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Evidence of parental bacterial transfer to larvae in *Argopecten purpuratus* (Lamarck, 1819)

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Argopecten purpuratus *is one of the most valuable molluscs in aquaculture activities in Chile. However, there are several problems for mass production of seeds under controlled condition. One of these is bacterial infection in early larval stages. In this study, breeders were analyzed as possible route for bacterial entrance to cultures. Bacteria associated with gametes were found, with predominance of genus* ***Pseudomonas****. Also the genera* ***Vibrio****,* ***Chromobacterium****,* ***Flavobacterium*** *and* ***Moraxella*** *were detected. In spawning induced under sterile condition, the transfer of bacteria from parents to early larval veliger stage was determined. Although bacterial concentration in gametes was low (O.24 CFU/ Ovum), bacteria show a clear tendency to increase in number at the veliger stage. Among the transferred bacteria, the presence of* ***Vibrio anguillarum****- related (VAR) bacteria as potential pathogen for marine bivalves is remarkable.*

Key words: ***Argopecten purpuratus****, bacterial transfer, bivalve mollusc, larval mortality,* ***Pseudomonas.***

INTRODUCTION

Chilean hatcheries of *Argopecten purpuratus* have several problems for sustained production. This is mainly due to a high larval mortality, which has been attributed to pathogen microorganisms (Disalvo, 1990; Navarro *et al*, 1991). For this reason, the urgent need of developing methods for controlling the larval mortalities in commercial hatcheries has acquired a great importance. In other bivalves of economical value, such as the oyster, having a culture technology clearly established,there still remain problems with production, because of bacterial infections in larval stages (Nottage and Birkbeck, 1986). Within the main routes of bacterial contamination to larval culture, such as incoming seawater, broodstock and microalgal food, breeders could be one important route of pathogens input to the culture by introducing gametes contaminated with bacteria. Elston (1989) stresses the difficulties encountered in avoiding infections of the cultures if the breeders are contaminated. In Chile, breeders of *A. purpuratus* contaminated specifically with vibrios have been detected in several bays (Chavez, 1991; Disalvo, personal communication).

The purpose of the present work was to determine bacterial contamination in gametes of *A. purpuratus* and to find out whether such bacteria are transmitted to larval stages.

MATERIALS AND METHODS

Qualitative determination of bacteria in gametes

In the present study a total of fifty breeders of *A. purpuratus*, obtained from natural beds of

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Northern Chile (23° 39' S) were analyzed. The specimens were selected considering mature and health conditions. Organisms in good and bad conditions were discriminated following indications described by Disalvo (1990) to determine anomalous breeders; these are: gonad fading, inflammation of kidneys, loss of tenderness of gill filaments, presence of pigments in gills, and mucopurulent faeces. The 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. Female and male contents of gonads were taken with a capillary and filtrated in 44 μm and 5 μm mesh for eggs and sperms, respectively.

The gametes were suspended in sterile marine saline solution (Austin, 1988) and homogenized in a Stomacher 80, in order to release the bacterial cells attached to the gametes and to assure an even distribution of the sample in marine general medium ST10 (Ishida *et al*, 1986). After one week incubation, the dominant strains and also the different colony types were isolated and characterized according to Muroga *et al* (1987) and Bergey's Manual of Systematic Bacteriology (Holt, 1984). For the identification of *Vibrio* strains the criteria of West and Colwell (1984) were also applied. Biochemical identification tests were carried out according to Hansen and Sorheim (1991). Additionally, the multitest system API 20E (Analytab) was used.

Determination of vertical bacterial transmission

Eight breeders of *A. purpuratus* obtained from natural beds were used. These were washed externally *in situ* and transported immediately to laboratory. After they were brushed and washed several times with filtered seawater (0.22 μm, Calix cartridge), the organisms were then kept for a few hours with circulating filtered seawater. The spawning was induced according the method described by Padilla (1979). The spawning organisms were put in sterile beakers of 2 litres with filtered seawater (0.22 μm Millipore). Since female and male spawnings are not simultaneous, both types of gametes may be obtained separately. Fertilization was made in one litre beaker with sterile seawater and maintained at 20° C. Control cultures of sterile seawater without gametes addition was maintained and analyzed in similar conditions as larval cultures.

