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BACTERIA INDUCED SHELL DISEASE OF LOBSTERS [□]

(Homarus americanus)

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Abstract: Chitin degrading species of bacteria in the genera *Pseudomonas, Vibrio,* and *Beneckea* were cultured from the lesions of lobsters *(Homarus americanus)* with a shell disease. A species of bacterium of the genus *Vibrio (Beneckea)* produced necrosis characteristics of shell disease in experimental lobsters when the integument had been damaged prior to inoculation.

INTRODUCTION

Hess[10] examined lobsters, *Homarus americanus,* bearing peculiar "pitted" lesions in a tidal pound in Yarmouth, Nova Scotia. Subsequent bacteriologic examination of the lesions revealed mixed groups of chitinoclastic bacteria. Hess called this condition a "shell disease caused by chitinovourous bacteria." However, the presence of these organisms in the necrotic lesions was not definitive evidence that chitin digesting bacteria were the causative agents. Indeed, investigations of this "burned spot disease" in European freshwater crayfish revealed that fungi were responsible for the epizootics.[18,26]

In contrast to the high prevalence of the disease in the crayfish[26], lobster shell disease appears to be quite rare in nature.[10] On the other hand, alteration of the natural habitat by sewage sludge apparently increases the incidence of shell disease in lobsters as well as crabs and shrimps.[29,9] When infected lobsters are held in captivity in high densities with healthy animals, the disease spreads quickly.[25] In addition, the rate of mortality is directly "related to the degree of infection or exposure to it, the heavily infected dying at the fastest rate and the controls at the slowest".[27] The mortality of the lobsters has been attributed to the destruction of the gill membrane.[19,8]

Scientists in the United States and Canada[17] have worked on the assumption that shell disease of lobster and other marine crustaceans is caused by bacteria. Bright[5] succeeded in proving that chitin degrading bacteria were responsible for the disease in the Alaskan king crab. In addition, Cook and Lofton[7] produced lesions on crabs with chitinoclasts, implicating a species of *Beneckea* as the infecting bacterium. Other workers have isolated similar bacteria from the necrotic pits of diseased crabs[17] and lobsters.[25,19,8] However, they were not successful in their attempts to reproduce the shell disease in lobsters in the laboratory. In the present investigation, some of the bacterial flora associated with shell disease and the experimental infection of lobsters are described.

MATERIALS AND METHODS

Area of study and source of specimens

The southern part of Nova Scotia was selected as the area of study because it is an ideal location for obtaining impounded and freshly caught

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lobsters. More importantly, the few reported cases of lobster shell disease have come from this region. Water samples and lobster specimens were collected at two pounds and the nearshore waters of Yarmouth and Cape Sable, Nova Scotia.

Sterile swabs of healthy, damaged, or diseased lobsters were placed in a broth containing 0.1% yeast extract, 0.5% casamino acids [2] and artificial seawater[24] and a 0.5 by 4.0 cm strip of purified chitin.[6,2] The tubes were incubated at 20 C until degradation of the chitin strip was observed. A loopful of broth was streaked for pure colonies on chitin agar. The chitin agar consisted of 0.1% yeast extract, 2.0% precipitated lobster chitin, 1.5% agar and artificial seawater. The pH was adjusted to 7.4-7.6. Chitin utilization was indicated by clearing of the opaque medium around the colonies.[14,11,12]

Isolates capable of utilizing chitin were classified at the generic level with the schemes of Shewan[21], Baumann, *et al.*[1], and Bergey's Manual.[4]

Cell shape and motility were determined on trypticase soy broth cultures by phase contrast microscopy. Gram stains were performed on 24 h trypticase soy agar cultures using the Hucker Modification.[23] Flagella were stained by the silver impregnation procedure of Blenden and Goldberg.[3]

The salt requirement for growth was determined on nutrient agar consisting of 0.5% peptone and 0.3% yeast extract [2] with and without 3% NaCl. Other test media were made with artificial seawater.

Indole production was detected using 2216E broth[16] supplemented with 1% Tryptone.[2] For detection of nitrate reduction, strains were grown on 2216E broth containing 0.2% KNO_3. Gelatinase activity was determined on nutrient agar containing 3.0% gelatin. Starch hydrolysis was detected by adding 0.2% filter-sterilized starch to nutrient agar.

The medium of Hugh and Leifson[13] was used with all strains to test their ability to utilize glucose.

Casein hydrolysis was observed on nutrient agar in which 30% skim milk was incorporated.[21] The plates were streaked and examined for clearing zones. This medium also was used for the detection of intracellular pigments.

