魚病研究 Fish Pathology, 33 (2), 79–84, 1998. 6

Pathogenicity of *Vibrio splendidus* biovar II, the causative bacterium of bacillary necrosis of Japanese oyster larvae

Gopalrajan Sugumar[1,2], Toshihiro Nakai[2,*], Yasushi Hirata[3], Danji Matsubara[3] and Kiyokuni Muroga[2]

[1]*Fisheries College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Tuticorin 628 008, India*
[2]*Faculty of Applied Biological Sciecnce, Hiroshima University, Higashihiroshima 739-8528, Japan*
[3]*Hiroshima Prefectural Farming Fisheries Association, Takehara 729-2313, Japan*

(Received February 25, 1998)

The potential pathogenicity of strains of *Vibrio splendidus* biovar II, which were isolated from bacillary necrosis of triploid larvae of Pacific oyster *Crassostrea gigas* in a hatchery in western Japan, was investigated. The course of experimental infection with virulent strains of *V. splendidus* biovar II was very rapid in 5-day-old veliger larvae with disease signs apparent within 6 to 12 h after exposure of larvae at doses of 10^4 to 10^6 CFU/m*l*, and mortalities up to 100% were recorded in 24 h at 10^5 and 10^6 CFU/m*l*. Although all the tested stages of larvae were experimentally infected by virulent strains of *V. splendidus* biovar II, a later stage (17 days post-hatching) was less susceptible than the earlier stages of development. Diploid and triploid larvae were almost equally susceptible to this pathogen. Extracellular products and intracellular components of the strains were lethal to larvae, but their lethality did not correlate with the virulence of live cultures. These results suggest that the ability to elaborate toxins is not the only virulence factor in the pathogenicity of *V. splendidus* biovar II. The ability of this pathogen to bring about significant mortalities in oyster larvae at densities of 10^4 CFU/m*l* and its long survival in seawater make this pathogen a potential threat to larval oyster productions in hatchery systems.

Key words: bacillary necrosis, vibriosis, *Crassostrea gigas*, veliger, *Vibrio splendidus*, pathogenicity, toxicity

Marine vibrios have often been implicated in larval and juvenile diseases of bivalve molluscs. Larval vibriosis or bacillary necrosis is the most serious problem limiting hatchery productions of bivalve larvae in several geographical locations (Sindermann, 1988). Although several species of *Vibrio*, like *V. alginolyticus*, *V. anguillarum*, *V. splendidus*, *V. tubiashii* and unidentified *Vibrio* spp., were incriminated in bacillary necrosis, only *V. tubiashii* has been referred to as primary pathogen while the others were considered only as opportunistic pathogens (Elston, 1993). Several studies have been attempted to explain the pathogenesis of bacillary necrosis. Some of the studies indicated exotoxins elaborated by the invading bacteria for the pathogenesis (Nottage and Birkbeck, 1986; Lodeiros *et al.*, 1987; Riquelme *et al.*, 1995), while other reports support the invasive potential of the bacteria as the significant factor in pathogenesis (Elston and Leibovitz, 1980; Jeffries, 1982). Recent studies suggested that the pathogenicity may be due to the combination of both invasive and toxic potentials of the pathogen (Nicolas *et al.*, 1996). However, the mechanism of pathogenicity is still far from clear and requires further detailed investigations.

Since 1993, triploid larvae of Japanese or Pacific oyster *Crassostrea gigas* has been successfully produced in the hatchery of the Hiroshima Prefectural Farming Fisheries Association (HPFFA) in western Japan (Akashige and Kusuki, 1996). However, it was frequently marred by occurrences of mass mortality in larval stages. The signs of the disease resemble those of bacillary necrosis reported in the USA and Europe (Tubiash *et al.*, 1965, 1970; Lodeiros *et al.*, 1987; Sindermann, 1988). On detailed bacteriological investigations the causative agent of the disease occurring in HPFFA hatchery was confirmed as *Vibrio splendidus* biovar II (Sugumar *et al.*, 1998). In this paper, we report studies on the pathogenicity of the causative bacterium of bacillary necrosis of Japanese oyster larvae. We also examined the toxicity of intracellular components and extracellular products of both virulent and avirulent strains to under-

* E-mail: nakaitt@ipc.hiroshima-u.ac.jp

stand their role in the pathogenicity.

