Poorly Lytic Bacteriophage from *Dactylosporangium thailandensis* (*Actinomycetales*)

M. L. HIGGINS¹ AND MARY P. LECHEVALIER

Institute of Microbiology, Rutgers-The State University, New Brunswick, New Jersey 08903

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Dactylosporangiophage A1 has a polygonal head (75 nm) with spherical capsomeres (3 nm) and a noncontractile tail (200 by 10 nm) with cross-striations which is terminated with at least three prongs which are used for attachment. It contains double-stranded deoxyribonucleic acid and produces very little lysis. Intracellular phage multiplication leads to the formation of crystalline aggregates of apparently complete virions. Plaques are formed only on certain substrains of *Dactylosporangium thailandensis* L1 and are always small (0.5 mm or less). They are clear on some substrains and turbid on others. Formation of plaques occurs only on one medium, Czapek agar with 0.2 to 0.4% yeast extract, 0.2% peptone, or a defined mixture of amino acids. Over 100 strains of bacteria, mainly actinomycetes, were screened in a futile attempt to find an indicator strain which is not a substrain of L1. The *Dactylosporangium*-phage system studied is considered to be a semiresistant carrier state.

Actinomycetes are filamentous, branching bacteria (8). Among these, some bear sporangia (*Actinoplanaceae*) which may contain either motile or nonmotile spores (10). One group of *Actinoplanaceae* form sporangia containing a single row of pyriform, lophotrichous spores (5). These organisms, now placed in the genus *Dactylosporangium* (16), have a type II cell wall which is also typical of members of the related genus *Actinoplanes* (8).

In the present study, we describe an actinophage growing in association with some substrains of *Dactylosporangium thailandensis* L1. Plaques are formed on these substrains only under special nutritional conditions, but virions are produced abundantly on most media.

MATERIALS AND METHODS

Organisms. The isolation of *D. thailandensis* L1, previously referred to as 9-41 (9), has already been described (5). Plaques have never been seen on this strain. Phage-producing substrains L2, A1, and A3 were spontaneously occurring, plaque-forming, single colony isolates obtained by plating out L1 on Czapek (Cz) agar or on Cz medium (18) plus 0.2% Difco yeast extract (YCz agar; Scheme 1). The phage carried by each of these strains bears the strain number of its host. Indicator strain C3_s was derived from *Dactylosporangium* A3 by the following procedure. Phage-free strains were obtained by successive isolation and restreaking of plaque-free single colonies on YCz

agar. Plate cultures were incubated for 7 to 10 days at 28 C between each transfer. One strain, C3_s, freed from phage after five such cloning procedures, was selected as an indicator strain. It never formed plaques unless reinfected by one of the three phage strains studied (L2, A1, and A3).

Media. The following media were used: Bennett's (5); TPC (5); YCz; yeast extract-dextrose (YD; 18); peptone-yeast extract-Ca(NO₃) (PY; 7). For solid media, agar was added at 1.5%. Nutritional assays were carried out using Cz agar with addition of appropriate amounts of sterile concentrates of yeast extract or peptone (Difco). Media used for physiological testing were described previously (11).

Preparation of phage concentrates. (i) For electron microscopy, batches of 30 to 100 petri dishes of TPC or YCz were inoculated with cell suspensions from 7-day-old slants of A1. After 5 to 6 days of incubation at 28 C, each plate was flooded with 5 ml of harvest medium (0.1 M NaCl, 0.25% tryptone, 0.001 M phosphate buffer, pH 7.0) and cell suspensions were produced by scraping. These were pooled and debris was removed by centrifugation for 10 min at 7,000 × g. The supernatant fluid was centrifuged for 120 min at 16,000 × g, and the pellet, after a 16-hr soaking in harvest medium at 5 C, was resuspended in buffer by gentle swirling. This last procedure was repeated twice. The final pellet was taken up into 1.0% ammonium acetate neutralized with ammonium hydroxide. (ii) In screening for detector strains or for reinfected cloned strains, a 0.9% NaCl solution was used to suspend biomasses from petri dish cultures. Such suspensions were centrifuged at 2,900 × g for 30 min, and the supernatant fluid was shaken with a small amount of distilled chloroform to kill residual host cells.

¹ Present address: Department of Microbiology, Temple University School of Medicine, Philadelphia, Pa. 19104.

SCHEME 1. Family tree of *D. thailandensis* strains.