Samples of gametes, post-fertilization stages and larvae were filtrated in sterile sieves with the proper pore size, and analyzed for determining heterotrophic culturable bacteria, using a similar procedure as that previously described for gametes.

Microscopic observations were carried out in samples of nonhomogenized gametes to detect associated bacteria. Gametes were fixed in 1% sterile formalin. These were stained with 1 μg/ml of fluorochromo 4'6-diamidino-2 phenylindol (DAPI) and observed in a epifluorescence microscope (Olympus BH2-RFCA), according to the method described by Longo and Scarpa (1991). Photomicrographs were taken with 40 and 100x objectives using Kodak film.

RESULTS

Results of bacteriological analyses of breeders obtained from natural beds are summarized in Table I. Culturable heterotrophic bacteria were found in both gonads portions, female and male, throughout the sampling period. The gonads of *A. purpuratus* were dominated by members of the genera *Vibrio, Pseudomonas, Chromobacterium, Flavobacterium* and *Moraxella.* Within the Vibrios strains, *V. anguillarum* occurred in September and October, while *V. alginolyticus* was present in January.

The presence of bacterial adherence to female gametes was detected through observation under epifluorescence microscopy (Fig 1). The males gametes showed scarce bacterial adherence (Fig 2). The presence of bacteria was not detected in control cultures.

In spawning induced under sterile conditions, a concentration of culturable bacteria in gametes of 0.24 CFU/ovum and 3.3×10^{-5} CFU/sperm was found. In veliger larvae a bacterial concentration of 2.9×10^{2} CFU/larvae was observed.

The qualitative changes of bacterial composition in gametes, post-fertilization and D-shape larval stages are shown in Figure 3. Isolates could be identified up to the genus

TABLE I

Dominant bacteria in gonads of *Argopecten purpuratus* breeders

Months	Aug	Sept	Oct	Nov	Jan
Sex	M F	M F	M F	M F	M F
Vibrio spp.	- -	+ +	+ +	- -	+ +
Pseudomonas					
Groups I - II	- +	- +	+ +	+ +	+ -
Groups III - IV	- -	- +	+ +	- -	+ -
Chromobacterium spp.	+ -	- -	- -	+ +	- -
Flavobacterium spp.	- -	+ +	- +	+ -	- -
Moraxella spp.	+ -	+ +	+ +	+ +	- -

M, males F, females
+, presence -, absence

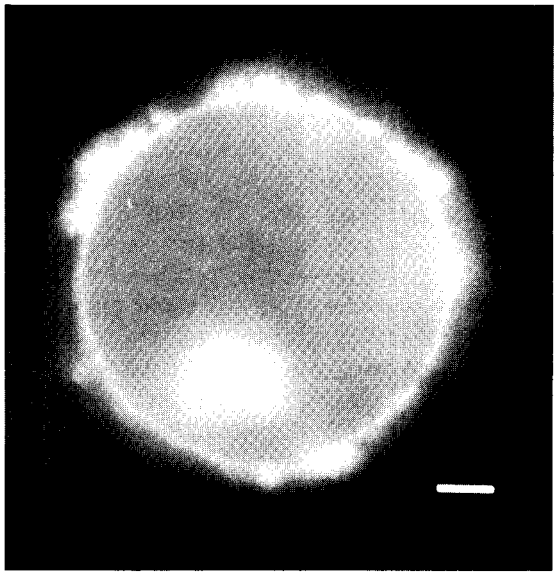

Fig 1. Ovum of *A. purpuratus* with bacterial adherence. Bar, 8 µm.

level and in some cases at the species level. The dominant bacteria in gametes and post-fertilization stages were *Pseudomonas* group I-II and III-IV, according Muroga *et al* (1987). At the veliger stage, several bacteria were observed, *Chromobacterium*, *Pseudomonas* I-II and III-IV, and *Vibrio*. Among vibrios strains, the occurrence of *Vibrio anguillarum*-related (VAR) bacteria was detected.

