Bacteriophages of the North Sea – state of the art

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ABSTRACT

Bacteriophages may play ecological key roles in the complex marine bacterial communities and habitats. It is assumed that phages control bacterioplankton through their lytic activities, and that temperate phages may contribute to horizontal gene transfer. However, these statements rely on a limited set of data. Current knowledge is based on microscopic virus-like particle (VLP) counts and some observations using classical plaque forming units (PFU) providing information of bacteriophages in selected marine habitats. Molecular approaches were applied rarely. Unfortunately, the required information in order to decipher the complex puzzle(s) is presently not available. There is no technique on-hand for quick virus determination. Furthermore, the dominant bacterial hosts are often not culturable. The development of a concept on the systematic elucidation of phage ecology is overdue. It should encompass molecular and classical approaches. This paper contributes some ideas to solve these problems.

INTRODUCTION

Recent publications stress that phages play an important role in the complex marine food webs as summarized in recent reviews by Proctor (1998), Wilhelm and Suttle (1999) and Wommack and Colwell (2000). Phages are abundant in the marine environment (up to 10^8 virus-like particles ml^{-1}) (Bergh *et al.*, 1989; Wommack and Colwell, 2000). The abundances are positively correlated to the trophic level and productivity of their habitats.

Usually their numbers are determined as VLP's PFU generated by their host bacteria. Microscopic approaches require transmission electron microscopy (Bergh *et al.*, 1989) or epifluorescence microscopy (Hennes and Suttle, 1995). Phages infect and lyse planktonic bacteria. It is assumed that besides the digestion of bacteria by heterotrophic nanoflagellates, phages account for 10 to 50% of bacterial mortality (Fuhrman and Noble, 1995; Hennes and Simon, 1995; Fuhrman, 1999). Simultaneously, the release of dissolved organic and inorganic nutrients derived from lysed bacteria may enhance bacterial substrate turnover and result in higher bacterioplankton biomass. Besides their quantitative growth control, phages can influence the community structure of the planktonic bacteria also qualitatively. This may be due to the high bacterial host specificity of many marine bacteriophages (Moebus, 1983; Hennes *et al.*, 1995; Wichels, 1997; Wichels *et al.*, 1998; Wommack and Colwell, 2000) and comprises complex and diverse phage activities, like specific lysis, resistance phenomena, and/or lysogeny of host bacteria, possibly also

resulting in horizontal gene transfer by transduction (Jiang and Paul, 1998; Chiura *et al.*, 2000). If information of lysogenic bacterial hosts from the North Sea is rare (Moebus, personal communication), a convenient example for lysogeny is represented by *Sphingomonas* sp. strain B18, isolated from Lake Pluss See (Ostholstein, Germany) (Witzel *et al.*, 1994; Wolf *et al.*, 2003). Also worthy of note, the fact that a global movement of ballast water by ships creates a worldwide distribution of microorganisms including virus particles (CIESM, 2002; Ruiz *et al.*, 2002). All these phenomena have an impact on the bacterial community structure.

New biotechnological approaches focus on the utilization of phages. Phages can function as tools, agents and/or chemical compounds. For example applications make use of phages to remove phosphate from activated sludge systems (Khan *et al.*, 2002). Or phages may be used as antifoam agents (Thomas *et al.*, 2002). In another instance bacteriophage H4489A provides specific lyases to digest the hyaluronan capsule of pathogen streptococci (Baker *et al.*, 2002). Brion and Silverstein (2001) describe a successful practice using non-pathogen phages to test the efficiency of water recycling systems. Probably, in the near future new applications will be detected for marine viruses.