Irradiation by ultraviolet light. Growth from a Bennett's agar slant that had been incubated for 1 week at 28 C was suspended in sterile 0.9% NaCl, shaken with glass beads for 3 min by use of a Vortex Mixer (Scientific Industries, Inc., Queens Village, N.Y.), and filtered through sterile Whatman no. 1 filter paper. Stirred thin layers of the filtered suspension were irradiated with a General Electric mercury vapor lamp (G15T8) at doses of 186 to 2,046 ergs per mm² per sec. Samples were taken at 30-sec intervals up to 330 sec, at which point the survival rate approximated 0.28%. Care was taken to avoid photoreactivation. Irradiated cells were grown on both solid YCz and liquid YCz and in YD at 28 C. YCz agar plates were observed for plaques and were also harvested for possible phage by flooding with 0.9% NaCl (see above). Assays for phage, both from plate preparations and liquid culture, were performed as described below.

Phage assays. The solution to be assayed was centrifuged at 1,580 $\times$ g for 3 min to sediment the cellular debris, and the supernatant fluid was diluted in physiological saline. A measured amount, usually 0.03 ml, was pipetted onto the surface of separate petri dishes containing YCz agar. After drying, usually overnight at room temperature, the indicator strain C3s was streaked over the dried area with a loop. The tough leathery consistency of the C3s culture precluded the use of other more conventional techniques. Plates were incubated at 28 C and plaque counts were made after 6 and 10 days.

Induction by antibiotics. Several chemicals were examined as inducers of plaque production by use of Sensitivity Disks (Difco) on YCz agar. These included bacitracin, chloramphenicol, chlortetracycline, dihy-

drostreptomycin, erythromycin, furadantin, gantrisin, isonicotinic acid hydrazide, neomycin, novobiocin, oleandomycin, oxytetracycline, penicillin, polymyxin, streptomycin, tetracycline, and viomycin.

Cell analyses. Cell wall preparations were analyzed by the procedures of Becker et al. (1). Whole-cell analyses for sugars were carried out according to Lechevalier (11).

Packed-cell volumes (PCV). A 4-ml amount of liquid culture was centrifuged in a 15-ml graduated centrifuge tube. After centrifuging for 3 min at 1,580 $\times$ g, the volume of packed cells was measured in milliliters.

Negative staining. Drops of phage concentrates were placed on carbon-stabilized, Formvar-coated, 400-mesh copper grids. The excess liquid was absorbed on filter paper and was replaced by a drop of 2.0% phosphotungstic acid adjusted to pH 6.2 with potassium hydroxide. The excess stain was immediately removed with filter paper, and, after air drying, the preparations were promptly examined with an electron microscope at instrument magnifications ranging from 10,000 to 30,000 $\times$.

Nucleic acid analysis. Phage concentrates were treated with nucleases in harvest medium (pH 6.2) fortified with 0.01 M MgCl₂ and were subjected to an additional cycle of centrifugation at 16,000 $\times$ g. Drops of the purified concentrate were dried on microscope slides, stained with acridine orange, and examined by fluorescent microscopy (2). As a control, some smears were exposed to nucleases before staining.

Preparation of ultrathin sections. Blocks (1 mm³) were cut from plate cultures of A1 grown on TPC. They were immersed for 30 min in 0.05 M phosphate buffer (pH 6.2) containing 3.0% glutaraldehyde and 0.01 M MgCl₂. After four 30-min rinses with phosphate buffer, the blocks were placed in acetate-Veronal buffer (pH 6.2), fixed in osmium tetroxide for 16 hr, and washed with uranyl acetate (6). After dehydration in ethyl alcohol, the blocks were embedded in Epon 812 (10). Sections were cut with a diamond knife and were stained for 2 hr in aqueous uranyl acetate followed by 20 min in lead citrate (14). Ultrathin sections were examined with an electron microscope at instrument magnifications of 10,000 $\times$.

The electron microscope used was an Akashi TRS 50E1 with 100- μ m apertures.

RESULTS AND DISCUSSION

The parent strain, *Dactylosporangium* L1, showed no sign of being infected with phage but gave rise to variants capable of functioning as carriers (Fig. 1). No spontaneous plaques were formed when it was grown on any of the media tried, nor were plaques formed after infection with either the clear-plaque A3 or the turbid-plaque L2 dactylosporangiophages. *Dactylosporangium* L1 did not produce phage in submerged culture, and phage production could not be induced by ultraviolet light or by a variety of antibiotics. When first isolated, it produced many sporangia; at present it is almost sterile.

Dactylosporangium strains A1 and A3 formed more sporangia than L1. The plaques observed in these strains were small (ca. 0.5 mm), clear, and were formed only under certain nutritional conditions. Virions produced by either dactylosporangia A1 or A3 gave rise, in the indicator, to clear plaques of irregular, angular outline.

