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Sewage Coliphages Studied by Electron Microscopy

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Sewage was enriched with 35 *Escherichia coli* strains, and sediments of enrichment cultures were studied in the electron microscope. They contained up to 10 varieties of morphologically different particles. T-even-type phages predominated in 14 samples. Thirteen phages were enriched, representing the families Myoviridae (seven), Styloviridae (two), Podoviridae (three), and Microviridae (one). Twelve of these corresponded to known enterobacterial phage species, namely, 121, K19, FC3-9, O1, 9266, T2, 16-19, χ, β4, N4, T7, and φX174. Cubic RNA phages and filamentous phages were not detected. Types 121 and 9266 have previously been observed only in Romania and South Africa. Identification by morphology is usually simple. Our investigative technique is qualitative and will not detect all phages present. Most enrichment strains are polyvalent, and electron microscopy is always required for phage identification. In a general way, electron microscopy seems to be the method of choice for investigation of phage geography and ecology.

Phages in water were first investigated in 1923 (25) and many times since. They were usually differentiated by host range and plaque type. The electron microscope was rarely used. Three electron microscopical studies, carried out between 1971 and 1980, used the enrichment technique. Before examination, phages were purified by isolation of single plaques. A total of 73 phages was described, namely, 16 phages from seawater enriched with *Vibrio* spp. and other marine bacteria (26, 27), 47 phages of yellow-pigmented enterobacteria from the Garonne River in France (F. Grimont, Ph.D. thesis, Université de Bordeaux II, Bordeaux, France, 1977), and 10 phages of enterobacteria and pseudomonads from a sewage treatment plant (36). In addition, quantitative methods that used filter concentration (45) or sedimentation onto agar blocks and positive staining with uranyl acetate (UA) (18, 19) were developed. Phages were not propagated. These studies are unsatisfactory for various environmental or virological reasons. (i) The isolation of single plaques is a highly selective procedure and cannot detect all phages present. (ii) The quantitative methods do not identify phage hosts. (iii) UA positive staining causes artifacts and makes phage identification difficult (5). (iv) In one study (19), phages were neither depicted nor described. It may be concluded that the potential of the electron microscope for studying phage ecology has not been fully explored.

Naturally occurring coliphages were investigated in several recent studies on water pollution (10, 15–17, 20, 21, 29, 37, 38, 40). Except for RNA phages, which are differentiated by serology, phages were poorly identified, if at all, and the number of indicator strains usually was small. Only one isolate was studied morphologically (15). This is regrettable, because coliphages are widely used experimental models, and knowledge of their ecology is desirable in itself. Classification schemes for enterobacterial phages are now available (2, 3). We wanted to apply these schemes to phage identification. This paper reports electron microscopical observations on 35 enrichment cultures of coliphages, carried out before phage isolation.

MATERIALS AND METHODS

Bacteria and bacteriophages. A total of 35 *Escherichia coli* strains used for enrichment are listed in Table 1. Phage FC3-9 and its host were obtained from M. Regué, Barcelona, Spain. The host, called *Citrobacter intermedius* in the original description of the phage (42), has been reclassified as *Klebsiella pneumoniae* subsp. *aerogenes* (M. Regué, personal communication). Bacteria and phages were propagated at 37°C on

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TABLE 1. Phages and phagelike particles observed

Strain no.	*E. coli* indicator strain[a]	Presence[b] of the following phages:						
		Myoviridae						
		121 (A1)[c]	K19 (A1)	FC3-9 (A1)	O1 (A1)	9266 (A2)	T2 (A2)	16–19 (A3)
1	O20:B84							
2	O26:B6, 1.2		++		*			
3	O26:B6, 1.3		++			+++		
4	O26:B6, 1.4		++		*	+		
5	O44:K74, 37.1					+++		
6	O44:K74, 37.2							
7	O55:B5							
8	O86:B7							
9	O112:B11	*			++		+	
10	O119:B14						+++	
11	O124:B19							+
12	O127:B8, 13.1						+++	
13	O127:B8, 6868						+++	
14	O127:B8, 14208						+	
15	O127:B8, 17036			*			++	
16	O127:B8, 17038						++	
17	O128:B12, 14.1			++			*	
18	O128:B12, 14.2	+					+	
19	O128:B12, 14.3			++			*	+
20	34.1	*	*		++			
21	70.1	+++		*	*		+	
22	72.1							
23	K12S						+++	
24	K12 C-600 (λ)						++	
25	K12 C-600		*		+		+++	
26	K12 58-161						+++	
27	ATCC 13303				+++		+	
28	ATCC 13706						*	
29	ATCC 15597							
30	ATCC 23226				*		+++	
31	ATCC 25922				*		*	
32	Glaxo 1572E						+++	
33	Glaxo 1573E	*		*	*		+++	
34	JC 6310						+++	
35	MRE 600				+		+++	

[a] Origin of strains: nos. 1 to 12 and 17 to 22, local isolates; nos. 13 to 16 and 23 to 26, Pasteur Institute, Paris, France; nos. 27 to 31, American Type Culture Collection, Rockville, Md.; nos. 32 to 33, Glaxo Research Ltd., Greenford, Middlesex, England; no. 34, M. H. Richmond, Department of Bacteriology, University of Bristol, England; no. 35, W. A. Anderson, Department of Biology, Faculty of Science, this university (see reference 13).

