6-8-2007

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Accepted version. *Journal of Biological Chemistry*, Vol. 282 (June 2007): 17101-17113. DOI.
This research was originally published in the *Journal of Biological Chemistry*. Wim D'Haeze, Christine Leoff , Glenn Freshour, K. Dale Noel and Russell W. Carlson. "Rhizobium etli CE3 bacteroid lipopolysaccharides are structurally similar but not identical to those produced by cultured CE3 bacteria." *Journal of Biological Chemistry*. 2007. Vol 282 :17101-17113.

Rhizobium etli CE3 Bacteroid Lipopolysaccharides Are Structurally Similar but Not Identical to Those Produced by Cultured CE3 Bacteria*

By Wim D'Haeze[1], Christine Leoff, Glenn Freshour, K. Dale Noel, and Russell W. Carlson[2]

***Rhizobium etli* CE3 bacteroids were isolated from *Phaseolus vulgaris* root nodules. The lipopolysaccharide (LPS) from the bacteroids was purified and compared with the LPS from laboratory-cultured *R. etli* CE3and from cultures grown in the presence of anthocyanin. Comparisons were made of the O-chain polysaccharide, the core oligosaccharide, and the lipid A. Although LPS from CE3 bacteria and bacteroids are structurally similar, it was found that bacteroid LPS had specific modifications to both the O-chain polysaccharide and lipid A portions of their LPS. Cultures grown with anthocyanin contained modifications only to the O-chain polysaccharide. The changes to the O-chain polysaccharide consisted of the addition of a single methyl group to the 2-position of a fucosyl residue in one of the five O-chain trisaccharide repeat units.This same change occurred for bacteria grown in the presence of anthocyanin. This methylation change correlated with the inability of bacteroid LPS and LPS from anthocyanin-containing cultures to bind the monoclonal antibody JIM28. The coreoligosaccharide region of bacteroid LPS and from anthocyanin grown cultures was identical to that of LPS from normal laboratory-cultured CE3. The lipid A from bacteroids consisted exclusively of a tetraacylated species compared with the presence of both tetra-and pentaacylated lipid A from laboratory cultures. Growth in the presence of anthocyanin did not affect the lipid A structure. Purified bacteroids that could resume growth were also found to be more sensitive to the cationic peptides, poly-L-lysine, polymyxin-B, and melittin.**

Root nodule development is orchestrated by a symbiotic molecular dialogue between Gram-negative *Rhizobium* bacteria (*e.g. Azorhizobium* sp., *Bradyrhizobium* sp., *Rhizobium* sp.,*Sinorhizobium* sp.) and specific legume host plants. Nodules are newly formed organs consisting of plant cells occupied with bacteroids that provide the host plant with fixed nitrogen. In the best studied symbiotic interactions, bacteria enter the roots via susceptible curled root hairs, and intracellular infection threads guide the bacteria toward *de novo* nodule primordia, where internalization into plant cells takes place. Initiation of nodule development and invasion

require the production of bacterial signal molecules, including fatty acylated chitin oligosaccharides known as Nod factors (1), and structurally complex surface polysaccharides $(SPS)^3$ (2, 3).

The outer surface of rhizobia typically consists of SPS that include extracellular polysaccharides (EPS) that are released into the media, capsular polysaccharides that are tightly associated with the bacterial surface, and lipopolysaccharides (LPS) that are anchored in the outer membrane (4). LPS are composed of lipid A, a core oligosaccharide, and an O-antigen polysaccharide. Accumulating data demonstrate the important role that rhizobial SPS play in invasion and nodule development and their involvement in the initiation of infection and invasion, suppression of plant defense, bacterial release from infection threads, bacteroid development and senescence, induction of plant gene expression, and protection against antimicrobial compounds (2, 3).

Various observations suggest that proper LPS synthesis is required for invasion and nodule development in various symbiotic interactions, including the interaction between *Rhizobium etli* and *Phaseolus vulgaris* (2, 4). An *R. etli* mutant that lacks the O-chain polysaccharide portion of its LPS elicited the formation of infection threads on *P. vulgaris*; however, the bacteria ceased to develop within the root hair that formed thick walls(5,6).The formation of nodule primordial was normal, but no bacteria were released from infection threads and internalized into plant cells (6). Occasionally, some bacteria were present in intercellular spaces. It was furthermore demonstrated that not only the presence of the O-chain polysaccharide on the LPS but also the abundance of O-chain polysaccharide was important for nodulation. For example, mutant strain *R. etli* CE166 produced, based on PAGE analysis of the LPS, only 40%LPS containing the O-chain polysaccharide compared with the parent strain, and the symbiotic phenotype of this mutant was the same as that observed for a mutant that entirely lacks the O-chain polysaccharide (7, 8).

A striking feature of LPS synthesis is that it is influenced by a variety of environmental factors (9). The LPS contained in bacteria isolated from the host (bean) nodules was diminished in its ability to bind monoclonal antibodies JIM28 and JIM29. In addition, the ability to bind these mAbs was also affected by pH,O_2, or phosphate concentrations and temperature. Mutants that produced O-chain polysaccharide-containing LPS that do not change in their ability to bind JIM28 or JIM29 were impaired in their nodulation frequency and development(9).In addition, it was shown that *R.etli* CE3, grown in the presence of *P. vulgaris* root or seed exudates, produced modified LPS that was no longer recognized by a particular monoclonal antibody (mAb), JIM28, specific for the O-chain polysaccharide of LPS from laboratory-cultured *R. etli* CE3 (10). Major

compositional differences between LPS produced by CE3 cultures grown at pH 7.2 and that of pH 4.8 cultures included replacement of 2,3,4-tri-*O*-methylfucose by 2,3-di-*O*-methylfucose and an increase of 2-*O*-methylfucose content (11). These results showed the importance of determining the molecular/genetic basis for these subtle structural changes to *R. etli* LPS.

Here we describe the preparation of LPS from *R. etli* CE3 bacteroids purified from the host root nodules, and we compare its structure to that produced by *R. etli* CE3 grown under normal laboratory conditions (Fig.1).Although LPS from CE3 bacteria and bacteroids were structurally similar, we observed that bacteroid LPS was antigenically different from that of bacteria and showed a doubling in 2-*O*-methylfucose within the O-chain polysaccharide. Mass spectrometry analyses also demonstrated that the lipid A from bacteroid LPS lacked a *β*-hydroxymyristic acid acyl residue. Our results also indicated that the *R. etli* CE3 bacteroid population that could resume growth was significantly more sensitive to cationic peptides than *R. etli* laboratory-cultured bacteria.

Experimental Procedures

Plant Growth and Nodule Preparation—For each aeroponic growth chamber (AGC), 180 *P. vulgaris* seeds (black turtle; Sacajawea Organic Foods) were surface-sterilized in 50 ml of95%ethanolfor4minwhileshakingthesolutionmanually.Theethanol was discarded, and the seeds were washed two times with sterilized deionized water. The seeds were then rinsed with 50 ml of 5% sodium hypochloride (Acros Organics) for 4min followed by several washes with sterilized deionized water (2 liter total volume). Seeds were transferred to plastic pots containing a 0.8% agarose layer (0.8 g per 100 ml of tap water) for germination (five seeds per pot to allow enough space for the seeds to germinate) and incubated in the dark at 30 ℃ for 4 days.

The AGC consisted of a polypropylene barrel that was not light-transparent and a lid with 150 holes through which plants could grow. A humidifier (505 Defensor from Axair AG, Pfäffikon, Switzerland) was placed on the bottom of the barrel. A tap was present in the barrel, which allowed changing of the nutrient solution in an efficient manner, and the lid-barrel contact was tight so that no nutrient solution was lost during plant growth. The entire ACG, including the humidifier, was cleaned with 98% ethanol prior to use and rinsed with 10 liters of sterilized nitrogen-free nutrient solution (12). Subsequently, the *P. vulgaris* seedlings were transferred to the AGC (one seedling per hole) and supported by some water-soaked horticultural rock wool. The latter also nicely sealed the space between the seedling and the lid material without damaging the hypocotyl. Remaining seed coats were removed manually prior to the transfer of the seedlings to the AGC.

R. etli CE3 was grown as described (8), and the pellets of two300-ml overnight late exponential phase cultures were extensively washed with nutrient solution and added to the AGC after seedlings were transferred. The nutrient solution, including the CE3 inoculum, was refreshed every other day for 4weeks. The AGCs were placed in an acclimatized plant growth room with a photoperiod of 14/10, a relative humidity of 60%, and a day and night temperature of 23 and 18 ℃, respectively. Mature nodules were manual ly harvested 4 weeks after seedlings were transferred to the AGC. The nodules were collected in 50-ml tubes and immediately frozen until bacteroids needed to be prepared for LPS purification.

R. etli CE3 Bacteroid Isolation—A slightly modified stepwise sucrose gradient-based ultracentrifugation approach as described by Ching *et al.* (13) was used for bacteroid isolation.Briefly,5gof frozen nodules were extensively ground using a mortar and pestle until a homogeneous paste was obtained. Ten milliliters of filter-sterilized grinding buffer (13) were added, and the mixture was manually stirred with a glass bar for a few minutes. Six polyallomer ultracentrifugation tubes with acapacityof12.2ml (Beckman Coulter) were prepared by adding the stepwise sucrose gradient (*i.e.* from bottom to top: 2.076 ml of 57% sucrose, 2.699 ml of 52% sucrose, 2.699 ml of 50% sucrose, and 2.076 ml of 45% sucrose). Care was taken to avoid mixing of different sucrose layers. The remaining space in the tube was filled with ~1.6 ml of the crushed nodule mixture in grinding buffer. The tubes were equilibrated, placed in an SW40Ti rotor, and ultracentrifuged at 100,000 X *g* for4hat 10 ℃ (Beckman Coulter). When the ultracentrifugation run was completed, tubes were carefully removed from the rotor, and the five bands were immediately transferred to a separate tube using a Pasteur pipette. The bacteroids (band 4) were washed twice (7,000 rpm for 20 min at 4 ℃) with phosphate buffer, pH 7.2, and after washing, the pellet was resuspended in a final volume of 200 *μ*l of phosphate buffer, collected in a 50-ml tube, and stored at -20 ℃. When a total volume of 50 ml was obtained, the LPS was prepared using the hot phenol/water extraction as described below.

