Analysis of Cyanophage Diversity in the Marine Environment Using Denaturing Gradient Gel Electrophoresis

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ABSTRACT

Cyanophages (viruses that infect cyanobacteria) are abundant in the marine environment and are thought to be a significant factor in determining the population dynamics of members of the unicellular phycoerythrin-containing cyanobacteria of the genus *Synechococcus*. In an effort to use molecular techniques to characterise cyanophage populations, a conserved region from the cyanophage genome was identified in 3 genetically distinct marine cyanomyoviruses and sequence analysis revealed that they exhibited significant similarity to a gene encoding a capsid assembly protein (gp20) from the enteric coliphage T4. Comparison of these sequences permitted the design of PCR primers which specifically amplified a region of 165 bp from cyanomyovirus isolates tested. Denaturing gradient gel electrophoresis (DGGE) was then used to separate 165 bp DNA fragments from a range of different cyanomyovirus isolates which had been PCR-amplified together. DGGE was subsequently used to investigate the population structure of cyanophages during the course of a south-north transect of the Atlantic ocean in April/May 1996. Cyanophage population structure changed dramatically in the surface (7 m) waters across the transect. It was also noted that some DNA fragments in the DGGE analysis were common throughout the transect suggesting that some genetically identical cyanophages have ubiquitous distribution in the surface ocean. DGGE analysis also revealed a high cyanophage diversity through all the depth profiles and changes in population structure were observed with depth. Maximum diversity was invariably correlated to maximum *Synechococcus* spp. abundances. In some stations where there was a deep mixed layer, cyanophage population structure was similar throughout the water column. In contrast, changes were observed in cyanophage population structure when water column stratification occurred. Environmental factors which shape cyanophage population structure such as nutrient availability, fluctuating light and changing physical conditions are discussed; all of which were measured on the south-north transect of the Atlantic ocean.

Introduction

Since the discovery of high concentrations of viruses in the marine environment [1], there has been a concerted effort to develop accurate techniques to analyse virus populations both quantitatively and qualitatively. Many classical viral analysis techniques relied on the culture of suitable hosts such as enteric bacteria which, when compared to hosts from the marine

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environment, are extremely easy to culture and have generation times from as short as 20 minutes. It is only recently that molecular techniques have been employed to investigate algal-virus populations (for review see Wilson & Mann [12]). One approach has been to identify algal virus-specific PCR primers which recognise specific sequences to investigate genetic diversity. Chen and Suttle [3] developed PCR primers based on B-family (α-like) DNA polymerases which could detect microalgal viruses. Algal-virus-specific PCR primers were subsequently used to amplify DNA polymerase gene fragments from seawater concentrated in the Gulf of Mexico [4], and it was demonstrated that there was a diverse community of both *Micromonas pusilla* viruses and other unknown members of the *Phycodnaviridae*.

Prior to the current study, similar techniques were developed to identify cyanophages in the marine environment [6]. A conserved region from the cyanophage genome was identified in 3 genetically distinct cyanophages and sequence analysis revealed that they exhibited significant similarity to a gene encoding a capsid assembly protein (gp20) from the enteric coliphage T4 [8]. Comparison of gene 20 sequences from three cyanophages and coliphage T4 permitted the design of two degenerate PCR primers, CPS1 and CPS2 (see Fuller et al. [6] for sequences), which specifically amplified a region of 165 bp from cyanophages of the genus cyanomyovirus (which correspond to Bradley group A [2]). Although Fuller et al. [6] were unable to develop a quantitative assay to enumerate natural cyanophage populations, they speculated that analysis of amplified products would provide important information on the diversity of marine cyanophages.

Despite the fact that *Synechococcus* spp. are ubiquitous through the worlds oceans [11], very few studies have actually tried to elucidate the ecological significance of cyanophages in regulating *Synechococcus* spp. abundance and community structure. In the current study we investigated the diversity of cyanophages in seawater samples collected during an Atlantic Meridional Transect (AMT) cruise which sailed from the Falkland Islands in the South Atlantic Ocean to the UK. Changes in cyanophage population structure in depth profiles, at individual stations, were examined by denaturing gradient gel electrophoresis (DGGE) [9]. In addition, vertical profiles of *Synechococcus* spp. abundance, nutrient concentration and temperature were measured and compared to DGGE profiles in an attempt to assess the parameters which affected cyanophage population dynamics.