Materials and Methods

Bacterial strains

Four strains of *V. splendidus* biovar II were used in this study. These strains were selected based on their LD_{50} against 5-day-old oyster larvae as reported previously (Sugumar *et al.*, 1998): two virulent strains, no. 58 and 60, were isolated from diseased larvae of *C. gigas*, their LD_{50} being 10^4 CFU/m*l*, one low virulent strain, no. 72, isolated from larval rearing tank water which could bring 25% mortality at 10^7 CFU/ml and one avirulent strain, no. J13, with no larval mortality even at 10^7 CFU/m*l*, isolated from natural seawater at the time of disease outbreak in the hatchery.

Pathogenicity assays

Healthy triploid larvae of *C. gigas* produced at the HPFFA hatchery were challenged with the above-mentioned 4 strains of *V. splendidus* biovar II. Static cultures of bacteria grown in marine broth (Difco) at 25°C for 24 h were centrifuged at 3,000 × *g* for 20 min, washed twice and resuspended in 0.01M PBS (1.5% NaCl, pH7.0). Ten-fold dilutions of the cell suspensions were prepared and 10^3 to 10^6 CFU/m*l* were added to 5 or 6-day-old veliger larvae (shell height of 5-day-old = 100.3 ± 11.2 µm and 6-day-old = 125.9 ± 16.1 µm) maintained at a density of 4 larvae/m*l* in 10 m*l* of filter sterilized natural seawater in 6-well cell culture chambers (Falcon). Chambers without addition of the bacterium served as controls. These assays were performed in duplicates at 25°C. The course of infection and mortality rate were studied by observing the larvae at 6 h intervals under a low power (60 ×) microscope. Swarming of bacteria around shell margin, sloughing of cilia and fragmentation of velum of larvae were further confirmed using a phase contrast microscope. Larvae sinking at the bottom with no apparant intravalvular movement as observed through low power microscope were considered dead and final larval survival was recorded after 24 h. Reisolation of the introduced bacterium was done by carefully picking up moribund/dead larvae from challenged wells and directly plating on TCBS (thiosulphate citrate bile salts sucrose) agar (Difco). Colonies that developed were characterized according to the scheme of Alsina and Blanch (1994a, b).

Susceptibility of diploid and triploid larvae to virulent strains were compared under identical conditions using 5-day-old larvae from the same batch at a density of 4 larvae/m*l* and survival rates were recorded after 24 h. Further, susceptibility of triploid larvae at different developmental stages were tested with 5-day, 10-day and 17-day-old larvae under similar conditions as above, except for the larval density which was maintained at 3 larvae/m*l* with 17-day-old larvae. Toxicity of extracellular products (ECP) and intracellular components (ICC) prepared from 1-day-old cultures of *V. splendidus* biovar II was also tested on oyster larvae by adding ECP or ICC in the culture chambers at 5 and 10% final concentrations (v/v). After 2 h of exposure, the water in the culture chambers was changed twice with fresh filter-sterilized seawater to remove ECP or ICC and the larval survival was recorded after 24 h.

Preparation of extracellular products (ECP) and intracelluar components (ICC)

The ECP of the 4 strains of *V. splendidus* biovar II were prepared on 70% artificial seawater based – trypticase soy agar (Eiken) by the cellophane plate method (Inamura *et al.*, 1984). Briefly, cellophane-overlaid agar plates were inoculated with precultured bacteria and incubated at 25°C for 24–96 h. Bacterial cells were harvested with 2 m*l* of 0.01M PBS (pH7.0) and centrifuged at 12,000 × *g* for 20 min. The supernatant was passed through a 0.45 µm membrane filter (Advantec), dialysed with PBS at 4°C overnight and stored at 4°C. The ICC were prepared from the bacterial pellet of cellophane plate culture. After washing twice with PBS and adjusting the cell concentration to 25 mg/m*l* in PBS, the cells were sonicated for 15 min (Branson sonicator, model 250) and centrifuged at 12,000 × *g* for 20 min, and the supernatant was filtered through a 0.45 µm membrane filter. The filtrate containing ICC was stored at 4°C. The protein contents of both ECP and ICC were measured with folin phenol (Lowry *et al.*, 1951).