Dot-blot Immunoblotting—Immunodot blot assays were prepared (14). Briefly, a fraction of an overnight CE3 culture or samples of the respective bands were washed with phosphate buffer, pH 7.2, and diluted to an A_{600} equal to 1. One microliter of the initial concentration of each sample and of 10-, 100-, and1000-fold dilutions were spotted on a nitrocellulose membrane (Sigma) and air-dried for 1h.The membrane was transferred to a small glass dish, which was put on a rocker set at low speed, and washed three times with TBS solution (50 mM Tris/HCl, 200 mM NaCl, pH 7.4) for 15 min. Blocking was performed by adding 20 ml of 2% (w/v) bovine serum albumin in TBS and incubation for 30 min. The membrane was incubated overnight after addition of nitrogenase antibodies (1/5000 dilution in 2%bovine serum albumin) (15). The membrane was

then washed with TBS solution for 2 h during which the solution was refreshed at least five times. The membrane was incubated in the presence of alkaline phosphatase anti-rat IgG (Sigma;1/5000 dilution in 2% bovine serum albumin) and subsequently washed for 30 min in TBS solution during which the solution was refreshed at least five times. The membrane was developed in alkaline phosphatase substrate solution, containing 9 ml of Tris/HCl buffer (100 mM Tris/HCl, pH 9.6), 1 ml of nitro blue tetrazolium (NBT) solution (1 mg/ml NBT in Tris/HCl buffer plus 2% dimethyl sulfoxide), 100 μl of 5-bromo-4-chloro-indolylphosphate (5mg/ml in dimethylformamide),and 40 μl of 1M $MgCl_2$.

Cationic Peptide Sensitivity Assay—Overnight bacterial cultures of CE3 and CE338, the latter is affected in the synthesis ofEPS (16), and freshly isolated bacteroids were extensively washed with phosphate buffer, pH 7.2, and diluted to an A_{600} equal to 1. The cationic peptides tested were melittin, polymyxin B, and poly-L-lysine (Sigma). For melittin, 1 μl of a 20 fg/ml stock solution was added to 800 μl of a solution of bacteria or bacteroids and incubated for 30 min at room temperature; for polymyxin B, 3 μl of a 20 fg/ml stock solution was added to 10 μl of bacteria or bacteroids and incubated for1hat room temperature; and for poly-L-lysine, 3 flofa50 fg/ml stock solution was added to 10 μl of bacteria or bacteroids and incubated for1hat room temperature. The viability was determined as described previously (17). This assay was repeated 10times for each bacterial or bacteroid preparation with each cat-ionic peptide, and a statistical analysis was performed using the Student's *t* test. Averages were not significantly different when $p > 0.05$.

MicroscopyTechniques—An initial microscopic examination of the various bands obtained after ultracentrifugation was done using a classical Gram staining. Material from bands 1through 5 and cultured CE3 bacteria were stained with crystal violet followed by a safranin staining (Sigma) and there after immediately examined using a light microscope (Olympus, Tokyo, Japan).

Transmission electron microscopy was employed to observe cultured CE3 bacteria (negative control), purified CE3 bacteroids (band 4), and sections through mature nodules (positive control). For the latter, thenodules were treated and embedded for transmission electron microscopy as described previously(18).The embedding of CE3 bacteria and bacteroids was done as follows (all procedures were carried out at 4 ℃ under rotation).Samples were extensively washed with 0.1 M cacodylate buffer(Sigma)and fixed bya gradual fixation approach. The pellets were consecutively resuspended in 0.5% formaldehyde, 0.5% glutaraldehydein 0.1 Mcacodylate buffer, 1.0% formaldehyde, 1.0% glutaraldehydein0.1Mcacodylatebuffer, 1.5% formaldehyde, 1.5%glutaraldehyde in 0.1 M cacodylate buffer, 2.0% formaldehyde,

2.0%glutaraldehyde in 0.1 M cacodylate buffer, and finally in 2.5%formaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Each time, the samples were incubated for 20 min. Then the pellets were washed three times with 0.1 M cacodylate buffer followed by a dehydration series, including2h in 30% ethanol,2h in 50% ethanol, overnight in 70% ethanol,2h in 95% ethanol, and overnight in 95% ethanol. The samples were then imbedded in LR White Hard Grade by resuspending the pellet overnight in ethanol/LR White (1/1 v/v), a step that was repeated two times. Finally, the pellets were resuspended in pure LR White and rotated overnight, which was repeated at least five times. The samples were transferred to capsules and incubated at 65 ℃ for 48 h to allow polymerization.

Sections were made using an MT 6000-XL ultramicrotome (RMC, Inc., Tucson, AZ). Routine control sections were 1 fm thick and were stained with toluidine blue. Sections for transmission electron microscopy were 90 nm thick and collected on gilded copper slot grids (Ted Pella, Inc., Redding, CA) that were placed on Formvar bridges to dry (19). Sections were post-stained for 2 min with 4% (w/v) aqueous uranyl acetate and for 0.5 min with lead citrate (20). Sections were examined at 80 kV with a Zeiss 902A electron microscope.

LPS Isolation—Crude LPS was obtained from the bacteria and bacteroids using the hot phenol/water extraction procedure (21), which was modified by Carlson e*t* al*.* (22). The water phase containing the LPS was treated with RNase, DNase, and proteinase K, dialyzed, and then lyophilized (22). The LPS extracted into the phenol phase was treated as described by Carrion *et al.* (23).The LPS was purified from these crude preparations with affinity chromatography using polymyxin B-Sepharose (Pierce) (24, 25). Briefly, the crude LPS was dissolved in 50 mM NH_4CO_3 and applied to the column. The column was then washed with 50 mM NH_4CO_3, followed by a solution of300 mM triethylamine adjusted to pH 6.4 with acetic acid, and then a solution of 0.1 MNH_4CO_3 in 2 Mureato remove any non-LPS material from the column. The LPS was finally removed using a solution of 1% deoxycholate (DOC) in 0.1 M NH_4CO_3. The LPS was extensively dialyzed against a solution of 50 mM Tris base with 10% ethanol, then against deionized water, and lyophilized.

For cultures grown in the presence anthocyanin, crude anthocyanin preparations were obtained by acid extraction, as described previously by Noel *et al.* (10), from *P. vulgaris* seed (cv. Midnight Black Turtle Soup supplied by Idaho Seed Bean, Twin Falls, ID). *R. etli* CE3 was grown in medium (8) to which the crude anthocyanin extract had been added as described (10). The LPS was isolated by hot phenol/water extraction as described above and purified by Sepharose 4B chromatography after dialysis and treatment with nucleases and proteinase K (21, 26).

Electrophoresis and Immunoblotting—The LPS preparations were analyzed using

DOC-PAGE, and the polyacrylamide gels were stained using the Alcian blue-silver staining procedure as described previously (27). Immunoblotting was also performed according to the method described by Reuhs *et al.* (27). Briefly, LPS-containing gels were soaked in transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol) and electrophoretically transferred to a nitrocellulose membrane using a Bio-Rad Transblot SD semi-dry transfer cell set at a current of 20V for 20min. The membrane was equilibrated in TBS(0.2 M NaCl, 20 mM Tris, pH 7.4) for 5 min, then blocked using 5% nonfat dry milk (Bio-Rad)in TBS, and then overlaid with a 1/100 dilution of one of the primary mAbs (JIM26, JIM27, JIM28, or JIM29) in blocking solution. The membrane was then washed (five times for 5 min in TBS) and incubated with alkaline phosphatase-conjugated secondary antibody, at 1/1000 dilution of the antibody. Finally, the membrane was equilibrated in substrate buffer (0.1 M Tris, 0.1 MNaCl, 5mMMgCl_2, pH9.5)and developed for5minusingadevelopingsolutionof20mlofsubstratebuffer,128flofNBT stock solution (50 mg/ml NBT in 70% dimethylformamide),and 66 μl of BCIP stock solution (50 mg/ml BCIP in 100% *N*,*N* dimethylformamide). Once the bands were visible, the reaction was stopped by washing with deionized water.

LPS Analysis—Compositions were determined by the preparation and gas chromatography-mass spectrometry (GC-MS) analysis of trimethysilyl methyl glycosides (28). This procedure was also used to determine the fatty acid composition of the LPS preparations (29). Glycosyl composition of the LPS preparations was also determined by the preparation and GC-MS analysis of alditol acetates (28).

The location of methyl ether groups and the link age positions of the various glycosyl residues were determined by the preparation and GC-MS analysis of partially methylated alditol acetates (PMAAs) as described by Ciucanu and Kerek (30). Methylation was performed using tri-deuteriomethyliodide so that analysis of the partially methylated alditol acetates by GC-MS would reveal the location of the naturally occurring methyl groups on the LPS. The per-trideuteromethylated polysaccharides were hydrolyzed using 2 M trifluoroacetic acid at 121 ℃ for 2 h (29). The resulting partially (trideu tero) methylated glycosyl residues were reduced using sodium borodeuteride and acetylated at 80 ℃ wit h a 1/1 mixture of acetic anhydride: pyridine (29). The partially (trideutero) methylated alditol acetates were then analyzed using GC-MS.

Analysis of the core oligosaccharides was determined by subjecting the LPS preparations to 1% acetic acid for1hat100 ℃, removing the lipid A by centrifugation, and analysis of the carbohydrates by HPAEC using a Carbo PacPA-1 (Dionex) with pulsed amperometric detection as described previously (24).Separation was achieved using a gradient of 3–90% sodium

acetate (1 M) in100mM NaOH at a flow rate of 1 ml/min over 50 min.

The lipid A was obtained from the LPS by mild acid hydrolysisin 1%SDS in 20 mM sodium acetate, pH4.5, as described by Caroff *et al.* (31). After hydrolysis, the SDS was removed by washing the dried hydrolysis product residue with a solution of2/1deionizedH_2O:acidifiedethanol (100 flof4 M HCl in20 ml of ethanol). The residue was collected by centrifugation and washed again with 95% ethanol. The ethanol washing steps were repeated several times, and the final residue was suspended in deionized water and lyophilized to givea white, fluffy lipid A preparation. MALDI-TOF MS was performed in the negative ion reflectron mode with a 337 nm nitrogen laser, operating at a 20-kV extraction voltage, and with time-delayed extraction. Approximately 2 μl of a 1 mg/ml lipid A solution inchloroform: methanol (3/1, v/v) was mixed with 1 μl of trihydroxyacetophenone matrix solution (93.5 mg of trihydroxyacetophenone/1 ml of methanol) and applied to the probe for mass analysis. Spectra were calibrated externally using *Escherichia coli* lipid A (Sigma).

Results

Efficient Production of Relatively High Numbers of R.etli CE3 induced P. vulgaris Root Nodules—Thus far, the conventional system to cultivate *P. vulgaris* (common bean) plants for nodulation experiments is with Leonard jars, in which the roots are grown in pots filled with vermiculite. This system works well for the symbiotic interaction between *P. vulgaris* and *R. etli* CE3 but is rather labor-intensive if one needs to scale-up plant growth, which was necessary in our study because of the fact that sufficient amounts of pure LPS are required to perform proper structural analyses and additional biological experiments. Therefore, we engineered an AGC in which 150 plants can be grown at once under semi-sterile conditions (Fig. 2). To demonstrate that nodulation under the AGC conditions is at least as efficient as in the conventional Leonard jars, we determined the average number of nodules per root system and also investigated the healthiness of the plants. Both systems produced vigorous green plants, and the average nodule numbers per root system were not significantly different, being 490 or 420 nodules per root system when plants were grown in the AGC or the Leonard jars, respectively (data not shown). No significant differences were observed among the average wet weight of nodules per root system, roots, or stem and leaves of plants grown in the AGC or in Leonard jars (data not shown). Two AGCs were used continuously and simultaneously, and nodules were harvested manually, and bacteroids were purified (see below) continuously for approximately 1 year in order to obtain a quantity of purified bacteroid LPS that was sufficient to perform the structural analyses described herein.

