French Scallops: A New Host for Ostreid Herpesvirus-1

Isabelle Arzul,* Jean-Louis Nicolas,† Andrew J. Davison,‡ and Tristan Renault* 1

*IFREMER, Laboratoire de Génétique et Pathologie, 17390 La Tremblade, France; †IFREMER, Laboratoire de Physiologie des Invertébrés, 29280 Plouzané, France; and ‡MRC Virology Unit, Church Street, Glasgow G11 5JR, United Kingdom

Received August 13, 2001; returned to author for revision September 4, 2001; accepted September 12, 2001

Sporadic high mortalities were reported among larval French scallops (Pecten maximus). Electron microscopy of moribund larvae revealed particles with the characteristics of a herpesvirus in association with cellular lesions. PCR and DNA sequencing showed that the virus is a variant of ostreid herpesvirus-1 that has already been described in clams and oysters. This is the first description of a herpesvirus infection of a scallop species. The virus was transmitted successfully from an extract of infected scallop larvae to uninfected scallop or oyster (Crassostrea gigas) larvae, demonstrating that it is able to infect both species. Detection of viral DNA in asymptomatic adult scallops by in situ hybridisation indicates that the herpesvirus may have been transmitted from adults to larvae. It is notable that, unlike most herpesviruses, this virus has a wide host range reflected by its ability to infect several species of marine bivalve.

Key Words: herpesvirus; scallop; Pecten maximus; OsHV-1; polymorphism; transmission; host range; bivalve.

INTRODUCTION

Viruses morphologically similar to members of the family Herpesviridae have been identified in various marine bivalve species around the world (Renault, 1998). The first observation was reported in Crassostrea virginica adults (Farley et al., 1972). Subsequently, herpes-like viruses were associated with high mortality rates in other cultivated oyster species including Ostrea edulis (Comps and Cochenec, 1993; Renault et al., 2000a) and C. gigas in French (Nicolas et al., 1992; Renault et al., 1994a) and New Zealand (Hine et al., 1992) hatcheries. Herpes-like viruses were also observed in haemocytes of O. angasi adults in Australia (Hine and Thorne, 1997) and in New Zealand in flat oysters, Tiostrea chilensis (Hine et al., 1998). One of these viruses, isolated from moribund larval C. gigas, has been characterised as ostreid herpesvirus-1 (OsHV-1) (Minson et al., 2000), and its 207-kb genome has been completely sequenced (A. J. Davison, unpublished data). Herpesviruses have also been characterised in nonostreid bivalves, the European clam, Ruditapes decussatus, and Manila clam, R. philippinarum (Renault and Arzul, 2001; Renault et al., 2001). A variant of OsHV-1 (hereafter termed OsHV-1var) was detected in Manila clam and Japanese oyster larvae which presented concomitant mortalities in 1997 in a French hatchery (Arzul et al., 2001b). OsHV-1 and OsHV-1var are representatives of a single viral species that may be the ubiquitous cause of observed herpesvirus infections of marine bivalves.

In September 2000, high mortality rates (100%) occurred among certain batches of scallop, Pecten maximus, larvae in a Breton commercial hatchery in France. Scallop aquaculture was developed locally in Brittany for restocking natural French environments, as this species had been seriously depleted in the 1980s owing to intense fishing pressure and unfavourable meteorological conditions. Breeding of larval and postlarval scallops is intensive and at risk from epizootic diseases, especially those caused by bacteria such as the Vibrio species (Nicolas et al., 1996). With the exception of small DNA-negative virus-like particles reported in P. noraeelandiae (Hine and Wesney, 1997), viral aetiology has not been linked to scallop mortalities. We describe here for the first time a herpesvirus that infects P. maximus and show that it is ostensibly the same at OsHV-1var. Herpesviruses are usually detected in larvae and spat, but some reports concern adults (Farley et al., 1972; Hine and Thorne, 1997), suggesting that bivalve herpesviruses are able to persist in adults without mortality (I. Arzul, unpublished data). Adult scallops belonging to the batch which produced infected larvae were analysed by in situ hybridisation and shown to be carrying the herpesvirus.

