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Acquisition and maintenance of resistance to viruses in eukaryotic phytoplankton populations

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Summary

Viruses are known to play a key role in the regulation of eukaryotic phytoplankton population densities; however, little is known about the mechanisms of how they interact with their hosts and how phytoplankton populations mediate their regulations. Viruses are obligate parasites that depend on host cell machinery for their dissemination in the environment (most of the time through host cell lysis that liberates many new particles). But viruses also depend on a reliable host population to carry on their replication before losing their viability. How do hosts cells survive when they coexist with their viruses? We show that clonal lines of three picoeukaryotic green algae (i.e. *Bathycoccus* sp., *Micromonas* sp., *Ostreococcus tauri*) reproducibly acquire resistance to their specific viruses following a round of infection. Our observations show that two mechanisms of resistance may operate in *O. tauri*. In the first resistant type, viruses can attach to their host cells but no new particles develop. In the second one, *O. tauri* acquires tolerance to its virus and releases these viruses consistently. These lines maintained their resistance over a 3-year period, irrespective of whether or not they were re-challenged with new viral inoculations. Co-culturing resistant and susceptible lines revealed resistance to be associated with reduced host fitness in terms of growth rate.

Introduction

Viruses are the most abundant and diverse pathogens known, and they pose a serious threat to all organisms. They are obligate parasites and thus both reduce the fitness of their host cells by multiplying within them, often causing lysis of the host cell to release their progeny, and yet they must allow their hosts to survive and evolve in a very competitive world (Bowie and Unterholzner, 2008). This paradox has led to the question of the stability of host–virus communities in the environment, leading to several non-exclusive hypotheses. Some studies suggest that the coexistence of the host and its associated virus(es) is based on the high dilution of both partners in the environment allowing a balance between the host growth rate and the viral lysis, or by a reduction in the virulence of lytic viruses (Suttle, 2007; Zhang and Shakhnovich, 2009). In the same way, other studies mention that virus-induced mortality can be low (Fuhrman, 1999), due to viral resistance or tolerance phenomena (i.e. chronic cycle or lysogenic cycle). Resistance to super-infection by a second virus is known in bacteriology when a first infection leads to integration of the phage into the host genome (lysogeny) conferring resistance to other phages (reviewed in Weinbauer, 2004). Some viruses (e.g. bacteriophages, mammalian retroviruses, herpesviruses) can remain latent in a host for long periods and subsequently give rise to an acute infection, often as a consequence of an environmental stimulus (Glaser *et al.*, 1985; Kapp, 1998; Coffin and Rosenberg, 1999).

The question of the stability of host–virus communities is highly relevant in the sea where viruses are very abundant (Fuhrman, 1999; Suttle, 2005; 2007). Marine viruses are thought to infect all oceanic prokaryotic and eukaryotic organisms, including primary producers (Lemke, 1976; Van Etten and Meints, 1999), and thereby strongly influence the production of marine biomass. The existence of prokaryotic marine microalgae resistant to viral infection was first hypothesized for *Synechococcus* by Waterbury and Valois (1993). These authors suggested that this process is widespread in marine ecosystems, participating in population renewal after a lytic crash. Several other mechanisms of phage resistance or immunity have been described (Stoddard *et al.*, 2007). The most common resistance appears to be an alteration of host surface receptors which reduces or eliminates the ability of phages to attach to the host cell and to establish an infection (Bohannan and Lenski, 2000). Other mechanisms include the inability of a bacterium to support viral replication and/or the presence of restriction–modification systems in which host restriction endonucleases degrade viral DNA upon entry in the host cell (Lenski and Levin,

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1985). More and more studies are now being performed on viruses of unicellular eukaryotic phytoplankton (protists). These eukaryotic phytoplankton are mostly infected by Phycodnaviridae (Brussaard, 2004), a family of large (100–300 nm in diameter; Massana *et al*., 2007; Fischer *et al*., 2010), icosahedral, double-stranded DNA viruses (Wilson *et al*., 2005). Phycodnaviruses are genetically diverse but morphologically similar (Dunigan *et al*., 2006; Bellec *et al*., 2009). With the exception of *Emiliania huxleyi* virus 86 (Mackinder *et al*., 2009), phycodnaviruses have no envelope, once the virus attaches to the host cell, the viral DNA is injected and the release of the virus occurs by lysis of the host cell.

Waters and Chan (1982) reported the incomplete lysis of host population and subsequent growth, suggesting that the whole population was not affected, even where water turbulence mixes all phytoplankton. Resistance has been reported after addition of DNA (Zingone *et al*., 2006) or RNA (Mizumoto *et al*., 2008) in viruses specific to the eukaryote microalgae *Heterocapsa* or *Micromonas*, respectively, but the processes which govern resistance are unknown. Resistance can be seen as a coevolutionary arms race (Lenski and Levin, 1985; Clarke *et al*., 1994; Frada *et al*., 2008), where exposure of diploid coccolithophoridae *Emiliania huleyi* to one of its virus induces transition to the resistant haploid phase. Recently, Tomaru and colleagues (2009) showed a new mechanism of resistance in a RNA virus–host interaction which may be related to an intracellular suppression mechanism barring viral genome replication. Here, we present evidence that resistance to viruses is a general phenomenon in the order *Mamiellales* (widespread green eukaryotic micro-algae) and does not depend on adhesion of viruses to host surfaces but may rather be associated with a specific host cellular response.