Purification of R. etli CE3 Bacteroids—Bacteroids were purified using an ultracentrifugation-based stepwise sucrose gradient. Characteristically, we obtained five bands of biological material, numbered 1 (top) to 5 (bottom) (Fig. 3*A*). To identify which band contained the CE3 bacteroids, the material obtained in each band was stained with crystal violet and safranin and observed using light microscopy. Only band 4 appeared to be pure and consisted solely of elongated structures with a shape that was similar to that of CE3 bacteroids (32, 33). All other bands were impure and mainly contained plant cell debris (bands 1 and 2) and clusters or single round-shaped bacteria-like structures (bands 3 and 5). In addition, dot blots using different concentrations of material derived from each of the bands were performed using anti-nitrogenase antibodies, and it was observed that the nitrogenase activity was predominantly present in the material found in band 4, a result that further supports that this band contained the CE3 bacteroids. A transmission electron microscopy analysis of band 4 material demonstrated that it consisted of elongated organisms primarily occupied with low electron-dense material(Fig.3*C*), reportedly identified as polyhydroxybutyrate typically found in *Rhizobium* bacteroids (32).ThisimagewassimilartothatobservedforCE3bacteroids present in the central nitrogen-fixing tissue of mature P. vulgaris nodules (Fig. 3*D*) but distinct from cultured bacteria that were smaller and more spherical in shape, and which did not contain the polyhydroxybutyrate-rich material (Fig. 3*C*, *inset*). Taken together, these observations demonstrate that band 4 consists of isolated CE3 bacteroids.

R.etli CE3 Bacteroid LPS PAGE Pattern—LPS from CE3 bacteria and bacteroids were purified using the hot phenol/water method and initially analyzed by DOC-PAGE. Two major clusters of LPS were observed as follows: the low molecular weight LPS II that does not contain O-antigen polysaccharide, and the high molecular weight LPS I that contains the O-antigen polysaccharide (26, 34). No differences in the LPS II banding pattern were observed (data not shown), whereas at least one band present in LPS I prepared from cultured CE3 bacteria was not present in the LPS I of CE3 bacteroids (Fig. 4, *top panel*).

R. etli CE3 Bacteroid LPS I Exhibit a Distinct Antigenicity— We investigated the binding of four mAbs (JIM26, JIM27, JIM28, and JIM29 (9)) to the LPS purified from *R. etli* CE3 bacteria, CE3 bacteroids, and CE3 grown in the presence of anthocyanin. A previous report demonstrated that LPS II does not react with any of these four antibodies (9), which was confirmed by our observations. Consequently, we focused only on the binding to LPS I (Fig. 4). All four antibodies reacted with LPS I from laboratory-cultured CE3. The LPS I from all preparations bound to mAbs JIM26 and JIM27, whereas the CE3 bacteroid LPS I did not bind JIM28 and showed reduced binding to both JIM29 and JIM26 compared with LPS I from CE3bacteria. Similar to bacteroid

LPS, the LPS I from CE3 cultured in the presence of anthocyanin showed reduced binding toJIM26 and JIM29 and did not bind JIM28. These observations strongly suggest that the JIM28 epitope present in LPS I ofculturedCE3bacteria is absent in CE3 bacteroid LPS I,and that this structural change also occurs when CE3 is cultured in the presence of host-derived anthocyanin. The reduced binding of bacteroid LPS I to JIM26 and JIM29 may also reflect structural changes to these epitopes during symbiosis.

The O-antigen Polysaccharide of R. etli CE3 Bacteroid LPSContains an Additional Methyl Group at the 2-Position in One of the Five Repeating Oligosaccharide Unit Fucosyl Residues—The glycosyl compositions of the total carbohydrates released from the LPS preparations by mild acid hydrolysis are shown in Table 1. These results show that the bacteroid LPS preparations and the LPS preparation from cultures grown in the presence of anthocyanin are increased in the level of 2-*O*-methylfucose in comparison to the LPS from cultures grown under standard laboratory conditions. There are also other minor quantitative differences between these LPS preparations; however, the increase in 2-*O*-methylfucose seems to be the consistent change that is observed in both the bacteroid LPS extracted into the water or into the phenol layers. This increase in 2-*O*-methylfucose is consistent with results previously reported for LPS from cultures grown at low pH, as well as for LPS from cultures grown in the presence of anthocyanin (35). The exact level of the increase in fucosyl 2-*O*-methylation was determined by computing the percentage of the total fucosyl residues that are 2-*O*-methylated for each LPS preparation (Fig. 5 and Table 2). The bacteroid LPS preparations and the LPS from cultures grown in anthocyanin have 34 and 31% of their total fucosyl residues as 2-*O*-methylfucose, respectively, although for LPS preparations from laboratory cultures, this percentage is 13–16%. Because the O-chain polysaccharide contains six fucosyl residues available for 2-*O*-methylation due to each of the 3,4-linked fucosyl residues in the five oligosaccharide repeat units and a sixth 3-linked fucosyl residue in the “outer core” region (Fig. 1), these percentages support the conclusion that one of six fucosyl residues (*i.e.* 16.7%) is 2-*O*-methylated in LPS from laboratory cultures, whereas two of six residues (*i.e.* 33.3%) are 2-*O*-methylated in the LPS from bacteroids and from cultures grown in the presence of anthocyanin.

To determine whether this additional fucosyl methylation occurred on the single 3-linked fucosyl residue in the outercore region of the O-chain polysaccharide or on one of the five repeating unit 3,4-linked fucosyl residues, PMAAs were prepared using tri-deuteromethyl iodide for methylation and analyzed by GC-MS. This enabled us to distinguish between and quantify the fucosyl residues that contained anendogenous 2-*O*-methyl group from those that were not methylated in that position, which would contain a 2-*O*trideuteromethyl group. Quantification was

accomplished using the relative levels of *m/z* 118 to *m/z* 121 ions for each of the PMAAs; these ions are because of fragments that contain an endogenous 2-*O*-methyl group or the chemically introduced 2-*O*-trideuteromethyl group, respectively. The results are shown in Table 3 and reveal that the major increase in2-*O*-methylation in the bacteroid LPS clearly occurs on a repeating unit 3,4-linked fucosyl residue. These results (glycosyl composition and methylation results) together with the composition results support the conclusion that during bacteroid formation there is an increase in 2-*O*-methylation from one to two of the 3,4-linked fucosyl residues in one ofthe five O-chain polysaccharide repeating units.

The R. etli CE3 Bacteroid LPS Contains No Observable Structural Changes to the Core Oligosaccharide—The core oligosaccharides from the different LPS preparations were compared by HPAEC of the carbohydrate components released by mild acid hydrolysis. The HPAEC profiles (Fig. 6)were identical to one another and identical to that reported previously for *R. etli* CE3 (36), showing that the core components produced by mild acid hydrolysis include the $GalA_2Kdo_1$ trisaccharide, the $Gal_1Man_1GalA_1Kdo_1$ tetrasaccharide, and its anhydro derivatives, as well as monomeric Kdo and GalA (Fig. 6). The identical profiles show that all of the LPS preparations have the same core structure (Fig. 1)reported previously for *R. etli* CE3 (36).

The Lipid A from R. etli CE3 Bacteroids Lacks a β*-Hydroxymyristic Acid Residue*—The lipid A from the various LPS preparations was released by mild acid hydrolysis at pH 4.5 in the presence of SDS and analyzed for fatty acid composition and by MALDI-TOF MS. All of the lipid A preparations contain the expected fatty acids (37), namely *β*-hydroxymyristate, *β*-hydroxypalmitate, *β*-hydroxystearate, 27-hydroxyoctacosanoate, and smaller amounts of *β*-hydroxypentadecanoate. The MALDI-TOF MS spectra for the lipid A from bacterial and bacteroidal LPS are shown in Fig. 7. Previous reports (29, 38, 39) have shown that the lipid Apreparation from *R. etli* CE3 contains several structures because of small differences in the fatty acylation patterns as well as to the fact that the proximal glycosyl residue can exist as glucosamine, 2-aminogluconic acid, or 2-aminogluconolactone. The lipid A preparation from *R. etli* CE3 bacteria (Fig. 7*A*) shows masses that are consistent with the reported structures (also shown in Fig. 7*A*). These structures consist of penta-and tetraacylated forms of lipid A, *e.g. m/z* = 2002.5 and 1758.7, respectively. However, the bacteroid lipid A preparation (either from the water or phenol phases) contains only the tetraacylated lipid A, *i.e.* it lacks a *β*-hydroxymyristate moiety. The tetraacylated lipid A could lack a *β*-hydroxymyristoyl residue at the 2-, 3-, or 3'-positions of the lipid A glycosyl backbone (Fig. 1), and at this time it is not known which position lacks this residue. The lipid A preparation from *R. etli* CE3 grown in the presence of anthocyanin was also analyzed by MALDI-TOF MS, and the results showed that it contained the

same profile of structures as that found for the lipid A from *R. etli* CE3 bacteria cultured under standard laboratory conditions (Fig. 7*C*). Thus, although growth in anthocyanin results in the same apparent changes to the O-chain polysaccharide as found in the bacteroid LPS, it does not result in the changes that occur in bacteroid lipid A.

R. etli CE3 Bacteroids Are More Sensitive for Cationic Peptides than Cultured CE3 Bacteria—It has been demonstrated that surface polysaccharides protect bacteria against harsh environmental conditions (17, 40). Particularly, a *Sinorhizobium meliloti* mutant affected in the synthesis of LPS was shown to be more sensitive to the exposure of cationic peptides than the parental strain (41). Because *R. etli* CE3 bacteroids exhibit structurally different LPS, we tested the sensitivity ofCE3 bacteria and bacteroids for exposure to melittin, poly-Llysine, and polymyxin B. CE3 bacteria and bacteroids were incubated in 20 ng/ml melittin, 6 μg/ml polymyxin B, and 15 μg/ml poly-L-lysine for 30 min and 1 h and 1 h, respectively, followed by the determination of the viability (17). *R. etli* CE3 bacteroids were significantly more sensitive to all three of the cationic peptides(Fig. 8).This effect did not appear to be due to qualitative or quantitative changes in the EPS because CE338 has the same degree of resistance to cationic peptides as laboratory-cultured CE3 (Fig. 8).