RESULTS

Analysis of moribund larvae

Detection of Herpes-like Virus Particles by TEM. Viral particles were observed in the nuclei and cytoplasm of infected cells in the connective tissues of moribund P. maximus larvae collected at 7 or 10 days old. Nuclear particles were circular or polygonal in shape, 74–86 nm in diameter (Fig. 1). Some nuclear particles contained an
electron-dense structure, and other particles appeared empty (Fig. 2). Naked nucleocapsids were also observed free in the cytoplasm of lysed infected cells (Fig. 3). Enveloped virions were detected in cytoplasmic vesicles (Fig. 4) and also in perinuclear spaces (Fig. 5). The occurrence of nuclear tubular structures 45 to 55 nm in diameter was also observed (Fig. 4). Extracellular particles were usually enveloped and measured approximately 110 nm in diameter. Infected cells showed hypertrophied nuclei and marginated chromatin densely packed around the nuclear envelope (Fig. 3). Degenerating and lysing infected nuclei and cells were observed frequently (Figs. 3 and 4).

Detection of Oyster Herpesvirus DNA by PCR Analysis. Four samples (1–4) were analysed using primer pairs Gp3/Gp4, B3/B4, and C2/C4. Samples 1 and 2 consisted of 7-day-old moribund *P. maximus* larvae and samples 3 and 4 consisted of 10-day-old moribund larvae. The Gp3/Gp4 and B3/B4 primers amplified fragments of the ex-
pected sizes from the four larval samples and OsHV-1 DNA (Figs. 6a and 6b). The C2/C4 PCR products were about 180 bp smaller than those obtained with OsHV-1 DNA (Fig. 6c). No PCR product was detected in negative controls.

Analysis of Viral DNA by Sequencing. The Gp3/Gp4 and C2/C4 PCR products derived from larval samples 3 and 4 were sequenced. Two independent plasmids were processed for each product to rule out errors induced by PCR amplification. The sequences of each pair were identical. The sequences of the Gp3/Gp4 fragments from samples 3 and 4 were identical to each other and 99% identical to the OsHV-1 sequence (Fig. 7a). The nucleotide differences resulted in seven amino acid residue substitutions, yielding 97% identity in the predicted protein sequences (Fig. 7b). The sequences of the C2/C4 fragments from samples 3 and 4 were identical to each other and to that of OsHV-1 var (Arzul et al., 2001b), but different from that of OsHV-1. Differences between OsHV-1 and OsHV-1 var included several single nucleotide substitutions, insertions, or deletions and, more notably, a deletion of 200 bp near the C2 primer site accompanied by an insertion of 27 bp. Arzul et al. (2001b) registered the possibility that OsHV-1 var lacks the region containing C2 and that this primer instead hybridises fortuitously to a partially matched region.

Transmission assays of viral infection

Intraspecies Transmission of Viral Infection. In the three replicates of assays 1 and 2, larva presented high mortality rates which reached 100% at 5 days after exposure to extract from moribund *P. maximus* larvae (Table 1). Two days after exposure, the mean mortality rates were 26.7 and 22.3% for the three replicates of assay 1 and assay 2, respectively (Table 1). TEM revealed intracellular virus particles in these larvae. In the three replicates of assay 3, larva not exposed to extract presented mean mortality rates of 2% at 2 days after exposure and 6% at 5 days after exposure (Table 1). No viral particle was observed in these larvae by TEM.

Interspecies Transmission of Viral Infection from Scallop Larva to Oyster Larvae. Three days after exposure to extract from moribund *P. maximus* larvae (assays 1–5) or sterile seawater (assays 6–7), *C. gigas* larvae were recovered, washed three times, and prepared for PCR. The initial inoculum, the three wash samples (S1–S3), and the larvae were analysed using primer pair C2/C6. Products from the inoculum appeared 180 bp smaller than those obtained with OsHV-1 DNA (Fig. 8). Samples S1–S3 in assays 6–7 did not yield product (Table 2). For assays 1–5, the products from S1 and S2 were the same size as those from the inoculum (Table 2 and data not shown), and S3 did not yield product (Table 2). Products from larvae in assays 1–5 were the same size as those from the inoculum (Fig. 8). Larvae from assays 6–7 and all the negative controls did not yield product (Fig. 8).

