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Immunohistochemistry of great scallop *Pecten maximus* larvae experimentally challenged with pathogenic bacteria

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ABSTRACT: Three challenge experiments were carried out on larvae of the great scallop *Pecten maximus*. Larvae were bath-challenged with *Vibrio pectenicida* and 5 strains resembling *Vibrio splendidus* and one *Pseudoalteromonas* sp. Unchallenged larvae were used as negative controls. The challenge protocol was based on the use of a multidish system, where the scallop larvae (10, 13 and 15 d post-hatching in the 3 experiments, respectively) were distributed to 2 ml wells with stagnant seawater and exposed to the bacterial cultures by bath challenge. Presence of the challenge bacteria in the wells was verified by polymerase chain reaction (PCR). A significantly increased mortality was found between 24 and 48 h in most groups challenged with *V. pectenicida* or *V. splendidus*-like strains. The exception was found in larval groups challenged with a *Pseudoalteromonas* sp. LT 13, in which the mortality rate fell in 2 of the 3 challenge experiments. Larvae from the challenge experiments were studied by immunohistochemistry protocol. Examinations of larval groups challenged with *V. pectenicida* revealed no bacterial cells, despite a high degree of positive immuno-staining. In contrast, intact bacterial cells were found in larvae challenged with *V. splendidus*. In the case of larvae challenged with the *Pseudoalteromonas* sp., positive immuno-staining was limited to visible bacteria inside the digestive area and cells of the mucosa. The experiments confirm that *V. splendidus* and *V. pectenicida* are pathogenic to scallop larvae, and that the *Pseudoalteromonas* strain is probably not a primary pathogen, although it cannot be ruled out as a secondary pathogen.

KEY WORDS: *Pecten maximus* · *Vibrio splendidus* · Immunohistochemistry · Challenge experiments · Larvae

INTRODUCTION

The great scallop *Pecten maximus* L occurs naturally along the coasts of Europe, north to the Lofoten islands in Norway. Due to good prices on the European market, suitable environmental conditions and a harvestable wild population, potential for commercial scallop production in Norway has been identified, and attempts to cultivate the species are currently underway in Norway and France (reviewed by Bergh & Strand 2001).

Aquaculture of the great scallop in Norway is based upon hatchery-produced spat. This production is commonly associated with highly variable survival during the larval stages. Mortalities of up to 100% during the early life stages have frequently been experienced at the only Norwegian hatchery, and the total annual production of 2 mm spat has been limited to about 2 million since 1996 (Torkildsen & Magnesen 2004).

Opportunistic bacteria are probably the primary cause of the mortalities (Nicolas et al. 1996, Robert et al. 1996, Torkildsen et al. 2000, 2002, 2005). This is a well-known problem in bivalve hatcheries, and several bacteria, especially *Vibrio* species, cause diseases in bivalves. *Vibrio pectenicida* has been isolated from moribund *Pecten maximus* larvae in French hatcheries (Lambert et al. 1998). In cultivated northern Chilean scallop *Argopecten purpuratus*, vibriosis caused by

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V. anguillarum (Riquelme et al. 1995) and *V. alginolyticus* (see Riquelme et al. 1996a,b) has caused high mortality. *V. splendidus* was the causative agent of annual summer mortalities of the Pacific oyster *Crassostrea gigas* in France (Lacoste et al. 2001, Le Roux et al. 2002, Waechter et al. 2002). The causative agent of brown ring disease (BRD), *V. tapetis* (Borrego et al. 1996) caused high mortalities in Manila clams *Ruditapes philippinarum*, especially in France. Mortalities up to 100% caused by *V. tapetis* occurred in a challenge study of Manila clams (Allam et al. 2002). Other bacterial species causing disease in bivalves are *Aeromonas hydrophila* (Riquelme et al. 1996a), *Chlamydia* (Leibovitz 1989), *Chlamydia*-like organisms (Morrison & Shum 1982, Renault & Cochennec 1995, Hine & Diggles 2002) and *Rickettsia*-like organisms (Elston 1986, Le Gall et al. 1988, 1991, Wu & Pan 1999).

However, bacteria are also a part of the diet of bivalves, which feed on microalgae, other microplankton, bacteria and particulate organic material (Hovgaard et al. 2001). When Samain et al. (1987) studied water quality, they found that feed particle sizes between 0.22 and 1 µm significantly improved the growth of *Pecten maximus* larvae and suggested that bacteria are an important part of the food supply. Comparable results were found in studies of the Chilean scallop *Argopecten purpuratus*. Larval growth and survival increased when this species was cultivated in water filtered though a 5 µm filter, compared with filtration though a 0.22 µm filter (Riquelme et al. 1997). In hatcheries, scallop larvae are normally fed various species of algae, typically *Pavlova lutheri*, *Isochrysis galbana*, *Skeletonema costatum*, *Chaetoceros calcitrans* and *Tetraselmis suecica*, in different combinations and concentrations (Ruiz-Ponte et al. 1999, Riquelme et al. 2001, Torkildsen & Magnesen 2004). The different algal cultures and the scallop larvae are associated with different bacterial communities (Sandaa et al. 2003). The use of bacterial supplements, as an addition to algal diets, was suggested by Douillet & Langdon (1993) and Douillet (1993a,b). By using ^{14}C-labelled live or heat-killed bacteria, Douillet (1993a,b) demonstrated that Pacific oyster, *Crassostrea gigas*, larvae can digest and assimilate bacterial carbon. Crosby et al. (1990) found similar abilities in the American oyster *C. virginica*. In early studies of the blue mussel *Mytilus edulis*, Birkbeck & McHenery (1982) concluded that mussels are capable of selecting lysozyme-sensitive bacteria for subsequent processing. Douillet & Langdon (1993) demonstrated that the addition of a particular bacterial strain, CA2, possibly an *Alteromonas* sp., enhanced the growth and survival of Pacific oyster larvae. The optimal bacterial concentration of CA2 was 10^5 cells ml^{-1} (Douillet & Langdon 1994). *P. maximus* larvae showed significantly lower mortality rates when cell extracts of *Roseobacter* strain BS107, were added to larval cultures (Ruiz-Pointe et al. 1999).

