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Robert Johns
Old Dominion University

Shane Ceraul
Old Dominion University

Daniel E. Sonenshine
Old Dominion University, dsonensh@odu.edu

Wayne L. Hynes
Old Dominion University, whynes@odu.edu

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TITLE: Tick immunity to microbial infections: control of representative bacteria in the hard tick *Dermacentor variabilis* (Acari: Ixodidae).

AUTHORS: Robert Johns, Shane Ceraul, Daniel E. Sonenshine and Wayne L. Hynes
Department of Biological Sciences, Old Dominion University, Norfolk, Virginia 23529

INTRODUCTION

Although ticks are known to transmit an impressive variety of protozoan, viral, bacterial (including rickettsial) and other microbial pathogens, little is known about their ability to resist microbial infection. Like other metazoan animals, ticks must be capable of resisting infection with naturally acquired microbes, e.g., common non-pathogenic bacteria acquired through injury or host skin bacteria taken up during blood feeding. However, if ticks have an immune system capable of resisting invasion by noninfectious microorganisms, how are the numerous infectious microbes transmitted by ticks able to proliferate in these arthropods without injuring or killing their hosts? Do they evade immune recognition or actively suppress the immune response?

Much has been learned about the ability of insects and other invertebrates to defend themselves against microbial invasion. In insects, a complex of humoral and hemocytic defenses cooperates to protect the animal from invading organisms. Innate proteins such as lysozyme (*ca.* 14 kDa), present an immediate barrier to invading bacteria. Lysozymes, which primarily attack gram positive bacteria are most effective when they act in combination with other, inducible antimicrobial peptides to disrupt bacterial cell walls and kill these microbes (Gotz and Bowman, 1985). Bacterial challenge also provokes a vigorous attack by hemocytes resulting in their destruction by phagocytosis or encapsulation. Finally, recognition of the microbes initiates synthesis of inducible polypeptides or proteins, absent in non-sensitized insects (Gillespie et al., 1997) that exhibit bacteriocidal/bacteriostatic activity against numerous bacterial genera. These

inducible antibacterial peptides of insects have been categorized into several major groupings, among the most important of which are members of the defensin family (Bulet et al., 1991; Hoffmann and Hetru, 1992; Chalk et al., 1994; Cociancich et al. 1994; Lowenberger et al. 1995). Insect defensins are mostly small peptides (*ca.* 4 kDa), containing 29--34 amino acid residues of which 6 are cysteine. These potent peptides attack primarily gram-positive bacteria, leading to cell lysis by the formation of membrane penetrating channels (Gillespie et al., 1997). Cecropins, found thus far only in lepidopteran and dipteran insects, are small peptides (*ca.* 4 kDa) that act on both gram positive and gram negative bacteria. Other major categories of insect inducible antibacterial proteins are also small peptides (4--6 kDa) such as the proline-rich drosocin or larger polypeptides (mostly > 20 kDa) such as the glycine-rich attacins and sarcotoxins that act primarily against gram negative bacteria (Cociancich et al.,1994). In addition to insects, antimicrobial proteins also occur in other arthropods. Horseshoe crab hemocytes secrete the 79-residue tachylepsin which attacks both gram positive and gram negative bacteria and the potent cysteine protease inhibitor cystatin (Saito et al.,1995). These proteins are believed to act synergistically with lysozyme to mount an effective defense against invading microbes (Agarwala et al., 1996). Similarly, antimicrobial proteins also occur in other invertebrates, such as the highly potent defensins mytilin A and B that attack both gram positive and gram negative bacteria (Charlet et al., 1996).

In contrast to the insects and other invertebrates, little is known about antimicrobial function in ticks. Aside from the brief reports of hemolymph lysozyme (Podboronov, 1991; Kühn and Haug, 1994) and lectins (Grubhoffer et al., 1991), no inducible antimicrobial proteins or peptides similar to those described in insects have been identified. Hemocytes are believed to play

a dominant role in defending ticks against invasive microbes (Zhioua et al. 1996). Microbes are phagocytized by plasmatocytes and type I granulocytes (Binnington and Obenchain, 1981; Kühn and Haug, 1994), and larger organisms by encapsulation by type II granulocytes (Eggenberger *et al.* 1990). Little is known about the hemocyte population response to microbial challenges, although Podboronov (1991) and (Kryuchechnikov, 1991) reported an increase in cell numbers after bacterial infection. Nothing is known about how the hemocytes recognize these nonself invaders. Similarly, little information is available concerning the presence of antimicrobial proteins in tick hemolymph. Johns *et al.* (1998) showed evidence of two fractions, one of which has been suggested to contain a lysozyme-like peptide, that inhibited growth of *B. subtilis*. These antimicrobial peptides were not identified. If antimicrobial proteins and peptides commonly occur in ticks, are they novel or similar to the well-described families of antimicrobial proteins found in insects? It is also important to know whether ticks have a repertoire of antimicrobial proteins that are always present (i.e., constitutive) versus others that are expressed only after microbial challenge (i.e., inducible).

