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Ecology of Bacteriophages Infecting Activated Sludge Bacteria

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Little is known about the endemic bacteriophages of activated sludge. In this investigation 49 virus-host systems were studied by isolating co-occurring bacteria and bacteriophages from the aeration basin of a sewage treatment plant during 5 successive weeks. The phage titers were high and fluctuated during the time period. The occurrence of phage-sensitive and -resistant hosts did not depend on the presence or absence of phages. Several phage-host systems expressed variable plating efficiencies. In addition, phages with broad host ranges were observed. These results show that phages are an active part of this ecosystem and that they may exert selection pressure for phage resistance on their bacterial host populations.

Phages are abundant in natural ecosystems (2, 18, 19, 21). However, our understanding of the ecology of bacteriophages has been largely based on theoretical considerations and laboratory-scale studies of several well-characterized type strains and their phages. The early chemostat studies of *Escherichia coli* and its phages T2, T3, and T4 (9, 16, 17) indicated that a stable equilibrium is reached. The theory emerging from this line of research (reviewed in reference 11) predicts that most of the bacteria are resistant to co-occurring phages, which are maintained by a subdominant phage-sensitive population. Dominant bacterial clones sensitive to co-occurring phages are transient phenomena.

There are difficulties in collecting data on bacteria living in natural environments. (i) It is not possible to cultivate and quantitate all bacteria in any available medium (5, 23). (ii) The bacterial flora of natural ecosystems like activated sludge is so divergent that on a nonselective medium only the most dominant strains can be isolated more than once (8). Thus, the dominance patterns of different bacterial strains or clones are not easy to elucidate. (iii) Studies on growth rates or competitive differences of bacterial clones cannot be carried out in a native environment. Such studies can be carried out in laboratory conditions, but it is not clear whether these results are relevant to the same isolates in natural communities.

As far as we are aware, the bacteriophages of activated sludge have not been studied, except for those that infect influx enterobacteria (for a review, see reference 4). The activation of sludge drastically decreases the numbers of these bacteriophages and their enterobacterial hosts. However, there is no information about the phage-host relations in this ecosystem.

The present study is based on a work (8) in which we classified activated sludge bacteria by analyzing their total cellular protein patterns by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. With this method we can identify and group isolates despite mutations changing their colony morphology, growth requirements, and/or phage sensitivity. The group of bacterial isolates that we call a strain is made up of specimens that are taxonomically very close (7, 8, 10). This allows us to conclude that the observed resistances to phage are due to single or at most a few mutations and not just reflections of general differentiation of host isolates. By using the bacterial collection of our previous work (8), we isolated co-occurring bacteriophages from the aeration basin of a sewage treatment plant for 5 successive weeks. We determined phage densities and efficiencies of plating (EOPs), the occurrence of phage resistance in isolated host bacteria, and the host ranges of phages on these bacteria. The methodology used here allowed us to obtain useful direct information about selected phage-host systems over time in activated sludge.

MATERIALS AND METHODS

Bacteria and phages. The bacterial strains used were described previously (8). The first number in the isolate number refers to the weekly sample from which the isolate originated. The first isolate of each bacterial strain is taken as a type isolate after which the strain is designated. Thus the four similar isolates 3006, 3010, 3063, and 3097 belong to strain 3006. In addition, *E. coli* B and K-12 (3, 6) and bacteriophage φ6 (22) were used in this investigation.

Samples for both bacterial and bacteriophage isolation were taken once a week on Wednesdays at 9:00 a.m. from the aeration basin of Viinikanlahti municipal treatment plant in Tampere, Finland, from 14 September through 12 October 1988. After settling for half an hour, the bacteria were isolated from the dispersed phase as described previously (8).

For phage isolations, 100-ml samples of the disperse phases were frozen at −20°C. When the bacterial strains were available, the samples were thawed and centrifuged 8,000 rpm for 20 min at 4°C (Sorvall SS34 rotor) and filtered through a Minisart NMR 0.2-μm-pore-size (Sartorius) filter. An aliquot was stored at −20°C, and the rest was concentrated 8.5-fold with 10% polyethylene glycol 6000 as described previously (24). The concentrated sample was stored at −20°C.

