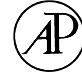
Fish & Shellfish Immunology (1996) **6,** 403–412



Specificity and some physicochemical characteristics of the antibacterial activity from blue crab *Callinectes sapidus*

EDWARD J. NOGA*, THOMAS A. ARROLL† AND ZHIQIN FAN

College of Veterinary Medicine, North Carolina State University, 4700 Hillsborough Street, Raleigh, NC 27606, U.S.A.

(*Received 14 April 1995, accepted in revised form 11 March 1996*)

Haemolymph of the brachyuran crustacean *Callinectes sapidus* possesses bactericidal activity which is highly inhibitory to Gram-negative bacteria cultured from blue crab carapace, including *Aeromonas hydrophila*, *Vibrio parahemolyticus*, *Vibrio alginolyticus* and *Vibrio vulnificus*. Several strains of *Escherichia coli* were also susceptible, but all other bacteria that were not isolated from blue crabs were resistant. No lysozyme-like activity was detected. A quantitative turbidometric assay using *E. coli* was used to measure antibacterial activity after exposure to various physical and chemical treatments. The activity was most active at low pH, heat-labile, inhibited by sodium chloride, and inactivated by protease. The antibacterial activity appeared to be confined to the haemocytes. © 1996 Academic Press Limited

Key words: brachyuran crustacean, humoral immunity, bactericidin, bacterial microflora.

I. Introduction

The blue crab, *Callinectes sapidus*, supports a very important commercial fishery from the mid-Atlantic region to the Gulf of Mexico coast of the United States. As is true of other animals, it is susceptible to a wide range of infectious diseases, including viruses, fungi and parasites (Johnson, 1983; Sparks, 1985). Various bacteria have also been reported to be important pathogens of the blue crab (Sindermann & Lightner, 1988). Despite the importance of infectious agents, very little is known of the mechanisms responsible for protecting against these pathogens in the blue crab.

There is increasing evidence that a wide array of nonspecific antibacterial compounds provide an important defence in arthropods, including crustaceans (Lackie, 1988; Hetru *et al.*, 1994; Smith & Chisholm, 1992; Soderhall & Cerenius, 1992). This paper describes a potent antibacterial activity in blue crabs which probably plays an important role in host defence.

*Corresponding author.
†Present address: Department of Pathobiology, University of Washington School of Medicine, Seattle, WA 95818, U.S.A.

1050–4648/96/060403+10 $18.00/0 © 1996 Academic Press Limited

II. Materials and Methods

ANIMALS AND HAEMOLYMPH

Blue crabs were obtained by trawling or from crab pots in the Albemarle-Pamlico Estuary, North Carolina. Haemolymph was collected by inserting a 22 G needle attached to a 3 ml syringe into the arthrodial membrane and gently aspirating into the syringe. Some samples were taken by severing the 5th pereiopod at the merepodite and collecting the haemolymph in a tube.

For serum, samples were allowed to clot at 4° C, homogenised to break up the clot, and centrifuged at 50 000 *g* for 20 min. The resulting supernatant from 5–20 crabs was pooled and frozen at −70° C until use.

BACTERIA USED FOR ASSAYS

Cultures were taken from blue crabs by swabbing the surface of the carapace with a sterile plastic loop and applying it to a culture plate having trypticase soy agar with 5% defibrinated sheep blood. The inoculum was then evenly spread on the plate using a sterile swab. Plates were incubated at room temperature and colonies were picked. Isolates were purified by restreaking three times and then identified using standard procedures (Krieg, 1986). Isolates of *Aeromonas hydrophila*, *Vibrio parahemolyticus*, *Vibrio alginolyticus* and *Vibrio vulnificus* were cultured.

Other bacteria used in this study included American Type Culture Collection (Rockville, MD) isolates of *Staphylococcus aureus* (ATCC no. 12598), *Micrococcus luteus* (ATCC no. 381), *Pseudomonas aeruginosa* (ATCC No. 19154) and *Serratia marcescens* (ATCC no. 990). *Escherichia coli* D31 and *Enterobacter cloacae* B12 were obtained from Dr M. Postlewaite, University of Oregon. *Escherichia coli* strains nos 90-8033 and 90-8035 were obtained from Dr P. Orndorff, North Carolina State University. *Staphylococcus faecum* (NCSU no. 90-8030), *Staphylococcus intermedius* (NCSU no. 90-8031) and *Streptococcus* Group G (NCSU no. 90-8024) were clinical isolates from the Clinical Microbiology Laboratory, North Carolina State University. Stock cultures were stored at −70° C in brain heart infusion broth with 10% glycerol. Working cultures were maintained at room temperature on trypticase soy agar with 1% NaCl.

