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Pathogenicity studies on a *Vibrio anguillarum*-related (VAR) strain causing an epizootic in *Argopecten purpuratus* larvae cultured in Chile

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ABSTRACT: A *Vibrio anguillarum*-related (VAR) strain, isolated in pure culture from an epizootic in a commercial hatchery producing *Argopecten purpuratus*, was characterized, and its potential pathogenicity to veliger larvae of *A. purpuratus* determined. Experimental challenges indicated that the bacterium affects larval survival at concentrations of 10^4 to 10^8 cells ml^{-1} The effect of water quality and temperature on pathogenicity was also evaluated. Larval survival in seawater filtered through 5 µm pore-size membranes was 45.6%, whereas using seawater passed through 1 and 0.2 µm filters, larval survival increased to 66.4 and 80.4% respectively. Temperature also affected pathogenicity as larval survival at 15°C for 24 h was 69.3% but decreased to 30 and 26.9% at 20 and 25°C, respectively. Toxic activity was found in cell-free supernatant of bacterial culture. Larval survival was reduced to 68.9 and 36.4% after 20 and 40% (v/v) of supernatant, respectively, was added to the rearing water. These results suggest that exotoxins produced by the VAR strain play an important role in its pathogenicity for scallop larvae.

KEY WORDS: *Argopecten purpuratus* · Larvae · *Vibrio anguillarum*-related (VAR) · Pathogenicity · Exotoxins

INTRODUCTION

Great importance is attributed to vibrios as pathogens of bivalves because of their association with high mortalities of larval cultures with consequent economic losses to the hatcheries (DiSalvo et al. 1978, Elston & Leibovitz 1980, Jeffries 1982, Brown 1983, Nottage & Birkbeck 1986, 1990, Lodeiros et al. 1987, Bourne et al. 1989, Navarro et al. 1991). Thus, there is an urgent need to develop methods for controlling sudden outbreaks of vibriosis in commercial hatcheries.

The occurrence of epizootics caused by vibrios in bulk bivalve cultures may be related to their high concentration in the environment, since low concentrations of vibrios do not necessarily imply a risk for larval culture (Jeffries 1982). Sudden proliferation of members of the Vibrionaceae associated with severe larval mortalities generally occurs in summer when organic matter increases in the sea and the seawater temperature exceeds 18°C (Sinderman 1990). In view of this, parameters such as the concentration of organic matter and temperature should be monitored during larval culture since they could be important contributing factors in the proliferation of potential pathogenic vibrios in a culture system.

Although vibriosis caused by *Vibrio anguillarum* and *V. tubiashii* has been considered the most important bacterial infection limiting the production of marine fish and shellfish throughout the world (see review by Toranzo & Barja 1990), other vibrios exist in the environment that are taxonomically and serologically related to these 2 species (Bryant et al. 1986, Fouz et al. 1990). These other variants have been traditionally considered to be of no pathogenic significance, but have been also associated with disease in larval and adult stages of marine fish (Masumura et al. 1989, Fouz et al. 1990, Toranzo et al. 1990, 1993, Myhr et al. 1991, Toranzo & Barja 1993) and shellfish (Baticados et al.

1990, Lavilla-Pitogo et al. 1990, Paillard & Maes 1990, Castro et al. 1992, Fujiwara et al. 1993). These vibrios correspond mainly to different biotypes of *V. splendidus* and *V. pelagius* species and can be grouped under the designation '*V. anguillarum*-like' or *V. anguillarum*-related (VAR) organisms (Larsen 1985, Fouz et al. 1990, Toranzo & Barja 1990, Myhr et al. 1991, Pazos et al. 1993).

The pathogenicity of some vibrios isolated from molluscs (oysters) has been assumed to be due to their invasive capacity and/or their ability to produce exotoxin(s) (DiSalvo et al. 1978, Elston & Leibovitz 1980, Elston et al. 1981, Birkbeck et al. 1987).

The aim of the current study was to evaluate the effect of temperature and water quality on the pathogenic capacity of a VAR strain isolated in pure culture from an epizootic in a commercial hatchery for *Argopecten purpuratus* (Lamarck, 1819). In addition, the role of exotoxins produced by this *Vibrio* strain on larval survival was investigated.

