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Abstract

Cyanobacteria constitute a versatile group of photosynthetic bacteria of immense commercial and ecological importance. Some species of this group are cultivated and sold as food because of their high nutritional value. This is typically the case for *Arthrospira platensis*. We describe for the first time a virus infecting this economically important filamentous cyanobacterium isolated from culture pools located in the South of France. This virus could be observed and discriminated easily from other particles with flow cytometry. Based on morphology and molecular investigation, it was proposed that the virus belongs to the cyanopodovirus group with a capsid and short tail of about 120 and 20 nm, respectively. Finally, the virus appeared to be highly specific (very narrow host range) to *A. platensis*.

Keywords

Cyanobacteria; cyanophage; culture; characterisation; *Arthrospira platensis*

Introduction

The genus *Arthrospira* (ex *Spirulina*) is a non-heterocystous filamentous cyanobacterium, characterised by multicellular, cylindrical and usually screwlike coiled trichomes, inhabiting diverse environments including those of high salinity (Anagnostidis and Komarek 1988; Manen and Falquet 2002). Several strains have been isolated worldwide, and made useful in a variety of fundamental and applied research studies: commercial mass cultures have indeed been developed for the food industry in local areas, but also for alternative biofuel feedstock, skin-care product resources, etc (Ciferri and Tiboni 1985; Belay et al. 1996; Fox 1996). To the best of our knowledge, nothing has been published yet on viruses associated to the dynamics of this species, despite its high commercial value.

Bacteriophages infecting cyanobacteria, namely cyanophages, are tailed and contain dsDNA. They belong to three families: the cyanomyoviruses (virus with a long contractile tail), the siphoviruses (virus with a long non-contractile tail) and the podoviruses (virus with a short or non-apparent tail) (Safferamn et al. 1983). Cyanophages were first studied in freshwater systems where a virus infecting a filamentous cyanobacterium had been isolated about 50 years ago (Safferman and Morris 1963). Following this discovery, the isolation and characterization of several freshwater cyanophages were studied extensively between the 1960's and early 80's (Brown 1972; Padan and Shilo 1973; Safferman 1973; Sherman and Brown 1978; Gromov 1983). Cyanophage description, infecting both unicellular and filamentous marine cyanobacteria, occurred after 1980. It was not until the early 1990's that cyanophages infecting the marine *Synechococcus* were isolated (Wilson et al. 1993; Suttle 2000). The literature has become relatively rich over recent years with the description of cyanophage structure and diversity, both for the ocean and for some lakes (Short and Suttle 2005; Wilhelm et al. 2006; Chen et al. 2009; Matteson et al. 2011). On the other hand, studies about the characterisation, ecological importance and dynamics of cyanophages infecting specific cyanobacteria remain relatively scarce (Sandaa and Larsen 2008; Yoshida et al. 2008).

Being alerted during the summer of 2011 by an episode of high *Arthrospira platensis* mortality cultured in some pools located in the South of France, we suspected that such event mortality could be due to a mass lytic process involving specific viruses, i.e. cyanophages. We obtained water samples from different pools with or without mortality to test for the presence of viruses. We discovered a cyanophage, able to infect and lyse *A. platensis* and for which a basic description is provided.

Materials and methods

Site and sample description A dozen of samples were obtained from "le chant de l'eau", an exploitation based in the south of France (Fuilla) and consisting of 8 pools of 70 -200 m^2. Growth conditions of the cyanobacterium have been described in Jourdan (2006). Briefly, *A. platensis* grew in outdoor and under glass pools inside which a soft agitation is provided with a temperature varying between 25°C and 35°C, natural light:dark cycles and nutrient concentrations as described thereafter (sodium bicarbonate = 8 g/l; potassium sulfate = 1 g/l ; sodium chloride = 5 g/l ; potassium nitrate = 2 g/l ; magnesium sulfate = 0.2 g/l ; calcium chloride = 0.1 g/l ; ammonium + ammoniac? = 0.2 g/l). Samples were taken from some of these different 15 cm depth pools where the cyanobacterium was observed to die (in a few hours to days) or not to die. The strain is referred to *paracas* from the species *A. platensis* owing to the original location where it was first isolated (i.e. Peru). The samples were subjected to flow cytometry and transmission electronic microscopy analysis, infection experiments and by various molecular techniques as described below. Alternatively, we also obtained samples from other farms of the South of France (referred to as Domaine algal, Carpio, Algosud) to test the infectivity of the virus and also to test for lysogenic induction.

