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ORIGINAL ARTICLE

Pathogenicity of a Highly Exopolysaccharide-producing *Halomonas* Strain Causing Epizootics in Larval Cultures of the Chilean Scallop *Argopecten purpuratus* (Lamarck, 1819)

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Abstract Mass mortalities of larval cultures of Chilean scallop *Argopecten purpuratus* have repeatedly occurred in northern Chile, characterized by larval agglutination and accumulation in the bottom of rearing tanks. The exopolysaccharide slime (EPS) producing CAM2 strain was isolated as the primary organism from moribund larvae in a pathogenic outbreak occurring in a commercial hatchery producing larvae of the Chilean scallop *Argopecten purpuratus* located in Bahía Inglesa, Chile. The CAM2 strain was characterized biochemically and was identified by polymerase chain reaction amplification of 16S rRNA as *Halomonas* sp. (Accession number DQ885389.1). Healthy 7-day-old scallop larvae cultures were experimentally infected for a 48-h period with an overnight culture of the CAM2 strain at a final concentration of ca. 10^5 cells per milliliter, and the mortality and vital condition of larvae were determined by optical and scanning electron microscopy (SEM) to describe the chronology of the disease. Pathogenic action of the CAM2 strain was clearly evidenced by SEM analysis, showing a high ability to adhere and detach larvae velum cells by using its "slimy" EPS, producing agglutination, loss of motility, and a posterior sinking of scallop larvae. After 48 h, a dense bacterial slime on the shell surface was observed, producing high percentages of larval agglutination (63.28±7.87%) and mortality (45.03±4.32%) that were significantly ($P<0.05$) higher than those of the unchallenged control cultures, which exhibited only 3.20±1.40% dead larvae and no larval agglutination. Furthermore, the CAM2 strain exhibited a high ability to adhere to fiberglass pieces of tanks used for scallop larvae rearing (1.64×10^5 cells adhered per square millimeters at 24 h postinoculation), making it very difficult to eradicate it from the culture systems. This is the first report of a pathogenic activity on scallop larvae of *Halomonas* species, and it prompts the necessity of an appraisal on biofilm-producing bacteria in Chilean scallop hatcheries.

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Introduction

The culture of Chilean scallop *Argopecten purpuratus* (Lamarck, 1819) is one of the most commercially important industries of Chilean mariculture and is mainly concentrated in the North region of the country. Although the major supply of scallops' seeds is from the natural environment, 30% comes from controlled condition cultures because of poor catches of natural seeds, which occur cyclically in Chile [14]. Therefore, it has been necessary to develop a hatchery system to provide a consistent supply of larvae under controlled conditions. Although culture techniques for efficient scallop larvae production have been developed, Chilean hatcheries occasionally suffer epizootic episodes,

often producing the complete loss of batches, causing the inability to ensure constant production.

Shellfish larval mortalities occurring in controlled cultures are mainly associated with high bacterial loads enhanced by culture conditions such as high temperatures, high food supplies, and high larval densities [22]. The most important problem for Chilean scallop culture is the occurrence of high larval mortalities caused by the specific development of pathogenic bacteria, mainly belonging to *Vibrio* and *Aeromonas* species [36, 38]. In addition, bacterial analysis of *A. purpuratus* gonads, gametes and larvae from natural environment and hatchery adults showed a high incidence of other Gram-negative bacteria [35]. In spite of studies reporting the bacterial production of toxins with ciliostatic action [30, 31], most bacterial infections occurring in shellfish larvae are of invasive etiology [16, 28, 33], and are mainly characterized by larval necrosis, velum distention or retraction, empty digestive glands, swimming cessation, and sudden death events in 18 h [19, 34, 39, 41].

Recently, in various Chilean scallop larvae commercial hatcheries have developed recurrent episodes of bacterial pathogenic outbreaks, usually characterized by the larval agglutination and consequent sinking and accumulation in the bottom of culture tanks producing high culture losses. Our observations suggested the active participation of biofilm-producing bacteria in these events of scallop larvae mortalities, but no studies concerning the identification of the etiological agent and its pathogenic activity have been developed. An understanding of the mechanisms by which bacterial agents produce disease in cultured larvae is essential for developing a management strategy to control outbreaks in intensive scallop larvae husbandry.

Considering that high losses in production of Chilean scallop would seriously affect the economy of north Chile and the absence of studies on the strategies of bacterial pathogens associated to Chilean scallop culture, the goal of this study was to identify the causative agent of an epizootic producing massive losses in the larval culture of Chilean scallop *Argopecten purpuratus* and to characterize the chronology of its pathogenic action.

Methods

Bacterial Strain Isolation

Larvae of the Chilean scallop *Argopecten purpuratus* Lamarck, 1819 (Bivalvia, Pectinidae) were sampled from culture exhibiting a massive mortality because of heavy larval agglutination and settlement. The hatchery was located on the coast of Bahía Inglesa in Chile. Serial dilutions of the homogenate of samples of moribund and dead larvae were prepared and 0.1-ml aliquots were spread-plated in triplicate onto 2216 Marine agar (Difco, Franklin Lakes, NJ) prepared with deionized distilled water, incubated for 7 d at 17°C, the usual temperature of water in scallop larvae-rearing tanks and examined each 24 h. A predominant colony appeared at a high level, almost in pure culture, and was isolated by streaking on Tryptic soy agar (Difco) supplemented with NaCl (2%). This strain, designated CAM2 (isolated from the scallop hatchery of Camanchaca Co.) was selected and tested for purity before storage.