DISCUSSION

Among the bacterial genera found in both gonads portions of breeders, the presence of *Pseudomonas* and *Vibrio* was notorious. Very limited information exists about the microflora present in reproductive organs or gametes of bivalves. *Pseudomonas* and *Vibrio* had been reported causing mortality in embryos and larvae of oyster (Brown, 1983). Both species of *Vibrio* found in gonads; *V. anguillarum* and *V. alginolyticus* are known by their pathogenicity for larvae of marine organisms (Nottage and Birkbeck, 1987; Sinderman, 1988). The incorporation of these pathogenic Vibrios into the gonads could be made through the intestine, because this organ crosses the two sections, female and male gonads in *A. purpuratus*. It has been found that *V. anguillarum* is able to colonize all regions of the intestine in fish (Horne and Baxendale, 1983). This is suggested as infections mechanism. In *A. purpuratus*, this invasive mechanism through intestine may be important, due to bioaccumulative properties of filters-feeders organisms as bivalves. The vibrios fluctuations in gonads of breeders obtained from natural environments could be explained by the change of vibrios in the water column. It is known that vibrios concentration is influenced by the phytoplankton succession in sea waters (Riquelme *et al*, 1988). *Moraxella*, *Flavobacterium* and *Chromobacterium* were also found as dominant bacteria in gonads. There is no information in the literature about the role, or effects of these genera on marine bivalves. *Moraxella* and *Flavobacterium* have been found associated with other organisms, such as fish, particularly attached to eggs of cod (*Gadus morhua*) and halibut (*Hippoglossus hippoglossus*) (Hansen and Olafsen 1989). Recently, Birkbeck and Gallacher (1993) reported that one strain of *Moraxella* produces the ciliostatic exotoxin, precluding swimming of bivalve larvae and, consequently, larval death. The ciliostatic toxin is a determinant virulence of bivalve pathogenic vibrios and it was found to be produced primarily by *V. alginolyticus*.

Bacteria associated with the surface of gametes were observed (Figs. 1 and 2). This is the first report of bacterial observation in bivalve gametes. Prieur *et al* (1990) observed gametes of *Mytilus edulis*, and they did not find bacteria associated with female and male gametes. However, in fish there are several reports of colonization of eggs and also intra-ovum infection (Barker *et al*, 1989; Hansen *et*

