The Isolation and Characterization of Bacteriophages Infecting Obligately Thermophilic Strains of *Bacillus*

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Twenty-four thermophilic bacteriophages have been isolated from diverse sources such as compost, soil, silage and rotting straw. Although considerable individual host specificity was observed, the phages were able to infect most of the major taxonomic groups of *Bacillus* thermophiles. The phages varied considerably in morphology and size; the phage heads were either cylindrical or polyhedral with tails varying in length between 15 and 500 nm. Most of the phages were stable at 50 °C for 4–5 h but at 70 °C the plaque-forming units decreased by between 10²- and 10⁷-fold in 2 h. The DNA of morphologically similar phages was examined by restriction enzyme analysis, and some differences in the DNA fragment patterns were found. Efficiency of plating data indicated that '*B. caldodenax*' has a restriction and modification system. These phages may be valuable for the study of the genetics of thermophilic bacilli: transduction of '*B. caldodenax*' and '*B. caldovelox*' by phage JS017 has been observed.

INTRODUCTION

A few thermophilic bacteriophages able to infect strains of *Bacillus stearothermophilus* have been isolated and characterized. These include $\phi\mu$ -4 (Thompson & Shafia, 1962), TP1C (Welker & Campbell, 1965), TP84 (Saunders & Campbell, 1965), T ϕ 3 (Egbert & Mitchell, 1967), D5 (Reanney & Wood, 1973), and the earlier isolates of White *et al.* (1955) and Koser (1926). *B. stearothermophilus* is an extremely heterogeneous group of micro-organisms, with strains described having DNA compositions varying from 42 to 71 mol % G + C (Sharp & Woodrow, 1982). Walker & Wolf (1971) described three major sub-groups of *B. stearothermophilus* and a number of minor groups based on biochemical and physiological character. '*Bacillus caldodenax*', '*B. caldovelox*' and '*B. caldolyticus*', isolated from hot springs in Yellowstone National Park (Heinen & Heinen, 1972), are closely related to the previously described obligately thermophilic sporeformer, '*B. kaustophilus*' (Prickett, 1928), now designated *B. stearothermophilus* ATCC 8005. Their minimum growth temperature is 42 °C and all grow at 75 °C; their spores are oval and terminal with swelling of the sporangia.

The aim of this research was to isolate phages able to infect the major taxonomic groups of *B. stearothermophilus* and to provide a relatively simple means of sub-group identification.

METHODS

Organisms. '*B. caldodenax*' DSM 406, '*B. caldovelox*' DSM 411, '*B. caldolyticus*' DSM 405, the *Bacillus* thermophile RS 93, and *B. stearothermophilus* strains NCA 1503, NW 10, EP 240, EP 262, EP 136, DSM 2334 and ATCC 12016 were used as phage-sensitive indicator strains. These strains have been described by Sharp *et al.* (1980) and Wolf & Sharp (1981).

Streptomycin-resistant (Str^r) strains of *B. stearothermophilus* NCA 1503 and the *Bacillus* thermophiles RS 239, RS 240, RS 241 and RS 242 were selected following repeated subculture on Tryptone Soya Agar (TSA, Oxoid CM31) supplemented with streptomycin at 15–100 µg ml⁻¹ (Sharp & Woodrow, 1982).

Additional strains used in examining phage host range were *B. stearothersophilus* NW 4S (Welker & Campbell, 1965), *B. coagulans* ATCC 8038 and ATCC 12245, *B. stearothersophilus* ATCC 8005, *Bacillus thermophile* strains RS 15, RS 108, RS 125 (Sharp & Woodrow, 1982), '*B. thermocatenulatus*' DSM 730, *B. subtilis* 168 and *B. amyloliquefaciens* ϕ 1.

Phages. TP1C and TP84 were from Dr N. E. Welker, North West University, Illinois, USA. Phage ATCC 12016B was obtained from the American Type Culture Collection. ϕ 29 was from Dr B. Reilly, University of Minnesota, USA. ϕ 1 was from Dr J. Spizizen, University of Arizona, Tucson, Arizona, USA. Phage JS001 was obtained by induction of *B. stearothersophilus* NCA 1503.

Media. Bacteria were routinely maintained and subcultured on TSA, which was also used as the basal medium for phage plating. Trypticase agar (Welker & Campbell, 1965) was used to overlay the phage and bacteria assay mixture.

Cultures were grown in Tryptone Soya Broth (TSB, Oxoid CM131) and BS medium (Sargeant *et al.*, 1971).

Phage maintenance and assay. Phages were maintained as suspensions in culture supernatant or PMN buffer (0.01 M-KH₂PO₄, 0.05 M-NaCl, 0.001 M-MgCl₂, pH 7.0 with KOH). Sterility was maintained by the addition of chloroform to the suspension. This was ineffective against spores from certain strains, e.g. RS 93, and some phages were chloroform sensitive. Alternatively phage suspensions were sterilized by filtration through a 0.45 μ m Millex disposable filter unit (Millipore).

Phages were titrated by the soft-agar double-layer technique (Adams, 1959) using 0.6% (w/v) trypticase soft agar supplemented with 0.015 M-CaCl₂. Phage dilutions were made in 0.09% (w/v) saline or PMN buffer and were incubated at 55 °C unless stated otherwise.

Isolation of phages from soil and compost samples using a range of indicator strains. Samples (5 g) of soil, mud, compost, etc., were added to 100 ml TSB and incubated at 55 °C for 7 h. After removal of the cells and debris by centrifugation, 10 ml samples were inoculated with a range of potential phage-sensitive indicator strains. Dilutions of filtered culture supernatant were plated with the respective potential host strains, using soft agars. Single plaques were transferred with tooth picks to 5 ml PMN buffer for replating. Three successive reselection steps were made for each plaque type isolated.