[b] +, ++, +++, Variable amounts of particles; *, traces.

[c] Phage species names are given with the morphotype in parentheses (see Fig. 1).

Trypticase soy agar or broth (BBL Microbiology Systems, Cockeysville, Md.).

Enrichment technique. Two sewage samples of 1 liter each were taken in July 1979 at the Central Pumping Station of Quebec City and were centrifuged for 15 min at 3,000 × *g* with a Beckman J2-21 centrifuge and a JA-14 rotor. Erlenmeyer flasks containing 20 ml of double-strength Trypticase soy broth and 20 ml of centrifuged sewage were inoculated with 1 ml of 3-h-old broth cultures of the respective indicator strains. After agitation for 3 h, lysates were filtered through membrane filters of 0.45-μm pore size (Millipore Corp., Bedford, Mass.) and were titrated. Titers usually were from 10^7 to 10^{10} PFU/ml. A few lysates with lower titers were propagated again. For control, 0.2-ml amounts of centrifuged sewage were plated on indicator strains chosen for their apparent specificity (nos. 19, 21, 31, and 33) or polyvalence (no. 2) (Table 1).

Electron microscopy. Lysates and filtered sewage were centrifuged at 72,400 × *g* for 90 min and washed twice in ammonium acetate (0.1 M, pH 7.0), using an International B-60 ultracentrifuge and a SW405 rotor. Sediments were deposited on copper grids with carbon-coated Formvar films, stained with 2% potassium phosphotungstate ([PT], pH 7.2) or 2% UA (pH 4.2), and studied in a Philips EM 300 electron microscope operating at 60 kV. Magnification was monitored with catalase crystals (Polaron Electron Opticals Ltd., London, England) (32).

TABLE 1—*Continued*

Presence[b] of the following phages:						No. of different observations of phagelike particles				
Styloviridae		Podoviridae			Microviridae					
χ (B1)	β4 (B1)	N4 (C1)	T7 (C1)	Esc-7-11 (C3)	φX174 (D1)	A1	B1	B2	C1	C3
			+++							
			*					1		
						2	4		1	
	*			+++						
	+		*							
	+++						2		1	
						1				
						2				
							2			
							1			
	*					2	1			
							1			
*						1				
			*	++		1	1			
						2	2	1		
++										
	+++		*							
							1		1	
			*			1	1			
						1		1		1
						1				
					+++	1	1			
	+++									
		+++				1	2			
			*			1	1		1	

RESULTS

All sediments contained phages and usually were a mixture of morphologically different particles. Centrifuged sewage showed traces of phages with isometric heads and long or short noncontractile tails. Phages in sediments belonged to the four families, Myoviridae, Styloviridae (name not yet approved by the ICTV; the corresponding family has official status [33]), Podoviridae, and Microviridae (33). Most phages were tailed and could be further subdivided into six morphotypes defined by head shape and tail structure (4) (Fig. 1). The number of different particles varied from one sediment to another. One sediment contained 10 types of particles (strain no. 2). Nine sediments showed only one type. These patterns may be summarized as follows, with the number of types of particles given first and the number of strains showing that number of types given in parentheses: 10 (1), 8 (2), 7 (1), 6 (2), 5 (3), 4 (4), 3 (4), 2 (9), 1 (9).

Thirteen phage types were frequently observed and clearly had been propagated (Fig. 2 to 14). Eleven of them corresponded to known enterobacterial phage species, namely, 121, K19, O1, 9266, T2, 16-19, χ, β4, N4, T7, and

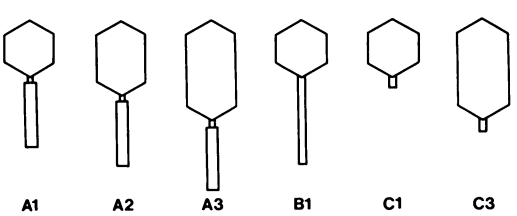

FIG. 1. Observed morphotypes of tailed phages.

