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Phage and Phage-Like Particles of *Bacillus pumilus* from an Animal Waste Lagoon

D. L. Berryhill and K. E. Irmen

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In the past several years, animal waste lagoons located at the Animal Research Center, North Dakota State University, have been shown to contain a number of bacteriophages. Berg et al. (3) isolated and characterized seven coliphages which had not been described previously. Lutgen (5) isolated 18 different bacteriophages for *Enterobacter*, 2 phages for *Citrobacter*, 2 phages for *Providencia*, and a single phage each for *Escherichia*, *Proteus*, and *Salmonella*.

In this report (a portion of a thesis presented by K. E. Irmen in partial fulfillment of the requirements for the M.S. degree in bacteriology at North Dakota State University), we describe a new temperate bacteriophage for a *Bacillus pumilus* lagoon isolate. In addition, the strain lysogenic for the new phage and the *B. pumilus* type strain (ATCC 7061) both harbor inducible particles which resemble "killer particles" reported for other *Bacillus* strains (1, 12). *B. pumilus* ATCC 7061 also produces a second type of phage-like particle upon induction.

MATERIALS AND METHODS

Bacteria.

B. pumilus strains 3 and 9 were isolated from lagoon B located at the Animal Research Center, North Dakota State University. Identification of the strains was based on the methods of Gordon et al. (11) and Buchanan and Gibbons (9). Both isolates require exogenous biotin for growth as determined by auxanography using a chemically defined medium (16). Only strain 9 is capable of utilizing citrate as a sole source of carbon.

B. pumilus ATCC 7061 was obtained from the American Type Culture Collection, Rockville, MD. *Bacillus subtilis* strains W23 and 168 *try*$^-$, used in host-range studies, were available in the Department of Bacteriology, North Dakota State University. All bacterial strains were maintained on trypticase soy agar (TSA; Baltimore Biological Laboratories) slants and stored at 4 C.

Induction Procedure.

Cells from an overnight slant culture were used to inoculate 100 ml of trypticase soy broth (TSB; Baltimore Biological Laboratories) to an optical density of 0.15 at 540 nm, and the broth culture was incubated, with shaking, at 30 C. When an optical density of 0.35 was attained, mitomycin C (Calbiochem) was added to the culture to achieve a final concentration of 4.0 μg/ml. After exposure to mitomycin C for 15 min, the cells were quickly pelleted by centrifugation, resuspended in 100 ml of fresh TSB, and incubated. When lysis of the culture was evident, cell debris was removed by centrifugation, and the supernatant fluid was filter-sterilized (type HA membrane; Millipore Corp., Bedford, MA) and stored at 4 C.

Propagation and Concentration of Phage.

Phage lysates containing greater than 1 x 10^{10} plaque-forming units (PFU) per ml were prepared by broth propagation. One hundred milliliters of TSB were inoculated with host bacteria and incubated at 30 C, with shaking, until logarithmic growth was attained. The culture was then infected with phage and allowed to incubate until lysis was complete. Bacterial debris was removed by centrifugation, and the lysate was stored at 4 C after being filter-sterilized. Phage titrations were performed according to the method of Adams (2) using TSA plates and nutrient soft agar (nutrient broth [Difco] containing 0.4% agar).

*Berryhill is associate professor and Irmen a former graduate student, Department of Bacteriology.

Phages were concentrated by centrifuging lysates at 48,000 X *g* for 90 min at 5 C, followed by resuspension of the phage pellet in a small volume of appropriate liquid overnight at 4 C. Aggregates which did not resuspend were removed from the concentrated lysate by low-speed centrifugation.

Electron Microscopy.

Concentrated particles, suspended in 0.1 M ammonium acetate, were negatively stained with 2% sodium phosphotungstate (pH 7.2) and observed with an AEI Corinth 275 electron microscope. Dimensions of particles were determined using the head diameter of coliphage λ as a standard (3).

RESULTS

Treatment of *B. pumilus* strain 9 cells with mitomycin C resulted in complete lysis of the culture within 3 h. The lysate was titrated using strain 9 indicator cells, and a few plaques formed during the 18-h incubation. A representative plaque was stabbed and purified by three successive single-plaque isolations, and a high-titer lysate was prepared. When the lysate was concentrated and examined by electron microscopy, two different types of particles belonging to Bradley's morphological groups A and C (7) were present in approximately equal numbers.

In order to determine the plaque-forming ability of each type of particle, preparative CsCl isopycnic centrifugation (4) was employed. Concentrated lysate was mixed with CsCl (initial density = 1.425 g/ml) and the mixture was centrifuged to equilibrium. The density gradient was then fractionated, and each fraction was examined by electron microscopy as well as for PFU and deoxyribonucleic acid (DNA) content (10). The particles belonging to morphological group C had a buoyant density of 1.43 g/ml (compared to 1.35 g/ml for the group A particles), formed plaques, and contained a substantial amount of DNA. Based on these results, the group C particles were designated phage ND-9. The group A particles (9A particles) were incapable of plaque formation; however, a small amount of DNA was detected in the 9A particle fractions.

Phage ND-9 (Fig. la) has an oblong head averaging 37 x 53 nm, and a tail structure about 50 nm long; the tail appears to have prongs projecting from a collar at the base of the head. The fluorescent staining procedure of Bradley (6) revealed that the DNA of phage ND-9 is double-stranded. In addition, the phage is stable in 5% chloroform and at 60 C for 10 min, although it is inactivated after exposure to 70 C for 10 min.

