

## The Isolation and Characterization of a Lipopolysaccharide-Specific *Pseudomonas aeruginosa* Bacteriophage

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### SUMMARY

The isolation and some properties of a lipopolysaccharide (LPS)-specific phage isolated against *Pseudomonas aeruginosa* is reported. The phage, designated  $\phi$ PLS-1, is a Bradley A type phage with a head diameter of 70 nm and a contractile tail 120 nm long. The average adsorption rate constant for  $\phi$ PLS-1 is  $4.48 \times 10^{-9}$  ml/min.  $\phi$ PLS-1 is inactivated by purified LPS from *P. aeruginosa* strain 1-1A, showing a  $\text{PhI}_{50}$  value of 1.25  $\mu\text{g/ml}$ .

### INTRODUCTION

As was the case with *Salmonella*, a true picture of the role of lipopolysaccharide (LPS) in *Pseudomonas* pathogenicity may be gained through the analysis of mutants defective in LPS biosynthesis or assembly (rough mutants). The isolation of these rough strains can be accomplished by selection for resistance to LPS-specific phages. The logic behind using LPS-specific phages to isolate spontaneous or induced rough mutants has been extensively reviewed (Rapin & Kalckar, 1971; Stocker & Mäkelä, 1971; Wilkinson *et al.* 1972; Lindberg, 1973). Analogous reasoning went into the isolation of a pilus-deficient (Bradley, 1973 *c*) mutant of *P. aeruginosa*.

Although a considerable number of *Pseudomonas* phages have been isolated, none have received the extensive attention paid to the coliphages, and in particular very few definitive studies into the cellular chemoreceptors for *Pseudomonas* phages have been reported. *P. aeruginosa* pili are the receptors for the filamentous phage Pf (Bradley, 1973 *b*), single-stranded RNA-containing phage PP7 (Bradley, 1966 *b*), Bradley B-type (Bradley, 1966 *a*) phages PO4 and PO2 (Bradley, 1973 *a, c*), and the temperate phage F116 (Pemberton, 1973). Although Bartell *et al.* (1971) reported that phage 2 was irreversibly inactivated by slime polysaccharide, Lindberg in his recent review on phage chemoreceptors (Lindberg, 1973) suggests caution in interpreting their results. Recently, Castillo & Bartell (1974) reported that phage 2 also interacts with host LPS.

The present communication reports the isolation and some properties of a LPS-specific phage active against *P. aeruginosa*.

### METHODS

*Bacteria.* The strains of *P. aeruginosa* used in this study were: 1-1A (Kropinski & Chadwick, 1975); ATCC 10145; and the Habs serotypes, H-1, 2a, 2b, 3, 4, 5c, 5d, 6, 7, 8, 9, 10, 11, Ps-11, H-12 and S-13 (obtained from Dr N. A. Hinton). The cultures were maintained at 4 °C in nutrient agar stab medium (Miller, 1972). Bacterial cultures were incubated in an Aquatherm water bath shaker (Model G-86, New Brunswick Scientific Co., New Brunswick, N.J.).

*Isolation of phage.* Bacteriophage  $\phi$ PLS-1 was isolated from the raw sewage influent at the Kingston Township Sewage Treatment Plant using the technique of Kropinski & Warren (1970), and purified by several serial single plaque pickings.

*Titration of phage.* Phage plaque assays were made by the agar overlay technique (Adams, 1959). The top and bottom layers consisted of LB containing 0.6 and 1.5% (w/v) agar, respectively.

*Preparation of high titre lysates.* A 125 ml Ehrlenmeyer flask containing 25 ml of LB was inoculated with 0.5 ml of an overnight culture of strain 1-1A which was incubated at 37 °C with shaking to an optical density at 650 nm of approx. 0.1. One plaque was added to the flask and incubation was continued until lysis (approx. 3 h). Whole cells and debris were removed by centrifuging at 10000 g for 10 min, and the supernatant fluid was stored over chloroform at 4 °C. This method routinely yielded lysates with titres of 5 to  $10 \times 10^{10}$  p.f.u./ml. Crude phage lysates were concentrated with polyethylene glycol (PEG) 10% (w/v) following the method of Yamamoto *et al.* (1970). The PEG precipitate was collected by low speed centrifugation and dissolved in 0.01 M-tris-HCl, pH 7.5. Prior to electron microscopy the phage were dialysed against 0.01 M-ammonium acetate, pH 7.2.