Results

Resistance is reproducibly observed in diverse genera of the Mamiellales

Ostreococcus tauri (RCC745), *Bathycoccus* sp. (RCC1105) and *Micromonas* sp. (RCC1109) were inoculated during exponential phases with their lytic viruses, OtV5, BpV2 and MpV1 (Derelle *et al*., 2008; Moreau *et al*., 2010) respectively. For the three algae, the growth rates measured by flow cytometry were 1.95 ± 0.07, 2.68 ± 0.16 and 2.01 ± 0.37 cell divisions day^{-1} respectively. Hosts cell lysis was observed within 2–10 days post inoculation (dpi), but lysis was never complete and a small fraction of the cells survived and could grow after addition of fresh medium, even if free infectious viral particles were still present in the culture (Fig. 1A–C). This phenomenon was reproducibly observed for the three genera. The resistance remained stable after serial subculturing (every 10 days) and no lysis was observed after addition of fresh virulent viruses.

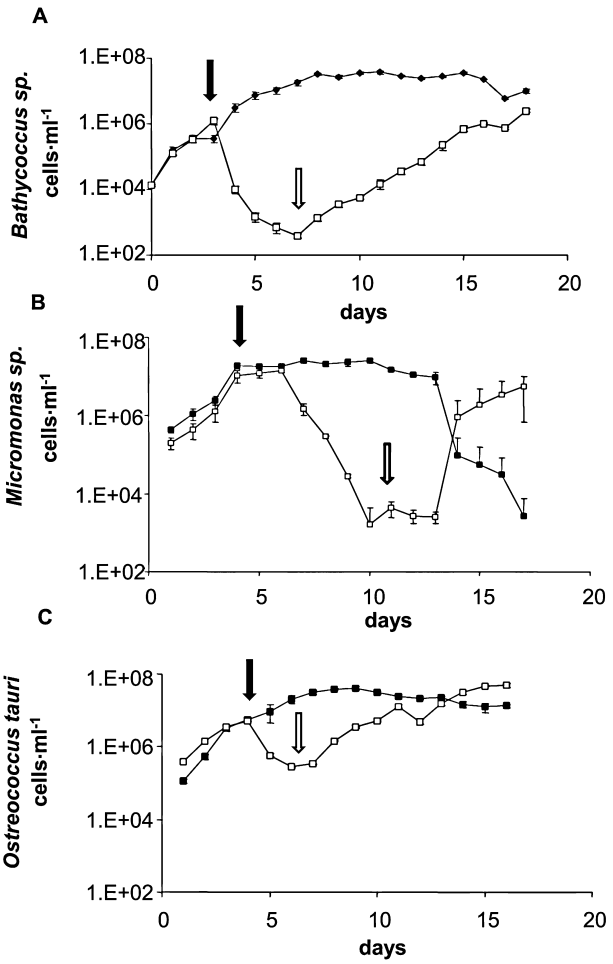

Fig. 1. Development of virus-resistant cells in *Mamiellales*. The addition of culture medium to lysed cultures allowed growth of resistant cells in the presence of remaining viable viruses. Positive controls show cultures that were not inoculated with their respective virus (black filled symbols). First arrows (filled) indicate viral inoculation and second arrows (open) indicate addition of fresh culture medium when lysis was finished. The assays were performed at least three times (error bars).
A. Development of resistance to BpV2 (*Bathycoccus* sp. Virus 2) in *Bathycoccus* sp.
B. Resistance in *Micromonas* sp. to MpV1 (*Micromonas* sp. Virus 1).
C. Resistance in *Ostreococcus tauri* to OtV5 (*O. tauri* Virus 5).

Two kinds of virus-resistant cell lines: virus producers and non-producers

To better characterize the resistance phenomenon, detailed studies were performed on the host–virus model system *O. tauri*–OtV5, since both the host and the virus genomes are completely sequenced (Derelle *et al*., 2006; 2008). Three independent cultures of *O. tauri*-resistant cells were plated out on agarose and 15 colonies were

