Methods for the Direct Isolation and Enumeration of Actinophages in Soil

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(Received 13 October 1981; revised 3 February 1982)

Existing direct isolation methods may seriously underestimate the actual numbers of actinophages in soil, since major 'losses' may be incurred during agitation, filter sterilization and, to a lesser extent, centrifugation. Two methods were devised for the isolation and enumeration of soil actinophages and were compared experimentally with two previously described methods. Numbers of up to 2×10^4 p.f.u. (g soil)⁻¹ were detected in compost soil.

INTRODUCTION

Phages for many bacterial genera may be readily isolated from most soils by enrichment of the soil with the potential host (Adams, 1959; Billing, 1969; Willoughby *et al.*, 1972; Dowding, 1973; Dowding & Hopwood, 1973; Zachary, 1974), with nutrients (Reanney & Marsh, 1973; Casida & Liu, 1974; Tan & Reanney, 1976; Germida & Casida, 1981) or with both host and nutrients (Newbould & Garrard, 1954; Retinskaya & Rautenstein, 1960). Phages thus isolated have been widely used in biochemical, genetic and taxonomic research. Ecological studies, however, may require knowledge of the numbers of phages present in natural, unamended systems. Reanney & Marsh (1973) have suggested that if phages occurred in soil at 0.1% of the titre obtained under optimum laboratory conditions, they must be the most numerous genetic objects in that habitat. Phages are, therefore, potentially agents of prime ecological importance.

Methods for the direct isolation of phages from soil usually yield extremely low titres (Khavina, 1954; Welsch *et al.*, 1955; Reanney, 1968; Collard, 1970; Casida & Liu, 1974; Tan & Reanney, 1976). Problems associated with direct isolation include: (i) inactivity of the hosts (Gray & Williams, 1971), hence the increases in phage numbers on enrichment; (ii) inactivation or loss of phages due to the use of inappropriate eluent, extraction and sterilization techniques; (iii) the inability of direct assays to detect phages in close physical association with resting cells (Casida & Liu, 1974) or adsorbed to soil colloids (Robinson & Corke, 1959; Sykes & Williams, 1978).

In the investigation reported here, some of the factors influencing the efficiency of direct isolation of actinophages from soil were assessed. New approaches were outlined for obtaining a more realistic estimate of the numbers of actinophages in soil.

METHODS

Actinomycetes and actinophages. Spore suspensions of the following streptomycetes in 10% (v/v) glycerol were prepared and stored at -18°C (Wellington & Williams, 1978). *Streptomyces lavendulae* (International Streptomyces Project 069), *S. michiganensis* (ISP 015), *S. griseus* (ISP 236), *Streptomyces* sp. (Mx 1), *Streptomyces* sp. (Mx 2), *Streptomyces* sp. (Mx 3). Pure stocks of actinophages ϕ 069 and ϕ Mx 2 were inoculated together with their hosts, *S. lavendulae* (ISP 069) and *Streptomyces* sp. (Mx 2) on plates of nutrient agar (NA) (Oxoid CM3), pH 7.0 and incubated at 25°C for 16 h. Areas of confluent lysis were transferred to vials containing nutrient broth (NB) (Oxoid CM69), pH 7.0, shaken at 800 oscillations per minute (o.p.m.) on a reciprocal flask shaker (Griffin and George Ltd) for 15 min and allowed to stand at 4°C for 16 h. The resulting phage suspensions (approximately 10^7 p.f.u. ml⁻¹) were filter-sterilized (Millipore type HAWP, pore size 0.45 μm).

Soils. Fresh soils were sieved through a 2 mm mesh and stored in polythene bags at 4°C . Sub-samples of sieved sand, arable and garden (1) soils (Table 1) were air-dried for 4 d and stored in polyethylene screw-capped bottles. The pH of soil samples was determined in water extracts using a soil : water ratio of 1 : 2 with a glass electrode and a

direct-reading pH meter. Water content was determined by loss in weight of soil samples dried to constant weight at 55 °C. Percentage organic matter was measured by loss in weight on ignition at 850 °C for 30 min (Ball, 1964). Particle size distribution was determined by a modification of the hydrometer method (Day, 1965). Soils were sterilized by autoclaving at 15 p.s.i. (103.5 kPa) for 20 min.

