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NOTE

Effect of cultivation broth pH, temperature and NaCl concentration on virulence of an *Enterococcus*-like bacterium to the giant freshwater prawn *Macrobrachium rosenbergii*

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ABSTRACT: The growth of a pathogenic *Enterococcus*-like bacterium (strain KM002) isolated from *Macrobrachium rosenbergii* was examined in brain heart infusion broth (BHIB) at different pHs, temperatures, and NaCl concentrations. It grew from pH 3 to 10 (optimum 7 to 8), from 5 to 45°C (optimum 25 to 30°C) and from 0.5 to 6.5% NaCl (optimum 0.5 to 1.0%). *M. rosenbergii* was then challenged by injection of either 2×10^6 (low dose) or 1×10^7 (high dose) cfu prawn^{-1} of KM002 previously incubated under various conditions for 24 h. After injection, the prawns were monitored for pathology for 168 h. Control prawns were injected with carrier solution only. Survival was 100% for all control groups. In pH tests, onset of mortality was earlier and total mortality was higher for bacteria grown at pH 7 to 8 than for those grown at pH 6 and 9. For pH 7 and 8, respective cumulative mortality was 40 and 43% at low dose challenge, and 50 and 90% at high dose challenge. In temperature tests, onset of mortality was earlier and total mortality was higher for bacteria grown at 27 and 30°C than for those grown at 23 and 35°C. At 27 and 30°C, respective cumulative mortality was 33 and 30% at low dose challenge and 90 and 60% at high dose challenge. In NaCl concentration tests, the onset of mortality was earlier and total mortality was higher for bacteria grown at 0.5 and 1.0% NaCl than for those grown at 1.5 and 2.0%. At 0.5 and 1.0% NaCl concentrations, cumulative mortality was 40% at low dose challenge and 70% at high dose challenge. It was concluded that incubation of KM002 at 27 to 30°C in BHIB containing 0.5 or 1.0% NaCl at pH 7 to 8 resulted in significantly enhanced virulence to *M. rosenbergii*.

KEY WORDS: *Macrobrachium rosenbergii* · *Enterococcus* · Virulence · Cultivation broth · pH · Temperature · NaCl

Fish farmers and scientists generally use disease information and data from challenge tests to decide whether immunization, therapy or environmental management is needed to control disease outbreaks (Anderson 1990). The use of antibiotics in the laboratory has been reported to be an effective therapy in combating bacterial infections in crustaceans (Lightner 1983, Baticados et al. 1990). However, field application is not very satisfactory, because more than 25% of prawns are often infected before the disease is recognized (Prayitno & Latchford 1995). In addition, long-term use of antibiotics may result in the development of antibiotic-resistant pathogens in culture ponds (Aoki & Egusa 1971, Supriyadi & Rukyani 1992). Therefore, understanding factors that control pathogen virulence and how management of environmental parameters can influence them may make it possible to develop alternative disease control measures.

Enterococcus-like muscle necrosis of *Macrobrachium rosenbergii* occurs only during the summer in Taiwan, and especially during phytoplankton blooms (Cheng & Chen 1998a). Previous research indicated that mortality of *M. rosenbergii* caused by strain KM002 of this bacterium was exacerbated by environmental parameters of temperature and pH that differed from those known to be optimal for prawn growth (Cheng & Chen 1998b). Salinity and pH have also been reported to affect the virulence of luminous bacteria to tiger prawn *Penaeus monodon* (Prayitno & Latchford 1995). The present study was carried out to determine whether variations in pH, temperature and NaCl concentration would affect the growth of KM002 and its virulence to *M. rosenbergii*.

Materials and methods. ***Growth of KM002 at various pHs, temperatures and NaCl concentrations:*** *Enterococcus*-like KM002 used in this study was isolated from moribund *Macrobrachium rosenbergii* (Cheng & Chen 1998a). It was cultured on tryptic soy agar (TSA, Difco) for 24 h at 30°C before being transferred to 10 ml of brain heart infusion broth (BHIB, Difco) containing 0.5% NaCl for 24 h at 30°C as a stock

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bacterial broth for growth tests. Inoculum for the growth tests consisted of 0.5 ml of this stock broth culture.