Strain Culture and Preservation

Stock cultures of the CAM2 strain were maintained at 4°C on Tryptic soy agar (Difco) supplemented with NaCl (2%), and subcultured every 2 wks. For long-term preservation, CAM2 strain cultures were frozen at −80°C in Tryptic soy broth (Difco) supplemented with 2% NaCl (*w*/*v*) and 20% glycerol (*v*/*v*) [15]. When required, frozen cultures were recovered by streaking onto Tryptic soy agar plates (Difco) supplemented with NaCl (2%), which were incubated at 20°C for 24 h.

Biochemical and Physiological Analysis

The CAM2 strain was examined with standard morphological, physiological, and biochemical tests. Phenotypical characteristics, including Gram stain, motility, oxidation/fermentation of d-glucose (O/F), *O*-nitrophenyl-β, D-galactopyranoside (ONPG), casein, esculin, starch and gelatin hydrolysis, nitrate reduction, growth on MacConkey agar, oxidase, catalase, acetoin and indole production were determined as described by Barrow and Feltham [7] in media supplemented with NaCl (2%). Møller decarboxylation of L-lysine and L-ornithine, and dihydrolation of L-arginine were performed and interpreted according to Hansen and Sörheim [17]. Growth at 4°, 15°, 20°, 30°, 37°, and 42°C were tested on Tryptic soy broth (Difco) supplemented with 2% NaCl and the salt requirement was tested using peptone broth (Difco) supplemented with 0, 1, 2, 4, 6, 8, 10, 15, 20, and 30% NaCl. The results were recorded after incubation at 20°C for 48 h. In addition, the CAM2 strain was characterized by using the commercially available test systems for the identification of nonfermentative Gram-negative rods, API 20NE (bioMérieux, Marcy-l'Etoile, France) and GN2 microplate (Biolog Inc., Hayward, CA) systems. API 20NE assay was performed according to the instructions of the manufacturer. The CAM2 strain was inoculated into 2.00% NaCl, and turbidity was adjusted to 0.5 MacFarland standard (bioMérieux, Marcy-l'Etoile, France). Bacterial inoculum was distributed into the API 20NE gallery, which was incubated

at 20°C and read at 24 and 48 h, but only results after 48 h of incubation were considered. Biochemical reactions were read as positive or negative, translated into numerical profiles and bacterial identification was performed by using the APILAB Plus identification software (bioMérieux, Marcy-l'Etoile, France). For the GN2 microplate (Biolog Inc.) assay, the CAM2 strain was inoculated into a solution containing 2.50% NaCl, 0.80% $MgCl_2$ and 0.05% KCl, and the microtitre plate was inoculated according to the instructions of the manufacturer. The plate was incubated aerobically in the dark at 20°C, and duplicate readings were made after 48 h and 72 h of incubation, but only results after 72 h of incubation were considered. Bacterial identification was performed by using the Microlog System 4.2 identification software (Biolog Inc.).

Polymerase Chain Reaction (PCR) Amplification of the Bacterial 16S rRNA Genes

Crude DNA extracts were obtained from pure bacterial isolates according to Valsecchi [43]. The 16S rRNA gene from each bacterial strain was partially amplified by the polymerase chain reaction (PCR) using primers and reaction conditions as described by Lee and Qian [21]. PCR products were analyzed by electrophoresis on 2% agarose gels with marker ϕx174/Hae (Gibco BRL, Life Technologies, Rockville, MD). The bands of about 700 bp were purified from the agarose gel using the Wizard preps DNA purification kit (Promega, Madison, WI) according to the manufacturer's instructions. The 16S rRNA gene amplicon was sequenced from both ends using 355F and 1055R primer and ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kits (ABI) [40] in an automated DNA sequencer (ABI 310, Applied Biosystems, Foster City, CA, USA) as previously described [2, 21]. Nucleotide sequence alignment and comparisons were carried out by using BLAST (Basic Local Alignment Search Tool) on the NCBI (National Center for Biotechnology Information) web server.

Culture of the CAM2 Strain

The CAM2 strain was grown for 24 h at 20°C in 200-ml flasks containing Tryptic soy broth (Difco) supplemented with 2% NaCl (Merck). Log-phase bacterial cultures were centrifuged at 5,500 rpm for 15 min and the pellets were resuspended in sterile seawater (salinity 36‰) and then resuspended again with a vortex mixer to break up clumps. Turbidity was adjusted to match a 0.5 McFarland standard (bioMérieux) to provide a stock culture for scallop larvae challenge. Before challenge assays, adequate serial dilutions were performed to inoculate 3 ml of a 10^7 CFU ml^{-1} solution.

Bacterial Pathogenicity on Scallop Larvae

Healthy 7-day-old scallop larvae obtained from the Marine Culture Lab of the Universidad Católica del Norte were reared at 17°C in a static system using 500-ml glass beakers with microfiltered (0.22 μm) UV-treated seawater (salinity 36‰) at an approximate concentration of 10 larvae ml^{-1}. The scallop larvae were fed with unicellular algae *Isochrysis galbana* because of its high reproduction rate and its sufficient nutritional quality [44]. Larvae were fed with an axenic culture of *Isochrysis galbana* at 30,000 cells ml^{-1} before the bacterial challenge assay [26]. Scallop larvae cultures (300 ml) in triplicate were inoculated with 3 ml of a log-phase culture of the CAM2 strain to obtain a final concentration of 10^5 CFU ml^{-1}. Also, scallop larval cultures without bacterial inoculation were considered as controls. Scallop larvae cultures were maintained for a 48-h period and gently agitated by hand each 30 min during the day to ensure that the bacterial inoculum was evenly suspended. Larval samples (5 ml) were collected at 0, 6, 12, 18, 24, 36, and 48 h and agglutination, mortality, and vital condition (swimming, vellum ciliar movement, and digestive gland status) [22] of approximately 50 larvae were determined by using a Zeiss Axiolab microscope. Scallop larvae that showed no apparent movement, closed valves, and no velar activity were considered dead, as described by Prado et al. [33]. In addition, larvae samples (5 ml) were collected for scanning electron microscopy analysis, and approximately 50 larvae from each replica were observed.