ASSAYS FOR ANTIBACTERIAL ACTIVITY

All bacterial isolates were examined for susceptibility to serum using both quantitative and qualitative assays. In the streak assay, various bacterial isolates were streaked onto a nutrient agar; 10 μl of either serum or saline were then spotted onto the streak and the plate incubated for 24–48 h. Inhibition was indicated by the absence of bacterial growth at the site of sample spotting, with no growth inhibition where saline was spotted.

Quantitative differences in antibacterial activity were measured using both a plating assay and a turbidometric assay. The plating assay was performed as described by Anderson & Chain (1982). Bacteria were inoculated into trypticase soy broth containing 1% NaCl (TSB) at 37° C. After 18–24 h the bacterial suspension was washed twice with 0·10 M sodium phosphate buffer with 1%

NaCl, pH 7·0 (PBS), and diluted to an O.D.$_{570}$ of about 0·100. This suspension was then diluted 1:100 with PBS. Various amounts of serum were added to known amounts of PBS and incubated with bacterial suspension (approx. 2×10^5 cells ml^{-1}, for 30 min at 25° C. Controls included samples that were inoculated with PBS instead of serum and samples that had no bacteria added. The reaction was then simultaneously stopped by placing the tubes in a 4° C water bath and diluting them with cold (4° C) PBS. One hundred μl of each sample was then spread onto duplicate plates of trypticase soy agar with 1% NaCl. Colonies were counted after 24 h and the percentage inhibition determined.

The turbidometric assay was performed as described by Noga *et al.* (1994). A volume (usually 2 μl) of each test sample (i.e., serum, plasma, or haemocyte lysate) was brought up to a total volume of 40 μl with PBS. Ten μl of a diluted bacterial suspension was then added (making a final bacterial concentration of approx. 2×10^5 cells ml^{-1}) and the tubes were incubated for 30 min at 25° C. The reaction was simultaneously stopped by placing all tubes in a 4° C water bath; 450 μl of cold (4° C) TSB was then added to all tubes. One hundred μl aliquots of each sample were added to quadruplicate wells of a 96-well tissue culture plate. The optical density of each well was recorded using an automated spectrophotometer (Dynatech, Chantilly, VA) when the optical density of the negative control cultures (having bacteria but no haemolymph sample added) reached an O.D.$_{570}$ of at least 0·090–0·110 (usually after 4–6 h of incubation).

CHARACTERISATION OF ANTIBACTERIAL ACTIVITY

The effect of pH on antibacterial activity was tested by incubating serum in PBS at a pH of 5·2, 5·5, 6·0, 6·5, 7·0, 7·5 or 8·0. Samples were incubated for 30 min and then diluted and cultured using the turbidometric assay as described previously.

The effect of temperature on antibacterial activity was tested by incubating serum at 7·8, 25·8, 37·8, 40·8, 53, 56·6, 60, 65 or 70° C for 30 min and then immediately cooling the samples in an ice bath. Samples were then incubated with bacterial suspension for 30 min, then diluted and cultured using the turbidometric assay as described previously.

The effect of protease on antibacterial activity was tested by incubating serum with 400 μg ml^{-1} Proteinase K (no. P-6556, Sigma Chemical Co., St Louis, MO) in 0·10 M sodium phosphate buffer, pH 7·0, for 1 h. Samples were then incubated with bacterial suspension for 30 min and diluted and cultured using the turbidometric assay as described previously.

The effect of salt concentration on antibacterial activity was tested by incubating various amounts (2–10 μl) of serum in 32 parts per thousand artificial seawater (Instant Ocean, Aquarium Systems, Mentor, OH) or in phosphate buffer with 0%, 1% or 3% NaCl with *E. coli* D31 (2×10^5 cells ml^{-1}). Samples were incubated for 30 min and then diluted and cultured using the turbidometric assay as described previously.