The purpose of this study was to determine if the tick, *Dermacentor variabilis* (Say), can control invasion by representative gram positive and gram-negative bacteria. This report also provides evidence for the existence of soluble hemolymph antimicrobial proteins with activity against *E. coli*, which differ from proteins described previously (Johns et al., 1998) that showed activity against *B. subtilis*. Hemolymph control of *Borrelia burgdorferi* is also compared.

MATERIALS AND METHODS

Ticks. The American dog tick, *Dermacentor variabilis*, the model species, was

maintained in an incubator at 27°C ± 1°C and 92 ± 1% RH. Larvae and nymphs were fed on rats (*Rattus norvegicus*); adults, on rabbits (*Oryctolagus cunniculus*). Tick rearing methods were those described by Sonenshine (1993).

Bacteria. Bacteria inoculated into ticks were 1) the gram-positive bacteria: *Bacillus subtilis (*ATCC strain 6051), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis, Corynebacterium xerosis,* and 2) the gram negative organisms: *Pseudomonas aeruginosa (* ATCC 27853), *Pseudomonas denitrifcans, Escherichia coli* (ATCC 25922), and *Enterobacter aerogenes.* To test for anti-*E. coli* activity, a sensitive strain of *E. coli* (ATCC strain 8739) as well as the more robust *E. coli* strain ATCC 25922 were used as the indicator species. To test for anti-*Borrelia* activity, a low passage (infectious) strain (B-31) of *Borrelia burgdorferi* was used. Except for *B. burgdorferi,* supplied by the Centers for Disease Control, U. S. Public Health Service, Fort Collins, CO, USA, all bacterial species were obtained from the American Type Culture Collection, Washington, D.C., USA, aliquoted, and stored frozen. Aliquots were thawed and subcultured onto agar plates and stored at 4^0 C. Prior to use in tick inoculations or for antimicrobial assays the organisms were cultured overnight.

Bacterial Inoculations and Hemolymph Collections. Bacterial inoculations were done only in virgin fed female ticks. Five µl bacterial suspensions (1×10^7 cells/ml) from 18 hr log phase cultures were inoculated into the hemocoel via the scutal foramen (accessed by depressing the capitulum), using 10 µl Hamilton syringes and ½ inch, 30 gauge hypodermic needles. Subsequently, hemolymph was collected from bacteria-challenged ticks from a surface-sterilized (alcohol) amputated leg using a Drummed glass micropipette (Drummed Scientific Co., Broomall, PA, USA). Hemolymph was collected in an equal volume of sterile Shen's saline (Oliver et al.,

1974) at 4°C and stored frozen (-85° C) until needed for analysis. The collection buffer was fortified with 2 mM phenyl methane sulfonyl fluoride (PMSF) to inhibit tick protease activity. Hemolymph obviously contaminated with guanine crystals and ruptured midgut contents was discarded.

Tick Survival Following Bacterial Challenge. Ticks were incubated and monitored for 5 days following bacterial inoculation for evidence of mortality. Hemolymph was collected from samples of surviving ticks and examined by phase contrast microscopy or plating on tryptose-soy agar (TSA) or culturing in BSK-II (for *B. burgdorferi*) to determine the presence of bacteria. Since *P. aeruginosa* was lethal to ticks, the experiment with this organism was repeated using heat killed (121° C for 20 min) bacteria to determine whether dead bacteria would also kill ticks.

Measuring Number of *E. coli* Bacteria in Tick Hemolymph following Injection. This was done by determining the number of colony forming units (CFUs) that appeared (each colony representing a single bacterial cell) when aliquots of hemolymph from surface-sterilized (70% ethanol) bacteria-inoculated ticks were plated on agar. The hemolymph aliquots were collected at 0.25, 1, 6, 24 and 48 hours after inoculation, diluted 1:100 in Shen's buffer, plated onto MacConkey agar (Difco Laboratories, Detroit, MI) (a selective and differential medium for gram negative organisms, e.g., *E. coli),* and incubated for 24 hours. Sampling was replicated 5 - 7 x for each time interval.