Discussion

In this study, we analyzed for the first time the structure of LPS isolated from purified *R. etli* CE3 bacteroids and have shown that CE3 bacteroids isolated from bean nodules are altered in a number of ways from laboratory-cultured bacteria. First, although the LPS of CE3 bacteria and CE3 bacteroids are structurally similar, the LPS from bacteroids (i) contains a singleadditionalmethylgroupatO-2onafucosylresidueinoneofthe five O-chain repeating units (Fig. 9), and (ii) its lipid A lacks a β-hydroxymyristic acid moiety. Second, the results indicate that bacteroids that can resume growth are significantly more sensitive to cationic peptides than are laboratory-cultured bacteria.

Prior reports have noted changes in the LPS during symbiosis by examining rhizobia obtained from their respective host root nodules (which consist of a mixture of bacteria and bacteroids) or during growth of rhizobial cultures under conditions that mimic those within the host root nodule (9, 11, 42, 43). These changes have included differences in the DOC-PAGE banding pattern of the LPS, the production of a secondary rhamnan O-chain in the case of *Rhizobium* sp. NGR234 (44, 45), the glycosyl composition and *O*-acetylation changes in *Rhizobium leguminosarum* LPS O-chain polysaccharide (43), and an increase in long chain fatty acylation of the lipid A of *R. leguminosarum* LPS (43). The changes reported for *R.*

leguminosarum and *Rhizobium* sp. NGR234 are more dramatic than the subtle changes we observe in the bacteroids of *R. etli* CE3. As mentioned previously, it has been reported that there is an increase in 2-*O*-methylfucosyl residues on the *R. etli* CE3 LPS O-chain during growth at low pH, from bacteria isolated from bean nodules and from bacteria grown in the presence of anthocyanin (9, 11). In this study, we found that the O-chain from isolated *R. etli* CE3 bacteroids contains exactly one additional methyl group located on one of the five possible O-chain oligosaccharide repeating unit fucosyl residues. Because this methylation results in the loss of binding to JIM28 mAb, we believe that it occurs on a specific repeating unit fucosyl residue and hypothesize that this residue is in the repeating unit that is adjacent to the capping fucosyl residue as shown in Fig. 9. This hypothesis is based on the fact that loss of the capping fucosyl residue also results in the loss of binding to JIM28 (as well as the loss of binding to JIM27 and JIM29) (46); therefore, it is likely that the JIM28 epitope involves both the capping fucosyl residue and the 2-hydroxyl group of the 3,4-linked fucose in the repeating unit that is in close proximity, namely in the adjacent repeat unit. This fucosyl 2-hydroxyl group is apparently not required for the binding of JIM26, JIM27, or possibly JIM29 m Abs because these bind to the bacteroid LPS. The symbiotic function of 2-*O*-methylation of this fucosyl residue is unknown; however, it has been reported that a mutant, CE395*α*395, which is completely defective in all fucosyl 2-*O*-methylation (35), shows delayed nodulation, and nodules that form are more widely dispersed on the lateral root than is the case for the CE3 parent. The mutation is in a gene in the O-chain polysaccharide synthesis gene cluster, *lpsα*, that encodes a putative methyltransferase (35). Another mutant in this same gene also lacks2-*O*-methylfucosyl residues in its LPS isolated from laboratory-grown cultures as well as from bacteria isolated from host root nodules.[4] It is not clear how this single putative methyltransferase can methylate two unique fucosyl residues on the O-chain, one of which occurs both *ex planta* and *in planta* and the second occurs only *in planta*. Further work is required to clarify the mechanism of this methylation reaction.

The other major change to *R.etli* CE3 LPS that occurs during bacteroid formation is the loss of a *β*-hydroxymyristic acid residue from, or the inability to add this fatty acyl residue to, its lipid A resulting in the production of only tetraacylated lipid A. Changes in lipid A fatty acylation have been reported for pathogens within their hosts, *e.g.* addition of palmitate observed in *Pseudomonas aeruginosa* isolated from infants with cystic fibrosis that developed a chronic airway infection with *P. aeruginosa* (48). It is known that *R. leguminosarum* contains a lipid A acylase that removes a *β*-hydroxymyristic acid from the 3-position of the lipid A glycosyl backbone (47). Therefore, it is possible that within the host there is an increase in the activity of

this enzyme. A lipid A 3-*O*-acylase from *Salmonella typhimurium*, PagL, has been reported (48). It is an outer membrane protein suggesting that de-*O*-acylation is a post-synthesis modification of the lipid A. However, we cannot identify a *pagL* ortholog in the recently published (49) *R. etli* CFN42 genome sequence. The inability to identify a pag*L* ortholog in *R. etli* is not surprising in view of the fact that the overall sequence similarity of several *pagL* genes is reported to be rather low (50). Despite this low sequence similarity, *pagL* has been identified in a number of bacterial species that are shown to exhibit lipid A-3-*O*-deacylation activity (50). However, in other species that exhibit this activity, one of which is *R. leguminosarum*, *pagL* has not yet been identified (50). Another enzyme has been identified in *S. typhimurium* that alters the lipid A fatty acylation pattern. This enzyme is encoded by *lpxR* and removes the lipid A 3'-acyloxyacyl moiety of the lipid A (51). Because we do not yet know the position on the *R. etli* bacteroid lipid A from which the *β*-hydroxymyristic acid is missing, such an enzyme in R*. etli*, if present, could account for the bacteroid tetraacylated lipid A. As with *pagL*, we have not identified an *lpxR* homolog in *R. etli.* Mutants that are defective in the *pagL* or *lpxR* homologs would certainly help determine whether one of theacylase enzymes is responsible for the lipid A structural change and elucidate the possible symbiotic function of this lipid A modification. However, it is first necessary to locate these genes on the *R. etli* CE3 genome.

Besides the structural differences outlined above, it is in fact remarkably interesting to notice that LPS from CE3 bacteria and bacteroids are structurally very similar. Conservation of the LPS structure during differentiation to bacteroids might be crucial to suppress or avoid induction of the plant defense response. Indeed, substantial changes in the structure of surface polysaccharides often have drastic effects, including the induction of a plant defense reaction accompanied by local production of antibacterial compounds, cell death, and physiological blockage of invading bacteria (3).

The observed changes to the bacteroid LPS indicated that there may be more global alterations to the bacteroid cell surface. Previous work had shown, for example, that *R. leguminosarum* biovar *viciae* bacteroids from pea were significantly increased in their hydrophobicity compared with laboratory-grown cultures (44). Although an extensive analysis of the surface hydrophobicity changes that take place during CE3 bacteroid differentiation remains to be performed, the results of a preliminary surface hydrophobicity test suggest that *R. etli* CE3 bacteroid cells from *P. vulgaris* nodules that could resume growth were more hydrophilic than laboratory-cultured *R. etli* CE3 or its EPS-minus mutant *R. etli* CE338 (data not shown). It is unlikely that this increase in bacteroid hydrophilicity is because of the removal of one *β*-hydroxymyristoyl residue or to the conversion of pentaacylated lipid A to a tetraacylated form.

Therefore, in addition to these LPS modifications, the bacteroid membrane apparently undergoes additional, as yet unknown, modifications that influence the surface hydrophobicity of bacteroids.

Structural and biophysical changes, among others, to surface components that occur during bacteroid differentiation may depend on the type of symbiotic interaction. In the case of pea, indeterminate nodules are formed in which there is synchronous division between the bacterial cell and the plant-derived membrane, known as the peribacteroid membrane (PM), resulting in a single occupancy symbiosome in which each bacteroid is surrounded by the PM. In the case of bean, which forms determinate nodules, multiple bacteroids can be surrounded by a single PM resulting in multioccupancy symbiosomes. This is thought to be due to either a lack of synchrony between bacterial cells and PM division or to the fusion of single occupancy symbiosomes (54). In the case of indeterminate nodules, it is possible that the maintenance of single occupancy symbiosomes results from more intimate contact between the dividing bacterial cell and the PM, whereas the latter determinate process involves detachment of the bacterial and PM cell. In the former case, we have reported that an *R. leguminosarum* biovar *viciae* mutant that is defective in the synthesis of the very long fatty acid moiety of its lipid A is also defective in bacteroid formation, and results in multiple occupancy symbiosomes (52, 53). It is important to investigate the characteristics of the surface polysaccharides of bacteroids in these and other symbiotic systems in order to be able to fully understand the mechanism of symbiosis.

The ability of a *Rhizobium* to form a nitrogen-fixing symbiosis with its host legume requires that the rhizobial cells survive or counteract the host defense response in some manner. Low molecular weight antimicrobial membrane-lytic peptides known as defensins are involved in one of the immediate host innate responses to potential pathogens (54). These molecules are found in both animals and plants (54). Generally, they are cationic, and perhaps the best known examples are polymyxin B and melittin. These molecules often act via a combination of their positive charge, which enables them to interact with anionic molecules on the bacterial surface, in combination with their hydrophobic character, which results in a disruption of the bacterial membrane(54,55).The LPS is the target molecule of defensins in Gram-negative bacteria, and bacteria that are resistant acquire this resistance by modification to their LPS structures(56–60); perhaps, one of the best known examples is the resistance acquired through the addition of aminoarabinose and ethanol amine groups to the LPS of *S. typhimurium* (57–60). Because of the structural alteration of the LPS during bacteroid formation, bacteria and bacteroids were compared for their resistance to poly-L-lysine, polymyxin B, and melittin. Inevaluating the

sensitivity of CE3 bacteroids against cationicpeptides, it should be noted that we were monitoring the sensitivity of viable bacteroids, *i.e.* those that can resume growth after isolation from the bean nodule. Mergaert *et al.* (61) recently reported that only 0.4% of the *R. leguminosarum* biovar *viciae* bacteroids isolated from *Vicia sativa* nodules and *S. meliloti* bacteroids isolated from *Medicago* (both *V. sativa* and *Medicago* form indeterminate nodules) resumed growth. This is in stark contrast with *R. leguminosarum* biovar *phaseoli* bacteroids isolated from *P. vulgaris* nodules and *Mesorhizobium loti* bacteroids isolated from *Lotus japonicus* nodules (both hosts form determinate nodules), of which 20% resumed growth. It was found that the bacteroid population that could resume growth was significantly more sensitive than bacteria. The interaction of the LPS with these peptides has not been investigated. However, it has been reported that disruptions in the fatty acylation pattern of the lipid A from *S. typhimurium* prevent the modification, *i.e.* addition of aminoarabinose, of the LPS required for increased resistance to polymyxin B (62). Thus, it is possible that the observed lipid A modification in bacteroids could also prevent some type of structural modification to the LPS resulting in increased sensitivity to these polycationic peptides. Another possibility is that bacteroids have a weakened outer membrane, *e.g.* because of loss of the lipid A hydroxymyristic acid moiety, which is simply more susceptible to the effects of the cationic peptides. The resistance of the laboratory cultures to any type of host cationic peptides would be expected for a successful symbiont, and this resistance is likely to be more important during early stages of infection of the host plant (63). The sensitivity of the bacteroids to these cationic peptides suggests that once surrounded by the PM the bacteroid may be sequestered from any further exposure to host defensins. The protection offered by the PM could be important so that the bacteroid can survive structural changes to its membrane that may be required for the exchange of metabolites with the host during an effective symbiosis. It is necessary to examine the type of interaction between these peptides from the host legume with bacteroidal and bacterial LPS to gain further insight into the molecular basis for the increased sensitivity of *R. etli* CE3 bacteroids.