Actinophage assay. This was carried out by a modification of the double-layer method (Adams, 1959). Actinophage suspension or sterile soil extract suitably diluted in NB (1 ml) was inoculated on to a basal layer of NA. NB (2.5 ml) containing 0.6% (w/v) agar (Oxoid L13), maintained at 45 °C, was inoculated with a spore suspension of the streptomycete host and poured over the basal layer. Five replicate plates were prepared and incubated at 25 °C for 16 h when plaques were counted.

Factors affecting the recovery of actinophages added to sterile soil. Some factors which might influence the recovery of added actinophages from sterile soil were studied. These included agitation procedure and extraction period, eluent, centrifugation and filter sterilization.

The basic method was as follows. Stock actinophage $\phi 069$ suspension was added to 20 g of sterile, air-dried sand in 100 ml flasks to give a concentration of approximately 10^2 p.f.u. (g sand)⁻¹. Eluent (50 ml) was added and extracts agitated, then allowed to stand at 25 °C for 30 min. The supernatants were centrifuged at 1200 g for 30 min, filter-sterilized, and the filtrate assayed for actinophage $\phi 069$. This method was progressively modified according to the results of the following sections.

(i) **Agitation procedure and extraction period.** Sand extracts in NB (pH 7.0) were agitated for 15, 30 and 60 min at 4 °C using an orbital incubator (Gallenkamp) at 200 rev. min⁻¹, a magnetic stirrer (Biotec, Solna, Sweden) at 300 rev. min⁻¹ and a reciprocal flask shaker at 200 o.p.m. Supernatants were centrifuged and assayed for actinophage $\phi 069$ without filtration.

(ii) **Eluent.** The following eluents were assessed: NB, pH 7.0; 3% (w/v) beef extract (BE) (Oxoid L29), pH 7.0; 1.0 M-glycine buffer (Sigma 600-5), pH 8.7; 1.0 M-sodium nitrate (BDH 10255); peptone/yeast/calcium broth (PYCa) (Bradley *et al.*, 1961), pH 7.0; glucose/Casamino acid/proline broth (GCP) (Sykes, 1977), pH 7.0. The effects of pH and the protein content of the eluent on actinophage recovery were also tested by adjusting the pH of NB with 0.1 M-NaOH to between 7.0 and 10.0. Bovine albumin (Sigma, A4503), gelatin (Oxoid L8) or egg albumen (Difco 0147-5-7) were added to NB, pH 8.0 at concentrations of up to 1000 mg l⁻¹.

Extracts were shaken on the reciprocal flask shaker for 30 min at 4 °C at 200 o.p.m., centrifuged, filter-sterilized and the filtrate assayed for actinophage $\phi 069$.

(iii) **Filter sterilization.** The titres of a suspension of actinophage $\phi 069$ in NB were compared before and after filtration. The filters compared were Gelman (type GA-6, cellulose triacetate, pore size 0.45 μ m; Gelman Hawksley, Lancing, Sussex) and Millipore (type HAWP, mixed cellulose acetate and nitrate, pore size 0.45 μ m). Pretreatments of the Millipore filter were compared. Filters were soaked for 4 h in 2% (v/v) Decon 90 (Decon Laboratories, Brighton, Sussex), 0.1% (v/v) Tween 80 (BDH 56023), 2% (w/v) gelatin, 3% (w/v) BE (pH 8.0), NB (pH 8.0) and NB with 0.1% (w/v) egg albumen (pH 8.0).

The effect of filtration of soil extracts on recovery of actinophage $\phi 069$ from sterile sand was also tested. Sand was shaken as before with 50 ml NB plus 0.1% (w/v) egg albumen (pH 8.0). Extracts were centrifuged and the supernatant assayed for actinophage $\phi 069$ either directly or after filtration through untreated Millipore filters or filters pretreated with 0.1% (v/v) Tween 80, 2% (w/v) gelatin, 3% (w/v) BE (pH 8.0) or NB (pH 8.0).

(iv) **Centrifugation.** Soil extracts were shaken as before and allowed to stand at 4 °C for 16 h, when half the supernatant was centrifuged at 1200 g for 30 min. The supernatants from centrifuged and uncentrifuged samples were assayed for actinophage $\phi 069$ without filtration.