Microbial Inhibition Assays. The presence of antimicrobial proteins or peptides was determined by the agar plate inhibition assay and the microtiter inhibition assay using hemolymph from bacteria-naive (control) and bacteria-sensitized tick hemolymph plasma collected 1 hour after bacterial inoculation as described by Johns et al. (1998).

Protein Fractionation by HPLC and Ultrafiltration. For hemolymph from *E. coli*-infected ticks, reversed phase high-pressure liquid chromatography (RP-HPLC) was done as described by Johns et al (1998). The column was a Vydac C_4 reversed phase protein column (Nest Group, Southboro, MA). The solvents were H_2O with 0.1% trifluoroacetic acid (TFA) and acetonitrile. A steep gradient, 85% H_2O to 15% H_2O over 45 min was used. Following HPLC, fractions were vacuum evaporated on a Centrivap Concentrator (Labconco, Kansas City, MO) reconstituted in sterile phosphate-buffered saline (PBS) and assayed for anti-microbial activity. Molecular weight ranges of the anti-microbial proteins or peptides were determined by ultrafiltration. Briefly, large samples (e.g., 1 ml) of crude hemolymph from *E. coli*-infected ticks were fractionated using Microcon ultrafiltration devices (Millipore, Bedford, MA). The fractions were filtered sequentially with 100, 50, and 30 kDa molecular weight cut off (MWCO) membranes. This separated the proteins into groups based on molecular weight. Filtrates collected following each fractionation were assayed against the challenge bacteria for evidence of antimicrobial activity. The retentates were assayed by HPLC using a Beckman Spherogel SW size exclusion column (SEC) (range 2,000 to $> 10^6$ daltons) (Beckman Instrument Co., Berkeley, CA) to evaluate filtration success.

RESULTS AND DISCUSSION

Tick Survival Following Bacterial Inoculations. Survival of ticks 5 days after bacterial inoculation is summarized in Table 1. Most of the ticks survived inoculation with the Gram positive bacteria. However, a mixed response was observed with the Gram negative bacteria; almost all ticks inoculated with *B. burgdorferi* survived, and most survived inoculation with *E. coli*, whereas 60% survived challenge with *E. aerogenes*. Few ticks survived inoculation with the

two *Pseudomonas* species. No bacteria were detected when samples of hemolymph from ticks (at 5 days) surviving inoculation with *B. subtilis, S. aureus, E. coli* or *B. burgdorferi* were plated on TSA agar or were cultured in BSK. However, large numbers of organisms were obtained from hemolymph taken from survivors of *P. aeruginosa* infection. With the latter, tick mortality appears to have resulted from bacterial viability since all ticks survived when the experiment was repeated with heat-killed *P. aeruginosa.* In contrast to ticks, insects, e.g., *Galleria mellonella*, express antimicrobial proteins that disrupt the LPS layer of *P. aeruginosa*, thereby facilitating access of hemolymph lysozyme capable of destroying the peptidoglycan layer of the bacteria cell wall and killing the microbes (Gagen and Ratcliffe, 1976).

Decline in Bacteria Inoculated into Tick Hemolymph. Perhaps the strongest evidence of the tick's ability to control *E. coli* infections is the rapid decline in CFUs on agar plates streaked with hemolymph from ticks at different intervals after bacterial challenge. Theoretically, in the absence of immune control, bacterial numbers will rise exponentially in view of their short doubling time (20 minutes). Therefore, injection of 50,000 *E. coli* cells into fed virgin *D. variabilis* females with a mean weight of *ca.* 250 mg (Ziv et al. 1981), of which an estimated 23% is hemolymph (Kaufman and Phillips, 1973) would produce an estimated 2.3×10^8 cells/µl at 6 hrs if growth were unrestricted. However, CFU's, although present at 0.25 and 1 hr, showed a decline rather than an increase with time and no bacteria were found at 6, 24 or 48 hours post-inoculation (Table 2). None were found by microscopic examination of hemolymph from *E. coli*-inoculated ticks at the three later time intervals. This reflects rapid clearing of *E. coli* from the tick's hemolymph, much more rapid than the nearly 24 – 48 hours reported for the gram positive *B. subtilis* (Johns et al., 1998). A possible explanation for the disappearance of *E. coli* from the

tick's hemolymph is suggested by the clumping phenomenon found in insects. In *Pieris brassicae* and *Galleria mellonella*, the number of circulating hemocytes declines almost immediately following infection as large clumps comprised of bacteria and hemocytes are formed (Gagen and Ratcliffe, 1976). This process effectively cleared the hemolymph within 5 minutes of several different gram- negative bacterial species in these insects. Lysis of bacterial cells by tick soluble hemolymph antimicrobial proteins, as suggested by evidence described below, may also play a significant role in removing these cells.

