Vol. 33: 111–118, 1998 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published June 19

Vibrio splendidus biovar II as the causative agent of bacillary necrosis of Japanese oyster *Crassostrea gigas* larvae

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ABSTRACT: Recurrent outbreaks of a disease leading to mass mortalities in an oyster (*Crassostrea gigas*) hatchery located in western Japan were investigated. The disease occurred regularly in 2- to 8-d-old larvae and has been experimentally controlled in the hatchery by treating the larval rearing water with streptomycin, without ascertaining the etiological agent. The signs of the disease and the course of infection resembled bacillary necrosis reported in oysters and other bivalve molluscs in the USA and Europe. Quantitative and qualitative examinations of the bacterial flora of hatchery samples including source water, broodstock, larval feed and larvae revealed a very high total bacterial load and presumptive vibrios in diseased larvae. Further, the bacterial profile revealed that *Vibrio* spp. constituted approximately 60 to 95% of the bacteria isolated from infected larvae and most isolates were identified as *V. splendidus* biovar II and *V. harveyi*, suggesting their possible role in the disease. However, experimental challenges proved the pathogenicity of *V. splendidus* II. Several isolates of *V. splendidus* II from infected larvae were highly pathogenic, producing 100% mortality at levels of 10^5 cfu ml^{-1} in 24 h, while isolates from other sources demonstrated a low degree of virulence. Detection of *V. splendidus* II from broodstock, especially in the gonad of a few breeders, suggests the probability that broodstock could be the source and route of transmission of this pathogen.

KEY WORDS: Bacillary necrosis · Vibriosis · *Crassostrea gigas* · Larvae · *Vibrio splendidus* II · Pathogenicity

INTRODUCTION

Bacillary necrosis, a disease of larval and juvenile bivalve molluscs, is one of the major problems in hatcheries, causing serious production losses. Marine vibrios have often been incriminated in such mortalities and the disease, also termed larval vibriosis, has been reported from several geographical locations, especially in the USA and Europe (Elston 1984). Bacillary necrosis of several species of larval bivalves was first described to be caused by Gram-negative motile rods, some of which were subsequently identified as *Vibrio alginolyticus* and *V. anguillarum* (Tubiash et al. 1965, 1970). Involvement of *V. anguillarum* in larval mortalities of oyster *Ostrea edulis* (DiSalvo et al. 1978) and *Vibrio* spp. in epizootic of *Crassostrea virginica* (Brown & Losee 1978, Elston et al. 1981) were reported in the USA. Jeffries (1982) isolated 3 different *Vibrio* species from diseased *C. gigas* larvae in the United Kingdom and experimentally found that strains of *V. anguillarum* were highly pathogenic, while *V. alginolyticus* and *V. splendidus* were, respectively, moderately and less pathogenic to oyster larvae. However, the isolates reported as *V. anguillarum* by Tubiash et al. (1970) and Jeffries (1982) were later classified as a new species under the name *V. tubiashii* (Hada et al. 1984). Recently, a *V. anguillarum*-related strain causing epizootics of scallop *Argopecten purpuratus* larvae was reported in Chile (Riquelme et al. 1995a) and *V. splendidus* was among the isolates from diseased scallop *Pecten maximus* larvae in France (Nicolas et al. 1996). Although bivalve larval vibriosis implicating

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these *Vibrio* species has been reported from diverse geographical locations, it is regarded only as an opportunistic disease occurring in crowded husbandry systems (Elston 1984, 1993).

The Japanese or Pacific oyster *Crassostrea gigas* has been intensively cultured along the Pacific coast of Japan since the 19th century and has depended fully on natural spats. In 1993, mass production of triploid larvae of *C. gigas* by the application of thermal shock and caffeine treatment to fertilized eggs (Akashige & Kusuki 1996) was initiated at the hatchery of the Hiroshima Prefectural Farming Fisheries Association (HPFFA) in western Japan along the Seto Inland Sea. However, there were always high mortalities, especially in early stages (2 to 8 d) of larval development, which seriously limited production. Experimental treatment of rearing water with antibiotic (7.2 µg ml^{-1} streptomycin), under the presumption that the mortality could be due to bacterial infection, controlled the disease, leading to successful rearing of larvae in the hatchery since 1995. Against this background, a bacteriological investigation was conducted at the HPFFA hatchery to determine the causative agent(s) of the disease and the source of infection.

