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Genetic Basis for *Rhizobium etli* CE3 O-Antigen O-Methylated Residues That Vary According to Growth Conditions▽

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The *Rhizobium etli* CE3 O antigen is a fixed-length heteropolymer with O methylation being the predominant type of sugar modification. There are two O-methylated residues that occur, on average, once per complete O antigen: a multiply O-methylated terminal fucose and 2-O methylation of a fucose residue within a repeating unit. The amount of the methylated terminal fucose decreases and the amount of 2-*O*-methylfucose increases when bacteria are grown in the presence of the host plant, *Phaseolus vulgaris*, or its seed exudates. Insertion mutagenesis was used to identify open reading frames required for the presence of these O-methylated residues. The presence of the methylated terminal fucose required genes *wreA*, *wreB*, *wreC*, *wreD*, and *wreF*, whereas 2-O methylation of internal fucoses required the methyltransferase domain of bifunctional gene *wreM*. Mutants lacking only the methylated terminal fucose, lacking only 2-O methylation, or lacking both the methylated terminal fucose and 2-O methylation exhibited no other lipopolysaccharide structural defects. Thus, neither of these decorations is required for normal O-antigen length, transport, or assembly into the final lipopolysaccharide. This is in contrast to certain enteric bacteria in which the absence of a terminal decoration severely affects O-antigen length and transport. *R. etli* mutants lacking only the methylated terminal fucose were not altered in symbiosis with host *Phaseolus vulgaris*, whereas mutants lacking only 2-*O*-methylfucose exhibited a delay in nodule development during symbiosis. These results support previous conclusions that the methylated terminal fucose is dispensable for symbiosis, whereas 2-O methylation of internal fucoses somehow facilitates early events in symbiosis.

O antigens typically constitute the distal portions of lipopolysaccharides (LPS) and help determine the diverse surface characteristics of Gram-negative bacteria. These repeat unit carbohydrate polymers vary tremendously in structure and, as a family, they exhibit all known sugars and sugar modifications, linked in myriad ways forming homopolymers and heteropolymers. Control of polymer length also varies, allowing highly uniform to completely random lengths. Great diversity of O-antigen structures even within a species is well known. Moreover, O antigens of a single strain can vary according to growth and environmental conditions. One such condition is the presence of a multicellular host (5, 18, 36, 40, 42, 44).

Rhizobium etli CE3 fixes nitrogen inside root nodules it incites on the common bean *Phaseolus vulgaris.* The O antigen of its LPS (Fig. 1) is essential for bacterial infection during development of this symbiosis (41). In addition, at least two alterations occur in the O antigen when *R. etli* CE3 is grown in the presence of either the host plant or plant exudates. The content of the multiply O-methylated terminal fucose is decreased (19, 44), whereas the 2-O methylation of internal fucoses (2OMeFuc) increases twofold (Fig. 1) (15, 44). In addition to the multiply O-methylated terminal fucose and 2OMeFuc, methylation occurs always on 6-deoxytalose and likely on glucuronic acid to yield 3-*O*-methyl-6-deoxytalose (3OMe6dTal) and methyl-esterified glucuronyl (MeGlcA) residues (Fig. 1) (22); however, the incidence of these methylations is not known to vary with growth condition. The genetics responsible for the variable O methylations and the additions of the residues they modify have not been elucidated.

Most mutations affecting the known *R. etli* CE3 O-antigen structure map to a 28-kb genetic cluster on the chromosome (Fig. 2) (previously referred to as *lps* region α [8, 19, 37, 40, 45]). Genes and mutations within this cluster previously have been given the designations *lps* (9) and *lpe* (19). Recently, the new designation *wre* has been sanctioned by the Bacterial Polysaccharide Gene Database for this genetic cluster and other genes specifically devoted to the *R. etli* CE3 O antigen, in keeping with the system of nomenclature for bacterial polysaccharide genes (47).

Duelli et al. (19) identified a 3-kb genetic locus that is required for the presence of the 2,3-di-*O*-methylfucose or 2,3,4-tri-*O*-methylfucose at the terminus of the O antigen. Now known to be near one end of the O-antigen genetic cluster (Fig. 2), the DNA sequence reported by Duelli et al. encompasses nucleotides 807701 to 810147 of the subsequently determined genome sequence (28). Sequence and annotation of the 3-kb locus have since been revised. In place of the four open reading frames (ORFs) suggested previously (19), the current annotation predicts two ORFs: *wreA* and *wreC* (Fig. 2). The *wreA* ORF is predicted to encode a methyltransferase (19), but the predicted WreC polypeptide sequence matches no known methyltransferase or glycosyltransferase or any other polypeptide sequence in the database (Fig. 3). When it became clear that this locus was part of the larger O-antigen genetic cluster, the nucleotide sequence suggested that three genes contiguous to *wreA* also might encode functions needed for synthesis and addition of the terminal fucose. The results to be shown bore out predictions of this hypothesis.

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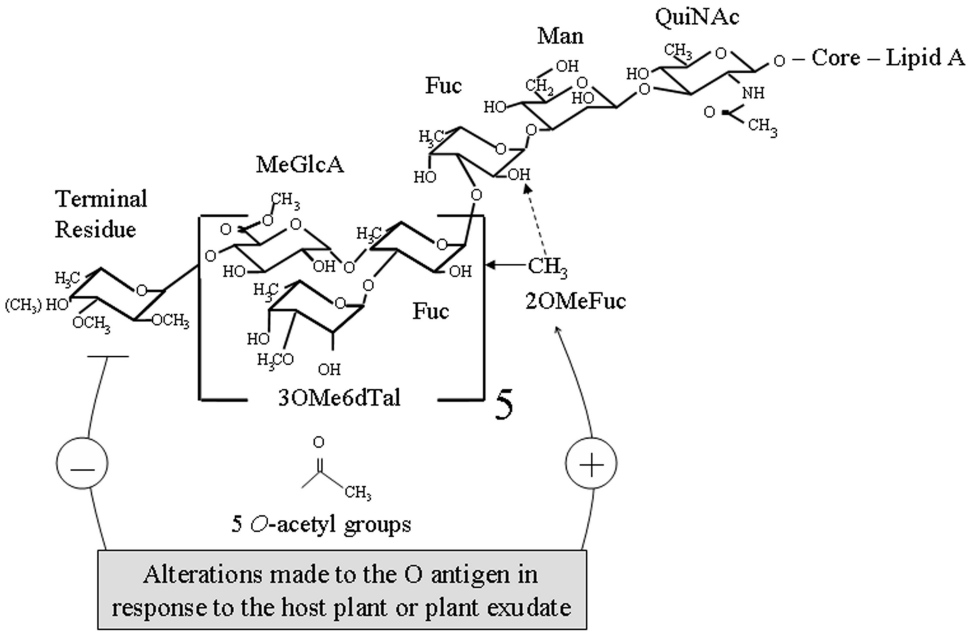

FIG. 1. *R. etli* CE3 O-antigen structure (22). The portion of the LPS conceptually defined as O antigen begins with *N*-acetyl-quinovosamine (QuiNAc) at the reducing end followed by a mannose (Man) residue and a fucose (Fuc) residue. Attached to this fucose is the repeating unit consisting of one fucose residue, one 3-*O*-methyl-6-deoxytalose residue (3OMe6dTal), and one glucuronyl methyl ester residue (MeGlcA). The sugars of the repeating unit are added sequentially exactly five times (in most molecules). An *O*-acetyl group is present in each of the repeating units, but its location is unknown at this time. Growth in TY culture results in one 2-*O*-methylfucose (2OMeFuc) per O antigen on average (22). The O-antigen backbone is capped with a 2,3-di-*O*-methylfucose (referred to as the terminal residue in this report) on which additional O methylation at the 4-position is variable as indicated by parentheses. Growth of the bacteria in the presence of the host plant or plant exudates induces the increase of 2-O methylation of internal fucose (2OMeFuc) residues and decreased relative amount of the terminal residue (44).