The phage tests were carried out on 10% TGYA (tryptone-glucose-yeast extract [TGY] [1] containing 15 g of Difco Bacto-Agar per liter) plates with 10% TGY soft agar (1 mM $CaCl_2$ and 7 g of Bacto-Agar per liter of 10% TGY). The presence of phages was tested by applying a 10-μl drop of each of the concentrated samples onto each of 629 bacterial lawns. The plates were incubated at 20°C. When plaques were observed, the unconcentrated phage samples were plated on appropriate hosts. The phages were purified from these plates by two successive single plaque isolations. Then

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all bacterial isolates with protein patterns identical to those of the original host were tested for EOP. The nonidentical bacteria of the same strain cluster that were sensitive to phage were also included in the EOP test. When variations in EOP were detected, the phage was plaque purified, when possible, with the other isolates and the EOP was determined once more on all sensitive host bacteria.

The nomenclature of viruses was derived from the nomenclature of their hosts. For example, a phage originally isolated and plaque purified on host 3010 was designated ϕ3010. Phages from two separate plaques purified on the same host isolate were designated, for example, ϕ5054.1 and ϕ5054.2.

To control the effects of freezing during the sampling procedure, we determined the titers before and after a freeze-thaw cycle for 24 plaque-purified phage isolates (data not shown). The phages capable of plaque formation after the initial freezing and thawing cycle did not show considerable sensitivity to freezing. In the test even the most sensitive isolate (ϕ5030) was inactivated only to one-fourth of its original titer.

Phage particle concentration and purification. Soft agar layers of 30 identical semiconfluent plates were collected and centrifuged at 10,000 rpm for 20 min at 4°C (Sorvall SS34 rotor). The pellet was resuspended in 10 ml of 10% TGY and centrifuged again; the two supernatants were combined. The phages were concentrated 75-fold with polyethylene glycol 6000 and resuspended in 400 μl of 10 mM potassium phosphate (pH 7.2) containing 1 mM $MgCl_2$ (buffer A). The remaining agarose in the sample was reduced by centrifuging in an Eppendorf microcentrifuge (10,000 rpm for 5 min at 4°C). Phages were purified by rate zonal centrifugation in 5 to 20% (wt/vol) sucrose gradient in buffer A (SW41 rotor, 23,000 rpm at 10°C for 25 or 120 min, depending on the phage). Ten fractions were collected, and the phage titers were determined. The highest-titer fractions were diluted twofold, and the phage were pelleted by centrifugation (Ti50 rotor, 32,000 rpm for 2 or 5 h at 5°C). The resulting pellet was resuspended in 50 μl of buffer A. When possible, the phage protein pattern was analyzed by sodium dodecyl sulfate-gel electrophoresis as described previously (15). The molecular sizes of major proteins were estimated by comparing their mobilities with those of the structural proteins of bacteriophage ϕ6, the genes of which have been sequenced (14). These molecular size markers cover the range from 9.5 to 85.0 kDa.

RESULTS

General observations. In the initial screening, in which the phage samples were pipetted on 629 bacterial lawns, we found plaques on isolates belonging to 56 of the 302 strains. The exact titer determinations with soft agar (50°C) succeeded only for 47 of these. This indicates that 16% of the phages were thermosensitive. The plaque morphology was usually clear, which is typical for virulent phages. This investigation is based only on the thermoresistant phages that survived a freeze-thaw cycle.

Density of phages in activated sludge. The total number of phage particles capable of forming visible plaques on the 629 hosts tested with these conditions varied from 3.3×10^4 to 1.7×10^5 PFU/ml in five different samples (Table 1). In the plaque assay 47 different strains, corresponding to about 15% of all strains available, were sensitive to these phages (Table 1). The titers determined for different phages varied from 1×10^1 to 5×10^4 PFU/ml in one sample. The numbers of phages fluctuated considerably during the 5-week period (Table 1).

