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The Interaction of R. leguminosarum 128C53 with Pea Root Proteins and a Comparison of the Surface Polysaccharide with a Mutant Defective in Nodulation

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The Interaction of R. leguminosarum 128C53 with Pea Root Proteins and a Comparison of the Surface Polysaccharide with a Mutant Defective in Nodulation.

(TITLE)

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Ru-Po Lee

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ABSTRACT

The Rhizobium extracellular polysaccharides (EPS) and lipopolysaccharides (LPS) are implicated in the symbiotic process. The interaction between host root protein fractions and symbiont EPS-polyacrylamide affinity support has been investigated. Ten percent root protein fractions had been observed to bind to the affinity support. The compositions of the polysaccharides from the parent R. leguminosarum and its Exo^{-1} mutant have also been compared. The Exo^{-1} mutant produces diminished amount of EPS and does not nodulate the host pea roots. Five kinds of polysaccharides from the parent were obtained: EPS, capsule polysaccharides (CPS I) washed from the bacteria with saline solution, LPS, small polysaccharides (SmPS) and the polysaccharides remaining in the supernatant from the ultracentrifugation of the impure viscous LPS solution (CPS 2a). The compositions of the EPS, CPS I, and CPS 2a are identical. The Exo^{-1} mutant also produces EPS (1-7%) and a small amount of CPS I. This EPS and CPS consists solely of LPS and SmPS. None of the parental type acidic EPS and CPS are produced by the Exo^{-1} mutant. In addition, the Exo^{-1} mutant produces LPS polysaccharide fragments. The LPS from the mutant and parent are identical to each other in their compositions, both the core and the O-antigen regions. The O-antigen sugar linkages were also compared by using methylation analysis and the linkages of the O-antigen from the parent and the mutant are also identical. The meaning of these results is discussed with regard to the role of Rhizobium surface polysaccharides in symbiosis.

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ABSTRACT

The Rhizobium extracellular polysaccharides (EPS) and lipopolysaccharides (LPS) are implicated in the symbiotic process. The interaction between host root protein fractions and symbiont EPS-polyacrylamide affinity support has been investigated. Ten percent root protein fractions had been observed to bind to the affinity support. The compositions of the polysaccharides from the parent R. leguminosarum and its Exo^{-1} mutant have also been compared. The Exo^{-1} mutant produces diminished amount of EPS and does not nodulate the host pea roots. Five kinds of polysaccharides from the parent were obtained: EPS, capsule polysaccharides (CPS I) washed from the bacteria with saline solution, LPS, small polysaccharides (SmPS) and the polysaccharides remaining in the supernatant from the ultracentrifugation of the impure viscous LPS solution (CPS 2a). The compositions of the EPS, CPS I, and CPS 2a are identical. The Exo^{-1} mutant also produces EPS (1-7%) and a small amount of CPS I. This EPS and CPS consists solely of LPS and SmPS. None of the parental type acidic EPS and CPS are produced by the Exo^{-1} mutant. In addition, the Exo^{-1} mutant produces LPS polysaccharide fragments. The LPS from the mutant and parent are identical to each other in their compositions, both the core and the O-antigen regions. The O-antigen sugar linkages were also compared by using methylation analysis and the linkages of the O-antigen from the parent and the mutant are also identical. The meaning of these results is discussed with regard to the role of Rhizobium surface polysaccharides in symbiosis.

INTRODUCTION

Rhizobium bacteria have the ability to infect the roots of legumes and form nitrogen-fixing nodules. Many reports have shown that the infection between the Rhizobium and the legume host is specific.[1] For example, Rhizobium japonicum only nodulates soybeans, while Rhizobium leguminosarum only nodulates peas. The Rhizobium, using the energy provided by the legume, fixes the nitrogen gas to ammonia and makes it available to the plant. The Rhizobia which fix the nitrogen in the roots of the legume are divided into two main groups according to their growth rate. The fast growing strains, which include Rhizobium leguminosarum, Rhizobium trifolii, and Rhizobium meliloti have a mean generation time of about 3-4 hours, and the slow growing strains which include Rhizobium japonicum have a mean generation time of about 6-7 hours.

The Gram-negative bacteria, which include Rhizobium, have two major cell surface polysaccharides, the lipopolysaccharide (LPS) and the extracellular polysaccharide (EPS). Both the LPS and the EPS are thought to participate in the infection and nodulation process.[2] But the real interaction between EPS and/or LPS with leguminous roots is not completely known. Therefore, my project is directed towards determining the molecular basis by which R. leguminosarum specifically infects pea roots. This work is based on the idea that EPS and/or LPS play an important role in symbiosis and may interact specifically with pea root proteins.

There are four discrete steps which are required in the infective symbiotic process. Those are: (1) The attachment of the Rhizobium to

the roots of the legumes, (2) root-hair curling, (3) the formation of infection threads which provide a route for the bacteria from the root surface to the inner cells and (4) the infection of the inner root cells and the bacteroid formation. Bacteroids are the form of the bacteria which fix nitrogen to ammonia. It is possible that when attachment or root hair curling occurs, some proteins from the roots bind to the EPS and/or LPS. Three kinds of crude protein fractions from pea roots were prepared: (1) Proteins which were washed from the root surface with a saline solution, (2) cellular proteins from the ultracentrifuge supernatant of root homogenates, (3) proteins which were extracted from the root cell membranes by using Triton X-100. Protein (1) was used because the first attachment between roots and Rhizobia occurs at the root surface. Proteins (2) and (3) were used because the molecular interaction which results in the root hair curling and infection thread formation probably involve the root cellular proteins located at the cell membrane. Affinity supports were prepared by using polyacrylamide to immobilize the EPS and separate the binding proteins from the non-binding proteins. Proteins, which specifically bind this polysaccharide should bind to the affinity support, while non-binding proteins should elute from the affinity column. All three protein fractions were labeled with fluorescein and the binding and non-binding proteins were collected separately. Using the fluorometer, the percentage of binding was determined.

As previously stated, the surface carbohydrates of Rhizobium including EPS and LPS are implicated as having a role in symbiosis. There are many reports which show that cell surface mutants of Rhizobia have altered symbiotic characteristics. An example is the mutant of R. leguminosarum which produces diminished amounts of EPS and does not nodulate its host

pea, while mutants which produce larger amounts of EPS will produce more nodules. The mutant of R. Leguminosarum which produces diminished amounts of EPS has been described by Sanders et. al.[2] This mutant, Exo^{-1}, has an LPS which is very similar to the parent LPS. These results suggest that Rhizobium EPS is important for the initial interaction between a Rhizobium and its host. However the results do not determine whether or not EPS plays an important role in the specific recognition process. Further studies have shown that there may be a correlation between the ability of the bacteria to form a capsule and the ability to nodulate the host plant.[3] A capsule is thought to be a form of EPS which is closely associated with the cell wall. The polysaccharide in this form is known as the capsular polysaccharide(s) (CPS). As the number of encapsulated cells is reduced, there is a concomitant decrease in the EPS and also in the efficiency of infection. The Exo^{-1} mutant, which produces between 1 to 7% of the parental EPS, produces no encapsulated cells and does not nodulate the host.

The LPS of Rhizobium have not been studied as much as EPS. Studies have shown that the EPS of Rhizobium leguminosarum do not vary in structure from strain to strain. Unlike the EPS, the LPS from R. leguminosarum are highly variable in their composition. The LPS are synthesized in the plasma membrane and are then transferred to the outer membrane.[4] The LPS contains three major regions: A lipid, core oligosaccharide, and an O-antigen polysaccharide. The core oligosaccharide is attached to the lipid through a ketosidic linkage involving 2-keto-3-deoxyoctanoic acid (KDO). The O-antigen repeating polysaccharide is attached to the core oligosaccharide.[5] The lipid-A moiety is the hydrophobic part of this large molecule. It contains fatty acids linked to glucosamine residues. The core oligosaccharide and O-antigen chain, in contrast with the fatty acyl part of lipid-

A, are highly hydrophilic. By using gel electrophoresis, we have shown that the R. leguminosarum 128C53 sm^r rif^r Exo^{+1} and Exo^{-1} strains have two kinds of LPS. One is the intact LPS, which contains lipid, core, and O-antigen. The other only contains lipid and core without O-antigen. A schematic of these two LPS types is shown in Figure (1).

It has been suggested that the LPS component may be involved in the specificity of host pathogen infection. Infection by the pathogenic Pseudomonas solanacearum and Agrobacterium tumefaciens bacteria is inhibited by LPS preparations.[6-8] A more recent report shows that only the rough forms of P. solanacearum infects its host, tobacco.[9] Since rough bacteria contain incomplete LPS that lack the O-antigen polysaccharide and contain only the lipid and core oligosaccharide,[9-12] this suggests that the core region of P. solanacearum LPS plays an important role in the infection process. Because of this example, it seems likely that LPS of Rhizobium also have some role in determining the specificity of symbiotic infection.

The major part of my project was to compare the cell surface polysaccharides between the parental R. leguminosarum 128C53 sm^r rif^r Exo^{+1} and its Exo^{-1} mutant. Five kinds of polysaccharides from the Exo^{+1} were obtained: EPS, polysaccharides washed from the bacteria with saline (CPSI), LPS, small polysaccharides (SmPS) and the polysaccharides remaining in the supernatant from the ultracentrifugation of the viscous lipopolysaccharide solution (CPS 2a). Four kinds of polysaccharides were obtained from the Exo^{-1} mutant, the CPS2a fraction was missing. The LPS was also characterized by determining the sugar linkages of the O-antigen region.