Flow cytometry analysis Samples were pre-filtered through GF/F (Whatman) and polycarbonate 0.2 µm (Millipore) filters in order to remove all cellular materials. Viruses were observed and

counted using a FACS Calibur flow cytometer (Becton Dickinson) equipped with an air cooled laser providing 15 mW at 488 nm. Samples were fixed with glutaraldehyde (0.5% final concentration, grade I, Merck) for 30 min, then diluted in 0.02 µm filtered TE buffer (0.1 mM Tris-HCL and 1 mM EDTA, pH 8), and incubated with SYBR Green I (at a final 10^{-4} dilution of the commercial stock solution; Molecular Probes), for 5 min at ambient temperature in the dark, followed by an incubation for 10 min at 75°C, and then another 5 min at room temperature, prior to FCM analysis. Note that the viruses could also be observed without heating but the discrimination was comparatively poor. Analysis was made on samples to which a suspension of 1-µm beads had been added (Molecular probes). Flow cytometer listmode files obtained were then transferred and analyzed on a PC using the custom-designed freeware CYTOWIN (Vaulot 1989).

Host-range experiment For this test, the samples with the virus of interest was filtered twice through a 0.45 µm polycarbonate mesh syringe sterile (Fischer Scient.) filter to remove all particles but not the viruses. Infection was processed classically by adding 500 µL of the cyanovirus isolate to 2 to 5 mL of a variety of cyanobacterial strains, in duplicate, from the Thonon Culture Collection (TCC) or other collections : 25 PE-rich *Synechococcus* spp (TCC 32, 185, 186, 789 to 808), 10 PC-rich *Synechococcus* (PCC 6301, 6311, 6707, 6715, 7917, 7918, 7941, 7952, 9004 and 9005), 4 PC-rich *Synechocystis* (PCC 6308, 6803, 6905, 7509), 1 colonial cyanobacterial form (*Microcystis aeruginosa*, TCC 80) and 1 filamentous cyanobacterial form (*Planktothrix rubescens*, TCC 29), all originated from freshwater ecosystems. Finally the virus was tested against *A. platensis* obtained from the other farms mentioned above. The infectivity of the virus was not tested on marine species or strains since *A. platensis* is a freshwater cyanobacterium that cannot be cultured in natural seawater even if it accommodates a high salinity range up to 25 mg/L. Only when a clear lysate was produced in the duplicates the infection was recorded as successful.

Infection of host cells The process of infection was studied by adding a suspension of <0.45 µm suspension of virus particles to 4 different cultures of *A. plantensis* and changes within the algal

host was followed by fluorescence using a FluoScan (Metrastat). Note that we neither tested different multiplicity of infection nor worked with many replicate treatments.

Induction of lysogenic A. platensis We addressed the prevalence of lysogeny within different cultures of *A. platensis* following Dillon and Parry (2008). Briefly, a 1 mg/L stock solution of mytomycin C (Sigma) was prepared in <0.02 µm filtered milliQ water and stored in the dark at 4°C. 10 ml aliquots of exponentially growing cultures of each *A. platensis* sample were incubated with mitomycin C at final concentrations of 0 (control containing only water), 1, 5, and 20 µg/mL under a 14:10 light: dark (L:D) cycle for 2 weeks in our temperature-controlled algal culture room. 1 mL sub-samples were taken at time 0, 7 and 14 days to count viruses with FCM (the cyanophage and the bacteriophages from the contaminating heterotrophic bacteria being easily discriminated by FCM parameters due to different side scatter and green DNA-dye complex fluorescence as shown on Figure 1).

Transmission Electron Microscopy (TEM). For visualization and characterisation of viral particles by TEM, glutaraldelhyde fixed samples (1% final concentration) stored at 4°C were then harvested by ultracentrifugation onto 400 mesh NI electron microscope grids with carbon-coated Formvar film, by using a Beckman Coulter SW40 Ti Swing-Out-Rotor run at 70,000 x g for 20 min at 4°C (Weinbauer and Peduzzi 1994; Sime-Ngando et al. 1996). Each grid was stained at room temperature (ca. 20°C) for 30 s with uranyl acetate (2% wt/wt), rinsed twice with 0.02 µm filtered distilled water and dried on a filter paper. Grids were then examined using a JEOL 1200EX TEM operated at 80 kV at a magnification of x 100,000. The photographic negatives were scanned with Adobe Photoshop and cynaophage dimensions were determined using IMAGEJ software.

Primers and Polymerase chain reaction (PCR) analysis To identity the virus, different primers (see Table 1) were used like the CPS1/CPS2, CPS4/CPS5, CPS1.1/CPS8.1, all targeting the portal-vertex-capsid-protein encoded gene *g20* of Cyanomyovirus (Fuller et al. 1998; Wilson et al. 2000; Sullivan et al. 2008), MZIA1bis/MZIA6 targeting the major-capsid-protein encoded gene *g*23 of Myoviridae (Filée et al. 2005), and *pol1* designed by Chen et al. (2009) and *pol2* designed in this

study (targeting the Cyanopodovirus specific DNA polymerase gene of cyanopodovirus). Briefly, the *pol2* primers were designed based on the sequences of marine and estuarine podoviral *pol* gene sequences available in GenBank (FJ872594 to FJ872816). The primers were designed using primer 3 software (http://frodo.wi.mit.edu/primer3/) and primer design software of NCBI (Primer-BLAST). A total of 16 primers were obtained and tested for the efficiency to amplify the viral concentrate. The primer pair initially called Jason 3Fa and 4R (hereafter referred to as *pol2*), was designed to give a positive amplicon of 389 bp. The *pol2* primer was tested for its specificity on a variety of viral concentrates (both cyanophage and algal virus isolates) supplied by different colleagues (see acknowledgements) as both positive or negative controls. Other primers were also tested such as MCP F/R and AVS1/2 targeting the major capsid protein of algal viruses (Chen and Suttle 1995, Larsen et al. 2008) to test the identity of the virus. All these tests were performed both on untreated sample but also on FCM sorted population after reconcentration using centrifugal filter units (Amicon UltraCell 10 Kda, Millipore) if necessary for PCR analysis.

