Micromonas pusilla reovirus: a new member of the family Reoviridae assigned to a novel proposed genus (Mimoreovirus)

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Micromonas pusilla reovirus (MpRV) is an 11-segmented, double-stranded RNA virus isolated from the marine protist Micromonas pusilla. Sequence analysis (including conserved termini and presence of core motifs of reovirus polymerase), morphology and physicochemical properties confirmed the status of MpRV as a member of the family Reoviridae. Electron microscopy showed that intact virus particles are unusually larger (90–95 nm) than the known size of particles of viruses belonging to the family Reoviridae. Particles that were purified on caesium chloride gradients had a mean size of 75 nm (a size similar to the size of intact particles of members of the family Reoviridae), indicating that they lost outer-coat components. The subcore particles had a mean size of 50 nm and a smooth surface, indicating that MpRV belongs to the non-turreted Reoviridae. The maximum amino acid identity with other reovirus proteins was 21%, which is compatible with values existing between distinct genera. Based on morphological and sequence findings, this virus should be classified as the representative of a novel genus within the family Reoviridae, designated Mimoreovirus (from Micromonas pusilla reovirus). The topology of the phylogenetic tree built with putative polymerase sequences of the family Reoviridae suggested that the branch of MpRV could be ancestral. Further analysis showed that segment 1 of MpRV was much longer (5792 bp) than any other reovirus segment and encoded a protein of 200 kDa (VP1). This protein exhibited significant similarities to O-glycosylated proteins, including viral envelope proteins, and is likely to represent the additional outer coat of MpRV.

INTRODUCTION

The family Reoviridae is a large family of viruses with genomes containing 10, 11 or 12 segments of double-stranded RNA (dsRNA). Members of the family Reoviridae have been isolated from a wide range of mammals, birds, reptiles, fish, crustaceans, insects, ticks, arachnids, plants and fungi and include a total of 75 virus species, with a further ~30 tentative species reported to date (Mertens et al., 2005). The family currently includes 12 distinct genera, which are Orthoreovirus, Orbivirus, Rotavirus, Coltivirus, Aquareovirus, Cypovirus, Fijivirus, Mycoreovirus, Phytoreovirus, Oryzavirus, Seadornavirus and Idnoreovirus (Mertens & Diprose, 2004; Mertens et al., 2005). Recently, three new genera were proposed to the International Committee on Taxonomy of Viruses (ICTV) for classification of a nine-segmented insect virus, 12-segmented crustacean viruses and an 11-segmented protist virus (described in this paper). These proposals received preliminary support from ICTV.

The morphology of some members of the family Reoviridae has been studied intensively (Prasad et al., 1988; Yeager et al., 1990, 1994; Grimes et al., 1998; Gouet et al., 1999; Hill et al., 1999; Reinisch et al., 2000; Diprose et al., 2001; Nason et al., 2004). The virus particles have icosahedral symmetry with a diameter of approximately 60–85 nm. They are usually regarded as non-enveloped, although some can acquire a transient membrane envelope during morphogenesis or cell exit (Murphy et al., 1968; Estes & Cohen, 1989; Martin et al., 1998; Mertens et al., 2000; Owens et al., 2004). Reoviruses can contain one, two or three concentric protein layers, identified here as 'subcore', 'core' and 'outer capsid', respectively. The inner-capsid layers and proteins are primarily involved in virus assembly and replication and
show a remarkable degree of structural conservation between different genera, exemplified by the subcore shell, constructed from 120 molecules of a single protein (Grimes et al., 1998; Reiniisch et al., 2000; Mertens, 2004). In contrast, the outer-capid proteins, which are involved in virus transmission, cell attachment and penetration, show greater variation, reflecting differences in the targeted host species, as well as responses to immune selective pressure by ‘neutralization’ antibodies.

Members of the family Reoviridae can be subdivided into two groups. The ‘turreted’ viruses have 12 icosahedrally arranged projections (called turrets) situated on the surface of the icosahedral core particle, one at each of the fivefold axes (e.g. orthoreoviruses or cypoviruses) (Baker et al., 1999; Hill et al., 1999). The cores of the ‘non-turreted’ viruses have a ‘protein-bilayer’ structure, with a smooth or bristly surface appearance (e.g. rotaviruses or orbiviruses; Grimes et al., 1998; Baker et al., 1999; Mertens et al., 2000, 2005).