Notes

* This work is supported in part by National Institutes of Health Grant GM39583 (to R.W.C.), Department of Energy Grant DE-FG02-98ER20307 (to K.D.N.), and a long term postdoctoral fellowship from the European Molecular Biology Organization (to W.D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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4. J. Box and K. D. Noel, personal communication.

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Appendix

Table 1: The glycosyl compositions of the carbohydrates released by mild acid hydrolysis of the CE3 LPS preparations.

Glycosyl residue	CE3 water phase	Bacteroid water phase	CE3/anthocyanin water phase	CE3 phenol phase	Bacteroid phenol phase
2,3,4-TOMFuc	ND	2.7	2.5	1.4	2.2
2,3-DOMFuc	3.8	2.4	1.6	3.0	2.3
2-MeFuc	4.0	10	8.0	4.2	11
3-Me6dTal	19	19	15	15	17
Fuc	22	14	14	18	16
Gal	8.3	12	5.1	4.7	5.2
Man	11	11	11	8.7	10
GalA	14	11	14	11	12
GlcUA	14	13	18	26	19
Kdo	2.5	1.5	3.5	3.3	2.1
QuiNAc	3.8	3.0	6.0	2.8	3.6

The compositions are given as relative mole percents of total carbohydrates. Due to the lack of standards for some glycosyl residues, and the many different glycosyl residues, we cannot be certain of the actual percent of mass accounted for by these various glycosyl residues in the various LPS samples. Compositions for the neutral glycosyl residues were determined by the preparation and analysis of alditol acetates and for GalA, GlcA, Kdo, and *N*-acetylquinovosamine by the preparation and analysis of trimethylsilyl (TMS) methyl glycosides. Standards were available and used to determine response factors for Fuc, Gal, Man, GalA, Glc, and Kdo. No standards were available for the remaining glycosyl residues, so the response factor for Fuc was used for the methylated Fuc and methylated 6-deoxytalose residues, and the response factor for GlcNAc was used for *N*-acetylquinovosamine. The abbreviations used are as follows: 2,3,4-TOMFuc, 2,3,4-tri-*O*-methylfucose; 2,3-DOMFuc, 2,3-di-*O*-methylfucose; 3-Me6dTal, 3-*O*-methyl-6-deoxytalose; GalA, galacturonic acid; QuiNAc, *N*-acetylquinovosamine; ND, none detected.

Table 2: Relative ratio of fucosyl residues from the various LPS preparations

Glycosyl residue	CE3 water phase	Bacteroid water phase	CE3/anthocyanin water phase	CE3 phenol phase	Bacteroid phenol phase
2,3,4-TOMFuc	ND	2.7	2.5	1.4	2.2
2,3-DOMFuc	3.8	2.4	1.6	3.0	2.3
2-MeFuc	4.0	10	8.0	4.2	11
Fuc	22	14	14	18	16
Total Fuc	30	29	26	27	32
2-Me Fuc of total Fuc (%)	13	34	31	16	34

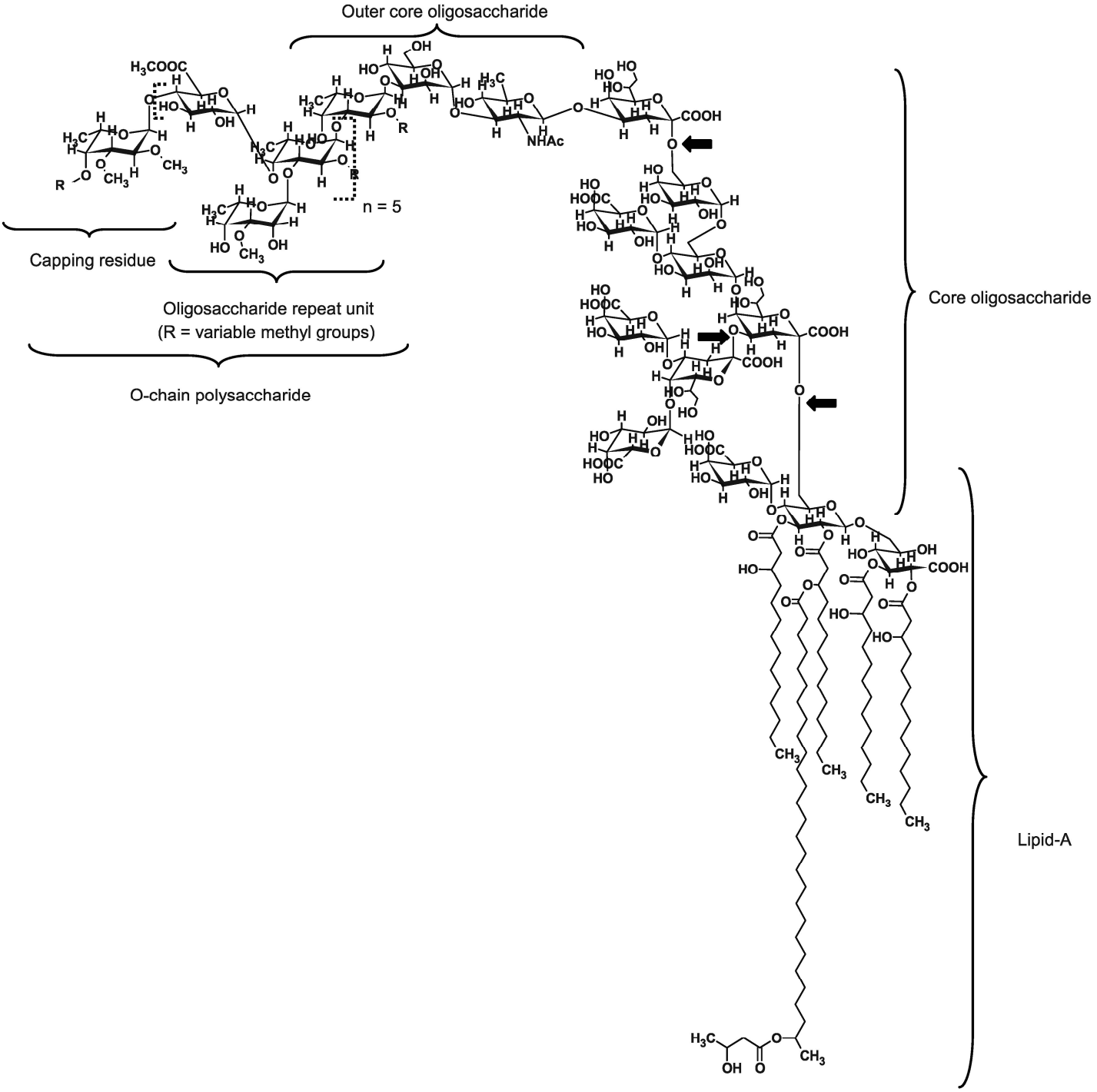
The abbreviations used are as follows: 2,3,4-TOMFuc, 2,3,4-tri-*O*-methylfucose; 2,3-DOMFuc, 2,3-di-*O*-methylfucose; ND, none detected.

Table 3: The glycosyl linkages of the O-chain polysaccharide fucosyl residues and the location of the endogenous methyl groups on the O-chain polysaccharide.

Glycosyl residue	Laboratory-cultured CE3 water phase	CE3 bacteroid water phase	Laboratory-cultured CE3 phenol phase	CE3 bacteroid phenol phase
Terminal TOM or DOMFuc	23	24	19	20
3-Linked Fuc	23	12	19	13
2-O-Me-3-Linked Fuc	2.7	6.7	2.9	3.2
3,4-Linked Fuc	37	22	41	25
2-O-Me-3,4-Linked Fuc	13	34	18	39

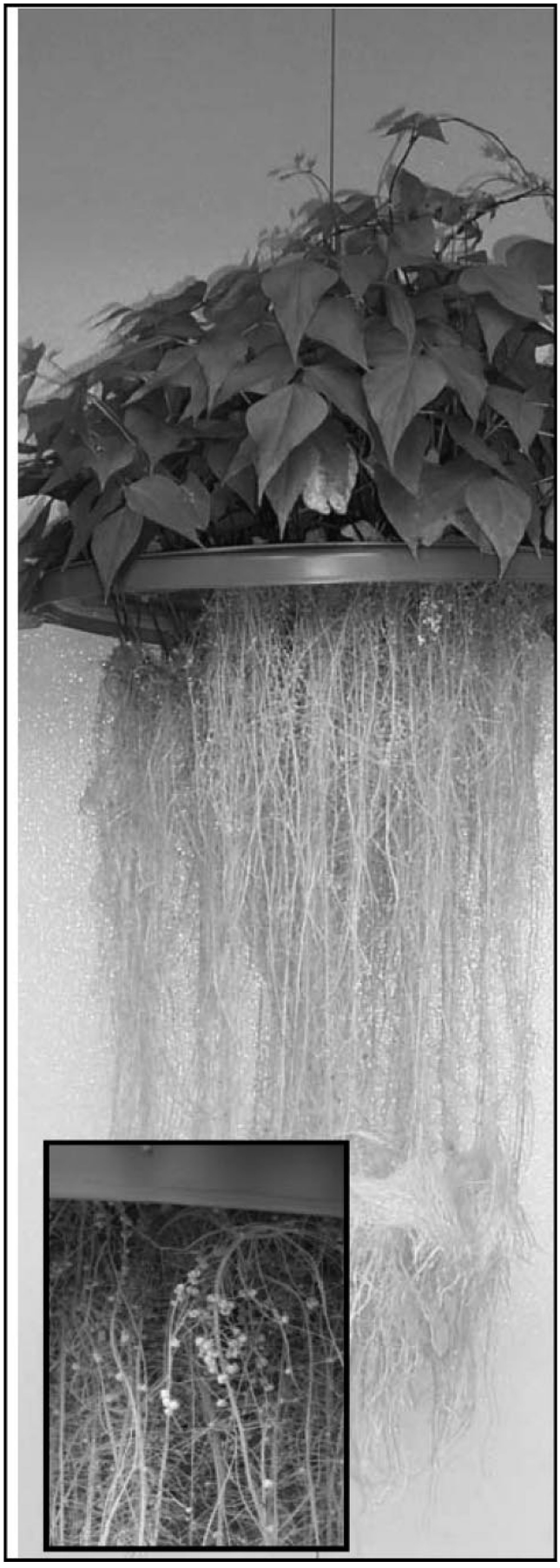
Values were calculated as relative percent of total fucosyl partially methylated alditol acetate peak areas. The abbreviations used are as follows: TOMFuc, 2,3,4-tri-*O*-methylfucose; 2,3-DOMFuc, 2,3-di-*O*-methylfucose.