Serum samples were tested for lysozyme activity using a commercial agar gel diffusion assay (Kallestad Diagnostics). Serum and haemocyte lysate

samples were placed into wells according to manufacturer's instructions and the plate was incubated at room temperature. Controls included buffer and hen egg white lysozyme.

LOCALISATION OF THE SOURCE OF ANTIBACTERIAL ACTIVITY

Unclotted (i.e., caffeinated) haemolymph was collected by aspirating haemolymph into a syringe having caffeine (no. C-0750, Sigma Chemical, St Louis, MO) in 3% NaCl, which inhibits clotting in some arthropods (Nakamura *et al.*, 1976). Final caffeine concentration was about 2 mM. Pyrogen-free reagents were used for all haemocyte separations. In blue crabs, this procedure delays clotting for up to 20 min, allowing the collection of intact haemocytes (Arroll & Noga, unpublished data).

Caffeinated haemolymph was immediately centrifuged at 2500 *g* at 23° C for 10 min and the plasma was gently aspirated. The haemocyte pellet was resuspended in an equal volume of Tris buffer (Nakamura *et al.*, 1976). The antibacterial activity of both plasma and haemocyte samples was compared to a serum sample collected simultaneously from the same animal.

STATISTICAL ANALYSIS

The effects of temperature and pH on antibacterial activity were analysed using a polynomial regression analysis (PROC.REG of SAS (6·09); SAS Institute, Cary, NC). The response variable was % bacterial growth. Orthogonal polynomial coefficients corresponding to the temperature or pH values were generated using PROC.IML of SAS and these were used as explanatory variables in the model. Analysis of the effect of protease on antibacterial activity, the antibacterial activity in various blood compartments and the antibacterial activity against various bacterial isolates was examined using an *F* test, to test for equality of variance between the test versus control groups. Based upon these results, a two-tailed Student's *t*-test (Microsoft Excel) was used to analyse for any significant differences among the various treatments.

III. Results

Results of all three assays for antibacterial activity gave similar results. Isolates which were resistant when using the spot assay were then tested with the turbidometric assay. The latter also gave negative results. All the Gram-negative bacterial isolates from blue crabs were susceptible to blue crab serum (Table 1). All *E. coli* isolates were also susceptible to serum; *E. coli* D31 was one of the most sensitive bacteria (Table 1, Fig. 1). All other isolates, including both Gram-negative and Gram-positive organisms, were resistant. Many of these organisms grew better in the presence of serum (e.g., *Staphylococcus aureus*). There was no evidence of lysozyme activity with the lysoplate assay.

The serum activity was bactericidal (Fig. 1). The effect of temperature data was analysed separately in the ranges 7·8–40·8° C and 40·8–70·0° C because a

Table 1. Inhibitory activity of blue crab serum against various bacteria

Bacterium	Strain or type	Gram	Bacterial growth (mean ± S.D.)	% Inhibition
Staphylococcus aureus	ATCC 12598	+	H: 0·216 ± 0·004 C: 0·102 ± 0·001	—
Staphylococcus faecum	NCSU 90-8030	+	H: 0·184 ± 0·005 C: 0·098 ± 0·006	—
Staphylococcus intermedius	NCSU 90-8031	+	H:0·121 ± 0·005 C: 0·078 ± 0·006	—
Streptococcus Group G	NCSU 90-8024	+	H: 0·069 ± 0·003 C: 0·030 ± 0·002	—
Micrococcus luteus	ATCC 381	+	H: 0·130 ± 0·004 C: 0·095 ± 0·004	—
Pseudomonas aeruginosa	ATCC 19154	−	H: 0·103 ± 0·002 C: 0·105 ± 0·004	—
Escherichia coli	D31	−	H: 0·003 ± 0·001 C: 0·111 ± 0·008	97**
Escherichia coli	NCSU 90-8033	−	H: 0·100 ± 0·010 C: 0·240 ± 0·050	58*
Escherichia coli	NCSU 90-8034	−	H: 0·193 ± 0·015 C: 0·293 ± 0·025	34**
Escherichia coli	NCSU 90-8035	−	H: 0·080 ± 0·020 C: 0·205 ± 0·055	61*
Enterobacter cloacae	B12	−	H: 0·180 ± 0·004 C: 0·131 ± 0·003	—
Serratia marcescens	ATCC 990	−	H: 0·111 ± 0·003 C: 0·091 ± 0·003	—
Aeromonas hydrophila	NCSU 88-5009C-1	−	H: 0·086 ± 0·005 C: 0·111 ± 0·004	23**
Aeromonas hydrophila	NCSU 88-5009C-2	−	H: 0·020 ± 0·001 C: 0·150 ± 0·003	87**
Aeromonas hydrophila	NCSU 88-5009C-3	−	H: 0·012 ± 0·001 C: 0·058 ± 0·023	79*
Vibrio parahemolyticus	NCSU 88-5009A-1-A	−	H: 0·165 ± 0·002 C: 0·207 ± 0·006	20*
Vibrio parahemolyticus	NCSU 88-5009B-1	−	H: 0·075 ± 0·007 C: 0·094 ± 0·002	20*
Vibrio alginolyticus	NCSU 88-5009A-2	−	H: 0·017 ± 0·004 C: 0·180 ± 0·016	91*
Vibrio vulnificus	NCSU 88-5008A-1	−	H: 0·164 ± 0·009 C: 0·190 ± 0·001	14*

