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To cite this version:
Sylvie Rousvoal, Betty Bouyer, Camilo López-Cristoffanini, Catherine Boyen, Jonas Collén. Mutant swarms of a totivirus-like entities are present in the red macroalga Chondrus crispus and have been partially transferred to the nuclear genome. Journal of Phycology, Wiley, 2016, 52 (4), pp.493-504. <10.1111/jpy.12427>. <hal-01320013>
MUTANT SWARMS OF A TOTIVIRUS-LIKE ENTITY ARE PRESENT IN THE
RED MACROALGA CHONDRUS CRISPUS AND HAVE BEEN PARTIALLY
TRANSFERRED TO THE NUCLEAR GENOME

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ABSTRACT

Chondrus crispus Stackhouse (Gigartinales) is a red seaweed found on North
Atlantic rocky shores. Electrophoresis of RNA extracts showed a prominent band
with a size of around 6,000 bp. Sequencing of the band revealed several sequences
with similarity to totiviruses, double-stranded RNA viruses that normally infect
fungi. This virus-like entity was named CcV. It should probably be regarded as an
extreme viral quasispecies or a mutant swarm since low identity (<65%) was found
between sequences. Totiviruses typically code for two genes: one capsid gene (gag)
and one RNA-dependent RNA polymerase gene (pol) with a pseudoknot structure
between the genes. Both the genes and the intergenic structures were found in the
CeV sequences. A non-identical gag gene was also found in the nuclear genome of
C. crispus, with associated EST and upstream regulatory features. The gene was
presumably horizontally transferred from the virus to the alga. Similar dsRNA bands
were seen in extracts from different life cycle stages of C. crispus and from all
geographical locations tested. In addition, similar bands were also observed in RNA
extractions from other red algae; however, the significance of this apparently
widespread phenomenon is unknown. No phenotype caused by the infection nor any
virus particles, or capsid proteins were identified; thus, the presence of viral particles
has not been validated. These findings increase the known host range of totiviruses to
include marine red algae.

Key words: Chondrus, dsRNA, mutant swarm, Rhodophyta, totivirus, virus

INTRODUCTION

Viruses play a key role in the functioning of marine ecosystems (Fuhrman 1999,
Suttle 2005, 2007). Their prevalence and diversity contribute to the control of the
abundance, the diversity, and the productivity of many marine organisms and
influence global nutrient and energy cycles. For example, it is estimated that viruses
are responsible for the mortality of 20% of the oceans’ biomass every day and that
their abundance is 15 times higher than that of prokaryotes, even if the biomass is
less. Because of the increasing understanding of the relevance of marine viruses a
substantial research effort has been made to study their ecology, physiology, and
molecular biology. Marine viruses have been shown to infect a multitude of various
organisms from cyanobacteria to whales. Viruses have also been suggested to represent the most genetically diverse biological entities in the ocean.

There are various types of viruses that differ in terms of their genetic material: single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), single-stranded RNA (ssRNA), and double-stranded RNA (dsRNA). The sizes of the viral genomes vary between a couple of thousand to several million base pairs with RNA viruses normally having smaller genomes (Philippe et al. 2013). Plant viruses can be divided into four types based on their lifestyle: persistent, chronic, endogenous and acute. Infections with persistent plant viruses typically do not induce any phenotype and are transmitted via gametes. In plants, this type of lifestyle has hitherto been known exclusively from dsRNA viruses. Acute viruses can normally be transported between plants and typically produce a phenotype (Roossinck 2010).

One area where effects of viruses on marine organisms have been more intensely studied is the interaction between viruses and phytoplankton (e.g. Etten et al. 1991, Reisser 1993, Brussaard et al. 2004, Lang et al. 2009). It has been observed (Suttle 2007) that viruses have a key role in controlling the progress, and sometimes the collapse, of many phytoplankton blooms (e.g. *Emiliania huxleyi*, *Phaeocystis globosa* and *Heterosigma akashiwo*). Another system that has been studied in more detail is the ecological consequences of the interaction between the green unicellular alga *Ostreococcus* and its DNA virus (e.g. Derelle et al. 2008, 2015, Moreau et al. 2010). It has therefore been suggested that viral dynamics are one of the explanations to the paradox for the plankton (Fuhrman 1999); successful plankton species, due to their abundance, are more likely to succumb to virus-induced collapses of the populations than are rare species. This preserves the diversity of the phytoplankton.
We have, thus, some information on the interaction between unicellular algae and viruses as well as its importance.