Since the literature cited above clearly documents that bacteria in bivalve cultures may represent both threats and valuable food components, it is important to describe the action of different bacteria found in larval cultures. An understanding of the processes involved in larval mortality is essential. The aim of the present study was to investigate the action of bacteria suspected of being pathogenic to scallop larvae. Larval groups were challenged with bacterial isolates from *Pecten maximus* larvae, resembling *Vibrio splendidus* and *Pseudoalteromonas* (Torkildsen 2004). Mortality was compared to that of larvae challenged with the known pathogenic bacterium *V. pectenicida* (Lambert et al. 1998) and an unchallenged control group. Immunohistochemistry was employed to assess uptake of the bacteria and their effect on larval tissues.

MATERIALS AND METHODS

Broodstock, oocytes and larvae. Scallop larvae were produced according to standard cultivation procedures at the scallop hatchery Scalpro A/S, Rong, near Bergen, as described by Torkildsen & Magnesen (2004). Broodstock originated from the County of Hordaland in western Norway (60° N). Oocytes were collected and hatched at the hatchery in April 2001. Spawning was induced by thermal shock, and the oocytes were fertilised as described by Gruffydd & Beaumont (1970). Larvae were kept in 800 l tanks with stagnant seawater at 18 ± 1°C taken from the nearby fjord at a depth of 60 m. The water was filtered through a 1 µm filter bag and renewed 3 times a week. Larvae were fed a diet consisting of mixed monocultures of the algae *Isochrysis galbana* (Parke) Tahitian strain, *Pavlova lutheri* (Droop), *Chaetoceros calcitrans* (Takano)/*C. mulleri*, *Skeletonema costatum and Tetraselmis suecica* at a ratio of 3:2:3:1:1 with a total concentration of 50 cells µl^{-1}.

Bacteria. The following bacteria were selected for challenge experiments: LT 06, LT 13, LT 21 and LT 73, isolated by Torkildsen et al. (2000, 2002, 2005) and PMV 18 and PMV 19 (C. Lambert unpubl.). LT 13 belongs to the genus *Pseudoalteromonas*, while the others (PMV 18, PMV 19, LT 06, LT 21 and LT 73) resemble *Vibrio splendidus* (Torkildsen et al. 2005). Challenge experiments included a negative control (unchallenged control) and a positive control challenged with *V. pectenicida* strain A496, a known pathogen of great scallop larvae (Lambert et al. 1998), referred to as challenged control. All bacteria were

stored at –80°C in a 20% glycerol/marine broth (Difco 2216) stock. They were incubated at 18°C and grown on petri dishes with Difco 2216 marine agar (MA) for 48 h. Colonies of the bacteria were transferred to Erlenmeyer flasks with 50 ml of marine broth (MB) (Difco 2216) and shaken at 90 rpm in a shaking incubator (INFORS AG) for 48 h. PMV 18 grows slower than the other strains and was therefore grown for 72 h. Bacteria were harvested by centrifugation (Heraeus Sepathec Megafuge 1.0 R) at 2772 × *g* for 10 min at 4°C, washed twice in phosphate-buffered saline (PBS) and resuspended in PBS. The number of live cells in the suspensions was determined by counting colony-forming units (CFU). Each bacterial suspension (100 µl) was plated on MA and grown for 48 h. Colonies were then counted, and total cell concentration was determined (Table 1). For each experiment, 2 parallel counts were made.

Challenge experiments. Three almost identical challenge experiments were performed. Scallop larvae were exposed to bacteria at different ages post hatching, 10 d in challenge Expt I, 13 d in Expt II and 15 d in Expt III. The multidish tray containing the control group of Expt I was lost during handling, and thus this experiment did not include a negative control. Larvae were transferred to 24-well polystyrene multidishes (Nunc) with 2 ml of sterile seawater (SSW) (28 ppt salinity), with about 20 to 40 larvae in each well. Ideally, the number of larvae per well should have been identical, but priority was given to minimizing handling. Given the small size (100 to 150 µm) and fragility of the larvae, an approximately equal number of larvae per well combined with a large number of replicates was considered preferable to the risk of losing larvae due to physical handling. To each well (except for the unchallenged control) 100 µl of the above-mentioned 48 h bacterial suspension (PBS+bacteria) was added. The cell concentrations of the bacterial suspensions were approximately 10^8 cells ml^{-1}, except for 3 suspensions that had a cell concentration of 10^7 cells ml^{-1} (Table 1). One multidish (i.e. 24 wells) was used for each bacterial strain. Inoculations were repeated with 10-fold and 100-fold dilutions of the bacterial culture. The larvae were incubated at 16°C in an air-conditioned room. Live and dead larvae were counted after 24 and 48 h using an inverted stereoscopic microscope (Leitz DM IL). Six wells were counted for each dilution and bacterial strain. Non-swimming larvae and larvae lying passively on the bottom were counted as dead. In some wells it was difficult to determine the exact number of live and dead larvae, especially when they were swimming rapidly. To reduce the possibility of error, the wells were counted twice. At the end of each counting session, 12 wells were emptied with a pipette, and larvae were fixed in 4% phosphate-buffered formaldehyde for further processing for immunohistochemistry. This procedure did not discriminate among live, moribund and dead larvae. The 12 wells that were emptied included the 6 counted wells.