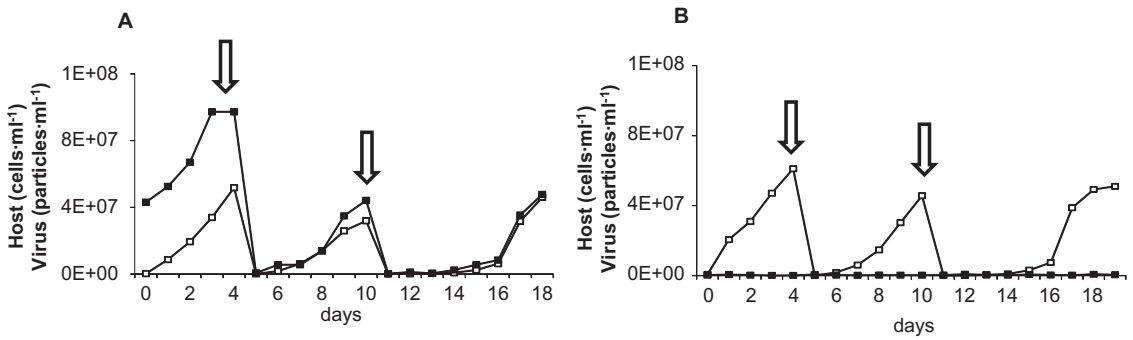

Fig. 2. Viral production by *Ostreococcus tauri*-resistant clones. Analysis of clones for the presence of viruses by flow cytometry.
A. Example of one clone which produce viruses.
B. Clone that did not produce viruses.
Open squares indicate host cell densities and filled squares represent virus particle densities. Arrows indicate subcultures.

isolated from each culture. After growth in liquid medium, each clone was tested both for its resistance to OtV5 and for its production of viral particles. All clones were resistant when inoculated with a fresh suspension of OtV5 particles. About 50% of them produced viruses, while the second half did not produce any detectable viral particle, neither infectious (measured by plating out for lysis plaques) nor uninfectious (as measured by flow cytometry) (Figs 2A and B and 3). The production of viral particles in the supernatants of producer clones remained constant over subcultures. Thus two kinds of clones were found, resistant non-producers (R^{NP}) and resistant producers (R^{P}). Flow cytometry analysis of R^{P} cells showed the existence of a free viral population (Fig. 3) and the overall production of viruses in R^{P} cells varied between one and three viruses per cell and per day, depending on individual clonal cell lines (Fig. 2A), which is more than one order of magnitude lower than in susceptible lysing cells (about 75 viruses per cell and per day estimated from the burst size of 25 viruses released per cell in 8 h) (Derelle *et al.*, 2008). Electronic microscopy confirmed the low production of viruses by R^{P} cells. Indeed, observations of viral particles inside cells were very rare (Fig. 4A–D), in contrast to susceptible cells (Fig. 4E and F). Furthermore, budding of a few viral particles has been observed (Fig. 4C and D), where a viral particle leaves the host cell in a vesicle, showing that lysis is not the process for the viral production in R^{P} clones, in contrast to susceptible cells (Fig. 4E and F). In addition, when two clones of R^{P} cells were plated and colonies isolated, all of these new isolated clones produced infectious viruses, suggesting that the production of viruses comes from resistant cells rather than by lysis of a minority of cells.

The cost of resistance

The potentially important advantage of host resistance in the environment might be balanced by its cost in terms of growth rate or competition with other organisms. To estimate this cost, the growth rates of both susceptible and resistant clones were first measured in our culture conditions. Susceptible and resistant cells (both R^{P} and R^{NP}) grew at 1.87 ± 0.09 and 1.95 ± 0.07 division day^{-1}, respectively, being not significantly different ($P = 0.15$). However, growth rate measurements may not be sufficiently sensitive to detect slight differences between cultures. To overcome this potential limitation, a second approach was investigated, by testing competition between the different clones during several months. Co-cultivation of susceptible *O. tauri* cells with resistant producers was not possible because the susceptible cells would all have been lysed by the viruses produced. In

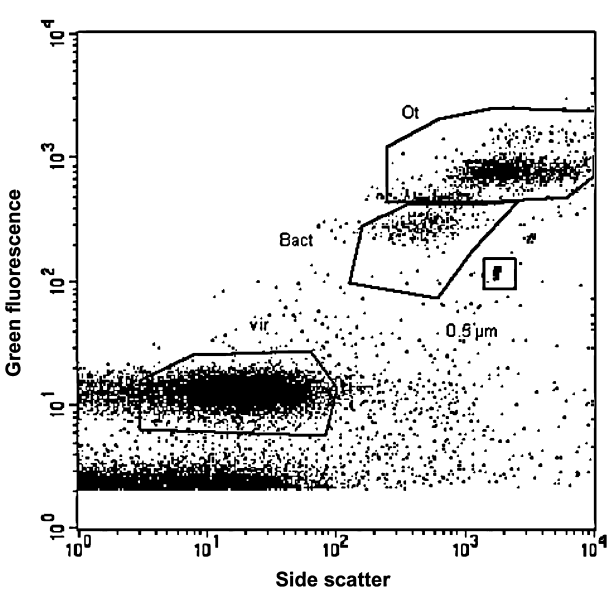

Fig. 3. Flow cytometric analysis of viral production. Three populations can be distinguished their side scatter (SSC) and their green fluorescence (Sybr Green I, DNA labelled): the virus population (Vir), bacteria (Bact) and *Ostreococcus tauri* (Ot). Beads provide an internal standard of 0.5 µm.

