Microbial Aspects of Penaeid Shrimp Digestion[1]

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ABSTRACT

Bacteria of the digestive tract of the white shrimp, *Penaeus setiferus*, comprise a limited number of generic types, characterized by rapid growth, tolerance to low pH and elaboration of an array of extracellular enzymes, especially chitinase. Mechanisms for bacterial survival and increase in cell biomass within the digestive tract are noted as is release of a selective bacteriolytic agent by the hepatopancreas. Elaboration of chitinase by specific bacteria within the microenvironment of the shrimp is indicated. Chitinase activity within the shrimp digestive tract is correlated with ingestion of dietary chitin, concomitant with an increase in chitinoclastic bacterial biomass. Bacteria may serve as a direct source of nutrients for shrimp as well as functioning in the elaboration of extracellular enzymes in the animal digestive processes.

INTRODUCTION

In this decade, increasingly greater attention is being given to crustacean culture, from pond-type operations to systems ultimately involving intensive cultivation on a quantitative controlled basis. Certainly, quality of water and realistic food conversion rates are essential considerations in order to achieve economic success of the particular enterprise. Nevertheless, often overlooked are the manifold effects that can be attributed to the microbiological component of the system, especially the direct role of microorganisms in the biology of the animal in question.

Microorganisms in natural and artificial aquatic environments may function in a diversity of ecologically effective capacities. Some organisms, as pathogens, may be deleterious, initiating mass mortalities or serving as incitants of diseases. Others may play a "neutral" role in the life cycle of the animal, affecting the

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animals neither beneficially or adversely. Still other species may be useful, serving in symbiotic, commensal or "protocooperative" relationships in digestive and other metabolic processes essential for substrate utilization and increments of growth. In all likelihood, microorganisms function in a variety of ways, affected directly and indirectly by physicochemical determinants in the immediate ecosystem of the shrimp as well as within the animal itself. This latter aspect unquestionably is a neglected area of invertebrate, and even of vertebrate, biology.

Studies reported here, developed as a part of our Sea Grant program, were designed to ascertain various contributory aspects of bacteria extant in penaeid shrimp digestive processes. In earlier reports from our laboratory (Hood, 1973; Hood et al., 1971), it was demonstrated that an indigenous bacterial biota occurs within the digestive tract of white shrimp (*Penaeus setiferus*) collected from natural estuarine waters in Louisiana. Results from other workers (Liston, 1957; Aiso et al., 1968; Sera and Ishida, 1972) support the concept of an indigenous microbiota in various aquatic fishes. To our knowledge, our studies are the first attempt to characterize such microbial systems in penaeid shrimp and to suggest their relative importance in metabolic processes of the animal.

MATERIALS AND METHODS

Survival of bacteria in the digestive tract of white shrimp: Twenty-two freshly collected juvenile white shrimp (*Penaeus setiferus*), approximately 100 mm in length, were removed from a main holding tank and put into partitioned 5-gallon aquaria. Animals were maintained without food for 48 hours following which they were provided diets containing cells of two bacteria, listed below. The crustacean food (designated FST-12-72), prepared as described by Butler (1969) and Meyers et al. (1972), was stabilized with an alginate binder allowing the extruded diet to remain intact during its manipulation and ingestion by the shrimp. The diet was a modification of FST 5-5/70 B (Meyers and Zein-Eldin, 1973), an effective grow-out ration, appropriately adjusted in ratio of ingredients to allow for the addition of the bacterial cell concentration. Into one position of FST-12-72 was mixed cells of *Bacillus firmus*, a dominant organism of the sediment, and into the other was added *Beneckea neptuna*, a bacterium isolated consistently from the shrimp digestive tract. Both bacterial species were grown in peptone (1%), yeast extract (0.05%), sea water broth flasks for 24 hours in shake culture. Following growth, cultures were centrifuged at 7,000 rpm, and cells resuspended in fresh sterile sea water (10 ml). The washed cell mass was then added to the crustacean diet. Ten animals were fed the diet containing the *Bacillus* organism, and ten, the *Beneckea* species, with each shrimp consuming 0.1 gm of the food-bacterial formulation. At various intervals after ingestion, two shrimp were sacrificed and the total bacteria in the proventriculus and the gut enumerated.

