Adsorption of a Phage Tail-Like Bacteriocin to Isolated Lipopolysaccharide of Rhizobium

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SUMMARY

Purified lipopolysaccharide (LPS) from the bacteriocin sensitive strain Rhizobium lupini 16–2 was shown to neutralize the killing activity of the bacteriocin. In the electron microscopical preparation the phage tail-like bacteriocin appears to be adsorbed to the LPS; the tail sheath is contracted and the fibres are oriented towards the LPS ribbon. In contrast, no interaction was observed between the bacteriocin and the LPS of two resistant strains of Rhizobium (16–2/I1 and 16–3). The inactivation of the bacteriocin by LPS depends on salt concentration, pH, and temperature. The receptor activity of LPS was destroyed by mild acid hydrolysis and by treatment with deoxycholate, which indicates that the micellar structure of the LPS is necessary for bacteriocin adsorption. The chemical composition of the 16–2 LPS was compared to that of the LPS of two resistant strains. In the case of 16–2/I1 LPS minor modifications suffice to confer resistance against the bacteriocin.

INTRODUCTION

In recent years, many bacterial strains have been shown to produce particles resembling substructures of bacteriophages. The particles are not able to propagate themselves but some of them can kill bacteria closely related to the producer strain (see Lotz, 1976). These particles with bactericidal activity are generally referred to as macromolecular bacteriocins (Bradley, 1967).

The adsorption of the bacteriocin to specific receptors on the bacterial cell surface is considered to be the first step of the interaction with a sensitive cell. The receptor substances have been characterized so far in the case of 'bacteriocin 45' from Proteus vulgaris (Smit, Hugo & DeKlerk, 1969) and of pyocin R from Pseudomonas aeruginosa (Ikeda & Egami, 1969, 1973; Ikeda & Nishi, 1973). Both bacteriocins adsorb to isolated lipopolysaccharide of sensitive strains, which indicates that this component of the outer membrane of Gram-negative bacteria is at least one receptor for the phage tail-like particles.

Strain Rhizobium lupini 16–3 produces INCO (incomplete) particles, which consist of a core tube, a surrounding contractile sheath, a base plate and six fibres (Lotz & Mayer, 1972). Unlike T4 fibres, these are not bent and carry two spherical bodies at each free end. Whereas the producer strain R. lupini 16–3 is resistant against its own bacteriocin, the particles kill cells of strain R. lupini 16–2. As seen in the electron microscope, the fibres of adsorbed

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particles are oriented towards the cell surface and the distance between cell surface and base plate of the adsorbed particles corresponds to the fibre length.

In this paper we give evidence that an analogous adsorption of INCO particles occurs with isolated lipopolysaccharide from strain *R. lupini* 16–2. Some properties of the *in vitro* interaction are described. The chemical composition of 16–2 LPS was described earlier (Pfister & Lodderstaedt, 1977; and Table 3). The lipid moiety is composed of hexosamine, phosphate and the fatty acids hydroxymyristic acid, myristic acid and palmitic acid. The polysaccharide contains rhamnose, hexosamine and KDO. The polysaccharide moiety of 16–2 LPS was compared with that of two bacteriocin resistant strains, in order to get more information about the receptor active region of 16–2 LPS.

**METHODS**

**Bacterial strains and media.** Strains 16–3, 16–2 and 16–2/1t of *Rhizobium lupini* (Lotz & Mayer, 1972) were a gift from W. Lotz. Strain 16–2/1t is a bacteriocin-insensitive spontaneous mutant of strain 16–2. *R. lupini* IS2 was isolated after u.v. mutagenesis (1200 erg/mm²) of strain *R. lupini* 16–3 and showed a tenfold higher bacteriocin production when compared with the wild type. The bacteriocin of *R. lupini* IS2 is identical to that of *R. lupini* 16–3, as far as morphology, bactericidal activity, host range and protein composition are concerned (H. Pfister, unpublished data). Media and general conditions for the cultivation of bacteria were described by Lotz & Mayer (1972).

**Isolation of the bacteriocin.** The bacteriocin was isolated as described previously (Lotz & Mayer, 1972) with the following modifications: in order to reach a higher yield, *R. lupini* IS2 was used instead of *R. lupini* 16–3. The crude lysate was purified in a preparative scale by sucrose gradient (5 to 20%) centrifugation in a SW25 rotor (4·5 h, 4 °C, 100000 g).