The gene responsible for the other conditionally variable O-antigen methylation, the 2-O methylation of internal fucose residues (2OMeFuc), had not been identified in prior published work. However, among mutants isolated by random Tn*5* mutagenesis, a few had been shown to lack 2OMeFuc entirely (44). We show here that the transposon insertions were located in the bifunctional gene *wreM*. Furthermore, results of directed insertion mutagenesis confirm two separate enzymatic domains encoded by this gene, with the α domain being required for the 2-O methylation activity and mutation of the other domain resulting in a truncated O antigen. Mutants from the directed mutagenesis that appeared to have no LPS defects

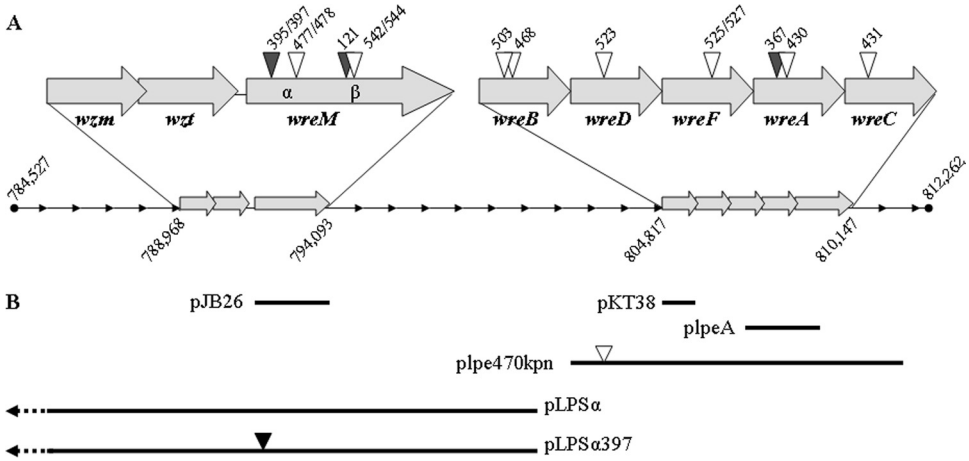

FIG. 2. *R. etli* CE3 O-antigen genetic cluster. (A) The *R. etli* CE3 chromosomal O antigen genetic cluster spans nucleotides 784527 to 812262 of the genome sequence (28) and consists of 25 putative ORFs. ORFs relevant to the present study are enlarged, and the relative locations of mutations are indicated. White triangles indicate mutations created by insertion of antibiotic cassettes, and black triangles indicate mutations created by Tn*5* mutagenesis. The strain numbers carrying these mutations are indicated above the triangles. (B) The solid bars represent the extents of *R. etli* CE3 DNA cloned for complementation analysis. The scale and positions match those of the lower map in panel A.

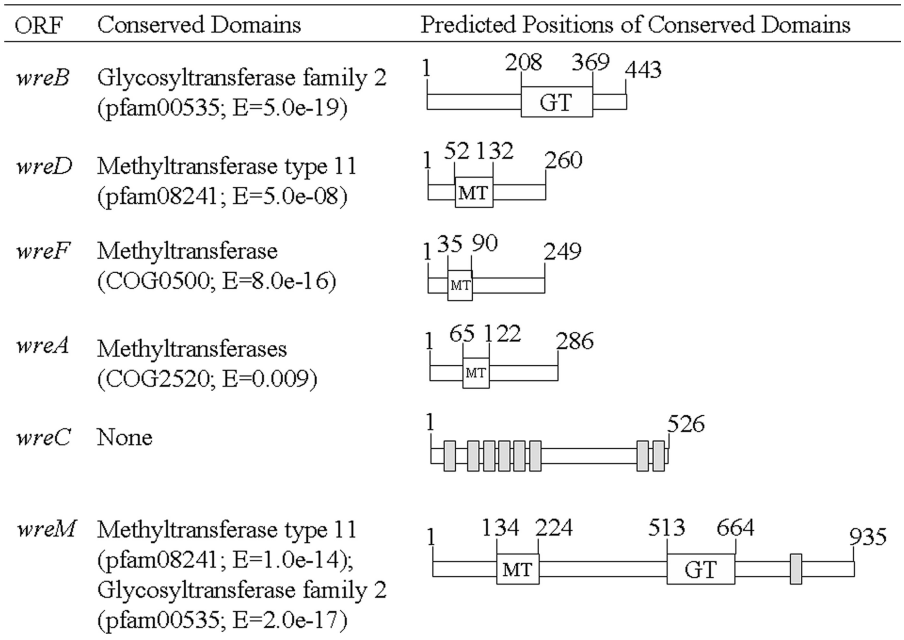

FIG. 3. Conserved domain predictions. Spanning nucleotides 804817 to 810147 of the genome sequence (28), ORFs RHE_CH00766, RHE_CH00767, RHE_CH00768, RHE_CH00769, and RHE_CH00770 were named *wreB*, *wreD*, *wreF*, *wreA*, and *wreC*, respectively. Previously, *wreF*, *wreA*, and *wreC* were referred to as *nlpe2*, *lpeA*, and *nlpe1*, respectively (19). ORF RHE_CH00755, spanning nucleotides 791286 to 794093, was named *wreM.* Predicted positions of conserved domains are indicated by amino acid positions. Abbreviations: GT, conserved glycosyltransferase domain; MT, conserved methyltransferase domain. Gray boxes indicate the predicted transmembrane domains.

other than the lack of 2OMeFuc served as tools to assess the importance of just this structural feature in the symbiosis with *P. vulgaris*.

MATERIALS AND METHODS

Growth of bacteria. *R. etli* strains were grown to stationary phase at 30°C on a rotating shaker in TY liquid medium (0.5% tryptone [Difco Laboratories], 0.3% yeast extract [Difco], 10 mM $CaCl_2$). *Escherichia coli* strains were grown in Luria-Bertani (LB) liquid medium (1.0% tryptone, 0.5% yeast extract, 0.5% NaCl) to stationary phase at 37°C on a rotating shaker (50). Agar medium contained 1.5% Bacto agar (Difco).

Computer analysis of predicted protein sequences. Amino acid sequences of ORFs deduced from the *R. etli* CE3 genomic DNA sequence (28; http://www.ccg.unam.mx/retlidb/) were compared to nonredundant (nr) protein sequences by BLAST search tool at the National Center for Biotechnology Information (NCBI) (2). Predicted transmembrane domains were determined by transmembrane helix prediction (TMHMM, version 2.0) at the Center of Biological Sequence Analysis (CBS) at the Technical University of Denmark (http://www.cbs.dtu.dk/index.shtml).

Materials and techniques for DNA isolation. Genomic DNA was isolated from *R. etli* strains by using an AquaPure Genomic DNA isolation kit (Bio-Rad) for use in cloning. *E. coli* JM109 (Promega) or INVαF′ (Invitrogen) competent cells were transformed (29), and plasmids were isolated from *E. coli* by using QIAprep Spin Miniprep (Qiagen). DNA was recovered from agarose gels by using a GeneClean II kit (MP Biomedicals, LLC) and modified with restriction enzymes purchased from New England Biolabs (NEB; Beverly, MA). Custom primers were synthesized by Operon Biotechnologies, Inc. (Huntsville, AL). DNA sequencing was performed by Agencourt Bioscience (Beverly, MA).

Cloning and site-directed mutagenesis of *wreB*, *wreD*, and *wreF*. Genomic DNA from mutant strain CE430 (19) was digested with enzyme KpnI. KpnI restriction sites at positions 801707 and 811521 of the *R. etli* CE3 genome sequence excise a 9.8-kb fragment that contains the *wreB*, *wreD*, *wreF*, *wreA*, and *wreC* ORFs and additional upstream DNA. The 9.8-kb fragment carrying a kanamycin (Km) cassette in *wreA* of CE430 was ligated to plasmid pBluescript II KS(−) (pBSKS−; Stratagene), creating plasmid plpe430kpn (17). For mutagenesis of *wreB*, plpe430kpn was digested with the enzymes PstI and SalI. The resulting 1.7-kb product containing *wreB* was ligated to plasmid pEX18Tc (30), and the gentamicin (Gm) cassette from plasmid pUCGm (52) was inserted in the SacI site of *wreB*, creating plasmid pKT35.

Using PCR, *wreD* was amplified from *R. etli* CE3 genomic DNA as a 1.2-kb product by using the primers *wreD*-XhoI (5′-GGCTCGAGAGCAAACAGATCCTACTTCG-3′) and *wreD*-PstI (5′-GGCTGCAGATATTCCAATATTCGCACCG-3′). The PCR product was ligated to pBSKS−, and a Gm cassette (52) was ligated between the HindIII sites, which delete 69 bp of the *wreD* ORF. *wreD* now carrying a Gm cassette was excised from pBSKS− and ligated to pEX18Tc (30), creating plasmid pKT49.

wreF was amplified from *R. etli* CE3 genomic DNA as an 850-bp PCR product by using the primers *wreF*-KpnI (5′-CGGTACCTTCGCCTCATTCTTCACAGT-3′) and *wreF*-XbaI (5′-GTCTAGAGACAGATAGCGAGCGATCAG-3′). The PCR product was digested directly with enzymes KpnI and XbaI and ligated to pEX18Tc (30). The Km cassette from plasmid pBSL86 (1) or a Gm cassette (52) was inserted in the BamHI site of *wreF*, creating plasmids pTW7 and pTW8, respectively.