Figure 1: The complete structure of the LPS from *R. etli* CE3.



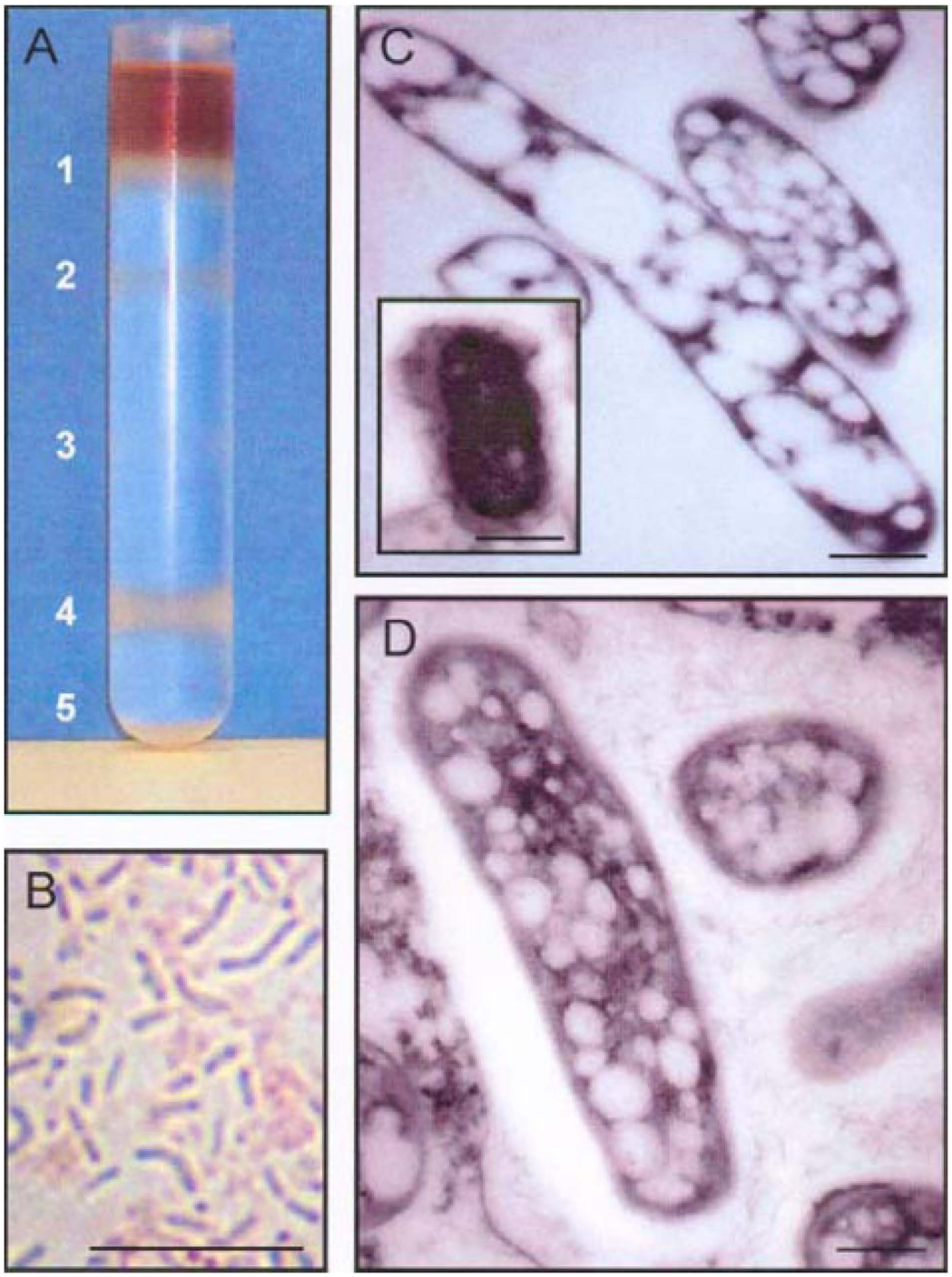
The *arrows* mark the positions of mildly acid-labile bonds that can be hydrolyzed with1%acetic acid at 100 ℃. The letter *R* designates the positions of variable methylation that occurs on the O-chain polysaccharide.

Figure 2: *R. etli* CE3-induced nodulation of *P. vulgaris* in aeroponic growth chambers.



Nodulated roots systems of a number of plants grown in an AGC are shown. Mainly the upper part of the roots (upper 50 cm) contains dense clusters with large nodules (*inset*) that were harvested – 4 weeks after the *P. vulgaris* seedlings were transferred to the AGC. Roots, hanging in the AGC, are continuously sprayed with an aerosol of nutrient solution mixed with *R. etli* CE3 bacteria.

Figure 3: Purification of *R. etli* CE3 bacteriods by stepwise sucrose gradient-mediated ultracentrifugation.



A, typical ultracentrifugation pattern. The various bands that appear inbetween two sucrose layers with a different sucrose concentration are indicated with *numbers 1-5*. Band 4 contains pure CE3 bacteroid. *B,* sample was taken from band 4 and analyzed by light microscopy using phase contrast optics. *C,* transmission electron microscopy image of purified bacteroids (band 4) that are long shaped and contain polyhydroxybutyrate (round-shaped bodies with low electron density). The *inset* represents an image of an *R. etli* CE3 cultured bacterium. *D,* transmission electron microscopy image of a CE3 bacteroid present in a mature *P. vulgarius* nodule harvested 3 weeks after nodulation as positive control. *Bars*: 10 *μ*m (B) and 0.25 *μ*m (*C* and *D*).
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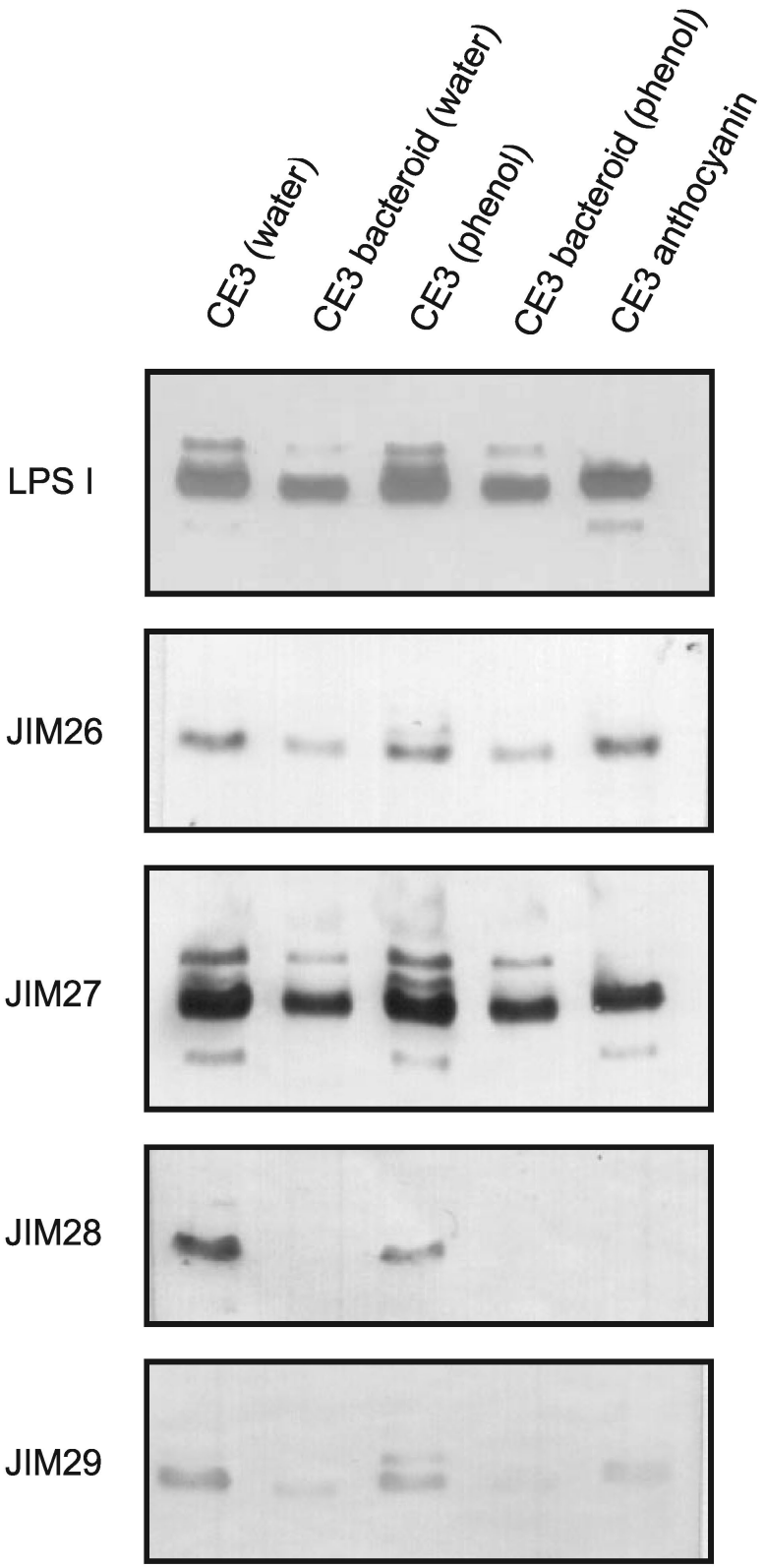
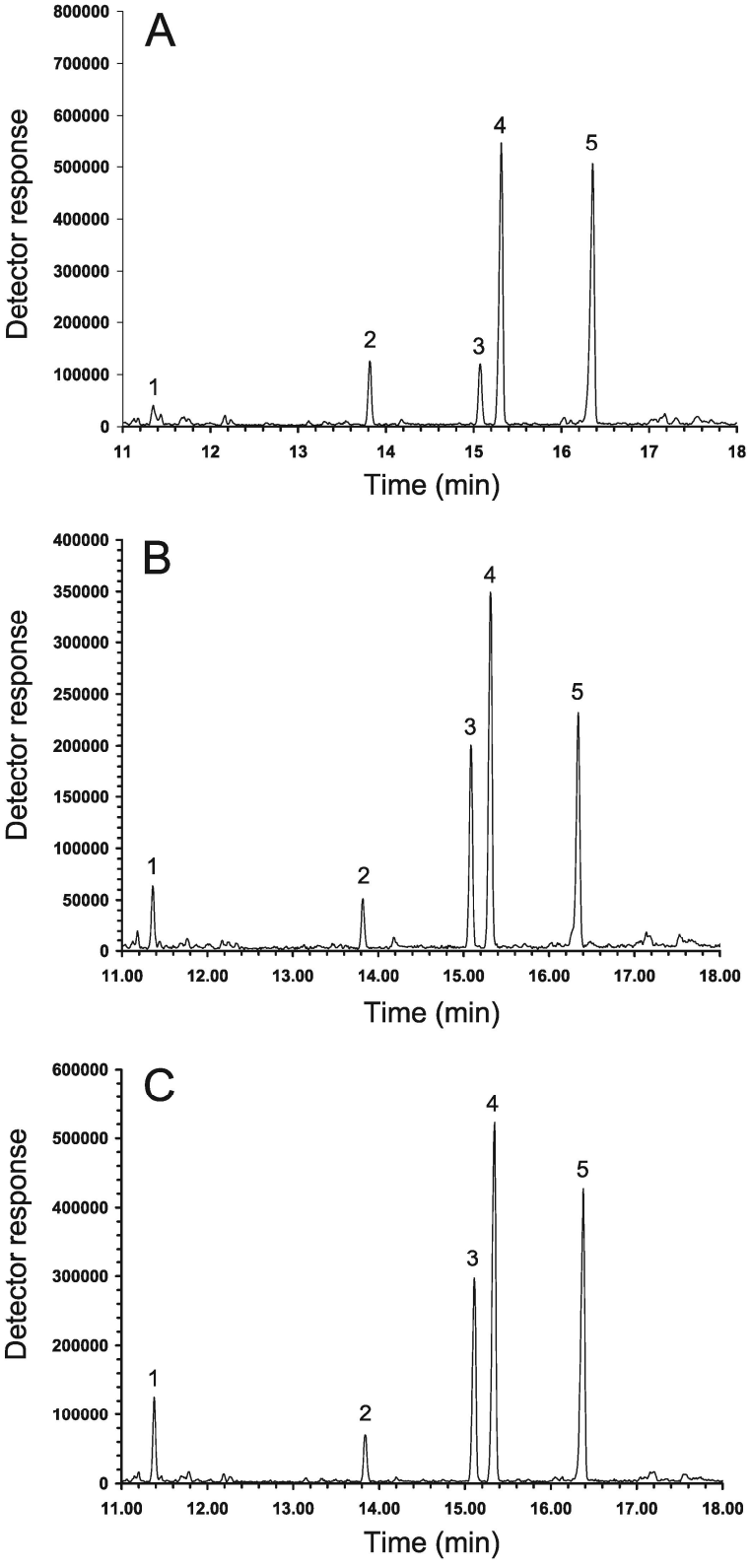
Figure 4: Immunoblot analyses of LPS I from *R. etli* CE3, CE3 bacteroids, and *R. etli* CE3 grown in the presence of anthocyanin.