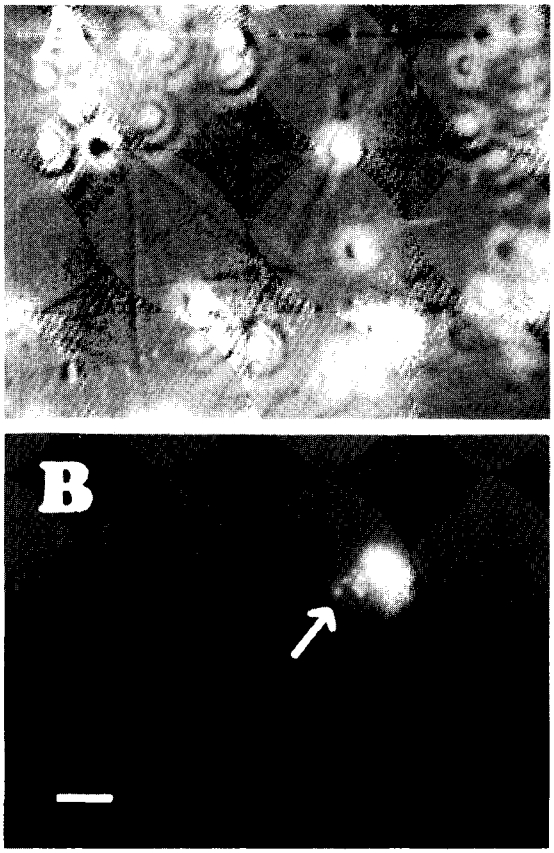

Fig 2. Photomicrographs of sperms of *A. purpuratus* in epifluorescence microscopy with DAPI staining. A. Sample obtained directly from gonad; observed through phase contrast-epifluorescence microscopy. B. Gametes obtained in spawning carried out in sterile conditions. Arrow, bacteria adhered to sperms. Bar, 4.5 μm.

al, 1992; Hansen, 1993). Furthermore, bacteria attached to ova, dissected from fish ovaries under sterile conditions, were observed (Hansen and Olafsen, 1989).

In the spawning carried out in sterile conditions, the larger number of bacteria found in ova as compared to sperms, revealed that the ova is the major responsible for transmission of bacteria from adult to larvae. This agrees with the large number of bacteria found in female gonads of mature breeders of *A. purpuratus* (Chavez, 1991). In the spawning the gametes go through the nephridium, in other scallop species (*Pecten maximus*) not all gametes are immediately evacuated from the nephridium in the spawn, allowing autofertilization to occur in this organ (Le Pennec, personal communication). In *A. purpuratus*, gametes infections may occur in the nephridium. This cannot be ruled out and needs further investigation.

Bacterial proliferation in the veliger stage, reaching values of 2.9 x 10^2 CFU/larvae, occurred due to the static condition of scallop culture. According to Bourne *et al* (1989), this kind of culture from post-fertilization until early veliger stage should not be aerated, because the probability of collecting embryos at windrows of the tank's edge is increased.

The presence of different bacteria associated with gametes could be explained by the different microenvironment surrounding both gametes, which selectively favours the adherence of specific bacterial groups. Members of the genus *Vibrio* were present only in small number in fish eggs, as in previous studies (Hansen and Olafsen, 1989; Hansen, 1993). It is known that *Vibrio* and *Pseudomonas* have different chemotaxis responses to carbon compounds (Riquelme and Ishida, 1988). In the post-fertilization stage, *Vibrio* and *Pseudomonas* I-II were not detected by culture methods. It is possible that these bacteria in the static conditions of post-fertilization stage, would be suppressed by the rapid growth or antagonisms of others bacterial strains.

The qualitative results of spawn in sterile conditions demonstrate the possibility of bacterial transfer from parents to larval stages of *A. purpuratus*. Among these bacteria there might be potential pathogens, such as *V. anguillarum*. Virulent strains of *V. anguillarum* (VAR) have been recently isolated from scallop culture from Chile (Pazos *et al*, 1993; Riquelme *et al*, unpublished results). These vibrios strains

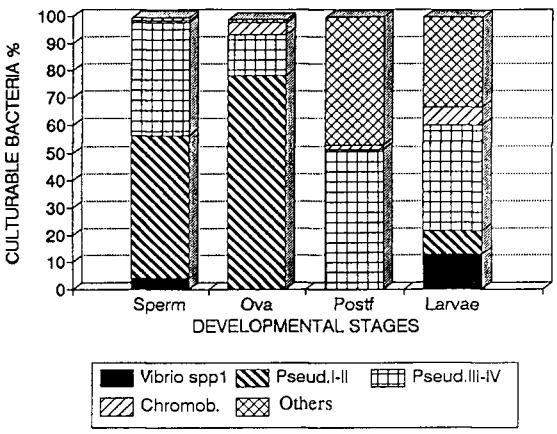

Fig 3. Composition of the different bacterial genera present in gametes and early developmental stages of *A. purpuratus*. **Postf**, post-fertilization stage.

are very lethal to larval culture due to their invasive capacity in the host and/or the ability to produce exotoxins (Birkbeck and Gallacher, 1993). Lodeiros *et al* (1987) suggest the possibility of parental bacterial transfer of *V. anguillarum* in *Ostrea edulis*, since this was detected in breeders tissues and larva.

