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A Comparison of the Surface Polysaccharides of Rhizobium trifolii SU843 with its Genetically Altered Mutant, 8002, Which is Defective at Root Hair Curling (HAC-)

Elroy Alfredo Turnbull

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A Comparison of the Surface Polysaccharides of Rhizobium trifolii SU843 with its Genetically Altered Mutant, 8002, Which is Defective at Root Hair Curling (HAC⁻).

BY

Elroy Alfredo Turnbull

THESIS

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I HEREBY RECOMMEND THIS THESIS BE ACCEPTED AS FULFILLING THIS PART OF THE GRADUATE DEGREE CITED ABOVE

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ABSTRACT

Bacteria of the genus Rhizobium are capable of forming a complex nitrogen fixing (fix⁺) symbiotic association with leguminous plants. The surface polysaccharides are believed to be essential in the symbiotic process. The surface polysaccharides of a genetically altered Rhizobium trifolii mutant, 8002—which does not cause root hair curling (HAC⁻), forms no nodules (Nod⁻) nor fixes nitrogen (Fix⁻)—is compared with its parent, SU843, which causes root hair curling (HAC⁺), forms nodules (Nod⁺), and fixes nitrogen (Fix⁺). Three types of surface polysaccharides—extracellular polysaccharide(s) (EPS), capsular polysaccharide(s) (CPS), and lipopolysaccharide(s) (LPS)—were obtained for each bacterial strain. Both bacterial strains produce EPS, which are very similar in composition. The mutant, 8002, produces a CPS which is 1/38 the amount produced by the parent, SU843. The 8002 CPS contains the sugars 2-O-methyl-6-deoxyhexose, rhamnose, and 2-keto-3-deoxyoctonic acid, whereas the parent CPS does not contain these sugars. The LPS of both bacterial strains are similar in sugar composition. The amount of SU843 LPS is five times greater than the amount of the mutant, 8002, LPS. The sugar composition of the O-antigens from both bacterial strains are similar, whereas the sugar compositions of their cores differ quantitatively. The small molecular weight polysaccharide(s) (SmPS) of both bacterial strains are different in sugar composition. The interpretation of these results is discussed in relation to the functions of the Rhizobium surface polysaccharides in the symbiotic process.
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INTRODUCTION

The genus Rhizobium is a member of the family of bacteria called Rhizobiaceae which consist of normally rod-shaped cells without endospores. The cells are aerobic, gram negative, and motile having either one polar flagellum or two to six peritrichous flagella, which do not play a role in symbiosis.¹,²

Rhizobium forms an intimate relationship with leguminae by infecting the roots thereby, leading to nitrogen fixing nodules, for which the bacterium is provided energy in the form of photosynthate. Because of this interaction with leguminous plants, Rhizobium is of both agricultural and economic importance.

Two major differences within the genus Rhizobium are host specificity and growth rate. An example of specificity is that Rhizobium trifolii nodulates only clover whereas Rhizobium japonicum nodulates only soybeans. This high degree of specificity seems to be, in part, due to plant lectins which interact with unique carbohydrate constituents on the surface of their Rhizobial symbionts.³ Fast growers, such as Rhizobium trifolii, Rhizobium phaseoli, Rhizobium meliloti and Rhizobium leguminosarum, in culture medium grow much faster—about one-half the doubling time—than slow growers, such as Rhizobium japonicum and Rhizobium lupini. Another distinguishable factor is that on yeast-manitol agar, the fast growers produce an alkaline end product.⁴

There are five major steps in the Rhizobium symbiotic process: (1) attachment of bacteria onto the surface of the root hair, (2) curling
of the plant root hairs, (3) formation of infection thread, (4) release of the bacteria into the inner cortex cell, and (5) formation of bacteroids which leads to nitrogen fixation.

However, the interactions between the legume host and strains of Rhizobium result in a wide variety of responses ranging from complete failure of the Rhizobium to invade the roots and form nodules to the production of nodules that vary greatly in their ability to fix nitrogen.

The molecular and biochemical mechanism of precisely what factors (polysaccharides, enzymes, etc.) are essential for each or all of the five major symbiotic steps mentioned earlier are not clearly defined or understood. Since the bacterium initially attaches and adheres to the plant root, surface factors are thought to be essential in the symbiotic process. The major cell surface factors of Rhizobium are polysaccharides--lipopolysaccharides (LPS) and extracellular polysaccharides (EPS).

Present evidence indicates that the EPS and the LPS from gram negative bacteria determine the specificity of their association with other organisms. The LPS of Salmonella and E. coli are the strain specific antigenic determinants of these bacteria and are known to be the determining factor in the specific infection of their hosts, whereas for certain types of E. coli, the EPS has been found to be the strain specific antigenic determinant and factor which determines infection. There are other cell surface factors, such as capsular polysaccharides (CPS)--a form of EPS--and a cell wall-associated glycan, which are thought to actively participate in the symbiotic process.

The composition of Rhizobial LPS differs, while the EPS is identical among several species of Rhizobium. The LPS consist of three major structural regions: a lipid region called lipid-A, a core oligosaccharide which is attached to the lipid-A through an acid labile ketosidic bond
involving 2-keto-3-deoxyoctonic acid (KDO), and an O-antigen polysaccharide which is a repeating oligosaccharide and is attached to the core oligosaccharide. The EPS, which is usually acidic since it contains uronic acid and pyruvic acid groups, is in the form of a slimy material which the bacteria excrete into the growth media.