MATERIALS AND METHODS

Bacterial isolation. In a hatchery located in northern Chile (27° 5' 42" S, 69° 51' 48" W) massive larval mortalities occur in spring-summer. On one of these occasions, culture tanks with more than 90% of larvae on the bottom were sampled. Swimming and bottom larvae were netted and washed with sterile seawater, then homogenized using a tissue grinder and spread on TCBS (thiosulfate-citrate-bile sucrose agar) (Oxoid) and marine general medium ST10 (Ishida et al. 1986). In addition, the bacterial flora of the hatchery water supply was determined: unfiltered, seawater in sedimentation tanks, filtered and UV treated seawater, and water from larval culture ponds. The bacterial flora of microalgal cultures (stock and production batches) used as larval food was also determined. All samples were spread in triplicate on TCBS and ST10 media and incubated at 20°C for 48 h on TCBS and for 7 d on ST10. Additionally, broodstock gonads were extracted and washed externally with 1% benzalkonium chloride. A small incision was made through the surface of the gonads with a heat sterilized scalpel. Gonad contents were removed with sterile Pasteur pipettes, then homogenized and spread on TCBS medium.

Biochemical and serological characterization. Pure cultures of the bacteria isolated from moribund larvae were subjected to standard morphological, physiological and biochemical tube and plate tests according to the procedures of West & Colwell (1984), Bryant et al. (1986), Fouz et al. (1990) and Hansen & Sorheim (1991). Plates and tubes were incubated at 22°C for up to 7 d. In addition, the commercial miniaturized API-20E system (Analytab) was employed using half strength seawater as diluent, and the results were scored after 48 h at 22°C.

The taxonomic position of the *Vibrio* strain recovered in pure culture from all diseased larvae was determined following the criteria of West & Colwell (1984), Bergey's Manual of Systematic Bacteriology (1984, 1986), Bryant et al. (1986), Myhr et al. (1991) and Austin & Lee (1992).

Drug resistance patterns were determined by the disc diffusion method (Barry & Thornsberry 1991) on Mueller-Hinton agar (bioMerieux) supplemented with 1.5% NaCl, using the following antimicrobial agents (μg $disc^{-1}$): ampicillin (10), streptomycin (10), chloramphenicol (30), tetracycline (30), oxytetracycline (30), erythromycin (15), kanamycin (30), oxolinic acid (2), Furazolidone (300), novobiocin (5) and trimethoprim-sulphamethoxazole (23.75-1.25). The vibriostatic agent O/129 (150) was employed only for taxonomic purposes.

Serological analysis was conducted by the slide-agglutination test as described by Sørensen & Larsen (1986) and Toranzo et al. (1987), using the thermostable bacterial 'O' antigens and rabbit antisera raised against the 10 'O' serotypes (from O1 to O10) of *Vibrio anguillarum* (Sørensen & Larsen 1986), *V. tubiashii* EX1 (Lodeiros et al. 1987) and *V. splendidus* biovar I ATCC 25914.

Pathogenicity studies. Virulence assays were performed to determine the pathogenicity of the *Vibrio* strain isolated as pure culture from moribund larvae. Healthy larvae of *Argopecten purpuratus* obtained from the hatchery were used for these assays.

Pathogenicity of the isolated strain was determined applying the following variables: (1) bacterial concentration, (2) water quality and (3) temperature. The assays were performed in triplicate according to a modification of methodology described by Brown (1983). *Argopecten purpuratus* larvae were added (at 2 larvae ml^{-1}) to sterile seawater filtered through 0.2 μm membranes (Millipore) contained in cell culture chambers of 15 ml capacity.

