

INVESTIGATION OF EPIZOOTIC SHELL DISEASE IN AMERICAN LOBSTERS (*HOMARUS AMERICANUS*) FROM LONG ISLAND SOUND: I. CHARACTERIZATION OF ASSOCIATED MICROBIAL COMMUNITIES

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ABSTRACT Epizootic shell disease (ESD) is a degradative process of the carapace in the American lobster, (*Homarus americanus*), putatively caused by bacterial infection, and potentially responsible for serious economic losses to the lobster fishery. In Long Island Sound (LIS), ESD is prevalent in lobsters from eastern LIS (ELIS), but almost absent in western LIS (WLIS), presenting a unique opportunity to examine the influence of microbial communities on the disease process among these subpopulations. Bacterial community compositions in diseased shell, healthy shell subsamples from lobsters exhibiting signs of disease, and carapace subsamples of healthy lobsters from ELIS, WLIS, and a coastal Maine reference site were profiled using terminal restriction fragment length polymorphism (TRFLP). Although overall bacterial community membership in diseased shell was not significantly different from healthy shell and healthy lobsters, prevalence of some individual terminal restriction fragments (TRFs) varied among disease state. Several TRFs were more abundant within lesions, whereas representation of other members appeared to be diminished, particularly members of the β - and γ -proteobacteria. One TRF linked to anaerobic bacteria was enriched in lesions, suggesting anoxic microenvironments within diseased tissues. Activities of 4 ectohydrolases among communities were also measured in replicate excised shell samples. Chitinase potentials were high in all samples, and were indistinguishable among sample types. In contrast, proteinase and cellulase potentials were significantly higher in diseased shell than healthy shell and healthy lobster. Lipase potentials in LIS samples were significantly higher than those from Maine, but similar among disease states. The absence of site-specific differences in microbial communities suggests that biogeographic variation in colonizing microbes is not a factor in disease susceptibility. Lesion development appears to induce compositional shifts in normal carapace microflora, with displacement of some community members as others become more prevalent. Protein and cellulose appear to be more important targets than chitin for bacterial degradation within lesions. Furthermore, lipase activity, degrading the epicuticle lipid layer, may play a key role in regions with high disease prevalence.

KEY WORDS: American lobster, *Homarus americanus*, terminal restriction length fragment polymorphism, 16S rDNA, ectohydrolase potentials, epizootic shell disease

INTRODUCTION

Epizootic shell disease (ESD) in the American lobster (*Homarus americanus*; Milne Edwards) appeared in Long Island Sound (LIS) during the late 1990s, and its prevalence increased dramatically after 1998 (Castro et al. 2006). The disease is restricted primarily to the southern reach of the lobster's inshore coastal range. However, low incidence of this disease has been observed in northern Massachusetts waters (Glenn & Pugh 2006), and outbreaks occurred in Maine in the summers of 2003 and 2004 (Glenn et al. 2007). ESD is one of numerous shell pathologies in crustaceans, known variously as shell disease, burned spot, black spot, and rust disease, and is characterized by necrotic lesions on lobsters, shrimp, and crab carapaces (Cook & Lofton 1973, Noga et al. 2000). Chitinoclastic bacteria are generally assumed to cause shell disease, although no single causative agent has been identified in most cases, and fungi have been implicated in some forms (Vogan et al. 2008). Lesions typically begin as shallow erosions in the outer cuticle, and then deepen and spread as the disease progresses, although complete loss of the cuticle and epidermis is rare, except in extremely severe cases (Noga et al. 2000, Smolowitz et al. 2005).

ESD is an erosion of the carapace associated with poly-microbial colonization (Tlusty et al. 2007, Quinn et al. 2009,

Quinn et al. 2012). Prevalence of the disease exceeded 20% in 1999 and has remained high in eastern LIS (ELIS) and Narragansett Bay (Castro et al. 2006). ESD contrasts with the earlier observed impoundment shell disease (ISD) and other forms of shell disease in that pillars of chitin remain intact in the deep lesions after removal of the protein/lipid matrix, rather than complete removal of the cuticle (Smolowitz et al. 1992, Smolowitz et al. 2005). In severe cases, the majority of the dorsum can be covered with lesions, but few appear on the ventral surface (Smolowitz et al. 2005). In 12 lobsters from eastern Canada, shell disease was associated with pathologies of several internal organs, including the testes, vas deferens, hepatopancreas, and gills (Comeau & Benhalima 2009). However, the significance of the link between shell disease and these disorders remains unclear. Endothelium and endocuticle damage do not appear until advanced stages of the disease, leading Comeau and Benhalima (2009) to conclude that lesions and internal pathologies were both signs of the same disease process, rather than a scenario of a secondary invasion of bacteria through the carapace causing a systemic infection.

Lesions exhibit greater bacterial densities than healthy carapace (Chistoserdov et al. 2005), and bacteria are abundant at the leading edge of the lesions and within the chitin pillars (Hsu & Smolowitz 2003). Chistoserdov et al. (2005) cultivated similar bacteria from both lesions and healthy portions of carapaces from diseased lobsters, including *Pseudoalteromonas gracilis* and bacteria from the Flavobacteriaceae family. Species of *Vibrio* were also

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isolated from a few lesions, but were not common. Chitinolytic bacteria were only a minor component of this community. Labyrinthomorphid-like protists were seen under histological examination (Smolowitz et al. 2005), and more recently various stramenopile sequences were obtained from 18S rDNA fingerprints, as well as nematode, bryozoan, and fungal sequences (Quinn et al. 2009). These organisms are likely secondary opportunistic invaders that may advance the course of the disease.

Unlike ISD, ESD lacks an obvious tie to pollution in LIS and elsewhere. In Massachusetts and Long Island waters, prevalence is highest in the relatively pristine areas, and lower in more contaminated areas (Castro et al. 2006, Glenn & Pugh 2006, Smolowitz et al. 2005). In Massachusetts, the disease prevalence appears linked to bottom water temperatures on a north-south gradient. Spread of ESD into northern MA waters corresponded to an 8-y period of above-average water temperatures, and the prevalence of disease in Buzzards Bay in spring before the molting period was significantly correlated to water temperatures greater than 20°C the previous summer (Glenn & Pugh 2006). Although, high temperatures could, in theory, affect cuticle formation, resulting in less dense shell and potentially higher susceptibility to bacterial degradation, increased temperature may play a more direct role by increasing bacterial growth (Tlusty et al. 2007, Tlusty & Metzler 2012). Alkyl phenols have also been implicated in disrupting cuticle formation (Laufer et al. 2012), with implications for microbial degradation. In LIS, varying prevalences may reflect different genetic stocks of lobster in the western and eastern basin (Crivello et al. 2005a, Crivello et al. 2005b, Landers 2005). The western population may be different as a result of the population bottleneck from the 1999 die-off and/or from more stringent selection pressures arising from stronger urban influences and higher bottom water temperatures. Prevalence is higher in larger lobsters and egg-bearing females, although disease prevalence may have shifted more recently to smaller individuals (Cobb & Castro 2006). Females delay molting while carrying eggs, and may suffer higher mortalities, or may lose their eggs to molt early and escape the disease (Waddy et al. 1995, Castro & Angell 2000, Castro et al. 2006). Extended intermolt duration may allow greater opportunity for physical damage to the cuticle and further bacterial degradation to occur (Tlusty et al. 2007, Vogan et al. 2008). ESD may result in exoskeletal defects in the new carapace or mortality during molting (Stevens 2009).

