

Withering Syndrome of the Small Abalone, *Haliothis diversicolor supertexta*, Is Caused by *Vibrio parahaemolyticus* and Associated with Thermal Induction

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The susceptibility of the small abalone *Haliothis diversicolor supertexta* to *Vibrio parahaemolyticus* 880915 strain and its extracellular products (ECP) at different temperatures was investigated. The strain was previously isolated from the haemolymph of the moribund small abalone with withering syndrome during an outbreak of mass mortality among the cultured animals in September 1999 in I-Lan, Taiwan. The bacterium and its ECP were lethal to the small abalone. Onset of the withering syndrome in the moribund or dead animals could be observed at 4–7 d post-bacterial challenge. The same bacterial strain could be isolated from the haemolymph of the moribund animals with or without the syndrome post-bacterial challenge. This syndrome could not be observed in the moribund or dead animals post-ECP challenge. The animals were more susceptible to the bacterium and ECP challenge at higher temperature (28 °C) indicating that the outbreak of the disease in warmer season is associated with thermal induction.

Introduction

Vibriosis is one of the major threats in marine shellfish aquaculture worldwide (Lightner, 1988; Austin and Austin, 1989; Lane and Birkbeck, 2000). A few reports regarding this disease have been published on abalone (Elston and Lockwood, 1983; Dixon *et al.*, 1991; Anguiano-Beltran *et al.*, 1998; Nishimori *et al.*, 1998; Liu *et al.*, 2000, 2001), and the causative agents were identified or suggested as *Vibrio alginolyticus*, *V. carchariae* and *V. parahaemolyticus*.

Furthermore, a withering syndrome of wild and farmed abalone has been reported in California, USA, and demonstrated to be associated with temperature, food availability, parasites and/or bacteria (Lafferty and Kuris, 1993; Gardner *et al.*, 1995; Altstatt *et al.*, 1996; Friedman *et al.*, 1997; Moore *et al.*, 2000). In a further study, a new Rickettsiales bacteria, *Candidatus Xenohaliothis californiensis*, has been described as the pathogen causing withering syndrome in abalone, *Haliothis* spp., along the west coast of North America (Friedman *et al.*, 2000).

Abbreviations: ECP, extracellular products; PBS, phosphate buffered saline; TSA, tryptic soy agar.

Recently, outbreaks of mass mortality among the cultured small abalone *Haliothis diversicolor supertexta*, manifesting withering syndrome, have occurred in Taiwan (Liu *et al.*, 2000, 2001). In the present study, we report the reproduction of withering syndrome and the implication of ambient water temperature with the outbreak of the disease in cultured small abalone using a *V. parahaemolyticus* 880915 strain originally isolated from the haemolymph of the diseased animals with the syndrome.

Materials and Methods

Bacterium and extracellular products (ECP)

Vibrio parahaemolyticus 880915 strain originally isolated from the haemolymph of diseased small abalone (*Haliothis diversicolor supertexta*) with withering syndrome during an outbreak of mass mortality among the cultured animals in I-Lan, Taiwan in September (water temperature 26 °C) 1999, was used in this study. The pure stock cultures were stored in phosphate buffered saline (PBS, pH 7.2) supplemented with 10% glycerol at –70 °C.

Stock cultures of the 880915 strain were grown on tryptic soy agar (TSA; Basingstoke, Oxoid, UK; supplemented with 3% NaCl) for 24 h at



25 °C and two swabs of the bacteria were suspended in 5 ml PBS. The suspension was spread onto TSA (+3% NaCl) overlaid with sterile cellophane and grown for 24 h at 25 °C. The ECP was harvested following a method previously described (Lee and Ellis, 1990). In brief, 10 ml of PBS was added to the surface of the cellophane overlaying TSA (+3% NaCl) and spread completely. The harvested bacterial suspension was then centrifuged (25,000 g for 60 min at 4 °C) and the pellet discarded. The supernatant was passed through a 0.22-µm filter (Millipore, Bedford, UK) and the ECP stored in 1-ml aliquots at -70 °C. Total protein was measured by the method of Bradford (1976) with bovine serum albumin as a standard.