Rhizobia contain naturally occurring plasmids [small circular molecules of extrachromosomal deoxyribonucleic acid (DNA)], which can supply supplementary information conferring properties such as resistance to antibiotic drugs. Genetic evidence has suggested that these plasmids might carry some of the information required in the determination and development of the symbiotic process.6-16

By alteration of the DNA within a gene or genes of the parental Rhizobium strain, a mutant can be created. The resulting mutation can thus influence the organism's biochemical and biological characteristics. The major objective of using genetically engineered mutants is to define factors and their functions which are responsible for the different steps in the symbiotic pathway.

A parental Rhizobium trifolii strain, SU843, and its genetically altered (Tn5 mutagenesis) mutant, 8002, will be compared. The R. trifolii SU843 causes root hair curling (HAC+), nodulates (Nod+) clover, and fixes nitrogen (Fix+), whereas the 8002 mutant has been shown by microscopic examination to be defective at root hair curling (HAC−), thus leading to its inability to form nodules (Nod−) and to fix nitrogen (Fix−) (B. Rolfe, personal communication).

By analyzing and comparing the cell surface polysaccharides of parental (SU843), and its mutant (8002), a relationship can be developed involving both genetics and biochemistry. Such an approach may identify some of the essential factors and their functions which are responsible
for the differing steps along the symbiotic pathway, thus aiding in the understanding of the molecular mechanisms of nitrogen fixation by the genus Rhizobium.

The major distinguishable aspect between the polysaccharides of the parental (SU843) and its mutant (8002) has been the CPS. The 8002 CPS shows considerable differences in both mass (mg) produced and sugar composition (type of sugars and their relative percentages) compared to the parental SU843 CPS.
EXPERIMENTAL

Three consecutive steps were executed for each bacterium (i.e. Rhizobium trifolii, SU843, and its genetically altered mutant, 8002) as follows:

(1) Growth and harvesting of the bacteria.

(2) Extraction and purification of the polysaccharides.

(3) Qualitative and quantitative analysis of the polysaccharides.

(1) **Growth and Harvesting of the Bacteria**

The bacteria were grown on modified Bergerson's media, which consisted of the following: CaCl₂·2H₂O, 5g/L; Na₂HPO₄·12H₂O, 36g/L; MgSO₄·7H₂O, 8g/L; FeCl₃, 0.3 g/L; Thiamine HCl, 0.2 g/L; Biotin, 0.02 g/L; Yeast Extract, 0.5 g/L; Glutamic acid, 0.5 g/L; Mannitol, 10.0 g/L; Trace elements (CaCl₂·2H₂O, 6.6 g/L; H₃BO₃, 145 mg/L; FeSO₄·7H₂O, 125 mg/L; CaCl₂·6H₂O, 59 mg/L; CuSO₄·5H₂O, 5 mg/L; MnCl₂·4H₂O, 4.3 mg/L; ZnSO₄·7H₂O, 108 mg/L; NaMoO₄, 125 mg/L). The pH was adjusted to 6.8 and the resultant media sterilized.

The bacteria were then transferred from a slant into a 125mL flask containing approximately 50mL of culture medium. The flask was then placed on a shaker for about 3-4 days at room temperature. The bacterial culture was then transferred to 12 litres of culture media and aerated. Five mL aliquots of culture media were removed at 4-6 hour intervals and its absorbance measured at 620nm using deionized water as the reference.

The bacteria were harvested at the late exponential or early stationary phase (determined graphically by absorbance versus time) and
separated from the media by centrifugation at 10,000 r.p.m. for 20 minutes.

(2) Extraction and Purification of the Polysaccharides.

Extracellular polysaccharides (EPS)

The bacteria free culture media was then concentrated by rotary evaporation under reduced pressure at approximately 40°C to about 200 mL. Three volumes of ethanol were added to precipitate the EPS from the culture media. The resulting mixture was then centrifuged at 9000 r.p.m. for 20 minutes. The precipitate-pellet was dissolved in deionized water, dialyzed at 4°C against deionized water and freeze dried.

Capsular polysaccharides (CPS)

The bacteria were washed with saline solution (0.43 g KH$_2$PO$_4$ per liter, 1.68 g Na$_2$HPO$_4$ per liter, 7.2 g NaCl per liter, and pH adjusted to 7.2) and centrifuged at 10,000 r.p.m. This was repeated until the supernatant did not contain any hexose as measured by the anthrone test. The saline supernatant solutions were combined and concentrated by rotary evaporation under reduced pressure at approximately 40°C to about 200 mL. Three volumes of ethanol were added and the resulting mixture centrifuged at 9000 r.p.m. for 20 minutes. The supernatant liquid was discarded and the precipitate-pellet was dissolved in deionized water, dialyzed against deionized water and freeze dried.