Seaweeds are an important component of the coastal ecosystem; however, much less is known about viruses that infect seaweeds or macroalgae than their unicellular counterparts (Schroeder 2011). One exception is a virus that was found in the brown alga *Ectocarpus siliculosus*. This dsDNA virus, EsV-1, belonging to the Phaeoviruses with a genome of 335,593 bp, becomes associated to the host’s genome (Müller et al. 1990, Klein et al. 1993, Delaroque et al. 2001, Cock et al. 2010). The virus particles are most expressed during reproduction and spread through infection of spores and gametes. It is manifested by malformed gametangia and lysis of swollen hyaline cells (Müller et al. 1990, 2000). Similar phaeoviruses have also been found in other brown algae, including, *Feldmannia irregularis*, *F. simplex*, (Müller and Frenzer 1993, Friess-Klebl et al. 1994), *Hincksia hincksiae* (Kapp et al. 1997), *Botrytella micromora* (as *Sorocarpus uvaeformis*) (Oliveira and Bisalputra 1978), *E. fasciculatus* (Sengco et al. 1996), *Streblonema* sp. (La Claire II and West 1977), and *Pylaiella littoralis* (Maier et al. 1998). Virus infections have also been observed in the green coenocytic macroalga *Bryopsis cinicola* (Koga et al. 1998, 2003). Here one 4.5 kbp sequence belonging to the dsRNA virus family Partitiviridae was found associated with mitochondria and for four others of about 2 kbp with chloroplasts (Ishihara et al. 1992).

Virus infections in red algae are much less studied than in brown algae; however, virus-like particles have been found, by electron microscopy-studies, in the cytoplasm of the freshwater red alga *Sirodotia tenuissima* (Lee 1971) and in the marine species *Acrochaetium* (Audouinella) *saviana* (Pueschel 1995), *Gracilaria epihippisora* (Apt and Gibor 1991), and *G. verrucosa* (Tripodi and Beth 1976).
addition, gall-like structures have been found in several species of Bostrychia
including B. kelanensis, B. radicosa, B. simpliciuscula, and B. tenella, and in some
cases the galls have been associated with virus-like particles (West et al. 2013). For
all of these possible red algal viruses the type of virus is unknown, no sequences are
known, and the only reported phenotype is the formation of galls. Thus, the
prevalence and importance of viruses in red algal biology is virtually unknown.

Chondrus crispus, or Irish moss, is a common, often intertidal red macroalga that has
been promoted as a model species for florideophyte red algae (Collén et al. 2014). It
has a triphasic life cycle with morphologically similar tetrasporophytes (diploid),
male, and female gametophytes (haploid) and morphologically different
carposporophytes (diploid) on the female gametophyte. The 105-Mbp genome was
recently sequenced and codes for 9,606 genes (Collén et al. 2013). During the
purification of RNA for the Chondrus genome project a prominent band of unusual
size was seen on agarose gels; sequencing of this band showed sequence similarities
to totiviruses, dsRNA viruses that normally infect fungi.