This present paper includes two major parts. Part One describes the work directed at isolating the root proteins which bind to the parent EPS.

Part Two compares the surface polysaccharides of R. leguminosarum 128C53 sm^r rif^r Exo^{+1} and its Exo^{-1} mutant.

EXPERIMENTAL

Growth of the Bacteria.

R. leguminosarum 128C53 sm^r rif^r and its Exo^{-1} mutant were grown on Vincent's medium,[13] which contains the following: 15g $MgSO_4 \cdot 7H_2O$, 5g $CaCl_2 \cdot 2H_2O$, 60g K_2HPO_4, 0.18g $FeCl_3$ (anhydrous), 5g NaCl, 20mg biotin, 10mg calcium pantothenate, 10mg thiamine·HCl, 10g mannitol and 1.1g glutamic acid per liter. The pH was adjusted to 6.8-7.0 with NaOH prior to autoclaving. The bacteria were transferred from a slant into a 125 mL flask containing about 50 mL culture medium. The flasks were placed on a shaker for 3-4 days at room temperature. The small flask was then transferred to 12 liters of culture media and harvested after growing about 3-4 days. Usually, this is the early stationary phase of growth. The bacteria were separated from the media by centrifugation at 10,000 g for 20 minutes.

Isolation of EPS.

The bacteria-free culture media was concentrated by rotary evaporation under reduced pressure to about 500 mL. In order to precipitate the extracellular polysaccharide from the culture, three volumes of ethanol were added. Normally, the solution was set overnight in the cold room to insure complete precipitation. The precipitate was harvested by centrifugation, dissolved in deionized water, and dialyzed in the cold room against deionized water, and freeze-dried.

Pea Root Protein Preparation.

Pea seeds (Laxton progress #9) were surface sterilized using a 20%

bleach solution for 15 minutes, rinsed with sterilized water 3 times and germinated in the growth chamber. The roots were removed and washed in saline solution[14] containing 0.43g KH_2PO_4, 1.68g Na_2HPO_4, 7.2g NaCl per liter at pH 7.2. The wash solution was dialyzed against deionized water and freeze-dried. This protein fraction is referred to as surface protein.[15] The roots were weighed. An equivalent amount of the following solution was added to the roots: 5mM $MgCl_2$, 50mM tris and 15% W/W sucrose at pH 7.6. The mixture was homogenized in a blender in the cold room. The homogenate was squeezed through a double layer of muslin and centrifuged at 1,000 g for 10 minutes to remove the cell walls and debris. The supernatant was ultracentrifuged at 100,000 g for 90 minutes to produce a supernatant and a pellet. The ultracentrifuge supernatant was dialyzed and freeze-dried to get the second protein preparations that is referred to as the soluble protein.[15] Twenty-five mL of a solution of 0.5% triton X-100, 0.2M glucose, 10mM tris, 5mM $MgCl_2$ at pH 7.0 were added to the pellet and sonicated for 1 hour. The solution was centrifuged at 100,000 g for 30 minutes and the supernatant dialyzed and freeze-dried. This is the third protein preparation, referred to as membrane protein.[15] All three proteins were labeled with fluorescein using the method of Coons and Kaplan.[16]

Isolation of EPS-Binding Root Proteins.

The EPS-polyacrylamide gel was made according to Horisberger's method,[17] and the gel was washed with buffer A (0.01M Na_3PO_4, 0.15M NaCl, 0.1mM $CaCl_2$ at pH 7.2), until the supernatant solution was free of carbohydrate. Another polyacrylamide gel without EPS was prepared to be used as a control.

The surface protein 28mg (preparation 1), 326mg of the soluble protein (preparation 2), and 102mg of membrane protein (preparation 3) were

obtained from the pea roots. In order to test the root proteins for binding to the EPS-polyacrylamide gel all the surface protein, 100 mg of soluble protein, and 30 mg of membrane protein were dissolved in buffer A solution separately. Each protein preparation was applied to an EPS-polyacrylamide gel column. By comparing the total fluorescense before and after passing through the column, we determined whether or not there are binding proteins in each of the root protein preparations. The fluorescense was measured at λexit 485 nm, λemission 510 nm.

Isolation and Analysis of Rhizobium Surface Polysaccharides.

The second aspect of my work was to compare the surface polysaccharide from the R. leguminosarum 128C53 sm^r rif^r Exo^{+1} with its Exo^{-1} mutant. The EPS was obtained according to the above description. The bacteria were washed with a saline solution and centrifuged to separate the bacteria from the saline wash solution. The above procedure was repeated three to four times, until the supernatant was free of hexose as measured by the anthrone test.[18] The saline supernatant solutions were combined and concentrated to about 300 mL. Three volumes of ethanol were added and the precipitated polysaccharide was dialyzed and freeze-dried to obtain the CPSI fraction.

The LPS were extracted from the wash bacteria by the phenol-water method.[19] Twenty to eighty grams of the wet bacteria were suspended in 100 mL of 65°C H_2O in which 100 mL of 65°C phenol was added. This mixture was stirred for 15 minutes at 65°C, then cooled on ice for 15 minutes and centrifuged at 13,000 g for 20 minutes at 4°C. The water layer was kept, and the phenol layer was heated to 65°C. One hundred mL of 65°C water was added again and the above procedures were repeated. The LPS, CPS, and nucleic acid would be found in the water layer. The water layers

were combined and dialyzed against deionized water.

The dialyzed LPS solution was concentrated to about 100 mL by rotatory evaporation under reduced pressure at 40°C. The nucleic acids were removed by digestion by adding 0.1 mL DNase (1 mg/mL) and 0.1 mL RNase (10 mg/mL) enzymes to the water layer. The water layer was made 0.1M tris, 0.01 M $MgSO_4$, and pH 7.2. This solution was allowed to stand overnight at room temperature and then dialyzed.

The dialyzed LPS-containing preparation was concentrated to about 50 mL by rotary evaporation, and then freeze-dried. After enzyme digestion, the LPS can be separated from the other polysaccharides by a Sepharose 4B gel filtration column.[20] However, the preparation from the parent R. leguminosarum Exo^{+1} strain was too viscous and it was not feasible to pass this solution directly to the Sepharose 4B column. Therefore, the LPS preparation from the parent bacteria were centrifuged at 100,000 g for 4 hours. The viscous supernatant solutions contained polysaccharides (CPS2a) which were not LPS, while the pellet LPS was dissolved in 10 mL of buffer solution (30 g/L ethylenediamine tetraacetic acid, 30 g/L triethylamine, pH 7.0), and applied to the Sepharose 4B gel filtration column (4 x 50 cm). The flow rate was approximately 0.2 mL/min. Fractions of 5.0 mL were collected and the concentration of hexose and KDO were measured in each fraction by using the anthrone method[18] and the thiobarbituric acid method[21] respectively. The void and included volume were determined with blue dextran (Sigma Chemical Co.) and glucose. The LPS material elutes in the partially included volume, while the small molecular polysaccharides elute near the included volume. The symmetry of the LPS peak and the constant KDO/hexose ratio suggest that the LPS is pure. Both carbohydrate peaks were dialyzed and freeze-dried. The LPS containing

preparations in Exo^{-1} were not viscous and can be put on the Sepharose 4B column directly without prior ultracentrifugation. All the purification steps are outlined in Figure 2.

The neutral hexose concentration of each polysaccharide was determined by using the anthrone method, uronic acid concentration was determined by the m-hydroxyl diphenyl method,[22] and the KDO was assayed by the thiobarbituric acid method.[21] The uronic acid in EPS and LPS were further analyzed by reduction of the uronic acid residues in the polysaccharides to the corresponding hexoses, followed by GC analysis of the alditol acetate derivative of the sugars which make-up the polysaccharide.[23] If the uronic acid is glucuronic acid an increase in the alditol acetate derivative of glucose is observed. If the uronic acid is galacturonic acid an increase in the alditol acetate of galactose is observed.

The EPS obtained from Exo^{-1} was found to have about 1% KDO. This means the EPS contains some LPS and is not the pure EPS. The EPS preparation in Exo^{-1} was applied on the Sepharose 4B column. Fractions of 5.0 mL were collected, and the concentration of hexose and KDO were measured in each fraction. Two peaks, a large molecular weight and a small molecular weight peak were obtained, dialyzed and freeze-dried separately. The Exo^{+1} EPS was also analyzed on the Sepharose 4B column, and hexose and KDO concentration of each fraction were also measured.

Aldoses were analyzed by gas chromatography as their alditol acetates.[24] Each kind of polysaccharide was hydrolyzed in 2N trifluoroacetic acid for 2 hours at 121°C. The monosaccharides were converted to the corresponding alditol acetates and identified by comparison of their gas chromatography retention times to the retention times of standards.[24]

The Rhizobium LPS polysaccharide usually consists of three regions: A lipid, a repeating oligosaccharide known as the O-antigen and a core oligosaccharide. The lipid can be removed by using mild acid hydrolysis (1% acetic acid at 100°C for 1 hour) which hydrolyzes the ketosidic bond between the KDO and lipid.[25] The core and O-antigen polysaccharide can be identified by separating these two LPS structural regions by Sephadex G-50 gel filtration column using a solution consisting of 4 mL pyridine, 10 mL acetic acid in 1000 mL H_2O as the solvent.[26] Fractions of 1.0 mL were collected and the hexose and uronic acid content of each fraction were measured colorimetrically. Polysaccharide peak I eluted after the void volume and is the O-antigen polysaccharide. Polysaccharide peak II eluted before the included volume is the core oligosaccharide. Both fractions were freeze dried.