Comparison of four procedures for the isolation and enumeration of actinophages in soils. The efficiencies of the following isolation methods were evaluated by comparison of recoveries of added actinophage $\phi Mx 2$ from sterile sand, arable and garden (1) soils. Actinophage $\phi Mx 2$ suspension was added to sterile soils in 100 ml flasks to give a final concentration of 116.5×10^2 p.f.u. (g soil)⁻¹.

Method A (Welsch *et al.*, 1955). Sterile distilled water (60 ml) was added to each flask which was then shaken frequently during a period of 2 to 3 h. Soil suspensions were filtered (Whatman no. 1) and sterilized by filtration (Millipore type HAWP, pore size 0.45 μ m). Seitz filters originally used in this method were unobtainable. The filtrates were assayed for actinophage $\phi Mx 2$.

Method B (Sykes, 1977). Sterile NB (60 ml, pH 7.0) was added to each flask, which was then shaken on an orbital incubator at 200 rev. min⁻¹ 25 °C for 60 min. Soil suspensions were allowed to stand at 25 °C for 30 min. The supernatants were centrifuged at 1200 g for 15 min, sterilized by filtration (Millipore type HAWP, pore size 0.45 μ m) and assayed for actinophage $\phi Mx 2$.

Method C (Devised procedure for chloroform-sensitive actinophage). NB (50 ml) containing 0.1% (w/v) egg albumen (pH 8.0) was added to each flask. Soil extracts were shaken on a reciprocal flask shaker at 200 o.p.m. for 30 min at 4 °C and allowed to stand at 4 °C for 16 h. The supernatants were centrifuged at 1200 g for 30 min, sterilized by filtration (Millipore type HAWP, pore size 0.45 μ m) and assayed for actinophage $\phi Mx 2$.

Method D (Devised procedure for chloroform-tolerant actinophage). The basic procedures were as for chloroform-sensitive actinophage. However, after centrifugation, 5 ml of the supernatant was transferred to a sterile vial and

Table 1. *Properties of soils used*

Soil	pH	Water content (% fresh weight)	Loss-on-ignition (% air-dry weight)	Particle size distribution (% oven-dry weight)			
				Coarse sand	Fine sand	Silt	Clay
Sand	8.6	1.8	3.0	65.5	34.5	0	0
Compost	7.6	44.4	19.3	39.1	51.9	9.0	0
Arable	6.9	26.0	10.2	44.3	42.1	10.7	2.9
Garden (1)	7.7	29.7	14.1	37.4	43.8	10.9	7.9
Garden (2)	7.1	12.3	6.9	54.9	36.5	1.0	7.6
Garden (3)	7.5	25.2	10.8	63.6	30.4	3.7	2.3
Garden (4)	8.2	19.4	11.1	34.3	49.7	10.6	5.4
Cultivated (1)	7.5	15.4	6.2	43.5	46.6	8.3	1.6
Cultivated (2)	7.6	22.0	6.7	47.0	46.9	6.1	0

0.1 ml chloroform added (Crosse & Hingorani, 1958). Vials were shaken on a reciprocal flask shaker at 200 o.p.m. for 5 min and allowed to stand at 4 °C for 2 min. The aqueous layer was assayed for actinophage ϕ Mx 2.

The four methods described above were used to isolate and enumerate indigenous actinophage for six *Streptomyces* spp. in eight fresh soils (Table 1).

RESULTS AND DISCUSSION

Factors influencing the recovery of actinophages from soil

Agitation. Maximum recovery (117%) of actinophage ϕ 069 added to sand was obtained when NB extracts were shaken at 200 o.p.m. on a Griffin reciprocal flask shaker for 30 min at 4 °C (Table 2). Fifty-eight percent were recovered from unshaken extracts, 43 and 4% after orbital shaking and magnetic stirring, respectively. Losses in the shaken samples may have been due to surface inactivation at the air/water interface, or to adsorption to the glass surface of the flasks, which occurs with bacteriophages and animal viruses (Troubworst *et al.*, 1974). Inactivation of certain physiologically active proteins such as enzymes and toxins by shaking is well known (Adams, 1948).

