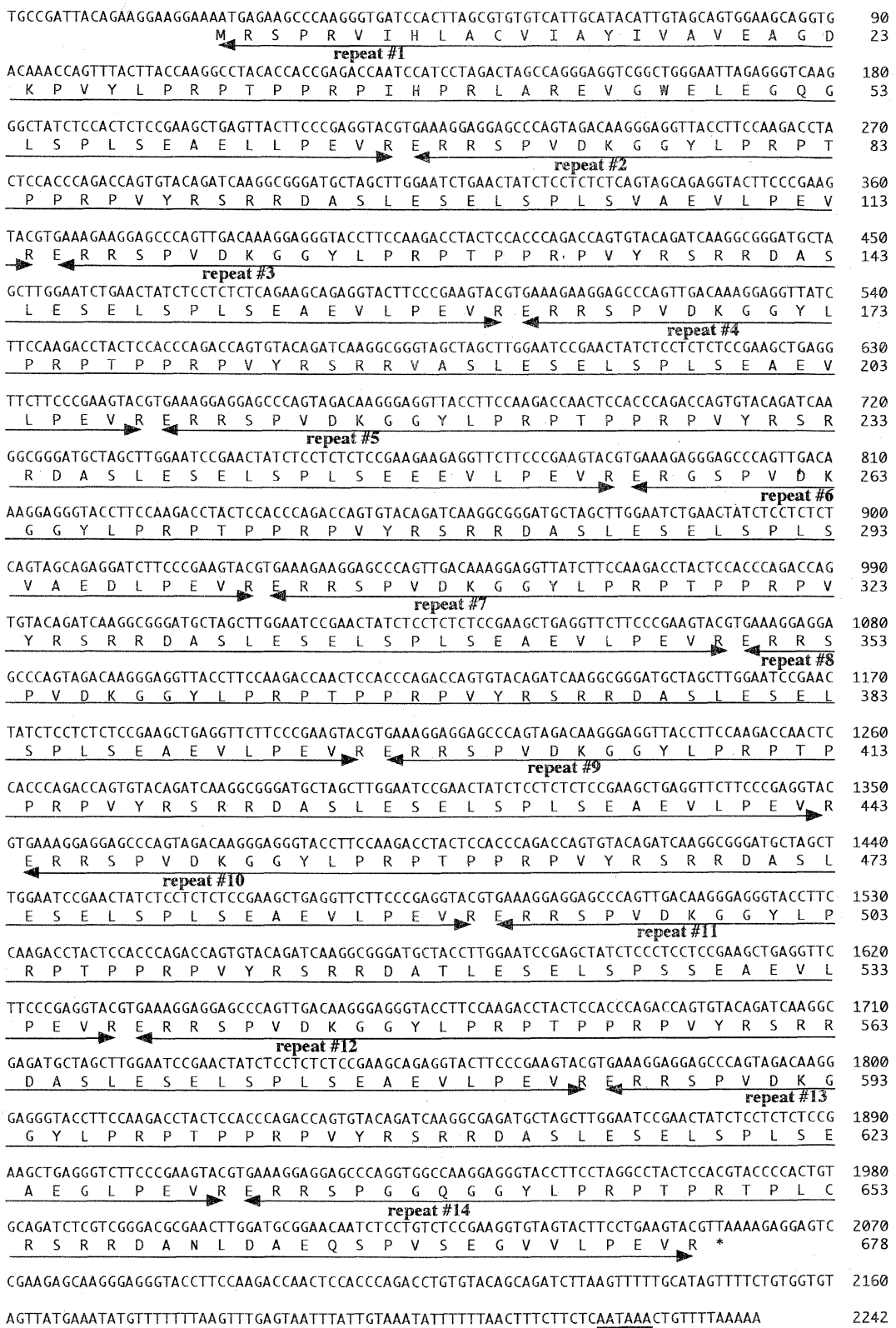

Fig. 2. Nucleotide and deduced amino acid sequences of λdiff2 cDNA clone. Fourteen tandem repeats of amino acid sequence are indicated with double-headed arrows. A putative polyadenylation signal is underlined. Asterisk represents the termination codon.

```
repeat #1     MRSP.VIHLACVIAYIVA.EA.DKPV.........IHP.LA.EVGWE..GQG.....E..L.....
repeat #2     --E-R-------RS---PVDKGG---YLPRPTPPRP-VYR-SRRDA-SLESE-LSPLSVAEVLPEVR
repeat #3     --.-.-------..---.........---.........-...-.....-.....-.....E........
repeat #4     --.-.-------..---.........---.........-...-..V.-.....-.....E........
repeat #5     --.-.-------..---.........---.........-...-.....-.....-.....EE.......
repeat #6     --.-.-------G.---.........---.........-...-.....-.....-.........D.....
repeat #7     --.-.-------..---.........---.........-...-.....-.....-.....E........
repeat #8     --.-.-------..---.........---.........-...-.....-.....-.....E........
repeat #9     --.-.-------..---.........---.........-...-.....-.....-.....E........
repeat #10    --.-.-------..---.........---.........-...-.....-.....-.....E........
repeat #11    --.-.-------..---.........---.........-...-.....-T....-...S.E........
repeat #12    --.-.-------..---.........---.........-...-.....-.....-.....E........
repeat #13    --.-.-------..---.........---.........-...-.....-.....-.....E..G.....
repeat #14    --.-.-------..---.GGQ..---.......RT.-LC.-.....-N.DA.-Q..V.EGV......
                *                 *    *******  *   *  *     *     ** *    *****
```