Separately, plasmids pKT35, pKT49, pTW7, and pTW8 were transferred into *R. etli* CE3 by triparental mating (26) with mobilizing plasmid MT616 (20). CE3 transconjugants containing these constructs were selected on TY agar plates supplemented with 30 μg of nalidixic acid/ml, 200 μg of streptomycin/ml, and either 30 μg of kanamycin/ml or 30 μg of gentamicin/ml. Among those that were also sensitive to 1 μg of tetracycline/ml and resistant to 8% sucrose on TY agar, colonies were saved after verifying by PCR the absence of the wild-type allele and presence of a mutant allele (data not shown). The PCR primers used for verification of *wreD* and *wreF* mutant alleles were the same primer sets used for cloning (mentioned above). For verification of the *wreB* mutant allele, the primers were *wreB*1 (5′-AATTCAGCACGGAGCCTATC-3′) and *wreB*2 (5′-CTCACCGTACCAATGCATCA-3′). The resulting *R. etli* strains were: CE503, CE523, CE527, and CE525, respectively (Table 1).

Complementation of *wreB*, *wreD*, and *wreF*. For complementation of *wreB*, the 1.7-kb PstI-SalI fragment from plpe430kpn was ligated to plasmid pFAJ1700 (16), creating plasmid pKT38. For complementation of *wreD* and *wreF*, CE470 (3) genomic DNA, which carries a Km cassette in the HindIII site of *wreE* (formerly *exoU*; RHE_CH00764), was digested with enzyme KpnI. The 9.8-kb fragment carrying a Km cassette in *wreE* of CE470 was ligated to pFAJ1700, creating plasmid plpe470kpn.

pKT38 was transferred into CE503 and plpe470kpn transferred into CE503, CE523, CE525, and CE527 by triparental mating, as described above. Strains

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description or genotype[a]	Source or reference
Strains		
R. etli		
CE3	Wild-type strain, *str-1*, Lps$^+$	43
CE3α	CE3 carrying pLPSα, Lps$^+$	44
CE121	CE3 derivative, *str-1*, *wreM*β::Tn*5*, Lps$^-$	9
CE367	CE3 derivative, *str-1*, *wreA*::Tn*5*, Lps$^+$	19, 53
CE395	CE3 derivative, *str-1*, *wreM*α::Tn*5*, Lps$^+$	44
CE397	CE3 derivative, *str-1*, *wreM*α::Tn*5*, Lps$^+$	This study
CE397α	CE397 carrying pLPSα, Lps$^+$	This study
CE397α397	CE397 carrying pLPSα397, Lps$^+$	This study
CE430	CE3 derivative, *str-1*, *wreA*::Km, Lps$^+$	19
CE431	CE3 derivative, *str-1*, *wreC*::Km, Lps$^+$	19
CE468	CE3 derivative, *str-1*, *wreB*::Km, Lps$^+$	3
CE470	CE3 derivative, *str-1*, *wreE*::Km, Lps$^-$	3
CE477	CE3 derivative, *str-1*, *wreM*α::Km, Lps$^+$	This study
CE477/pJB26	CE477 carrying plasmid pJB26, Lps$^+$	This study
CE478	CE3 derivative, *str-1*, *wreM*α::Gm, Lps$^+$	This study
CE478/pJB26	CE478 carrying plasmid pJB26, Lps$^+$	This study
CE503	CE3 derivative, *str-1*, *wreB*::Gm, Lps$^+$	This study
CE503/pKT38	CE503 carrying plasmid pKT38, Lps$^+$	This study
CE503/plpe470kpn	CE503 carrying plasmid plpe470kpn, Lps$^+$	This study
CE523	CE3 derivative, *str-1*, *wreD*::Gm, Lps$^+$	This study
CE523/plpe470kpn	CE523 carrying plasmid plpe470kpn, Lps$^+$	This study
CE525	CE3 derivative, *str-1*, *wreF*::Gm, Lps$^+$	This study
CE525/plpe470kpn	CE525 carrying plasmid plpe470kpn, Lps$^+$	This study
CE527	CE3 derivative, *str-1*, *wreF*::Km, Lps$^+$	This study
CE527/plpe470kpn	CE527 carrying plasmid plpe470kpn, Lps$^+$	This study
CE538	CE367 derivative, *str-l*, *wreA*::Tn*5*, *wreM*α::Gm, Lps$^+$	This study
CE538/plpeA	CE538 carrying plasmid plpeA, Lps$^+$	This study
CE538/pJB26	CE538 carrying plasmid pJB26, Lps$^+$	This study
CE540	CE468 derivative, *str-1*, *wreB*::Km, *wreM*α::Gm, Lps$^+$	This study
CE540/pKT38	CE540 carrying plasmid pKT38, Lps$^+$	This study
CE540/pJB26	CE540 carrying plasmid pJB26, Lps$^+$	This study
CE542	CE3 derivative, *str-1*, *wreM*β::Km, Lps$^-$	This study
CE542/pJB26	CE542 carrying plasmid pJB26, Lps$^+$	This study
CE544	CE3 derivative, *str-1*, *wreM*β::Gm, Lps$^-$	This study
CE544/pJB26	CE544 carrying plasmid pJB26, Lps$^+$	This study
E. coli		
INVαF′	F′ end*A1* rec*A1 hsdR17* (r_K^- m_K^+) sup*E44* thi-*1* gyr*A96 relA1* ϕ80lacZΔM15 Δ(lac*ZYA*-argF)U169λ$^-$	Invitrogen
JM109	*endA1 recA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *relA1 supE44* Δ(*lac-proAB*) [F′ *traD36 proAB laqI*^{q}ZΔM15]	Promega
MT616	*pro thi endA hsdR supE44 recA-J6* pRK2013Km::Tn*9*	20
Plasmids		
pBluescript KS(−)	Cloning vector; Amp^r	Stratagene
plpe430kpn	9.8-kb KpnI fragment of *R. etli* CE430 in pBSKS−	This study
pEX18Tc	Suicide plasmid, Tc^r, *oriT*$^+$, *sacB*$^+$	30
pUCGm	*aaC1* gene cassette, Gm^r	52
pKT35	1.7 kb of plpe430kpn with Gm cassette inserted in SacI site of *wreB* in pEX18Tc	This study
pKT49	*wreD* with Gm cassette inserted between HindIII sites in pEX18Tc	This study
pBSL86	*nptII* gene cassette, Km^r	1
pTW7	*wreF* with Km cassette inserted at BamHI site in pEX18Tc	This study
pTW8	*wreF* with Gm cassette inserted at BamHI site in pEX18Tc	This study
pFAJ1700	Expression vector, Tc^r	16
pKT38	1.7 kb of plpe430kpn carrying *wreB* in pFAJ1700	This study
plpe470kpn	9.8-kb KpnI fragment of *R. etli* CE470 in pFAJ1700	This study
pJB2	*wreM*α with Km cassette inserted at BamHI site in pEX18Tc	This study
pJB3	*wreM*α with Gm cassette inserted at BamHI site in pEX18Tc	This study
pKT66	*wreM*β with Gm cassette inserted at SalI site in pEX18Tc	This study
pKT67	*wreM*β with Km cassette inserted at SalI site in pEX18Tc	This study
pJB26	4.4-kb PCR product of *wreM* inserted in pFAJ1700	This study
plpeA	Wild-type *wreA* in pRK404E1	19
pLPSα	pCOS109.11, 30 kb of CE3 chromosomal O-antigen genetic cluster in pLAFR1	8
pLPSα397	pLPSα in which *wreM397* replaces the WT allele	This study
pCR2.1	Cloning vector, Amp^r Km^r	Invitrogen
pJB40	PCR product containing Tn*5* sequence and *wreM*α sequence from CE397 genomic DNA in pCR2.1	This study
pJB41	PCR product containing Tn*5* sequence and *wreM*α sequence from CE395 genomic DNA in pCR2.1	This study
pJB49	PCR product containing Tn*5* sequence and *wreM*α sequence from CE121 genomic DNA in pCR2.1	This study

[a] *str-1*, confers resistance to streptomycin; Lps$^{+/-}$, presence or absence of LPS I; ::Km, insertion of kanamycin resistance cassette; ::Gm, insertion of gentamicin resistance cassette. Gm^r, gentamicin resistance; Tc^r, tetracycline resistance; Amp^r, ampicillin resistance; Km^r, kanamycin resistance.

containing these constructs were selected on TY agar plates supplemented with 30 μg of nalidixic acid/ml, 200 μg of streptomycin/ml, 5 μg of tetracycline/ml, and either 30 μg of kanamycin/ml or 30 μg of gentamicin/ml. Colonies were purified and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (data not shown).

