Three Genes Encoding for Putative Methyl- and Acetyltransferases Map Adjacent to the *wzm* and *wzt* Genes and Are Essential for O-Antigen Biosynthesis in *Rhizobium etli* CE3

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The elucidation of the structure of the O-antigen of *Rhizobium etli* CE3 predicts that the *R. etli* CE3 genome must contain genes encoding acetyl- and methyltransferases to confer the corresponding modifications to the O-antigen. We identified three open reading frames (ORFs) upstream of *wzm*, encoding the membrane component of the O-antigen transporter and located in the *lpsA*-region of *R. etli* CE3. The ORFs encode two putative acetyltransferases with similarity to the CysE-LacA-LpxA-NodL family of acetyltransferases and one putative methyltransferase with sequence motifs common to a wide range of S-adenosyl-L-methionine-dependent methyltransferases. Mutational analysis of the ORFs encoding the putative acetyltransferases and methyltransferase revealed that the acetyl and methyl decorations mediated by these specific enzymes are essential for O-antigen synthesis. Composition analysis and high performance anion exchange chromatography analysis of the lipopolysaccharides (LPSs) of the mutants show that all of these LPSs contain an intact core region and lack the O-antigen polysaccharide. The possible role of these transferases in the decoration of the O-antigen of *R. etli* is discussed.

Additional keywords: lipopolysaccharide (LPS).

The establishment of the symbiotic relationship between *Rhizobium* spp. and their host plants is a complex process involving the exchange of a series of signals between the plant and bacteria, ultimately leading to the formation of a nitrogen-fixing root nodule (Spaink 2000). Certain bacterial polysaccharides have been shown to be essential for the successful invasion of nodules by rhizobia in several *Rhizobium*–legume symbioses. In the case of the *Rhizobium etli*–*Phaseolus vulgaris* L. (common bean) interaction, bacterial mutants that fail to produce the O-antigen polysaccharide portion of the lipopolysaccharides (LPSs) are substantially impaired in their ability to invade developing root nodules and, thus, primarily yield root nodules devoid of bacteria and bacteroids (Kannenberg et al. 1998). LPS is the major structural component of the gram-negative bacterial outer membrane and consists of three main

regions. The lipid A region forms the outer leaflet of the outer membrane lipid bilayer. External to this is the KDO-core oligosaccharide region, and the most distant from the membrane is the O-antigen, of which the structure is strain specific. The O-antigenic part of the LPS of *R. etli* CE3 is composed of a trisaccharide repeating unit linked to a capping fucosyl residue at the nonreducing end and a nonrepeating sequence consisting of three glycosyl residues at the reducing end (Fig. 1). Interestingly, the polysaccharide is O-acetylated and contains a variety of O-methylated glycosyl residues that renders the native glycan somewhat hydrophobic. This hydrophobicity is most pronounced at the nonreducing end, where the O-chain repeating units are capped by the terminal deoxysugar in which all of the hydroxyl groups are methylated (Duelli et al. 2001).

Thus far, at least six genomic regions involved in biosynthesis of the LPS of *R. etli* CE3 have been identified (Noel and Duelli 2000). Most of the information is located in the *lpsA* region of the *R. etli* CE3 chromosome, spanning 17 kb of DNA (Cava et al. 1989). Among the genes located in this *lpsA* region are genes required for O-antigen transport (*wzm* and *wzt*) and for O-antigen modification (*lpsB*) (Fig. 2; Table 1). The identification of *wzm* and *wzt* in *R. etli* CE3 (Lerouge et al. 2001) indicates that the O-antigen is transported across the cytoplasmic membrane by an ATP-binding cassette transporter, after “monomeric” assembly of the repeating units on the cytosolic face of the inner membrane. The biosynthetic point at which the methyl and acetyl groups are added to the O-antigen is unknown (i.e., during synthesis of the sugar nucleotides, after assembly of the repeating units, or after transport of the O-antigen through the inner membrane). *LpsB* is responsible for the variable O-methylation of the internal fucose residues of the O-antigen (Duelli et al. 2001; Noel and Duelli 2000; Noel et al. 2000). The absence of this methylation in mutant strain CE395 leads to a 50% decrease in the relative number of LPS molecules carrying the O-chain (LPSI). Strain CE395 elicits sparsely distributed pseudonodules that contain few bacteria and lack nitrogen-fixing activity (*Inf*[−] phenotype). Interestingly, growth of *R. etli* wild type in the presence of plant-released anthocyanins leads to an increased 2-O-methylation of the internal fucose residues, which is correlated with loss of JIM28 reactivity. JIM28 is a monoclonal antibody that recognizes the LPSI of *R. etli* CE3 but not the LPSI of other *Rhizobium* strains that have been tested (Noel et al. 2000). Conversely, the absence of this 2-O-methylation on internal fucosyl residues in strain CE395 is correlated with an increased binding of the JIM28 antibody (Duelli et al. 2001). Unlike the wild type, the LPS of mutant CE395 still binds the antibody after growth in anthocyanins.

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The nucleotide sequences for *orf1*, *orf2*, and *orf3* have been deposited in the GenBank database under GenBank accession number AF348454.

Fig. 1. Structure of the O-antigen of the *Rhizobium etli* CE3 LPS. Based on the O-antigen structure of *R. etli* CE3, methylation and acetylation sites are proposed. Anthocyanins from the host plant promote a specific methylation of the O-antigen of *R. etli* CE3. Gene *lpsB* is required for this methylation (Noel and Duelli 2000; Noel et al. 2000). Abbreviations: QuiNAc, *N*-acetylquinovosamine; 3-Me-dTal, 3-methyl deoxytalose; GlcA, glucuronic acid; Fuc, fucose; Man, mannose. Gene *lpeA* is required for the *O*-methylation of the 2,3,4-tri-*O*-methyl-fucosyl capping residue (Duelli et al. 2001).