Antibiotic sensitivity was tested on freshly seeded trypticase soy agar plates with the following antibiotic discs: (10 I.U. Penicillin, 10 mcg Streptomycin, and 30 mcg Tetracyline)[3]. In addition, vibriostat 0/129 (2:4 diamino - 6:7 di-isopropylpteridine) assay discs were prepared by saturating 5 mm sterile blank discs with the vibriostat compound [4] at 0.1% concentration in acetone.

Infection Experiments

During the course of this study four experiments were initiated to establish shell disease on lobsters obtained at Yarmouth, Nova Scotia. Healthy lobsters were tagged, weighed, and then acclimated at the experimental temperatures in flowing aerated seawater. The seawater was not sterilized or filtered to remove the natural heterotrophic population. After scrubbing the animals with 70% ethanol, patches approximately 2×3 cm were abraded on the carapace and the ventral surfaces of the claws with sterile sandpaper. An equal number of control animals, which had been injured as described but not in-

[2] Difco Laboratories, Inc. Detroit, Michigan 48201, USA.

[3] BBL, Division of Becton, Dickinson and Co., Cockeysville, Maryland 21030, USA.

[4] Allen and Hansbury, Ltd., Ware, Herts, England.

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oculated, were placed in separate tanks. In the second experiment, however, both halves of the lobsters' carapace and each claw were damaged. The test bacterium was applied to sanded half of the animals bearing the larger chela while the opposite side served as control.

The bacterial inoculum used in the infection experiments was prepared by growing the test organism for 3-4 days at 20 C in a nutrient broth culture containing 0.1% glucosamine HCl or strips of purified chitin. The heavily turbid culture was centrifuged and the pellet resuspended in 3% NaCl to a paste-like consistency. The slurry was then rubbed onto the sanded surfaces with sterile cotton-tipped swabs.

RESULTS

Eighteen lobsters having medium to advanced stages of shell disease were examined at St. Andrews Biological Station, New Brunswick, Canada, in early summer of 1973. Swabs from these specimens yielded a diverse mixture of chitinovorous bacteria (Table 1).

The general classification schemes divided the bacteria into three groups: the *Vibrio*, *Pseudomonas*, and *Beneckea*. One feature which distinguishes *Vibrio* from *Beneckea* is peritrichous flagellation in the latter.[1] However, most of the isolates in this study and the six *Beneckea* type strains obtained from the American Type Culture Collection possessed polar flagella.

Laboratory Infection

In the first experiment, groups of three lobsters were inoculated with isolates 228-16A and 299-4C and maintained at 5 and 15 C. Shell disease developed and progressed to an advanced stage (Figs. 1A, B) on the specimen which had been smeared with 228-16A and maintained at 5 C. The three lobsters incubated at 15 C as well as the six inoculated with isolate 299-4C failed to show any breakdown of the integument.

TABLE 1. Properties of Chitinoclasts Isolated from Diseased Lobsters at St. Andrews, N.B.

Experiment	Duration	Total No. of Lobsters Inoculated	No. Developing Disease on- Carapace	Claw	Temperature
General	November 13, 1973-	3	3	1	5C
	January 7, 1974	3	0	0	15C
Optimum	December 12, 1973-	5	3	0	2C
Temperature	April 12, 1974	5	2	1	5C
		5	1	0	10C
		5	1	0	15C
Optimum	May 25, 1974-	4	0	0	2C
Temperature	July 20, 1974	4	0	0	5C
		4	0	0	10C
		4	0	0	15C
Optimum[1]	July 25, 1974-	5	0	-	3C
Temperature	September 5, 1974	5	1	-	5C
		5	0	-	10C
		5	0	-	15C