Isolation of phages for specific strains (after Romig & Brodetsky, 1961). Samples (5 g) of soil, mud or compost were added to 15 ml water and incubated at 50 °C for 24 h. The samples were shaken and then allowed to stand for 30 min to allow debris to settle. A sample (5 ml) from the top layer was added to 10 ml TSB (supplemented with 0.015 M-CaCl₂) and incubated at 55 °C. After 4 h, 10 ml fresh TSB, 1.5 ml of a 6–8 h culture of a Str^r strain of *B. stearothersophilus* and 50 μ g streptomycin ml⁻¹ were added. Incubation was continued for a further 6 h. After centrifugation at 4000 g for 20 min the culture supernatant was diluted and plated on the appropriate Str^r strain. Single plaques were isolated and purified as described above.

Production of phage lysates. Phage suspensions of up to 10⁹ p.f.u. ml⁻¹ were prepared after lysis in the soft agar layer of double-layered plates.

The host strain was incubated in 10 ml BS broth at 55 °C for 5–6 h, giving a cell density of 5×10^7 – 10^8 viable cells ml⁻¹. Cell suspension (0.5 ml) and phage suspension (0.5 ml; 10^5 – 10^6 p.f.u. ml⁻¹) were added to 3 ml trypticase soft agar at 55 °C, spread over TSA plates and incubated overnight at 55 °C. The soft agar layer was removed using a 10 μ l disposable loop to a 50 ml centrifuge tube, mixed with 10 ml PMN buffer (approximately 5 ml additional buffer was added for each extra plate used) and centrifuged at 6000 g for 15 min. For transduction studies, or stock suspensions, the supernatant was generally sterilized by filtration and not purified further.

To obtain sufficient phage for DNA isolation, infections were carried out in liquid medium. The host strain was grown in TYF broth (Welker & Campbell, 1965) in 500 ml or 1000 ml Erlenmeyer flasks at 55 °C, shaken at 150 r.p.m. At mid-exponential phase (OD₄₂₀ 0.55–0.6) the phage suspension was added (to give a multiplicity of infection of 0.1) together with CaCl₂ (0.015 M) and glucose (0.4%, w/v). Incubation was continued for a further 3–4 h before collecting the culture supernatant. Phage titres were in the region of 10¹⁰–10¹¹ p.f.u. ml⁻¹.

When phage preparations were to be used for electron microscopy, or for DNA isolation or buoyant density studies, they were concentrated and further purified. The lysate supernatant was treated with DNAase, RNAase and lysozyme (each 10 μ g ml⁻¹) plus 203 μ g MgCl₂ · 6H₂O ml⁻¹, and incubated for 2 h at 37 °C. The phage were then sedimented by centrifugation at 30000 g for 3 h. The pellet was resuspended in 2–5 ml PMN buffer and dialysed against 2 $\times$ 2 litres PMN buffer for 48 h. The phage particles were centrifuged for 24 h on a 5 ml CsCl gradient (7.5 M-CsCl in 0.02 M-Tris buffer pH 7.2) at 160000 g at 6 °C. Phage bands were collected and dialysed for 24 h against 2 $\times$ 2 litres PMN buffer.

Study of phage plaque morphology and host range. Plaque morphology was examined after plating phage dilutions with the appropriate indicator strain and incubating at 60 °C for 16–18 h.

Phage suspensions (10⁶ p.f.u. ml⁻¹) were prepared after infection of the most suitable host available (generally '*B. caldotenax*', *B. stearothersophilus* NCA 1503, or strains RS 239 to RS 242). The preparation was serially diluted and dilutions were plated against a range of test strains.

Examination of host restriction and modification of phages. Phages were harvested following lysis on two independent host strains. The two phage populations were then titrated on both host strains. The phage titre on the

original host strain was considered to represent an e.o.p. of 1 and was compared with the e.o.p. on the second host strain.

Thermal inactivation of phages. Suspensions of bacteriophage in PMN buffer were adjusted to 10^7 – 10^8 p.f.u. ml⁻¹; 3 ml volumes were placed in 5 ml Pyrex tubes in 50 °C, 60 °C and 70 °C water baths, and 0.5 ml samples were titrated on the appropriate host at intervals up to 4.5 h.

Determination of buoyant density of phage particles. Percoll (Pharmacia) (49.5 ml) was mixed with 1.5 M-NaCl (5.5 ml) to prepare an iso-osmotic stock solution. This solution was mixed with an equal volume of 0.1 M-NaCl and 7 ml added to ultracentrifuge tubes (7 ml). Concentrated phage preparations (100 µl) were loaded on the top of Percoll gradients. Two control tubes, used to prepare a calibration curve for each centrifuge run, were prepared with 10 µl each of nine density marker beads (Pharmacia) ranging from 1.017 to 1.143 g ml⁻¹. After centrifugation at 60000 g for 15 min the distance from the base of the tube of the phage bands in the Percoll gradients was measured and buoyant densities were determined by reference to the calibration curve.