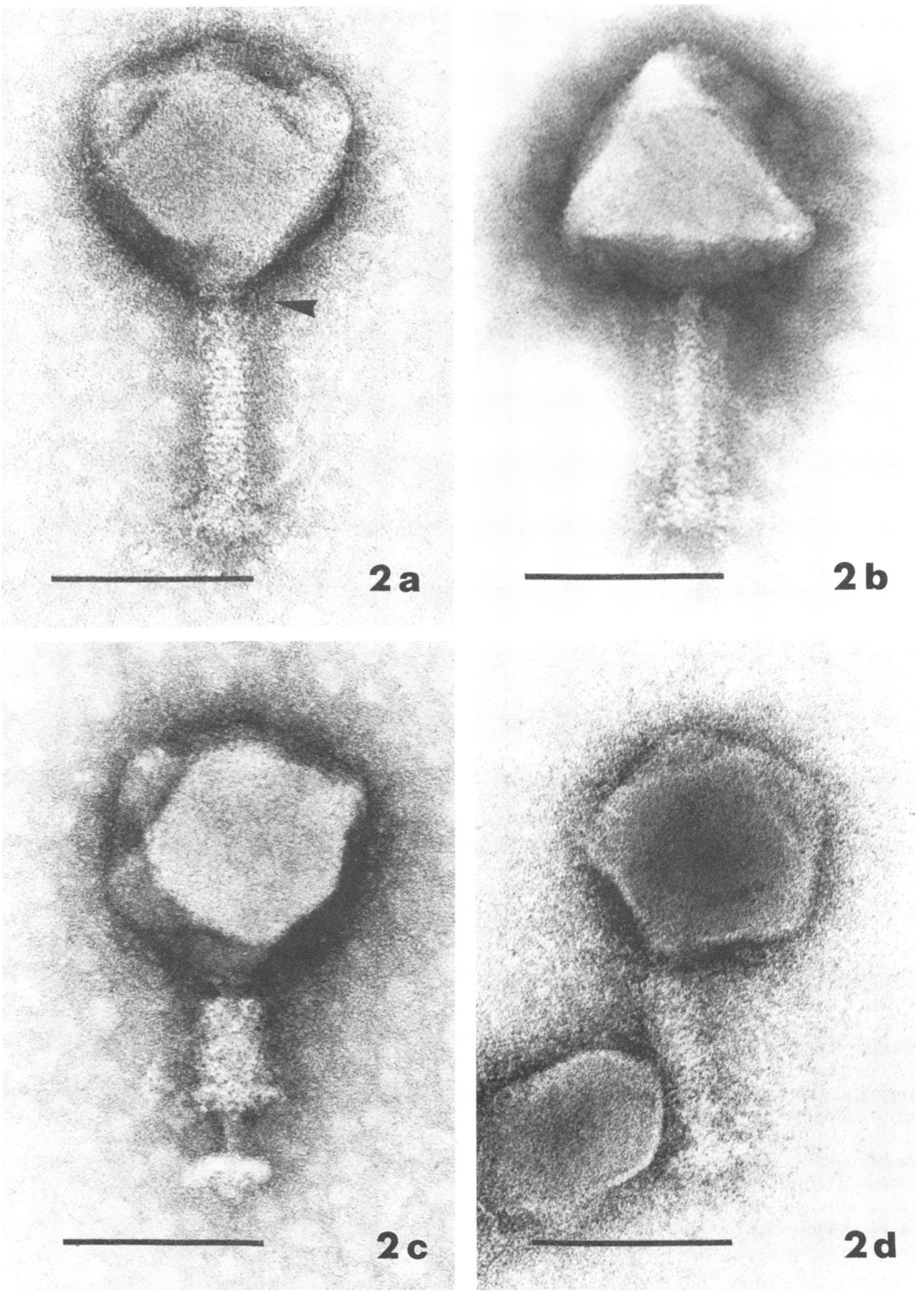

FIG. 2. Type 121. (a) Normal phage with extended tail and collar (arrow); UA. (b) Same after staining with PT; note the evanescent tail sheath. (c) Phage with contracted tail adsorbed to bacterial debris; (d) Phage with pentagonal head; UA.

