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Aerococcus viridans var. *homari*: The presence of capsule and the relationship to virulence in American lobster (*Homarus americanus*)

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ABSTRACT

The relationship between virulence and encapsulation of *Aerococcus viridans* var. *homari* was evaluated by growing virulent (Rabin's) and avirulent (ATCC 10400) strains under varying culture conditions, and during challenge trials. Changes in capsule thickness were monitored using a modified lysine-ruthenium red (LRR) fixation method and transmission electron microscopy. The virulent Rabin's strain possessed a prominent capsule of 0.252 μm ± 0.061 μm that was diminished by *in vitro* growth conditions to 0.206 μm ± 0.076 μm. The ATCC 10400 strain capsule thickness decreased from 0.157 μm ± 0.043 μm to 0.117 μm ± 0.043 μm after 10 *in vitro* passages. The virulent Rabin's strain capsule was significantly thicker than the avirulent ATCC 10400 strain under all growth conditions. Rabin's strain, regardless of pre-challenge growth conditions or dose (high dose 10^7 or low dose 10^2), was able to kill lobsters in 7 days at 15 °C. ATCC 10400 strain, regardless of pre-challenge growth conditions, killed lobster only at high doses (10^7) with varying median time to death of ~15 days, while at low doses (10^2) all lobsters survived and no bacteria were present after 42 days. This work demonstrates the importance of the thickness of the *A. viridans* capsule to virulence in the American lobster.

1. Introduction

Aerococcus viridans var. *homari* is a free-living, gram positive, tetrad-forming coccus bacterium that causes gaffkemia, a systemic disease of homarid lobsters (*Homarus americanus* and *Homarus gammarus*) found in the coastal waters of both North America and Europe (Wood, 1962; Stewart et al., 1966; Audouin and Léglise, 1971; Lavallée et al., 2001; Stebbing et al., 2012). Historically, gaffkemia has contributed to significant economic losses in commercial lobster holding facilities where the non-motile bacterium enters through exoskeletal defects caused by lobster fighting or poor handling practices post-harvest (Hitchner and Snieszko, 1947; Stewart, 1980; Cawthorn, 2011). Once infected by *A. viridans* var. *homari*, the lobster's innate immune system is overwhelmed and the lobster dies after the bacterium completely depletes the lobsters' energy reserves (Stewart, 1980).

Stewart et al. (1966) were the first to suggest that 'variation in virulence' of *A. viridans* var. *homari* strains was a distinct possibility as several naturally infected lobsters, acquired during a survey of Atlantic Canada, were found to survive and clear the bacterium. Initial characterization of virulent and avirulent strains revealed that virulent *A. viridans* var. *homari* strains were not agglutinated while avirulent strains were agglutinated by lobster serum (Stewart, 1984). Stewart (1984) further demonstrated, by light microscopy with alcian blue staining, that the amount of capsular polysaccharide determined whether an *A. viridans* strain was virulent (substantial capsule) or avirulent (trace amounts of capsule) and it was this feature that conferred resistance to agglutination. Subsequent observations of *A. viridans* var. *homari* cultures indicated that when isolates were cultured axenically in tryptic soy broth (TSB) for long periods of time (3–6 years) or were lyophilized for extended periods (2 years), that virulence was lost (Stewart et al., 2004b). Surprisingly, this loss of virulence was transient as the *A. viridans* var. *homari* capsule could be restored after only a seven day culture in filter-sterilized lobster serum (Stewart et al., 2004b).

Previously, we were able to differentiate the virulent and avirulent *A. viridans* var. *homari* isolates using a combination of random amplification of polymorphic DNA (RAPD) and 16S rDNA

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sequencing (Greenwood et al., 2005). The avirulent ATCC 10400 strain however was identical, based on this limited amount of molecular information, to the virulent Rabin's strain. This suggested that the variation observed in virulence among the different strains of *A. viridans* var. *homari* could be a function of changes in proteins and gene expression. Using proteomic analyses, we found distinct differences in protein expressions patterns between the virulent (Rabin's) and avirulent (ATCC 10400) *A. viridans* var. *homari* strains (Clark and Greenwood, 2011). Importantly, there was no within strain protein differences regardless of whether the bacteria were cultured serially or non-serially in either TSB or lobster serum (Clark and Greenwood, 2011).

In the present study, we revisit the issue of virulence within *A. viridans* var. *homari* by exploring phenotypic differences between the virulent Rabin's and avirulent ATCC 10400 strains under different growth conditions to evaluate the role of the polysaccharide capsule. Transmission electron microscopy with a modified lysine-ruthenium red (LRR) fixation protocol (Hammerschmidt et al., 2005) was used to examine the capsule of *A. viridans* at high resolution.