Examination of phages by electron microscopy. Phage suspensions were purified on CsCl gradients followed by dialysis for 48 h against 2 × 1 litre PMN buffer. A drop of the purified suspension (10^{10} p.f.u. ml⁻¹) was placed on a 200 mesh copper specimen grid coated with a Formvar/carbon film. After approximately 30 s the drop was gently removed by touching the edge of the grid with a piece of filter paper. A drop of negative stain (1%, w/v, phosphotungstic acid, pH 6.8) was then placed on the grid and similarly removed after approximately 10 s. The grids were examined in either a Philips EM300 or a Philips EM400T electron microscope operated at an accelerating voltage of 80 kV. Micrographs were recorded on Ilford EM technical film at magnifications of × 28000–× 46000. Negatives were developed in Kodak D163 developer and printed on Ilford print photographic paper, enlarging × 2 or × 3.

DNA isolation and purification. After overnight dialysis against 1000 vols PMN buffer, phage preparations were adjusted to a minimum of 10^{11} p.f.u. ml⁻¹. Proteinase K (BDH) dissolved in PMN buffer was added (20 µg ml⁻¹) and the mixture was incubated at 37 °C for 30 min. The phages were then dialysed for 6 h at 4 °C against 500 vols PMN buffer. The dialysis buffer was then changed to formamide buffer (200 mM-Tris/HCl, pH 7.5; 10 mM-Na₂EDTA; 50% formamide; pH to 8.5 with 4 M-HCl) for 16 h, followed by 48 h dialysis against 2 × 1000 vols 20 mM-Tris, 1 mM-EDTA buffer, pH 7.5. Phage DNA was assayed by absorbance at 260 nm and its purity assessed by the absorbance ratio at 260:280:230 nm.

A phenol extraction method derived from that of Saunders & Campbell (1965) was also used. An equal volume of 90% (v/v) phenol equilibrated twice against PMN buffer was added to the phage suspension in saline EDTA (0.15 M-NaCl, 0.1 M-EDTA, pH 8.0) and shaken for 30 min at 4 °C. The aqueous phase was extracted three times with diethyl ether and the DNA was precipitated with 2 vols cold 95% (v/v) ethanol. The DNA was dissolved in 0.1 × SSC buffer (Marmur & Doty, 1962) and dialysed for 48 h at 4 °C against 2 × 1000 vols 20 mM-Tris, 1 mM-EDTA buffer, pH 7.5.

The DNA was purified further by CsCl/ethidium bromide density centrifugation. A total of 5 ml DNA suspension in 20 mM-Tris, 1 mM-EDTA (pH 7.5) was added to 5 g CsCl with 50 µl ethidium bromide solution (10 mg ml⁻¹). The mixture was transferred to 10 ml polycarbonate centrifuge tubes and centrifuged at 150000 g at 6 °C for 20 h. The DNA bands were visualized under UV light and were collected with a 2 ml syringe. Ethidium bromide was removed by washing twice with an equal volume of isoamyl alcohol. The DNA was then dialysed for 24 h against 2000 vols PMN buffer.

Examination of phage DNA with restriction endonucleases. DNA samples were dialysed overnight against a low-salt buffer (10 mM-Tris, 10 mM-MgSO₄, 1 mM-dithiothreitol, pH 7.4). DNA (1 µg) was incubated in 20 µl of this buffer with 1–2 units of enzyme for 3 h at 37 °C (or 60 °C when using endonuclease *Bcl*I). The digested samples were heated at 70 °C for 5 min to inactivate the enzyme. Electrophoresis was done in a Protean double-slab electrophoresis cell (Bio-Rad). Vertical slab gels with 15 wells (3 mm wide) were prepared using 0.8% (w/v) agarose dissolved in Tris/borate buffer (90 mM-Tris; 90 mM-boric acid; 3 mM-Na₂EDTA; pH 8.0). An equal volume of loading buffer (0.02%, w/v, bromophenol blue; 20%, w/v, sucrose; 0.5%, w/v, Ficoll; 10 mM-EDTA; 5 mM-Tris/HCl; pH 7.5) was added to each sample. Gels were electrophoresed in Tris/borate buffer (pH 8) for 3 h at 30 mA, stained in ethidium bromide (2 µg ml⁻¹) for 0.5 h and destained for 16 h before examination on a UV transilluminator (254 nm). They were photographed using a Polaroid MP-4 land camera with an orange Wratten gelatin filter (Kodak).

RESULTS

Phage isolation and maintenance

Ten samples of organic material, including compost, soil and rotting vegetation, were examined for thermophilic phages using ten indicator strains. Many of the phage plaques evident in the initial isolation were lost on transfer to fresh media: all phages isolated on *B. stearothermophilus* strains NCA 1503 and EP 240 were lost, together with a number able to infect strain RS 93, '*B. caldotenax*' and '*B. caldovelox*'.

Thirteen phages, JS004–JS015 and JS027, were isolated by the method of Romig & Brodetsky (1961). Seven source materials were examined (compost, silage, rotting straw, river sludge, stable manure, soil and digested sewage sludge) for the presence of phage using five Str^r strains (*B. stearothermophilus* NCA 1503 and the *Bacillus thermophiles* RS 239, RS 240, RS 241 and RS 242). No phages were isolated from samples of river sludge or digested sewage sludge. One plaque type was isolated from stable manure, one from soil, three from silage, four from rotting straw and six from compost.

A number of thermophilic phage isolates were lost when chloroform was used to sterilize the preparations. Studies on the long-term survival of JS017 in PMN buffer with chloroform indicated a tenfold reduction in viability over 10–20 d with little further reduction over the next 200 d. The control sample showed a slight (two- to threefold) decrease in viability over 250 d. Suspensions of JS017 in filtered culture supernatant without chloroform showed no loss in p.f.u. after one year at 4 °C. Sterilization with chloroform was unsuccessful in killing spores, particularly in preparations harvested from *B. stearothermophilus* RS 93.