In order to determine the sugar linkages of the O-antigen, the polysaccharides are usually methylated and subjected to acid hydrolysis.[27] Prior to methylation, dimetheylsulfoxide anion was prepared as follows: NaH (1.4 g) in oil was washed three times with 15 mL of hexane (each time) under a stream of N_2 and then blown dry using N_2. The DMSO, 4 mL, was added dropwise to the NaH. This reaction was allowed to proceed for 30 minutes at room temperature. A standardized NaOH solution was used to titrate a solution containing 20 mL of the 0.1 N HCl and 100 µL DMSO anion. In this way the DMSO anion normality was found to be 4.99N. In the methylation procedure, 1 mg of O-antigen was dissolved in 500 µL of DMSO and set overnight. Forty µL of DMSO anion was added and the solution was sonicated for 1 hour. Methyl iodide (12.4 µL) was then added to make the equivalent equal to the DMSO anion equivalent. The above procedure was repeated and the samples were left overnight. On the next day, 40 µL of DMSO anion were added again and the above procedure was repeated two

more times, and the second time an excess methyl iodide (five times) was added. The resulting reaction mixture was allowed to stand overnight at room temperature. The remaining DMSO and excess methyl iodide were separated from the methylated polysaccharides by using an LH 20 column. The solvent for the column was a 1/1 methanol/chloroform solution. The methylated polysaccharides now can be hydrolyzed to the methylated monosaccharides and reduced with sodium borodeuteride to the alditols and acetylated with acetic anhydride in pyridine. Then they are separated and identified directly by combined gas chromatography and mass spectrometry. This was done at the NIH regional facility at Washington University, St. Louis, Missouri.

The core oligosaccharides of the LPS from R. leguminosarum Exo^{+1} and Exo^{-1} strains were further purified by applying them onto a Sephadex G-25 gel filtration column using the same solvent as described for the G-50 column. Fractions of 1.0 mL were collected and the uronic acid was measured colorimetrically. The core region was hydrolyzed to the monosaccharides reduced with sodium borohydride to the alditols, acetylated and analyzed by gas chromatography. Protein determinations were done according to Lowry method by using the bovine serum albumin as the standard.

Viable counts of bacteria were determined by plating 10^{-7} and 10^{-8} dilution of the original bacterial solution on agar media. Bacteria were grown in 125 mL flasks, which contains 50 mL Vincent's media with shaking for 3-4 days at room temperature. The culture media, 0.1 mL, was then transferred to a screw test tube which contains 9.9 mL sterilized water and shaken vigorously to get a 10^{-2} dilution. Using the 10^{-2} dilution to repeat the above procedure two times to get the 10^{-6} dilution. One-half of the 10^{-6} diluted solution was transferred to a screw test tube which contains 4.5 mL of sterilized water to get a 10^{-7} dilution. The above

procedure was repeated again using the 10^{-7} dilution to obtain a 10^{-8} dilution. One-tenth mL of the 10^{-7} and 10^{-8} dilutions were then transferred to solid agar medium. After 3-4 days of its growth, the number of the bacterial colonies were easily counted from the solid agar medium.

The LPS from the R. leguminosarum 128C53 sm^r rif^r Exo^{+1} and Exo^{-1} (from phenol-water extraction and from medium) were analyzed by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate. Concentrated running buffer, stacking gel, and running gel solutions are given in Table I. A slab gel was poured consisting of 10 mL of the running gel solution with 0.15% ammonium persulfate and 0.05% N,N,N',N'-tetramethylethylenediamine. When polymerization was completed, 5 mL of the stacking gel solution with 0.15% ammonium persulfate and 0.05% TEMED were put on the top.

The 25 µL of buffer solution (Table I) containing some bromphenol blue dye was used for each kind of LPS (100 µg). Before applying the LPS to the gel, the LPS samples were sealed in a glass tube and heated in a boiling water bath for 10 minutes. Electrophoresis was carried out at constant current using a buchler 3-1500 power supply. About 400 mL of running buffer was used in each electrode compartment. The samples were applied to the gel sample wells, and the current was 35 mA. The running time under these conditions was about 4 hours, until the bromphenol blue dye tracking band reached the end of the gel.

The gel was removed from the electrophoresis apparatus, and placed in a glass dish. In order to remove the SDS from the gel, the following solutions were used:[28] (1) 25% isopropyl alcohol, 10% acetic acid; overnight; (2) 10% isopropyl alcohol, 10% acetic acid; overnight; (3) 10% acetic acid; several hours. The gel was then stained by using the procedure as follows:[28] (1) 0.5% sodium meta-periodate ($NaIO_4$); 2 hours;

(2) 0.5% sodium arsenite in 5% acetic acid; 30-60 minutes; (3) 0.1% sodium arsenite in 5% acetic acid; 20 minutes; repeated twice; (4) 5% acetic acid; 10-20 minutes. The gel was then transferred to the Schiff reagent[28] (Table I) and left overnight. The pink bands appeared after 10 minutes and intensified as the Schiff reagent penetrated into the gel. (5) The gel was soaked in a solution which contains 0.1% sodium meta-bisulfite ($Na_2S_2O_5$) in 0.01 N HCl until the solution did not appear pink. The LPS bands could be seen at this time.

RESULTS

Pea Root Proteins Which Bind to R. leguminosarum EPS.

The three protein preparations were applied to the EPS-polyacrylamide column separately to determine whether or not there were interactions between the EPS and the pea root proteins. None of the protein from the surface protein preparation bound to the EPS-polyacrylamide column. One hundred percent of the fluorescein labeled protein eluted from the column. The membrane protein, 30 mg, was then applied to the column. Only 88.5% of the applied protein was recovered and a yellow band could be seen on the top of the column. In order to remove the binding membrane protein from the column, the following solutions were applied to the column: 0.5 M glucose, 1.0 M glucose, 1.0 M NaCl. Unfortunately, none of the above solutions removed the binding protein. The buffer A solution containing 5% SDS and 8 M urea was applied to the column and 0.5% of the binding protein was removed. However, it is difficult to remove the SDS from the binding protein. Figure 3 shows the interaction between the membrane protein and the EPS-polyacrylamide column.

The soluble protein, 100 mg, was applied to another column. This time 89.4% of the soluble protein was recovered, suggesting that 10.6% was bound to the column. The fluorescein band was produced on the top of the column at the same place as the membrane protein. All the above methods used to try to remove the binding protein from the membrane protein also did not remove the binding protein from the soluble protein preparation. An EDTA-TEA solution (EDTA 30g/L, triethylamine 30g/L, pH 7.0)

was also applied to the column and did not remove the binding protein. As a control the soluble protein which did not bind to the EPS column was reapplied to a fresh EPS column. None of this protein was bound showing that all of the EPS binding proteins were removed by the first EPS column. Furthermore, the soluble protein do not bind to a control column which does not contain EPS. The EPS was dissolved in buffer A solution and applied to the column in an attempt to remove the binding protein from the column. However, the EPS is too viscous, therefore the column stopped flowing. The portion of the column containing the EPS binding protein was transferred to a tube. To the tube, 1 mL of a solution (0.01 M NH_4OAC, 0.1% SDS, 1% mercaptoethanol, using NH_4OH to adjust pH to 7.0) was added. The tube was sealed and heated at 100°C for 15 minutes, centrifuged and the supernatant was freeze-dried. The binding protein (9.4 mg) was saved for further analysis.

Cell Surface Polysaccharides.

The amount of each kind of polysaccharide isolated from R. leguminosarum 128C53 sm^r rif^r Exo^{+1} and Exo^{-1} is shown in Table II. The mutant strain produces very small amounts of EPS and CPSI fractions compared to the parent strain and also does not produce CPS2a. The amounts of LPS and small polysaccharides produced by the parent strain are lower than the mutant. Since some LPS and small polysaccharides are in the CPS2a fraction this difference in LPS and SmPS amounts between the parent and the Exo^{-1} mutant is probably not significant.

The polysaccharides were hydrolyzed in 2N trifluoroacetic acid, and the alditol acetates were analyzed by gas chromatography. The sugar compositions are given in Table III and Table IV. The parent strain EPS, CPSI and CPS2a fractions consist of a large amount of galactose, glucose, and uronic acid.[29] The presences of LPS sugars rhamnose, fucose, and

mannose in those fractions suggest that the EPS, CPSI and CPS2a contains some LPS. It is known that Gram-negative bacteria excrete cell wall fragments into the media.[30,31] Therefore, it is not unusual that the EPS and CPS fractions from Rhizobia contain some LPS.

In order to show that the CPS and EPS fractions do contain LPS, both fractions were further purified. It has been shown that the more acidic EPS can be separated from the LPS by precipitation with cetyltrimethylammonium bromide (CTAB). The EPS and CPS2a fractions were dissolved in deionized water and a 3% CTAB solution was added. The solution was kept at 37°C for 2 hours.[32,33] This precipitate was separated by centrifugation and the sugar compositions of the precipitate and supernatant were determined as shown in Table V. From these results we find that the CTAB supernatants from both the CPS2a and EPS are greatly enriched in LPS sugars and that the purified EPS (CTAB precipitate) are very similar in their sugar compositions. The presence of LPS sugars in the CTAB precipitates shows that the LPS was not completely separated by this method.

It was found that the LPS in the water layer can be separated from a glucose rich small molecular weight polysaccharide by Sepharose 4B gel-filtration column. The diagram for the gel-filtration of the LPS containing ultracentrifugation pellet from the Exo^{+1} strain is shown in Figure 4. The Exo^{-1} strain gives identical results.

