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First description of a cyanophage infecting the cyanobacterium *Arthrospira platensis* (*Spirulina*)

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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

Arthrospira (*Spirulina*) is a non-heterocystous filamentous cyanobacterium, characterised by multicellular, cylindrical and usually screw-like coiled trichomes, inhabiting diverse environments including those of high salinity (Anagnostidis and Komarek 1988; Manen and Falquet 2002). Several species and 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 genus, 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) (Safferman 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 characterisation of several freshwater cyanophages were studied extensively between the 1960s and early 1980s (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 1990s 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 2006; Yoshida et al. 2008).

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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 and discovered a cyanophage, able to infect and lyse *A. platensis* and for which a basic description is provided.

Materials and methods

A dozen samples was obtained from *Le Chant de l'Eau*, a company based in the South of France (Fuilla), consisting of eight 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 the following nutrient concentrations (in g L^{-1}): sodium bicarbonate=8; potassium sulfate=1; sodium chloride=5; potassium nitrate=2; magnesium sulfate=0.2; calcium chloride=0.1; ammonium chloride=0.2 . 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. The *Arthrospira* 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 (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 flow cytometry (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 were 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), ten PC-rich *Synechococcus* (PCC 6301, 6311, 6707, 6715, 7917, 7918, 7941, 7952, 9004 and 9005), four PC-rich *Synechocystis* (PCC 6308, 6803, 6905, 7509), one colonial cyanobacterium (*Microcystis aeruginosa*, TCC 80) and one filamentous cyanobacterium (*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^{-1}. Only when a clear lysate was produced in the duplicates was the infection 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 four different cultures of *A. platensis*, 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, 1 mg L^{-1} stock solution of mitomycin C (Sigma) was prepared in <0.02 μm filtered MilliQ water and stored in the dark at 4°C. Ten-mL aliquots of exponentially growing cultures of each *A. platensis* sample was incubated with mitomycin C at final concentrations of 0 (control containing only water), 1, 5 and 20 μg mL^{-1} under a 14:10 light/dark cycle for 2 weeks in our temperature-controlled algal culture room. One-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 Fig. 1).

Transmission electron microscopy

For visualisation and characterisation of viral particles by transmission electron microscopy (TEM), glutaraldehyde 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 × *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 % *w/w*), 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 × 100,000. The photographic negatives were scanned with Adobe Photoshop and cyanophage dimensions were determined using IMAGEJ software.

Primers and polymerase chain reaction analysis

To identity the virus, different primers (see Table 1) were used, like the CPS1/CPS2, CPS4/CPS5 and 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 polymerase chain reaction (PCR) analysis.

The PCRs were performed by using the DNA Thermal Cycler T-Professional (Biometra) with the optimised conditions for each primer (Zhong and Jacquet, in preparation). Briefly, 25 μL reaction mix contained 1× 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 0.5× TBE (89 Tris–base, 89 boric acid, 2 mM EDTA; pH 8.0) and visualised by ethidium bromide staining on a UV transilluminator (Tex-35 M, Bioblock Scientific) and photographed using a gel documentation system (BioRad, Germany).

Table 1 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-349 F/533Ra/533Rb)	CCAAAYCTYGCMCARGT/CTCGTCRT GSACRAASGC/CTCGTCRTGDATRAASGC	DNA polymerase gene of cyanopodovirus	Chen et al. 2009
6	*pol2* (designed)	ACTGCAACGCCTGGGATGGTG/ AGCAATGCGGCGACCGTCAA	DNA polymerase gene of cyanopodovirus	This study
7	MCP F/R	GGYGGYCARCGYATT/TGIARYTGYTCRA YIAGGTA	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

Results and discussion

Use of cyanobacteria as a food supplement has a long history (Gantar and Svircev 2008) dating back to antiquity. Referred to wrongly as "*Spirulina*", *Arthrospira* Stizenberger 1852, which is in fact more related to *Planktothrix* and *Lyngbia* (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* (Vonshak 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 et al. 2004; Duhamel et al. 2006; Personnic et al. 2009). Such 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 with 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. 2). The capsid and the tail were about 120 and 20 nm, respectively, which is expected for such a cyanophage (Suttle 2000).

