Abundance of T4-type bacteriophages in municipal wastewater and sewage

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Several different sources of environmental water were analysed for isolation of bacteriophages with T4-type morphology. We found the highest abundance of coliphages in wastewater and sewage. An interesting plating profile of the samples was detected: the optimal plaque-forming temperature was about 37 °C for the municipal wastewater sample and around 30 °C for local sewage. Electron microscopy of sewage phage stocks resembled the virion morphology of the family *Myoviridae*, A2. SDS-PAGE of virion proteins confirmed a close phylogenetic relationship of the selected viruses to the T4-type bacteriophages. Positive PCR amplification signals using phage T4 gene *regB* (translational regulation) and gene 26 (baseplate morphogenesis) specific primers were obtained for 2 of 15 phage isolates tested.

Key words: T4-type bacteriophages, plating profile, virion proteins, electron microscopy, polymerase chain reaction (PCR)

INTRODUCTION

Recent estimates for the number of prokaryotic species range from 10^5 to 10^7 with the total amount of cells being $4–6 \times 10^{30}$ (Brüssow and Hendrix, 2002; Whitman et al., 1998). Large population size and rapid growth of microorganisms provide an enormous capacity of genetic diversity. Bacteriophages are important players in aquatic ecosystems. They often exceed bacterial abundance by one order of magnitude, being probably the most abundant biological entities on the planet (Hennes and Simon, 1995). Until recently, remarkably little has been known about phage diversity, because most research was focused on only a few laboratory isolates. At present, over 5000 bacteriophages have been studied by electron microscopy and can be attributed to 11 virus families (Ackermann and Krish, 1997). About 150 of them have the virion morphology that generally resembles phage T4, one of the best-characterized bacterial viruse (Tétart et al., 2001). Comparative analysis of T4-related phages is now being used to gain insight into both the evolutionary origins and interrelationships of these phage genes and the functions of their protein products. The genomes of T4-type phages isolated from Tbilisi hospitals, Long Island sewage, the Denver zoo show basic similarity, but have substantial insertions and deletions, showing horizontal gene transfer as a major factor in the evolution (Tétart et al., 1998). Knowledge of the diversity and ecology of tailed phages in natural ecosystems may contribute to the development of better methods for using bacteriophages as indicators of virological fecal pollutions in waters. It may well be that the phages of yet unknown morphotypes exist in nature.

The aim of our present work was to make an initial investigation of the abundance of T4-like bacteriophages in aquatic environments of Lithuania and isolation of a set of new phages that may contribute to a more complete understanding of the principles governing bacteriophage morphogenesis and diversity.

MATERIALS AND METHODS

Collection of environmental samples. Samples were taken at different sites: a pond from Šilutė district on July 16, 2000; a water reservoir from Plunge district on July 23, 2000; Naujasodis pond from Radviliškis district on November 23, 2000; slough near Alksniupiai, Radviliškis district on the same day; pond at Alksniupiai on September 3, 2001; Alksniupiai wastewater treatment plant on November 23, 2000 and on April 1, 2002; sewage from a small private live-stock farm at Alksniupiai on November 23, 2000, September 3, 2001 and April 1, 2002. Half- to hundred-milliliter samples were collected in sterile test tubes or bottles and mixed with the 1/10 volume of chloroform. Processed samples were stored at 4 °C until use.

Bacteriophage techniques. The host *Escherichia coli* B^E strain was routinely used for coliphage detection and multiplication. Phage growth and titrations were carried out using the double-layer agar method described by Adams (1959). Briefly, 0.5 ml of exponential host cells (~4 × 10^8 c.f.u./ml) in H broth (Clowes and Hages, 1968) was mixed with 2.5 ml of 0.6% (w/v) soft agar equilibrated at 46–49 °C, then 0.2 ml of environmental sample (after clearing by centrifugation at 4000 rpm for 20 min) or phage stock was added from appropriate dilution in H broth. The resulting mix was overlain over bottom nutrient agar EHA (1.2% w/v). Following 15 min at room temperature the plates were inverted and incubated at appropriate temperatures. After 18–24 h of development all resulting plaques were enumerated. Rapid screening and preliminary determinations (spot-tests) of the efficiency of plating (e.o.p.) were done by dropping 10 or 20 µl (with 10^2 to 10^4 plaque forming units (p.f.u.)) from appropriate dilutions of phage stocks or picked plaques on the plates poured with host cells and dried for 30 min at 37 °C in advance. Bacteriophage stocks were prepared from lysates made by the double-layer agar method, concentrated by two cycles of differential centrifugation. The resulting pellets were suspended in phosphate buffer (Clowes and Hages, 1968) and stored at 4 °C. The efficiency of plating at each temperature was calculated as a number of the p.f.u. at appropriate temperature/number of p.f.u. at 23 °C.