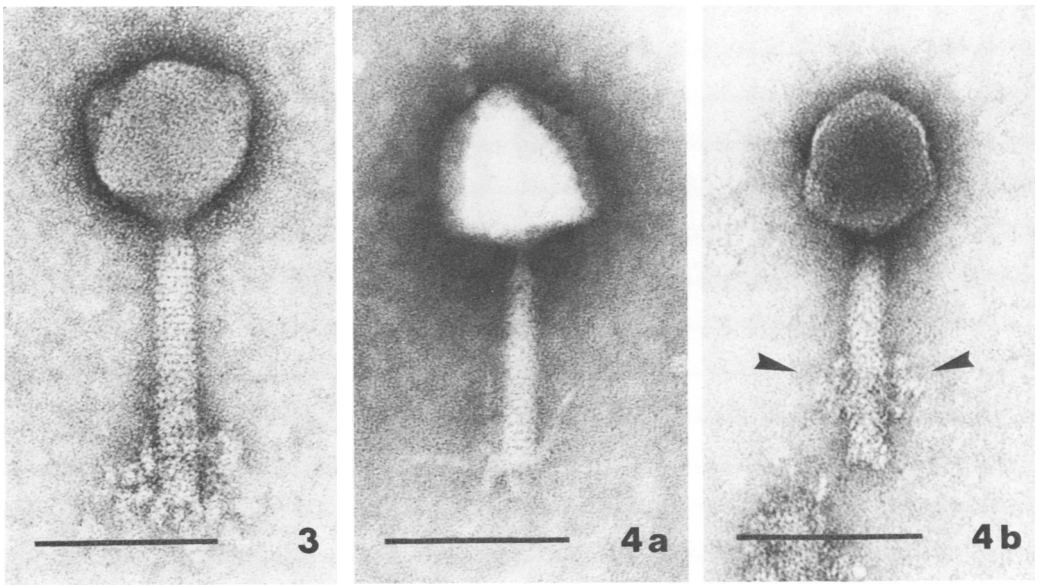

FIG. 3. and 4. (3) Type K19. Phage with extended tail and numerous tail fibers; UA. (4) Type FC3-9. Two particles with extended tails, representing (a) the local isolate with straight tail fibers and (b) the original phage FC3-9 with filaments (arrows) extending from the sheath; PT and UA. The relatively small size of the phage head in (b) is due to UA staining.

φX174 (2, 3). Types FC3-9 and Esc-7-11 were new entities. Subsequently, types 121, FC3-9, 9266, T2, β4, N4, and Esc-7-11 were isolated by three successive subcultures of single plaques. In addition to enriched phages, many sediments contained small numbers of phagelike particles (Fig. 15b to e) which were detected by electron microscopy only and presumably had not been enriched. Observations of phages and phagelike particles are summarized in Table 1. (In Fig. 2 to 15, the bar represents 0.1 μm, and unless otherwise stated, UA-stained phages were negatively stained.)

T2-type phages occurred in 23 sediments and sometimes were the only phages detected. K19-type phages were selectively enriched by strains of serotype O26:B6. Types 9266, 16-19, χ, N4, Esc-7-11, and φX174 were relatively rare. The latter was detected with the strain used for maintenance of the original phage φX174 (American Type Culture Collection Catalogue of Strains I, 15th ed., Rockville, Md., 1982). Cubic RNA phages (Leviviridae) and filamentous phages (Inoviridae) were not observed with certainty. One sediment, of strain no. 12, contained a few flexible filaments which could be either filamentous phages or pili. Some phagelike particles corresponded to known enterobacterial phages, for example, P2 (not shown). Others corresponded to phage KSY1 of *Streptococcus* spp. (43) or 3A of *Staphylococcus* spp. (1) (Fig. 15b and e). Except for particles positively stained with UA (Fig. 6b), phage identification generally was easy.

Main dimensions of types 121 to φX174 are reported in Table 2. Types 121, FC3-9, 9266, and Esc-7-11 were further investigated, because they represented new or insufficiently known phages. Type 121 has been observed in Romania only (35), and the original isolate is no longer available (N. Năcesco, Bucharest, personal communication). Type FC3-9 differed from other enterobacterial phages and was thought to represent a new species until we learned that a similar phage had been isolated in Spain (42). Type 9266 is known from South Africa (41) and perhaps Japan (36). The original phage 9266 is no longer available (J. N. Coetzee, Pretoria, personal communication). Types 121 and 9266 are kept in our collection as neotypes. Phage Esc-7-11 represents a new species and has been described elsewhere (6). Minor morphological parameters of types 121, FC3-9, and 9266, usually measured on a few privileged particles, are reported in Table 3.

Type 121 (Fig. 2) is one of the largest enterobacterial phages known (2). Phage heads show hexagonal or pentagonal outlines, indicating that the capsid is an icosahedron. The tail is contractile and has a neck with a tiny collar, about 27 transverse striations, a baseplate, and at least two short fibers. PT-stained tails are curiously thin and seem to be surrounded by a fibrous network, suggesting that the tail sheath is dam-