It is likely that other transferred bacteria genera, such as *Flavobacterium* and *Chromobacterium* can have beneficial effects on larvae, because, in sea water, particles of size between 0.22 and 1 μm (possibly bacteria) increase growth of scallop larvae (*Pecten maximus*) up to 20% (Samain *et al*, 1987). Also the capacity of a high proportion of bacteria of bivalve larvae to produce extracellular enzymes, such as proteases and lipases (Prieur, 1982) make these bacteria able to help in the digestion process of microalgal food. Alternatively to the nutritional role, bacteria may also enhance larval cultures by removing toxic metabolites (Douillet and Langdon, 1993).

It is, however, necessary to better clarify the role or effect of transferred bacteria on larval development of *A. purpuratus*, suggested by the observations of this study.

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REFERENCES

AUSTIN B (1988) Marine Microbiology. London: Cambridge University Press. 222 pp

BARKER G, SMITH S, BROMAGE N (1989) The bacterial flora of rainbow trout, *Salmo gairdneri* Richardson, and brown trout -*Salmo trutta* L; eggs and its relationships to developmental success. J Fish Dis 12: 281-293

BIRKBECK H, GALLACHER S (1993) Interaction of pathogenic vibrios with marine bivalves. In: R GUERRERO, C PEDROS-ALIO (eds) Trends in Microbial Ecology. Spanish Society for Microbiology. pp 221-226

BOURNE N, HODGSON C A, WHYTE J N C (1989) A manual for scallop culture in British Columbia. Can Tech Rep Fish Aquat Sci 1964: 215

BROWN C (1983) Bacterial diseases in bivalve larval cultures and their control. In: Culture of Marine Invertebrates. Selected Readings. Mar Biol Lab Massachusetts 21: 230-242

CHAVEZ P (1991) Determinación cuantitativa y cualitativa del género *Vibrio* en dos poblaciones de invertebrados marinos: *Concholepas concholepas* (Brugiere, 1789) y *Argopecten purpuratus* (Lamarck, 1819), en Bahía Mejillones del Sur. Tesis, Universidad de Antofagasta. Antofagasta, Chile. 60 pp

DISALVO LH (1990) Problemática del cultivo de ostiones. Enfermedades. In: FLORES H, MIRANDA C (eds) VI Taller de Acuicultura. Universidad Católica del Norte. Coquimbo, Chile. pp 78-82

DOUILLET P, LANGDON J (1993) Effects of marine bacteria on the culture of axenic oyster *Crassostrea gigas* (Thunberg) larvae. Biol Bull 184: 36-51

ELSTON R (1989) Bacteriological methods for diseased shellfish In: B AUSTIN, DA AUSTIN (eds) Methods for the Microbiological Examination of Fish and Shellfish. England: Ellis Horwood Ltd. pp 187-215

HANSEN GH (1993) Bacteriology of early life stages of marine fish. Dr Scient Thesis. University of Bergen

HANSEN GH, OLAFSEN JA (1989) Bacterial colonization of cod (*Gadus morhua*) and halibut (*Hippoglossus hippoglossus*) eggs in marine aquaculture. Appl Environ Microbiol 55: 1435-1446

HANSEN GH, SORHEIM R (1991) Improved method for phenotypical characterization of marine bacteria. J Microbiol Meth 13: 231-241

HANSEN GH, BERGH O, MICHAELSEN J, KNAPPSKOG D (1992) *Flexibacter ovolyticus sp nov*; a pathogen of eggs and larvae of Atlantic halibut *Hippoglossus hippoglossus* L. Intl J Syst Bacteriol 42: 451-458