Fig. 2. Physical and genetic organization of the 4,787-bp *lps* region of *Rhizobium etli* CE3, containing *orf1*, *orf2*, *orf3*, *wzm*, and *wzt* (Cava et al. 1990; Carlson et al. 1995; Lerouge et al. 2001) and located in the *lpsA* region. Only part of the *lpsA* region is represented, which is indicated by the dotted line. The relative locations of the Tn5 insertions or base pair insertions of the mutants identified in this or previous studies are indicated by circles (polar mutants) or rectangles (nonpolar mutants), respectively. Mutant CE395 completely lacks the 2-*O*-methylation of the internal fucose residues and has a Tn5-insertion in the *lpsB* gene. The level of blackness in the circles or rectangles represents the degree of lipopolysaccharide molecules carrying the O-chain-synthesis deficiency. Abbreviations: E, *Eco*RI; B, *Bam*HI; H, *Hind*III; X, *Xho*I. Not all restriction sites are indicated.

Another genetic locus, designated *lpe3*, spans five open reading frames (ORFs) (*orf1*, *lpeA*, *orf2*, *orf3*, and *orf4*) and is involved in O-methylation of the capping fucosyl residue. The O-chain of an *lpeA* mutant (CE367) lacks not only the methyl groups of 2,3,4-tri-O-methylfucose (TOM-Fuc) but also the entire residue, in which case the O-chain terminates in a glucuronic acid (GlcA) residue. With regard to LPS synthesis, it also is interesting that the absence of TOM-Fuc and 2,3-di-O-methylfucose (DOM-Fuc) does not seem to decrease the relative amount of LPSI. Mutations of the *lpe* genes do not prevent normal nodule development on *Phaseolus vulgaris* and have very little effect on the competition for nodule occupancy with the wild-type strain (Duelli et al. 2001).

In this article, we present three additional ORFs located upstream of the *wzm* and *wzt* genes and which may be responsible for methyl and acetyl decoration of the O-antigen in *R. etli* CE3. In contrast to the previously identified decoration genes *lpsB* and *lpeA*, the ORFs described in this article are essential for O-antigen biosynthesis because mutations in these ORFs lead to the loss of O-antigen. Nevertheless, composition analysis and high performance anion exchange chromatography (HPAEC) analysis of the LPSs of the mutants show that all of these LPSs contain a complete core region.

RESULTS

Sequence analysis of *R. etli* CE3 *orf1/orf2/orf3*.

To identify new O-antigen genes from *R. etli*, pFAJ1227 (Table 2; Fig. 2), containing DNA upstream of *wzm* in the *lpsA* region, was subcloned and partially sequenced. A 2,830-nucleotide stretch upstream of *wzm* was analyzed for the presence of putative ORFs. Three open reading frames (*orf1*, *orf2*, and *orf3*) were identified. The derived amino acid sequence of the three ORFs showed similarity with previously identified acetyltransferases (*orf1* and *orf2*) and methyltransferases (*orf3*). The position of the different ORFs is shown schematically in Figure 2. The ORFs probably are coupled translationally because there is an overlap of several base pairs between *orf1* and *orf2*. It also should be noted that the third-letter GC content of the three ORFs is in the range of 50 to 60% (51.9, 54.9, and 58.5%), which is considerably lower compared with the higher overall third-letter GC content of the *R. etli* genome (73.58%). Analogous to the *wzm* and *wzt* genes, the GC content of the entire coding region is between 50 and 55%, which also is lower than that of *R. etli* (60.64%). This finding may indicate that these genes have arisen by horizontal gene transfer.

Sequence comparisons.

Putative functions for the identified ORFs are assigned on the basis of similarity to other proteins. The putative acetyl-

transferases encoded by *orf1* and *orf2* share similarities in the C-terminal part to members of the CysE-LacA-LpxA-NodL family of acetyltransferases from a wide range of bacteria (Table 3). Members of this family O-acetylate quite different substrates, including serine (*Buchnera aphidicola* CysE), thiogalactoside (*Escherichia coli* LacA), O-antigen (*E. coli* WbnC), N-acetylglucosamine (*Rhizobium* NodL), and antibiotics, such as chloramphenicol (*E. coli* Cat) and streptogramin A (*Staphylococcus aureus* Vat and *Enterococcus faecium* Sat). These proteins have been shown to contain a repeat structure composed of tandem repeats of a hexapeptide (hexapeptide-repeat containing-transferases signature [PDOC00094]) also termed the isoleucine patch which, in the tertiary structure of LpxA, has been shown to form a left-handed parallel β -helix (Raetz and Roderick 1995).

orf1 and *orf2* both show similarity to NodL of *Sinorhizobium meliloti* (Baev and Kondorosi 1992) and *R. leguminosarum* (Surin and Downie 1988), which O-acetylates the C-6 of the terminal nonreducing glucosamine of the nodulation factors; WbnC from *E. coli* O113, which is likely to be responsible for O-acetylation of the O113 O-antigen (Paton and Paton 1999); and EpsH from *Streptococcus thermophilus*, which is suggested to be involved in acetylation of exopolysaccharides (Stingele et al. 1996). Acetylation of many of the above-mentioned substrates has a profound effect on the interaction of the bacterium with the corresponding host organism. The gene products of *orf1* and *orf2* are likely to be responsible for O-acetylation of the *R. etli* CE3 O-antigen polysaccharide, although the precise sugars modified are yet to be determined. Also, *orf1* and *orf2* both have hexapeptide repeats starting with isoleucine or valine, often followed by a glycine residue within the NH₂-terminal region (Fig. 3). Most of the enzymes that display the hexapeptide repeat sequence motif use acyl-coenzyme A or acyl-acyl carrier protein as substrates (Allignet and el Solh 1995; Raetz and Roderick 1995).