Scanning Electron Microscopy (SEM) analysis

Samples of larvae and bacteria were fixed with 2% glutaraldehyde (Merck, Damstadt, Germany), dehydrated in increasing concentrations of ethanol until 100%, and then were critical point dried using liquid CO_2 as transitional fluid. Later, the samples were mounted on double scotch tape and gold coated. Samples were examined on a JSM-T300 (JEOL) scanning electron microscope and photographed using a Nikon camera model FM10 (Tokyo, Japan).

Adherence of the CAM2 Strain to Fiber-glass

Adherence of the CAM2 strain on the surface of fiber-glass used in the scallop-rearing tanks was analyzed. Triplicate sterile pieces (4 cm^2) of fiberglass were deposited in Petri plates containing a suspension of a log-phase culture of the CAM2 strain at an approximate concentration of 10^6 cells ml^{-1}. Plates containing microfiltered (0.22 μm) seawater (salinity 36‰) were used as controls. Pieces of fiberglass were sampled after 24 h and the number of adhered bacterial cells was determined. Non-adhered bacteria were

aseptically removed washing ten times with sterile seawater and bacterial cell concentration in the biofilm was determined by removing the biofilm from the entire piece using a sterile swab and transferring to a sterile tube containing 50 ml of sterilized seawater (salinity 36‰). Homogenates were vigorously shaken and dilutions were inoculated by the spread plate technique onto plates with Tryptic soy agar supplemented with NaCl (2%). In addition, pieces of fiberglass (20 mm^2) in triplicate were fixed with 2% glutaraldehyde (Merck) for scanning electron microscopy (SEM) analysis. Samples were visualized at a final magnification from ×50 to ×50,000.

Statistical Analyses

For larval assays, agglutination and mortality percentages were transformed as *arcsin* (square root {percentage agglutination or mortality 100^{-1}]) and were analyzed using one-way analysis of variance (ANOVA). Normality of the variables was determined by using the test of Kolmogorov–Smirnov, whereas homogeneity of the variances was tested by the Levene's test. [45]. When overall differences were significant (less than 5% level), a Tukey's multiple range test [45] was used to determine significant differences ($P< 0.05$) among the proportions of agglutination and mortality of challenged and control scallop larvae. All statistical analyses were performed using the SPSS version 12.0 computer program [29].

Results

Bacterial Pathogen

Physiological and biochemical characteristics of the CAM2 strain are listed in Table 1. The CAM2 strain was characterized as a moderately halophilic, non-fermenter, Gram-negative bacilli. When the CAM2 strain was characterized by using the API 20NE system, the resulting code of 1475244 was interpreted as an "unacceptable profile", and no valid identification was obtained. It corresponded to positive reactions for nitrate reduction to nitrite, gelatinase, β-galactosidase, esculin hydrolysis, and utilization of glucose, arabinose, mannitol, maltose, and malate, whereas negative reactions for indole production, glucose fermentation, arginine dihydrolase, urease, and utilization of mannose, *N*-acetyl-glucosamine, gluconate, caprate, adipate, citrate, and phenyl-acetate. Furthermore, the metabolic fingerprinting pattern of the CAM2 strain obtained by using the Biolog GN2 Microplate identification test was also unable to obtain identification at genus level of the CAM2 strain. According to the Biolog identification kit, the CAM2 strain utilizes dextrin, glycogen, tween 40, tween 80, *N*-acetyl-D-glucosamine, L-arabinose, D-arabitol, D-cellobiose, i-erythritol, D-fructose, D-galactose, gentiobiose, α-D-glucose, *m*-inositol, α-D-lactose, lactulose, maltose, D-mannitol, ß-methyl-D-glucoside, D-sorbitol, sucrose, D-trehalose, turanose, pyruvic acid methyl ester, *cis*-aconitic acid, citric acid, D-gluconic acid, D-glucuronic acid, ß-hydroxybutyric acid, γ-hydroxybutyric acid, D,L-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, succinic acid, bromosuccinic acid, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-glutamic acid, glycyl-L-glutamic acid, L-leucine, L-phenylalanine, L-proline, L-serine, L-threonine, γ-amino butyric acid, urocanic acid, putrescine, 2-aminoethanol and glycerol when used as the sole carbon source and does not utilize α-cyclodextrin, *N*-acetyl-D-galactosamine,

Table 1 Phenotypic characteristics of the *Halomonas* CAM2 strain isolated from diseased Chilean larvae scallops (*A. purpuratus*) in a commercial hatchery

Characteristic	Result
Gram stain	–
Motility	+
ß-galactosidase (ONPG)	–
Oxidase production	+
Catalase production	+
O/F (D-glucose)	I
Casein hydrolysis	–
Esculin hydrolysis	+
Gelatin hydrolysis	+
Starch hydrolysis	–
Arginine decarboxylase	+
Lysine decarboxylase	–
Ornithine decarboxylase	–
Acetoin production	–
Indole production	–
Nitrate reduction	+
Growth on MacConkey agar	+
Growth in:	
0% NaCl	–
1% NaCl	+
2% NaCl	+
4% NaCl	+
6% NaCl	+
8% NaCl	+
10% NaCl	+
15% NaCl	–
20% NaCl	–
30% NaCl	–
Growth in:	
4°C	–
15°C	+
20°C	+
30°C	+
37°C	–
42°C	–

I: Inert

adonitol, L-fucose, D-mannose, D-melibiose, D-psicose, D-raffinose, L-rhamnose, xylitol, succinic acid mono-methyl-ester, acetic acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, α-hydroxybutyric acid, *p*-hydroxy phenylacetic acid, itaconic acid, α-keto butyric acid, α-keto glutaric acid, α-keto valeric acid, sebacic acid, succinamic acid, glucuronamide, L-alaninamide, L-aspartic acid, glycyl-L-aspartic acid, L-histidine, hydroxy-L-proline, L-ornithine, L-pyroglutamic acid, D-serine, D,L-carnitine, inosine, uridine, thymidine, phenyethyl-amine, 2,3-butanediol, D,L-α-glycerol phosphate, α-D-glucose-1-phosphate, and D-glucose-6-phosphate.