Plaque morphology of the thermophilic phages

Table 1 details the plaque morphologies of the thermophilic bacteriophages when plated in trypticase soft agar supplemented with 0.015 M-CaCl₂ and incubated overnight at 55 °C. Considerable variations in plaque morphology were observed using identical conditions for plating and incubation. The plaques of phage JS004, following its initial isolation and purification by single-plaque transfer, varied from 0.5 to 2 mm in diameter, appearing either hazy or clear, and round or irregular. After approximately 12 consecutive single-plaque transfers the morphology stabilized to that described in Table 1. Similar initial variations in plaque morphology were observed with JS006, JS007 and to a lesser extent the other phages isolated using strains RS 239 to RS 242.

Host restriction and modification of the thermophilic phages

The host ranges of the phages are shown in Table 2. Evidence was collected during host range studies and titrating of phage preparations which indicated that restriction and modification occurs in thermophilic species of *Bacillus* (Table 3).

Examination of the e.o.p. of phage JS001 on '*B. caldotenax*' and *B. stearothermophilus* ATCC 12016 (Table 3) indicates restriction by '*B. caldotenax*' of phage harvested from ATCC 12016. The latter strain, however, did not appear to restrict (e.o.p. 0.75) phage JS001 previously harvested from '*B. caldotenax*'. Phage JS001 harvested from '*B. caldotenax*' appeared to be modified, permitting a high level of infection when plated back to '*B. caldotenax*'. Plating of phage JS001 from '*B. caldotenax*' with '*B. caldovelox*' gave an e.o.p. of 0.8, indicating that the latter did not restrict phage from '*B. caldotenax*' (if dilution and plating variations are taken into account). An e.o.p. of 0.8 was not considered significantly different from an e.o.p. of 1. The data for phage JS001 harvested from '*B. caldotenax*' and plated on strains RS 15, RS 93, RS 108, RS 125 and EP 262 illustrate the variation in e.o.p. These data alone, while suggesting restriction, may indicate some inherent resistance in the population to phage infection.

Phages JS019, JS024 and JS025 indicated no evidence of restriction of phages harvested from '*B. caldotenax*' when plated on '*B. caldovelox*'. Conversely, these phages harvested from '*B. caldovelox*' when plated with '*B. caldotenax*' were restricted, with e.o.p. values of 0.28, 0.12 and 0.125, respectively. Phages harvested from '*B. caldotenax*' had a low e.o.p. on '*B. caldolyticus*'.

Plating of phage JS017 from '*B. caldotenax*' to '*B. caldovelox*' again showed no evidence of restriction, but plating on '*B. caldolyticus*', '*B. thermocatenulatus*' and *B. stearothermophilus* ATCC 8005 all indicated the presence of a restriction system. Phage JS017 harvested from '*B. caldovelox*' also appeared restricted by '*B. thermocatenulatus*' and ATCC 8005. Phage JS017 isolated from '*B. thermocatenulatus*' was restricted by ATCC 8005 but unusually gave an e.o.p. of 20 when plated on '*B. caldotenax*'.

Table 1. *Plaque morphology of thermophilic phages*

Phages were plated in trypticase soft agar supplemented with 0.015 M-CaCl₂ and overlaid on TSBA plates, which were incubated overnight at 55 °C.

Phage	Source	Host	Plaque description	
			Diameter (mm)	Appearance
JS004	Silage	<i>Bacillus thermophile</i> RS 239	0.5-1	Circular, hazy
JS005	Rotting straw	<i>Bacillus thermophile</i> RS 239	0.5-1	Circular, slightly hazy
JS006	Compost	<i>Bacillus thermophile</i> RS 239	0.5-0.75	Circular, clear
JS007	Silage	<i>Bacillus thermophile</i> RS 240	0.5-2	Circular, hazy
JS008	Rotting straw	<i>Bacillus thermophile</i> RS 241	1-3.5	Circular, hazy
JS009	Stable manure	<i>Bacillus thermophile</i> RS 242	0.5	Circular, hazy centre
JS010	Compost	<i>Bacillus thermophile</i> RS 242	3	Irregular, hazy with clear areas at edge
JS011	Silage	<i>Bacillus thermophile</i> RS 239	0.7	Circular, slightly hazy
JS012	Compost	<i>Bacillus thermophile</i> RS 239	0.5-2	Irregular, clear
JS013	Soil	<i>B. stearothermophilus</i> NCA 1503	0.75-1	Circular, hazy
JS014	Rotting straw	<i>B. stearothermophilus</i> NCA 1503	0.5-1	Circular, slightly hazy
JS015	Compost	<i>B. stearothermophilus</i> NCA 1503	0.5-2	Circular, slightly hazy
JS017	Compost	' <i>B. caldotenax</i> '	1-1.5	Circular, turbid/hazy centre
JS018	Rotting vegetation	' <i>B. caldotenax</i> '	1	Circular, clear centre
JS019	Rotting vegetation	' <i>B. caldotenax</i> '	1-1.5	Circular, clear centre, hazy at edges
JS020	Rotting vegetation	' <i>B. caldotenax</i> '	1-2	Circular, clear centre, hazy outer halo 4-8 mm
JS021	Rotting vegetation	' <i>B. caldotenax</i> '	2-3	Circular, clear centre, hazy outer halo
JS022	Compost	' <i>B. caldotenax</i> '	1-2	Circular, clear centre
JS023	Compost	' <i>B. caldotenax</i> '	0.5-1.5	Circular, clear centre, hazy edge
JS024	Compost	' <i>B. caldotenax</i> '	0.5-2	Circular, clear centre, hazy edge with less hazy outer edge
JS025	Compost	' <i>B. caldotenax</i> '	1-1.5	Circular, hazy
JS026	Compost	' <i>B. caldotenax</i> '	1-1.5	Circular, hazy
JS027	Compost	<i>Bacillus thermophile</i> RS 241	0.75	Circular, clear