MATERIALS AND METHODS

Sampling. Two batches of triploid larvae produced in the hatchery during May and June 1997 were used for the study to determine the causative agent of the disease. The experimental strategy included bacteriological examinations of cultured larvae in experimental tanks (500 l) and in regular production tanks (20 m^3) with and without streptomycin treatment of rearing water as described below: One batch of larvae was reared at a density of 4 larvae ml^{-1} in 2 experimental tanks and another batch of larvae was reared in the regular production tanks at a density of 2 larvae ml^{-1}. Both the batches were reared in 1 µm-filtered seawater at 25 ± 1°C as routinely performed in the hatchery. A concentration of 7.2 µg (concentration of active ingredient) ml^{-1} of streptomycin was maintained in larval rearing water in 1 of the 2 experimental and 1 of the 2 regular production tanks while the other 2 served as controls. Streptomycin level was maintained by replacing approximately 50% of tank water with freshly medicated water every 2 or 3 d and a 100% change during mid-cycle of larval production. Samples of larvae and water were drawn from both the treated and untreated tanks with the first sign of disease indicated by visible aggregation of larvae on tank bottom. In addition, samples of raw seawater, filtered seawater, larval feed (*Chaetoceros calcitrans* and *Pavlova lutheri*), broodstock and breeder tank water were also analysed bacteriologically. The shell length of larvae was determined by measuring a sample of 50 larvae using a Nikon profile projector, and larval survival was estimated by counting the larvae in sample volumes from each tank at the beginning and end of the production cycle (18 d).

Bacteriology. Inactive larvae, which settled to the bottom of the tanks with the onset of the disease, were siphoned out and filtered through a plankton net of 50 µm mesh size. Healthy larvae, which were swimming in the water column of the tanks, were collected by filtering water through a plankton net. Larval samples were washed with 0.2 µm-filtered seawater and resuspended in 2 ml of sterile seawater, and the larvae in 100 µl of the suspension were counted using a Neubauer chamber under a low power (60×) microscope. Homogenates of larvae, algae and adult oysters and samples of water were serially diluted, plated on Marine Agar 2216 (MA, Difco) and Thiosulfate Citrate Bile salt Sucrose (TCBS) agar (Difco) and incubated at 25°C. Colonies on TCBS and MA were counted after 48 and 72 h, respectively. Gonads of broodstock were sampled using an inoculation wire loop after making an incision in the gonad with a sterile scalpel, and were streaked directly on TCBS agar plates. For qualitative analysis, colonies on MA plates were picked randomly, purified and subjected to a battery of standard biochemical tests that identified them to generic level following the scheme described by the Oceanographical Society of Japan (1990). Presumptive vibrios including all representative morphotypes on TCBS and those on MA confirmed as *Vibrio* species were further assigned to various species according to Alsina & Blanch (1994a, b).

Drug sensitivity. Drug sensitivity of isolates of *Vibrio splendidus* II was assayed by the disc diffusion method (Barry & Thornsberry 1991) on Mueller-Hinton agar (Eiken Co., Ltd, Tokyo) supplemented with 1.5% NaCl, against the following 15 antibiotics and chemotherapeutics (in µg per disc): streptomycin (50), kanamycin (50), vancomycin (5), ampicillin (30), oxycillin (30), colistin (150), oxytetracycline (30), sulfactum-cefaperazolin (30), cefaperazolin (30), cephalexin (30), cefazolin (30), erythromycin (30), novobiocin (20), nalidixic acid (50) and chloramphenicol (100). Depending upon the zone of inhibition, as per manufacturer's guidelines, the isolates were classified as sensitive (S), weakly sensitive (WS) and resistant (R).