Cloning and site-directed mutagenesis of *wreM*. The *wreM* ORF was amplified from *R. etli* CE3 genomic DNA as a 2.9-kb PCR product by using the primers *wreM*1 (5′-GCTCCTCGTGATTGTTGATG-3′) and *wreM*2 (5′-AGGCTGGAT CGTCGATAAGT-3′). The PCR product was digested with enzyme SalI, which produced a 2.2-kb fragment containing the entire portion of *wreM* matching

methyltransferases (α), while the portion matching glycosyltransferases (β) was truncated. The fragment was ligated to pEX18Tc (30), and a Km cassette (1) or a Gm cassette (52) was inserted at the BamHI site in the *wreM* α domain, creating plasmids pJB2 and pJB3, respectively.

The domain *wreM*β was amplified from *R. etli* CE3 genomic DNA as a 1.2-kb PCR product by using the primers *wreM*β1 (5′-CCGGCACAGGTGGATATA AC-3′) and *wreM*β2 (5′-AGACCGGCAACCGAATGATG-3′). The PCR product was ligated to pEX18Tc (30), and a Gm cassette (52) or Km cassette (1) was inserted at the SalI site, which deletes 141 bp of *wreM*β. This resulted in plasmids pKT66 and pKT67, respectively.

Plasmids pJB2, pJB3, pKT66, and pKT67 were transferred into *R. etli* CE3, and double recombinants having replaced the wild-type allele with a mutant allele were selected, screened, and verified as described above. Mutant strains CE477, CE478, CE542, and CE544, respectively, were created (Table 1).

Complementation of *wreM*. The entire *wreM* ORF (including both α and β domains) was amplified from *R. etli* CE3 genomic DNA as a 4.4-kb fragment by using the primers *wreM*αβ1-KpnI (5′-CGGTACCTCGGACATTCAGTTCTCG CA-3′) and *wreM*αβ2-KpnI (5′-CGGTACCACCGCACGAACATCTTCGAC-3′). The 4.4-kb fragment was ligated to pFAJ1700 (16), creating plasmid pJB26. pJB26 was transferred into CE477, CE478, CE542, and CE544 by triparental mating, and strains containing these constructs were selected and analyzed as described above.

Determination of Tn*5* insertion locations. Isolation of strains CE121 and CE395 after Tn*5* mutagenesis and their mutant phenotypes were reported previously (9, 44). Strain CE397 has not been described previously. Like strain CE395, it was isolated after Tn*5* mutagenesis by screening for colonies that did not lose reactivity to monoclonal antibody (MAb) JIM28 after growth with *P. vulgaris* seed exudates as described previously (44), and its mutant phenotypes are reported here. To locate the Tn*5* insertion sites, genomic DNA from strain CE121 was used as a template for PCR by using the primers *wre*Mαβ2-KpnI (described above) and Tn*5*-out (5′-GCCGCACGATGAAGAGCAGAAG-3′), which is complementary to a portion of the IS*50* elements from the *E. coli* transposon Tn*5* (accession no. U00004) (38). Genomic DNA from strain CE395 or strain CE397 was used as a template for PCR by using the primer *wreM*αβ1-KpnI and the Tn*5*-out primer. In each case, the resulting PCR product, containing a portion of *wreM* sequence and a portion of Tn*5* sequence, was ligated to plasmid pCR2.1 (Invitrogen), creating plasmids pJB41 (CE397), pJB40 (CE395), and pJB49 (CE121). Each plasmid construct was sequenced by using the Tn*5*-out primer.

Genetic complementation and suppression of Tn*5* mutants. For construction of the strain CE397α, plasmid pLPSα (also referred to as pCOS109.11 [8, 44]) was transferred into CE397 by triparental mating, as described above. Colonies were selected on TY agar plates supplemented with 30 μg of nalidixic acid/ml, 200 μg of streptomycin/ml, 5 μg of tetracycline/ml, and 30 μg of kanamycin/ml. These colonies showed full restoration of the wild-type phenotype.

Plasmid pLPSα397 was isolated after *in vivo* homologous recombination in *R. etli* mutant CE397α that replaced the corresponding wild-type allele on pLPSα with the mutant allele *wreM397*, by using *E. coli* to trap the recombinant plasmid in the manner described previously (44). Plasmid pLPSα397 was transferred into CE397 by triparental mating, and colonies were selected as described directly above. The resulting strain was CE397α397 (Table 1). The effects of the Tn*5* mutations are partially suppressed in strain CE397α397, even though all *wreM* alleles are identically mutated, presumably because of the meripolyploid condition conferred by the multicopy plasmid pLPSα397. Similar genetic suppression in strain CE395α395 was reported in detail previously (44).

Construction of *wreM*α/*wreA* and *wreM*α/*wreB* double mutants. Plasmid pJB3 was transferred into *R. etli* CE367 (19, 53) and CE468, which carries a Km cassette in the HindIII site of *wreB* (3), by triparental mating as described above. Transconjugants containing these constructs were selected on TY agar plates supplemented with 30 μg of nalidixic acid/ml, 200 μg of streptomycin/ml, 30 μg of kanamycin/ml, and 30 μg of gentamicin/ml. Double recombinants having replaced the wild-type allele with a mutant allele were selected, screened, and verified as described above. The resulting *R. etli* strains were CE538 and CE540, respectively (Table 1).

Plasmids pJB26 and plpeA (19) were transferred separately into strain CE538 and plasmids pJB26 and pKT38 into strain CE540 by triparental mating, and colonies were selected on TY agar plates supplemented with 30 μg of nalidixic acid/ml, 200 μg of streptomycin/ml, 30 μg of kanamycin/ml, 30 μg of gentamicin/ml, and 5 μg of tetracycline/ml. Complementation with pJB26, plpeA, or pKT38 restored the respective O-methylated residue to the O antigen (see Fig. 7; also, data not shown).

SDS-PAGE and analysis of LPS antigenicity. Bacterial cells from 0.5 ml of fully grown cultures were pelleted, resuspended homogeneously in 0.1 ml of SDS-PAGE sample buffer (9) without mercaptoethanol, and heated at 100°C for 3 min. The soluble SDS extracts were subjected to discontinuous SDS-PAGE (44), in which the resolving gel was prepared with 18% acrylamide. After SDS-PAGE, gel contents were electrotransferred to nitrocellulose, and residual LPS in the gel was stained by a periodate-silver procedure (44). The dried nitrocellulose blots were immunostained by using MAb JIM28 (a generous gift from N. J. Brewin, Norwich, United Kingdom) and anti-rat immunoglobulin M conjugated with alkaline phosphatase (Thermo Scientific) as previously described (42, 53).

LPS sugar compositions. The combined aqueous layers from two sequential hot phenol-water extractions (PWE) of washed bacterial cell pellets or bacteroid preparations were processed as described before (6). Hydrolysis of the LPS sugar linkages, preparation of alditol acetate derivatives, and analysis on a 60-meter SP2330 column (Supelco) in a Hewlett-Packard 5890 gas chromatograph were performed as previously described (22, 44). In Table 2, the values for wild-type strain CE3 are based on five independent experiments; those for CE3α and the *wreM*α mutant are based on three independent experiments; those for the *wreB*, *wreD*, *wreF*, *wreA*, and *wreB*/*wreB*$^+$ mutants are based on two independent experiments; and those for *wreC*, *wreM397*, *wreM397*/α, *wreM397*/α*397*, *wreM*α/*wreA*, and *wreM*α/*wreB* mutants are representative of a single experiment. LPS analyses from the wild-type and *wreB*, *wreD*, *wreF*, *wreA*, and *wreC* mutant strains were verified by combined gas chromatography-mass spectrometry (GC-MS) of alditol acetates (19; data not shown).

Bacteroid preparation. Nodules were harvested from *P. vulgaris* at 18 days postinoculation (dpi) and processed as previously described with minor modifications (53). Briefly, ice-cold extraction buffer (10 mM Tris-HCl, 5 mM $MgCl_2$ [pH 7.5]) was added to nodules (0.3 ml/100 mg), and nodules were crushed vigorously in a tube on ice. Samples were centrifuged at 380 × *g* for 1 min at 4°C, and the supernatant was further centrifuged at 16,000 × *g* for 5 min at 4°C. The resulting pellet was washed twice with 1.0 ml of ice-cold extraction buffer and centrifuged at 16,000 × *g* for 1 min at 4°C. For subsequent LPS sugar compositions, 0.3 to 0.35 g of bacteroids were prepared per strain.