[1]Claws were not inoculated

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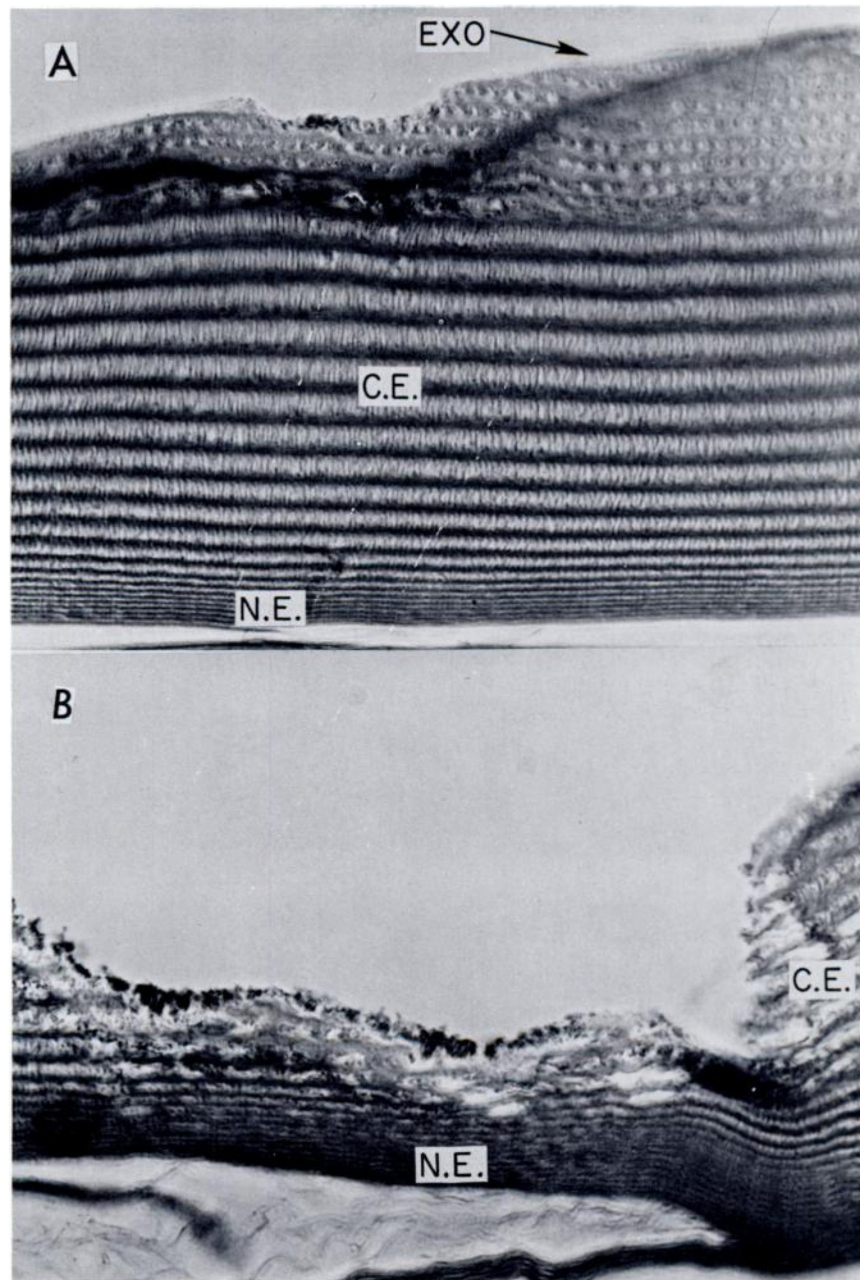

FIGURE 1. Histopathological sections showing the progression of shell disease through the exoskeleton of an infected lobster. EXO., exocuticle; C.E., calcified endocuticle; N.E., noncalcified endocuticle (X 160)

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Table 2 summarizes the results of all the infection attempts with bacterium 228-16A. Eighteen per cent (11/62) of the inoculated lobsters developed shell disease on the injured carapaces while 4.7% (2/42) of the animals had necrosis of the chelipeds. Bacteria reisolated from the lesions had the same properties as *Vibrio (Beneckea)* 228-16A. Control specimens were free of the pitting and necrosis characteristic of shell disease.

The investigations designed to determine the optimum temperature for infection were inconclusive. The data in Table 2 suggests that temperatures between 2 and 5 C yield higher prevalences of infection.

In general, the progression of the disease on these experimental animals was characteristic of Rosen's[18] description. The exoskeleton became spongy concurrent with the appearance of

TABLE 2. Summary of Infection Experiments.

	Vibrio (Beneckea)	*Vibrio*	*Pseudomonas*			
	228-16A	SA-14	299-4C	SA-4A	ML-1	321-10B
Gram Reaction	-	-	-	-	-	-
Motility	+	+	+	+	+	+
Kovac's Oxidase	+	+	+	+	+	+
Cytochrome Oxidase	+	+	+	+	+	+
Starch Hydrolysis	+	+	+	3	+	+
Gelatin Hydrolysis	+	+	+	+	+	+
Indole Production	+	+	+	-	-	+
Nitrate Reduction	+	+	-	-	-	+
Casein Hydrolysis	N/D	N/D	+	+	+	N/D
Glucose	F	F	O	O	O	+
Sucrose	F	F	O	O	O	+
Chitin Hydrolysis	+	+	+	+	+	+
Antibiotic Sensitivity						
10 I.U. Penicillin	±	±	-	2	-	-
10 mcg Streptomycin	+	+	+	+	+	+
30 mcg Tetracyline	+	+	+	+	+	+
0/129 Vibriostat	+	+	-	1	-	-
Salt Requirement	+	-	+	3	+	+
Color on Skim Milk	cream	cream	cream	cream	cream	cream
Diffusable Pigment	N/D	N/D	N/D	green	-	N/D
Number of cultures tested	4	1	1	4	4	3

