Isolation, Characterization, and Classification of Three Bacteriophage Isolates for the Genus *Levinea*

DAIEL E. MARKEL and CURTIS EKLUND

Department of Biological Sciences, University of Texas at El Paso, El Paso, Texas 79968

Bacteriophages for the genus *Levinea* were isolated from sewage samples. On the basis of plaque morphology and host specificity, three different phage isolates were obtained and purified by standard techniques. Cross-neutralization experiments with homologous and heterologous phage antisera showed no serological relatedness among the three phages and, therefore, they were placed into three serological groups. Electron micrographs showed the isolates to have different and distinctive morphological types, one of which was unique in that it possessed an elongated "bullet-shaped" capsid. Nucleic acid types for the three phages were determined by the acridine orange-fluorescent staining technique to be double-stranded deoxyribonucleic acid. Based on serological grouping, the three phages were classified and designated DM-11, DM-21, and DM-31. The value of the phage isolates in defining the species of the genus *Levinea* is discussed.

In 1971 Young et al. (7) proposed the name *Levinea* for a new genus in the family *Enterobacteriaceae* to include bacteria differing distinctly from the other genera, particularly *Enterobacter* and *Citrobacter*, by a number of key biochemical reactions. The *levineae* are gram-negative, peritrichous bacilli. *Levineae* differ from the other enteric bacteria in that they produce indole, give a positive methyl red reaction, and do not produce acetylmethylcarbinol. Also, hydrogen sulfide is not produced and aesculin is not hydrolyzed. The metabolic inability of *levineae* to accomplish the latter biochemical reactions particularly distinguishes them from *Citrobacter freundii*. *Levineae* are differentiated from *Enterobacter cloacae* in that gelatin is not liquefied and sucrose and raffinose are not fermented by the former. There is no serological relatedness among the genus *Levinea* and genera of the family *Enterobacteriaceae*. Two species, *L. amalonatica* and *L. malonatica*, were differentiated on the ability to utilize malonate.

On the other hand, Ewing and Davis (5) considered *levineae* to be hydrogen sulfide-negative variants of *Citrobacter*. They reported that the reactions of *L. amalonatica* correspond to those given by the hydrogen sulfide-negative, indole-positive biotype of *C. freundii*, whereas the reactions of *L. malonatica* correspond to *C. diversum* (sic) first described by Werkman and Gillen (6). *C. diversum* is composed of bacteria

that are adonitol, indole, and ornithine positive and KCN negative. Ewing and Davis (5) corrected the specific epithet in the name of this organism to *diversus* in accordance with current rules of nomenclature.

Brenner and co-workers (Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 58, 1972) reported that the two *Levinea* species had a 40 to 60% nucleotide base sequence relatedness with *Citrobacter*, *Enterobacter*, and other representative enterobacteria.

This paper describes the isolation, characterization, and classification of three *Levinea* phages. (This investigation was presented at the March 1973 meeting of the Texas Branch of the American Society for Microbiology, San Antonio, Texas.

MATERIALS AND METHODS

Bacterial strains. A strain of *Levinea amalonatica* and a strain of *L. malonatica* both obtained from the laboratory of Hotel Dieu Hospital, El Paso, Texas, were used.

The type strains *L. amalonatica* ATCC 25405 and *L. malonatica* ATCC 25408 were used as references for confirming the identities of the other *Levinea* strains used in this study.

Medium. Heart infusion (HI, Difco served as the growth medium. Incubation was at 37 C.

Isolation of phages. Three sewage samples were obtained from the El Paso Sewer facility. The samples were pooled and then passed through a membrane filter (0.45 μ m; Millipore Corp.). A 1-ml sample of

filtrate was added to 5 ml of each enrichment culture of *L. amalonatica* and *L. malonatica* during exponential growth of the cells. The mixtures were incubated for 2 h with gentle shaking, and the nonlysed bacteria and debris were sedimented by centrifugation ($8,000 \times g$ for 10 min). The supernatant fluid was assayed for phages by the double-agar overlay technique (1) with the indicator strain *L. amalonatica* or with *L. malonatica*. Plaque picking and subsequent purification were achieved by the usual methods (1).

Phage assay. Phage dilutions were made in sodium phosphate buffer (0.1 M, pH 7). Plaque counts were obtained by the overlay method (1). Incubation was at 37 C for 18 to 24 h.

Preparation of indicator strains. The strains used for the indicator lawns were grown in HI to obtain an absorbance of 0.1 at 550 nm.

Propagation of phage lysates. Each initial phage isolate was diluted and plated by the double-agar layer method so that the cells of the lawns exhibited almost confluent lysis. To the surface of each plate, 5 ml of HI broth was added and allowed to stand for 5 min to loosen the soft agar. An L-rod was then used to scrape the surface, and the soft agar slurry was collected. The agar was pelleted by centrifugation at $2,500 \times g$ for 5 min. The supernatant fluid was decanted and centrifuged at $8,000 \times g$ for 10 min, to pellet the bacterial debris. The supernatant fluid was then filtered through a membrane filter (0.45 μ m; Millipore Corp.). The filtrates were stored at 4 C.

