

Pseudoalteromonas spp. phages, a significant group of marine bacteriophages in the North Sea

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ABSTRACT: The occurrence and distribution of specific bacteriophages of marine *Pseudoalteromonas* spp. in the North Sea (*North Sea phages*) and their genetic relationship to several previously isolated marine phage species from waters of the Helgoland Roads (German Bight, *Helgoland phages*) were investigated. During 3 cruises from the Elbe estuary to western Norwegian waters, phages were concentrated by ultrafiltration. Detection and isolation of *North Sea phages* were performed by plaque assay, with 70 host bacteria of the genus *Pseudoalteromonas*. The genetic relationship between *North Sea phages* from different stations and *Helgoland phages*, formerly described as *Pseudoalteromonas* spp. phages, was assessed by DNA-DNA hybridization. DNA probes were prepared using whole phage DNA derived from 13 *Helgoland phages*. This approach provides the first information on the distribution of specific *Pseudoalteromonas* spp. phage-host systems (PHS) in the North Sea. The occurrence of *Pseudoalteromonas* spp. phages, which are specific for the tested *Pseudoalteromonas* spp. host bacteria, was restricted to a narrow geographical region of the German Bight between 53° 30' and 57° 00' N latitude. Most of the previously isolated *Helgoland phages* were highly host specific (54%), whereas this was true for only some of the 39 *North Sea phages* (16%). The most common *Pseudoalteromonas* spp. phage species found in the North Sea belong to the virus family *Siphoviridae* (species H103/1). Several phage strains within this phage species displayed different host sensitivity patterns.

KEY WORDS: Marine phages · *Pseudoalteromonas* · Phage DNA probes · North Sea

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INTRODUCTION

The last decade has provided substantial information on the occurrence and abundance of virus-like particles (VLPs), as investigated by electron microscopy and epifluorescence direct counts. It is well established that VLPs are ubiquitously distributed and highly abundant, with concentrations of 10^7 to 10^{10} VLPs l^{-1} in the aquatic environment (Bergh et al. 1989, Fuhrmann & Suttle 1993, Bratbak et al. 1994, Wommack & Colwell 2000). Despite this basic information there are still many open questions concerning their ecological role and functions in the bacterial communities. There is nearly no information on the distribution and abun-

dance of specific natural marine phage-host systems (PHS) and their genetic relationship to other phages in the marine environment. Kellogg et al. (1995) have investigated the genetic diversity and distribution of vibriophages of the host bacterium *Vibrio parahaemolyticus*. The authors presented evidence for a widespread occurrence of genetically related phages in various marine habitats over a geographical distance of some 4500 miles (~7241 km). These *Vibrio* sp. phages were members of the virus family *Myoviridae*, which were highly host specific. In contrast, by comparing host sensitivity patterns of marine phages isolated at different locations (i.e. west and east off the Azores), Moebus & Nattkemper (1981) found PHS that were geographically restricted to different regions.

In the present report we describe the occurrence and geographical distribution of specific marine phage

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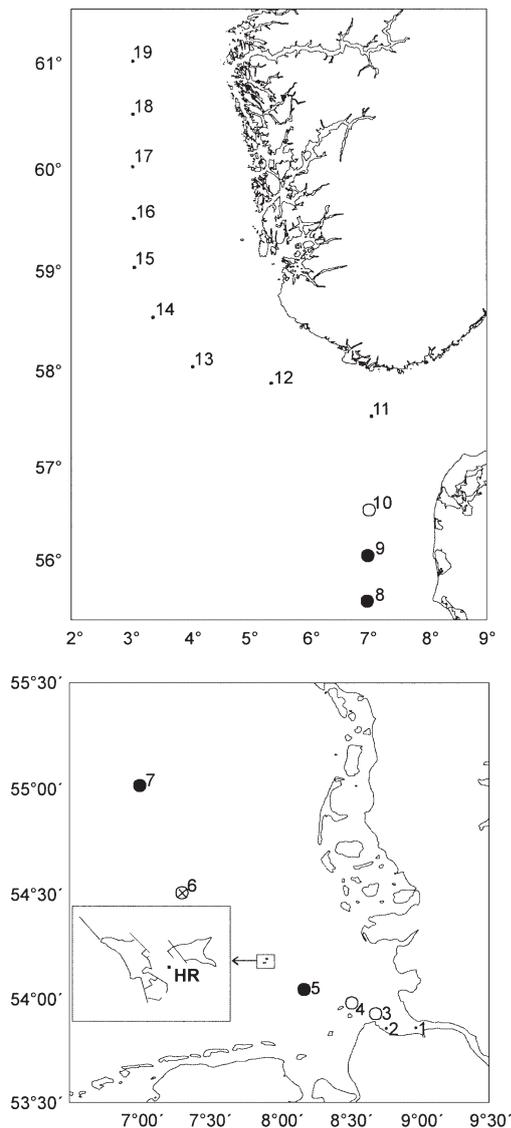