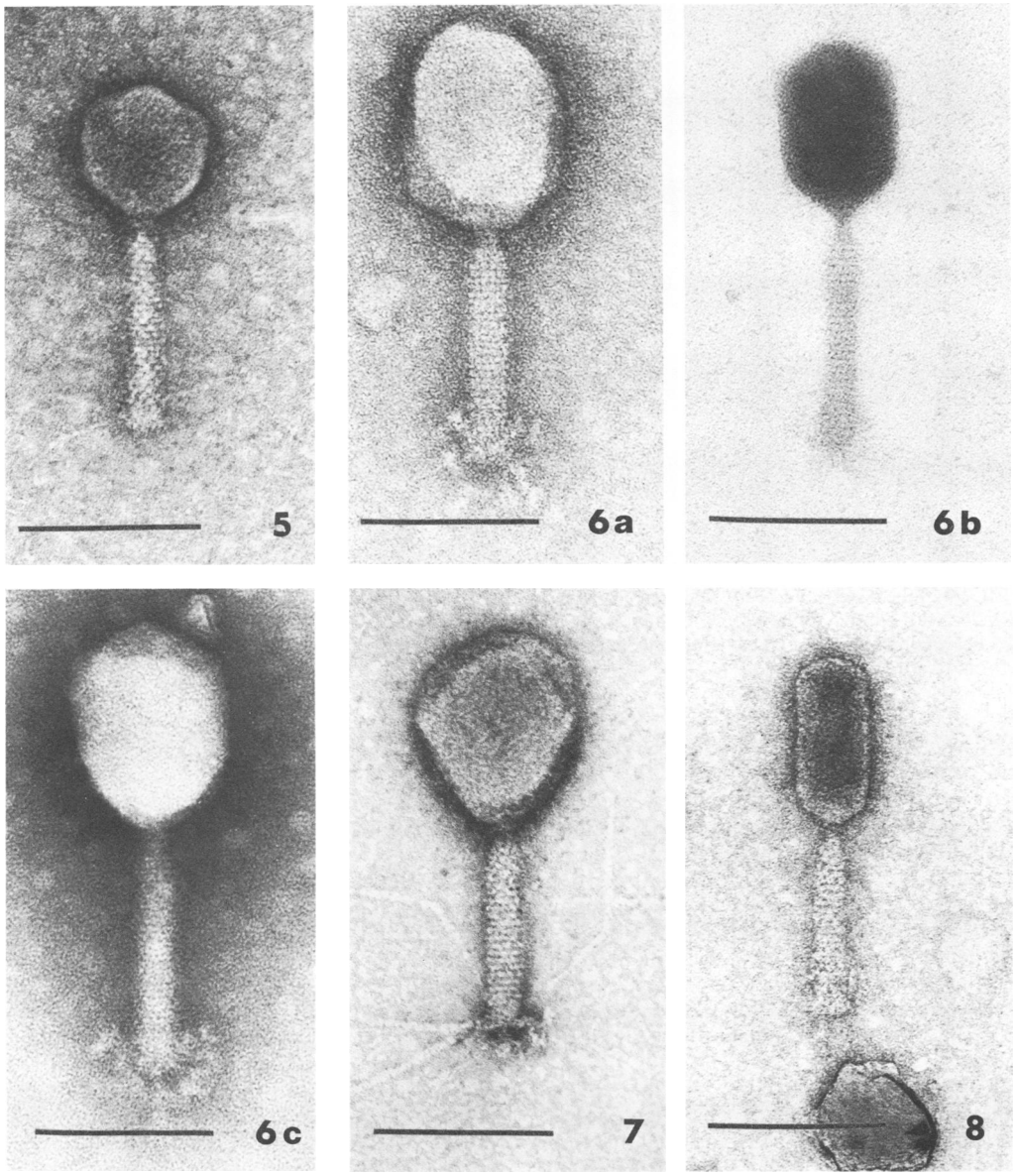

FIG. 5–8. (5) Type O1. Phage with extended tail and tail fibers; UA. (6) Type 9266. (a) Phage with extended tail, poorly visible tail striations, and short terminal fibers; UA. (b) Same after UA positive staining; note the shrunken head and the apparent absence of tail fibers. (c) Normal phage in PT. (7) Type T2. Phage with clearly visible tail striations and long, kinked tail fibers. (8) Type 16-19. Phage with extended tail and characteristically small narrow head; UA positive and negative staining.

aged by PT (Fig. 2b).

Type FC3-9 (Fig. 4) closely resembles type O1 except for its larger head, requiring measurements for differentiation. As in type 121, phage heads have hexagonal or pentagonal outlines. The contractile tail has a neck, a baseplate, and at least four short fibers. No collar has been found. Particles resemble the original phage FC3-9 (Fig. 4b) in shape and dimensions. However, the tail of the latter almost constantly shows fibrillar material which extends from the sides of the distal half of the sheath. This material is visible after PT and UA staining. It may derive from damaged tail sheaths or represent a new type of organite.