Phage antisera preparation. Rabbits were immunized intraperitoneally, with 5-ml doses of a phage filtrate with a titer of 10^8 to 10^{11} plaque-forming units (PFU)/ml, every 3 days for a period of 3 weeks. The animals were then bled through the marginal ear vein 5 days after their last injection. All sera were collected, processed, and stored at -20 C without preservatives.

Serological testing. The rates of inactivation of the various phages by their homologous and heterologous antisera were determined by the following procedure. Stock phages were diluted in phosphate buffer to about 10^5 to 10^6 PFU/ml. The sera were diluted to a concentration, determined by preliminary tests, that permitted approximately 1 to 10% survival of the original PFUs after sera and phage dilutions were mixed and incubated at 37 C for 5 to 10 min. Phage and serum dilutions were preheated to 37 C in a water bath. At time zero, 0.2 ml of the phage dilution was added to 1.8 ml of diluted antiserum. At specified time intervals, 0.1-ml samples were removed and diluted in cold phosphate buffer to stop the neutralizing action of the antiserum. Samples were plated in duplicate. Two controls were also included. One tube containing 1.8 ml of phosphate buffer served as a basis for determining the percentage of survival of phages. The second control was a tube containing 1.8 ml of normal serum to show that it did not inactivate the phages.

Host specificity assays. The routine test dilution (RTD) was determined for each phage on its homologous bacterial type (1). Each of the indicator strains was overlaid by the double-agar overlay technique.

After agar solidification, 0.02 ml of the RTD was spotted on the surface. After absorption of the RTD, the plates were incubated for 18 to 24 h at 37 C and observed for the presence or absence of lysis.

One-step growth experiments. Phage growth characteristics were determined by a stationary, one-step growth experiment (1) with the following modification. A tube containing 9 ml of HI broth was substituted for the usual 9.9-ml second growth tube. This substitution was made because the burst size of the phages was so small.

Assay of nucleic acid. The technique described by Bradley (3) for fluorescent staining of nucleic acid for type determination was used. T-1 phage, mouse liver deoxyribonucleic acid (DNA), and denatured mouse liver DNA were used as controls.

Electron microscopy. Each phage sample was concentrated and partially purified by differential centrifugation. The resultant pellet was suspended in phosphate buffer, mounted on Formvar-coated, 200-mesh grids, and negatively stained with 2% uranyl acetate (pH 4.3). Phage dimensions were obtained by taking the averages of 10 measurements.

RESULTS

Isolation of phages from sewage. Sewage samples were pooled, and phage plaques were obtained by the method previously described. On the basis of plaque morphology, three different phages were isolated and purified by further single-plaque selection.

Plaque morphology. Plaque morphologies of the three phages on their respective indicator organism are given in Table 1. The plaques observed were of two types: (i) minute, with a clear floor, represented by DM-11 and DM-21; and (ii) a slightly larger size with a turbid floor, represented by DM-31. This latter plaque type did not give any indication of a halo; however, when the plates were refrigerated overnight, a pronounced halo was observed.

Host infectivity. Host specificity assays were performed to ascertain the range of infectiveness of the phages. Two of the phages, DM-21

TABLE 1. Plaque sizes of *Levinea* phages

Phage	Plaque diameter (mm)	Plaque morphology	Lawn indicator
DM-11	1-2	Minute, clear	<i>Levinea amalonatica</i>
DM-21	1-2	Minute, clear	<i>L. malonatica</i>
DM-31	2-3	Turbid with halo	<i>L. malonatica</i>

TABLE 2. Growth characteristics of *Levinea* phages

Phage	Latent period (min)	Avg burst size
DM-11	28-32	8
DM-21	68-72	20
DM-31	36-38	48

and DM-31, could only infect *L. malonatica*, whereas DM-11 could only infect *L. amaltonatica*.

Serological relatedness studies. Each phage was tested against its homologous and two heterologous antisera to detect possible cross-neutralization reactions (Fig. 1). Controls consisted of normal rabbit serum and phosphate buffer. Results did not indicate any serological relatedness; therefore, three serological groups were established, each containing one phage.

One-step growth curves. The latent period and average burst size for each phage are presented in Table 2.

Nucleic acid type assay. Staining and enzyme tests were performed on each phage (Table 3). All three phage isolates proved to be double-stranded DNA by this method.

Phage morphology. The three phage isolates demonstrated different particle morphology (Fig. 2). Averages were taken on the dimensions and are presented in Table 4. Phages DM-11, DM-21, and DM-31 could be classified into the morphological groups A, B, and C, respectively, as proposed by Bradley (4). Phage DM-11 demonstrated a contractile tail, phage DM-21 possessed a long, curved, noncontractile tail, and DM-31 exhibited an unusual shape in that the capsid was elongated into a "bullet-shaped" structure with a short, stubby tail.

