Bacterial Assemblages involved in the Development and Progression of Shell Disease in the American Lobster

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Sampling. 25 lobsters with lesions and 6 healthy lobsters were collected from the Eastern Long Island Sound (ELIS) by Connecticut DEP and were made available for this research. Five lobsters, provided by NY DEC, were collected from Long Island coastal waters (LICW). Ten lobsters with shell disease collected from the Buzzards Bay (BB) and one lobster with lesions from the Vineyard Sound (VS) was a gift from Bruce Estrella (MA Division of Marine Fisheries). All lobsters had various degrees of severity of cuticular lesions and primarily substages C3 and C4 of cuticular development. The lobsters were sacrificed and used for (1) collection of hemolymph for microbiological analysis, (2) collection of lesion material for microbiological analysis, (3) histological examination.

A half of the carapace lesion(s) was used to collect bacterial biomass and a half was preserved in 4% formaldehyde for further histopathological examination. The scraped material from healthy carapaces was also suspended in sterile seawater to optical densities similar to those of lesion material suspensions and was used for DNA isolation. Typically, we collected material only from carapace lesions. However, for nine lobsters (eight from ELIS and one from LICW) we collected material from both carapace and tail lesions. Approximately, 5 ml of hemolymph were drawn directly from hearts of each lobster into sterile Vacutainer® tubes and refrigerated.

Materials collected for gross and microscopic histopathological examination included: fragments of shell with lesions, hepatopancreas, nerve cord, portions of stomach, gonads, heart, green gland and antennae. This material was fixed in 10 % formalin in seawater. Fixed tissues were trimmed, decalcified, processed in paraffin and hematoxylin and eosin slides were prepared for examination using standard histological methods.

Culture-dependent microbiological analyses of shell lesion material. Our experiments have shown that Seawater Agar II (SAII; seawater with 1.7% of agar, 0.1% peptone, 0.01% Tween 80 and vitamin mix) and Marine Agar 2216 are the most adequate media for isolation of chitinolytic and non-chitinolytic bacteria associated with shell lesions. Chitinolytic bacteria were selectively cultured in media containing crude chitin powder from crab shells, which is an adequate imitation of lobster shell material in its biochemical composition, since it contains not only chitin but also proteins and lipids.

We could successfully isolate from each lobster three to eight unrelated bacterial strains. Bacterial strains isolated from different lobsters, colonies of which appeared very similar, turn out to be either identical or closely related (based on 16S rDNA analysis). Generally, shell lesion material from many but not all lobsters contained chitinolytic bacteria. However, their isolation in pure cultures was difficult, due to a gliding motility of non-chitinolytic bacteria and a long time response in the development of positive reactions (sometimes up to two weeks). During this time, gliding bacteria completely engulf colonies of chitinolytic bacteria. Through multistage re-streaking, however, we managed to isolated pure cultures of chitinolytic bacteria from five lobsters one of which was from ELIS and four from BB and one horseshoe crab.

Culture-dependent microbiological analyses of hemolymph. 5 µl of hemolymph from all lobsters and a horseshoe crab was streaked on rabbit or sheep blood agar plates. One set of the plates was incubated at room temperature and another at 37 °C. No growth was observed on plates incubated at 37 °C. Some bacterial growth was detected on plates incubated at room temperature with plated hemolymph from ELIS lobsters #2, 3, 6, 12 and 13 (Table 1). We concluded that there is no correlation between shell disease and

Table 1. Bacteria in hemolymph of lobsters:

Animal #	Location	Isolates	Density in hemolymph (cells ml^{-1})
1, 4, 7-11, 14,15	Fishers Island	sterile	<200
2	Fishers Island	*Pseudomonas fragi* H2	1200
3	Fishers Island	*Brochothrix thermosphacta* H3	$>2\times10^6$
5, 27-30	Long Island Coast	sterile	<200
12	Fishers Island	isolateH12.1*, H12.2, H12.3, H12.4**	2×10^4
13	Fishers Island	isolate H13.1*, H13.2, H13.3**	3200
16-25	Buzzards Bay	sterile	<200
26	horseshoe crab from MBL aquarium	sterile	<200

* - isolates H12.1 and H13.1 are identical;
** - isolates H13..3 and H12.4 are identical.

hemolymph infection. Hemolymph of only four lobsters contained some bacterial contamination and only one lobster (#3) was heavily infected.