An 11-segmented dsRNA virus has been isolated from the marine protist Micromonas pusilla (Brussaard et al., 2004) and the isolate was originally designated Micromonas pusilla RNA virus (MpRV). This virus was renamed and is now recognized as Micromonas pusilla reovirus (MpRV). Although 11-segmented dsRNA viruses infecting aquatic animals are known (belonging to the genus Aquareovirus), the isolation of a dsRNA virus from M. pusilla constitutes the first case of isolation of a reovirus from a protist. The polysegmented dsRNA genome of MpRV identified it as a member of the family Reoviridae (Brussaard et al., 2004). Among various algal species, including different strains of M. pusilla, this virus was found to replicate only in strain LAC38 of M. pusilla. We report here a molecular study of MpRV. Sequence and phylogenetic analyses show clearly that MpRV does not belong to any of the genera identified to date.

**METHODS**

**Virus preparation.** The algal host *M. pusilla* (strain LAC38) was grown in enriched artificial seawater (Harrison et al., 1980; Cottrell & Suttle, 1991) at 15 °C under white light as described previously (Brussaard et al., 2004). Algal suspension (20 l; 2 x 10^7 cells ml^-1) was infected with MpRV and incubated at 15 °C until complete lysis occurred after 1 week.

Viruses were concentrated by ultrafiltration on Vivaflow 200 (molecular weight cut-off, 30 kDa; Vivascience), after which Tween 80 was added to a final concentration of 0.007%. The viruses were subsequently purified by removing cell debris from fresh lysate using low-speed centrifugation at 7000 g for 30 min at 4 °C. The supernatant was decanted and viral particles were concentrated by ultracentrifugation at 100 000 g for 2 h at 8 °C using an SW28 rotor. The viral pellets were resuspended in 150 µl SM buffer [0-1 M NaCl, 8 mM MgSO_4_, 50 mM Tris/HCl (pH 7.5), 0.005% (w/v) glycerol; Wommack et al., 1999] and stored at -80 °C until use.

**Virus purification and electron microscopy.** MpRV particles were purified by layering the suspension onto a preformed linear Percoll (Amersham Biosciences) gradient in a dilution buffer [150 mM NaCl, 250 mM sucrose, 1 mM MgCl_2, 4 mM CaCl_2, 10 mM Tris/HCl (pH 8.0)] followed by centrifugation at 110 000 g (45 min, 10 °C). The virus band was recovered and processed as described previously (Mohd Jaafar et al., 2005). These particles were subsequently purified on a discontinuous caesium chloride gradient (40/55%) as described by Burroughs et al. (1994), at 10 °C for 2 h at 210 000 g.

Cores of MpRV were prepared by treating 100 µl Percoll-purified virus with 100 µl CaCl_2 (3 M; final concentration, 1-5 M) (Estes & Cohen, 1989) for 30 min at 37 °C. The cores were then purified on the 40/55% discontinuous caesium chloride gradient as described above. MpRV core particles were recovered at the interface, diluted with an equal volume of 100 mM Tris/HCl (pH 8.0) and processed for electron microscopy.

The virus was adsorbed onto Formvar/carbon-coated grids, stained with 2 % potassium phosphotungststate for 30 s and dried prior to being examined by electron microscopy using a Philips Morgagni 268 transmission electron microscope.

**Isolation and purification of the genomic dsRNA for cloning.** Virus dsRNA was extracted from the suspended viral concentrate by using a commercially available guanidinium isothiocyanate-based protocol (RNA NOW reagent; Biogentex). Briefly, samples (150 µl) were dissolved in 1 ml reagent by vigorous mixing. Chloroform (200 µl) was added and the mixture was shaken for 1 min and kept for 10 min on ice, followed by centrifugation at 12 000 g for 10 min at 4 °C. The supernatant was recovered, mixed with 900 µl 100 % 2-propanol and incubated overnight at –20 °C. The RNA was pelleted by centrifugation at 18 000 g for 10 min at 4 °C, washed with 75 % ethanol, dried and dissolved in 50 µl water. The dsRNA was further purified by precipitating high-molecular-mass single-stranded RNAs in 2 M LiCl, as described elsewhere (Attoui et al., 2000a).

