Peptidoglycan-bound Polysaccharide Associated with Resistance of *Rhizobium loti* Strain NZP2037 to *Lotus pedunculatus* Root Flavolan

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Resistance of *Rhizobium loti* strain NZP2037 to the prodelphinidin-rich flavolan (condensed tannin) present in the roots of *Lotus pedunculatus* was associated with the presence of a peptidoglycan-bound flavolan-binding polysaccharide (FBP) in the outer cell membrane of this bacterium. The outer cell membrane of a flavolan-sensitive strain, NZP2213, did not contain this polysaccharide. FBP was extractable in highest concentration from NZP2037 cells during exponential growth of the bacteria. This correlated with exponential-phase cells of NZP2037 showing maximum resistance to flavolan. When added to cultures of NZP2213, FBP afforded a concentration-dependent protection of this strain to the bactericidal effect of the flavolan. ELISA and immunogold labelling of *Rhizobium* cells using FBP-specific antibodies confirmed the presence of FBP on the outer cell surface of NZP2037 cells and its absence from NZP2213 cells. Co-inoculation of *L. pedunculatus* with antibiotic-resistant mutants of NZP2037 and NZP2213 resulted in the formation of N₂-fixing nodules containing both strains.

INTRODUCTION

The ability of certain strains of *Rhizobium loti* (Jarvis *et al.*, 1982) to form effective N₂-fixing (Nod⁺ Fix⁺) nodules on *Lotus* species is correlated with their 'in vitro' sensitivity to flavolans (condensed tannins) present in high concentration in the roots of this legume (Pankhurst & Jones, 1979). A representative strain, NZP2037, which forms Nod⁺ Fix⁺ nodules on *Lotus pedunculatus*, was found to be four times more tolerant ('in vitro') of the flavolan extracted from the roots of this *Lotus* species than was strain NZP2213 which forms ineffective (Nod⁺ Fix⁻) nodules (Pankhurst *et al.*, 1979; Pankhurst & Jones, 1979). The Nod⁺ Fix⁻ nodules formed by NZP2213 on *L. pedunculatus* are tumour-like and contain no intracellular rhizobia (Pankhurst *et al.*, 1979). The ability of strain NZP2037 to tolerate higher concentrations of the flavolan 'in vitro' was associated with the capacity of the outer cell membrane of NZP2037 bacteria to bind more flavolan molecules than the outer cell membrane of NZP2213 bacteria (Pankhurst & Jones, 1979; Pankhurst *et al.*, 1982).

The initial *Rhizobium*–flavolan interaction is bacteriostatic. This is associated with cessation of growth and binding of flavolan molecules to the outer cell membrane of the *Rhizobium* cells. Subsequently the cells become swollen, irregularly shaped, and lose viability, possibly because cell division is inhibited (Pankhurst *et al.*, 1982).

In order to define further the nature of the flavolan resistance of strain NZP2037, we have identified surface components of NZP2037 and NZP2213 cells that bind flavolan molecules.

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Abbreviations: FBP, flavolan-binding polysaccharide; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

Flavolans can cross-link with protein and polysaccharide molecules to form large aggregates which are solubilized but not dissociated by SDS (Jones & Mangan, 1977). Thus, molecules reacting with flavolans can be identified by their inability to enter polyacrylamide gels or by their reduced mobility due to aggregation. Using this approach we show that flavolan binds in small amounts to a lipoprotein component of the outer cell membrane of both strains, but in large amounts to a strain-specific peptidoglycan-bound polysaccharide component of NZP2037 cells.

METHODS

Organisms and growth. *Rhizobium loti* strains NZP2037, NZP2213 and PN236 [a Nod⁺Fix⁻ Tn5-induced mutant of NZP2037 (Chua *et al.*, 1985)] were maintained on a mannitol/salts/yeast extract (M⁺) medium (Pankhurst, 1977). A modified form of this medium [M⁻, with yeast extract replaced with (g l⁻¹) sodium glutamate (1.0), thiamin hydrochloride (0.0005), biotin (0.005) and calcium pantothenate (0.01)] was used as a minimal medium for growing the rhizobia. *Rhizobium* growth in this medium was followed by measurement of optical density at 600 nm. Cell numbers were calculated from a graph of optical density values versus log total cell number [determined by direct counts using a Thoma (Gallenkamp) counting chamber]. Nodulation tests were done on *Lotus pedunculatus* Cav. 'Grasslands Maku' and *L. tenuis* Waldst. et Kit with plants grown under axenic culture conditions in test tubes (Pankhurst, 1981). A spontaneous spectinomycin-resistant mutant of NZP2037 (strain PN4001) and a spontaneous streptomycin-resistant mutant of NZP2213 (strain PN4115) were used in 'co-inoculation' experiments where it was necessary to identify the two strains. These mutants were identical to their respective parent strains in both their growth and nodulation characteristics. Rhizobia isolated from nodules (Pankhurst, 1981) were streaked onto M⁻ agar plates containing, where appropriate, spectinomycin (20 µg ml⁻¹) or streptomycin (250 µg ml⁻¹) for strain identification.

