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EXPOSURES OF *HOMARUS AMERICANUS* SHELL TO THREE BACTERIA ISOLATED FROM NATURALLY OCCURRING EPIZOOTIC SHELL DISEASE LESIONS

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ABSTRACT Epizootic shell disease (ESD) is an emerging form of shell disease of the American lobster (*Homarus americanus*) that has had detrimental effects on the fishery in southern New England. Three bacteria commonly isolated from lesions of wild lobsters with ESD—a novel *Aquimarina* sp. (*A.* '*homaria*' I32.4), a novel Rhodobacteraceae species ('*Thalassobius*' sp. I31.1) and a *Pseudoalteromonas* sp. (*Pseudoalteromonas* '*gracilis*' ISA7.3)—were applied directly to normal and abraded juvenile lobster carapaces, and then monitored for persistence over time and for the development of shell-disease lesions at 3 different temperatures (10°C, 15°C, and 20°C). Without abrasion of the carapace, no lesions developed in the exposures. After abrasion and exposure with a pure culture of *A.* '*homaria*' I32.4, lesions developed at all 3 temperature and *A.* '*homaria*' was detected in the lesions of all animals tested. Surprisingly, '*Thalassobius*' sp. I31.1 also colonized these lesions. A coexposure with all 3 bacteria also demonstrated lesion development and the persistence of *A.* '*homaria*' I32.4 and '*Thalassobius*' sp. I31.1. The bacterium *P.* '*gracilis*' ISA7.3 was not able to persist in any of the challenged lesions. Abraded areas of the cuticle with no bacteria added directly were also colonized by *A.* '*homaria*' and '*Thalassobius*' sp., and moderate lesions developed; however, the directly exposed lesions were significantly more severe ($P < 0.05$). The bacterium *A.* '*homaria*', but not '*Thalassobius*' sp., was detected in spontaneous lesions that developed independent of any abrasion and/or bacterial exposures. A novel bacterium, '*Candidatus* Kopriimonas aquarianus' was also detected in spontaneous lesions. This study shows that 2 bacteria isolated from ESD lesions of wild lobsters are able to persist in and act together as important components of lesion development on abraded surfaces of American lobsters. This indicates that they are likely major contributors to lesion development in the ESD polymicrobial infection and may represent significant pathogens of the American lobster.

KEY WORDS: shell disease, lesion, *Aquimarina*, *Thalassobius*, lobster, denaturing gradient gel electrophoresis, epizootic shell disease

INTRODUCTION

Shell disease is a ubiquitous disease of Crustacea that causes a degradation of the cuticle, producing dark, melanized lesions. Three forms of shell disease have been described in the American lobster (*Homarus americanus,* H. Milne Edwards, 1837), including impoundment shell disease, endemic burn spot shell disease, and epizootic shell disease (ESD) (Stewart 1980, Smolowitz et al. 1992, Ziskowski et al. 1996, Castro & Angell 2000). A fourth form of shell disease, termed enzootic shell disease, has also been proposed, representing the normal low incidence and low severity of shell disease that has been described for years (Smolowitz et al. 2005a). ESD is a severe form of shell disease that has emerged on the southern coast of New England during the past 8–10 y, and it has a poorly understood etiology (Cobb & Castro 2006). Population modeling has indicated that ESD has caused increased mortality to *H. americanus* in this area, as the inverse relationship between the amount of prerecruits and landings was better predicted with the inclusion of a shell-disease term (Wahle et al. 2009).

ESD is characterized by severe, deep erosions of the cuticle that extend laterally and, more characteristic of this disease than others, spreads irregularly over the dorsal carapace of the animal (Smolowitz et al. 2002, Smolowitz et al. 2005a). The lesions usually begin on the cephalothorax and the rostrum, but can affect the animal at all locations. Histologically, the lesions present as a degradation of the epicuticle and exocuticle. As lesions progress, they may erode through the calcified and uncalcified endocuticle, ultimately causing ulceration by destroying the underlying cuticular epithelium (Smolowitz et al. 2005a).

The average prevalence of ESD from south of Cape Cod to central Long Island Sound was approximately 20–30% from 1998 to 2005 (Cobb & Castro 2006). However, the prevalence has been recorded as more than 80% in ovigerous females in eastern Long Island Sound in 2002 (Cobb & Castro 2006), likely harming overall larval production (Landers 2005, Wahle et al. 2009). ESD prevalence has a significant correlation with water temperature, sexual maturity, and intermolt duration (Castro & Angell 2000, Glenn & Pugh 2006), and is highest immediately prior to the late-spring and late-fall molt. It has been hypothesized that an environmental stressor, such as high water temperature or pollutants, or physiological malfunction in the lobster cuticle, allows for an opportunistic infection by bacteria (Tarsitano & Lavalli 2005, Tlusty et al. 2007), but few studies addressed this theory directly. However, Bethoney et al. (2011) have shown that fish used as a bait do not contribute to ESD outbreaks.

There is a highly diverse community of microorganisms living in the lesions of lobsters with ESD, including bacteria, protistans, fungi, and nematodes (Chistoserdov et al. 2005, O'Kelly 2005, Chistoserdov et al. 2009, Quinn et al. 2009). The general consensus among researchers is that ESD is a polymicrobial infection for which bacteria are the primary

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agents causing lesion development (Fisher et al. 1976, Malloy 1978, Chistoserdov et al. 2005). The culturable bacterial community of ESD lesions contained members of the Flavobacteriaceae and *Pseudoalteromonas* spp.; some less commonly cultured bacteria included various alpha- and gammaproteobacteria (Chistoserdov et al. 2005). Another study also cultured members of the Flavobacteriaceae from surfaces unaffected by shell disease and diseased lobster surfaces, in which the authors proposed that the resistance of these bacteria to protistan grazing may select for their presence and facilitate their role in shell disease development (O'Kelly 2005). Culture-independent techniques verified the presence of alpha- and gammaproteobacteria and Bacteroidetes (specifically Flavobacteriaceae) in ESD lesions (Chistoserdov et al. 2009, Bell et al. 2012, Chistoserdov et al. 2012, Meres et al. 2012). Of particular interest was the presence of a specific species of *Aquimarina* (*A*. '*homaria*'), a group of strains belonging to Rhodobacteraceae, related to *Thalassobius* spp. and *Pseudoalteromonas* '*gracilis*', all of which were highly associated with the lesions of ESD (Chistoserdov et al. 2009, Chistoserdov et al. 2012).