Detection of oyster herpesvirus DNA in adult scallops by *in situ* hybridisation

To investigate the presence of the herpesvirus in adult *P. maximus*, 10 specimens were processed for *in situ* hybridisation using digoxigenin-labelled DNA probes. Positive reactions characterised by a purple precipitate (dark area on Fig. 9) were detected in seven animals. A similar signal was detected in infected spat, but no signal was detected when digoxigenin-labelled probe or antibody were omitted or when a digoxigenin-labelled probe specific to SVCV was used.
The abundance and intensity of the hybridisation signal in positive adult scallops varied between individuals and tissues. Labelling consisted of a small number of cells in the connective tissues of gills, gonad (Fig. 9), mantle, and muscle. Staining was detected in both the cytoplasm and the nuclei, and some stained cells exhibited anomalous chromatin patterns, including margination. In some cases, signal was also detected in epithelial cells of the gills.

**DISCUSSION**

Several descriptions of herpes-like virus infections in marine bivalves have been reported since 1972 in various species of oyster (Farley et al., 1972; Hine et al., 1992; Nicolas et al., 1992; Comps and Cochenne, 1993; Renault et al., 1994a; Hine and Thorne, 1997; Hine et al., 1998; Renault et al., 2000a) and clam (Renault and Arzul, 2001; Renault et al., 2001). We now describe for the first time the nucleotide and amino acid sequences of the Gp3–Gp4 fragment from scallop larvae (S) and reference OsHV-1 DNA (R). Nucleotide or amino acid substitutions are underlined in the S sequence. Primer sequences are in lower case.
time a herpesvirus infecting a species of scallop, *P. maximus*, in association with high larval mortality rates.

The virus particles observed by TEM in moribund *P. maximus* larvae collected from a commercial hatchery resemble those of herpesviruses of higher and lower vertebrates in morphology, virogenesis, and size range (Roizman, 1982, 1990; Roizman and Baines, 1991). These general characteristics of virus particles are similar to those documented previously in other bivalve species (Renault et al., 1994b, 2001; Hine and Thorne, 1997; Hine et al., 1998). A variety of morphological forms corresponding to replicating virus was noted in intranuclear and intracytoplasmic locations. Two classes of nucleocapsid were observed. One had an electron-dense core and corresponds to DNA-containing capsids, and the other lacked the core. Enveloped capsids (virions) were observed in perinuclear spaces, cytoplasmic vesicles, and extracellular locations. Virus replication was associated with various cellular lesions, including cell lysis. Thus, the herpesvirus exhibits all the microscopic features of productive replication in scallop larvae. This is in contrast to another virus (infectious pancreatic necrosis virus), which has been described as being sequestered in the digestive tissues of *P. maximus* without replicating and being progressively transmitted through food chains to brown trout (Mortensen, 1993; Mortensen et al., 1998).

The high mortality rate (100%) associated with detection of virus particles 5 days after exposure of healthy *P. maximus* larvae to infected larval extract fulfills Koch’s postulates. Low mortality rates (6%) were observed 5 days after exposure of healthy larvae to sterile water, and no virus particles were detected. These results demonstrate that the herpesvirus induces larval mortalities and

![FIG. 8. PCR analysis of samples subjected to interspecies transmission assays using primer pair C2/C6. I: inoculum of moribund scallop larval extract; 1–5: *C. gigas* larvae exposed to the inoculum; 6–7: *C. gigas* larvae exposed to sterile water; A–C: negative controls (distilled water); P: positive control (OsHV-1 DNA); L: 100-bp ladder (Eurogentec). The upper inverted comma indicates the positive control. The lower inverted comma indicates PCR products from samples.](image)

![FIG. 9. Positive cells characterised by a nuclear dark staining in gonad connective tissues from adult scallop (arrows).](image)
can be transmitted horizontally from infected to healthy scallop larvae. Variable mortality rates (0–62%) in larvae at 2 days after infection were probably due to variation in the quantity of infectious virus particles present in the inocula.