Isolation of anthocyanidins. A procedure devised by Duelli et al. (17) was modified as follows. Seeds, 360 g of *P. vulgaris* cv. Midnight Black Turtle Soup, were rinsed with distilled H_2O until liquid was clear. Then, 120 g of rinsed seeds was added separately to Fernbach flasks containing 250 ml of sterile 0.1% HCl and incubated at 30°C on a shaker at 180 rpm for 23 h. The resulting liquid phase was centrifuged at 2,000 × *g* for 20 min at 4°C. The supernatant was filtered over 2 sterile 15.0-cm Whatman filter papers. To the filtrate, concentrated HCl was added to a final concentration of 2.0 N, and the solution was boiled in a 100°C water bath for 40 min. After cooling to room temperature, 1-pentanol was added to 10% of the total volume. After manual shaking for about 1 min, the layers were allowed to separate, and the 1-pentanol layer was removed and dried on a rotary evaporator. Sufficient 0.01% HCl in methanol was added to completely dissolve the sample. As previously described (18), anthocyanidin concentration was calculated by using the molar extinction coefficient for delphinidin (3.09×10^4 at 555 nm in 0.01% HCl in absolute ethanol).

Growth of bacteria with anthocyanidins. Bacteria were first grown to stationary growth in TY medium (described above). For induced samples, these fully grown cultures were inoculated (1:50) into YEC liquid medium (0.5% yeast extract, 10 mM $CaCl_2$ [pH 6.8]) containing anthocyanidins at a final concentration of 0.4 mM (44). Control cultures were grown at the same time in YEC medium lacking anthocyanidins. Cultures were grown to stationary phase at 30°C on a rotating shaker, and SDS-PAGE samples were prepared as described above.

Nodulation tests. *P. vulgaris* cv. Midnight Black Turtle Soup seeds were transferred into vials without pregermination and inoculated as previously described (44). Plants were harvested at 13 and 16 dpi, and the nitrogenase activity of the acetylene reduction catalyzed by intact nodulated roots was measured as previously described (41, 44). After the acetylene reduction assay, the nodules were stripped off the roots, counted, and weighed.

In Table 4, the average values per plant for the wild type are based on at least 10 plants at both 13 and 16 dpi in each case; those for the *wreB* mutant are based on 11 plants at both 13 and 16 dpi; and those for the *wreM*α mutant are based on 8 plants at 13 dpi and 7 plants at 16 dpi. Confidence levels were calculated by using unpaired *t* tests with SigmaPlot software, version 9.0.

RESULTS

Mutations in ORFs near *wreA* and analysis of the mutant LPS. BLAST analysis of ORFs near *wreA* in the genome sequence (28) revealed that *wreB* was a probable glycosyltransferase gene, and *wreD* and *wreF* best matched methyltrans-

TABLE 2. O-antigen neutral and amino sugar compositions

Strain[a]	Relative content (%)[c]					3OMe6dTal/Gal ratio (%)[d]
	Terminal residue[b]	2OMeFuc	3OMe6dTal	Fuc	QuiN	
WT	7	9	31	47	6	100
WT/α	6	13	40	37	7	123
Mutants deficient in the terminal residue						
wreB mutant	ND[f]	10	36	47	7	101
wreD mutant	ND	14	34	44	8	93
wreF mutant	ND	12	31	50	7	95
wreA mutant	ND	11	39	42	8	91
wreC mutant	ND	9	37	47	7	98
wreB/*wreB*$^{+}$[e] mutant	7	9	37	40	8	95
Mutant with nonpolar insertion in *wreM* α domain						
*wreM*α mutant	8	ND	35	50	7	77
*wreM*α/*wreM*$^{+}$ mutant	7	2	37	47	6	101
Mutant with Tn*5* insertion in *wreM*						
wreM397 mutant	10	ND	24	58	8	21
wreM397/α mutant	6	12	40	36	6	122
wreM397/α*397* mutant	7	ND	34	51	7	51
*wreM*α/*wreA* and *wreM*α/*wreB* double mutants						
*wreM*α/*wreA* mutant	ND	ND	33	59	8	93
*wreM*α/*wreB* mutant	ND	ND	33	61	6	78

[a] WT, CE3; WT/α, CE3α; *wreB* mutant, CE503; *wreD* mutant, CE523; *wreF* mutant, CE525; *wreA* mutant, CE367; *wreC* mutant, CE431; *wreB*/*wreB*$^{+}$ mutant, CE503 carrying pKT38; *wreM*α mutant, CE477; *wreM*α/*wreM*$^{+}$ mutant, CE477 carrying pJB26; *wreM397* mutant, CE397; *wreM397*/α mutant, CE397α; *wreM397*/α*397* mutant, CE397α397; *wreM*α/*wreA* mutant, CE538; and *wreM*α/*wreB* mutant, CE540.

[b] Terminal residue, 2,3,4-tri-*O*-methylfucose and 2,3-di-*O*-methylfucose; 2OMeFuc, 2-*O*-methylfucose; 3OMe6dTal, 3-*O*-methyl-6-deoxytalose; Fuc, fucose; QuiN, quinovosamine; Gal, galactose.

[c] Values are calculated as relative percentages of those sugars found only in the O antigen.

[d] The ratio of 3OMe6dTal to Gal is calculated as a percentage of the wild-type ratio to show the relative abundance of LPS I to LPS II of each mutant compared to the wild type. The higher values in the WT/α and *wreM397*/α*397* mutants reflect the extra copies of *wre* DNA carried on plasmid pLPSα.

[e] Restoration of the terminal residue to the complemented *wreB* mutant (*wreB*/*wreB*$^{+}$) is representative of the *wreD*, *wreE*, *wreA*, and *wreC* mutants carrying DNA containing the respective wild-type gene (19; data not shown).

[f] ND, not detected.

ferase genes (Fig. 3). ORFs *wreB*, *wreD*, and *wreF* were mutated separately by *in vitro* insertion of nonpolar antibiotic resistance cassettes at nucleotides 805518 (CE503), 806519 to 806588 (CE523), and 807512 (CE525 and CE527) of the genomic sequence. LPS from mutant strains constructed with these specific mutations were analyzed.

Neither of the two forms of the terminal residue were detected among the neutral and basic sugars of the *wreB*, *wreD*, *wreF*, *wreA*, and *wreC* mutant LPS (Table 2, section I) (19). The other known O-antigen sugars were present in normal amounts.

SDS-PAGE resolves the LPS of *R. etli* CE3 into two distinct mobility classes (6): LPS I (containing the O antigen as well as the lipid A and core regions) and LPS II (containing only lipid A and core regions). These designations are comparable to the traditional terms S-LPS and R-LPS, respectively, with the distinction that the O antigen of most LPS I molecules has exactly five repeat units (Fig. 1) (22). LPS I from the *wreB*, *wreD*, *wreF*, *wreA*, and *wreC* mutants migrated slightly faster than that of the wild type, a finding consistent with the loss of one residue (Fig. 4A) (19). According to visual inspection of silver staining of LPS bands, all of these mutants produced approximately the same amount of LPS I as the wild-type strain (Fig. 4A). This conclusion was supported by the ratio of 3-*O*-methyl-6-deoxytalose (3OMe6dTal; a relatively invariant sugar of the O antigen found only in LPS I) to galactose (Gal; a core sugar found in both LPS I and LPS II) (Table 2). Therefore, both the sugar compositions (Table 2) and SDS-PAGE analysis (Fig. 4) indicated that the loss of the terminal residue did not otherwise affect O-antigen structure or abundance.

The LPS I of *R. etli* CE3 binds to four characterized MAbs: JIM26, JIM27, JIM28, and JIM29 (53). Duelli et al. (19) reported that LPS I of *wreA* mutants did not bind MAbs JIM27, JIM28, and JIM29, which have overlapping epitopes. Similar results were seen with all of the mutants lacking the terminal residue. Showing MAb JIM28 as a representative, no binding of this antibody to the LPS I of *wreB*, *wreD*, *wreA*, or *wreC* mutants was detected on immunoblots of SDS-PAGE gels (Fig. 4B). The binding of MAb JIM28 to the LPS I of *wreF* mutants was greatly reduced compared to that of the wild type (Fig. 4B, lane 4). Introduction of the wild-type genes on a low-copy plasmid into each mutant strain restored the normal amount of the terminal residue (Table 2 and data not shown) and the wild-type level of binding of MAbs JIM27, JIM28, and JIM29 to LPS I (Fig. 4D and data not shown).