H=haemolymph-treated; C=sham-treated (PBS).
*P<0·05; **P<0·01 (Student's t-test).

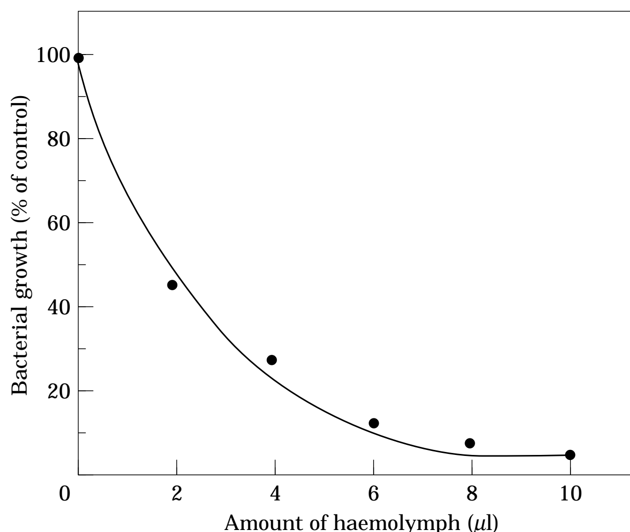


Fig. 1. Dose-response curve of serum antibacterial activity against *Escherichia coli* D31 as measured by the plating assay. Two replicate experiments yielded similar results.

plot of percent antibacterial activity versus temperature indicated that heating the serum to 40·8° C did not affect its activity. Above that temperature, activity decreased from 35% (53·0° C) to 100% (65 or 70° C). The activity was most active at low pH (5·2–6·0), with a slight inhibition at higher pH (maximum of 19% inhibition at pH 8·0). The change in activity with pH was best fit with a quadratic equation (% bacterial growth=0·006286+ [0·020517 × pH]+[0·007427 × $(pH)^2$].

The bactericidin was also inactivated by protease. The optical density of bacterial cultures having protease-treated serum (0·086 ± 0·005) was significantly ($P<0·05$) less than the optical density of those with buffer-treated serum (0·000 ± 0·001), but was slightly greater than the optical density of bacterial cultures treated with buffer alone (0·123 ± 0·004) or buffer plus protease (0·125 ± 0·007) ($P<0·05$). These data indicate that the activity is mainly, if not entirely, a polypeptide.

The activity was also inhibited by sodium chloride in a dose-responsive manner (Fig. 2). Sea water was also inhibitory. Serum activity was also retained after at least five freeze-thaw cycles and remained stable after at least 12 months at − 70° C. Antibacterial activity was confined to the haemocytes; no activity was detected in the plasma (Table 2).