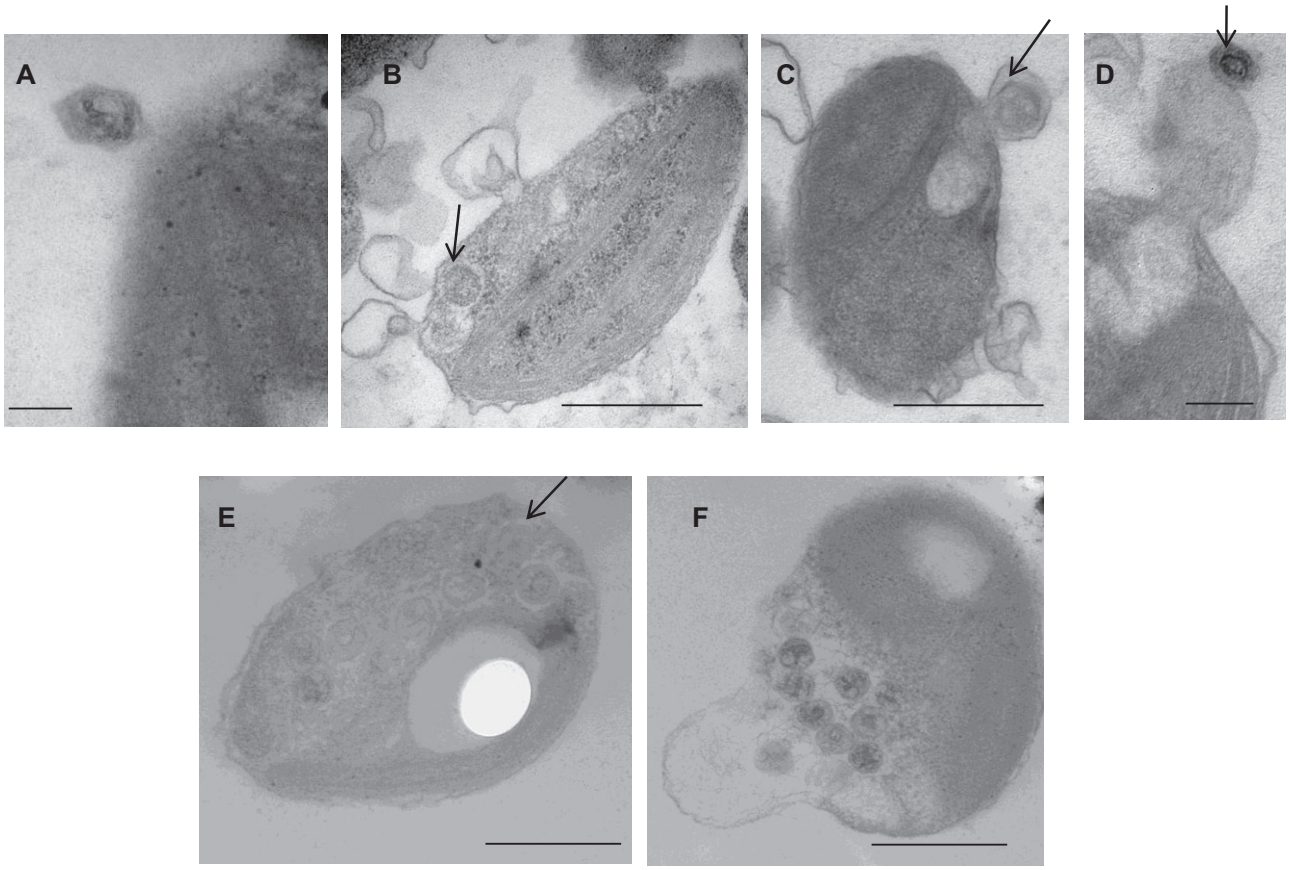

Fig. 4. Transmission electronic microscopy of susceptible and resistant producers (R^P) cells.
A–D. Resistant *Ostreococcus tauri* cells producing virus. (A) Virus particle attached to the cell membrane of the *O. tauri*-resistant cell. (B) OtV5 particle inside resistant-producer (R^P) cell. (C) Virus particle leaving a R^P cell. (D) Virus particle out of the R^P cell. Black arrows indicate OtV5 particles.
E and F. Susceptible *O. tauri* cells. (E) The cell cytoplasm is full of viruses. (F) Lysis of susceptible cells: after bursting, viruses are released. (B, C, E and F) The bar represents 500 nm. (A and D) The bar represents 100 nm.

contrast, competitions could be tested between susceptible and R^{NP} cells, and also between R^P and R^{NP} cells. In R^P : R^{NP} co-cultures, the decrease of viral particles in the culture indicates a decrease of R^P, over two subcultures (about 30 days) and the latter were completely eliminated after 20 subcultures (Fig. 5B). In susceptible : R^{NP} co-cultures, susceptible cells became dominant after eight subcultures (about 90 days) (Fig. 5A). In contrast, each clone grown alone was stable and no transformation from R^P to R^{NP} or susceptible cells was observed (data not shown). Consequently, these data established a competitive hierarchy between these clones, being lower in R^P than in R^{NP} and in R^{NP} than in susceptible cells.