The PCRs were performed by using the DNA Thermal Cycler T-Professional (Biometra) with the optimised conditions for each primer (Zhong et al. in preparation). Briefly, 25-µl reaction mix contained 1X PCR buffer, 2 mM $MgCl_2$, 200 µM of each dNTP, 0.4 µM of each primer, 0.5 U of Platinium ® *Taq* DNA polymerase (Invitrogen), and 1 µl of sample. The virus concentrates provided by colleagues (see acknowledgments) were also used either as positive or negative controls for PCR. PCR products were subjected to electrophoresis on a 1.5 % (w/v) agarose gel in 0.5X TBE (89 mM Tris-base, 89 mM boric acid, 2 mM EDTA, pH 8.0) and visualized by ethidium bromide staining on a UV transilluminator (Tex-35M, Bioblock Scientific) and photographed using a gel documentation system (BioRad, Germany).

Results and Discussion

Use of cyanobacteria as a food supplement has a long history (Gantar and Svircev 2008) dating back to Antiquity. Referred wrongly to as “Spirulina”, *Arthrospira* Stizenberger 1852, which is in

fact more related to *Planktothrix* and *Lyngbia* following Manen and Falquet (2002), have already been collected and used by Aztec populations (Ciferri 1983; Pulz and Gross 2004). Even today, malnutrition, especially due to a protein-poor diet is widespread in many parts of the world. The use of cyanobacteria as a non-conventional source of food and protein is a reality (Pulz and Gross 2004; Gantar and Svircev 2008; Rosenberg et al. 2008). At present, *Arthrospira* represents the second most important commercial microalga for the production of biomass as a health food and animal feed, after *Chlorella* (Voshak and Tomaselli 2000). Thus, in addition to purely fundamental aspects, it can be crucial to know the existence of factors such as viruses susceptible to impact severely culture systems and leading to economic losses.

The analysis with FCM (Fig 1) revealed a typical viral signature with a high FL1 (DNA-dye complex) level, different to what is usually observed in natural samples with the "classical" VLP1 to VLP4 (Larsen en al. 2004; Duhamel et al. 2006; Personnic et al. 2009). Such a FCM signature immediately suggested it could be a cyanophage, based on our own FCM cytogram experience and compared with different signatures of other cyanophage isolates (not shown). Obviously FCM signatures are not enough to characterise viruses (Brussaard et al. 2000) and other techniques were used to identify this particle unambiguously. The identity of the virus was further determined using both TEM and PCR. Such viruses can be observed easily with FCM that gives precise counts and thus the dynamics of such a virus in different culture conditions could be tested and followed.

TEM analysis revealed that there was a variety of phages in the different ponds with a majority of Podoviruses compared to the two other dsDNA Myo- and Siphovirus representatives. Once sorted using FCM and reconcentrated, TEM revealed that the virus infecting *A. platensis* was characterised by a short non contractile tail as it is the case for Cyanopodoviridae (Fig **5**). The capsid and the tail were about 120 and 20 nm, respectively, which is expected for such a cyanophage (Suttle 2000).

As a latter matter of proof, the PCRs conducted with primers targeting various groups of viruses (i.e. Myovirus, Podovirus, algal viruses) were negative for all the primers except for *pol1*

and *pol2* (Fig 3) from all the pools (Table 2). Here please change the titles inside the table as pol1 and pol2 instead of pol chen and new pol. The pool without culture (i.e. with just the water medium before to be cropped) was negative to all genes including *pol* gene PCRs performed in this study (Fig 3). At last, only VLP2 was positive to *pol* primers compared to VLP1 and VLP3. All these results thus supported that the virus was a cyanopodovirus. We refer to this cyanopodovirus obtained in this study as SPIRUVIR hereafter. We sequenced the PCR product of pol2 of SPIRUVIR to confirm its identity. We obtained a sequence of 365 bp whose BLAST search revealed 83 to 96% similarity to various DNA polymerase genes of cyanopodovirus sequences in the GenBank. The evolutionary history was inferred using the Neighbor-Joining method [1]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [2]. The evolutionary distances were computed using the Kimura 2-parameter method [3] and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences other than SPIRUVIR (Fig 4). Evolutionary analyses were conducted in MEGA5 [4].