Eluent. NB containing 0.1% (w/v) egg albumen at pH 8.0 was selected as the optimum eluent. Greater numbers of phages were recovered from sand extracted with complex media such as NB, GCP and PyCa broth than with deionized water, glycine buffer or NaNO₃ (Table 3). Most bacterial viruses are stable when diluted in broth, serum, ascitic fluid and similar diluents, but all are less stable and some totally unstable when diluted in salt solutions or chemically defined media (Adams, 1948).

Maximum recovery (70%) of actinophage ϕ 069 was obtained when NB was adjusted to pH 8.0 (Table 3). Isolation of actinophages from soils has previously been shown to depend on the pH of the eluent (Khavina & Rautenstein, 1959). Actinophages were rarely isolated when the pH of the eluting medium was < 7.0. Similarly, elution of *Arthrobacter* phages added to loam was most efficient when the eluent (deionized water or beef extract/glucose broth) was adjusted to pH 7.8 to 8.2 (Ostle & Holt, 1979). Adsorption of virus on to biological and non-biological surfaces depends largely on the ionic composition and pH of the medium in which they are suspended (Bitton, 1975; Duboise *et al.*, 1976). The use of an eluent with high pH might therefore be expected to increase desorption of negatively charged phage particles from the cation-exchange sites in soils. The stability of actinophages, however, is generally sensitive to pH values greater than 9.0 (Sykes *et al.*, 1981), thus the extremely high pH values used to isolate poliovirus and enterovirus (Landry *et al.*, 1978; Hurst *et al.*, 1978; Hurst & Gerba, 1979) were not appropriate.

The addition of bovine albumin or gelatin to NB pH 8.0 had little effect on actinophage recoveries (Table 4). The addition of 0.1% (w/v) egg albumen, however, significantly increased the recovery of actinophage ϕ 069 from 43 to 83% ($P < 0.05$) probably by reducing inactivation during shaking and promoting desorption of phage particles from clay minerals. The adsorption

Table 2. *Effect of agitation method and extraction period on the recovery of actinophage $\phi 069$ from sterile sand*

The number of phage added was 30.5×10^2 (g air-dried sand) $^{-1}$. Tukey's L.S.D._{0.05} (Winer, 1962) = 5.42. The numbers in parentheses indicate the percentage of added phage recovered.

Method	Time . . .	No. of phage recovered [$10^{-2} \times$ p.f.u. (g air-dried sand) $^{-1}$]		
		10 min	30 min	60 min
Unshaken		16.7 (55)	17.7 (58)	17.3 (57)
Orbital incubator, 200 rev. min $^{-1}$		17.0 (56)	13.0 (43)	9.0 (29)
Magnetic stirrer, 300 rev. min $^{-1}$		5.7 (19)	1.3 (4)	0 (0)
Reciprocal flask shaker, 200 o.p.m.		21.7 (71)	35.7 (117)	22.3 (73)

Table 3. *Effect of different eluents on the recovery of actinophage $\phi 069$ from sterile sand*

The number of phage added was 171.0×10^3 (g air-dried sand) $^{-1}$. Tukey's L.S.D._{0.05} (Winer, 1962) = 12.44.

Eluent	No. of phage recovered [10 ⁻² × p.f.u. (g air-dried sand) ⁻¹]	Percentage of added phage recovered
Deionized water	56.3	33
GCP broth	97.3	57
1.0 M-glycine buffer, pH 8.7	86.7	51
BE (3%, w/v)	75.3	44
PYCa broth	93.0	54
1.0 M-NaNO ₃	0.7	0.4
NB, pH 7.0	106.0	62
NB, pH 8.0	120.0	70
NB, pH 9.0	101.0	59
NB, pH 10.0	14.3	8

Table 4. *Effect of addition of protein to the eluent (NB, pH 8.0) on the recovery of actinophage $\phi 069$ from sterile sand*

The number of phage added was 247.0×10^2 (g air-dried sand) $^{-1}$. Tukey's L.S.D._{0.05} (Winer, 1962) = 15.42.

Protein additive (mg l ⁻¹)	No. of phage recovered [10 ⁻² × p.f.u. (g air- dried sand) ⁻¹]	Percentage of added phage recovered
Bovine albumin		
0	137.3	55
10	122.0	49
100	166.3	67
1000	163.0	66
Gelatin		
0	136.7	55
10	144.7	58
100	137.7	56
1000	127.0	51
Egg albumen		
0	107.0	43
10	132.7	54
100	168.0	68
1000	205.0	83

Table 5. *Effect of filtration on the titre of a suspension of actinophage $\phi 069$* Tukey's L.S.D._{0.05} (Winer, 1962) = 17.52.