Electron microscopy. Environmental samples were cleared by low speed centrifugation (3× at 6000 r.p.m. for 10 min) and diluted in deionized water to reduce salt content before subjecting to examination. Phage stocks were diluted to approximately 10^{11} particles per 1 ml in distilled water, 5 µl of the sample were spotted onto a grid, excess liquid was drained with filter paper before staining with two succsesive drops of 2% (pH 4.5) uranyl acetate, dried and examined in a JEM-100S Jeol transmission electron microscope.

SDS-PAGE. The SDS-PAGE system according to Laemmli (1970) was used. Phage particles were suspended in sample buffer, boiled for 5 min, and electrophoresed on 13% (w/v) polyacrylamide gel in the presence of SDS. After migration proteins were visualized by Coomassie brilliant blue staining.

PCR amplification. PCR detection of T4-type specific sequences in environmental coliphage isolates was done essentially as described (Jozwik and Miller, 1994). Briefly, the PCR protocol involved 20 cycles with a program of 60 s for denaturation at 92 °C, 1 min at 10 °C below the calculated melting temperature of the shorter primer for annealing, and 1 min at 72 °C for polymerization. The DNA template for the reaction was denatured phage particles (10^5–10^6) from plaques or stocks heated at 92 °C for 4 min. DNA amplification kits were from Fermentas AB.

RESULTS AND DISCUSSION

Positive for viable coliphage were found samples from Naujasodis pond (~20 p.f.u./ml) and, not surprisingly, the concentrations more than 100× higher in wastewater and sewage samples were detected. Coliphages were not found (the limit of detection of the method employed being ~1 p.f.u./100 ml) in the remaining environmental samples tested on the bacterial host strain *Escherichia coli* B^E at 30 °C. For a more detailed investigation the wastewater and sewage samples were chosen. Electron microscopy of the sewage sample revealed a broad spectrum of bacteriophages and other virus-like particles. These phages morphologically mostly belong to the phage families *Myoviridae* and *Siphoviridae* (Ackermann, 1996), most of them being with isometric heads. Figure 1A illustrates some representatives of virions of the sample examined by electron microscopy. Typical T4-type morphology virus particles were absent on the area of the electron microscopy grid analysed. In order to select T4-type bacteriophages, several representative plaques with readily distinguished morphology were picked from initial plates incubated at 30 °C and purified at least 2 times to ensure colony homogeneity. Fifteen and 18 phage stocks were made from sewage sample and from wastewater, respectively. The phages were designated from VR1 to VR17 (sewage sample isolates) and from VR20 to VR37 (wastewater sample origin). Purified particles of phages VR1, VR2, VR4, VR5, VR6, VR7 and VR8 resembled the virion morphology of the family *Myoviridae*, A2 morphotype, indistinguishable from bacteriophage T4 (Fig. 1B).

Comparison of the structural protein profiles of the isolated phages showed that all of them displayed a similar protein electrophoretic migration profile with the major capsid protein gp23 of bacteriophage T4 (see Fig. 2). Distinct protein patterns in the segments of the gels, corresponding to location of tail fiber proteins, may be seen with bigger total virion protein loads (not shown).

