Preliminary Characterization of Bacteriophages Infecting the Thermophilic Actinomycete *Thermomonospora*

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Bacteriophages lysing strains of *Thermomonospora alba* and *T. fusca* were isolated, following specific enrichment, from vegetable composts. Four *Thermomonospora* phages were distinguished by plaque morphology and host range. Electron microscopy of phage particles, termperature inactivation profiles, and electrophoretic analyses of major virion proteins and genomic DNA were used in the comparison and initial characterization of these phages. The four phages studied possessed polyhedral heads and long tails; genomes were linear double-stranded DNA molecules, 35 to 45 kilobases in length, which probably contain cohesive ends. Transfection of *Thermomonospora* protoplasts with purified genomic DNA from one of the phages was demonstrated.

The genus *Thermomonospora* (8) includes aerobic sporeforming actinomycetes that possess a type III cell wall (15) and produce single heat-sensitive spores at the termini of branched or unbranched sporophores on the aerial or on both aerial and substrate hyphae (7). A recent taxonomic study (19) identified a group of three species (*Thermomonospora alba*, *T. curvata*, and *T. fusca*) which produce extracellular enzymes for hydrolysis of recalcitrant polysaccharides, including cellulose and xylans.

A growing interest in biomass conversion strategies has brought attention to the metabolic capabilities of *Thermomonospora* species. Cellulases from strains of *T. curvata* (27) and *T. fusca* (5) have been studied in some detail, and xylanase activities of *T. curvata* and *T. fusca* have recently been characterized (20). *Thermomonospora* species exhibit several features which recommend them as experimental subjects for research on biomass degradation. Thermomonosporas grow rapidly on defined cellulose media, produce abundant spores, and secrete a complex of enzyme activities for the degradation of crystalline cellulose (27). The identity of thermomonosporas as sporeforming actinomycetes may facilitate genetic analysis of these organisms, through modification of strategies and techniques currently used with antibiotic-producing streptomycetes. Production of viable *T. fusca* protoplasts and genetic transformation of *T. fusca* with a streptomycete plasmid cloning vector have been demonstrated (26).

Thermomonosporas are frequently recovered from self-heated organic materials such as moldy hays (12), municipal wastes (28), and mushroom soils (6), in which they may be the dominant antinomycetes (13). It was expected that such soils would also yield bacteriophages active against *Thermomonospora* species. This article reports the first isolation, from compost, of bacteriophages that lyse strains of *Thermomonospora* and the preliminary characterization of four of these phages.

MATERIALS AND METHODS

Organisms and growth conditions. *T. fusca* YX (1) was obtained from the Biological Energy Corporation, King of

Prussia, Pa. *T. alba* IPV 1900 (16), *T. chromogena* N2900 (10), and *T. fusca* ATCC 27730 (190Th) (4) were obtained from M. P. Lechevalier, Waksman Institute, Rutgers University, Piscataway, N.J. *T. curvata* ATCC 19995 (8) and *T. mesophila* ATCC 27303 (23) were obtained from the American Type Culture Collection, Rockville, Md.

The complete medium for growth and maintenance of actinomycetes was a supplemented Luria-Bertani broth containing, per liter, 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, 1 g of glucose, and 10 ml of a mineral salts solution (22); the pH was adjusted to 7.5 with NaOH. Agar was added to 2% for plates and to 0.5% for top agar. Actinomycetes were grown at 37°C (*T. mesophila* ATCC 27303), 45°C (*T. alba* IPV 1900), or 55°C (all other thermophilic actinomycetes). Mycelial cultures were grown in baffled flasks shaken at 250 rpm on a rotary table. Stocks were maintained as single colonies on plates of complete agar, stored at 4°C, and as spore suspensions in 20% (vol/vol) glycerol in water stored at −20°C.