16S rRNA analysis of bacterial isolates. 16S rRNA genes from bacteria isolated from hemolymph and chitinolytic bacteria isolated shell lesions were amplified as described by Borneman *et al.* (*Appl. Environ. Microbiol.* 62:1935-1943.). On average a 700 bp portion of 16S rRNA gene was sequenced (approximately bases 600 through 1300, *E. coli* numbering). The sequence information was used to identify close relatives of our isolates in the GenBank and Ribosomal DNA project II databases. Based on the similarity with 16S rRNA sequences of closest relatives, the isolates were given either genus (less than 98% identity) or species (98% or more of identity) designation. For the hemolymph isolates H12.1-12.4 and H13.1-13.3 and the shell lesion isolate BA2, identification was inconclusive. The 16S rRNA analysis data are summarized in Tables 1 and 2.

No typical bacterial pathogens (*Aerococcus viridans* or *Vibrio fluvialis*) were found among hemolymph isolates. Chitinolytic bacteria forming yellow colonies, belong to one of the four closely related strains of *Cytophaga* sp., indicating that these bacteria are ubiquitous in shell lesions. Identical bacteria, i.e. *Cytophaga* sp. strain 23c1, was isolated from ELIS and BB lobsters as well as the horseshoe crab. All *Pseudoalteromonas gracilis* isolates were identical, indicating that this bacterium is also ubiquitous in shell lesions. It appears that microbial communities found in lesions of lobsters from ELIS and BB are similar to each other. However, the microbial community in lesions of the Vineyard Sound lobster was different.

Table 2. 16S rDNA analysis of isolates from lesions of lobsters with shell disease.

Animal #	Location	Number of independent isolates	Name of the isolate	Number of independent identical isolates from from all animals (≥99% identity)	Reaction with chitin
7	Fishers Island	1	*Psychroserpens* sp. AN7.1	2	NT*
		4	*Pseudoalteromonas gracilis* AN7.3	13	positive
11	Fishers Island	3	*Cytophaga* sp. 11a2	4	positive
		1	*Psychroserpens* sp. MA11.3	2	NT*
		5	*Pseudoalteromonas gracilis* 11b1white	10	positive
		1	*Shewanella figidimarina* AN11.1		NT*
		1	*Alteromonas arctica* AN112.2		NT*
18	Buzzards Bay	4	*Cytophaga* sp. 23c1	12	positive
19		4	*Cytophaga* sp. 19b2	3	positive
		1	*Pseudoalteromonas gracilis* 19b1white	11	positive
23	Buzzards Bay	3	*Cytophaga* sp. 23c1	12	positive
24	Buzzards Bay	8	*Cytophaga* sp. 23c1	12	positive
		3	*Cytophaga* sp. 11a2	4	positive
26 (horseshoe crab)	MBL aquarium	1	*Cytophaga* sp. 23c1	1	positive
		3	*Cytophaga* sp. 26a	2	positive
BA	Vineyard Sound	1	*Shewanella fidelia* BA1		negative
		1	*Vibrio* sp. BA2		negative
		1	*Vibrio ienrus* BA3		negative

* NT, not tested.

Culture-independent microbiological analyses. Denaturing gradient gel electrophoresis (DGGE) was a method of choice to compare microbial communities in the lesions and individual isolates. PCR and DGGE were carried out as described by Ferris *et al.* (AEM, 1996, 62:340-346) under conditions, which we optimized earlier. The best results with PCR products of DNA from shell disease lesions were achieved using the following conditions: temperature of run 60 °C, 14 hours at 100 v, 40-55% gradient of the UF solution, and 9% acrylamide gel. The gel shown in Figure 1 depicts a DGGE gel of the whole community from the lobster #11 along with individual isolates from the lesions of this animal (C - *Cytophaga sp.*, P - *P. gracilis*, A - *Alteromonas arctica*; S - *Shewanella frigidimarina*). Both *Cytophaga* sp and *P. gracilis* strains used in these experiments are chitinolytic and clearly present as members of the community. *P. gracilis* appears to be a dominant component of the community. Overall, the composition of bacterial communities in the lesions of BB and ELIS lobsters are very similar in that they have few individual bacterial strains and at least two or three of these strains are present in all analyzed animals. The composition of the microbial community in the lobster #5 (from LICW), however, was very different from those of ELIS and BB lobsters.

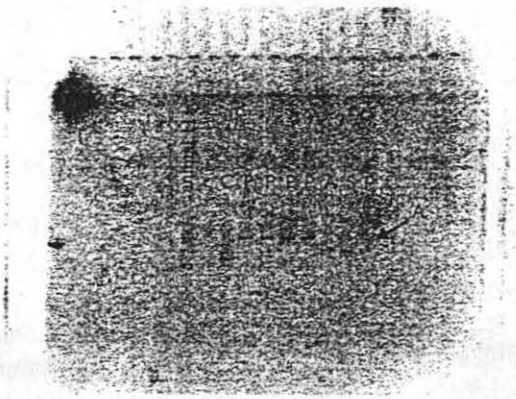

Figure 1. A DGGE gel of the whole bacterial community from lobster #1, along with individual isolates from the lesions of this animal.