Materials and Methods

Virus communities were concentrated from both surface seawater and depth profiles from 25 locations on a transect from the Falkland Islands to the UK between 24 April and 19 May 1996 during the second Atlantic Meridionial Transect (AMT-2) cruise. Samples featured in this report were taken from station numbers 9, 19°54’S 35°31’W; 14, 00°12’S 26°24’W; 18, 19°54’N 21°19’W and 20, 30°55’N 21°16’W. Samples were collected using a Neil Brown Mark IIIB (Instrument Systems, Inc.) CTD instrument with a rosette sampling system, fitted with 12 (10 l) General Oceanics water bottles. The CTD system was deployed to 200 m (where depth allowed) at each station. Nutrients (nitrate and phosphate) from vertical profiles were analysed onboard using a 4 channel Technicon segmented-flow auto-analyser.

Prior to sample concentration, phycoerythrin-containing *Synechococcus* spp. were enumerated by epifluorescence microscopy. 1 liter volumes of seawater were gently filtered

through 2 µm (nominal pore size) Nuclepore filters to remove zooplankton and larger phytoplankton. Resulting filtrates were concentrated 100 times by tangential flow filtration using a mini-ultrasette with a 30 kDa cut off membrane (Flowgen, Instruments Ltd. UK). Concentrates (ca. 10 ml) containing both cyanophage and *Synechococcus* communities were stored at -20°C until PCR amplification.

For DGGE analysis, concentrates were subjected to similar PCR conditions to that previously described [6] with the exception of primers: 8 pmol of each primer CPS4GC and CPS5 (Cyanophage Specific) were used in the reaction. Non-degenerate cyanophage specific primers were based on sequence data from cyanophage genes sequenced in Fuller et al. [6], the upstream primer (CPS4GC) was altered with a 40-nucleotide GC-rich sequence (GC clamp) added to the 5' end (necessary for DGGE analysis [9]), thus:
CPS4GC 5' –
CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGGTAGA
ATTTTCTACATTGATGTTGG - 3'. CPS5 5' – GGTAACCAGAAATCTTCAAGCAT - 3'. PCR amplification was carried out as described in Fuller et al. [6] with the exception of a lower annealing temperature of 50°C. DGGE analysis was performed using the Bio-Rad DGene system according to the manufacturers instructions. Briefly, PCR products (30 µl) were loaded onto a 10% polyacrylamide gel in 1x TAE (40 mM Tris base, pH7.4; 20 mM sodium acetate; 1 mM Na_2-EDTA) which contained a 15% - 35% denaturing gradient. Electrophoresis was performed at a constant voltage of 150 V and a temperature of 60°C for 5 hours. Separated PCR products were visualised by ethidium bromide staining.

Results and Discussion

We have previously reported the characterization of a set of homologous genes from myoviruses infecting members of the phycoerythrin-containing marine *Synechococcus* strains [6]. This permitted the development of PCR primers capable of specifically detecting these viruses in natural assemblages and has facilitated the analysis of the dynamics and diversity of viral populations in response to changes in host populations and nutrient availability in the present study.

PCR and DGGE analysis was carried out on all samples collected from the transect, however, only 4 stations are featured in this report. PCR products of ca. 200 bp in length (the product is usually 165 bp when the GC-clamp is not used [6]) were obtained from most samples (results not shown). Products were not observed from below the photic zone or from where *Synechococcus* spp. were undetectable. DGGE analysis revealed that some fragments are common throughout the transect. This was more noticeable when all the surface (7 m) PCR products were run together (results not shown), suggesting that some genetically identical cyanophages have a ubiquitous distribution in the surface ocean.

It is clear from the DGGE analysis of vertical profiles that cyanophage diversity is high in most water samples, between 2 and 10 distinct fragments were observed in each track (Fig. 1). High diversity in marine viruses is not unusual, similar extensive ranges in diversity have been observed in viruses which infect the photosynthetic flagellate *Micromonas pusilla* [5] and vibriophages isolated from seawater off Florida and Hawaii [7]. It is interesting to note the changes in cyanophage population structure reflected both nutrient and *Synechococcus* spp. concentrations at stations 18 and 20 (Fig. 2). At station 18, cyanophage diversity appears the same in each sample down the water column suggesting a well-mixed vertical