**Cloning of the dsRNA segments.** The genome segments of MpRV were copied into cDNA, cloned and sequenced according to the single-primer amplification technique described previously (Attoui et al., 2000a, b). Briefly, the viral dsRNA was separated on 1% agarose gel and purified by using an RNAid kit (Bio101). A previously described 3′-amino-blocked oligodeoxyribonucleotide (Attoui et al., 2000a, b) was ligated to both of the 3′ ends of the purified dsRNA segments by using T4 RNA ligase, followed by reverse transcription and PCR amplification using a complementary primer. PCR amplions were analysed by agarose-gel electrophoresis, ligated into the pGEM-T cloning vector (Promega) and transfected into competent XL-Blue *Escherichia coli*. Insert sequences were determined by using M13 universal primers, a D-rhodamine DNA sequencing kit and an ABI Prism 377 sequence analyser (Perkin Elmer).

**Assays of virus replication in insect-, mammalian- and fish-cell lines.** The virus was inoculated into the fish-cell line FHM (fathead minnow), which was grown in Leibovitz’s L-15 medium at 28 °C. The inoculated FHM cells were incubated at either 20 °C (a temperature which is closer to the growth temperature of MpRV in *M. pusilla*) or 28 °C.

Other cell lines tested included mosquito-cell lines and mammalian-cell lines. The mosquito-cell lines C6/36 and AA23 (both from *Aedes albopictus*), A20 (*Aedes aegypti*), AE (*Aedes aegypti*) and A w-albus (*Aedes w-albus*) were all grown in Leibovitz’s L-15 medium at 28 °C. The inoculated cells were incubated at either 20 or 28 °C. The mammalian-cell lines L-929 (mouse fibroblast), BHK-21 (hamster kidney), BGM (monkey kidney), HEP-2 (human adenocarcinoma) and MRC5 (human embryo lung) were all grown at 37 °C in Eagle’s minimal essential medium supplemented with 5 % fetal bovine serum. The inoculated cells were incubated at either 37 or 32 °C (a lower temperature, as MpRV grows at low temperature in *M. pusilla*).
For the purpose of adsorbing the virus to the cells, 100 μl MpRV concentrate was added to the cell monolayers in a 25 cm² flask and incubated at 28 °C (mosquito or fish cells) or at 37 °C (mammalian cells) for 1 h. The cells were washed twice with PBS and the culture medium was added. At day 5 post-infection (p.i.), the cells were scraped and half of the scraped-cell suspension was pelleted. The supernatant was discarded and the RNA was extracted by using RNA NOW (Biogenex). An aliquot of the remaining scraped-cell suspension was used to infect fresh cells in a second passage. Two more passages were subsequently performed.

The extracted RNA was processed for agarose-gel electrophoresis and RT-PCR, using specific MpRV primers as described below.

**RT-PCR of the RNA extract from the cell lines.** The RNA was copied into cDNA by using random hexanucleotide primers as described previously (Attoui et al., 1998). Briefly, the RNA was denatured in 15% DMSO by heating at 99 °C for 1 min and incubated immediately on ice. Reverse transcription was performed by using Superscript III reverse transcriptase (Invitrogen) at 42 °C. The resulting cDNA was PCR-amplified using first-round primers MpRVSeq2s1 (forward, positions 2259–2284; 5′-CACGGCGACG-CAACGTTCTTATAGAC-3′) and MpRVSeq2r1 (reverse, positions 2758–2733; 5′-CGTACACTGTCTAGATGCGTAACATG-3′) to produce an amplicon of 500 bp, and second-round primers MpRVSeq2s2 (forward, positions 2337–2362: 5′-CAGCGTCTGTAGCAATAACCTCGCGC-3′) and MpRVSeq2r1 (reverse, positions 2636–2611: 5′-CGTACACTGTCTAGATGCGTAACATG-3′) to produce an amplicon of 300 bp.

**Sequence analysis and phylogenetic comparisons.** Analysis of the MpRV sequence was performed by comparing each segment sequence with a database constructed with all available sequences from the family Reoviridae, using the local BLAST program implemented in the DNATools package (version 5.2.018; Rasmussen, 1995).

The predicted sequences of the proteins encoded by the 11 segments were also analysed by using the NCBI’s online BLAST program (http://www.ncbi.nlm.nih.gov/blast/).