Concentrations of bacterial cells were enumerated within the food as well as within the digestive tract of two of the starved animals that served as controls. Bacterial biomass was tabulated on standard plate count media using appropriate dilutions to obtain proper colony distribution (Collins and Lyne, 1970).

Bacteriolytic properties of the hepatopancreas: The digestive glands from four freshly collected shrimp were removed with micro-dissection tools and placed in cold sterile sea water. A final dilution of 1/10, i.e., 1 gm tissue/9 ml sea water, was obtained. The tissue was homogenized in a stainless steel micro-blender at 8,000 rpm for 2 minutes with the blender jar maintained in an ice bath to avoid possible heat damage to the cellular material. Cells of *Bacillus firmus* and *Beneckea neptuna,* grown on Marine Agar 2216 (Difco) slants for 24 hours at 22° C, were scraped off aseptically and added to 10 ml sterile sea water. Plate counts and direct microscopic counts were made on the suspensions to determine total concentration of bacterial cells. The hepatopancreas extract (2 ml) and 2 ml of sterile sea water were mixed, and the pH of the solution adjusted to 7.2 with KOH. Bacterial suspensions (2 ml) were added to the hepatopancreas extract. The tubes were incubated at 22° C on a roller drum (New Brunswick Scientific Co., Model TC-5), and at intervals of 0, 5, 50, 120 and 180 minutes, 1 milliliter aliquots were removed and both direct counts and plate counts performed.

Determination of pH of digestive tract: A number of standard pH indicators were prepared and mixed into the basal crustacean diet, FST-12-72. The latter was consumed readily by the starved shrimp and color changes were noted within the animal. Liquid indicators were also injected into the shrimp digestive tract. Color changes were carefully observed in the proventriculus (stomach) and intestine. The pH indicators used were as follows: thymol blue (pH 1.2-2.8), bromcresol green (pH 3.8-5.4), methyl red (pH 4.2-6.2), bromcresol purple (pH 5.4-7.2) and phenol red (pH 6.9-8.5).

Enzyme preparation and cultural conditions: Beneckea neptuna, isolated from the digestive tract and exhibiting chitinolytic activity, was transferred to slants of Marine Agar 2216 and stored at 4° C. Cells of the isolate were inoculated into peptone (5%), yeast extract (0.05%), and sea water (pH 7.6), and incubated at 22° C for 24 hours. One ml of the bacterial suspension was added to the chitin medium containing purified ball-milled chitin, 0.05% yeast extract, and sea water. The chitin (Calbiochem Grade B purified chitin) was ball-milled for approximately 72 hours at 4° C.

Enzyme activity was measured in media with initial pH values of from 6.0-8.5, at temperatures of 20-30° C and at intervals of from 1 to 10 days. The assay system used has been described by Okutani (1966). Total hexosamines were measured by the Elson-Morgan method as modified by Boas (Good and Bessman, 1964).

Effect of diet on chitinase production: Eight shrimp (approximately 100 mm in length) maintained in partitioned aquaria, were fed an alginate-bound extruded diet containing 10% chitin in a rice bran formulation. Eight other animals were supplied the rice bran without added chitin. The molted shrimp exoskeletons were carefully removed from the tanks to prevent consumption of this chitinaceous substance by the shrimp.

After 2 weeks, the animals were sacrificed and the proventriculus and the intestine removed. These organs were weighed and placed in distilled water to

give final dilutions of 1/10 gm of tissue to volume of water. Proventriculus and intestinal material was homogenized by blending at 8,000 rpm for 5 minutes with the blender jar maintained in an ice bath. The homogenate was centrifuged at 8,000 rpm in a refrigerated centrifuge. The supernatant obtained was used as the crude enzyme extract. The procedure for the determination of the shrimp chitinase was comparable in all respects with methodology used for the bacterial enzyme. Enumeration of chitinoclastic bacteria were determined as described by Hood and Meyres (1972).