*Electron microscopy.* A drop of purified phage (about  $10^{12}$  p.f.u./ml) was placed on grids pre-coated with Parlodion and an amorphous coat of carbon and stained with 1% phosphotungstic acid containing 0.1 mg egg albumin per ml, pH 6.8. A Forggflo EMU-4C electron microscope operated at 50 kV was used.

*Adsorption kinetics.* An overnight culture of strain 1-1A was diluted 1/50 into fresh LB and grown at 37 °C and 200 rev/min to mid-log phase (approx. 3 h). The culture was chilled and diluted with ice cold LB to an  $E_{650}$  of 0.1 to 0.2. Cells (9.0 ml) in a 125 ml flask were incubated at 37 °C for 5 min, infected by the addition of 1 ml of phage (approx.  $1.3 \times 10^6$  p.f.u./ml) and immediately shaken (75 rev/min). Samples (0.1 ml) were removed at intervals into ice cold LB plus a few drops of chloroform, agitated vigorously and maintained on ice until the end of the experiment when 0.1 ml portions were plated on strain 1-1A. The adsorption rate constant ( $k$ ) was calculated using the equation of Schlesinger (1960).

*Purification of lipopolysaccharide.* Lipopolysaccharide (LPS) was extracted from cells grown in Tryptic soy broth (Difco) using the technique of Westphal & Jann (1956) incorporating the modification of Key, Gray & Wilkinson (1970) to remove mucopeptide.

*Neutralization kinetics with LPS.* The neutralization of phage infectivity by LPS was determined using a modification of the procedure of Lindberg (1967). A 25 ml flask containing 0.8 ml of LB, and 0.1 ml of LPS (dissolved in distilled water) was incubated at 37 °C for 3 min when 0.1 ml of phage ( $3 \times 10^4$  p.f.u./ml) was added. The flask was incubated at 75 rev/min and 0.1 ml samples were plated at intervals.

*Effect of surface active agents on phage neutralization by LPS.* Neutralization studies had indicated that a final LPS concentration of 10  $\mu$ g/ml was sufficient to inactivate 90% of the phage within 1 h and therefore this concentration of LPS was chosen for the experiments with the surface active agents. The EDTA and SLS were added to a final concentration of 5 mM, sodium deoxycholate (DOC) to a final concentration of 2.5 mM and polymyxin B to 25  $\mu$ g/ml. The pH of the EDTA was adjusted to 6.7, the pH of the LB. The pH of the DOC and SLS were not adjusted. Reaction mixtures contained: 0.7 ml LB, 0.1 ml phage, 0.1 ml LPS and 0.1 ml of the chemical agent, in a 25 ml flask. The flask was shaken at 75 rev/min and at intervals 0.1 ml samples were removed and plated on strain 1-1A.