Lipopolysaccharides (LPS)

To the saline-washed bacteria (approximately 33.2 g for R. trifolii SU843, and 10.9 g for 8002) were added deionized water until the total volume was 100 mL. This was then placed in a water-bath at 65°C, which also contained 100 mL of 95% phenol, until it had equilibrated to the
temperature of the water-bath. Both the phenol and the saline-washed bacteria were combined and stirred constantly for 15 minutes at 65°C. The mixture was then immediately placed in an ice-bath for a further 15 minutes and thereafter centrifuged at 11,000 r.p.m. for 20 minutes.

The water layer was removed and retained, whereas the phenol layer was placed in the water-bath at 65°C until it equilibrated to the temperature of the water bath. One hundred mL of deionized water, also at the temperature of the water-bath, was added to the phenol layer and the previous procedure repeated. Both water layers containing the LPS and nucleic acids were combined and dialyzed against deionized water.

To the nucleic acid-LPS solution were added per 100 mL 0.1mL Dnase (1mg/mL) and 0.1 mL Rnase (10mg/mL) with 10 mL of 0.1M MgSO₄/0.5M tris base (pH adjusted to 7.5). The solution was then allowed to stand at room temperature for approximately 18 hours, then dialyzed against deionized water. It was then concentrated by rotary evaporation under reduced pressure at 40°C to approximately 50mL and freeze dried.

One hundred mg of this polysaccharide was then dissolved in 7.0 mL of buffer solution (30g/L ethylenediaminetetracetic acid and 30g/L triethylamine, pH 7.0). The resulting mixture was not viscous and was therefore applied directly to a sepharose 4B gel filtration column (4 x 50cm) with a flow rate of approximately 0.2mL/minute. The void and included volumes were determined by using blue dextran and glucose. Five mL fractions were collected and assayed for hexose (anthrone test) and KDO (thiobarbituric acid method). The LPS elutes near the void volume whereas the small molecular weight polysaccharide (SmPS) elutes near the included volume. The two peaks obtained (LPS and SmPS) were dialyzed at 4°C against deionized water and freeze dried.
The LPS is hydrolyzed (1% acetic acid at 100°C for 45 minutes) then placed in an ice-water bath for approximately 15 minutes. This resulted in a precipitate containing the lipid which has been detached from the polysaccharide by the rupture of the ketosidic bond between the lipid and the KDO. The precipitate mixture was centrifuged at approximately 5,000 r.p.m. for about 30 minutes. The polysaccharide supernatant containing the O-antigen and the core polysaccharides was removed and freeze dried. The lipid was then washed with deionized water, freeze dried and weighed.

Separation of the O-antigen from the core was achieved by dissolving the polysaccharide from the above centrifugate in 500µL of deionized water and applying this to a Sephadex G-50 filtration column. One mL fractions were collected and assayed for hexose (anthrone test) and uronic acid (m-hydroxybiphenyl method). Two peaks were obtained; one, the O-antigen that elutes near the void volume and the second, the core that elutes near the included volume; collected and freeze dried.

(3) Qualitative and Quantitative Analysis of the Polysaccharides.

Viable counts of bacteria were made by plating 10⁻⁷ and 10⁻⁸ dilution of the original bacterial solution on agar media. The bacteria were grown in 125-mL flasks containing modified Bergerson's media by shaking on a shaker at room temperature. The culture media (0.1mL) were then transferred to a screw capped test tube containing 9.9mL of sterilized deionized water and shaken to obtain a 10⁻² dilution. The above procedure was repeated twice to obtain a 10⁻⁶ dilution. One-half of the 10⁻⁶ diluted solution was transferred to a screw test tube containing 4.5mL of sterilized deionized water to obtain a 10⁻⁷ dilution. The above procedure was repeated to obtain a 10⁻⁸ dilution. One-tenth mL of the 10⁻⁷ and 10⁻⁸ dilution were
transferred to solid agar medium. After 3-4 days of bacterial growth, the number of colonies were easily counted on the solid agar medium. The weight (mg) of each polysaccharide obtained was noted and the ratio of mg per viable bacteria determined.

A solution of 1 mg/mL of each polysaccharide was made. This was then assayed to determine the percentage of hexose (anthrone test), uronic acid (m-hydroxyl biphenyl method), KDO (thiobarbituric method) and protein (Lowry method).  

Aldoses were analyzed by gas chromatography as their alditol acetates. Each polysaccharide was hydrolyzed in 2N trifluoroacetic acid for two hours at 121°C. By converting the monosaccharides to their corresponding alditol acetates, they were identified by comparison of their gas chromatographic retention times to the retention times of standards. Hexosamine was quantitatively assayed as in another report. Combined GC/mass spectrometry was done on the polysaccharide fractions of both strains at the NIH regional center at Washington University, St. Louis, Missouri.
RESULTS

Amount per viable bacteria.

The amount of each polysaccharide fraction per viable bacteria of Rhizobium trifolii SU843 and 8002 is shown in Table I. Both strains produce similar amounts of EPS. The amount of CPS from SU843 is 38 times greater than that from 8002, which is a significant difference. The amount of LPS and SmPS from SU843 are 5 and 15 times greater than the 8002 LPS and SmPS, respectively.

Surface Polysaccharides.