Isolation of bacteriophages from soil. Samples of vegetable composts, prepared from agricultural wastes and manure, were obtained from H. Danton, Rodale Research Center, Kutztown, Pa. A specific enrichment technique was used for isolation of bacteriophages. Fresh soil (1 g) was suspended in 9 ml of complete broth in a 50-ml Erlenmeyer flask. After addition of 1 ml of dense mycelial culture of a potential host, suspensions were incubated with shaking at 50°C for 6 h. Suspensions were filtered through glass wool and centrifuged at 8,000 × *g* for 10 min to sediment debris. Cleared suspensions were passed through a 0.2-μm-pore-size cellulose–mixed-ester membrane filter and centrifuged at 25,000 rpm in a Beckman type 40 rotor for 2 h to sediment phage. Pellets were suspended in 2 ml of SM buffer (18) by gentle agitation overnight at 4°C. These soil concentrates were filter sterilized and diluted through SM. A diluted sample (0.1 ml) was mixed with 10^6 to 10^7 spores of a potential host in 3 ml of top agar and poured onto a plate of complete agar. Bacteriophages were identified as plaques on mycelial lawns after incubation of plates at 55°C (45°C for *T. alba*) for 24 h. Agar plugs from isolated plaques were removed with a Pasteur pipette and transferred to 1 ml of SM for elution of phages. Single-plaque purification was repeated twice during the preparation of stocks. Bacteriophage

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TABLE 1. Distinguishing characteristics of *Thermomonospora* bacteriophages

Bacteriophage	Plaque morphology[a]	Host range[b]
Tb1	Clear, sharp	*T. alba* 1900
Tf2	Clear, sharp	*T. fusca* 27730, *T. fusca* YX
Tf3	Clear, diffuse	*T. fusca* 27730, *T. fusca* YX
Tf4	Clear, sharp	*T. fusca* YX

[a] Plaque morphology is given as morphology of the plaque center followed by morphology of the plaque margin.

[b] Phages were assayed for ability to infect *T. alba* IPV 1900, *T. chromogena* N2900, *T. curvata* ATCC 19995, *T. fusca* ATCC 27730, *T. fusca* YX, and *T. mesophila* ATCC 27303.

stocks were maintained in SM at 4°C as agar plugs from single plaques.

Preparation of lysates and purification of phage particles. For preparation of phage lysates, 10^3 to 10^4 PFU were mixed with 10^6 to 10^8 spores of an appropriate host in top agar, poured onto a plate of complete agar and then incubated for 20 h to give confluent lysis. Phage were eluted by the addition of 5 ml of SM followed by gentle agitation at 4°C for 18 h. Lysates, containing 10^7 to 10^{11} PFU/ml, were treated with chloroform (0.1 ml per 5 ml of phage suspension) and centrifuged at low speed to remove agar and cell debris.

For preparative purification of phage particles, phage from 10 lysates were precipitated by the addition of an equal volume of SM containing 2 M NaCl and 20% polyethylene glycol 8000, followed by incubation at 0°C for 2 h. Precipitates were recovered by centrifugation at 10,000 × g for 20 min and were suspended in 8 ml of SM. Crude phage suspensions were extracted twice with chloroform, and then 0.8 g of CsCl was added per ml of suspension. After centrifugation at 35,000 rpm in a Beckman type 40 rotor for 24 h, the resulting gradient was illuminated from above and the light-scattering phage band was removed through a 20-gauge needle. Purified phage preparations were stored in CsCl solution at 4°C.

Electron microscopy of purified phage particles. For electron microscopy, 2-μl samples of phage preparations were placed directly onto carbon-coated copper grids, which were rinsed gently with distilled water and negatively stained with 2% uranyl acetate. Microscopy was performed on a Philips 300 transmission electron microscope at 80 to 100 kV.

Temperature inactivation. For determination of temperature inactivation profiles, high-titer phage stocks (10^8 to 10^9 PFU/ml) were prepared by elution of phage from confluently lysed plates. These stocks were cleared with chloroform and by low-speed centrifugation and then diluted 10-fold with SM buffer and mixed gently. Aliquots of the dilutions were exposed to experimental temperatures for 30 min, removed to room temperature, and mixed briefly before plating with appropriate host spores for plaque scoring after incubation.

Analysis of phage proteins. For structural protein analysis, purified phage preparations were diluted with SM and then centrifuged at 35,000 rpm in a Beckman SW41 rotor for 90 min at 4°C. Phage pellets were suspended in 50 μl of 0.065 M Tris hydrochloride (pH 6.8) containing 10% (wt/vol) glycerol, 5% (wt/vol) 2-mercaptoethanol, and 2.3% (wt/vol) sodium dodecyl sulfate. The suspensions were allowed to stand overnight at room temperature and were then incubated at 100°C for 5 min.

Samples were loaded onto 11% polyacrylamide gels, and electrophoresis was performed by the method of Laemmli (14). Gels were stained with silver by the method of Wray et al. (31).

