Biochemical Characterization of Sinorhizobium meliloti Mutants Reveals Gene Products Involved in the Biosynthesis of the Unusual Lipid A Very Long-chain Fatty Acid*

Received for publication, March 2, 2011, and in revised form, March 25, 2011. Published, JBC Papers in Press, March 26, 2011, DOI 10.1074/jbc.M111.236356

Andreas F. Haag, Silvia Wehmeier, Artur Muszyński, Bernhard Kerscher, Vivien Fletcher, Susan H. Berry, Georgina L. Hold, Russell W. Carlson, and Gail P. Ferguson

From the School of Medicine and Dentistry, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, United Kingdom and the Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia 30602

Sinorhizobium meliloti forms a symbiosis with the legume alfalfa, whereby it differentiates into a nitrogen-fixing bacteroid. The lipid A species of S. meliloti are modified with very long-chain fatty acids (VLCFAs), which play a central role in bacteroid development. A six-gene cluster was hypothesized to be essential for the biosynthesis of VLCFA-modified lipid A. Previously, two cluster gene products, AcpXL and LpxXL, were found to be essential for S. meliloti lipid A VLCFA biosynthesis. In this paper, we show that the remaining four cluster genes are all involved in lipid A VLCFA biosynthesis. Therefore, we have identified novel gene products involved in the biosynthesis of these unusual lipid modifications. By physiological characterization of the cluster mutant strains, we demonstrate the importance of this gene cluster in the legume symbiosis and for growth in the absence of salt. Bacterial LPS species modified with VLCFAs are substantially less immunogenic than Escherichia coli LPS species, which lack VLCFAs. However, we show that the VLCFA modifications do not suppress the immunogenicity of S. meliloti LPS or affect the ability of S. meliloti to induce fluorescent plant defense molecules within the legume. Because VLCFA-modified lipids are produced by other rhizobia and mammalian pathogens, these findings will also be important in understanding the function and biosynthesis of these unusual fatty acids in diverse bacterial species.

The α-proteobacterium Sinorhizobium meliloti forms a symbiosis with the agriculturally important legume Medicago sativa (alfalfa) (for recent reviews, see Refs. 1 and 2). The symbiosis is initiated by the legume, which secretes flavonoids in the absence of a suitable nitrogen source within the soil. S. meliloti then produce noduleation factors and colonize the root hairs, where they induce the formation of plant-derived structures called infection threads. The bacteria then traverse down these infection threads and are eventually endocytosed into cortical root cells and encompassed in plant-derived, acidic membrane compartments called symbiosomes. Within the symbiosome compartment, S. meliloti differentiates into a morphologically distinct cell type known as a bacteroid (3), which fixes nitrogen gas into ammonia for the legume. The lipopolysaccharide (LPS) structure of S. meliloti has been found to play a key role in bacteroid development within legumes (4, 5).

LPS forms the outermost leaflet of the outer membrane of Gram-negative bacteria, such as S. meliloti, and is composed of lipid A, a sugar core, and O-antigen. The biosynthesis of LPS has been most comprehensively studied in Escherichia coli (6). However, unlike E. coli LPS, the LPS species of S. meliloti, other rhizobia, and Brucella species differ, because they are modified with an unusual very long-chain fatty acid (VLCFA)4 of either 27-OHC28:0, 27-O (2Ome C4:0) C28:0, or 29-OHC30:0 (7, 8). The S. meliloti acpXL and lpxXL genes, which encode a VLCFA acyl carrier protein and a VLCFA acyl transferase, respectively, are essential for the biosynthesis of VLCFA-modified lipid A in cultured S. meliloti (9, 10). The LpxXL VLCFA acyl transferase was also found to be essential for the biosynthesis of VLCFA-modified lipid A in S. meliloti bacteroids within alfalfa (5). Both S. meliloti AcpXL and LpxXL proteins were demonstrated to confer a competitive advantage to S. meliloti during the alfalfa symbiosis and play central but distinct roles in bacteroid development (5, 9, 10). S. meliloti acpXL and lpxXL mutants also displayed an increased sensitivity to environmental stresses in their free living state relative to the parent strain (9, 10). Therefore, S. meliloti VLCFA-modified lipid A is important for the legume symbiosis and for survival of S. meliloti within the rhizosphere. It was also recently determined that the lipid A VLCFA modification is essential for the symbiosis of Sinorhizobium NGR234 with different types of legume hosts (11), further confirming the importance of identifying the other gene products involved in VLCFA biosynthesis.