PHAGE RESEARCH AT MARINE STATION HELGOLAND

Extensive investigations at the Biologische Anstalt Helgoland have demonstrated a high diversity of bacteriophages in the open waters (Moebus and Nattkemper, 1981 und 1983; Moebus, 1987; Wichels *et al.*, 1998; Wichels *et al.*, 2002). All bacteriophages investigated contained double stranded DNA. Electron microscopy studies revealed that phages collected from the waters around Helgoland (German Bight) were members of the order Caudovirales with the families Myovirides, Siphoviridae and Podoviridae including different species and some conspicuous morphotypes (Fig. 1). The host specificity was striking. Seventy-three percent of a total

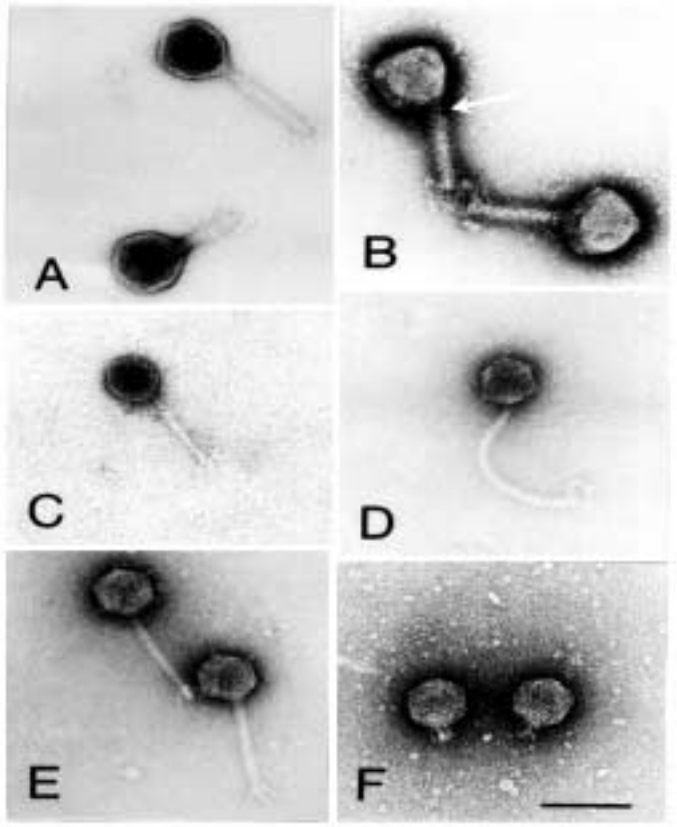

Fig. 1. Marine bacteriophages of three different virus-families, isolated from water samples near Helgoland (German Bight). **A:** Myoviridae, morphotype 1: head without antennae; short appendages on the tail; (phage H106/1); **B:** Myoviridae, morphotype 2: collar like structure between head and tail (see arrow); short appendages on the tail (phage H7/2; 15); **C:** Sipho-viridae, morphotype 1: head and tail without appendages (phage 10-77a); **D:** Siphoviridae, morphotype 2: knob-like appendages on the head, tail with a hook at the end (phage 11 68c); **E:** Siphoviridae, morphotype 3: knob-like appendages on the head, tail with short appendages (phage H105/1); **F:** Podoviridae, morphotype 1 (phage H100/1); bar: 100 nm.

of eighty-five phages investigated lysed exclusively their original bacterial host. The determination of host bacteria indicated that the bacterial isolates belong to the subgroup of proteobacteria. Phage diversity was elucidated by DNA-DNA-cross-hybridization (all phages were hybridised against each other), restriction analysis and the comparison of RAPD patterns. Cross-hybridization data unveiled a high phage diversity. Fourteen out of twenty-two phages showed no genetic relationship to the other particles tested (Fig. 2). The analysis of total phage protein patterns and restriction patterns was less helpful. Inactive restriction enzymes, and low phage protein concentrations caused major problems. The ecological approach showed that some of the phages Moebus (1983) isolated a decade earlier could still be detected in the waters of Helgoland and the German Bight. However, in using the bacterial host strains from Moebus, newly isolated lytic phages were only localized in a narrow regional area north and south of Helgoland (Fig. 3). These data suggest stable community structures over a long space of time of specific phage host systems. These findings are different from the data of Kellog *et al.* (1995). The authors detected specific *Vibrio* phages in the Atlantic Ocean which are distributed over a distance of several thousands nautical miles, whereas bacterial hosts from the German Bight were mostly members of the *Pseudoalteromonas* group (γ–Proteobacteria). The different data may not be contradictory. Presumably both the Vibrio and the *Pseudoalteromonas* group of bacterial hosts are well adapted to their marine environments. However, the German Bight with the major estuaries of the Elbe and Weser River can be regarded as an enormous melting pot, which differs nearly in every oceanographic and biological respect from the characteristics of the open Atlantic.