The deduced amino acid sequence of *orf3* contains three motifs which are conserved in several methyltransferases (Kagan and Clarke 1994). The presence of these motifs in the identified putative methyltransferase (Fig. 4) may imply S-adenosyl-methionine (SAM) binding and indicate a putative function as a SAM-utilizing methyltransferase. Similarity was observed to sterol methyltransferases (SMTs) in higher plants such as *Zea mays* and *Arabidopsis thaliana*, and to methyltransferases involved in ubiquinone/menaquinone and biotin biosynthesis, including *ubiE* (C-methylation), *ubiG* (O-methylation), and *bioC*. The characteristic sequence motifs also are found in methyltransferases involved in biosynthesis of specific carbohydrates in *Rhizobium* spp.: galactoglucan or EPSII (*expE3*) and Nod-Factors (*nodS*) (Geelen et al. 1993).

Table 1. Summary of previously and newly characterized mutants with mutations in the *lpsA* region

Mutant	LPS phenotype ^a	Putative function of mutated open reading frame	Plant phenotype of mutant	Reference
FAJ1201	No LPSI	Uniform acetylation of QuiNac and a not yet identified sugar of the repeating unit	Sparsely distributed pseudonodules	This study
FAJ1202	No LPSI	...	...	...
FAJ1204	No LPSI	...	...	...
FAJ1203	No LPSI	Uniform methylation of 3-O-methyl-6-deoxytalose or glucuronic acid	Sparsely distributed pseudonodules	This study
FAJ1200	No LPSI	Transport of O-antigen through inner membrane	Sparsely distributed pseudonodules	Lerouge et al. 2001
CE374	Normal amounts of LPSI but antigenically abnormal LPSI	?	Fewer but normal nodules (Fix ⁺)	Noel et al. 2000
CE395	Lower amounts of LPSI and no 2-O-methylation of fucose	Variable methylation of internal Fucose residues	Sparsely distributed pseudonodules	Noel et al. 2000

^a Lipopolysaccharides (LPS) and LPS molecules carrying the O-chain (LPSI).

Mutagenesis of *orf1*, *orf2*, and *orf3* and analysis of phenotypes on SDS-PAGE.

To evaluate the contribution of the putative transferases encoded by *orf1*, *orf2*, and *orf3* in the decoration of the O-antigen, nonpolar mutants of these three ORFs were constructed. In

each ORF, one or two base pairs were inserted to induce a translational frameshift. Together with the insertion of these extra base pairs, an additional *EcoRI* site was created in order to select the mutants through *EcoRI* digestion of the corresponding DNA regions (Fig. 2). For the selection of the mu-