Abalone and virulence tests

Small abalone (*H. diversicolor supertexta*) weighing between 10 and 14 g were held in tanks (2,500 litre) supplied with air-lifted 33 ppt salinity sea water at 25–26 °C, and were acclimated at 18, 23 or 28 °C for 24 h prior to bacterial or ECP challenge. The LD₅₀ tests (Trevors and Lusty, 1985), with batches of five abalone per treatment, were conducted by intramantle injection of 0.1 ml bacterial suspension (10⁷, 10⁶, 10⁵ or 10⁴ colony forming unit g⁻¹ abalone body weight) or ECP (15.0, 7.5, 5.0 or 1.5 µg protein g⁻¹ abalone body weight) into the animals at the right side of mantle (Liu *et al.*, 2000, 2001). Sterile PBS was injected into the controls. Mortality of the animals was recorded daily for 1 week post-injection. Isolation and identification of the bacteria from the haemolymph of moribund abalone post-bacterial challenge was carried out using TSA (+3% NaCl) and thiosulphate citrate bile salt sucrose agar (TCBS; Oxoid) plates.

Results

Extracellular products

The ECP of *V. parahaemolyticus* 880915 strain was harvested after 24 h of incubation of the culture at 25 °C. The total protein of the ECP was 1500 µg protein ml⁻¹.

Virulence tests

The injection of the bacterial cells or the ECP into small abalone was lethal to the animals held

at different temperature treatments (see Table 1). Moribund animals could be observed within 7 d post-challenged with bacterial cells or ECP. In addition, occurrence of withering syndrome (Fig. 1) (exhibiting gross signs of shrunken foot muscle, discolouration of the epipodium and retraction of visceral tissues, and reduced activity and inability to tightly adhere to the substratum), as seen in natural outbreak, in the moribund or dead animals could be observed at 4–7 d post-bacterial challenge but was not observed within the first 3 d. This syndrome could not be observed in the moribund or dead animals injected with ECP within 7 d.

The bacteria were reisolated from the haemolymph of the moribund abalone with or without withering syndrome injected with bacterial cells and reconfirmed to be the same species. No mortality or withering syndrome was observed in the controls injected with PBS.

Tables I shows the LD₅₀ values of the bacterial and ECP challenges in small abalone at 18, 23 and 28 °C ranging from 5.7 × 10⁵ to 3.7 × 10³ colony forming units and 9.3 to 2.3 µg protein g⁻¹ abalone body weight, respectively. The animals were more susceptible to the bacterium and ECP challenge at higher temperature (28 °C).

Discussion

Aquaculture for the small abalone (*H. diversicolor supertexta*) has been practiced for more than two decades in Taiwan (Chen, 1990). However, only recently mass mortality of the animals associ-

Table I. Virulence tests of bacterial cells and extracellular products (ECP) of *V. parahaemolyticus* 880915 strain injected in a volume of 0.1 ml into small abalone *Halopis diversicolor supertexta* weighing 10–14 g, held at 18, 23 or 28 °C.

Temperature (°C)	Sample	LD ₅₀ value* (g ⁻¹ abalone body weight)
18	bacterial cells	3.3 × 10 ⁵ colony forming units
18	ECP	9.3 µg protein
23	bacterial cells	5.7 × 10 ⁵ colony forming units
23	ECP	3.5 µg protein
28	bacterial cells	3.7 × 10 ³ colony forming units
28	ECP	2.3 µg protein

* The virulence tests, with batches of five abalone per treatment, were conducted by intramantle injection into the animals and observed for one week; dead animals were all observed within 7 d of challenge.