Pathogenicity assays. Healthy oyster larvae were challenged with strains of dominant *Vibrio* species to determine their pathogenicity. A total of 23 isolates consisting of strains of *Vibrio splendidus* II, *V. harveyi*, *V. alginolyticus* and a *V. anguillarum*-like organism were tested. Static cultures of bacteria grown in Marine Broth 2216 (Difco) at 25°C for 24 h were cen-

trifuged at 3000 × *g* for 20 min, washed twice and resuspended in phosphate-buffered saline (PBS) (1.5% NaCl). Ten-fold dilutions of the cell suspensions were prepared and approximately 10^3 to 10^7 colony-forming units (cfu) ml^{-1} were added to 5- to 8-d-old larvae (shell length = 107.8 ± 12.1 to 133.4 ± 18.2 µm) maintained at a density of 4 larvae ml^{-1} in 10 ml of filter sterilized seawater in 6-well cell culture chambers (Falcon). Chambers without addition of bacteria served as controls. These assays were performed in duplicates at 25°C and larval survival was recorded after 24 h. Larvae sinking to the bottom of the chamber with no apparent intravalvular movement as observed by a low power microscope were considered dead. Based on larval mortality at the end of 24 h exposure, the 50% lethal dose (LD_{50}) was determined in all possible cases by the method of Reed & Meunch (1938). Reisolation of the introduced bacteria in experimentally infected larvae was also performed. Moribund/dead larvae from challenged wells and larvae from control wells were carefully picked up with a pasteur pipette, washed in sterile seawater and the whole larvae directly plated on TCBS agar. Colonies that developed were characterized biochemically as described earlier.

RESULTS

Larval survival and dominant bacteria

High larval mortalities occurred in untreated groups in both experimental tanks (89%) and regular production tanks (72%), while in streptomycin-treated groups the cumulative mortality was 31 and 53%, respectively, in experimental and regular tanks. In experimental tanks, larval mortality escalated on Day 5, when dead and moribund larvae aggregated on the bottom of the untreated tank. Compared with actively swimming larvae, dead and moribund larvae had an about 1000-fold higher bacterial load (Table 1). However, the difference in bacterial load between active and inactive larvae in the streptomycin-treated tank was not so marked. The number of presumptive vibrios was very high in inactive larvae in the untreated tank with 10^3 cfu $larva^{-1}$ (Table 1). Qualitative analysis of bacterial flora of both the study groups revealed a high proportion of vibrios in inactive larvae, while in the tank water vibrios were totally absent (Fig. 1a). A large number of the presumptive vibrios in all samples were identified as *Vibrio splendidus* II, and strains of *V. harveyi* were also found associated with infected larval samples from the untreated tank (Table 2).

In regular production tanks, increased rate of larval mortality was observed on Day 4, but the total bacterial load and presumptive vibrios in inactive larvae were lower than those in the experimental tanks maintained at higher larval density (Table 3). However, 95% of the isolates on MA from inactive larvae in the untreated tank were identified as *Vibrio* species (Fig. 1b). *Vibrio* was also the most dominant population from samples of active larvae (65%) and water (35%) in the untreated tank and inactive larvae (45%) in the treated tank. *V. splendidus* II was detected from all samples examined, and was particularly dominant in inactive larvae (Table 4). *V. harveyi* was also detected in larvae of treated and untreated groups.

Most strikingly, all the samples of inactive larvae were found to contain a higher proportion of sucrose-negative vibrios (green-coloured colonies on TCBS) than sucrose-fermenting vibrios, constituting 72 to 90% of the total presumptive vibrios (Tables 1 & 3). Except in active larvae, where the

Table 1. Quantitative bacterial analysis of samples from experimental tanks

Sample	Total viable count (cfu g^{-1} or ml^{-1})	(cfu $larva^{-1}$)	Presumptive vibrio count[a] (cfu g^{-1} or ml^{-1})	(cfu $larva^{-1}$)
Untreated tank				
Inactive larvae	1.4×10^{11}	1.1×10^5	1.9×10^9 (79)	1.5×10^3
Active larvae	4.9×10^8	3.9×10^2	2.1×10^6 (54)	1.7×10^0
Tank water	8.3×10^4		6.0×10^1 (100)	
Streptomycin-treated tank				
Inactive larvae	8.4×10^8	6.6×10^2	3.7×10^7 (72)	2.9×10^1
Active larvae	5.8×10^9	4.6×10^3	4.5×10^6 (23)	3.5×10^0
Tank water	1.5×10^5		$<1.0 \times 10^1$	
Raw seawater	1.5×10^3		$<1.0 \times 10^1$	