It is noteworthy that we could also expect a positive response to *psbA* gene investigation since this photosynthesis gene has been found in many cyanophages except for the cyanosiphoviridae which have been reported not to carry the photosynthesis genes, both *psbA* and *psbD* (Huang et al. 2011).

The susceptibility of the various cultures was tested by the clarity of culture and development of the virus compared to controls. The podovirus caused lysis of only *A. platensis* cultures as against cyanobacterial and bacterial and eukaryotic hosts (not shown), demonstrating the high host specificity of this phage. This result is not surprising since cyanopodoviruses are generally reported as host specific, usually infecting only the host of isolation, compared to myoviruses typically that display a broader host range (Chen and Lu 2002; Mann 2003; Sullivan et al. 2003), even if Deng and Hayes (2007) also reported once that one podovirus could have a very broad host range. It was also interesting to note that the lysis was clearly different between the

different cultures and that some resistance phenomena could be observed (Fig 6), even if we did not work on reasons and mechanisms behind such a resistance that can dress different aspects (Thomas et al. 2012). From the lab experiments, we were unable to confirm the short latent period as suggested by the rapid biomass decay observed *in situ* in the pools (in one night) by the producers and also generally reported for cyanopodoviruses compared to the cyanomyo- and cyanosiphoviruses (Sullivan et al. 2003; Wang and Chen 2008).

Techniques we employed revealed that the virus was a cyanophage belonging to the Podoviridae family, i.e. a cyanophage with an isometric capsid head and short tail, a virus responding only to (cyanopodovirus-derived) *pol* gene PCR primers and with typical characteristics of this family referred earlier to the LPP (for *Lyngbya, Plectonema, Phormidium*) viruses, i.e. a narrow host range acting as parasite unique to a specific host (only *Arthropsira* was indeed infected by this virus). This virus is thus of particular interest since it is well known that most algal (including cyanobacterial) viruses have been largely isolated from marine provinces (Nagasaki and Bratbak 2010). It is also noteworthy that most cyanophages isolated to date belong to the family Myoviridae (Mann 2003) which has been reported to be easier to isolate, has a greater proportion of lytic cycle and a host range broader than the two other families (Suttle 2005).

In contrast to Padan et al. (1972), Cannon et al. (1971), Ohku and Fujita (1996), Hewson et al. (2001) who documented lysogeny in a variety of filamentous cyanobacteria or again Dillon and Parry (2008) who reported a high level of induction of lysogenic cells and release of temperate cyanopohages in the freshwater cyanobacterim *Synechococcus*, we did not obtain clear induction whatever the culture and the concentration of Mitomycin C used to induce cell lysis. As reported for other cyanobacterial genomes (Kettler et al. 2007; Dufresne et al. 2008), it is possible that there was intact prophages in *A. platensis*. The analysis of the genome of *Arthropsira platensis* using the Genbank database suggested however that a certain type of prophage may be present in the cyanobacterium, as in other cyanobacteria (Palenik et al. 2003; Sullivan et al. 2005), even if we failed to make the prophage element inducible if it is. If a cyanophage is integreated in the *A.*

platensis genome, the reason behind our incapacity to produce the virus and thus of this remarkable resistance is not known. Other inducible agents should be tested such as hight light and temperature or UV radiation before any clear conclusion could be advanced. As the mitomycin treatment did not lead to the production of new particles, it is unlikely however that the observed phage resistance was due to lysogeny.

We can only suggest here that such a virus could be indeed involved in the cyanobacterial culture mass mortality following favourable conditions for the virus to become lytic. In forthcoming studies, it will be important to be able to follow *in situ* population dynamics (both viruses and *A. platensis*) but also many other parameters that are likely to be important in growth and mortality processes of the cyanobacterium, such as light and nutrients, particularly nitrogen, both in terms of quantity and quality, but also zooplanktonic predation or eukaryotic parasitism. Interactions between cyanobacteria and cyanophages are complex and it will be also interesting to look in detail what can be the effect of the virus on the morphology shape and helix architecture of the cyanobacterium, known to be highly dependent on growth and environmental parameters (Vonshak and Tomaselli 2000). Several viruses, belonging to several families, can infect one single species in some cases. In other situations only one virus is virulent for one specific host. The environment is likely to play an important regulating factor on lytic *vs*. lysogenic processes in these interactions. We also know that cyanophages have probably played a key role in shaping some key metabolic processes such as photosynthesis in cyanobacteria. Cyanobacteria can be responsible for important resistance phenomena and associated costs. Differently said, cyanophages and cyanobacteria have a long history of co-evolution, an arm-race where both entities win and loss tour a tour and for which such interactions strongly depend on the environment.

Conclusion

We described for the first time a virus infecting one of the most commercially important species of cyanobacteria, *Arthrospira*, which is used for health food and animal feed and also reported as a

source of food additives, fine chemicals or again for the production of biofuels. This cyanophage may be an important regulating factor of this cyanobacterium but we do not know what are the main factors intervening in such a regulation and if, for instance, some important resistance mechanism, have been developed by the cyanobacterium. Improving our knowledge of such a virus is obviously a key issue.