The bacteria were incubated under various conditions in 50 ml BHIB in 250 ml flasks following the method described by Al-Harbi (1994). Temperature tests were carried out at pH 7.2 and 0.5% NaCl, pH tests were carried out at 30°C and 0.5% NaCl, and tests with different NaCl concentrations were carried out at pH 7.2 and 30°C. In total, there were 31 tests (9 pH tests, 9 water temperature tests and 13 NaCl tests). Each test was conducted in triplicate and bacterial growth was monitored at 12, 24, 48 and 120 h incubation by measuring absorbency at 686 nm using a Model U-200 spectrophotometer (Hitachi, Tokyo, Japan).

For pH tests, the medium was adjusted from pH 3 to 11 with 1 N HCl or 1 N NaOH solution. Temperature tests were carried out from 5 to 45°C in 5°C steps. NaCl concentrations tested were from 0.5 to 6.5% in 0.5% steps.

Virulence tests: After 24 h cultivation, KM002 in test media was harvested by centrifugation at 7155 × *g* for 15 min at 4°C. The pellet was resuspended in saline solution (0.85% NaCl) at 5×10^8 and 1×10^8 cfu ml^{-1} as stock bacterial suspension for injection challenges. Bacteria from the following media were tested for virulence: pH 6, 7, 8 and 9; temperatures 23, 27, 30 and 35°C; and NaCl concentrations of 0.5, 1.0, 1.5 and 2.0%.

Macrobrachium rosenbergii (10 to 15 g in the intermolt stage) were obtained from 2 commercial farms in Pingtung, Taiwan, and acclimated in the laboratory for 1 wk prior to experimentation. Two challenge trials were conducted. The first trial was conducted in only 1 replicate with a dose of 1×10^7 cfu $prawn^{-1}$ while the second was conducted in triplicate with a dose of 2×10^6 cfu $prawn^{-1}$. The test and control groups comprised 10 prawns each (600 tests in all). Challenge tests were conducted following the method of Cheng & Chen (1998b). However, prawns were kept in water at 31 to 32.5°C at pH 7.5 to 7.7 with aeration. Control prawns were injected with an equal volume of sterile saline solution. With the exception of the first trial, all data were subjected to 1-way analysis of variance (Steel & Torrie 1980). If significant differences were indicated at the 0.05 level, then the Duncan Multiple Range test was used to identify significant differences among the treatments (Duncan 1955).

Results. ***Effect of different parameters on growth of KM002:*** KM002 grew from pH 3 to 10 with optimum growth at pH 7 to 8. The maximum stationary phase remained longer at pH 5 to 6, and log phase occurred at pH 10 for 24 to 48 h. The bacterial density was highest after 12 h incubation at pH 6, 7 and 8 (Fig. 1). It

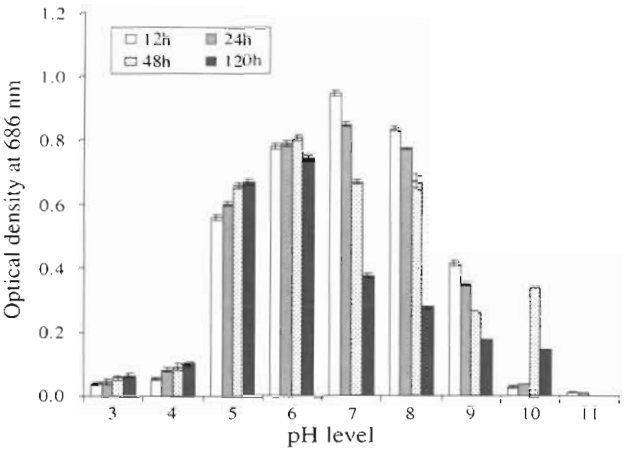

Fig. 1. Effect of pH on growth of KM002 at 30°C in BHIB medium containing 0.5% NaCl. Bacterial numbers were determined by optical density at 686 nm at 12, 24, 48 and 120 h