Mutations in *wreM* and analysis of the mutant LPS. *R. etli* CE3 gene *wreM* has a primary sequence domain matching methyltransferases (α domain) and a domain matching glycosyltransferases (β domain) (Fig. 3). The α and β domains of *wreM* were mutated separately with nonpolar antibiotic-resis-

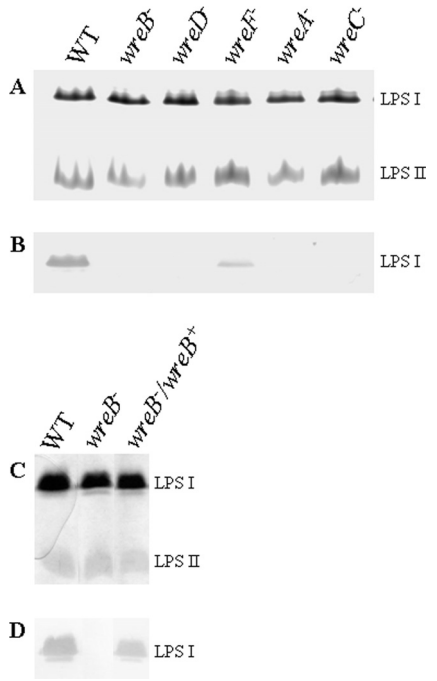

FIG. 4. SDS-PAGE and immunoblot analysis of the LPS of mutants deficient in the terminal residue. (A and C) SDS-PAGE of bacterial SDS extracts with LPS stained by silver-periodate. LPS I (containing O antigen) and LPS II (lacking O antigen) are indicated. (B and D) Immunoblot of gels in panels A and C decorated with MAb JIM28. Only LPS I is shown because LPS II is not bound by this antibody. Lanes for panels A and B: lane 1, WT (CE3); lane 2, *wreB* mutant (CE503); lane 3, *wreD* mutant (CE523); lane 4, *wreF* mutant (CE525); lane 5, *wreA* mutant (CE367); lane 6, *wreC* mutant (CE431). Lanes for panels C and D: lane 1, WT (CE3); lane 2, *wreB* mutant (CE503), lane 3, *wreB*/*wreB*$^+$ mutant (CE503 carrying pKT38). Restoration of MAb JIM28 binding to the complemented *wreB* mutant (*wreB*/*wreB*$^+$ mutant) is representative of the *wreD*, *wreE*, *wreA*, and *wreC* mutants carrying DNA containing the respective wild-type gene (19; data not shown). Like MAb JIM28, binding of MAbs JIM27 and JIM29 is restored to the LPS I of the complemented mutants (data not shown).

tance insertions at nucleotides 792025 (CE477 and CE478) and 793030 to 793173 (CE542 and CE544) of the genomic sequence. An insertion in the α domain (*wreM*α mutant) eliminated 2-O methylation of internal fucose residues (2OMeFuc) of the O antigen (Table 2). Contents of the other O-antigen sugars were unaffected, including the terminal residue. SDS-PAGE indicated that the LPS I of a *wreM*α mutant had the same mobility and amount as that of the wild type (Fig. 5A, lane 2). Introduction of the full-length, wild-type *wreM* gene (both α and β domains) partially restored 2OMeFuc (Table 2). A nonpolar insertion in the β domain of *wreM* (*wreM*β mutant) resulted in a faster-migrating LPS band on SDS-PAGE, greatly decreased in amount relative to wild-type LPS I (Fig. 5C, lane 2). Introduction of the full-length, wild-type *wreM* gene restored LPS I to this mutant (lane 3).

The Tn*5* insertion mutations of strains CE121 and CE395, whose LPS phenotypes have been described extensively (7, 9, 44, 53), also were mapped to the *wreM* ORF (Fig. 2) (see Materials and Methods). The Tn*5* of CE121 was located in the glycosyltransferase domain (β) at nucleotide 792975 of the genomic sequence, and its LPS phenotype (7, 9, 53) was identical in all known respects (compositions, SDS-PAGE profile, and antigenicity) to that of mutants with nonpolar insertions in this domain (see Fig. 5C, lane 2). Strains CE395 (44) and CE397 were isolated from a screen for mutants defective in the modification of LPS structure induced by anthocyanidins (44). The Tn*5* insertions of these strains were located in the methyltransferase domain (α) at nucleotides 791647 (CE395) and 791654 (CE397) of the genomic sequence. Like the nonpolar insertion mutations located in this domain of *wreM* (see Table 2), these Tn*5* insertions eliminated 2OMeFuc from LPS I (Table 2) (44). However, unlike the nonpolar mutations in this domain (see Fig. 5A, lane 2), these Tn*5* insertions also caused a reduced amount of LPS molecules that carry full-length O antigen (Fig. 5D, lane 3; Table 2) (44).

Mutants having little or no 2OMeFuc exhibited stronger JIM28 binding to LPS I (Fig. 5B, lanes 2 and 3; Fig. 5E, lanes 3 and 5). Conversely, when extra copies of *wre* DNA (plasmid pLPSα) in the wild type led to a greater relative content of 2OMeFuc (Table 2, strain WT/α), relative binding of MAb JIM28 was decreased (Fig. 5E, lane 2). Immunoblots with the LPS of strain CE397 and two strains derived from it further supported the conclusion that the degree of MAb JIM28 binding was inversely correlated with the relative content of 2OMeFuc in LPS I (Fig. 5E, lanes 3 to 5; Table 2).

Symbiotic phenotype associated with the absence of 2OMeFuc. Although it appears that the *wreM* α domain is required for 2OMeFuc of the O antigen when *R. etli* CE3 is grown in TY culture, it was conceivable that another methyltransferase induced by symbiosis mediates the increased incidence of 2OMeFuc observed in bacteroids or in bacteria cultured in the presence of host anthocyanins (15, 44). To test this possibility in bacteroids, strain CE477 carrying a nonpolar *wreM*α mutation was inoculated onto plants, and LPS was isolated from bacteroids within the resulting nodules at 18 dpi. 2OMeFuc was completely absent from the O antigen of mutant bacteroids (Table 3). The corresponding role of the *wreM* α domain in the effect of anthocyanins on bacteria cultured *ex planta* was assessed by immunoblot assay with MAb JIM28. The LPS I of wild-type bacteria, after culture in the presence of anthocyanins, exhibited decreased binding of MAb JIM28 (Fig. 6, lane 2), as would be predicted from the effect of increased 2OMeFuc on JIM28 binding noted in the foregoing section and documented in Fig. 5. *wreM*α mutants exhibited no antigenic change triggered by anthocyanins (Fig. 6, lane 4). Thus, the *wreM* α domain was required in the O-antigen changes observed under both of these tested conditions.

The *wreM*α mutation also caused a delay in symbiotic development (Table 4). The greatest deficiency was observed when seeds were planted without pregermination and inoculated with bacteria at the time they were planted on agar in vials. At 13 dpi, a point at which nodules containing the wild type have begun to fix nitrogen, the *wreM*α mutant had significantly reduced nitrogen fixation, likely due to significantly less nodule development (as monitored by number and weight in Table 4). This significant deficiency in symbiotic development at 13 dpi was reproduced in two additional independent experiments (data not shown). On the other hand, plants harvested at 16 dpi displayed no difference between the wild type and *wreM*α mutant (Table 4). Hence, the effect appeared to be