Resistance is not due to defective adsorption

Several approaches were undertaken to explore the mechanism of resistance. First, viral adsorption was measured on susceptible, R^P and R^{NP} cells at different multiplicities of infection (moi) ranging from 1 to 0.1. No significant difference could be observed between susceptible, R^{NP} and R^P, at any moi tested ($P = 0.026$ for moi of 1, $P = 0.03$ for moi of 0.7, $P = 0.0463$ for moi of 0.5, $P = 0.9927$ for moi of 0.1, one-way ANOVA, 1%) (Fig. 6), indicating that resistance was not due to a loss of viral adhesion following a possible modification of surface receptors. The specificity of our test was controlled by measuring the adsorption of a virus specific to *Micromonas* (i.e. MpV1 which does not lyse *O. tauri* cells) on *O. tauri* cells. Unspecific adsorption was low and non-significant compared with values found with specific host (i.e *Micromonas*) and this virus ($P = 0.8215$ at moi of 1).

In the second experimental set, R^{NP} cells were tested for the absence of the OtV5 viral genome inside the host cells, by using both pulsed field gel electrophoresis (PFGE) and polymerase chain reaction (PCR). R^P culture, for which the presence of infectious and lytic virus was clear, was used as positive control for the presence of the viral genome. As expected, a clear band corresponding to the viral genome was obtained from R^P cells by PCR

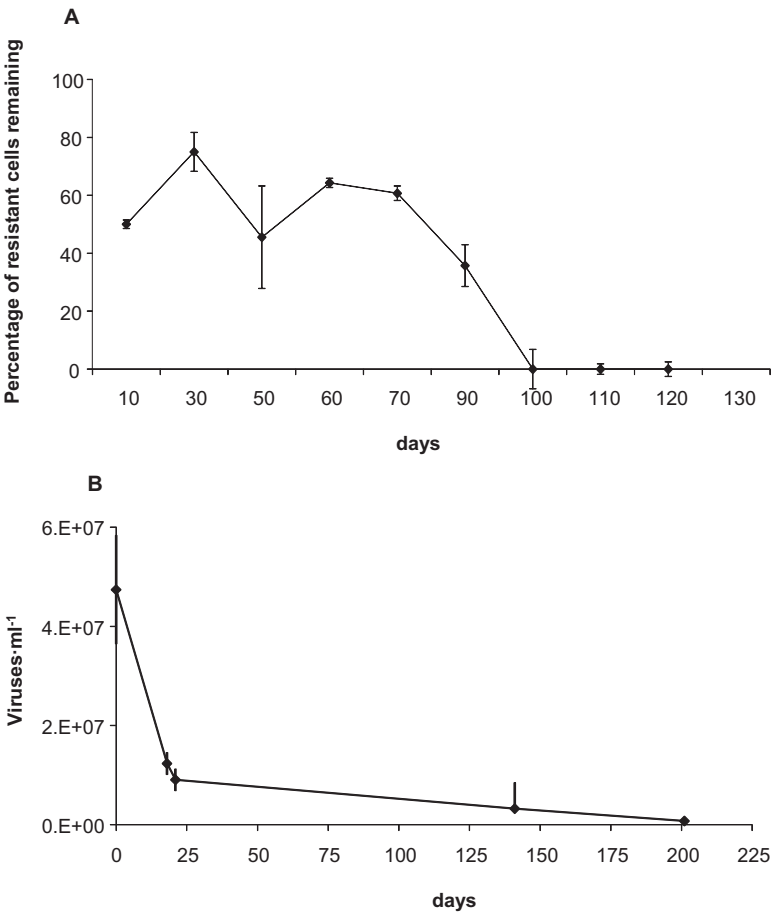

Fig. 5. Competition experiments for estimating the cost of resistance.
A. Competition between susceptible *Ostreococcus tauri* cells and one clonal non-producer (R^{NP}). Percentage of remaining resistant cells in the co-culture over time (days) in serial subcultures.
B. Competition between resistant non-producer (R^{NP}) and resistant producer (R^{P}). Concentration of viral particles coming from R^{P} cells in the co-culture over days.

whatever the primers used, whereas no band was seen from R^{NP} cells (Fig. 7A). The absence of the viral genome in R^{NP} cultures and its presence in R^{P} cells was also confirmed by PFGE (Fig. 7B) and by radiolabelled hybridization (data not shown).

Discussion

Viruses specific to several microalgal species have been described and incomplete lysis was reported for some of them including *Micromonas* sp. (Waters and Chan, 1982; Zingone *et al*., 2006), *Chlorella* sp. (Van Etten *et al*., 1991), *E. huxleyi* (Frada *et al*., 2008), *Pyramimonas orientalis*, *Chrysochromulina ericina*, *Heterosigma akashiwo* (Tarutani *et al*., 2000; 2006), *Phaeocysitis puchetii* (Thyrhaug *et al*., 2003) and *Heterocapsa circularisquama* (Tomaru *et al*., 2009). This phenomenon seems general and can explain how hosts and viruses can coexist in the same environments (Thyrhaug *et al*., 2003). However, the mechanism(s) underlying this resistance remain(s)