Filter type	Pretreatment	Phage titre ($10^{-2} \times \text{p.f.u. ml}^{-1}$)	Percentage of unfiltered phage titre
None	None	302.3	
Gelman*	None	135.7	45
Millipore†	None	219.7	73
Millipore	'Decon 90' (2.0%, w/v)	21.7	7
Millipore	Gelatin (2.0%, w/v)	195.7	65
Millipore	BE (3.0%, w/v) pH 8.0	154.0	51
Millipore	NB, pH 8.0	165.0	55
Millipore	NB + egg albumen (0.1%, w/v) pH 8.0	124.3	41
Millipore	'Tween 80' (0.1%, w/v)	161.0	53

* Gelman (type GA-6, cellulose triacetate, pore size 0.45 μm)† Millipore (type HAWP, mixed cellulose acetate and nitrate, pore size 0.45 μm).Table 6. *Effect of filtration on the recovery of actinophage $\phi 069$ from sterile sand*The number of phage added was 280.3×10^2 (g air-dried sand)⁻¹. Tukey's L.S.D._{0.05} (Winer, 1962) = 36.95.

Pre-treatment of Millipore filters	No. of phage recovered [$10^{-2} \times \text{p.f.u. (g air-dried sand)}^{-1}$]	Percentage of added phage recovered
No filter	259.0	92
None	122.3	44
Gelatin (2.0%, w/v)	92.3	33
BE (3.0%, w/v) pH 8.0	119.0	42
NB, pH 8.0	127.0	45
'Tween 80' (0.1%, w/v)	124.0	44

of virus to clay minerals may be reduced or prevented by the presence of proteinaceous materials such as egg albumen and bovine albumin (Carlson *et al.*, 1968; Burge & Enkiri, 1978; Ostle & Holt, 1979), foetal bovine serum (Jakubowski, 1969) or foetal calf serum (Gerba *et al.*, 1977; Ramia & Sattar, 1979). The surface inactivation of virus and phage on shaking may also be reduced by the addition of small amounts of protein to the medium (Adams, 1948; Troubworst *et al.*, 1974).

Filter sterilization. Filtration of a suspension of actinophage $\phi 069$ resulted in losses of 55 and 27% of the original titre with Gelman cellulose triacetate (type GA-6) and Millipore cellulose acetate and nitrate (type HAWP) filters, respectively (Table 5). Pretreatment of filters with proteins such as gelatin, BE and NB or surface-active agents such as Tween 80 and Decon 90, reduced the number of actinophage $\phi 069$ in the filtrate. De Vicente (1959) also found considerable differences in the degree of adsorption of phages to different filter types. Ninety-two percent of actinophage $\phi 069$ added to sterile sand were recovered from an unfiltered extract in NB plus 0.1% (w/v) egg albumen, pH 8.0 (Table 6). Filtration of the extract with untreated filters or filters pretreated with selected protein solutions or surface-active agents gave significantly reduced recoveries (33 to 45%) of added actinophage $\phi 069$. Collard (1970) showed that membrane filtration of a soil extract in water resulted in a 90% reduction and Seitz filtration, a 99% reduction in numbers of actinophages detected. Willoughby *et al.* (1972) also observed a considerable loss of *Actinoplanes* phage activity when host-enriched soil suspensions were filtered.

Centrifugation. Centrifugation of soil extracts at 1200 *g* for 15 min resulted in small reductions ($P \leq 0.05$) in the numbers of added actinophage $\phi 069$ recovered from sterile arable and garden soils (Table 7). Centrifugation had no effect on the number of actinophage $\phi 069$ recovered from

Table 7. *Effect of centrifugation on the recovery of actinophage ϕ 069 from sterile soils*

The number of phage added was 150.0×10^2 (g air-dried soil) $^{-1}$. Tukey's L.S.D._{0.05} (Winer, 1962) = 12.72.