A major hindrance to the understanding of shell disease in the American lobster has been the lack of a testable laboratory model of the disease (Prince & Bayer 2005). Polymicrobial infections such as ESD are notoriously difficult to reproduce and study *in vitro* (Drake & Brogden 2002). Lobster cohabitation experiments did not facilitate transfer of the disease from diseased to apparently healthy lobsters (Cawthorn 2011). However, a model of diet-induced shell disease was developed by Tlusty et al. (2008), in which they showed that feeding juvenile lobsters herring exclusively over a 352-day period induced high levels of mortality and shell disease. The study described here is the development of a challenge model of shell disease using juvenile lobsters with compromised epicuticles. Physical removal of the epicuticle may be required to emulate natural conditions, because adult lobsters often abrade their cuticles as a result of physical contact with their habitat, such as entering and exiting shelters. In addition, lobsters lose their epicuticles during late stages of cuticular formation (C4/DO) as a result of the regression of tegmental glands before molting (Smolowitz, unpubl. obs.). Juvenile lobsters with abraded epicuticles were exposed to cultures of 3 bacteria commonly isolated from wild ESD lesions: *A*. '*homaria*,' '*Thalassobius*' sp., and *P*. '*gracilis*'. The animals were then monitored for lesion development. Sections of the abraded surface were used for histology, and scrapings of the abraded surfaces were analyzed for the presence of the 3 bacteria used in the challenges as well as any additional species using denaturing gradient gel electrophoresis.

MATERIALS AND METHODS

Lobster Rearing and Care

All lobsters used in this study were raised at the Lobster Research and Rearing Facility (LRRF) at the New England Aquarium (Boston, MA). Because of the limited number of animals available in the facility and the lengthy time required to grow lobsters from larvae to juvenile size, 2 groups of lobsters were used for these experiments; the first 8 lobsters were separated into 3 temperature treatments—10°C, 15°C, and 20°C ($n = 2$, 4, and 2, respectively)—within the LRRF system and kept at the temperature treatments from 104–443 days and fed growth diet (Tlusty et al. 2005) before being used for exposures. The second group of 17 lobsters had been maintained in the temperature treatments for approximately 896–1,014 days (an additional $n = 7$, 6, and 4 for the 10°C, 15°C, and 20°C treatments, respectively). The lobsters were 4–5 y old, but of similar size as the initial group of lobsters. These lobsters had been fed a gelatin-based diet consisting of 98.5% Economac, an extruded diet with highly unsaturated fatty acids (Aquafauna Bio-Marine Inc., Hawthorne, CA), and 1.5% Naturose (natural astaxanthin) bound with dissolved gelatin (3 g gelatin in 38 mL distilled water for 30 g total ingredients). Although the groups were held at the initial temperatures for different lengths of time, they were in equally good health and used for exposure trials at the same molt stage (the beginning of C4). The total number of individual lobsters used for exposure trials were 10°C, $n = 9$ (5 challenged, 4 control); 15°C, $n = 10$ (6 challenged, 4 control); 20°C, $n = 6$ (5 challenged, 1 control); and they averaged 30.2 mm in carapace length (±0.66 mm SE) and 7.71 g (±1.38 g SE) in size when used for exposures. Sample sizes were lowest at 20°C, because at the LRRF, lobsters often die when they develop shell disease at this temperature. Some samples were lost during DNA extraction; the total number of lesions tested by PCR is listed in Table 1. Temperatures were monitored daily as well as logged every 5 min using Tidbit v.2 data loggers (Onset Computer Corporation, Bourne, MA).

Shell Exposures

In our initial pilot experiment, 2 lobsters were used for exposures to bacteria without shell abrasion. All subsequent lobsters had shells abraded, whether control animals (sterile filters) or challenged (bacteria on filters). To maximize sample numbers, the animals (control or exposed) were abraded on both lateral sides of the carapace, and each side was considered an independent sample. For the challenged lobsters, the left side of the carapace was used for the exposure of *A*. '*homaria*' only and the right side was used for the coexposure, and for the control lobsters both sides of the carapace were mock challenged. The 3 bacterial strains used for exposure—*A*. '*homaria*' strain I32.4, a '*Thalassobius*' sp. strain I31.1, and *P*. '*gracilis*' strain SA7.3—were isolated from ESD lesions of wild lobsters on marine agar by Chistoserdov et al. (2005). For the exposures, a suspension of each bacterial strain in sterile-filtered seawater was prepared, and direct counts were conducted by plating on marine agar (Difco, Detroit, MI). Bacterial densities for all strains averaged 5.8×10^8 cells/mL and ranged from 6.5×10^7–1×10^9 cells/mL. Suspensions (0.6 mL total volume) were centrifuged at 10,400 rpm for 5 min (Clay Adams Triac Centrifuge) and then filtered onto an Isopore 13-mm-diameter, 0.2-μm membrane filter. Prior to exposure, the lobster carapaces were rinsed with sterilized filtered seawater before filter placement, and the filters were then placed onto the lateral carapace of the lobsters. The left side of the carapace was challenged with *A*. '*homaria*' I32.4 (0.6 mL) and the right side of the carapace was challenged with all 3 strains—*A*. '*homaria*' I32.4, '*Thalassobius*' sp. I31.1, and *P*. '*gracilis*' ISA7.3 (0.2 mL of each strain, approximately 10^8 cells). Filters were held on the lobster by wrapping a rubber band around the cephalothorax of the lobster between the second and third pereiopods. Control animals were treated the same way as challenged animals, except filters contained only 0.6-mL sterile

TABLE 1.