Table 2. Bacterial strains and plasmids

Bacterial strains and plasmids	Relevant characteristics	Reference or source
Strains		
<i>Rhizobium etli</i>		
CE3	Wild type, Nal ^r	Noel et al. 1984
FAJ1200	CE3 <i>wzm::mTn5</i> mutant, Nal ^r , Km ^r (ABC transporter), Inf	Lerouge et al. 2001
FAJ1201	CE3 <i>orf1</i> nonpolar mutant, Nal ^r (acetyltransferase 1), Inf	This study
FAJ1202	CE3 <i>orf2</i> nonpolar mutant, Nal ^r (acetyltransferase 2), Inf	This study
FAJ1203	CE3 <i>orf3</i> nonpolar mutant, Nal ^r (methyltransferase), Inf	This study
FAJ1204	CE3 <i>orf1orf2</i> nonpolar double mutant, Nal ^r (acetyltransferase 1 + acetyltransferase 2), Inf	This study
CMPG13010	FAJ1201 carrying pCMPG13001	This study
CMPG13011	FAJ1202 carrying pCMPG13002	This study
CMPG13012	FAJ1203 carrying pCMPG13003	This study
CMPG13013	FAJ1204 carrying pCMPG13004	This study
<i>Escherichia coli</i>		
DH5 α	<i>hsdR17 endA1 thi-1 gyrA96 relA1 recA1 supE44 ΔlacU169(ϕ80lacZΔM15)</i>	Sambrook et al. 1989
Plasmids		
pUC19	ColE1 replicon, <i>lacZ</i> , Ap ^r cloning vector	Yanisch-Perron et al. 1985
PUCBM20	pUC derivative with extended polylinker, Ap ^r	Boehringer Mannheim
pUC18 <i>NorI</i>	pUC derivative with two <i>NorI</i> sites at both ends of the polylinker, Ap ^r	Herrero and Timmis 1990
pJQ200uc1	Suicide vector, containing <i>sacB</i> gene of <i>Bacillus subtilis</i> , Gm ^r	Quandt and Hynes 1993
pCOS109.11	<i>R. etli</i> CE3 <i>lpsa</i> region in 31-kb insert	Cava et al. 1990
pFAJ1227	pUCBM20, with a 7.8-kb <i>EcoRI</i> fragment of the <i>lpsa</i> region containing the <i>wzm</i> locus	This study
pFAJ1235	pUCBM20, with the 3.1-kb <i>HindIII</i> fragment of pFAJ1227	This study
pFAJ1236	pFAJ1235 containing a single bp insertion (T) into <i>orf1</i> , thereby creating an <i>EcoRI</i> -site in <i>orf1</i>	This study
pFAJ1237	pFAJ1235 containing a single bp insertion (G) into <i>orf2</i> , thereby creating an <i>EcoRI</i> -site in <i>orf2</i>	This study
pFAJ1238	pFAJ1235 containing a double bp insertion (TC) into <i>orf3</i> , thereby creating an <i>EcoRI</i> -site in <i>orf3</i>	This study
pFAJ1239	pUC18 <i>NorI</i> , with the <i>HindIII</i> fragment from pFAJ1236	This study
pFAJ1240	pUC18 <i>NorI</i> , with the <i>HindIII</i> fragment from pFAJ1237	This study
pFAJ1241	pUC18 <i>NorI</i> , with the <i>HindIII</i> fragment from pFAJ1238	This study
pFAJ1242	pFAJ1240, containing a single bp insertion (T) into <i>orf1</i> and a single bp insertion (G) into <i>orf2</i> , thereby creating an <i>EcoRI</i> -site in both <i>orf1</i> and <i>orf2</i>	This study
pFAJ1243	pJQ200uc1, with the <i>NorI</i> fragment from pFAJ1239	This study
pFAJ1244	pJQ200uc1, with the <i>NorI</i> fragment from pFAJ1240	This study
pFAJ1245	pJQ200uc1, with the <i>NorI</i> fragment from pFAJ1241	This study
pFAJ1246	pJQ200uc1, with the <i>NorI</i> fragment from pFAJ1242	This study
pFAJ1708	RK2 replicon, <i>nptII</i> promoter, Tet ^r expression vector	Dombrecht et al. 2001
pCMPG13001	pFAJ1708, with a 0.794-kb PCR ^a fragment containing <i>orf1</i> (primers RHI-578 + RHI-579) in the <i>AscI/PmeI</i> sites	This study
pCMPG13002	pFAJ1708, with a 0.874-kb PCR fragment containing <i>orf2</i> (primers RHI-580 + RHI-581) in the <i>AscI/PmeI</i> sites	This study
pCMPG13003	pFAJ1708, with a 1.038-kb PCR fragment containing <i>orf3</i> (primers RHI-582 + RHI-583) in the <i>AscI/PmeI</i> sites	This study
pCMPG13004	pFAJ1708, with a 1.419-kb PCR fragment containing <i>orf1</i> and <i>orf2</i> (primers RHI-578 + RHI-581) in the <i>AscI/PmeI</i> sites	This study

^a Polymerase chain reaction = PCR.

Table 3. Summary of identified open reading frames (ORF) of *Rhizobium etli* CE3

ORF	No. of amino acids	G+C content (%)	Third letter GC (%)	Similar proteins (reference or accession no.)	Iden./sim (%) (no. aa) ^a	Putative function of protein
<i>orf1</i>	205	52.3	51.9	<i>orf2</i> , <i>Rhizobium etli</i> CE3 WbnC, <i>Escherichia coli</i> (AAD50488.1) NodL, <i>Rhizobium leguminosarum</i> (P08632) EpsH, <i>Streptococcus thermophilus</i> (AAC44015.1)	25/40 (214) 24/33 (179) 22/33 (190) 21/34 (159)	O-acetyltransferase ...
<i>orf2</i>	214	51.5	54.9	<i>orf1</i> , <i>Rhizobium etli</i> CE3 NodL, <i>Rhizobium leguminosarum</i> (P08632) WbnC, <i>Escherichia coli</i> (AAD50488.1) EpsH, <i>Streptococcus thermophilus</i> (AAC44015.1)	25/40 (205) 22/33 (190) 18/32 (179) 15/28 (159)	O-acetyltransferase ...
<i>orf3</i>	286	54.5	58.5	ESMT1_ZEA, <i>Endosperm C-24</i> sterol methyltransferase, <i>Zea mays</i> (AAB70886.1) UBIE_SALTY, <i>Salmonella typhimurium</i> (NP_462855) EXPE3_SMEL, <i>Sinorhizobium meliloti</i> , (NP_437428) NodS, <i>Rhizobium etli</i> CE3 (Villalobos et al. 1994)	18/28 (344) 16/26 (251) 13/24 (281) 11/21 (201)	Methyltransferase ...

^a Percent identical/similar and number of amino acids (in parentheses).

tants, a polymerase chain reaction (PCR) on the putative mutants was done with the primers IL-106 and IL-107 that generates a fragment covering the three ORFs. This fragment then was digested by *Eco*RI and showed two bands on agarose gel for the double recombinants instead of one band for the wild-type strains. The four different transconjugants were designated FAJ1201 (insertion in *orf1* encoding for acetyltransferase1), FAJ1202 (insertion in *orf2* encoding for acetyltransferase2), FAJ1203 (insertion in *orf3* encoding for methyltransferase), and FAJ1204 (insertion in *orf1* and *orf2* encoding for the two acetyltransferases). Finally, the structure of the mutated region was verified by Southern hybridization.

The LPSs of the four mutants were analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining (Fig. 5). The LPSs of the four mutants were devoid of O-antigen because no LPS I band could be detected on the gel. These results suggest that the acetyl and methyl decorations mediated by the acetyl- and methyltransferases identified in this study are essential for O-antigen synthesis. Both acetyltransferases are essential for O-antigen synthesis because none of the single acetyltransferase mutants produces O-antigen. Therefore, no functional redundancy between the two acetyltransferases is observed.