Our field experiments in the waters around Helgoland underscore the high diversity of different shapes and sizes of virus-like particles. Fig. 4 provides an example of the natural virioplankton. Seasonal phage distributions, determined by epifluorescence direct counting with YO-PRO stain (Molecular Probes), followed the seasonal bacterioplankton characteristics (Fig. 5). Low

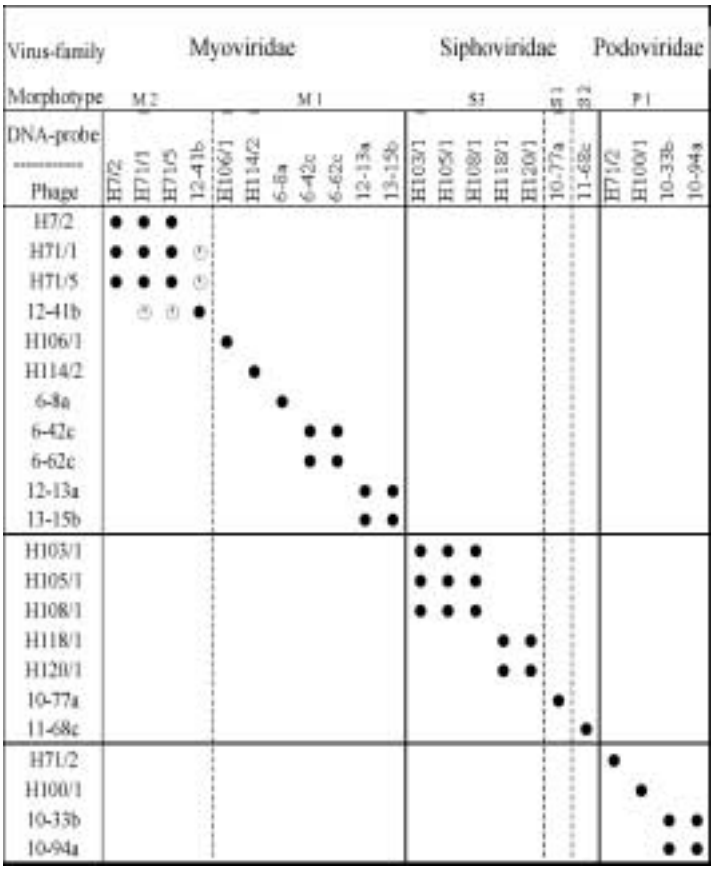

Fig. 2. DNA-DNA-cross-hybridizations of 22 marine bacteriophages collected from waters around Helgoland. l: strong hybridization signal. m: weak hybridization signal. Phages and DNA-probes were assigned to their families and morphotypes.

