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Isolation of Two Bacteriophages from *Bacillus larvae*, PBL1 and PBL0.5, and Partial Characterization of PBL1

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SUMMARY

Two temperate bacteriophages have been isolated from *Bacillus larvae*: PBL1 and PBL0.5. Strains lysogenic for either of these phages are immune to lysis by the same phage but are sensitive to the other phage. PBL1 has an oval head, a non-contractile tail, and a base plate with a pin structure but no apparent tail fibres. The genome of PBL1 consists of double-stranded DNA with a molecular weight of $24.1(\pm 0.6) \times 10^6$, a $G + C$ content (derived from melting temperature) of 41.5%, and cohesive ends. Restriction enzyme analysis permitted construction of a physical map of the genome.

*Bacillus larvae* is a pathogen of the larvae of the honeybee (*Apis mellifera* L.), causing a fatal disease called American foulbrood. A bacteriophage of *B. larvae* was first isolated by Smirnova (1953) from decaying larvae of bees killed by American foulbrood. Gochnauer (1955) isolated a phage from a lysogenic culture of *B. larvae*. Differences were found between Gochnauer's phage and that isolated by Smirnova (1954) in their ability to pass through asbestos filters, heat stability and plaque morphology (Gochnauer, 1970). In addition to describing some properties of the one phage isolated from the strain now known as *B. larvae* NRRL B-3553 (Gochnauer & L'Arrivee, 1969), Gochnauer (1970) presented evidence suggesting that other phages were present in other strains of *B. larvae*. This conclusion was drawn from sensitivity tests using culture filtrates from different *B. larvae* cultures and lawns of many different strains. No efforts were made to isolate the different phages. Gochnauer (1970) was unable to concentrate or purify the phage from strain B-3553, and, hence, was unable to observe the morphology or analyse the nucleic acid component of this phage.

A phage specific for *B. larvae* was isolated from a soil sample from a park in Plodiv, Bulgaria (Popova et al., 1976; Valerianov et al., 1976). This phage, named L3, lysed 10 of 15 strains of *B. larvae* tested. It did not lyse *B. cereus* or *B. anthracis*.

A phage, termed BLA, was isolated in Czechoslovakia from several *B. larvae* strains obtained from combs containing bee larvae killed by American foulbrood (Drobniková & Ludvík, 1982). All of the phage preparations from different cultures were considered to be identical, based on the sole criterion of their appearance in electron micrographs.

The main goal of the present study was purification and characterization of the phage isolated by Gochnauer (1955) from *B. larvae* NRRL B-3553. Gochnauer & L'Arrivee (1969) reported that when a culture filtrate of strain B-3553 was plated on lawns of *B. larvae* NRRL B-3555, both large plaques (2 to 3 mm) and pinpoint plaques appeared. Subculturing of both resulted in a uniform plaque size (1 to 2 mm), however, and the authors concluded that both plaque types were caused by the same phage. Evidence presented in this paper indicates that strain B-3553 contains two distinct phages.

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B. larvae NRRL strains used in this study were B-2605, B-3553, B-3554, B-3555, B-3558 and B-3650. All were kindly provided by Dr L. Nakamura (Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill., U.S.A.) except for strain B-3553 which was provided by Dr T. A. Gochnauer (Canada Department of Agriculture, Ottawa, Canada). All of these strains are independent isolates (Gordon et al., 1973). B. larvae NRRL B-3553, which is naturally lysogenic for the phage described by Gochnauer (1970), was grown in MYPGP broth [Mueller Hinton broth (Difco), 1-0%; yeast extract (Difco), 1-5%; K2HPO4, 0-3%; glucose, 0-2%; sodium pyruvate, 0-1%] to a density of 10⁸ to 2 × 10⁹ cells/ml, and the cells were removed by centrifugation. The culture supernatant was plated on a lawn of B. larvae NRRL B-3554 by use of a standard soft agar overlay technique. Bottom agar (25 ml) consisted of MYPGP broth plus 2-0% agar (Difco); soft top agar (2 ml) consisted of MYPGP broth plus 0-6% agar.

Two different types of plaques were observed: a large (2 mm) turbid plaque and a small (0-5 mm) plaque. The large plaques were the source of the phage we refer to as PBL1. The phage was subcultured by numerous transfers from single plaques. Initially, we did not know whether the small plaques were due to a variant of PBL1 or to a different phage.

It was decided to check the other B. larvae strains for lysogeny. It was assumed (i) that culture supernatant of lysogenic strains would contain phages and (ii) that strains lysogenic for a phage would not show plaques when exposed to the same phage. The phage(s) in strain B-3553 produced plaques on all the other five strains. The supernatant from strain B-3558 produced plaques on all the other strains except B-3553. The plaques produced by the supernatant of strain B-3558 were very small (approx. 0-5 mm). These observations were consistent with the hypothesis that strains B-3553 and B-3558 had a common phage that produced a small plaque and was not PBL1, or contained different phages that shared the same immunity mechanism. (Additional supporting evidence is presented below.) There was no evidence of strains B-2605, B-3554, B-3555 and B-3650 containing phages infective for the other strains tested.