The EPS from Exo^{-1} was found to contain large amounts of LPS sugars. Therefore the EPS from the Exo^{-1} strain was applied to a Sepharose 4B column. The result is shown in Figure 5. Figure 5a shows a typical elution profile for the parent EPS. This EPS is heterogenous in molecular weight and therefore its elution is spread out over the entire range of the column. Figure 5b shows the Sepharose 4B column chromatograph of the Exo^{-1} EPS. The first peak elutes in the partially included volume, while

the second peak elutes near the included volume. Those two peaks were dialyzed, freeze-dried and analyzed. These results are shown in Table IV. From these results we find that the first peak which contains rhamnose, fucose, mannose and KDO is the same as LPS composition. The second peak is a glucose-rich polysaccharide and is the same as the small polysaccharide separated from the LPS. The uronic acid has also been identified and the LPS from both strains contains galacturonic acid while the EPS contains glucuronic acid.

The SmPS fractions from both the Exo^{+1} and Exo^{-1} strains contain a large amount of glucose. In addition the Exo^{-1} SmPS (separated from EPS and LPS) also contain LPS sugars. The SmPS from the parent strain does not contain LPS sugars. This suggests that the SmPS from the mutant strain include some LPS fragments. The SmPS fractions have been further analyzed by applying them onto a Sephadex G-50 gel filtration column. Figure 6a is the elution profile of the parent strain SmPS and Figure 6b shows the mutant. All three peaks were analyzed by gas chromatography, and these results are shown in Table VI. The composition of the first peak from the Exo^{-1} strain SmPS is just like the O-antigen composition except it contains a small amount of uronic acid. The second peak of the Exo^{-1} strain and the parent SmPS peak only have glucose and a small amount of uronic acid.

The polysaccharide regions of Rhizobium LPS can be separated from the lipid by mild acid hydrolysis. The core oligosaccharide sugars and O-antigen polysaccharide sugars can be identified by separating these two regions using Sephadex G-50 column chromatography. Figure 7 shows a Sephadex G-50 elution profile of the LPS polysaccharides from the parent strain. Identical results are obtained for Exo^{-1} LPS and the LPS separated from the Exo^{-1} EPS. It has been shown that Sephadex G-50 column chromatography of the mild acid hydrolyzed polysaccharides from E. coli LPS also results

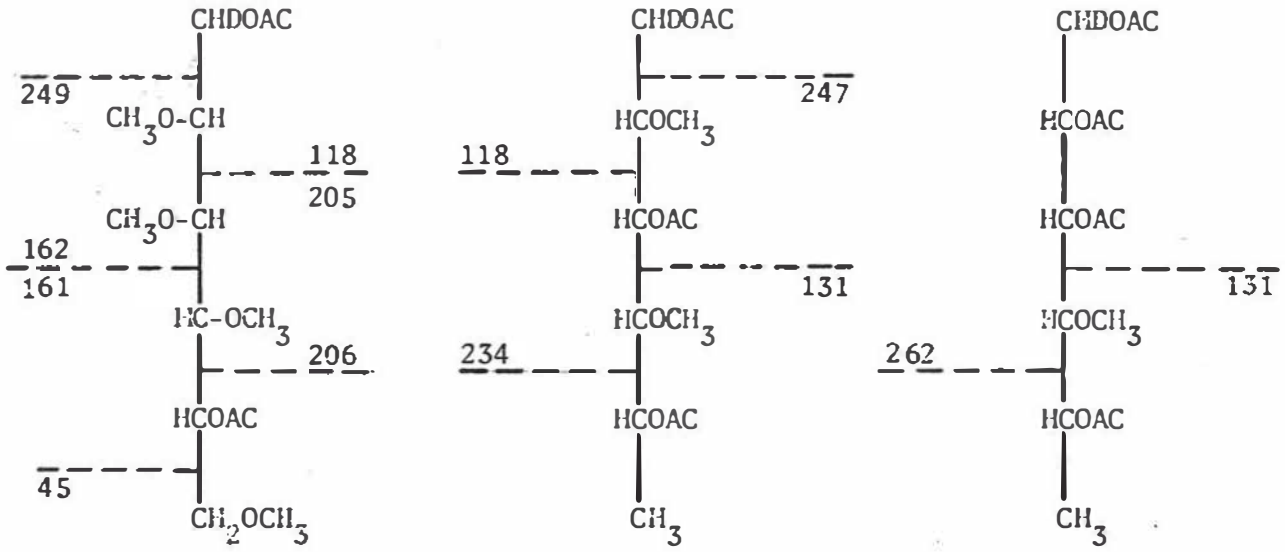
in two major polysaccharide peaks.[34,35] Polysaccharide peak I eluted after the void volume and is the O-antigen polysaccharide region. Polysaccharide peak II eluted just before the included volume and is the core oligosaccharide.

The core and O-antigen polysaccharides were hydrolyzed in 2N trifluoroacetic acid, and the resulting monosaccharides were converted to their corresponding alditol acetates and analyzed by gas chromatography. Table VII gives the relative sugar compositions of the core for the Exo^{+1} and the Exo^{-1} strains and the O-antigens for Exo^{+1}, Exo^{-1} strains and the LPS excreted in the Exo^{-1} EPS. The results show that the O-antigens are identical to each other and that the core oligosaccharides are also very similar to one another in composition.

The LPS has been further analyzed by using gel electrophoresis. Gel electrophoresis of the Exo^{+1} and Exo^{-1} LPS resulted in two bands, implying that the original sample contained two different sizes of LPS. Figure 8 shows the gel electrophoresis picture of the Exo^{+1} LPS, the Exo^{-1} LPS and the LPS from the Exo^{-1} EPS. These results support the idea that the Rhizobium LPS consist of two molecular species. The top band has the lower mobility and is probably the intact LPS which contains lipid A, core and O-antigen. The bottom band has the higher mobility and probably contains only lipid A and core without O-antigen.

The O-antigen from both Exo^{+1} and Exo^{-1} were methylated and acetylated to determine their linkages. The aim of methylation is to methylate all the free hydroxyl groups in the O-antigen polysaccharide. This was achieved by repeating the reaction with DMSO anion and methyl iodide. Determination of the sugar linkages of the polysaccharide also requires the hydrolysis of this methylated polysaccharide, followed by reducing with sodium borodeuteride and then acetylation with acetic anhydride/pyridine. Analysis

was done using the combined application of gas chromatography and mass spectrometry. This was carried out by the regional GC/MS facility at Washington University. On reduction of methylated sugars, the sodium borodeuteride is used to label the C-1 carbon so one can distinguish between a 2-O-methyl and a 4-O-methyl pentose or a 3-O-methyl and a 4-O-methyl hexose. The gas chromatography of methylated O-antigen from Exo^{+1} and Exo^{-1} and their linkages are shown in Figure 9, and the different mass spectra are as follows:



t-mannose 1,3 linked-6-deoxyhexose 1,2,3 linked-6-deoxyhexose

The O-antigens from the parent and Exo^{-1} strains are identical to each other.

Table I: Formulas for gel electrophoresis solutions.

(A)	Running gel solution (5 mL):		
	Tris buffer (22.71g/100 mL)	1 mL	pH 8.8
	10% SDS	50 µL	
	50% Acrylamide 1.3% Bisacrylamide	1.5 mL	
	Deionized water	2.45 mL	
(B)	Stacking gel solution (5 mL):		
	Tris buffer (7.69g/100 mL)	1 mL	pH 6.8
	10% SDS	50 µL	
	50% Acrylamide 1.3% Bisacrylamide	0.7 mL	
	Deionized water	3.25 mL	
(C)	Running buffer:		
	Tris buffer (30.28 g/L)		
	glycine (144.13 g/L)		pH 8.3
	dilute 1:10		
(D)	Dye for LPS:		
	Mercaptoethanol	1 mL	
	Sucrose	5 g	
	Disodium ethylenediamine tetraacetate	5 mM	
	Bromphenol blue	0.1 g	
	dilute to 10 mL (using stacking buffer)		
(E)	Schiff reagent:		
	Basic fuchsin	2.5 g	
	sodium meta-bissulfite	5 g	
	HCl 1N	50 mL	
	Deionized water	500 mL	
	Charcoal	2 g	

Table II: Amounts (mg x 10^{-12}) of the Polysaccharide Fractions from *R. leguminosarum* 128C53 sm^r rif^r and its Exo^{-1} Mutant.

Strain	EPS	CPS1	CPS2a	LPS	SmPS
Exo^{+1}	447	7.49	40.9	7.79	3.44
Exo^{-1}	32.7	0.303	0	18.4	10.9

Table III: The Relative Sugar Composition of *R. leguminosarum* 128C53 sm^r rif^r Polysaccharide Fractions.

	Rha	Fuc	Man	Gal	Glc	Uronic Acid	KDO
EPS	0.35	0.28	3.0	10	63	23	0.21
CPS1	1.1	0.90	1.5	11	63	21	0.45
CPS2a	6.5	6.3	4.2	6.1	56	20	1.0
LPS	23	28	21	3.8	2.4	17	4.8
SmPS	0	0	1.7	3.1	84	11	0

Table IV: The Relative Sugar Composition of the Polysaccharides from *R. leguminosarum* 128C53 sm^r rif^r Exo^{-1}.