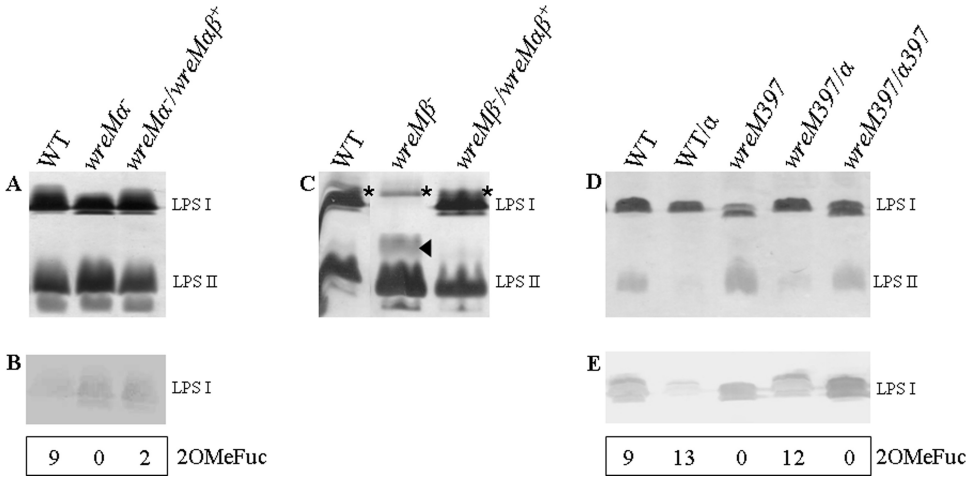

FIG. 5. SDS-PAGE and immunoblot analysis of the LPS of insertion mutations in *wreM*. (A, C, and D) SDS-PAGE of bacterial SDS extracts with LPS stained by silver-periodate. (B) Immunoblot of gel in panel A decorated with MAb JIM28. Lanes for panels A and B: lane 1, WT (CE3); lane 2, *wreM*α mutant (CE477); lane 3, *wreM*α/*wreM*$^+$ mutant (CE477 carrying pJB26). Lanes for panel C: lane 1, WT (CE3); lane 2, *wreM*β mutant (CE542); lane 3, *wreM*β/*wreM*$^+$ mutant (CE542 carrying plasmid pJB26). The arrowhead indicates the LPS band uniquely apparent in the *wreM*β mutant. In panel C, prolonged silver staining reveals bands (asterisk) running slightly above LPS I in the wild type and mutants. (E) Immunoblot of gel in panel D decorated with MAb JIM28. Lanes for panels D and E: lane 1, WT (CE3); lane 2, WT/α (CE3α); lane 3, *wreM397* mutant (CE397); lane 4, *wreM397*/α mutant (CE397α); lane 5, *wreM397*/α*397* mutant (CE397α397). The amount of 2OMeFuc in each strain, as displayed in Table 2, is shown below panels B and E.

on the rate of symbiotic development rather than sustained abrogation of the symbiosis.

The absence of the terminal residue had no effect on symbiosis. A *wreB* mutant (and, not shown, *wreC* and *wreF* mutants) lacking only this residue showed no discernible delay in nodulation nor in any symbiotic measure at 13 and 16 dpi (Table 4, section II). This symbiotic behavior was consistent with the idea that the observed decrease in this residue, induced by plant compounds, may help promote symbiosis with the *R. etli* host (19, 44).

Analysis of the LPS of strains lacking both the terminal residue and 2-O methylation of internal fucose. Mutant LPSs that lack the terminal residue do not bind MAb JIM28, whereas those lacking 2OMeFuc bind the antibody more strongly. These opposing effects indicate that both of these features of the O antigen are important in determining the degree of antibody binding. To further investigate their relative contribution to the MAb JIM28 epitope, mutant LPS lacking both 2OMeFuc and the multiply O-methylated terminal residue were created by constructing double mutants defective in the *wreM* α domain and *wreA* or in the *wreM* α domain and *wreB*. Sugar compositions of the LPS from these double mutants lacked 2OMeFuc and the terminal residue, whereas the other O-antigen sugars were in normal amounts (Table 2). SDS-PAGE analysis indicated that the LPS I resulting from these double mutations had nearly the same mobility and amount as that of the wild type (Fig. 7A, lanes 2 and 3). The LPS I from either double mutant was not bound by MAb JIM28; however, introduction of only the respective wild-type terminal residue gene restored antibody binding to LPS I (Fig. 7B, lanes 4 to 7). When these mutants were inoculated onto plants, the phenotype was similar to that of the *wreM*α mutants (data not shown).

TABLE 3. O-antigen neutral and amino sugar compositions of *wreM*α mutant strain isolated from bacteroids[a]

Strain[b]	Relative content (%)					3OMe6dTal/Gal ratio (%)
	Terminal residue	2OMeFuc	3OMe6dTal	Fuc	QuiN	
WT	6	10	49	28	7	100
*wreM*α mutant	4	ND[c]	36	54	6	129

[a] See Table 2 footnotes for definition of abbreviations and an explanation of the column headings.
[b] WT, CE3; *wreM*α mutant, CE477.
[c] ND, not detected.

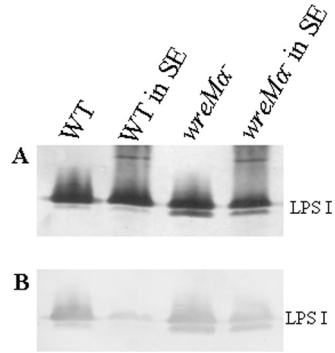

FIG. 6. SDS-PAGE and immunoblot analysis showing the effect of *P. vulgaris* anthocyanidins on the JIM28 epitope. (A) SDS-PAGE of bacterial SDS extracts with LPS stained by silver-periodate. (B) Immunoblot of gel in panel A decorated with MAb JIM28. Lanes: 1 and 2, WT (CE3); and lanes 3 and 4, *wreM*α mutant (CE477). In lanes 2 and 4, the strains had been grown in the presence of seed-anthocyanidin extract (SE), which is responsible for the streaking above LPS I in the silver-stained gel.

TABLE 4. Measures of symbiotic proficiency[a]

Time postinoculation (days)[b]	Inoculant[c]	Symbiotic proficiency (SD)[d]		
		Acetylene reduction[e]	No. of nodules[f]	Nodule wt (mg)
13	WT	2.6 (3.0)†	28.4 (11.3)‡	23.9 (14.0)†
	*wreM*α mutant	0.1 (0.2)†	19.3 (9.9)‡	10.3 (8.1)†
16	WT	5.5 (3.7)	34.5 (20.6)	27.6 (17.2)
	*wreM*α mutant	6.7 (6.3)	34.4 (14.0)	32.8 (15.7)
13	WT	6.8 (5.1)	33.5 (12.1)	29.1 (14.9)
	wreB mutant	7.1 (6.5)	38.5 (10.0)	31.7 (12.7)
16	WT	12.1 (3.5)	43.9 (21.5)	49.6 (20.8)
	wreB mutant	11.6 (5.0)	35.9 (12.7)	39.8 (14.5)

[a] Results are expressed per plant.
[b] That is, the number of days after inoculation that the plants were harvested.
[c] The data shown are from the following strains: WT, CE3; *wreM*α mutant, CE477; and *wreB* mutant, CE503.
[d] The values for mutant and wild type in these pairs are significantly different at 95% (†) and 90% (‡) confidence, as determined by Student *t* test (with 17 degrees of freedom).
[e] Expressed as nanomoles of ethylene produce per minute per plant.
[f] Only developed nodules were counted. Some nodules were underdeveloped at 13 dpi and were not counted.

DISCUSSION

Five genes—*wreB*, *wreD*, *wreF*, *wreA*, and *wreC*—are required for synthesis and linkage of the multiply O-methylated terminal residue, whereas one domain (α) of the multidomain gene *wreM* is specifically required for the 2-O methylation of internal fucose residues. All of the other ORFs within the *R. etli* CE3 chromosomal O-antigen genetic cluster (Fig. 2) have been mutated (8, 19, 36, 37; unpublished data), and none have mutant phenotypes affecting only these residues.

Significance of WreM α domain. *wreM* is a very long gene with at least two distinct enzymatic domains. The activity specified by its first domain (α) could be confirmed unambiguously because of the nonpolar insertions that affected only this domain. The most probable explanation for allowing expression of the downstream domain (β) in these mutants is that the insertion elements allow transcription outward from the element, and there are three possible translational start codons in the proper reading frame downstream of the insertion and upstream of the β domain. Each possible start codon has a very good consensus ribosome binding site for *R. etli* (27) at the appropriate distance upstream, the best being for an AUG codon. In fact, it is a better match than the best combination of ribosome binding site and start codon upstream of the α domain. This situation raises the question of whether this translation start is used in the wild-type bacteria, perhaps at a higher frequency than translation of the entire ORF. This would result in a population of WreM consisting only of the β domain, which might explain why 2-O methylation of fucose occurs only in one-fifth of the repeating units, on average.