Fig. 3. Alignment of amino acid sequences of fourteen (#1–#14) tandem repeats of λdiff2. The repeats are aligned with repeat #2. Identical amino acid residues are shown by dots. Horizontal bars represent inserted gaps. Residues conserved in every repeat are indicated by asterisks.

```
TCAAAGACCTCTCGCAACTAAGAAAGGAAAAATGCATATTGCAAGATTTTGTTTGCTCTCTTCGATGGCCGTCCTGGCTCTGTCAGCTGG    90
                          M  H  I  A  R  F  C  L  L  S  S  M  A  V  L  A  L  S  A  G    20

ATACGTATCTGGGGCAGTCATTGAGATCCCTGACGAAATTTTGGACAGCGCCAGATTCATTTCTCTTTATTCAGATGGCCTCAGACAGAA   180
 Y  V  S  G  A  V  I  E  I  P  D  E  I  L  D  S  A  R  F  I  S  L  Y  S  D  G  L  R  Q  K    50

GCGTCAGTTAAATTTGAGCGGACCTGGATCAGAGCACGCAGGAACCATAAGACTGGATGGGCAGAGGAACATCTTCGACAACGGTAGAAC   270
 R  Q  L  N  L  S  G  P  G  S  E  H  A  G  T  I  R  L  D  G  Q  R  N  I  F  D  N  G  R  T    80

CCGAGTAGATGGGACGGGATCCTACCAGCTGGACTATGCCCGTGGAATGAAGCCTATTCATGGAGCAGGCCTGGGTGCTGAGGTCAACCA   360
 R  V  D  G  T  G  S  Y  Q  L  D  Y  A  R  G  M  K  P  I  H  G  A  G  L  G  A  E  V  N  H   110

TAACATCTGGAGAGGTAGAGGAGGCCAGTCCCTGGATCTCTATGGTGGAGCAACCAGACAGTTCAACTTTGGAAATAGACCTAATGAGTG   450
 N  I  W  R  G  R  G  G  Q  S  L  D  L  Y  G  G  A  T  R  Q  F  N  F  G  N  R  P  N  E  W   140

GGGTGCTCATGGAGGCATCAGATATAACTTCTAAAATCAAATAACGTACTGTATTAATATGTAAATATGAATTGTAATATATTTTATACA   540
 G  A  H  G  G  I  R  Y  N  F  *                                                             150

TATATATTTGTAAATAAAATTTTTTCCTAAAACA
```

Fig. 4. Nucleotide and deduced amino acid sequences of cDNA insert of λdiff16 clone. A putative polyadenylation signal is underlined. Asterisk represents the termination codon.

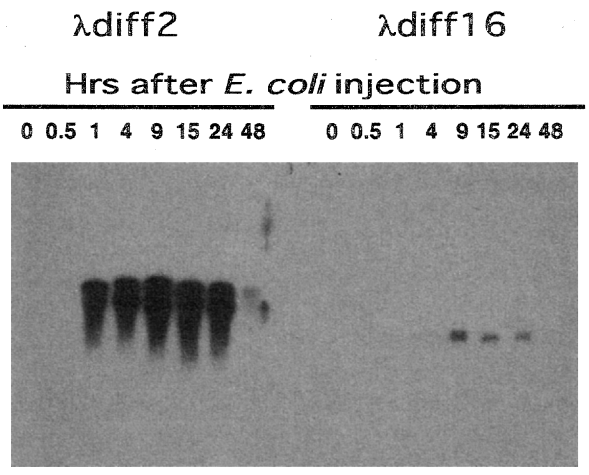

Fig. 5. Accumulation of λdiff2 (left) and λdiff16 (right) transcripts after *E. coli* injection. Five micrograms of total RNA prepared from the fat body at indicated time intervals were run. The probes for Northern blot hybridization were prepared from the λdiff2 and λdiff16 cDNA clones, respectively, and the probed blots exposed to X-ray films overnight at −80°C.

```
λdiff2 repeat#1 core   ...EAGDK--PVYLPRPT-PPRPIHPRLARE...
λdiff2 repeat#2 core   ...SPVDK--GGYLPRPT-PPRPVY-RSRRD...
pyrrhocoricin             VDK--GSYLPRPT-PPRPIYNRN
drosocin                  G-KPRP-YSPRPTSHPRPI--RV
apidaecin                 G-NNRPVYIPQ----PRPPHPRL
```