To prove that the phenotype of the different mutants was due to the mutation in the respective locus and not the result of

polar effects on putative downstream genes, complementation experiments were done. Therefore, the three genes identified in this study each were amplified by PCR and subsequently cloned in the cloning vector pFAJ1708 carrying the *npfII* promoter, resulting in pCMPG13001 (carrying *orf1*), pCMPG13002 (carrying *orf2*), and pCMPG13003 (carrying *orf3*). Also, *orf1* and *orf2* were amplified together and cloned into pFAJ1708, resulting in pCMPG13004 in order to complement the double mutant FAJ1204. The four constructed clones then were mobilized into the respective mutants to determine if supplying the genes *in trans* could restore the wild-type phenotype. The complemented mutant strains were able to synthesize LPSI as the parental strain (Fig. 5), thus demonstrating that the mutant phenotype observed was due to a nonpolar frameshift mutation in the respective loci.

Composition analysis and HPAEC analysis of the LPSs from *R. etli* CE3 and from its mutants, FAJ1201, 1202, 1203, and 1204.

The glycosyl composition for the various LPSs is given in Table 4. The values are relative mole ratios normalized to the level of glucosamine (i.e., GlcNAc in Table 4), a component of the lipid A portion of the LPS. Based on the known structure of the LPS from *R. etli* CE3 (Forsberg et al. 2000), there is 1 mol of glucosamine per mole of LPS. The results show that mutants

Fig. 3. Multiple protein sequence alignment of the *Rhizobium etli* *orf1* and *orf2* with acetyltransferases of the LacA-NodL family. Only the common region of 60 amino acids near the C-terminus in all of these proteins is shown. The alignments were generated from CLUSTAL W (Thompson et al. 1994). Residues identical or similar in all sequences are shaded. Gaps introduced for optimal alignment are marked by dashes. Arrows indicate the first residue of the isoleucine patch. Residues identical or similar in all sequences are shaded. Gaps introduced for optimal alignment are marked by dashes. Re, *R. etli* CE3; Mja, *Methanococcus jannaschii*; Aer, *Aeromonas hydrophila*; Ecoli, *Escherichia coli* O113; Strep, *Streptococcus thermophilus*; Rleg, *R. leguminosarum*; Staph, *Staphylococcus aureus*; Vchol, *Vibrio cholera*; Mloti, *Mesorhizobium loti*; Bfrag, *Bacteroides fragilis*; Prot, *Proteus mirabilis*.

Fig. 4. Multiple protein sequence alignment of part of the *Rhizobium etli* CE3 *orf3* with similar methyltransferases. Only the region with the three motifs of sequence similarity present in AdoMet-dependent methyltransferase and identified by Kagan and Clarke (1994) is shown. The alignments were generated from CLUSTAL W (Thompson et al. 1994). Residues identical or similar in all sequences are shaded. Gaps introduced for optimal alignment are marked by dashes. Residues identical or similar in all sequences are shaded. Gaps introduced for optimal alignment are marked by dashes. RE, *R. etli* CE3; XANAC, *Xanthomonas axonopodis*; SALTY, *Salmonella typhimurium*; BACSU, *Bacillus subtilis*; ERWHE, *Erwinia herbicola*; SMEL, *Sinorhizobium meliloti*; YEAST, *Saccharomyces cerevisiae*; ZEA, *Zea mays*.

FAJ1200 (Lerouge et al. 2001) through FAJ1204 have identical compositions. The LPSs from all of these mutants are devoid of all of glycosyl residues that are characteristic of the O-chain polysaccharide (i.e. Fuc, 3-*O*-methyl-6-deoxytalose [3-Me-6-deoxyTal] and GlcA). Some of the LPS preparations contain trace levels of GlcA and glucose (Glc) which are most likely due to slight contamination by extracellular polysaccharide. All the mutants contain galacturonosyl (GalA), galactosyl (Gal), and mannosyl (Man) residues in a ratio that is consistent with the reported core structure (Carlson et al. 1995; Forsberg and Carlson 1998). The lack of O-antigen glycosyl residues is consistent with the PAGE pattern that shows a lack of LPSI in these mutant strains.

The HPAEC profiles of the oligosaccharides released by mild acid hydrolysis from the LPSs of FAJ1201 and the wild type are shown in Figure 6. The HPAEC profiles of the other mutants are very similar to the profile of FAJ1201 and, therefore, are not shown. The results show that all of the LPSs, on mild acid hydrolysis, produce monomeric Kdo, GalA, the tetrasaccharide (and its anhydro Kdo versions), and trisaccharide core oligosaccharides. These are the common core components that previously have been reported for *R. etli* and *R. legumino-*

sarum LPSs (Carlson et al. 1995; Forsberg and Carlson 1998). The monomeric Kdo is due to the external Kdo residue when it does not contain O-chain polysaccharide at position *O*-4. Monomeric GalA is thought to be released due to some degradation of the core region during mild acid hydrolysis (Carlson et al. 1995). These results show that all of the mutant LPSs have a complete core region.

In summary, these results support the conclusion that mutants FAJ1201, 1202, 1203, and 1204 have LPSs that lack the O-chain polysaccharide, but contain an intact core structure.

Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the mutants and complemented strains. Lipopolysaccharide (LPS) samples were prepared for electrophoretic analysis by the method described by Valverde (1997). The samples were loaded on a 15% SDS-PAGE and the gel was silver stained by the method of Tsai and Frasch (1982). Lane 1, CMPG13010 (FAJ1201 complemented with *orf1*); lane 2, FAJ1201 (insertion in *orf1* encoding for acetyltransferase1); lane 3, CMPG13011 (FAJ1202 complemented with *orf2*); lane 4, FAJ1202 (insertion in *orf2* encoding for acetyltransferase 2); lane 5, CMPG13012 (FAJ1203 complemented with *orf3*); lane 6, FAJ1203 (insertion in *orf3* encoding for methyltransferase); lane 7, CMPG13013 (FAJ1204 complemented with *orf1* and *orf2*); lane 8, FAJ1204 (insertion in *orf1* and *orf2* encoding for the two acetyltransferases); lane 9, wild-type CE3.