Protease assay

The proteolytic activity of the ECP and ICC was determined using azocasein as the substrate as described by North and Walker (1984). A 100 µ*l* of the sample was incubated with 0.5 m*l* of azocasein (10 mg/m*l*) and 0.5 m*l* of buffer (0.1M Tris-HCl, pH8.0) at 37°C for 2 h. To terminate the reaction, 0.2 m*l* of 50% trichloroacetic acid was added and the resulting precipitate was removed by centrifugation. The supernatant was made alkaline by addition of 0.2 m*l* of 4M NaOH and the absorbance was measured at 520 nm. Under these conditions an absorbance increase of 1.0 was equivalent to

the hydrolysis of 0.71 mg of azocasein. Specific proteolytic activity was expressed as amount of azocasein hydrolyzable by 1 mg of protein in the sample.

Survival of V. splendidus *biovar II in seawater*

Survival of virulent and avirulent strains in coastal seawater was tested by inoculating low numbers (10^1 ~ 10^2 CFU/m*l*) of the precultured and washed cells in 100 m*l* flasks containing autoclaved seawater. Flasks were gently agitated at 50 rpm in a shaker incubator maintained at 25 ± 1°C. Samples of seawater were spread-plated on marine agar (Difco) for viable count every day for about a week and later once in a week.

Results and Discussion

Course of infection and susceptibility of larvae

The progression of the disease and mortality rate were found to be rapid when the larvae were infected with higher initial doses of the strains. As shown in Fig. 1, little difference was seen in the overall mortality pattern or in the progression of the disease between the two virulent strains tested. When 5-day-old veliger larvae were challenged with a dose of 10^6 CFU/m*l*, the larval survival at 6 h post-challenge was only about 67% and 30% with strains no. 58 and 60, respectively, and in both cases mortalities higher than 90% were seen in less than 18 h. Even on exposure at the bacterial concentration of 10^4 CFU/m*l*, the larval survival was as low as 10% against strain no. 58 and 43% against strain no. 60 at 24 h. In most cases, swarming of bacterial cells was observed around shell margin and velum of the larvae before cessation of intravalvular movements. The bacterial cells not only swarmed around the moribund larvae but also freely moved inside and out of the shell of larvae. Bacterial swarming was followed by loss of cilia of the velum and/or fragmentation of velar tissues. Occassionally, dead larvae were also observed with their velum and other tissues intact. The progressive disease signs of larvae were seen at bacterial doses of 10^4 to 10^6 CFU/m*l*, but the signs of disease appeared at different incubation time depending on the bacterial doses. Even with the infection dose of 10^4 CFU/m*l*, the disease signs could be observed within 12 to 18 h of exposure. Infection dose of 10^3 CFU/m*l* was too low to cause either disease signs or mortality in larvae. Such a rapid course of experimental infection with appearance of disease signs in 4 to 5 h after exposing different species of bivalve larvae to *V. tubiashii* has already been documented (Tubiash *et al.*, 1965; Sindermann, 1988). Mortalities in cockle *Fulvia mutica* larvae by an unidentified *Vibrio* sp. with similar disease signs were also reported in Japan (Fujiwara *et al.*, 1993).

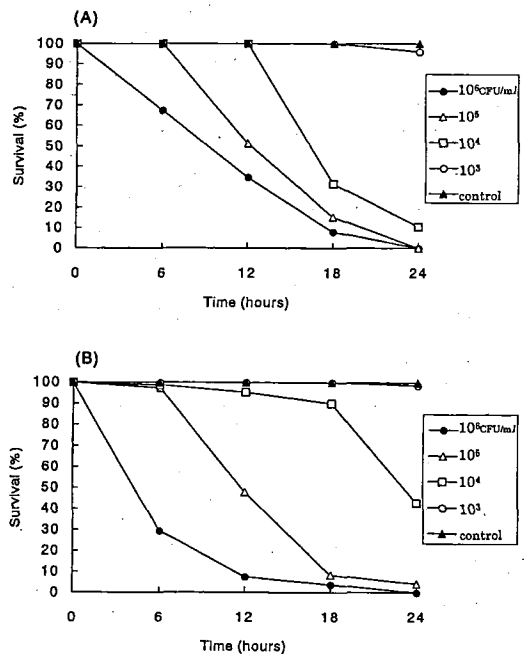

Fig. 1. Course of infection in 5-day-old oyster larvae challenged with *V. splendidus* biovar II. (A) strain no. 58, (B) strain no. 60

It was found that all stages of larvae tested were susceptible to virulent strains of *V. splendidus* biovar II (Table 1). However, when infected with 10^4 CFU/m*l*, the survival of advanced larvae (17-day-old) was 88 to 92% while the other two tested stages exhibited a survival percentage of 11 to 54, thereby, late stages of larvae were relatively less susceptible than the early stages. In the hatchery also, natural infection always occurs at early stages of production especially within the first week of development of the larvae, after which the mortality rate was less.