(1) Effect of bacterial concentration: Bacteria cultured in ST10 broth were washed by centrifugation (3840 × *g* for 15 min) and suspended in Marine Saline Solution (MSS) (Austin 1988). Bacterial cells in this suspension were stained with 1 μg ml^{-1} of DAPI (4',6-diamidino-2-phenylindole) (Porter & Feig 1980) and counted under epifluorescence microscope (Austin 1988). Dilutions of the cell suspension were then added to the larvae. Final concentrations of *Vibrio* used were ca 10^4, 10^5, 10^6, 10^7 and 10^8 cells ml^{-1}. Chambers without addition of bacteria were used as controls. These bioassays were carried out at 20°C, and larval survival at 19 and 24 h was recorded.

(2) Effect of water quality: *Argopecten purpuratus* larvae were suspended in seawater filtered through different pore-size membranes: (a) 5 µm (Nytal), (b) 1.2 µm (Millipore) and (c) 0.2 µm (Millipore). A final concentration of bacteria 10^6 cells ml^{-1} was added to each chamber. Controls without bacteria were used for each treatment. Larval survival was recorded at 10, 19 and 24 h.

(3) Effect of temperature: To evaluate the effect of temperature on the pathogenicity of this strain, a bacterial concentration of 10^6 cells ml^{-1} was added to each chamber containing larvae in filtered (0.2 µm) seawater. The temperatures used for bioassays were 15, 20 and 25°C. Controls without bacteria were used for each treatment. Larval survival was recorded at 10, 19 and 24 h.

Effect of cell-free supernatant of bacterial culture on larval survival. Bacterial cultures in ST10 were centrifuged for 15 min at 3840 × *g*. The supernatant was filtered through 0.2 µm membranes (Millipore), and added at 20 and 40% v/v to each chamber containing larvae in filtered (0.2 µm) seawater. Chambers without filtered supernatant and with 10^5 and 10^6 viable cells ml^{-1} of bacteria were used as controls. Larvae were maintained in these conditions at 20°C and survival recorded after 24 h.

Record of larval survival. In all the experimental trials, larval survival was quantified by means of observation of the organisms under a microscope. Larvae on the bottom of chambers that showed no apparent movement, closed valves and no velar activity were considered dead.

The presence of bacteria on moribund larvae was verified by epifluorescence microscopy using DAPI staining.

All assays were repeated at least twice. Results were analyzed using the statistical *G* test (Zar 1984).

Table 1 Vibrios and total culturable bacteria (CFU ml^{-1}) in different samples analyzed in the hatchery

Samples	Vibrio (TCBS)	Total bacteria (ST10)
Entrance seawater	2.0×10^2	9.3×10^3
Sedimentation tanks	1.0×10^2	4.9×10^3
Filtered water treated with UV	$<10^a$	7.0×10^2
Water from larval tanks	1.2×10^4	1.3×10^4
Microalgae	$<10^a$	nd
Breeders	$2.4 \times 10^{3\ b}$	nd

[a]Below detection limit of the spread-plate method
[b]CFU g^{-1} of gonad tissues
nd: not determined

RESULTS

Results of bacteriological analysis of samples in the hatchery are presented in Table 1. The filtered and UV-treated water entering larval culture tanks contained a low bacterial concentration and undetectable number of *Vibrio* spp. However, after the filtration and UV treatment, the *Vibrio* populations increased in the culture tanks, reaching values of 92.3% of the total culturable bacteria. This increase is very high compared to the 2.2% composition of vibrios present in the intake seawater. In microalgal cultures used as food for the larvae, *Vibrio* strains were also not detected.

Bacteriological analysis of moribund larvae revealed the presence of a *Vibrio* strain in pure culture, which was identified as *V. anguillarum* related (VAR) (Table 2). This isolate shared characteristics with *V. anguillarum*, *V. tubiashii* and *V. splendidus* biovar I. However, serological tests indicated that this isolate did not share antigens with the reference strains of these 3 species. The bacterium was resistant to several antimicrobial agents (Table 2).

The bioassays on the pathogenicity of the VAR to larvae showed that a concentration of 10^6 cells ml^{-1} was necessary to produce a decrease in larval survival (Table 3). The highest mortality (0% larval survival) was recorded at 24 h with a concentration of 10^8 cells ml^{-1} ($p < 0.05$). However, with a bacterial concentration of 10^4 cells ml^{-1} survival was 94% at 24 h, not significantly different from the control ($p < 0.05$). The number of colony-forming units (CFU) in the initial bacterial inoculum was determined in selective medium (TCBS) and the number of culturable *Vibrio* strain corresponded to ca 0.1% of the total cells.

