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Viral degradation of marine bacterial exopolysaccharides

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Abstract :

The identification of the mechanisms by which marine dissolved organic matter (DOM) is produced and regenerated is critical to develop robust prediction of ocean carbon cycling. Polysaccharides represent one of the main constituents of marine DOM and their degradation is mainly attributed to polysaccharidases derived from bacteria. Here, we report that marine viruses can depolymerize the exopolysaccharides (EPS) excreted by their hosts using 5 bacteriophages that infect the notable EPS producer, Cobetia marina DSMZ 4741. Degradation monitorings as assessed by gel electrophoresis and size exclusion chromatography showed that 4 out of 5 phages carry structural enzymes that depolymerize purified solution of Cobetia marina EPS. The depolymerization patterns suggest that these putative polysaccharidases are constitutive, endo-acting, and functionally diverse. Viral adsorption kinetics indicate that the presence of these enzymes provides a significant advantage for phages to adsorb onto their hosts upon intense EPS production conditions. The experimental demonstration that marine phages can display polysaccharidases active on bacterial EPS lead us to question whether viruses could also contribute to the degradation of marine DOM and modify its bioavailability. Considering the prominence of phages in the ocean, such studies may unveil an important microbial process that affects the marine carbon cycle.

Keywords : marine phage, EPS, polysaccharidase, DOM, ocean

Introduction

The dynamics of dissolved organic matter (DOM) in seawater plays a pivotal role in ocean biogeochemistry. Marine DOM is derived from different biological processes, including phytoplankton release particularly during bloom events (Carlson & Hansell., 2014), excretion of capsular polysaccharides or exopolysaccharides (EPS) by heterotrophic bacteria (Stoderegger & Herndl 1998, 1999, 2001), sloppy feeding by metazoan grazers (Møller *et al*., 2005) or biodegradation of macroalgal biomass (Krause-Jensen & Duarte (2016). Viral-mediated lysis of microbes is also recognized as one of the largest source of DOM in the ocean (Middelboe *et al*., 1996, Fuhrman 1999, Suttle 2005). Regardless of the source of DOM, extracellular enzymes encoded by microbial communities initiate the degradation of DOM in the sea (Arnosti 2011). These enzymes efficiently depolymerize high-molecular-

weight organic matter into compounds of lower molecular weight. Part of these hydrolysis products is converted into bacterial biomass that supports higher trophic levels and inorganic nutrients that can be recycled by primary producers. Another part consists of refractory forms of organic matter that persist in the ocean for extended periods of time and contribute to carbon sequestration (Jiao *et al*., 2010; Krause-Jensen & Duarte, 2016). Extracellular enzymes influence the fate of most of the organic matter and thereby, they play a pivotal role in marine carbon cycling (Arnosti 2011).

High molecular weight acidic polysaccharides represent one of the main constituents of marine DOM (McCarthy *et al*., 1993; Panagiotopoulos & Sempéré, 2005). Despite their key-role in marine carbon cycling, the interactions between extracellular enzymes and marine DOM are not well understood. Studies, mostly conducted on terrestrial microorganisms, showed that Carbohydrate Active enZymes (CAZymes, Henrissat 1991; Davies & Henrissat, 1995; Lombard *et al*., 2013) are used to bind, modify, and degrade these polymers for consumption. Among these CAZYmes, the proteins that specificaly catalyze the breakdown of polysaccharides are designated as polysaccharide depolymerases or polysaccharidases. These enzymes are either constitutive or induced; they act either on the terminal end of the polymers (exo-acting mode) or cleave the polymer mid-chain (endo-acting mode) (Sutherland, 1995). They can be found attached to the cell or they can be released into the surrounding environments (Arnosti, 2011). Studies of biological models from variable origins evidenced a notable diversity within these enzymes, which are currently classified among 304 protein families based on sequence homology in the CAZy database (Lombard *et al.*, 2013; http://www.cazy.org/). In marine environments, polysaccharidases are ubiquitous and there is no doubt that they degrade much of the DOM in ocean (Arnosti 2011a; Arnosti 2011b). It is commonly assumed that polysaccharidase activity derives mainly from bacteria. The recent advances in genomics clearly demonstrated that other microbes encode polysaccharidases.

Among these, viruses, which usually outnumber bacteria by one order of magnitude in the ocean, can display such enzymes (Pires *et al.*, 2014).

A wealth of biomedical studies reports that viruses of bacteria (bacteriophages or phages) use polysaccharidases to depolymerize their host capsule/ slime/ biofilms in order to gain access to their primary receptors (Bayer *et al.*, 1978; Sutherland 1999; Sutherland *et al.*, 2004). These enzymes, typically located on phage tail, display a high level of functional and molecular diversity (Sutherland 1999; Scholl *et al.*, 2005; Cornelissen *et al.*, 2011, 2012; Yele *et al.*, 2012) and can influence biological traits of viruses such as host specificities (Leiman *et al.*, 2007). To the best of our knowledge, there is no experimental evidence of polysaccharidase activity in marine phages. Yet, marine bacteria are active producers of polysaccharides or exopolysaccharides (EPS) that are either associated with the bacterial cell surface or excreted into the environment (Biersmith & Benner 1998; Heissenberger *et al.*, 1996; Casillo *et al.*, 2018). This led us to question as to whether marine phages also carry polysaccharidases to infect their host. The finding of polysaccharidases associated to marine viruses could provide novel fundamental knowledge about the ecology of these prominent microbes. Because polysaccharidases initiate the infection cycle, we could expect that their intrinsic properties (life-time, substrate specificity, activity velocity) and their ability to withstand environmental changes (salinity, temperature, pH, pressure) directly influence the rates of infection and subsequent viral induced mortality or even the life-time of a viral particle in the ocean. Importantly, the passive degradation of dissolved bacterial EPS by viruses could have important biogeochemical implications, such as affecting the size spectrum, the reactivity and the bioavailability of marine DOM.