We undertook a culture-independent comparative analysis of bacterial communities in both diseased and apparently healthy lobsters from ELIS, western LIS (WLIS), and Maine using terminal restriction fragment length polymorphism (TRFLP) of the 16S rDNA gene to identify key members of the lobster epibiont community that may be important in ESD. We also used community ectohydrolase potentials to assess the role of bacterial ectoenzymes in the degradation of polymers in the lobster carapace. Ectoenzymes are bacterial enzymes that hydrolyze specific bonds in extracellular biotic polymers such as lipids, chitin, peptides, and polysaccharides, so that breakdown products can be transported into the cell. Ectoenzyme potentials profiles can indicate substrate availability, important substrates for growth, as well as changes in community composition in environmental samples (Sinsabaugh & Foreman 2001, Taylor et al. 2003, Williams & Jochem 2006).

MATERIALS AND METHODS

Sampling

Lobsters were collected using double-entry vinyl-coated wire pots (76 × 51 × 30 cm, 2.5-cm mesh) from LIS. Eighty-eight lobsters were collected from ELIS by Millstone Environmental Laboratory on June 22, 2007, August 23, 2007, October 18, 2007, and June 12, 2008. Twenty-four lobsters were collected from WLIS on June 28, 2007, and June 27, 2008 by the New York State Department of Environmental Conservation. Throughout the remainder of this article, lobsters are referred to as “diseased” or “healthy” according to whether they show signs of ESD or not, respectively. Forty lobsters from ELIS were diseased, 48 were healthy; all WLIS lobsters were healthy. Thirteen lobsters (9 diseased and 4 healthy) were collected near Boothbay Harbor, ME, by the Maine Department of Marine Resources, shipped overnight on ice, and processed on July 11, 2007. Lobsters from LIS were kept cool overnight on ice and processed the day after collection. Diseased and healthy lobsters were kept in separate coolers to avoid cross-contamination. All lobsters used in this study were alive at the time of tissue processing and sample collection.

Disease severity was ranked on a scale from 0–10, with 0 representing healthy lobsters, and a score of 10 representing the most severe cases observed, in which lesions extended through the carapace and exposed underlying tissue. Weight, length, and gender were recorded before lobsters were sacrificed. Shell fragments were excised from the dorsal cephalothorax, and underlying tissue was removed. In diseased lobsters, samples from the lesions (diseased shell (DS)) and samples of shell located in areas free of lesions (healthy shell (HS)) were removed. Shell sections were also collected from healthy lobsters.

DNA Extraction

Approximately 5 × 10-mm shell fragments were stored frozen in 100 µL enzymatic lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM sodium EDTA, 1.2% TritonX-100, and 20 mg/mL lysozyme). After thawing, shells were vortexed on a Vortex Genie TurboMix attachment for 2 bursts of 2 min, incubated at 37°C for 30 min, with an additional 2 bursts of vortexing during the incubation. Extractions were then processed according to the DNeasy tissue kit protocol for Gram-positive bacteria (Qiagen, Valencia, CA).

16S rDNA TRFLP

Twenty-five-microliter PCRs were done in triplicate for each template. Each reaction contained 1× Qiagen PCR buffer, 1.5 µL MgCl₂, 5 µL Solution Q (Qiagen), 250 µM each dNTP, 200 µM of the forward primer 63F-FAM (5'-CAGGCCTAACACATG CAAGTC-3'), 200 µM of the reverse primer 778R (5'-AGGGT ATCTAATCCTGTTTGC-3') (Marchesi et al. 1998, Rosch & Bothe 2005), 0.5 µL 10 mg/mL bovine serum albumin, 2.5 µL DMSO, and 1 U HotStartTaq DNA polymerase. Primers were synthesized by Integrated DNA Technologies (Coralville, IA). 63F-FAM is labeled with the fluorochrome 5-carboxyfluorescein. PCR was run in a Teche gradient thermocycler (Minneapolis, MN), and Taq polymerase was activated at 95°C for 20 min during the initial denaturation step, then 30 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, followed by a final extension

at 72°C for 7 min and a 4°C hold until further processing. PCR product of the correct size was visualized on 1.8% agarose gels. Replicate PCR products were pooled and purified with the QIAquick PCR purification kit. The DNA was quantified by PicoGreen staining (Invitrogen, Carlsbad, CA) on a Turner Designs Aquafluor handheld fluorometer (Sunnyvale, CA). Fluorescently labeled PCR products (100 ng) were digested in duplicate with the restriction enzymes AluI, MspI, RsaI, Sau96I (NEB), and MvnI (Roche) with the provided buffers according to manufacturer instructions. Digests were ethanol precipitated and resuspended in 10 µL molecular-grade water. Ten microliters Hi-Di formamide and 0.5-µL MapMarker Rox-1000XL size standard (Bioventure, Murfreesboro, TN) were added to 5-µL samples and analyzed on an ABI3100 capillary electrophoretic genetic analyzer. The size of FAM-labeled DNA fragments may be underestimated as a result of differences in migration between fluorescein and rhodamine dyes; the error is typically 2–3 bp for 100–500-bp size fragments, and increases for larger fragments (Schutte et al. 2008).

Data Processing

Electropherograms were processed with GeneMapper version 3.7 software (ABI, Carlsbad, CA). Fragments between 50 bp and 700 bp were included in the analysis. Peaks appearing in only one of the digests were removed manually. T-REX (Culman et al. 2008) was used to filter noise according to the method of Abdo et al. (2006), using a 1-SD cutoff from the mean peak height to identify true peaks. Electropherograms were aligned in T-REX using the algorithm developed by Smith et al. (2005) and allowing more than 1 peak to be merged into a single terminal restriction fragment (TRF).

Phylogenetic Associations

TRFs were processed against a database of 71,581 16S rDNA sequences created within MiCA: Virtual Digest (ISPaR) and allowing 3 mismatches within 15 bases from the 5' end of the primers (Shyu et al. 2007). A sizing error of 3 bp was used to match sample TRFs to the predicted TRFs of database organisms.