RESULTS AND DISCUSSION

The microbiota of the shrimp digestive tract was characterized by a fairly constant standing bacterial biomass, with little change in abundance noted during different collection periods. Compared with the microbiological features of the natural environment of the animals, the microbial biomass within the digestive tract was a magnitude higher (2.9×10^7 cells/gm) than the sediment (2.9×10^6 cells/gm), and more than two magnitudes greater than the waters (1.5×10^5 cells/ml) (Table 1). Significantly, the bacteria of the sediments and waters comprised numerous genera while those from the digestive tract were typically composed of a restricted number of taxa. Sediments were characteristically abundant in *Bacillus* species, while adjacent waters included species of *Pseudomonas, Flavobacterium, Chromobacterium, Micrococcus, Aeromonas, Alginomonas, Vibrio* and other genera. In contrast, species isolated from the penaeid digestive tract were representatives of *Pseudomonas, Vibrio* and *Beneckea*, especially the latter two taxa. Elsewhere, Sera and Ishida (1972) noted that the indigenous bacterial flora in the digestive tracts of marine fish comprised a specific *Vibrio* group. Briefly, species of the shrimp intestinal tract were characterized by: active production of proteolytic, amylolytic, lipolytic and chitinolytic extracellular enzymes; growth at a relatively low pH (i.e., 5.0); short generation times (i.e., 30 minutes) at relative low temperatures (i.e., 22° C). Of 16 isolates characterized biochemically, all exhibited chitinoclastic properties while none were able to degrade cellulose (Table 1).

The high concentration of bacteria in the shrimp intestine and the magnitude of activity exhibited *in vitro*, compared with sediment isolates, suggested that the bacteria play a biologically active role within the digestive tract of the penaeids. To examine this possibility, shrimp were fed extruded diets containing: (1) a predominant sediment bacterium (*Bacillus* sp.) comprising more than 10% of the total microbiota of the sediment and (2) a gut isolate (*Beneckea* sp.) comprising more than 50% of the total microbiota of the digestive tract. The diets contained an initial concentration of approximately 4.0×10^8 bacterial cells/0.1 gm of crustacean food. The actual recovery of the two species from the digestive tract at various time intervals after food ingestion is shown in Figure 1.

One hour after ingestion, the concentration of *Bacillus* and *Beneckea* cells recovered from the digestive tract decreased. After 12 hours, cells of the sediment organism could not be recovered, whereas the quantity of *Beneckea* cells increased and remained constant up to 24 hours. Even after 3 days, with no

TABLE I

CHARACTERISTICS OF BACTERIA ASSOCIATED WITH THE DIGESTIVE TRACT OF P. SETIFERUS AND ASSOCIATED WATER AND SEDIMENT HABITAT

P. setiferus	sediments	waters
Biomass		
2.9×10^7 cells/gm	2.9×10^6 cells/gm	1.5×10^5 cells/ml
Characteristic Genera of Bacteria Present		
Vibrio Pseudomonas Beneckea	Bacillus Pseudomonas Vibrio Aeromonas others	Pseudomonas Flavobacterium Chromobacterium Micrococcus Aeromonas Alginomonas Vibrio others
Percentage of Total Bacteria Showing Enzymatic Characteristics		
Proteolytic 60	19	--
Amylolytic 60	2	--
Lipolytic 40	1	--
Cellulolytic 0	1	1
Chitinolytic 100-85	10	10
Generation Time at 22 C in Peptone Sea Water Medium*		
30 minutes	2 hours	--
Minimum pH for Growth*		
5.0	6.0	--

*Average for three selected species.

supplemental food supplied to the shrimp, cells of the digestive tract *Beneckea* isolate were still present. At such time, the number of cells had decreased to approximately 10^4 cells/digestive tract. Based on these observations, it appears that certain bacteria are able to survive and reproduce in the microenvironment of the shrimp digestive tract.