MATERIALS AND METHODS

Algal material and culture. Unless explicitly stated otherwise studies were carried
out on a strain of the red alga Chondrus crispus Stackhouse (Gigartinales) grown in
unialgal culture (Collén et al. 2013) at the Station Biologique de Roscoff, from a male
gametophyte collected at Peggy’s Cove, Nova Scotia, Canada (44°29′N, 63°55′W)
in 1985 by Juan Correa. For the other specified C. crispus life cycle stages, samples
were collected near Roscoff or in other specified locations.
RNA extraction and purification. Chondrus crispus RNA was extracted based on a protocol adapted from Apt et al. (1995). Briefly, frozen tissue (~1 g) was ground with a small amount of sand and liquid N₂ in a mortar and pestle. The powder was transferred to a 50 ml Teflon tube, and extracted with 15 ml extraction buffer (100 mM Tris–HCl pH 7.5, 1.5 M NaCl, 50 mM EDTA, 50 mM DTT, 2% CTAB) for 60 min, with continuous shaking at 200 rpm at room temperature. Cellular debris was removed after centrifugation with one volume of chloroform–isoamylic alcohol (24:1) at 10,000 g and 4 °C for 20 min. The aqueous phase was transferred to a Teflon tube and polysaccharides were precipitated with 0.25 volumes of 100% ethanol and centrifuged with one volume of chloroform–isoamylic alcohol (24:1) at 10,000 g and 4 °C for 20 min. Total RNA was precipitated overnight with 3 M LiCl and 1% of β-mercaptoethanol; after centrifugation, the pellet was dissolved in TE buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA). RNA quality was assessed on 1.5% agarose gel stained with 0.1 µg/ml ethidium bromide (EtBr) and quantified using a NanoDrop ND-2000 spectrophotometer (NanoDrop products, Wilmington, DE, USA). CsCl separation of total nucleic acids was used to purify dsRNA in 2 ml polyallomer tubes with 0.95 g/ml CsCl in TE and 50 µl of 10 mg/ml EtBr solution. The solution was centrifuged for 24 h at 90,000 rpm. The dsRNA band was recovered and the EtBr was extracted with TE-saturated butanol. The remaining aqueous phase containing the dsRNA was diluted three times and isopropanol-precipitated over-night at -20 °C. After centrifugation for 45 min at 18,000 g and 4 °C, the pellet was washed with 200 µL 80% ethanol and recentrifuged for 30 min at 18,000 g and 4 °C; the pellet was finally dissolved in 100 µL of TE and kept at -20 °C until use.
Cloning and sequencing. Cloning of RNAs was adapted from Djikeng et al. (2008) using random-tagged primers and RNA purified from agarose gels. Briefly, 100 ng of RNA and 2 pmol of random tagged primers were denatured for 5 min at 70 °C. The first cDNA strand was synthetized using the Improm-RT polymerase Kit (Promega), after annealing 5 min at 25 °C, elongation 1 h at 38 °C, and inactivation. 15 min at 70 °C the RNA strand was degraded with 2 units of RNase H, 20 min at 37 °C. The second strand was generated using 2 pmol of random tagged primer and 2.5 units of Klenow. After purification cDNAs were amplified by PCR and cloned in TOPO-TA (Invitrogen). Race PCR was performed to obtain longer sequences using 75 ng of gel-purified viral RNA with 1.2 μM specific primers, drawn from a first sequence alignment in the polymerase region, and 1.2 μM universal primers. Reverse transcription was carried out at 42 °C for 90 min, using SmartScribe reverse transcriptase Protocol (Clontech). After a 1/12.5 dilution, 1 μl of cDNA was amplified in a total volume of 25 μl, with 400 nM of each specific and universal primers, 0.2 mM dNTP, Pfu buffer, using 1.25 units Pfu DNA polymerase (Invitrogen). After a denaturation step for 4 min at 94 °C, samples were cycled in an ABI2700 thermocycler as following: 30 sec at 94 °C, 30 sec at 60 °C, 6 min at 72 °C for 5 cycles followed by 5 cycles of 30 sec at 94 °C, 30 sec at 56 °C, 6 min at 72 °C and 25 cycles of 30 sec at 94 °C, 30 sec at 52 or 56 °C, depending on the considered specific primer, 6 min at 72 °C and a final step of 10 min at 72 °C. PCR products (2 μL) were directly cloned with TOPO-TA cloning (Invitrogen) and transformants were screened for size by PCR and sequenced using universal primers M13Forward and M13Reverse. Tail-PCR experiments were performed using combined random and specific primers; 200 ng RNA were reverse transcribed using 200 nM GSP1 and 2 μM random primer RP1, using one step Access quick RT-PCR 2-fold master Mix.
(Promega) in a 20 µl total volume, according to the manufacturer specifications. The second step included 2.5 µl of the first 50-fold diluted amplified sample, 2 µM of RP1 and 200 nM internal GSP2, 3 mM MgCl₂, 0.2 mM dNTP, Go-Taq buffer and 1 unit Go-Taq polymerase (Promega). The third step is consisted in the same protocol as the second step, including a 50-fold dilution of the sample and 2 µM of RP1 and 200 nM of internal GSP3.

The cloning and sequencing effort generated only small sequence fragments in the capsid part and in the polymerase sequence region; in addition, race or tail-PCR never permitted amplification of the intergenic part, which can be described as a complex pseudoknot structured sequence. Because we demonstrated that at least seven different sequences could be cloned, with regard to the partial sequences motif obtained, we decided to try to extend some of the contigs obtained; using specific primers and one-step RT-PCR cross amplification between ends of the capsid part and the RdRp part to test whether extremities were from the same virus fragment or not. We started from 30 ng of CsCl purified dsRNA, denatured for 4 min at 99 °C, reverse transcribed for 45 min at 45 °C and the PCR cycling parameters were programmed as follows: 4 min at 95 °C, and 32 cycles of 30 sec at 95 °C, 30 sec at 54 °C, 3 min at 72 °C in a 2700 ABI thermocycler. Sequencing was performed using capillary electrophoresis on an ABI prism 3100 sequencer (Applied Biosystems, Foster City, CA, USA) with 50-cm capillaries.