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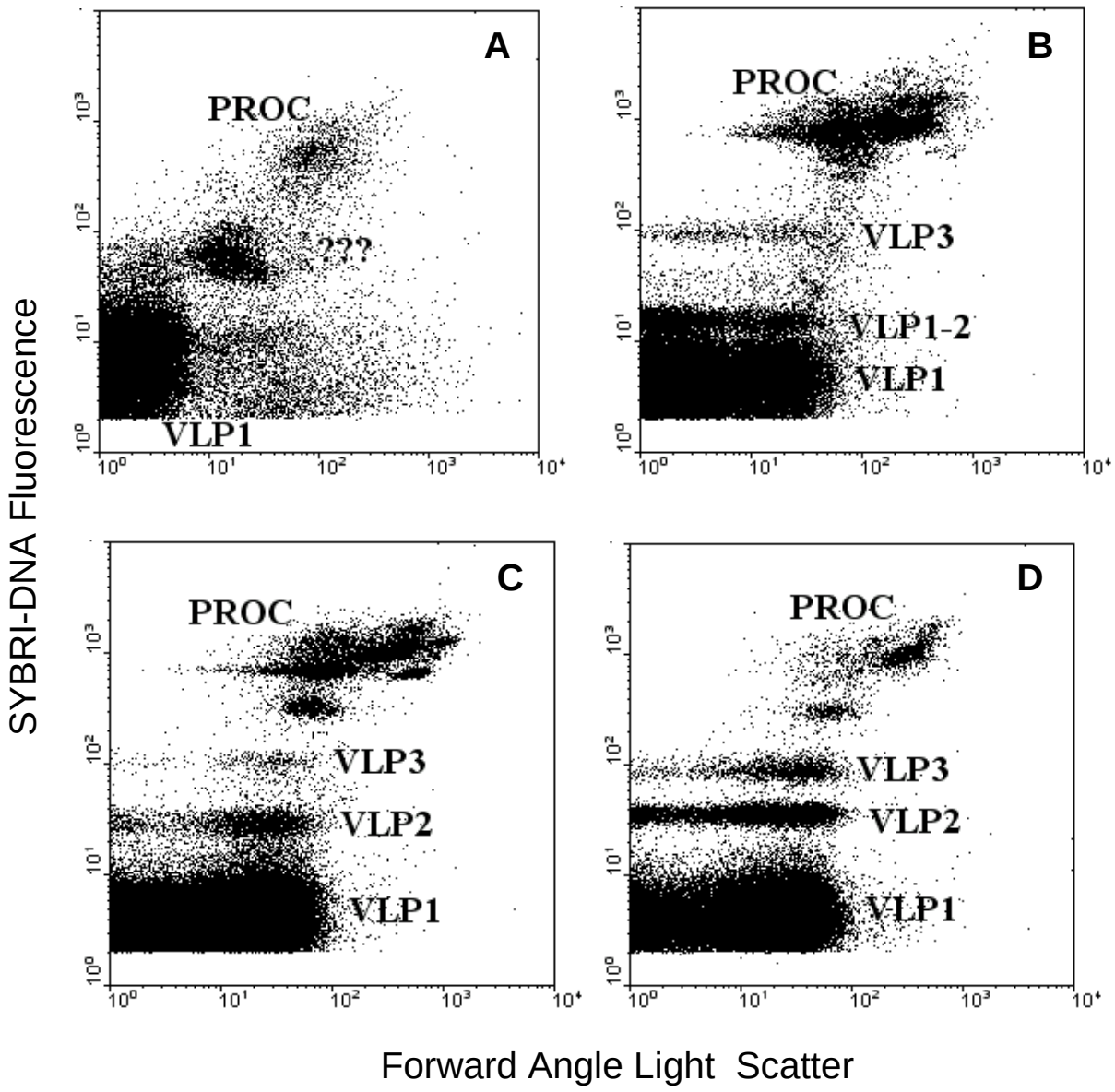

Fig1 FL1 (Sybr Green I – DNA complex fluorescence) *vs*. FSC (Forward Angle Light Scatter) cytograms showing typical viral signatures from the original samples received from *le chant de l'eau*. Samples were issued from a pool where there was no cyanobacterial mortality and recently fed with new medium (A), an important cyanobacterial mortality (B), recently inoculated with a new cyanobacterial culture but on a former contaminated site (C) and the beginning of symptoms of cyanobacterial mortality (D). Different signatures of viruses were referred to as VLP1, VLP1-2, VLP2 and VLP3. The prokaryotic (bacteria, cyanobacteria and archaea) community was referred to as PROC. VLP2 was the signature of the virus infecting *A. platensis*.

