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Characterization of a temperate actinophage, MPphiWR-1, capable of infecting *Micromonospora purpurea* ATCC 15835

Bruce C. Tilley[1], Janise L. Meyertons[2] and Mary P. Lechevalier[3]

Waksman Institute of Microbiology, Rutgers University, Piscataway, NJ, U.S.A.

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SUMMARY

A temperate actinophage was isolated from soil using the gentamicin-producing microorganism, *Micromonospora purpurea* ATCC 15835 as host. The characterization of the phage represents the initial step in its development as a cloning vector. The phage isolated, MPphiWR-1, formed red- to purple-pigmented turbid plaques. Cells isolated from these plaques were resistant to superinfection with lytic mutants of MPphiWR-1. Southern blots of genomic DNA from a resistant culture showed that MPphiWR-1 integrated into the host genome. The phage was UV- or Mitomycin C-inducible. The integration, resistance to superinfection and inducibility indicated a lysogenic relationship with the host.

Using MPphiE-RCPM, a lytic derivative, the phage host range was demonstrated to include members of three genera: one species each of *Ampullariella* and *Catellatospora*, and 12 species of *Micromonospora*. The phage belonged to Ackerman's B1 morphotype having an isometric head and a flexible noncontractile tail. The density of the phage was 1.525 g/cc. Restriction site mapping demonstrated that the phage DNA was 57.9 kb long and had cohesive ends. Using EDTA enrichment, viable mutants with deletions of at least 3.5 kb were isolated and mapped. Phage adsorption, sensitivities and plating efficiency were investigated. Non-liposome PEG-mediated transfection was demonstrated.

[1] Present Address: American Cyanamid Co., Lederle Labs, Middletown Rd., Pearl River, NY 10965
[2] Present Address: Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, NY 11724
[3] Correspondence: M.P. Lechevalier, Waksman Institute of Microbiology, Rutgers University, P.O. Box 759, Piscataway, NJ 08855-0759, U.S.A.

INTRODUCTION

Two-thirds of the known aminoglycoside antibiotics are produced by non-*Streptomyces*, with about 150 of these isolated from *Micromonospora* [32]. Twelve *Micromonospora* species produce gen-

tamicin-type antibiotics [4]; gentamicin itself is produced commercially using *M. purpurea* and *M. sagamiensis* [35]. Of the more than 20 aminoglycoside antibiotics commercially available, gentamicin comprises about one half of the total sales [4].

In spite of their commercial importance and their ability to produce and modify a large variety of antibiotics [4,41], reports on the genetics of *Micromonospora* are few [5]. To address this lack of knowledge, techniques and systems need to be developed for this genus. A broad host range temperate phage could provide the basis for of a cloning system similar to the streptomycete specific phiC31 derivatives [13,37], or the phage R4 derived cosmid [31].

The first described *Micromonospora* specific actinophages were the lytic phages phiUW21 and phiUW51 [22,23]. Several other lytic *Micromonospora* phages were used to screen for the presence of restriction enzymes [29]. Temperate phages specific for *Micromonospora* species were partially characterized [8] and used to demonstrate liposome mediated transfection [9]. We report on a temperate phage (MPphiWR-1) capable of infecting/transfecting *M. purpurea* ATCC 15835 and suitable for development as a cloning vector.

MATERIALS AND METHODS

Organisms and phages

All strains of actinomycetes used in this study are listed in Table 1. The phages studied included MPphiWR-1 and its mutants, MPphiE, MPphiE-RCPM, MPphiE-RCPM DM4, and MPphiE-RCPM DM8.

Actinomycete cultures were maintained at −20°C, in the medium in which they were grown. Phages were stored at 4°C either as filtered growth medium, buffer stocks, or suspended in CsCl.

Media and buffers

Tryptone Yeast (TY) [29]; Glucose (20 g/l), tryptone (5 g/l), yeast extract (5 g/l), 3-[*N*-morpholino]propanesulfonic acid (MOPS buffer) (0.025 M) pH 7.2, $MgSO_4$ (5 mM). TYa is TY with 10 mM $MgSO_4$. The millimolar concentrations of Ca $(NO_3)_2$, when added, are represented by the number after the medium abbreviation. *Starch Tryptone Yeast* (STY) [29]; TY with Maltrin M-040 (40 g/l) (Grain Processing Corp., Muscatine, IA). *P Medi-*

Table 1

Host range of MPphiE-RCPM

Susceptible Organisms
Ampullariella lobata (ATCC 15350), *Catellatospora* sp.* (LL-26-29), *Micromonospora brunnea* (NRRL B-16079), *M. carbonacea* subsp. *aurantiaca* (NRRL 2997), *M. chalcea* subsp. *izumensis* (ATCC 21561), *M. coerulea** (NRRL B-16092), *M. echinospora* subsp. *echinospora* (ATCC 15837), *M. echinospora* subsp. *pallida* (ATCC 15838), *M. halophytica* subsp. *halophytica** (ATCC 27596), *M. inositola** (NRRL 16095), *M. purpurea** (ATCC 15835), *M. purpureochromogenes* subsp. *halolerans** (RV101), *M. rhodorangea* (NRRL 5326), *M. scalabitana* subsp. *rubra* (NRRL B-16085), *M. scalabitana* subsp. *sporogenes* (NRRL B-16086), *M. zionensis** (thia$^-$, R$^+$, M$^+$) (LL-100-125)
* These strains probably contained restriction-modification systems (See text).
Nonsusceptible organisms
Actinoplanes azureus (ATCC 31157), *A. ferrugineus* (ATCC 29868), *A. missouriensis* (ATCC 14538), *A. multisporangius (LL-A60)*, *A. philippinensis* (NRRL 5462), *Ampullariella* sp. (LL-37Q-46), *Catellatospora* sp. (LL-L31), *Catenuloplanes japonicus* (ATCC 31637), *Dactylosporangium thailandense* (LL-9-41), *D. thailandense* (LL-9-41C), *D. thailandense* (LL-D449), *D. aurantiacum* (LL-D748), *Glycomyces* sp. (LL-I17), *Micromonospora aurantiaca* (NRRL B-16091), *M. carbonacea* (ATCC 27114), *M. chalcea* (ATCC 12452), *M. citrea* (NRRL B-16101), *M. coerulea* (ATCC 27081), *M. echinoaurantiaca* (NRRL B-16102), *M. fulvoviolacea* (NRRL B-16103), *M. fulvoviridis* (NRRL B16104), *M. globosa* (NRRL 11299), *M. grisea* (MG-A001), *M. grisea* (MG-A023x), *M. halophytica* subsp. *nigra* (NRRL 3097), *M. inyoensis* (ATCC 27600), *M. lacustris* (NRRL B-11050), *M. lilacina* (NRRL B-16080), *M. megalomicea* subsp. *nigra* (ATCC 27598), *M. olivoasterospora* (ATCC 21819), *M. parva* (NRRL B-16093), *M. peucitica* (NRRL B-16082), *M. purpureochromogenes* (ATCC 27007), *M. rosaria* (NRRL 3718), *M. sagamiensis* var. *sagamiensis* (ATCC 21826), *M. saitamica* (NRRL B-16084), *M. viridifaciens* (NRRL B-16087)
LL = Lechevalier Culture Collection, NRRL = Northern Regional Research Laboratories, ATCC = American Type Culture Collection

um [33]; containing 10 mM $MgCl_2$ and no $CaCl_2$ [33]. *P+ Medium* [33]; P medium with 25 mM $CaCl_2$. *R Medium* [33]; with the following modifications; sucrose (100 g/l), glucose (20 g/l), MOPS buffer pH 7.2 (25 mM), $MgCl_2$ (5 mM), $CaCl_2$ (25 mM) and Difco yeast extract (2 g/l). *RM Medium*; R medium without sucrose. *S Broth* [33]; TY medium containing monobasic (2 g/l) and dibasic (4 g/l) potassium phosphate replacing MOPS. *PB-20 Buffer* [9]; Phage storage and dilution buffer with 5 mM $MgSO_4$ and 20 mM Ca $(NO_3)_2$. PB buffer with other levels of $MgSO_4$ or $Ca(NO_3)_2$ are identified as with TY medium.