The presence of a DGGE band for *A. 'homaria'* 132.4 and *'Thalassobius'* sp. 131.1 in lesions at the 3 temperatures and the mean severity score of the lesions for the lobsters from each treatment.

	10°C				15°C				20°C			
			No. of Lobsters for which Lesions Were Positive for				Number of Lobsters for which Lesions Were Positive for				No. of Lobsters for which Lesions Were Positive for	
Infection	No. of Lobsters in Experiment	Mean Severity Score	*A. 'homaria'*	*'Thalassobius'* sp.	No. of Lobsters in Experiment	Mean Severity Score	*A. 'homaria'*	*'Thalassobius'* sp.	No. of Lobsters in Experiment	Mean Severity Score	*A. 'homaria'*	*'Thalassobius'* sp.
A. 'homaria'	5	2.3	5	5	5	1.7	5	5	5	1.5	5	5
Co-infection	5	2.5	5	5	6	1.5	6	6	3	2.4	3	3
Control*	4	1.6	0	1	4	1.0	8	2	2	1.5	2	0
Spontaneous	N/L	N/L	N/L	N/L	N/L	N/L	N/L	N/L	3	N/L	3	0

* Control lobsters represent animals abraded, but not exposed to bacteria. There were twice the number of lesions sampled as the number of lobsters indicated for these controls because both sides of the carapace were abraded and mock infected. N/L, lesions did not develop.

filtered seawater (hereafter referred to as control lesions). After challenge, lobsters were held in individual plastic containers in 1 of 3 different recirculation tanks (193 × 53 × 7.5 cm, 390 L each, 20% flow-through daily) fed from a common water source according to their respective incubation temperature. Lobsters were checked weekly for the development of shell-disease lesions. If disease had not developed or lesions were not yet an appropriate size, filters were removed and the exposure process was repeated each week until lesions approximately 1 cm^2 developed. After lesions developed to this size, the lobsters were sampled for DNA analysis. The numbers of exposures required to develop a lesion at different temperatures were recorded and analyzed statistically (*t*-test).

Because no lesions developed when bacteria were applied to intact carapace surfaces ($n = 2$; each challenged and rechallenged 3 times for 3 wk), to facilitate lesion development, a method was developed to erode the epicuticle layer by abrading the lateral carapaces with fine (400-grit) sandpaper prior to filter placement. Lobsters were rinsed with sterile filtered seawater, and an area approximately 1 cm^2 on each side of the carapace was rubbed with sandpaper for 20–25 sec. The carapaces were checked under a stereomicroscope at 20× to ensure the epicuticle was removed. The abraded areas were rinsed again with sterile filtered seawater and exposed to bacteria as described earlier. Lobsters were checked on a weekly basis for lesion development. Water temperatures during the exposure trials were maintained at their designated temperatures (mean ± SE of the mean): 10.04 ± 0.19°C (10°C), 14.50 ± 0.27°C (15°C), and 19.80 ± 0.45°C (20°C).

Sample Collection

Lobsters were rinsed with sterilized filtered seawater at the time of sampling. Lesions were also rinsed with sterilized filtered seawater to remove unattached or loosely associated bacteria. Sampling of unaffected shell and lesions consisted of scraping the lesions and abraded surfaces of each lobster and intact shell surfaces with a sterile razor blade, and collecting the scraped material in 500 µL sterile 50 mM Tris-HCl and 50 mM EDTA buffer (pH 8.8). Half of each lesion and abraded surface was kept intact for histological examination. The lobster was then sacrificed and the abdomen was removed, and the cephalothorax bisected tissues were fixed in 10% sodium phosphate-buffered formalin for histological examination (Howard et al. 2004).

DNA Extraction

To detect bacteria in exposed lesions, DNA was extracted for polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE). Egg white lysozyme (Amresco, Solon, OH) was added to the shell samples to a final concentration of 1 mg/mL, and the mixtures were then incubated at 37°C for 30 min. A sodium dodecyl sulfate solution was added to a 2% final concentration, followed by the addition of proteinase K (Fisher Bioreagents, Fair Lawn, NJ) at 1.25 mg/mL, and then incubated at 50°C for 15 min. The samples were then subjected to 3 freeze–thaw cycles at 50°C, then –80°C and bead beating in a Mini-Beadbeater-8 with 0.10-mm Zirconia/Silica Beads (BioSpec Products, Bartlesville, OK). DNA was extracted consequently using phenol and chloroform. Buffered

phenol (pH 6.8) (Sigma-Aldrich Inc., St. Louis, MO) was added to bacterial lysate at a 1:1 ratio, the mix was emulsified vigorously, then centrifuged for 5 min at 5,000 rpm, and the aqueous phase was removed. An equal volume of chloroform (FisherBiotech, Fair Lawn, NJ) was added to the aqueous phase and spun at 5,000 rpm for 15 min. DNA was precipitated from this aqueous phase by adding 1/10 volume of 3 M sodium acetate and 2.2× volume of 100% cold ethanol. The sample was then frozen overnight at –80°C and spun at 5,000 rpm for 35 min in an Eppendorff microcentrifuge. The DNA pellet was then washed with 70% ethanol, dried, and resuspended in 200 μL of sterile ddH_2O.