	Rha	Fuc	Man	Gal	Glc	Uronic Acid	KDO
CPS1	16	22	23	5.3	16	17	2.0
LPS	23	30	22	4.2	2.2	17	3.3
SmPS	6.6	7.9	4.5	2.3	67	10	1.5
Peak I separated from EPS	25	25	20	4.9	0.85	21	4.0
Peak II separated from EPS	4.6	2.4	2.5	4.2	73	12	0.4

Table V: The Relative Sugar Composition of the Polysaccharides Separated by CTAB Parent EPS and CPS2a Fractions.

	Rha	Fuc	Man	Gal	Glc	Uronic Acid	KDO
EPS, CTAB precipitate	Tr	Tr	0.70	14	54	31	0
EPS, CTAB supernatant	8.0	8.0	6.0	9.0	46	21	2.5
CPS2a, CTAB precipitate	2.9	3.1	2.8	9.9	52	28	0.80
CPS2b, CTAB supernatant	21	24	15	0.80	25	11	3.8

Tr = trace amounts present.

Table VI: The Relative Sugar Composition of the G-50 Polysaccharides from the Parent and Exo^{-1} SmPS Fractions.

	Rha	Fuc	Man	Gal	Glc	Uronic Acid	KDO
Parent SmPS	0	0	0	0	89	11	0
Exo^{-1} SmPS Peak 1	28	35	18	Tr	4.6	9.3	4.8
Exo^{-1} SmPS Peak 2	0	0	0	0	88	9.4	2.2

Table VII: The Relative Sugar Compositions of the O-Antigen and Core Polysaccharides Isolated from the Various LPS Fractions.

		Rha	Fuc	Man	Gal	Glc	Uronic Acid	Protein
O-antigen	Exo^{+1}	35	41	23	0	0.7	0	0
	Exo^{-1} (LPS)	35	41	24	0	Tr	0	0
	Exo^{-1} (EPS)	34	40	24	0	1.3	0	0
Core	Exo^{+1}	0	0	12	9.5	Tr	70	8.8
	Exo^{-1}	0	0	8.9	7.0	0.8	69	15

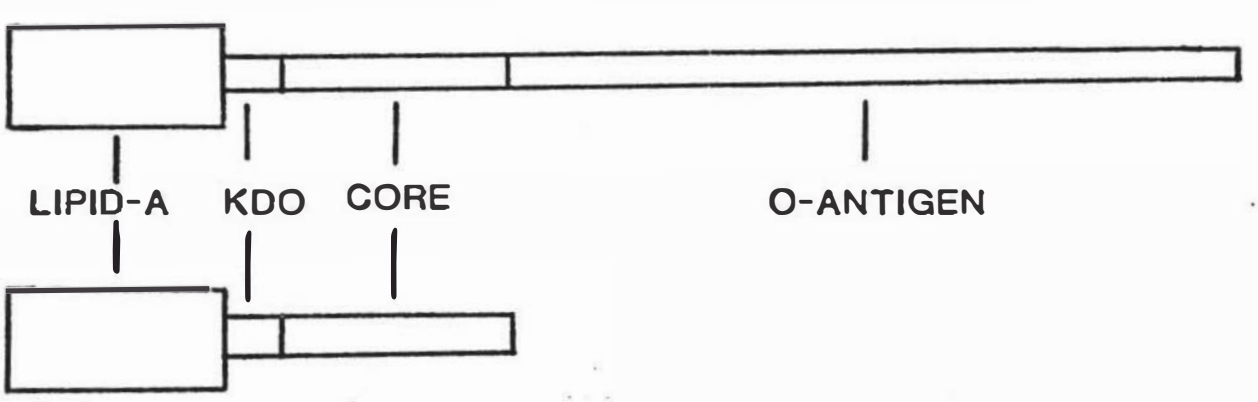


Figure 1. Two general types of LPS present on the surface of Gram-negative bacteria.

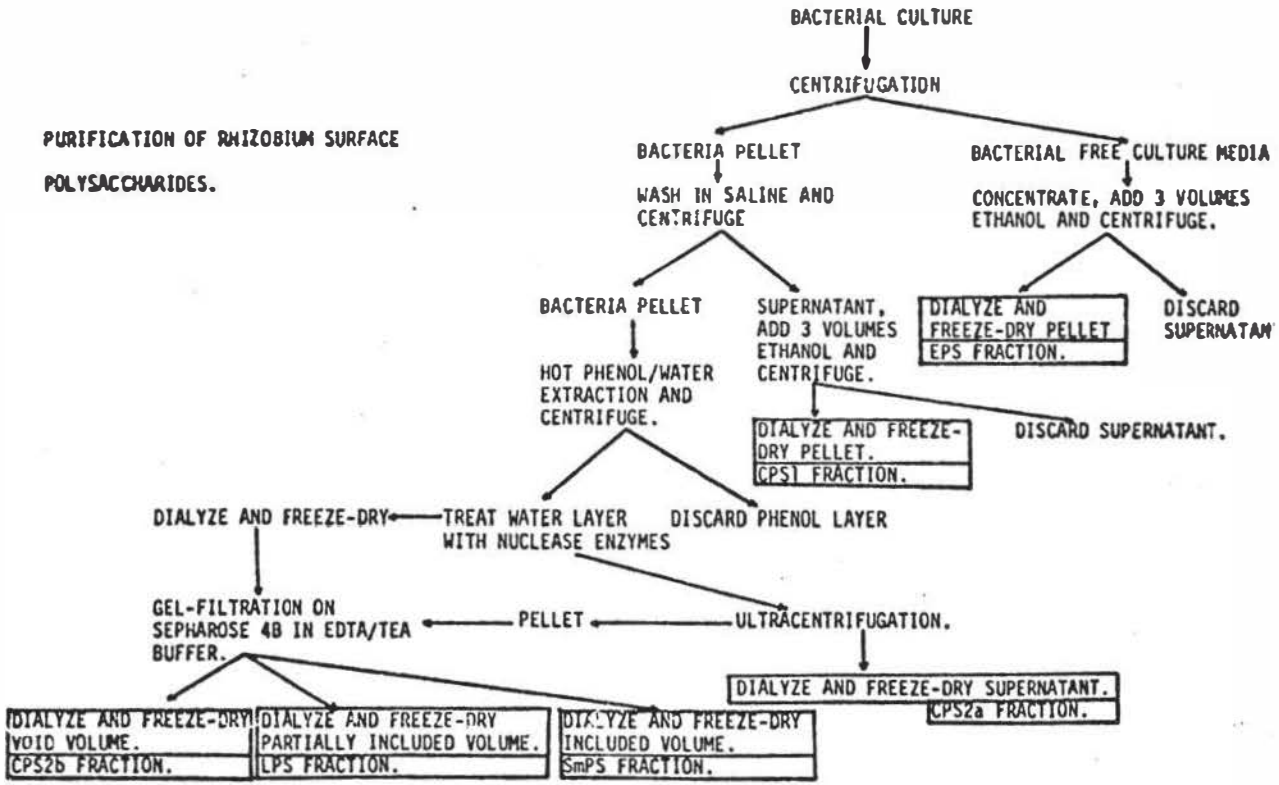


Figure 2. The purification scheme for the various polysaccharide fractions..

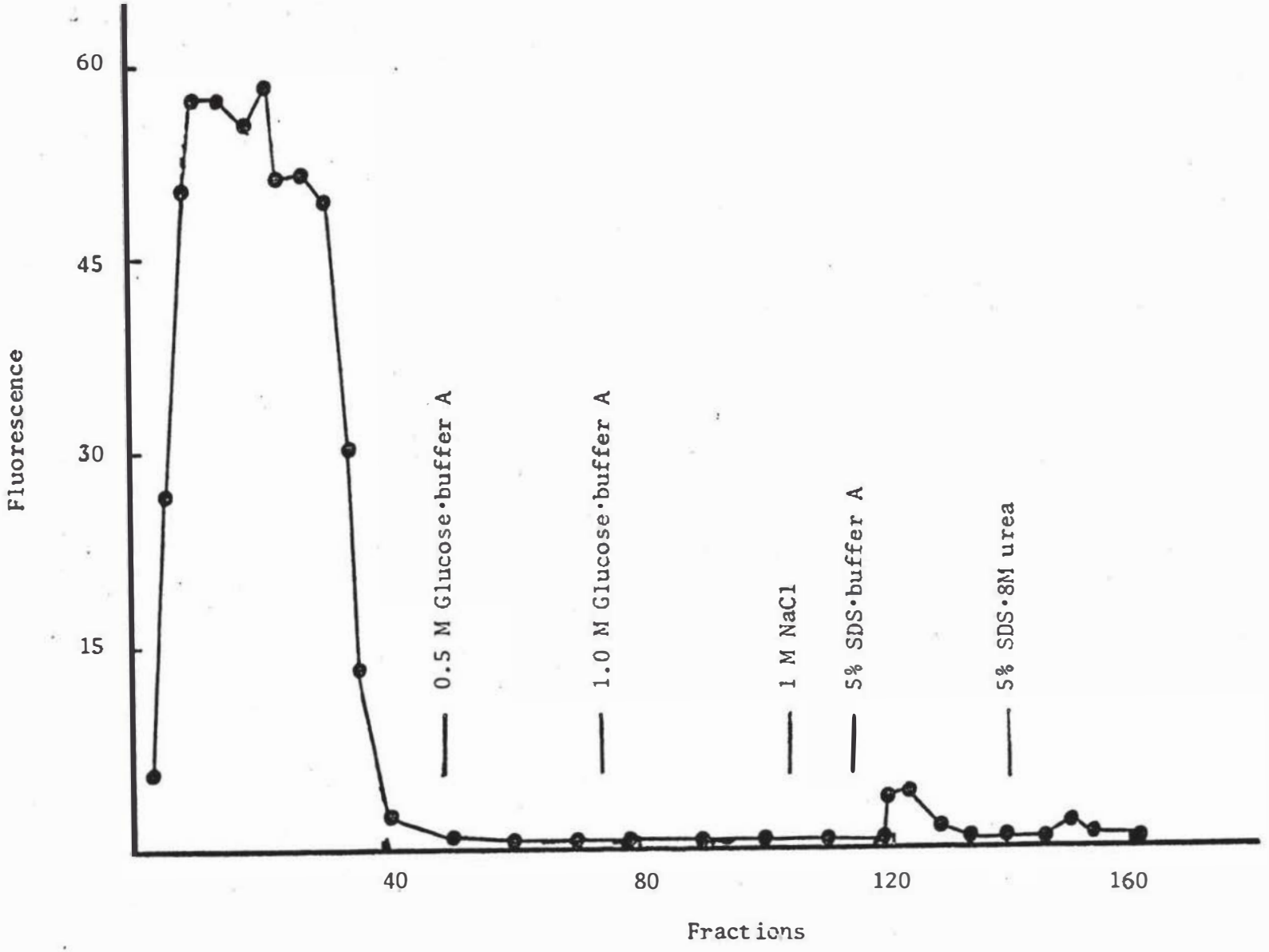


Figure 3. The membrane protein fractions eluted from EPS-polyacrylamide affinity support.