Otherwise, PCR amplification of the 16S rRNA gene and sequencing analysis of the amplified PCR products of the CAM2 strain evidenced a 99% identity (599/603 residues identical) to *Halomonas* sp. NT N97 (AB085658.1), and the data were deposited in the GenBank nucleotide sequence database under accession number DQ885389.1.

Bacterial Pathogenicity on Scallop Larvae

The chronology of the infectious disease produced by the CAM2 strain was clearly visible by using optical and SEM analysis. The pathogenic effect was time-dependent and the number of larvae showing pathological signs increased over time. Optical microscopy observations of scallop larvae exposed for 24 h to the CAM2 strain showed a decreased ciliar movement, evidencing the spotting phenomenon, defined as an accumulation of larvae agglutinated in the bottom of the beakers (Fig. 1A). In addition, after 24 h postinoculation SEM micrographs evidenced a dramatic larval agglutination mediated by the accumulation of bacterial exopolysaccharide on the assayed larval shells (Fig. 1B).

Using scanning electron microscopy, before bacterial inoculation, scallop larvae were characterized by a clean shell surface and intact velum (Fig. 2A–B), whereas 6 h postinoculation with the CAM2 strain larvae showed bacterial cells adhered to their surface (Fig. 2C–D), but no differences in vital condition and mortality with control larvae were observed. Twelve hours after bacterial inoculation, scallop larvae exhibited high levels of bacterial colonization on scallop shell surface (Fig. 2E–F). After 18 h, CAM2 strain cells attached to the scallop shell surface began to form microcolonies (Fig. 2G–H), with a progressive bacterial colonization on scallop shell surface until the production of a dense biofilm mediated by the production of an exopolysaccharide matrix at 24 h postinoculation (Fig. 2I–J). After 36 h, challenged scallop larvae evidenced dense bacterial slimes on the shell surface (Fig. 2K–L), showing an important increase in the larval agglutination. In addition, scanning electron microscopy observations evidenced that an important number of scallop larvae velum ciliar cells were adhered to bacterial cells attached to the shell surface by the action of EPS adhesive filaments produced by the CAM2 strain (Fig. 3A). This produced a blocking of ciliar movement, with a posterior detachment of ciliar cells (Fig. 3B), causing the scallop larvae to stop swimming and sink because of ciliary arrests and remain inactive on the bottom of culture beakers. However, after a 36-h period, control scallop larvae not inoculated with the CAM2 strain showed normal swimming behavior, intact velum, and low bacterial loads (Fig. 4A), exhibiting a clean shell surface without bacterial cells adhered to their shell surface (Fig. 4B).

Agglutination and mortality of challenged larvae scallops were significantly higher ($P<0.05$) than those from the unchallenged control larval groups after 12 h, and these differences were maintained throughout along the pathogenicity bioassay (Fig. 5).

After 12 h, low percentages of challenged larvae were agglutinated (larvae grouping, 18.92±10.01%, mean ± SD, 3 replicates), and some dead larvae were observed (9.78±4.91%). Challenged larval groups exhibited an increase in the larval agglutination (43.12±3.26%) and mortality (31.63±3.96%) 24 h postinoculation. After a period of postinoculation of 48 h, agglutination and mortality affected the 63.28±7.87% and 45.03±4.32% of challenged larvae, respectively, whereas the control cultures exhibited only 3.00±2.45% of dead larvae and no larval agglutination (Fig. 5).

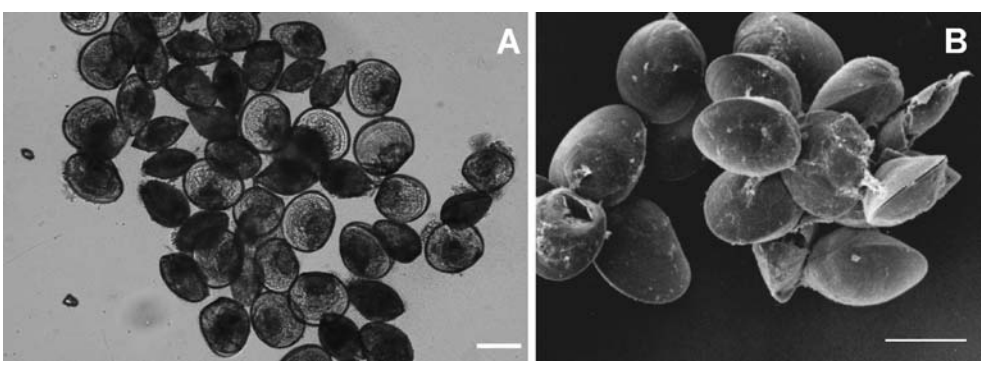


Figure 1 Optical (A) and scanning electron (B) micrograph showing agglutination of scallop larvae inoculated with the CAM2 strain after a 24-h period of incubation (bar in A= 100 μm; bar in B=100 μm)