**DNase and RNase essays.** To discriminate between DNA and RNA, we compared DNase and RNase digestion on total nucleic acid extracts. We subjected 1.5 µg of total nucleic acid to 2 units of Turbo DNaseI (Ambion) or 1 unit of RNaseA (Macherey Nagel) for 1h at 40 °C. After precipitation, the samples were loaded on 1% agarose gel in TAE stained with EtBr.
RESULT AND DISCUSSION

Native agarose gel electrophoresis of RNA extracts from *Chondrus crispus* showed, in addition to rRNA and mRNA (and some DNA contamination), one prominent band with a size of approximately 6,000 bp. This band was not affected by DNase treatment but disappeared after treatment with RNase (Figure 1A). The bands were much less visible after denaturation of the RNA extract on a denatured gel (Figure 1B) than on a native gel. This sensitivity to RNase indicated that the bands were made of RNA and likely double-stranded since the intensity was greatly diminished on a denatured gel. This is caused by the stronger affinity of the ethidium bromide stain to double-stranded RNA than single-stranded RNA (Sambrook and Russell 2006).

The probably double-stranded RNA band was purified, reverse transcribed using random priming, and partially sequenced. Several sequences were attained which showed very high sequence diversity and could, thus, not be assembled in a continuous sequence. Several fragments were found to be similar to *Xanthophyllomyces dendrorhous* virus (XdV) (Figure 2) and this sequence was used to align the sequences from *C. crispus*. XdV is a double-stranded RNA virus, belonging to the totivirus (Baeza et al. 2012). The genome of totiviruses, typically with a genome size 4.6–6.3 kbp, normally codes for two proteins, one capsid (*gag*) gene and one RNA-dependent RNA polymerase (*pol*) gene with partially overlapping open reading frames (ORFs). The sequences from the tentative *C. crispus* virus (CcV), contained partial sequences from both genes (Figure 3 and 4).

Considerable efforts were made to try to obtain a full sequence, but each approach,
including SISPA method, race- or tail PCR, with degenerated, specific, or random PCR primers gave sequences that corresponded to new viral sequences instead of one specific sequence. This indicated a considerable sequence diversity within the CcV. It should be noted that the host strain of *C. crispus* used was artificially forced through a bottleneck of reduced size to reduce the possibility of the strain being a chimera before the genome sequencing (Collén et al. 2013) and thereafter grew vegetatively. The origin of the strain represents milligrams of tissue so that no nuclear sequence diversity was detected during genome sequencing.

The high error rate of viral replication with an RNA-dependent RNA polymerase compared to a DNA polymerase generates sequence diversity within viral populations that is higher than in cellular organisms. This causes the formation of quasispecies or mutant swarms (Andino & Domingo 2015). The CcV seems to be an extreme example of this with very high sequence diversity, represented by the 20-65% amino acid identity found between sequences (Table 1). Compared with other dsRNA viruses, such as the polio virus with two nucleotide mutations per genome or less than 0.03% difference in nucleotidic sequences (Vignuzzi et al. 2006) and a similarly low diversity in the St. Louis encephalitis virus (Ciota et al. 2011). The genetic diversity of CcV is thus several orders of magnitude higher. One explanation for this higher sequence diversity could be the absence (or rarity) of horizontal spreading of totiviruses compared to the polio and encephalitis viruses. To our knowledge, the sequence diversity of totiviruses within one host has only been reported as different viruses e.g. the *X. dendrorhous* virus XdVL1 and XdVL2 (Baeza et al. 2012) which are less divergent than the CcV sequences. It should be noted that despite the large sequence variability only one distinct band was seen, implying sequence diversity rather than size diversity and also that size is conserved.
even though the sequence is not. In addition, the acquired sequences typically coded for open reading frames. The CcV system is therefore a potentially interesting system to study the biology of quasispecies and a model to study viral evolution with previously unknown features.

Compared to totiviruses from, for example, *X. dendrorhous, Saccharomyces cerevisiae* and *Tuber aestivum*, the major conserved amino acid residues described in the consensus sequence of the totivirus *pol* gene (Baeza et al. 2012) are present in the CcV sequence (Figure 3). This is also true for domains 5 and 6, which have been demonstrated to be essential for viral function (Ribas 1992, Routhier 1998). Together, this strongly indicates that functional dsRNA from a totivirus-like entity is present in *C. crispus*.

The totiviruses belong to the mycoviruses which are widespread in yeasts and filamentous fungi but have not been reported from algae. Typically, they do not possess extracellular infections modes, are not associated with obvious disease symptoms, and do not exhibit harmful effects (Ghabrial and Suzuki, 2009). Only a few reports of phenotype modifications like hypovirulence or antifungal activity have been described (Pearson et al, 2009, Magliani 1997). Moreover, natural vectors are so far unknown (Ghabrial and Suzuki, 2009). It has been suggested that the dominant mode of diversification in most totiviruses is codivergence, parallel diversification between host and parasite (Göker et al, 2011). The large sequence diversity found in CcV indicates that other phenomena are probably occurring in the interaction between *C. crispus* and CcV.