The S. meliloti acpXL and lpxXL genes form a six-gene cluster (Fig. 1A), where the other four genes are also hypothesized to encode proteins involved in the biosynthesis of the lipid A VLCFA modification (Fig. 1A) (9, 10, 12). This six-gene cluster is also conserved in Rhizobium leguminosarum, where it was shown that the fabZXL, fabF2XL, and fabF1XL genes are part of the same operon and that the FabF2XL gene product is involved

*The abbreviations used are: VLCFA, very long-chain fatty acid; HMW, high molecular weight; LMW, low molecular weight; ACP, acyl carrier protein.

1 Supported by an IMS Ph.D. studentship.
2 Both authors contributed equally to this work.
3 To whom correspondence should be addressed. Tel.: 44-1224-437372; Fax: 44-1224-437348; E-mail: g.ferguson@abdn.ac.uk.

4 The abbreviations used are: VLCFA, very long-chain fatty acid; HMW, high molecular weight; LMW, low molecular weight; ACP, acyl carrier protein.
**S. meliloti Lipid A VLCFA Biosynthesis**

### Strains and plasmids

| Strain or plasmid | Relevant characteristic(s) | Source or reference |
|-------------------|-----------------------------|---------------------|
| **S. meliloti strains** |                           |                     |
| Rm1021            |                             | Ref. 36             |
| acpXL             |                             | Ref. 9              |
| lpxXL             |                             | Ref. 9              |
| adhA2XL           |                             | This study          |
| fabF1XL           |                             | This study          |
| fabF2XL           |                             | This study          |
| fabZXL            |                             | This study          |
| mshA2             |                             | Ref. 25             |
| **E. coli strains** |                           |                     |
| DH5a              |                             | Bethesda Research Laboratories |
| M7616             |                             | Ref. 17             |

### Plasmids

| Plasmid | Relevant characteristic(s) | Source or reference |
|---------|-----------------------------|---------------------|
| pKk000  |                             | Ref. 17             |
| pk18mobGI |                             | Ref. 16             |
| pRF771       |                             | Ref. 18             |