[a]Values in parentheses are % of sucrose-negative vibrios

Table 2. *Vibrio* species associated with samples from experimental tanks. n = number of isolates picked for species characterization

Species	Untreated tank			Streptomycin-treated tank	
	Inactive larvae (n = 20)	Active larvae (n = 8)	Tank water (n = 7)	Inactive larvae (n = 17)	Active larvae (n = 5)
V. aestuarinus	1	–	–	–	–
V. anguillarum-like	1	–	–	–	1
V. harveyi	4	–	1	–	–
V. mediterranei	–	3	–	4	3
Vibrio Phenon 14	1	–	–	–	–
V. splendidus II	13	5	6	13	1

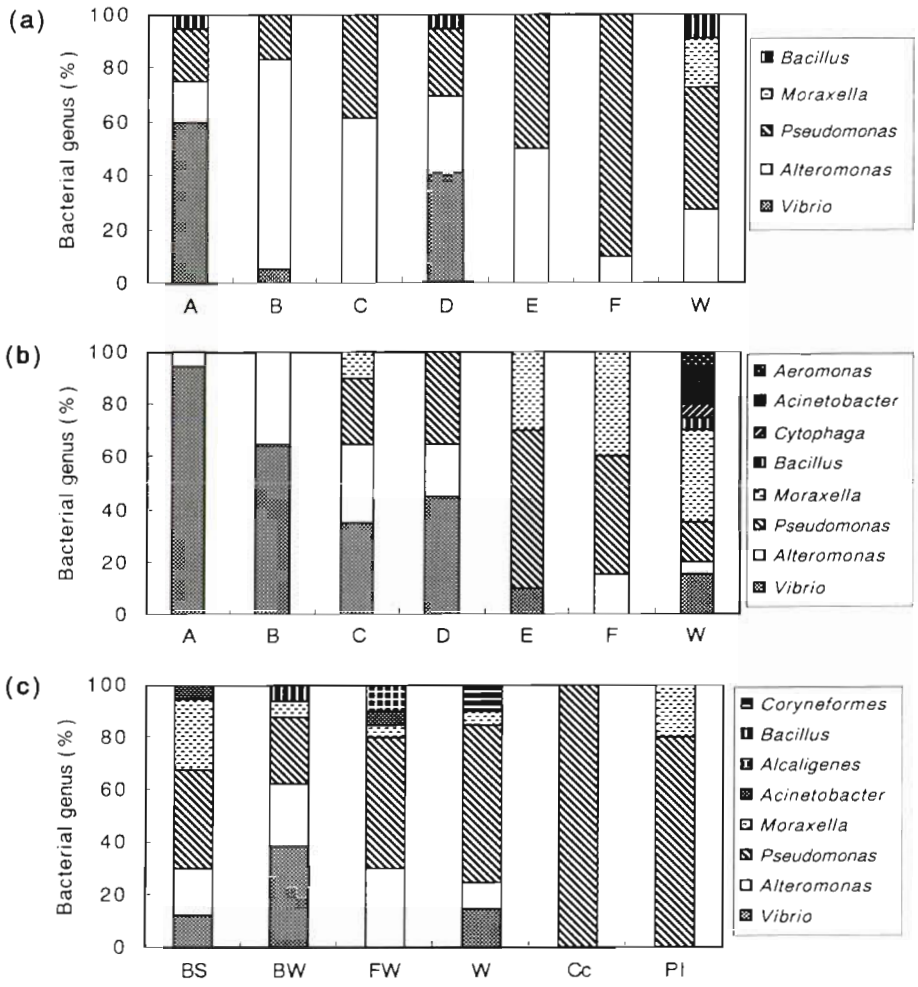

Fig. 1. Qualitative analysis of bacterial flora. (a) Samples from experimental tanks, (b) samples from regular production tanks, and (c) samples from other sources. A, B and C: inactive larvae, active larvae and water, respectively, from untreated tanks; D, E and F: inactive larvae, active larvae and water, respectively, from streptomycin-treated tanks. W: raw seawater; BS: broodstock; BW: breeder tank water; FW: filtered seawater; Cc: *Chaetoceros calcitrans*; Pl: *Pavlova lutheri*

sucrose-positive vibrios outnumbered sucrose-negative vibrios, all other samples, including raw seawater, had a higher proportion of sucrose-negative vibrios.