Phage-specific PCR primers designed from published sequences (Gruidl et al., 1988; Nivinskas et al., 1990; Valerie et al., 1986) were used to detect T4 phage homologous DNA in our environmental isolates. The following primers for *regB* region (translational control) and for gene *26* (baseplate morphogenesis) were employed, respectively: 5'-CGATAATCTTCTACCGTAAATGG and 5'-

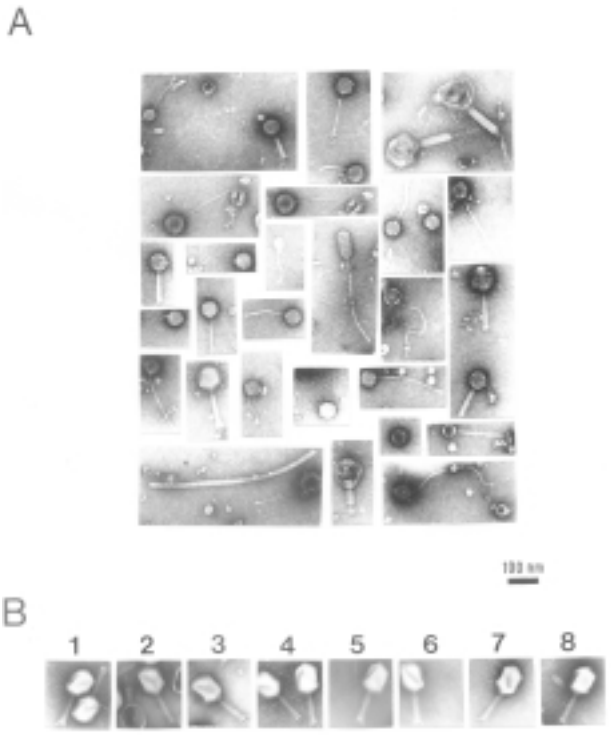

Fig. 1. Transmission electron micrographs of bacteriophages and virus-like particles from cleared sewage sample (panel **A**) and purified bacteriophage virions from sewage sample coliphage stocks and wild-type phage T4 (panel **B**): *1* – phage VR1; *2* – phage VR2; *3* – phage VR4; *4* – phage VR5; *5* – phage VR6; *6* – phage VR7; *7* – phage VR8; *8* – phage T4. Scale bar represents 100 nm

TACTCTATTACTGGAAGCCAGC; 5'-GCTTCGTCGTCACTTTGAGTCGG and 5'-GAAGCCTTTCCTGGTAAACGACG. DNA amplification products of predicted size (~0.4 kb and ~1.0 kb for *regB* and gene *26*, respectively) were detected with 2 of 15 sewage sample isolates – phages VR1 and VR8. Interestingly, only in the case of phage VR1 and VR8 the plaque morphology and the efficiency of plating closely resemble those of wild type bacteriophage T4 (data not shown). Phage VR8 exhibits a high rate of multiplication, at least in the case of the agar layer method, and an unusually high percentage of petite heads of virions as seen by electron microscopy (micrograph not shown).

Unexpectedly, we have determined an optimal temperature of plaque forming for raw wastewater to be about 37 °C and that for sewage sample around 30 °C (see Fig. 3 for details). Express spot-tests of the e.o.p. at different temperatures of the whole set of selected phages in this study (data not shown) showed that only 3 sewage phage isolates (VR1, VR2 and VR8) exhibited their optimum at ~37 °C, while 17 of 18 wastewater isolates preferred a high temperature of incubation for plaque development. Typical representatives of two samples (phage VR7 and phage VR29) were subjected to the

Table. **Efficiency of plating of bacteriophages on *Escherichia coli* B^E**

Phage	Relative efficiency of plating at the following temperatures:			
	23	30	37	44
T4	1.0	1.1	1.1	0.9
VR7	1.0	0.9	$<10^{-6}$	$<10^{-6}$
VR29	1.0	1.8	2.4	1.9

Note. Values are the average of two separate determinations on at least two plates.