Table 2. *Host range of thermophilic phages*

Phage suspensions (10^6 p.f.u. ml⁻¹) were prepared from lysates of '*B. caldopenax*' or the most suitable phage host available. Dilutions were then plated with the test strains and observed for plaque formation following overnight incubation at 55 °C. Results were recorded as: 4+, e.o.p. 1; 3+, e.o.p. 0.1–0.01; 2+, e.o.p. 0.01–0.001; +, e.o.p. <0.001; –, no evidence of phage infection; *, not tested. No phages examined formed plaques on *B. coagulans* ATCC 8038 and ATCC 12245, *B. subtilis* 168 and *B. amyloliquefaciens* ϕ 1. ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; NW, Neil Welker; for EP and RS designated strains see Sharp (1982).

Host strain	Phage . . .	JS 004	JS 005	JS 006	JS 007	JS 008	JS 009	JS 010	JS 011	JS 012	JS 013	JS 014	JS 015
' <i>B. caldopenax</i> ' DSM 406		–	–	–	–	–	–	–	–	–	–	–	–
' <i>B. caldovelox</i> ' DSM 411		–	–	–	–	–	–	–	–	–	–	–	–
' <i>B. caldolyticus</i> ' DSM 405		–	–	–	–	–	–	–	–	–	–	–	–
<i>B. stearothermophilus</i> NCA 1503		–	–	–	–	–	–	–	–	–	3+	3+	3+
<i>B. stearothermophilus</i> ATCC 12016		*	*	*	*	–	*	*	*	*	–	–	–
<i>B. stearothermophilus</i> NW 10		–	–	+	–	–	–	–	*	*	2+	2+	2+
<i>B. stearothermophilus</i> NW 4S		*	*	*	*	*	*	*	*	*	–	–	–
<i>B. stearothermophilus</i> EP 136		–	–	*	*	–	*	*	*	*	–	–	–
<i>B. stearothermophilus</i> RS 93		3+	–	3+	4+	–	4+	+	*	*	+	–	–
<i>B. stearothermophilus</i> EP 262		–	–	+	*	–	*	*	*	*	*	*	*
<i>B. stearothermophilus</i> EP 240		–	–	*	*	–	*	*	*	*	*	*	*
<i>B. stearothermophilus</i> ATCC 8005		*	*	*	*	*	*	*	*	*	*	*	*
' <i>B. thermocatenulatus</i> ' DSM 730		*	*	*	*	*	*	*	*	*	*	*	*
<i>Bacillus thermophile</i> RS 239		4+	4+	–	4+	4+	*	–	*	*	*	*	*
<i>Bacillus thermophile</i> RS 240		–	–	–	2+	–	*	–	*	*	*	*	*
<i>Bacillus thermophile</i> RS 241		2+	2+	–	4+	4+	4+	+	*	*	*	*	*
<i>Bacillus thermophile</i> RS 242		+	–	2+	+	–	2+	4+	*	*	*	*	*

Thermal stability of the thermophilic phages

The temperature sensitivity of phages JS005, JS007, JS014, JS017, JS019, JS024 and JS025, held for 4–5 h at 50, 60 and 70 °C, was examined. After 1 h at 70 °C all the phages showed a considerable reduction in p.f.u., which then declined only slightly with continued incubation. At 60 °C a smaller but significant decrease in p.f.u. was observed within 1–2 h, followed by a levelling off of the p.f.u. Phage JS014 differed from the other phages in that it showed similar rates of inactivation at 60 °C and 50 °C. At 50 °C most of the phages showed little decrease in p.f.u. (less than tenfold) after 4 h. Exceptions were JS024 (99% decrease) and JS025 (99.9% decrease).

Estimation of buoyant density of the thermophilic phages

Bouyant density estimations using calibrated Percoll gradients indicated that several of the phages formed two or more bands in the gradients (Table 4). The bands were collected with a syringe and examined for the presence of viable phages. The bouyant density values in Table 4 correspond to either single phage bands or several bands with a high p.f.u. content. The bands with a low p.f.u. content were considered to contain phage debris or ghosts. Although none of these bands were examined by electron microscopy, electron micrographs of phage preparations have shown the presence of many phage ghosts and large numbers of free tails.

Size and morphology of the thermophilic phages

Electron micrographs (Fig. 1) of 10 of the thermophilic phages show four distinct morphological types (Table 5). Phages JS004 and JS027 were characterized by a comparatively short stubby tail and no evidence of a tail plate. Phages JS019, JS022, JS024 and JS025 all had icosahedral heads of approximately 40–65 nm diameter with tails between 120 nm and 135 nm long, and all showed evidence of a tail plate. Phage JS014 had an icosahedral head 85–90 nm in diameter and a long (400 nm) flexible tail with helical symmetry. Phages JS017 and JS026 had cylindrical heads with rounded ends, with tails varying between 115 nm and 120 nm long.