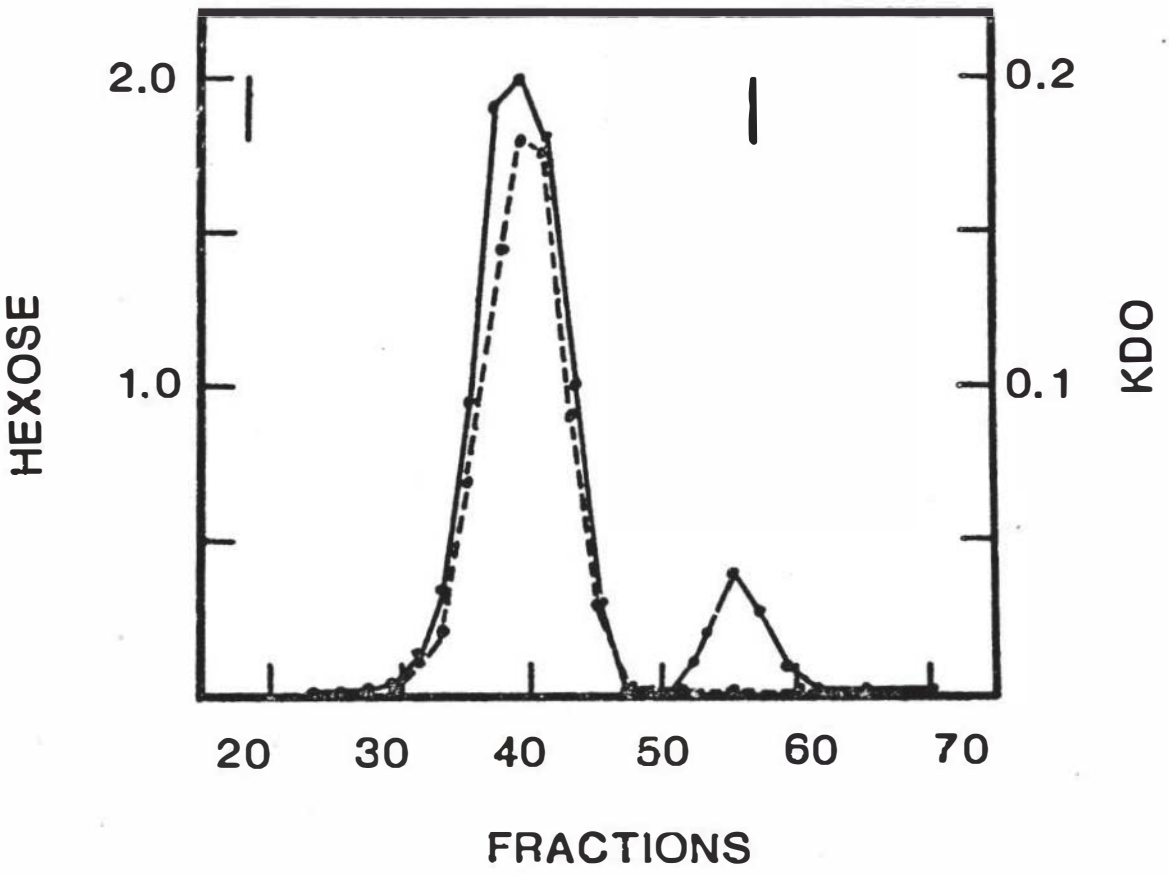


Figure 4. Sepharose 4B column chromatography of R. leguminosarum 128C53 sm^r rif^r Exo^{+1} LPS. The solvent is EDTA/TEA buffer. Hexose was assayed by using the anthrone test and the KDO was assayed by using thiobarbituric acid method.

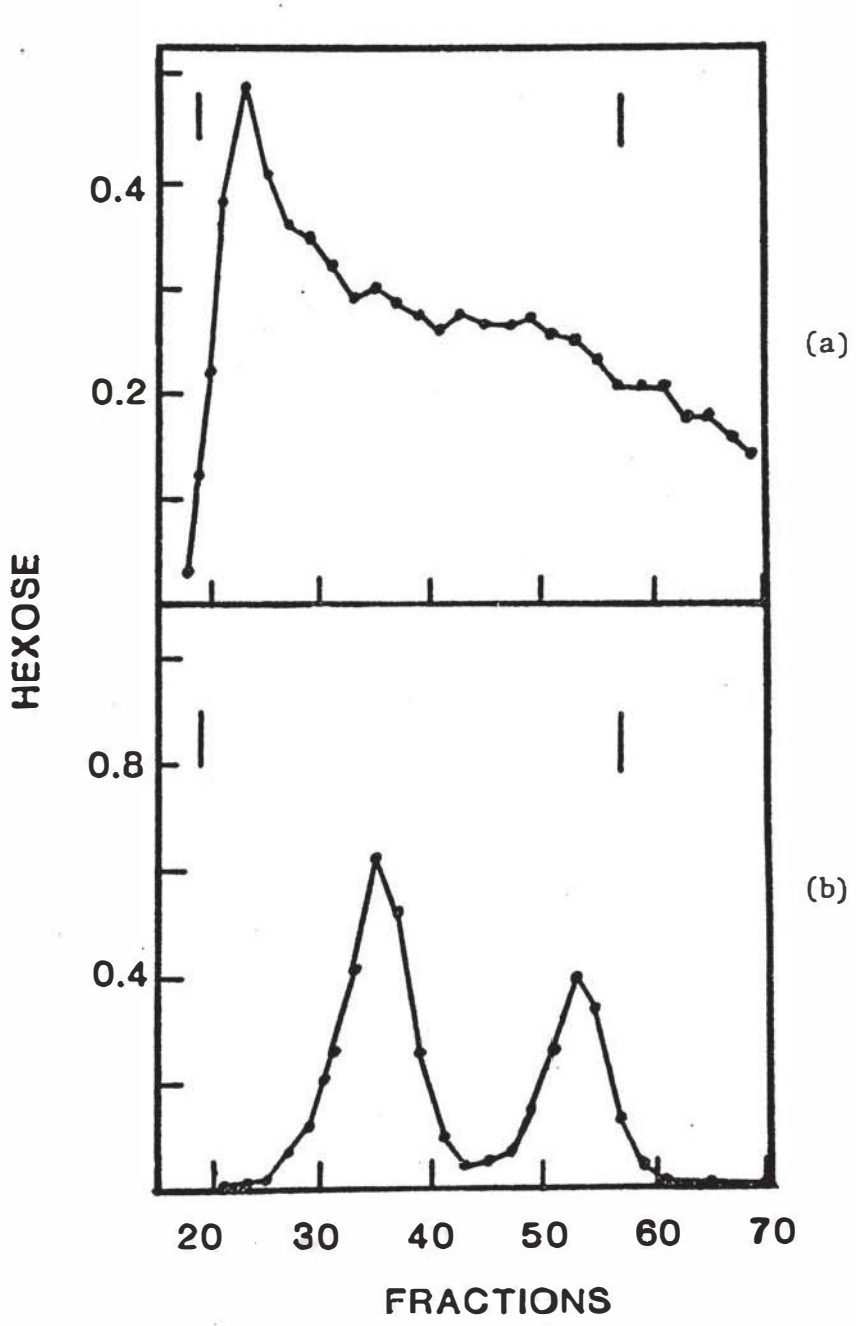


Figure 5: The Sepharose 4B elution profile of the EPS from the (a) parent and (b) Exo^{-1} mutant.