The results of different treatments of water filtration indicated that in seawater filtered through 5 µm pore-size membranes, larval survival decreased to 67.3% in 10 h and 45.6% in 24 h (Fig. 1A). However, using water filtered through 1 µm and 0.2 µm pore-sizes, larval survival of 66.4 and 80.4% respectively was reached at 24 h. Larval survival in these 3 treatments of seawater to which the VAR was added was significantly lower ($p < 0.05$) than in the respective controls (Fig. 1B).

The pathogenicity of the VAR to larvae of *Argopecten purpuratus* was affected by culture temperature (Fig. 2). At 25°C the pathogenic effect was considerable, because larval survival was reduced to 36.8 and 26.9% at 10 and 24 h respectively (Fig. 2A). In contrast, in the controls (without bacteria added) survival of larvae greater than 70% was observed at both times (Fig. 2B). At 20°C, larval survival of 70.4 and 30% at 10 and 24 h respectively was observed, both of which were lower ($p < 0.05$) than controls (Fig. 2B).

In the presence of the VAR strain, best larval survival ($p < 0.05$) was found at 15°C, reaching 81.9 and 69.3%

Table 2. Identification of *Vibrio anguillarum* (VAR) strain

Characteristic	Response
Gram stain	–
Motility	+
Oxidase	+
Catalase	+
Voges-Proskauer	–
Indole production	+
Citrate utilization	–
H_2S production	–
O/F (glucose)	F
Gas from glucose	–
Growth at 5°C	–
15°C	+
25°C	+
37°C	–
42°C	–
Growth in 0% NaCl	–
3% NaCl	+
5% NaCl	+
8% NaCl	–
10% NaCl	–
Growth on TCBS	+yellow
Arginine dihydrolase	+
Lysine decarboxylase	–
Ornithine decarboxylase	–
Lipase (Tween 80)	+
β-Galactosidase (ONPG)	+
Urease	–
Gelatinase	+
Amylase	+
Haemolysis (sheep blood)	+
Acid production from:	
Glucose	+
Mannose	+
Galactose	+
Fructose	+
Sucrose	+
Rhamnose	–
Arabinose	–
Amygdaline	+
Melibiose	–
Mannitol	+
Inositol	–
Sorbitol	–
Sensitivity/resistance to:	
O/129	S
Novobiocin	R
Ampicillin	R
Chloramphenicol	I
Tetracycline	R
Oxytetracycline	R
Streptomycin	I
Erythromycin	R
Kanamycin	I
Oxolinic acid	R
Furazolidone	S
Trimethoprim-sulphamethoxazole	R

F: fermentative strain; R: resistant; S: sensitive; I: intermediate

Table 3. *Argopecten purpuratus*. Effect of different concentrations of *Vibrio anguillarum* (VAR) on larval survival

Bacterial concentration added (cells ml^{-1})	Larval survival (%) at 19 h	24 h
10^4	98	95
10^5	98	87
10^6	76	70
10^7	11	3
10^8	0	0
Control (no *Vibrio* added)	100	100