Viruses from the totiviridae family are mainly found in fungi and protozoa. Penaeid shrimp infectious myonecrosis virus (IMNV), isolated from the Pacific white shrimp,
was the first totivirus described infecting a host other than a fungus or a protozoan (Nibert, 2007). Another virus, DTV, genetically related to IMNV was found to infect a Drosophila cell line (Wu et al., 2010). Totiviruses have also been isolated from mosquitoes (Armigeres subalbatus) (Zhai et al., 2010, Isawa et al., 2011). The viruses isolated from mosquitoes are genetically similar to IMNV and DTV (Isawa et al., 2011). A totivirus, related to the Giardiavirus, has also been found in salmon (Haugland et al., 2011). Together with our data presented here, this shows that the host range for totivirus is relatively large within the eukaryotes.

The totivirus genome organization is characterized by a non-segmented dsRNA, with two genes, gag and pol, encoding two overlapping proteins, a capsid protein (CP) and an RNA-dependent RNA polymerase (RdRp). In most totiviruses, the (+) strand viral transcript is flanked by a 5' and a 3'-UTR and directs the translation of the CP protein and a smaller CP-RdRp fusion protein via a -1 ribosomal frameshift. The ribosomal -1 frameshift site consists of a heptamer with the consensus motif XXXYYYY, (X represents any nucleotide, Y represents A or U, and Z represents A, C or U) preceded by a GC dinucleotide (Jacks et al., 1988; Bekaert et al., 2003). The heptamer is followed by a short region preceding a stable RNA secondary structure, such as a pseudoknot or hairpin. The translational efficiency has been demonstrated to be directly related to the stability of these structures (Bidou et al. 1997). Slippery sites can promote a low level of frameshifting and downstream pseudoknot structures stimulate this process (Giedroc et al. 2000). In the 166 bp overlapping part of the sequence of CcV2 a putative slippery site, GGGUUUU, is located 18 nucleotides upstream a pseudoknot type structure. The structure has an estimated free energy at -18.85 kcal/mol (Figure 4). Thus, the CcV genome is organized as described in other totiviruses with two overlapping ORF including a 5'-ORF coding a capsid protein
and a 3'-ORF coding an RNA-dependent RNA polymerase. This involves an ORF disruption with an internal stop codon and -1 ribosomal frameshift.

Viral sequences from various families are sometimes found integrated into the host organism’s genome (Holmes 2011). A search using the CcV sequence in the sequenced *C. crispus* nuclear genome showed that one non-identical copy of the *gag* gene was present (Fig 4B). RNAseq data showed expression of this gene in *C. crispus* (not shown). Sequence analysis suggests that the gene also contains regulatory sequences (see below). This indicates that a transfer has occurred from the virus to the *C. crispus* genome. This type of transfer between endogenous viral elements from non-retroviral RNA viruses is known from insects (Chrochu et al. 2004), other animals (Katzourakis and Gifford 2010), and plants (Chiba et al. 2011).

For the viral genes found in plant genomes they represented both single- and double-stranded viruses of various families. There is evidence that totiviral and partitiviral genes are transferred frequently into the nuclear genome of eukaryotes, and that some transferred genes have functions in the recipient genomes since totivirus-like sequences have been identified in, for example, *Medicago truncatula*, *Lotus japonica*, and *Populus trichocarpa*, where they have been matched to the *gag* gene of the *Vicia* cryptic virus M (Liu et al. 2010). ESTs from totiviruses have also been found in a cDNA library from the diatom *Phaeodactylum tricornutum* (Liu et al. 2012), but it is unknown if this was due to incorporation in the genome or caused by the presence of viral entities. Normally, after integration of viral gene sequences in a genome the genes start to degenerate (Zwart et al. 2014). Considering that the *gag* gene in the nuclear genome is full length and has EST support, this suggests that either the gene provides an advantage to *C. crispus* or that the integration is recent. It could be noted that transgenic maize expressing the totiviral protein KP4 is more
resistant to fungal infection (Allen et al. 2011) and thus, incorporation of viral genes may have positive effects. No ESTs or presence in the genome were found for the \textit{pol} gene in \textit{C. crispus}.

A close analysis of nuclear genome regions around the \textit{gag} gene gave unexpected results (Figure 6). Consensus sequences of prokaryote-type promoters were found in the upstream part of the gene. This includes a sigma70 type promoter located -11 of the TATAAA-box and -38 of the TTGAGA-box preceded by a CAAT-box located -67 before a putative start codon. Using yeast mitochondrial codon table instead of the eukaryote nuclear one results in a unique reading frame for the \textit{cap} gene from the methionine codon found downstream the TATA-box. Interestingly, 35 aa preceding the putative \textit{gag} start codon show similarities with a bacterial protein from \textit{Simkania nevegensis}. Furthermore, in the same reading frame 24 aa sequence homologous to the cytochrome P450 (CYP) CYP808A2 (XP_005710778.1) from \textit{C. crispus}.