In this study, we investigated the capacity of marine phages to depolymerize their host EPS and attempted to relate this to important biological traits of marine viruses. Therefore, we used 5 phages (*CobetiA maRINa* phages: Carin-1 to 5) that infect the globally distributed γ-

Proteobacteria Cobetia marina (DSMZ 4741, hereafter *C. marina*) known for its ability to produce EPS (Arahal *et al*., 2002; Yumoto *et al*., 2004; Ivanova *et al*., 2005, Lelchat *et al*., 2015) and its potential contribution to the carbon export (Guidi et al. 2016). Our study revealed that marine phages do possess enzymes active on bacterial dissolved EPS, which may influence virus ecology and the recycling of bacterially derived DOM in the ocean.

Material and methods

- Bacterial host

The marine gram-negative Gammaproteobacteria *Cobetia marina* DSMZ 4741 was used as reference host to isolate phages from seawater. This strain belongs to a halotolerant mesophilic, heterotrophic and aerobic genus routinely grown on liquid and agar Zobell medium (Baumann *et al*., 1983; Romanenko *et al*., 2013).

- Phage isolation

Cobetia marina phages were isolated from surface water of the bay of Brest at the long-term monitoring station SOMLIT (4° 33' 07.19 W, 48° 21' 32.13 N) from June to September 2011. Seawater samples (20 L) were filtered through 0.7 µm glassfiber filter (Whatmann®) and concentrated (100-fold) using a 100 kDa ultrafiltration cartridge (Pellicon, Sartorius®). Once concentrated, samples were filtered through a PES syringe filter (0.2 µm cut-off, Sartorius®) and directly used in a plaque assay by spreading a mixture of the reference host and viruses in sterile molten Zobell agar (noble agar 0.6%, 5 $g.L^{-1}$ peptone, 1 $g.L^{-1}$ yeast extract dissolved in seawater) heated at 37°C on a layer Zobell agar (5 $g.L^{-1}$ peptone, 1 $g.L^{-1}$ yeast extract, 15 $g.L^{-1}$ agar dissolved in seawater). The plate was incubated at 20°C (Swanstrom & Adams, 1951; Kropinski *et al*., 2009). After 24 h incubation, well-resolved plaques (PFU - plaque forming unit) were picked from the lawn of host cells, eluted in sterilized ultrafiltered seawater, and combined with host culture in a new plaque assay. This procedure was repeated two more

times to ensure isolation of a clonal population for each phage. Clonal lysates of each viral strain was stored in SM buffer (NaCl: 100 mM; $MgSO_4$, $7H_2O$: 8 mM; TrisHCl: 50 mM) at 4°C until further study.

- Phage purification

Bacteriophages were purified according a protocol adapted from Bachrach & Friedmann (1971). Phages solution was purified on sucrose linear gradients (10 to 40 % w/v). First, 500 µL of freshly prepared viral lysate was treated with 50% (v/v) chloroform for 1 h to remove organic contaminants such as vesicles which can contain enzymes including polysaccharidases (Arntzen *et al.*, 2017; Li *et al.*, 2016). Then, the aqueouos phase was separated by low-speed centrifugation (3000 x g, 5 min at room temperature) and loaded on the top of the gradient before ultracentrifugation (134000 g, 45 min, 4°C) using a SW 41 Ti rotor. The viral band was extracted using a sterile syringe needle. The viral particles were diluted in SM buffer or sterilized ultrafiltered seawater and they were pelleted by a second ultracentrifugation (2 h, 352000 g, 4°C) using a 70.1 Ti rotor °C to separate them from sucrose. After resuspension of the viral pellet in SM buffer, bacteriophages were then filtered through a 0.2 µm PES filter (Sartorius®).

- Transmission electronic microscopy (TEM)

A drop (10 µL) of clonal phage suspension was applied to a copper EM grid (400 mesh size) with a nitrocellulose backed carbon surface (Ackermann, 2007; 2009). The sample was adsorbed on the grid for 10 sec after which the grid was blotted with filter paper and stained with 2% (w/v) uranyl acetate for 45 sec, blotted again and allowed to air dry. Specimens were imaged using a JEOL® JEM 1400 transmission electron microscope operating at 100 keV at a magnification of 50,000X. Pictures were analysed using ImageJ software.

- Host range

CobetiA maRINa phages: Carin-1 to 5 host specificities were determined by plaque assay using 3 strains of *C.* marina and 6 strains of the related *Halomonas* genus. Dilution series of Carin-1 to 5 suspensions (10^2 to 10^8 PFU.mL^{-1}) were incubated with the potential host cultures in exponential growth phase for 15 min. After incubation, samples were mixed with the molten agar, plated onto a lawn of Zobell agar and incubated at 20°C in darkness for 24 h.

- EPS production

Cobetia marina EPS (hereafter referred to as EPS L_6) were produced as described previously (Lelchat *et al*., 2015). Briefly, a pre-culture was grown in ZoBell medium (1 g.L^{-1} yeast extract, 5 g.L^{-1} peptone diluted in 80% filtered seawater and 20% MilliQ) supplemented with glucose (30 g.L^{-1}) at 25°C under agitation. After 10h incubation, pre-culture was inoculated (10% v/v) in a 3 L fermenter (INFORS®) containing Zobell medium supplemented with Tris-base (1.5 g.L^{-1}) and glucose (30 g.L^{-1}). The pH medium was adjusted to 7.6 and maintained at this value by addition of H_2SO_4 or NaOH. The culture was grown at 25°C for 33 h until the culture reached the late exponential phase. During the stationary growth phase, the temperature was decreased to 20°C for 48 h in order to enhance the EPS production. The oxygenation was monitored and regulated by stirring and air flux. The consumption of glucose was monitored with the enzyplus kit (St Gobain®, France). At the end of the fermentation, the culture medium was centrifuged (1 h, 14000 g, 4°C) and the supernatant was filtered with a Buchner through a 0.45 µM glass filter (Whatman®). Sodium azide (NaN_3, 0.4 g.L^{-1} final concentration) was added to the filtrate to prevent bacterial regrowth. The filtered culture supernatant containing the soluble EPS was then purified by tangential

ultrafiltration with a 100 kDa cut-off cartridge (Millipore®) against MilliQ water. The purified EPS was then frozen, freeze dried and stored away from light and moisture.