Dominant bacteria in other samples

Quantitative and qualitative analyses of samples of adult oysters, breeder tank water, feed consisting of 2 algal cultures (*Chaetoceros calcitrans* and *Pavlova lutheri*), raw seawater and 1 µm-filtered seawater demonstrated the presence of vibrio populations in broodstock, breeder tank water and raw seawater, with a high proportion of sucrose-negative vibrios (Table 5, Fig. 1c). *Vibrio harveyi* and *V. splendidus* II were dominant among vibrios of these 3 samples. Strains of *V. splendidus* II were also isolated from gonad samples of broodstock upon direct streaking on TCBS. In contrast, vibrios were not detected from the normal flora of algal cultures, which primarily consisted of *Pseudomonas*.

Table 3. Quantitative bacterial analysis of samples from production tanks

Sample	Total viable count (cfu g^{-1} or ml^{-1})	(cfu $larva^{-1}$)	Presumptive vibrio count[a] (cfu g^{-1} or ml^{-1})	(cfu $larva^{-1}$)
Untreated tank				
Inactive larvae	7.2×10^8	5.7×10^2	9.9×10^7 (90)	7.8×10^1
Active larvae	6.4×10^6	5.0×10^0	1.3×10^6 (41)	1.0×10^0
Tank water	2.0×10^3		4.0×10^1 (88)	
Streptomycin-treated tank				
Inactive larvae	6.9×10^7	5.4×10^1	1.4×10^7 (75)	1.1×10^1
Active larvae	1.1×10^7	8.4×10^0	1.3×10^5 (39)	0.1×10^0
Tank water	1.2×10^4		1.5×10^1 (50)	
Raw seawater	5.2×10^2		1.0×10^1 (100)	

[a] Values in parentheses are % of sucrose-negative vibrios

Table 4. *Vibrio* species associated with samples from regular production tanks. n = number of isolates picked for species characterization

Species	Untreated tank			Streptomycin-treated tank			Raw seawater
	Inactive larvae (n = 29)	Active larvae (n = 22)	Tank water (n = 10)	Inactive larvae (n = 15)	Active larvae (n = 7)	Tank water (n = 2)	(n = 3)
V. aestuarinus	–	4	–	–	2	–	–
V. alginolyticus	1	–	–	–	1	–	–
V. anguillarum-like	–	–	–	3	–	–	–
V. harveyi	4	2	–	2	1	–	–
V. mediterranei	–	6	–	–	1	–	–
V. pelagius I	–	–	–	–	–	–	1
Vibrio Phenon 14	–	–	1	–	–	1	–
V. splendidus I	3	–	2	2	–	–	–
V. splendidus II	21	10	7	8	2	1	2

Table 5. Quantitative bacteriological analysis of hatchery samples

Sample	Total viable count (cfu ml^{-1} or g^{-1})	Presumptive vibrio count[a] (cfu ml^{-1} or g^{-1})
Breeder	1.9×10^5	1.8×10^5 (60)
Breeder tank water	6.2×10^4	2.5×10^2 (69)
Chaetoceros calcitrans	3.0×10^{10}	$<1.0 \times 10^6$
Pavlova lutheri	1.2×10^{10}	$<1.0 \times 10^6$
Raw seawater	1.3×10^3	3.0×10^1 (83)
Filtered seawater	1.5×10^3	$<1.0 \times 10^1$

[a]Values in parentheses are % of sucrose-negative vibrios

Pathogenicity of *Vibrio* spp.

