Role of *O*-Acetyl Groups in the Lipopolysaccharide Receptor for *Rhizobium* Phage 1P

By ELŻBIETA ZAJĄC AND Z. LORKIEWICZ*

Institute of Microbiology, M. Curie-Skłodowska University, Akademicka 19, 20–033 Lublin, Poland

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Rhizobium trifolii lipopolysaccharide (LPS) preparations lost their receptor properties for phage 1P after deacetylation by alkali. LPS treated with acetyl esterase isolated from *Lactobacillus plantarum* showed a simultaneous decrease in the amount of acetyl groups and phage receptor activity. The content of *O*-acetyl groups in the LPS of a phage-resistant mutant was significantly decreased when compared to that in LPS from the parent phage-sensitive strain 24SM. Phage 1P could cleave acetyl groups from the extracted LPS without any detectable formation of reducing groups. Binding of phage 1P to the LPS receptor was irreversible. It was demonstrated by electron microscopy that phage DNA was ejected *in vitro* in the presence of LPS from *R. trifolii* 24SM but not of that from the phage-resistant mutant.

INTRODUCTION

Various components of the cell envelope are known to serve as receptor sites for bacteriophages in Gram-negative bacteria (Lindberg, 1977). The recognition of the receptor is a highly specific process. The mechanism of interaction of the phage with the receptor has been elucidated for a number of phages but little information is available on phage receptors in *Rhizobium*. Barnet & Vincent (1970) suggested that the receptor site for the phages 7, 7cr and 8 was associated with the O-antigen of *Rhizobium*. Atkins & Hayes (1972) detected alterations in LPS and lipoprotein of a *R. trifolii* strain that adsorbed phage poorly. The phage 16-12-1 for *Rhizobium lupini* was found to be pilus dependent (Lotz & Pfister, 1975). Depolymerases specific for exopolysaccharides were detected in a number of phages specific for *R. trifolii* (Barnet & Humphrey, 1975) and *R. leguminosarum* (Dandekar & Modi, 1978). We have demonstrated that the receptor specificity for phage 1P resides in the LPS of *R. trifolii* 24SM (Zajac *et al.*, 1975a). The sites of phage attachment as well as the mechanism of the interaction between the phage and its *Rhizobium* receptor have not however been determined in detail.

In this report we show that *O*-acetyl groups are essential components in the phage receptor, and that phage 1P cleaves *O*-acetyl groups from isolated LPS.

METHODS

Bacterial and phage strains. *Rhizobium trifolii* 24SM and its derivative 15, resistant to phage 1P (Zajac *et al.*, 1975a) were used. They were grown in medium 4 as described by Zajac *et al.* (1975a). Phage 1P propagated on *R. trifolii* 24SM was employed in assays of receptor activity of LPS.

LPS. This was prepared and deacetylated as described previously (Zajac *et al.*, 1975a).

Gas-liquid chromatography. This was performed as described by Zajac *et al.* (1975b).

Preparation of acetyl esterase. The enzyme was isolated from *Lactobacillus plantarum* according to Oterholm *et al.* (1972). Its activity was determined by mixing 5 ml of an homogenized suspension of penta-*O*-acetyl-D-glucose (0.2 M) at pH 6.5 with 0.2 ml of the enzyme preparation (equivalent to 100 µg protein ml⁻¹) and incubating at 37 °C for 1 h with subsequent pH measurements.

Abbreviation: LPS, lipopolysaccharide.

Quantitative assay of O-acetyl groups. O-Acetyl groups were measured by the procedure of Hestrin (1949) and by gas chromatography (Fromme & Beilharz, 1978).

Infrared analysis. Samples of LPS (3 mg; prepared as described by Westphal & Jann, 1965) in KBr were analysed in a Zeiss IR 20 apparatus.

Assay of reducing groups. This was done by the procedure of Park & Johnson (1949), using D-glucose as the standard.

Sonication. LPS of *R. trifolii* 24SM with attached phage 1P was sonicated using an MSE sonicator, 150 W, 8 × 15 s, 0 °C.

Electron microscopy. LPS suspension (2 mg ml⁻¹) was incubated with phage (2 × 10¹¹ p.f.u. ml⁻¹) at 37 °C for 1 h in the presence of 0.2% albumin. Then the material was placed on Formvar-coated electron microscope grids. After application of 2% (w/v) phosphotungstic acid at pH 7.0 for 2 min at 4 °C, the grids were dried and examined in a JEM 100B electron microscope.