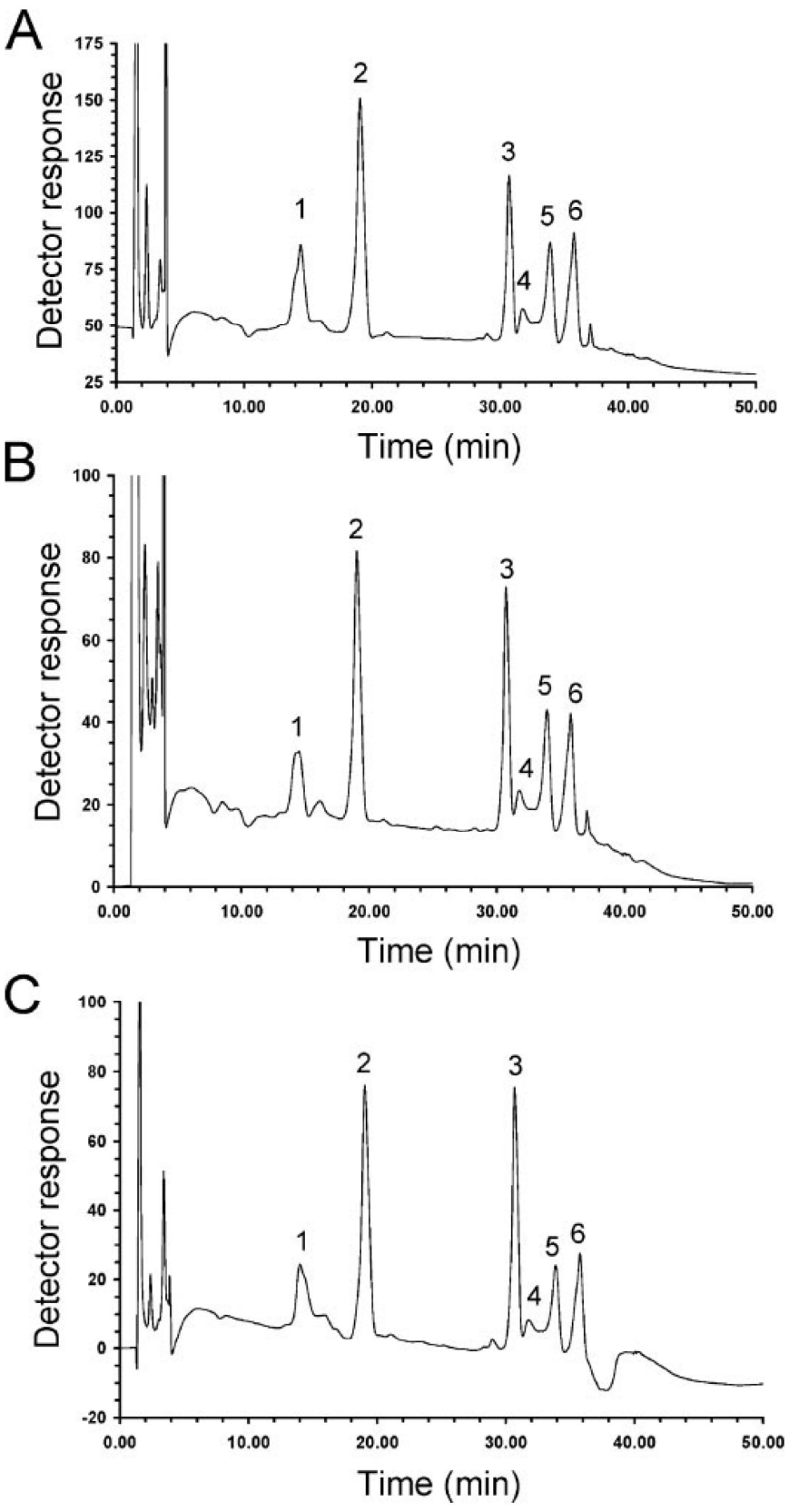
Immunoblots from DOC-polyacrylamide gels were silver-stained (*top panel*) or subjected to immunoblot analysis using antibodies JIM26, JIM27, JIM28, and JIM29.

Figure 5: The gas chromatographic profiles showing the alditol acetates of the methylated and 6-deioxyglycosyl residues for the O-chain polysaccharides from the LPS of cultured CE3 bacteria (*A*), from the LPS of CE3 bacteroids (*B*), and from the LPS of CE3 cultured in the laboratory in the presence of anthocyanin (*C*).



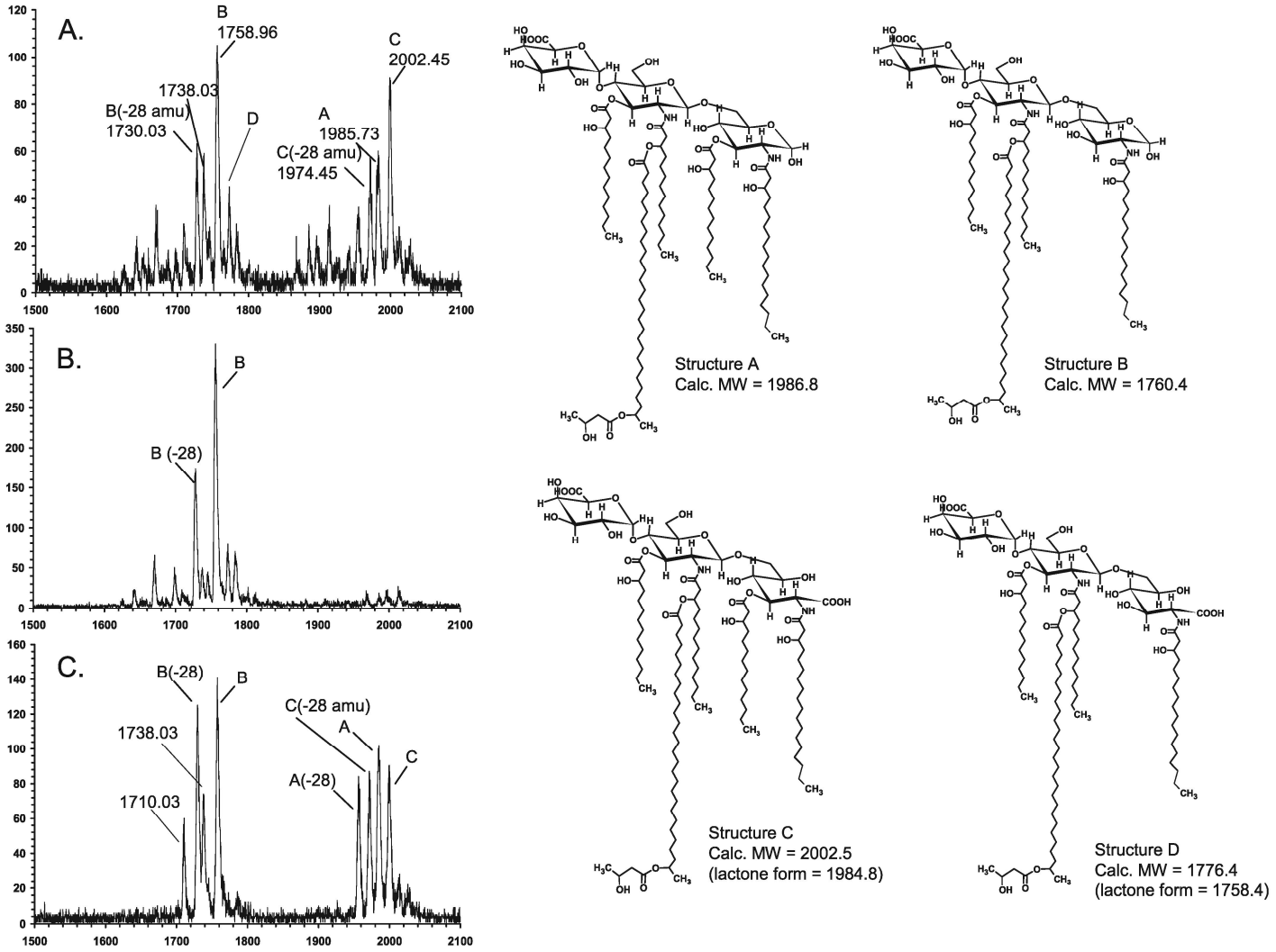
The profiles shown are for the LPS extracted into the water phase during hot phenol/water extractions. Identical, respective, results were obtained for the LPS found in the phenol layer from laboratory-cultured CE3 bacteria and bacteroids. The phenol-extracted LPS from the culture grown in the presence of anthocyanin was not analyzed. The peaks are as follows: *1,* *2,*3,4-tri-*O*-methylfucose; *2,* 2,3-di-*O*-methylfucose; *3,* 2-*O*-methylfucose; *4,* 3-*O*-methyl-6-deoxytalose; *5* = fucose.