Bacteriocin containing fractions were stored at 4 °C over several drops of chloroform. The bactericidal activity was determined by end-point dilution (Ozeki, 1968). The titre given in the text represents the reciprocal of the highest dilution, a 0·02 ml drop of which caused a visible growth inhibition in a lawn of bacteriocin-sensitive bacteria.

**Isolation and purification of lipopolysaccharide.** LPS was isolated from bacteria grown in 2·5 l flasks with vigorous air-flow and harvested during the logarithmic growth phase by low speed centrifugation. The LPS was isolated by phenol–water extraction (Westphal & Jann, 1965). In the case of strain 16–3 a phenol–chloroform–petroleum ether extraction (Galanos, Lüderitz & Westphal, 1969) at room temperature was preferred. Purification was achieved by repeated centrifugation at 100000 g.

**Degradation of the lipopolysaccharides and purification of the sugar moieties.** Lipid and sugar moieties of the LPS were cleaved employing mild hydrolysis in 1% aqueous acetic acid (1 h, 100 °C). The water-insoluble fraction was obtained by low-speed centrifugation. The sugar components were purified on a Sephadex G50 column (1·8 cm inner diam., 90 cm length) by elution with pyridine/acetic acid/water = 10/4/986 at a velocity of 2·4 ml/h cm². Sugar containing fractions of each peak were pooled and lyophilized.

Hydrazinolysis (Reske, Wallenfels & Jann, 1973) of the LPS was carried out with water free hydrazine for 16 h at 100 °C in sealed glass tubes. After evaporation of the hydrazine the residue was taken up in water and the insoluble hydrazides of the fatty acids were removed by centrifugation. The supernatant was lyophilized and stored for further application.

**Sugar analysis.** The sugar content of a sample was determined by the phenol–H₂SO₄ method (Dubois et al. 1955). After hydrolysis of LPS with 1 N-H₂SO₄ (1 h, 100 °C) and
neutralization with $\text{Ba(OH)}_2$ the sugar components were separated by thin layer chromatography on cellulose coated plates (Merck, Darmstadt, W. Germany) in ethylacetate/pyridine/water = 60/25/20 and by high-voltage paper electrophoresis (42 V/cm) in pyridine acetate (pH 5.3). Sugar spots were visualized with alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950). Quantitative determinations of individual sugars of the native LPS were carried out as follows: rhamnose content was measured by the cysteine-HCl method (Dische & Shettles, 1948), hexosamine according to Strominger, Park & Thompson (1959) and KDO as described by Waravdekar & Saslaw (1959).

Test for receptor activity. Unless otherwise stated incubations of bacteriocin and LPS were carried out in saline buffer (0.01 M-tris-HCl, pH 7.5, 0.5 M-NaCl) at 30 °C for 30 min. The receptor activity of the LPS is given as the quotient of the bacteriocin titre of an untreated control and the bacteriocin titre of the LPS treated sample.

Electron microscopy. All preparations were carried out on carbon-coated copper grids. Negative staining was performed according to Valentine, Shapiro & Stadtman (1968) with 2% uranyl acetate (pH 4.8). Electron micrographs were taken with a Jeol JEM 7A instrument.

RESULTS

Interaction of bacteriocin and LPS

The adsorption of phages to isolated receptor molecules is known to inactivate the phage (Lindberg, 1973). Therefore, in order to study the receptor activity of *Rhizobium* LPS, INCO-particles were incubated with purified LPS of the INCO-sensitive strain *R. lupini* 16–2 and were tested afterwards for loss of bactericidal activity. With increasing amounts of 16–2 LPS the bactericidal activity of INCO particles was finally reduced to 0.1% (Fig. 1). In contrast, no inactivation occurred after incubation with LPS of the INCO resistant strains 16–2/I and 16–3, which indicates a specific inactivation of the bacteriocin by 16–2 LPS.
Fig. 2
Fig. 2. (a, b) Electron micrographs of INCO particles incubated with (a) 16–2 LPS and (b) 16–2/I1 LPS. (c, d) Electron micrographs of INCO particles incubated with sensitive 16–2 cells after pre-incubation with (c) 16–2 LPS and (d) 16–2/I1 LPS.
During the first 10 min of incubation inactivation kinetics were exponential (Fig. 4). After that time inactivation slowed down and even with LPS concentrations of 30 μg/ml and after incubation times of 8 h, 0.1% of the initial activity was observed.