Assay for testing the effect of flavolans on the growth of *Rhizobium*. Culture conditions and assay procedures for testing the sensitivity of rhizobia to flavolans in liquid culture were as described by Pankhurst *et al.* (1982). The effect of a putative flavolan-binding polysaccharide on the *Rhizobium*-flavolan interaction was determined as follows. A washed M⁻ broth suspension of rhizobia containing 10⁶ cells ml⁻¹ was diluted 10-fold with M⁻ medium containing flavolan and polysaccharide and incubated with shaking (100 r.p.m.) at 28 °C. After 24 h, samples were removed and the number of viable bacteria determined by dilution plating into M⁻ agar medium.

Identification of flavolan-binding components from *Rhizobium* cells. Rhizobia grown in M⁻ broth to mid-exponential phase were harvested by centrifugation (10 min at 20000 g, 4 °C), washed twice with 0.1 M-potassium phosphate buffer, pH 7.2, and resuspended in the same buffer to give 10¹¹ cells ml⁻¹ (total cell count). A portion of this suspension was then mixed rapidly with an equal volume of phosphate buffer containing flavolan (500 µg ml⁻¹). After mixing, the suspension was incubated at 28 °C for 4 h. After incubation the bacterial cells were collected by centrifugation and washed once with phosphate buffer containing 1% (w/v) polyvinylpyrrolidone (*M*_w 10000; Sigma), and then resuspended with the aid of brief sonication (15 s; MSE Ultrasonic Disintegrator) in 0.125 M-Tris/HCl buffer, pH 6.8, containing 1% (w/v) SDS and 5% (v/v) 2-mercaptoethanol. The suspension was then heated in a boiling water bath for 5 min and centrifuged at 40 000 g for 1 h. The pellet was resuspended in fresh extraction buffer and the process of extraction repeated a further three times. The supernatants from each extraction were then combined and stored for electrophoretic analysis. The residue, consisting of *Rhizobium* cell envelopes, was washed three times with 0.125 M-Tris/HCl buffer, pH 6.8, and then resuspended in the same buffer containing 20 µg lysozyme ml⁻¹ (39000 units mg⁻¹; Sigma) and 370 µg EDTA ml⁻¹ and incubated (with shaking, 100 r.p.m.) for 12 h at 28 °C. After incubation the suspension was centrifuged at 40000 g for 1 h. The pellet was washed twice with Tris buffer and then extracted three times with Tris/SDS/2-mercaptoethanol, as above. The combined supernatants from these extracts were retained. The final residue was washed twice with Tris buffer and then resuspended in this buffer. Residues and SDS extracts were analysed for flavolans (see below). Soluble extracts were examined by SDS-PAGE for loss of components from flavolan-treated cells compared to untreated cells.

Isolation of a flavolan-binding polysaccharide (FBP) from NZP2037 cells. NZP2037 cells grown to mid-exponential phase in M⁻ media were extracted exhaustively with SDS buffer to solubilize membrane and soluble molecules and the residual cell envelopes treated with lysozyme as described above. The residue obtained after the lysozyme treatment was extracted with phenol/water (Westphal & Jann, 1965) to extract the polysaccharide. The water layer was extracted 10 times with an equal volume of diethyl ether, dialysed (twice) against distilled water, centrifuged (48000 g for 10 min) and freeze-dried. The freeze-dried material was shown by PAGE to contain the FBP as the major constituent (> 90%) and to be free of protein.

Analysis of flavolans and binding of flavolan to FBP. Flavolan was prepared from the roots of *L. pedunculatus* as an off-white polymer (prodelphinidin : procyanidin ratio 77 : 23) as described by Jones *et al.* (1976). Free flavolan was measured by the vanillin/HCl method (Broadhurst & Jones, 1978). Flavolan bound to bacterial cells or membranes or contained within SDS extracts was measured as anthocyanidin after boiling in butanol/HCl (Jones *et al.*, 1976).

To study the binding of flavolan to FBP, 50 µl of FBP (1 mg ml⁻¹ in distilled water) or 50 µl of water (control) were added to 50 µl of water containing flavolan (0 to 1000 µg) and incubated at 20 °C for 1 h. After this incubation 0.9 ml of water was added to each sample which was then centrifuged (48 000 g, 10 min). A sample (0.5 ml) of the supernatant was then analysed for flavolan. Bound flavolan was estimated as the difference between the flavolan content of samples with or without added FBP.

Measurement of FBP and a flavolan-binding lipoprotein in NZP2037 and NZP2213 cells at different stages of growth. To quantify the amount of FBP and a flavolan-binding lipoprotein produced by *Rhizobium* cells at different stages of growth small samples of cells (about 5×10^9) were first extracted with SDS and treated with lysozyme as described above. SDS extracts of the lysozyme-treated cells were then electrophoresed on 15% (w/v) polyacrylamide gels. The polysaccharide was stained with periodic acid/Schiff's reagent and the lipoprotein stained with Coomassie blue. The gels were then cut into strips and the optical density of the stained bands measured using a Gilson gel scanner attached to a Beckman DU spectrophotometer. The optical density (peak height) of the polysaccharide band was measured at 500 nm and the optical density of the major 'lipoprotein' band (M_r 44 000; Fig. 1) was measured at 595 nm.