Soil	Centrifugation of soil suspension	No. of phage recovered	Percentage of added phage recovered
		[10 ⁻² × p.f.u. (g air- dried soil) ⁻¹]	
Sand	None	146.0	97
	1200 g, 15 min	142.3	95
Arable	None	140.0	93
	1200 g, 15 min	124.3	83
Garden (1)	None	139.7	93
	1200 g, 15 min	119.0	79

Table 8. *Comparison of the efficiency of four isolation methods by the recovery of actinophage ϕ Mx 2 added to sterile soils*

The number of phage added was 116.5×10^2 (g air-dried soil) $^{-1}$. Details of Methods A to D are given in Methods. Tukey's L.S.D._{0.05} (Winer, 1962) = 27.90. The numbers in parentheses indicate the percentage of added phage recovered.

Soil	No. of phage recovered [$10^{-2} \times$ p.f.u. (g air-dried soil) $^{-1}$]			
	Method A	Method B	Method C	Method D
Sand	21.0 (18)	29.5 (25)	101.7 (87)	111.2 (96)
Arable	15.9 (13)	21.3 (18)	85.8 (74)	108.7 (93)
Garden (1)	37.5 (32)	35.1 (30)	39.9 (34)	138.3 (118)

sterile sand. This is consistent with sand having fewer adsorption sites than clay soils (Bitton, 1975; Burge & Enkiri, 1978).

Comparison of four methods for the isolation and enumeration of actinophages in soils

Between 96 and 100% of added actinophage ϕ Mx 2 were recovered from the three test soils using Method D (Table 8). This method was the most efficient. Method C was generally more efficient than Methods A and B ($P \leq 0.05$). Eighty-seven and 74% of added actinophage ϕ Mx 2 were recovered from sand and arable soils, respectively, but only 34% from the garden soil. The latter contained the highest proportion of clay and any method involving filtration will thus result in low phage recovery due to the adsorption to the filter of the phage/colloid complexes. The recovery efficiencies of Methods A and B were extremely low (13 to 32%) for all soils.

The four methods may be ranked in terms of their efficiency for the direct isolation and enumeration of actinophages for six streptomycetes in eight soils as follows: Method D > Method C > Method B > Method A (Table 9). The greatest number of actinophages were obtained from the compost soil. Method D yielded approximately thirty times the number of actinophages obtained by Method A and three times that obtained by Methods B and C (*Streptomyces* sp. Mx 3). Similar results were obtained for arable soil (*Streptomyces* sp. Mx 2, Mx 3, ISP 236) and garden (1) soil (*Streptomyces* sp. Mx 1, Mx 3).

In some soils there was no significant difference in the numbers of actinophages obtained by Methods C and D, e.g. arable (*Streptomyces* sp. Mx 1), garden (2) (*Streptomyces* sp. Mx 1, Mx 2, Mx 3, ISP 236) and cultivated (1) (*Streptomyces* sp. ISP 069). Assuming that approximately 50% of actinophages may be retained by the filter (Method C) there could have been a high proportion of chloroform-sensitive actinophages for the given host in these soil populations. Alternatively, there may have been a large proportion of small actinophages which can pass readily through the filter. Actinophages for *Streptomyces michiganensis* (ISP 015) were isolated from cultivated (2) soil by Methods B and C, but not by Method D. This suggests that all members of the actinophage population for *S. michiganensis* (ISP 015) in this soil were chloroform-sensitive.

Streptomycete hosts Mx 1, Mx 2 and Mx 3 were isolated from compost, arable and garden

Table 9. Comparison of four methods for the direct isolation and enumeration of actinophages in natural soils

Tukey's L.S.D._{0.05} (Winer, 1962) = 86.4.