The interaction of bacteriocin and 16-2 LPS could be demonstrated directly in the electron microscope. For these experiments 0.3 ml of an INCO lysate (titre 4096) were mixed with 50 μg of LPS. As shown in Fig. 2(a), a large number of INCO particles appeared associated with LPS. Their tail sheaths were contracted and the fibres were directed towards the LPS ribbons. After incubation of INCO particles with LPS of the INCO resistant strain R. lupini 16-2/I1 no adsorption and no sheath contraction was observed (Fig. 2b). Incubation with LPS of strain 16-2 under the conditions described above led to an inactivation of 99% of the bactericidal activity. In contrast, up to 80% of INCO particles were in a native, extended state, when observed in the electron microscope. This is due to a high percentage of biologically inactive, extended particles in the bacteriocin stock. Quantitative interaction of 16-2 LPS with bactericidal particles could be confirmed by the following experiment: no phage tails were adsorbed to sensitive cells after pre-incubation with 16-2 LPS (Fig. 2c), whereas 16-2 cells were densely occupied with phages after pre-incubation with LPS from strain 16-2/I1 (Fig. 2d). As expected in both cases, a large number of inactive, extended particles was present.

Dependence of INCO inactivation on salt concentration, pH and temperature

The inactivation of INCO particles by LPS was highly dependent on salt concentration. Addition of NaCl up to 0.5 M led to an increase of receptor activity from 32 to 1024 (Table 1). These results did not change when phosphate buffer was used instead of tris-HCl (0.01 M, pH 7.5 in all cases) or when 0.2 M-MgCl₂, MgSO₄ or Na₂SO₄ were added instead of NaCl. Therefore, there is no evidence for ion-specificity of the reaction.

The pH-dependence of the inactivation reaction is shown in Fig. 3. Blocking of the bactericidal activity was most efficient in the pH range from 7.0 to 8.0. If the LPS was pre-incubated at pH 5 or 10 and dialysed against pH 7.5 before mixing with INCO particles, no loss of receptor activity was observed. This indicates that there was no irreversible destruction of LPS structure in this pH range.

The inactivation of INCO particles depends on temperature (Fig. 4). The reaction at 0 °C was significantly slower than at 30 °C.

Characterization of the structural prerequisites for receptor activity of the LPS

In order to demonstrate the location of the receptor activity in the saccharide moiety, 16-2 LPS was treated with periodate, as oxidation of single sugars should destroy the ability to inactive INCO-phages. A solution of 0.4 mg LPS in 0.3 ml 0.2 M sodium acetate was mixed with 0.1 ml of periodate (0.2 M). After 5 h at room temperature, 0.12 ml of ethylene glycol was added to stop the reaction. The receptor activity was tested after dialysis and proved to be completely destroyed (Table 2). This gives strong evidence, that a proper sugar configuration is essential for the adsorption of INCO particles. The receptor activity was also sensitive to alkali treatment (pH 13 for 5 h at room temperature), acetic acid hydrolysis and hydrazinolysis (see Methods).

The bacteriocin was not inactivated by 16-2 LPS in the presence of 1% deoxycholate. After removing the detergent by dialysis against buffer, the receptor activity could be totally recovered (Table 2). This result was confirmed by examination in the electron microscope. No LPS structures were detectable in the presence of 1% deoxycholate and all phage-tails
Table 1. Dependence of INCO inactivation by R. lupini 16–2 LPS on salt concentration