Both the triploid and diploid larvae were susceptible with only minor differences to the virulent strains of *V. splendidus* biovar II. As shown in Fig. 2, survival rates of diploid were always higher than those of triploid larvae, though the differences were not significant. Therefore, it is unlikely that the triploid larvae have inherently higher susceptibility to this bacterial infection than diploids.

Table 1. Susceptibility of oyster larvae at different developmental stages to *V. splendidus* biovar II

Age (days post-hatching)	Shell height (μm)	larval density (no/ml)	Survival rate (%) at 24 h									
			against strain no. 58 at					against strain no. 60 at				
			10^6	10^5	10^4	10^3	control	10^6	10^5	10^4	10^3	control
			(CFU /ml)*					(CFU /ml)*				
5	100.3 ± 11.2	4	0	0	11	96	100	0	4	43	99	100
10	202.1 ± 26.6	4	0	0	54	100	100	0	0	11	100	100
17	321.5 ± 29.6	3	0	8	92	100	100	0	3	88	99	100

* Coefficient of bacterial density was 8 ~ 9 × 10^3 CFU/ml in all cases.

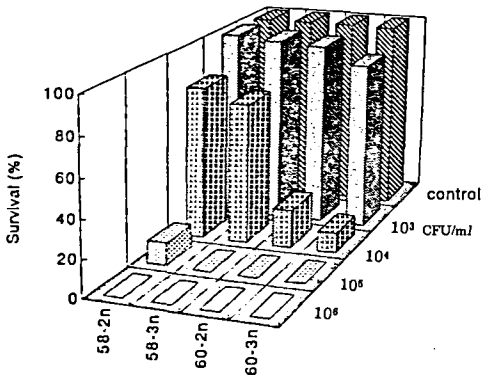

Fig. 2. Susceptibility of diploid (2n) and triploid (3n) oyster larvae (5-day-old) to strains (no. 58 and 60) of *V. splendidus* biovar II.

Toxicity and pathogenesis

Cellophane plate cultures demonstrated higher level of ECP as measured by protein content and higher protease activity than that of broth cultures. Specific protease activity obtained from cellophane plate assay was 20.5 in 1-day-old culture of strain no. 72, while it was 6.9, 6.3 and 2.0 respectively for 1-day-old cultures of strains no. 58, 60 and J13 (Table 2). Although the protein content of ECP increased progressively with time, the specific protease activity was maximum with ECP of 1-day-old cultures and then decreased. There was no correlation between the amount of production of ECP or protease and the virulence of live cultures. However, the ECP of both virulent and less virulent cultures exhibited toxicity against oyster larvae, while the ECP of avirulent strain, no. J13 did not cause significant lethality (Table 3). The lack of lethality of ECP of no. J13 could be related to the least protease activity. Unlike the ECP, ICC of these strains did not exhibit protease activity, although larval toxicity was observed (Table 3). ICC of strain no. J13 was lethal to larvae, however, the live culture of the strain could not cause mortality of oyster larvae even at 10^7 CFU/ml and therefore, no correlation could be established between the virulence of live cultures and the toxicity of their ICC. These results combined with degeneration of velar tissues and rapidity of larval mortality suggest that there is

Table 2. Protease assay of ECP of *V. splendidus* biovar II

Strain no.	Incubation time											
	Day 1			Day 2			Day 3			Day 4		
	PC	TPA	SA	PC	TPA	SA	PC	TPA	SA	PC	TPA	SA
58	1.4	9.6	6.9	1.9	8.0	4.2	1.5	3.6	2.4	2.2	3.8	1.7
60	1.0	6.3	6.3	1.5	6.2	4.1	1.6	4.5	2.8	1.9	2.9	1.5
72	0.8	16.4	20.5	1.3	18.0	13.8	1.5	15.0	10.0	1.8	14.0	7.8
J13	0.3	0.6	2.0	1.7	0.6	0.4	2.0	0.2	0.1	2.8	0.2	0.1

PC: Protein content of ECP in mg/ml
TPA: Total protease activity = Amount (mg) of azocasein hydrolyzed by 1 ml of ECP
SA: Specific activity = Amount of azocasein hydrolyzable by 1 mg of protein in ECP