Fig. 1. Sampling area of the RV 'Heincke' Cruises HE49 (September 1993), 54 and 65 (April and October 1994) in the North Sea (German Bight to Norwegian waters). Stn Helgoland Roads (HR) is the origin of 70 phage-host systems used in this study (Moebus 1992a,b). Circles represent the occurrence of *Pseudoalteromonas* phages, as determined by plaque assay, at each station (●: 1993; ○: 1994; ⊗: both years)

species infecting *Pseudoalteromonas* spp. in the North Sea. Our previous work on DNA homologies among 22 different marine phages, as determined by DNA-DNA hybridization, showed that the whole DNA of each phage was specific for only 1 phage species (Wichels et al. 1998). These results suggest that phage DNA can be used as specific DNA probes in order to identify single phage species. Data elucidating the genetic relatedness and host range of *Helgoland phages* and *North Sea phages* are presented.

MATERIALS AND METHODS

Water samples were collected during 3 cruises to the North Sea. Samples were tested for plaque formation on 70 bacterial isolates belonging to the genus *Pseudoalteromonas*. After isolation and purification of the new *Pseudoalteromonas* spp. phages, they were characterized by DNA hybridization and host range cross-reaction test, in order to assign them to a virus family and a phage species.

Bacterial strains, phages and media. Seventy marine bacterial host strains and 13 lytic phages were provided by Moebus (1992a,b). They were isolated from water samples collected at Helgoland Roads (HR; 54° 09' N, 7° 52' E; Fig. 1), which is located approximately 55 km offshore in the German Bight. In order to characterize the 70 host bacteria, colony hybridization was performed under stringent conditions with group-specific 5' labeled digoxigenine DNA probes (EUB 338, GAM 42a, ALT 1413, PSA 184; Table 1; Eilers et al. 2000).

Thirteen PHS from this Moebus collection were chosen randomly to investigate the occurrence of specific PHS in the North Sea. The phages had been characterized previously by electron microscopy, DNA-DNA hybridization and host range (Wichels et al. 1998). They represent 3 virus families within the order *Caudovirales* (tailed bacteriophages): 6 *Myoviridae*, 4 *Siphoviridae* and 3 *Podoviridae*. Each phage lyses at least 1 of the 70 bacterial strains tested. According to their host range, they were assigned to host sensitivity groups (SGs) as follows: SGI, highly host specific (1 host); SGII, broad host range (2 to 10 host bacteria); and SGIII, broadest host range (11 to 36 host bacteria) (Table 2). These phages are now termed *Helgoland phages*.

Sampling of seawater. For the isolation of marine phages during 3 North Sea cruises with RV 'Heincke' in September 1993 (Cruise HE49), in April (Cruise HE54) and October 1994 (Cruise HE65), water samples were collected at 19 stations on a transect from the Elbe estuary to west Norwegian coastal waters (Fig. 1). At each of the stations, 100 l of water was pumped from 1 m depth through a pre-filter cascade (100, 50 and 10 µm gauze filters) followed by 3 µm filtration (cellulose nitrate). Water was concentrated by using an ultrafiltration system (Amicon hollowfiber cartridge, molecular cut off 100 000 Da) as described by Suttle et al. (1991). The final volume of the phage-containing samples was ca. 100 ml (i.e. concentration factor 1000). In order to remove bacteria, they were filtered through 0.15 µm cellulose nitrate filters. In avoiding phage adsorption, filters were pre-washed with sterile 3% beef-extract solution. Phage-containing samples were stored at 4°C until use. Filtration units were sterilized between samplings.

Plaque detection and isolation of phages.