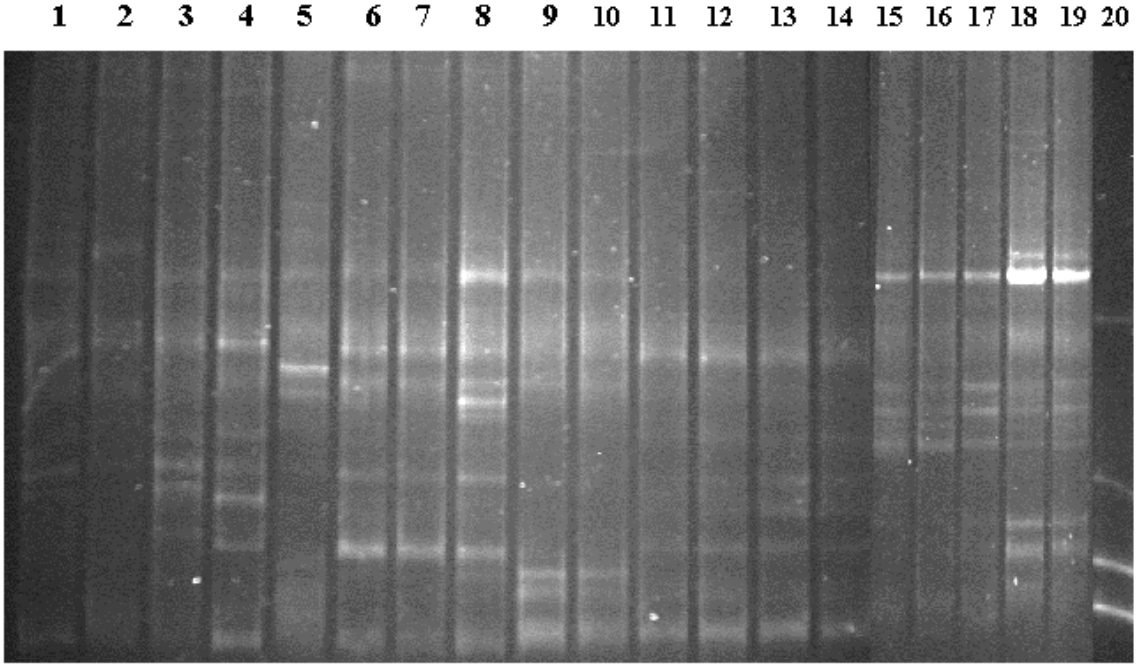

Fig. 1. DGGE analysis of cyanophage population structure from 4 depth profiles on the AMT-2 cruise transect, using the cyanophage-specific primers CPS4GC and CPS5 (this study). Concentrates were amplified from station numbers and depths, thus: station 9, depth 7 m (lane 1); 9, 30 m (lane 2); 9, 50 m (lane 3), 9, 80 m (lane 4); 9, 100 m (lane 5); 14, 7 m (lane 6); 14, 20 m (lane 7); 14, 40 m (lane 8); 14, 70 m (lane 9); 14, 80 m (lane 10); 18, 7 m (lane 11); 18, 30 m (lane 12); 18, 50 m (lane 13); 18, 70 m (lane 14); 20, 7 m (lane 15); 20, 50 m (lane 16); 20, 60 m (lane 17); 20, 80 m (lane 18); 20, 100 m (lane 19). Cyanophage strains S-PM2, S-BM1, S-WHM1 and S-BnM1 [6] were all amplified in the same reaction (lane 20). PCR products were subject to DGGE analysis using a 15-35% urea/formamide gradient at 150 V for 5 h in 10% acrylamide and visualised by ethidium bromide staining.

profile (Fig 1. lanes 11-14). In support of this there seems to be no strong thermal stratification (Fig. 2a) and nutrient concentrations are undetectable in the surface 80 m (Fig. 2b). There is a peak in *Synechococcus* spp. concentrations at 70 m (Fig. 2c), however, there is no corresponding change in cyanophage diversity.

In comparison, there is marked stratification of the cyanophage population structure down the water column at station 20 (Fig. 1 lanes 15-19). In the 3 surface samples there are only 4 – 5 distinct fragments compared to the deeper samples at 80 m and 100 m where there is a higher diversity of 7 – 10 fragments. The highest diversity at station 20 coincides with an increase in nutrient concentrations measured below 80 m (Fig. 2e). In addition, there is measurable thermal stratification in the water column (Fig. 2d). At stations 9 and 14, maximum diversity (Fig. 1, lanes 4 and 8 consecutively) was observed at the same depth as maximum *Synechococcus* spp. concentration (results not shown).