Gel electrophoresis. SDS extracts were electrophoresed through 15% polyacrylamide gels according to Laemmli (1970). Proteins were stained with 0.25% (w/v) Coomassie blue R-250 (Sigma) in methanol/water/acetic acid (4:4:1, by vol.) and destained in the same solvent. Polysaccharides were stained with periodic acid/Schiff's reagent and basic fuchsin (Zacharius *et al.*, 1969) and lipid with Sudan black.

Western blotting. After PAGE, proteins and polysaccharides were transferred to nitrocellulose (Towbin *et al.*, 1979), using a Tris (0.125 M)/glycine (0.04 M) buffer, pH 8.3, containing 10% (v/v) methanol and 0.003% (w/v) SDS, overnight at 70 V and 10 °C. The nitrocellulose was stained for protein with 0.1% (w/v) Amido black in methanol/water/acetic acid (68:25:7, by vol.) and destained in the same solvent. For probing with antibodies, the nitrocellulose was blocked with 3% (w/v) BSA (Fraction V; Sigma) in Tris-buffered saline (TBS; 50 mM-Tris, 0.8% (w/v) NaCl, pH 7.5) for 1 h at 37 °C. The nitrocellulose was probed with antibodies from test and pre-immune sera in dilution buffer (TBS containing 3%, w/v, BSA and 0.05%, v/v, Tween 20) for 1 h at 37 °C, washed four times for 5 min in wash buffer (TBS containing 0.05% Tween 20) and probed with alkaline-phosphatase-labelled anti-(rabbit IgG) (Sigma) (diluted 1000-fold in dilution buffer) for 1 h at 20 °C. The nitrocellulose was then washed three times for 5 min in wash buffer, once in 0.2 M-Tris/HCl, pH 8.2, and the phosphatase activity developed in 0.2 M-Tris/HCl, pH 8.2, containing Naphthol AS-MX (0.4 mg ml⁻¹; Sigma) and Fast violet B salt (2.5 mg ml⁻¹; Sigma). The nitrocellulose was then washed with distilled water to stop the reaction and air-dried.

Gel filtration. Gel filtration was done on Ultragel ACA 22 (LKB) in 25 mM-Tris/HCl, pH 7.4, containing 1% SDS. The column size was 100 cm $\times$ 1.6 mm and the flow rate 7 ml h⁻¹. The column was calibrated using the following proteins: bovine salivary band 4 protein, M_r 70 000 (Jones *et al.*, 1982); BSA, M_r 65 000; ovalbumin, M_r 43 000; carbonic anhydrase, M_r 29 000 and lysozyme, M_r 14 000. The void volume of the column was determined using dextran blue (Pharmacia). The fraction size collected was 0.6 ml. M_r values of FBP and a flavolan-binding lipoprotein were determined from the relationship between K_{av} and $\log M_r$.

Production of antibody to FBP. Purified FBP (500 µg) was dissolved in sample buffer (Laemmli, 1970) and separated on a 15% polyacrylamide gel containing a single well. After separation, two columns of holes (1 cm apart) were punched down both sides of the gel (1 cm from the edge of the sample well into the sample region) and the gel sliced vertically through the holes. These two gel slices were then stained for polysaccharide as described above. The stained slices were then lined up with the unstained portion of the gel by matching the holes to locate the FBP. The region of the gel containing the FBP was dissected, homogenized into phosphate-buffered saline [PBS; 80 g NaCl, 11.5 g Na₂HPO₄, 2.0 g KH₂PO₄ (l⁻¹)] using a glass homogenizer and the suspension injected subcutaneously into New Zealand White rabbits at 2 week intervals (Harboe & Ingild, 1974). The IgG fraction of the collected serum was purified by ammonium sulphate precipitation followed by ion exchange chromatography (Harboe & Ingild, 1974). Specificity of the antibody was tested by Western blot analysis using pre- and post-lysozyme SDS extracts of mid-exponential phase NZP2037 cells (Fig. 1). There was no reaction between the antibody and components of the pre-lysozyme treated SDS extracts. A strong reaction, however, was observed between the antibody and the polysaccharide component of the post-lysozyme treated SDS extract corresponding to FBP. A weak background smear was observed surrounding the major FBP band on the nitrocellulose. The antibody was thus regarded as being specific for the FBP complex and used in the reported studies.

ELISA. The wells of microtitre plates (Dynateck) were coated with poly-L-lysine by incubation with 0.1% (w/v) poly-L-lysine in sodium carbonate buffer, pH 9.6 [1.59 g Na₂CO₃, 2.93 g NaHCO₃ (l⁻¹)] for 2 h at 37 °C. *Rhizobium* cells (50 µl of a suspension containing 2×10^9 cells ml⁻¹) were added to each well and the plates centrifuged for 10 min at 1000 g. The plates were incubated for 2 h at 4 °C and recentrifuged. They were immersed in 0.25% (v/v) glutaraldehyde in PBS at 0 °C for 5 min, washed six times with PBS and blocked with sodium carbonate buffer, pH 9.6, containing 1% (w/v) BSA and 100 mM-glycine. Antibody to the *Rhizobium* polysaccharide (FBP) in dilution buffer (PBS containing 0.5%, v/v, Tween 20 and 1%, w/v, BSA), was then added to the wells and the plates were incubated at 37 °C for 2 h. The plates were washed six times with wash buffer (PBS containing 0.1%, v/v, Tween 20) and flicked dry. Peroxidase-labelled goat anti-(rabbit IgG) in dilution buffer was