Fig. 6. Alignment of λdiff2 repeat #1 and #2 core sequences to three other established insect proline-rich antibacterial peptides. Conserved residues between λdiff2 repeats and other peptides are double-underlined. Horizontal bars represent inserted gaps. Sequences of pyrrhocoricin, drosocin and apidaecin are from Cociancich *et al.* (1994), Bulet *et al.* (1993) and Casteels *et al.* (1989), respectively.

and apidaecin from the honeybee, *Apis mellifera* (Casteels *et al.*, 1989) in addition to pyrrhocoricin (Fig. 6). Both repeat #1 and #2 were shown to have very high amino acid identity with pyrrhocoricin and drosocin. The threonine-11 residues of both pyrrhocoricin and drosocin are *O*-glycosylated and this substitution is reported to be necessary for the full bactericidal activity in the case of drosocin (Bulet *et al.*, 1993). This threonine residue was also conserved in every repeat of λdiff2 (Figs. 3 and 6). From these results the authors conclude that λdiff2 encodes for a multipeptide precursor of proline-rich antibacterial peptides of *R. clavatus*. Assuming that the precursor peptide is processed to be biologically active molecules having the similar structure to pyrrhocoricin, three types of variants could be generated (see Fig. 3). Among insect antibacterial peptides so far cloned, apidaecin was the only case to have multipeptide precursor structure (Casteels-Josson *et al.*, 1993). Interestingly, the core region of the repeat #1 was very similar to apidaecin while the repeats #2–#14 were not, suggesting that the apidaecin genes might have evolved from the ancestral multipeptide genes of Hemimetabola through selective exon amplifications and rearrangements.

Preliminary expression experiments were done using baculovirus vector and *Bombyx mori* culture cells to check the bactericidal activity of the product from λdiff2 clone. The results were negative: there was no detectable bactericidal activity in either culture medium or in cell lysate by growth inhibition assay (data not shown). These results possibly arose for the following reasons: 1) The activity of products were beneath threshold of the detection methods used because of their limited quantities. 2) The *B. mori* culture cells lacked enzymes necessary for proteolytic processing and/or an addition of sugar chains on the multipeptide precursor of the λdiff2 product.

The induction profile of bactericidal activity in immunized hemolymph corresponds well to changes in transcript abundance of the λdiff2 and λdiff16 (Figs. 1 and 5), suggesting that products of the two genes are responsible for the bactericidal activity. However, it has not been evidenced whether the active peptides derived from the two genes occur *in vivo*. It awaits the analysis at protein level to clarify this point.

In the present study we have succeeded in direct isolation of cDNA clones of antibacterial peptides by using differential screening without purification and analysis of corresponding peptides. This "direct" method is suitable especially for materials that are difficult to obtain in sufficient quantities. The PCR-based differential display method (Liang and Pardee, 1992; Asling *et al.*, 1995) may be a good alternative for differential screening. As far as the authors can determine, this is the first report of the mRNA sequences of antibacterial peptides in hemimetabolous insects, and the λdiff2 is the second case of multipeptide precursor structure in insect antibacterial peptide genes.

SEQUENCE AVAILABILITY

The nucleotide sequence data of λdiff2 and λdiff16 reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the accession numbers D49415 and D49929, respectively.

ACKNOWLEDGMENTS

We thank Dr. DeMar Taylor of Tsukuba University for reading the manuscript. DNA sequencer was provided by Center for Molecular Biology and Genetics, Mie University. This work was supported in part by Grant-in-Aid for Scientific Research to KM (Nos. 04660047 and 07660056) from the Ministry of Education, Science, Sports and Culture, Japan.

REFERENCES

Ando K, Natori S (1988) Inhibitory effect of sarcotoxin IIA, an antibacterial protein of *Sarcophaga peregrina*, on growth of *Escherichia coli*. Biochemistry 27: 1715–1721

Asling B, Dushay MS, Hultmark D (1995) Identification of early genes in the *Drosophila* immune response by PCR-based differential display: the attacin A gene and the evolution of attacin-like proteins. Insect Biochem Mol Biol 25: 511–518

Boman HG, Faye I, Gudmundsson GH, Lee JY, Lidholm DA (1991) Cell-free immunity in *Cecropia*-a model system for antibacterial proteins. Eur J Biochem 201: 23–31

