Preliminary observations of a bacteriophage infecting *Xenorhabdus luminescens* (Enterobacteriaceae)

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Summary. A bacteriophage infective to *Xenorhabdus luminescens*, a bacterial symbiont of heterorhabditid nematodes, was recovered from insects that supported poor nematode development. Plaque tests showed the phage particles to be infective only to primary and not secondary colonies of *X. luminescens*. The phage was not infective to *X. nematophilus* primaries or secondaries. The bacteriophage particles ranged 80–90 nm in length, with the head ranging from 40 to 50 nm in diameter. Restriction analysis was performed on isolated bacteriophage DNA. This first report of a bacteriophage from *Xenorhabdus* species has practical implications since it could be detrimental to cultures of *Heterorhabditis* nematodes that are being produced throughout the world for the biological control of insects.
Key words. Bacteriophage; *Xenorhabdus; Heterorhabditis*; bacteria; nematoda.

Bacteria of the genus *Xenorhabdus* are found in a specialized area of the alimentary tract of entomogenous rhabditoid nematode infective juveniles and can be isolated from the hemocoel of insects which have been parasitized by these nematodes[1]. *Xenorhabdus* bacteria have no clear affinities with any known family. The genus has been placed in the Enterobacteriaceae although it is only distantly related to the genera that comprise the core of the family[2]. A species displaying luminescence, *X. luminescens*, is associated with nematodes of the genus *Heterorhabditis*, including *H. heliothidis*.

When released into the hemocoel of an insect host, the *Xenorhabdus* bacteria multiply, leading to septicemic death of the insect within 48 h. The nematodes feed on the bacteria and breakdown products of the insect cadaver. Successful nematode development depends on the presence of a flourishing bacterial population in the insect cadaver.

It has been shown that species of *Xenorhabdus* occur in two forms, which can be distinguished by colony morphology and by color on specific growth media. The primary form is the principal form transmitted into new hosts by the infective-stage nematode. The secondary form appears during nematode reproduction. The primary form does not disappear during this time, however, and is selectively retained by progeny infective juveniles. Conversion of the primary form into the secondary form may take place both in vivo and in vitro[3]. The cause of this conversion is not understood. Conversion of the secondary form into the primary form has not been documented.

During routine investigations in which *H. heliothidis* was cultured on *Galleria mellonella* (wax moth) larvae, two of us (S. K. and W. L.) examined hosts containing poorly developed nematodes. A bacteriophage infective to *X. luminescens* was isolated from these insects. In this paper, we present electron microscopic analysis of the bacteriophage particle morphology and agarose gel electrophoretic analysis of the bacteriophage DNA genome.

Materials and methods. *Galleria mellonella* infected with *H. heliothidis* turn a chracteristic dark red color. *Galleria* cadavers which were pale red, yellow, brown or black were chosen as indicative of poor *Xenorhabdus* growth. Initial bacteriophage material was recovered from these abnormal insect cadavers by homogenizing 20 corpses in 10 ml buffered saline plus gelatine (BSG) with a tissue homogenizer. The homogenate was centrifuged at 5000 rpm for 15 min in a clinical centrifuge. The supernatant was removed and a few drops of chloroform added. After shaking 20 min, the supernatant was filtered through a glass prefilter, followed by 1.5-, 0.45- and 0.22-μm filters. The resultant filtrate (2.5 ml) was placed in 2.5 ml of a 12-h trypticase soy broth with a 0.5% yeast extract (TSY) culture of primary *X. luminescens*. This was incubated for 20 min, then 0.5-ml aliquots were added to 4.5 ml of melted soft TSY agar and poured onto eight TSY agar plates. Control plates had an overlay of 4.5 ml melted nutrient agar, 0.25 ml healthy primary bacteria and 0.25 ml sterile saline. All plates were inverted and incubated at 25 °C. Additional tests using the above described experimental plan were performed using secondary forms of *X. luminescens* as well as primary and secondary forms of *X. nematophilus*.