PCR Amplification

For DGGE analysis, 16S rRNA variable regions V3, V4, and V5 were amplified using the universal primer set 341FM-GC (5′-CCTACGGGDGGCWGCAG-3′, *Escherichia coli* position 341 bp) and 907RM (5′-CCGYCWATTCMTTTGA GTTT-3′, *E. coli* position 907) adjusted from Muyzer et al. (1998). The forward primer was modified by the addition of a 40-bp GC-rich sequence (Muyzer et al. 1998). This PCR contained 25 μL GoTaq Green Master Mix (Promega, Madison, WI), 1.5 μM of the forward and 0.5 μM of the reverse primers, an additional 1.0 mM $MgCl_2$ and 1.5 μL template (quantities of a template were variable, but, on average, ~10 ng for lesions and 1 ng for melanized spot) in a 50-μL reaction volume. A touchdown protocol was used of 5 min at 95°C, followed by 20 cycles of 1 min at 95°C, 1 min at 65°C to 55°C (touchdown, –0.5°C per cycle), and 3 min at 72°C, followed by 15 cycles of 1 min at 95°C, 1 min at 55°C, and 3 min at 72°C, and was concluded with a final extension of 7 min at 72°C. Some samples had very low DNA concentrations after extraction and were therefore amplified using nested PCR. The first reaction of the nested PCR used the primers AC18.1F (5′-AGAGTTT GATCHTGGCTYAG-3′, *E. coli* position 8–27 bp) and AC22R (5′-ACGGNTACCTTGTTACGACTT-3′, *E. coli* position 1492–1512 bp) modified from Weisburg et al. (1991). This reaction contained 12.5 μL GoTaq Green Master Mix (Promega), 2 μM of both the forward and reverse primer, and 1.5 μL template DNA (<1 ng) in a 25-μL reaction. The product of this reaction was used as a template for the 341RM-GC-907R touchdown PCR for samples that would not amplify directly. All PCR products were run in a 1% agarose gel and visualized after staining with ethidium bromide (0.5 μg/mL) on a transilluminator.

Denaturing Gradient Gel Electrophoresis and Band Processing

DGGE was carried out using a CBS Scientific DGGE system (CBS Scientific Co., Del Mar, CA) in 1× Tris-acetate EDTA buffer (pH 7.8; FisherBiotech, Fair Lawn, NJ) at 60°C. All DGGE gels were 6% polyacrylamide of dimensions 20 × 17.6 cm, 1.5-mm thick, and contained an increasing denaturant concentration (7 M urea and 40% formamide is 100% denaturant) of 20–80%. The electrophoresis was carried out at 80 V for 14 h. After electrophoresis, the gel was stained with ethidium bromide (0.1 μg/mL) for 20 min and visualized using a transilluminator. Bands of interest were excised from the gel using a sterile razorblade and were then placed into a microcentrifuge tube with 0.2 g sterile 2-mm glass beads (BioSpec Products, Inc.) and 500 μL ddH_2O. The excised acrylamide/bead mixture was then bead beaten in a Mini-Beadbeater (BioSpec Products, Inc.) at high speed for 3 min. The sample was then kept at 4°C overnight to allow diffusion of DNA.

DNA Sequencing

A 1-μL aliquot of the aqueous portion of the homogenized acrylamide band was reamplified with the same initial primer set from which it was derived, but using a forward primer without a GC clamp. The reactions contained 25 μL GoTaq Green Master Mix, 0.5 μM of each forward and reverse primer, and 1.5 μL of the DNA sample. The thermocycling conditions were the same as outlined for the same DGGE amplifications. The PCR product was then purified with a Wizard SV Gel and PCR Clean Up System (Promega). The purified product was sequenced using the forward primers and a BigDye terminator cycle sequencing kit v.3.1 on an Applied Biosystems 3130 DNA sequencer (Applied Biosystems, Foster City, CA). Sequences were searched against the GenBank database using the BLAST tool to determine the best sequence matches, and then against the RDP database to determine appropriate phylogeny and identity. Computer analyses of DNA sequences was carried out using Lasergene 5.3 from DNA*Star (Madison, WI). All nucleotide sequences have been deposited into the NCBI GenBank database under the accession nos. JN987166–JN987178.

Histopathological Assessment of Lesions

Tissues from 4 or more areas were sampled for histological evaluation from each evaluated lobster. These included 9 animals held at 10°C (4 controls and 5 challenged), 8 animals at 15°C (2 controls and 6 challenged), and 7 animals held at 20°C (2 controls and 5 challenged). Tissues of affected carapace were removed from the half of each lesion that was not sampled for bacterial evaluation. Carapace still attached to the underlying epithelium and connective tissues was taken from the lesions by cutting 2–3-mm-thick slices perpendicular to the surface of the lesion and/or normal carapace surface. Tissues (fixed previously in 10% sodium phosphate-buffered formalin) were decalcified using an EDTA decalcification solution (Howard et al. 2004). Tissues were processed in paraffin, cut as 5-μm sections and stained using hematoxylin and eosin stains (Smolowitz et al. 2005a).

Each animal was evaluated for stage of cuticle formation, lesion severity, depth of lesions, and presence of pseudomembranes (Smolowitz et al. 1992), inflammatory membranes (Smolowitz et al. 2002), epithelial proliferation, melanization, and hemocytic inflammation in the dermis underlying the lesions. A lesion severity score from 1–4 was provided for each histological section (1, mild; 2, moderate; 3, severe; and 4, ulcerated through the cuticle). Mere evaluation of the depth of the lesion is not accurate, because the initial abrasion depth may vary between animals, the occurrence of the proliferative inflammatory membrane is inconsistent between animals, and because the interface between the inflammatory membrane and the inner layers of the uncalcified endocuticle is not distinct. The severity index takes into account multiple aspects of the host response to and the bacterial action in the lesions. Severity scores from replicates of both treatments appeared to be distributed normally; thus, a Student's *t*-test was used to assess whether the mean scores differed between the treatments.

RESULTS

Lesion Development

Initial bacterial exposures to lobsters with intact carapace (no abrasion), yielded no development of lesions at the exposure site after 7 wk ($n = 2$ lobsters). Removal of the waxy epicuticle layer of the lobster shell was hypothesized to be necessary for lesions to develop. Indeed, all abraded areas led to the development of lesions at all temperatures (Fig. 1). Control lobsters took longer than challenged lobsters across all temperatures to achieve lesion melanization of an appropriate size for sampling (1 cm^2; 2.64 ± 0.59 wk (SE) vs. 1.56 ± 0.23 wk). The average number of times the lobsters needed to be reexposed to develop sufficient lesion size varied by temperature: 1.8 times at 10°C, 1.3 times 15°C, and 1.4 times at 20°C (significantly longer by Student's *t*-test at 10°C, $P < 0.05$). Lesions formed from *A.* '*homaria*' I32.4 looked grossly similar to lesions formed from coexposure of *A* '*homaria*' I32.4, '*Thallasobius*' sp. I31.1, and *P.* '*gracilis*' ISA7.3 (Fig. 1). Lesions from challenged lobsters and lesions from abraded areas on control lobsters also appeared grossly similar (Fig. 1). Lesions developed independent of abrasion or bacterial exposure on 3 animals at 20°C (herein referred to as spontaneous lesions). The spontaneous lesions had a marked difference in appearance compared with the induced lesions (Fig. 1).