Preceding the putative CYP gene are several putative regulatory boxes, three successive adjacent E-Boxes, followed by a G-Box, and a GCC-Box.

An E-Box is a cis-acting element, a conserved DNA sequence involved in regulation of gene expression linked to environmental and physiological signaling. These elements serve as binding sites for transcriptional activators, and have been demonstrated to be important for circadian gene expression (Giuliano et al., 1988, Staiger et al, 1989, Williams et al., 1992). For example, in the green alga \textit{Chlamydomonas reinhardtii}, upregulation of the C3 subunit of the CHLAMY1 RNA-binding protein is mediated by an E-box element in its promoter; causing temperature-dependent up-regulation of C3, its circadian expression, and its co-regulation by C1 (Seitz et al, 2010). The G-Box is a conserved DNA sequence in plants that has been described as a putative sugar sensor involved in starch
catabolism (Reinhold et al., 2011). A close proximity of the G-Box and the GCC-box is often found in tobacco and linked to the regulation of gene expression during plant defense responses (Buttner, 1997). GCC-box sequences in plants are promoter elements involved in the regulation of expression of plant defense genes against biotic or abiotic stressors and play an important role in relaying pathogen-initiated signals and the activation of plant defense responses (Solano et al, 1998, Brown et al, 2003; Shenk et al, 2000; Reymond et al, 1998). GCC boxes have also been characterized in tomato endochitinase, glucanase and osmotin genes (Tournier et al, 2003). This gene structure rich in sensor motifs and a viral-type sequence indicates that gene expression is modulated by changes in environmental or physiological conditions.

We used the gag and pol sequences from CcV and the nuclear gag sequence to understand the phylogeny of the CcV. The sequences for the gag and pol genes were compared with other sequences of totiviruses found in GenBank in December 2015. The pol genes from CcV cluster within the totivirus and is most closely related to the XdV gene (Figure 7). It is to be noted is that all of these sequences are from terrestrial organisms and most from Fungi. It is, however, unclear if the sequences obtained from terrestrial plants are due to fungal infections or if they are using the plant cells as hosts (Roossinck 2010). Similarly, the CcV gag genes cluster with the XdV gene. However, the genomic gag gene did not cluster with the CcV sequences, but rather with the Saccharomyces and Scheffersomyces virus gene sequences. The gag gene it is sometimes reported to be of viral origin and sometimes to be of nuclear origin (Figure 7), but no clear phylogenetic pattern emerged.

Even though the nuclear gag gene was expressed and dsRNA coding for the gag and pol genes were present in C. crispus, inspections of TEM images showed no clearly
identifiable virus-like particles (not shown) nor were any attempts to purify viral particles successful. Therefore, we are not presently convinced that viral particles are produced in *C. crispus* during normal conditions.

After finding the CcV in our gametophytic laboratory strain we wanted to know if this was specific for this strain and this life cycle stage. We therefore collected *C. crispus* from various locations and in different life cycle stages. The dsRNA could be visualized in all life stages tested, including tetrasporophytes and male and female gametophytes of *C. crispus* from Roscoff and in each part of the thallus tested. A band could also be seen in *C. crispus* samples from every location tested; in Europe from the northernmost location, Tjärnö in Sweden, via Plymouth in the UK, to the southernmost location, Porto in Portugal (Table 2). The band was also present with similar intensity in *C. crispus* with visible epiphytes and lesions. This indicates that the presence of dsRNA is wide-spread within *C. crispus*, at least along the European coast. For the laboratory strain with an origin in Canada, we cannot exclude an "infection" after the arrival of the strain in the laboratory in 1986.

We also wanted to verify if the presence of dsRNA was a phenomenon specific for *C. crispus* or if it was a more general occurrence. We therefore collected samples of other red algae around Roscoff, France (Table 3). A visible band was found in most of the red algae, except *Palmaria palmata* and *Furcellaria lumbricalis*, including one member of the Bangiales, a *Porphyra/Pyropia* species. This indicates that the presence of dsRNA identities of probable viral origin is wide-spread in red algae.

When the first sequence signatures showed similarities with totiviruses, which are mainly found in fungi or protozoa, we first suspected a contamination of *C. crispus* by epi- or endophytes in the culture medium. To exclude this, we decided to extract
nucleic acids from protoplasts. In protoplasts, the cell wall is enzymatically digested
with cellulase and carrageenase (Le Gall et al, 1990). The total nucleic acid
extraction from protoplast was then obtained and a band of similar intensity was seen
(Figure 7).