Culture/host manipulations and studies

Standard broth growth conditions. Broth cultures were grown in 25 × 150 mm test tubes containing 10.0 ml of medium, with or without a freely moving 5 × 64 mm glass rod. Erlenmeyer flasks were used for larger volumes as follows; 2.0 liter flasks contained 500 ml of broth and 250 ml flasks contained 40 ml. The tubes or flasks were agitated on a rotary shaker with a 2.54 cm stroke at 400 RPM. Unless otherwise indicated, incubation was at 30°C.

Cell mass measurements. Broth cultures were diluted and the optical density (OD) measured in Klett units (KU) using a Klett-Summerson colorimeter with a #54 (green) filter [18, 40].

Protoplast preparation. Glycine-sensitized cells were prepared under standard tube growth conditions by inoculating TY broth containing 0.4% glycine with 250 units (Klett OD units/ml × volume in milliliters) of an 18–20 h old culture of *M. purpurea.* After 18–22 h of growth, the cells (500–1500 KU) were washed 2 × with P medium (no $CaCl_2$) ($3000 \times g$, 10–20 min at RT). Cells were then resuspended at 20% of the original volume in P^+ medium, mixed with an equal volume of filter sterilized lysozyme (20 mg/l; Sigma) in P^+ medium and shaken (30°C, 200 RPM) until adequate protoplasting was achieved (usually 30–45 min). Protoplasts were then filtered through non-absorbent cotton to remove osmotically-stable mycelial fragments, centrifuged as above and resuspended in fresh P^+ medium. Prepared protoplasts were used immediately or stored at 0°C.

Transfection. Freshly prepared protoplasts were incubated 16–20 h at 0°C in P^+ containing 50 mM $CaCl_2$ (P-50). Protoplasts (2.0×10^{10}) were collected by centrifugation (10–20 min, $3000 \times g$, 0°C in 15 ml Corex tubes, resuspended in P-50 and mixed with DNA diluted in P-50 to a final volume of 5.0 ml (all materials and procedures at 0°C). After 15–45 seconds, an equal volume (5.0 ml) of 40% PEG-6000 (Fluka) in P-50 (RT) was then mixed into the DNA-protoplast suspension, centrifuged as above and the pellet resuspended in 1.0 ml P^+ medium. The transformed protoplasts were diluted in P^+ medium, spread in a soft agar overlay on R medium, then incubated at 30°C. The overlay contained 0.1 ml of protoplasts, 4.0 ml of 45°C medium and 0.3 ml of indicator *M. purpurea* cells where desired [40].

Indicator cells were standard 16–22 h tube cultures grown in TY broth. Cells were washed 2 × with P medium and resuspended in P^+ medium to an OD of 400–500 KU.

Phage isolation and manipulation:

Soil wash isolation. (after Dowding [17]). Air-dried soil (1.6 g), was shaken for 90 min in 10 ml of TYa-5 at 320 RPM at 28°C. A 5.0 ml aliquot of this material was centrifuged at $8000 \times g$ for 15 min at 4°C. The drained soil pellet was loosened by resuspending using a Vortex mixer (without liquid addition) and then incubated statically at 28°C. After 72 h it was mixed thoroughly with 10 ml of TYa-5 broth, centrifuged for 15 min at $4000 \times g$ at 4°C, then filtered through a 0.22 micrometer Millipore membrane filter and plated.

Standard phage plating procedure. Cells were grown overnight in TY-0 or STY-0, adjusted to an OD of 400–500 KU/ml and $Ca(NO_3)_2$ added just prior to use. A suitable phage preparation (0.1 ml) was mixed with 0.3 ml of cells, then 4 ml of top agar and spread over 25 ml of homologous base agar in a 15 × 100 mm petri dish [3] and incubated at 30°C unless otherwise stated.

Phage plating optimization: Ca^{2+} and Mg^{2+}. A stock of MPphiE-RCPM was diluted in PBa-5 buffer to a titer of 500 PFU/ml and then plated on *M. purpurea* ATCC 15835 on variously modified TY.

The TY was modified to contain $MgSO_4$ and Ca $(NO_3)_2$ in combinations of levels from 0 to 50 mM [17].

Phage host range evaluation. Potential hosts were evaluated by dilution plating and spot testing [22] on TYa-5. Plating (0.1 ml) and spot testing (0.01 ml) of a phage preparation was done at 10^0, 10^{-2}, 10^{-4} and 10^{-6} dilutions. Positive results were indicated when isolated plaques were seen at any dilution. When inhibition of growth occurred where the drop was spotted but isolated plaques were not discernable, the area of inhibition was picked, soaked in PBa-5 and retested.

Phage stocks. Single plaques were picked or lawns were scraped from plates having nearly confluent plaques, and allowed to soak. Cells and debris were removed by filtration through Durapore HVLP or GVLP low protein binding membranes (Millipore Corp.) and stored at 4°C [20].

Lytic phage were propagated in *M. purpurea* ATCC 15835 cells grown in TY-20 broth. The cells, diluted to a concentration of 30–50 KU/ml, were inoculated with 10^5 PFU/ml of phage stock or a single isolated plaque, shaken for 24 h, then centrifuged and membrane-filtered.

Lysogen induction: mitomycin C. Eighteen to 20 h old *M. purpurea* AA3 cells were exposed to 5 mcg/ml of mitomycin C for various lengths of time. The cells were then diluted 10^{-2} into TY broth (10 ml/tube) and shaken overnight at 30°C. Phage in cell-free portions were titered on *M. purpurea* ATCC 15835.

Ultraviolet light. Twice water washed, 24 h old *M. purpurea* AA3 cells (OD 900–1700 KU) were diluted to 400 KU OD and exposed to 254 nm light at 0.787 Joules/m^2/s. One ml samples were taken and diluted 10^{-1} into TY-20 in tubes and shaken at 30°C for 21 h under standard conditions. Induced phage stocks were membrane-filtered and titered on *M. purpurea* ATCC 15835.

Lysogen resistance to a lytic phage variant. Lysogens were grown to an optical density of 200–1000 KU. The clear-plaque forming derivative, MPphiE-RCPM, was titered on the lysogenic hosts. The presence of clear plaques indicated phage sensitivity.

Phage temperature sensitivity. Phage stock was diluted 2.0×10^{-2} into TY-20 and 0.5 ml portions were incubated at various temperatures. At 0 h (control) a 4°C sample, and after 23 h, each preparation, was first diluted sixfold with PB-20 buffer, then titered on *M. purpurea* ATCC 15835.