Dactylosporangium L2 grew more rapidly than the parent and gave rise to a mixture of sterile, sectoring, and sporangia-forming colonies on YCz. The plaques formed on this culture by the phage it carries were tiny, irregular, and turbid. These plaques were visible only under the microscope. As on dactylosporangia A1 and A3, plaque formation on *Dactylosporangium* L2 was subject to nutritional control. However, no nutritional conditions were found that supported the formation of clear plaques on this organism; either the plaques were turbid or there were none at all. Phage L2 gave rise to tiny late-appearing turbid plaques in the indicator. Furthermore, superinfection of *Dactylosporangium* L2 with the "clear-plaque" A3 phage not only yielded fewer plaques than would be expected on the basis of assays, but the plaques were turbid. We conclude that *Dactylosporangium* L2 is semi-resistant to dactylosporangiophage. It is possible that the mechanism of this semiresistance is related to a host-controlled modification of the phage muramidase production that is both genetic and nutritional.

As previously stated, dactylosporangia L2, A1, and A3 show plaques reproducibly only under certain nutritional conditions. Of numerous

media and conditions of growth that were investigated, only Cz agar to which either 0.2 to 0.4% yeast extract or 0.2% peptone had been added supported the formation of clear-cut plaques. Addition of various carbohydrates, vitamins, or mineral salts or variation in agar concentration, temperature, pH, or humidity had no effect on plaque formation. Of various amino acids examined, only a combination of histidine, isoleucine, leucine, and methionine (40, 120, 140, and 40 mg/liter, respectively) added to Cz agar, supported plaque formation equivalent to that obtained on Cz agar fortified with yeast extract or peptone. None of these amino acids alone, tried at various concentrations, could substitute for the combination of the four.

The mechanism by which plaque formation was enhanced by YCz was studied in shaken liquid cultures (Table 1). In media, such as YD or TPC, virions were only released once the stationary phase had been reached. Cell wall synthesis during log phase presumably outpaced phage production and/or lysozyme induction or synthesis. In contrast, in YCz, virions were released early and showed evidence of readsorption and release cycles. An explanation of the fact that maximum phage titer is often observed after 1 day in YCz, although this is during the lag phase of cellular growth, is that the late log-phase cells employed as inocula for these studies had been grown in liquid YD. Presumably, virions present in the YD-grown cells were immediately re-

TABLE 1. Phage titer (plaque count) and growth (PCV) of two substrains of *D. thailandensis* L1 during incubation at 28 C on three media^a

Strain	Medium	Day							
		1	2	3	4	5	6	7	8
A1	YCz	200,000 (N) ^b	156,000 (0.05)	450,000 (0.12)	72,500 (0.08)	39,600 (0.08)	36,100 (0.08)	240,600 (0.08)	145,000 (0.08)
	TPC	70 (N)	0 (0.05)	2,100 (0.1)	12,600 (0.2)	13,000 (0.1)	14,500 (0.1)	20,000 (0.1)	20,000 (0.1)
	YD	0 (N)	70 (0.05)	0 (0.45)	0 (1.4)	0 (1.6)	455,000 (1.6)	326,000 (1.1)	700,000 (0.9)
A3	YCz	393,000 (0.15)	99,000 (0.2)	125,000 (0.25)	56,000 (0.18)	185,000 (0.15)	208,000 (0.15)	117,000 (0.15)	89,000 (0.15)
	TPC	1,220 (0.1)	450 (0.12)	380 (0.2)	8,000 (0.2)	4,900 (0.2)	4,200 (0.15)	9,100 (0.15)	8,750 (0.15)
	YD	0 (0.5)	2,640 (1.3)	760 (1.3)	13,200 (1.1)	33,000 (1.0)	82,500 (0.75)	128,000 (0.7)	53,000 (0.7)

^a Upper figures indicate number of phage particles per milliliter of culture. Lower figures in parentheses represent milliliters of PCV per 4 ml of culture.

^b Less than 0.05 ml.

leased on transfer to YCz as a result of enhanced muramidase production.

Peak counts of free phage were usually lower in YCz than in YD and frequently were considerably lower. Growth curves showed that very little, if any, net cellular synthesis occurred in YCz, the amount of cells present at the peak of growth being only about 10 to 25% of that in YD.

The mechanism of action of YCz is probably not so much that it restricts cell wall synthesis (as can be judged by the growth on solid medium) but that it permits synthesis, release, and readorption of large amounts of phage during the log phase, preventing an increase in biomass of the actinomycete host. That these early bursts in YCz may be directly related to enhanced production of muramidase rather than to increased phage production is strongly suggested by (i) the crystalline arrays of apparently complete virions in sections of hyphae on solid TPC (Fig. 6) and (ii) the appearance of virions in liquid TPC or YD only after normal cellular autolysis has begun.