IV. Discussion

The microbicidal activity in blue crab haemolymph is active against at least several Gram-negative organisms (Table 1). Its activity against bacteria that inhabit the surface of the blue crab shell suggests that it may play a role in defence against endogenous flora if the activity is also present in the shell. It has recently been shown that most of the bacteria residing on the carapace of

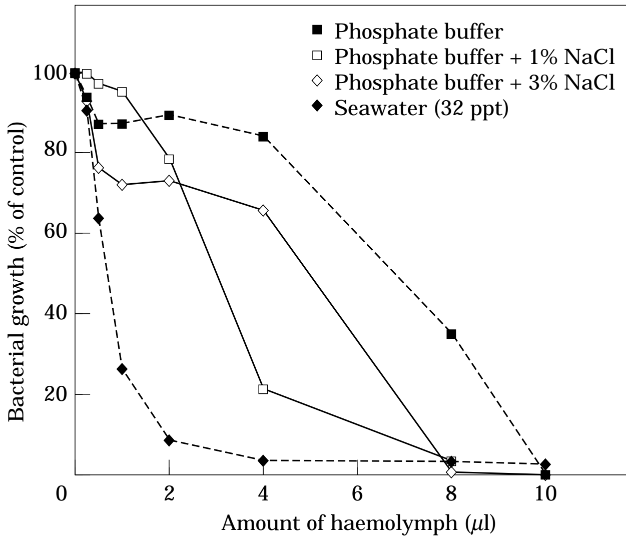


Fig. 2. Effect of sodium chloride and seawater on the antibacterial activity of blue crab serum against *Escherichia coli* D31. Two replicate experiments yielded similar results.

Table 2. Antibacterial activity of blue crab serum, plasma, and haemocyte lysate

Treatment	Bacterial growth ((O.D.$_{570}$) (± S.D.)
Plasma+haemocyte lysate	0·007 ± 0·001*
Serum	0·009 ± 0·001*
Haemocyte lysate	0·018 ± 0·001**
Plasma	0·073 ± 0·008***
Caffeine+PBS	0·081 ± 0·003***
PBS	0·082 ± 0·003***

*, **, ***Indicates values that are significantly different ($P<0·05$) from each other.

blue crabs are susceptible to this antibacterial activity (Noga *et al.*, 1994). Furthermore, it has also been found that blue crabs having depressed levels of this antibacterial activity are at increased risk of developing shell disease (Noga *et al.*, 1994). Thus, if this activity can enter the carapace, it may be an important shell defence.

The resistance of live *Micrococcus luteus* suggested that the blue crab antibacterial activity was not a lysozyme. The negative results with the lysoplate assay, and activity only against Gram-negative organisms further substantiates this observation. Its high degree of activity against *E. coli* D31 also suggests that the bactericidin may be a membrane-active agent, since D31 is especially sensitive to membrane active-antibiotics (Boman *et al.*, 1974).

The enhanced activity in low (less than physiological) salt concentrations suggests that the bactericidal activity might function in a hypotonic cell vacuole or some other low tonicity site, as is true for some antimicrobial peptides (Selsted & Harwig, 1987; Lehrer & Ganz, 1990). However, this finding needs further substantiation, since the conditions used in this experiment may not be reflective of those present *in vivo*.

There may be more than one substance present. Studies in insects have shown that some species possess many types of antibacterial polypeptides (Bowman, 1991; Hetru *et al.*, 1994). However, most insect factors are inducible (often not detectable in 'unstimulated' animals), and are present in the plasma, not the haemocytes.

The bactericidin is mainly (and possibly exclusively) present in the haemocytes. While not all activity was recovered during the haemocyte separation procedure (as indicated by a slightly lower amount of antibacterial activity in the haemocytes versus serum), activity was only detected in the haemocytes. Plasma had no significant activity (Table 2). The slightly lower activity in haemocyte lysate may be due to the very fragile nature of the haemocytes, some of which may have been damaged, releasing some activity during handling. Interestingly, caffeine, which is an effective anticoagulant in chelicerates, also slowed the clotting of blue crab haemolymph. The coagulation mechanisms of crustaceans and chelicerates are quite different and the mechanism responsible for this effect in blue crabs remains to be determined.

Chisholm & Smith (1992) found that the shore crab (*Carcinus maenas*) had potent haemolymph antibacterial activity which was localised to the granular haemocytes. This activity was heat-stable, not dependent upon divalent cations, and non-lytic. Susceptible bacteria included both Gram-positive and Gram-negative marine environmental isolates.