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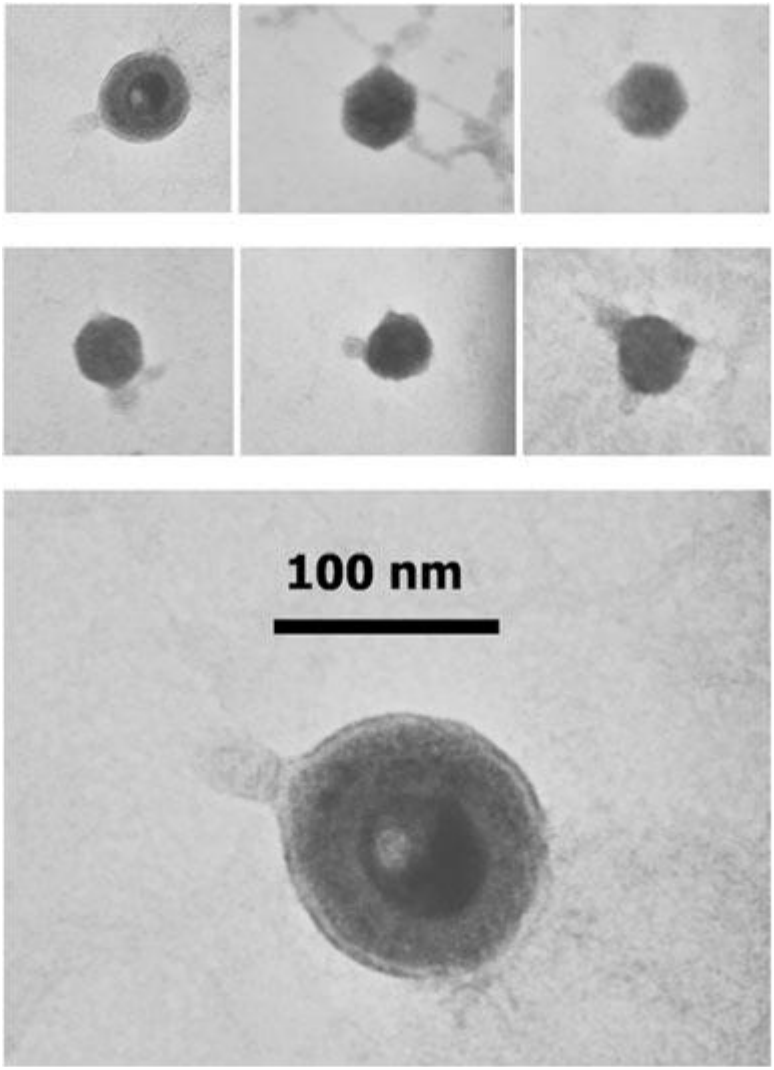