This data set was collected over a large spacial area and the structure of the water column was different at each station over the cruise transect, hence, it is difficult to make any general conclusions on the observations made. Analysis of data does suggest that either physical structure of the water column, nutrient availability or host concentration, or more likely a combination of all three, will influence cyanophage population structure and diversity. It has been previously established that there is considerable genetic diversity within natural assemblages of host *Synechococcus* spp. [10]. Cyanophage selection pressure

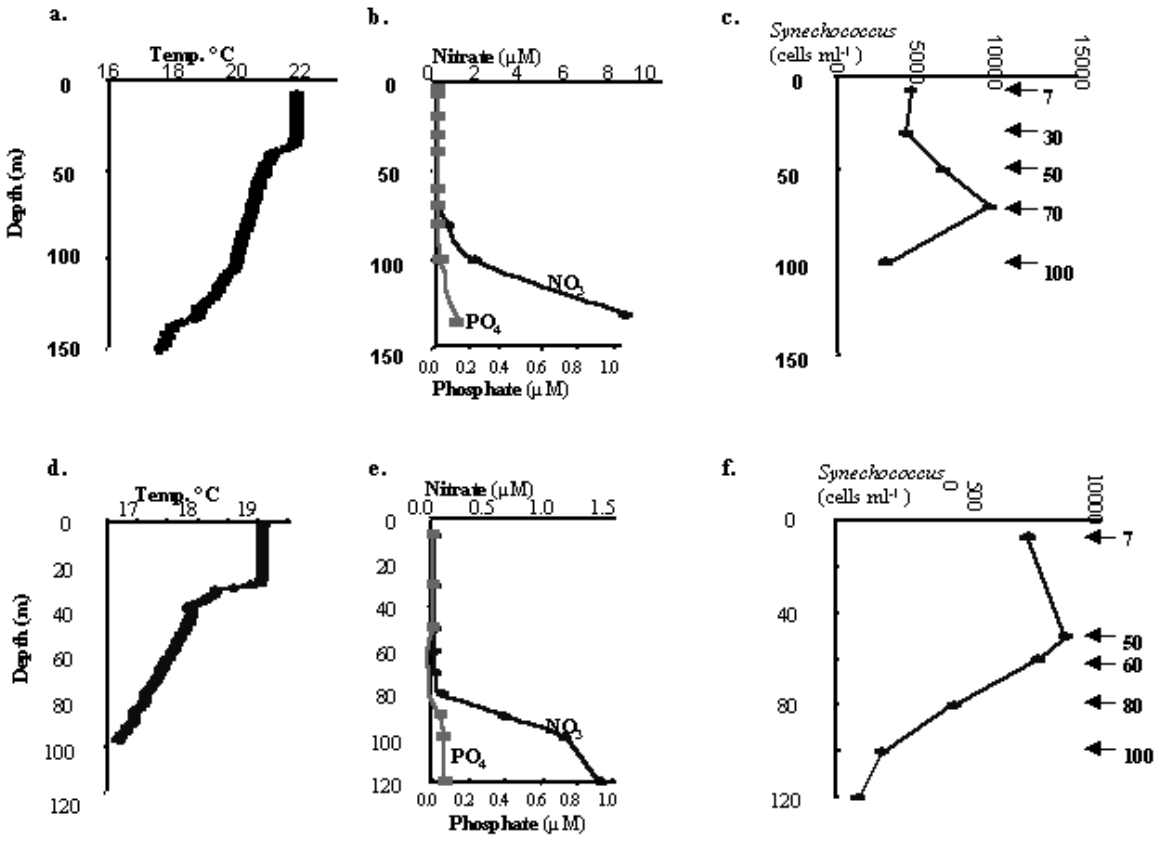

Fig. 2. Analysis of vertical profiles from stations 18 (a – c) and 20 (d – f). Measurements recorded included temperature (a, d), nutrients (b, e) [nitrate and phosphate profiles are labelled on each graph] and *Synechococcus* spp. (c, f) [actual depths where counts were carried out are labelled accordingly on the right hand side of each graph].

will undoubtedly influence the *Synechococcus* spp. clonal structure via infection and lysis of sensitive cells or through horizontal gene transfer events via lysogeny and/or transduction. These events will, in turn, be influenced by nutrient availability (for review, see Wilson and Mann [12]), fluctuating light and changing physical conditions. Consequently, the clonal structure of the cyanophage standing stock will be very diverse as was observed from the DGGE analysis in the current study. DGGE represents a powerful technique to rapidly assess changes in virus population structure and consequently correlate these changes with fluctuating environmental parameters.

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