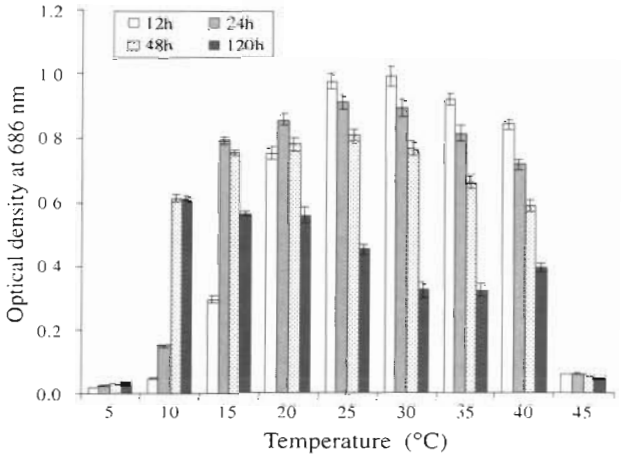

Fig. 2. Effect of temperature on growth of KM002 at pH 7.2 in BHIB medium containing 0.5% NaCl. Bacterial numbers were determined by optical density at 686 nm at 12, 24, 48 and 120 h

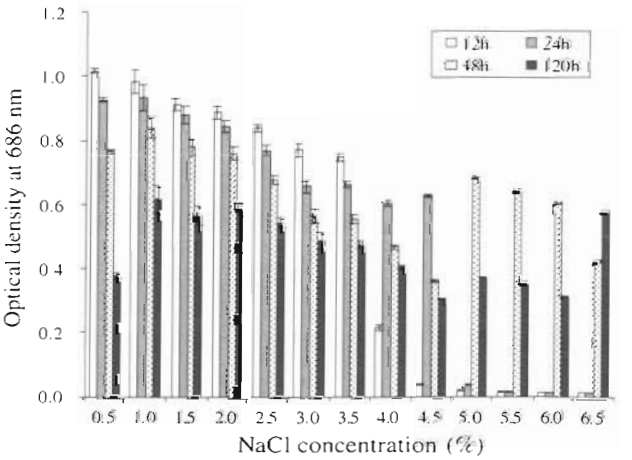

Fig. 3. Effect of NaCl concentration on growth of KM002 in BHIB medium at pH 7.2 and 30°C. Bacterial numbers were determined by optical density at 686 nm at 12, 24, 48 and 120 h

grew from 5 to 45°C with optimum growth at 25 to 30°C. Density was highest after 12 h at 25 to 40°C (Fig. 2). It grew from 0.5 to 6.5% NaCl with optimum growth at 0.5 to 1.0% NaCl, and density was highest after 12 h incubation at 0.5 to 3.5% NaCl. Growth was delayed with NaCl concentrations higher than 4.0%. The maximum stationary density decreased and the time to maximum was longer with increasing NaCl concentration (Fig. 3). In summary, optimum growth was at pH 6 to 8, 25 to 30°C and 0.5 to 1.0% NaCl.

Effects of different parameters on virulence of KM002: All the unchallenged control prawns survived. In contrast to these saline-injected controls, all the challenged prawns stopped feeding after injection. In the pH tests (Table 1) at a challenge dose of 1×10^7 cfu prawn^{-1}, the onset of mortality occurred earlier with bacteria grown at pH 7, 8 and 9 (16 h) than at pH 6 (24 h). Cumulative mortality over 168 h was 40, 50, 90 and 20% for pH 6, 7, 8 and 9, respectively. At a challenge dose to 2×10^6 cfu prawn^{-1}, the onset of mortality occurred earlier for pH 7 and 8 (8 h) than for pH 6 and 9 (16 h). The cumulative mortality was significantly lower for bacteria grown at pH 6 (23%) and pH 9 (20%) than at pH 7 (40%) and pH 8 (43%).