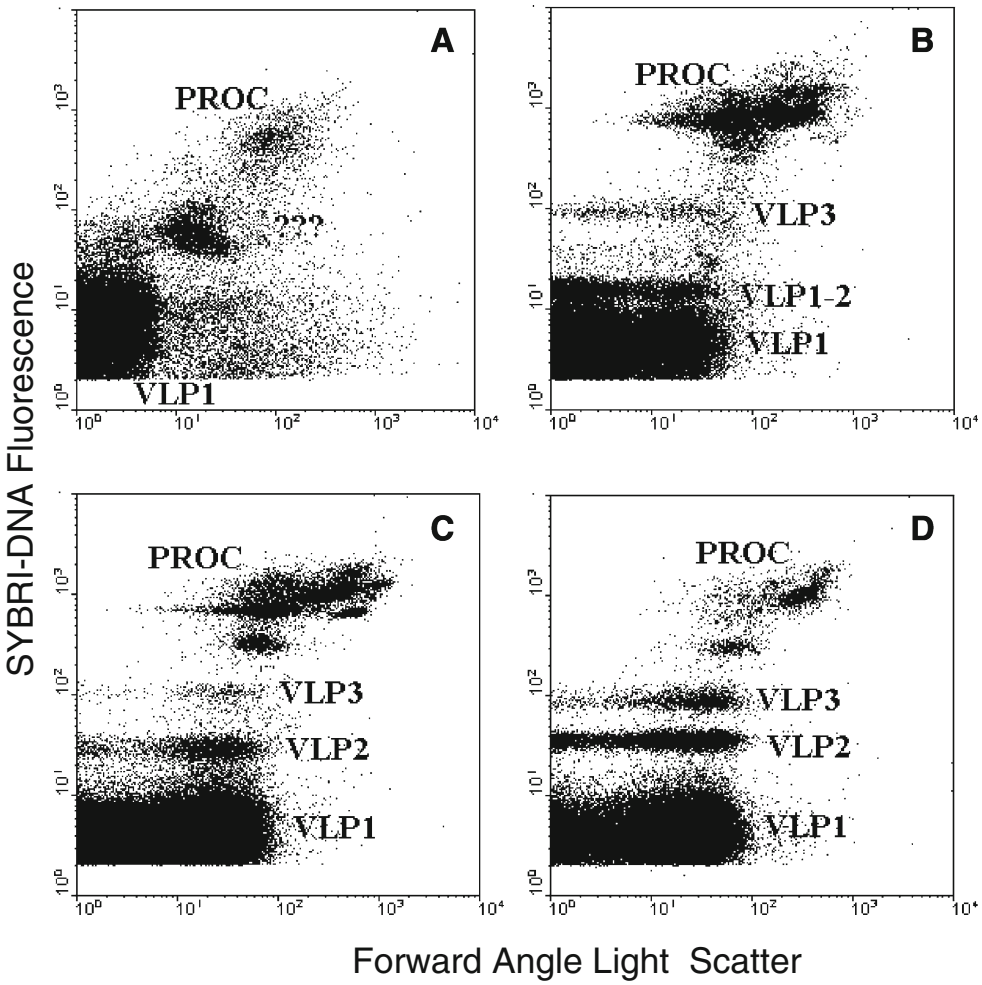

Fig. 1 FL1 (Sybr green I–DNA complex fluorescence) versus FSC (forward angle light scatter) cytograms showing typical viral signatures from the original samples received from *Le Chant de l'Eau*. Samples were issued from a pool where there was no cyanobacterial mortality and recently fed with new medium (**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*