DISCUSSION

Plaques formed by the phages DM-11 and DM-21 were extremely difficult to enumerate due to their small size. It was found that if the base agar was thin the plaques were more easily viewed; they appeared very similar in morphology. DM-31 plaques, however, were easily observed since the plaque size was larger and there was a turbid floor.

Host specificity showed DM-11 to be specific for *Levinea amaltonatica* and DM-21 and DM-31

FIG. 1. Serological relatedness. All antisera diluted 1:50. Symbols: ●, homologous antisera; ○, heterologous antisera and controls. (A) DM-11, (B) DM-21, (C) DM-31.

TABLE 3. *Results of fluorescent-stained specimens*

Specimen	After Na ₂ HPO ₄	Molybdic acid	After tartaric acid	RNase ^a	DNase ^b	Nucleic acid type
T-1	Green	Green	Orange	—	+	2-DNA
ML-DNA ^c	Green	Green	Orange	—	+	2-DNA
ML-DNA (denatured)	Red	Green	Orange	—	+	1-DNA
DM-11	Green	Green	Orange	—	+	2-DNA
DM-21	Green	Green	Orange	—	+	2-DNA
DM-31	Green	Green	Orange	—	+	2-DNA

^a —, No color change after treatment.^b +, Spot rendered colorless.^c ML, Mouse liver. FIG. 2. *Phage morphology. (A) DM-11, (B) DM-21, (C) DM-31. Bars represent 100 nm.*TABLE 4. *Dimensions of Levinea phages*

Phages	Head width (nm)	Head length (nm)	Tail length (nm)	Total length (nm)
DM-11	54.0	54.5	138.0	192.5
DM-21	65.2	73.0	165.0	238.2
DM-31	42.0	158.0	12.0	170.0

to be specific for *L. malonatica*. These results indicate the possibility that the phages may be used to differentiate between the two species, and an investigation of this possibility is currently being pursued.

The small burst size of the three phages accounted for the reason that clear broth lysates were never obtained. High-titer phage stocks could only be acquired by the confluent-lysis overlay method.

Electron micrographs revealed that the three phages were morphologically unrelated and belonged to either Bradley group A, B, or C. Some *Caulobacter* phages (2) have a capsid morphology similar to that of DM-31, but the former possesses a much longer tail.

Because of the unrelated morphological types of the three phages, it was not at all unusual to find that they were serologically unrelated. The experiments undertaken were done primarily to study serological relatedness and not inactiva-

tion kinetics. Primary emphasis was placed on serology for classification of the *Levinea* phages, since serological relatedness is the most widely used taxonomic criterion recommended for defining the species category in bacteriophages (1). Cross-neutralization assays indicated three serological groups which were designated I, II, and III. The individual phages were given numbers preceded by the number of the serological group. This two-digit number was preceded by the initials DM. Thus, new phage isolates belonging to serological group I would be assigned a number between 11 and 19. The same could be applied to those new phage isolates belonging to serological group II or III. This allows not only for the addition of new isolates but also for additional serological groups.

The possibility that the *Levinea* phages reported here, especially DM-11, could be used to show either a relatedness or nonrelatedness

between *Citrobacter* species and *Levinea* species is apparent. Studies are currently being undertaken to elucidate this conjecture.

ACKNOWLEDGMENTS

We are sincerely grateful for the electron micrographs of the phage isolates made by Leodocia Pope, The University of Texas at Austin. We acknowledge the technical assistance of Melinda Jimenez.

LITERATURE CITED

1. Adams, M. H. 1959. Bacteriophages. Interscience Publishers, Inc., New York.
2. Agabian-Keshian, N., and L. Shapiro. 1970. Stalked bacteria: properties of deoxyribonucleic acid bacteriophage CbK. J. Virol. 5:795-800.
3. Bradley, D. E. 1966. The fluorescent staining of bacteriophage nucleic acids. J. Gen. Microbiol. 44:383-391.
4. Bradley, D. E. 1967. Ultrastructure of bacteriophages and bacteriocins. Bacteriol. Rev. 31:230-314.
5. Ewing, W. H., and B. R. Davis. 1972. Biochemical characterization of *Citrobacter diversus* (Burkey) Werkman and Gillen and designation of the neotype strain. Int. J. Syst. Bacteriol. 22:12-18.
6. Werkman, C. H., and G. F. Gillen. 1932. Bacteria producing trimethylene glycol. J. Bacteriol. 23:167-182.
7. Young, V. M., D. M. Kenton, B. S. Hobbs, and M. R. Moody. 1974. *Levinea*, a new genus of the family *Enterobacteriaceae*. Int. J. Syst. Bacteriol. 21:58-63.