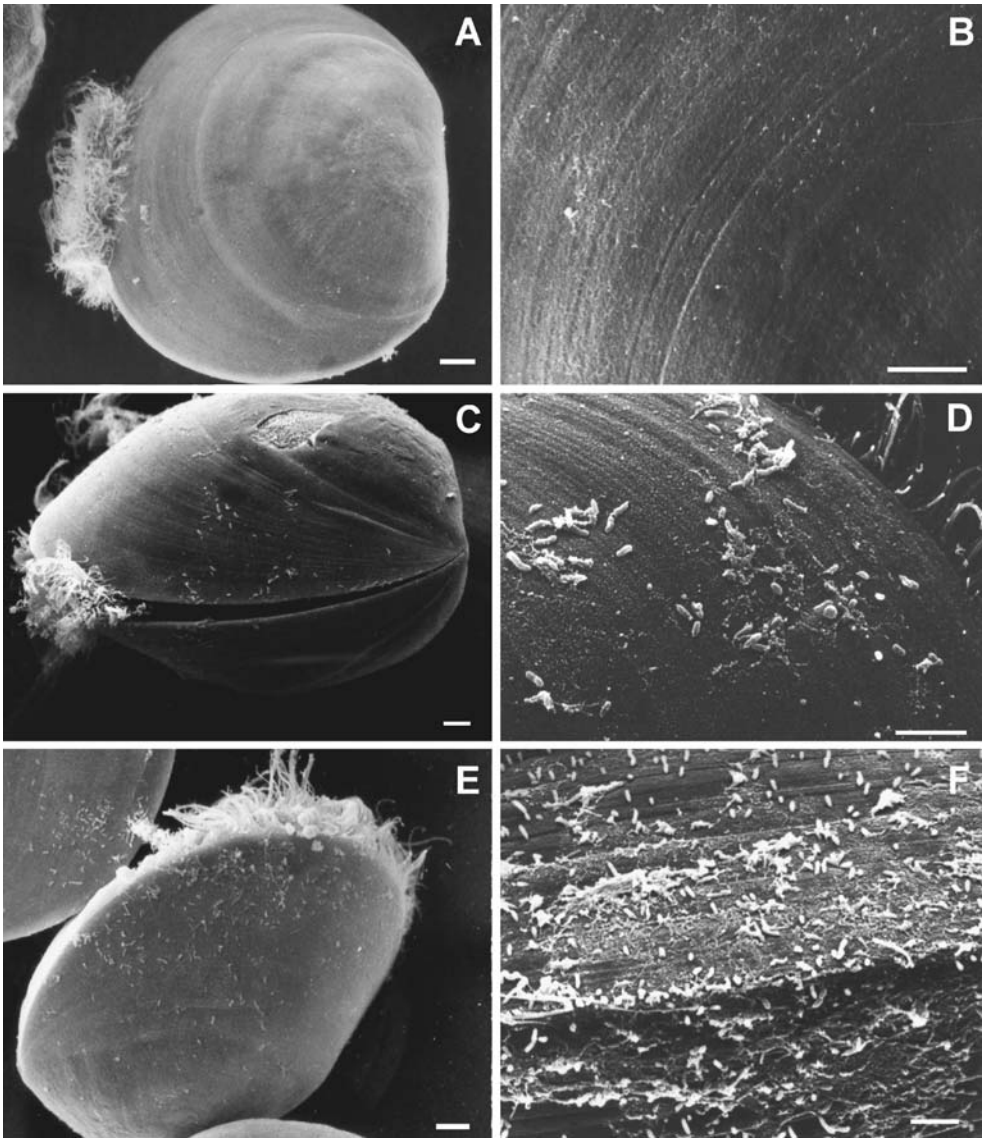


Figure 2 Scanning electron micrograph of 7-day-old *Argopecten purpuratus* scallop larvae before bacterial inoculation (A–B, bar in A=10 μm; bar in B=5 μm), and challenged with the CAM2 strain after 6 h (C–D, bar in C=10 μm; bar in D=5 μm), 12 h (E–F, bar in E=10 μm; bar in F=5 μm), 18 h (G–H, CAM2 microcolony formation marked by white arrow, bar in G=10 μm; bar in H=5 μm), 24 h (I–J, bar in I=25 μm; bar in J=1 μm) and 36 h (K–L, bar in K=5 μm; bar in L=1 μm)

Adherence of the CAM2 Strain to Fiberglass

The CAM2 strain showed a high *in vitro* ability to adhere to fiberglass, exhibiting levels of $1.64\times10^5\pm7.71\times10^4$ cells adhered per square millimeter fiberglass after a 24-h period. However, no biofilm or exopolysaccharide filaments were observed (Fig. 6).