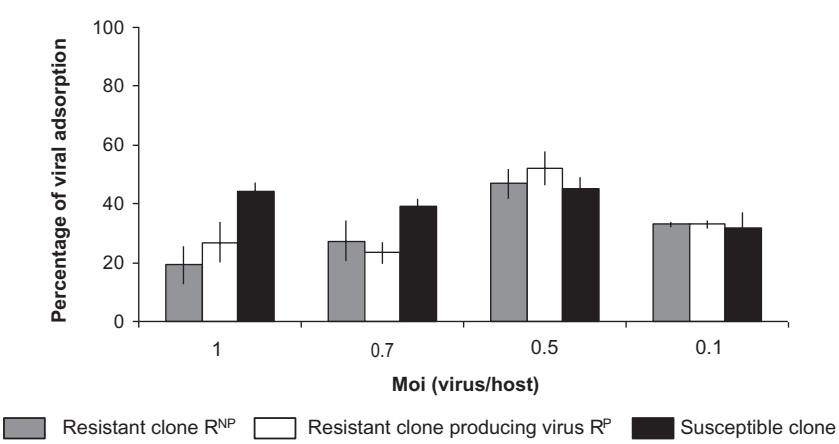

Fig. 6. Viral adsorption on susceptible and resistant *Ostreococcus tauri* cells. Percentage of adsorbed viruses was measured by plaque forming units, at four multiplicities of infection (moi 1, 0.7, 0.5 and 0.1). Percentage of viral adsorption was measured on resistant non-producer (R^{NP}) clone, resistant producer (R^{P}) and susceptible.

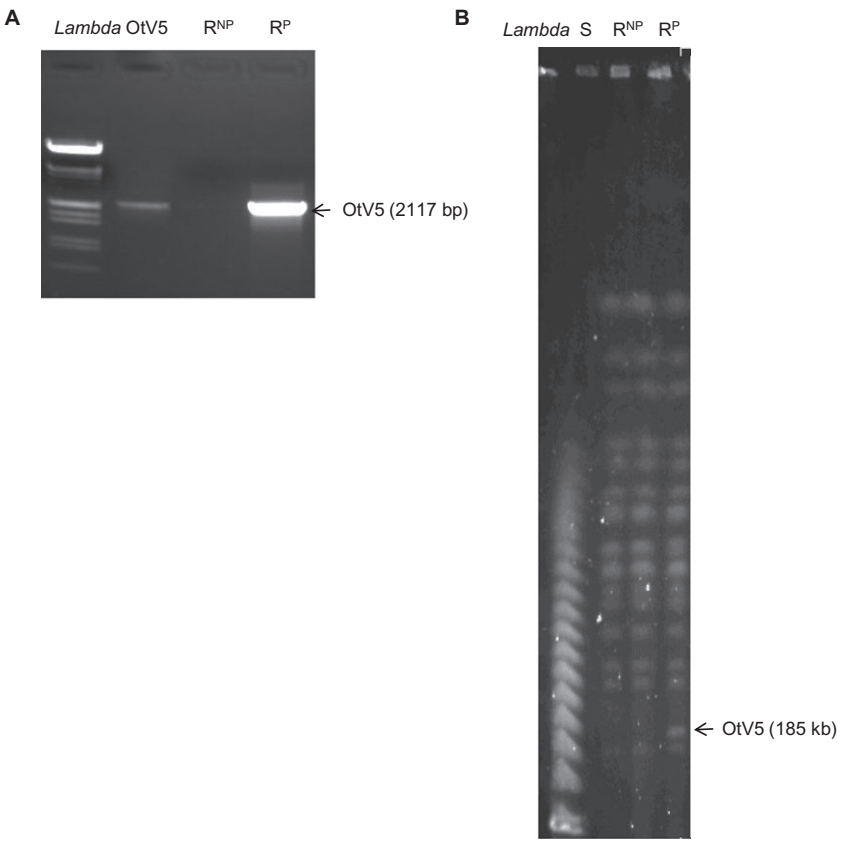

Fig. 7. Presence or absence of viral genomes in resistant producer (R^P) and non-producer (R^{NP}) *Ostreococcus tauri* clones.
A. PCR amplification of OtV5 fragment (2117 bp) in purified OtV5, R^{NP} and R^P cells respectively. Left lane is EcoRI + HindIII-digested *lambda* phage MW standard.
B. Pulsed field gel electrophoresis (PFGE) of *O. tauri* susceptible strain: S, R^{NP} and R^P cells respectively. Left lane: concatenated *lambda* phage DNA MW ladder. The arrow indicates OtV5 genome (185 kb).

unclear and the common general phenotype of 'resistance' could correspond to various unrelated mechanisms, which can be classified in two main categories. The first one intimates that resistant cells must pre-exist in culture, perhaps arising by spontaneous mutations, and are selected under viral infection. Here the stability of resistant cells suggests an existing genetic change in the population which is stable over generations. Alternatively, the second category proposes that once the virus attacks the first host cells, some signals allow susceptible host cells to shift towards resistant phenotypes. This prompt shift could result by induced mutation or from differential regulation of metabolic processes for example by expression of different proteins.