Table 3. Toxicity of ECP and ICC of *V. splendidus* biovar II to oyster larvae

Strain no.		Larval mortality (%) at concentrations (v/v) of		
		0%	5%	10%
58	ECP	0	13	17
	ICC	0	5	28
60	ECP	0	7	20
	ICC	0	8	26
72	ECP	0	11	11
	ICC	0	2	15
J13	ECP	0	3	3
	ICC	0	11	27

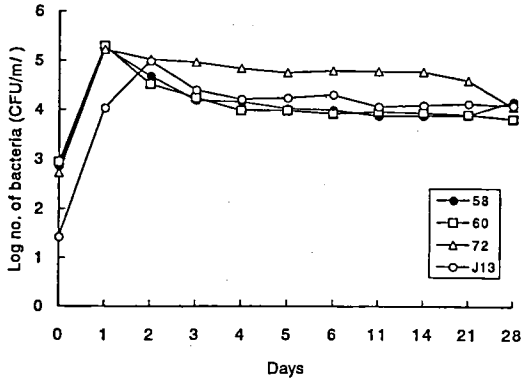

Fig. 3. Survival of strains of *V. splendidus* biovar II in coastal seawater.

possible involvement of toxins in the pathogenicity, however, it is also clear that the ability to elaborate toxins is not the only criterion to make a strain more virulent. Proteinase (s) and/or cytolytic factors of the culture supernatant of a *Vibrio* strain were suggested to be involved in the disintegration of gill tissues and pathogenicity of *C. gigas* spats (Nottage and Birkbeck, 1986). ICC seems to have less significance in the pathogenicity of the isolated *V. splendidus* biovar II. The lack of correlation between the toxicity of ICC and the virulence of these cultures suggests to differ from the opinion raised by Nicolas *et al.* (1996) that vibrios ingested by the larvae release toxins when lysed by the digestive enzymes of the larvae, which degrade the tissues leading to mortality.

Based on histological and ultrastructural observations of infected oyster larvae, Elston and Leibovitz (1980) reported three types of pathogenesis caused by two different unidentified *Vibrio* strains. The extensive visceral atrophy in late stages and velar damage in early stages of *C. virginica* larvae due to *Vibrio* sp. invasion were referred to as type III and type II pathogenesis, respectively. Another *Vibrio* strain was reported to grow preferentially along mantle, invade visceral cavity overwhelming phagocytosis in all stages of larvae, which was termed as type I pathgenesis. The course of infection by *V. splendidus* biovar II in the present study resembled the type I pathogenesis reported by Elston and Leibovitz (1980).

All the tested strains of *V. splendidus* biovar II grew in autoclaved seawater without supplemented nutrients. As shown in Fig. 3, during the first day of incubation all strains of the bacteria increased by about 3 log numbers, followed by one log reduction in numbers between days 2 and 3 and later maintained almost at the same level for up to 4 weeks of observation. A similar trend of growth and survival was seen both in coastal seawater and off-shore water. This long survival in seawater suggests that this pathogenic bacterium could be one of the autochthonous microflora of seawater capable of causing infection in oyster larvae. In the hatchery, although strains of *V. splendidus* biovar II were present in larval rearing water, their relative numbers were far less when compared with their load found along with the larvae and were totally absent in algal cultures (Sugumar *et al.*, 1998). Therefore, these strains may probably have preference for larval hosts or the nutrients derived from the larvae could have facilitated rapid multiplication.

Although larval vibriosis is generally considered as an opportunistic disease of intensively cultured molluscs, *V. tubiashii* alone has so far been regarded as a specific or primary pathogen of bivalve larvae because of their specific occurrences in oyster larval disease from widely geographical areas and ability to cause larval mortalities at concentrations less than 10^5 CFU/m*l* (Elston, 1993). In the present case, virulent strains of *V. splendidus* biovar II were highly pathogenic to *C. gigas* larvae, and the pathogenicity is comparable to that of *V. tubiashii* reported earlier (Tubiash *et al.*, 1965; Jeffries, 1982). Even after repeated subcultures over a period of 5 months, the culture (strain no. 58) did not lose its virulence and caused 100% larval mortality at 10^5 CFU/m*l* within 24 h. It is difficult to differentiate a pathogen as primary or opportunistic, however, a key distinction is that a pathogen has an inherent capacity to breach host cell barriers whereas a commensal organism or opportunistic pathogens do not (Falkow, 1997).