Extracellular polysaccharides (EPS). The relative sugar composition of the EPS from R. trifolii SU843 and 8002 are shown in Table II. The major sugars—galactose, glucose, and uronic acid—are in a ratio of 1/4/2 and 1/4/2 for the EPS from SU843 and 8002, respectively. The SU843 EPS does not contain 3-N-methyl-3-amino-3,6-dideoxyhexose nor heptose, whereas the 8002 contain detectable quantities of these sugars. These sugars are characteristic of the LPS (results for LPS are given below).

Both the SU843 and 8002 EPS are further purified by Sepharose 4B column chromatography. The EPS from both the SU843 and the 8002 elute close to the void volume (Figures 1 and 2). These peaks were collected, dialysed and freeze dried, hereafter referred to as SU843 EPSI (from SU843 EPS) and 8002 EPSI (from 8002 EPS). The relative sugar compositions of SU843 EPSI and 8002 EPSI are shown in Table III. The major sugars, galactose, glucose and uronic acid, are in a ratio 1/4/2 and 1/5/2 from
The SU843 EPSI and the 8002 EPSI respectively. Both EPSI fractions contain significant amounts of mannose. The 8002 EPSI contains about twice the mannose of SU843 EPSI. These data suggest that the SU843 EPSI and the 8002 EPSI are similar.

Capsular polysaccharides (CPS). The relative sugar compositions of the CPS of SU843 and 8002 are shown in Table IV. The CPS of 8002 shows a significant difference from that of SU843. The SU843 does not contain 2-0-methyl-6-deoxyhexose, rhamnose nor KDO, whereas the 8002 does contain these sugars. An amino sugar, 3-N-methyl-3-amino-3,6-dideoxyhexose, is present in the 8002 CPS, but was not detected in the SU843 CPS. The major sugars, mannose, galactose, glucose, heptose and uronic acid are in a ratio 1/1.5/3.3/0.92/0.92 and 1/1.4/11/2.2/9.5 for the CPS from SU843 and 8002 respectively.

Both the SU843 CPS and the 8002 CPS were further purified by Sepharose 4B column chromatography and their elution profiles are shown in Figures 3 and 4 respectively. Two peaks were obtained from the SU843 CPS. One polysaccharide elutes near the void volume, called SU843 CPSI, and the second polysaccharide elutes after the void volume, called the SU843 CPSII. Three polysaccharides were obtained for the 8002 CPS. One elutes at the void volume, the second elutes just after the void volume and the third elutes close to the included volume. These polysaccharides are referred to as 8002 CPSI, 8002 CPSII, and 8002 CPSIII respectively. All five polysaccharides (8002 CPSI, 8002 CPSII, 8002 CPSIII, SU843 CPSI and SU843 CPSII) were collected, dialyzed and freeze dried.

The relative sugar compositions of the Sepharose 4B polysaccharide fractions, SU843 CPSI, SU843 CPSII (from SU843 CPS), and 8002 CPSI, 8002 CPSII and 8002 CPSIII (from 8002 CPS) are shown in Table V. The SU843 CPSI does not contain 3-N-methyl-3-amino-3,6-dideoxyhexose nor
heptose, whereas the SU843 CPSII does contain these sugars. The SU843 CPSI contains smaller quantities of mannose and uronic acid, but larger quantities of galactose and glucose than SU843 CPSII. Three of the major sugars, galactose, glucose, and uronic acid are in a ratio 1/4.5/2.2 and 1/4.2/9.3 from SU843 CPSI and SU843 CPSII respectively. The SU843 CPSI is very similar in sugar composition to the SU843 EPS and the 8002 EPS fractions.

The 8002 CPSI is different in its sugar composition from the 8002 CPSII and the 8002 CPSIII. Both the 8002 CPSII and the 8002 CPSIII fractions contain some of the sugars which characterize LPS, such as heptose and 2-0-methyl-6-deoxyhexose. However, the 8002 CPSI does not contain these LPS type sugars. The 8002 CPSII and the 8002 CPSIII are similar to each other in the type of sugars present. The 8002 CPSI contains larger quantities of mannose, galactose and glucose, but smaller quantities of uronic acid than both the 8002 CPSII and 8002 CPSIII. The amino sugar, 3-N-methyl-3-amino-3,6-dideoxyhexose, which is found in the 8002 CPS before the Sepharose 4B column purification, is not detected in the 8002 CPSI nor in the 8002 CPSIII. However, trace amounts are present in the 8002 CPSII.

**Lipopolysaccharides (LPS).** The relative sugar compositions of the EPS from SU843 and 8002, purified by Sepharose 4B column chromatography (Figures 5 and 6), are shown in Table VI. The LPS of both SU843 and 8002 contain the same type of sugars. The sugars are 2-0-methyl-6-deoxyhexose, rhamnose, mannose, galactose, 3-N-methyl-3-amino-3,6-dideoxyhexose, heptose, KDO, uronic acid and hexosamine. Most of these sugars found in the SU843 LPS and the 8002 LPS are in approximately the same ratio and this suggests that the LPS from both strains are very similar.
On mild acid hydrolysis the lipid of Rhizobium becomes detached from the polysaccharide regions. These other polysaccharide regions, the O-antigen polysaccharide and the core oligosaccharide, are separated by application to a Sephadex G-50 column. The relative sugar compositions of the O-antigens and the cores of both SU843 and 8002 are shown in Table VII and their G-50 column profiles are shown in Figures 7 and 8. The SU843 and 8002 O-antigens (Figures 6 and 7) elute after the void volume, whereas both cores (Figures 7 and 8) elute close to the included volume. The SU843 O-antigen is similar to the 8002 O-antigen. The major sugars, 3-N-methyl-3-amino-3,6-dideoxyhexose, glucose, heptose and uronic acid are in a ratio 1/6.3/3.5/9.6/19 and 1/6.1/3.3/10/15 for the O-antigen from SU843 and 8002 respectively. The cores of SU843 and 8002 do contain the same type of sugars, but are different quantitatively. The major sugars, mannose, galactose, glucose, heptose and uronic acid are in a ratio 1/0.94/0.33/0.29/9.5 and 1/1.1/0.77/0.47/12 for the cores from SU843 and 8002 respectively. The presence of heptose, which is usually found in the O-antigen, in both cores suggests that either both cores may contain some O-antigen fragments or that heptose is part of the core.