Fig. 6. High performance anion exchange chromatography profiles of the oligosaccharides released from the indicated lipopolysaccharides by mild acid hydrolysis. These various oligosaccharides have been identified previously from the core structure of *Rhizobium etli* CE3 LPS (Carlson et al. 1995; Forsberg and Carlson 1998). Peak 1 = Kdo, peak 2 = galacturonosyl, peak 3 = tetrasaccharide, peak 4 = anhydrotetrasaccharide, peak 5 = another anhydrotetrasaccharide, peak 6 = trisaccharide.

Table 4. Composition analysis of lipopolysaccharides (LPSs) from *Rhizobium etli* CE3, FAJ1200, FAJ1201, FAJ1202, FAJ1203, and FAJ1204 by gas chromatography-mass spectrometry^a

Residues	FAJ1200 LPS	FAJ1201 LPS	FAJ1202 LPS	FAJ1203 LPS	FAJ1204 LPS	CE3 LPS
2,3,4-Tri- <i>O</i> -MeFuc	—	—	—	—	—	0.10
2- <i>O</i> -MeFuc	—	—	—	—	—	0.30
3- <i>O</i> -Me-6-deoxy-Tal	—	—	—	—	—	1.30
Fuc	—	—	—	—	—	1.70
GlcA	—	*Tr	*Tr	*Tr	*Tr	2.60
GalA	2.4	2.6	2.7	2.9	3.0	2.40
Man	1.7	1.8	1.9	1.7	1.9	1.30
Glc	*0.2	*0.4	*0.5	*0.5	*0.4	0.10
Gal	1.0	1.1	1.0	1.1	1.0	1.20
GlcNAc	1.0	1.0	1.0	1.0	1.0	1.00
GlcN-onate	1.0	0.9	1.0	0.9	0.9	0.90
QuiNAc	—	—	—	—	—	0.10

^a Values are relative moles normalized to the amount of glucosamine quantified as *N*-acetylglucosamine (GlcNAc). 2,3,4-Tri-*O*-MeFuc = 2,3,4-tri-*O*-methylfucose, 2-*O*-MeFuc = 2-*O*-methylfucose, 3-*O*-Me-6-deoxy-Tal = 3-*O*-methyl-6-deoxytalose, Fuc = fucose, GlcA = glucuronic acid, GalA = galacturonic acid, Man = mannose, Glc = glucose, Gal = glucose, GlcN-onate = 2-aminogluconic acid, QuiNAc = *N*-acetylquinovosamine. — = Absent, Tr = traces, * = glucose and the trace amount of glucuronic acid are common contaminants found in these LPS preparations due to the presence of a small amount of extracellular polysaccharide. These glycosyl residues are not part of these LPS molecules.

Whenever an *R. etli* mutant lacks the O-antigen, it is defective in infection on bean and gives rise to widely dispersed pseudonodules (Lerouge et al. 2001; Noel and Duelli 2000). Inoculation of the different LPS mutants on bean revealed an Inf⁻ phenotype.

DISCUSSION

Only a few studies have addressed the biological significance of *O*-acetylation or methylation of bacterial polysaccharides. Aside from the obvious consideration of hydrophobicity, the biological significance of the various partially or fully *O*-methylated and *O*-acetylated residues in the *R. etli* O-antigen is not clear. Furthermore, several reports argue for a shift toward more hydrophobic LPS with higher levels of acetylation or methylation during symbiosis (Bhat and Carlson 1992; Kannenberg and Carlson 2000, 2001). The function of this increase in hydrophobicity may be necessary for the invasion of host cells which occurs by an endocytosis.

Some of the changes observed in the LPS from nodule bacteria are believed to be host specific (changes in LPS are induced by host exudates (anthocyanins) and changes are dependent on the type of nodules). Currently, no information is available on the genetic mechanisms that underlie these observed changes. The higher degree of methylation or acetylation during symbiosis may be the result of a symbiosis-dependent upregulation (for instance, by anthocyanins) of the genes encoding proteins that introduce these O-antigen decorations. Most likely, the plant compounds trigger modifications in bacterial enzyme activity or gene expression. However, further analysis of the regulatory mechanisms underlying LPS modifications induced by plant exudate factors, such as the anthocyanin identified by Duelli and Noel (1997), could lead to possible clues as to how rhizobial LPS synthesis is regulated in planta (Spaink 2000). In pathogenic bacteria, the O-antigen and the acetylation state clearly is important for infection. The acetylated O-antigen is the dominant antigen in many immune responses and it has been shown in a number of species to be critical for protection (Lerouge and Vanderleyden 2002; Reeves 1995).