The pathogenicity of the tested *Vibrio* spp. against oyster larvae is presented in Table 6. Five of the tested strains of *V. splendidus* II (strain nos. 58, 59, 60, Q3 and 102) caused 100% or near 100% mortality at 10^5 cfu ml^{-1} and their LD_{50} values were equal to or less than 5.2×10^4 cfu ml^{-1}. None of the strains of *V. harveyi* caused remarkable mortalities, even at concentrations of 10^7 cfu ml^{-1}, nor did strains of *V. anguillarum*-like and *V. alginolyticus*. Strain-to-strain variations in pathogenicity were observed among *V. splendidus* II, and

Table 6. Pathogenicity of *Vibrio* species against oyster larvae

Strain no.	Species	Source	Coefficient of bacterial density	Larval survival at (10^6	10^5	10^4	10^3 cfu ml^{-1})	Control	LD_{50} (cfu ml^{-1})
31	*V. splendidus* II	Breeder	3.4×	89	83	90	98	99	$>3.4 \times 10^6$
V11	*V. splendidus* II	Active larvae	5.4×	83	100	100	100	100	$>5.4 \times 10^6$
J13	*V. splendidus* II	Seawater	13.5×	100	100	100	99	100	$>1.3 \times 10^7$
43	*V. splendidus* II	Breeder tank	3.1×	96	100	100	100	100	$>3.1 \times 10^6$
72	*V. splendidus* II	Larval tank	5.4×	97	99	100	100	100	$>5.4 \times 10^6$
58	*V. splendidus* II	Inactive larvae	9.2×	0	0	0	76	100	4.2×10^4
59	*V. splendidus* II	Inactive larvae	6.9×	0	1.5	26	96	97	2.9×10^4
60	*V. splendidus* II	Inactive larvae	9.3×	0	0	0	49	100	$<9.3 \times 10^3$
63	*V. splendidus* II	Inactive larvae	3.2×	65	82	96	100	100	$>3.2 \times 10^6$
Q1	*V. splendidus* II	Inactive larvae	1.0×	82	93	94	99	100	$>1.0 \times 10^6$
Q3	*V. splendidus* II	Inactive larvae	10.6×	0	0	21	95	100	4.3×10^4
Q4	*V. splendidus* II	Inactive larvae	2.5×	96	95	98	98	98	$>2.5 \times 10^6$
102	*V. splendidus* II	Inactive larvae	2.2×	0	4	79	96	99	5.2×10^4
105	*V. splendidus* II	Inactive larvae	3.3×	14	59	100	100	100	5.9×10^5
36	*V. harveyi*	Breeder	17.5×	88	98	96	95	99	$>1.8 \times 10^7$
N14	*V. harveyi*	Breeder tank	10.0×	94	98	99	100	100	$>1.0 \times 10^7$
56	*V. harveyi*	Inactive larvae	18.7×	93	92	95	100	100	$>1.9 \times 10^7$
Q6	*V. harveyi*	Inactive larvae	18.2×	72	88	95	100	100	$>1.8 \times 10^7$
76	*V. harveyi*	Larval tank	20.0×	94	97	97	99	99	$>2.0 \times 10^7$
B3	*V. harveyi*	Active larvae	6.9×	100	100	100	100	100	$>6.9 \times 10^6$
62	*V. anguillarum*-like	Inactive larvae	15.7×	100	100	100	100	100	$>1.6 \times 10^7$
O6	*V. anguillarum*-like	Inactive larvae	4.0×	100	100	100	100	100	$>4.0 \times 10^6$
37	*V. alginolyticus*	Breeder	9.1×	92	96	100	100	100	$>9.1 \times 10^6$

Table 7. Drug sensitivity of isolates of *Vibrio splendidus* II. S = sensitive; WS = weakly sensitive; R = resistant