The relative sugar compositions of the SU843 SmPS and the 8002 SmPS, purified by Sepharose 4B column chromatography (see Figures 5 and 6), are shown in Table VIII. The SmPS of 8002 is different from that of SU843 in that it does contain some typical LPS sugars. The SU843 SmPS does not contain LPS-type sugars and is largely glucose, about 85%.

The sugars, 2-O-methyl-6-deoxyhexose and 3-N-methyl-3-amino-3,6-dideoxyhexose, for which there were no available standards, have been identified by GC/mass spectrometry which was done at the NIH regional center at Washington University, St. Louis, Missouri. The unique fragments produced and detected by GC/mass spectrometry are shown in Figure 9.
DISCUSSION

Summary of Results

Similarities between parent (SU843) and mutant (8002).

(1) Both strains produce similar quantities of EPS.

(2) The LPS of both strains are similar quantitatively in sugar compositions. The O-antigens from both strains are also similar quantitatively in sugar composition, whereas both cores are different quantitatively in their sugar compositions.

Differences between parent (SU843) and mutant (8002).

(1) The impure EPS from both bacterial strains differ in the type of sugars present, however, the purified EPS from both strains is identical.

(2) The mutant (8002) produces significantly smaller quantities of CPS, which contains different sugars than the parental (SU843) CPS.

(3) The SmPS of the mutant (8002) contains sugars which are not present in the parent (SU843).

Interpretation of Results

Extracellular Polysaccharides (EPS):

The SUS43 and 8002 impure EPS are different in the types of sugars present, but after both are applied to a Sepharose 4B column their polysaccharide fractions, SU843 FPSI and 8002 EPSI, are identical to each
other. Since the impure 8002 EPS contained the typical LPS sugar, 3-N-methyl-3,6-dideoxyhexose, then this suggests that it may contain some LPS or LPS polysaccharide fragments.

**Capsular Polysaccharides (CPS)**

The SU843 CPS is different qualitatively and quantitatively from the 8002 CPS. When the CPS from both bacterial strains are applied to a Sepharose 4B column the polysaccharide fractions from SU843 CPS (SU843 CPS I and SU843 CPS II) are different in sugar composition from those of 8002 CPS (8002 CPS I, 8002 CPS II and 8002 CPS III). This suggests that the 8002 CPS may not be a typical parental CPS. There is also a significant reduction in the quantity of 8002 CPS produces, only about 2.5% of the amount of SU843 CPS. The sugars, 2-O-methyl-6-deoxyhexose, rhamnose and KDO, which are present in the SU843 LPS, 8002 LPS and 8002 CPS, are not present in the SU843 CPS. These data suggest that either (1) strain 8002 may not produce a capsule, or (2) if it does, then it may not be a typical parental CPS, and/or it may contain some LPS fragments.

**Lipopolysaccharides (LPS)**

The SU843 LPS and the 8002 LPS are very similar in sugar composition. The LPS from SU843 and 8002 contain smaller quantities of KDO in comparison to the LPS from several other Rhizobium strains, which suggests that either the number of KDO molecules per LPS molecules joining the lipid to the core is small or the O-antigen chain is long. The O-antigens from SU843 and 8002 are identical. The cores from SU843 and 8002 differ quantitatively in sugar composition. These data suggest that if the LPS plays a role in root-hair curling, it is the core region of the LPS and not the O-antigen which is important. More structural work needs to be done on the core regions to verify these differences.
The SmPS of 8002 contains typical LPS sugars, mannose, heptose and KDO, and this suggests that it may contain some LPS fragments. The SmPS of SU843 contains larger quantities of glucose than that of 8002 and does not contain the typical LPS sugars. This suggests that it may be a small molecular weight glucan.

Functions of the Rhizobium Surface Polysaccharides

The surface polysaccharides of Rhizobium, EPS, CPS, and LPS, are believed to participate in the symbiotic process. The EPS and LPS are thought to be responsible for different symbiotic steps ranging from attachment of the bacteria to the plant root hairs to the formation of nodules on the legumes by the bacteria. For example, it has been proposed that the capsular polysaccharide (CPS) of Rhizobium trifolii carries determinants which are antigenically cross-reactive with a surface component on clover seedling roots. These cross antigenic determinants, possibly LPS, CPS or EPS, are thought to be exposed on strains of Rhizobium which infect clover.