Phage sensitivity — chloroform and diethyl ether. MPphiE-RCPM phage stocks were diluted into TY-20 then mixed with 1% (v/v) of test material on a vortex mixer for two seconds. After five min, samples from the aqueous phases were titered on *M. purpurea* ATCC 15835. EDTA, EGTA (Ethylene glycol-bis (beta-aminoethyl ether) *N*, *N*, *N'*, *N'*-tetraacetic acid), Octyl-beta-D-glucopyranoside (OBDG) and Triton X-100: One-tenth ml of dilutions (10^{-3}-10^{-6}) of phage stocks in PB-20 buffer were mixed with 0.1 ml of double-strength test material in PB-20 buffer. After mixing, the samples were allowed to stand ten minutes, then plated on *M. purpurea* ATCC 15835. The final concentrations of tested materials after plating were below the toxic level for the host.

Cell-associated phage adsorption. (after Adams [3] and Dowding [17].) Overnight tube cultures (OD) of 400–1500 KU of *M. purpurea* ATCC 15835 grown in TY-0 broth were washed once with TY medium or PB-0 buffer, resuspended in ⅛ to ½ their original volume with TY-0, dispensed into tube, and then the Ca^{2+} level was adjusted to 20 mM with a 1.0 M solution $Ca(NO_3)_2$.

Cells were infected with phage diluted to approximately 10^7 PFU/ml in the same medium used to resuspend the cells. The phage volume added never exceeded 1/50 of the cell suspension. The phage-cell mixture was then shaken at 200 RPM, 30°C (13×100 mm test tubes with 0.5 ml).

Samples were diluted 1/6 with PB-20 buffer, then ½ membrane filtered (Durapore HVLP). Both the filtered and unfiltered materials were titered on *M. purpurea* ATCC 15835. Colony-forming units present were also determined on TY-20. The number of adsorbed phage producing a viable infection was determined by subtracting the titer of phage in the filtered sample portion from the titer of phage in the unfiltered sample portion.

Phage electron microscopy. Filter-sterilized

broth culture phage stocks or dialyzed cesium chloride gradient-purified phage were used. Samples were processed as described [29].

Phage density determination. MPphiE-RCPM density was determined by centrifuging the phage to equilibrium in CsCl (starting densities of 1.473–1.500 g/cm^3), fractionating the gradient and then determining the density of the sample containing the highest concentration of phage particules. Preparations were centrifuged at 35,000 RPM, 4°C in a Type 40 rotor (Beckman Instruments). Refractive indices of fractions were measured with a refractometer (Carl Zeiss) and the density of the sample determined from a standard table. Phage concentration was determined by titering the fractions on *M. purpurea* ATCC 15835.

A correlation of fractions containing viable phage to those containing the major portion of the DNA was done by testing fractions semi-quantitatively for the presence of DNA by UV fluorescence. Each fraction was spotted on an agarose plate containing 50 mM EDTA and 1 mcg/ml ethidium bromide. After the spots had dried the gel was photographed under long wave UV irradiation and the most intensely fluorescing spots identified [40].

Phage deletion mutant isolation. Phage stock was mixed with 10× EDTA stock to yield a final concentration of 50 mM. After 15 min incubation at room temperature, an equivalent amount of 10× $Ca(NO_3)_2$ was added to each sample and mixed. The samples were then titered on *M. purpurea* ATCC 15835 in TY-20 agar. Phage stock was prepared from plates having nearly confluent plaques.

The enrichment for EDTA-resistant phage was repeated until the ratio of the phage titer after treatment, to the phage titer without treatment showed little relative difference between successive phage stock preparations (four times). DNA from isolated EDTA-selected phage plaques was prepared and analyzed.

DNA isolation and manipulation:

Genomic DNA isolation. Unfiltered protoplasts were prepared from overnight cultures of *M. purpurea* ATCC 15835 or AA3. After formation, protoplasts were collected by centrifugation (10–20 min, 3000 × *g*) and the pellets resuspended to 2.5% of the starting overnight culture volume with P$^+$ medium.

The protocol of Gross-Bellard et al., [19] for isolation of high molecular weight DNA from eukaryotic cells was used with slight modification. After incubation, the DNA preparations were extracted with phenol/chloroform [27], extracted once with an equal volume of chloroform, then dialyzed against one liter of TE + NaCl (10 mM) four times, or until the OD at 270 nm of the final dialysis buffer was less than 0.05. The purified DNA was quantitated using UV absorption at 260 nm and stored at 4°C.

Small scale phage DNA preparation. Cell-free, membrane-filtered phage stocks propagated in broth were used since DNA from non-CsCl-purified phage preparations propagated in agar contained restriction enzyme-inhibiting substances. Cell-free filtered phage lysates were treated with ribonuclease (50 U/ml) (RNase A, Worthington Biochemicals) and deoxyribonuclease (40 U/ml) (DNase I, Sigma) and allowed to incubate at room temperature. After two h, the phage was precipitated using PEG 6000 (11% w/v) [42], and the material processed as described [27], but without $CHCl_3$. The phage-containing pellets were resuspended in 0.5 ml water, adjusted to 50 mM EDTA and the whole preparation extracted with phenol [20,27]. After ethanol precipitation, the dried DNA pellets were dissolved in 55 microliters of TE.

Large-scale phage isolation and DNA purification. Phage preparations were plated on *M. purpurea* ATCC 15835 at titers yielding confluent or nearly confluent plaques. After development of the lawn, phage was eluted by soaking (3–18 h at room temperature) into an overlay of water or buffer [27] or the soft agar layer was removed and processed using buffer [20]. After PEG 6000 precipitation, the phage were resuspended in PB-20 buffer and centrifuged through a CsCl step gradient [30], and/or banded to equilibrium in CsCl (density 1.50–1.52 g/cc).

A portion of the CsCl-purified phage stock (20–50 OD units at 260 nm) was diluted with phage buffer up to 2.5–4.0 ml, then dialyzed against at least two changes of PB-20 buffer (2 l) at 4°C for

18–24 h each. After dialysis, EDTA (500 mM) was added to a final concentration of 50 mM. The phage DNA was then phenol-extracted [20,27,30].

DNA manipulations and electrophoresis. Standard procedures for manipulation and electrophoresis of DNA were used [20,27].

DNA homology analysis. DNA homology was demonstrated using Southern blots [38]. The transfer of DNA from the agarose gel to the support material material (Gene Screen Plus) was carried out as recommended by the manufacturer (NEN-DuPont).

Hybridization of the probe to the bound DNA followed the procedure of Church and Gilbert [14]. Biotinylated probe DNA was prepared using bio-11-dUTP as the labeled nucleotide according to the procedures of manufacturer of the nick translation kit (ENZO). Detection of bio-11-dUTP-labeled material used a DETEK 1-hrp kit following the procedures of the manufacturer (ENZO).

Restriction endonuclease digestion of DNA. Enzyme digestions were performed as recommended by the manufacturer (Bethesda Research Laboratories, New England Biolabs, IBI and Boehringer Mannheim Biochemicals). Reactions were stopped by heating to 65°C for 15 min.

RESULTS/DISCUSSION

Phage isolation. MPphiWR-1 was isolated from an Israeli soil sample (Schering Corporation # 3787) previously demonstrated to contain strains of *Micromonospora*. The presence of a phage was indicated by easily visible turbid red to purple plaques on an orange lawn of *M. purpurea* ATCC 15835. These were eluted and replated with the appearance of two morphological types of plaques: the turbid red to purple type originally seen, and a clear-plaque derivative.