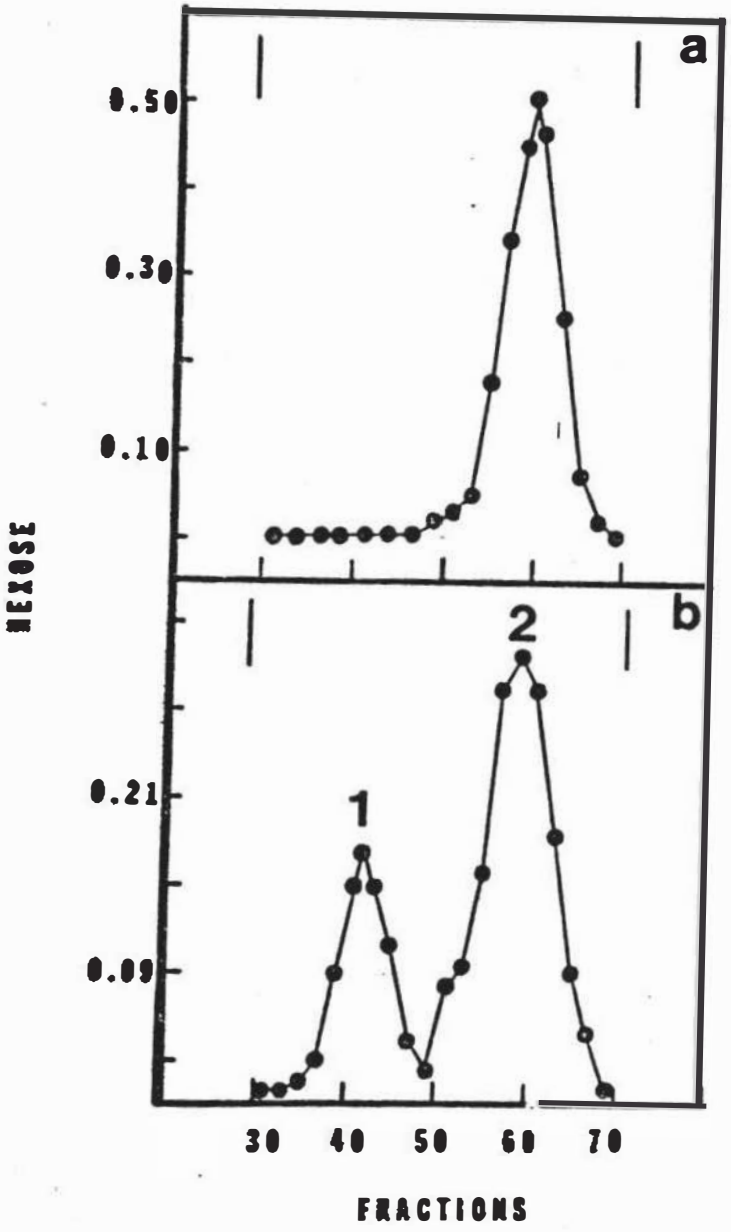


Figure 6. Sepharose G-50 elution profile of the SmPS from the a) parent and b) Exo^{-1} mutant.

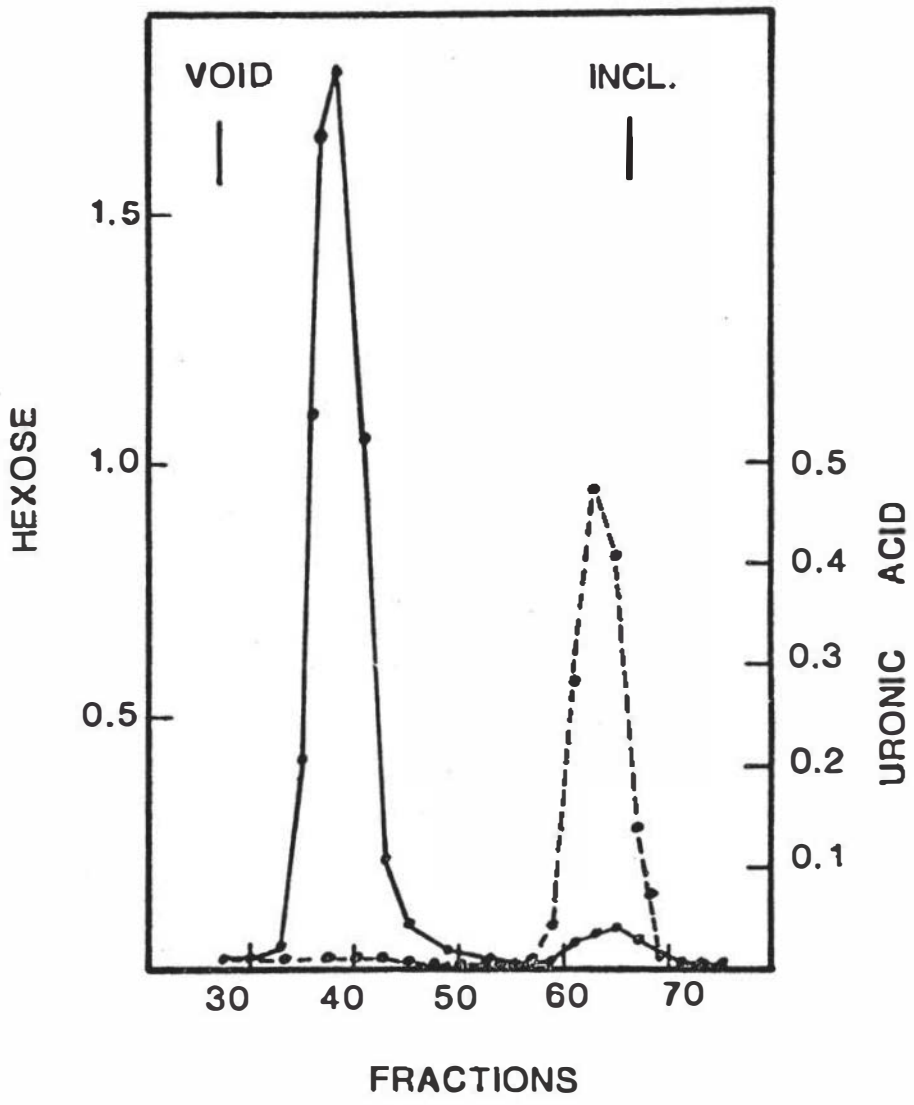


Figure 7. A Sephadex G-50 elution profile of the polysaccharide released from the parent LPS by mild acid hydrolysis.

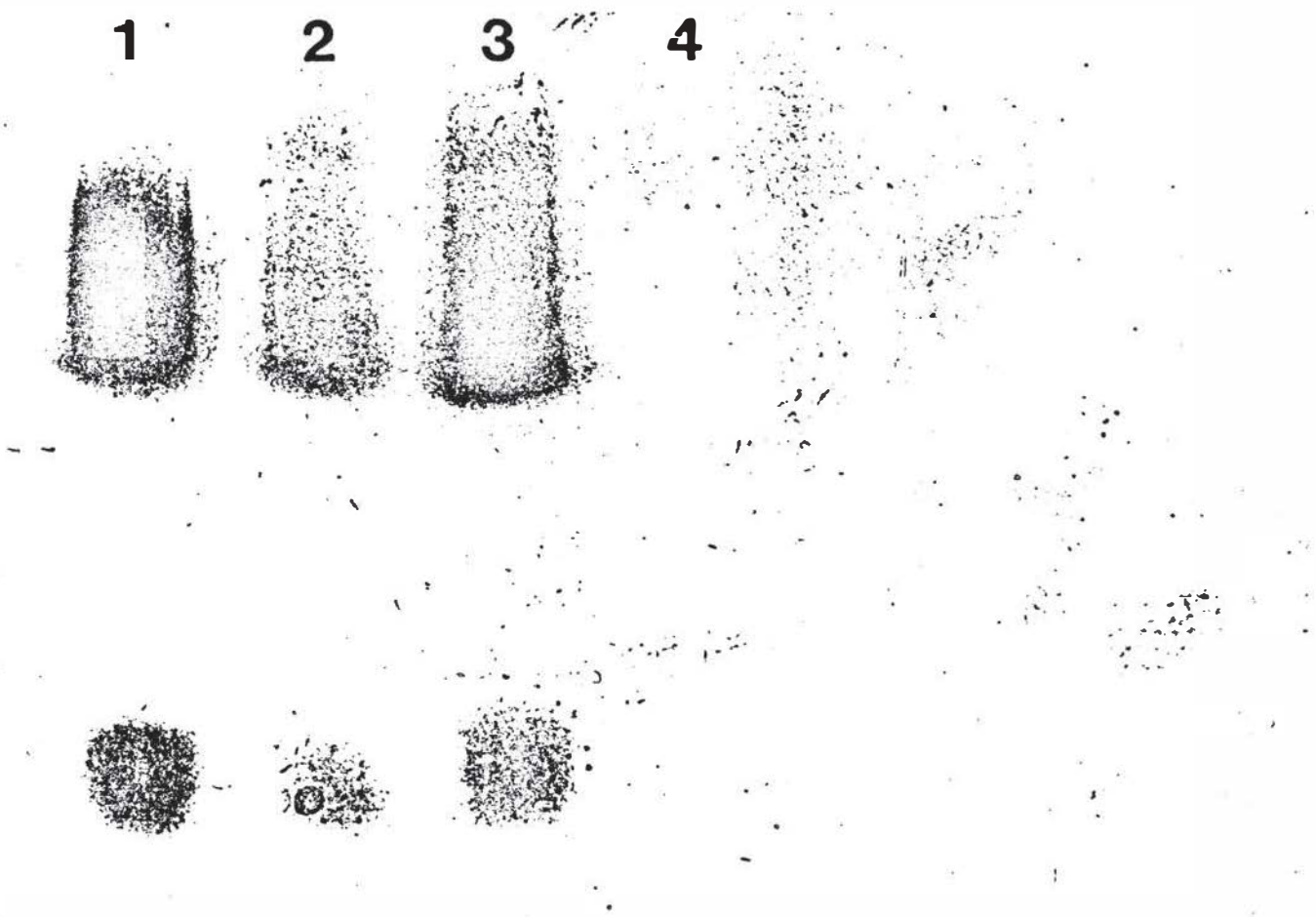


Figure 8. The gel electrophoresis picture of the (1) parent, (2) mutant LPS and (3) the LPS separated from the mutant EPS and (4) the parent LPS after removal from the lipid region.