- Enzymatic screening

Enzyme assays were realized in triplicate. Purified viruses (10^{10} PFU.mL^{-1}) were mixed at 4°C with EPS L_6 (0.2 % w/v final concentration) in incubation buffer (Tris-HCl 50 mM, NaCl 0.5 M, $MgSO_4$ 8 mM, pH 8 at 20°C), filtered through a 0.2 µm filter (Sartorius®) and incubated 24 h at 25°C under agitation (120 rpm). Controls containing viral suspension, *C. marina* lysate extract and EPS as well as EPS alone were processed and incubated under similar conditions to check for potential auto-hydrolysis phenomenon according Lelchat *et al* (2014).

The polysaccharide degradation was visualized using agarose gel electrophoresis according to the method of Lee and Cowman (1994). With L_6 being an acidic polysaccharide, runs were conducted at pH 8.5. Briefly, the agarose gel (1%) was prepared in Tris/acetic acid / EDTA (TAE) buffer (TAE 10X: Tris-Acetic acid 0.4 M, Na_2 EDTA 10 mM, pH 8.5). Samples (12 µL) were loaded on the gel and electrophoresis was run for 75 min at 100 V.

The gel was stained overnight in a Stains All solution (Lee & Cowman 1994; Volpi & Maccari 2002) (10 mL of 3,3'-diethyl-9-methyl-4,5,4',5'-dibenzothiacarbocyanine 0.1% (w/v) in *N*,*N*-dimethylformamide, 10 mL of *N*,*N*-dimethylformamide; 50 mL of isopropanol; 10 mL of 300 mM Tris HCl pH 8.8, completed to 200 mL with MilliQ water). The gel was then destained for 2 h under natural light in distilled water.

Polysaccharide degradation was also monitored using size exclusion chromatography (SEC) on an Akta Fast Protein Liquid Chromatography apparatus (GE Healthcare Life Sciences®)

equipped with a Superdex 200 10/300 column (GE Healthcare 200®, optimum separation range: 1000-100000 $g.mol^{-1}$ dextran equivalent) as described by Lelchat *et al*. 2015. Samples were recovered after incubation, then injected with a 200 µL loop, separated with the incubation buffer (Tris-HCl 50 mM, NaCl 0.5 M, $MgSO_4$ 8 mM, pH 8 at 20) at 0.5 $mL.min^{-1}$ and detected using UV and refractometry to discriminate signals that could be assigned to proteins or polypeptides, but not sugars.

Low molecular weight degradation (50kDa > x >5 kDa) products were sampled, purified by centrifugal ultrafiltration (5 kDa cutt-off, Amicon Ultra 15, Merckmillipore®) and analyzed by ^{1}H-NMR according Lelchat *et al*., (2015). About 10 mg of depolymerized L_6 fractions were analyzed by NMR after three exchanges/dehydration cycles in deuterated water (99.9%). EPS were resuspended in 700 µL D_2O. Spectra were recorded at room temperature in the Laboratory of Nuclear Magnetic Resonance Spectroscopy (University of Western Brittany) on a 500 MHz Bruker® spectrometer.

- Enzyme kinetics

The depolymerization kinetics of the EPS L_6 by Carin-1 and Carin-5 was monitored during a 32 h enzyme kinetic according Lelchat *et al*. 2014. Purified bacteriophages (10^{10} $PFU.mL^{-1}$) were incubated with EPS L_6 (0.4 % w/v) in incubation buffer (Tris-HCl 50 mM, NaCl 0.5 M, $MgSO_4$ 8 mM, pH 8 at 20°C) according the modified incubation conditions optimized previously but with addition of NaN_3 as bacteriostatic (6 mM final concentration). Samples were taken every hour from 1 to 17 h and at 23 h, 25 h, 29 h and 32 h, flash frozen and stored at -80°C. Blanks containing virus solution alone, *C. marina* protein extract and EPS as well as EPS alone were incubated in the buffer to assess of the absence of auto-hydrolysis phenomenon. Depolymerization kinetics were visualized by 1% agarose gel electrophoresis

and SEC through a superdex-200 column (GE Healthcare Life Sciences®) as described above.

- Phage adsorption kinetics

The adsorption constant was determined for each phage with the host which was either induced or not induced to produce EPS following a protocol adapted from Hyman & Abedon (2009). A bacterial culture in exponential growth (5.10^8 bacteria.mL^{-1}) was incubated with each strain of bacteriophages at a multiplicity of infection (MOI) of 1 in a total volume of 40 mL. Samples (1 mL) were taken every 3 min for 30 min, and immediately centrifuged (7000 rpm, 1 min, 20°C). A supernatant aliquot containing the non-adsorbed phages was fixed with glutaraldehyde (final concentration 0.5 %) at 4°C for 15 min and flash-frozen in liquid N_2. Non-adsorbed viruses were enumerated by flow cytometry (FACs CANTO II, Beckton Dickinson®) upon SYBR-Green I staining (10 min, 80°C) according to Brussaard (2004). Blanks containing only viruses or bacteria were taken in parallel and processed as described above. Cytograms were analyzed using Flowing Software®. Adsorption coefficients (Cd) were calculated by dividing the regression coefficient of the natural logarithm of the curve describing the variation of viral abundance in 30 min ($x_{[virus]}$) by the average bacterial abundance [Bacterial abundance]: $Cd = - x_{[virus]}$/ [Bacterial abundance].