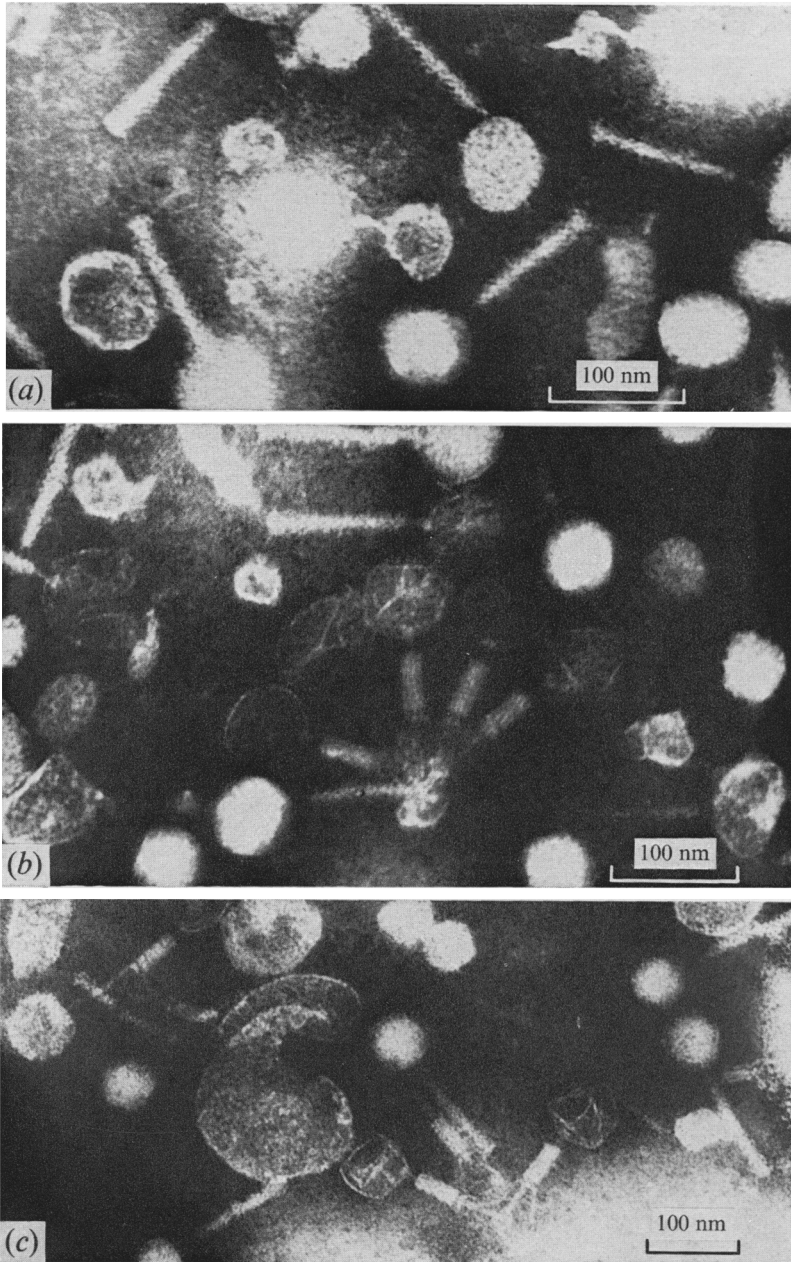


Fig. 1. (a) Electron micrographs of  $\phi$ PLS-1 after staining with 1% phosphotungstic acid. (b) Cluster of  $\phi$ PLS-1 particles showing empty heads, contracted sheaths and prominent tail pins. Subunits in vertical array are visible in the contracted sheaths. (c) Phage particles showing indications of tail fibres and extended cores.

## RESULTS

### *Morphology*

$\phi$ PLS-1 is a Bradley A type phage (Bradley, 1967), subclass A1 (Ackermann, 1973) possessing an overall length of 190 nm. It has a hexagonal head 70 nm in diam., and a contractile tail 120 nm long by 11 nm wide at its midpoint (Fig. 1a). Subunit structure is

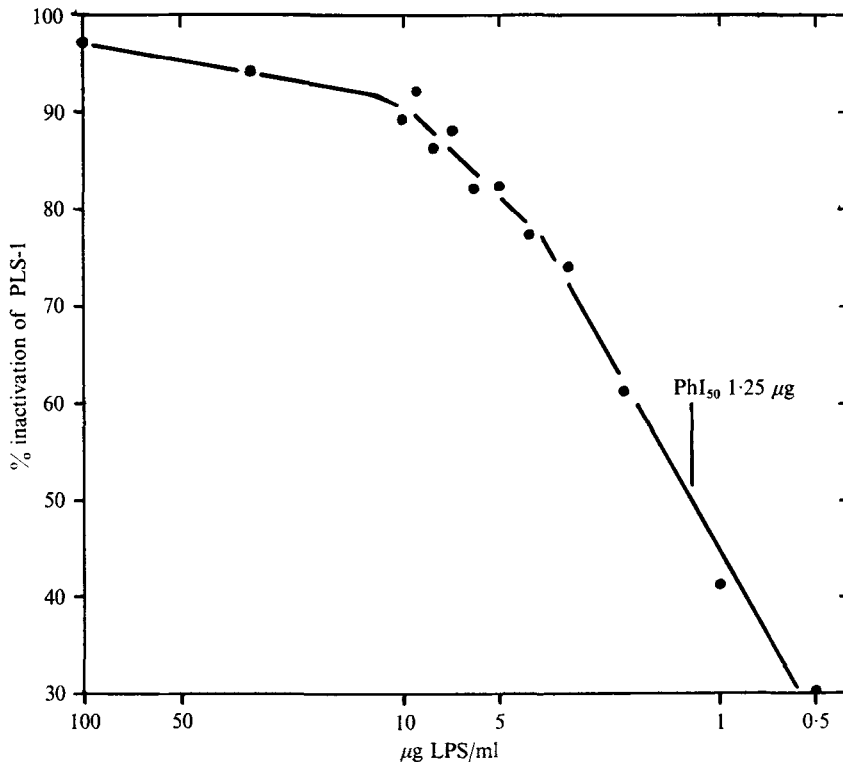


Fig. 2. The  $\phi$ PLS-1 inactivating capacity of phenol+water-extracted lipopolysaccharide from *P. aeruginosa* 1-1A. The activity ( $\text{PhI}_{50}$ ) of the lipopolysaccharide (LPS) is expressed as the concentration of LPS (dry w/v) necessary to inactivate 50% of the phage.

apparent in the sheath, with an apparent conformational change occurring upon contraction. The contracted sheath measures 40 nm long by 16 nm wide, and displays prominent tail pins (Fig. 1*b*). Cores and possibly tail fibres are observable in Fig. 1*c*.