Figure 6: The DIONEX chromatographic profiles of the oligosaccharides released by mild acid hydrolysis of the LPS from CE3 bacteria (*A*), (*B*) bacteroids, and (*C*) bacteria grown in the presence of anthocyanin.



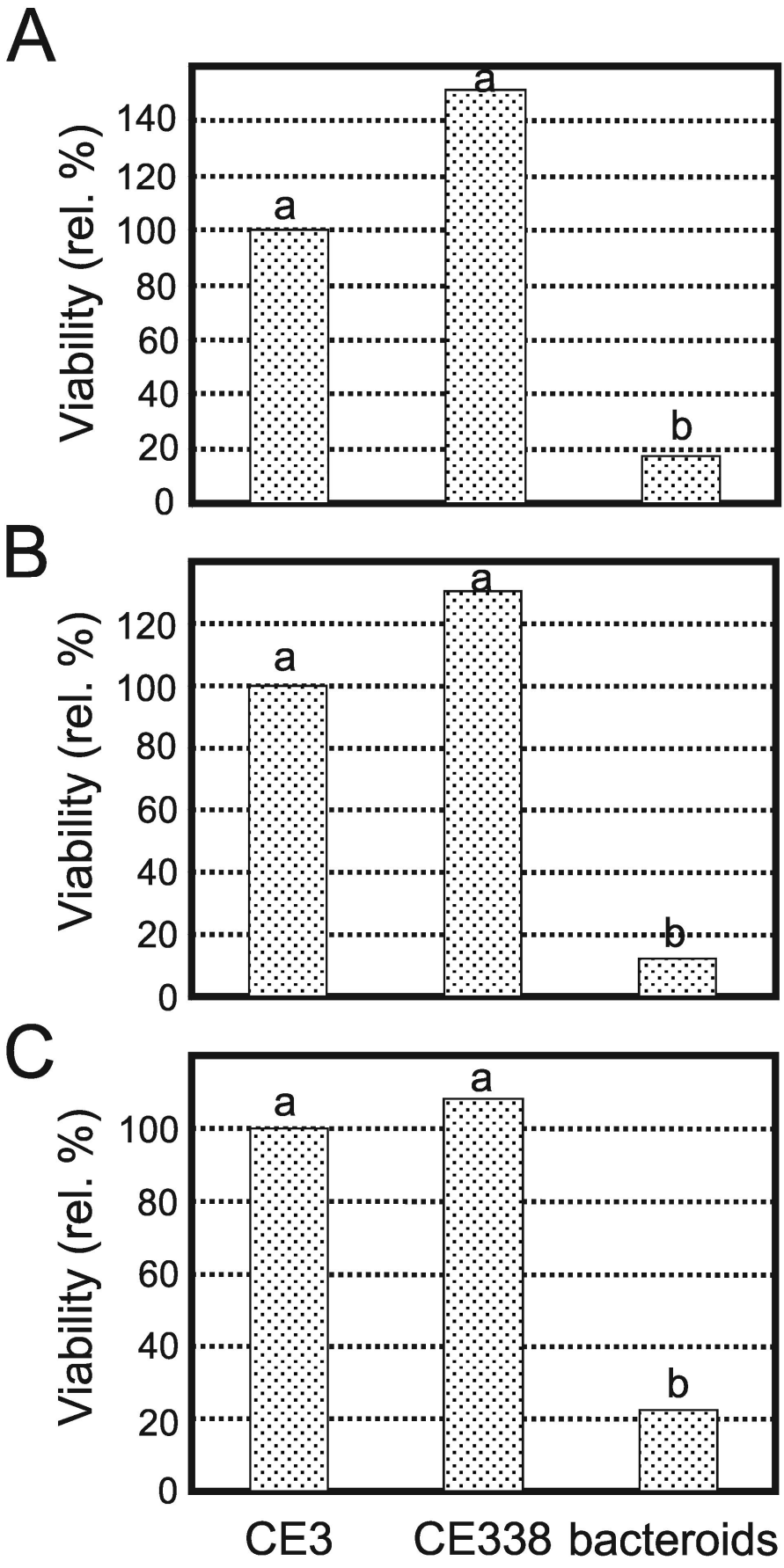
The profiles shown are for the LPS extracted into the water phase during hot phenol/water extractions. Identical, respective, results were obtained for the LPS found in the phenol layer from laboratory-cultured CE3 bacteria and bacteroids. The phenol-extracted LPS from the culture grown in the presence of anthocyanin was not analyzed. The identity of the oligosaccharide peaks have been identified and reported previously (36). *Peak 1*, Kdo; *peak 2*, GalA; *peak 3*, GalMan(GalA)Kdo tetrasaccharide; *peak 4*, anhydro version of the tetrasaccharide; *peak 5*, a second anhydro version of the tetrasaccharide; *peak 6*, $GalA_2$Kdo trisaccharide.

Figure 7: The MALDI-TOF MS spectra for the lipid A from CE3 bacteria water phase LPS.



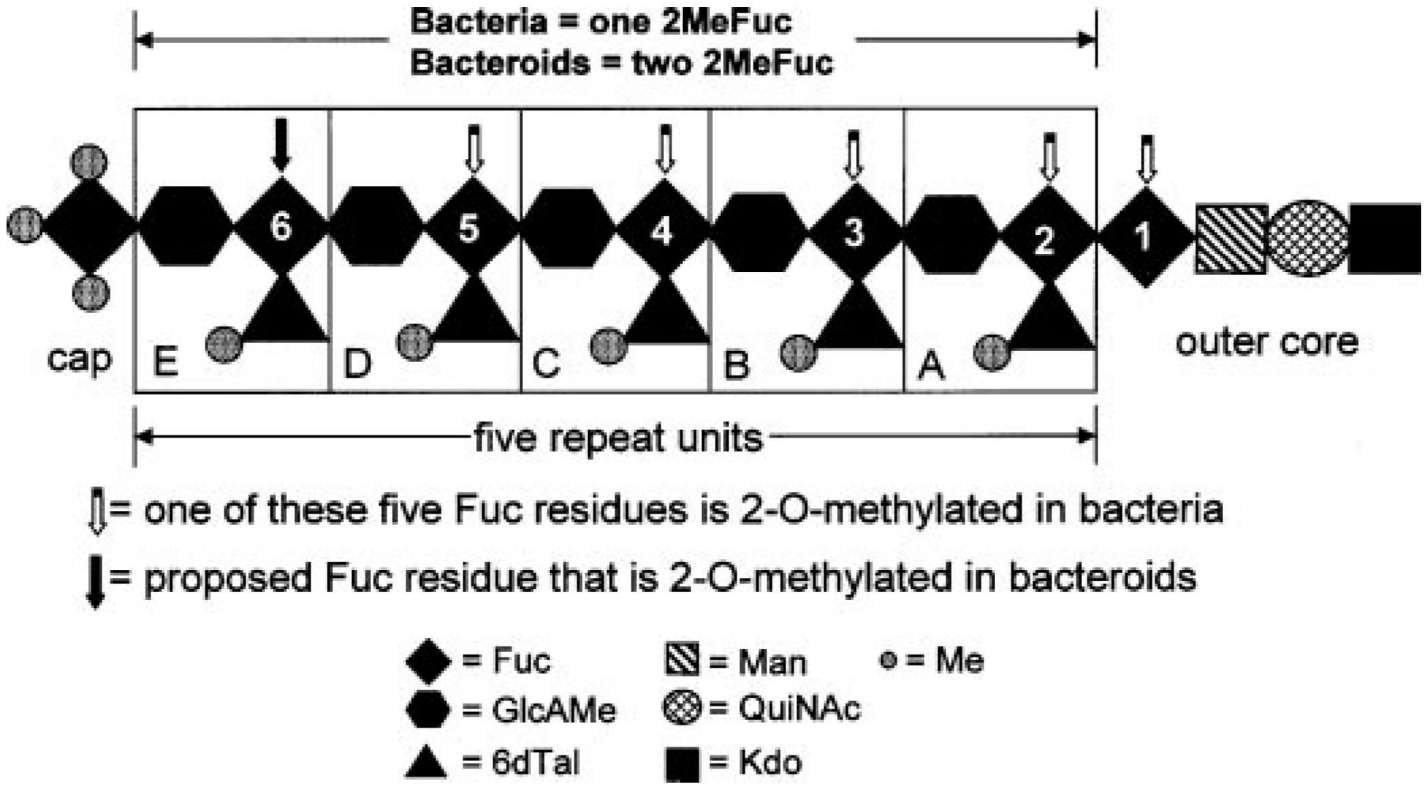
The results were identical for the lipid A from LPS that extracted into the phenol phase. *A,* spectrum of lipid A from CE3 bacteria cultured under normal laboratory conditions. *B,* spectrum of lipid A from C3 bacteroids. *C,* lipid A from CE3 bacteria cultured in the laboratory in the presence of anthocyanin. Different ions are observed in each cluster of ions because of variation in fatty acyl chain length. The structures for which the proximal residue is 2-amingogluconic acid (structures *C* and *D*) can also form lactones (*i.e.* molecular weights of 1984.8 and 1758.4, respectively). During mild acid hydrolysis of the LPS to obtain the lipid A, the lactone forms of the lipid A can eliminate *β*-hydroxymyristic acid from the 3-position (*i.e.* 1984.8 – 245 = 1739.8), which probably accounts for the observed ion at *m/z* 1738.03. The structures for these ions have been reported previously (38, 39).

Figure 8: Sensitivity of *R. etli* E3, CE338, and CE3 bacteroids for cationic peptides.



Aliquots of *R. etli* CE3, the EPS mutant CE338, and CE3 bacteroids were incubated with the cationic peptides melittin (*A*), polymyxin B (*B*), and poly-L-lysine (*C*) followed by determining the relative (*rel.*) surviving colony-forming units, which are indicative for the viability. The percentage of viable *R. etli* CE3 bacteria was set at 100. Averages that are not significantly different are indicated with the same letter ($p > 0.05$).

Figure 9: This figure shows a schematic diagram of the O-chain polysaccharide from CE3.



The data indicate that bacteroids have two 2-*O*-methylfucosyl residues, whereas bacteria have one 2-*O*-methylfucosyl residue. The *open arrows* mark the five possible positions for fucosyl 2-*O*-methylation in the bacterial O-chain polysaccharide. The *closed arrows* shows the proposed position of the additional 2-*O*-methylation that occurs on the O-chain from bacteroids. The rationale for this proposed methylation position is described in the text.