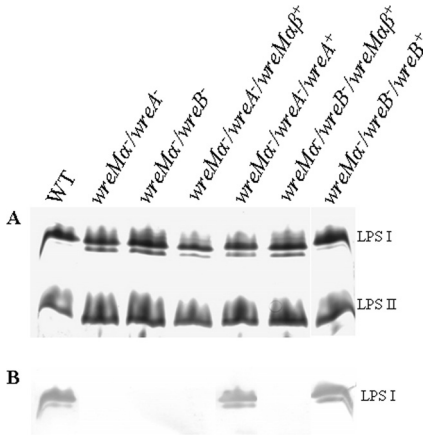

FIG. 7. SDS-PAGE and immunoblot analysis of the LPS of double mutants. (A) SDS-PAGE of bacterial SDS extracts with LPS stained by silver-periodate. (B) Immunoblot of the gel sections in panel A decorated with MAb JIM28. Lanes: lane 1, WT (CE3); lane 2, *wreM*α/*wreA* mutant (CE538); lane 3, *wreM*α/*wreB* mutant (CE540); lane 4, *wreM*α/*wreA*/*wreM*$^+$ mutant (CE538 carrying pJB26); lane 5, *wreM*α/*wreA*/*wreA*$^+$ mutant (CE538 carrying plpeA); lane 6, *wreM*α/*wreB*/*wreM*$^+$ mutant (CE540 carrying pJB26); and lane 7, *wreM*α/*wreB*/*wreB*$^+$ mutant (CE540 carrying pKT38).

2OMeFuc is also absent in *wreM*α mutants under conditions (occupancy in nodules and growth in anthocyanins) that, in the wild type, trigger a doubling in its content. Therefore, the same activity that is responsible for the basal methylation is apparently the target for this upregulation, but the step in the expression process that is regulated remains to be determined. A further indication that the anthocyanin effect is mediated through this gene is that all Tn*5* insertion mutants isolated in a screen for retention of MAb JIM28 binding after growth in anthocyanins were mutated in *wreM*. Two of these mutations (in CE395 and CE397) were in the *wreM* α domain, while a third mutation is located at the C terminus of *wreM* (V. J. Bonne, J. M. Box, and K. D. Noel, unpublished results).

Previous work with Tn*5* insertions in the *wreM* α domain suggested that eliminating 2OMeFuc by mutation delayed symbiotic development (44). However, the mutant investigated in that study (CE395) had the complex LPS phenotype exhibited by CE397 in the present study (see Fig. 5D, lane 3; Table 2), which is probably due to the polar effects of the Tn*5* on at least the *wreM* β region. Because symbiotic development is strictly dependent on the amount of O antigen per cell (45), the delay in nodule development could be attributed to deficient O antigen amount or other LPS defects in this mutant besides the absence of 2OMeFuc. Therefore, it was important to obtain the nonpolar *wreM*α mutations that apparently did not affect the expression of any other genes, not even the

downstream domain of the same gene. Because the nonpolar mutant has wild-type amounts of LPS I (Fig. 5A), symbiotic proficiency can be attributed more confidently to impairing only the *wreM* α domain and the consequent loss of 2OMeFuc.

In the midst of all of the other molecules and signals required to establish a successful symbiosis, it is surprising that the loss of this single methylation event has an observable phenotype. Because plant compounds released from both seeds and roots could induce the increased 2-O methylation while the bacteria are still outside the plant, the effect may be on a very early event in the infection process, perhaps in the efficiency of initiation or progression of the infection thread. This scenario is one explanation for the mutant phenotype being strongest when the bacteria are inoculated on ungerminated seeds, thereby allowing the greatest exposure to anthocyanins released from the seed coats (18, 44). It has been proposed (40) that the O antigen is a ligand for a plant receptor that helps direct the cytoskeleton and vesicular traffic to where the closest impinging bacterium is located. Thus, the plasma membrane grows at that location, and the exterior plant components of the infection thread are released for assembly at that point. According to this hypothesis, modification of the O antigen structure may facilitate interaction with the hypothetical receptor. Besides these plant-induced changes in the O antigen, there is one other mutant whose phenotype suggests that a specific structure of the *R. etli* CE3 O antigen is important in symbiosis. An alteration affecting only the structure of the quinovosamine residue also causes delayed nodule development (23, 45), a more severe delay than that caused by the *wreM*α mutation in the present study.

These mutant phenotypes certainly do not preclude other ideas about the importance of the O antigen in symbiosis. One recurrent idea proposed for all bacterial surface polysaccharides is that they defend against or suppress host defenses (24, 39). Along these lines, the anthocyanins that induce changes in *R. etli* CE3 O antigen are toxic to the bacteria at higher concentrations (17, 18, 42). However, the *wreM*α mutants are no more sensitive to these compounds (data not shown) in an agar diffusion assay (25) that was developed to test whether increased LPS fucose methylation helped resist the toxicity of anthocyanins.

JIM28 epitope. Although the O-antigen structures affected were unknown in early work (42, 53), three MAbs, including JIM28, have been instrumental in studying the variable features of the CE3 O antigen, whose genetic basis is the subject of the present study. The terminal residue and 2OMeFuc affect the JIM28 epitope but in opposite directions. Loss of the terminal residue results in no binding (Fig. 4B) (19), whereas loss of 2OMeFuc leads to stronger binding (Fig. 5B and 5E) (44). Double mutants that have lost both the *O*-methyl moieties indicate that the terminal residue is essential for MAb JIM28 binding; the potential increase in affinity afforded by the *wreM*α mutation could not even partially restore binding when *wreA* or *wreB* mutations also eliminated the terminal residue (Fig. 7B).

Inasmuch as binding requires the terminal residue, it is most likely that the antibody binds to this residue and the last repeat unit. An extension of this idea is that when the prior fucose is 2-O methylated, MAb JIM28 binding is eliminated or greatly decreased. Indeed, one explanation regarding the WreM α domain 2-O methylation activity induced by host anthocyanins is that it could be mainly directed to the last repeat unit by an unknown but fascinating mechanism (15, 44). Alternatively, increased activity that is stochastic with respect to the repeat units might give the same qualitative result, especially if attachment of the terminal residue is simultaneously decreased (44), and could explain why binding of MAb JIM28 to LPS I has not been completely eliminated in wild-type cells tested after growth *ex planta* in the presence of host compounds (42, 44).

Significance of genes for the terminal residue. Of the five genes required for production and attachment of the terminal residue, four would be predicted if every presumed chemical activity required a separate gene, i.e., three methyltransferase genes (*wreD*, *wreF*, and *wreA*) and one glycosyltransferase gene (*wreB*). Why is a fifth gene, *wreC*, required? One hypothesis is that WreC acts as a membrane anchor and scaffold for assembly of a methylating complex, possibly including the glycosyltransferases as well (17). A further speculation is that assembly of the complex is very poor if one of the proteins is lacking or has a highly perturbed structure because of an insertion. If true, this idea explains why the mutations in *wreC*, *wreD*, *wreF*, and *wreA* all resulted in basically the same phenotype (Fig. 4B and Table 2).

O antigens are synthesized by one of two principal pathways (reviewed in reference 46). *R. etli* CE3 apparently synthesizes its O antigen by the type of pathway in which an ATP-binding cassette (ABC) transporter translocates a fully polymerized O antigen from the cytoplasmic to the periplasmic face of the inner membrane (4, 21, 32, 33). The basis for this inference is that homologs of the subunits of the ABC transporter, *wzm* and *wzt* (48, 49, 51), are present in the chromosomal O-antigen genetic cluster (see Fig. 2) (28, 37) and homologs of the signature genes of the other main synthesis pathway—*wzy*, *wzx*, and *wzz*—are absent. *E. coli* O8, O9, and O9a O antigens also are synthesized by the ABC transporter pathway, and biochemical studies strongly imply that the modification at the termini of these O antigens is required for controlling chain length and for transport of the completed O antigen across the membrane (12–14). However, this cannot be true in *R. etli* CE3. Mutants lacking the terminal residue have no defects in the size or amount of the O antigen (Table 2; Fig. 4) (19).

In *K. pneumoniae* O2a, the O antigen lacks a unique terminal modification, and evidence indicates that length regulation and transport are both conferred by the ABC transporter (34). Supporting evidence includes overexpression of genes encoding the ABC transporter subunits, which results in O antigens with shorter lengths. Although some version of this second proposed mechanism deserves scrutiny in *R. etli* CE3, the first test of this specific idea was negative. When extra copies of the *R. etli wzm* (wzm_{RE}) and wzt_{RE} genes were introduced into *R. etli* CE3, the length of the O antigen was unaffected (data not shown).

One distinction is that the *E. coli* O8, O9, and O9a and *K. pneumoniae* O2a O antigens appear to be of modal (varied) length, whereas the *R. etli* CE3 O antigen is of fixed length. It is possible that these two different types of O antigens would require different mechanisms to regulate their length. Nevertheless, something related to ABC transporter structure and/or function may be involved because all of the known fixed-length

O antigens appear to be synthesized by the ABC transporter pathway or at least contain homologs of *wzm* and *wzt* (10, 11, 31, 35, 54). It seems likely that termination of *R. etli* CE3 O antigen synthesis is controlled by a third mechanism, one that may be representative of other strains having extremely uniform length of the O antigen.

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