For phylogenetic analysis, the putative RNA-dependent RNA polymerase (RdRp) sequence of MpRV was compared with the amino acid sequences of putative RdRps of representative strains of viruses representing the 12 genera of the family Reoviridae. GenBank accession numbers are provided in Supplementary Table S1 (available in JGV Online). Sequence alignments were performed by using the CLUSTAL W (version 1.84) software program (Thompson et al., 1994). Phylogenetic analyses were carried out with the software program MEGA3 (Kumar et al., 2004) using the Poisson-correction or the gamma-distribution algorithms and the neighbour-joining method for tree building.

**RESULTS**

**Electron microscopy**

The virus particles that were pelleted from supernatant or purified on a Percoll gradient had a mean diameter of 90–95 nm, which is larger than that of any previously described member of the family Reoviridae. Some damaged particles (data not shown) showed an outermost thin layer of protein (~15 nm thick) surrounding a more compact internal structure (~75 nm).

The particles that were purified on CsCl have a mean diameter of 75 nm (Brussaard et al., 2004), which suggests that these particles had lost the outermost thin layer of protein. However, this size is similar to that described for whole particles of other viruses of the family Reoviridae.

Particles that had been treated with CaCl₂ and purified on a Percoll gradient had a diameter of 50 nm, showing that they had lost outer capsid proteins. They also had a smooth outline (Fig. 1), similar to that observed for the subcores (the pseudo-T=2, also known as modified T=1, layer) of rotaviruses, orbiviruses and seadornaviruses (Mertens et al., 2005; Mohd Jaafar et al., 2005), which are non-turreted viruses.

**Cloning of MpRV cDNA**

The 11 dsRNA segments of the MpRV genome (Fig. 2) were all cloned, sequenced and deposited in GenBank under accession numbers listed in Table 1. The lengths of the segments and their corresponding encoded proteins are shown in Table 1. Analysis of the 5′ and 3′ non-coding regions (NCRs) showed that all of the segments share five conserved nucleotides at their 5′ ends and six conserved nucleotides at their 3′ ends (5′-GAAGAAA--AAAGUC-3′; Table 1). Moreover, the first and last 2 nt of all of the segments are inverted complements.

**Sequence analysis**

A comparison of the genome sequence of MpRV with those of characterized members of the family Reoviridae was performed. MpRV could not be classified within any of the existing genera of the family. In particular, MpRV could not be assigned to either of the genera Rotavirus or Aquareovirus, which both contain viruses with 11-segmented dsRNA genomes. The maximal amino acid identity with aquareovirus and rotavirus proteins was found in the polymerase gene (the most conserved gene between viruses belonging

![Fig. 1. Electron micrographs of MpRV. Particles pelleted from the clarified lysate of infected *M. pusilla*. Some particles (indicated by arrows) have a larger diameter. At the upper left corner (inset), core particles treated with 1-5 M CaCl₂ are shown to have a smooth outline (turrets are absent). Bars, 100 nm (main image); 50 nm (inset).](http://vir.sgmjournals.org)
to distinct genera of the family Reoviridae). Amino acid identity with aquareovirus polymerases (species Aquareovirus A and Aquareovirus C) was found to be 8–10%, whereas with rotaviruses polymerases (species Rotavirus A, Rotavirus B and Rotavirus C), maximal amino acid identity was 21%. In both cases, these values are compatible with those calculated for viruses belonging to distinct genera (Attoui et al., 2002a).

The usual size of segment 1 (Seg-1) for viruses of the family Reoviridae is approximately 4000 bp. The Colorado tick fever virus (CTFV) genome has previously been reported to have the largest Seg-1 of all sequenced reoviruses (4350 bp; Attoui et al., 2005). The Seg-1 of MpRV determined in this study was found to be 5792 bp long, which is unusually longer than any Seg-1 in other members of the family Reoviridae. Sequence analysis of MpRV Seg-1 showed that it contains a single open reading frame (ORF) encoding the VP1 protein, which is 1897 aa long.

The BLAST search showed that VP1 exhibited significant homology (as indicated by the E values of the BLAST program) to viral, bacterial and yeast haemagglutinins. This analysis showed that aa 88–321 exhibited 24% identity with the minor capsid protein sigma-1 of orthoreoviruses (a haemagglutinin responsible for cell attachment; GenBank accession number AAA47276).