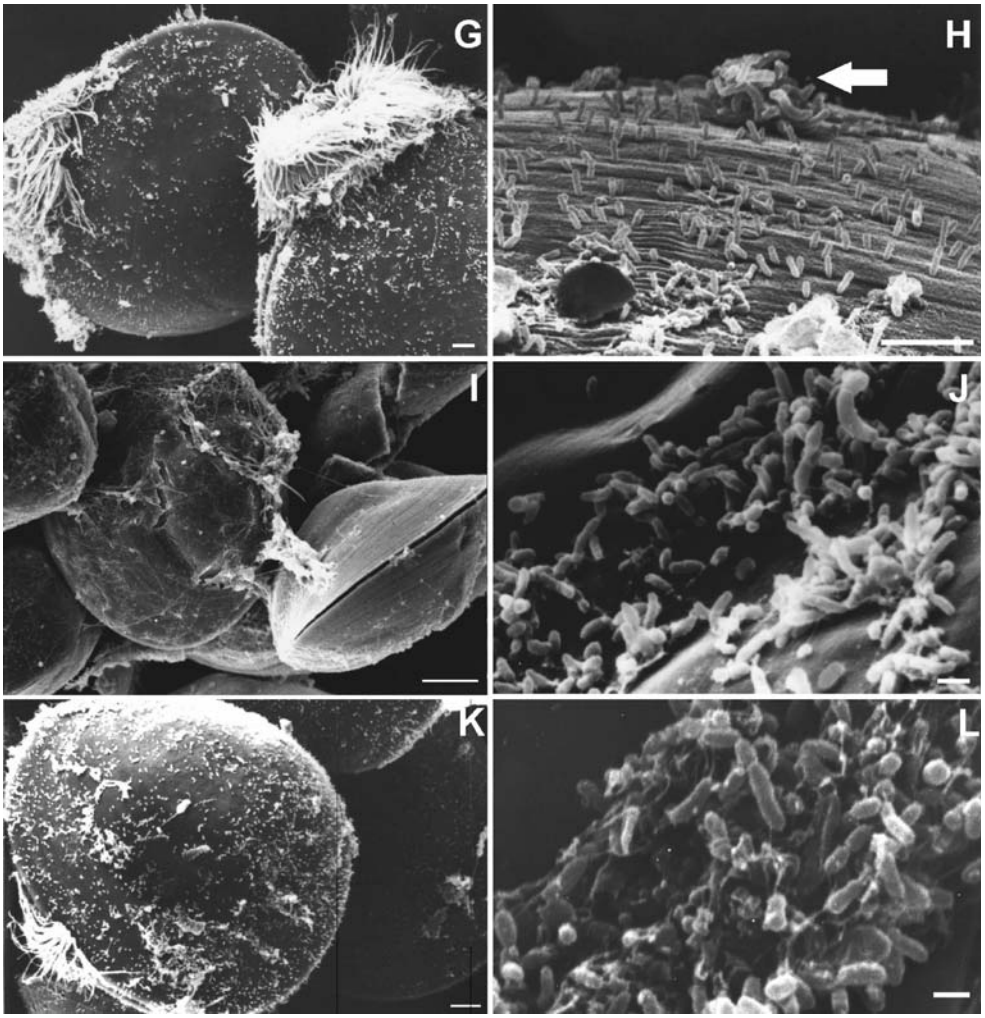
Discussion

As expected, CAM2 profiles generated by using the API 20NE and Biolog systems were unable to identify the CAM2 strain, considering that *Halomonas* species are not covered in the API 20NE and Biolog databases. It appears that these identification systems should expand their databases to include *Halomonas* species to be useful to identify this pathogen, but the high phenotypic heterogeneity of the family Halomonadaceae is a clear challenge for the identification of representatives of this group at the genus or species level, requiring a high number of characteristics to obtain reliable results, as stated by Mata et al. [25].

At present, only sequence comparison of rRNA genes (16S and 23S) has proved to be a valuable tool for the taxonomic classification of this group [4]. Although, it is very useful for identification purposes, this method exhibits an important limitation in the *Halomonas* identification at the species level [3] as a result of limited availability of *Halomonas* spp. sequences lodged in Genbank.

Halomonas are ubiquitous and most of the *Halomonas* species are a constituent of a bacterial group that is very abundant in marine environments, hence its eradication from a hatchery environment is impossible. Various studies

Figure 2 (continued)



demonstrated the exopolysaccharide production properties of *Halomonas* species [9, 24], but, to our knowledge, this is the first time that *Halomonas* strains have been associated with larval scallop mortality. It seems that *Halomonas* pathology appears to be directly related to poor husbandry, suggesting that sources of bacterial infection are broodstock, algal cultures, and incoming seawater, as previously stated by Elston [13]. These sources must be further investigated to identify the primary sources of this pathogen in scallop hatcheries. Although *Halomonas* strains have not been previously reported among culturable microbiota from *A. purpuratus* adults [6, 37], isolation of *Halomonas venusta* strains from geoduck clam and oyster adults have been recently reported [42].

The high ability to adhere to scallop shells enables the *Halomonas* strain to become highly predominant on this surface, enhancing its high proliferation by using the nutrients accumulated on larval shells. This explains why that diseased larvae recovered from hatcheries exhibited almost pure cultures of the pathogen.

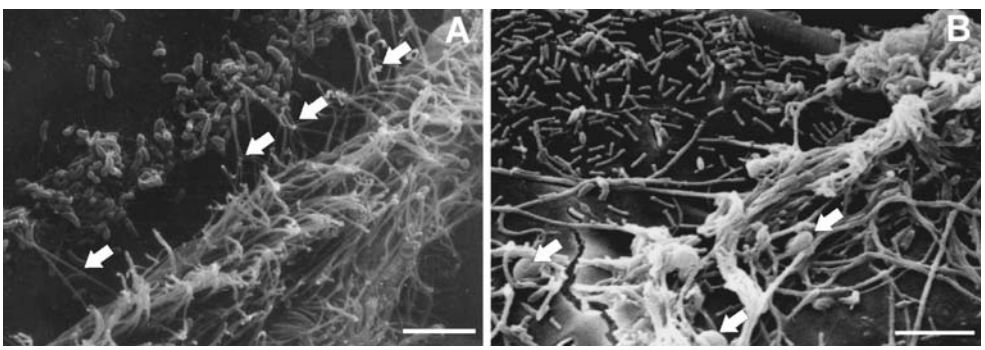


Figure 3 Scanning electron micrograph showing adherence (A, 12 h postinoculation) (bacterial exopolysaccharide filaments marked by white arrows), and detachment (B, 24 h postinoculation) (detached velum ciliar cells marked by white arrows) of velum ciliar cells of scallop larvae by the CAM2 strain (bars=5 μm)