Table 1. Cell concentration (cells ml^{-1})in all bacterial suspensions for each experiment, based on viability counts. CFU = colony-forming units on plates 1 and 2. 100 µl was added to each plate. A496 = *Vibrio pectenicida*. The bacterial strains PMV18, PMV 19, LT 06, LT 21 and LT 73 resemble *Vibrio splendidus*, while LT 13 resembles *Pseudoalteromonas*

Bacteria	Expt	Dilution plated	CFU Plate 1	CFU Plate 2	Cell conc.
A496	I	1.00×10^7	3	3	3.00×10^8
PMV 18	I	1.00×10^6	9	7	8.00×10^7
PMV 19	I	1.00×10^7	1	4	2.50×10^8
LT 06	I	1.00×10^7	3	3	3.00×10^8
LT 13	I	1.00×10^7	4	6	5.00×10^8
LT 21	I	1.00×10^7	4	2	3.00×10^8
LT 73	I	1.00×10^7	3	3	3.00×10^8
A496	II	1.00×10^6	3	5	4.00×10^7
PMV 18	II	1.00×10^7	1	3	2.00×10^8
PMV 19	II	1.00×10^7	3	6	4.50×10^8
LT 06	II	1.00×10^7	3	4	3.50×10^8
LT 13	II	1.00×10^7	5	7	6.00×10^8
LT 21	II	1.00×10^7	5	6	5.50×10^8
LT 73	II	1.00×10^7	4	4	4.00×10^8
A496	III	1.00×10^6	6	5	5.50×10^8
PMV 18	III	1.00×10^7	4	6	5.00×10^7
PMV 19	III	1.00×10^7	9	8	8.50×10^8
LT 06	III	1.00×10^7	8	9	8.50×10^8
LT 13	III	1.00×10^7	7	6	8.50×10^8
LT 21	III	1.00×10^7	5	5	5.00×10^8
LT 73	III	1.00×10^7	9	8	8.50×10^8

For bacterial samples, 100 µl water was taken from randomly selected wells from each group, and plated on MA. The remaining larvae were returned to the air-conditioned room. The counting procedure lasted about 15 min. Bacterial colonies grown from the wells were inoculated in Erlenmeyer flasks with 50 ml MB, incubated at 18°C and shaken at 90 rpm for 48 h. The cultures were frozen at –80°C in 20% glycerol until the polymerase chain reaction (PCR) was performed. Three counts were made for each experiment.

Statistical considerations. The survival/mortality data were not normally distributed; non-parametric tests were used for statistical analyses. Testing for several proportions ($\alpha = 0.025$, critical region: $\chi^2 < 12.832$ for $\nu = 5$ df) (Walpole et al. 2002) was performed to test the homogeneity among the 6 wells that were counted for each larval group, after 24 and 48 h. A 2×2 contingency table ($p < 0.05$, df = 1), performed

in Statistica v 5.0 (StatSoft), was used to test for mortality differences between the 2 control larval groups and the challenged test larval groups.

Polymerase chain reaction (PCR), DNA amplification. This method was used to verify that the bacteria present in the wells actually were LT 06 and LT 13. Specific primers were used for LT 06 f1: 55–82 (ACAGTAACAATCCTTCGGGTGCG), r1: 452–476 (TCAAGAGGCGCCGCTATTAACTAC) and LT 13 f1: 55–80 (AACAGAAAGTAGCTTGCTACTTGGC), r1: 435–460 (TCACAGCTAGCAGGTATTAACTACT). The fragment size produced by the LT 06 primers is 417 bp and the fragment produced by the LT 13 primers is 405 bp. The PCR was performed in reaction mixtures of 25 µl in 0.2 ml 8-strip PCR tubes (Axygen). The mixture contained 14.85 µl of distilled water, 3 µl of $MgCl_2$, 2.5 µl of PCR buffer, 2.5 µl of 1.25 mM dNTP (Promega Madison), 0.5 µl of each primer, 0.15 µl of *Taq* polymerase (Promega) and 1 µl of bacterial sample. The mixture without bacterial sample was used as a negative control, and LT 06 and LT 13 were used as positive controls. Reactions were carried out in a Gene Amp, PCR systems 9700 (Perkin Elmer) with an initial denaturation step of 94°C for 2 min, 35 cycles of denaturation (94°C for 1 min), annealing (62°C for 30 s), extension (72°C for 1 min) and final extension at 72°C for 5 min. Amplified DNA, 5 µl, was examined by horizontal 1% agarose (SeaKem LE). Gene Mass Ruler DNA ladder mix (MBI Ferments), 2 µl, was used as a nucleic acid standard. Visualisation was obtained by UV illumination after staining with ethidium bromide.

Immunohistochemistry. All microscope slides used for testing the different bacteria against the antiserum were coated with a diluted Poly-L-Lysine solution (Sigma Diagnostics). Coating was carried out to allow the bacteria to attach to the slides during the staining procedure.

Antisera were made for the bacterial strains *Vibrio pectenicida* (anti-A496), LT 06 (anti-LT 06) and LT 13 (anti-LT 13) and were produced according to the method of Oeding (1957). Formaldehyde-killed, washed bacteria were administered by intravenous injection to the rabbits. The polyclonal rabbit antisera were absorbed by the method of Knappskog et al. (1993), to minimize the possibility of cross-reaction. Each absorbed antiserum (anti-A496, anti-LT 06 and anti-LT 13) was tested for cross-reaction with bacterial strains and larval tissue samples prior to the immunohistochemistry. The dilution used on tissue samples of each antiserum was determined by testing a range of antiserum dilutions on bacterial samples.

Larval samples fixed in 4% phosphate-buffered formaldehyde were dehydrated in ethanol and embedded in paraffin. Larvae were sectioned at 3 µm (Leica Jung Biocut 2035), incubated at 58°C for 30 min, dewaxed in xylene, rehydrated in a series of ethanol baths and washed in running water. The absorbed polyclonal rabbit antisera, anti-A496, anti-LT 06 and anti-LT 13, were diluted 1:10 in Tris-buffered saline (TBS) with 2.5% bovine serum albumin (BSA). To prevent non-specific antibody binding, sections were blocked by using 5% BSA in Tris-buffered formaldehyde for 20 min. Avidine-biotin-alkaline phosphatase complex (ABC/AP) reaction kit (DAKO A/S) and New Fuchsin Substrate system (Dako) were used to visualise positive staining. Shandon's haematoxylin was used for counterstaining. At each stage of staining, 2 controls were used. Unchallenged larvae were used as negative controls and bacterial smears on microscope slides were used as positive controls. The same procedure was used to stain both larvae and bacteria. During the staining procedure, tissue sections and bacterial samples were kept separately in order to prevent cross-contamination. To ensure complete staining of all the larvae, each microscope slide was totally covered with the immunohistochemistry kit. All incubations were performed at room temperature (20°C) in a humidity chamber. A Leica DMBE microscope equipped with a Leica Wild MPS52 phototube was used to photograph the sections. Films used were Fujichrome 100 and Fujichrome 200.