HOLT JG (chief ed) (1984) Bergey's Manual of Systematic Bacteriology. Baltimore: Williams and Wilkins. 1: 162-218; 2: 516-549

HORNE M, BAXENDALE A (1983) The adhesion of *Vibrio anguillarum* to host tissues and its role in pathogenesis. J Fish Dis 6: 461-471

ISHIDA Y, EGUCHI M, KADOTA H (1986) Existence of obligately oligotrophic bacteria as a dominant population in the South China Sea and the West Pacific Ocean. Mar Ecol Prog Ser 30: 197-203

LODEIROS C, BOLINCHES J, DOPAZO C, TORANZO A (1987) Bacillary necrosis in hatcheries of *Ostrea edulis* in Spain. Aquaculture 65: 15-29

LONGO F, SCARPA (1991) Expansion of the sperm nucleus and association of the maternal and paternal genomes in fertilized *Mulinia lateralis* eggs. Biol Bull 180: 56-64

MUROGA K, HIGASHI M, KEITOKU H (1987) The isolation of intestinal microflora of farmed red seabream (*Pagrus major*) and black seabream (*Acanthopagrus schegeli*) at larval and juvenile stages. Aquaculture 65: 79-88

NAVARRO R, STURLA L, CORDERO O, AVENDAÑO M (1991) In: S SHUMWAY (eds) Scallops: Biology, Ecology and Aquaculture. NY: Elsevier. pp 1001-1015

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

NOTTAGE A, BIRKBECK H (1987) Production of proteinase during experimental infection of *Ostrea edulis* L. larvae with *Vibrio alginolyticus* NCMB 1339 and the antigenic relationship between proteinases produced by marine vibrios pathogenic for fish and shellfish. J Fish Dis 10: 265-273

PADILLA M (1979) Desarrollo larval del ostión del Norte *Chlamys (Argopecten) purpuratus* Lamarck (1819) en condiciones de laboratorio (Mollusca: Pelecypoda). Ciencia y Tecnología del Mar, CONA 4: 1-52

PAZOS F, SANTOS Y, MAGARIÑOS B,BANDIN I,NUÑEZ S, TORANZO A (1993) Phenotypic characteristics and virulence of *Vibrio anguillarum*-related organisms. Appl Environ Microbiol 59: 2969-2976

PRIEUR D (1982) Les bactéries hétérotrophes dans les élevages experimentaux et industriels des larves de bivalves marins. Oceanis 8: 437-457

PRIEUR D, MEVEL G, NICOLAS JL, PLUSQUELLEC A, VIGNEULLE M (1990) Interactions between bivalve molluscs and bacteria in the marine environment. Oceanogr Mar Biol Annu Rev 28: 277-352

RIQUELME C, ISHIDA Y (1988) Chemotaxis of bacteria to extracellular products of marine bloom algae. J Gen Appl Microbiol 34: 417-423

RIQUELME C, FUKAMI K, ISHIDA Y (1988) Effects of bacteria on the growth of a marine diatom, *Asterionella glacialis*. Bull Japan Soc Microb Ecol 3: 29-34

SAMAIN JF, COCHARD JC, CHEVELOT L, DANIEL J, JEANTHON C, LE COZ JR, MARTY Y, MOAL J, PRIEUR D, SALAUN M (1987) Effet de la qualité de l'eau sur la croissance larvaire de *Pecten maximus* en écloserie: observations préliminaires. Haliotis 16: 363-381

SINDERMAN CJ (1988) Vibriosis of larval oysters. In: CJ SINDERMAN, DV LIGHTNER (eds) Disease diagnosis and control in North American marine aquaculture. NY: Elsevier. pp 271-274

WEST PA, COLWELL R (1984) Identification and classification of Vibrionaceae, an overview. In: COLWELL RR (ed) Vibrios in the Environment. NY: Wiley & Sons. pp 285-363