Results and Discussion

In this study, 5 phages lytic to the EPS-producing bacteria *C. marina* DSMZ 4741 were isolated *(*referred to as Carin-1 to 5) from the coastal Atlantic Ocean. The TEM examination of clonal suspensions of these phages indicated that they belong to the *Caudovirales* order characterized by an icosahedral head connected to a tail of variable morphology (Figure 1).

The strain Carin-1 exhibited a short tail and it was assigned to the *Podoviridae* family (head: $59_{+/-3.2}$ nm, tail: $12.8_{+/-2.1}$ nm) Carin-2 (head: $113.1_{+/-15.4}$ nm, tail: $137.5_{+/-18.9}$ nm), Carin-4 (head: $80_{+/-3.4}$ nm, tail: $140.3_{+/-6.8}$ nm), and Carin-5 (head: $69.7_{+/-3}$ nm, tail: $130.7_{+/-11.3}$ nm) displayed a contractile tail characteristic of the *Myoviridae* family while Carin-3 (head: $59_{+/-3.2}$ nm, tail: $12.8_{+/-2.1}$ nm) belonged to the *Siphoviridae* family with a long and non-contractile tail.

Evidence of marine phage mediated EPS depolymerization

Two phenotypic assays, that are typically used to screen for phages with biofilm disrupting abilities (Bessler *et al*., 1975), suggested that *Carin* phages possessed polysaccharidases active on their host EPS. All 5 phage isolates maintained their ability to form plaques on *C. marina* lawn even when the host EPS production was over stimulated using a glucose-enriched culture medium for growth. Furthermore, the 5 Carin phages formed clear plaques surrounded by an opaque halo zone that expanded in diameter over time (Figure S1). The increasing halo diameter is usually generated by phage-associated enzymes that depolymerize bacterial EPS as the phages spread out of the lysis zone by diffusion (Miyake *et al*., 1997; Glonti *et al*., 2010; Pleneteva *et al*., 2011; Cornelissen *et al*., 2011, 2012).

To confirm the hypothesis that Carin phages possess EPS polysaccharidases, we incubated *Cobetia marina* EPS L_6 (hereafter L_6) with and without viruses and visualized the depolymerization pattern on agarose gel electrophoresis. The migration of native L_6 showed 2 EPS populations with distinct molecular weight (Figure 2). The incubation of L_6 with purified Carin particles resulted in 3 degradation patterns. Carin-1 and -5 induced a nearly complete depolymerization of L_6 into products of low molecular weight. Carin-2 and -4 induced a partial degradation of the L_6, while Carin-3 did not seem to affect L_6 migration profile. The ^{1}H-NMR analysis of degradation products (50kDa > x >5 kDa) showed no difference of

spectra compared to native L_6 (Figure 3, Lelchat *et al*., 2015), indicating that Carin-1 and 5 generated L_6 oligosaccharides.

Detailed degradation kinetics as monitored by agarose gel eletrophoresis (Figure 4) and size exclusion chromatography (Figure S2) suggest that even Carin-1 and Carin-5, which generated a nearly complete L_6 degradation, possess different polysaccharidases. The kinetics show that both phages produced the formation of intermediate molecular weight species during the course of L_6 depolymerization, which suggests that they possess endo-active polysaccharidases as consistently reported in literature for phages of biomedical interest (Sutherland 1995; 1999). Endo-active enzymes can cleave glycosidic bonds all along the polysaccharidic chain and thereby enable phages to efficiently penetrate the EPS layer, even for polymers of high viscosity (Sutherland 1999). Despite similar endo-active modes of action, comparison of the migration (or elution) profiles suggest that Carin-1 and -5 polysaccharidases act differently on L_6. Carin-1 was able to significantly depolymerize the population of HMW species within 2h while products of high molecular weight were still apparent for Carin-5 after this incubation period. Depolymerization occurs within a few hours and seems to start very fast even when the incubation mixture is still at 4°C. At T0, the high molecular weight spot is fainter for incubation with Carin-1 compared to incubation with Carin-5 and despite a similar EPS initial concentration and all the possible precautions during the kinetic preparation. It is likely that Carin-1 can degrade the EPS of its host at cold temperature in order to adsorb and initiate its lytic cycle. *C. marina* DSMZ 4741 can indeed grow at 5°C (Yumoto *et al*., 2004)..

The results described above provide unequivocal evidence that marine phages can have the ability to degrade dissolved polysaccharides derived from their marine host. Because these assays were conducted with purified phage suspension, free of contaminants from the viral lysate, it is very likely that the polysaccharidases are bound to the virus particle. In the

literature, the presence of structural polysaccharidases have often been described in *Podoviridae* (Miyake *et al*., 1997; Linnerborg *et al*., 2001; Jakobsson *et al*., 2007; Leiman *et al*., 2007; Shaburova *et al*., 2009; Glonti *et al*., 2010; Pleneteva *et al*., 2011; Cornelissen *et al*., 2012; Roach *et al*., 2013) and *Myovirirdae* (Cornelissen *et al*., 2011; Shaburova *et al*., 2009; Elsässer-Beile & Stirm, 1981; Kwiatkowski *et al*., 1983; Nimmich *et al*., 1992) that infect pathogenic bacteria but are also reported in *Siphoviridae* (Niemann *et al*., 1976, 1977; Rieger-Hug & Stirm, 1981; Nimmich *et al*., 1992; Smith *et al*., 1994; Miyake *et al*., 1997; Chertkov *et al*., 2011; Gutierez *et al*. 2010, 2012; Roach *et al*., 2013). Nevertheless, we cannot rule out that virion-free polysaccharidases are excreted during the course of Carin-3 infection cycle as suggested by the formation of plaques surrounded by halo zones. It is also possible that Carin-3 uses another type of enzyme, such as lysine murein hydrolase, which degrades the protein moieties of bacterial biofilm as reported previously (Fischetti 2008). The different degradation patterns observed for Carin phages suggest that their polysaccharidases are functionally diverse. This diversity does not appear to be related to the classification of the selected isolates. Indeed, Carin-2, -4, and -5 are all members of the *Myoviridae* family but display different L_6 degradation patterns. A detailed molecular and biochemical characterization would certainly help understanding whether this functional variability arises from the activity of different types of polysaccharidases, from a variable number of polysaccharidase copies or from a combination of different enzymes bound to phage particle as reported for the coliphage K1-5 (Leiman *et al*., 2007).