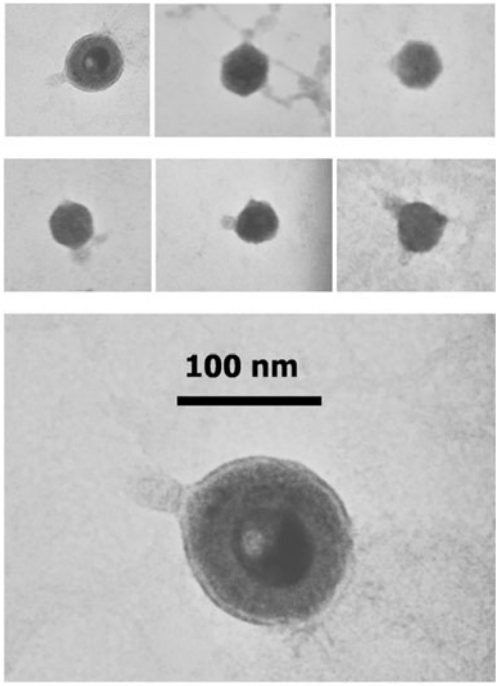

Fig. 2 TEM micrographs of the cyanopodivirus infecting *A. platensis*

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). The pool without culture (i.e. with just the water medium before inoculation) was negative for all genes, including *pol* in PCRs performed in this study (Fig. 3). Only VLP2 was positive to *pol* primers compared with VLP1 and VLP3. All these results thus supported that the virus was a cyanopodovirus. Hereafter, we refer to this cyanopodovirus obtained in this study as SPIRUVIR. 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 (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) (Felsenstein 1985) are shown next to the branches. The evolutionary distances were computed using the Kimura two-parameter method (Kimura 1980) 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 (Tamura et al. 2011).

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 with controls. The podovirus caused lysis of only *A. platensis* cultures as against cyanobacterial and bacterial 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 with myoviruses typically that display a broader host range (Chen and Lu 2002; Mann 2003; Sullivan et al. 2003), even if Deng and Hayes (2008) 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. 5), even if we did

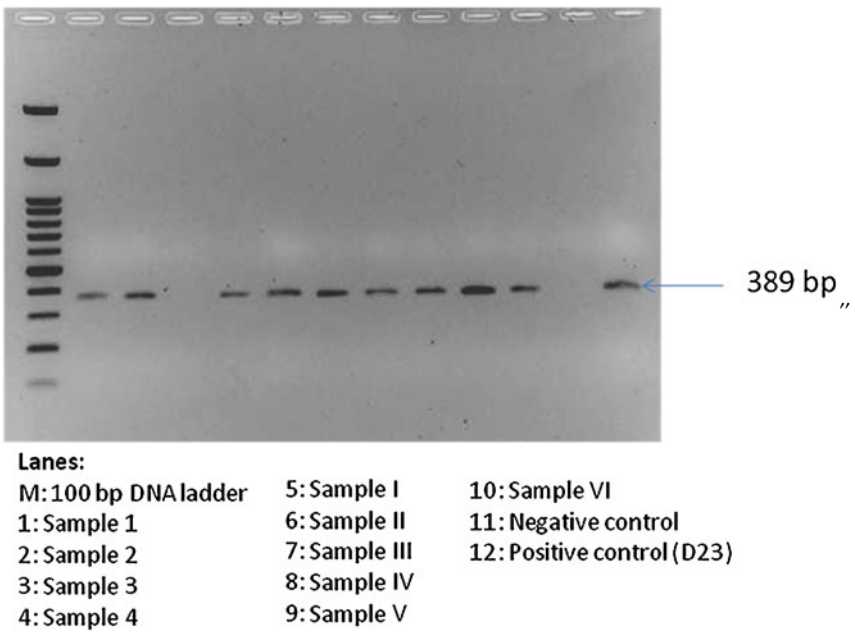

Fig. 3 PCR amplification using *pol2* primers for the cyanopodovirus resulting in an amplicon of 389 bp. All the samples received from different pools of the farms were subjected to PCR with *pol2* primers. *Lanes 1*: S1, *2*: S2, *3*: S3, *4*: S4, *5*: SI, *5*: SII, *6*: SIII, *7*: SIV, *8*: SV, *9*: SVI, *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

Table 2 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 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	pol1	g23	CPS1.1/8.1	CPS1/2	CPS4/5	pol2
S1	−	−	−	+	−	−	−	−	+
S2	−	−	−	+	−	−	−	−	+
S3	−	−	−	−	−	−	−	−	−
S4	−	−	−	+	−	−	−	−	+
SI	−	−	−	+	−	−	−	−	+
SII	−	−	−	+	−	−	−	−	+
SIII	−	−	−	+	−	−	−	−	+
SIV	−	−	−	+	−	−	−	−	+
SV	−	−	−	+	−	−	−	−	+
SVI	−	−	−	+	−	−	−	−	+

not work on reasons and mechanisms behind such a resistance that can dress different aspects (Thomas et al. in press). 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 with the cyanomyo- and cyanosiphoviruses (Sullivan et al. 2003; Wang and Chen 2008).