The initial decrease in bacteria after ingestion, noted above, suggested further tests. The decrease probably reflects destruction of bacterial cells during various digestive processes, i.e., mechanical grinding, lowering of pH as well as possible

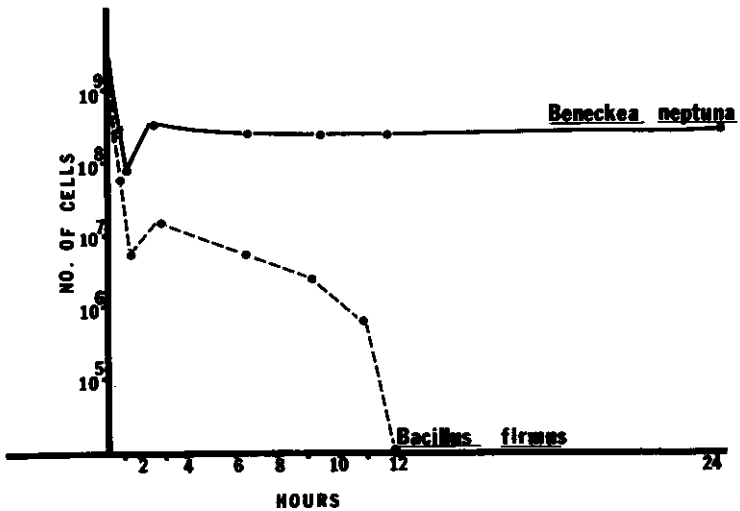

Fig. 1. Survival of the bacteria *Beneckea neptuna* and *Bacillus firmus* through digestive tract of *Penaeus setiferus*. Initial cell concentration at 0 hours was 4.0 x 10^9 cells/0.1 gm diet.

bacteriolytic agents within the proventriculus. To determine if bacteriolytic substances are secreted by the hepatopancreas, cells of *Bacillus* sp. and *Beneckea* sp. were exposed to an extract of hepatopancreas tissue. An initial decline in total number of cells was noted (Table 2) together with actual cell lysis (observed microscopically). Thus, the bacteria may serve as a source of food for the shrimp in that the bacterial protein, released upon cell lysis, may be utilized by the shrimp. Elsewhere, ZoBell and Feltham (1938) reported the presence of a bacteriolytic agent in oysters and mussels. Diets of bacteria exclusively were also shown to support growth of sand crabs and sipuculid worms, certain bottom feeders (MacGinite, 1932) and larval frogs (Burke, 1933).

With the use of various indicators (mixed with crustacean food as well as injected directly into the digestive tract) the pH of the stomach and gut was determined for starved and fed shrimp. In 48-hour starved animals, the pH of the stomach and gut was approximately pH 5.0 and 5.5, respectively. With actively feeding animals, the pH varied little from that of the food. Using a food whose pH was 7.0, no change in the color of the indicator was noted either in the

Table 2. Survival of *Bacillus firmus* and *Beneckea neptuna* in Hepatopancreas Extract from *Penaeus setiferus*

Time of Observations	Number of Cells/ml of Extract	
	B. neptuna	*B. firmus*
Initial	7.9 x 10^8	1.8 x 10^8
After 5 minutes	2.1 x 10^6	6.0 x 10^7
After 20 minutes	3.7 x 10^7	7.0 x 10^7
After 120 minutes	8.1 x 10^7	1.2 x 10^8

stomach or the intestine during passage of the food through the digestive tract. While pH probably has little effect on bacteria initially ingested, it may be a significant factor allowing certain bacteria to remain within the digestive tract. As noted previously, bacteria isolated from the digestive tract typically were more tolerant of an acidic pH. The indigenous bacteria may survive simply because they are able to grow at a pH of 5.5 or as low as pH 5.0.