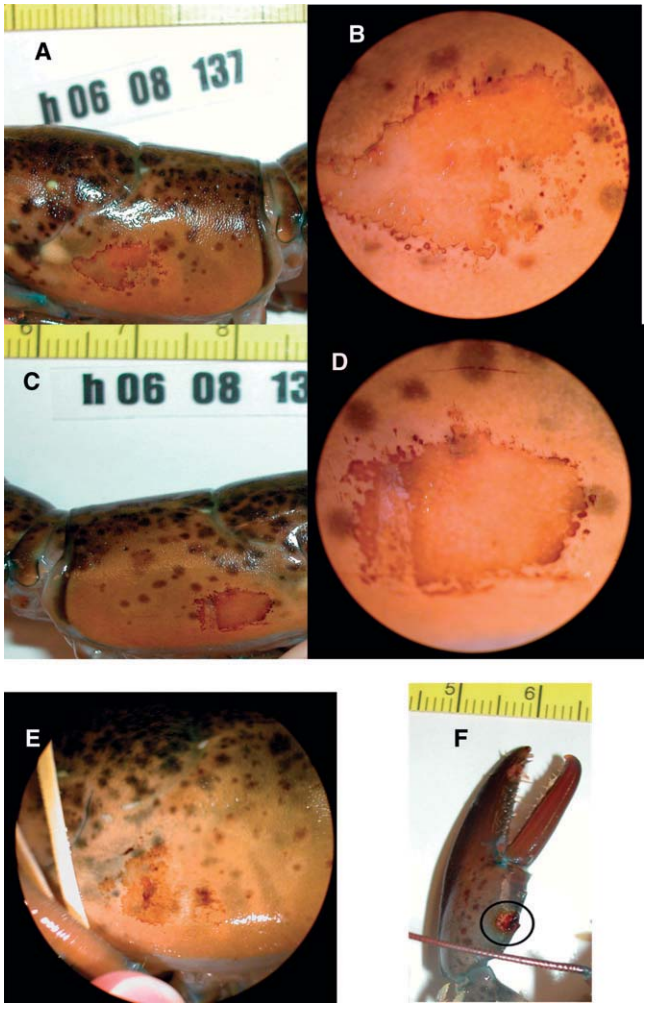

Figure 1. Lesion types that developed on the lobsters in exposure experiments. (A) Lobster held at 15°C was abraded and exposed to *A.* '*homaria*' I32.4 only. (B) Same lesion at 20× magnification. (C) Lobster held at 15°C was abraded and coexposed to *A.* '*homaria*' I32.4, '*Thalassobius*' sp. I31.1, and *P.* '*gracilis*' ISA7.3. (D) Same lesion at 20× magnification. (E) Lobster held at 15°C was abraded but unchallenged. (F) Spontaneous lesion on a lobster cheliped that developed independently of abrasion on a lobster held at 20°C.

Histopathological Assessment of Lesions

The mean severity score for control abrasions was 1.4, and for exposed lesions was 1.9. This difference was deemed significant by a Student's *t*-test ($P < 0.05$). Thus, histological evaluation of lesions showed that erosions were more severe in animals that had been challenged with bacteria compared with animals that had sterile filters placed on the abraded carapace (Figs. 2 and 3). The severity scores were significantly different between controls and challenged lesions at 10°C and 15°C, but not at 20°C. There was no significant difference between severity of lesions in which *A.* '*homaria*' I32.4 alone was applied or when a mixture of bacteria was applied. Although not directly quantifiable, there was a trend in difference in host response to the lesions at different temperatures and different stages of carapace formation. Challenged lobsters held at 15°C in stage C4 of carapace formation (uncalcified endocuticle had been secreted by the cuticular epithelium (Smolowitz et al. 1992)) tended to develop layers of inflammatory cells (a pseudomembrane) between the affected cuticle and challenged animals. Two animals in which pseudomembranes were noted also showed formation of a thin layer of an inflammatory membrane internal to the pseudomembrane that had been secreted by the cuticular epithelium (Fig. 3). All animals, held

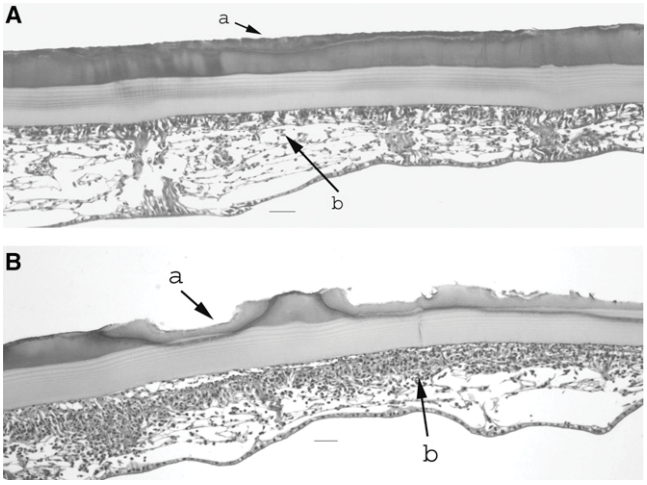

Figure 2. Photomicrographs of hematoxylin and eosin-stained 6-μm paraffin sections of experimental lobster carapace. (A) Section through abraded area of the cuticle of a control animal showing loss of epicuticle and necrosis of exocuticle with melanization (a; clear line) and sparse hemocytes in the dermis underlying the cuticular epithelium (b). (B) Section through abraded area of cuticle challenged with a mixed population of bacteria showing deep erosions into the exocuticle and necrosis that extends into the calcified endocuticle (a; black line represents melanization), and moderate, diffuse hemocytic inflammation in the dermis underlying the hypertrophic cuticular epithelium (b). Bars = 100 μm.