Implications of EPS polysaccharidase activity for phage ecology

In the literature, the characterization of phage-associated polysaccharidases is mostly dedicated to the discovery of novel enzymes active on the biofilm or polysaccharidic capsule formed by pathogenic bacteria (Pires *et al*., 2016). By contrast, very few studies have

investigated how EPS polysaccharidases influence phage biological traits and to a larger extent their ecology. The finding that four out of five of the Carin phages we isolated possessed EPS polysaccharidases suggests that this property may provide a competitive advantage in the natural environment.

Because the assumed primary role of phage-associated polysaccharidases is to degrade their host capsular/biofilm EPS in order to attain membrane receptors (Bayer *et al*., 1979), it is reasonable to expect that the presence of these enzymes may affect the adsorption kinetics of Carin phages, particularly upon intense host EPS excretion. To address this question, we determined the adsorption constant (Cd) of Carin isolates (data missing for Carin-4) in culture conditions mimicking normal and stimulated EPS production (Table 1). The phage Cd in control conditions varied between 5.08 $x10^{-8}$ to 3.51 $x10^{-10}$ $mL.min^{-1}$ for Carin-2 and Carin-1, respectively, which ranged within the values reported in the literature (Fujimura & Kaesberg, 1962; Olkkonen & Bamford, 1989; Murray & Jackson 1992; Moldovan *et al*, 2007; Storms *et al*., 2010; Gallet *et al*., 2011). The observed Cd variation between phage isolates may arise from differences in viral particle diameter, morphology, viral electric charge but also the number of viral receptors. Nonetheless, the stimulation of host EPS synthesis decreased Cd values (Table 1) regardless of the viral isolate, suggesting that intensive EPS production acts as a physical barrier for phage adsorption. Interestingly, the amplitude of the changes in Cd varied depending on the ability of Carin phages to degrade *C. marina* EPS. While the Cd value of Carin-1, -5 and -2, which encode EPS polysaccharidase, were increased upon induction of EPS synthesis 5, 7, and 15-fold respectively, that of the phage Carin-3 was drastically augmented with a calculated 527-fold increase. The resulting Cd values show that phages that display polysaccharidase activity generally adsorb faster on their host under conditions mimicking intense EPS production. In nature, the amount and the chemical composition of EPS excreted by bacteria can be influenced by environmental parameters such

as temperature, nutrient, pH, but also by the bacterial lifestyle (planktonic versus biofilm) (Sutherland, 1972; Suresh Kumar *et al*., 2007). Under such conditions, the presence of polysaccharidases may provide a competitive advantage to efficiently propagate on their hosts. Likewise, the isolation of both EPS-polysaccharidase-encoding and EPS-polysaccharidase-lacking phages from the same geographical area could also reflect the dynamics or the variability of EPS production by *Cobetia marina* under natural settings.

Besides influencing phage adsorption kinetics, the presence and the diversity of polysaccharidase may influence host specificities as reported by Leiman *et al*. (2007). This previous study showed that the acquisition of several polysaccharidases by a given phage broaden its host range. In the light of this finding, we determined the host specificities of Carin phages using 3 C. *marina* strains and 6 strains of the genetically related genus *Halomonas* (Table S1) . This assay indicates that the 5 Carin phages have a narrow host range. In addition to *C. marina* DSMZ 4741, the phages Carin-1, 2, 3, and 4 could infect *C. marina* LMG 6798 whereas Carin-5 could not replicate on any alternate host. Hence, we could not relate the divergent EPS degrading abilities of Carin phages with their specificity pattern. The host spectrum assay was however conducted on a limited number of strains.

Concluding remarks

Viruses are undoubtedly the most abundant biological entites in the ocean (Suttle 2007). So far, their role has mainly been attributed to the mortality and the subsequent cell lysis that they impose on their host. Viral lysis mediates the release of up to 150 Gt C per year, and as such, viruses are responsible for one of the main fluxes of DOM in the ocean (Suttle 2005). Although the ecological and biogeochemical implications of marine viruses are acknowledged internationally, the regulation of viral activities are still poorly understood. Our study provides evidence that viruses of marine bacteria can display structural

polysaccharidases that likely initiate the infection cycle. In the model system *C. marina* – specific viruses, these enzymes were dectected in 4 out of 5 virus isolates, they appeared to be constitutive, endo-acting and functionally diverse. The detailed biochemical characterization of these molecules, that is the spectrum of substrates they can degrade, their regulation by environmental factors (*i.e.* temperature, pH, pressure, salinity), their life-time, or their structure, should provide novel insights into the functional mechanisms and the ecology of marine viruses.