Type 9266 (Fig. 6) has the general morphology

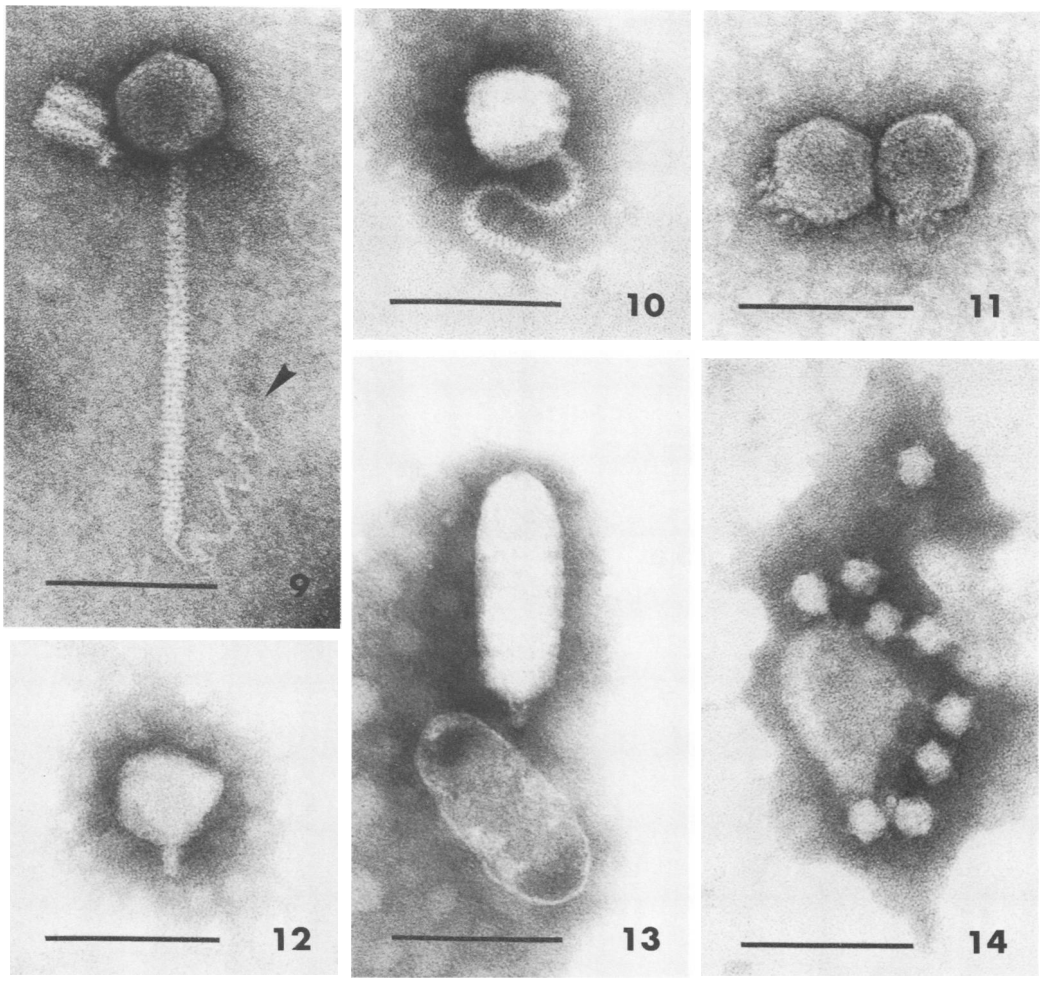

FIG. 9–14. (9) Type χ. Note the long, wavy tail fiber (arrow). The particle at the left of the phage head is a contracted tail sheath from another phage; UA. (10) Type β4. The extremely flexible tail is typical; PT. (11) Type N4. Two phages with spikes; UA. (12) Type T7. Particle showing a head with facets and a short tapering tail; PT. (13) Type Esc-7-11. Phages with full and empty heads; PT. (14) Type ϕX174. Phages with apical capsomers adsorbed to bacterial debris; PT.

of T-even phages (Fig. 7), but differs from them by its slightly larger size and the absence of a collar, a baseplate, and long, kinked tail fibers. The extended tail has about 32 striations and at least 10 short fibers with enlarged tips. The aspect of contracted tails (not shown) suggests that these fibers are attached to the sheath. Because of the close resemblance of types 9266 and T2, the presence of T-even-type phages should not be diagnosed without visualization of their characteristic tail fibers.

Type Esc-7-11 (Fig. 13) was named after the 7-11 species (2), which includes a series of bacillus-shaped phages with elongated heads and short tails. It differs from other phages of the 7-11 group by a shorter head (133 against 154 nm), a lower DNA content (45×10^6 against 57×10^6 to 58×10^6), its host range, and the absence of serological relationships and DNA-DNA hybridization (6, 22).

DISCUSSION

Phage identification. The enriched phages of types 121 to ϕX174 can be considered as enterobacterial phages. The identity of phagelike particles is less clear. Since sewage samples were cleared by centrifugation only, it is possible that these particles derived from sewage bacteria which participated in the enrichment process or that they represented chance observations of the original sewage flora.