Ectohydrolase Potentials

Approximately 10 × 10-mm carapace sections were stored overnight in filter-sterilized LIS seawater at 4°C. Fluorogenic analogs were used to assay ectohydrolase potentials on 4 major substrate classes: peptidase–peptides, cellulase–polysaccharides, chitinase–chitin, and lipase–lipids. The substrates for these ectoenzymes were leucine–methylcoumarynyl (MCA)–amide, methylumbelliferyl (MUF)–D-glucoside, MUF–N-acetyl–D-glucosaminide, and MUF–oleate, respectively. Fluorogenic tracers were prepared in 10:1 sterile water:methyl cellosolve. Shell pieces were then divided into 4 sections, placed in 15-mL Falcon tubes and overlain with 2.5-mL sterile seawater. Fluorogenic substrates were added to a final concentration of 20 µM (Taylor et al. 2003) and incubated for 4 h in a 25°C water bath in the dark. The reactions were then stopped with the addition of 2.5-mL ice-cold 100% ethanol (Mayr et al. 1999). Samples were stored at –20°C until fluorescence measurement (within 4 days). A sterile seawater control was incubated alongside the samples. Inactivated seawater and shell controls were run by adding 2.5 mL ethanol immediately after the addition of

substrate. Samples were brought to room temperature, vortexed, and fluorescence in the seawater measured on a Shimadzu RF-551 Fluorescence HPLC monitor. Calibration curves were generated by 4 subsequent additions of 20 µL of 5-µM free MUF or MCA fluorochrome to the final sample measured. The shell fragments were then rinsed with ethanol, dried at 55°C, weighed, and photographed. Surface areas were calculated from digitized images using ImageJ software. Hydrolysis rates were corrected for sterile seawater and inactivated controls, and converted to µmol hydrolysate released per square millimeter per hour and µmol hydrolysate released per milligram per hour to normalize by shell area and shell mass, respectively. Given that ambient substrate concentrations are difficult to measure, fluorogenic substrates were added at presumably saturating concentrations, and rates represent maximum hydrolytic potentials, rather than ambient rates (see Taylor et al. 2003).

Statistical Analysis

TRFLP data matrices were exported from T-REX as both presence/absence and relative peak area, and were analyzed with PC-ORD version 5. Ordinations using nonmetric multidimensional scaling (NMS) were performed with Sorensen's dissimilar distance matrix in the "Slow and Thorough" mode. Two-way cluster analysis was performed using the unweighted-pair group using arithmetic averages (UPGMA) and Sorensen distances.

TRF relative area and ectohydrolase potentials were compared among DS, HS, and HL carapace fragments using Kruskal-Wallis 1-way analysis of variance. Tests with significant results continued with Dunn's post hoc test for pairwise comparisons. Paired DS and HS fragments from the same lobster were compared using Wilcoxon's signed rank test. A statistical threshold of $P < 0.05$ was considered significant, and a Bonferroni correction for familywise error was applied in multiple comparisons. Data were analyzed in SigmaStat statistical software (v.3.5 Systat Software, Inc., San Jose, CA).

RESULTS

Community Fingerprinting: TRFLP

Initially, we screened TRFLP profiles for unique community members within lesion samples. In each of the 5 restriction enzyme digests, no TRF was unique to lesions and absent in HS or HL and vice versa. Differences among disease states were, however, found in MspI profiles (Fig. 1), in which HL profiles had significantly ($P < 0.05$) fewer TRFs than DS or HS profiles (15 ± 0.8 , 17 ± 1.6 , and 19 ± 1.6 TRFs, respectively). Despite observed differences in individual profile appearance and TRF number, community profiles failed to group in either cluster analysis or by NMS ordination on the basis of disease state, disease severity, gender, sampling site, or collection date using presence/absence of TRF peaks. To reduce the influence of rare peaks on the analysis, peaks occurring in 4 or fewer community profiles were removed, and the analysis repeated, but results remained statistically indistinguishable. However, a small but significant positive correlation was found between TRF number and disease severity (Spearman's rank correlation $r = 0.15$, $P < 0.05$, $n = 542$). The median number of TRFs (\pm SE) for all