RESULTS

Challenge experiments

Survival and mortality data were pooled if there were no significant differences among the 6 wells. From these results, 8 out of 46 of the chi-square statistical analyses rejected the H_o hypothesis, i.e. rejected that all populations had equal survival rates. We concluded that there were differences in mortality among the 6 wells (Tables 2 & 3).

In most challenged larval groups, mortality (i.e number of non-motile larvae) was higher at 48 than at 24 h after challenge (Table 3). The exception was larval groups challenged with LT 13, in which the mortality rate decreased in Expts II and III. In the unchallenged control group, there was no difference between 24 and 48 h (Table 3). Mortality within the unchallenged control larval groups was higher in Expt III than in Expt II.

Mortality in the unchallenged control larval groups was between 18.2 and 31.4% (average mortality was approximately 25%) (Table 3). In challenged control larval groups (larvae challenged with *Vibrio pectenicida*) the mortality rate varied between 17.6% and 65.7% (average mortality 37%) (Table 3). The difference in mortality rates between the 2 control groups was only significant in Expt III after 24 h (2×2 contin-

Table 2. Treatments found to be significantly different in mortality. The bacterial strains LT 21, LT 73 and PMV 19 resemble *Vibrio splendidus*, while LT 13 resembles *Pseudoalteromonas*

Expt	Time (h)	Bacteria	χ^2
I	24	LT 72	14.87
I	48	LT13	16.43
I	48	LT21	13.87
II	24	LT73	15.75
II	48	PMV 19	14.71
III	24	LT2 21	14.85
III	48	PMV 19	11.25
III	48	LT21	20.64

gency table, p = 0.0162). At that time, mortality rates in all challenged groups were significantly different from the unchallenged control larval group (Table 3).

Larvae challenged with LT 06 suffered an average mortality rate of approximately 40%, ranging between 28.6 and 64.7% (Table 3). Mortality in these groups was significantly different from the mortality in the unchallenged control larval groups in Expt III after 24 h (2 × 2 contingency table, p = 0.0110). When compared with mortality in the challenged control larval groups, a significant difference in mortality was found in Expt I after 24 h of challenge (2 × 2 contingency table, p = 0.0051).

In general, the challenge with LT 13 resulted in low mortality in most larval groups and the average percentage mortality was lowest of all challenged groups (approximately 29%). Mortality in these larval groups was the same as or less than in the unchallenged control groups (Table 3), and was significantly different only in the Expt III after 24 h (2 × 2 contingency table, p = 0.0286). Mortality in larval groups challenged with LT 13 was significantly different from the challenged control larval groups in Expts II and III, 48 h post-challenge (2 × 2 contingency table, p = 0.0019 and p = 0.0258, respectively)

Challenge with LT 21 caused an average mortality rate of approximately 45% (Table 3), and mortality was significantly different from that in the unchallenged controls in Expt II, 24 and 48 h after the challenge (2 × 2 contingency table, p = 0.0294 and p = 0.0000, respectively) and Expt III after 24 and 48 h (2 × 2 contingency table p = 0.0010 and p = 0.0000). In comparison with the challenged control larval groups, the difference in mortality was significant in Expt I, 24 h post challenge (2 × 2 contingency table, p = 0.0002) and in Expt II and III after 48 h of challenge (2 × 2 contingency table, p = 0.0126 and p = 0.0026, respectively).

Challenge with the bacterial strain LT 73 resulted in high mortality in all challenge experiments, especially in Expt III after 48 h of challenge (86.4%) (Table 3). Average mortality was approximately 49%, which was the highest of all groups. The mortality was significantly different from that in the unchallenged control larvae in Expt II after both 24 and 48 h (2 × 2 contingency table, p = 0.0349 and p = 0.0000, respectively), and in Expt III after 24 and 48 h of challenge (2 × 2 contingency table, p = 0.0127 and p = 0.0000, respectively). When compared to the mortality experienced in the challenged control larval groups, significant differences were found in Expts I and III after 24 h (2 × 2 contingency table, p = 0.0000 and p = 0.0162, respectively).

PMV 18 caused an average mortality of approximately 38% (Table 3). Mortality in larval groups challenged with PMV 18 and the unchallenged control larval groups was significantly different in Expts II and III after 24 h (2 × 2 contingency table, p = 0.0000 and p = 0.0001, respectively). Compared with the challenge control larval groups, the mortality in larval groups challenged with PMV 18 was significantly different in Expt I at both 24 and 48 h (2 × 2 contingency table, p = 0.0245 and p = 0.0009) and in Expt II after 24 and 48 h

Table 3. Percent mortality of challenge Expts I, II and III including unchallenged and challenged control groups. Challenged control: larval groups challenged with *Vibrio pectenicida*. ND: no data (Challenge Expt I did not include an unchallenged control group). *Significantly different from the unchallenged control larval groups (p < 0.05). ^Significantly different from the challenged control larval groups (p < 0.05). The bacterial strains PMV18, PMV 19, LT 06, LT 21 and LT 73 resemble *Vibrio splendidus*, while LT 13 resembles *Pseudoalteromonas*

Expt	Time (h)	Unchallenged control	Challenged control	PMV 18	PMV19	LT 06	LT 13	LT 21	LT 73
I	24	ND	17.6	29.4^	36.8^	31.5^	26.3	33.3^	36.5^
I	48	ND	65.7	40.7^	75.0	64.7	50.5	65.9	53.0
II	24	18.2	24.1	60.6*^	18.5	28.6	18.0	27.2*	27.9*
II	48	19.3	26.6	12.8^	18.8	29.4	10.4^	39.9*^	46.8*^
III	24	31.4^	46.4*	50.0*	40.8*	44.3*	42.6*	47.1*	43.8*
III	48	31.0	39.3	36.9	49.6*^	39.7	23.6^	53.6*^	86.4*^
Average		20.5	36.6	38.4	39.9	39.7	28.6	44.5	49.1

(2×2 contingency table, $p = 0.0000$ and $p = 0.0005$, respectively).