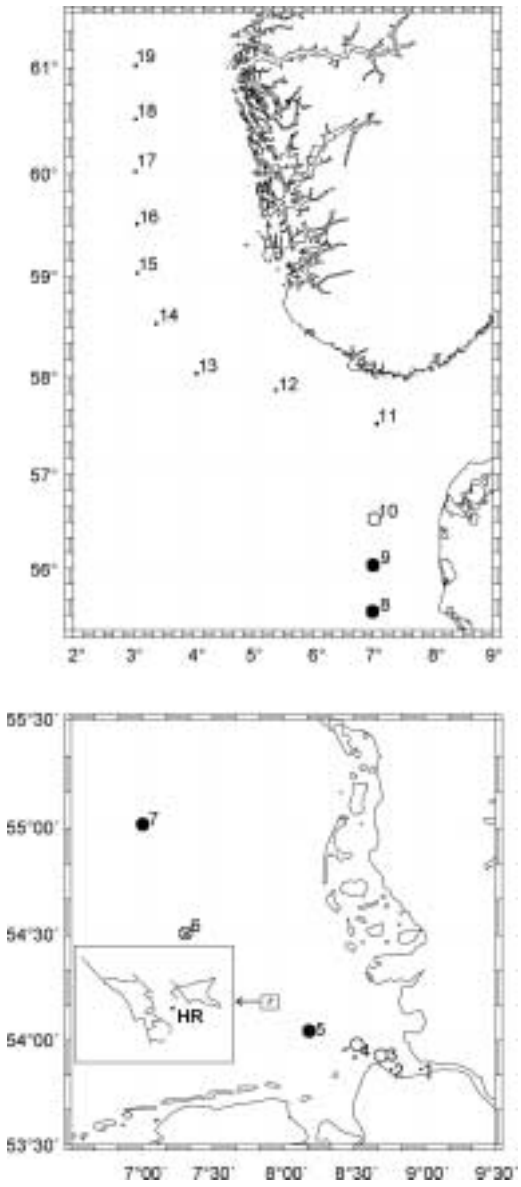

Fig. 3. Sampling sites of the RV *Heincke* cruises HE49 (September 1993), 54 and 65 (April and October 1994) in the North Sea (German Bight to Norwegian waters). Station Helgoland Roads (HR) is the origin of 70 phage host systems used in this study (Moebus 1992a,b). Circles represent the occurrence of Pseudoalteromonas phages, as determined by plaque assay, at each station (l 1993; m 1994; U both years; other stations no plaques formation).

numbers phages and bacteria were found during the cold seasons; high numbers were detected during the productive seasons from May to October.

NEEDS AND NEW APPROACHES

Presumably phages play ecological key roles in controlling marine bacterioplankton and gene transfer. However, presently there is no concept for unveiling the ecological role with lytic and temperate activities of phage host systems. The functions of the zillions of marine virus particles, which represent an exciting block of the “mare incognitum”, are still enigmatic.

Generally, there are two fundamental complexes of questions concerning the bacterial hosts and their phages. (i.) Bacterial hosts: systematic new approaches to quantify, culture and determine the dominant host bacteria in the various natural habitats are urgently needed. Since bacterial hardware is still essential in laboratory experiments, the development of new culture techniques is indispensable. In order to reach realistic answers on quality and frequencies of virus mediated natural gene transfers, thorough investigations of lysogenic bacteria and its temperate phages are required. That kind of approaches should include the development of specific primers for selected phage genes. (ii.) Phages: progress in virus research will depend on the availability of quick determination procedures for phages. In this field universal viral probes from polymerases, lyases, etc. are needed.