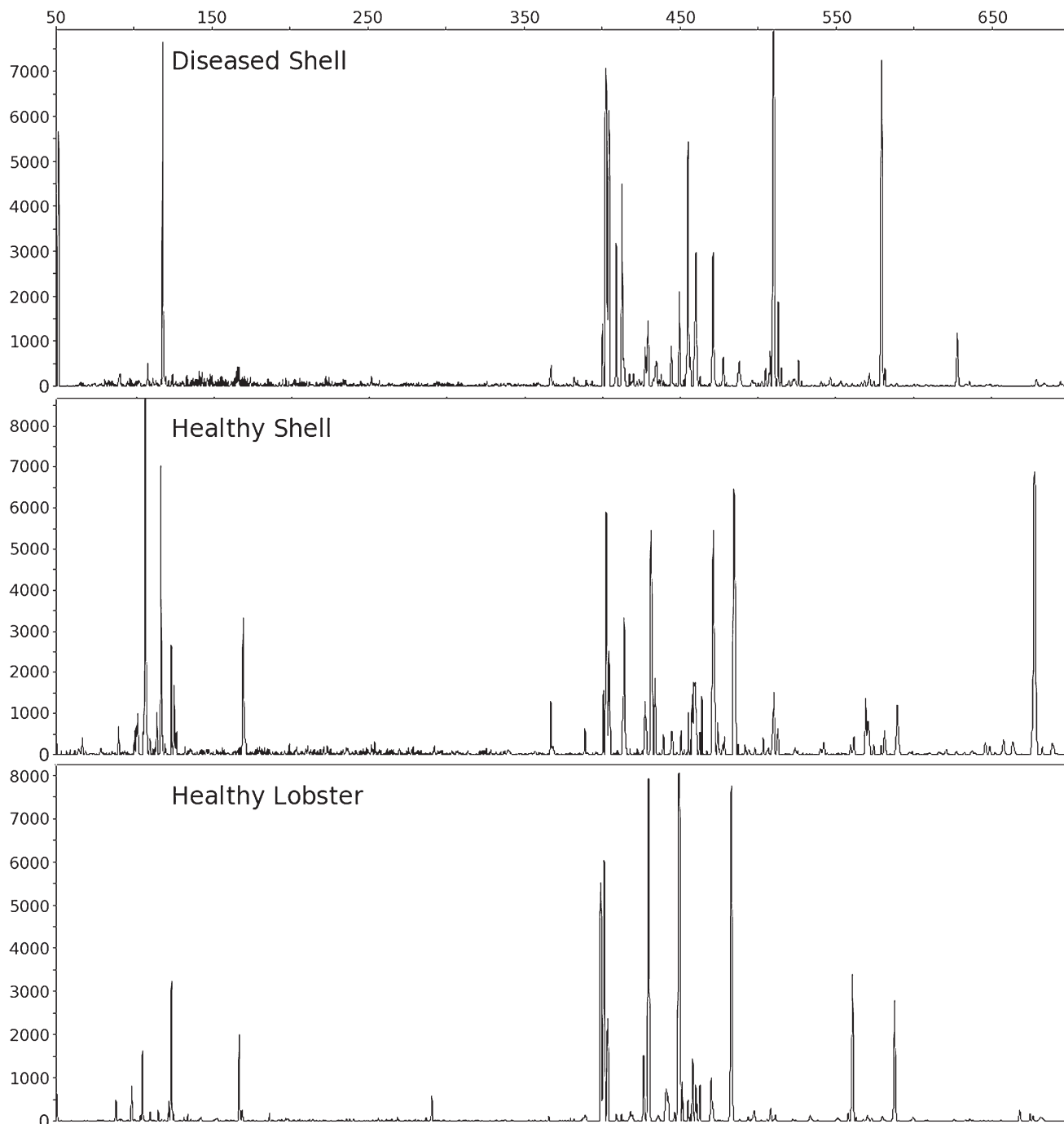


Figure 1. Example of TRFLP fingerprints generated with restriction enzyme *MspI*. Diseased shell and healthy shell fragment profiles from a single lobster collected in June 2008; healthy lobster fragment collected on the same sampling date. Fragment length in base pairs is shown on the x-axis.

restriction profiles was 17 ± 0.3 . Kruskal-Wallis revealed that *MvnI* profiles were significantly ($P < 0.05$) more populated with TRFs (20 ± 0.5) than *AluI*, *RsaI*, *Sau96I*, or *MspI* profiles (17 ± 0.8 , 16 ± 0.8 , 17 ± 0.5 , and 16 ± 0.7 , respectively).

Among all digests, relative abundances of 39 TRFs varied significantly among sample types. Of these, 4 peaks in DS samples contributed significantly higher proportions to total electropherogram peak area than in HS and HL shell samples, (i.e., were overrepresented in lesions (Kruskal-Wallis, $P < 0.05$)). None of the TRFs could be assigned definitively to a single

species based on the MICA database, because multiple species can produce equivalent fragments using TRFLP protocols. However, some tentative associations can be made higher in the taxonomic hierarchy (Table 1). The *RsaI* peak at 400 bp matched the restriction site in several γ -proteobacteria, including that of 37 *Vibrio* species, as well as several more species from Firmicutes and α -proteobacteria. Interestingly, numerous members of the class Clostridia and other members of the Firmicutes phylum have a restriction site at 218/219 bp in virtual *AluI* digests, which may correspond to our *AluI* 217-bp

TABLE 1.

TRFs listed are significantly overrepresented in diseased shell samples relative to healthy shell and healthy lobster samples.

Enzyme and TRF expected size (bp)	Bacterial Phylum/Subphylum
Alu 217	α -proteobacteria (6), β -proteobacteria (3), γ -proteobacteria (2), Firmicutes (7)
Msp 412	α -proteobacteria (5), ϵ -proteobacteria (2)
Msp 455	β -proteobacteria (39), γ -proteobacteria (146)
Rsa 400	α -proteobacteria (3), γ -proteobacteria (41), Firmicutes (4)

Bacteria listed are expected to amplify with the PCR primers used in this study and predicted to generate TRFs of the appropriate size using a 3 bp sizing error. Numbers in parenthesis indicate the number of species within each family containing the correct restriction site.

peak, although 2 species of *Vibrio* also share this site. The MspI 455-bp TRF matched restriction sites in both the β - and γ -proteobacteria, including 17 species of *Pseudoalteromonas* and 112 species of pseudomonads. The MspI peak at 412 bp matches 3 genera in the α -proteobacteria—*Acidiphilium*, *Roseobacter*, and *Thalassospira*—as well as 2 species of *Helicobacter*.

Six peaks were overrepresented in HL samples relative to DS and HS samples (Table 2), indicating that abundances of some members of the normal microflora were depressed in lesions. No

TABLE 2.

TRFs listed were significantly overrepresented in healthy lobster samples relative to diseased shell samples and healthy shell from lobsters exhibiting signs of disease.

Enzyme and TRF Expected Size (bp)	Bacterial Phylum/Subphylum
Rsa 415	γ -proteobacteria (6), ϵ -proteobacteria (16), Actinobacteria (4), Bacteroidetes (2), Chloroflexi (2), Firmicutes (57), Fusobacteria (9), Tenericutes (4)
Msp 452	β -proteobacteria (46), γ -proteobacteria (49), ϵ -proteobacteria (7), Bacteroidetes (1), Firmicutes (7)
Msp 564	β -proteobacteria (1), γ -proteobacteria (2)
Msp 589	No exact matches, closest peaks uncultured Nitrospirae
Sau 162	β -proteobacteria (15), γ -proteobacteria (138), ζ -proteobacteria (1), Aquificae (1), Bacteroidetes (1), Firmicutes (4)
Sau 281	α -proteobacteria (3), β -proteobacteria (71), γ -proteobacteria (31), ϵ -proteobacteria (4), Actinobacteria (5), Aquificae (1), Chlorobi (8), Firmicutes (7), Fusobacteria (3), Tenericutes (2)

Bacteria listed are expected to amplify with the PCR primers used in this study and are predicted to generate TRFs of the appropriate size using a 3-bp sizing error. Numbers in parentheses indicate the number of species within each family containing the correct restriction site.

matches in the database were found to a peak in MspI profiles at 589 bp. Allowing a sizing error of more than 10 bp indicates the closest-size fragments belong to uncultured Nitrospiraceae. Only 3 organisms match the peak at Msp 564 bp: *Azospira* (β -proteobacteria), and *Acidithiobacillus* and *Cellvibrio* (γ -proteobacteria). Potential matches to the remaining peaks at RsaI 415 bp, MspI 452 bp, Sau96I 162 bp, and Sau96I 281 bp were far more numerous than those to peaks overrepresented in lesions. Members of the β - and γ -proteobacteria dominate the list of potential matches, whereas α -proteobacteria are notably absent, with the exception of the tentative assignment of Sau96I 281 bp to the genus *Ehrlichia*, which can probably be dismissed because all known members are obligate intracellular pathogens of mammals.