Strain no.	SM	KM	VCM	ABPC	MPIPC	EM	OTC	CEX	CPZ	CEZ	SBT-CPZ	NA	CP	NB	CL
31	S	WS	R	S	WS	S	S	S	S	WS	S	S	S	WS	S
43	WS	S	R	S	R	S	S	S	S	S	S	S	S	S	S
58	S	WS	R	WS	R	S	S	WS	R	S	S	S	S	S	S
59	S	S	R	WS	R	S	S	WS	R	WS	S	S	S	S	S
60	S	S	R	WS	R	S	S	WS	S	WS	WS	S	S	S	S
63	S	S	R	WS	R	WS	S	WS	S	R	S	S	S	WS	WS
72	WS	S	R	WS	R	S	S	WS	WS	WS	S	S	S	S	S
Q1	S	S	R	WS	R	S	S	WS	S	WS	S	S	S	S	S
Q4	S	WS	R	WS	R	S	S	WS	WS	WS	S	S	S	S	S
J13	WS	WS	R	S	WS	S	S	WS	R	S	WS	S	S	S	S
102	S	S	R	WS	R	S	S	WS	S	WS	WS	S	S	S	S
105	S	S	R	S	R	S	S	WS	S	S	S	S	S	S	S
E7	S	WS	R	S	R	S	S	WS	WS	S	WS	S	S	S	S

SM = Streptomycin (50 µg)
KM = Kanamycin (50 µg)
VCM = Vancomycin (5 µg)
ABPC = Ampicillin (30 µg)
MPIPC = Oxycillin (30 µg)
EM = Erythromycin (30 µg)
OTC = Oxytetracycline (30 µg)
CEX = Cephalexin (30 µg)
CPZ = Cefaperazolin (30 µg)
CEZ = Cefazolin (30 µg)
SBT-CPZ = Sulfactum-cefaperazolin (30 µg)
NA = Nalidixic acid (50 µg)
CP = Chloramphenicol (100 µg)
NB = Novobiocin (20 µg)
CL = Colistin (150 µg)

generally isolates from infected larvae were more virulent than those from other sources. Even among the strains of *V. splendidus* II isolated from infected larval samples, LD_{50} for 5- to 8-d-old larvae ranged from less than 9.3×10^3 (strain no. 60) to greater than 2.5×10^6 cfu ml^{-1} (strain no. Q4). Inoculated bacteria could be reisolated from moribund/dead larvae in all experimental infections and larvae maintained in control wells were free of *V. splendidus* II. Signs of the disease in all challenge experiments were similar to those of natural infections with intense bacterial swarming around the shell margin, subsequent loss of cilia and velum followed by death of larvae. In some cases, dead larvae were observed with an intact velum.

Drug sensitivity patterns of the isolates of *Vibrio splendidus* II demonstrated that all the 13 isolates examined were sensitive to the majority of the drugs tested including streptomycin (Table 7). However, the isolates were resistant to vancomycin (peptide group) and oxycillin (penicillin group) and most of them were weakly sensitive to ampicillin (penicillin group) and cephalexin (cephem group).

DISCUSSION

Use of antibiotics for controlling the bacterial numbers in rearing water and improving the survival of bivalve larvae has been reported previously (Walne 1958, Tubiash et al. 1965, Brown & Losee 1978, DiSalvo et al. 1978, Jeffries 1982, Nicolas et al. 1996). In the present study, the effect of streptomycin was clearly demonstrated by an increased larval survival in the antibiotic-treated group in both experimental and regular production tanks. Quantitative and qualitative analyses of bacterial flora associated with *Crassostrea gigas* larvae showed a high incidence of *Vibrio*, which was distinctly higher in diseased (dead/moribund) larvae reared without streptomycin treatment. Although these results gave a strong indication of the involvement of *Vibrio* in the disease, the presence of several species of *Vibrio* made it difficult to incriminate a particular causative agent in the recurrent outbreaks.