More importantly, polysaccharidases are widely distributed in the ocean and they play an important role in biogeochemical cycles, digesting much of the marine DOM (Arnosti 2011). To date, it is broadly assumed that these enzymes mostly derive from bacteria. The finding that tailed marine phages, that are widespread and abundant in the ocean, can display constitutive polysaccharidases active on bacterial EPS lead us to question whether viruses could also contribute to the degradation of marine DOM, and, therefore, the marine carbon cycling in the ocean. Most marine bacteria indeed produce EPS, which potentially could fuel the pool of DOM in the ocean (Carlson & Hansell., 2014). Yet, the fate of these compounds remains largely unknown (Zhang *et al*., 2015). Complex bacterial EPS are thought to be rather recalcitrant to microbial degradation suggesting that they have long resident time in the ocean (Jiao *et al*., 2010). The depolymerization of bacterial EPS by viral polysaccharidases could provide an unexpected process affecting size distribution and the recycling of these compounds even at cold temperature. Thus, viral polysaccharidases, according to their mode of action and polysaccharide specificities, could be unexpected additional players in the size-reactivity continuum model in DOM biogeochemistry (Benner & Amon., 2015). Bacterial EPS, ususally negatively charged, are also involved in the complexation of trace metals such as iron, which limits primary production in more than 40% of the ocean (Martin *et al.,* 1990; Falkoswki *et al.,* 1998; Boyd *et al.,* 2000; Boyd & Ellwood, 2010; Moore *et al.,* 2013).

Besides the possible direct impact on DOM recycling, virally mediated degradation could also modify the bioavailability of EPS-bound trace metals. Considering the numerical dominance of viruses in the ocean, future studies should focus on the extent of virally mediated EPS degradation in the sea, and the bioavailability of the degradation products. Over the past decades, the bacteria *C. marina* has been used as a model for studies on EPS production and biofilm remediation (Maréchal *et al*., 2004; Ivanova *et al*., 2005; Mieszkin *et al*., 2012; Salaün *et al*., 2012). Given the structural singularity of its EPS (Lelchat *et al*., 2015) and the recent finding that members of the genus *Cobetia* may actively contribute to the carbon export in the global ocean (Guidi *et al*. 2016), this bacterium and its associated phages emerge as a model of considerable interest for such biogeochemical studies.

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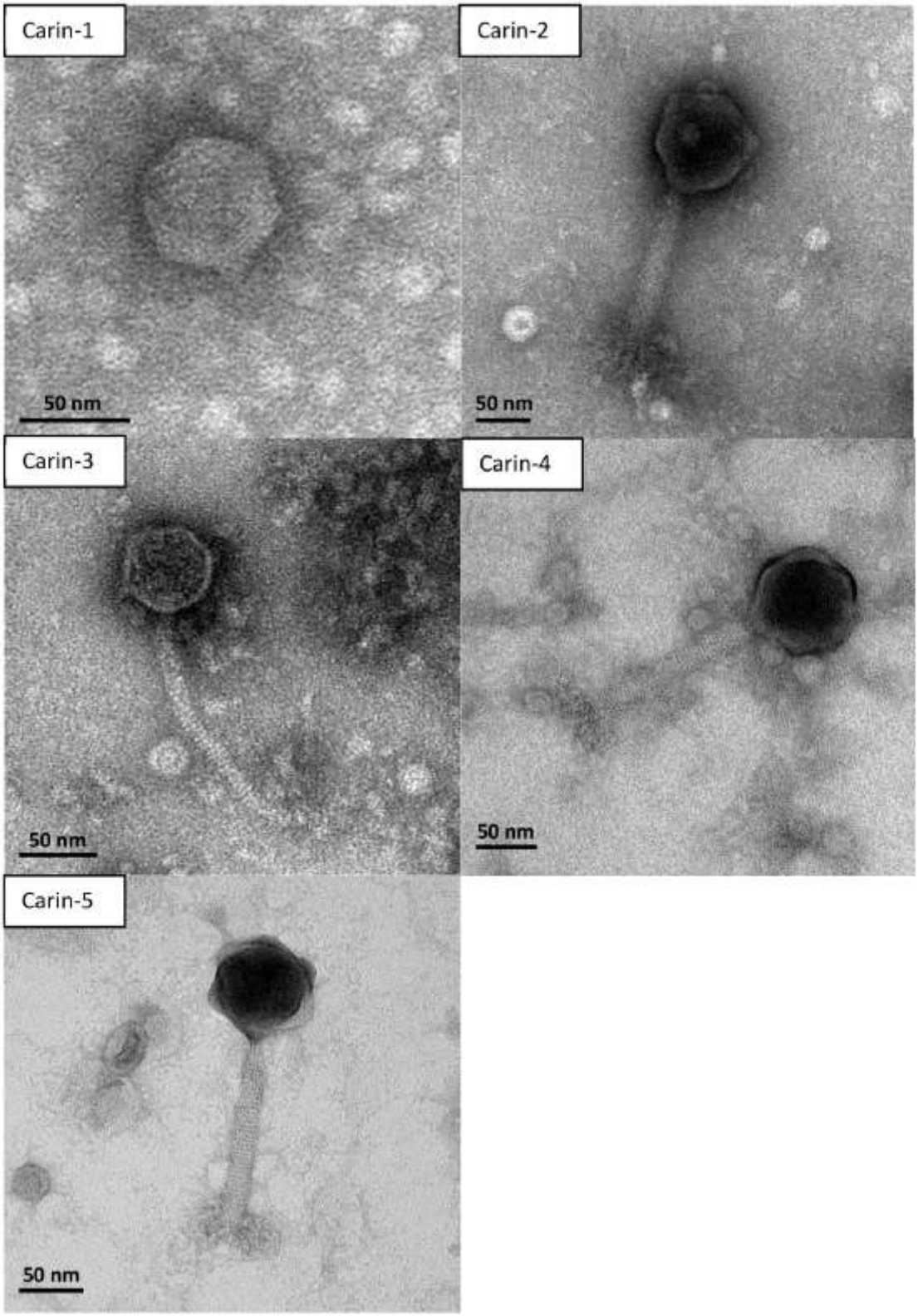