RESULTS

We had previously shown that receptor sites for phage 1P are located on LPS of *R. trifolii* 24SM (Zajac *et al.*, 1975a). L-Glycero-D-mannoheptose, 3-O-methylheptose, 3,6-di-O-methylheptose and mannose are major sugar components of this LPS (Russa & Lorkiewicz, 1979), which suggested that heptoses might form part of the phage receptor. However, L-glycero-D-mannoheptose was found to be the major component of LPS from the phage-resistant mutant 15.

Many phages that bind to LPS have endoglycosidic activity, usually splitting a specific sugar bond in the LPS (Iwashita & Kanegasaki, 1973; Reske *et al.*, 1973). To test this for the phage 1P, LPS of *R. trifolii* 24SM (10 mg ml⁻¹) was suspended in 0.01 M-Tris/HCl buffer, pH 7.4, and incubated with phage (6 × 10¹⁰ p.f.u. ml⁻¹) for 16 h. Every four hours the amount of reducing groups was determined and compared with results for LPS incubated under the same conditions but without phage. No increase in reducing groups was observed.

In a further search for components of the phage receptor we examined the possible involvement of acetyl groups. The infrared spectrum of LPS of the phage-sensitive strain *R. trifolii* 24SM showed a strong absorption peak at 1740 cm⁻¹ (Fig. 1, A), which is characteristic for ester-bound acetyl groups. The spectrum of the corresponding LPS from the phage-resistant mutant 15 did not show such a peak (Fig. 1, B). The content of O-acetyl groups measured by either gas chromatography or the Hestrin procedure was also significantly lower in LPS from mutant 15 than in LPS from strain 24SM (Table 1). This suggested that acetyl groups may be involved in the attachment of the phage to the receptor. Alkali-treated LPS preparations showed a simultaneous decrease in O-acetyl groups and phage receptor activity (Table 1).

Fig. 1. Infrared absorption spectra of LPS isolated from the phage-sensitive strain 24SM (A) and that of the phage-resistant mutant 15 (B). The C=O stretching vibration of an O-acetyl group gives very strong absorption in the region 1725–1749 cm⁻¹. The peak of acetyl group frequencies was not detected in LPS from mutant 15.

Table 1. Content of *O*-acetyl groups in LPS preparations and their phage-inactivating capacity

LPS from:	<i>O</i> -Acetyl groups ($\mu\text{mol per mg LPS}$)		Percentage of phage inactivation per $\mu\text{g LPS}\dagger$
	Gas-liquid chromatography	Hestrin procedure	
Strain 24SM	0.34	0.3	81
Strain 24SM (+0.02 M-NaOH)*	0.04	—	3
Mutant 15	0.078	0.092	0

* Treatment was for 1 h at 37 °C.

† Final concentration of LPS was $1 \mu\text{g ml}^{-1}$ and of phage 1P $3 \times 10^3 \text{ p.f.u. ml}^{-1}$; incubation was for 15 min at 37 °C.

Fig. 2

Fig. 2. Inactivation of phage 1P by acetylcholinesterase-treated LPS from strain 24SM. ○, Untreated LPS; ●, LPS treated with acetylcholinesterase (equivalent to $100 \mu\text{g protein per mg LPS}$), 1 h, 37 °C; □, phage 1P incubated with acetylcholinesterase, 1 h, 37 °C. For testing phage receptor activity of LPS the final concentration of LPS was $5 \mu\text{g ml}^{-1}$ and of phage 1P $3 \times 10^3 \text{ p.f.u. ml}^{-1}$.

Fig. 3

Fig. 3. Infrared spectra of LPS from strain 24SM: A, untreated; B, pretreated with 0.02 M-NaOH, 1 h, 37 °C; C, incubated with acetylcholinesterase (equivalent to $100 \mu\text{g protein per mg LPS}$), 1 h, 37 °C; D, incubated with phage 1P ($6 \times 10^{10} \text{ p.f.u. per mg LPS}$), 1 h, 37 °C.

Alkaline deacetylation is a rather drastic procedure, which might lead to additional LPS alterations, especially to hydrolysis of some ester-bound fatty acids and to substitution of glycosidic bonds by ester linkages at carbons 3 and 6. We therefore used a more specific technique of deacetylation by acetylcholinesterase (see Methods). After the *Rhizobium* LPS had been incubated with the enzyme for 1 h, a significant decrease in its phage receptor activity was observed (Fig. 2). In a control sample of phage 1P incubated with acetylcholinesterase the virus titre remained unaltered. The spectrum of the LPS treated with the enzyme showed a decline in absorption at 1740 cm^{-1} .