Several clear plaques were selected and purified by repeated rounds of single-plaque isolations. The size of the plaques varied (1–5 mm) regardless of the size of the progenitor. One chosen for further study was designated as MPphiE. When a single plaque of MPphiE was eluted and plated on *M. purpurea* ATCC 15835 growing on TYa-5, from 10^5 to 10^6 clear plaque-forming units (PFU) could be obtained. A spontaneous mutant of MPphiE which formed clear plaques on protoplast regeneration medium (R medium), MPphiE-RCPM, was isolated and used in most of the subsequent studies requiring clear plaque formation.

When turbid plaques were purified further, they yielded up to four morphological types; three turbid and one clear. Isolated representative plaques picked from the turbid variants yielded from 10^3 to 10^4 PFU when plated on *M. purpurea* ATCC 15835 and consisted predominantly of the parental types. One turbid plaque phenotypically like that initially seen, was picked, the cells allowed to grow out in broth, and the phage associated with these cells designated MPphiWR-1.

Host range. Eight genera of actinomycetes with a type II cell wall composition (52 total), including thirty-seven representatives of thirty different species of *Micromonospora*, were tested for sensitivity to the clear-plaque forming mutant MPphiE-RCPM (Table 1). Fifteen strains (three genera) were found to be phage-sensitive. Seven of these demonstrated significant efficiency-of-plating increases when MPphiE-RCPM was propagated on an homologous host rather than *M. purpurea* ATCC 15835. This effect was considered in this study to be caused by restriction-modification systems [21,29].

Phage morphology. Electron micrographs of MPphiE-RCPM show an actinophage with a long flexible tail and an isometric head. A spike-like structure was seen occasionally at the end of the tail. The diameter of the head from vertex to vertex was 78 nm and the tail length was 218 nm (Fig. 1).

Restriction enzyme sensitivity. DNA isolated from MPphiE-RCPM was tested for its sensitivity to fifty-three restriction enzymes. Thirty-eight enzymes digested the DNA to various degrees (Table 2). All the four-base-recognizing restriction enzymes tested cut the phage DNA and all the enzymes which recognize six base sequences that have an adenine at the 5′ position do not cut the phage DNA (*Bgl*II, *Cla*I, *Hind*III, *Mlu*I, *Stu*I).

Restriction mapping. Single-enzyme and dou-

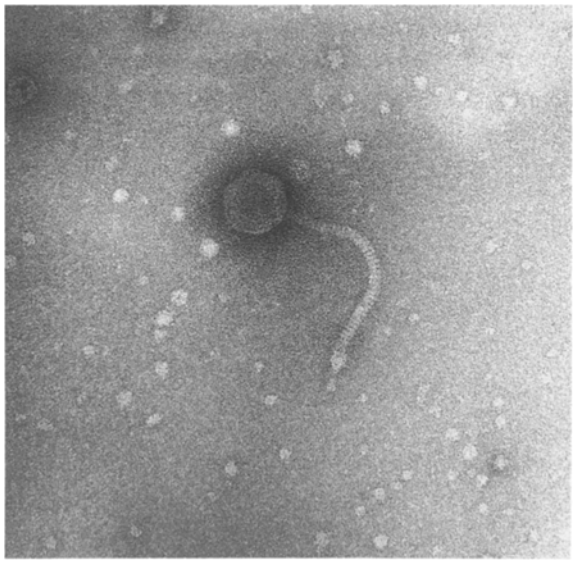

Fig. 1. Electron micrograph of MPphiE-RCPM. (150 000 × Magnification).

Table 2

Restriction digestion of MPphiE-RCPM DNA

No restriction sites:

*Bam*HI, *Bgl*I, *Bgl*II, *Cla*I, *Eco*RV, *Hind*III, *Hpa*I, *Mlu*I, *Nde*I, *Nru*I, *Pst*I, *Sac*I, *Stu*I, *Xba*I, *Xho*I.

1–6 Restriction sites (# fragments):

*Pvu*II (2), *Eco*RI (3), *Nar*I (3), *Sac*II (4), *Kpn*I (7)

>6 Restriction sites:

*Acc*I, *Alu*I, *Ava*II, *Ava*I, *Ban*I, *Bbv*I, *Bcl*I, *Bst*N1, *Fnu*4H1, *Fnu*DII, *Hae*III, *Hae*II, HhaI, *Hinc*II, *Hinf*I, *Hpa*II, *Mbo*II, *Mbo*I, *Mnl*I, *Msp*I, *Nae*I, *Nci*I, *Nco*I, *Pvu*I, *Rsa*I, *Sal*I, *Sau*3A1, *Sma*I, *Sph*I, *Tag*I, *Tth111*I, *Xma*III, *Xmn*I

ble-enzyme digests of MPphiE-RCPM DNA with *Eco*RI (3 bands, 2 cuts), *Pvu*II (2 band, 1 cut) and *Sac*II (4 bands, 3 cuts) were used to position the restriction sites (Fig. 2). The larger *Pvu*II fragment was arbitrarily designated as the left end, and the smaller the right end. The sizes of each restriction fragment are the averages from several experiments. The value generated for the size of the phage genome, 57.9 kb, is the averaged sum of the fragment sizes.

Identification of phage ends: cohesive ends. Results from the restriction mapping of MPphiE-RCPM DNA suggested the presence of cohesive ends. The gel in Fig. 3 compared *Eco*RI, *Eco*RI-*Sac*II and *Sac*II-digested preparations of MPphiE-RCPM DNA. These samples had been incubated at 4°C overnight to allow annealing of any cohesive ends. Portions of these materials were then heated at 78°C for 15 min and compared to unheated portions. In every lane containing heated DNA, there was a loss of a band and an increase in two other bands corresponding to the connected and separated cohesive phage DNA end fragments respectively [16,6]. Careful visual inspection of the gel and densitometric scanning of the gel photographs indicated that the band decreased by heating is not completely eliminated, possibly indicating long and/or high G-C containing sticky ends [24]. These results supported other studies indicating that the 13.3 and 10.7 kb *Eco*RI-generated and 24.8 and 12.1 kb *Sac*II-generated fragments are the end fragments of the phage DNA.

Phage density determination. The density of MPphiE-RCPM was determined using isopycnic centrifugation in CsCl. The averaged phage density value was 1.524 g/cc (range 1.516 to 1.528 g/cc, standard deviation 0.005, coefficient of variation 0.003).

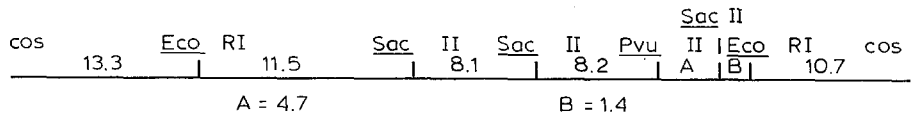

Fig. 2. Restriction map of MPphiE-RCPM. cos = cohesive end; Fragment sizes are in kb.