Fig. 2 TEM micrographs of the cyanopodivirus infecting A. platensis

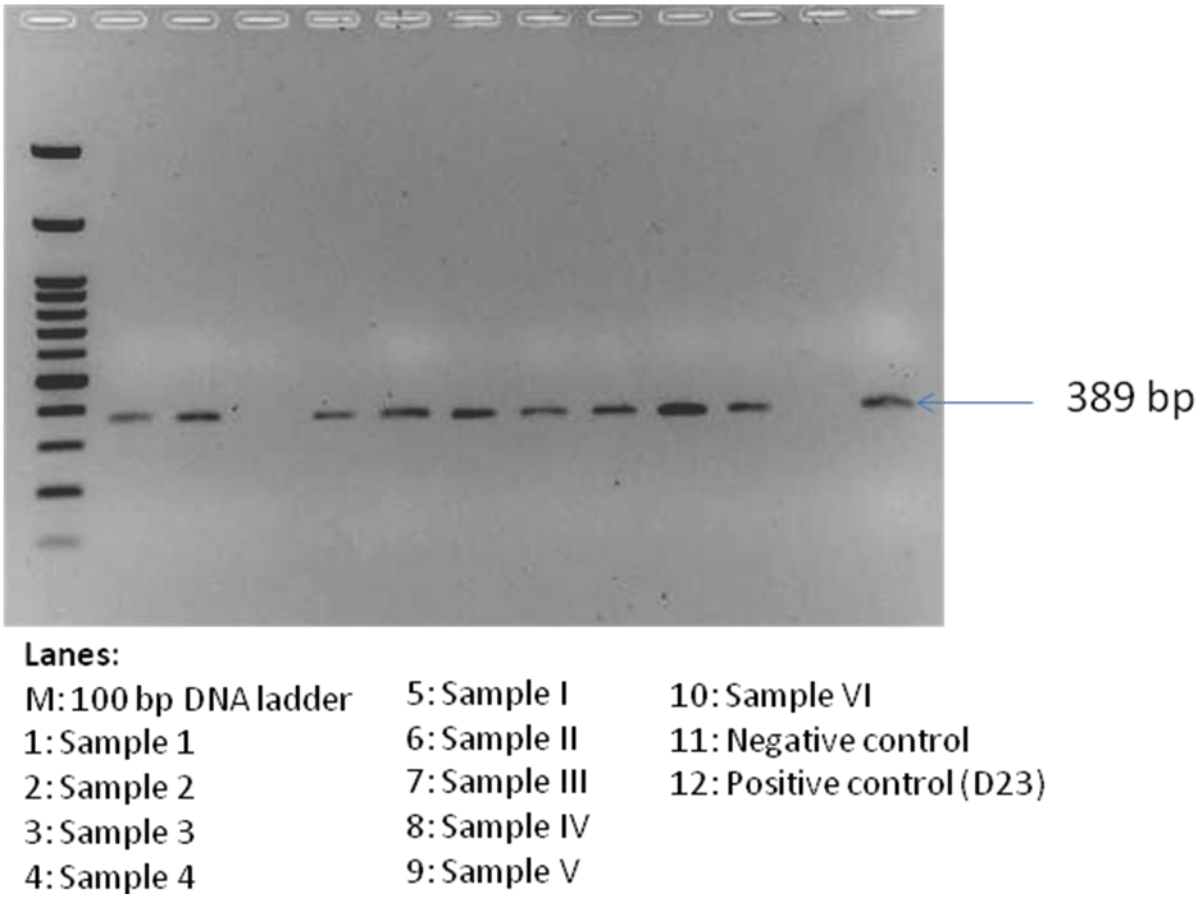

Fig3 PCR amplification using *pol2* primers for the cyanopodovirus resulting in an amplicon of 389 bp. All the samples received from different pools of the farms were subjected to PCR with pol2 primers. Lanes 1: S1, 2: S2, 3:S3, 4: S4, 5:SI, 5:SII, 6: SIII, 7: SIV, 8: S V, 9: S VI, N: Negative control 10: Positive control (D23), M: 100 bp ladder. Band absence is only observed in the negative control and for the pool in which only medium water was added.

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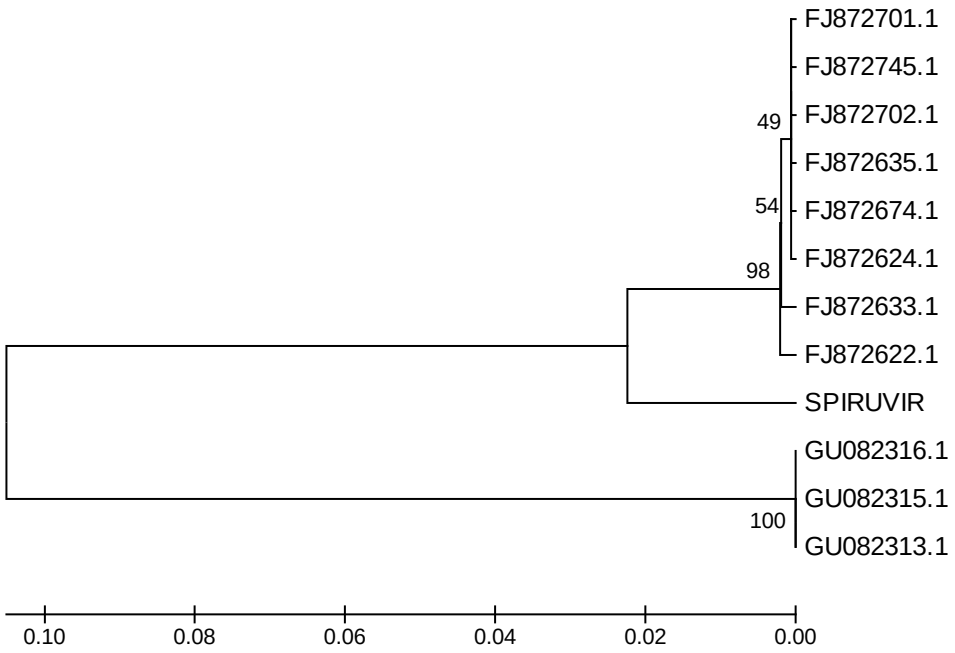

Fig4 Phylogenetic (Neighbour joining tree) tree showing the similarity of Spiruvir DNA polymerase gene to the podoviral DNA polymerase sequences in NCBI. The bootstrap value was given as 1000 using Kimura 2 parameter model for constructing the tree.