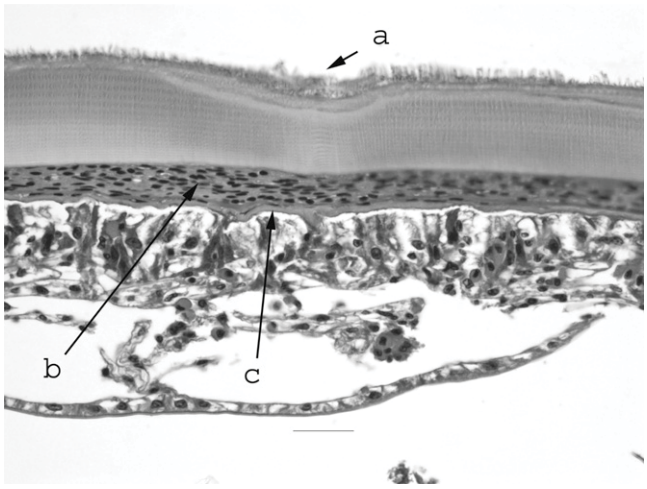

Figure 3. (A–C) Photomicrograph of hematoxylin and eosin-stained 6-μm paraffin section of experimental lobster carapace showing loss of the outer layers of the cuticle (epicuticle and exocuticle) and irregular surface of the exposed calcified endocuticle (A), pseudomembrane formation by transformed hemocytes (B), and a thin layer of inflammatory membrane (C). Bar = 50 μm.

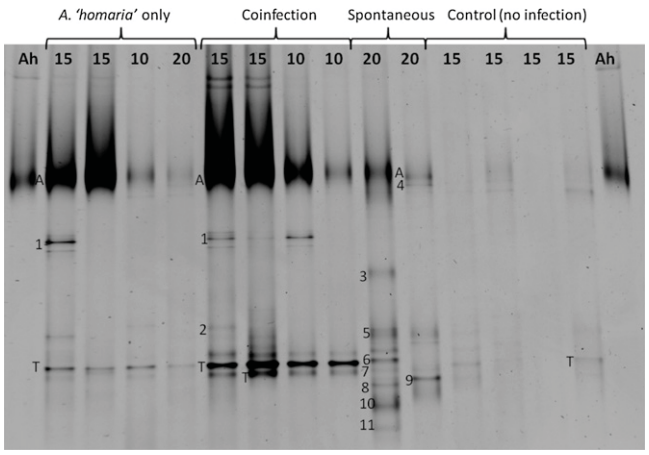

Figure 4. A representative DGGE gel of 16S rDNA amplified from DNA isolated from lobster lesion communities with the 341FM-907RM universal primer set. All 4 lesion types are represented and indicated on the gel: *A*. '*homaria*'-only exposures, coexposure, spontaneous lesions, and control (not challenged lesions). Each lane is labeled by the temperature at which the lobsters were held for the exposures except lane Ah, which is a gel reference control of 16S rDNA amplified from DNA of a pure culture of *A*. '*homaria*' I32.4. Bands sequenced on the gel are labeled to their left with either A for *A*. '*homaria*' sequence or T for '*Thalassobius*' sp. sequence, or numbered as referred to in the text.

at 20°C showed similar findings to those held at 15°C, but more animals—both controls and challenged—showed the formation of an inflammatory membrane in response to the lesions. Only rarely were pillar formations noted in these lesions compared with adults in the wild with ESD (Smolowitz et al. 2002). This may be the result of the use of filter material over the lesions, which would apply shearing pressure to the lesion surface and potentially wear away any residual spiraled chitin (pillars) in the lesions, or because chitin pillars are specific to ESD in wild lobsters.

Detection of Bacteria in Lesions of Challenged Lobsters

DGGE was used to detect all 3 bacterial strains used for exposures as well as other members of the bacterial community in the lesions of control and challenged lobsters. A lesion was considered positive for a specific bacterial strain if it had a band matching the migration of the control band or a sequenced representative of such control in the community (Fig. 4). The bacterium *P*. '*gracilis*' was never detected in the DGGE bacterial community profiles, indicating its absence from all lesions. The community profile of the challenged lesions at all temperatures was simple, with only 3 predominant bands seen in most samples (Fig. 4). At 20°C the community profile had a lighter band-staining intensity than at the other 2 temperatures. Control lesions also showed a simple community (2–4 bands); however, the community members were different and the band-staining intensity was far lower than challenged lesions, indicating a much lower bacterial load. Attempts to amplify 16S rDNA of bacteria from surfaces unaffected by shell disease were not successful perhaps because of their very low bacterial loads (Chistoserdov et al. 2005).

The lesions in lobsters challenged with *A*. '*homaria*' I32.4 alone showed a uniform community profile in DGGE (Fig. 4). The community profile was the same at the 3 temperatures, where it was dominated by a band matching that of *A*. '*homaria*' I32.4, 2 bands matching the '*Thalassobius*' sp. I31.1, and a few other bands representing other bacteria. At 15°C the *A*. '*homaria*' band was often so intense that is smeared in the gel, indicating an extremely high concentration of DNA (Fig. 4). The lesions coexposed to all 3 bacteria showed an almost identical profile to the *A*. '*homaria*' I32.4-only exposed lesions. Three lobsters at 20°C developed lesions spontaneously at different locations independent of abrasion and application of bacteria. This has been a common occurrence at the LRRF and has been documented previously (Tlusty et al. 2008). These spontaneous lesions were also analyzed by DGGE for comparison with challenged lesions. The community of the spontaneous lesions was much more diverse, with up to 10 different bands observed (Fig. 4). The bacterium *A*. '*homaria*' I32.4 was detected in all 3 spontaneous lesions by band sequencing, but '*Thalassobius*' sp. I31.1or *P*. '*gracilis*' ISA7.3 was not.