Larvae challenged with PMV 19 experienced an average mortality of approximately 40% (Table 3). These larval groups were significantly different from the unchallenged control larval groups in Expt III after 24 and 48 h (2×2 contingency table, $p = 0.0481$ and $p = 0.0003$, respectively). When compared to larval groups challenged with *Vibrio pectenicida*, significant differences in mortality were found in Expt I after 24 h (2×2 contingency table, $p = 0.0002$) and Expt III after 48 h (2×2 contingency table, $p = 0.0370$).

PCR verified that the randomly sampled bacteria taken after each counting (24 and 48 h) and challenge experiment, were LT 06 and LT 13 that were used for challenge (Figs. 1 & 2). The use of specific primers against these 2 bacterial strains produced fragments similar in size to the fragment size produced by the primers LT 06 (417 bp) and LT 13 (405 bp). Some variations in the intensity of the fragments were detected.

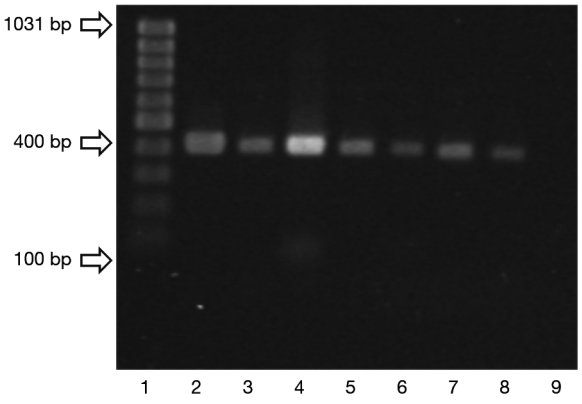


Fig. 1. Agarose gel electrophoresis photo of PCR-amplified 16S rDNA in bacterial isolates sampled from the 24-well multidishes challenged with LT 06. Lanes 2 to 7: bacterial isolates taken from the 24-well mulitidishes from the different challenge experiments randomly sampled after each count; Lane 8: LT 06 used as positive control; Lane 9: Strain LT 13 used as negative control; Lane 1: standard marker. All templates are diluted 1:100

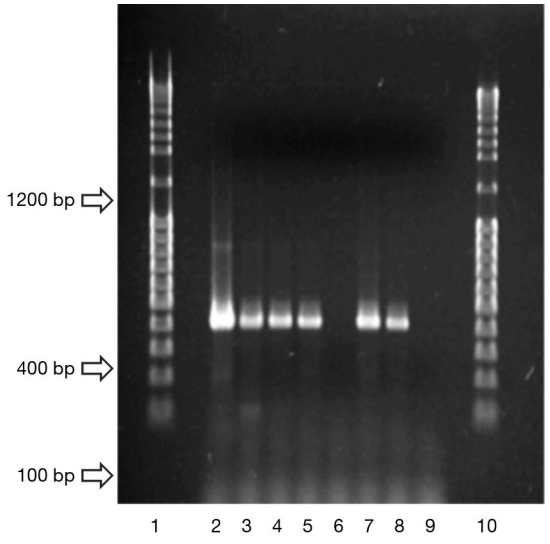


Fig. 2. Agarose gel electrophoresis photo of PCR amplified 16S rDNA in bacterial isolates sampled from the 24-well multidishes challenged with LT 13. Lanes 2 to 5: bacterial isolates taken from the 24-well multidishes from the different challenge experiments randomly sampled after each count; Lane 6: bacterial strain LT 06 used as negative control; Lanes 7 and 8: LT 13 used as positive control; Lane 9: core mix with no template; Lanes 1 and 10: standard markers. All templates are diluted 1:100

Immunohistochemistry

Formaldehyde fixation produced contraction of the larvae prior to embedding, complicating the reading of slides and interpretation of immunohistochemical staining. The structure of larvae, before and after fixation, is shown in Fig. 3. Fig. 3c was drawn from Fig. 4, and represents an explanation of Figs. 4 to 13.

Cross-reactions among the 3 antisera, anti-A496, anti-LT 06 and anti-LT 13 were not found, either when tested directly on bacteria or on larval tissue samples. The unchallenged control larvae did not display positive immuno-staining when stained with anti-A496 and anti-LT 06 (Fig. 4). However, approximately 50% of the unchallenged control larvae displayed specific immuno-staining inside the stomach and digestive area and endothelial cells when stained with anti-LT 13 serum (see Fig. 13). This staining was generally weaker than in larvae challenged with LT 13. The morphology of all control larvae was normal and displayed no signs of tissue damage (Fig. 4). The immunohistochemical examinations showed no relationship between pathogenesis and larval age.

Immuno-staining revealed large differences among the 3 larval groups; challenged control larval groups (larvae challenged with *Vibrio pectenicida*) and larval groups challenged with LT 06 or LT 13. Larvae challenged with *V. pectenicida* and LT 06 displayed similarities when immuno-stained (Figs. 5 & 8) and showed similar signs of infection in comparison with larvae challenged with LT 13. In addition, most larvae were positively stained on the outer shell and mantle surfaces.