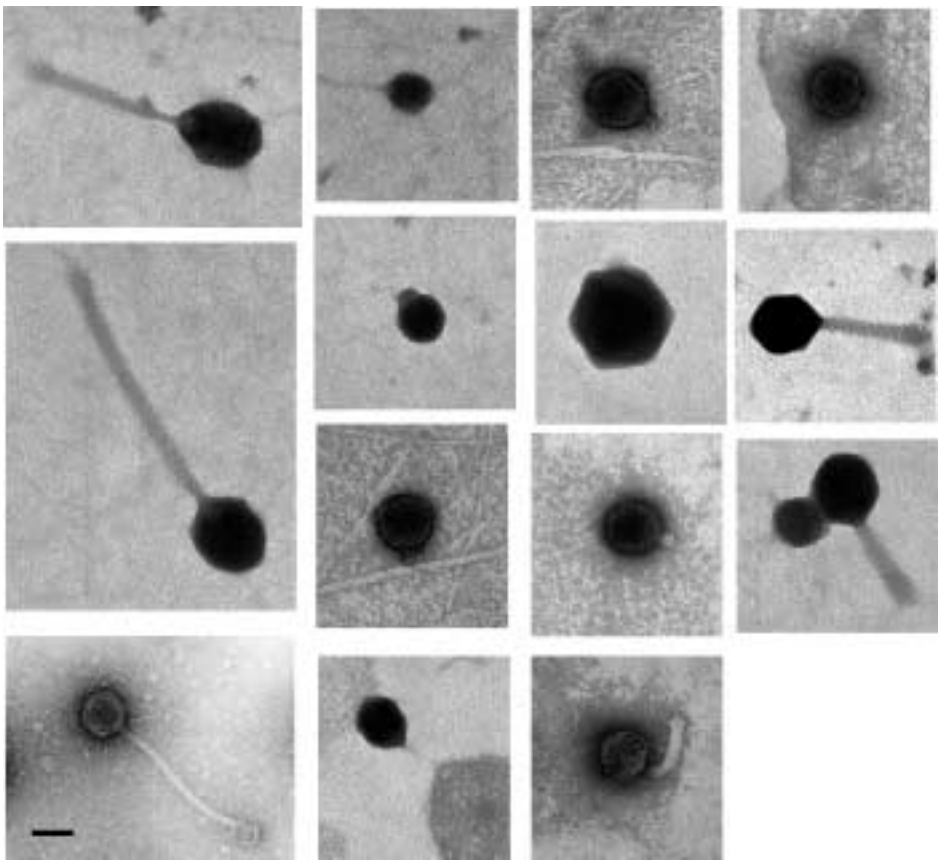

Fig. 4. Natural virioplankton in water samples from Helgoland (German Bight); sampling from April to June 2001 (bar: 80 nm).

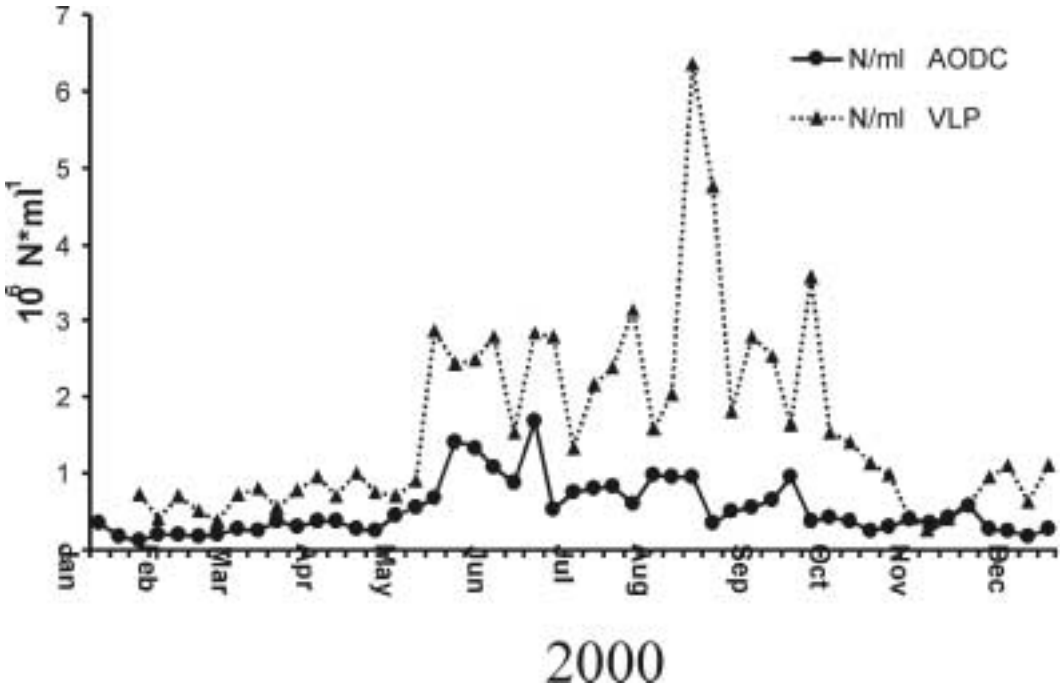

Fig. 5. Bacterioplankton (AODC) and virioplankton (VLP, YOPRO) total counts in 2000; weekly sampling near Helgoland (German Bight).