In the algae *E. huxleyi* virus-susceptible diploid cells are observed to undergo meiosis upon exposure to a compatible virus, the emerging haploid phase being resistant, providing *E. huxleyi* with a mechanism of escaping viral attack (Frada *et al*., 2008). Another indirect mechanism of resistance related to this second category has also been described in *E. huxleyi*, based on apoptosis of surrounding cells belonging to the same clone, triggered by the secretion of a sphingolipid (Pagarete *et al*., 2009). The suppression of viral genome replication has also been observed in the marine dinoflagellate *Heterocapsa,* where the transfected RNA HcRNAV34 viral genome was replicated only in susceptible cells but not in resistant ones (Tomaru *et al*., 2009).

Here, we report the appearance of viral resistance in the three main *Mamiellales*' genera, i.e. *Bathycoccus*, *Micromonas* and *Ostreococcus*. A shift of ploidy was not observed in these resistant cells, as shown by flow cytometric analysis using a DNA fluorescent marker (data not shown). The original population of *O. tauri* was clonal; however, we cannot exclude that a small proportion of cells carried mutations in genes affecting susceptibility/resistance (first hypothesis for resistance) or it may be possible that viral infection promoted mutation or a shift in physiology suggesting the emergence of a small subpopulation of resistant cells (second hypothesis for resistance).

Cellular responses to viral attack could occur on at least five levels: (i) the internalization of the viral DNA, (ii) the replication of the viral DNA, (iii) the interference with the expression or function of viral proteins, (iv) the encapsidation or (v) the lysis mechanism. Contrary to previous studies (Waters and Chan, 1982; Tarutani *et al*., 2006), viral adsorption experiments showed that binding of OtV5 to its host is not significantly different between susceptible and resistant cells, showing that resistance is not due to a

change of cell surface receptors. In our experiments, the existence of resistant producers (R^P) and non-producers (R^{NP}) clones potentially involves two independent resistance mechanisms. In R^P cells, viruses entered into the cells, the viral genome was replicated and viruses were encapsidated and released. Consequently, the resistance mechanism might act by controlling the growth and replication of viruses and/or by controlling host lysis. Indeed, to maintain producer clones, the viral cycle must be inhibited to a large extent, so that viral replication occurs at low level (one to three viruses per cell and per day), 25-fold lower than after lysis (75 viruses per cell and per day). Furthermore, in R^P clones where cells release viral particles regularly, the lytic process itself must also be inhibited. Here, instead of release through lysis, the virus is released from the host through budding; in this way, both host and virus are preserved and the virus may be influencing host metabolism in a way to ensure its longer-term survival. Here we present an alternative for the virus between low-level replication, where vertical transmission is prevalent and viral productivity is low, and lysis, where both horizontal transfer and viral productivity are high. The R^P strategy might thus better be described as tolerance rather than resistance.

In R^{NP} cells, adsorption occurs but no viral DNA was observed inside the host, so the resistance mechanism is likely to interfere with an early stage of the viral life cycle. If suppression of viral growth in R^{NP} is sufficient to ensure that virus-free host cells are produced more frequently at cell division, then establishment of resistant virus-free cultures would rapidly ensue.

This is the first description of two kinds of resistance to one type of virus in algae, but, as mentioned above, might be suspected in some cases as for *Micromonas pusilla* for which infected cultures were reported to manifest a complex pattern of resistant strains (Waters and Chan, 1982). Both resistance phenotypes most likely represent a cellular response to viral attack and R^P clones are reminiscent of chronic infections described in other well-known dsDNA viruses such as herpes viruses (Knipe and Cliffe, 2008; Virgin *et al*., 2009). Whether this mechanism involves any of the complex defences deployed in plants (Soosaar *et al*., 2005; Truniger and Aranda, 2009) or by metazoans (Field *et al*., 1968) remains to be established. The ecological consequences of this phenomenon are probably important for the regulation of microalgal populations in the sea.

Even resistant *O. tauri* cells remain stable over time and no shift between resistant and susceptible phenotypes has been observed, the low mutation rate estimated by Grimsley and colleagues (2010) in *O. tauri* of 10^{-7} and the observation that numerous independent *O. tauri*-resistant populations show two types of cells suggests that the resistance observed is not due to pre-existing mutation but rather to an intracellular response for suppression of the viral virulence in R^{NP} and for viral tolerance in R^P. However, we cannot exclude that an alternative explanation involving spontaneous mutations might exist. Further genetic approaches are required to elucidate the mechanisms of these intriguing processes.

Experimental procedures

Strains and cultures conditions

The algal strains used were *O. tauri* (RCC745, isolated from Thau lagoon, Courties *et al*., 1994), *Bathycoccus* sp. (RCC1105, isolated from Mola sea station) and *Micromnonas* sp. (RCC1109, isolated from Leucate lagoon). Algal cultures were grown in Keller medium (Sigma-Aldrich, Saint-Quentin Fallavier, France) diluted in 0.22 μm filtered and autoclaved sea water (NaCl 36 g l^{-1}) with antibiotics (Kanamycin, Neomycin, Penicillin, 1/1000 final concentration) under continuous light (100 μmol photon m^{-2} s^{-1}) at 20 ± 1°C. Exponentially growing cultures (20 ml) of each algal strain were usually inoculated in triplicate with a viral concentration of 2.10^6 viral particles ml^{-1} (final concentration) and a moi of 1. Then, clonal host cell lines were obtained by plating out a resistant cell culture. Individual colonies were then picked off. Thirty clones were analysed for the presence of viruses by flow cytometry. Samples for enumeration of algae and viruses were collected every day and analysed by flow cytometry (Becton Dikinson, San Jose, CA, USA) (see below).