All challenged control larvae examined displayed positive immuno-staining. Infection was located in oesophagus, stomach and rectum, spreading in the areas around the digestive mass, especially the endothelial mucosa, and to the surrounding tissues. In most of the larvae, necrotic tissue and pycnotic cells could be observed (Figs. 5 & 6). In challenge Expt III a

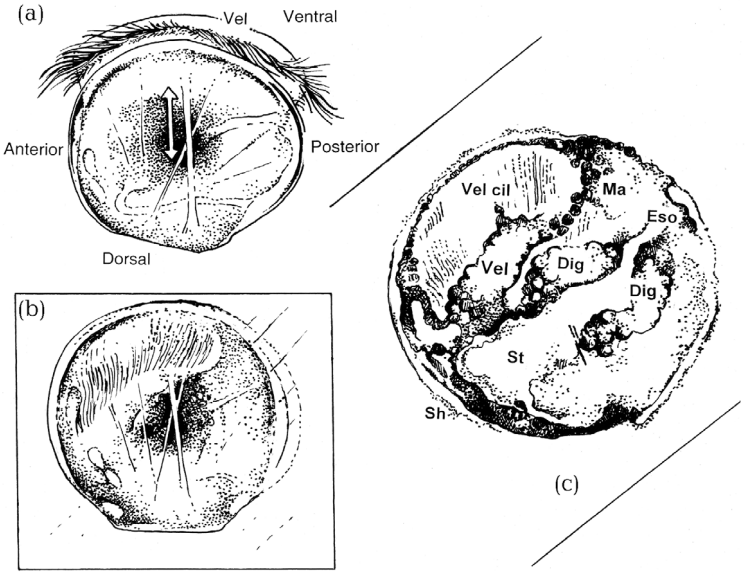


Fig. 3. *Pecten maximus*. Orientation and tissues seen in the sections (see Figs. 4–13). (a) illustrates the larva prior to fixation and how the velum contracts (arrows). (b) illustrates the larvae after fixation, with the contracted velum inside the thin shell, folded inwards with a longitudinal furrow and ciliar rim, underlying the mantle lobes. The longitudinal section that cuts through the approximate centre of the larva (indicates orientation of the larva section, cut to illustrate [c]), includes parts of stomach and gut. The ventral part includes parts of the contracted velum and mantle. (c) Tissues and body compartments of the larva corresponding to the larva shown in Fig. 4 (where strong staining covers the details and makes difficult any differentiation between the mantle cells, the thin epithelium of the velum roof and the larger cells of the velum basis). Lines indicate counter-clockwise turning, to the same orientation as Fig. 4. Eso: esophagus; St: stomach; Dig: digestive cells; Sh: shell matrix; Vel cil: velum cilia; Ma: mantle cells

clear difference was seen in the positive immuno-staining between 24 and 48 h (Figs. 6 & 7). All larvae challenged for 48 h were generally infected and appeared positively immuno-stained in most or all tissues (Fig. 7). This clear difference was not found in the 2 previous challenge experiments or in larval groups challenged with LT 06 and LT 13. Despite the positive intracellular immuno-staining of mucosal cells (Fig. 6, arrow), no positive identification of bacterial cells was confirmed in any of the individuals examined, either inside the digestive mass or intracellularly in epithelial cells.

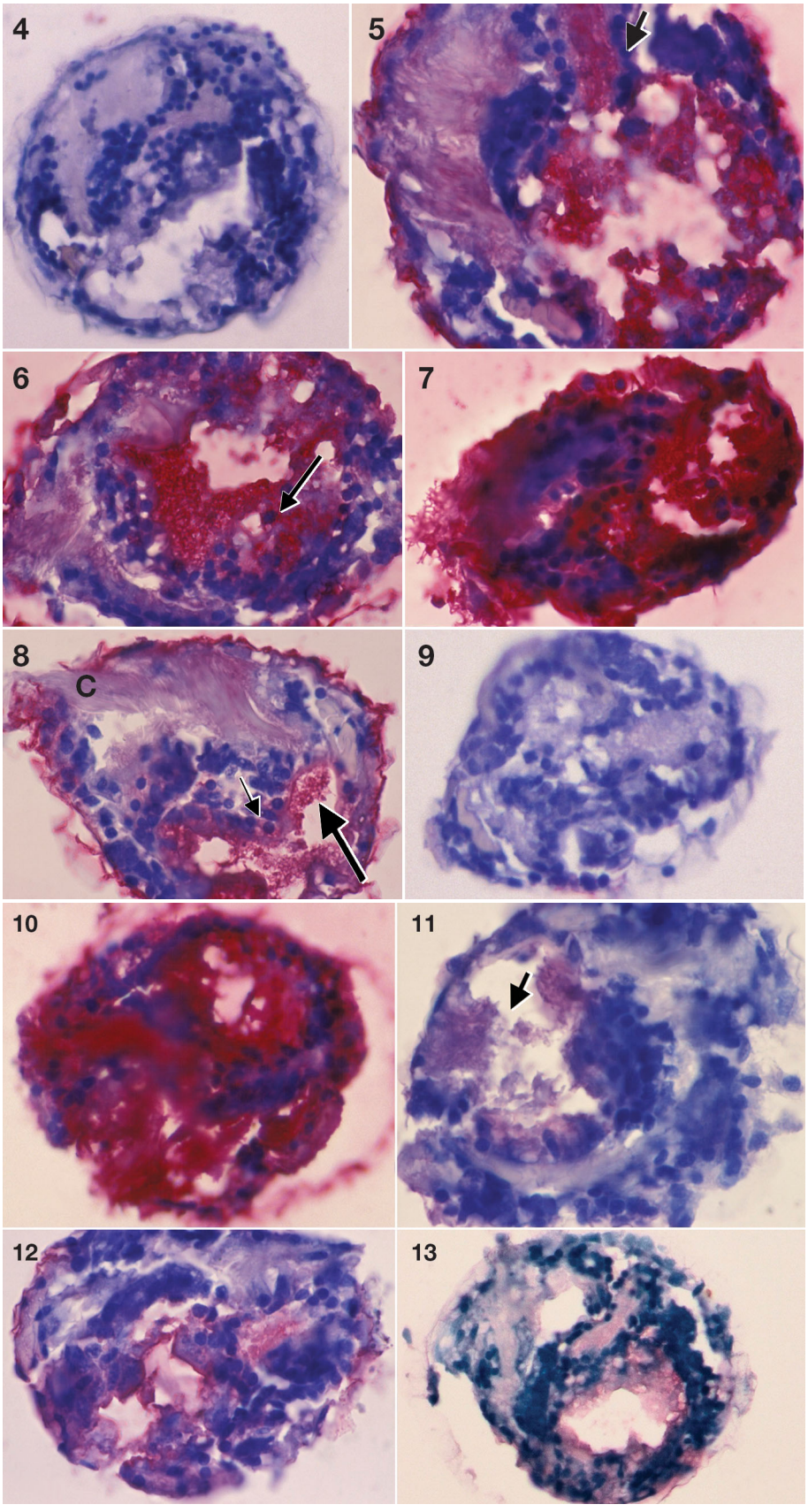
In contrast to larvae challenged with *Vibrio pectenicida*, examination of larvae challenged with LT 06 revealed apparently intact bacterial cells inside the digestive mass and inside mucosal cells (Fig. 8, indicated with small arrow). Otherwise, the challenge results with LT 06 apparently had similar impact on the scallop larvae as *V. pectenicida*. Infection was basically located in the gut and the digestive area, although spread to the surrounding area in some larvae. Although a few larvae were generally infected, larvae challenged for 48 h did not appear to be more severely affected by the infection than larvae challenged for 24 h. Despite challenge, 2 larvae, one in challenge Expt I (48 h) and one in challenge Expt III (48 h), displayed a total absence of specific immuno-staining (Fig. 9). Other larvae on the same sections, adjacent to these unstained larvae, were all positively stained (Fig. 10).