Bulet P, Cociancich S, Dimarcq JL, Lambent J, Reichhart JM, Hoffmann D, Hetru C, Hoffmann JA (1991) Insect immunity. Isolation from a coleopteran insect of a novel inducible antibacterial peptide and of new members of the insect defensin family. J Biol Chem 266: 24520–24525

Bulet P, Cociancich S, Reuland M, Sauber F, Bischoff R, Hegy G, van Dorsselaer A, Hetru C, Hoffmann JA (1992) A novel insect defensin mediates the inducible antibacterial activity in larvae of the dragonfly *Aeschna cyanea* (Paleoptera, Odonata). Eur J Biochem 209: 977–984

Bulet P, Dimarcq JL, Hetru C, Lagueux M, Charlet M, Hegy G, van Dorsselaer A, Hoffmann JA (1993) A novel inducible antibacterial peptide of *Drosohipla* carries an *o*-glycosylated substitution. J Biol Chem 268: 14893–14897

Casteels P, Ampe C, Jacobs F, Vaeck M, Tempst P (1989) Apidaecins: antibacterial peptides from honeybees. EMBO J 8: 2387–2391

Casteels P, Ampe C, Riviere L, van Damme J, Elicone C, Fleming M, Jacobs F, Tempst P (1990) Isolation and characterization of abaecin, a major antibacterial response peptide in the honeybee (*Apis mellifera*). Eur J Biochem 187: 381–386

Casteels-Josson K, Capaci T, Casteels P, Tempst P (1993) Apidaecin multipeptide precursor structure: a putative

mechanism for amplification of the insect antibacterial response. EMBO J 12: 1569–1578

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol chloroform extraction. Anal Biochem 162: 156–159

Cociancich S, Dupont A, Hegy G, Lanot R, Holder F, Hetru C, Hoffmann JA, Bulet P (1994) Novel inducible antibacterial peptides from a hemipteran insect, the sap-sucking bug *Pyrrhocoris apterus*. Biochem J 300: 567–575

Dickinson L, Russell V, Dunn PE (1988) A family of bacteria-regulated, cecropin D-like peptides from *Manduca sexta*. J Biol Chem 263: 19424–19429

Dimarcq JL, Keppi E, Dunbar B, Lambert J, Reichhart JM, Hoffmann D, Rankine SM, Fothergill JE, Hoffmann JA (1988) Insect immunity. Purification and characterization of a family of novel inducible antibacterial proteins from immunized larvae of the dipteran *Phormia terranovae* and complete amino-acid sequence of the predominant member, diptericin A. Eur J Biochem 171: 17–22

Fujiwara S, Imai J, Fujiwara M, Yaeshima T, Kawashima T, Kobayashi K (1990) A potent antibacterial protein in royal jelly. Purification and determination of the primary structure of royalisin. J Biol Chem 265: 11333–11337

Hultmark D (1993) Immune reactions in *Drosophila* and other insects: a model for innate immunity. Trends Genet 9: 178–193

Hultmark D, Engström A, Andersson K, Steiner H, Bennich H, Boman HG (1983) Insect immunity. Attacins, a family of antibacterial proteins from *Hyalophora cecropia*. EMBO J 2: 571–576

Hultmark D, Engström A, Bennich H, Kapur R, Boman HG (1982) Insect immunity. Isolation and structure of cecropin D and four minor antibacterial components from *Cecropia* pupae. Eur J Biochem 127: 207–217

Lambert J, Keppi E, Dimarcq JL, Wicker C, Reichhart JM, Dunbar B, Lepage P, van Dorsselear A, Hoffmann JA, Fothergill JE, Hoffmann D (1989) Insect immunity. Isolation from immune blood of the dipteran *Phormia terranovae* of two insect antibacterial peptides with sequence homology of rabbit lung macrophage bactericidal peptides. Proc Natl Acad Sci USA 86: 262–266

Liang P, Pardee AB (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257: 967–971

Matsuyama K, Natori S (1988) Molecular cloning of cDNA for sapecin and unique expression of the sapecin gene during the development of *Sarcophaga peregrina.* J Biol Chem 263: 17112–17116

Okuda M, Natori S (1985) Primary structure of sarcotoxin I, an antibacterial protein induced in hemolymph of *Sarcophaga peregrina* (flesh fly) larvae. J Biol Chem 260: 7174–7177

Qu X, Steiner H, Engström A, Bennich H, Boman HG (1982) Insect immunity. Isolation and structure of cecropin B and D from pupae of the Chinese oak silk moth, *Antherea pernyi*. Eur J Biochem 127: 219–224

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York, 2nd ed, pp 10.44–10.45

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467

Taniai K, Kato Y, Hirochika H, Yamakawa M (1992) Isolation and nucleotide sequence of cecropin B cDNA clones from the silkworm, *Bombyx mori*. Biochim Biophys Acta 1132: 203–206