<table>
<thead>
<tr>
<th>Reaction buffer</th>
<th>LPS-activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01 M-tris-HCl, pH 7.5</td>
<td>32</td>
</tr>
<tr>
<td>0.01 M-tris-HCl, pH 7.5, 0.05 M-NaCl</td>
<td>64</td>
</tr>
<tr>
<td>0.01 M-tris-HCl, pH 7.5, 0.01 M-NaCl</td>
<td>128</td>
</tr>
<tr>
<td>0.01 M-tris-HCl, pH 7.5, 0.20 M-NaCl</td>
<td>256</td>
</tr>
<tr>
<td>0.01 M-tris-HCl, pH 7.5, 0.50 M-NaCl</td>
<td>1024</td>
</tr>
<tr>
<td>0.01 M-tris-HCl, pH 7.5, 0.75 M-NaCl</td>
<td>1024</td>
</tr>
<tr>
<td>0.01 M-tris-HCl, pH 7.5, 1.00 M-NaCl</td>
<td>1024</td>
</tr>
<tr>
<td>0.01 M-tris-HCl, pH 7.5, 0.20 M-MgCl₂</td>
<td>256</td>
</tr>
<tr>
<td>0.01 M-tris-HCl, pH 7.5, 0.20 M-MgSO₄</td>
<td>256</td>
</tr>
<tr>
<td>0.01 M-tris-HCl, pH 7.5, 0.20 M-Na₂SO₄</td>
<td>128</td>
</tr>
</tbody>
</table>

Fig. 3. pH-dependence of INCO inactivation by 16–2 LPS. pH was adjusted with phthalate buffer (0.01 M) between pH 5 and pH 7 and with tris-HCl buffer (0.01 M) between pH 7 and pH 10. Both buffer systems led to equal values for LPS receptor activity at pH 7.

appeared extended. Lowering the deoxycholate concentration by dialysis against buffer led to spherical LPS structures and after total removal of the detergent the LPS showed typical ribbon-like structures again. INCO-phages were adsorbed to these ribbons as described above. It was concluded from these results, that the micellar structure of the LPS is necessary for its function as a phage receptor.

Chemical composition of LPS

The chemical composition of 16–2 LPS is given in Table 3. The composition of the polysaccharide part of LPS from bacteriocin-sensitive and bacteriocin-resistant strains was compared in order to identify the receptor active region of the LPS. Like 16–2 saccharide, 16–2/It saccharide could be separated by chromatography on Sephadex G50 into a polymer (mol. wt. 8000) and an oligomer (mol. wt. 900) fraction (Fig. 5). Only a low mol. wt. saccharide (1400) arose from the LPS of the INCO producer strain R. lupini 16–3. It was concluded from this that R. lupini 16–3 is a rough strain.
Fig. 4. Effect of temperature (□, △, 0°C; ■, ▲, 30°C) on the kinetics of INCO inactivation, shown with two concentrations of 16-2 LPS (□—□ and ■—■, 3 μg/ml; △—△ and ▲—▲, 30 μg/ml).

Table 2. Sensitivity of R. lupini 16-2 LPS receptor activity to chemical modifications

<table>
<thead>
<tr>
<th>Pre-treatment of LPS</th>
<th>LPS-Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>64</td>
</tr>
<tr>
<td>Periodate oxidation</td>
<td>1</td>
</tr>
<tr>
<td>Hydrolysis with 1% acetic acid</td>
<td>1</td>
</tr>
<tr>
<td>Alkali (pH 13)</td>
<td>1</td>
</tr>
<tr>
<td>Hydrazinolysis</td>
<td>1</td>
</tr>
<tr>
<td>Deoxycholate (1%)</td>
<td>1</td>
</tr>
<tr>
<td>Deoxycholate (1%), followed by dialysis</td>
<td>64</td>
</tr>
<tr>
<td>against tris-HCl, pH 7.5</td>
<td></td>
</tr>
</tbody>
</table>

* See Methods for definition of LPS-activity.

Table 3. Chemical composition of the R. lupini 16-2 LPS*

<table>
<thead>
<tr>
<th>Component</th>
<th>Weight percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxymyristic acid</td>
<td>4.3</td>
</tr>
<tr>
<td>Myristic acid</td>
<td>1.6</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>0.6</td>
</tr>
<tr>
<td>Hexadecenoic acid</td>
<td>0.9</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>32.0</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>5.9</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>0.7</td>
</tr>
<tr>
<td>KDO (2-keto-3-deoxyoctonate)</td>
<td>0.8</td>
</tr>
<tr>
<td>Heptose</td>
<td>0.3</td>
</tr>
<tr>
<td>Phosphate</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Fig. 5. Purification of the saccharide moieties of 16–2/1t LPS (● – ●) and 16–3 LPS (▲ – ▲) on Sephadex G50. The mol. wt. were estimated from the elution volume after calibration of the column with dextran blue and glucose. A, polysaccharide of 16–2/1t (mol. wt. 8000); B, oligosaccharide of 16–2/1t (mol. wt. 900); C, oligosaccharide of 6–3 (mol. wt. 1400).