An interesting question is at what stage of LPS synthesis acetylation or methylation of the O-antigen takes place. As can be derived from the *R. etli* O-antigen structure (Forsberg et al. 2000), two general types of methylation exist. The first type is variable methylation, which occurs in methylation of the capping fucosyl residue, and of the 3- and 3,4-linked fucosyl residues. The second type is constant methylation. This is illustrated by the fact that the terminally linked 3-*O*-methyl-6-deoxytalose, and the GlcA methyl ester, in the repeat unit are always methylated (Fig. 1). Therefore, we speculate that the 3-*O*-Me-6-deoxy-Tal is methylated at the sugar nucleotide level, whereas the variable fucosyl methylations occur postpolymerization. As mentioned in the introduction, LpsB and LpeA are involved in the variable methylation of the fucosyl residues, which was confirmed by chemical analysis, and the mutants still have LPS with O-antigen (Duelli et al. 2001; Noel et al. 2000). However, a mutation in the *lpeA* locus that is involved in the 2,3,4-tri-*O*-methylation of the fucosyl capping residue results in the absence of the entire fucose residue and not just the absence of the methyl groups on that residue (Duelli et al. 2001). Therefore, it may be that the synthesis and addition of the unique tri-*O*-methylfucosyl capping residue occur separately from the synthesis of the rest of the O-chain polysaccharide. Likewise, the structural data show that there are five repeat units and five *O*-acetyl substituents suggesting that *O*-acetylation of a repeating unit sugar is probably required for the synthesis of the repeat unit. Thus, the methylation genes

identified in this study may be involved in the methylation of the 6-deoxytalose residue (not the fucosyl residues) or the GlcA methyl esterification, and the *O*-acetylation genes may be required for acetylation of a repeating unit sugar (Fig. 1).

Interestingly, both the methylation and acetylation decorations mediated by the loci identified in this study are essential for the formation of the O-antigen polysaccharide. Contrary, the absence of methylation of the fucosyl capping residue does not affect LPSI synthesis (Duelli et al. 2001), and the absence of 2-*O*-methylation of the internal fucose residues in strain CE395 leads only to a reduction (50%) in the relative amount of LPSI (Noel et al. 2000). In enteric bacteria, there also are examples of decorations that are not necessary for O-antigen polysaccharide synthesis (Slauch et al. 1995) as well as examples that are required for O-antigen synthesis (Rocchetta et al. 1999).

It is not yet clear why two different acetyltransferases are present in the identified O-antigen gene cluster. The two identified acetyltransferases in *R. etli* show little sequence similarity to each other (25% amino acid identity; Table 3), suggesting that they acetylate different sugars or recognize another specific sugar environment. In general, little homology exists between the different acetyltransferases of various rhizobia (Berck et al. 1999). However, the presence of these two acetyltransferases can be discussed in more detail with regard to the structure of the O-chain. Analysis of the O-chain structure revealed that one of the acetyl groups is the *N*-acetyl of QuiNAc. The ratio of *O*-acetyl groups to *N*-acetyl groups is exactly five, which is the number of repeating units. As discussed above, it is likely that there is a single *O*-acetyl group per repeating unit, and, therefore, a single location for that *O*-acetyl group. Thus, from the structure, we would predict the presence of two acetyltransferases; one which *N*-acetylates QuiN, and a second which *O*-acetylates one of the three glycosyl residues in the repeat unit. Although no *N*-acetyltransferase genes were identified in this DNA region, there is a precedent for *O*-acetyltransferases catalyzing *N*-acetyl transfer in the urinary pathogen *Providencia stuartii* (Payie and Clarke 1997; Rather et al. 1993), in *Streptococcus thermophilus* Sfi6 (Stingle et al. 1996), and in *Pseudomonas aeruginosa* serotype O5 (Rocchetta et al. 1999).

In conclusion, the three ORFs *orf1*, *orf2*, and *orf3* have been assigned putative functions on the basis of sequence similarity and mutational analysis. Based on similarity analysis and O-antigen structure analysis, *orf1* and *orf2* are proposed to encode acetyltransferases, responsible for acetylation of quinovosamine and of a repeating unit sugar, whereas *orf3* is proposed to encode a methyltransferase responsible for methylation of the 6-deoxytalose residue or the GlcA methyl esterification. Overexpression of enzymatically active methyl- and acetyltransferases and the development of an in vitro assay to measure methyl- or acetyltransferase activity on these specific sugar substrates will be necessary to formulate more detailed functions. Interestingly, in contrast to previously described *R. etli* CE3 mutants affected in O-antigen methyltransferase genes, the *orf3* mutant described in this study does not produce any O-antigen (LPSI), pointing toward the essential role in O-antigen assembly.

MATERIALS AND METHODS

Bacteria and media.

The bacterial strains and plasmids used in this study are listed in Table 2. *Escherichia coli* strains were maintained on LB agar at 37°C and grown in Luria-Bertani (LB) broth supplemented with the appropriate antibiotics (Miller 1972). *R. etli* CE3 and the described mutants (Table 2) were maintained on tryptone-yeast (TY) medium with added calcium dichloride

(Beringer 1974) and supplemented with the appropriate antibiotics. Triparental conjugations were done as previously described (Michiels et al. 1998).

Nucleic acid manipulations and analysis.

Nucleic acid manipulations were done as previously described (Ausubel et al. 1987; Sambrook et al. 1989). Plasmid DNA was prepared, and DNA sequencing was determined with a Tn5 primer, the universal pUC-primer, and the reverse pUC-primer on an ALF automated sequencer (Pharmacia Biotech, Uppsala, Sweden). The nucleotide sequences for *orf1*, *orf2*, and *orf3* have been deposited in the GenBank database under GenBank accession number AF348454.

Cloning of the *lps* genetic region and construction of mutants and complemented mutants.