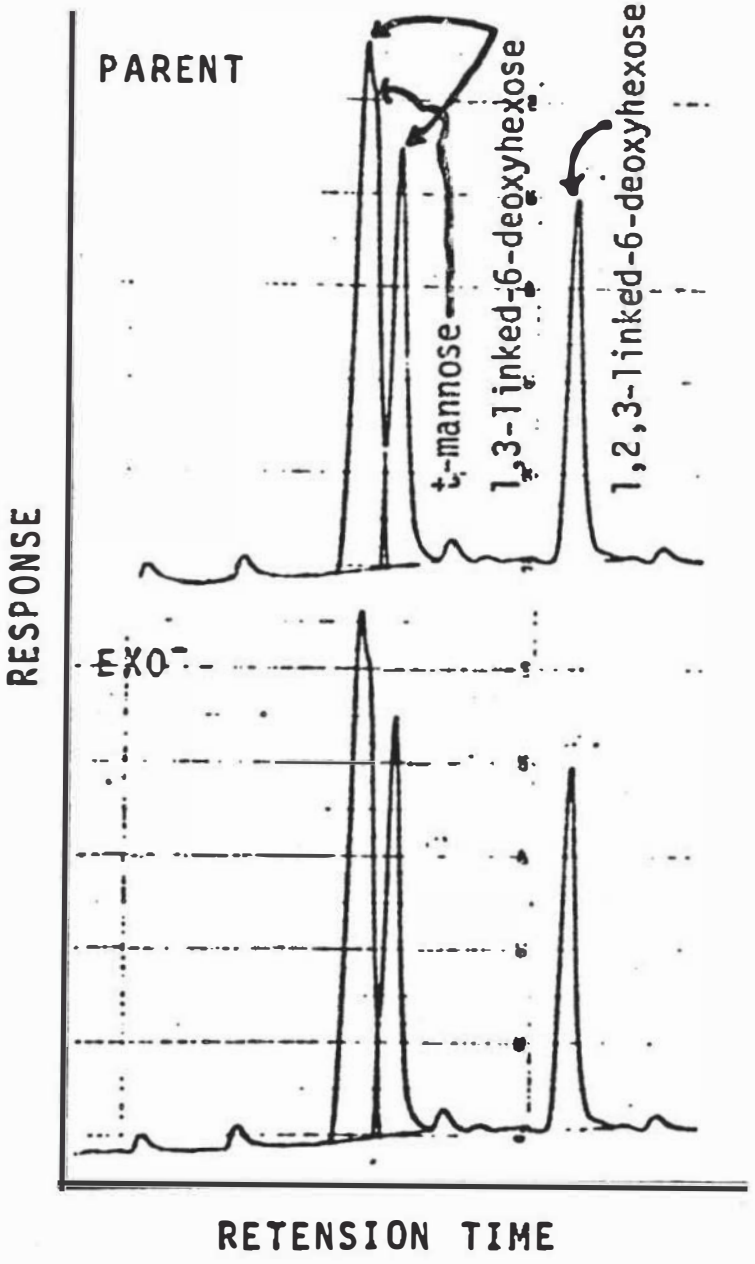


Figure 9. The GC trace of methylated O-antigen from the parent and Exo^{-1} mutant.

DISCUSSION

Isolation of Rhizobium Polysaccharide Binding from Legume Roots:

Summary of Results. From the data we have shown that about 10% of the soluble protein (preparation 2) and the membrane protein (preparation 3) fractions adhere to the EPS-polyacrylamide affinity support column. It is necessary to isolate these root proteins which interact with the EPS-polyacrylamide affinity column and then analyze the binding proteins and non-binding proteins. However, it has proved difficult to remove the binding proteins from the affinity column. At the present time, we have not compared host binding proteins with the non-host binding proteins.

Specificity of binding protein. In order for a Rhizobium to infect its host root, the bacterium must first attach to the host root via the Rhizobium EPS and/or LPS. It has been suggested that the Rhizboium attach to the host root by a specific process.[36] By using protein preparation from a non-host such as soybean and applying them to the R. leguminosarum EPS-polyacrylamide column, we should be able to determine whether or not this interaction is specific.

Analysis of Binding Proteins. The hypothesis that the interaction between root proteins and Rhizobium is specific is based on the idea that the lectin from the host legume specifically binds the symbiont Rhizobium.[37] Lectins are plant proteins with high affinity for specific sugar residues and are commonly present in the seeds of legumes. Several reports suggest that the lectin receptor resides in the capsule which surrounds

the Rhizobium. Since the capsule is composed of EPS, it is possible that a receptor for this lectin resides on the surface of the Rhizobium, and the receptors may be the Rhizobium EPS. In addition, other data suggest that lectin binding to the surface of Rhizobia does not correlate with symbiotic specificity.[38] Therefore, it is important to isolate the binding protein fractions from the affinity column and by using the gel electrophoresis to determine whether or not this binding protein is lectin. Perhaps one of the root protein binding fractions may be the lectin, or the results may show that the lectin is different from the binding protein fractions. This work is in progress.

Binding Analysis. The reason we used glucose to try to remove the binding protein from the EPS-polyacrylamide affinity column is that glucose is a hapten for pea lectin. Unfortunately, this did not work, even when the concentration of glucose had been increased. A further experiment would be to mix glucose with the root protein fractions before applying onto the affinity column. In this case we should not find any binding to the EPS-polyacrylamide column since all the root protein fraction binding sites would be occupied by glucose. Other haptens could be tested in a similar manner.

Cell surface Polysaccharide comparison:

Summary of Results. The data presented above shows that the Exo^{-1} mutant produces small amounts of EPS and CPS in comparison to the parent strain. This data verifies the original data reported by Sanders et al.[2] The Exo^{-1} strain produces about twice the LPS of the parent strain. However, there exists significant amounts of LPS sugars in the CPS2a fraction suggesting that not all the parental LPS were removed during ultracentrifugation. Therefore, this difference in the LPS amounts between the parent

and mutant may not be important. All the results we obtained regarding the R. leguminosarum 128CS3 sm^r rif^r Exo^{+1} and its Exo^{-1} mutant are as follows:

Similarities between Parent and Mutant.

(1) The LPS are excreted into the media for both strains.

(2) The LPS from the mutant and the parent are identical to each other in their sugar compositions, both the core and the O-antigen regions. In addition the O-antigen from the parent and the Exo^{-1} mutant have identical sugar linkages.

Differences between the Parent and Mutant.

(1) The mutant strain produced diminished amounts of EPS and CPS. The EPS and CPS produced by exo^{-1} are not the typical parental EPS and CPS. These mutant EPS and CPS fractions contain only LPS and SmPS.

(2) The SmPS for Exo^{-1} mutant contains a significant amount of LPS fragments. These fragments were not produced in the Exo^{+1} strain.

Role of EPS and CPS. Sanders et al suggested that the reduced production of extracellular carbohydrate by the mutant Exo^{-1} may be related to the inability of this strain to nodulate pea seedlings. Our data shows that the mutation in Exo^{-1} results in the total lack of parental type EPS and CPS. Therefore, it may be that the parental type of the EPS and CPS is essential for symbiosis.

Our data also verify the earlier microscopic results which show that the Exo^{-1} mutant does not produce a capsule.[3] These reports also suggest that there may be a correlation between the presence of capsules and the infectivity of R. leguminosarum.[3] The parent strain produces both CPS

and EPS, and the data suggest that CPS is identical to the EPS. It is reasonable that the inability of Exo^{-1} to synthesize typical EPS also results in the inability to synthesize typical CPS. It may be that the ability of the bacteria to form a capsule is essential for symbiosis. However, we cannot say if the capsule is important for the specificity of symbiosis.

Role of LPS. The Rhizobium LPS has been suggested to be involved in symbiotic process. Therefore, the LPS has been examined to determine whether or not there has been any alteration in the structure of the LPS from the mutant strain. From our data, the LPS from the mutant appears to be identical to the parent strain as previously reported by Sanders et al.[2] The LPS has been further analyzed by separating the intact LPS into O-antigen polysaccharide and core oligosaccharide. The LPS regions from the parent are identical in composition to the mutant. The O-antigen polysaccharides have been further analyzed to determine the sugar linkages. The O-antigen from the parent and mutant are identical to one another in their linkages. The parent strain has the ability to infect the pea roots and form nodules while the mutant strain does not attach to the root and does not form root-hair curls. These data implicate that the LPS may not be directly involved in the symbiotic process. However, it is still possible that the LPS have a role in later symbiotic steps such as bacteroid formation, etc.

Role of LPS fragments. Our results also show that when the bacteria are growing, the parent and mutant strains excrete the LPS into the media. The mutant EPS consists of about 70% LPS. It is possible that the LPS polysaccharide fragments excreted by Exo^{-1} inhibit the attachment of the bacteria. However, when the peas are inoculated with a mixture of wild type and Exo^{-1} mutant, there is no effect on the nodulation of the pea

(R. Carlson, unpublished data). Thus it seems that these LPS fragments do not have any inhibitory action on the nodulation process. In order to determine if the Rhizobium LPS could have a role in determining the specificity of host-symbiotic interaction, more studies, i.e. O-antigen structure analysis and core structure analysis, should be done. We also are studying a series of R. trifolii mutants which have more defined mutations affecting the symbiotic process.

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