We then tested whether phage 1P actually deacetylated its receptor, by incubating the LPS with phage 1P under the conditions used in experiments testing for the appearance of new

Fig. 4. (a) Electron micrograph of phage 1P particles interacting with LPS from strain 24SM; the phage DNA is ejected. (b) Phage 1P particles incubated with LPS from the phage-resistant mutant 15; the phage particles are filled with DNA. The bar markers represent 0.1 μm .

reducing groups. The incubated material was analysed by infrared spectrophotometry. A significant decrease in the content of acetyl groups was observed compared with the untreated LPS preparation, demonstrating that the phage 1P did deacetylate the LPS (Fig. 3).

Binding of phage 1P to the LPS of strain 24SM was irreversible, as indicated by the failure to release active phage by Triton X-100, sonication or blending, or by transfer of LPS with adsorbed phage 1P from 4 °C to 37 °C. This suggested that the *in vitro* reaction of phage 1P with LPS resulted in the ejection of phage DNA. This was confirmed by electron microscopy: empty ghosts of phage 1P were found in the preparation of phage 1P incubated with LPS from strain 24SM (Fig. 4a), whereas phage particles incubated with LPS from mutant 15 were filled with DNA (Fig. 4b).

DISCUSSION

Bacteriophage receptors have been found in the LPS of numerous representatives of *Salmonella* (Iwashita & Kanegasaki, 1973, 1975, 1976; Kanegasaki & Wright, 1973), *Shigella* (Przondo-Hessek *et al.*, 1974; Przondo-Hessek & Romanowska, 1978; Romanowska *et al.*, 1976), *Pseudomonas* (Jarrel & Kropiński, 1977; Meadow & Wells, 1978) and *Rhizobium* (Zajac *et al.*, 1975a, b). Phages ϵ^{15} , ϵ^{34} , g341 and P22 attach to the O-side chains of *Salmonella*. The linkage between successive repeating units is thought to be an essential component of the receptor (Iwashita & Kanegasaki, 1975; Reske *et al.*, 1973).

The purpose of the present study was to determine the essential components of the receptor for *Rhizobium* phage 1P, and to investigate the mechanism of the phage-receptor interaction. We had previously found that the receptor for phage 1P resided in the LPS of *R. trifolii* 24SM. L-Glycero-D-mannoheptose was the major component of the LPS of *R. trifolii* 24SM (Zajac *et al.*, 1975b). Analysing various LPS preparations of *R. trifolii* and *R. leguminosarum* we noticed some correlation between the content of L-glycero-D-mannoheptose and the ability of LPS to inactivate phage 1P. Furthermore, degradation of heptose by oxidation with NaIO_4 reduced the capacity of LPS to bind (unpublished results). Tamaki *et al.* (1971) demonstrated that in *Escherichia coli* K12, susceptibility to phage T7 was dependent on the heptose in the core of LPS. However, in further studies these authors found that some heptose-defective mutants were also susceptible to the phage, suggesting that some factor in LPS other than heptose was required for phage T7 adsorption. We observed that the phage-resistant mutant 15, derived from *R. trifolii* 24SM, had 5% less L-glycero-D-mannoheptose than its parent, but this can hardly be a significant difference (Zajac *et al.*, 1975b).

Our further studies (Zajac *et al.*, 1975a; Russa *et al.*, 1982) indicated that there is an LPS component other than heptose which is needed for phage attachment. Since phage 1P did not have endoglycosidic activity we examined in detail the role of acetyl groups in phage 1P adsorption. Acetyl groups are essential for the attachment of numerous phages. Shaw & Chatterjee (1971) found that *O*-acetyl groups were components of the bacteriophage 52A receptor on *Staphylococcus aureus* cell walls. Phage c341 was shown to cleave acetyl groups from *Salmonella anatum* *O*-side chains (Iwashita & Kanegasaki, 1976). It was also found that Vi-phages I, II and III had deacetylase activity (Taylor, 1965; Szczeklik & Taylor, 1973; Beilharz *et al.*, 1978). Phage E79 infected *Pseudomonas aeruginosa* PAO only if its LPS contained acetyl groups (Jarrel & Kropiński, 1977). In the present study we detected a significant decrease in *O*-acetyl groups in LPS of the phage-resistant mutant 15. LPS preparations of *R. trifolii* 24SM lost their capacity for phage 1P inactivation after alkaline deacetylation. Although the alkaline deacetylation of LPS was confirmed by infrared analysis and gas chromatography, some other alterations of LPS might occur which could affect phage receptors. Thus the involvement of acetyl groups was also examined by application of the specific enzyme acetylsterase, which decreased the phage receptor activity of the LPS. Phage 1P adsorbs irreversibly to the LPS receptor in host cells.

It is concluded that *O*-acetyl groups are essential for phage 1P attachment. During this process phage 1P deacetylates its LPS receptor.

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