We consider CcV to be associated with C. crispus and not other eukaryotic
organisms since it was found in extractions of RNA performed simultaneously with
the DNA extractions for the genome project and no DNA from other eukaryotes were
found during the genome analysis. The cultures of C. crispus are not axenic, so it is
possible that the dsRNA virus is associated with bacteria. However, the virus was
also present in extractions of RNA from protoplasts (Figure 7), where bacteria
associated with the cell wall were drastically reduced. Totiviruses are also unknown
from bacteria (Ghabrial and Suzuki 2009) and considering the amounts of dsRNA
found on the gels we find it is highly likely that CcV is associated with C. crispus
and not an associated organism. This is, at least, true for the Peggy’s Cove strain. For
the other samples microscopic analysis to localize endophytic fungi gave negative
results (data not shown), but we cannot formally exclude that endophytic fungi
associated with algae might be the host of totiviruses for these strains and species.

CONCLUSIONS

A totivirus-like entity closely related to viral strains that mostly infect fungal species
exists in the red macroalga Chondrus crispus. Double-stranded viral RNA was
found in relatively high quantities, but no viral particles were found, nor has any
phenotype been identified. It is present in all tested life cycle stages and ubiquitous in
C. crispus isolates from different geographic areas. In a limited sample very large
sequence diversity was seen. Similar bands of dsRNA are present in other red algae. To our knowledge, this is the first time a virus-related sequence has been reported from a red alga and the first time a totivirus is reported from algae. The CcV genome is organized as described in other totiviruses with two overlapping ORFs including a 5'-ORF coding a capsid protein (CP) and a 3'-ORF coding an RNA-dependent RNA polymerase. This involves an ORF disruption with an internal stop codon and a -1 ribosomal frameshift. A copy of the gene encoding the capsid was identified in the nuclear genome of *C. crispus*. Despite the fact that the presence of dsRNA seems to be widespread in red algae nothing is known about the physiological and ecological consequences of the association with totivirus-like entities.

**ACKNOWLEDGMENTS**

Work at the *Station Biologique de Roscoff* was supported by the *Centre National de Recherche Scientifique*, the University Pierre and Marie Curie, and IDEALG Grants ANR-10-BTBR-04-02 and 04-04 “*Investissements d’avenir, Biotechnologies-Bioressources*”. We also want to thank Marion Azidrou and Léa Cabioch for work performed early in the project, Anne-Claire Baudoux for expertise on viral particle precipitation, Gabriel Markov for CYP analysis, Simon Dittami for helpful comments, and Gunilla Toth and Isabel Sousa Pinto for collecting *Chondrus crispus* in Sweden and Portugal.
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Quasispecies diversity determines pathogenesis through cooperative interactions in a

West, J. A., Pueschel, C. M., Klochkova, T. A., Kim, G. H., de Goër, S. &
Zuccarello, G. C. 2013. Gall structure and specificity in Bostrychia culture isolates


al. 2010. Isolation and full-length sequence analysis of Armigeres subalbatus
totivirus, the first totivirus isolate from mosquitoes representing a proposed novel

31:121-34.
Table 1. Comparisons of % identity between amino acid sequences obtained by sequencing a dsRNA band from *Chondrus crispus* and comparing multiple sequences for the *pol* gene. Sequences were translated to amino acids, aligned and compared pairwise using Clustal Omega (www.ebi.ac.uk).

<table>
<thead>
<tr>
<th></th>
<th>CcV1</th>
<th>CcV2</th>
<th>CcvP5</th>
<th>CcvP6</th>
<th>CcvP7</th>
<th>CcvP8</th>
<th>CcvP9</th>
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<tr>
<td>CcV1</td>
<td>100</td>
<td></td>
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<tr>
<td>CcV2</td>
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<td>CcvP8</td>
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<td>19</td>
<td>26</td>
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</tr>
<tr>
<td>CcvP9</td>
<td>50</td>
<td>61</td>
<td>52</td>
<td>32</td>
<td>65</td>
<td>27</td>
<td>100</td>
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</table>
Table 2. The presence of a detectable dsRNA band around 6 kbp in RNA extractions from *Chondrus crispus*.