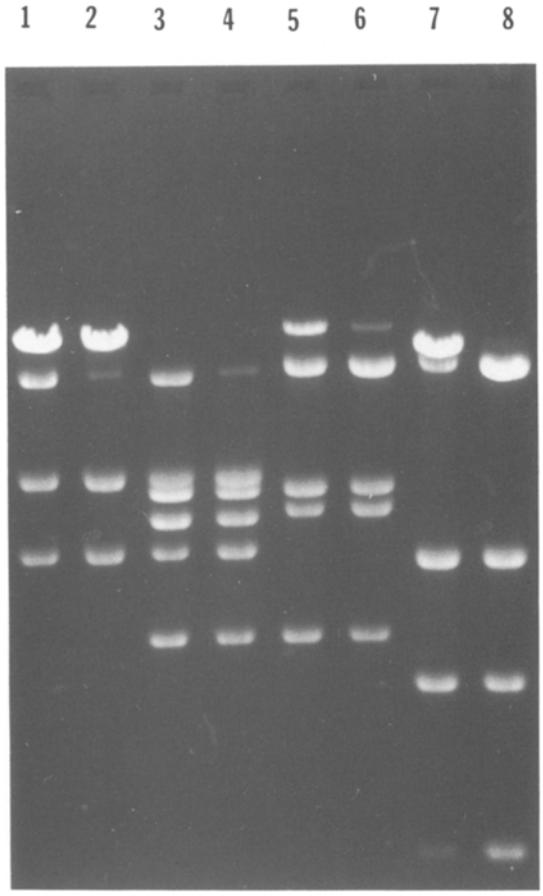

Fig. 3. Comparison of heated and unheated restriction enzyme digests of MPphiE-RCPM DNA. Lanes are identified from left to right, the origin of the gel is at the top. Separation was achieved with a 0.5% agarose gel. Each lane contained 0.3 micrograms of DNA. Heating was for 78°C for 15 min. Lane 1, MPphiE-RCPM DNA cut with *Eco*RI and not heated, fragments are 32.5, 24.0, 13.3, 10.7 kb; lane 2, as in lane 1 but heated, fragments are 32.5, 13.3, 10.7 kb; lane 3 MPphiE-RCPM DNA cut with *Eco*RI and *Sac*II and not heated, fragments are 24.0, 13.3, 12.9, 12.1, 11.5, 8.1 kb; lane 4 as in lane 3 but heated, fragments are 13.3, 12.9, 12.1, 11.5, 8.1 kb; lane 5, MPphiE-RCPM DNA cut with *Sac*II, fragments are 36.9, 24.8, 12.9, 12.1, 8.1 kb; lane 6 as in lane 5 but heated, fragments are 24.8, 12.9, 12.1, 8.1 kb; lane 7, Lambda DNA cut with *Hin*dIII, unheated fragments are 27.5, 23.1, 9.4, 6.6, 4.4 kb; lane 8, as in lane 7 but heated (65°C), fragments are 23.1, 9.4, 6.6, 4.4 kb.

Phage plating efficiency: temperature and media. The phage, MPphiE, was tested for propagation on three different media (R, RM and TYa-5) at 28 and 35°C. Plating MPphiE at 35°C resulted in a plaque count decreased substantially (99.4%) regardless of the medium, compared to the 28°C controls. In addition, when the phage was titered on the high sucrose protoplast renegeration medium (R medium), two different plaque phenotypes were seen. The predominant type formed turbid dark plaques at 28°C; the others were clear. MPphiE-RCPM was isolated from these plaques on R medium as a clear plaque-forming, spontaneous mutant of MPphiE.

Media optimization Ca^{2+} and Mg^{2+} levels. Kikuchi and Perlman [22] used 6 mM Ca^{2+} for *Micromonospora* phage isolation, hence the media initially used in these studies had 5 mM Ca^{2+}. Chater and Carter [11] optimized the cation levels for phage infection for a given medium by using the agar-solidified version of that medium and determining the phage plating efficiencies on it.

The best plating efficiencies for MPphiE-RCPM in a TY medium was attained with Mg^{2+} levels up to 10 mM and Ca^{2+} levels from 10 to 50 mM (Table 3). The largest plaques were seen when Ca^{2+} levels were between 5 and 20 mM. The levels of Ca^{2+} and Mg^{2+} chosen for further use were 20 mM and 5 mM, respectively.

Phage stability and resistances. Sensitivities to different physical and chemical agents are often used to characterize phages [1,2]. The results of sensitivity tests on MPphiE-RCPM are given in Table 4. It was fairly resistant to the surfactants OBDG (0.01% w/v) and Triton X-100 (0.1% v/v). Ether reduced the phage titer by 99.897% and chloroform ($CHCl_3$) by 99.9997%. As the phage is somewhat sensitive to ether, it thus may contain lipids essential to its survival. Sensitivity to $CHCl_3$ was nearly absolute, eliminating its use as a preservative with this phage.

EDTA and pyrophosphate, non-specific chelating agents, and EGTA, a chelating agent with a greater affinity for calcium ions than magnesium ions, were all toxic at various levels. In phage buffer, MPphiE-RCPM is very sensitive to EDTA. Above a point where all the divalent cations are chelated (20–30 mM) (Table 5), the titer of the phage drops by more than 99.9859%. With EGTA at 30 mM (Table 5), the titer of MPphiE-RCPM

Table 3

MPphiE-RCPM plating optimization—Ca^{2+} and Mg^{2+} levels

PFU per plate

Mg^{2+}	Ca^{2+} levels							
Levels	0 mM	5 mM	10 mM	15 mM	20 mM	25 mM	30 mM	40 mM
0 mM	0	28	49	43	50	37	42	43
5 mM	3	39	39	46	50	46	55	36
10 mM	2	27*	37	39	28	46	50	27
15 mM	4	28	37	39	31	38	44	31
20 mM	5	21	33	30	37	35	29	29

* = control

only drops 59.2%, indicating that divalent cations, but not necessarily calcium ions, are required for the stability of the phage. Pyrophosphate at 50 mM (data not shown) was as effective as EDTA for the inactivation of MPphiE-RCPM particles.

MPphiE-RCPM, after exposure (23 h) to temperatures up to 42°C, is somewhat stable (77% inactivated), but at 52°C, all the virions were inactivated (Table 6).

Phage DNA deletion mutants. Enrichment of phage populations for deletion mutants has been achieved by treatment with a chelating agent [10,34]. Purified EDTA-resistant mutants of MPphiE-RCPM isolated from the fourth successive enrichment were analyzed for the presence of deletions. Digestion of phage DNA with *Sal*I produced >15 bands in an agarose gel, permitting small deletions to be readily observed. All of the putative deletion mutants tested differed from MPphiE-RCPM in a single band.

To define the length and location of the deletions, a double digestion of the DNA from the mutant phages was done with *Eco*RI and *Sac*II which would produce fragments of 1.4, 8.1, 10.7, 11.5, 12.9, 13.3 kb from normal MPphiE-RCPM (Fig. 2). Deletion mutants DM4 and DM8 were tested as representative of two classes of mutants isolated. The results of the *Eco*RI–*Sac*II double-restriction enzyme digest on DM4 and DM8 are shown in Fig.

Table 4

Sensitivities of MPphiE-RCPM to various organic solvents and surfactants

Material	Conc	Control titer(1)	Final titer(1)	Surviving fraction
OBDG(2)	0.01%	1.91×10^6	1.39×10^6	0.728
Triton X-100	0.10%	1.91×10^6	1.14×10^6	0.597
Chloroform	1.0%	7.80×10^6	2.00×10^1	0.00000256
Ether	1.0%	1.21×10^7	1.26×10^4	0.00104

(1) Titer is in pfu/ml; (2) OBDG = Octyl beta-D-glucopyranoside.