Dominant bacterial hosts: improvement of cultivation techniques

Recent studies revealed that the isolation techniques used so far (ZoBell medium, marine broth) are highly selective for the isolation of fast growing γ–Proteobacteria (*Pseudalteromonas* sp.). However, results of fluorescence in-situ hybridization (FISH) showed that these *Pseudalteromonas* species do not represent the dominant bacterial groups in the natural marine environment (Eilers *et al.*, 2000). In general, results of the standard cultivation procedures used so far do not reflect the structure of the natural bacterial composition and the majority of planktonic bacteria in the marine habitats detected by FISH, cannot be cultured by using current techniques (Giovannoni and Rappé, 2000). Among these "uncultivated" bacteria are specific phylogenetic clusters of the a Proteobacteria (e.g., SAR11, SAR116, *Roseobacter*), γ–Proteobacteria (e.g. SAR-86) and those of the *Bacteroidetes* group. There are only very few cultures available of the *Roseobacter* group, and one single isolate of the SAR11 cluster (Rappé *et al.*, 2002), which is very difficult to culture. Our culture collections do contain some strains closely related to the mentioned clusters, generated through enrichment cultures. Consequently only very few phages were isolated from these bacterial groups. One example represents the *Roseobacter*-specific podovirus, Roseophage SOI1. This phage is highly host specific. Its genome has been completely sequenced (Rohwer *et al.*, 2000).

Phage diversity: molecular approaches

It is assumed that presently uncultured but *in situ* dominant bacteria will use the same infection mechanisms as other isolates in nature, but the role and importance of lytic and/or virulent life cycles of marine phages still need to be evaluated. For a better understanding of marine phage ecology, natural dominant bacteral groups should be used in future experimental approaches. Additionally the development of new techniques is indispensable. It can be assumed that pulsed field electrophoresis (PFGE) will provide a better resolution of the diversity of marine phage communities. The genome of the total phage population, separated by PFGE, displayed significant variation in size, and showed specific band patterns due to seasonal variations (Wommack *et al.*, 1999a, 1999b; Steward and Azam, 2000). By using a combination of plaque assay and DNA-DNA-hybridization, Wichels *et al.* (2002) demonstrated the existence of a restricted distribution of specific *Pseudoalteromonas* phages for the North Sea (see above). Another promising approach of the analysis of the diversity of natural phage and virus assemblages is the denaturing gradient gel electrophoresis (DGGE) of PCR fragments. While Short and Suttle (1999) demonstrated the diversity of natural phytoplankton-viruses by DGGE from PCR-amplified viral DNA-polymerase gene fragments, there is nearly no information for marine bacteriophage diversity. Only Rohwer *et al.* (2000) showed first results of sequence homologies among DNA-polymerases genes of phages of different habitats.

Classical virology and molecular approaches

Besides the elucidation of phage diversity in marine habitats, quantification of specific groups of phages is of high interest to estimate their influence on bacterial populations in nature. Plaque tests on their own are insufficient, since this method does not differentiate between specific phage groups. Only a detailed analysis of all plaques will generate quantitative data about specific phages. An option would be the time consuming DNA-DNA-hybridization or as an excellent alternative the real time PCR. Quantitative approaches by real time PCR in detecting specific virus particles have been successfully conducted in clinical virology (Aberham *et al.*, 2001; Japs *et al.*, 2001; Najioullah *et al.*, 2001), but have not been used so far in phage ecology.

The powerful techniques (PFGE, DGGE) discussed above, including the development of specific primers and probes for dye detection of phage genes (quantitative PCR), will broaden our insight of phage ecology and of the interactions between phages and their hosts. This includes both, the quantitative assessment of phages and their hosts and additionally the qualitative impact of specific phages on the composition and dynamics of the bacterioplankton. These new technologies will provide exciting information on the potential transfer of functional genes between phages and bacteria.

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