All the examined larvae examined from the challenge with LT 13 displayed positive immuno-staining. The infection was apparently less severe, as they were less positively stained than larvae challenged with *Vibrio pectenicida* and LT 06. Positive immuno-staining was limited to visual bacteria inside the digestive area and mucosal cells (Figs. 11 & 12). Surrounding tissues were not infected and appeared normal. Intracellular bacteria could not be observed.

DISCUSSION

Scallop larva challenged with *Vibrio pectenicida* and the *V. splendidus*-like strain LT 06 showed many similarities regarding pathology, immuno-staining and mortality. Apparently our study is the first attempt to characterise such infections in bivalves by immunohistochemistry. This method enabled us to visualise affected tissue in the digestive mass and gut area of larvae challenged with these bacteria. Infection seemed to spread from these areas to the surrounding tissues. Together with the mortality results, these observations demonstrate that LT 06, like *V. pectenicida*, is pathogenic to scallop larvae. This supports the results of Nicolas et al. (1996) who described a strain resembling *V. splendidus* associated with mortality in scallop *Pecten maximus* larval cultures.

Immunohistochemical examinations of larval groups challenged with *Vibrio pectenicida* revealed no bacterial cells, despite a high degree of positive immuno-staining. A general experience at our laboratory in studies of fish larvae with identical immuno-staining protocols (Ø. Bergh unpubl.) is that tissue samples exposed to pathogenic *Vibrio* spp. will be positively stained in areas around the stained bacteria. This is probably due to staining of partially dissolved extracellular bacterial products. Lambert et al. (1998, 2001)



Figs. 4 to 13. *Pecten maximus*. Immunohistochemical staining of paraffin sections from larvae. Avidine-biotin-alkaline phosphatase method, rabbit anti-A496, -LT 06 and -LT 13 serum and Shandon haematoxylin counterstained. Positive immunohistochemistry is visualised by red colour. Counterstaining gives tissues different tones of blue. Larval diameters range between 90 and 150 µm. Fig. 4. Unchallenged, healthy larva stained with anti-LT 06. Figs. 5, 6 & 7. Larva challenged with *Vibrio pectenicida* for 24 h (Fig. 6) and 48 h (Figs. 5 & 7) stained with anti-A496. In Fig. 5 note intracellular staining in mucous cells (arrow). Red area inside digestive mass is probably a cluster of bacteria. Figs. 6 & 7 show variation in infectivity between 24 and 48 h observed in Expt III. In Fig. 6 the esophagus (arrow) and digestive areas, in particular, are clearly positively stained together with the shell surface. Fig. 7. Totally infected larvae 48 h post challenge. Figs. 8, 9 & 10. Larvae challenged with LT 06 for 48 h, stained with anti LT 06. Fig. 8. Mantle surface, shell matrix and stomach wall are positively stained, and bacteria are visible and positively stained in the stomach (large arrow). Intracellular bacteria are verified in mucous cells (small arrow). The cilia (C) are also positively stained. Fig. 9. Note absence of immuno-staining in spite of bacterial exposure for 48 h. Larvae show no signs of infection. Fig. 10. Larvae situated next to larvae in Fig. 8. Note the generally positive immuno-staining. Figs. 11 & 12. Larvae challenged with LT 13 for 24 and 48 h, respectively, stained with anti-LT 13. Fig. 11. Bacteria are seen in stomach and digestive area (arrow). Positive staining is restricted to digestive mass, showing no signs of infiltrating the surrounding area. Fig. 12. Positive immuno-staining is seen on shell surface, on stomach wall and in esophagus. Fig. 13. Unchallenged control larvae stained with anti-LT 13. Positive immuno-staining is seen in esophagus, stomach lumen and endothelial cells

demonstrated that *V. pectenicida* is highly toxic to *Pecten maximus* larvae due to an intracellular release of toxins that inhibit the respiratory burst activity of the haemocytes. These toxins may infiltrate the larval tissue. It is likely that polyclonal antiserum anti-A496 labelled some of these released toxins and possibly other compounds, and thereby positively stained the larval tissue, even though no bacterial cells were found. Verification of the presence of bacterial cells was difficult in all challenged larvae, partly due to clustering of bacterial cells. The large red area inside the digestive mass in some larvae (see Fig. 6) could be clusters of bacteria. The size of the scallop larvae (approximately 100 to 150 µm) and the low number of cells made observation of tissue degeneration and necrosis difficult. Most challenged larvae displayed positive immuno-staining on the shell surface and mantle. This could be due to bacterial adhesion to the surface of the larvae, but could also be due to lack of washing of the larvae prior to fixation.