In temperature tests (Table 2) at a challenge dose of 1×10^7 cfu prawn^{-1}, the onset of mortality was earlier for bacteria grown at 27, 30 and 35°C (16 h) than for those grown at 23°C (24 h) and cumulative mortality was 30% (23°C), 90% (27°C), 60% (30°C) and 60% (35°C) over 168 h. At low dose challenge (2×10^6 cfu prawn^{-1}), mortality onset was earlier at 30°C (16 h) than at other temperatures (24 h) and cumulative mortality was significantly higher for bacteria grown at 27°C (33%) and 30°C (30%) than for those grown at 23°C (13%) and 35°C (17%).

In the NaCl concentration tests (Table 3) at a challenge dose of 1×10^7 cfu prawn^{-1}, the onset of mortality was earliest with bacteria grown at 0.5 and 1.0% NaCl (16 h) followed by 1.5% NaCl (24 h), and 2.0% NaCl (48 h). Cumulative mortality was 70% (0.5% NaCl),

Table 1. Susceptibility of *Macrobrachium rosenbergii* to *Enterococcus*-like (KM002) incubated in BHIB containing 0.5% NaCl at different pH levels and at 30°C. In the second trial, data in the cumulative mortality column having different letters are significantly different ($p < 0.05$)

Trial	Bacterial dose (cfu prawn^{-1})	Treatment's pH	Number of deaths 8	16	24	48	72	96	120	144	168	Cumulative mortality
First	Control	Saline										0/10 (0%)
	1×10^7	6			2		2					4/10 (40%)
	1×10^7	7		1	1	2	1					5/10 (50%)
	1×10^7	8		1	2	1	4	1				9/10 (90%)
	1×10^7	9		1	1							2/10 (20%)
Second	Control	Saline										0/30 (0%)
	2×10^6	6		2	1	2	1	1				7/30 (23%)^b
	2×10^6	7	1	1	1	1	3	1	4			12/30 (40%)^a
	2×10^6	8	1	1	1	4	4		2			13/30 (43%)^a
	2×10^6	9		1			3	1	1			6/30 (20%)^b

Table 2. Susceptibility of *Macrobrachium rosenbergii* to *Enterococcus*-like (KM002) incubated in BHIB containing 0.5% NaCl at pH 7.2 but at different temperatures. In the second trial, data in the cumulative mortality column having different letters are significantly different ($p < 0.05$)

Trial	Bacterial dose (cfu prawn^{-1})	Treatment's temperature (°C)	Number of deaths 8	16	24	48	72	96	120	144	168	Cumulative mortality
First	Control	Saline										0/10 (0%)
	1×10^7	23			2				1			3/10 (30%)
	1×10^7	27		8	1							9/10 (90%)
	1×10^7	30		3	1	2						6/10 (60%)
	1×10^7	35		2	1	3						6/10 (60%)
Second	Control	Saline										0/30 (0%)
	2×10^6	23			1	2		1				4/30 (13%)^b
	2×10^6	27			2	3	2	1	1	1		10/30 (33%)^a
	2×10^6	30		1	1	2	3	1	1			9/30 (30%)^a
	2×10^6	35			1	2	2					5/30 (17%)^b

Table 3. Susceptibility of *Macrobrachium rosenbergii* to *Enterococcus*-like (KM002) incubated in BHIB at pH 7.2 and 30°C but at different NaCl concentrations. In the second trial, data in the cumulative mortality column having different letters are significantly different ($p < 0.05$)