The 7.8-kb *EcoRI* fragment from pCOS109.11 (cosmid containing the *lps* α region) (Cava et al. 1990) was first cloned into pUCBM20, yielding pFAJ1227. A 3.1-kb *HindIII* fragment of pFAJ1227 containing *orf1*, *orf2*, and *orf3* was subcloned in pUCBM20, yielding pFAJ1235. The nonpolar point mutations and an additional frameshift in *orf1*, *orf2*, and *orf3* were introduced via the QuickChange site-directed mutagenesis (Stratagene, La Jolla, CA, U.S.A.) using the following primer combinations on pFAJ1235: Rhi81 (5'-GGTCGATGGAATTC GATTACGATCGGTAATCGCG-3') and Rhi82 (5'-CGCGAT TACCGATCGTAATCGAATTCATCGACC-3') for mutation in *orf1*, Rhi83 (5'-GGCGCATGCGAATGCAGAATTC AACC GTTGGC-3') and Rhi84 (5'-GCCAACGGTTGAATTC TGCA TTCGCATGCGCC-3') for mutation in *orf2*, and Rhi85 (5'-GGCAGATGCTGAATTCGGAAGCTATTGCGGCGC-3') and Rhi86 (5'-GCGCCGCAATAGCTTTCCGAATTCAGCA CTCTGCC-3') for mutation in *orf3*. As a result of the mutation, a new and unique *EcoRI* site was created in *orf1*, *orf2*, and *orf3* (Fig. 2; Table 2) (GAATTC in *orf1*, GAATTC in *orf2*, and GAATTC in *orf3*; mutations are shown in bold: insertion of T, G, and TC, respectively), resulting in three new clones, pFAJ1236, pFAJ1237, and pFAJ1238, respectively. Subsequently, the 3.1-kb *HindIII* fragment containing the mutated *orfs* was inserted into pUC18*NotI*, resulting in pFAJ1239, pFAJ1240, and pFAJ1241. A mutation into *orf1* of pFAJ1240 (already containing a mutation in *orf2*) was inserted, using primers Rhi81 and Rhi82, thereby creating an *EcoRI* site in both *orf1* and *orf2* and resulting in clone pFAJ1242. Finally, the *HindIII* fragments of pFAJ1239, pFAJ1240, pFAJ1241, and pFAJ1242 were recloned as *NotI* fragments in the suicide vector (Table 2), creating pFAJ1243, pFAJ1244, pFAJ1245, and pFAJ1246. These plasmids were introduced into *R. etli* CE3, and double recombinants were selected as described previously (Rosemeyer et al. 1998) creating a mutant in *orf1* (FAJ1201), *orf2* (FAJ1202), and *orf3* (FAJ1203) and a double mutant in *orf1* and *orf2* (FAJ1204). To distinguish between wild-type strains and double recombinants, PCR was done on the putative mutants with primers IL-106 (5'-GTCGGCAAAAGG CAATTGCTCGG-3') and IL-107 (5'-CGCAATGATTCCTGG GGTATCC-3'), yielding a fragment containing the three *orfs*. *EcoRI* digestion on this PCR fragment was performed and showed two bands on agarose gel for the double recombinants instead of one band for the wild-type strain.

In order to complement each mutant *in trans* with the respective wild-type allele, DNA fragments containing the respective genetic loci were amplified by PCR with platinum *Pfx* DNA polymerase (Life Technologies, Gaithersburg, MD, U.S.A.) using several primer combinations: RHI-578 (5'-AGGCGCGCCGCGGATACGGCGCTGCGAACG-3') and RHI-579 (5'-TCCCCCGGGGGCCAGCAAGGCGCCATTGG C-3') for amplification of *orf1*, RHI-580 (5'-AGGCGCGCCG

GACGATGTTTGGATCAGCTTCGG-3') and RHI-581 (5'-TC CCCCCGGCGTAGTGC GGCGCCTTCCAACG-3') for amplification of *orf2*, RHI-582 (5'-AGGCGCGCCCGCTGGATCT GTCGTCACAAAGG-3') and RHI-583 (5'-TCCCCCGGGCG AACAGGCCCCAACGCTTCGC-3') for amplification of *orf3*, and RHI-578 and RHI-581 for amplification of *orf1* and *orf2*. These PCR products then were ligated to the pFAJ1708 vector (Dombrecht et al. 2001), resulting in pCMPG13001, pCMPG13002, pCMPG13003, and pCMPG13004, respectively. These plasmids were mobilized into FAJ1201, FAJ1202, FAJ1203, and FAJ1204 respectively, resulting in the transconjugants CMPG13010, CMPG13011, CMPG13012, and CMPG13013.

Database searching and multiple alignments.

Database searches were performed using the programs BLASTX and BLASTP (Altschul et al. 1990). The ClustalW algorithm (Thompson et al. 1994) was used for multiple protein alignments and the alignment layout was prepared by the Genedoc program (Nicholas et al. 1997).

LPS purification and characterization.

One liter of each of the mutant strains was grown in TY-calcium medium. The bacteria were harvested by centrifugation, and the LPS extracted by hot phenol-water extraction and further purified from the aqueous layer as described by Lerouge and associates (2001).

Crude LPS extracts were further purified as described by Valverde and associates (1997) and separated by SDS-PAGE using a 15% resolution and a 5% stacking gel, according to Laemmli (1970). LPS profiles were visualized by silver staining (Tsai and Frasch 1982).

The purified LPS from CE3 and the mutant strains were hydrolyzed in 1% acetic acid at 100°C for 1 h. The oligosaccharides released by mild acid hydrolysis were analyzed by HPAEC as previously described (Carlson et al. 1995).

The glycosyl composition analysis was performed essentially as described by Lerouge and associates (2001). The glycosyl compositions of the purified LPS were determined by the preparation and gas chromatography-mass spectrometry analysis of trimethylsilyl methylglycosides and alditol acetates.

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