Isolation and analysis of phage DNA. For purification of bacteriophage DNA, phage preparations were dialyzed against TM buffer (18) for removal of CsCl. Phage were incubated with 20 mM EDTA–0.5 mg of proteinase XIV (Sigma Chemical Co., St. Louis, Mo.) per ml–0.5% sodium dodecyl sulfate at 37°C for 1 h. Phage DNA solutions were extracted with phenol-chloroform for removal of protein and dialyzed against TE buffer (10 mM Tris hydrochloride, 1 mM EDTA [pH 8.0]). The DNA concentration was estimated from the A_{260} by using the approximation that 1 A_{260} unit is equivalent to 50 μg of double-stranded DNA per ml (18). Phage DNA was precipitated with ethanol and suspended in TE to 50 μg/ml.

Phage DNA was digested with restriction endonucleases at 37°C in buffers provided by the supplier (IBI, New Haven, Conn.). Digests were analyzed by electrophoresis in agarose gels. Samples, containing 0.5 μg of DNA, were mixed with 0.4 volume of tracking dye (21) and loaded into 0.8% agarose gels prepared in TBE running buffer (18). After electrophoresis at 1 to 4 V/cm for 4 to 16 h, gels were stained for 45 min in running buffer containing 0.5 μg of ethidium bromide per ml and destained for 1 h in 1 mM MgSO₄. DNA in gels was visualized with a UV transilluminator and photographed through a Hoya O(G) filter onto Kodak Panatomic X film. Fragment lengths were estimated by comparing the migration of fragments on gels with that for *Hind*III fragments of bacteriophage lambda DNA.

Transfection of *Thermomonospora* protoplasts. For transfection, protoplasts of *T. fusca* YX were prepared as described previously (26) and suspended in a drop of P medium (24). Phage DNA (5 μg) suspended in 10 μl of TE and mixed with 10 μl of P medium, was added to the protoplasts, 0.5 ml of 20% polyethylene glycol 1000 in P medium was added immediately, and they were mixed by pipette. After incubation at room temperature for 1 min, transfection mixtures were diluted with 5 ml of P medium. Protoplasts were recovered by centrifugation at 1,000 × g for 20 min, suspended in 1 ml of P medium, and spread onto plates of R2YE regeneration agar (30). Plates were overlaid with 3 ml of top agar (R2YE prepared with 0.5% agar) containing spores of an appropriate host strain. Transfection events were detected as plaques after incubation at 45°C.