TABLE 1. Detection of phages in five samples

Host isolate	PFU (10^3) in 1 ml of sample from week:				
	1	2	3	4	5
3002	ND[a]	ND	0.15	0.07	ND
3010	ND	0.13	1.5	0.64	0.29
3038	ND	0.41	24.80	32.80	4.76
3064	ND	ND	ND	0.09	50.00
3085	0.08	2.50	0.39	0.24	0.01
3109	0.02	0.04	ND	0.01	0.10
3122	ND	ND	ND	ND	0.02
3125	ND	ND	0.02	0.02	ND
4001	10[b]	10	10	10	10
4041	ND	0.03	7.52	29.20	1.22
4065	0.01	0.07	0.02	0.01	0.01
4085	0.62	0.13	0.01	ND	ND
4086	0.01	0.01	0.52	0.72	0.07
4091	0.25	0.02	ND	ND	ND
4094	ND	ND	0.05	0.41	0.11
4101	15.00	11.00	20.00	40.00	2.96
5001	0.02	ND	ND	ND	ND
5022	0.02	ND	3.90	20.00	3.40
5030	0.01	0.03	0.43	0.30	0.07
5033	0.88	ND	0.01	ND	0.02
5054	0.01	ND	ND	ND	2.08
5060	ND	ND	0.06	0.09	2.36
5078	ND	ND	0.01	ND	ND
5087	0.13	ND	ND	ND	ND
5098	ND	ND	ND	0.08	ND
5100	ND	ND	ND	0.01	0.95
5105	0.03	ND	ND	ND	ND
6003	ND	0.02	0.01	0.06	2.00
6004	0.08	0.03	0.18	0.86	3.54
6009	0.07	ND	ND	ND	ND
6016	ND	ND	ND	0.15	0.04
6026	ND	ND	ND	0.01	ND
6043	ND	ND	ND	0.05	2.90
6044	0.10	0.11	0.33	0.64	0.23
6057	ND	ND	ND	ND	0.05
6058	ND	0.04	0.01	2.00	0.02
6077	ND	0.01	0.16	0.05	0.07
6115	ND	0.04	0.13	2.00	0.16
7015	ND	ND	0.03	0.03	ND
7022	0.01	ND	ND	ND	ND
7027	ND	ND	ND	ND	0.06
7039	0.02	8.96	1.36	1.78	0.16
7050	2.80	4.32	8.08	3.44	0.95
7051	2.56	1.04	0.67	0.36	0.10
7066	0.76	3.84	2.40	24.00	3.76
7087	ND	ND	0.01	ND	0.02
7104	ND	0.05	0.23	0.13	0.05

[a] ND, not detected (density of virus particles lower than 10 PFU/ml).
[b] Accurate titers on strain 4001 could not be determined due to unclear plaque morphology.

Phage resistance. The occurrence of phage resistance was tested when bacterial strains with isolates sensitive to a particular phage were isolated more than once during the 5-week study (8). The phages infecting these 18 strains formed three categories in respect to their host ranges. The largest group comprised phage isolates that were able to infect only a few of the isolates belonging to the same host strain (12 cases). Six phage isolates infected all of the independent isolates of the host strain. In three cases the phage growth was supported by more than one strain (ϕ4094, ϕ7066, ϕ3122, and similar phages; see below).

TABLE 2. Phages infecting isolates of strain 3006

Phage	Test isolate	EOP
ϕ3010	3006	0
	3010	1
	3063	0
	3097	0
ϕ3063	3006	0
	3010	0.1
	3063	1
	3097	1
ϕ3097	3006	0
	3010	0.1
	3063	1
	3097	1

An important question in understanding the effect of phages on their hosts is to determine the occurrence of resistance and sensitivity in relation to the occurrence of the phage. This was tested with phage-host systems in bacteria isolated from samples that did not have the maximal density of phages infecting other isolates of that strain (55 sensitive and 16 resistant isolates). The occurrence of resistant and sensitive hosts in these samples did not differ from random distribution (χ^2 test).

Observed differences in EOP. Three of the four isolates of strain 3006 supported plaque formation in the primary screening of the activated sludge (isolates 3010, 3063, and 3097). When a plaque on strain 3010 was purified and that phage stock was tested for the infectivity to the other three isolates (Table 2), isolates 3063 and 3097 in addition to 3006 were resistant to purified phage ϕ3010. Starting from the original sterilized activated sludge, plaques were purified on 3063 and 3097. Both of these phages (ϕ3063 and ϕ3097) were able to infect each other's hosts and also to infect strain 3010, but at a lowered EOP. From these results we concluded that two separate phages were isolated, one infecting only host 3010 and the other infecting all of the sensitive hosts but infecting 3010 with a lowered EOP. The purification of virus particles did not succeed for these phages, and it was not possible to verify our conclusion at the molecular level.

Phage ϕ3064 was infective to all six isolates of the host strain. The isolates, however, fell in two categories: the EOPs on 4002, 4036, and 5058 were 6 orders of magnitude lower than those on 3064, 4035, and 4064. The plaques on low-EOP hosts could not be purified to test their EOPs on the higher-EOP isolates; thus the genetic basis for variation in EOP remains unknown. The variations of EOP of phages ϕ4094 and ϕ7087.1, which infect several host strains, are discussed below.