Few papers describe scallop larval anatomy, and the majority are based on electron microscopy of veliger larvae (Cragg & Crisp 1991). However these examinations have limited relevance to immunohistochemical examinations. The development of the immunohistochemical protocol described herein was optimised for the visualisation of the presence and modes of action of certain bacteria. However, the method is clearly suboptimal for purposes of precise morphological studies.

The challenge experiments showed that all challenged larval groups suffered higher average mortality than the unchallenged control groups. In comparison with the larval groups challenged with *Vibrio pectenicida*, all challenged groups, except those challenged with LT 13, experienced average higher mortality. From the mortality results, we consider that the bacterial strains PMV 18, PMV 19, LT 06, LT 21 and LT 73 are pathogenic to scallop larvae.

In contrast, larval groups challenged with LT 13 suffered relatively low average mortality and the immunohistochemical examinations revealed a lesser degree of infection compared with larvae challenged with *Vibrio pectenicida* and LT 06. However, Denaturating Gradient Gel Electrophoresis (DGGE) showed bacteria apparently identical to LT 13 to be frequently present in larval cultures suffering from high mortality (R. A. Sandaa unpubl.). Thus this bacterium may act as a secondary opportunistic pathogen that causes disease and mortality in already weakened larvae. Many other factors, such as broodstock condition, egg quality, larval condition and feed affect larval growth, development and survival in mussels (Phillips 2002) and scallops (Seguineau et al. 1996, 2001, Soudant et al. 1998).

Some of the unchallenged control larvae were positively stained with the antiserum anti-LT 13 in the digestive mass and gut area. Compared with larvae challenged with LT 13, this positive staining was weaker. Although neither bacterial samples nor larval tissue samples revealed cross-reaction, cross-reaction with other bacteria present inside the larval lumen is possible. The scallop larvae in our experiment were not axenic, and were not kept in a sterile environment prior to the challenge experiments. When specific primers for the bacterial strains LT 06 and LT 13 were used, no other bacteria present in the wells were detected. Recently, characterisations of bacterial flora associated with *Pecten maximus* larvae have been performed by DGGE of PCR-amplified 16S rDNA (Sandaa et al. 2003). This method provides an overview of the bacterial community, including both culturable and non-culturable components. Full sequencing of the 16S rDNA and Restriction Fragment Length Polymorphism (RFLP) for genotyping 16S rRNA are 2 methods for characterising unknown cultured bacteria. The latter was used by Jensen et al. (2002) to characterise bacteria cultured from halibut *Hippoglossus hippoglossus* fry. LT 13 has been isolated from the microalgae *Chaetoceros calcitrans*, which is used as feed for scallop larvae (Torkildsen et al. 2005). The bacterium could be inside the larvae as a consequence of feeding. Bacteria accumulating in the algal cultures may influence the bacterial flora associated with filter-feeders such as bivalve larvae. Skjermo & Vadstein (1993) found that adding algae to the rearing water of halibut larvae increased the bacterial concentration by 45% and that the bacterial flora associated with fish larvae was related to the flora in the water. The DGGE profile of the bacterial community in a mixed algal culture resembled the DGGE profile of the algae present in the highest concentration (Sandaa et al. 2003). This suggests that additions of algae may influence the bacterial community. However, a clear difference in the DGGE profile of bacteria associated with the scallop larvae and the DGGE profile of the water samples was also found.

The bacterial strains PMV 18, PMV 19, LT 06, LT 21 and LT 73 all resemble *Vibrio splendidus* (Torkildsen et al. 2005). *V. splendidus* strains are widely spread in the marine ecosystem and cause disease in various aquatic organisms including the Pacific oyster (Lacoste et al. 2001, Waechter et al. 2002), great scallop (Nicolas et al. 1996), turbot *Scophthalmus maximus* larvae (Gatesoupe et al. 1999), gilt-head sea bream *Sparus aurata* (Balebona et al. 1998) and juvenile giant tiger shrimps *Penaeus monodon* (Leaño et al. 1998). The *V. splendidus* biovar II-related strain TNEMF6, caused a cumulative mortality of 80% in Pacific oyster spat (Waechter et al. 2002). *V. splendidus* infect a wide

range of animals. Not all *V. splendidus*-related strains are pathogenic (Waechter et al. 2002). This could explain the differences in mortality among the strains used in this challenge experiment. The number of larvae inside the wells likely affected larval survival. Wells containing large amounts of larvae may provide better growth for the bacteria and an increased in infection rates.

Three of the bacterial suspensions were at a concentration of 10^7 cells ml^{-1} instead of 10^8 when they were added to the wells. In Expt II the concentration of bacteria in the suspension of *Vibrio pectenicida* was 4.0×10^7 CFU ml^{-1}, and in Expts I and III the bacterial concentrations were 3.0×10^8 and 5.5×10^8 CFU ml^{-1}, respectively. The low mortality in Expt II compared to the mortality in Expt I may have been related to the differences in cell concentration of the bacterial suspensions. However, concentration differences between the suspensions used in Expts II and III did not affect the mortality. Cell concentrations of the PMV 18 suspensions added in Expts I and III were 8.0×10^7 and 5.0×10^7 CFU ml^{-1}, respectively. In Expt II the concentration was 2.0×10^8 CFU ml^{-1}. PMV 18 caused similar mortality rates in all 3 challenge experiments. Thus it cannot be concluded if differences in bacterial concentrations may have affected larval mortality. In a similar multidish challenge experiment with *Tenacibaculum ovolyticum* (previously *Flexibacter ovolyticus*), *V. anguillarum* and *Aeromonas salmonicida* subsp. *salmonicida*, on eggs and larvae of Atlantic halibut *Hippoglossus hippoglossus* and Atlantic cod *Gadus morhua*, mortality increased with increasing challenge dose (Bergh 2000).

In conclusion, immunohistochemistry can be a powerful tool for studies of diseases of larval bivalves. Our results indicate that the *Vibrio splendidus*-like strains tested are pathogenic to scallop larvae. The pathology resembles infections with *V. pectenicida.* The *Pseudoalteromonas*-like strain LT13 is probably not a primary pathogen, but could act as a secondary opportunistic bacterium.

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