RESULTS

Phage isolation and host range. Bacteriophages which lysed strains of *T. alba* and *T. fusca* were readily isolated from samples of vegetable composts. The abundance of phage varied between 10^5 and 10^9 PFU/g of fresh soil, measured after specific enrichment of soil suspensions. Four phages were isolated on *T. alba* IPV 1900, two were isolated on *T. fusca* ATCC 27730, and six were isolated on *T. fusca* YX. No phage lysing *T. chromogena* N2900 could be isolated from four soil suspensions incubated with *T. chromogena* mycelia. On the basis of plaque morphology and host range among laboratory strains of *Thermomonospora* species (assayed by spotting lysates onto mycelial lawns), four phages were chosen for further study. These phages were given the trivial names Tb1, Tf2, Tf3, and Tf4; distinguishing characteristics of the four phages are presented in Table 1. Tb1 was maintained on *T. alba* IPV 1900, Tf2 and Tf3 were maintained on *T. fusca* ATCC 27730, and Tf4 was maintained on *T. fusca* YX. Lysates of Tb1, Tf2, Tf3, and Tf4 were also spotted onto lawns of 20 thermophilic actinomycetes which had been isolated from compost by the technique of Hirsch and Christensen (9); of
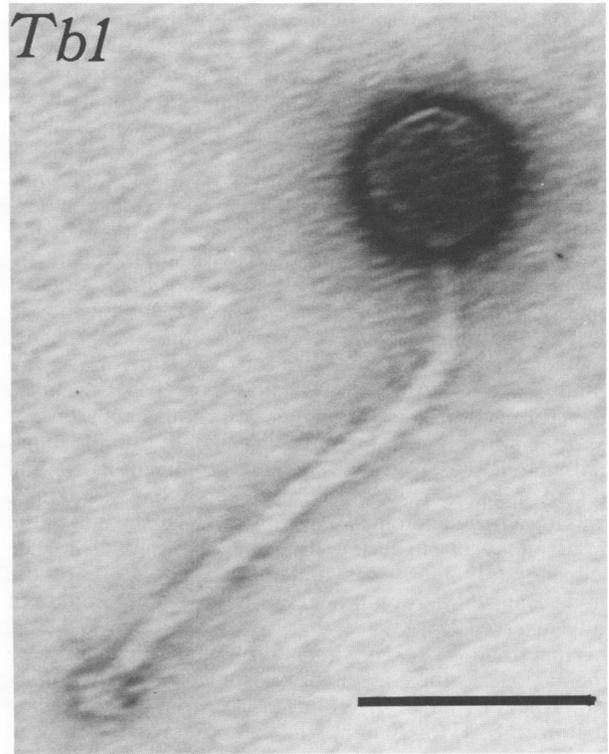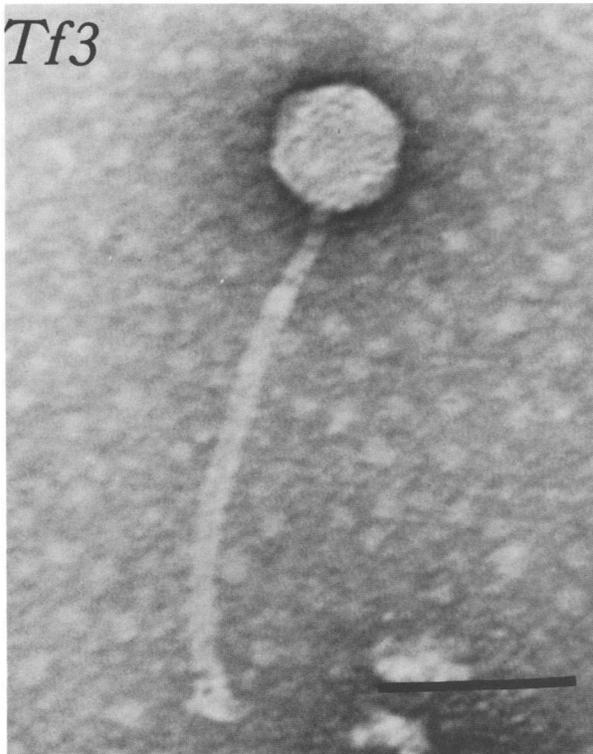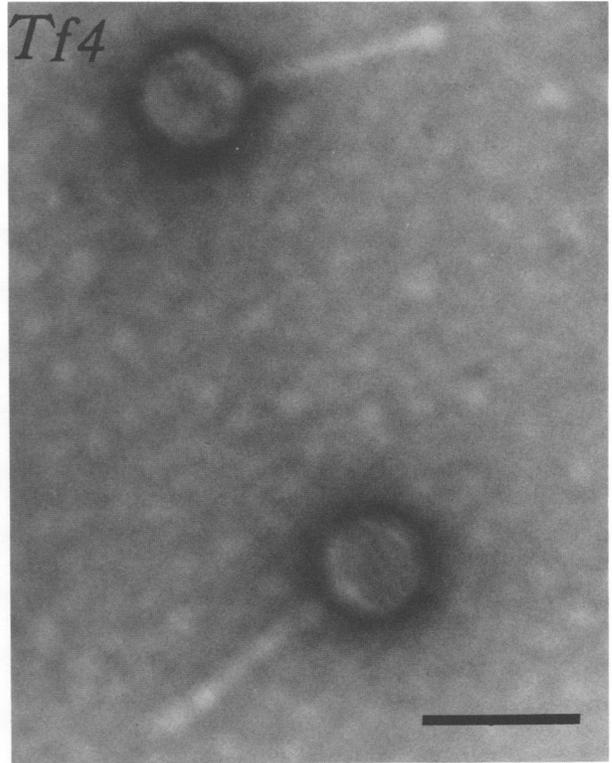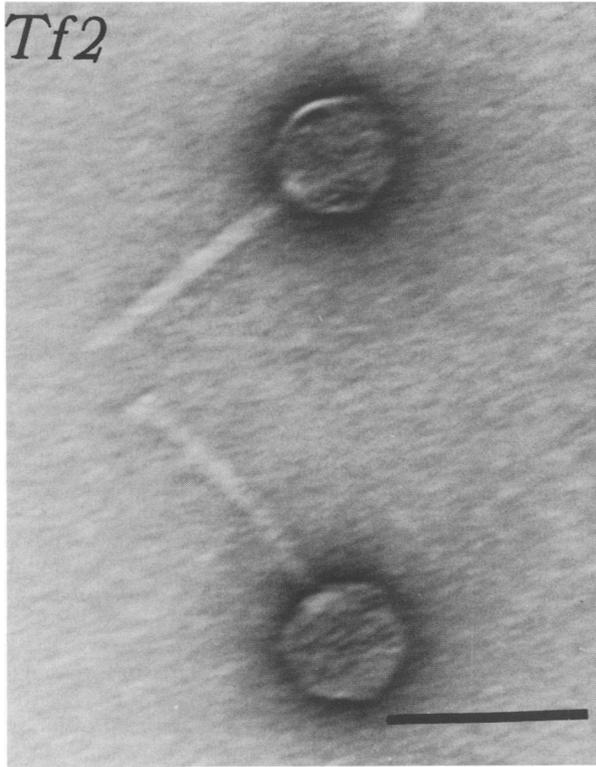
FIG. 1. Negatively stained particles of *Thermomonospora* bacteriophages. Bar, 0.1 μm.
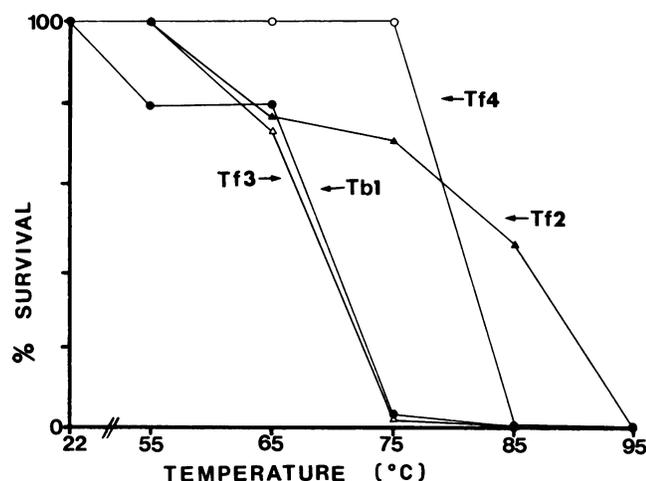
FIG. 2. Temperature inactivation of *Thermomonospora* bacteriophages after 30-min thermal treatments in SM buffer. Datum points represent averages of three or more experiments performed with each of the phages.