The most dominant community peaks (comprising 15–25% of relative area) across all samples were located at AluI 215 bp, Sau96I 156 bp and 257 bp, MspI 403 bp, MvnI 57 bp, and RsaI 68 bp (Table 3). The AluI TRF at 215 bp could correspond to a common *Vibrio* spp. restriction site; however, it also matches genera within the α -, β -, and γ -proteobacteria, and Firmicutes phyla. The Sau96I 156-bp TRF is distributed broadly among the α -, β -, and γ -proteobacteria, and Firmicutes phyla as well, whereas the Sau96I peak at 257 bp appears to be restricted to 7 genera within the α -proteobacteria. The 68-bp peak in RsaI profiles is restricted to 4 genera in the α -proteobacteria, all being members of the order Rhizobiales. Similarly the 57-bp peak in MvnI profiles occurs in 3 genera belonging to Rhizobiales. The MspI 403-bp TRF also appears restricted to members of the α -proteobacteria.

Based on finding peaks associated with the Firmicutes phylum (a spore-forming group of obligate anaerobes), 6 shell fragments previously rinsed with ethanol and stored at -20°C were placed in MCP agar (Acumedia), specific for cultivation of *Clostridium*, and incubated under anaerobic conditions at room temperature. Growth was slow, but after several weeks, acidification of the media and gas production were observed, indicative of fermentation. Fermentation was not observed in MCP agar inoculated with 5 HS and HL shell fragments. Restriction digests of one culture indicated a match to 3 species

TABLE 3.

TRFs listed are the dominant peaks across all disease states (mean abundance, 15–25% of sample peak areas).

Enzyme and TRF Expected Size (bp)	Bacterial Phylum/Subphylum
Alu 215	α -proteobacteria (63), β -proteobacteria (1), γ -proteobacteria (27), Firmicutes (6)
Msp 403	α -proteobacteria (42)
Mvn 57	α -proteobacteria (9)
Rsa 68	α -proteobacteria (19)
Sau 156	α -proteobacteria (68), β -proteobacteria (21), γ -proteobacteria (56), Firmicutes (6)
Sau 257	α -proteobacteria (98)

Bacteria listed are expected to amplify with the PCR primers used in this study and are predicted to generate TRFs of the appropriate size using a 3-bp sizing error. Numbers in parentheses indicate the number of species within each family containing the correct restriction site.

of *Clostridium*—*Clostridium frigoriophilum*, *Clostridium frigoris*, and *Clostridium algoriphilum*—as well as uncultured members. Thus, our spore germination experiments support our assignment of Alu 217 to Clostridia.

Ectohydrolase Potentials

Samples from all sites and dates were pooled to assess differences in ectohydrolase potentials among disease states. Trends in mass- and surface area-normalized ectohydrolase potentials were generally similar, but only the latter are presented here because we deem surface area more relevant to bacterial activity than shell mass. Peptidase potentials varied from below detection limits to $450 \mu\text{mol}/\text{mm}^2/\text{h}$. Median peptidase potentials ($\pm\text{SE}$) of DS (78 ± 14), HS (27 ± 4.4), and HL (15 ± 3.8) were significantly different from one another (ANOVA, $P < 0.05$; Fig. 2). Cellulase potentials were statistically higher in DS (60 ± 25) than in HS (13 ± 6) and HL samples (19 ± 43). Chitinase potentials were an order of magnitude higher than other hydrolysis potentials, ranging from below detection limits to $13,000 \mu\text{mol}/\text{mm}^2/\text{h}$, but no significant differences ($P > 0.05$) among groups were found ($2,180 \pm 457$, $1,290 \pm 543$, and $1,300 \pm 246 \mu\text{mol}/\text{mm}^2/\text{h}$ for DS, HS, and HL samples, respectively). Lipase potentials varied from below detection limits to $2,410 \mu\text{mol}/\text{mm}^2/\text{h}$, but were statistically similar among DS, HS, and HL samples from all locations with medians of 91 ± 28 , 101 ± 29 , and $122 \pm 73 \mu\text{mol}/\text{mm}^2/\text{h}$, respectively.

Among ELIS lobsters exclusively, peptidase and cellulase potentials were significantly higher in DS than in HS and HL samples. In contrast, lipase potentials were significantly lower in DS and HS samples than in HL samples. Notably, chitinase potentials did not differ between disease states in the ELIS sample pool. Although low sample sizes limit the statistical power, patterns within Maine lobsters alone were not significantly different from ELIS samples. Results from tests pairing DS and HS shell samples from individual lobsters did not differ from unpaired tests.

Spatial Patterns

To examine whether hydrolytic potentials varied among lobster populations, we compared samples from WLIS, ELIS, and Maine. Samples were broken into 7 categories based on sampling site and disease state. Peptidase, cellulase, and chitinase potentials were similar within disease states among the 3 sampling sites (Kruskal-Wallis; $P > 0.05$). Lipase potentials in HL samples from ELIS and WLIS were significantly higher than in samples from Maine (Fig. 3). DS and HS lipase potentials from ELIS were statistically higher than those from Maine.

Temporal Patterns

Temporal patterns in ectohydrolase potentials varied among ectohydrolases as well as disease state. Pairwise comparisons of potentials among sampling dates in ELIS samples were conducted within each disease state (Table 4). Peptidase potentials were significantly higher in DS samples in June 2008 and October 2007 than potentials in June 2007 samples (Fig. 4). Peptidase potentials were highest in HS samples in June 2008, but did not differ significantly from activities on other dates. Potentials in HL samples were significantly lower in June 2007 than in August 2007 or June 2008. Lipase potentials in DS samples were lowest in June 2007 and highest in June 08. A similar pattern occurred in HS and HL samples, but differences were not statistically significant. In HL samples, potentials initially decreased from June 2007 to August 2007 and then increased again through October 2007 and June 2008, with June 2008 significantly higher than all other dates. For cellulase, potentials in DS samples were statistically lowest in August 2007 and highest in October 2007. In HS samples, activities also were lowest in August 2007 and increased through October 2007 and June 2008. HL samples followed the same trends as HS samples. In chitinase, potentials in DS samples were highest in June 2007 and October 2007. In HS samples and HL samples, potentials were highest in June 2007 and remained low for the remaining sampling dates. General seasonal patterns