Comparison of dactylosporangia L1 and A1 showed no differences in temperature range for growth, patterns of acid production from various carbohydrates, or utilization of various compounds. Comparisons of cell wall and whole-cell sugar patterns of dactylosporangia L1, L2, A1, A3, and C3_g grown in YCz and YD liquid media, showed no differences in the major constituents that could be related to susceptibility to lysis. About 100 strains of bacteria, mainly actinomycetes, were tested without success as potential detector strains.

Examination of negatively stained preparations of dactylosporangiphage obtained from *Dactylosporangium* A1 showed that it was a bacteriophage of Bradley's type B (3); that is, a phage with a polygonal head and a long, non-contractile tail (Fig. 1 to 4). Actinophages of this general morphology have been observed by Bradley and Ritzi (4). The heads of dactylosporangiphage A1 measured 75 ± 3 nm in diameter. Spherical capsomeres about 3 nm in diameter could be resolved in many of the head ghosts (Fig. 5). Although capsomeres have been observed in other bacteriophages of this general morphological type, for example coliphage λ , such observations are rare (3). The tails were 200 ± 10 nm long and 9 ± 2 nm in diameter. Although some tails were smooth (Fig. 1), cross-striations were observed in most cases (Fig. 2, 3). These markings were similar to those previously reported for a temperate actinophage (13). The presence of a longitudinal canal within the tail was seen in some ghosts (Fig. 4). Tails

were terminated by a fixation organelle, the exact structure of which was not satisfactorily resolved. In some cases, a three-prong forked structure was observed (Fig. 1a).

Dactylosporangiphage A1 is a double-stranded deoxyribonucleic acid virus. This conclusion is based on a yellow-green fluorescence of acridine orange-stained smears which disappeared after deoxyribonuclease treatment. Ribonuclease had no effect. Preparations of coliphage T1 and Rous sarcoma virus were run as controls.

Phage multiplication was followed by the examination of ultrathin sections of *Dactylosporangium* A1 harvested periodically from TPC plate cultures. First stages of growth of the actinomycete were observed only at 48 hr. Details of the cytology of *Dactylosporangium* A1 have been reported (M. L. Higgins, Ph.D. Thesis, Rutgers-The State Univ., New Brunswick, N.J., 1968).

After 72 hr of incubation at 28 C on TPC agar, only normal cells were observed. After 96 hr, numerous virions, often packed in crystalline arrangements, could be detected in the substrate mycelium (Fig. 6). Immature sporangia forming on the aerial mycelium did not show any visible sign of phage proliferation, but virions and ghosts were occasionally seen extracellularly (Fig. 7).

Dactylosporangium A1 formed large spherical bodies on the substrate mycelium. These structures of unknown function, at first suspected to be a host response to phage infection, were never observed to contain virions.

After 120 hr of incubation, the picture was very similar to that at 96 hr except that sporangia were maturing on the aerial mycelium. Phage particles were finally visible on the aerial mycelium after 144 hr. At that time, virion-bearing hyphae enlarged and some lysis could be detected on the substrate mycelium together with signs of mycelial regrowth. Crystalline arrangements of phage virions have been described for a number of other bacteriophages which do not lyse their hosts before a critical density of about 2,000 to 3,000 viruses per bacterial cell has been reached (15). Although a few cells of *Dactylosporangium* A1 did lyse, the basic mechanism of phage release appeared to be a slow leakage of particles from usually enlarged cells. At no time was there any indication of the peripheral migration of nuclear pools as observed with T-even phage infections (6, 12).

The observations presented here reinforce the observation that, in actinomycetes, the carrier state is a very stable condition (17). Evidently, this is due to their heterokaryotic, filamentous organization, in which many cells within a colony

FIG. 1-5. *Actinophage A-1* negatively stained with phosphotungstic acid. (1) Phage particle with a smooth tail. (1a) Terminal portion of a tail showing a three-pronged fixation structure. (2) Phage particle with a striated tail. Note the resolution of individual capsomeres. (3) Phage particle with an hexagonal head and striated tail. (4) Ghost particle. (5) Tailless ghost demonstrating individual capsomeres.

FIG. 6. Substrate cell of *D. thailandensis* A1 containing phage particles arranged in a crystalline lattice. From a 96-hr TPC agar culture.

FIG. 7. Immature sporangium of A1 removed from a 96-hr TPC agar culture.

can undergo lysis without any serious structural damage being visible with the light microscope.

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