the 20, 18 that resembled *Thermomonospora* in colony morphology were susceptible to one or more of the four phages.

Plate lysates were routinely used as a source of *Thermomonospora* bacteriophages; lysates containing 10^8 to 10^{12} PFU could be eluted from a single confluently lysed plate. The viability of purified phage suspensions recovered from CsCl gradients was significantly lower than that predicted from lysate titers or concentrations of DNA that could be recovered from such preparations, indicating inactivation during manipulations associated with phage purification.

Morphology. Negative-stain electron-microscopic analyses of phage particles recovered from CsCl gradients (Fig. 1) showed all four of the characterized virions to be of the same general morphology, similar to coliphage lambda and belonging to group B of Bradley (2). The heads appeared polyhedral with distinct hexagonal outlines, all with approximate dimensions of 64 to 65 nm, both laterally and longitudinally. Symmetric protuberances and indentations (data not shown) suggested the multicomponent nature of the virion heads. The tail lengths of the virions were 260 to 280 nm for Tb1 and Tf3 and 122 to 125 nm for Tf2 and Tf4. Tail flexibility, obvious for the longer-tailed phages, was not as apparent for the shorter-tailed types. A central dark zone, running longitudinally along the tail, was especially prominent in Tf3 and suggested the presence of a core canal. A base structure, resembling a plate and pin, was visible at the distal end of the Tb1 tail and was suggested by some views of Tf3. The presence of tail fibers was undetermined; by use of the methods reported here, tail fibers of coliphage T4 could not be fully resolved.