Each challenged or control lesion was scored for the presence or absence of each bacterial strain used in these exposures (Table 1). The bacteria *A*. '*homaria*' I32.4 and '*Thalassobius*' sp. I31.1 were detected in all exposed lesions at all temperatures whether in coexposure or pure culture. In the control lesions, *A*. '*homaria*' I32.4 and '*Thalassobius*' sp. I31.1 were detected, but variably at the different temperatures. At 10°C, *A*. '*homaria*' I32.4 was not detected in any control lesions, whereas, '*Thalassobius*' sp. I31.1 was detected in one (Table 1). At 15°C, *A*. '*homaria*' I32.4 was detected in all lesions, whereas '*Thalassobius*' sp. I31.1 was detected in only 2. Of the bacteria *A*. '*homaria*' I32.4 was the only 1 of the 2 to be detected in 20°C control lesions. These results indicate that both '*Thalassobius*' sp. I31.1 and *A*. '*homaria*' I32.4 can persist in lesions of lobsters with abraded cuticles and can also colonize lesions where they were not applied directly.

At 10°C, the lesions contained only *A*. '*homaria*' I32.4 and '*Thalassobius*' sp. I31.1, but other bacteria were present in the challenged and control lesions at 15°C and 20°C. In the *A*. '*homaria*'

I32.4-only challenged lesions, a *Saprospira* sp. (band 1, Fig. 4) was detected at 15°C. In the cochallenged lobsters, the same *Saprospira* sp. and an unclassified Bacteroidetes species. (band 2, Fig. 4) were detected at 15°C. The 20°C challenged lesions also contained a *Vibrio* sp., and the control lesions contained a *Silicibacter* sp., an unidentified Bacteroidetes sp. and an *Aquimarina* sp. (bands from gels not shown). The spontaneous lesions contained a more diverse community, including the presence of *Maribacter polysiphoniae* (band 3, Fig. 4), a *Tenacibaculum* sp. (band 4, Fig. 4), a *Kiloniella* sp. (band 5, Fig. 4), a *Sneathiella sp*. (band 6, Fig. 4), a *Pelagibaca* sp. (band 7, Fig. 4), an unidentified alphaproteobacterium (band 8, Fig. 4), a *Ruegeria* sp. (band 9, Fig. 4), *Loktanella agnita* (band 10, Fig. 4), and an unidentified deltaproteobacterium (band 11, Fig. 4).

DISCUSSION

This study demonstrated that a form of shell disease was developed successfully in the laboratory by abrading the lobster surface, and then applying cultures of bacteria isolated from wild lobsters with ESD. More specifically, this study has demonstrated that *A*. '*homaria*' and a '*Thalassobius*' sp. cultured from ESD lesions could develop lesions actively on the surface of lobsters, and thus may represent important pathogens of the disease. Furthermore, these bacteria colonized abrasions readily where they were not applied directly, indicating they can colonize shell lesions very easily. Histological analysis showed more severe lesions developed when bacteria were applied as opposed to sterile filters, and this difference was statistically significant. These results indicate that the bacterial exposures increased the lesion severity (Fig. 2), which is reflected in the DGGE gels, where the bacterial load in control lesions is much lower (Fig. 4), as evidenced by the fainter banding pattern in unexposed lesions. In addition, cuticular melanization occurred 1 wk faster in exposed lesions, indicating aspects of the lobster immune response were more active when these pathogenic bacteria were applied directly.

However, lesions did not develop on lobster surfaces unless the epicuticle was removed. The epicuticular layer of the lobster carapace is very thin, devoid of chitin, and noncalcified; it contains waxy lipids, proteins, and calcium salts (Waddy et al. 1995). Thus, it represents a very different barrier than the underlying chitinous layers. Our results and those of Malloy (1978), in which lesions also only developed after removal of the epicuticle by abrasion, implicate this layer as critical to shell disease development. Kunkel et al. (2012) have also emphasized the importance of the epicuticle to bacterial infection in their work on the innate defense of the lobster shell chemistry. A phenomenon similar to the artificial abrasions here may be occurring in the natural environment. Lobsters can lose portions of their epicuticle over time as a result of abrasion with surfaces, and lobsters with longer intermolt durations may have an increased likelihood of this occurring. Accordingly, ESD incidence is known to be highest and most severe in lobsters with large intermolt durations (especially ovigerous females) (Cobb & Castro 2006). Other external stressors likely contribute to ESD susceptibility (Tlusty et al. 2007)—specifically, temperature—as certain stress conditions were required for cultured *H. americanus* to be susceptible to chitinolytic bacteria *in vivo* (Fisher et al. 1976, Malloy 1978). In this study, bacterial loads were highest at 15°C, which may indicate that this temperature is optimal for the bacterial community. This temperature was also found to induce the highest levels of shell disease in a laboratory study (Tlusty & Metzler 2012).