Although most enriched phages were easily

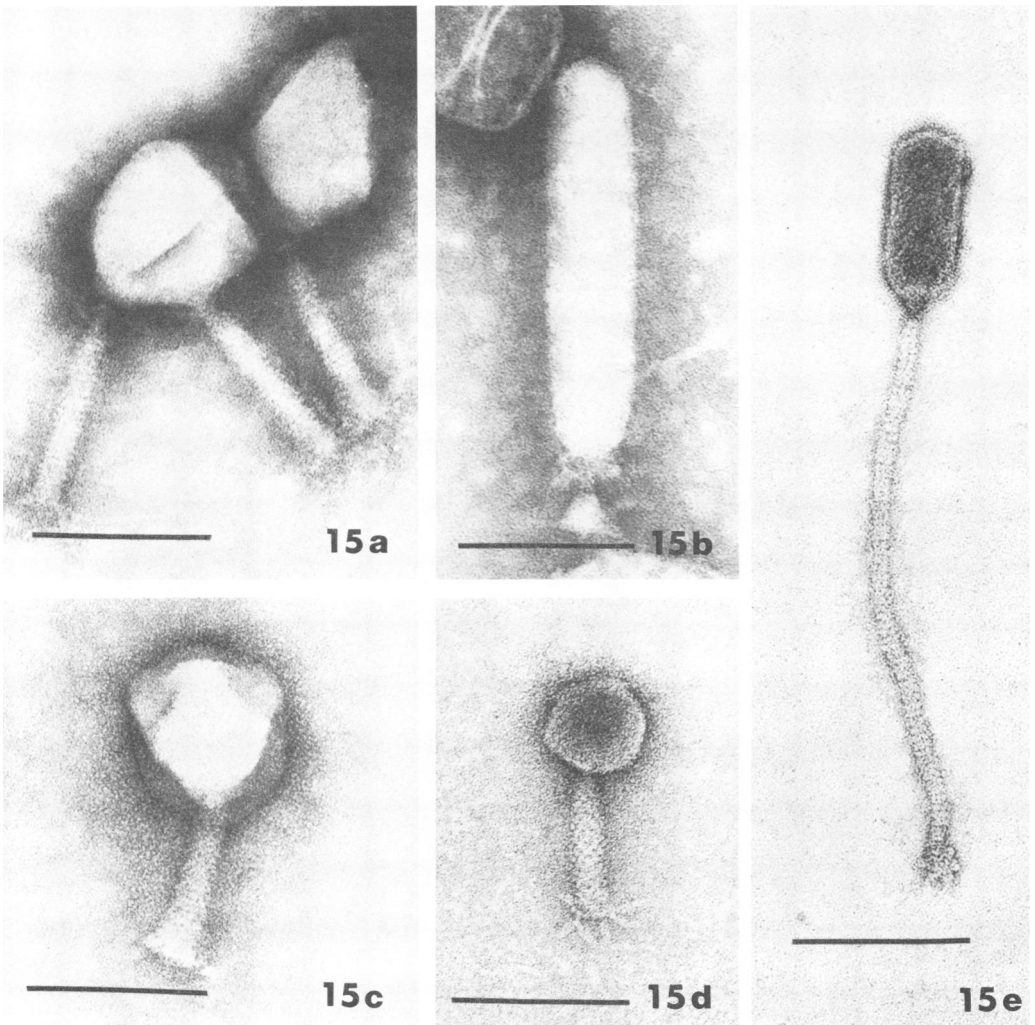

FIG. 15. Various observations. (a) T2-type particle with two tails, representing an extremely rare morphological aberration. The abnormal shape of the head is typical for phages with two or more tails; PT. (b to e) Phagelike particles of variable morphology; PT (b) or UA (c to e). Particles in (b) and (e) resemble certain *Streptococcus* and *Staphylococcus* phages. The particle in (d) has a tail of 80 nm in length only and may be the smallest known phage with a contractile tail.

identified, some reservations must be made. (i) Differentiation of types FC3-9 and Esc-7-11 from type O1 and the 7-11 phage group (2), respectively, required measurements on prints. (ii) Identification of types N4 and T7 is tentative because of their simple structure. (iii) Identity of type FC3-9 is uncertain because of the fibrillar material associated with the original phage FC3-9. Phage dimensions usually corresponded to those reported in the literature (2). Exceptions are types 121 and K19. Phage 121 was described as having a head 150 nm in diameter and a tail 165 nm in length (35). The head size of phage K19 was reported as 120 nm and its tail length as 183 nm (30, 31). Both phages had apparently been measured without magnification control, and we believe that their dimensions were overstated. Finally, type χ was found to be smaller than reported by one group of workers (9), but corresponded to the original description of phage χ (34) and viruses of this type isolated by Grimont (Ph.D. thesis).

In conclusion, phage identification by electron microscopy is rapid and simple. Identification by host range and plaque size is taxonomically unacceptable, and identification by antisera (17) is prohibitive, at least in tailed phages. Electron microscopy is of limited value in phages of

TABLE 2. Main dimensions of enriched phages

Species	Stain	No. of particles measured	Dimensions (nm) of:	
			Head	Tail
121	PT	20	115	114 by 16
	UA	20	116	116 by 22
K19	PT	10	89	146 by 17
FC3-9	PT	20	87	113 by 16
	UA	20	81	116 by 19
O1	PT	10	72	113 by 16
9266	PT	20	116 by 84	131 by 19
	UA	20	120 by 82	131 by 23
T2	PT	20	107 by 74	110 by 16
16–19	PT	10	102 by 57	94 by 15
χ	PT	10	63	229 by 12
β4	PT	10	61	161 by 8
N4	PT	10	60	10 by 8
T7	PT	10	56	14 by 9
Esc-7-11[a]	PT	50	133 by 53	12 by 9
	UA	50	134 by 42	
φX174	PT	10	30	None

[a] From reference 6.

uncharacteristic morphology, for example, actinophages (11). In enterobacterial phages, which are morphologically varied, it appears as the method of choice for phage identification.