Fig. 1. Testing of specificity of antibodies to FBP by Western blotting. Samples were separated on 15% (w/v) SDS polyacrylamide gels according to Laemmli (1970) and stained either for protein and polysaccharide or transferred to nitrocellulose (Towbin *et al.*, 1979). Antibodies were detected with phosphatase-labelled antirabbit IgG and naphthol AS-MX phosphate and Fast violet B. Arrows (→) indicate the position of FBP. Lanes 1–6 are polyacrylamide gels, lanes 7–9 are nitrocellulose after Western blotting. Lanes 1, 4 and 7 are pre-lysozyme SDS extracts of NZP2037 stained for protein, polysaccharide or probed with rabbit anti-FBP antibodies respectively; lanes 2, 5 and 8 are post-lysozyme SDS extracts of NZP2037 stained for protein, polysaccharide or probed with rabbit anti-FBP antibodies respectively. Lanes 3, 6 and 9 are *M*, marker proteins (from top to bottom): β -galactosidase, 116000; phosphatase b, 97400; BSA, 66000; egg albumin, 45000; and carbonic anhydrase, 29000. Lanes 3 and 6 were stained with Coomassie blue and lane 9 with Amido black.

added to each well and the plates were incubated for a further 1 h at 37 °C. After six washings with wash buffer, 200 μ l of substrate [40 mg *O*-phenylenediamine and 30 μ l H_2O_2 , (30%, v/v) in 100 ml phosphate/citrate buffer, pH 6.0 (7.3 g Na_2HPO_4 l⁻¹, 5.1 g citric acid l⁻¹)] was added to each well and the plates were incubated for 30 min at 20 °C in the dark. Peroxidase was inactivated by addition of 50 μ l 4 M- H_2SO_4 to each well. The optical density (at 495 nm) of the wells was then recorded with an automatic plate reader (Flow Multiscan MC).

Immunogold staining of *Rhizobium* cells and bacteroids. Samples of *R. loti* cells (harvested at different stages of growth) and *L. pedunculatus* root nodules formed by *R. loti* strain NZP2037, were prepared and processed for electron microscopy in Lowicryl K4M resin using the low temperature procedures described by Robertson *et al.* (1984). Thin sections were cut with a diamond knife and processed as follows for immunogold staining on 200 mesh gold grids bearing a carbon-Parlodion film. The sections were immersed in anti-FBP antibody (20 μ g ml⁻¹ in solution A: 10 mM-Tris/HCl, pH 7.5; 0.8%, w/v, NaCl; 2%, w/v, BSA; 1%, v/v, Tween 20) for 1 h at 37 °C, washed three times with solution A, without BSA, at room temperature, immersed in goat anti-rabbit IgG-Au (20 (Janssen), diluted 1:10 with solution A for 1 h at 37 °C, washed five times with 10 mM-Tris/HCl, pH 7.5, containing 0.8% (w/v) NaCl at room temperature and finally washed with distilled water. The sections were post-stained with saturated uranyl acetate for 2 min, followed by lead citrate for 2 min and viewed in a Phillips EM200 electron microscope.

RESULTS

Flavolan-binding components of NZP2037 and NZP2213 cells

Extraction of intact flavolan-treated NZP2037 and NZP2213 cells with SDS removed 70% of the bound flavolan from NZP2213 cells but none from the NZP2037 cells. As this extraction would have removed most cell components (proteins, polysaccharides) not covalently linked to the peptidoglycan layer of the bacterial cell walls (Braun, 1975) we concluded that the component of NZP2037 cells reacting with flavolan was covalently linked to the peptidoglycan layer. This result also indicated that the component on the surface of the NZP2213 cells reacting

with the flavolan must be different to that binding flavolan on the NZP2037 cell surface. Gel electrophoresis of the proteins removed from the flavolan-treated cells during this extraction gave patterns identical to those of control (untreated) cells similarly extracted (data not shown).

The *Rhizobium* cell envelope 'ghosts' remaining after the first round of SDS extraction were then treated with lysozyme and subjected to a second round of SDS extraction. This treatment removed 77% of the flavolan bound to the NZP2037 cells and a further 9% from the NZP2213 cells. When these SDS extracts were compared by gel electrophoresis with similar extracts from control cells, two major and one minor component present in the control cell extract were either present in diminished quantity or absent from the flavolan-treated cell extract. Two components (one major, one minor), both proteins, were present in reduced amounts in the extracts of flavolan-treated cells of both strains (Fig. 2*a*). These proteins reacted positively with Sudan black (Fig. 2*c*) suggesting that they were lipoproteins. The second major component, a polysaccharide, was present in NZP2037 cell extracts but was absent from NZP2213 (Fig. 2*b*) extracts and from extracts of NZP2037 pretreated with flavolan (Fig. 2*d*). A new high M_r polysaccharide, associated with flavolan (Fig. 2*d*), was observed at the top of the separating gel in extracts of NZP2037 cells pretreated with flavolan.