The bacterium *A*. '*homaria*' I32.4 is a member of the Flavobacteriaceae, which includes common pathogens of marine animals (Bernardet 1998, Avendano-Herrera et al. 2005), and has been cultured from the ESD lesions of all lobsters tested to date from a variety of locations in New England as well as lesions of other forms of shell disease (Chistoserdov et al. 2009, Chistoserdov et al. 2012). In these experiments, *A*. '*homaria*' I32.4 was clearly present in the lesions of all lobsters after challenge regardless of temperature, and its presence significantly increased the lesion severity. It was especially prevalent in challenges done at 15°C, where there was so much of its DNA that the band smeared as a result of insolubility in the DGGE gel, indicating a high cell density (Fig. 4). The bacterium was also detected in abrasions where bacteria were not applied directly, which is evidence that *A*. '*homaria*' could adhere to and colonize the abraded lobster surface. However, this did not occur at 10°C, indicating adherence and colonization may be temperature dependent. The bacterium *A*. '*homaria*' is a gliding bacterium, and this motility may be inactive at a temperature below its optimum (i.e., 10°C). Furthermore, lobsters that developed lesions independent of any abrasion or exposure (spontaneous lesions) also had *A*. '*homaria*' present in all cases, which demonstrates the bacterium could colonize lesions that were independent of manual epicuticle removal and could develop in a more natural manner. All these properties of *A*. '*homaria*' provide evidence for its pathogenic nature in shell disease of *H. americanus*. Although our experimental design could not determine which bacteria initiated the lesions, the fact that only it and '*Thalassobius*' sp. were dominant in the lesion bacterial community indicates they are the likely candidates. A second bacterium somewhat related to *Kiloniella* sp. ('*Candidatus* Kopriimonas aquarianus') was detected in all spontaneous lesions. Quinn et al. (2012) demonstrated that this bacterium is always present, along with *A*. '*homaria,*' in diet-induced lesions, and is indicative of diet-induced shell disease.

The '*Thalassobius*' sp. is also a likely pathogenic member of the ESD community because it was detected in all the same lesions that *A*. '*homaria*' was, except for the ''spontaneous'' lesions and in not all control lesions. Nevertheless, of particular importance was its consistent presence in lesions that were challenged with *A*. '*homaria*' only, indicating the '*Thalassobius*' sp. was also able to adhere to and colonize lesions where it was not applied directly. This may explain the histological results that showed no significant difference in lesions that were exposed to *A*. '*homaria*' only or all 3 bacteria, as both lesions subsequently contained *A*. '*homaria*' and '*Thalassobius*' sp. Future experiments need to be done in a sterile environment to determine whether lesions develop differently or at all in the complete absence of *A*. '*homaria*', '*Thalassobius*' sp., or any other bacterium.

Interestingly, *P*. '*gracilis*' was never detected after exposure, indicating that it was cleared from the lesions and is not a significant pathogenic member of the community. Reasons for its absence are unknown, but may provide evidence that, although this bacterium is commonly present in ESD lesions, it merely colonizes the lesions as a secondary opportunist. These results also suggests that *A*. '*homaria*' I32.4 and the '*Thalassobius*' sp. I31.1 were not passive survivors in the carapace abrasions but actively colonized them. If they merely survived

in the lobster lesions then *P.* '*gracilis*' ISA7.3 would be expected to have survived as well.

Histologically, there was no difference in lesion severity between the application of either *A.* '*homaria*' alone and the coexposure of the 3 bacteria. Pseudomembranes and inflammatory membranes were only noted in animals held at 15°C and 20°C. These are important inflammatory responses to carapace erosions, because the continual buildup of the inner surface of the carapace is the only way to prevent breakthrough of the bacterial erosion into the underlying epithelium, causing ulceration. Ulceration can lead to adhesion and can prevent a successful molt. Adult animals with ESD examined in late spring/early summer often show thick inflammatory membranes (Smolowitz et al. 2002). Adult animals in early stages of new carapace formation (C2 to early C4) are more likely to produce pseudomembranes in response to infections, and if successful in limiting further erosion, will be shored up by epithelial deposition of more normal carapace layers on the inner surface of the pseudomembrane (Smolowitz et al. 1992). The production of inflammatory membranes occurs in late stages of carapace formation (C4 and D). The difference between the occurrence of inflammatory membranes versus pseudomembranes may be a function of age, length of time since bacterial exposure (carapace is being produced continually in early stages of carapace formation), condition of the animal, or some unknown cause, and may represent a difference in the disease appearance/effects between adults and juvenile lobsters when using juveniles as a model for disease.

In contrast to what was seen in a study of wild lobsters with shell disease in which the microbial community was diverse, containing at least dozens of bacterial species (Chistoserdov et al. 2012, Meres et al. 2012, Bell et al. 2012), in our exposed lesions, the community was not as diverse. The purpose of the coexposure with all 3 bacteria was to mimic the polymicrobial nature of ESD that is known to exist in natural infections and to mimic the effect of such diversity (Cawthorn 2011). However, in most instances, only the *A.* '*homaria*' and '*Thalassobius*' sp. were detected, indicating that our infections were enriching for these 2 bacteria. Polymicrobial infections are difficult to reproduce and study in a manner addressing Koch's postulates, often requiring *in vivo* and *in vitro* studies to identify parameters associated with infection and disease (Drake & Brogden 2002), and are further complicated by the phenomenon of secondary infections (Brook 2002). The simple community in lesions of challenged lobsters indicates that the bacterial diversity seen in natural ESD is likely the result of the development of the community over time and shifting microbial dynamics in the lesion. The lobster shell disease lesion is likely a dynamic and diverse microenvironment that changes during progression of the polymicrobial infection. Nevertheless, in this study we have demonstrated that *A.* '*homaria*' and '*Thalassobius*' sp. represent 2 important components within the polymicrobial community of the lesions. Dissemination of aquatic animal disease requires three factors: (1) pathogen(s), (2) environment, and (3) host (Sneiszko 1974). In the case of ESD, the first factor is the presence of a pathogenic consortium. However, not every chitinolytic bacterium is capable of initiating or joining the ESD community. For example, *Vibrio* spp. long suspected to be involved in shell disease development (Fisher et al. 1976, Malloy 1978) turned out to be associated inconsistently with lesions, and were isolated only because they are easy to culture and are ubiquitous in the coastal ocean; the *Pseudoalteromonas* sp. tested here did not persist in the lesions. An aspect of the second factor is the temperature optimal for both lobster host and pathogens, and a likely aspect of the third factor is a breach in the epicuticle. Future application of the method described here could continue to identify important members of the pathogenic consortium, to understand which bacteria in the shell disease community are active pathogens and which may be opportunistic or mere inert colonizers. Furthermore, expansion of experiments in which lesions are induced in the absence of the bacteria used in this study will shed light on whether these bacteria are indispensible components of lobster shell disease.

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