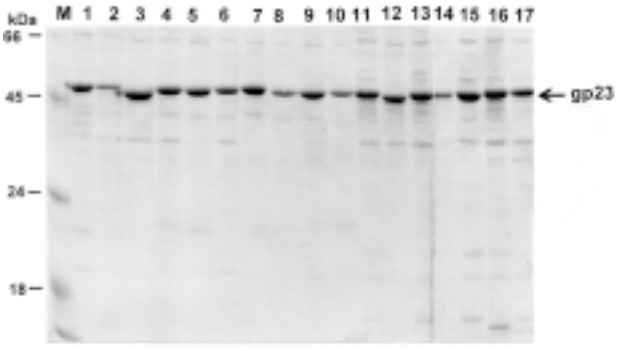

Fig. 2. SDS-PAGE (13%) of intact virions of some phages isolated from sewage (lanes 2 to 11) and wastewater treatment plant isolates (lanes 12 to 17). Lanes: M – molecular mass marker proteins; *1* – phage T4; *2* – phage VR1; *3* – phage VR2; *4* – phage VR4; *5* – phage VR5; *6* – phage VR7; *7* – phage VR10; *8* – phage VR12; *9* – phage VR14; *10* – phage VR16; *11* – phage VR21; *12* – phage VR23; *13* – phage VR24; *14* – phage VR29; *15* – phage VR32; *16* – phage VR33; *17* – phage VR34

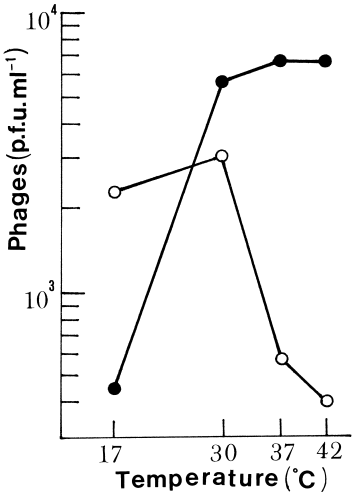

Fig. 3. Dependence of plating efficiency of bacteriophages on incubation temperature of: wastewater (filled circles) and sewage (open circles) samples. *Escherichia coli* B^E strain served as a host

determination of quantitative efficiency of plating (Table). As expected, the optimal temperature of plaque formation was 37 °C for phage VR29. In the case of phage VR7, an unusual temperature-sensitive phenotype was observed (Table). Moreover, temperature sensitivity was detected even from 34 °C in additional spot-tests (data not shown). The unusual temperature sensitivity phenomenon detected may be due to the adaptation of coliphages to a particular range of bacterial hosts. Different temperature optimums in various environmental habitats were reported for coliform bacteria (Leclerc et al., 2000; Leclerc et al., 2001). Thermotrophic bacteria from man and from numerous warm-blooded domestic animals are abundant in raw municipal wastewater, while the properties of the local sewage from a small private live-stock farm are closer to natural environmental habitats with a substantial abundance of psychrotrophic bacteria.

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T4-TIPO BAKTERIOFAGŲ GAUSUMAS KOMUNALINĖSE NUOTĖKOSE IR SRUTOSE

S a n t r a u k a

Atlikta T4 tipo bakteriofagų paieška įvairaus bakterinio užterštumo lygio vandens šaltiniuose Lietuvoje. Didžiausia gyvybingų kolifagų koncentracija nustatyta komunalinėse nuotėkose ir srutose. Nustatyta įdomi šių bakteriofagų mėginių išsėjimo efektyvumo priklausomybė nuo inkubavimo temperatūros: srutų atveju optimumas buvo apie 30°C, o nuotėkų mėginio – maždaug 37°C. Elektroninė mikroskopija parodė, kad dauguma iš srutų išskirtų bakteriofagų priklauso morfologinei *Myoviridae,* A2 šeimai. SDS-PAGE duomenys, įvertinant pagrindinio T4 tipo bakteriofagų kapsidės baltymo gp23 elektroforetinį judrumą, rodo galimą atrinktų fagų filogenetinį artumą. Iš 15 testuotų bakteriofagų 2-ų PGR amplifikacijos signalai, naudojant specifinius T4 fago *regB* geno (transliacijos reguliacija) ir geno *26* (bazinės plokštelės morfogenezė) pradmenis, buvo teigiami.

Raktažodžiai: T4 tipo bakteriofagai, išsėjimo efektyvumas, viriono baltymai, elektroninė mikroskopija, polimerazės grandininė reakcija (PGR)