Phage detection. The examination of enriched sediments is a purely qualitative approach. Its efficiency in detecting phages probably lies between the plating method and particle counts by electron microscopy. Our plate counts indicated very low titers of 0 to 10^3 PFU/ml, reflecting both the small volumes plated (0.2 ml) and the apparent specificity of indicator strains (Table 1). Similar low plate counts were reported elsewhere (10, 28, 40, 44). By contrast, a group of Japanese investigators found phage titers which were frequently 10^6 and occasionally 10^8 PFU/ml (20, 21, 37, 38). Electron microscopical phage counts indicated titers of 10^3 to 10^4/ml after filter concentration (45) and up to 10^7/ml after ultracentrifugation (18, 19).

It is unlikely that all coliphages present were detected, because only 10 of 24 known species of tailed enterobacterial phages (2) were observed, cubic RNA phages and filamentous phages were not found, and some phages were possibly overgrown by others.

The absence of RNA and filamentous phages was disturbing. One of the indicator strains, no. 29, is used for propagation of RNA phage MS2 (American Type Culture Collection Catalogue of Strains I, 15th ed., Rockville, Md., 1982). In our experience, lysates of RNA phages usually contain phage-coated pili. This highly conspicuous feature would not have gone unnoticed. Sewage was shown to contain up to 10^4 male-specific phages (16) and 10^3 to 10^5 RNA phages per ml, representing 5 to 90% of total coliphages (20, 21, 37, 38). On the other hand, all 47 isolates of Grimont (Ph.D. thesis) were tailed. We suspect that none of our indicator strains had grown sex pili or that RNA and filamentous phages were overgrown by others. Indeed, no pili were observed by electron microscopical examination of strains no. 25 to 30.

TABLE 3. Minor dimensions of types 121, FC3-9, and 9266

Species	Dimensions (nm) of:								
	Head (side length)	Tail							
		Collar	Neck	Sheath			Tail tube	Baseplate	Fibers (length)
				Extended	Contracted	Striations (width)			
121	59	20 by 2	8 by 8	106 by 16	40 by 25	3.5	114 by 88	34 by 4	20?
FC3-9	49	—[a]	8 by 7	103 by 16	42 by 21	3.3	108 by 7	20 by 5	47
9266	74 and 44[b]	—	12 by 8	119 by 19	47 by 30	3.5	131 by 8	—	23

[a] —, Absent.

[b] UA, used. All other data are from PT-stained particles.

TABLE 4. Geographical distribution of observed phage species

Species	Country	References[a]
121	Romania, France?	35, [b]
K19	France, Poland	30, 31, [b]
FC3-9	Spain	42
O1[c]	England, ubiquitous?	2, 8, 24, [b]
9266	South Africa, Japan?	36, 41
T2	Ubiquitous	2, 42
16-19	Canada, France	7, 23, 39, [b]
χ	England, France, South Africa	9, 14, 34, [b]
β4	Ubiquitous	2, 12, 14
N4[c]	Italy, ubiquitous?	2
T7[c]	USA, ubiquitous?	2
Esc-7-11	Canada	6
φX174	Ubiquitous	3

[a] References 2 and 3 are review papers.

[b] F. Grimont, Ph.D. thesis, Université de Bordeaux II, 1977.

[c] Many uncertain identifications.

The apparent specificity of some indicator strains suggested that they could be used for detection of certain phages without subsequent electron microscopy. However, when we tested our phage isolates, all but type N4 had much larger host ranges than expected from electron microscopical observations. The specificity of certain indicator strains is equally explained by overgrowing, and host range cannot replace electron microscopy for phage identification.

The advantages of examining enriched sediments are that this approach gives an instant and probably more complete view of phage populations than the isolation of single plaques and that phage hosts are known with a high degree of probability. The latter would be impossible in electron microscopical phage counts. Limitations are the qualitative nature of our technique, the nature and number of indicator strains, and the limit of visualization in the electron microscope. In our experience, phages with titers of less than 10^5 PFU/ml are difficult to detect.

Geographical distribution of phages. Types 121, K19, and 9266 are apparently rare and have been observed in such distant countries as Romania, Poland, or South Africa (30, 31, 35, 41). Similarly, the phagelike particle depicted in Fig. 15b corresponds to a streptococcal phage found in Finland only (43). Other types, namely, T2, β4, N4, T7, and φX174, correspond to well-known phage groups. Even allowing for uncertain identifications, they are clearly ubiquitous. Table 4 shows the geographical distribution of the phage types found in this work. Because of the large numbers of references for ubiquitous phages, the reader is referred to review papers (2, 3). Data on the geographical distribution of phages are so far available for RNA phages only (20, 21, 37, 38). We hope that electron microscopy will provide similar data for tailed phages.

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