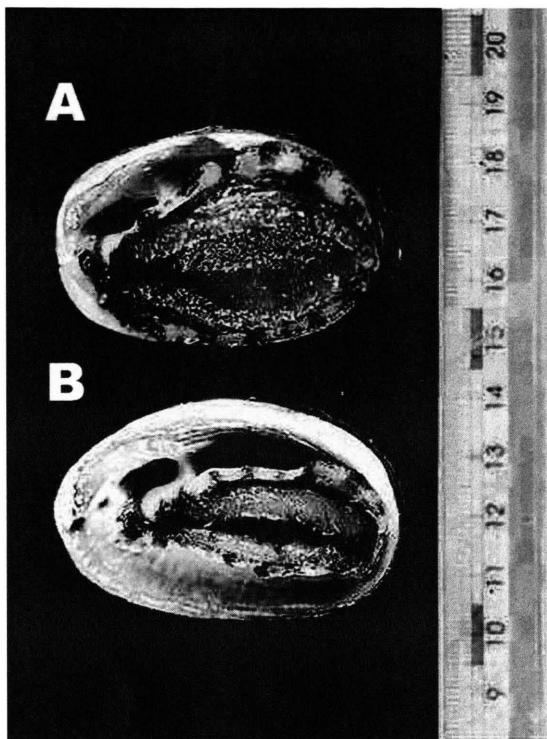


Fig. 1. (A) Normal small abalone *Haliothis diversicolor supertexta* (B) Withering syndrome reproduced in the moribund or dead small abalone observed at 4–7 d post-challenged with *V. parahaemolyticus* 880915 strain. The gross signs of the syndrome were shrunken foot muscle, discoloration of epipodium and retraction of visceral tissues.

ated with outbreaks of vibriosis i.e. withering syndrome or abscess/ulcers in the mantle, has occurred especially in warmer season (Liu *et al.*, 2000, 2001). In a previous study, gross signs of withering syndrome has not been reproduced in the moribund or dead small abalone after injection with bacterial cells or ECP of the same *Vibrio* species (B4 strain) within 2 d (Liu *et al.*, 2000). Similar results were also found here injecting with bacterial cells and ECP of 880915 strain within 2 d, respectively. However, the syndrome manifesting shrunk foot muscle, discolored epipodium, retracted visceral tissues, reduced activity and inability to tightly adhere to the substratum could be observed in the moribund animals post-challenged with bacterial cells in longer duration (4–7 d) (Fig. 1). These symptoms of withering syndrome found here were similar to those described in other studies on black and red abalone (Lafferty

and Kuris, 1993; Gardner *et al.*, 1995; Altstatt *et al.*, 1996; Friedman *et al.*, 1997; Moore *et al.*, 2000). The present results reveal that the reproduction of gross signs of withering syndrome in small abalone post-challenged with bacterial cells is simply a time-course effect.

Our present finding that the injection of bacterial cells could reproduce withering syndrome and the same bacterial strain could be reisolated from the moribund animals indicate that *V. parahaemolyticus* is the causative agent of the disease. Furthermore, that the injection of ECP failed to reproduce gross signs of the syndrome in the animals suggests that the presence of bacterial cells rather than ECP produced *in vitro* may be prerequisite to the onset of gross signs of the syndrome. Presumably, unknown factor(s) which may play an important role in the disease process may be secreted in small amount or not secreted at all by the bacteria in *in vitro* condition.

As shown in Table I, the small abalone were more susceptible to the bacterium and ECP challenge at 28 °C. Therefore, thermal induction may indeed trigger the outbreak of the disease as that suggested in previous studies in small abalone (Liu *et al.*, 2000) and in black and red abalone (Friedman *et al.*, 1997; Moore *et al.*, 2000).

Recently, *Candidatus Xenohaliotis californiensis* has been described as a pathogen causing withering syndrome in abalone, *Haliothis* spp. (Friedman *et al.*, 2000). In our opinion, reproduction of the syndrome by infecting with the Rickettsiales pathogen in the animals has not been validated using acceptable criteria. However, different pathogens causing similar syndromes in animals are not uncommon especially in fish and shellfish (Austin and Austin, 1989).

In conclusion, our results confirm that *V. parahaemolyticus* is one of the causative agents of withering syndrome in small abalone and thermal induction is a triggering factor for the outbreak of the disease. These results are useful in farming management for small abalone particularly in warmer season.

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