Figure 1: Transmission electron micrographs of the 5 bacteriophages that infect the marine bacterium *Cobetia marina* DSMZ 4741 (Carin phages)

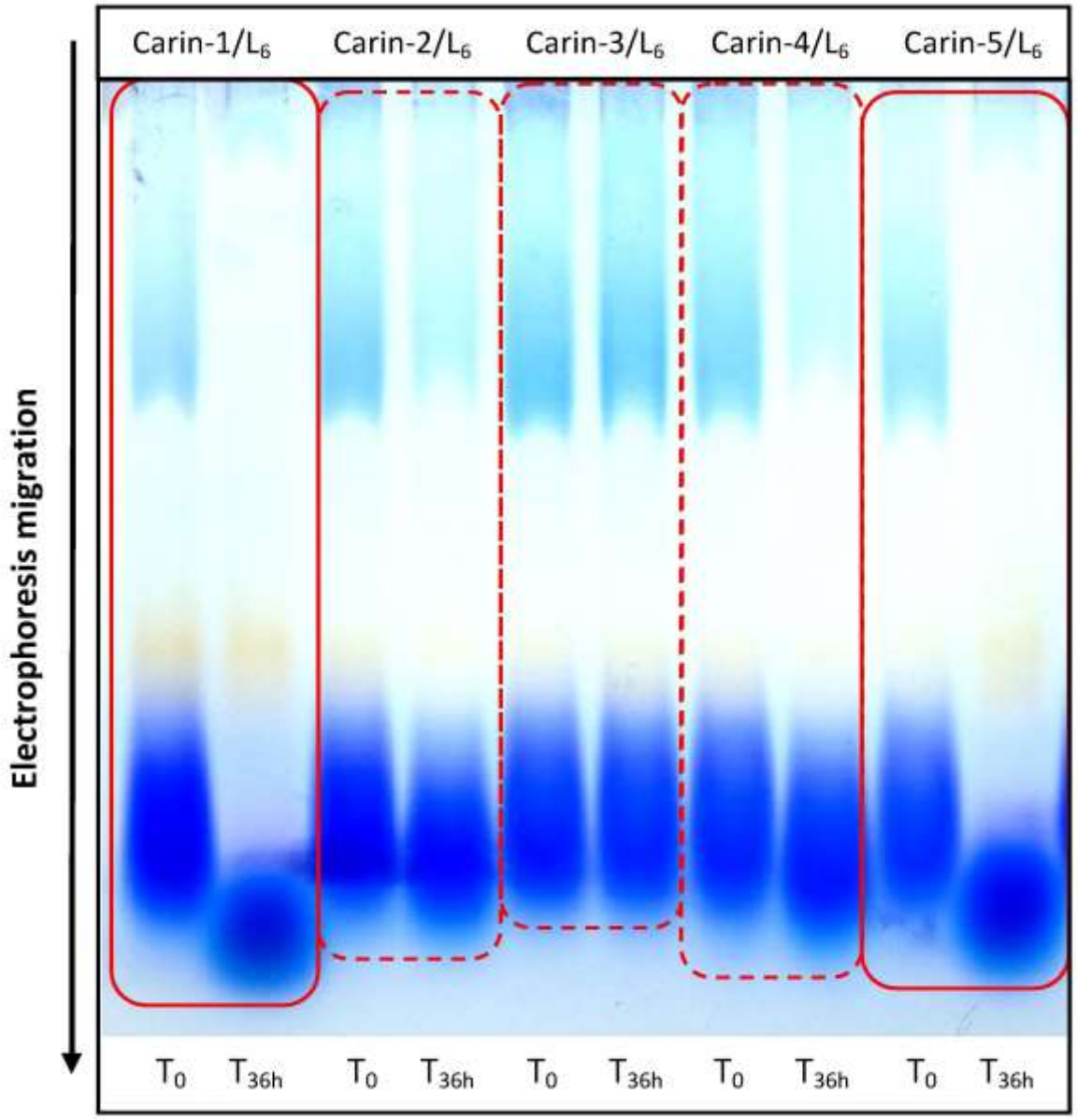

Figure 2: Migration pattern of *Cobetia marina* L_6 EPS (0.4 $g.L^{-1}$) with and without purified Carin phage suspensions (10^9 PFU) as assessed by agarose gel electrophoresis after Stains all coloration (blue smear: polysaccharides, yellow smear: proteins). This assay indicate that Carin-1, -2, -4 and -5 were able to depolymerize, *C. marina* EPS into products of low molecular weight.