Thermal stability. *Thermomonospora* bacteriophages were distinguishable in their levels of resistance to thermal inactivation (Fig. 2). Phage Tf2 retained >45% viability following incubation at 85°C for 30 min, while all others tested showed <1% survival at this temperature. Phage Tf4 was stable over the largest temperature range, but abruptly lost >99% of its plaque-forming ability between 75 and 85°C. Phages Tf3 and Tb1 exhibited lower thermostabilities than Tf2 and Tf4 and differed only in the slight loss of viability of phage Tb1 at 55°C.

Electrophoretic analysis of virion proteins and genomic

DNA. Protein analysis of *Thermomonospora* bacteriophages (Fig. 3) revealed several similarities between phages Tb1 and Tf3. The molecular weight of the main protein of these phages was 30,000. The other major bands of Tb1 and Tf3 were in the range of 35,000 to 45,000. Two sets of minor bands were observed at the molecular weight regions of 60,000 and 116,000. Two very low molecular-weight bands were observed for both phages. The protein bands of Tf2 and Tf4 also resembled each other. Tf2 had one major band at 45,000; Tf4 had one at 40,000. Tf2 showed seven minor bands between 50,000 and 100,000, while Tf4 showed 13 minor bands in the same region; these are almost certainly aggregates, and their presence, following treatment for separation of polypeptides, may be related to the high thermal stability of these two phages. The major bands presumably represent phage coat proteins

Purified genomic DNA from *Thermomonospora* bacteriophages was digested with each of five restriction endonucleases having hexanucleotide recognition sites and then analyzed by agarose gel electrophoresis; data are presented in Table 2, and *Bam*HI digests are shown in Fig. 4. With one exception, the production of *Hin*dIII fragments of approximately 23 and 12 kilobases from DNA of both Tf2 and Tf4, restriction fragment patterns obtained for each of the phage genomes were unique. Genomes of phages Tf2 and Tf4 possessed numerous *Bam*HI and *Pst*I sites, distinguishing them from Tb1 and Tf3. Genomic DNA of phage Tb1 was not digested with *Pst*I, which may be the result of modification of Tb1 DNA at these sites. Samples of genomic DNA of *Thermomonospora* bacteriophages were extracted with acidic phenol (32), which quantitatively removes linear double-stranded DNA from aqueous solutions, and electrophoresed. No ethidium bromide-stained material was detected, demonstrating that the genomes are linear molecules. The presence of cohesive termini was suggested by minor fragments, in some restriction digests, which were eliminated when the digests were heated prior to electrophoresis. This is shown for an *Eco*RI digest of Tf2 genomic DNA in Fig. 5;
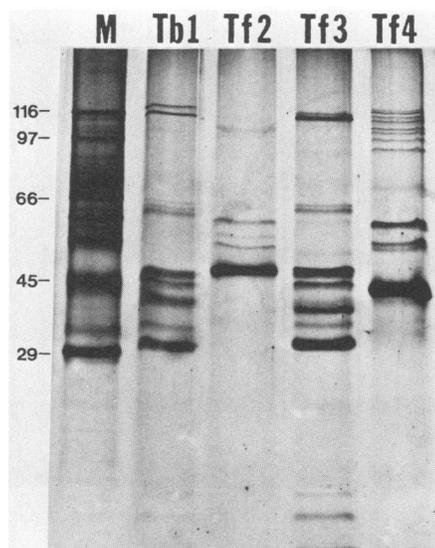
FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of major virion proteins of *Thermomonospora* bacteriophages. Lane M, molecular weight standards. Molecular masses, in kilodaltons, are indicated in the left margin.

similar phenomena were noted for genomic digests of phages Tf3 and Tf4.

Transfection of *T. fusca* YX protoplasts. *T. fusca* YX protoplasts were transfected at a frequency of approximately 50 transfection events per μg of DNA by using genomic DNA of phage Tf2. Single plaques (transfection frequency, <5 events per μg of DNA) were observed on two occasions following transfection of *T. fusca* YX protoplasts with genomic DNA of phage Tf3. Transfection of *T. fusca* YX with genomes of Tb1 and Tf4 has not been detected. No plaques could be detected when phage DNA solutions were mixed with spores of an appropriate host strain and plated onto complete agar, demonstrating that the transfection events observed were the result of uptake and expression of purified DNA by *T. fusca* YX protoplasts.