For the detection of plaques, 70 bacterial host strains were used. Double layer agar plates (10 ml bottom layer, 3 ml soft agar upper layer) were prepared as described by Wichels et al. (1998). Phage-containing samples obtained from the 3 North Sea cruises were tested for plaque formation. All 70 bacterial host strains were used as targets. The soft agar was mixed with ca. 10^8 bacteria (liquid culture). Immediately after solidification each phage-containing sample was dotted (10 μ l) onto the upper layer with a multi-inoculator (Moebus 1980). Plaque formation was determined after incubation overnight at 18°C in the dark. If no plaques were formed, 1 ml of each phage-containing sample was plated onto a second set of double layer agar plates. For isolation and purification of single plaques, dilution series were prepared from positive phage-containing samples. Plaques were examined after incubation (for conditions, see above) and isolated. Purified phage stocks (lysates) were prepared according to Moebus (1980). We will further refer to these phages isolated from water samples taken during the cruises as *North Sea phages*. For phage-host cross-reaction tests, double layer agar plates were prepared. Phage stocks of the *North Sea phages* were dotted in a dilution series of 10^0 to 10^{-6} onto the upper layer. This was essential for differentiation between clear lysis reaction (plaque formation) and inhibition of the bacterial lawn. Depending on the number of lysed bacterial isolates, phages were assigned to host SGs (Wichels et al. 1998).

Isolation of phage DNA. For each of the isolated *North Sea phages*, stock solution lysates of 20 ml were prepared (Moebus 1980). DNA was isolated according to Sambrook et al. (1989) and by omitting the chloroform step of that method.

Phage DNA probes and DNA-DNA hybridization. Phage DNA probes of the 13 *Helgoland phages* (Table 2) were made (Wichels et al. 1998). For dot blot hybridization, whole phage DNA of each of the *North Sea phages* (5 to 10 μ l) was dotted onto nylon membranes (Hybond N, Amersham) and immobilized by UV light ($\lambda_{312\text{nm}}$, 7 min). DNA-DNA hybridization was carried out under stringent conditions (68°C for 16 h). Hybridization was detected according the manufacturer's protocol (Boehringer Mannheim). Positive hybridization signals occurred after 1 to 4 h of incubation.

Table 1. Group-specific 16S rDNA probes for the phylogenetic analysis of host bacteria

DNA probe	Sequence	Source
EUB 338	5'-AGA GTT TGA TCM TGG CTC AG	Amann et al. (1990)
GAM 42a	5'-AAC AGG ATT AGA TAC CCT GG	Manz et al. (1992)
ALT 1413	5'-TTT GCA TCC CAC TCC CAT	Eilers et al. (2000)
PSA 184	5'-CCC CTT TGG TCC GTA GAC	Eilers et al. (2000)

Table 2. Phage species used as species-specific DNA probes for DNA-DNA hybridization. SGI: highly host specific (1 host); SGII: broad host range (2 to 10 host bacteria); SGIII: broadest host range (11 to 36 host bacteria)

Phage species	Virus family	Number of host strains infected ^a	Host sensitivity group (SG)
H7/2	Myoviridae	23	III
H106/1		1	I
H114/2		1	I
6-8a		1	I
6-42c		18	III
12-13a		1	I
H103/1	Siphoviridae	3	II
H118/1		7	II
10-77a		3	II
11-68c		4	II
H71/2	Podoviridae	1	I
H100/1		1	I
10-33b		1	I

^a70 *Pseudoalteromonas* spp. were tested

RESULTS

Occurrence of plaque-forming units in North Sea water samples

Water samples taken during 3 cruises with RV 'Heincke' in 1993 and 1994 (Fig. 1) and were tested for plaque formation with 70 bacterial strains. The characterization of these bacteria, carried out by colony hybridization with group-specific digoxigenine labeled DNA probes, showed that all 70 bacterial host strains belong to the genus *Pseudoalteromonas* (data not shown).

In water samples taken in September 1993 (cruise HE49), plaque formation was observed at 4 stations (Stns 3, 4, 6 and 10) in the German Bight and in Danish coastal waters (Fig. 1, Table 3). In total, 13 plaque reactions occurred with at least 1 of the 70 bacterial host strains tested. Twelve new phages were isolated; 1 phage was lost during the isolation procedure.

In water samples taken in October 1994 (Cruise HE65), plaque formation was detected at 5 stations (Stns 5 to 9) (Fig. 1, Table 3). In total, 33 plaque reac-

tions were observed with at least 1 of the 70 bacterial isolates tested. Most plaque reactions were detected at Stn 6 near Helgoland.