Qualitative sugar analysis revealed, that 16–2 and 16–2/1t polysaccharide is composed of rhamnose, hexosamine and KDO, whereas 16–3 oligosaccharide was completely void of rhamnose, containing only hexosamine and KDO. Rhamnose amounted to 37% (w/w) of the total LPS in the case of 16–2 and 16–2/1t and represented 71% of the polysaccharide fractions. This indicates, that rhamnose is the main component of the polysaccharide side chain and its absence from 16–3 LPS is in line with the data of chromatography.

**Test for enzymic activity of INCO particles**

Bacteriophages ɛ16 P 22, Ə8 and ɛ8 are known to degrade the polysaccharides, to which they adsorb, enzymically (Iwashita & Kanegasaki, 1973; Kanegasaki & Wright, 1973; Reske et al. 1973; Iwashita & Kanegasaki, 1975). In order to test the enzymic activity, INCO particles were incubated with 100 mg of 16–2 LPS for 20 h at 30 °C. During this period the amount of reducing sugars remained constant. Furthermore, after high-speed centrifugation of the incubation mixture, no defined oligosaccharides were detectable by chromatography of the supernatant on Sephadex G50. Therefore, INCO-phages probably are not able to cleave the 16–2 polysaccharide.

**DISCUSSION**

Purified LPS from strain *R. lupini* 16–2 was shown to neutralize the bactericidal activity of INCO phages, whereas LPS of the bacteriocin resistant spontaneous mutant *R. lupini* 16–2/1t and of the bacteriocin producer strain *R. lupini* 16–3 did not inactivate INCO phages. Therefore, the LPS represents very probably at least one INCO receptor at the cell surface of *R. lupini* 16–2. It is possible that further receptor substances are necessary for final adsorption as described in the case of bacteriophage T4 (Wais & Goldberg, 1969).
However, as revealed in the electron microscope, all conformational changes, which occur after contact of INCO phages with intact cells, are triggered by isolated LPS, too.

The ribbon-like structure of LPS did not alter after INCO adsorption, as observed after interaction of a Proteus bacteriocin with LPS of the sensitive strain (Smit et al. 1969). Furthermore, INCO phages were not able to cleave the polysaccharide chain of the LPS. Consequently, neither conformational changes nor chemical changes seem to be necessary prerequisites for adsorption. The narrow pH-optimum for INCO inactivation by 16-2 LPS may indicate that electrostatic interaction plays an important role in binding.

The first step of bacteriocin adsorption is supposed to be a contact of INCO base plate and receptor, which leads to sheath contraction and to an interaction of the tail fibres with complementary cell surface structures (Lotz & Mayer, 1972). This model requires the well defined orientation of a base plate and six tail fibre receptors and may explain why only complex LPS with micellar structure showed receptor activity.

Chemical analysis of the LPS of two bacteriocin resistant strains indicated that in the case of R. lupini 16-3 the lack of rhamnose containing polysaccharide confers resistance to the bacteriocin. However, the rhamnose polysaccharide is not sufficient for INCO adsorption, as seen from the sugar composition of 16-2/lL LPS.

Our results suggest that minor sugar modifications might be sufficient to inactivate the specificity of the INCO adsorption site. Further studies are required to define the receptor active region of 16-2 LPS more exactly.

The knowledge about the structure of Rhizobium LPS is also of interest in another respect. Wolpert & Albersheim (1976) have shown that isolated LPS reacts specifically with lectins from those plants, which can be infected by the respective rhizobial strains. Therefore, the LPS structure may be a molecular basis for the specificity of Rhizobium plant symbiosis. Bacteriophages and bacteriocins could be most useful to study LPS structure by analysis of resistant mutants. This would be possible on a large scale, as phages and bacteriocins are widespread among Rhizobia (Lotz et al. 1974) and several of them are already known to adsorb to the LPS of their host (H. B. Nordhoff & H. Pfister, unpublished data).

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Bacteriocin adsorption to LPS


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