9A particles (Fig. lb) are composed of a head 33 nm in diameter, a contractile tail 198 nm in length, and tail fibers. It was discovered that samples for the electron microscopic examination of 9A particles which were free of phage ND-9 could be simply prepared by concentrating particles in the growth medium of uninduced strain 9 cultures. Apparently 9A particles are spontaneously produced at a much higher frequency than is phage ND-9.

The biological activities of pure phage ND-9 and 9A particles were determined by titrations and the "spot test" (5). Phage ND-9 formed plaques on *B. pumilus* strains 3 and 9 with equal efficiency, but had no activity on *B. pumilus* ATCC 7061 or *B. subtilis* strains W23 and 168 *try*⁻. Undiluted 9A particles produced lysis on lawns of all *B. pumilus* and *B. subtilis* strains tested except *B. pumilus* strain 9; no plaques were detected by titration.

To obtain a further indication of the frequency at which *B. pumilus* strains harbor inducible particles, cells of strain ATCC 7061 were treated with mitomycin C and the lysate was concentrated. Electron microscopy revealed two types of phage-like particles in the lysate: particles similar to 9A particles, and particles belonging to morphological group B. The latter particles (Fig. 1c) have heads 57 nm in diameter and flexible tails 117 nm long. The lysate containing the two types of particles did not possess biological activity with any of the strains used in this study.

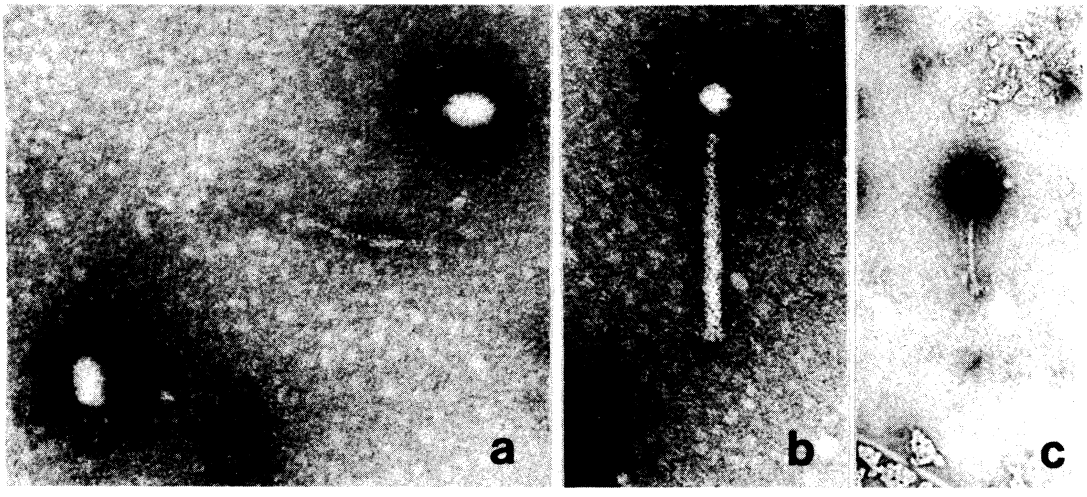

Fig. 1. Phage and phage-like particles of *B. pumilus*. (a) Phage ND-9, X 150,000. (b) 9A particles, X 150,000. (c) Group B particle from *B. pumilus* ATCC 7061, X 110,000.

DISCUSSION

Strain 9 of *B. pumilus* is lysogenic for phage ND-9 which, on the basis of morphology, is a new phage. The only other phages of *B. pumilus* for which morphologies have been described, PBP1 (13) and ϕ75 (8), belong to Bradley's morphological group B (7); phage PBP1 is flagella-specific, while ϕ75 infects only asporogenic variants of *B. pumilus*. Because phage ND-9 can be propagated on strain 9 cells, either prophage immunity is lacking in this phage-host system or we have isolated a mutant phage no longer susceptible to prophage immunity. The fact that phage ND-9 was detected at a very low frequency in induced cultures of strain 9 supports the latter possibility.

9A particles are similar to killer particles described for *B. subtilis* (1, 12) and *Bacillus licheniformis* (1), and to a bacteriophage-like particle induced from *B. pumilus* NRRL B-3275 (14). All of these particles have nearly identical morphologies, and all exhibit killer activity, but no plaque formation, against heterologous *Bacillus* strains. In addition, 9A particles contain a small amount of DNA — a property observed for some *B. subtilis* killer particles (1). Whether or not killer particles are best classified as "bacteriocins", "particulate bacteriocins", or "defective phages" remains the subject of debate.

The demonstration that the type strain of *B. pumilus*, ATCC 7061, harbors killer particles and morphological group B particles suggests that lysogeny and/or bacteriocinogenicity is widespread in *B. pumilus*. Unfortunately, the ATCC 7061 particles could not be further characterized because of the lack of suitable indicator systems for their biological activities.

SUMMARY

B. pumilus strain 9, isolated from a farm animal waste lagoon, is lysogenic for phage ND-9, a new phage. Strain 9 also produces killer particles which have been described for a number of other *Bacillus* strains. Induction of *B. pumilus* ATCC 7061 yields two types of phage-like particles, one of which morphologically resembles killer particles.

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