DISCUSSION

Information on bacteriophages of thermophilic actinomycetes in general, and of *Thermomonospora* species in particular, is limited. Patel (25) reported phagelike particles from cultures of a lysogenic strain identified as *Thermomonospora*, but neither the phage nor its host was described in detail. Kurup and Heinzen (11) isolated several actinophages from lysogenic strains of *Micropolyspora*; none of these infected any of five *Thermomonospora* strains tested. As reported here, bacteriophages infecting strains of *Thermomonospora* appear to be abundant in organic composts. While the phages described were isolated following specific enrichment, phages infecting *T. fusca* YX were isolated from fresh soils at concentrations of >10⁴ PFU/g (data not shown).

The general morphology and genome structures of the four *Thermomonospora* bacteriophages do not distinguish them greatly from the majority of actinophages described for *Streptomyces* species (17), most of which fall into group B of Bradley (2). Published electron micrographs of phi C31 (29) show the presence of a baseplate pin on the tail analogous to that of *Thermomonospora* phage Tb1, while R4 (3) resembles the other *Thermomonospora* actinophages in lacking such a structure. As with all bacteriophages reported to infect actinomycetes, the phages described here possess double-stranded DNA genomes. These phages display a high degree of thermostability, which may help to ensure their survival in organic composts, in which temperatures may exceed 70°C (12).

While the bacteriophages described here clearly represent distinct forms, several lines of evidence suggest a division of the phages into two classes, one containing Tf2 and Tf4 and the other containing Tb1 and Tf3. Criteria forming the basis for such a classification include tail length, genome length, thermostability, and similarities in molecular weight distributions of major virion proteins. The structural similarities

FIG. 4. Agarose gel electrophoresis of purified genomic DNA from *Thermomonospora* bacteriophages (−) and phage DNA digested with BamHI (+). Lanes M, fragment length standards, HindIII digest of bacteriophage lambda DNA, giving fragments of 23.1, 9.4, 6.6, 4.3, 2.3, and 2.0 kilobases.

are not reflected in the observed host ranges of the phages. For example, while Tb1 and Tf3 are quite similar structurally, only one *Thermomonospora* strain, a soil isolate from this laboratory, has been shown to be susceptible to both phages. Similarly, several strains, including *T. fusca* ATCC 27730, were shown to be susceptible to Tf2 but resistant to Tf4. It should be noted that the assay used to determine host range may not have been sufficiently sensitive to detect efficient restriction (coefficient, <10⁻⁵) by any host on any phage. The failure of the actinophages described here, which were isolated on strains of *T. alba* and *T. fusca*, to infect type strains of *T. chromogena*, *T. curvata*, and *T. mesophila* suggests that the phages may be taxon specific; isolation of
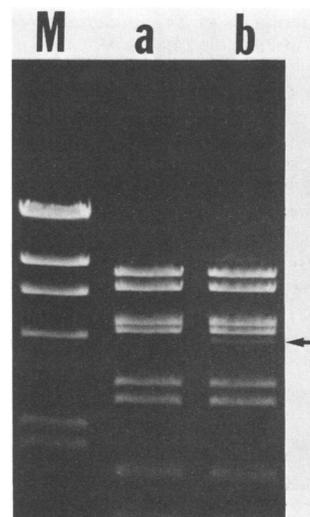
FIG. 5. Electrophoretic evidence for cohesive termini in DNA of *Thermomonospora* bacteriophage Tf2. Purified Tf2 DNA was digested with an excess of *Eco*RI, stored overnight at 4°C, and then divided into two samples. Lanes: a, sample incubated at 65°C for 10 min prior to electrophoresis; b, sample loaded directly into gel; M, fragment length standards, HindIII digest of bacteriophage lambda DNA (fragment lengths as in Fig. 4). Arrow indicates the position of the suspected termini-containing fragment.

TABLE 2. Physical analysis of *Thermomonospora* bacteriophage DNA

Bacteriophage	No. of target sites for[a]:					Estimated length[b] ± SE
	BamHI	BglII	EcoRI	HindIII	PstI	
Tb1	13	6	12	9	0	43 ± 3
Tf2	>12	3	8	1	>12	35 ± 1
Tf3	12	8	8	8	12	45 ± 2
Tf4	>12	2	5	1	>11	36 ± 1

[a] Number of sites assumes a linear, nonpermutated molecule.

[b] Lengths (in kilobases) were estimated from the summation of fragment lengths from three separate digests of each genome.

phages infecting these species and susceptibility testing of a large number of identified *Thermomonospora* strains would help to clarify this.

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