Broad host range. The host ranges of phages were tested by plating them on nonidentical but related hosts that had supported phage growth during the initial screening phase of the study. Phage ϕ4094 infected the isolate 5078 of strain 3112 with an EOP of 10^{-6}. When ϕ4094 was plaque purified on 5078, it gave a stock with a low titer (3×10^6) and infected 4094 with a low EOP (about 10^{-6}). The low infectivity of phage stocks on these isolates when produced in different hosts suggests that particles that infect either host differ by one mutation. Most of the known host range mutations are actually extended host range mutations (11), but this system seems to represent a true host range mutation because the mutant can no longer grow on the original host.

TABLE 3. Phages infecting 3122-like bacteria

Phage	Propagation strain	Test strain	EOP
ϕ3122	3122	3122	1
		7066	1
		7087	1
ϕ7066	7066	3122	0
		7066	1
		7087	0.001
	7087	7066	1
		7087	1
ϕ7087.1	7087	3122	0.5
		7066	0.5
		7087	1
ϕ7087.2	7087	3122	1
		7066	1
		7087	1

A more complicated situation was obtained for strains 3122, 7066, and 7087, which are closely related (8). Phages plating on strain 7066 were found in all five weekly samples, whereas phages plating on strains 3122 and 7087 were found only in one and two samples, respectively (Table 1). ϕ7066 infected strain 7087, although at a lowered EOP (Table 3). This explains the titer dissimilarities on strains 7066 and 7087 in different samples (Table 1). When ϕ7066 was plaque purified on strain 7087, the EOP was the same for both hosts. A possible explanation for this might be a restriction-modification system acting in strain 7087 but not in 7066.

ϕ3122 infected both isolates of the host strain (3122 and 4037) and strains 7066 and 7087. ϕ7087.1 and ϕ7087.2 infected strains 3122 and 7066, although ϕ7087.1 had a 50% reduced EOP on these hosts. In addition, there were plaque morphology and EOP differences between ϕ7087.1 and ϕ7087.2 on Luria-Bertani medium (13): ϕ7087.1 formed small plaques with an EOP that was several orders of magnitude lower than that of ϕ7087.2, which formed large plaques with an almost unaltered EOP on host 7087. The similarity of these four phage isolates infecting the same hosts was tested by using particle sedimentation and protein composition analysis. The fast sedimentation of ϕ7066 indicated that it was unrelated to ϕ3122, ϕ7087.1, and ϕ7087.2. The protein gel analysis of purified ϕ3122, ϕ7087.1, and ϕ7087.2 confirmed them all to be similar slowly sedimenting small phages with major capsid protein of approximately 16 kDa (data not shown). The protein composition and sedimentation behavior of these phages suggest that they are single-stranded RNA-type phages like the MS2 phage of *E. coli*.

Plaque morphology variation. When the phage ϕ4101 was plaque purified on all sensitive isolates of its host strain (4081, 4101, 4102, and 4115), the EOP was the same on all isolates and independent of the host used to propagate the phage. However, the plaque morphology was dependent on both the host used for titer determination and the host used to propagate the phage.

Return of phage. A phage that formed plaques on strain 5054 was obtained with samples from the first and last weeks (Table 1). Phages isolated from both samples infected both isolates of the host strain (5054 and 5055). They also had similar plaque morphologies. The protein gel analysis of purified phage particles showed that these two phage isolates

had identical protein patterns, identifying them as the same phage with a major capsid protein of about 35 kDa (data not shown). Other examples of strains with phages detected twice with a sample lacking detectable phages in between are 3109, 5022, 5033, and 7087 (Table 1). In all of these cases the phage had titers close to our detection limit either before or after its disappearance.

DISCUSSION

The bacteriophages isolated here are undoubtedly reproducing in this ecosystem, which suggests that their hosts are also active. This was verified by determining titers of coliphages, which decline in activated sludge but are known to be frequent in communal sewage (4). The phage titers on two different strains of *E. coli* were 60 PFU/ml at most and in most samples were below our detection limit and thus lower than those of most of the phages studied here.