Antimicrobial Peptides or Proteins in Tick Hemolymph Plasma. When 25 μl of hemolymph plasma from *E. coli*-challenged and sham-inoculated ticks was tested by the whole plate assay using *E. coli* ATCC 8739 as the indicator strain, a10 mm zone of inhibition was found around the well containing the sample from the challenged ticks, but no inhibition was found around the well containing the sample from the non-stimulated (control) ticks. This finding suggests that soluble anti-microbial proteins or peptides are present in tick hemolymph following challenge with *E. coli*, but not in non-stimulated ticks. Thus, no constitutive anti-microbial activity against *E. coli* challenge is evident in these ticks. This response is very different from the observations made by Johns *et al.* (1998) for ticks challenged with the gram-positive *B. subtilis*. In the latter case, these authors found constitutive anti-microbial activity in bacteria-naïve ticks, as well as enhancement (or induction) of the response in bacteria-challenged ticks. This sugests that different proteins or peptides are responsible for control of gram positive versus gram negative bacteria.

When hemolymph from *E. coli*-stimulated ticks was fractionated by RP-HPLC, anti-microbial activity was found consistently in two fractions when they were tested against *E. coli*

strain ATCC 8739. Examination of the HPLC chromatogram representing the hemolymph from the *E. coli*-challenged ticks shows a major peak in the first fraction (Fr-1), which eluted at 23.4 minutes (48% acetonitrile) and a much smaller peak in the second fraction (Fr-2), which eluted at 43.0 minutes (74% acetonitrile) (Fig. 1). In contrast, when Johns et al. (1998) examined hemolymph from *B. subtilis*-challenged *D. variabilis* females by RP-HPLC using the same protein column, solvents and run method, they only found anti-microbial activity in two fractions eluting early in the acetonitrile:water gradient, indicating polar proteins or peptides. Repeating Johns et al.'s study with hemolymph from *B. subtilis*-challenged ticks, we confirmed the existence of two early eluting anti-*B. subtilis* fractions. One contained a very small peak that eluted at 4 minutes (Fr-1, 17% acetonitrile), while the other latter contained a larger peak that eluted at 14.5 minutes (Fr-2, 38% acetonitrile) (Fig. 1, insert). Neither fraction inhibited growth of *E. coli*. Similarly, neither of the *E. coli* inhibiting fractions separated from the hemolymph of the *E. coli*-challenged ticks inhibited growth of *B. subtilis*. Clearly, different antimicrobial proteins or peptides are expressed against *E. coli* and possibly other gram negative bacteria than against gram positive bacteria such as *B. subtilis*. This is similar to responses seen in many insects, where defensins are induced to control gram positive bacteria and different peptides such as cecropins, attacins and others are induced to control gram negative bacteria (Gillespie et. al., 1997).

To determine the molecular weight range of the anti-*E. coli* proteins or peptides, *E. coli*-stimulated tick hemolymph was fractionated by ultrafiltration. Anti-*E. coli* activity was found in the filtrates of the 100, 50 and 30 MWCO membranes. Analysis of the filtrates by SEC-HPLC revealed that filtration was incomplete, i.e., some of the proteins smaller than the MWCO for each membrane remained in the retentate. This problem was solved by repeated filtration of the

retentates until analysis of the retentates showed almost no proteins greater than the corresponding MWCO's, respectively, remaining (less than 0.3%). Anti-microbial activity was correlated with the smallest proteins, all less than 30 kDa molecular weight. Further purification is needed to isolate and identify the specific anti-microbial peptides or proteins