Table 2. (continued)

Host strain	Phage . . .	JS 017	JS 018	JS 019	JS 020	JS 021	JS 022	JS 023	JS 024	JS 025	JS 026	JS 027
' <i>B. caldotenax</i> ' DSM 406		4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	—
' <i>B. caldovelox</i> ' DSM 411		4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	—
' <i>B. caldolyticus</i> ' DSM 405		+	4+	+	4+	3+	+	—	3+	3+	3+	—
<i>B. stearothermophilus</i> NCA 1503		—	—	—	—	—	—	—	—	—	—	—
<i>B. stearothermophilus</i> ATCC 12016		3+	—	—	—	+	—	—	—	—	2+	—
<i>B. stearothermophilus</i> NW 10		2+	—	—	—	—	—	—	—	—	—	—
<i>B. stearothermophilus</i> NW 4S		*	*	*	*	*	*	*	*	*	*	—
<i>B. stearothermophilus</i> EP 136		—	—	—	+	—	—	+	+	—	—	—
<i>B. stearothermophilus</i> RS 93		3+	3+	3+	3+	3+	4+	3+	3+	3+	3+	—
<i>B. stearothermophilus</i> EP 262		2+	—	—	—	—	—	—	—	—	—	—
<i>B. stearothermophilus</i> EP 240		+	+	+	+	+	+	—	+	+	—	—
<i>B. stearothermophilus</i> ATCC 8005		2+	*	*	*	—	—	—	—	—	—	—
' <i>B. thermocatenulatus</i> ' DSM 730		2+	*	*	*	—	—	—	—	—	—	—
<i>Bacillus thermophile</i> RS 239		*	*	*	*	—	—	—	—	—	—	3+
<i>Bacillus thermophile</i> RS 240		*	*	*	*	—	—	—	—	—	—	—
<i>Bacillus thermophile</i> RS 241		*	*	*	*	—	—	—	—	—	—	4+
<i>Bacillus thermophile</i> RS 242		*	*	*	*	—	—	—	—	—	—	—

Table 3. Restriction and modification of phages by thermophilic hosts based on the e.o.p.

Phages were titrated using the soft agar plating technique; plates were incubated overnight at 60 °C.

Phage	Host used for preparation of phage	Hosts used for titration of phage	Plaque count	E.o.p.
JS001	' <i>B. caldotenax</i> '	' <i>B. caldotenax</i> '	8×10^8	1
		<i>B. stearothermophilus</i> ATCC 12016	6×10^8	0.75
JS001	<i>B. stearothermophilus</i> ATCC 12016	<i>B. stearothermophilus</i> ATCC 12016	1×10^6	1
		' <i>B. caldotenax</i> '	1×10^2	0.0001
JS001	' <i>B. caldotenax</i> '	' <i>B. caldotenax</i> '	2.7×10^5	1
		' <i>B. caldovelox</i> '	2.2×10^5	0.8
		<i>Bacillus thermophile</i> RS 15	5.0×10^4	0.19
		<i>Bacillus thermophile</i> RS 108	1.25×10^4	0.046
		<i>Bacillus thermophile</i> RS 125	4.0×10^3	0.015
		<i>B. stearothermophilus</i> RS 93	1.3×10^2	0.0005
		<i>B. stearothermophilus</i> EP 262	1.0×10^1	0.00004
JS019	' <i>B. caldotenax</i> '	' <i>B. caldotenax</i> '	2×10^4	1
		' <i>B. caldovelox</i> '	2×10^4	1
JS019	' <i>B. caldovelox</i> '	' <i>B. caldovelox</i> '	1×10^6	1
		' <i>B. caldotenax</i> '	2.8×10^5	0.28
JS017	' <i>B. caldotenax</i> '	' <i>B. caldotenax</i> '	5×10^7	1
		' <i>B. caldovelox</i> '	5×10^7	1
		' <i>B. caldolyticus</i> '	1.2×10^2	0.000002
		' <i>B. thermocatenulatus</i> '	1.4×10^5	0.003
		<i>B. stearothermophilus</i> ATCC 8005	1.5×10^5	0.003
JS017	' <i>B. caldovelox</i> '	' <i>B. caldovelox</i> '	3.2×10^5	1
		' <i>B. thermocatenulatus</i> '	4.0×10^2	0.001
		<i>B. stearothermophilus</i> ATCC 8005	7.0×10^2	0.002
JS017	' <i>B. thermocatenulatus</i> '	' <i>B. thermocatenulatus</i> '	3.0×10^4	1
		' <i>B. caldotenax</i> '	6.0×10^5	20
		<i>B. stearothermophilus</i>	2.0×10^3	0.07
JS024	' <i>B. caldotenax</i> '	' <i>B. caldotenax</i> '	1×10^7	1
		' <i>B. caldovelox</i> '	7×10^6	0.7
		' <i>B. caldolyticus</i> '	1×10^5	0.01
JS024	' <i>B. caldovelox</i> '	' <i>B. caldovelox</i> '	1.2×10^7	1
		' <i>B. caldotenax</i> '	1.4×10^6	0.12
JS025	' <i>B. caldotenax</i> '	' <i>B. caldotenax</i> '	1×10^7	1
		' <i>B. caldovelox</i> '	1×10^7	1
		' <i>B. caldolyticus</i> '	4×10^5	0.04
JS025	' <i>B. caldovelox</i> '	' <i>B. caldovelox</i> '	8×10^6	1
		' <i>B. caldotenax</i> '	1×10^6	0.125

Fig. 1. Electron micrographs of thermophilic phages. (a) JS004, (b) JS007, (c) JS014, (d) JS017, (e) JS017 showing variation in length of the phage heads, (f) JS017 with an unusually long tail, (g) JS019, (h) JS022, (i) JS024, (j) JS025, (k) JS026, (l) JS027. Bars, 100 nm.