Trial	Bacterial dose (cfu prawn^{-1})	Treatment's NaCl (%)	Number of deaths 8	16	24	48	72	96	120	144	168	Cumulative mortality
First	Control	Saline										0/10 (0%)
	1×10^7	0.5		4	1	1		1				7/10 (70%)
	1×10^7	1.0		5	2							7/10 (70%)
	1×10^7	1.5			2		1	1	1			5/10 (50%)
	1×10^7	2.0				2	1		1			4/10 (40%)
Second	Control	Saline										0/30 (0%)
	2×10^6	0.5		6	1	3	1	1				12/30 (40%)[a]
	2×10^6	1.0		1	1	5	2	2	1			12/30 (40%)[a]
	2×10^6	1.5			1	2	1		1			5/30 (17%)[b]
	2×10^6	2.0			2	2	1	1	1			7/30 (23%)[b]

70% (1.0% NaCl), 50% (1.5% NaCl) and 40% (2.0% NaCl). At a challenge dose of 2×10^6 cfu prawn^{-1}, the mortality onset was 16 h for bacteria grown at 0.5% and 1.0% NaCl and 24 h for those grown at 1.5% and 2.0% NaCl. Cumulative mortality was significantly higher for bacteria grown at 0.5% NaCl (40%) and 1.0% NaCl (40%) than for those grown at 1.5% NaCl (17%) and 2.0% NaCl (23%).

Discussion. It is well known that environmental parameters affect the growth of pathogens and their production of toxins (Weinberg 1985, Arp 1988). Smith (1990) indicated that fast-growing pathogens can overwhelm the initial, non-specific defense and cause disease before more powerful immune defenses can operate fully. Slow-growing pathogens are more prone to both types of defense. Riquelme et al. (1995) reported that water temperature and other parameters affected the pathogenicity of *Vibrio* strains in Chilean scallop *Argopecten purpuratus* larvae. Prayitno & Latchford (1995) reported that exposure of luminous bacteria *Vibrio harveyi* to low salinity levels (10 and 15 ppt) for 12 h before use in immersion challenge tests with *Penaeus monodon* larvae resulted in a significantly higher rate of mortality. They also reported that exposure of *V. harveyi* to pH 5.5 significantly reduced its pathogenicity. Ramesh et al. (1989) reported that environmental parameters influence the growth of luminous bacteria. Weinberg (1985) reported that environmental variables affecting toxin production are considerably narrower than those for growth of producer cells. These reports indicate that pathogenic bacteria grown under specific environmental conditions can cause disease and death in cultured animals.

The optimal levels for rearing juvenile *Macrobrachium rosenbergii* are salinity 0 to 10 ppt, pH 7.0 to 8.5 and temperature 29 to 31°C (New 1995). Environmental parameters such as pH and temperature have been shown to affect the resistance of *M. rosenbergii* to KM002 (Cheng & Chen 1998b) in that susceptibility to infection was highest when prawns were exposed to a pH of 8.8 to 9.5 at 33 to 34°C. The present study has shown that conditions near optimum for growth of KM002 (pH 6 to 8, at 25 to 30°C and at 0.5 to 1.0% NaCl) also enhanced virulence to *M. rosenbergii*. Brady & Lasso de la Vega (1992) observed a direct relationship between the concentration of bacteria in water and the concentration of bacteria in the hemolymph of prawns. Thus, environmental parameters can trigger disease outbreaks not only by affecting the health and defense mechanisms of the host, but also the virulence and density of bacterial pathogens.

The resistance of *Macrobrachium rosenbergii* to *Enterococcus*-like disease is low at high temperatures (e.g. 33°C) and high pH (e.g. 9.0) during the occurrence of phytoplankton blooms (Cheng & Chen 1998b). It is more resistant at pH 7.5 to 7.7, temperature 0 to 31°C and salinity 5 to 10 ppt. However, the present study indicated that the environmental parameters which enhance its resistance are all in the range for good growth and high pathogenicity of KM002. The environmental parameters of pond water fluctuate continuously, and KM002 growth can occur whenever they are adequate for its growth. However, disease outbreaks will occur only when the environmental parameters increase the susceptibility of *M. rosenbergii*. In other words, KM002 is an opportunistic pathogen, and it may be possible to manage environmental parameters in prawn ponds such that they promote the resistance of *M. rosenbergii* while decreasing bacterial concentrations and virulence.

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