Activity against *Borrelia burgdorferi*. Hemolymph (plasma) from partially fed *D. variabilis* females sensitized with *E. coli* was found to kill *B. burgdorferi* when incubated with these bacteria (replicated 3 times). Low passage spirochetes (CDC variety of B-31 strain) transferred from a 4-day old culture maintained at 33°C in a CO_2 incubator were destroyed when passaged to fresh BSKII (H) medium and incubated with whole hemolymph plasma. Spirochetes were also completely absent or present in greatly reduced numbers when incubated with the hemolymph filtrate from the 100 MWCO microconcentrator. Large numbers of spirochetes, indicating growth, were present when the same size inoculum was incubated with Shen's solution or merely passaged in fresh medium. The results of these experiments are summarized in Table 3. We also found that *B. burgdorferi* inoculated into *D. variabilis* females had no effect on tick survival during a 5-day observation period (Table 1). In nature, *B. burgdorferi* ingested by *D. variabilis* are believed to be destroyed in the midgut of that tick before or during the molt to the next life stage, since none are found when these ticks feed again. Therefore, *D. variabilis* is not vector competent (Piesman and Sinsky, 1988). Even if some spirochetes were able migrate to the hemolymph of these ticks, it is likely that they would be destroyed by the tick's antimicrobial proteins before they could be passed to vertebrate hosts. It would be of interest to determine whether the hemolymph of *Ixodes scapularis* also inhibits or lyses cultured *B. burgdorferi*. Several studies (Ribeiro, et al. 1987; Zung et al. 1989) have shown that *B. burgdorferi* migrate

from the midgut across the hemocoel to the salivary glands during tick feeding. However, these spirochetes have acquired host proteases such as plasmin or its precursor, plasminogen (Plg) (the latter is converted to the active plasmin by host plasminogen activator) that entered the gut lumen with the blood meal. Using an anti-Plg IFA, Coleman *et al.* (1997) found that *Borrelia* spirochetes in the midgut of *I. scapularis* acquired Plg during tick feeding. Some of the spirochetes retained this protein as they disseminated from the midgut across the hemolymph to the salivary glands, although relatively few (less then 5% of midgut population) survived to reach the salivary glands. In contrast, spirochetes in ticks fed on mice deficient in Plg lacked this protein and virtually none were found in the hemolymph. A few spirochetes were found bound to hemocytes, presumably undergoing phagocytosis, in the ticks fed on Plg competent mice, suggesting that even Plg-bound spirochetes failed to evade immune detection.

Tick salivary secretions may also facilitate the survival of these microbes. *I. scapularis* saliva contains an anti-complement protein that inactivates serum complement in blood from *B. burgdorferi*-infected rabbits, thereby preventing the destruction of midgut spirochetes by ingested host antibodies against *B. burgdorferi*. Mather et al (1996), who could not find this same anticomplement protein in *D. variabilis* saliva, suggested that spirochetes may be destroyed in non-vector competent tick species by vertebrate host immunoglobulins and complement, which might explain why spirochetes are lost when the tick molts following the spirochete-infected blood meal (Piesman and Sinsky, 1988). However, Berenberg et al. (1971) showed that *D. variabilis* saliva (from adult ticks) readily cleaved host complement *in vivo*. The salivary anticomplement activity may be sufficient to prevent the ingested anti-*Borrelia* immunoglobulins from destroying the spirochetes in feeding adult *D. variabilis*, some of which may traverse the

midgut during the long feeding period. Unless destroyed by hemolymph antimicrobial agents or phagocytes, such spirochetes could reach the salivary glands, although they are unlikely to be transmitted unless the adults detach prematurely or detach during mate-finding activity. Additional evidence of tick borreliocidal activity, perhaps due to hemolymph antimicrobial proteins, is suggested by the finding that even in vector-competent *I. scapularis*, where spirochetes reached densities in the tens of thousands/tick during the ecdysial period, fewer than 10% of the spirochetes survived following the breakdown and reformation of the tick's body tissues that accompanies the molt (Piesman et al. 1990).

SUMMARY

Fed female ticks, *Dermacentor variabilis,* survived for at least 5 days without significant mortality following intra-hemocoelic inoculation of three bacterial species, *Bacillus subtilis, Staphyloccocus aureus, Micrococcus luteus, Escherichia coli*, and *Borrelia burgdorferi*. In contrast, survival of ticks 5 days after inoculation with *Pseudomonas aeruginosa* was only 12% and the few surviving individuals appeared moribund. However, survival of ticks inoculated with heat-killed *P. aerurginosa* was 100%, suggesting that lethality of this bacterial species required viable cells. *D. variabilis* was found to control *E. coli*, a representative gram negative bacterium, very rapidly. Some control was evident in as little as 1 hour after bacterial inoculation, and all *E. coli* cells were cleared from the hemolymph within 6 hours. This is a much more rapid response than that observed by Johns et al. (1998) for a representative gram positive bacterium, *B. subtilis*.