Table 4. *Determination of buoyant density of phages using calibrated Percoll gradients*

Percoll gradients were calibrated with density marker beads. When more than one band was apparent in the gradient, each band was examined for the presence of high numbers of viable phage particles. When the band was wide or consisted of several closely spaced bands with a high p.f.u. content the buoyant density range was recorded.

Phage	Buoyant density of high-p.f.u. band	Buoyant density of low-p.f.u. band where present
λ	1.04–1.055	–
T7	1.025–1.05	–
TP1C	1.1	1.035–1.045
TP84	1.075	–
JS002	1.08	1.05
JS005	1.04	–
JS017	1.0, 1.02, 1.025, 1.035	–
JS019	1.045	–
JS022	1.065	1.04
JS025	1.02	1.045

Table 5. *Size and morphology of thermophilic phages*

Phage	Group assignment	Shape of head	Size of head (nm)	Length of tail (nm)	Evidence of tail base plate	Comments
JS004	1	Icosahedral	55-60	15	-	Short cylindrical tail with collar
JS007	3	Icosahedral	80-85	400-420	-	Long flexible filamentous tail with cross-striations; fine fibre at tip of tail visible on some particles
JS014	3	Icosahedral	85-90	400	-	Long flexible filamentous tail with cross-striations
JS017	4	Cylindrical	80 x 40	115	+	Rigid tail with cross-striations; triangular base plate
JS019	2	Icosahedral	50	130	+	Rigid tail
JS022	2	Icosahedral	60-65	135	+	Rigid tail
JS024	2	Icosahedral	60	120	+	Tail shows limited flexibility
JS025	2	Icosahedral	45-50	120	+	Rigid tail
JS026	4	Cylindrical	80 x 45-50	120	+	Tail shows limited flexibility
JS027	1	Icosahedral	55-60	13	-	Signs of a very short stubby tail on a few particles

Fig. 2. Diagrammatic representation of restriction endonuclease cleavage fragment patterns of the DNA of four thermophilic phages. All endonuclease digestions were done at 37 °C (*Bcl*I at 60 °C) for 3 h. Enzymes were inactivated at 70 °C for 5 min and the samples were loaded onto a 0.8% agarose gel and run at 30 mA for 3 h. A *Hind*III digest of λ DNA was included as a size calibration marker. The asterisks indicate smearing of the gel, showing general digestion of the DNA.

Between 1% and 5% of the JS017 population had heads of twice the normal length (Fig. 1*e*). The occurrence of phage JS017 with longer tails was also observed (Fig. 1*f*); the phage head was slightly narrower than the normal head and the tail showed clear evidence of striations or segmentation. Phage ghosts of JS026 and JS017 showed evidence of being waisted at the centre and some of the larger phage heads of JS017 appeared to have striations around the head. Both phages appeared to have a triangular tail plate.

Examination of phage DNA with restriction endonucleases

DNA from four thermophilic phages was examined after digestion with restriction endonucleases and agarose gel electrophoresis (Fig. 2). This technique enabled the differentiation of phages which appeared morphologically identical. Phage JS017, which was morphologically distinct from the other three phages, had a distinctive restriction endonuclease cleavage pattern. Ten *Bam*HI sites and 18 *Bcl*I sites were evident, whereas none of the remaining three phages showed evidence of DNA cleavage with these enzymes (Fig. 2). A number of enzymes appeared to cause general digestion of the phage JS017 DNA, resulting in smearing of the gel. Phages JS022, JS024 and JS025, which were all morphologically similar, showed several differences in their endonuclease cleavage patterns. JS024 had a minimum of 22 *Bgl*I sites compared with four and three for JS022 and JS025, respectively. JS024 also had no *Xba*I site compared with three and four for JS022 and JS025 respectively. The differences between phages JS022 and JS025 were small and mainly involved slight differences in the number of restriction fragments produced by the different enzymes.

DISCUSSION

Thermophilic phages infecting a wide range of thermophilic strains of *Bacillus* were readily isolated from soil and compost. The host range data indicate considerable host specificity. Four distinct taxonomic groups of obligately thermophilic strains of *Bacillus*, with five subgroups, were described by Sharp & Woodrow (1982). The phages isolated in this study are specific to three of these taxonomic groups. Phages JS004 to JS012 and JS027 all infect members of subgroup 1a, which are typified by their inability to hydrolyse starch. Those phages were specific to this group and did not infect strains in other taxonomic groups. Phages JS013 to JS015 showed close host specificity to *B. stearothermophilus* strains NCA 1503 and NW 10,

which are both members of taxonomic group 2b. This group represents the classical characteristics of *B. stearrowthermophilus* and contains the type strain. Phages JS017 to JS026 all infected '*B. caldopenax*', '*B. caldovelox*', '*B. caldolyticus*' and *B. stearrowthermophilus* ATCC 8005, all members of taxonomic group 2a. None of the phages infected the two strains of *B. coagulans* examined, or *B. subtilis* and *B. amyloliquefaciens*.

The host range of a phage is influenced by the presence of restriction and modification systems in the host cells. Catterall *et al.* (1976) speculated that restriction and modification systems in thermophilic bacilli were the rule rather than the exception. Our evidence suggests that '*B. caldopenax*' has a restriction system which is able to reduce by up to 10^{-4} the e.o.p. of phages harvested from some strains of *B. stearrowthermophilus*. Restriction of phages harvested from '*B. caldovelox*', which is taxonomically very similar to '*B. caldopenax*', is evident but at a much lower level. A number of other strains appear to restrict phage infection, including '*B. caldolyticus*' and *Bacillus thermophiles* RS 15, RS 93, RS 108 and RS 125.