1

2

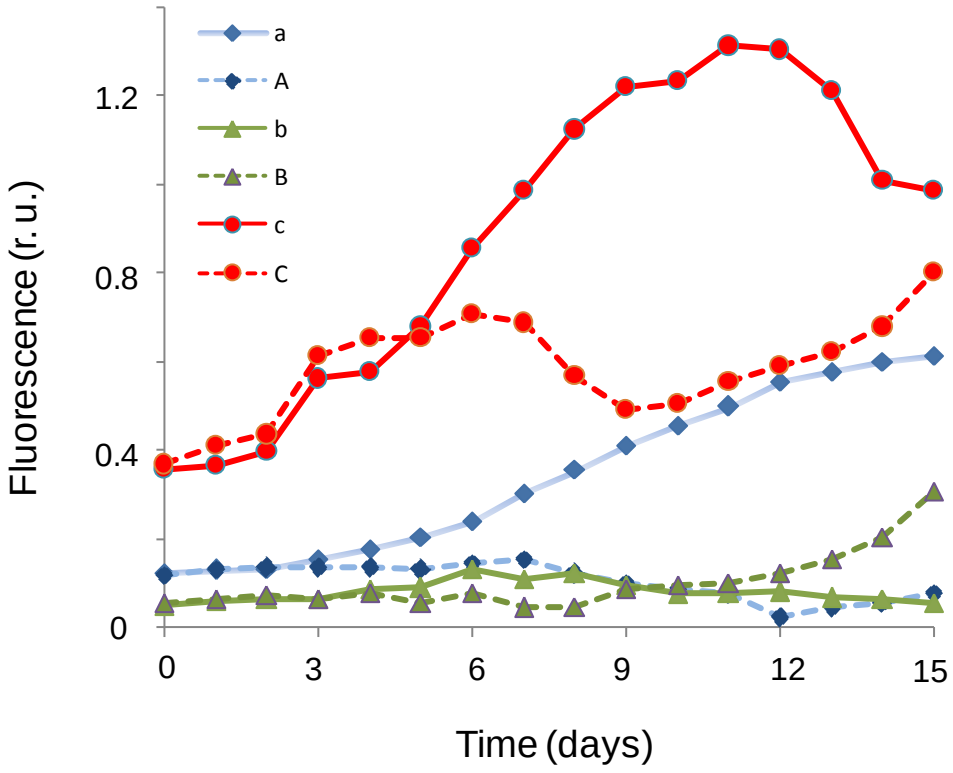

Fig5 Different cultures of *A. platensis* were inoculated with the virus at day 1 with a MOI of 1. It was clear from these infection experiments that infectivity and mortality processes differed in the cultures and that resistance could also be detected. A, B; C: Infected; a, b, c : not infected

1

Tab1 Table showing the details of the various primers used in this study.

Sl. No	Primer name	Primer sequence (5'-3') (F/R)	Target	Reference
1	CPS1/CPS2	GTAGWATTTTCTACATTGAYGTTGG/ GGTARCCAGAAATCYTCMAGCAT,	Portal-vertex-capsid-protein encoded gene *g20* of Cyanomyovirus	Fuller et al. 1998
2	CPS4/CPS5	GTAGAATTTTCTACATTGATGTTGG / GGTAACCAGAAATCTTCAAGCAT	Portal-vertex-capsid-protein encoded gene *g20* of Cyanomyovirus	Wilson et al. 2000
3	CPS1.1/CPS8.1	GTAGWATWTTYTAYATTGAYGTWGG/ ARTAYTTDCCDAYRWAWGGWTC	Portal-vertex-capsid-protein encoded gene *g20* of Cyanomyovirus	Sullivan et al. 2008
4	MZIA1bis/MZIA6	GATATTTGIGGIGTTCAGCCIATGA/ CGCGGTTGATTTCCAGCATGATTTC	Major-capsid-protein encoded gene *g*23 of Myoviridae	Filée et al. 2005
5	*pol1* (CP-DNAP-349F/533Ra/533Rb)	CCAAAYCTYGCMCARGT/ CTCGTCRTGSACRAASGC/ CTCGTCRTGDATRAASGC	DNA polymerase gene of cyanopodovirus	Chen et al. 2009
6	*pol2* (New pol)	ACTGCAACGCCTGGGATGGTG/ AGCAATGCGGCGACCGTCAA	DNA polymerase gene of cyanopodovirus	This study
7	MCP F/R	GGYGGYCARCGYATT / TGIARYTGYTCRAYIAGGTA	Major capsid protein of algal viruses	Chen and Suttle 1995
8	AVS1/2	(GARGGIGCIACIGTIYTIGAYGC / GCIGCRTAICKYTTYTTISWRTA)	Major capsid protein of algal viruses	Larsen et al. 2008

Tab2 Table showing PCR results for different primers targetting different viral groups, for all the samples received from different pools. The samples were positive only for the *pol* primers (referred in the table as pol-Chen and New pol, *i.e. pol1* and *pol2* in the text) targeting the cyanopodovirus. The absence of positive result is only observed for S3 that corresponded to a pool just filled with the culture medium and in which no cyanobacteria (and viruses) were added.

Samples	Primer Sets								
	mcp R/F	avs 1/2	psbA F/R	pol-Chen	g23	CPS1,1/8,1	CPS1/2	CPS4/5	New pol
S1	-	-	-	+	-	-	-	-	+
S2	-	-	-	+	-	-	-	-	+
S3	-	-	-	-	-	-	-	-	-
S4	-	-	-	+	-	-	-	-	+
SI	-	-	-	+	-	-	-	-	+
SII	-	-	-	+	-	-	-	-	+
SIII	-	-	-	+	-	-	-	-	+
SIV	-	-	-	+	-	-	-	-	+
SV	-	-	-	+	-	-	-	-	+
SVI	-	-	-	+	-	-	-	-	+