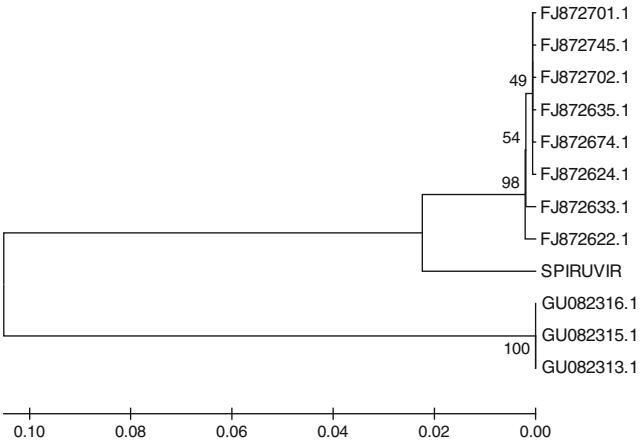

Fig. 4 The evolutionary history was inferred using the neighbor-joining method. The optimal tree with the sum of branch length= 0.23914466 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura two-parameter method and are in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. Codon positions included were first + second + third + noncoding. All positions containing gaps and missing data were eliminated. There were a total of 351 positions in the final dataset. Evolutionary analyses were conducted with MEGA5. References for FJxxx and GUxxx are Chen et al. (2009) and Huang et al. (2010), respectively

The 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 and has a greater proportion

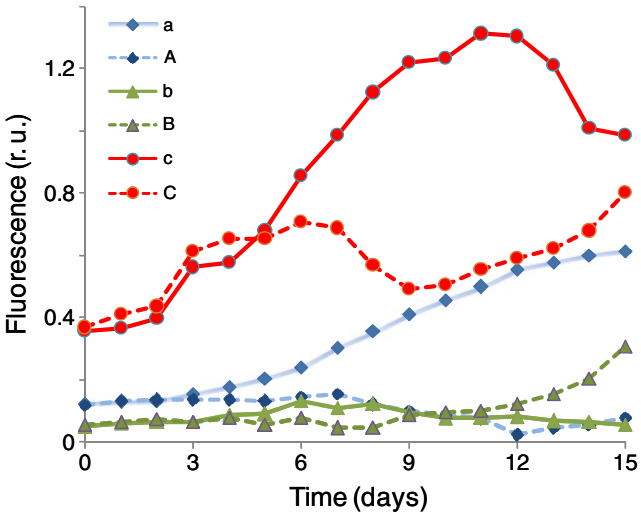

Fig. 5 Different cultures of *A. platensis* were inoculated with the virus at day 1 with a MOI of 1. It was clear from these infection experiments that infectivity and mortality processes differed in the cultures and that resistance could also be detected. *A*, *B*, *C*: infected; *a*, *b*, *c*: not infected

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); Ohki and Fujita (1996) and Hewson et al. (2001) who documented lysogeny in a variety of filamentous cyanobacteria or 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 were intact prophages in *A. platensis*. Analysis of the genome of *A. platensis* using the Genbank database suggested, however, that a certain type of prophage may be present in this cyanobacterium, as in other cyanobacteria (Palenik 2003; Sullivan et al. 2005), even if we failed to make the prophage element inducible if it is. If a cyanophage is integrated 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 high light and temperature or UV radiation before any clear conclusion is reached. 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 as to what the effect of the virus can be 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 versus 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 lose in turn and for which such interactions strongly depend on the environment.

In conclusion, we describe, 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. This cyanophage may be an important regulating factor of this cyanobacterium, but we do not know what main factors are intervening in such a regulation and if, for instance, some important resistance mechanisms have been developed by the cyanobacterium. Improving our knowledge of such a virus is obviously a key issue.

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