Infected larvae were analysed by PCR and DNA sequencing to assess whether the scallop herpesvirus is similar to OsHV-1. The ability to generate Gp3/Gp4 and B3/B4 PCR products of the expected sizes using primers based on the OsHV-1 sequence indicates that the scallop virus is very similar to OsHV-1. However, the sequence of the Gp3/Gp4 product shows that the scallop virus is closely related, but not identical, to OsHV-1. Unusually, each of the seven nucleotide substitutions is predicted to result in an amino acid substitution. The C2/C4 product was the same size as that for OsHV-1var predicted to result in an amino acid substitution. The sequence of the Gp3/Gp4 product shows that the scallop virus is very similar to OsHV-1. However, the sequence indicates that the scallop virus is closely related, but not identical, to OsHV-1. Unusually, each of the seven nucleotide substitutions is predicted to result in an amino acid substitution. The C2/C4 product was the same size as that for OsHV-1var (Arzul et al., 2001b), 180 bp smaller than that for OsHV-1, and identical in sequence to that of OsHV-1var. Thus, it appears that OsHV-1var, which has been shown previously to infect oyster and clam larvae, also infects scallop larvae. It is not known whether OsHV-1 can infect scallop larvae.

The original source of OsHV-1var in the Breton hatchery, in which P. maximus is the only species bred, is unknown. However, the observations that this virus can infect several species in the field and that the scallop virus could be transmitted to C. gigas larvae under laboratory conditions are entirely consistent with acquisition from another bivalve species. A possible role for vertical transmission from adults to larvae is supported by the detection of viral sequences in asymptomatic scallops from the same batch of adults that produced the larvae. It is notable that the connective tissue of the gonad was among the regions that contained infected cells. Vertical transmission has also been implicated for OsHV-1 in C. gigas (Le Deuff et al., 1996), and viral proteins and DNA have been detected in healthy adult specimens of this species (I. Arzul, unpublished data).

This report extends the number of marine bivalve species that can be infected by OsHV-1var and adds to the pathogens already faced by P. maximus in commercial aquaculture. It highlights the risks of breeding different species in the same hatchery and indicates the importance of exercising strict precautionary measures to prevent the spread of herpesvirus infections between monocultures. It also stimulates further speculation that OsHV-1 and OsHV-1var are derived from a parental, and perhaps benign, bivalve herpesvirus that has been selected for virulence and wide host range by the practices of modern shellfish farming (Arzul et al., 2001b).

MATERIALS AND METHODS

Animals

Moribund P. maximus larvae, 7 or 10 days old, were collected in September 2000 from a hatchery located in Brittany (France). Before sampling, the larvae presented high mortality rates, which reached 100% in all batches by 10 days after fertilisation.

Ten adults from the same batch that produced the larvae were collected for in situ hybridisation analysis. These animals did not present any mortality or other symptoms of disease.

Intraspecies transmission

Seven-day-old P. maximus larvae from a Breton hatchery were placed at a concentration of five larvae per millilitre in 2-l flasks containing 0.22-μm filtered seawater. They were incubated at 18°C and fed with a mixture of three algae (Pavlova lutheri, Isochrysis affinis galbana, and Skeletonema costatum or Chaetoceros calcitrans) at a concentration of 20 cells/μl for each species. Algae and antibiotic (see below) were added three times per week, and the culture water was completely renewed three times per week.

Three different assays, each replicated three times, were carried out using 7-day-old healthy larvae. In assays 1 and 2, larvae were exposed to 1 ml of extract obtained by grinding 105 moribund P. maximus larvae in 20 ml of sterile seawater using a Dounce homogeniser. In assay 1, the ground larvae were filtered through a 0.22-μm filter to remove bacterial contaminants. In assay 2, the ground larvae were not filtered. In assay 3 (the negative control), healthy larvae were maintained without adding ground larval extract. Chloramphenicol (4 mg/l) was included in all assays to reduce mortalities related to bacterial infections (Nicolas et al., 1996).

Interspecies transmission

Moribund P. maximus larvae (400 mg) from a Breton hatchery were ground in 20 ml of seawater using a Dounce homogeniser and filtered through a 0.22-μm filter to remove bacterial contaminants. One microlitre of inoculum was sampled for PCR. Five 1-l flasks containing 3-day-old axenic C. gigas larvae were inoculated with 8 ml of ground larval extract (assays 1–5), and two 1-l flasks containing 3-day-old axenic C. gigas larvae were inoculated with 8 ml of sterile seawater (assays 6–7). Three days after exposure, the larvae were recovered, concentrated, and washed three times in filtered seawater by centrifugation (200 g for 15 min) as described by Arzul et al. (2001a). One microlitre of seawater was collected for PCR analysis after each wash. After washing, larvae were treated for PCR analysis (Renault et al., 2000b).