Accordingly, although there are not enough evidences to understand the pathogenesis, virulent strains of *V. splendidus* biovar II possess a definite mechanism to breach the defences of healthy larvae and capable of destroying the host completely. With this potential virulence and ability to survive in seawater, this pathogen poses a real threat to larval oyster productions unless its entry into the hatchery system is effectively prevented.

References

Akashige, S. and Y. Kusuki (1996): Artificial induction and larval survival of triploids in the oyster *Crassostrea gigas*. *Bull. Hiroshima Fish. Exp. Station*, **19**, 1–20.

Alsina, M. and A. R. Blanch (1994a): A set of keys for biochemical identification of environmental *Vibrio* species. *J. Appl. Bacteriol.*, **76**, 79–85.

Alsina, M. and A. R. Blanch (1994b): Improvement and update of a set of keys for biochemical identification of *Vibrio* species. *J. Appl. Bacteriol.*, **77**, 719–721.

Elston, R. A. (1993): Infectious diseases of the Pacific oyster, *Crassostrea gigas*. *Ann. Rev. Fish Dis.*, **3**, 259–276.

Elston, R. A. and L. Leibovitz (1980): Pathogenesis of experimental vibriosis in larval American oysters, *Crassostrea virginica*. *Can. J. Fish. Aquat. Sci.*, **37**, 964–978.

Falkow, S. (1997): What is a pathogen? *ASM News*, **63**, 359–365.

Fujiwara, M., Y. Uyeno and A. Iwao (1993): A *Vibrio* sp. associated with mortalities in cockle larvae *Fulvia mutica* (Mollusca: Cardiidae). *Fish Pathol.*, **28**, 83–89.

Inamura, H., K. Muroga and T. Nakai (1984): Toxicity of extracellular products of *Vibrio anguillarum*. *Fish Pathol.*, **19**, 89–96.

Jeffries, V. E. (1982): Three *Vibrio* strains pathogenic to larvae of *Crassostrea gigas* and *Ostrea edulis*. *Aquaculture*, **29**, 201–226.

Lodeiros, C., J. Bolinches, C. P. Dopazo and A. E. Toranzo (1987): Bacillary necrosis in hatcheries of *Ostrea edulis* in Spain. *Aquaculture*, **65**, 15–29.

Lowry, O. H., N. J. Roseborough, A. L. Farr and R. J. Randall (1951): Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

Nicolas, J. L., S. Corre, G. Gauthier, R. Robert and D. Ansquer (1996): Bacterial problems associated with scallop *Pecten maximus* larval culture. *Dis. Aquat. Org.*, **27**, 67–76.

Nottage, A. S. and T. H. Birkbeck (1986): Toxicity to marine bivalves of culture supernatant fluids of the bivalve-pathogenic *Vibrio* strain NCMB 1338 and other marine vibrios. *J. Fish Dis.*, **9**, 249–256.

North, M. J. and M. Walker (1984): An analysis of intracellular proteolytic activities of *Tetrahymena pyriformis* GL. *J. Gen. Microbiol.*, **130**, 1977–1985.

Riquelme, C., G. Hayashida, A. E. Toranzo, J. Vilches and P. Chavez (1995): Pathogenicity studies on a *Vibrio anguillarum*-related (VAR) strain causing an epizootic in *Argopecten purpuratus* larvae cultured in Chile. *Dis. Aquat. Org.*, **22**, 135–141.

Sindermann, C. J. (1988): Vibriosis of larval oysters. In "Disease diagnosis and control in North American aquaculture", 2nd ed. (ed. by C. J. Sindermann and D. V. Lightner). Elsevier, Amsterdam, pp. 271–274.

Sugumar, G., T. Nakai, Y. Hirata, D. Matsubara and K. Muroga (1998): *Vibrio splendidus* biovar II as the causative agent of bacillary necrosis of Japanese oyster *Crassostrea* gigas larvae. *Dis. Aquat. Org.*, (in press).

Tubiash, H. S., P. E. Chanley and E. Leifson (1965): Bacillary necrosis, a disease of larval and juvenile bivalve mollusks. I. Etiology and epizootiology. *J. Bacteriol.*, **90**, 1036–1044.

Tubiash, H. S., R. R. Colwell and R. Sakazaki (1970): Marine vibrios associated with bacillary necrosis, a disease of larval and juvenile bivalve mollusks. *J. Bacteriol.*, **103**, 271–272.