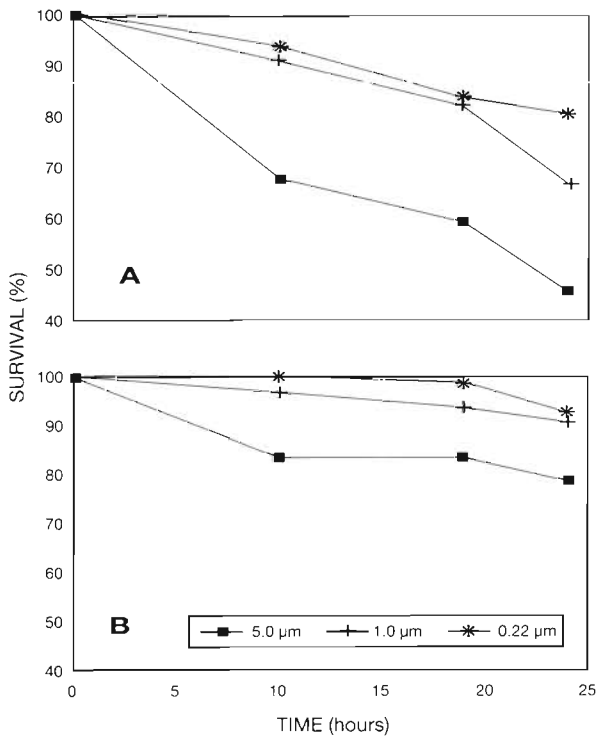

Fig. 1. *Argopecten purpuratus*. Effect of differential filtration of seawater on larval survival. (A) Larvae with addition of VAR strain; (B) control

at 10 and 24 h, respectively (Fig. 2A). However, at this temperature larval survival in the control was significantly lower than in the tanks containing the *Vibrio* strain (Fig. 2B).

The addition of cell-free supernatant of *Vibrio* cultures to tanks of larvae showed a clear lethal effect causing a decrease of larval survival to 68.9 and 36.4% after 24 h following addition of 20 and 40% v/v, respectively (Fig. 3). These values were significantly lower than those obtained in controls as well as in tanks containing 10^5 viable cells ml^{-1} Colonization of bacteria was observed on moribund larvae exposed to the VAR strain for 24 h (Fig. 4).

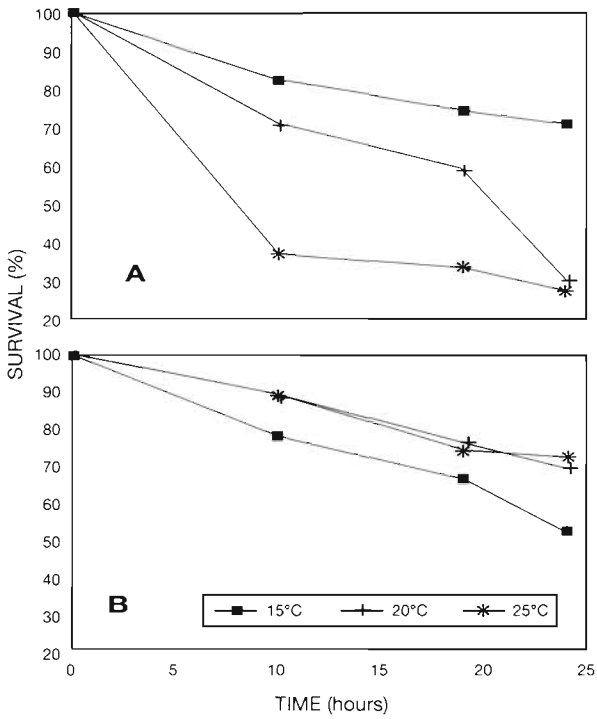

Fig. 2. *Argopecten purpuratus*. Effect of temperature on larval survival. (A) Larvae with addition of VAR strain; (B) control

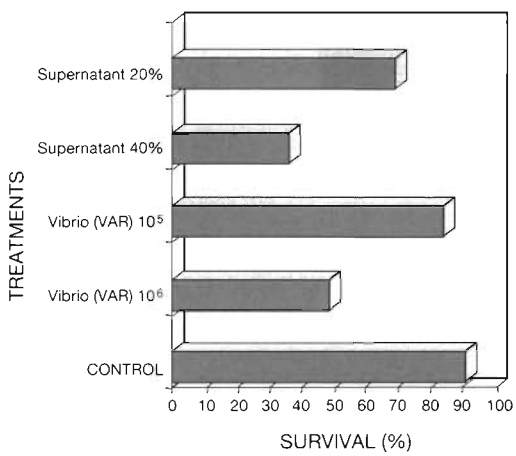

Fig. 3. *Argopecten purpuratus*. Survival of larvae exposed to: 10^5 and 10^6 cells ml^{-1} of vibrios (VAR) and 20% and 40% (v/v) of culture supernatant of this strain

DISCUSSION

The results of the survey in the hatchery suggest that the probable incorporation route of vibrios to the system could be the broodstock which contained a significant load of vibrios (2.4×10^3 CFU g^{-1}) in their reproductive organs. Similar findings of vertical transmission of *Vibrio* strains have been reported previously for *Ostrea edulis* (Lodeiros et al. 1987) and *Argopecten purpuratus* (Riquelme et al. 1994).