2. Materials and methods

2.1. Experimental animals

Adult American lobsters, *H. americanus*, (n = 50, weighing 450–550 g) were obtained from a local live seafood supplier (Charlottetown, PEI, Canada) and subjected to health assessments following the protocol Acorn et al. (2011) incorporating the challenge protocol of Battison et al. (2004). Briefly, lobsters were assessed for vigor and inspected for visible exoskeletal defects while haemolymph was evaluated for total haemocyte counts, and tested for the presence of bacteria or ciliates through inoculation of phenylethyl alcohol (PEA), tryptic soy broth (TSB) or modified ATCC 1651 MA medium. Only healthy, pathogen-free lobsters were selected for inclusion in the study. Lobsters were individually housed in a recirculating artificial seawater (ASW) (Instant Ocean, 30 ppt) system equipped with UV filtration (Aquabiotech, Coaticook, QC, Canada) in the Aquatic Animal Facility of the Atlantic Veterinary College in accordance with the University of Prince Edward Island Animal Care Committee approved animal care protocol #07-042. Lobsters were acclimated to 15 °C by increasing the water temperature from 5.5 °C by 1 °C per day to mimic the natural water temperature optimal for *A. viridans* challenges. Lobsters were held for two weeks prior to the beginning of the experiment. Water quality parameters including pH, oxygen, ammonia and nitrite were monitored daily.

2.2. Sterile lobster haemolymph preparation

Sterile lobster haemolymph was prepared as described by Clark and Greenwood (2011) based on a modification of the protocol of Cornick and Stewart (1968, 1973). Briefly, 30–40 mL of haemolymph was drawn from the ventral abdominal sinus of two male lobsters. Haemolymph was screened to ensure that it was free of bacterial and ciliated pathogens, one mL was used to inoculate PEA at 28 °C and 15 °C, TSB at 28 °C and 15 °C, and modified ATCC 1651 MA medium at 5 °C. The haemolymph was incubated at 5 °C until a non-retracting, firm clot had formed prior to being homogenized using a 7 mL glass Tenbroeck tissue homogenizer. The supernatant was collected by centrifugation at 1200 g for 5 min, filter-sterilized (0.45 μm syringe filters, Sarstedt) and stored at 5 °C.

2.3. A. viridans in vitro culture in sterile lobster serum

A. viridans var. *homari* Rabin's strain (Rabin, 1965) was acquired from Dr. James Stewart, (Department of Fisheries and Ocean, Bedford Institute of Oceanography, Dartmouth, NS, Canada) and American Type Culture Collection (ATCC) strain ATCC 10400 (Snieszko and Taylor, 1947) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Both strains' phenotypic and molecular characterizations have been confirmed previously (Greenwood et al., 2005). Bacteria were maintained by serial passage every 24 h in TSB with 3% (w/v) NaCl at 28 °C at 90 RPM in an incubator shaker (Max^{Q} 4000, Barnstead) as previously described (Greenwood et al., 2005). Colony forming units (CFU) were calculated by serially diluting *A. viridans* cultures 1/10 to 1/10^6 with sterile 3% NaCl and plating 6 × 25 μL aliquots on blood agar plates. Plates were incubated at 28 °C for 48 h and colonies were then counted (Battison et al., 2004).

Bacteria were transferred to sterile lobster serum and cultured under serial and non-serial conditions (Clark and Greenwood, 2011). Briefly, *A. viridans* cultures (Rabin's and ATCC 10400) that were serially passaged for >32 times in TSB were used to inoculate (7.5 × 10^5 CFU of bacteria) 1.5 mL of sterile lobster serum in clean, autoclaved 25 mL Erlenmeyer flasks. Cultures were incubated at 28 °C at 90 RPM for 24 h in an incubator shaker (Max^{Q} 4000, Barnstead). This process was repeated for 10 consecutive days by using the previous day's culture to inoculate a new 1.5 mL aliquot of sterile lobster serum.

Similarly, for non-serial passage of *A. viridans* cultures (Rabin's and ATCC 10400), 35 mL of sterile lobster serum was added to a 250 mL Erlenmeyer flask and either inoculated with 1.8 × 10^6 CFU of *A. viridans* culture that had been serially passaged in TSB (Rabin's or ATCC 10400) or not inoculated (control). The cultures were grown at 28 °C at 90 RPM for ten consecutive days.

2.4. A. viridans challenge experiments

2.4.1. A. viridans var. homari challenge trial

Lobsters (12 females and 13 males) were randomly assigned to six treatment groups and injected into the ventral abdominal sinus with 200 μL that contained a high dose (7.0 × 10^7 CFU/mL) or a low dose (7.0 × 10^2 CFU/mL) of *A. viridans* var. *homari* ATCC 10400 strain in sterile 3% NaCl, or 200 μL of sterile 3% NaCl as a control (Supplementary Table 1). Five lobsters that did not receive injections were used as sentinels in the study. The ATCC 10400 challenge trial lasted 42 days.

Similarly, lobsters (13 females and 10 males) were randomly assigned to six treatment groups and injected, via the ventral abdominal sinus, with 200 μL that contained a high dose (7.0 × 10^7 CFU/mL) or a low dose (7.0 × 10^2 CFU/mL) of *A. viridans* var. *homari* Rabin's strain in sterile 3% NaCl, or 200 μL of sterile 3% NaCl as a control (Supplementary Table 1). Three lobsters that did not receive injections were used as sentinels in the study. The Rabin's challenge trial was terminated after 14 days due to the death of all of the *A. viridans* challenged animals.

Complete physical assessments were performed on each lobster on the day of injection and every 7 days thereafter. In addition, every lobster was visually checked for vigor and defensive posture at least twice daily. If a lobster was found to have haemocyte counts less than 3.0 × 10^9/L, it was classified as moribund and euthanized. Euthanasia was performed by severing the ventral nerve cord anterior to the chelae-thorax junction. All animals that survived until the end of the 42 day trial were euthanized. Complete necropsies were performed on all challenge trial animals.