+ = all positive;
- = all negative;
Numbers indicate positive responses if less than total;
± = slightly sensitive;
N/D = test not done;
F = Fermentative;
O = Oxidative.

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numerous small pits. These pits increased in size developing depressed centers which eventually merged to form continuous craters.

Cratering of the lesions was accompanied by development of a yellow slime layer. Isolates from the layer were highly agarolytic. The slime layer may be analogous to the orange pigmented material seen by Young and Pearce.[29] Furthermore, small planktonic animals were observed in the lesions and pits. These animals may be simple commensals or parasites actively attacking the exoskeleton and exacerbating the disease.

DISCUSSION

Eleven of the 62 (17.7%) lobsters developed lesions on the carapace when inoculated with a *Vibrio (Beneckea)* type bacteria. Control animals were free of disease. The prevalence of infection on the ventral surface of the claws was only 4.7%. The higher prevalence of disease on the carapace is consistent with the observations that commensals more readily attach themselves to the dorsal surface of crustaceans.[17,28,15] This preferential attachment suggests that there are more "imperfections or pores" in the polyphenolic covering of the dorsal exoskeleton, which might permit bacterial penetration.

Bacteria similar to strain 228-16A were reisolated from the lesions. Previous attempts to reinfect lobsters with chitinoclastic bacteria had failed. The results of the present research represent the first successful experimental infection of lobsters with bacteria causing shell disease and implicate a *"Vibrio (Beneckea)"* type as the infecting organism.

Rosen[18] has reviewed most of the existing literature on shell disease in aquatic crustacea and has outlined some of the problems involved in understanding the infection process. His studies suggested that bacterial infection was dependent upon a breaching of the epicuticlar defenses. In the present research, necrotic lesions developed on experimental lobsters only after this layer had been scraped away. Rosen indicated that the innermost noncalcified endocuticle was never penetrated on diseased animals. Similarly, this layer remained intact on lobsters infected with isolate 228-16A. Gopalan and Young[9] however, have observed necrosis and penetration of the epidermis of shrimp.

Lobster fishermen in Yarmouth have observed shell disease in varying degrees of severity over a number of years (pers. comm.). They noted that the disease appeared only after large numbers of lobsters had been held in the pounds and floating cars. Very few animals with the disease were reported from direct landings. Fishermen attributed the disease to mechanical abrasion and injury caused by the lobsters' habit of chewing or gnawing at one another under crowded conditions. In closed floating cars and crates of the pounds, the exchange of water and oxygen is retarded. Thus, under these stressful conditions, chitin decomposing bacteria associated with voided waste products and those normally found on lobster exoskeletons[20] might enter punctures and injuries and initiate an epizootic among weakened animals.

Young and Pearce[29] and Gopalan and Young[9] induced shell disease on lobsters and shrimp incubated on sewage sludge without first mechanically damaging their exoskeletons. Their results indicate that an injury or abrasion is not necessarily a prior condition for the establishment of shell disease. Pits and lesions were scattered over the entire bodies of the diseased lobsters examined at St. Andrews, New Brunswick. These necrotic surfaces were not always continuous but separated with solitary pits located

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from the rostrum to the telson. This separation into distinct lesions seems also to argue against the surface injury as the route of infection. However, the apparent inhibition of shell disease with tetracycline[9] does support the evidence that bacteria are the causative agents.

In summary, several lobsters, *H. americanus,* were infected experimentally with shell disease using a *Vibrio (Beneckea)* type bacterium. Establishment of the disease in laboratory animals was dependent upon the removal of the epicuticle. However, in the natural environment, the bacteria may also employ some other route of entry and spread the disease to distal parts of the lobster by means of a sub-epicuticular tunnelling process.

Future research in this area should be directed toward:

(1) the susceptibility of the various stages of the life and molting cycles of the lobster to shell disease;
(2) the possible synergistic action of the different groups of bacteria on the lesions; and
(3) the determination of the natural incidence of the disease for lobsters inhabiting "abrasive vs. soft," and polluted vs. non-polluted environments.

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