The microbiological analysis allowed us to identify a VAR strain as responsible for the larval mortalities. In agreement with previous reports (Lodeiros et al. 1987, Myhr et al. 1991, Castro et al. 1992, Pazos et al. 1993) this strain is taxonomically related to *Vibrio anguillarum*, *V. tubiashi*, and *V. splendidus*. Until now, only 2 occurrences of pathogenic *V. anguillarum* and related strains (VAR) have been reported as responsible for mortalities of mollusc larvae in Chile. *V. anguillarum* has been recovered from *Mytilus chilensis* (Vial et al. 1988), and VAR organisms were isolated from *Argopecten purpuratus* and *Concholepas concholepes* culture (Pazos et al. 1993). The multiresistance to antimicrobial agents of *V. anguillarum* isolated is remarkable (Table 2), and may be attributed to the frequent use of drugs in larval culture in the hatchery. The assays of pathogenicity conducted with this *V. anguillarum* indicated that a concentration of 10^6 cells ml^{-1} is necessary to cause a pathogenic effect in *A. purpuratus* larvae.

The lower larval survival observed in bioassays with seawater filtered through 5 µm membranes can be attributed to the increase of this bacterium because of the copiotrophic characteristic of vibrios (West & Colwell 1984) and by the highest availability of particles and nutrients in this filtered seawater.

The increase of temperature affected the pathogenicity of this *Vibrio* strain, with 69.3% larval survival at 15°C in 24 h, but 30 and 26.9% respectively at 20 and 25°C. These findings may be attributed to the higher temperatures favouring bacterial proliferation. In fact, a typical feature of the majority of *Vibrio* species is their improved growth at temperatures above 15°C (West & Colwell 1984). Moreover, at 25°C the heat stress could adversely affect the larvae and predispose them to *Vibrio* attack. In rearing scallop larvae, temperatures between 18 and 24°C are used (Navarro et al. 1991).

In control cultures larval survival was lower at 15°C (Fig. 2B). This could be explained by the fact that 15°C is below the optimal temperature for the development of *Argopecten purpuratus* larvae. However in 15°C cultures inoculated with the VAR strain, larval survival was higher than in controls. Apparently the VAR strain is less virulent at 15°C, and also partially satisfies some nutritional requirements for larval survival. This point requires further investigation.

The overall results for the effects of water quality and temperature on the pathogenicity of the VAR strain in *Argopecten purpuratus* larvae indicate that both parameters are of great importance for controls against the increase of Vibrionaceae in larval cultures.

The result that the cell-free supernatant of *Vibrio* cultures decreased the larval survival suggest that a probable exotoxin(s) produced by this strain play a role