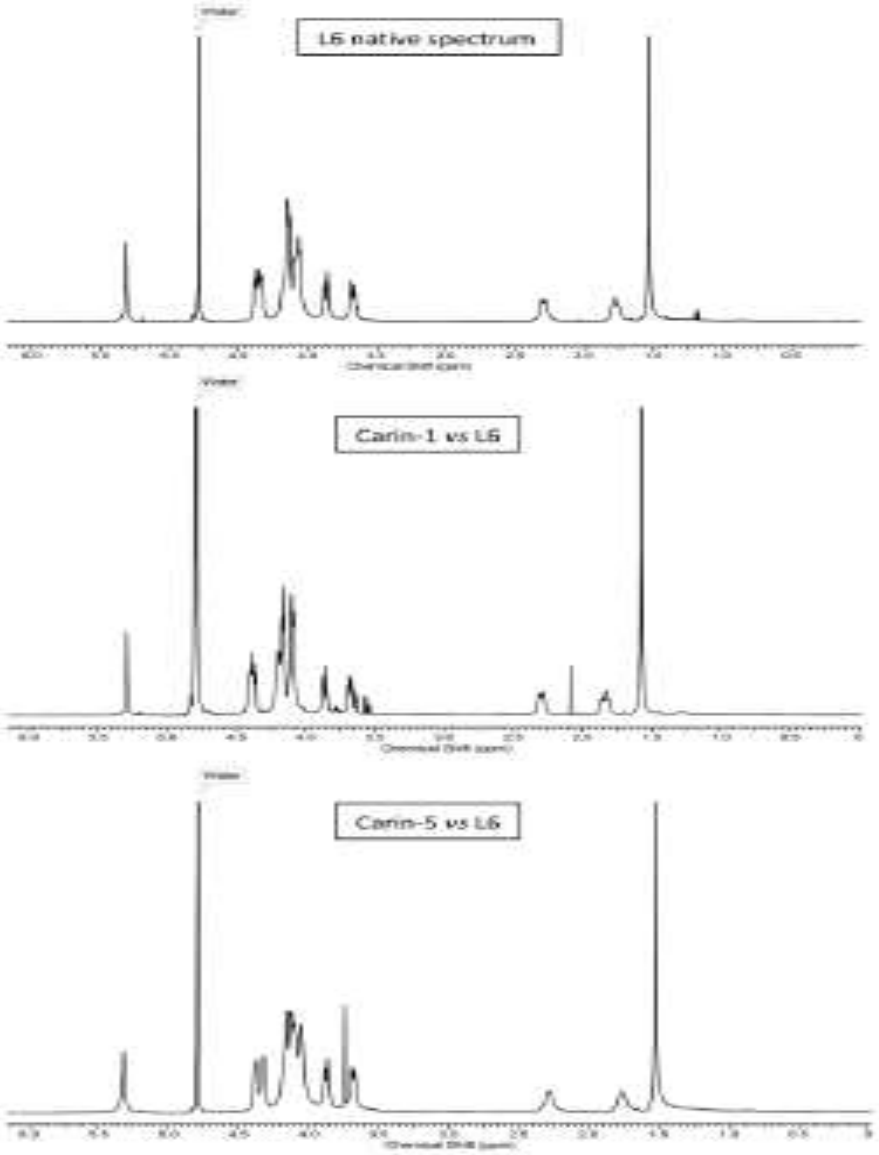

Figure 3: ^{1}H-NMR spectra of L_6 degradation products after Carin-1 and Carin-5 mediated depolymerization

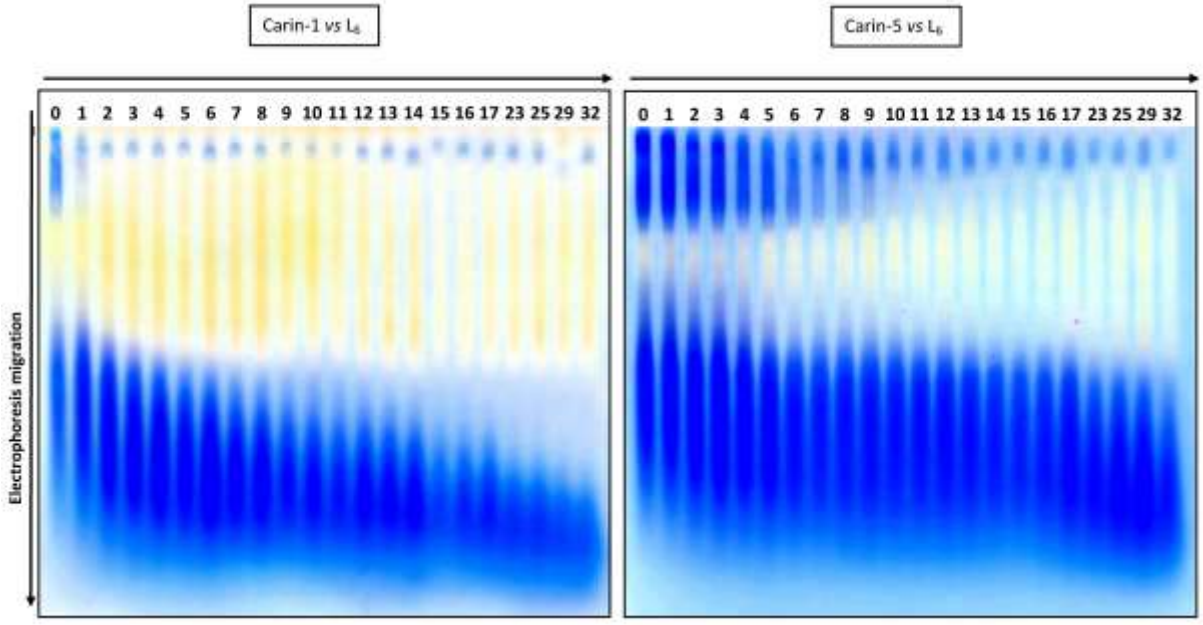

Figure 4: Degradation kinetics of the L_6 EPS (0.4 $g.L^{-1}$) by Carin-5 and Carin-1 (10^9 PFU) as assessed by agarose gel electrophoresis (Stains all coloration, blue smear: polysaccharides, yellow smear: proteins). The migration profiles suggest that both phages induced the formation of intermediate molecular weight species during the course of L6 depolymerization, yet, at different velocity.

Table 1: Adsorption coefficient of Carin-1, 2,3 and 5 under control and EPS induced host growing conditions

	Adsorption coefficient Cd ($ml.min^{-1}$)		
Bacteriophage	EPS synthesis non-induced	EPS synthesis induced	Ratio $Cd_{non\text{-}ind}/Cd_{ind}$
Carin-1	$3{,}51.10^{-10}$	$7{,}78.10^{-11}$	4,51
Carin-2	$5{,}08.10^{-8}$	$3{,}32.10^{-9}$	15,30
Carin-3	$1{,}75.10^{-8}$	$3{,}32.10^{-11}$	527
Carin-4	nd	nd	nd
Carin-5	$1{,}56.10^{-9}$	$2{,}07.10^{-10}$	7,53