Soil	Streptomycete host	No. of phage recovered [p.f.u. (g soil) ⁻¹]			
		Method*:			
		A	B	C	D
Compost	Mx 1	514.5	2173.0	2916.7	12387.3
	Mx 2	669.0	5820.0	3916.7	12008.3
	Mx 3	785.0	6885.0	7262.5	22950.0
	ISP 069	0	0	0	0
	ISP 015	0	0	0	0
	ISP 236	87.0	566.0	397.3	806.0
Arable	Mx 1	17.0	82.0	225.0	275.0
	Mx 2	50.0	117.0	641.7	1283.3
	Mx 3	56.0	181.0	666.7	1858.3
	ISP 069	0	0	0	0
	ISP 015	0	0	0	0
	ISP 236	16.0	107.0	277.0	660.0
Garden (1)	Mx 1	13.0	87.0	233.3	1516.7
	Mx 2	16.0	35.0	207.0	558.3
	Mx 3	41.0	98.0	558.3	2868.3
	ISP 069	0	0	0	0
	ISP 015	0	0	0	0
	ISP 236	0	0	0	0
Garden (2)	Mx 1	0	2.0	10.7	64.7
	Mx 2	1.0	2.0	5.0	49.0
	Mx 3	5.0	4.0	32.3	90.7
	ISP 069	0	0	0	0
	ISP 015	0	0	0	0
	ISP 236	3.0	22.0	36.3	86.7
Garden (3)	Mx 1	0	127.0	121.3	316.7
	Mx 2	5.0	66.0	58.7	195.7
	Mx 3	37.0	111.0	164.0	416.7
	ISP 069	0	0	0	0
	ISP 015	0	0	0	0
	ISP 236	0	0	0	0
Garden (4)	Mx 1	0	0	0	0
	Mx 2	0	0	0	0
	Mx 3	48.0	3.0	15.7	104.0
	ISP 069	0	0	0	0
	ISP 015	0	0	0	0
	ISP 236	0	0	0	0
Cultivated (1)	Mx 1	0	0	0	0
	Mx 2	0	0	0	0
	Mx 3	0	0	0	0
	ISP 069	6.0	124.5	64.7	104.0
	ISP 015	0	0	0	0
	ISP 236	0	0	0	0
Cultivated (2)	Mx 1	0	0	0	0
	Mx 2	0	0	0	0
	Mx 3	0	0	0	0
	ISP 069	0	0	0	0
	ISP 015	0	1.0	17.0	0
	ISP 236	0	0	0	0

* For details of the Methods A, B, C and D, see Methods.

soils and the large numbers of actinophages counted in these soils may reflect host activity. Fewer actinophages were obtained for *Streptomyces* sp. ISP 069, ISP 015 and ISP 236 which were type cultures not originating from the soils under study.

In order to understand the ecological significance of phages in natural soil, it is first necessary to have an efficient method for their detection and enumeration. The results of this study suggest that existing direct isolation procedures may seriously underestimate the actual numbers of actinophages in soil due to major 'losses' incurred during agitation, centrifugation, filter sterilization and the use of unsuitable eluents. The two suggested procedures, Methods C and D for chloroform-sensitive and chloroform-resistant actinophages, respectively, minimize these losses.

Method C is more efficient than existing direct isolation procedures tested. Although losses of up to 67% may be incurred by adsorption of phage particles to the filter, filter sterilization is probably the only available method for the isolation of chloroform-sensitive actinophages. Alternative methods for preventing growth of soil bacteria on assay plates, such as drying soil, heat, thymol, phenol, antibiotics, lysozyme, selective centrifugation and modification of the assay medium were found to be unsuitable (unpublished data).

Soil extracts may be effectively sterilized by treatment with 2% (v/v) chloroform applied to the supernatant after centrifugation and this does not inhibit growth of the streptomycete host (Method D). This method is therefore particularly useful for actinophage enumeration in soils with a high clay content. When filtration is used most phages will be lost by adsorption of phage/colloid complexes to the filters. However, some phages are inactivated by chloroform (Eisenstark, 1967; Billing, 1969) so only the chloroform-resistant phages from the natural soil population will be enumerated. This method yielded considerably greater numbers of actinophages than have previously been reported using direct isolation procedures (Khavina, 1954; Welsch *et al.*, 1955; Collard, 1970).

It is impossible to provide optimal conditions for the isolation of all actinophages present in a given sample, since phage-host systems differ considerably in their susceptibility to inactivation by a range of environmental conditions. However, the suggested procedures should facilitate a more realistic estimate of numbers of actinophages in the soil mass or in microsites of host activity. This will provide a basis for more detailed studies of actinophage-host interactions in soil. Hopefully the methods will also be applicable to ecological studies of the phages of other bacteria.

This work was supported by N.E.R.C. Research Grant (GR3/3672). We are grateful for helpful discussions with Dr S. B. Primrose and Dr N. Seeley, Department of Biological Sciences, University of Warwick.

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