Table 5

Sensitivity of MPphiE-RCPM to the chelating agents EDTA and EGTA

Chelating agent treatment				
Level	EDTA (5 min)		EGTA (10 min)	
(mM)	Titer(1)	Survival	Titer(1)	Survival
0	2.9×10^6	1.00×10^0	1.9×10^6	1.00×10^0
5	2.5×10^6	8.62×10^{-1}	nd	nd
10	3.0×10^6	1.03×10^0	1.4×10^6	7.37×10^{-1}
20	1.5×10^6	5.17×10^{-1}	1.3×10^6	6.84×10^{-1}
30	5.9×10^1	1.72×10^{-5}	7.8×10^5	4.11×10^{-1}
40	4.1×10^2	1.41×10^{-4}	nd	nd
50	2.7×10^2	9.31×10^{-5}	nd	nd

(1)Titer is in pfu/ml; nd = not done.

Table 6

Stability of MPphiE-RCPM at various temperatures

23 h at Temperature (°C)	4	25	28	30	31.5	34	37	42	52
Titer($\times 10^6$)	5.6	3.6	2.9	2.0	3.4	3.0	1.5	1.3	0.0
Survival %	100	64	52	36	61	54	27	23	0.0

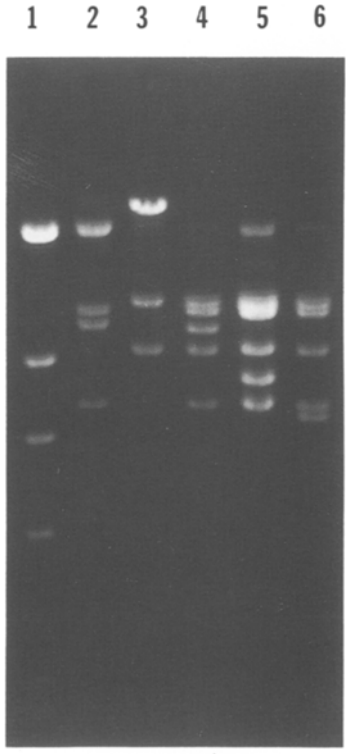

Fig. 4. Agarose gel comparison of DNA from MPphiE-RCPM to deletion mutants DM4 and DM8. Lanes are indicated from left to right. Agarose concentration of gel was 0.5%. Origin is at the top. Lane 1, Lambda DNA cut with *Hind*III, fragments are 23.1, 9.4, 6.6, 4.4, 2.3, 2.0 kb; lane 2, MPphiE-RCPM DNA cut with *Sac*II, fragments are 24.8, 12.9, 12.1, 8.1 kb; lane 3, MPphiE-RCPM DNA cut with *Eco*RI, fragments are 33.9, 13.3, 10.7 kb; lane 4, MPphiE-RCPM DNA cut with *Eco*RI and *Sac*II, fragments are 13.3, 12.9, 11.5, 10.7, 8.1 kb; lane 5, DM4* DNA cut with *Eco*RI and *Sac*II, fragments are 13.3, 12.9, 10.7, 8.8, 8.1 kb; lane 6, DM8 DNA cut with *Eco*RI and *Sac*II, fragments are 13.3, 12.9, 10.7, 8.1, 7.9 kg. *Note the 24.0 kb fragment formed by the unmelted cohesive end fragments.

4. In the mutants (lanes 5 and 6), the 11.5 kb fragment found in MPphiE-RCPM is missing, and a new smaller fragment is found: an 8.8 kb fragment in DM4 and a 7.9 kb fragment in DM8. These data indicate that the 11.5 kb fragment contains the deletions and these could be up to 3.5 kb long. It is not known whether the deletions are contiguous or are overlapping. The 11.5 kb fragment also contains the phage attachment site for the temperate phage in the AA3 lysogen (see Phage Integration section).

Phage adsorption. The adsorption of phage to a cell can be monitored by determining the number of cells with adsorbed phage, or by determining the number of free unadsorbed phage present at any given time. MPphiE-RCPM was used with *M. purpurea* ATCC 15835 to study and maximize the adsorption process.

Filtration to remove cells with adsorbed phage [17] was the best procedure to allow the determination of unadsorbed phage. The use of mixed cellulose ester membranes (Millipore Corporation) resulted in unacceptable binding/inactivation of the phage. Low protein binding Durapore membranes (Millipore Corporation) were used and did not bind/inactivate phage.

Using the standard conditions, measurement of phage adsorption (Table 7) showed that after 90 min there is an increase in viable phage over the titer obtained at the previous time point, indicating that productive infections had occurred. The data also indicated that there exist two components responsible for the decrease in the phage titers seen in the filtrate, inactivation and adsorption. Inactivation of phage particles was reflected by a decrease in total phage in the unfiltered samples. This inactivation accounted for about 88% (at 60 min) of the

Table 7

Adsorption of MPphiE-RCPM to *M. purpurea* ATCC 15835

Adsorption time	Unfiltered titer(1)	Viab.(2)	Filtered titer(1)	Filtered/ unfiltered	Control titer(1)
0 min	5.7×10^4	1.000	5.1×10^4	0.895	6.0×10^4
30 min	1.3×10^4	0.228	2.9×10^3	0.223	nd
60 min	6.9×10^3	0.121	7.0×10^2	0.101	nd
90 min	2.3×10^4	0.404	6.3×10^2	0.027	nd
120 min	8.1×10^3	0.142	5.5×10^2	0.068	3.2×10^4

Overnight culture density = 850 KU/ml; Cfu/ml in adsorption was 1.5×10^7 (5200 KU/ml); (1) titer in pfu/ml, all data is 1/6 of actual counts due to the dilution of samples prior to filtering; (2) viability calculated as (total phage at any time point)/(total phage at 0 min).

total input viable phage. The inactivation effect was probably not due to multiple adsorption to one colony-forming-unit (CFU) since the phage to CFU ratio was always >100:1. The other component, adsorption of the phage to the cells, accounted for up to 90% of viable phage at 60 min of incubation. If only the pfu present in the filtrate were considered in calculating adsorption, and inactivation ignored, at 60 min the finding of 1.2% of the original viable phage population in the filtrate would lead to the erroneous conclusion that 99.8% of the phage were adsorbed. Therefore, only the difference between the filtered and unfiltered titers were considered to be adsorption.

Various conditions were tried to enhance adsorption or inhibit inactivation. Cells were grown in modified TY media such as S, S with 0.2% glycine and S with 5 mM Ca^{2+} with no difference in adsorption or inactivation relative to the control medium observed. This suggested that phage binding sites were not inducible under the conditions tested.

Adsorption and inactivation were eliminated or significantly decreased when chloroform-killed or autoclaved cells were used (data not given). Normal adsorption apparently requires viable cells or the phage receptors were destroyed when the cells were killed [3]. In any instance, some or all of the inactivation seen here may be due to normal adsorption to dead cells.