Stewart & Zwicker (1972) reported that haemolymph antibacterial activity in the American lobster (*Homarus americanus*) resided in the plasma, but required a factor(s) in the haemocytes to be activated. While there was more activity in lobster plasma than in haemocytes, it is also possible that some factor(s) in the haemocytes was activated/potentiated when plasma and haemocytes were mixed together. Pistole and co-workers also found in the American horseshoe crab (*Limulus polyphemus*) that neither plasma nor haemocytes had any antibacterial activity alone, but were very potent when mixed together (Furman & Pistole, 1976; Pistole & Britko, 1978). However, the lack of detectable activity in haemocytes may have been due to inadequate sensitivity of their assay, since potent antibacterial activity has recently been isolated from the haemocytes of this species (Warren *et al.*, 1992).

While haemolymph-associated antibacterial activity has been described in other invertebrates, the great majority of research has been performed in insects (Hetru *et al.*, 1994). Relatively few studies have described such activity in marine invertebrates. Other crustaceans where antibacterial activity has been reported include the tiger shrimp *Penaeus monodon* (Adams, 1991), the spiny lobster *Panulirus argus* (Evans *et al.*, 1968), and the stomopode *Squilla martis* (Danielli *et al.*, 1989). Potent antibacterial activity has also been identified in white shrimp (*Penaeus setiferus*) (Noga *et al.*, in press). Recently, researchers have isolated potent antibacterial polypeptides

from haemocytes of a chelicerate arthropod, the Japanese horseshoe crab *Tachypleus tridentatus* (Nakamura *et al.*, 1988, Toh *et al.*, 1991).

Blue crab serum also contains an agglutinin that recognises sheep erythrocytes (Pauley, 1974). However, the bactericidal activity described in the present study differs in several ways from agglutinin. The bactericidin is most active below pH 6·0, still active after heating to 50° C, and totally inactivated by protease, whereas the crab agglutinin is partially to totally inhibited below pH 6·0, completely inactivate by heating to 50° C, and resistant to trypsin (Pauley, 1974). While the bactericidin and the agglutinin appear to be different molecules, it is possible that they may act synergistically in host defences, such as with the agglutinin recognising the foreign agent and the bactericidin killing the invader after phagocytosis.

Supported by the U.S. Environmental Protection Agency under Cooperative Agreement no. 821584010, NCSU College of Veterinary Medicine Grant no. 53-0043, and the University of North Carolina Sea Grant project NA46-RG-0087 to EJN. We thank C. Lemons for supplying the clinical bacterial isolates, M.-S. Yang and C. Wang for technical assistance, and J. Jayawickrama for assistance with statistical analysis.

References

Adams, A. (1991). Response of penaeid shrimp to exposure to *Vibrio* species. *Fish & Shellfish Immunology* **1,** 59–70.

Anderson, R. S. & Chain, B. M. (1982). Antibacterial activity in the coelomic fluid of a marine annelid, *Glycera branchiata. Journal of Invertebrate Pathology* **40,** 320–326.

Boman, H. G. (1991). Antimicrobial peptides: key components needed in immunity. *Cell* **65,** 205–277.

Boman, H. G., Nilsson-Faye, I., Paul, K. & Rasmuson, T. (1974). Insect immunity I. Inducible cell-free antibacterial reaction in hemolymph of *Samia cynthia* pupae. *Infection and Immunity* **10,** 136–145.

Chisholm, J. R. S. & Smith, V. J. (1992). Antibacterial activity in the haemocytes of the shore crab, *Carcinus maenas. Journal of the Marine Biological Association of the United Kingdom* **72,** 529–542.

Danielli, E., Ferrero, E. A. & Marzani, R. (1989). Antibacterial activity and induced immunological responses in *Squilla martis* (Crustacea, stomatopoda) haemolymph. *European Aquaculture Society* (Spec. Publ.) **10,** 81–82.

Evans, E. E., Painter, B., Evans, M. L., Weinheimer, P. & Acton, R. Y. (1968). An induced bactericidin in the spiny lobster *Panulirus argus. Proceedings of the Society for Experimental Biology and Medicine* **128,** 394–398.

Furman, R. M. & Pistole, T. G. (1976). Bactericidal activity of hemocytes from the horseshoe crab, *Limulus polyphemus. Journal of Invertebrate Pathology* **31,** 376–382.

Hetru, C., Bulet, P., Cociancich, S., Dimarcq, J.-L., Hoffman, D. & Hoffman, J. A. (1994). Antibacterial peptides/polypeptides in the insect host defense: A comparison with vertebrate antibacterial peptides/polypeptides. In *Phylogenetic Perspectives in Immunity: The Insect Host Defense* (J. A. Hoffman, C. A. Janeway, Jr. & S. Natori, eds) pp. 43–65. Austin, TX: R. G. Landes.