The bacteria from the digestive tract had one noteworthy characteristic in common, namely, the ability to elaborate a very active extracellular chitinase. A chitinase system may be advantageous to penaeids since a moderate percentage of the shrimp's diet is composed of chitinous material. Furthermore, glucosamine, a breakdown product from chitin, has been shown to be a growth factor for prawns (Kitabayashi et al., 1971), and acetylglucosamine, another degradation product, has been implicated in biosynthesis of crustacean exoskeleton (Stevenson, 1972). The question was posed then, can the bacteria produce chitinases within the digestive tract? To answer this question, cultural conditions (temperature, pH and time) were determined for chitinase production by an isolate found in high concentrations within the digestive tract. Although maximum chitinase was produced by *Beneckea neptuna* at 25° C, considerable yield was obtained at 20° C (Figure 2). Very little difference was noted in

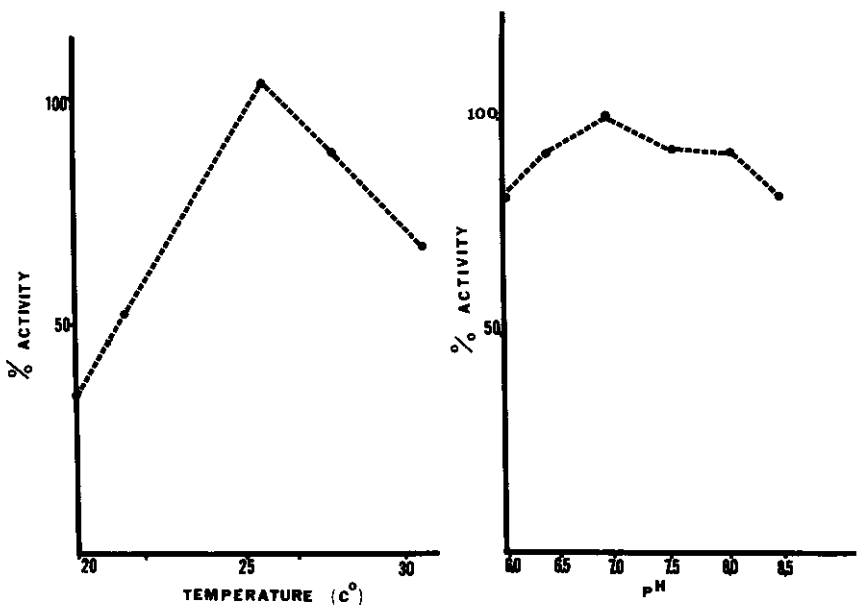

Fig. 2. Yield of chitinase by *Beneckea neptuna* at various temperatures. Cells grown for 4 days in chitin medium. Activity expressed as percent of hexosamines produced divided by amount of hexosamines produced at optimal temperatures (25° C).

Fig. 3. Yield of chitinase by *Beneckea neptuna* at various pH levels. Cells grown for 5 days in chitin medium at 22° C. Activity is expressed as percent of hexosamines produced divided by hexosamines at optimal pH (7.0).

chitinase yield at pH 6.0 and 7.0 (Fig. 3). Although five days were required (Fig. 4) for maximum chitinase activity, after only 1 day, chitinase yield was relatively high. Therefore, chitinase yield from the bacteria isolated from within the digestive tract was detectable (1) at temperatures similar to those within the tract, (2) at a pH that is common within the tract and (3) within a time period of 24 hours. This latter aspect is important since food remains in the shrimp digestive tract for comparatively short periods, i.e., from 6 to 24 hours. It is reasonable to assume that the bacterial enzyme is elaborated within the digestive tract.

To obtain a direct measurement of chitinase activity within the digestive tract, shrimp were fed a diet containing 10% chitin and one entirely devoid of chitin. Chitinase activity was determined in the digestive tract after feeding the diets for 2 weeks. An increase in activity was observed in the digestive tract of shrimp fed the chitin diet, concomitant with an increase in the number of chitin decomposing bacteria (Table 3). These preliminary data suggest that chitinase is produced by bacteria within the digestive tract of the shrimp.