Fifteen percent of the bacterial strains isolated were sensitive to co-occurring phages. This number should be taken as a minimum estimate, because (i) the sensitivity of the detection is about 12 PFU/ml, (ii) two freeze-thaw cycles are applied to the sample, (iii) some phages adsorb to the sterilization filter, (iv) all phages do not form plaques in the conditions used (this became evident as the plaque morphology of different phages varied from big clear plaques to very tiny ones that could not be counted), and (v), since most strains were isolated once, the phages were not detected when the only isolate belonged to a resistant clone. If we assume that 90% of the phages were not detected (which still might be an underestimate), and if we take into account the estimated total number of strains in activated sludge (several thousands [8]) and suppose that the same proportions of all strains are infected with comparable numbers of viruses, the total number of phages in the dispersed phase of activated sludge would be about 10^6 to 10^8 PFU/ml. This figure is still smaller than the estimate for fresh waters based on electron microscopy (10^8 PFU/ml [2]) and strengthens our assumption that the given number is an underestimate.

The phage infection cycle seems to be very slow in activated sludge. Typically it takes a few weeks to rise from the first emergence to highest phage density (ϕ3038, ϕ3064, ϕ4041, ϕ4086, ϕ5022, ϕ6004, ϕ6058, ϕ6115, ϕ7050, and ϕ7066). The observation of Tan and Reanney (21) that phage infection in enrichment cultures of soil is much slower than in laboratory conditions is in accordance with our results. The time scale of Tan and Reanney is in hours, whereas our observations indicate much slower dynamics. We conclude that in natural ecosystems phage infections are very slow compared with those in laboratory conditions. Since we do not know the exact densities or the generation times of the hosts, it is not possible to speculate whether this reflects slow adsorption kinetics or slow life cycles of the host. Another feature emerging from our data is the slow disappearance of phages (ϕ3038, ϕ3085, ϕ4041, ϕ4085, ϕ4091, ϕ4101, ϕ5022, ϕ5030, ϕ6058, ϕ6115, ϕ7039, ϕ7050, ϕ7051, and ϕ7066), but also the reappearance of phages was detected (ϕ3109, ϕ5022, ϕ5033, ϕ5054, and phages against 7087). In all of these examples the phage had titers close to our detection limit either before or after its disappearance, which makes the interpretation of this result difficult. The phages isolated before and after disappearance were in two cases (ϕ5054 and ϕ7087.1, ϕ7087.2) confirmed to be the same by using purified phage preparations. These observations indicate that the number of phages oscillates or stays for weeks at low densities in this ecosystem. Possible explanations for these phenomena are the circulation of sludge during plant operation and a probable equilibrium of phages between flocculated and dispersed phases, although the mixing of activated sludge is rigorous.

The chemostat studies show that host resistance evolves within a short time as a response to the presence of phage (11, 12). Our results are in accordance with this prediction; (i) phages were found in only 15% of all strains, and (ii) only about 40% of the phage-sensitive bacterial strains that were isolated several times were composed of isolates which were all sensitive to the appropriate phages. It should be pointed out here that, because not all of the phages were detected (see above), the number of sensitive isolates is somewhat underestimated.

The vast microbial diversity of this ecosystem hampered our attempt to study many isolates of each strain in a single sample, but the randomized isolation procedure used (8) allows us to conclude that most (if not all) of the isolates represent dominant or codominant clones and that subdominant clones were not detected. One of the predictions of the theoretical work is that the sensitive hosts should dominate before phage infection and that the resistant forms should dominate after phage infection. However, there was no relation between the occurrence of phage and resistant bacteria. It can be speculated that phage-resistant bacteria have evolved during an earlier oscillation cycle of phage infection. The lack of relation with the occurrence of phages and sensitive hosts indicates either that these bacteria can survive during the infection or that they can grow back to high numbers after the infection. Thus, some of our observations agree with and some contradict the theoretical model.

The observation that the phage isolated from an earlier sample for strain 7087 was less virulent to its host than to a latter isolate is in accordance with the chemostat results of Spanakis and Horne (20), who showed that, as a phage becomes more virulent to its host, the host evolves to become more resistant. In our case, however, it is not possible to exclude the possibility that a later phage has not evolved from the former one but is a separate but very closely related species.

Most of the phages infected only a single strain. However, some phages (ϕ4094, ϕ7066, and ϕ3122 and similar viruses isolated on 7087) were able to infect more than one strain, suggesting that broad-host-range phages are common in natural ecosystems. Thus it can be argued that the classical approach of studying single phage-single host systems is too narrow to obtain a comprehensive understanding of the phage ecology.

We show here that phages are an active part of the activated sludge. Although it is well known that phages are parasites, their effect on host populations is not properly understood. Even less understood is their effect on the activated sludge process itself. This investigation does not indicate that they are strong regulators of species composition. Instead, they seem to modify specific, phage resistance-related, genetic properties of bacteria by exerting selection pressure for phage resistance.

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