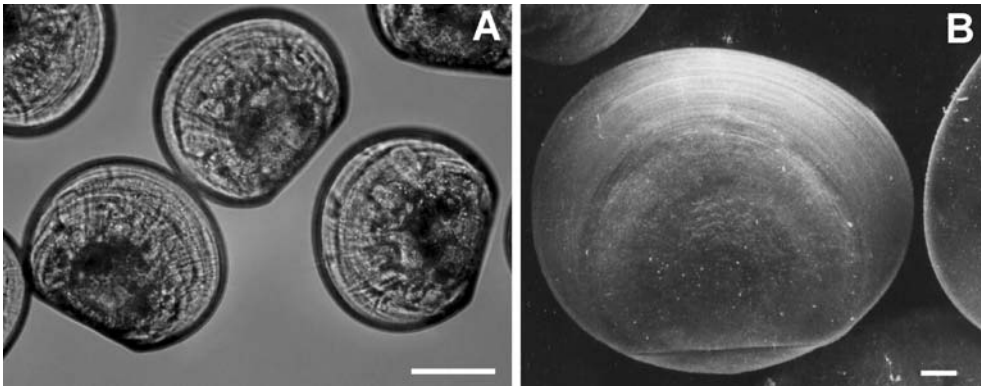


Figure 4 Optical (A) and scanning electron (B) micrograph of scallop larvae not inoculated with the CAM2 strain after a 36-h period of incubation (bar in A=50 μm; bar in B=10 μm)

During the hatching process, scallop larvae are usually reared in static water, in dense numbers and fed with large amounts of microalgae. These conditions are highly favorable for the quick proliferation of opportunistic pathogens occasionally present in the culture system and a high increase of the organic matter content [19]. It is possible to assume that CAM2 attachment to scallop shells is an opportunistic strategy to increase the rate of substrate uptake producing the exopolymer matrix to trap and concentrate nutrients, not intending to cause harm to scallop larvae.

As the CAM2 strain seemed to multiply mainly on shell larvae, its pathogenic action is only indirect causing a larval agglutination, without a systemic infection of the scallop soft tissues or production of toxins. Furthermore, SEM analysis evidenced the absence of the CAM strain inside the scallop larvae during the pathogenicity assay. It is evident that the susceptibility of scallop larvae to the CAM2 strain is not dependent on the quality of larval brood, or on defense mechanisms exhibited by the scallop larvae. In addition, the involvement of fluctuations of physical and chemical factors that could stress, highly important for the development of larval pathologies caused by opportunistic bacteria, did not seem to be important in the *Halomonas* pathogenicity action.

The balance between the planktonic and biofilm modes of existence of marine bacteria, and the factors that trigger them, should provide insight into microbial lifecycle strategies and would be helpful in understanding the hatchery conditions that could be important in the biofilm formation of the *Halomonas* strain.

Various scallop-rearing hatcheries in the North zone of Chile have registered massive larval losses with clinical signs very similar to those reported in this study. Chilean scallop farm results have shown that when scallop larvae belonging to batches suffering high agglutination and mortality were treated with antibiotic solutions (mainly florfenicol and oxytetracycline), daily mortality rates did not decrease within 24 or 48 h. This is not rare, considering that bacterial infections that involve a biofilm mode of growth are generally chronic and are often difficult to treat because of the recognized resistance to antibacterials in biofilms [23, 32]. In addition, the administration of drugs to treat the disease could only favor the *Halomonas* predominance in the scallop larvae and promote its proliferation,

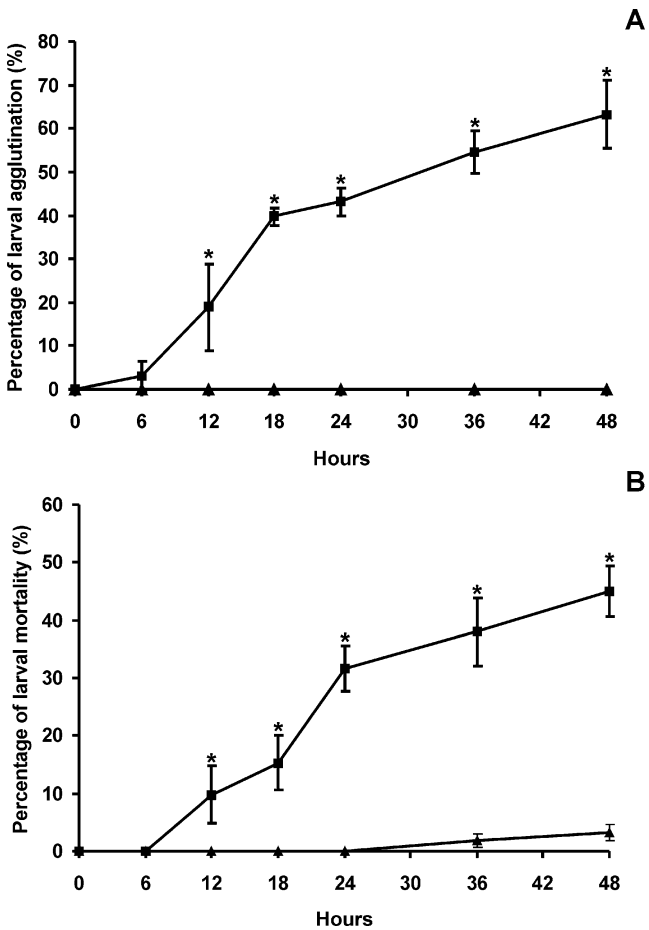


Figure 5 Agglutination (A) and mortality (B) of 7-day-old scallop larvae unchallenged (*up triangles*) and challenged with 10×10^5 CFU ml^{-1} of the *Halomonas* CAM2 strain (*squares*), over a 48-h period. Values are means (±SD) of three replicates. Significant differences between control and challenged larvae according to a Tukey test (ANOVA: $P<0.05$) are indicated by an asterisk above CAM2 challenged values