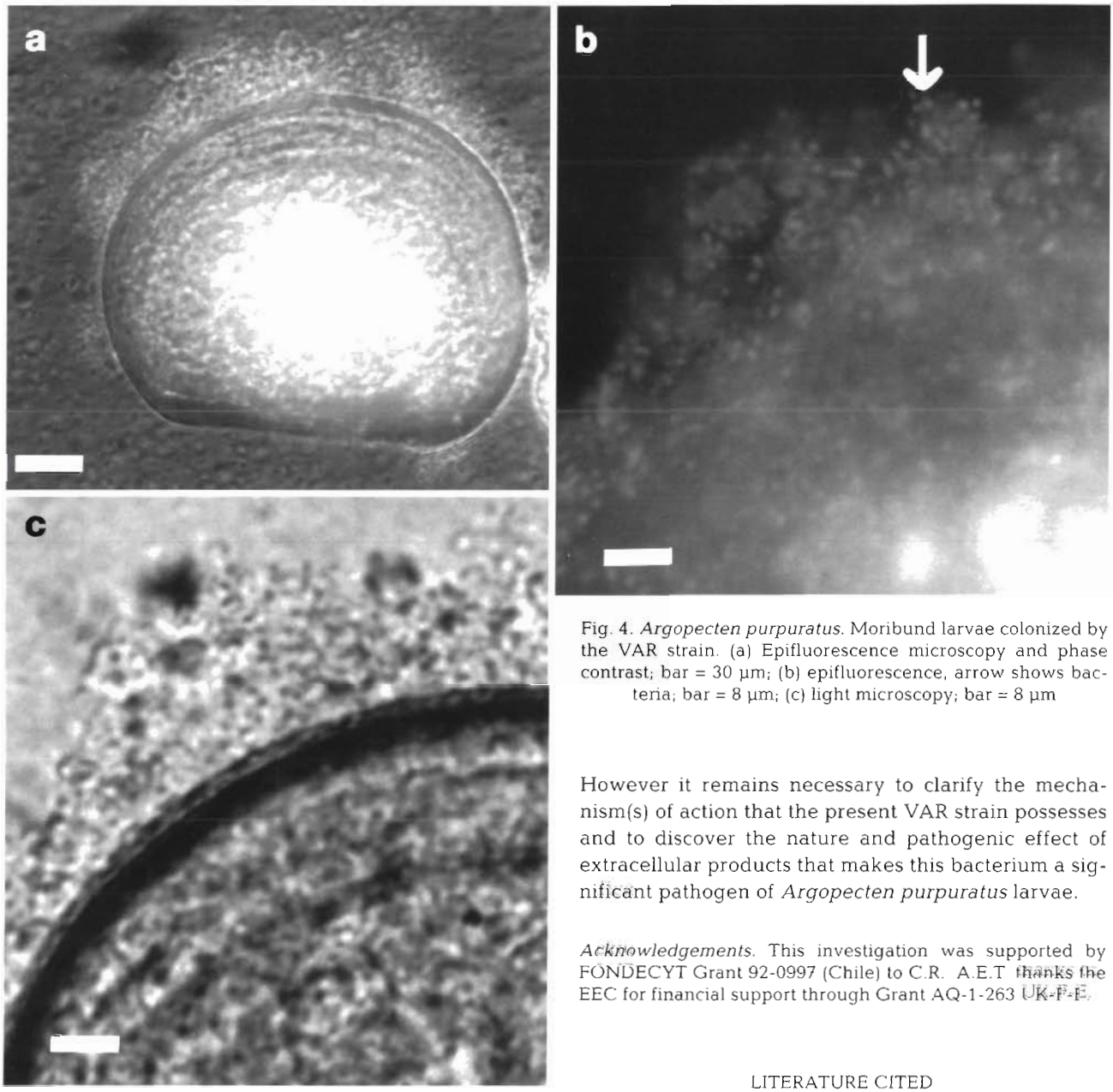

Fig. 4. *Argopecten purpuratus*. Moribund larvae colonized by the VAR strain. (a) Epifluorescence microscopy and phase contrast; bar = 30 µm; (b) epifluorescence, arrow shows bacteria; bar = 8 µm; (c) light microscopy; bar = 8 µm

in its pathogenicity. A review of the literature indicates that some species of *Vibrio* produce toxins that adversely affect mollusc larvae (Brown & Roland 1984, Nottage & Birkbeck 1986, 1987).

Epifluorescence microscopy revealed the presence of bacteria colonizing the moribund larvae (Fig. 4). The possibility of direct invasion by the bacterium cannot be ruled out, but needs verification.

Pathogenicity of a bacteria is usually determined by the capacity to release a toxin(s), attack the host or both (Elston & Leibovitz 1980, Brown & Roland 1984, Nottage & Birkbeck 1986, 1987, Birkbeck et al. 1987).

However it remains necessary to clarify the mechanism(s) of action that the present VAR strain possesses and to discover the nature and pathogenic effect of extracellular products that makes this bacterium a significant pathogen of *Argopecten purpuratus* larvae.

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