Electron micrographs were prepared from material obtained by infecting *X. luminescens* cells (m.o.i. = 1) in TSY broth cultures and incubating 5 h at 25 °C. The broth was centrifuged at 15,000 rpm for 5 min in a microcentrifuge. Bacteriophage particles were precipitated by mixing with 0.2 volumes of 20% PEG 4000, 2.5 M NaCl. After sitting 30 min at room temperature, the mixture was centrifuged 5 min at 15,000 rpm in a microcentrifuge. Bacteriophage particles in the pellet were resuspended in a small volume of 10 mM Tris, pH 8, 1 mM EDTA (TE). The suspension was prepared for electron microscopy by staining with saturated aqueous uranyl acetate and examined in a Philips EM 300 electron microscope.

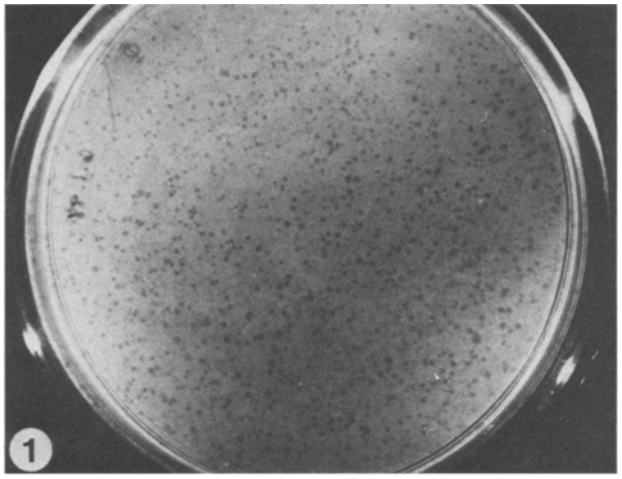
Bacteriophage DNA was purified from the particle suspension described above by extracting three times with neutralized phenol and once with chloroform: isoamyl alcohol (24:1). The DNA was then precipitated with ethanol and resuspended in TE. Restriction endonucleases *Hind* III and *Eco*RI were obtained from New England Biolabs and were used as described by the supplier.

During our investigation a plasmid was discovered in the bacterial cultures. Steps were taken to characterize this plasmid and show its distinctness in relation to XLP. Plasmid DNA was isolated by the method of Birnboim and Doly[4].

Results. After incubating *X. luminescens* primary overnight with the bacteriophage suspension, plaques were clearly evident (fig. 1). All plaques were similar in size and morphology. No plaques were present on the controls or when *X. luminescens* secondary or *X. nematophilus* primary or secondary were used in place of *X. luminescens* primary.

Electron micrographs revealed a typical bacteriophage particle which will be referred to here as the XLP (*Xenorhabdus luminescens* primary) bacteriophage (fig. 2). The head is hexagonal in lateral view and ranges from 40 nm in the shortest diameter to 50 nm in diameter from apex to apex. In most cases, the tail was broken off at the base plate below the collar. The plate was located from 16 to 18 nm below the head and the total tail was approximately 40 nm long. Base plates and tail pins could be made out in a few whose tail was not broken (fig. 2, insert).

Electrophoretic analysis of XLP bacteriophage DNA after digestion with *Eco*RI resulted in 5 fragments of approximately 15, 12, 8, 7 and 1.8 kb (fig. 3). Thus, the total estimated genome is approximately 44 kb (lambda is 48 kb). Many fragments were produced by *Hind* III XLP treatment. Since the sizes of the fragments sum to 60 kb, it is concluded that digestion by the enzyme was incomplete.