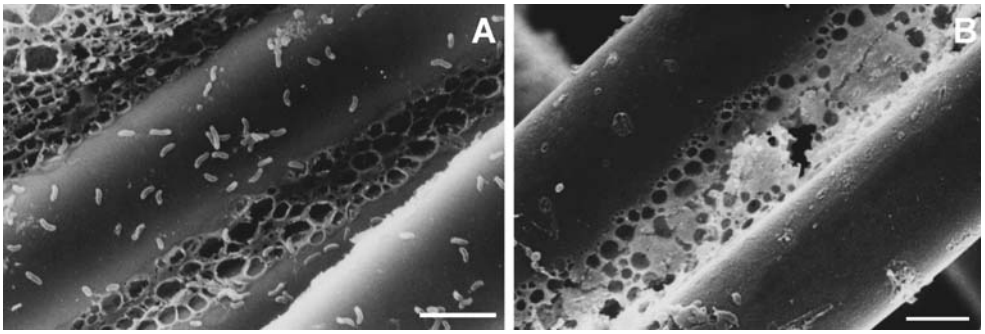


Figure 6 Scanning electron micrograph of fiberglass used in the scallop rearing tanks inoculated (A) and not inoculated (B) with the CAM2 strain after a 24-h incubation period (bars=5 μm)

considering that *Halomonas* biofilms are not susceptible to the antibacterial treatments (unpublished results). So it seems that the prevention of *Halomonas* adhesion to scallop shells is essential to eliminate this threat for early growing phases of scallop. Because many bacterial species control their growth, adhesion, and biofilm formation by means of a regulatory mechanism named quorum sensing [8], the inhibition of quorum-sensing signals leads to the disruption of biofilm formation and the inhibition of bacterial pathogenicity [5, 12, 18]. Thus, it is possible to suggest the possibility to use naturally produced quorum-sensing blockers such as furanone compounds derived from many marine organisms [11, 20] to prevent biofilm formation by *Halomonas* strains in scallop hatcheries.

In addition, the routine antibiotic treatment or UV sterilisation as a preventive measure in scallop hatcheries could also be effective to control planktonic cell numbers of *Halomonas* before bacterial adhesion on scallop shells, thus reducing the potential to form biofilms.

The pathogenicity assays demonstrated that the CAM2 strain exhibits a high ability to specifically attach to the shell of Chilean scallop, evidencing that biofilm formation is very rapid. The ability of the CAM2 strain to produce high mortality rates (45%), in a short period of time (48 h), is of particular importance because water in scallop larvae rearing tanks at Chilean hatcheries is renewed every 1 to 2 d and most of the batch could die within this period. Furthermore, the fact that scallop larvae rearing tanks are made of the same fiberglass assayed in this study suggests the possibility that this strain is able to survive the water changes, forming biofilms on the inner tank surfaces. Therefore, this kind of bacteria can probably remain in the culture system for a long period of time, increasing the risk of larval infection, but attempts to isolate *Halomonas* strains from sources other than moribund scallop larvae, including tank water sampled during epizootics, have been unsuccessful to date. All these facts emphasize that proper cleaning of tanks is critical for control of pathogen persistence in the culture systems. In addition, the feasibility of using nontoxic "self-cleaning" silicone polymer surfaces that have been developed to reduce fouling in the shipping industry [1, 10, 27] in the Chilean scallop hatcheries must be considered.

An extrapolation of our results to predict the potential effect of *Halomonas* sp. on larval scallop aquaculture would depend on the actual concentration of this bacterium in the culture systems, and also on other culture conditions.

The potential seeding of subclinically infected scallop juveniles in open waters would permit their transfer to grow-out areas where the seed could disseminate the bacteria to nearby scallops, also affecting wild stocks. Therefore, it would be valuable to determine the presence of this microorganism in scallop seeds before transfer to avoid placing contaminated seed in field locations.

Although it has been demonstrated that a highly specific interaction occurs between a biofilm-forming bacteria and a shellfish larvae, the ecological significance of most naturally occurring epiphytic bacterial communities in marine environments is still unclear. As stated by Prieur et al. [34], because of their economic importance in many countries, marine bivalves have probably been studied more than other invertebrates, revealing interesting and sometimes unusual interactions with marine bacteria. The ubiquity of *Halomonas* strains in marine environments makes it likely that other invertebrate larvae could be also be colonized by this bacterium in natural environments. Bacterial attachment to bivalve shells may play an important role for invasive bacteria in marine invertebrate larvae having a profound impact on the composition and activity of the epiphytic community; this needs further investigation.

It is clear that the interaction of bacteria with marine animals and plants should be more considered in future work, as it represents an important component of the microbial ecosystem and will probably lead to the discovery of other types of interactions causing detrimental effects on colonized animals.

Conclusions

In conclusion, our study demonstrates that CAM2 strain, belonging to the *Halomonas* genus, exhibits a high ability

to colonize scallop larvae shell and to adhere to scallop ciliar cells, causing a sudden larvae agglutination that remained active with a progressive blocking of ciliar movement and distended velum. This produced a loss of motility and a posterior settlement and accumulation in the bottom of the tanks, without signs of necrosis or tissue destruction. Furthermore, the CAM2 strain exhibits an important ability to adhere to fiberglass, suggesting that this bacterium can remain in culture systems for a long period of time, considering that larvae culture tanks used in Chilean scallop hatcheries are made of the same fiberglass. This fact emphasizes that proper cleaning of culture tanks is critical for control of its persistence in the culture systems and suggests the necessity of developing novel therapies for biofilm-based disease occurring in Chilean pectiniculture. Finally, on the basis of our data, we conclude that a direct relationship between the CAM2 strain and collapses of Chilean scallop larval cultures exists, confirming the pathogenic status of *Halomonas* species being responsible for important losses in Chilean scallop hatcheries.

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