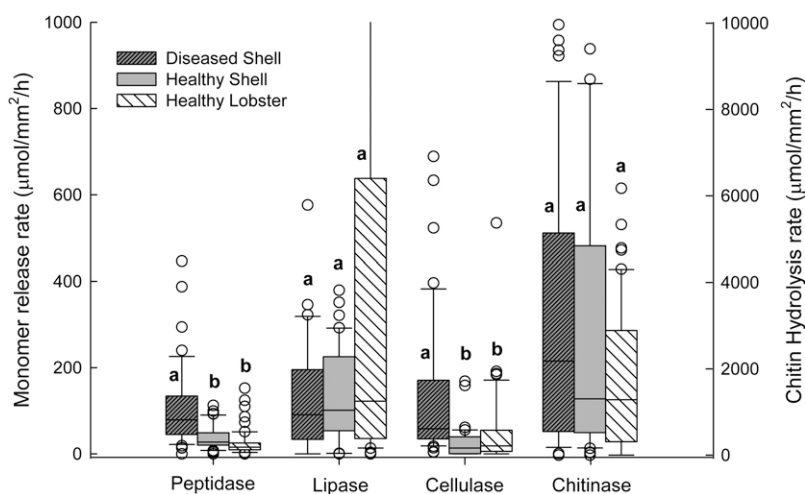


Figure 2. Ectohydrolase potentials in diseased shell samples, healthy shell samples, and healthy lobster samples. Peptidase, lipase, and cellulase potential rates are scaled on the left axis; chitin hydrolysis potentials are scaled on the right axis. Boxes represent the interquartiles of all samples (25th–75th percentiles), internal horizontal lines are medians, and whiskers are the 90th percentile. o, statistical outliers. Letters indicate which disease states shared statistically equivalent (same letters) ectohydrolase potentials or diverged significantly (different letters; Kruskal-Wallis, $P < 0.05$).

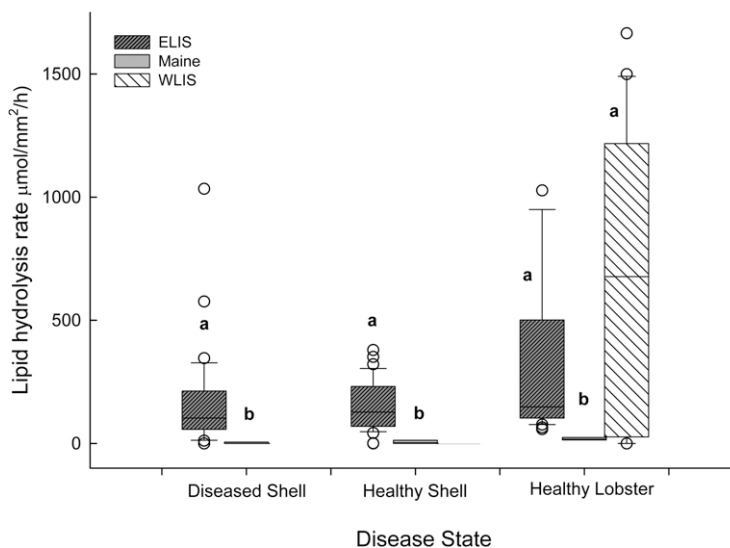


Figure 3. Spatial patterns for lipase potentials among disease states. Box and whisker criteria are the same as in Figure 2. No diseased lobsters were collected from WLIS. Letters indicate which sampling sites shared statistically equivalent (same letters) ectohydrolase potentials or diverged significantly (different letters; Kruskal-Wallis, $P < 0.05$).

were not apparent, and enzyme potentials do not appear to reflect general environmental parameters, such as ambient temperature.

Gender

There was no statistical difference between males and females within disease classes in any of the ectohydrolase potentials either for all sampling sites combined or ELIS only. Sample sizes were too small for statistically valid comparisons on individual dates.

DISCUSSION

Although bacteria are typically implicated in shell disease syndromes in various crustaceans, rarely has a specific etiological agent been identified (Goarant et al. 2000, Noga et al. 2000). Absence of a TRF clearly associated with lesions and lacking in healthy carapace samples corroborates earlier culture-dependent research on lobsters, which failed to isolate any single causative agent (Chistoserodov et al. 2005). More recently, a putative pathogen, *Aquimarina 'homaria'*, has been identified in ESD and ISD lesions, as well as from a much rarer form of

TABLE 4.

Post hoc pairwise comparison of ectohydrolase potentials between sampling dates on ELIS disease states using Dunn's test with Bonferroni correction for familywise error in multiple comparisons.

Ectohydrolase	Sampling date	DS				HS				HL			
		June 2007	August 2007	October 2007	June 2008	June 2007	August 2007	October 2007	June 2008	June 2007	August 2007	October 2007	June 2008
Peptidase	June 2007	—	1.20	2.73*	3.73*	—	0.48	0.47	2.18	—	3.14*	2.54	3.07*
	August 2007	—	—	1.47	2.57	—	—	0.92	2.52	—	—	0.29	0.08
	October 2007	—	—	—	1.26	—	—	—	1.74	—	—	—	0.23
	June 2008	—	—	—	—	—	—	—	—	—	—	—	—
Lipase	June 2007	—	1.55	1.57	0.59	—	1.50	1.80	1.03	—	1.37	1.59	3.10*
	August 2007	—	—	3.17*	2.08	—	—	3.29	2.46	—	—	2.98*	4.77*
	October 2007	—	—	—	0.89	—	—	—	0.69	—	—	—	1.29
	June 2008	—	—	—	—	—	—	—	—	—	—	—	—
Cellulase	June 2007	—	1.58	0.99	1.34	—	1.28	2.45	3.17*	—	1.52	0.92	2.53
	August 2007	—	—	2.76*	2.96*	—	—	3.51*	4.13*	—	—	2.53	4.77*
	October 2007	—	—	—	0.48	—	—	—	0.85	—	—	—	1.52
	June 2008	—	—	—	—	—	—	—	—	—	—	—	—
Chitinase	June 2007	—	2.49	0.49	3.66*	—	3.80*	2.88*	2.01	—	1.81	1.14	0.61
	August 2007	—	—	2.12	1.22	—	—	1.08	1.66	—	—	0.50	2.49
	October 2007	—	—	—	3.35*	—	—	—	0.68	—	—	—	1.73
	June 2008	—	—	—	—	—	—	—	—	—	—	—	—

Values reported are Q test statistic; difference in means scaled to variance. Statistical significance: * $P < 0.05$. DS, diseased shells; HL, healthy lobsters; HS, healthy shells.