SUMMARY

Unquestionably the role of microorganisms in the *in vivo* biology of penaeid shrimp is diverse; they may be a pathogenic species or they may be organisms serving advantageously as direct sources of food and as producers of a range of metabolic byproducts of nutritive value to the developing crustacean. Studies reported here, and earlier work, have shown the occurrence of a significant

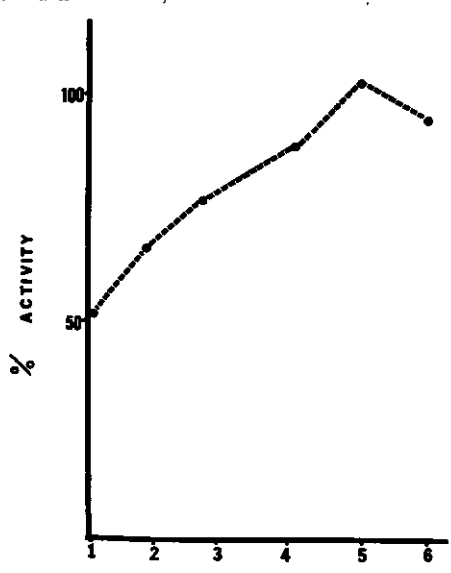

Fig. 4. Yield of chitinase by *Beneckea neptuna* at various times. Cells grown in chitin medium at 22°C. Activity expressed as percent of hexosamines produced divided by hexosamines at optimal time (5 days).

Table 3. Chitinase Activity in *P. setiferus* Digestive Tract with Chitinous and Non-Chitinous Diets

Enzyme Activity	Enzyme Units* Non-chitinous diet	Chitinous diet (10%)
Initial	1.6	1.5
After two weeks of feeding	1.7	2.1
Concentrations of chitinoclastic bacteria after two weeks of feeding	4.8×10^4 cells/gm	3.7×10^5 cells/gm

*One enzyme unit equals 0.05 mg hexosamine/ml

indigenous microbial biota within the white shrimp (*P. setiferus*) obtained from estuarine waters of Barataria Bay, Louisiana. These bacteria are characterized by rapid generation times and notably active utilization of a broad spectrum of biodegradable substrates. In addition, the gut bacteria are restricted to a comparatively few genera, notably *Beneckea* and *Vibrio*, compared with the diverse number of species prevalent in the natural environment of the shrimp. This limitation of species types, in all likelihood, reflects the ability of certain bacteria to survive passage through the shrimp digestive tract and remain viable in the microenvironment of the animal. Although a percentage of the bacterial cells are destroyed upon initial ingestion, and possibly used as a food source, the remaining cells of certain bacterial species may provide enzymes which contribute to the digestive processes of the shrimp.

Studies have demonstrated the occurrence of a bacteriolytic agent within the hepatopancreas of *P. setiferus*. Along with mechanical grinding processes associated with digestion, bacterial protein may be released within the proventriculus for subsequent utilization by the animal.

Perhaps one of the most significant physiological aspects of the bacteria is their elaboration of an extracellular chitinase enzyme(s). Of the microorganisms within the digestive tract of the shrimp, approximately 85% are able to produce chitinase. Chitinase activity within the intestine increases with supplementation of finely ground crustacean chitin to the diet of the animals. In all likelihood, this increase in enzymatic activity is due to the accompanying rise in biomass of the chitinoclastic intestinal bacteria. While the implication of this microbial chitinase in the nutrition of the shrimp has not been shown conclusively, as noted earlier, other workers have demonstrated that glucosamine, an end product of chitin degradation, is a growth factor required by certain prawns.

It is apparent that the bacteria within the shrimp gut serve in at least two general capacities: (1) they provide a source of nutrition in the form of microbial biomass, and (2) they actively elaborate enzymes, especially chitinase, which function in digestion of a range of substrates. With further intensification of crustacean culture, the contribution of chitinoclastic and other metabolically-active microorganisms to penaeid digestive processes and overall shrimp growth

deserves no small attention. In their recent discussion of problems in shrimp nutrition, Zein-Eldin and Meyers (in press) emphasized the need to consider the microbiological aspects of the shrimp digestive tract in order to obtain a total picture of the animal's nutrition. This aspect will increase in importance as various formulated diets are proposed for grow-out rations, since evidence suggests that the microbial complement of the shrimp digestive tract may be determined in part by the characteristics of the ingested food particles. It is entirely conceivable that these microbial processes may be modified or accelerated to the advantage of the mariculturist in his efforts to effect more economically advantageous food conversion rates.

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