It is noteworthy that VP1 was found to be related to various haemagglutinins, such as those of the bacterial pathogens Burkholderia spp. (amino acid identity, 20%; similarity, 40%; E value, 4 × 10^-16) and Staphylococcus spp. (amino acid identity, 19%; similarity, 38%; E value, 3 × 10^-4) and the

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**Table 1. Lengths of dsRNA segments 1–11, encoded putative proteins and 5' and 3' NCRs of MpRV**

<table>
<thead>
<tr>
<th>Segment</th>
<th>GenBank accession no.</th>
<th>Length (bp)</th>
<th>G+C content (mol%)</th>
<th>Protein length (aa)</th>
<th>Mass* (Da)</th>
<th>Length (bp)</th>
<th>Terminal sequences</th>
<th>3' NCR Terminal sequences</th>
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<td>49.5</td>
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<td>49.0</td>
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<td>---GAAAGUC-3</td>
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</tbody>
</table>

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*Calculated theoretical molecular mass. In case of potential read-through.
yeasts Candida albicans (identity, 20 %; similarity, 39 %; E value, $3 \times 10^{-4}$) and Saccharomyces cerevisiae (identity, 20 %; similarity, 37 %; E value, $7 \times 10^{-6}$). VP1 also matched the envelope proteins of viruses such as those of equine herpesvirus gp2 (identity, 20 %; similarity, 32 %) or that of Acholeplasma bacteriophage (identity, 26 %; similarity, 46 %).

It is interesting to note that VP1 has a high serine and threonine content (≥11 % of each), compared with 1–7.5 % for other amino acids. This is characteristic of glycoproteins and, in particular, for mucin and mucin-like proteins (Byrd & Bresalier, 2004) and cell-wall adhesins. Such serine- and threonine-rich proteins are usually heavily O-glycosylated. In summary, VP1 might form an extra coat at the outermost surface.

Amino acid sequence repeats were identified within VP1. Interestingly, each repeat was found to align best with a protein sequence immediately N-terminal to it in VP1. The repeated sequences were not identical to the matching sequences. This is evocative of what has been described as sequence duplication in viral genes, followed by distinct evolution of the parental and the daughter repeated sequences (Gibbs & Keese, 1995). Examples of such repeats are shown in Fig. 3.

Segment 2 of MpRV probably encodes the viral RdRp. The RdRp core motifs identified in the protein encoded by this segment include the motif SG (position 801–802) and the motif GDD (position 835–837). Interestingly, a partial match (aa 647—962; identity, 21 %) was found between MpRV RdRp and that of the human isolate of species Rotavirus A (GenBank accession no. CAC44891), which is also an 11-segmented dsRNA virus belonging to the family Reoviridae.

The VP5 of MpRV was found to partially match (aa 214–318; identity, 21 %) the outer-capsid spike protein VP4 of Rotavirus A. It also showed 24 % identity to the killer toxin protein (GenBank accession no. S51548) of yeast M28 dsRNA virus (an unclassified virus). Segment 5 shows an ORF spanning nt 44–2005. This ORF is interrupted by an in-frame TGA stop codon at position 1571–1573. A similar situation has been described in segment 9 of CTFV (genus Coltivirus), in which a read-through has been identified. The occurrence of a read-through in segment 5 of MpRV remains to be identified experimentally by cloning segment 5 in a eukaryotic expression vector under the control of a strong promoter and identification of possible long and short forms of the proteins, as has been realized for CTFV segment 9 (Mohd Jaafar et al., 2004).

The VP7 of MpRV was found to partially match (aa 130–209; identity, 32 %) the non-structural protein NS1 of Cypovirus 1, whereas the VP8 of MPRV partially matched (aa 42–66; identity, 28 %) the NSP2 of human Rotavirus A (NSP2 has a dsRNA helix-distabilization activity, binds RNA and is an NTPase).

The VP9 was found to partially match (aa 269–338; identity, 28 %) the segment 7-encoded protein of Nilaparvata lugens reovirus (a fijivirus), which is a core protein and possesses a nucleotide-binding activity (Mertens et al., 2005).

To summarize, based on sequence comparisons with genomes of members of the family Reoviridae, putative functions deduced from the best-fit analyses were attributed to the various MpRV proteins and the information is presented in Table 2.

**Phylogenetic analysis based on viral polymerase sequences**

It has previously been found that an amino acid identity of ≤30 % in the polymerase sequence is suitable to distinguish between genera of the family Reoviridae (Attoui et al., 2002a). However, there are two exceptions to this rule. The first is the rotavirus B polymerase, which is only 22 % identical to other rotaviruses. However, the inclusion of Rotavirus B within the genus Rotavirus together with Rotavirus A and Rotavirus C does not rely primarily on genetic distances between the polymerases of these viruses. It was based on the morphological resemblance between these viruses and the type of disease that they cause. The second exception is the aquareoviruses and orthoreoviruses, which are 42 % identical (Attoui et al., 2002a). This significantly high amino acid identity is evidence of a common ancestry. However, the distinct hosts and the different econiches of these viruses justify their classification within two distinct genera.