Johnson, P. T. (1983). Diseases caused by viruses, bacteria, rickettsia, and fungi Vol. 6. In *Biology of Crustacea* (A. J. Provenzano, ed.) pp. 1–78. New York: Academic Press.

Krieg, N. R. (Ed.) (1986). *Bergey's Manual of Systematic Bacteriology*, Vol. 1. Baltimore: Williams and Wilkins.

Lackie, A. M. (1988). Immune mechanisms in insects. *Parasitology Today* **4,** 98–105.

Lehrer, M. & Ganz, T. (1990). Antimicrobial peptides of human neutrophils. *Blood* **76,** 2169–2181.
Nakamura, S., Furunaka, H., Miyata, T., Tokunaga, F., Muta, T., Iwanaga, S., Niwa, M., Takao, T. & Shimonishi, Y. (1988). Tachyplesin, a class of antimicrobial peptide from the hemocytes of the horseshoe crab (*Tachypleus tridentatus*). *Journal of Biological Chemistry* **263,** 16709–16713.
Nakamura, S., Iwanaga, S., Harada, T. & Niwa, M. (1976). A clottable protein (coagulogen) from amoebocyte lysate of Japanese horseshoe crab (*Tachypleus tridentatus*). *Journal of Biochemistry* **80,** 1101–1021.
Noga, E. J., Arroll, T. A., Bullis, R. A. & Khoo, L. (in press). Antibacterial activity in the hemolymph of the white shrimp (*Penaeus setiferus*). *Journal of Marine Biotechnology*.
Noga, E. J., Engel, D. P., Arroll, T. W., McKenna, S. & Davidian, M. (1994). Low serum antibacterial activity coincides with increased prevalence of shell disease in blue crabs *Callinectes sapidus. Diseases of Aquatic Organisms* **19,** 121–128.
Pauley, G. B. (1974). Comparison of a natural agglutinin in the hemolymph of the blue crab, *Callinectes sapidus*, with agglutinins of other invertebrates. In *Contemporary Topics in Immunobiology*, Vol. 4, (E. L. Cooper, ed.) pp. 241–260. New York: Plenum Press.
Pistole, T. G. & Britko, J. L. (1978). Bactericidal activity of amoebocytes from the horseshoe crab, *Limulus polyphemus. Journal of Invertebrate Pathology* **31,** 376–382.
Selsted, M. E. & Harwig, S. S. L. (1987). Purification, primary structure and antimicrobial activities of a guinea pig neutrophil, defensin. *Infection and Immunity* **55,** 2281–2286.
Sindermann, C. J. & Lightner, D. F. (1988). *Disease Diagnosis and Control in North American Marine Aquaculture*, 2nd Ed. New York: Elsevier.
Smith, V. J. & Chisolm, J. R. S. (1992). Non-cellular immunity in crustaceans. *Fish & Shellfish Immunology* **2,** 1–31.
Soderhall, K. & Cerenius, L. (1992). Crustacean immunity. *Annual Review of Fish Diseases* **2,** 3–23.
Sparks, A. K. (1985). *Synopsis of Invertebrate Pathology*. New York: Elsevier.
Stewart, J. E. & Zwicker, B. M. (1972). Natural and induced bactericidal activities in the hemolymph of the lobster, *Homarus americanus*: products of hemocyte-plasma interaction. *Canadian Journal of Microbiology* **18,** 1499–1509.
Toh, Y., Mizutani, A., Tokunaga, F., Muta, T. & Iwanaga, S. (1991). Morphology of granular hemocytes of the Japanese horseshoe crab *Tachypleus tridentatus* and immunocytochemical localization of clotting factors and antimicrobial substances. *Cell and Tissue Research* **266,** 137–147.
Warren, H. S., Glennon, M. L., Wainright, N., Amato, S. F., Black, K. M., Kirsch, S. J., Riveau, G. R., Whyte, R. I., Zapol, W. M. & Novitsky, T. J. (1992). Binding and neutralization of endotoxin by *Limulus* antilipopolysaccharide factor. *Infection and Immunity* **60,** 2506–2513.