D. variabilis hemolymph plasma from *E. coli*-challenged ticks inhibited microbial growth, but hemolymph from non-challenged ticks was not constitutively anti-microbial, i.e., plasma from non-infected individuals did not inhibit growth of *E. coli*. This differs from studies with ticks challenged with *B. subtilis,* which were found to be constitutively anti-microbial. Anti-*E. coli* activity of hemolymph plasma from challenged ticks was correlated with two relatively non-polar fractions separated by HPLC using a reversed phase Vydac (C_4) protein column. However, these fractions had no effect on *B. subtilis*. Similarly, fractions separated by HPLC from *B. subtilis*-challenged ticks had no effect on *E. coli* growth. In addition to its activity against the non-vectored, non-pathogenic bacteria, *D. variabils* hemolymph from *E. coli*-stimulated ticks was also

found to be borreliocidal against the Lyme disease spirochete, *Borrelia burgdorferi*. Further study is needed to purify and identify the anti-microbial proteins or peptides active in tick hemolymph against this array of different types of microbes.

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Table 1. Survival of *Dermacentor variabilis* Partially-fed Virgin Females Five Days After Inoculation with 5 x 10^4 Bacterial Cells

Type	Species of Bacteria	No. in sample	Percent Survival
Gram +	*Bacillus subtilis*	57	97.9
Gram +	*Staphylococcus aureus*	57	100
Gram +	*Staphylococcus epidermidis*	12	80.0
Gram +	*Micrococcus luteus*	12	100
Gram +	*Corynebacterium xerosis*	12	60.0
Gram -	*Escherischia coli*	48	98.0
Gram -	*Enterobacter aerogenes*	12	60.0
Gram -	*Pseudomonas aeruginosa*	57	12.0
Gram -	*Pseudomonas aeruginosa* (heat-killed)	12	100
Gram -	*Pseudomonas denitrificans*	12	20.0
Gram -	*Borrelia burgdoferi*	12	95.0
-------	Shen's solution (controls)	42	100

Table 2. Survival of *Escherichia coli* in hemolymph of bacteria-challenged partially-fed females of *Dermacentor variabilis* as determined by colony forming units (CFU's) on agar[1]

Hours after inoculation	Mean (± S.D.) CFU's/ microliter hemolymph[2]
0.25	1238.9 ± 987.9
1	992.0 ± 616.8
6	0
24	0
48	0

1. An estimated 5 x 10^4 bacteria were inoculated per tick.
2. Each colony forming unit represents one *E. coli* bacterium. One microliter hemolymph samples were diluted 1:100 in tick saline, plated onto agar, incubated overnight and the number of CFU's determined per microliter of hemolymph. Counts for each time interval were replicated 5 times. Controls were sham-inoculated ticks, replicated 4 times. No CFU's were found in the controls.

Table 3. Experiment to test the borreliocidal activity of tick hemolymph (plasma) and protein fractions from *E. coli*-stimulated *Dermacentor variabilis* fed females.