The insoluble residue remaining after the second round of SDS extraction, which still contained about 20% of the flavolan originally bound to the intact cells of both NZP2037 and NZP2213, was not further analysed.

Partial characterization of the flavolan-binding lipoprotein and polysaccharide

The M_r values of the two flavolan-binding protein components, thought to be lipoprotein as they both stained positively with Sudan black (Fig. 2*c*), were estimated by SDS-PAGE to be 88 000 and 44 000. The higher M_r protein may possibly be a covalently linked dimer of the 44 000 unit. Exclusion chromatography on Ultragel ACA22 (LKB) in 1% SDS gave M_r values of 90 000 and 45 000 for these two proteins, in close agreement with the results from the SDS gels.

The polysaccharide isolated from phenol/water extraction of lysozyme treated NZP2037 cell envelope 'ghosts' was a water-soluble product. It appeared as a poly-dispersed material on polyacrylamide gels (Fig. 2*b*) and was found by gel exclusion chromatography to have an approximate average M_r of 55 000.

Binding of flavolan to the NZP2037 FBP

The ability of the FBP extracted from exponential-phase cells of NZP2037 to bind *L. pedunculatus* root flavolan is shown in Fig. 3. Purified FBP bound up to four times its weight of flavolan. Assuming an M_r of 55 000 for FBP and 7000 for the flavolan (Jones *et al.*, 1976), this binding represents a molar ratio of flavolan : FBP of 28 : 1. Flavolan did not bind to extracellular polysaccharide extracted from NZP2037 (Pankhurst *et al.*, 1982) (data not shown).

Presence of FBP in NZP2037 cells at different stages of growth

Pankhurst *et al.* (1982) showed that exponential-phase (24 h) cells of NZP2037 were more resistant to *L. pedunculatus* flavolan and able to bind three times more flavolan than exponential-phase cells of NZP2213. In contrast, stationary-phase cells of both strains were equally sensitive to the flavolan. To examine what relationship these observations had to the FBP found on NZP2037 cells, samples of cells were removed from an actively growing M^- broth culture of NZP2037 and assayed for the presence of this polysaccharide. It was found that FBP could be extracted from cells only when they were in the exponential-phase of growth (Fig. 4). Cells in early stationary-phase (48 h) and stationary-phase (72 h) yielded no extractable FBP. Cells of NZP2213 removed at various times during growth in M^- medium contained no extractable FBP. In contrast, the amount of lipoprotein present in SDS extracts of either NZP2037 (Fig. 4) or NZP2213 (data not shown) closely followed the growth curve.

Results of ELISA in which a polyclonal antibody raised against purified FBP (and shown to be specific for this component on NZP2037 cells; see Methods) was reacted with NZP2037 and NZP2213 cells at various stages of growth are presented in Fig. 5. FBP was not detected by ELISA on NZP2213 cells at any stage of growth. In contrast, NZP2037 reacted with the FBP

Fig. 2. Polyacrylamide gels of SDS extracts of NZP2037 and NZP2213 cell envelope 'ghosts' after lysozyme treatment. Arrows indicate proteins (▶) and polysaccharides (→) aggregated by reaction with flavolans. M_r markers were as in Fig. 1. (a) Lane 1, extracts of NZP2037 control; lane 2, NZP2037 cells pretreated with flavolan; lane 3, extract of NZP2213 control; lane 4, NZP2213 cells pretreated with flavolan. (b) Lane 1, NZP2213 extract stained for polysaccharide; lane 2, NZP2037 extract stained for polysaccharide; lane 3, M_r marker proteins. After staining gels were equilibrated in 7% acetic acid. (c) Lane 1, M_r markers; lane 2 NZP2037 cells extract stained for protein; lane 3, as lane 2 but stained for lipid. (d) Lane 1, M_r markers; lane 2, extract of NZP2037 control cells, and gel stained for polysaccharide; lane 3, extract of NZP2037 cells pretreated with flavolan and the gel stained for polysaccharide; lane 4, as lane 3 but gel stained for flavolan with vanillin/HCl.

Fig. 3. Binding of *L. pedunculatus* root flavolan to NZP2037 FBP. The dotted line indicates the maximum flavolan bound per 50 µg of added FBP.

Fig. 4

Fig. 4. Growth (●), lipoprotein content (▲) and FBP content (○) of *R. loti* strain NZP2037. Lipoprotein and FBP content were determined after SDS and lysozyme treatment of a standard number (5×10^9) of cells and gel electrophoresis as described in Methods. The optical density values are arbitrary units only.

Fig. 5

Fig. 5. ELISA of NZP2037 (●) and NZP2213 (○) bacteria (10^8 cells per sample well) with anti-(NZP2037 FBP) antibody and preimmune antisera (▲). The FBP and preimmune antibodies were used at 1/800 dilution and the peroxidase anti-(rabbit IgG) antibodies at 1/2000 dilution. Optical density was measured at 495 nm. Bars represent the range of four determinations.

antibody at all stages of growth. Exponential-phase cells (24 h) of NZP2037 showed a small but reproducible increase (about 10%) in the amount of antibody bound relative to cells in the other phases of growth (Fig. 5).