Histopathological assessment. Gross examination of affected animals show moderate to deep erosions in the hard cuticle. Lesions are most common along the dorsum of the cephalothorax and abdomen, but in severe cases may extend to the claws and lateral and ventral hard carapace. Early lesions appear symmetrically, but further work needs to be done to verify this. Deeper lesions are often brown to black and result in softened carapace tissue overlying internal connective tissues of the lobster.

Histopathologically, carapace erosions are of variable depth; but deep, extensive erosions are common. Early erosions into the epicuticle and exocuticle show invasion primarily around the carapace pores and vertical extension deep into the exocuticle. These erosive areas are melanized. As the lesions deepen, and the exocuticle is eroded, they leave behind cuticular matrix that forms skeletonal pillars in the holes (Figure 2). This contrasts with impoundment shell disease, where erosions do not routinely occur as thin vertical erosions into the carapace, but rather appear as progressive areas of scooped out cuticle that leave no cuticular matrix behind.

More severe lesions show erosions that extend deeper into the calcified and uncalcified endocuticle. Melanization of the affected tissue is diffuse and in the deeper lesion the pillars of cuticle break off. Variable but often extensive layers of new uncalcified carapace (endocuticle/membranous layer) are produced by the intact hyperplastic epithelium underling the eroded site. This mechanism appears to prevent eventual ulceration into the underlying soft tissues of the animals body that could result from progressively deepening erosions.

Inflammation in epizootic shell disease is composed of increased numbers of mixed populations of hemocyte types in the underlying connective tissues and accumulations of usually necrotic hemocytes between layers of carapace. Inflammation, and cuticular proliferation, as well as melanization, of the affected cuticle are also seen in impoundment shell disease and are general responses to erosion of the cuticle for any reason.

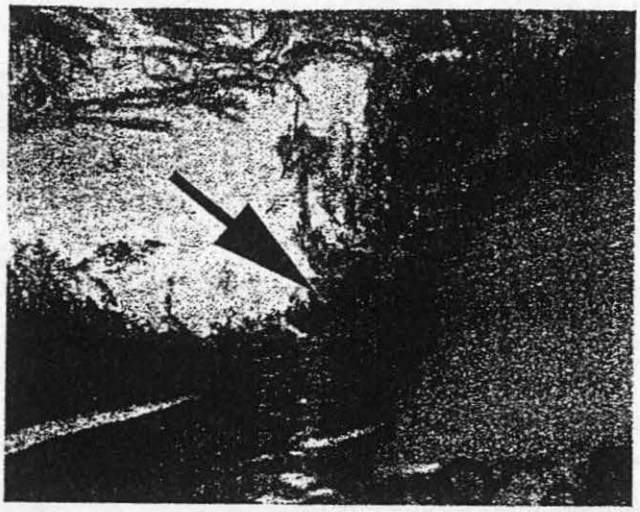

Figure 2. Early erosions into the epicuticle and exocuticle show invasion primarily around the carapace pores and vertical extension deep into the exocuticle. These erosive areas are melanized. As the lesions deepen, and the exocuticle is eroded, they leave behind cuticular matrix that forms skeletonal pillars in the holes.

Ulceration, focal loss of all carapace and invasion into underlying lobster connective tissues is very rare in tissues examined to date. Once ulceration occurs, intense inflammatory reaction produces a melanized pseudomembrane that covers the lesions. These foci can inhibit molting by causing attachments between old and new carapace.

No other disease has consistantly been identified to date in animals affected by epizootic shell disease (determined by examination of other body tissues), thus indicating epizootic shell disease is not secondary to some other primary disease (such as parameoba infections or gaffkemia).

Various organisms are identified in the shell erosions. Nematodes, sponge, algae and ciliates are occasionally seen. An as yet unidentified smaller protozoan is often seen in lesions and may be secondarily important in lesion development. But, by far the predominate organisms found at the interface of necrotic and live shell in both shallow and deep erosions into the cuticle are bacteria. Gram staining shows Gram negative bacteria in this position.

Infection experiments. A series of infection experiments has been commenced at the Flax Pond Marine Laboratory. Groups of healthy lobsters (5 individual each) were exposed to isolated *P. gracilis* and individual *Cytophqga* strains (10^6 cells of each per liter of seawater) for 24 hours. The carapace of two out of the five lobsters in each experiment was mechanically breached. To elucidate transmittance of shell disease, in a separate experiment, four healthy lobsters (epicuticle of two of them was mechanically damaged) are kept in the same tank with two lobsters with shell disease. No transmission of infection occurred after 6 month of incubations.

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