The results of the polymerase sequence analysis of MpRV are illustrated by a radial neighbour-joining tree (Fig. 4). MpRV
Table 2. Putative functions of the proteins deduced from coding regions of MpRV segments

The putative functions described in this table resulted from a best-fit analysis using a database built with sequenced genomes of members of the family Reoviridae. For details, see text. NI, No significant identity with any reovirus protein.

<table>
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<th>Segment</th>
<th>Protein designation</th>
<th>Putative function (possible location)</th>
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<tr>
<td>1</td>
<td>VP1</td>
<td>Haemagglutinin and cell attachment (outer coat)</td>
<td>Sigma-1 of orthoreoviruses</td>
</tr>
<tr>
<td>2</td>
<td>VP2</td>
<td>Putative viral RNA polymerase (core)</td>
<td>Reovirus polymerases</td>
</tr>
<tr>
<td>3</td>
<td>VP3</td>
<td>Putative pseudo-T=2 layer (core)</td>
<td>Pseudo-T=2 layer of phytoreoviruses</td>
</tr>
<tr>
<td>4</td>
<td>VP4</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>VP5</td>
<td>Putative outer-coat protein (outer coat)</td>
<td>Rotavirus VP4</td>
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<tr>
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<td>VP6</td>
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<td></td>
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<td>Possible non-structural protein</td>
<td>NS1 of cypovirus</td>
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<td>Putative outer layer of core (core)</td>
<td>Similarity to VP7 of fijiviruses</td>
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<td>11</td>
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does not cluster with any of the known, characterized members of the family Reoviridae. It clustered with neither aquareoviruses (11-segmented), which are turreted viruses, nor with rotaviruses (11-segmented), which are non-turreted. Rather, MpRV stands as a representative of a separate phylogenetic group. This is confirmed by the low amino acid identity values (5–21 %) between MpRV polymerase and those of member viruses of other genera of the family Reoviridae.

Analysis of the replication of MpRV in insect-, mammalian- and fish-cell lines

Analysis of RNA extracted from cells that were inoculated with MpRV did not reveal any dsRNA profile following agarose-gel electrophoresis. RT-PCR analysis of these extracts using primers specific to segment 2 of MpRV was negative upon first-round and nested PCR, demonstrating that purified MpRV could not replicate in the tested cell lines.

DISCUSSION

When MpRV was isolated (Brussaard et al., 2004), the identification of the 11-segmented dsRNA profile of its genome, the morphological characteristics of the virions and its physicochemical properties led to the classification of MpRV as a tentative member of the family Reoviridae. The present study provides several additional arguments for the classification of MpRV as a full member of the family Reoviridae. Full characterization of the dsRNA genome by cloning and sequencing revealed that it has a total length of 25 563 bp. This is similar to the genome length of other viruses of the family Reoviridae, which range between ~18 500 and 29 210 bp (Attoui et al., 2002b; Mertens et al., 2005). However, segment 1 of MpRV is significantly longer than segments 1 of other sequenced members of the family Reoviridae.

In addition, the putative polymerase of MpRV partially matches rotavirus polymerase and contains the signature motifs of putative RdRps of viruses belonging to the family Reoviridae. In compliance with the criteria defining the family Reoviridae, analysis of the terminal sequences revealed that all 11 segments of MpRV have conserved termini within the 5’ and 3’ NCRs. Such terminal sequences are recognized as one of the defining species parameters within the family Reoviridae. These conserved termini in the MpRV genome are different from any of those described previously within the family Reoviridae, showing that MpRV does not belong to any previously described species within this family.

The 11-segmented genome is a characteristic of rotaviruses and aquareoviruses within the family Reoviridae. Being isolated from an aquatic organism, it would be reasonable to suppose that MpRV is a tentative member of the genus Aquareovirus (infecting fishes, crustaceans and molluscs). As an aquatic reovirus, it would be interesting to know whether this virus can infect other aquatic species and whether it has only one host, or whether M. pusilla might represent a vector that transmits the virus among other aquatic species. Our results indicated that MpRV does not replicate in fish-, insect- or mammalian-cell lines that were tested and that it is a member of neither the genus Aquareovirus nor the genus Rotavirus, as MpRV has features unique among the family Reoviridae.