Immunogold staining of FBP

Thin sections of NZP2037 exponential- and stationary-phase cells and NZP2213 exponential-phase cells stained with the FBP antibody and the secondary goat anti-rabbit IgG-Au (20) antibody are shown in Fig. 6. Extensive gold staining is centred over the cell-wall/cell-membrane complex of NZP2037 exponential-phase cells but absent from NZP2213 cells.

Fig. 6. Immunogold staining for FBP of thin sections of (a) NZP2037 exponential-phase cells, (b) NZP2037 stationary-phase cells, (c) NZP2213 exponential-phase cells and (d) PN236 exponential-phase cells. The anti-FBP antibody was used at a concentration of $20 \mu\text{g ml}^{-1}$. PHB, poly- β -hydroxybutyrate; P, polyphosphate. Bars, $1 \mu\text{m}$.

NZP2037 stationary-phase cells exhibited a level of staining equivalent to that of NZP2037 exponential-phase cells (Fig. 6a cf. Fig. 6c) but exponential-phase cells of PN236, a $\text{Nod}^+ \text{Fix}^-$ mutant of NZP2037 (Chua *et al.*, 1985) showed a greatly reduced level of staining. Bacteroids of NZP2037 present in a 6-week-old *L. pedunculatus* root nodule also showed extensive staining over the cell-wall/cell-membrane region (Fig. 7). In addition, significant staining was observed within the peribacteroid space (surrounding the bacteroids) (Fig. 7).

Protection of NZP2213 cells from flavolan by addition of NZP2037 FBP

If the FBP from exponentially growing NZP2037 cells was primarily responsible for enabling NZP2037 to tolerate higher concentrations of flavolan '*in vitro*', it might be expected that addition of this material to a suspension of NZP2213 cells would protect them from the

Fig. 7. Immunogold staining for FBP of thin sections of 21-day-old *L. pedunculatus* root nodules formed by NZP2037. (a) Unstained, control section; (b) section stained with anti-FBP antibody ($20 \mu\text{g ml}^{-1}$). The NZP2037 bacteroids (B) are individually enclosed within a peribacteroid membrane (PBM). The space between the bacteroid and the peribacteroid membrane is called the peribacteroid space (PBS). Bars, $1 \mu\text{m}$.

Table 1. *Effect of the addition of NZP2037 FBP on the viability of NZP2037 and NZP2213 cells grown in the presence of L. pedunculatus root flavolan*

The number of viable cells was determined 24 h after addition of flavolan and/or FBP to a suspension containing $1.6 \times 10^5 \text{ cells ml}^{-1}$ (NZP2213) and $2.1 \times 10^5 \text{ cells ml}^{-1}$ (NZP2037). The final flavolan concn was $50 \mu\text{g ml}^{-1}$. Each value is the mean of four determinations. ND, Not determined.

Treatment	Total number of viable cells ml^{-1}	
	NZP2213	NZP2037
Control	5.2×10^6	7.6×10^6
Flavolan	0.0	5.4×10^6
Flavolan + FBP ($50 \mu\text{g ml}^{-1}$)	8.2×10^3	ND
Flavolan + FBP ($100 \mu\text{g ml}^{-1}$)	1.8×10^6	ND
Flavolan + FBP ($200 \mu\text{g ml}^{-1}$)	2.1×10^6	5.6×10^6

bactericidal effect of the flavolan. To test this hypothesis NZP2037 FBP ($50, 100$ or $200 \mu\text{g ml}^{-1}$ final concn) was added to a suspension of washed NZP2213 cells ($10^5 \text{ cells ml}^{-1}$) in M^- broth. Flavolan ($50 \mu\text{g ml}^{-1}$ final concn) was added and the mixture incubated with shaking (100 r.p.m.) at 28°C . After 24 h incubation the total number of viable cells in each mixture was determined by dilution plating onto M^+ medium: $50 \mu\text{g flavolan ml}^{-1}$ completely killed NZP2213 cells but had little effect on NZP2037 cells (Table 1). However, addition of NZP2037 FBP ($100\text{--}200 \mu\text{g ml}^{-1}$ final concn) to NZP2213 cell suspensions gave significant protection against the flavolan (Table 1).

Table 2. *Effect of inoculation of L. pedunculatus with NZP2037/NZP2213 mixtures on plant growth and strain occupancy of individual nodules*

A spectinomycin-resistant mutant of NZP2037 (strain PN4001) and a streptomycin-resistant mutant of NZP2213 (strain PN4115) were used. Plants were harvested and the contents of individual nodules examined 6 weeks after inoculation.

No. of bacteria added per plant*		Shoot dry wt (mg per plant)	No. of nodules per plant	No. of nodules tested	No. of nodules yielding isolates†	Number of nodules containing:		
PN4001	PN4115					PN4001	PN4115	PN4001 + PN4115
10 ²	10 ⁶	1.2	11	30	19	19	(11)†	—
10 ⁴	10 ⁶	2.2	12	30	28	28	(2)†	—
10 ⁶	10 ⁶	4.7	10	30	30	12	—	18
10 ⁶	10 ⁴	4.5	13	30	30	27	—	3
Uninoculated		1.1	—	—	—	—	—	—

* Average number for six individual plants.