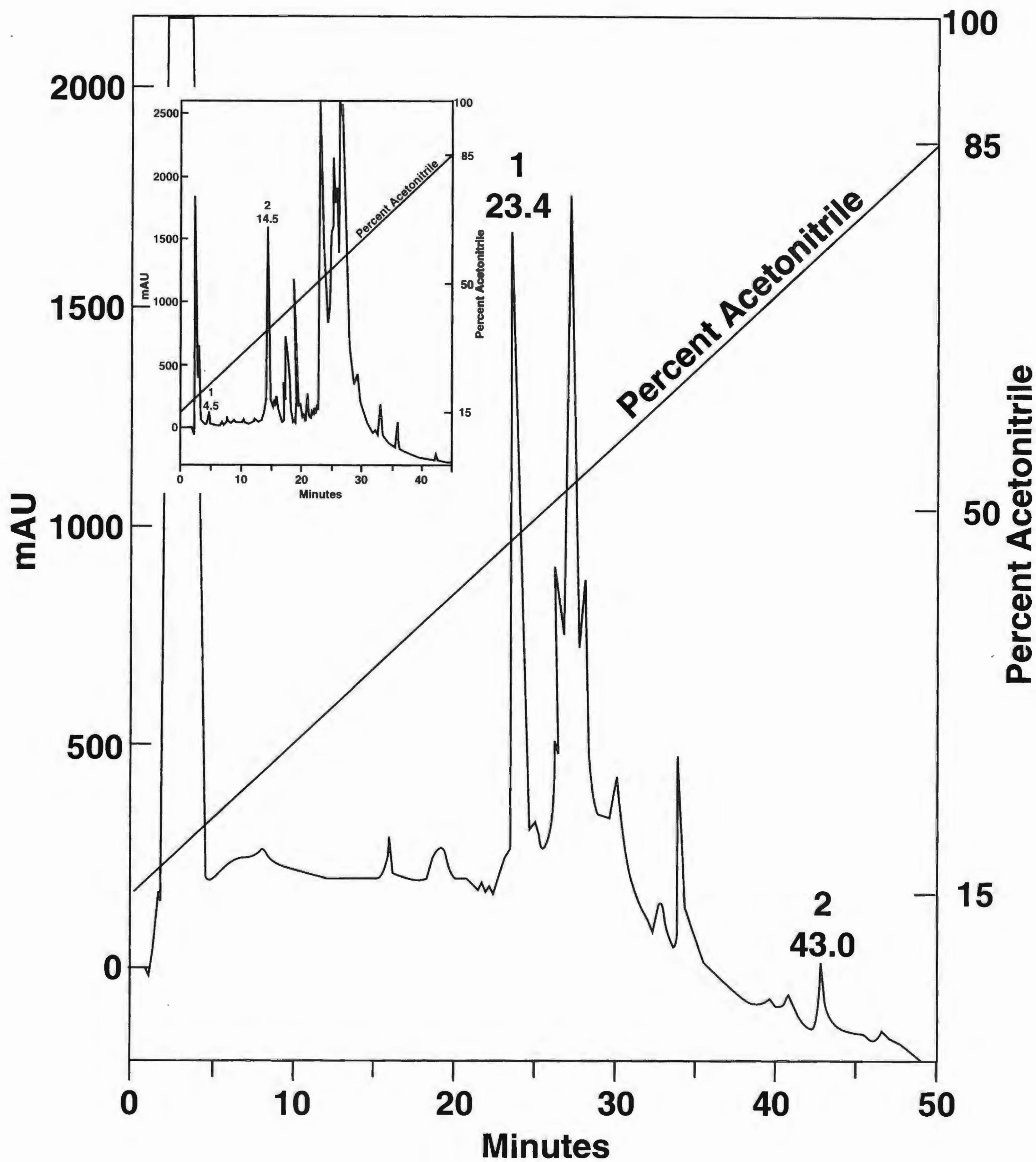
Replicate No.	Tick saline (PMSF)	Tick saline (no PMSF)	BSK II	Tick Hemolymph plasma Unfractionated	Tick Hemolymph 100 MWCO filtrate
1	Growth +	Growth +	Growth +	0.00	0.00
2	Growth +	Growth +	Growth +	0.00	3 spirochetes
3	Growth +	Growth +	Growth +	0.00	0.00

Abbreviations: Growth +; Barbour/Stoner/Kelly Culture medium II (Harvard)

Legend

Figure 1. Chromatogram illustrating the peaks observed when hemolymph plasma (4.1 mg total protein) from bacteria-challenged ticks was separated by reversed phase high-performance liquid chromatography (RP-HPLC). Two fractions were found to be anti-microbial against *E. coli,* one (1) with a peak at 23.4 minutes, the second (2) with a peak at 43.0 minutes. MAU = milliabsorbance units.

Insert. Hemolymph from *B. subtilis*-stimulated ticks containing 3.0 mg total protein was separated by RP-HPLC using identical solvents and run conditions. Two fractions were found to be anti-microbial against the indicator organism, *M. luteus*, one (1) with a peak at 4 minutes, the second (2) with a peak at 14.5 minutes.



mAU
2000
1500
1000
500
0
100
85
50
15
Percent Acetonitrile
Percent Acetonitrile
1
23.4
2
43.0
0
10
20
30
40
50
Minutes
2500
2000
1500
1000
500
0
mAU
2
14.5
1
4.5
Percent Acetonitrile
100
85
50
15
Percent Acetonitrile
0
10
20
30
40
Minutes