#### *Plaque morphology, host range and efficiency of plating*

$\phi$ PLS-1 formed small plaques about 2.5 mm in diam., with clear centres and a slightly turbid halo, on strain 1-1A. The host range was very limited. The phage lysed only three of eighteen *P. aeruginosa* strains: 1-1A, H-1 and H-6. The e.o.p. was 1.4 on strain H-1 and 0.5 on H-6 when compared to strain 1-1A.

#### *Adsorption to whole cells*

In LB, the concentration of unadsorbed phage decreases exponentially with first order kinetics for over 90% of the adsorption of the phage. The average adsorption rate constant was  $4.48 \times 10^{-9}$  ml/min with a range of  $3.98 \times 10^{-9}$  ml/min to  $4.97 \times 10^{-9}$  ml/min.

Following the procedure of Olsen, Metcalf & Todd, (1968), it was shown that  $\phi$ PLS-1 does not require divalent cations for adsorption to whole cells. The plaque count of the phage in media containing 0.5 and 1.0% (w/v) sodium citrate was the same as that determined using media supplemented with 0.001 M-CaCl<sub>2</sub>. However, the plaque morphology did change. In citrate plates the plaques remained the same size but the turbid halo was now much more evident.

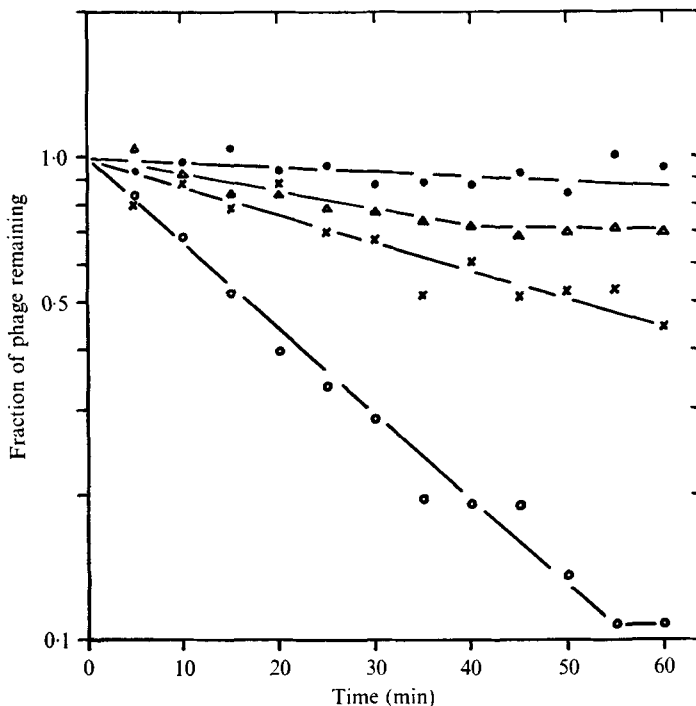


Fig. 3. The effect of chemical agents on  $\phi$ PLS-1 inactivation by phenol-water-extracted LPS: ●—●,  $\phi$ PLS-1+LPS+EDTA (5 mM);  $\Delta$ — $\Delta$ ,  $\phi$ PLS+LPS+SLS (5 mM);  $\times$ — $\times$ ,  $\phi$ PLS-1+LPS+DOC (2.5 mM); ○—○,  $\phi$ PLS-1+LPS. When either EDTA or SLS were incubated alone with  $\phi$ PLS-1 there was no decrease in phage titre. With the DOC concentration used, 62 % of the phage were inactivated in the control after 60 min. In the presence of LPS 55 % of the phage were inactivated suggesting that the LPS might be exhibiting a protective effect. PLS-1 is not inactivated by incubation at 37 °C for 1 h.