Function of the Extracellular Polysaccharides (EPS)

The EPS of SU843 and 8002 are comparatively more similar than either the CPS or LPS of these bacterial strains. Since attachment of the bacteria to the plant roots is common to both strains, it may be possible that the EPS might play a functional role in this symbiotic step. Recent studies with a Rhizobium leguminosarum mutant have also indicated that the EPS might play a functional role in attachment of the bacteria to the plant root hairs. The R. leguminosarum mutant which does not attach to pea roots does not synthesize EPS.
Function of Lipopolysaccharide (LPS)

The LPS of SU843 is very similar in sugar composition to that of 8002. However, SU843 produces five times as much LPS per viable bacteria as does the 8002 mutant. These data suggest that whatever function or functions the LPS may play in symbiosis, it is expected to be common to both bacterial strains but may be reduced in efficiency in the 8002 strain. Three possibilities exist:

(a) The LPS may play a functional role in attachment of the bacteria to the plant roots. If this happens then presumably the 8002 strain may not attach as effectively to the plant root hairs as the SU843. This supposedly ineffective attachment is expected to lead to further symbiotic steps occurring, but reduced in efficiency. Since the 8002 still attaches to the plant roots (B. Rolfe, unpublished data) but is defective at root hair curling and does not cause further symbiotic steps to occur, then it is very unlikely that the LPS is important for attachment. R. Lee has shown that the LPS from an R. leguminosarum mutant, which does not attach to the plant roots, has the same structure as the LPS of its parent (which carries out normal nodulation). This also supports the idea that the LPS is not important for bacterial attachment to the plant root hairs.

(b) The LPS may play a functional role in root-hair curling. Since the SU843 strain causes root hair curling, whereas the 8002 strain does not, it might be assumed that quite a significant difference in the respective LPS should be expected. Since the LPS from both bacterial strains are very similar, the possibility that LPS initiates root hair curling is also very small.

(c) The LPS may play a role in symbiotic steps beyond root hair curling. Since the 8002 strain is defective at root hair curling, but
has an LPS very similar to the parent SU843 strain, it is very likely that the LPS plays a role after root-hair curling. Examination of other mutants, defective in nodule formation but normal in root hair curling, is in progress to determine possible functional role or roles of the LPS in symbiosis. Preliminary evidence suggests that the LPS of one such mutant is very different from the LPS of the parent strain (Turnbull and Carlson, unpublished data).

Function of the Capsular Polysaccharide (CPS)

In comparing the surface polysaccharides of SU843 with those of 8002, the 8002 CPS shows the greatest alteration. Since the 8002 bacterial strain is defective at root-hair curling (HAC\(^-\)), the data suggest that the 8002 CPS is very likely to be important for this symbiotic step. Dazzo et al has proposed that in Rhizobium trifolii strains, the capsular polysaccharide could be responsible for initial attachment of high numbers of Rhizobia to the plant root hairs via cross-bridging lectins at an early recognition event.\(^3\)\(\textsuperscript{1}\) This would be followed by post-attachment recognition events requiring the host range-specific binding of lectins between localized sites on the root hairs and the LPS of the bacteria which triggers a cascade of invasive steps.\(^3\)\(\textsuperscript{1}\) Our data suggest that it is the CPS which may be important for triggering this cascade of invasive steps. Possibly then, at root hair curling in the Rhizobium trifolii SU843 bacterial strain all systems are positive and the bacteria progresses to further symbiotic steps. However, in the case of the 8002 strain, which is defective at root hair curling (HAC\(^-\)), the systems for this step are negative and consequently, further symbiotic steps are inhibited or blocked. Possibilities for the functional role of CPS include the following:
(a) In the absence of CPS, the plant may form cell wall structures in response to the 8002 strain, which restrict further invasion by the bacteria.

(b) In the absence of CPS, the plant may produce toxic or inhibitory substances in response to the 8002 bacterial strain, which also restrict further invasion of the bacteria, or,

(c) In the absence of CPS, the necessary recognition sites normally present in the bacterial strain, which are required to interface with those receptors on the plant roots, may either be absent or are significantly altered and therefore, further invasive steps cannot occur.
Figure 1. Sephadex 4B column chromatography of R. trifolii SU843 EPS. The solvent is EDTA/TEA buffer.
Figure 2. Sephadex 4B column chromatography of R. trifolii 8002 EPS. The solvent is EDTA/TEA buffer.
Figure 3. Sephadex 4B column chromatography of *R. trifolii* SU843 CPS. The solvent is EDTA/TEA buffer.
Figure 4. Sephadex 4B column chromatography of R. trifolii 8002 CPS. The solvent is EDTA/TEA buffer.
Figure 5. Sepharose 4B column chromatography of R. trifolii SU843 LPS. The solvent is EDTA/TEA buffer. Hexose assayed by using the anthrone test and KDO by the thiobarbituric acid method. Hexose, ---- and KDO, ----.
Figure 6. Sepharose 4B column chromatography of R. trifolii 8002 LPS. The solvent is EDTA/TEA buffer. Hexose assayed by using the anthrone test and KDO by the thiobarbituric acid method. Hexose, ---; KDO, ----.
Figure 7. Sephadex G-50 elution profile of the polysaccharides released from SU843 LPS by mild acid hydrolysis. Hexose and uronic acid.