Phylogenetic analysis based on the polymerase amino acid sequence, for example, demonstrated clearly that MpRV is not a member of the genera Rotavirus or Aquareovirus, despite its genome being made of 11 dsRNA segments. Amino acid identity of the polymerase of MpRV is 8–10 % with aquareovirus polymerase and 19–21 % with rotavirus polymerase. These are values comparable to those found between viruses belonging to distinct genera of the family Reoviridae. The morphology of the particles is also not identical to that of other members of the family Reoviridae. The diameter of whole particles purified under conditions not affecting the integrity of the outer coat is significantly larger (90–95 nm) than the diameters usually observed in
viruses of the family *Reoviridae*. In a previous study, when MpRV was purified on a CsCl density gradient, two virus bands were visible (Brussaard *et al.*, 2004). The less-dense virus band was found to contain eight structural proteins, including traces of a protein at approximately 200 kDa that was absent from the denser virus band, suggesting that this protein is removed at high ionic strength. This size is compatible with the deduced size of VP1 of MpRV. This protein is likely to be responsible for the unusually large size of the whole virus particles. VP1 showed similarities within its amino-terminal sequence to Sigma-1 (a haemagglutinin and cell-attachment protein) of MRV (genus *Orthoreovirus*). This suggested that VP1 might represent the virus attachment protein to the cell wall of *M. pusilla*. VP1 was also found to exhibit similarities to glycoproteins of viral and non-viral agents. The high serine and threonine content of VP1 suggests that it might be O-glycosylated, as is the case of mucin and mucin-like proteins.

Transient envelope structures have been described for orbiviruses (Martin *et al.*, 1998; Owens *et al.*, 2004), coltiviruses (Murphy *et al.*, 1968; Attoui *et al.*, 2002b), rotaviruses (Estes & Cohen, 1989) and seadornaviruses (Mohd Jaafar *et al.*, 2005) as a consequence of budding of virus particles from
the cell membrane or budding into the endoplasmic reticulum during morphogenesis. If MpRV VP1 forms such a structure, MpRV would be the first member of the family Reoviridae to possess a constitutive additional outer coat. This requires further investigation by techniques such as cryoelectron microscopy.

The length of the VP1 of MpRV is unusual among sequenced members of the family Reoviridae. The presence of many repeats within this sequence evokes the possibility that VP1 could have arisen from amino acid fragment duplication, which diverged afterwards. The precise mechanism and the constraints that have driven such an evolution are unknown.

The existence of the remaining seven structural proteins in the purified MpRV particles (Brussaard et al., 2004) is compatible with that described for non-turreted reoviruses, where particles are composed of seven structural proteins, such as in seadornaviruses and orbiviruses (Mertens et al., 2005; Mohd Jaafar et al., 2005). The MpRV particles that were treated with CaCl₂ generated structures that could be compared with the smooth, non-turreted subcore layer of orbiviruses, indicating that MpRV belongs to the non-turreted group within the family Reoviridae. This is further evidence that MpRV is not a member of the genus Aquareovirus, as members of this genus are turreted viruses.

It is interesting that, within the RdRp phylogenetic tree, the branch of MpRV digests the tree, separating the groups of turreted and non-turreted viruses. It has been found that M. pusilla is evolutionarily older than the hosts of other members of the family Reoviridae (Bhattacharya & Medlin, 1998; Doolittle, 1999; Cavalier-Smith, 2002). The location of MpRV at the node separating the turreted and non-turreted viruses suggests that it belongs to a third branch (although non-turreted), which is possibly ancestral. The presence of an additional protein coat or a potential pseudo-envelope structure may be an ancestral character lost by other members of the family Reoviridae or a specific adaptable character of the reoviruses infecting protists.

Based on all of these findings, MpRV should be classified as a member of a novel genus within the family Reoviridae. This genus could be designated Mimoreovirus (Micromonas pusilla reovirus). A formal proposal was made to the ICTV session during the International Congress of Virology held in San Francisco, CA, USA, in July 2005 to create the genus Mimoreovirus and to designate MpRV as its type species. This proposal received initial support and was passed for wider consultation.

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**REFERENCES**