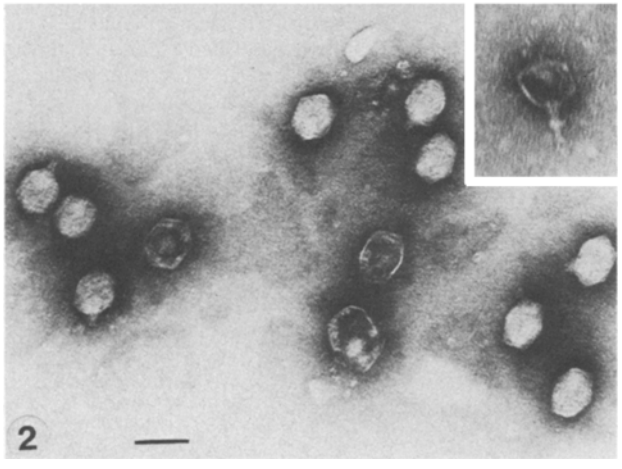


Figure 1. Plaques showing the presence of the XLP bacteriophage on a lawn of *Xenorhabdus luminescens* primary bacteria grown on TSY agar plates.

Figure 2. Electron micrograph showing heads and heads with partial tails of the XLP bacteriophage (bar = 50 nm). (Insert shows a bacteriophage particle (stain has penetrated the head) with collar and tail pins (same magnification).

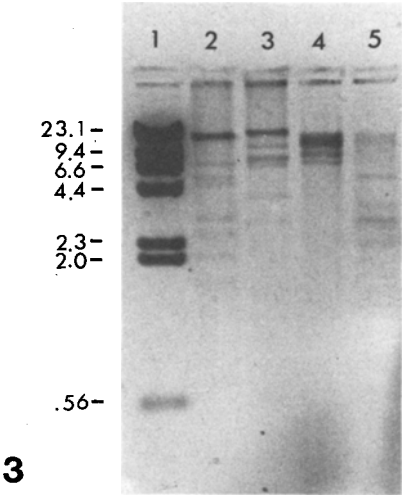


Figure 3. Results of agarose gel electrophoresis. Lane 1: Bacteriophage lambda marker DNA digested with *Hind* III. Lane 2: Plasmid DNA from *Xenorhabdus luminescens* primary cells treated with *Eco*RI. Lane 3: Plasmid DNA treated with *Hind* III. Lane 4: XLP bacteriophage DNA treated with *Eco*RI. Lane 5: XLP bacteriophage DNA treated with *Hind* III.

A restriction analysis of plasmid DNA isolated from a culture of primary *Xenorhabdus luminescens* which had not been exposed to XLP bacteriophage is also presented (fig. 3). This control experiment was undertaken in order to confirm that the bacteriophage DNA isolated was not an artifact due to plasmid DNA being released from lyzed bacterial cells. The restriction patterns for the plasmid DNA and the bacteriophage DNA are distinct. The plasmid DNA yields 8 fragments with *Eco*RI (23, 9, 7, 5, 4, 3, 2.5 and 2 kb) and 7 fragments with *Hind* III (23, 10, 8, 4, 3, 1.4 and 1.1 kb). The total size of the plasmid is therefore 50–56 kb.

Discussion. The present report is the first documentation of a bacteriophage attacking bacteria of the genus *Xenorhabdus*. An earlier study showed that both *X. luminescens* and *X. nematophilus* primary forms were capable of producing defective bacteriophage [5].

Of special interest regarding the XLP bacteriophage is its specificity not just to *X. luminescens*, but to the primary form of the bacterium, since the secondary form of *X. luminescens* is actively dividing and thus should be able to produce bacteriophage particles.

The discovery of a *Xenorhabdus* phage also has an important practical implication since *Heterorhabditis* nematodes are now being grown in large quantities as biological control agents for insects. Introduction of XLP or other *Xenorhabdus* bacteriophages into culture facilities would have disastrous results on nematode production as one of their major nutrients became depleted.

The restriction analyses in figure 3 rule out the possibility that the plasmid DNA observed is a cryptic form of bacteriophage DNA. The relationships between the primary and secondary forms of *Xenorhabdus*, the selective infectivity of XLP for primary bacteria, the selective retention of primary bacteria in entomogenous nematodes and the function of the plasmid in *Xenorhabdus luminescens* primary bacteria are promising topics for future molecular genetic studies.

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