The effect of selected TY-20 medium components

Table 8

TY-20 medium component effect on adsorption and inactivation of MPphiE-RCPM with *M. purpurea* ATCC 15835

Adsorption time	Unfiltered titer (1)	Viability (2)	Filtered titer (1)	Filtered/ unfiltered	Adsorption (Klett) (3)
Complete TY-20					
0 min	4.5×10^4	1.000	1.5×10^4	0.333	12 000
75 min	5.0×10^3	0.111	1.1×10^3	0.220	
Without Ca^{2+}					
0 min	2.8×10^4	1.000	2.3×10^4	0.821	10 600
75 min	2.7×10^3	0.096	2.0×10^3	0.741	

The density of the overnight culture was 1100; Starting phage titer was 5.2×10^4. (1) titer in pfu/ml, all data presented as 1/6 of actual counts due to sample dilution prior to filtering; (2) viability was calculated as (total phage at any time point)/(total phage at 0 min); (3) OD in KU/ml.

upon adsorption and inactivation was tested by eliminating Ca^{2+}, $Mg2^{+}$, MOPS and glucose individually. Only the deletion of Ca^{2+} from the medium was effective and that decreased adsorption (Table 8). This result confirmed those from the plating optimization. No condition effectively blocked phage inactivation. Even when adsorption was inhibited (Ca^{2+} deleted) inactivation was significant (Table 8).

These phage adsorption studies indicate that the adsorption of MPphiWR-1 (as represented by MPphiE-RCPM) was complex: the phage readily adsorbs/binds to a cell as measured by the differences between the filtered and unfiltered titers. The adsorption rate cannot be determined due to the inability to distinguish whether phage inactivation occured before or after adsorption. Adsorption is dependent on Ca^{2+} ions and inactivation is not; therefore, inactivation most likely happens prior to, and is probably not related to the adsorption phenomenon which leads to productive infections.

Lysogen phage production and resistance. The lysogenic substrain (AA) of *M. purpurea* ATCC 15835 was isolated from a turbid plaque. It was tested, without further purification, for the spontaneous production of infectious phage in STYa-5 broth. Phage were detected in cell free supernatants at both 24 and 48 h ($2.1–3.5 \times 10^5$ pfu/ml with about 70% of the plaques clear).

M. purpurea AA as a lysogen was resistant to the clear plaque mutant of MPphiWR-1, MPphiE-RCPM. To test for infectious centers (phage release), AA was sonicated, diluted and plated in a lawn of *M. purpurea* ATCC 15835. A total of 4.2×10^8 cfu were present, of which 40% were infectious centers; thus, either (1) some of the colonies were not lysogens; (2) detection of the phage was difficult, or (3) the lysogenic association was extremely stable.

The clarify this, fifty colonies from the control population used in the infectious center study were picked and tested for sensitivity to MPphiE-RCPM by plating 1.4×10^8 pfu on each isolate. Since no indication of plaques or plate clearing was detected on any, all clones were considered lysogens. Apparently phage detection from lysogen colonies is difficult and/or the lysogenic relationship was very stable. One isolate, number three, from *M. purpurea* AA (AA3) was selected and used in all subsequent lysogen studies. The temperate phage associated with *M. purpurea* AA3 was designated MPphiWR-1.

Prophage induction. Mitomycin C and ultraviolet (UV) irradiation were used to induce phage from the *M. purpurea* AA3 lysogen. Exposing the cells to 5 mcg/ml mitomycin C for various lengths of time resulted in phage induction (Table 9). UV light irradiation also induced phage production (Table 9) with maximum induction and growth inhibition seen at the longest exposure (240 s).

Restriction comparison of DNA from MPphiWR-1 and clear plaque-forming mutants. The most efficient means to produce the temperate phage was to elute lawns of confluent turbid plaques with phage buffer. Using this method, enough MPphiWR-1 was obtained to purify in a CsCl gradient. Unexpectedly, two bands were found in the preparation, one with the expected density for MPphiWR-1 found in the middle of the

Table 9

Mitomycin C and UV induction of MPphiWR-1 from the lysogen *M. purpurea* AA3.

Exp.	Inducer	Exposure	pcv(1)	Klett(2)	pfu/ml(3)
A	none	none	nd	653	3.5×10^4
A	mmc	0 min[4]	nd	353	9.5×10^4
A	mmc	5 min	nd	63	4.9×10^5
A	mmc	10 min	nd	51	3.8×10^5
A	mmc	20 min	nd	51	3.9×10^5
A	mmc	40 min	nd	51	4.4×10^5
A	mmc	80 min	nd	51	7.2×10^5
B	UV	0 s	0.80	nd	0
B	UV	30 s	0.80	nd	3.0×10^1
B	UV	60 s	0.70	nd	1.1×10^2
B	UV	120 s	0.60	nd	2.4×10^2
B	UV	240 s	0.35	nd	2.2×10^5

mmc = mitomycin C at 5 mcg/ml; UV = ultraviolet light; nd = not done; (1) pcv = packed cell volume; (2) Klett = turbidity measured as Klett units/ml. Starting culture OD were A = 950 and B = 1700; (3) Up to 1% of the plaques scored were clear; (4) 0 min exposure, the cells were immediately diluted into TY after mixing in the mmc.

gradient, and the other with a density of approximately 1.435 g/cc found at the top. This low density band was a previously unknown phage, MPphiWR-2, associated with *M. purpurea* ATCC 15835 (Tilley et al., in preparation).

The *Eco*RI, *Sal*I and *Sac*II generated restriction patterns of DNA from gradient purified MPphiWR-1 and MPphiE-RCPM, gave virtually identical results. Identical *Sal*I and *Kpn*I-generated restriction patterns were also seen when MPphiE-RCPM was compared to ten independently isolated clear-plaque forming mutants of MPphiWR-1. This showed that the clear plaque mutants which arose from MPphiWR-1, were not due to extensive phage genomic alterations. Thus, the clear plaque mutants were considered reasonable substitutes for the original temperate phage.

Phage integration. The restriction enzyme *Sac*II was used in all genomic DNA preparations for Southern blots because it gave extensive digestion of *M. purpurea* ATCC 15835 and *M. purpurea* AA3 genomic, as well as reasonable digestion of the phage DNA. When Southern blots of *Sac*II-digested genomic DNA from *M. purpurea* ATCC 15835 (367 mcg) and AA3 (78 mcg) were probed with MPphiE-RCPM DNA, homologous sequences were only found in the lysogen (AA3). An increase in size of two bands in the lysogen compared to the free phage (Fig. 5, Lane 5) is consistent with the presence of integrated prophage. The bands altered in size are those which would correspond to the one fragment (36.9 kb) formed by the joining of the phage DNA ends (24.8 + 12.1 kg); therefore, the phage attachment site is located somewhere within this fragment.

In order to determine more precisely where the phage attachment site was located, *Sac*II–*Eco*RI and *Sac*II–*Pvu*II double digestions of the free phage MPphiE-RCPM DNA (Fig. 5) were carried out. The single *Pvu*II site cuts the 12.9 kb fragment of the free phage as expected (Fig. 5, Lanes 6 and 7). The 12.9 kb fragment in the lysogen was cut by *Pvu*II (Fig. 5, Lanes 5 and 8) demonstrating that it was the same 12.9 kb fragment as that produced by digestion of MPphiE-RCPM with *Sac*II.