<table>
<thead>
<tr>
<th>Life cycle stage</th>
<th>Location</th>
<th>Virus detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gametophyte male</td>
<td>Roscoff, France</td>
<td>Yes</td>
</tr>
<tr>
<td>Gametophyte female</td>
<td>Roscoff</td>
<td>Yes</td>
</tr>
<tr>
<td>Tetrasporophyte</td>
<td>Roscoff</td>
<td>Yes</td>
</tr>
<tr>
<td>Gametophytes</td>
<td>Roscoff</td>
<td>Yes</td>
</tr>
<tr>
<td>stressed*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gametophyte</td>
<td>Tjärnö, Sweden</td>
<td>Yes</td>
</tr>
<tr>
<td>Gametophyte</td>
<td>Plymouth, UK</td>
<td>Yes</td>
</tr>
<tr>
<td>Gametophyte</td>
<td>Helgoland, Germany</td>
<td>Yes</td>
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<tr>
<td>Gametophyte</td>
<td>Chaucre, France</td>
<td>Yes</td>
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<td>Gametophyte</td>
<td>Lorient, France</td>
<td>Yes</td>
</tr>
<tr>
<td>Gametophyte</td>
<td>Viana do Castelo, Portugal</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Algae with visible epiphytes and lesions.*
Table 3. The presence of a detectable dsRNA band with a size around 6681 kbp in different species of red macroalgae collected around Roscoff, France.

<table>
<thead>
<tr>
<th>Species</th>
<th>Class</th>
<th>Band detectable</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dumontia contorta</em> (S.G. Gmelin) Ruprecht</td>
<td>Florideophyceae</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Grateloupia turuturu</em> Yamada</td>
<td>Florideophyceae</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Chondracanthus acicularis</em> (Roth) Fredericq</td>
<td>Florideophyceae</td>
<td>Yes</td>
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<tr>
<td><em>Porphyra/Pyropia sp</em></td>
<td>Bangiophyceae</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Laurencia pinnatifida</em> (Hudson) J.V.</td>
<td>Florideophyceae</td>
<td>Yes</td>
</tr>
<tr>
<td>Lamouroux</td>
<td></td>
<td></td>
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<tr>
<td><em>Mastocarpus stellatus</em> (Stackhouse) Guiry</td>
<td>Florideophyceae</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Polysiphonia elongata</em> (Hudson) Sprengel</td>
<td>Florideophyceae</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Palmaria palmata</em> (L.) Weber &amp; Mohr</td>
<td>Florideophyceae</td>
<td>No</td>
</tr>
<tr>
<td><em>Corallina elongata</em> J. Ellis &amp; Solander</td>
<td>Florideophyceae</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Furcellaria lumbricalis</em> (Hudson) J.V.</td>
<td>Florideophyceae</td>
<td>No</td>
</tr>
<tr>
<td>Lamouroux</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. Agarose gel electrophoresis of nucleic acid extract from *Chondrus crispus*:
A. native control (1), native control incubated 1h at 40°C (2), DNase1 treated (3), RNase treated (4); L Smart Ladder (Eurogentec). B. Native control with 10% DMSO (1), after denaturing 5 min at 95 °C with 10% DMSO.

Figure 2. Schematic representation of the *Chondrus crispus* viral sequences compared to the sequences of related viral sequences of *Xanthophyllumyces dendrorhous* (XdL1b, XdL2) and *Saccharomyces cerevisiae* (ScVL-A(L1)); note the presence of the viral capsid sequence integrated into the *C. crispus* genome (CcVGe). The capsid coding open reading frame (ORF) in blue, RNA dependent RNA polymerase coding ORF in red, and overlapping ORF in purple.

Figure 3. Alignment of domains in the RNA-dependent RNA polymerase (pol) gene from totiviruses as determined in Bruenn (2003) and Koonin (1991) with the *Chondrus crispus* dsRNA sequences. The consensus conserved domains described in Baeza et al. (2012) are highlighted.

Figure 4. Pseudoknot structure prediction of the CcV2 sequence using DotKnot method (based on Cao and Chen 2006, 2009) and drawn using PseudoViewer3 (Byun et al. 2009).
Figure 5. Genome region of *Chondrus crispus* containing the *gag* gene homolog.

CYP: fragment of a cytochrome P-450 gene similar to a *C. crispus* nuclear gene (in blue); E-Box, G-Box and GCC-Box (in grey boxes): cis-regulatory elements located upstream the *gag* gene CcVGe (in red); open reading frame related to an unknown protein found in *Simkania nevegensis* (in green); putative sigma-type promoters (underlined in black); universal genetic code/yeast mitochondrial genetic code (in italics).

Figure 6. A. An unrooted neighbor-joining tree of the capsid sequence (n, reported as nuclear gene; v, reported as viral gene). B. An unrooted neighbor-joining tree of RNA dependent RNA polymerase sequences. The alignment was based on entire sequences using Muscle and neighbor-joining analysis was carried out in MEGA.

Figure 7. Gel electrophoresis of nucleic acid extracts from *C. crispus* protoplasts on a 1% agarose gel stained with EtBr. L: smart Ladder Eurogentec.
Figure 1

A

L | 1 | 2 | 3 | 4

← Host gDNA
← Viral RNA
← Host RNAs

B

L | 1 | 2

← Host gDNA
← Viral RNA
← Host RNAs
Figure 2