During Cruises HE49 and HE65, no plaques were detected in samples collected from Norwegian waters (HE49: Stns 11 to 19 and HE65: Stns 10 to 19). This was

Table 3. Comparison of 45 *North Sea phages* (Cruises HE49 and HE65) by DNA-DNA hybridization. Phages were isolated from seawater samples of RV 'Heincke' Cruises HE49 and HE65 (September 1993 and October 1994). SG: sensitivity group; SGI: highly host specific (1 host); SGII: broad host range (2 to 10 host bacteria); SGIII: broadest host range (11 to 36 host bacteria); ●: positive hybridization signal

Stn	Virus family		Host SG	<i>Helgoland phages</i>												
	Host	Probe phage		Myoviridae					Siphoviridae				Podoviridae			
				H7/2	H106/1	H114/2	6-8a	6-42c	12-13a	H103/1	H118/1	10-77a	11-68c	H71/2	H100/1	10-33b
HE 49																
3	13-15	FI/15	II													●
4	H100	No lysatec ^a	II													
4	H114	FI/3	II						●							
4	H114	FI/12	II													●
4	H103	FI/4	II							●						
4	H103	FI/5	II							●						
4	H105	FI/6	II							●						
4	H105	FI/7	II					●								
4	10-94	FI/8	II													●
4	10-94	FI/13 ^b	II													●
4	13-15	FI/14 ^b	II													●
6	H114	FI/2	II													●
10	H114	FI/1	II													●
HE 65																
5	H106	FIII/8	I		●				●							
5	M109	FIII/33	II													●
6	12-4	FIII/28	III													●
6	1-23	FIII/14	I													
6	10-103	FIII/26	I													●
6	10-52	FIII/23	III													●
6	10-94	FIII/25	III													●
6	11-68	FIII/27	III													●
6	13-15	FIII/30	III													●
6	5-34	FIII/16	III													●
6	5-68	FIII/17	III													●
6	6-42	FIII/18	III													●
6	6-62	FIII/19	III													●
6	6-92	FIII/20	III													●
6	H118	FIII/11	III													●
6	H74	FIII/31	III													
6	M109	FIII/32	III													
7	5-34	FIII/15	III													●
7	H118	FIII/10	II													●
7	H137	FIII/12	III													●
7	H139	FIII/13	II													●
7	H71	FIII/1	I													
8	9-12	FIII/22	I													●
9	9-12	FIII/21	II													●
9	10-77	FIII/24	I													●
9	12-13	FIII/29	II													●
9	H100	FIII/2	II													●
9	H101	FIII/3	II													●
9	H102	FIII/4	II													●
9	H103	FIII/5 ^b	II													●
9	H105	FIII/6 ^b	II													●
9	H106	FIII/7	II													●
9	H108	FIII/9 ^b	II													●

^aPhage lost during isolation procedure

^bDNA hybridization signal occurred with 2 phage families

Table 4. Host-phage cross-reaction tests of 12 phages (Cruise HE49) with 70 *Pseudoalteromonas* spp. bacterial strains (from Helgoland waters; 1990). Only bacterial strains with positive results are depicted. •: positive cross-reaction; nd: not determined

Stn:	North Sea phages (HE49)											
	3	4	4	4	4	4	4	4	4	4	6	10
Phage:	FI/15	FI/3	FI/4	FI/5	FI/6	FI/7	FI/8	FI/12	FI/13	FI/14	FI/2	FI/1
Genetically related phage species:												
	H118/1	12-13a	H103/1	H103/1	H103/1	H114/2	H118/1	H118/1	nd	nd	12-13a	H118/1
Host bacteria												
H103		•	•	•	•	•		•	•		•	•
H105		•	•	•	•	•	•	•	•		•	•
H108		•	•	•	•	•	•	•	•		•	•
H114		•	•		•			•		•	•	•
10-94	•				•	•	•		•	•		
13-15	•				•	•	•		•	•		

also true for the samples of the Elbe estuary (HE49: Stns 1 and 2, and HE65: Stns 1 to 4). No plaque reactions were observed in samples taken during Cruise HE54 (April 1994). In total, 45 *North Sea phages* were isolated (HE49: 12 phages; HE65: 33 phages).

Genetic relationship of newly isolated phages

Forty-five *North Sea phages* were compared with the 13 *Helgoland phages* by DNA-DNA hybridization (Wichels et al. 1998). Hybridization was performed with whole phage DNA (Table 3). Thirty-nine of the *North Sea phages* showed DNA homology with one of the *Helgoland phages*, which comprise the 3 virus families (Table 2) tested. Accordingly, 19 *North Sea phages* were assigned to the family *Myoviridae* (Cruise HE49: 3; Cruise HE65: 16) and 15 to the family *Siphoviridae* (Cruise HE49: 7; Cruise HE65: 8). Three *North Sea phages* (Cruise HE65) showed DNA homologies with *Podoviridae*. In samples of cruise HE49 no *Podoviridae* were found. Five phages displayed DNA homologies with the DNA of different families (Phages FI/13, FI/14 and FIII/5, FIII6 and FIII9; Table 3). These results are ambiguous, since even fresh phage lysates from single plaques dis-

played the same results. At Stn 6 near Helgoland (Cruise HE65), most hybridization signals occurred with the DNA probe of phage species 6-42c (*Myoviridae*).