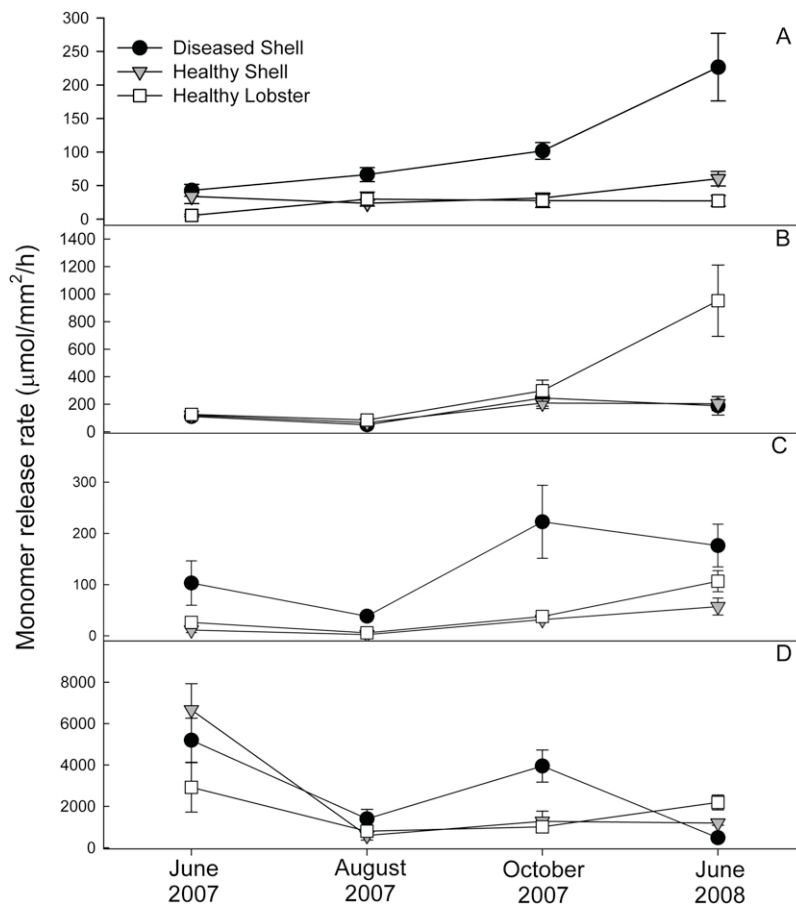


Figure 4. (A–D) Temporal patterns in ectohydrolase potentials for peptidase (A), lipase (B), cellulase (C), and chitinase (D) in ELIS. Symbols indicate means of diseased shell samples, healthy shell samples, and healthy lobster samples. Error bars = SE.

shell disease—enzootic shell disease or EnSD—but was only detected in 45% of HS carapace samples (Chistoserdov et al. 2012). In recent laboratory challenge experiments with young lobsters, the bacteria *A. 'homaria'* and *Thalassobius* sp. were detected in all induced lesions when exposed to inocula of these organisms (Quinn et al. 2012). The bacterium *A. 'homaria'* was also detected in uninoculated lesions developed at temperatures greater than 10°C. Surprisingly, bacterial diversity in spontaneous lesions was far higher according to denaturing gradient gel electrophoresis (DGGE) patterns, and the typical chitin pillars seen in traditional ESD lesions were absent in all induced lesions. However, pyrosequencing of healthy and diseased lobsters indicates that *A. 'homaria'* may be a normal member of the healthy biofilm that increases under disease conditions. Nearly a third of the operational taxonomic units identified in that study significantly discriminated healthy from diseased populations, pointing to a polymicrobial etiology of shell disease (Meres et al. 2012). Unfortunately, we were unable to mine our TRFLP data for matches to *A. 'homaria'* because the available partial 16S rDNA sequence was obtained from amplicons derived from a forward primer downstream from the 63F-FAM we used, and the intervening segment length is unknown.

TRFLP data appear to corroborate previous culture-dependent studies by identifying members of the α -proteobacteria as prominent members for the normal community (Chistoserdov

et al. 2005). The dominant TRFs derived from 2 restriction digests appear to be linked to members of the order Rhizobiales. Although typically thought of as nitrogen-fixing plant symbionts, members of the genus *Mesorhizobium* have been detected in cultures of the dinoflagellate *Gymnodinium catenatum* (Green et al. 2004) isolated from the marine sponge *Phakellia ventilabrum* (Krick et al. 2007) and in bulk water, suspended aggregates, and the oxic sediment layer from a tidal flat (Stevens et al. 2009). The *Roseobacter* genus was a potential match to 4 TRFs of interest, which is unsurprising given that the *Roseobacter* clade is a major member of coastal bacterioplankton, contributing up to 20% of clones in 16S rDNA libraries (Buchan et al. 2005, Gonzalez & Moran 1997). Numerous species within the *Vibrio* genus emerged as potential matches to 4 peaks of interest; TRFs that were overrepresented in DS samples, as well as peaks that were dominant normal community members. Although *Vibrio* spp. were only rare members in a culture-dependent study, they are typical members of coastal communities (Caruso et al. 1996, Jones et al. 2007). Some *Vibrio* spp. are pathogenic, including *Vibrio fluvialis*, which is considered to be the etiological agent causing limp lobster disease. Food pathogens, such as *Vibrio parahaemolyticus*, have been isolated from lobsters as well as other seafood (Wong et al. 1999, Tall et al. 2003, Terzi et al. 2009). Shell disease in Caribbean spiny lobster appears to be associated with the normal shell microflora, of which a novel species of *Vibrio* was a dominant member (Porter et al. 2001).

Culture studies also identified *P. gracilis* as a common member of American lobster shell communities (Chistoserdov et al. 2005), and this organism was among the species that were a potential match to the MspI 455-bp fragment, a peak that was more abundant in DS samples, but also present in HS and HL samples. Although members of Flavobacteriaceae were identified as important members of lobster shell communities using culture-dependent methods (Chistoserdov et al. 2005, Chistoserdov et al. 2012), no members of the *Cytophaga-Flavobacterium* group were matched to peaks in our TRFLP profiles, likely because of a mismatch in the 68F primer discriminating against most members of the *Cytophaga-Flavobacterium* group (Glockner et al. 1999).

TRFLP data also indicated a decline in the prevalence of some components of the normal microflora, especially members of the β - and γ -proteobacteria. Whether this represents a decline in absolute abundance or only a relative decline resulting from an increased absolute abundance in disease-associated members cannot be determined with current data. The nature of the TRFLP analysis makes definitive identification of TRFs difficult, because numerous unrelated bacteria groups may share restriction sites, and fluorochrome dye labels and purine content can influence sizing errors during analysis (Schutte et al. 2008).

Previous work has shown that ESD differs from ISD in key ways, including breakdown of important carapace components such as chitin, lipids, and proteins (Smolowitz et al. 2005). Bacterial degradation of these polymers in the environment is mediated by activity of ectohydrolases converting large polymers into monomeric products for uptake (Taylor et al. 2003). Given the metabolic cost of manufacturing ectoenzymes, regulation is theorized to be tightly controlled by both the availability of target substrates for hydrolysis and the scarcity of other more labile compounds to fuel productivity (Chróst 1989). As such, hydrolysis potentials are expected to reflect overall turnover rates of substrates and the importance of specific substrates to bacterial growth.