† Bacteria could not be isolated from the Nod⁺ Fix⁻ nodules formed by PN4115 following surface sterilization. Such nodules were morphologically tumour-like and therefore assumed to be formed by PN4115.

Role of FBP in nodulation

If the presence of FBP on NZP2037 cells and its absence on NZP2213 cells was in any way correlated with the Nod⁺ Fix⁺ phenotype of NZP2037 and the Nod⁺ Fix⁻ phenotype of NZP2213 on *L. pedunculatus* (Pankhurst & Jones, 1979) it might be expected that addition of FBP to *L. pedunculatus* inoculated with NZP2213 may alter the nodule phenotype of this strain for this host. To test this possibility FBP was added to the nutrient solution bathing the roots of individual *L. pedunculatus* seedlings at the time of inoculation with NZP2213. The number of NZP2213 bacteria added per plant was initially low (about 10³ bacteria per plant) and FBP was added in quantities ranging from 100–1500 µg per plant. No change to the Nod⁺ Fix⁻ phenotype of NZP2213 was observed. However, after 6 weeks the tumour-like structures formed by NZP2213 on *L. pedunculatus* were noticeably larger on plants treated with FBP than on control untreated plants.

In another experiment *L. pedunculatus* seedlings were 'co-inoculated' with a bacterial suspension containing antibiotic-resistant mutants of NZP2037 and NZP2213 in different ratios. This was done to see if the continued production of FBP by NZP2037 could assist NZP2213 into a Nod⁺ Fix⁺ nodule on this host. Nod⁺ Fix⁺ nodules containing both strains were readily obtained but only when a high (about 10⁶ bacteria per plant) number of both strains was initially added to the plants (Table 2). Lowering the number of bacteria of one strain relative to the other resulted in the formation of nodules containing only NZP2037 or tumour-like structures (presumably formed by NZP2213) from which no bacteria could be isolated after surface sterilization (Table 2).

DISCUSSION

The principal result of this study was the demonstration that resistance of *R. loti* strain NZP2037 to the prodelfinidin-rich flavolan from *L. pedunculatus* was associated with the presence of a strain-specific polysaccharide component in the outer cell membrane complex of this bacterium. *R. loti* strain NZP2213, which is sensitive to this flavolan, did not contain this polysaccharide component in its outer cell membrane.

With NZP2037, stepwise extraction of flavolan-treated exponential-phase cells with SDS, and SDS after lysozyme treatment, showed that all the flavolan was tightly bound to the cells and released only after lysozyme treatment. Approximately 70% of the flavolan was bound to a specific polysaccharide (termed the FBP), 10% to lipoprotein and 20% to further unidentified

components. In contrast, about 70% of the flavolan bound to NZP2213 cells was released by the initial SDS extraction. The remaining 30% was, as in NZP2037, bound to lipoprotein (10%) and unidentified components (20%). These results suggest that the outer surfaces of exponentially growing NZP2037 and NZP2213 cells are different, and that whereas with NZP2213 the bulk of the flavolan binds to components (polysaccharides, proteins) essential for cell viability the bulk of the flavolan molecules bind to FBP (a component apparently not essential for cell viability) on the NZP2037 cell surface. This 'preferential binding' of flavolan to FBP on the surface of exponentially growing NZP2037 cells correlates with the ability of these cells to remain viable in the presence of flavolan (Pankhurst *et al.*, 1982).

The apparent growth-phase-dependent nature of flavolan-resistance shown by NZP2037 cells (Pankhurst *et al.*, 1982) correlated with our ability to extract FBP from the cells. Resistance and concentration of extractable FBP were both highest in cells in the exponential-phase of growth (Pankhurst *et al.*, 1982; Fig. 4). Stationary-phase cells of NZP2037, which are as sensitive to the flavolan as NZP2213 cells (Pankhurst *et al.*, 1982), contained no extractable FBP (Fig. 4). This change in ability to extract FBP from NZP2037 cells as they progress from exponential- to stationary-phase growth is of interest and suggests that major changes occur to the surface characteristics of NZP2037 cells as they age. Changes to the lipopolysaccharide and capsular polysaccharide components of *R. trifolii* and *Bradyrhizobium japonicum* cells that bind clover and soybean seed lectins respectively have been reported as these bacteria progress through the growth cycle (Hrabak *et al.*, 1981; Bhuvaneswari *et al.*, 1977). A decline in lectin-binding activity of *B. japonicum* cells as they enter stationary-phase coincides with methylation of the galactose residues reducing the ability of the polysaccharide to bind the lectin (Mort & Bauer, 1980).

ELISA and immunogold staining of intact NZP2037 and NZP2213 cells with a polyclonal antibody specific for FBP confirmed the presence of FBP on the surface of NZP2037 cells and its absence from NZP2213 cells. In addition, both of these experiments demonstrated the presence of anti-FBP determinants on NZP2037 stationary-phase cells (Figs 5 and 6) whereas FBP could not be extracted from cells in stationary-phase (Fig. 4). A possible explanation for this is that FBP moieties become increasingly cross-linked as the NZP2037 cells progress from exponential- to stationary-phase growth. This cross-linking could reduce the flavolan-binding capacity of the FBP and make it refractory to extraction, but it may not alter its capacity to bind to the FBP antibody. The antibody, because it is polyclonal, is likely to bind to many sites on FBP that are not specifically involved in binding flavolan.