By combining both of the techniques—DNA homology studies and host range cross-reaction test—phages of a single species were differentiated into phage strains as follows:

(1) Phages of the same species (i.e. showing DNA homologies) also displaying identical host range were considered to be identical strains of 1 species. For example, Phages FI/2 and FI/3 (Table 3) were assigned to species 12-13a, although they were isolated at different stations (Stns 4 and 6, respectively). Since these 2 phages had the same host ranges (Table 4), they were considered to be identical. Another 3 *North Sea phages* of Cruise HE65 (FIII/25, FIII/26 and FIII/30; Table 3) that showed identical host sensitivity patterns (data not shown) were also considered to be identical. Three phages isolated at Stn 9 (FIII/5, FIII/6 and FIII/9) that showed DNA homologies with 2 virus families (Table 3) had identical host sensitivity patterns and thus were considered to be identical.

(2) Phages of one species that exhibited different host ranges were considered to display different phage strains (e.g. 3 phages of phage species H103/1 isolated from Stn 4: FI/4, FI/5 and FI/6; Table 4). Finally, in total 39 of the 45 *North Sea phages* isolated during Cruises HE49 and HE65 were considered to belong to different phage strains.

Phage-host cross-reaction tests of these 39 different *North Sea phages* showed that the majority of the phages (33 phages) lyse more than one of the bacterial strains (Table 5). Thirteen phages had a very broad host range and were assigned to SGIII (i.e. those that lyse more than 11 of the

Table 5. Comparison of host ranges of the 2 groups of *Pseudoalteromonas* spp. phages investigated: 13 *Helgoland phages* (isolation 1990) and 39 *North Sea phages* (isolation 1993 and 1994). Host ranges of both phage groups were tested on 70 marine bacteria of the genus *Pseudoalteromonas*

Host SG	Helgoland phages (%)	North Sea phages (%)
SGI, highly host specific (1 host)	54	16
SGII, broad host range (2 to 10 hosts)	31	51
SGIII, broadest host range (11 to 36 hosts)	15	33

bacterial strains of all 70 bacteria tested). Twenty phages were assigned to SGII (those that lyse 2 to 10 bacterial strains of all 70 bacteria tested). The 13 *Helgoland phages* showed significant differences in host range from the *North Sea phages* (Table 5). Seven *Helgoland phages* belonged to SGI, whereas only 6 of the 39 *North Sea phages* were highly host specific (SGI). Statistical analysis (Mann-Whitney rank sum test, SigmaStat, version 1.0) revealed that the 2 groups are significantly different ($T = 237.5$, $p < 0.0244$).

DISCUSSION

The mean concentration of VLPs in the aquatic environment ranges between 10^7 and 10^{11} VLPs l^{-1} (Bergh et al. 1989, Cochlan et al. 1993, Wommack & Colwell 2000). However, these marine VLPs are still considered a 'black box'. It is not clear which groups of viruses are present in the natural environment (bacteriophages and viruses of phyto- or zooplankton) and how they are distributed. Furthermore, it is unknown whether these particles are infectious and which bacterial groups can be infected. In comparison to VLP counts, the determination of phage titer (plaque assays) is an additional sensitive tool for the detection of bacteriophages. However, the detection and isolation of specific phages by plaque assays is limited since bacterial hosts have to be available for this. On the other hand, plaque assays are limited, as they do not provide further information on the phages (e.g. morphological or genetic affiliation). *Pseudoalteromonas* spp. phages representing all 3 virus families show a very diverse morphology with regard to DNA composition and host range. On the basis of a species concept for bacteriophages (Ackermann et al. 1992), Wichels et al. (1998) showed that the whole phage DNA can be used as a species-specific DNA probe. Their studies on the DNA homologies of the marine *Pseudoalteromonas* spp. phages demonstrated that positive hybridization signals were detected exclusively among phages of the same virus family. In the present study this concept was used to investigate the occurrence of *Pseudoalteromonas* spp. phages in the North Sea by DNA-DNA hybridization using specific phage DNA probes. However, from 45 isolated phages, 85% were assigned to marine phage species already described. From their DNA homology, 27 phages were assigned to individual phage species. In contrast to the species concept of Ackermann et al. (1992), 5 phages showed DNA homologies with 2 virus families. This may be due to DNA fragments, which can be transferred to different phage species via double infection with 2 phages and homologue recombination (Botstein 1980, Gibbs 1987). Double infection of a bacterial host by different phages and homologue