Four morphological phage types were observed; the majority (JS019, JS022, JS024 and JS025 from lysates of '*B. caldopenax*') had polyhedral heads (45–65 nm in diameter) with tails (120–135 nm long). Their morphology closely resembles that of the *B. stearrowthermophilus* phages previously reported: TP1C from lysates of strain 4S (Welker & Campbell, 1965), TP84 infecting strain NW 10 (Bassel *et al.*, 1971), $\phi\mu$ -4 infecting strain NU 10 (Rabussay *et al.*, 1970) and T ϕ 3 from lysates of ATCC 8005 (Egbert, 1969). Two phages, JS004 and JS027, were distinct in having icosahedral heads, 55–60 nm in diameter, but short stubby tails, 13–15 nm long. Phage JS014, which infected *B. stearrowthermophilus* NCA 1503, had an icosahedral head 90 nm in diameter, with a long (400 nm) flexible tail, showing helical symmetry. Reanney & Wood (1973) described a phage (D5) isolated from compost, which infected *B. stearrowthermophilus* NRS T91, a strain derived from the original NCA 1503 isolate (Gordon *et al.*, 1973). Phage D5 was reported to have an icosahedral head 87 nm in diameter and a striated tail, 180 nm long (Reanney & Wood, 1973). Phage JS007, which infected starch-negative strains, had a head of similar diameter (80–85 nm) and a flexible tail up to 420 nm long. This appears to be exceptional; Hemphill & Whitely (1975) reviewed the morphology of 16 *B. subtilis* phages having tails of between only 30 nm and 250 nm in length. The cylindrical heads of phages JS017 and JS026 were morphologically distinct from all previously described thermophilic phages. JS017 showed variable morphology, with some phage heads double the normal length of 80–90 nm. In some preparations these variants numbered between 1 and 5% of the population and it was initially considered that they might be transducing particles (Munster *et al.*, 1982). Subsequent examination of a number of transducing phage preparations by electron microscopy indicated that whereas the proportion of these variants ranged from 0 to 5%, the transducing ability of the individual preparations did not vary significantly.

B. subtilis phages have been characterized or 'fingerprinted' by examination of the restriction endonuclease digestion pattern of their DNA; ϕ 3T, SP02 and ϕ 105 were examined by Wilson *et al.* (1974), and ϕ 29 and ϕ 15 by Ito & Kawamura (1976). Phages JS022, JS024 and JS025 were morphologically identical (Table 5) and all had similar host ranges. Examination of their restriction endonuclease 'fingerprints' (Fig. 2) indicated a close similarity between JS022 and JS025, with small, but significant differences between these two and JS024. Such 'finger-printing' of thermophilic phages has not been previously reported, but it does offer a relatively simple means of distinguishing between phages with identical host range and morphology.

The buoyant densities of the thermophilic phages in Percoll gradients ranged from 1.0 to 1.1 g ml⁻¹. The occurrence of multiple, or two or three separate bands from individual phage preparations indicated the occurrence of multiple density classes of phage particles. These were probably the result of damaged phage particles which banded separately in the gradient as phage heads and tails. In the examination of several phage preparations by electron microscopy phage ghosts were often predominant; the absence of DNA within the phage heads would result in phage particles with different densities. Karamata (1970) identified multiple density classes of phage P1 which were the result of tetramer formation through binding at phage base plates. In this form the phages were not infective, although they were protected from inactivation by phage antiserum. The multiple-density bands found in some preparations of thermophilic

phages were not examined by electron microscopy, although only one such band showed evidence of a high p.f.u. content. The four bands of similar density found for JS017 may be the result of variations in the size of the phage heads and tails as previously observed. A re-examination of the buoyant density of phage JS017 in a Percoll gradient showed two bands, with densities of 0.98 and 1.017 g ml⁻¹.

Thermal stability studies on seven phage isolates showed few significant differences between them. The p.f.u. of most phage preparations fell less than tenfold after 4–5 h at 50 °C; the exceptions were JS007, JS024 and JS025, whose p.f.u. decreased by factors of 10², 10² and 10³, respectively. After 4 h at 70 °C the p.f.u. had decreased by between 10²- and 10⁷-fold. These results were comparable with those reported for most other thermophilic phages. Phage D5 showed little reduction in p.f.u. after 3 h at 60 °C but there was a 10³-fold reduction at 70 °C (Reanne & Wood, 1973). Welker & Campbell (1965) reported a 52% decrease in p.f.u. of phage TP1C in TYF medium after 30 min at 65 °C, followed by a levelling out of the survival curve. Phage $\phi\mu$ -4 suspended in distilled water showed little decrease in p.f.u. after 1 h at 75 °C, but there was a 10²-fold reduction in p.f.u. when the phage were suspended in Tris buffer (Shafia & Thompson, 1964). Onodera (1961) reported the isolation of an unusual bacteriophage from compost; this infected a thermophilic *Bacillus* sp. and consisted of spherical particles only 20 nm in diameter which were resistant to heating at 100 °C for 2 h.

Bacteriophage JS017 has been reported to carry out specialized transduction of thymine and methionine auxotrophs of '*B. caldotenax*' (Munster *et al.*, 1982). Other phages are being examined for their ability to carry out generalized transduction, which may be used in support of transformation data in establishing a preliminary genetic map of '*B. caldotenax*'.

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