The experiments involving the immunogold staining of exponential-phase cells of the Tn5-induced mutant of NZP2037, strain PN236, and of NZP2037 bacteroids, were both of potential significance. Strain PN236 is similar to NZP2213 in that it forms tumour-like, bacteria-free structures rather than true nodules on *L. pedunculatus* (Chua *et al.*, 1985). This strain has a level of resistance to the *L. pedunculatus* flavolan *in vitro* that is approximately 3-fold lower than that of NZP2037, i.e. approximating that of the flavolan-sensitive strain NZP2213 (C. E. Pankhurst, unpublished results) and, as shown in Fig. 6(d), it appears to have a reduced amount of FBP in its outer cell membrane. This result provides further evidence linking the presence of FBP to flavolan-resistance and flavolan-resistance to ability to develop normal nodules; it also further demonstrates the specificity of the anti-FBP antibody. In direct contrast to the reduced staining of PN236 cells with anti-FBP, NZP2037 bacteroids showed a strong reaction (Fig. 7). Of interest was the occurrence of anti-FBP components in the peribacteroid space. This is possibly a consequence of the pronounced changes that occur to the *Rhizobium* cell membrane complex during bacteroid development. Included in this differentiation process is the potential sloughing-off of the outer cell membrane (Bal *et al.*, 1980), which could result in the release of FBP into the peribacteroid space.

The observation that FBP was only released from exponential-phase cells of NZP2037 following lysozyme treatment of cell envelope ghosts suggests that it is covalently linked to the peptidoglycan layer or gel (Hobot *et al.*, 1985) separating the outer and inner cell membrane. Similarly, the lipoprotein that bound to flavolan is also probably covalently linked to the peptidoglycan. As the FBP has not been characterized beyond an approximate M_r of 55000 it is

not known what particular characteristic affords it the capacity to effectively bind flavolan both *in vivo* and *in vitro* (Fig. 3). However, as both NZP2037 and NZP2213 are resistant to procyanidin-rich flavolans (Pankhurst & Jones, 1979), and as procyanidin differs from prodelphinidin only with respect to carrying two rather than three hydroxyl groups on the B ring of the molecule (Jones *et al.*, 1976), there must be some specificity between FBP and the trihydroxy groups of the B ring of prodelphinidin.

The biological efficacy of the NZP2037 FBP was demonstrated by its capacity to afford a concentration-dependent protection of NZP2213 cells to the bactericidal effect of the *L. pedunculatus* flavolan (Table 2). However, addition of FBP to *L. pedunculatus* seedlings inoculated with NZP2213 did not alter the Nod⁺Fix⁻ phenotype of NZP2213 for this host. While it may have been expected that if sensitivity of NZP2213 to the *L. pedunculatus* flavolan is the reason for its failure to infect the roots of this plant (Pankhurst & Jones, 1979), and that the presence of FBP in the growth medium could therefore assist in overcoming this, it is likely that any interaction between NZP2213 cells and flavolan that will have a direct effect on the outcome of the symbiosis will occur at the site of infection and/or during the infection process itself. The presence of FBP in the growth medium is therefore unlikely to prevent this. However, the presence of NZP2037 rhizobia in the rhizosphere along with NZP2213 did permit NZP2213 rhizobia to gain entry into a *L. pedunculatus* nodule (Table 2). Such 'pick-a-back' entry of a non-invasive *Rhizobium* strain in the presence of an invasive strain has been reported by Rolfe *et al.* (1980). With NZP2037 and NZP2213 dual occupancy of nodules depended on equal numbers of both rhizobia being present in the inoculum. It could be argued that the continued production of FBP by live NZP2037 rhizobia was responsible for the entry of NZP2213 into the *L. pedunculatus* nodule.

The Nod⁺Fix⁻ nodules formed by NZP2213 and PN236 on *L. pedunculatus* are tumour-like and contain no intracellular rhizobia (Pankhurst *et al.*, 1979; Chua *et al.*, 1985). Similar structures, containing no intracellular rhizobia, have since been described for mutants of *R. meliloti* (Finan *et al.*, 1985) and *R. phaseoli* (Vandenbosch *et al.*, 1985) deficient in exopolysaccharides, and for transconjugants of *Agrobacterium tumefaciens* and *Escherichia coli* containing the *R. meliloti* symbiotic plasmid or regions of it carrying nodulation genes (Wong *et al.*, 1983; Truchet *et al.*, 1984; Hirsch *et al.*, 1985). In all cases it appears that while certain nodulation genes, e.g. the 'common' *NodABCD* genes (Egelhoff & Long, 1985), are being expressed by these bacteria in association with the legume root thus producing a 'nodule-like' or 'tumour-like' structure, genes coding for essential steps in the infection process *per se* are either absent or non-functional. With *R. loti* strain NZP2213 it may be suggested that it is its sensitivity to the flavolan present in *L. pedunculatus* roots that blocks its entry into the root cells of this *Lotus* species, but that as a consequence of other nodulation genes becoming activated a 'tumour-like' structure is still developed.

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