Transmission electron microscopy

Moribund larvae were fixed directly in 3% glutaraldehyde in 0.2 M sodium cacodylate (pH 7.2). Larval samples were then processed as described previously (Renault et al., 1994a). Ultrathin sections were stained
with uranyl acetate and lead citrate and examined by transmission electron microscopy (TEM) using a JEOL JEM 1200EX instrument operating at 80 kV.

PCR and sequence analysis

Larval samples were prepared by grinding frozen larvae in double-distilled water, boiling, and centrifuging (Renault et al., 2000b). Supernatants were immediately diluted 10-fold in double-distilled water and frozen at −20°C. Oligonucleotide primers were designed on the basis of the OsHV-1 genome sequence. Four primer pairs were used, as follows: Gp3 (5’-GGT TGT GGG TTT GGA AAT GT-3’) and Gp4 (5’-GGC GTC CAA ACT CGA TTA AA-3’) (698-bp product), B3 (5’-GTG GAG GTG GCT GTT GAA AT-3’) and B4 (5’-ACT GGG ATC CGA TCA AC-3’) (207-bp product), C2 (5’-CTC TTT ACC ATG AAG ATA CCC ACC-3’) and C4 (5’-GGA GTT GTG GTA TAC TCG AGA TTG-3’) (352-bp product), and C2 and C6 (5’- GTG CAC GGC TTA CCA TTT TT-3’) (710-bp product) to cover the C2/C4 product. The Gp locus (at approximately 134,000 bp in the genome) encodes part of a putative glycoprotein; the B locus (146,900 bp) encodes part of a putative inhibitor of apoptosis protein, and the C locus (present twice in the genome at 4500 and 178,500 bp) encodes parts of two proteins of unknown functions (Arzul et al., 2001b). PCR reactions were carried out and the products analysed on 1% agarose gels as described previously (Renault et al., 2000b). The positive control consisted of 10 ng/µl OsHV-1 DNA extracted from purified virus particles (Le Deuff and Renault, 1999).

Gp3/Gp4 and C2/C4 PCR products derived from two moribund larval scallop samples were ligated into linearised pTzAdv vector (Clontech). Ligated DNA was transformed into competent Escherichia coli TOP10F’. Relevant portions of selected plasmids were sequenced using an ABI PRISM sequencing kit (Perkin-Elmer).

In situ hybridisation

In situ hybridisation was performed according to a previously described protocol (Renault and Lipart, 1998), except that 896-bp probes were produced by PCR using OsHV-1 DNA as template (0.1 ng/reaction), primers C1 (5’-TTC CCC TCG AGG TAG CTT TT-3’) and C6 and digoxigenin-11-dUTP (Boehringer Mannheim) in a buffer containing 1.5 mM MgCl2. Detection was performed using an alkaline phosphatase conjugated mouse IgG antibody against digoxigenin (1:500) incubated in NBT/BCIP (1:50). The slides were stained with Bismarck brown yellow and mounted in Eukitt resin. Negative controls included samples processed without including digoxigenin-labelled probes or without antibody. An additional negative control consisted of samples hybridised with a digoxigenin-labelled probe specific to Spring Viraemia of Carp Virus (SVCV) kindly supplied by Dr. R. M. Le Deuff (CEFAS, Weymouth, U.K). A positive control consisted of C. gigas juveniles shown to be infected with OsHV-1 by TEM and PCR.

ACKNOWLEDGMENTS

We acknowledge Dr. A. Gérard for facilitating this work at the IFREMER Station in La Tremblade, France. We are grateful to Dr. J. R. Bonami for his scientific insights. This research was supported in part by European Union contracts (FA-S2 9052 and FAIR-CT-4334). This study would not have been possible without the valuable contribution of French private shellfish farmers. We also thank Dr. D. M. Stone and Dr. R. M. Le Deuff (CEFAS, Weymouth, U.K) for their hospitality and help in sequencing and for valuable critical comments on the manuscript.

REFERENCES