recombination or incorrect excision and packaging of the phage DNA may lead to the transfer of DNA fragments of different origin. This is discussed as a fundamental idea for a model of modular evolution for viruses (Kim & Davidson 1974, Mise 1976, Schwarz et al. 1983, Gibbs 1987, Jarvis 1995).

The *North Sea phages* as detected by plaque assays occurred in water samples of the German Bight between $53^{\circ}30'$ and $57^{\circ}00'$ N latitude. The *Pseudoalteromonas* spp. host strains were isolated near Helgoland. Since phages depend on the presence of their specific host bacteria, these results may indicate the distribution limits of the *Pseudoalteromonas* spp. host strains. One of the limiting factors may be the low salinity in the river Elbe and its estuary, with 15 PSU at low and 20 PSU at high tide. Salinity in the German Bight ranges between 29 and 36 PSU. In regions north of $57^{\circ}00'$ N latitude it can be assumed that the influence of the Atlantic and the Skagerrak may function as a natural barrier causing the limiting geographical PHS distribution.

At present no information exists concerning the distribution patterns of specific bacteria in the North Sea. Hence, until now there has also been insufficient information concerning the geographical distribution of phage species. The most 'common' phage species found in this study was *Siphovirus* H103/1. This species was detected in samples of Cruises HE49 and HE65 at 4 different stations (Stns 4, 7, 8 and 9). Remarkably, the different strains of *Siphovirus* H103/1 displayed various host sensitivity patterns representing all 3 host SGs. Another common phage species, *Myovirus* 6-42c, was detected at only a single station. Within this species, strains of different SGs were also present. These data underscore the high diversity of *Pseudoalteromonas* spp. phages on a high phylogenetic level. Moebus (1983), who isolated bacteria from the Bay of Biscay and the Atlantic, observed the restricted distribution of specific PHS indirectly by comparison of phage-host cross-reaction tests. Kellogg et al. (1995) investigated the distribution of genetically related vibriophages, which lyse *Vibrio parahaemolyticus*. These phages were found over great geographical distances of ca. 4500 miles (~7241 km) in the Atlantic and Pacific Oceans. However, our data provide clues for the varying distribution of different *Pseudoalteromonas* spp. phage species in space and time. Some of the specific phage species were detected in a narrow region between $53^{\circ}30'$ and $57^{\circ}00'$ N latitude. Other phage species were found at only a single station. Some phage species occurred over an extended period of time (1993 and 1994); others were detected only once. Wommack et al. (1999) investigated the community structure of viroplankton in Chesapeake Bay. These authors also found temporal

changes of virus populations by using the pulsed field gel electrophoresis (PFGE) fingerprint technique. PFGE analysis of natural complex viroplankton allows the separation of individual virus strains according to their genome size.

Statistical analysis of host ranges revealed that the *Helgoland* phages, which are highly host specific, were significantly different from the *North Sea* phages, with broader host ranges. These differences may be a consequence of an isolation strategy. The *Helgoland* phages were isolated using enrichment cultures (Moebus 1980), while the *North Sea* phages were isolated after enrichment of VLPs by ultrafiltration. On the other hand, the *North Sea* phages, which exhibit a broader host range, might be 'host range mutants' of the original phage species. The *North Sea* phages were isolated in 1993 and 1994, and the genetically related *Helgoland* phages were isolated in 1990 in the same general geographical area. Lenski (1987) showed that phage mutants may acquire the potential of an extended host range. The extended host range can improve the ability of propagation and thus may be interpreted as a survival strategy.

The *North Sea* phages isolated during Cruises HE49 and HE65 are genetically related to the *Helgoland* phages. Since these *Pseudoalteromonas* spp. phages were found frequently within several years in a narrow geographical region of the North Sea, it can be assumed that this group constitutes a significant and stable phage population in this area, but changes in phage sensitivity and host range frequently occur. Hence, further investigation is needed to elucidate by which quantitative and qualitative means *Pseudoalteromonas* spp. phages control their host bacteria.

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