The *Sac*II–*Eco*RI and *Sac*II digestions of *M. purpurea* AA3 DNA (Fig. 5, Lanes 3 and 5), and show that the only difference between these two digestions are in the sizes of the largest fragments produced. The *Eco*RI digestion of *Sac*II-digested *M. purpurea* AA3 DNA (Fig. 5, Lane 3) results in the formation of a band at 24.0 kb from the band located at the 33.9 kb size region of the gel. This 24.0 kb band (lane 3) represents the joined cohesive ends of MPphiE-RCPM phage which are bounded by *Eco*-RI restriction sites (lane 4). The 14.3 kb fragment represents one of the new fragments expected to be formed upon lysogenization and consists of a portion of the 11.5 kb fragment (Lane 2) along with a

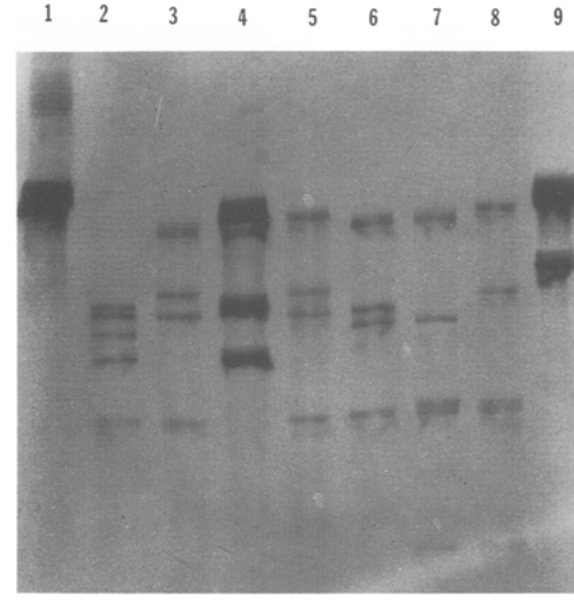

Fig. 5. Southern blot comparison of restriction digests of genomic DNA from *M. purpurea* AA3 and MPphiE-RCPM. Lanes are from left to right, origin is at the top. Probe DNA was MPphiE-RCPM. Lane 1, MPphiE-RCPM DNA uncut; lane 2, MPphiE-RCPM DNA cut with *Eco*RI and *Sac*II, fragments are 13.3, 12.9, 11.5, 10.7, 8.1 kb; lane 3, *M. purpurea* AA3 DNA cut with *Eco*RI and *Sac*II, fragments are 24.0, 14.7, 12.9, 8.1 kb; lane 4, MPphiE-RCPM DNA cut with *Eco*RI, fragments are 33.9, 24.0, 13.3, 10.7 kb; lane 5, *M. purpurea* AA3 DNA cut with *Sac*II, fragments are 33.9, 14.7, 12.9, 8.1 kb; lane 6, MPphiE-RCPM DNA cut with *Sac*II, fragments are 24.8, 12.9, 12.1, 8.1 kb; lane 7, MPphiE-RCPM DNA cut with *Sac*II, and *Pvu*II, fragments are 24.8, 12.1, 8.2, 8.1, 4.7 kb; lane 8, *M. purpurea* AA3 DNA cut with *Sac*II and *Pvu*II, fragments are 32.7, 14.7, 8.2, 8.1, 4.7 kb; lane 9, MPphiE-RCPM DNA cut with *Pvu*II, fragments are 41.1, 16.8 kb.

Table 10

DNA level effects on protoplast transfection

DNA Levels	pfu	Adjusted pfu (1)	pfu/mcg DNA (2)	Rel. pfu (3)	mol proto. (4)	pfu/ proto. (5)
10 mcg	3.3×10^3	2.2×10^5	2.2×10^4	1	8	1.1×10^{-5}
20 mcg	4.3×10^4	2.9×10^6	1.5×10^5	12	16	1.4×10^{-4}
40 mcg	5.6×10^5	3.8×10^7	9.4×10^5	170	32	1.8×10^{-3}
80 mcg	9.1×10^5	6.2×10^7	7.7×10^5	280	64	3.0×10^{-3}

Protoplasts used were 2.07×10^{10}. The viable fraction was 0.0148 (cfu/protoplast plated). (1) Adjusted pfu; the averaged number of plaques obtained/ml of transfection sample, divided by the protoplast viable fraction. This represents the number of plaques that should be obtained at 100% protoplast viability assuming DNA interacts similarly with viable and nonviable protoplasts. (2) Pfu/mcg; the adjusted number of plaques/mcg of DNA. (3) Relative pfu; the number of pfu obtained in any transfection/pfu of the control. (4) Molecules of DNA/protoplast; DNA (g) $\times 1.60 \times 10^{16}$ (phage DNA molecules/g)/# protoplast. Phage DNA molecular weight; 3.76×10^7 daltons per phage molecule (i.e. 57 900 base pairs $\times$ 650 daltons/bp). (5) pfu/protoplast; adjusted pfu/# protoplasts used in transfection (2.07×10^{10}).

portion of *M. purpurea* genomic material. The other undetected fragment consisted of the complementary portion of the 11.5 kb fragment plus *M. purpurea* genomic DNA. This is possible if the material removed from the largest *Sac*II fragment upon *Eco*RI digestion contains mostly host genomic DNA, that is, not enough phage DNA to be detected and/or this material co-migrates with another phage DNA band. These data are consistent with the hypothesis that the temperate phage, upon infection, circularized and then integrated into the host genome as described in the Campbell model [7]. This integration occurs at the attachment site of the phage found within the 11.5 kb *Sac*II–*Eco*RI fragment.

Transfection. The effect of increasing levels of DNA upon the transfection of a constant number of protoplasts was tested (Table 10). The system was not saturated up to 80 mcg, but at this level the ten-fold increase in pfu seen for each doubling of lower DNA levels was decreased. This suggests a requirement for more than one phage genome per cell, and may be due to the quality of phage DNA used, incomplete modification of the DNA [12], some other interaction with the restriction system(s) present in *M. purpurea* ATCC 15835 [23] or a peculiarity of the phage.

The liposome-free, PEG-6000 transfection of *M. purpurea* ATCC 15835 used here is at least as efficient as that reported for liposome and non-liposome mediated transfection for other *Micromonospora* [9,15,26,28] and *Streptomyces* [20,36,39] at high DNA levels. The transfection does not, however, appear to be as efficient as others in that at lower DNA concentrations, fewer plaques were obtained for each mcg of phage DNA used [9,36,39].

CONCLUSIONS

A novel temperate actinophage, MPphiWR-1, was isolated from soil. In the prophage state it exists integrated into the host genome like the streptomycete phage phiC31 [25]. The lysogen is inducible with mitomycin C or UV irradiation. In *M. purpurea* ATCC 15835, the host used for isolation, a cryptic phage, MPphiWR-2, may be affecting efficient MPphiWR-1 propagation upon induction.

The actinophage, MPphiWR-1, belongs to the common B1 morphotype [2] and the DNA has co-

hesive ends. EDTA-selected deletion mutants up to 3.5 kb long were isolated implying that the phage DNA is packaged by a site-specific mechanism. The phage has a broad host range, infecting representatives of three actinomycete genera and 14 out of the 37 *Micromonospora* tested. Included among these actinophage-sensitive microorganisms are producers of several classes of antibiotics [41].

The ability to delete regions of the phage genome, as well as the broad host range would allow MPphiWR-1 or its derivatives to be developed into effective cloning vectors. As a vector, the phage could be used with heterologous DNA inserted into the genome like phiC31 [10,37]. In addition, by using the cohesive ends, the phage may be suitable for development into a cosmid as was done with R4 [31]. A mutant of MPphiWR-1 was used to demonstrate non-liposome-mediated transfection conditions at least as efficient for *M. purpurea* ATCC 15835 as was liposome-mediated transfection in other *Micromonospora*. In view of its demonstrated characteristics, this actinophage could be developed into an effective tool to manipulate or further explore the poorly-understood molecular biology/genetics of this group of commercially important microorganisms.

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