SU843 O-antigen

SU843 Core

FRACTIONS
Figure 8. Sephadex G-50 elution profile of the polysaccharides released from 8002 LPS by mild acid hydrolysis. Hexose ---- and uronic acid ------.
Figure 9. The unique fragments resulting from GC/mass spectrometry of 2-O-methyl-6-deoxyhexose and 3-N-methyl-3-amino-3,6-dideoxyhexose.
Table I. Amounts per viable bacteria of the polysaccharide fractions from *R. trifolii* strains SU843 and 8002.¹

<table>
<thead>
<tr>
<th>Strain</th>
<th>EPS</th>
<th>CPS</th>
<th>LPS</th>
<th>SmPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SU843</td>
<td>25</td>
<td>5.9</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>8002</td>
<td>18</td>
<td>0.16</td>
<td>0.26</td>
<td>0.10</td>
</tr>
</tbody>
</table>

¹Amounts are given as mg per $10^{12}$ viable bacteria.
Table II. The relative sugar composition of the EPS of R. trifolii SU843 and 8002.\textsuperscript{1,2,5}

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SU843</td>
<td>2.4</td>
<td>13</td>
<td>0</td>
<td>56</td>
<td>0</td>
<td>27</td>
<td>1.1</td>
</tr>
<tr>
<td>8002</td>
<td>3.7</td>
<td>11</td>
<td>6.7</td>
<td>47</td>
<td>Tr\textsuperscript{4}</td>
<td>28</td>
<td>0.80</td>
</tr>
</tbody>
</table>

\textsuperscript{1}The above sugars of the table account for 77\% and 82\% by mass of SU843 and 8002 EPS respectively.

\textsuperscript{2}Protein accounts for 3.8\% and 3.9\% by mass of SU843 and 8002 EPS respectively.

\textsuperscript{3}Refers to 3-N-methyl-3-amino-3,6-dideoxyhexose. This sugar was quantitated using the response factor of glucose.

\textsuperscript{4}Tr refers to trace amounts present.

\textsuperscript{5}Compositions are given as relative weight percents.
Table III. The relative sugar composition of the Sepharose 4B column polysaccharides from SU843 EPS and 8002 EPS.

<table>
<thead>
<tr>
<th></th>
<th>Man.</th>
<th>Gal.</th>
<th>Glu.</th>
<th>Uronic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>SU843 EPSI</td>
<td>3.9</td>
<td>14</td>
<td>53</td>
<td>28</td>
</tr>
<tr>
<td>8002 EPSI</td>
<td>7.9</td>
<td>15</td>
<td>46</td>
<td>30</td>
</tr>
</tbody>
</table>

1Compositions are given as relative weight percents.
Table IV. The relative sugar composition of the CPS of R. trifolii SU843 and 8002.\(^1,2,5\)

<table>
<thead>
<tr>
<th></th>
<th>20M-3(^3)</th>
<th>Rha.</th>
<th>Man.</th>
<th>Gal.</th>
<th>3NM3A-4</th>
<th>Glu.</th>
<th>Hep.</th>
<th>Uronic Acid</th>
<th>KDO</th>
<th>Hexos-Amine</th>
</tr>
</thead>
<tbody>
<tr>
<td>SU843</td>
<td>0</td>
<td>0</td>
<td>13</td>
<td>19</td>
<td>0</td>
<td>43</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>1.2</td>
</tr>
<tr>
<td>8002</td>
<td>1.2</td>
<td>.43</td>
<td>3.7</td>
<td>5.2</td>
<td>3.1</td>
<td>39</td>
<td>8.3</td>
<td>55</td>
<td>0.57</td>
<td>2.9</td>
</tr>
</tbody>
</table>

\(^1\) The above sugars account for 42\% and 72\% by mass or weight of SU843 and 8002 CPS respectively.

\(^2\) Protein accounts for 2.9\% and 7.4\% by mass of SU843 and 8002 CPS respectively.

\(^3\) Refers to 2-0-methyl-6-deoxyhexose. This sugar was quantitated using the response factor of rhamnose.

\(^4\) Refers to 3-N-methyl-3-amino-3,6-dideoxyhexose. This sugar was quantitated using the response factor of glucose.

\(^5\) Compositions are given as relative weight percents.
Table V. The relative sugar composition of the Sephargse 4B column polysaccharides from SU843 CPS and 8002 CPS.\(^5\)

<table>
<thead>
<tr>
<th></th>
<th>2OM6DH(^1)</th>
<th>Rha.</th>
<th>Man.</th>
<th>Gal.</th>
<th>3NM3A-3,6DDH(^2)</th>
<th>Glu.</th>
<th>Hep.</th>
<th>Uronic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>SU843 CPSI</td>
<td>0</td>
<td>0</td>
<td>0.56</td>
<td>13</td>
<td>ND(^4)</td>
<td>58</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>SU843 CPSII</td>
<td>0</td>
<td>0</td>
<td>7.0</td>
<td>5.7</td>
<td>1.9</td>
<td>24</td>
<td>7.8</td>
<td>53</td>
</tr>
<tr>
<td>8002 CPSI</td>
<td>0</td>
<td>2.3</td>
<td>21</td>
<td>11</td>
<td>ND(^3)</td>
<td>47</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>8002 CPSII</td>
<td>1.9</td>
<td>0.83</td>
<td>3.2</td>
<td>6.2</td>
<td>Tr(^3)</td>
<td>26</td>
<td>3.5</td>
<td>58</td>
</tr>
<tr>
<td>8002 CPSIII</td>
<td>1.8</td>
<td>0.95</td>
<td>4.3</td>
<td>6.0</td>
<td>ND(^4)</td>
<td>23</td>
<td>8.4</td>
<td>55</td>
</tr>
</tbody>
</table>

\(^1\)2-O-methyl-6-deoxyhexose.