Pathogenicity assays conducted with different *Vibrio* isolates indicated *V. splendidus* biovar II as the causative agent of the disease in *Crassostrea gigas* larvae. *V. alginolyticus*, although reported to be pathogenic to oyster larvae (Tubiash et al. 1965, Jeffries 1982), did not produce larval mortalities in the present study, perhaps due to strain-to-strain variation. Interestingly, not all tested strains of *V. splendidus* II were virulent and most virulent strains were isolated from diseased larvae. Strains from sources other than diseased larvae were either less virulent or avirulent. Further detailed investigations are required to establish any clear relationship between the virulence of the cultures and their source of isolation. Virulent strains of *V. splendidus* II were pathogenic to *C. gigas* larvae at concentrations of 10^4 cfu ml^{-1} in rearing water and at higher concentrations the mortality rate was more drastic. These high rates of mortality could also be due to the high water temperature (25°C) maintained in all infection experiments, which favours rapid multiplication of bacteria, as observed by Riquelme et al. (1995a) in

infection experiments with scallop larvae. The virulent isolates of *V. splendidus* II in this study were equally pathogenic as those of *V. tubiashii*, which is reported to be a primary pathogen on oyster larvae (Jeffries 1982), and as that of an unidentified *Vibrio* sp. against cockle *Fulvia mutica* larvae (Fujiwara et al. 1993).

The progressive signs of the disease included an abnormal circular pattern of swimming of larvae, bacterial swarming around the velum and shell margin, detachment of cilia and velum and eventual death of larvae similar to earlier descriptions (Tubiash et al. 1965, Brown & Losee 1978, Elston & Leibovitz 1980, Jeffries 1982, Lodeiros et al. 1987). Therefore, it is concluded that this disease was another case of bacillary necrosis caused by *Vibrio splendidus* biovar II. In all previous cases of bacillary necrosis, sucrose-positive vibrios have been reported to be involved, and Jeffries (1982) suggested that controlling the concentration of sucrose-fermenting vibrios (yellow colonies on TCBS) below 100 ml^{-1} in rearing water could minimize losses attributable to vibriosis. However, the case reported here is quite different and sucrose-negative vibrios were dominant in all infected larval samples and in most other samples. Involvement of *V. splendidus* as an opportunistic pathogen in scallop larvae has recently been considered (Nicolas et al. 1996). However, the species was not detected in all outbreaks of disease and an unidentified *Vibrio* species was concluded to be the major pathogen of scallop larvae.

Sources of possible bacterial infections of bivalve larval cultures in hatcheries are generally the broodstock, algal feed and raw seawater (Elston 1984). During the present investigations, vibrios were not detected from algal cultures fed to both larvae and broodstock, making algal feed unlikely to be the source of infection. Strains of *Vibrio splendidus* II were detected in raw seawater as well as in broodstock. Detection of *V. splendidus* II strains in the gonad of adult oysters suggests the probability that broodstock could be the source and route of transmission of this pathogen, as already pointed out for *Ostrea edulis* (Lodeiros et al. 1987) and *Argopecten purpuratus* (Riquelme et al. 1995b). In the hatchery, unlike the larvae which are reared in 1 µm-filtered seawater, the broodstock are maintained and conditioned only in sand-filtered seawater before spawing. Therefore, there is a possibility that the strains of *V. splendidus* II entering the system from raw seawater could build up along with normal flora in adult oysters. When passed on to larvae, these strains might proliferate in stressed individuals and eventually infect healthy individuals, thus bringing about mass mortality in crowded culture systems. When treated with streptomycin, this pathogen is under control and mortality rates are low. The mechanism of infection may be a combination of invasive and toxic pathways as suggested in earlier studies (Elston & Leibovitz 1980, Lodeiros et al. 1987, Nicolas et al. 1996). The involvement of toxins in the pathogenicity of *V. splendidus* II, although implicated in our preliminary studies, requires further investigation.

At present, there is no evidence of the emergence of streptomycin-resistant strains among the *Vibrio splendidus* II isolated in this study, although streptomycin has been used experimentally in the hatchery since 1995, and this treatment has been effective in preventing disease. Development of alternative methods to control the disease becomes imperative as prolonged use of antibiotics might result in the selection of antibiotic-resistant bacteria. Biological control of this pathogen and/or other effective means of preventing the build-up of *Vibrio* spp. by better management practices are necessary in the hatchery system.

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Editorial responsibility: Albert Sparks, Seattle, Washington, USA

Submitted: December 20, 1997; Accepted: March 21, 1998
Proofs received from author(s): June 10, 1998