A lysogenic strain of B. larvae NRRL B-3555 [B-3555(PBL1)] was obtained from the centre of a turbid plaque after infection of this strain with PBL1. The culture was transferred several times from single colonies to avoid the possibility of phage carry-over. This culture was immune to PBL1, and culture supernatant contained phage producing plaques on all of the other strains except B-3553. Strain B-3555(PBL1), used as an indicator strain, showed small plaques when supernatants from either strain B-3553 or B-3558 were added. This result constituted further evidence that the small-plaque phages in these strains were not PBL1.

The phage in strain B-3558, which we designated PBL0.5 because of its small plaque size, was isolated from plaques on strain B-2605 and purified by repeated single-plaque picking. The purified phage produced plaques on all strains except B-3553 and B-3558. We isolated a strain of B-2605 made lysogenic for PBL0.5c (a clear-plaque mutant of PBL0.5). This strain was immune to PBL0.5 and to supernatant from strain B-3558. Plating of supernatant from strain B-3553 on strain B-2605 (PBL0.5c) yielded large turbid plaques from the resident PBL1, as expected. From all of the above data we concluded that strain B-3558 contains PBL0.5, while strain B-3553 contains PBL1 and PBL0.5 (or a related phage having the same immunity mechanism as PBL0.5). All the other strains lack these phages.

As stated previously, PBL1 plaques are turbid. Occasionally, clear plaques were observed. A phage, which we designated PBL1c, was isolated from a clear plaque on a lawn of strain B-3555 and purified. Like PBL1, it produced plaques on all strains except B-3553 and B-3555(PBL1). PBL1c is still capable of lysogenizing B. larvae, even though it produces apparently clear plaques on strains B-2605, B-3554, B-3555, B-3558 and B-3650. When a large number of the phage was added to each of the above strains, and the mixtures were plated, confluent lysis occurred. Two to 3 days later, small colonies appeared. The apparent lysogens were transferred several times using single colonies as inocula. These strains were immune to infection by PBL1 and PBL1c, and the culture supernatants contained PBL1c.

PBL1 titres of 1 × 10⁹ to 1 × 10¹⁰ p.f.u./ml were routinely obtained by elution from confluent lysis plates (10⁶ phages/plate; 37 °C incubation for 24 h) or by infection of broth cultures. Lysates of broth cultures were obtained by addition of PBL1 (m.o.i. 5) to a culture of strain B-3555 grown in MYPGP broth to a density of 3 × 10⁷ to 6 × 10⁷ cells/ml. Incubation was...
Fig. 1. Electron micrograph of PBL1 with a full head (left) and an empty head (right). The additional structure on the left is probably a bacterial flagellum. Prior to electron microscopy, the phage were purified by the following procedure. Phage obtained from lysates of broth cultures or confluent lysis plates were centrifuged (72670 g, 90 min, 4 °C), resuspended in 1 ml sterile water, and layered on top of a CsCl step gradient. This had been prepared in a 5 ml Beckman nitrocellulose tube (0.5 × 2 in) by adding 1.5 ml, 1.0 ml and 1.0 ml of CsCl solutions of density 1.5, 1.3 and 1.1 g/ml, respectively, all in 0.02 M-Tris-HCl buffer pH 7.4. The sample was overlaid with paraffin oil and centrifuged (242922 g, 2 h, 15 °C). The main band containing phage was removed and dialysed, consecutively, at 4 °C: (i) 2 h against 50 mm-Tris–HCl pH 7.4, 10 mm-MgSO4, 1.0 m-NaCl; (ii) as (i) but 0.5 m-NaCl; (iii) as (i) but without NaCl; (iv) overnight against H2O.

continued at 37 °C with shaking until lysis was complete. PBL1 was stable in MYPGP broth or water at 4 °C for at least 1 year.

PBL1 was purified as described in the legend to Fig. 1. It was negatively stained with 2% sodium phosphotungstate on a carbon support film and was viewed at a magnification of 45000 to 50000 with a JEOL-100B electron microscope operating at 60 kV. An electron micrograph of PBL1 (Fig. 1) shows the head as an oval structure without any apparent angularity. The tail seems to be non-contractile. A base plate with a pin structure exists, but no tail fibres are visible. Many of the phages appeared to aggregate, forming rosettes (data not shown). The reason for this clustering is unknown. Using as an internal standard phage T4, for which the dimensions are firmly established (Luftig, 1968), we determined the dimensions of PBL1. The length and width of the head were 95.9 ± 2.6 nm and 52.5 ± 1.9 nm (standard deviations are given). The length and width of the tail were 131.8 ± 1.5 nm and 9.4 ± 0.6 nm and the length and width of the base plate were 37.9 ± 1.3 nm and 6.4 ± 0.5 nm.