\(^2\)3-N-methyl-3-amino-3,6-dideoxyhexose.

\(^3\)Tr = trace

\(^4\)ND = not detectable due to low sensitivity in the response factor.

\(^5\)Compositions are given as relative weight percents.
Table VI. The relative sugar composition of the LPS fractions of SU843 and 8002. 1,2,3,6

<table>
<thead>
<tr>
<th></th>
<th>2OM-6DH</th>
<th>Rha.</th>
<th>Man.</th>
<th>Gal.</th>
<th>3NM3A-3,6DDH</th>
<th>Glu.</th>
<th>Hep.</th>
<th>KDO</th>
<th>Uronic Acid</th>
<th>Hexos-Amine</th>
</tr>
</thead>
<tbody>
<tr>
<td>SU843 LPS</td>
<td>3.4</td>
<td>1.8</td>
<td>3.6</td>
<td>10</td>
<td>10</td>
<td>15</td>
<td>22</td>
<td>0.34</td>
<td>30</td>
<td>3.2</td>
</tr>
<tr>
<td>8002 LPS</td>
<td>2.3</td>
<td>1.3</td>
<td>3.0</td>
<td>5.7</td>
<td>8.5</td>
<td>13</td>
<td>19</td>
<td>0.31</td>
<td>40</td>
<td>4.9</td>
</tr>
</tbody>
</table>

1 The above sugars in the table represent 80% and 69% by mass or weight of the SU843 KPS and the 8002 LPS respectively.

2 Protein accounts for approximately 4.1% and 1.5% of the SU843 LPS and the 8002 LPS respectively.

3 The lipid-A accounts for approximately 16% and 30% by mass of the SU843 LPS and the 8002 LPS respectively.

4 2-0-methyl-6-deoxyhexose.

5 3-N-methyl-3-amino-3,6-dideoxyhexose.

6 Compositions are given as relative weight percents.
Table VII. The relative sugar composition of the O-antigen and core polysaccharides isolated from the LPS of SU843 and 8002.

<table>
<thead>
<tr>
<th></th>
<th>2ON6DH(^1)</th>
<th>Rha.</th>
<th>Man.</th>
<th>Gal.</th>
<th>3NM3A-3,6DDH(^2)</th>
<th>Glu.</th>
<th>Hep.</th>
<th>Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>0-antigens</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SU843</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O-antigen</td>
<td>3.8</td>
<td>1.8</td>
<td>1.8</td>
<td>2.4</td>
<td>15</td>
<td>8.3</td>
<td>23</td>
<td>44</td>
</tr>
<tr>
<td>8002</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O-antigen</td>
<td>5.3</td>
<td>2.3</td>
<td>2.0</td>
<td>2.6</td>
<td>16</td>
<td>8.7</td>
<td>26</td>
<td>37</td>
</tr>
<tr>
<td><strong>Cores</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SU843 Core</td>
<td>0</td>
<td>0</td>
<td>8.3</td>
<td>7.8</td>
<td>0</td>
<td>2.7</td>
<td>2.4</td>
<td>79</td>
</tr>
<tr>
<td>8002 Core</td>
<td>0</td>
<td>0</td>
<td>6.4</td>
<td>7.1</td>
<td>0</td>
<td>4.9</td>
<td>3.0</td>
<td>78</td>
</tr>
</tbody>
</table>

\(^1\)2-O-methyl-6-deoxyhexose.

\(^2\)3-N-methyl-3-amino-3,6-dideoxyhexose.

\(^3\)Compositions are given as relative weight percents.
Table VII. The relative sugar composition of the SmPS fractions of *R. trifolii* SU843 and 8002.\(^1,2,3\)

<table>
<thead>
<tr>
<th></th>
<th>Man</th>
<th>Gal</th>
<th>Glu</th>
<th>Hep</th>
<th>Uronic Acid</th>
<th>KDO</th>
<th>Hexos-Amine</th>
</tr>
</thead>
<tbody>
<tr>
<td>SU843</td>
<td>0</td>
<td>2.7</td>
<td>84</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>8002</td>
<td>3.5</td>
<td>4.4</td>
<td>42</td>
<td>15</td>
<td>34</td>
<td>0.34</td>
<td>0.78</td>
</tr>
</tbody>
</table>

\(^1\)The above sugars in the table represent 42% and 70% by mass or weight of SU843 and 8002 SmPS respectively.

\(^2\)Protein accounts for 4.1% and 1.5% by mass of SU843 and 8002 SmPS respectively.

\(^3\)Compositions are given as relative weight percents.
REFERENCES