Enumeration of algae and viruses

All analyses were performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an air-cooled laser providing 15 mW at 488 nm. For enumeration of algal cells, fresh samples were discriminated by chlorophyll autofluorescence. Viral counts were performed on fixed samples (2% glutaraldehyde, final concentration) diluted in TE buffer (10 mM Tris HCL, EDTA, pH = 8) and stained for 10–15 min with SYBRGreen-I (Molecular probe) (Marie *et al*., 1999; Brussaard *et al*., 2000). Concentrations of infectious viruses in the viral suspensions were measured by plaque forming units. We used a plate-gel technique for plating *O. tauri* embedded in a plate containing agarose (1.5% final concentration). Lysis 'plaques' could then be visualized as cleared circular regions extending through the whole depth of the gel (Derelle *et al*., 2008).

Transmission electron microscopy

For transmission electron microscopy, resistant and susceptible *O. tauri* cells were prepared according to Chretiennot-Dinet and colleagues (1995) and Derelle and colleagues (2008). Cells contained in 50 ml aliquots of 1% glutaraldehyde-fixed samples were harvested by centrifugation (Centrikon Beckman) at 3000 *g* for 20 min. The centrifuged material was then mixed with 1% liquid agarose using a microrepetor (SMI, Emerville, CA, USA). Once the agarose in the disposable glass micropipette had solidified, a 'noodle'

containing the cells was obtained and fixed for 2 h in 2.5% glutaraldehyde with one volume cacodylate buffer (0.4 M) and two volumes culture medium (Keller medium). The noodle was then washed three times for 15 min in a one volume 0.4 M cacodylate buffer and one volume culture medium. Post-fixation was carried out in 1% OsO_4 in 0.2 M cacodylate buffer for 1 h. After three washes in 0.2 M cacodylate buffer, the noodle was cut in small pieces, dehydrated in a series of ethyl alcohol and embedded in Epon 812. Thin sections were then stained with uranyl acetate and lead citrate before examination on 7500 Hitachi transmission electron microscope.

Competition experiments

Co-cultures of susceptible : R^{NP} and R^{P} : R^{NP} cells were initially started at 50%:50% and the percentage of each clone was then followed for 7 months, a period including around 20 subcultures and 480 cell divisions. The ratio of susceptible cells over time in the susceptible : R^{NP} co-cultures was estimated by flow cytometry by observing the proportion of cells lysed by OtV5. The evolution of the R^{P} cells in R^{P} : R^{NP} co-cultures was determined both by flow cytometry measurement of viral particles and by plating out culture supernatants on susceptible cells to check for the presence of infectious viral particles, at each subculture. Controls were performed on each clone grown individually (in triplicates), to check if there is no reversion between clones.

Virus adsorption measurement

We used two resistant clones (one producer and one non-producer) and one susceptible clone. Aliquots of OtV5 were inoculated into exponentially growing cultures of these clones at various moi corresponding to 1, 0.75, 0.5 and 0.1. Multiplicity of infection ratios were modified according to the number of infectious viruses. To measure unspecific adsorption, we used a *Micromonas* virus (MpV1, Moreau *et al*., 2010) on *O. tauri* cells, at moi of 1. Each experiment was carried out in quadruplet. Thirty minutes after viral inoculation, samples were centrifuged at 8000 *g* during 10 min. Aliquots of 250 μl of supernatant were then incubated with 10 ml of an *O. tauri* susceptible strain, 10 ml of Keller medium with antibiotics (as above) and agarose (1.5% final concentration). Each sample was then poured into a 90-mm-diameter Petri dish. Plaque forming units were counted 2 days after, and we counted the number of viruses that are not adsorbed compared with the number of total viruses in the incubation.

PFGE and PCR

Agarose embedded *O. tauri* were analysed by PFGE as described by Mead and colleagues (1988). Chromosomes of all resistant clones were separated by PFGE using a CHEF-DR III System (Bio-Rad) and a two-step programme, as follows: step 1, 60 s pulse for 15 h; step 2, 90 s pulse for 9 h. The included angle was 120° and the voltage was 6 V cm^{-1} (Derelle *et al*., 2002).

Polymerase chain reactions were made on concentrated cultures heated at 100°C during 10 min, with primers designed on the basis of OtV5 genome (Derelle *et al*., 2008). These primers (5′-ATGGGTTCATCTCAGTAGAA-3′ and 5′-CTCAACTACGACTGGGACGC-3′) amplified fragments of 2117 bp long. PCR conditions were as described previously (Bellec *et al*., 2009) with a melting temperature of 55°C.

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