PBL1 DNA was isolated by the procedure of Ito et al. (1976). The melting temperature (Tm) of the DNA in 1 × SSC buffer (0.15 m-NaCl, 0.015 m-sodium citrate, pH 7.0) was determined (Mandel & Marmur, 1968) to be 86.3 °C, a value that corresponds to a G + C content of 41.5%.

The molecular weight of PBL1 DNA was found to be 24.1(±0.6) × 106 from the sum of the molecular weights of the DNA fragments resulting from treatment with restriction enzymes BamHI, BglII, SacI, EcoRI, HindIII, HpaI, PsiI, SalI, SmaI and XhoI. The buffers and reaction temperatures used for each enzyme were those described by Davis et al. (1980). All DNA
fragment sizes were determined by comparing the mobility of DNA bands during agarose gel electrophoresis with the mobility of DNA molecules of known molecular weights [e.g. HindIII fragments (Robinson & Landy, 1977) and ρ11 BglII fragments (Mizukami et al., 1980)]. BamHI, BglII and SacI cut the phage genome into four, five and four fragments, respectively. Digestion with EcoRI did occur, but the resulting pattern was uninterpretable. No digestion was observed with HindIII, HpaI, PstI, SalI, SmaI and XhoI.

A physical map of the phage genome was constructed (Fig. 2) by comparison of the fragment sizes of the BamHI, BglII and SacI digests and the fragment sizes of BamHI/BglII and SacI/BglII double digests. Ambiguity concerning the order of the BglII B and BglII E fragments was resolved by determination of DNA fragment sizes after partial digestion with BglII.

A peculiarity in the band pattern was noted after agarose gel electrophoresis of the DNA fragments resulting from digestion with BglII. The DNA band pattern was different depending on whether the BglII digest was heated (70 °C for 5 min) just before electrophoresis. The heated sample exhibited five bands, and the sum of the molecular weights of the fragments was 24.1 × 10^6, a molecular weight consistent with other results. The unheated sample exhibited six bands, and the sum of the molecular weights of the fragments was 30.5 × 10^6. Our hypothesis is that the terminal fragments BglII C and D have cohesive ends, some of which anneal with each other in non-heated digests. Thus, the extra DNA band in the unheated digest was C plus D. The molecular weight of this band (6.2 × 10^6) is consistent with this conclusion. The sum of the molecular weights of BglII C and BglII D is 6.44 × 10^6. This observation is similar to that made with ϕ105, a B. subtilis phage (Perkins et al., 1978). The existence of cohesive ends suggests that in the host cell the phage genomes form circular molecules or concatemers.

Clear-plaque mutants of both PBL1 and PBL0.5 were isolated. They were still capable of lysogenizing host strains, but at a frequency much lower than the corresponding wild-types. For possible use as prophylactic agents to prevent the occurrence of American foulbrood, these phages at present are less than ideal, because they will not lyse cultures that carry them as prophages. Further research to isolate virulent mutants is needed to meet this goal. Smirnova (1953, 1954, 1956, 1961) has reported some success in prophylactic use of phage against American foulbrood.

PBL1 is probably the phage originally isolated from B. larvae NRRL B-3553 and studied by Gochnauer (1955, 1970) and Gochnauer & L’Arrivee (1969). The latter authors noticed the ‘pin-point’ plaques due to phage in culture supernatant from B. larvae NRRL B-3553, but mistakenly concluded that the plaques were due to PBL1. We now know that the small plaques are due to PBL0.5, or a phage related to PBL0.5 by having a common immunity mechanism. Further studies must be conducted to ascertain whether PBL0.5 and the small-plaque-forming phage in strain B-3553 are identical.

We do not know whether there is any relationship between the phages that we have studied and those studied by investigators other than Gochnauer. Drobnikova & Ludvik (1982) presented electron micrographs of a phage isolated in Czechoslovakia, designated BLA, which morphologically resembles PBL1. However, the reported dimensions of BLA differ considerably from those of PBL1.
PBL1 and PBL0.5 are of potential value as cloning vectors in *B. larvae*. They could also be of value in studying transcriptional regulation during sporulation of *B. larvae*. Such studies with phages of *B. subtilis* provided the initial clues that led investigators to the hypothesis that a cascade of sigma factors was involved in selective gene expression during sporulation (Losick & Pero, 1981).

A summary of the work was presented previously (Field *et al.*, 1982).

**REFERENCES**


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