#### Neutralization experiments with LPS

LPS preparations from strains 1-1A and ATCC10145 were tested for their inactivating capacity towards  $\phi$ PLS-1. The LPS from the type culture had no inactivating effect on  $\phi$ PLS-1 but the LPS from strain 1-1A inactivated about 90 % of the phage after 1 h when the LPS was present at a final concentration of 10  $\mu$ g/ml. The  $PhI_{50}$ , the concentration of LPS needed to inactivate 50 % of phage, was 1.25  $\mu$ g/ml (Fig. 2). Even at high LPS concentrations (200  $\mu$ g/ml), a fraction of the phage was not affected. Similar results have been shown by Jann *et al.* (1971).

#### Treatment of LPS with surface active agents

The effect of the various chemicals on phage viability and on phage neutralization by LPS is shown in Fig. 3. EDTA (5 mM) and SLS (5 mM) had no effect on phage viability but they essentially abolished adsorption of the phage to the LPS. Polymyxin B (25  $\mu$ g/ml) had no effect on phage viability or on neutralization of infectivity by LPS. DOC (2.5 mM) reduced the adsorption of the phage to the LPS to some degree, probably by a dissociation of the LPS but as indicated in Fig. 3 possibly due simply to the action of the DOC on phage viability.

## DISCUSSION

The virulent phage  $\phi$ PLS-1 (Kropinski & Chadwick, 1975) falls into Bradleys' morphological classification group A which contains all phage with a contractile tail (Bradley, 1967). The contractile tail of  $\phi$ PLS-1 and its larger head diameter distinguishes this LPS-specific phage from the temperate phage 2 of Bartell *et al.* (1971) which adsorbs to slime polysaccharide and lipopolysaccharide. The possibility that  $\phi$ PLS-1 also interacts with slime polysaccharide could not be tested since strain 1-1A does not produce slime.

The results show that the sites of attachment for  $\phi$ PLS-1 are present in the LPS of *P. aeruginosa* strain 1-1A as judged by the ability of the LPS to neutralize the phage. This has been confirmed by examining the effects of surfactants on phage neutralization by LPS.

As expected, the addition of EDTA abolished phage neutralization by the purified LPS of strain 1-1A. A similar effect of EDTA on phage-LPS interactions was reported by Takeda & Uetake (1973). Since  $\phi$ PLS-1 does not require divalent ions for adsorption to whole cells the EDTA cannot be affecting adsorption co-factors.

Polymyxin B (25  $\mu$ g/ml) had no effect on the ability of the LPS to neutralize the phage and this suggests that the polymyxin B is not dissociating the LPS, a result in agreement with the work of Rubio, Portolés & Lopez, (1973). This inactivity of polymyxin B does not however appear to be general since Cooperstock (1974) found that the crude endotoxins from a variety of aerobic gram-negative bacteria, though not *Pseudomonas*, were considerably inactivated by as little as 1  $\mu$ g/ml polymyxin B. Of course, polymyxin B may act differently *in vivo* and *in vitro*. However, the inhibition of LPS-specific phage adsorption in polymyxin-treated bacteria (Monner, Jonsson & Boman, 1971) is direct evidence that polymyxin-induced alterations of LPS do occur *in vivo*.

Beer *et al.* (1965) showed that SLS can affect LPS without destroying biological activity. Our results, however, indicate that it abolished the ability of LPS to neutralize  $\phi$ PLS-1. In this regard they agree with the findings of Oroszlan & Mora (1963) who showed that SLS dissociates LPS with concomitant loss of biological activity.

DOC has a partial effect on phage neutralization by the LPS, presumably due to DOC acting on the LPS. It has been reported (Ribi *et al.* 1966) that DOC can dissociate endotoxin into subunits and destroy its biological activity, but since DOC also caused some type of phage inactivation the true reason for the DOC effect is obscure.

The  $\text{PhI}_{50}$  for LPS in the current study (1.25  $\mu$ g/ml) more closely resembles the values obtained by Castillo & Bartell (1974) with phage 2 and Monner *et al.* (1971) with coliphage  $\phi$ - $\omega$  (1  $\mu$ g/ml) than the results with *Salmonella* phage Felix 0-1 (Lindberg, 1967). In the latter study values as low as 0.002  $\mu$ g/ml were recorded. It has been reported by Monner *et al.* (1971) that additional purification of *Escherichia coli* LPS was sometimes found to give rise to significant losses of phage blocking activity, presumably through destruction of some 2° or 3° structures. It may be that *Pseudomonas* LPS is also sensitive to purification.

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