Variations in hydrolysis of carapace polymers have implications on disease progression given the distinct structural roles they play and their specific locations within the carapace layers. Elevated lipase potentials in LIS lobsters of all disease states are totally consistent with the hypothesis that disruption of the epicuticle lipid layer would make the cuticle more susceptible to degradation by bacteria (Tlusty et al. 2007, Vogan et al. 2008) and may help explain the high prevalence of disease in ELIS. In addition, physical abrasion of the epicuticle layer was shown to be necessary to produce infection of the carapace in laboratory-held lobsters (Malloy 1978, Quinn et al. 2012). Longer intermolt duration may result in a greater opportunity for abrasion of the epicuticle to occur in the wild. Lipase potentials fail to explain differences in disease prevalence between ELIS and WLIS because potentials in ostensibly HL in WLIS were similar to those in ELIS. Related work on shared lobsters demonstrates significant differences in immune competency between WLIS and ELIS lobsters, and associations between reduced immune response and disease state, suggesting that intrinsic lobster immunological properties may be crucial to disease susceptibility (Homerding et al. 2012).

Differences in ectohydrolase potentials were apparent between disease states. Elevated lipase potentials in HL from ELIS were primarily driven by high potentials in June 2008, and may indicate conditions favorable for lipid degradation on that

sampling date. Although not significantly different, the lower lipase potentials in DS and HS samples from the ELIS and Maine sample groups may indicate that, as the lipid epicuticle layer is lost, other biopolymers become targets for the ESD bacterial community. Loss of the epicuticle would expose the protein and chitin-rich exocuticle, consistent with significantly higher peptidase potentials in lesions versus HS and HL shell samples, as well as the loss of the protein/lipid matrix seen in scanning electron microscopy (Smolowitz et al. 1992). Elevated peptidase activity could also potentially play a role in protecting bacteria from antibacterial peptides in the cuticle. Antimicrobial peptides have been recognized in shells of shrimp (Destoumieux et al. 2000), horseshoe crabs (Iijima et al. 2005), and insects (Brey et al. 1993). Elevated cellulase potentials in lesions are more difficult to explain, because cellulose is not a component of lobster cuticle. Glucosidase activity may not be as specific as expected and may target other (1,4)-glycosidic bonds in polysaccharides, such as those present in biofilms (Sutherland 2001).

High chitinase potentials observed among all shell samples were surprising in light of the low proportion of chitin-degrading organisms that have been isolated from lobster shells previously and in this study (data not shown). These potentials are also surprising given that chitin in lesions of field-collected specimens remains somewhat intact as the protein/lipid matrix is undermined during ESD's progression. However, several bacterial groups commonly found in lobster shell communities, such as Vibrionaceae, Flavobacteriaceae, α -proteobacteria, including *P. gracilis* as well as Clostridiaceae are known chitin degraders (Cook & Lofton 1973, Chistoserdov et al. 2005, Vogan et al. 2008). With the exception of *Clostridium*, these groups appear to be important members of the normal carapace microflora and have been isolated from both lobsters with and without signs of shell disease (Chistoserdov et al. 2005). Contributions of TRFs associated with Firmicutes members were minor in HL, and spore germination was not observed in our anaerobic MCP agar enrichments from archived frozen healthy carapace. However, Firmicutes TRFs were elevated in lesions, and germination of clostridial spores was demonstrated from archived samples. These anaerobes probably represent secondary infections that occur after heterotrophic bacteria draw down oxygen levels within lesions. High chitinase potentials on lobster shells may simply reflect that chitin degradation is commonly expressed in marine microbes, because only trace amounts of chitin are found in sediments, despite production rates estimated at 1.3×10^9 mt/y (Cauchi 2002, Vogan et al. 2008). In addition, although median chitinase potentials did not differ significantly across disease state, variability was high (Fig. 2; $rsd = 94\%$, 119% , and 104% in DS, HS, and HL samples, respectively). The exceptionally high chitinase potentials found in statistical outliers of the DS and HS disease classes (maximum potentials, $13,000 \mu\text{mol}/\text{mm}^2/\text{h}$ and $9,970 \mu\text{mol}/\text{mm}^2/\text{h}$, respectively) could indicate that although bulk chitinase degradation may not be the primary cause of ESD, temporally variable or localized chitin degradation could play a role in the initiation or advancement of lesions.

The host susceptibility hypothesis put forward by Tlusty et al. (2007) posits that lesion formation is dependent on loss of chitin exceeding its deposition by the host, with large disparities between rates leading to rapid lesion formation. This model can be extended to lipid and protein components of the carapace as well. High rates of lipid degradation in ELIS and WLIS imply

that some lobsters are able to maintain their epicuticle layer with new deposition of lipid whereas others are unable to secrete enough cuticle maintenance products and hence develop lesions. Differences in the ability to deposit new cuticle material among individual lobsters likely depend on physiological stress and health, as suggested by Tlusty et al. (2007) and Vogan et al. (2008), as well as genetic factors, given that different populations inhabit ELIS and WLIS (Crivello et al. 2005a, Crivello et al. 2005b).

Although ESD ultimately is likely caused by bacteria, given the distribution of rod-shaped bacteria in micrographs, and elevated bacterial abundances in lesions (Hsu & Smolowitz 2003, Chistoserdov et al. 2005, Smolowitz et al. 2005.), non-infectious stressors undoubtedly play an important role in ESD and other forms of shell disease. ISD in lobsters can be induced by poor diet and is exacerbated by crowded conditions (Malloy 1978, Stewart 1984, Tlusty et al. 2008, Myers & Tlusty 2009). A form of shell disease in spiny lobsters was much more prevalent immediately outside the boundary of a marine reserve than within, implicating repeated handling as a cause (Freeman et al. 2009). Similarly, Comeau and Benhalima (2009) reported changes in the hepatopancreas in lobsters with ESD, indicative of either environmental stress or reduced feeding, as well as pathologies of the gills that would limit respiration leading to further metabolic stress. This body of evidence lends itself to the more complex explanation of shell disease as an interaction between the shell's bacterial community, the host immune system, and environmental stressors.

CONCLUSIONS

Ectohydrolase potential measurements support previous speculation on an important role for lipolytic and protein-degrading bacteria during development of lesions. The presence of obligate anaerobes in lesions indicates that the elevated heterotrophic bacterial activity may be creating anoxic micro-niches within the carapace. Our results indicate that no specific

bacterial group is associated exclusively with ESD in the wild, and that the dominant bacterial groups on lobster carapaces do not appear to differ substantially between lobsters with ESD and apparently healthy lobsters. However, significant changes do occur in the relative abundance of a few groups and may support a polymicrobial scenario in disease development. Similar broad bacterial groups were found to dominate communities in lobsters from all 3 sampling locations and constitute the normal microflora, whereas rarer members vary widely among individuals. The dominant community members appear to include bacteria previously isolated in culture studies of ESD, as well as common marine bacterioplankton. Our results support disease models that take into account recent work on stress and immunosuppression of the host, and interactions with the normal bacterial microflora as key components to the progression of ESD.

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