### Primers

| Primer name | Sequence (5’→3’) |
|-------------|------------------|
| pkX_DS_R     | CCAAAAGGCGATCCTGTCG |
| smc04279−33_PstI_F | GCGGATCCATCGGCAAGGTCGAC |
| smc04279−stop_XbaI_R | GCGGATCCTGTCGACGACGATC |
| smc04279+78_EcoRI_F | GCGGATCCTGTCGACGACGATC |
| smc04279+451_HindIII_R | GCGGATCCTGTCGACGACGATC |
| smc04279−270_F | GCGGATCCTGTCGACGACGATC |
| smc04279−35_PstI_R | GCGGATCCTGTCGACGACGATC |
| smc04279−stop_XbaI_R | GCGGATCCTGTCGACGACGATC |
| smc04279+31_PstI_R | GCGGATCCTGTCGACGACGATC |
| smc04279+406_HindIII_R | GCGGATCCTGTCGACGACGATC |
| smc04279−155_F | GCGGATCCTGTCGACGACGATC |
| smc04279−31_Nol_F | GCGGATCCTGTCGACGACGATC |
| smc04279+1307_XbaI_R | GCGGATCCTGTCGACGACGATC |
| smc04279+36_PstI_R | GCGGATCCTGTCGACGACGATC |
| smc04279+446_HindIII_R | GCGGATCCTGTCGACGACGATC |
| smc04279−126_F | GCGGATCCTGTCGACGACGATC |
| smc04279−54_PstI_R | GCGGATCCTGTCGACGACGATC |
| smc04279−stop_XbaI_R | GCGGATCCTGTCGACGACGATC |
| smc04279+36_PstI_F | GCGGATCCTGTCGACGACGATC |
| smc04279+277+204_HindIII_R | GCGGATCCTGTCGACGACGATC |
| smc04279−241_F | GCGGATCCTGTCGACGACGATC |
| smc04279−stop_XbaI_R | GCGGATCCTGTCGACGACGATC |
| smc04279−40_PstI_F | GCGGATCCTGTCGACGACGATC |
| smc04279−stop_XbaI_R | GCGGATCCTGTCGACGACGATC |
| smc04279+352_R | AAGGGGGGATGTGCTGC |
| smc04279+960_F | CAGCGCGATCCATGAAG |
| smc04279+235_R | CAGCGCGATCCATGAAG |
| smc04279+698_F | CAGCGCGATCCATGAAG |
| smc4268+149_R | CAGCGCGATCCATGAAG |
| smc4275+188_R | CAGCGCGATCCATGAAG |
| _8_F | GAGGGGATTGCCGACGACGATC |
| _1492_R | GAGGGGATTGCCGACGACGATC |
Constructions of Insertional Mutants—All primers used in this study are listed in Table 2. The insertional mutant in \textit{adha}2XL was obtained by amplifying a 374-bp internal fragment of the gene using primers smc04270(+78) EcorI F and smc04270(+451) HindIII R. For the mutant in \textit{fabF}1XL a 374-bp internal fragment was used with primers smc04273(+33) PstI F and smc04273(+406) HindIII R, for the mutant in \textit{fabF}2XL a 411-bp internal fragment using primers smc04275(+36) PstI F and smc04275(+446) HindIII R, and for the \textit{fabZ}XL mutant a 169-bp internal fragment using primers smc04277(+36) PstI F and smc04277(+204) HindIII R was amplified by PCR. The PCR fragments were then purified, digested with the respective restriction enzymes according to the manufacturer’s protocols, ligated into the respective restriction sites of \textit{pRF}771 (18) at the appropriate restriction sites, under the control of the \textit{acp}XL gene and a 35-bp region upstream of its start codon were amplified using primers smc04273(+40) PstI F and smc04276 stop XbaI R; the \textit{smc}04275 stop XbaI R; and the \textit{smc}04277 stop XbaI R. The \textit{fabZ}XL gene was amplified, including 35 bp upstream of the \textit{acp}XL gene and a 35-bp region upstream of its start codon were amplified using primers smc04277 stop XbaI R; the \textit{fabF}2XL gene and a region 54 bp upstream of it were amplified using primers smc04275 stop XbaI R; and the \textit{fabF}1XL gene was amplified, including 31 bp upstream of the start codon, using primers smc04273 stop XbaI R; the \textit{adha}2XL gene and 35 bp upstream of the start codon were amplified using primers smc04270 stop XbaI R; and the \textit{lp}xXL gene was amplified, including 33 bp upstream of the start codon, using primers smc04278 stop PstI F and smc04278 stop XbaI R. All genes were then cloned into the PstI and XbaI sites of \textit{pRF}771 to generate \textit{pSmacp}XL, \textit{pSmfab}2XL, \textit{pSmfab}F1XL, \textit{pSmadh}A2XL, and \textit{pSm}lpxXL, respectively.

**LPS Isolation**—The LPS species were isolated using either SDS lysis followed by SDS-PAGE or hot water/phenol extraction as described previously (10, 19). For SDS lysis, the strains were grown for 48 h (72 h for the \textit{S. meliloti} \textit{lp}xXL mutant) to mid-exponential phase (\(A_{600} \approx 2.0\)), and then 1 ml of culture was used for the extraction procedure. The hot water/phenol extraction was performed from 4 liters of the strains grown in LB/MC supplemented with the relevant antibiotics until mid-exponential phase as earlier. After extraction, the lyophilized water phase fractions were dissolved in deionized water at a concentration of 10 \(\mu\)g/ml and then purified by ultracentrifugation (100,000 \(\times\) g, 8 °C) for 3.5 h. The purified LPS pellets were then resuspended in deionized water and lyophilized.

**Lipid A Analysis by MALDI-TOF MS**—The LPS species isolated into the aqueous phase were washed with 95% (v/v) ethanol at 4 °C to remove phospholipids, resuspended in water and freeze-dried. Lipid A was released from 2 mg of LPS by mild acid hydrolysis using 1% (v/v) HOAc. Briefly, samples (5 mg/ml in 1% (v/v) HOAc) were hydrolyzed at 100 °C for 1 h with constant stirring, freeze-dried, and extracted with Bligh-Dyer solvent (2 CHCl\(_3\), 2 MeOH, 1.8 H\(_2\)O, v/v/v). The organic phase was collected, and extraction was repeated with chloroform only. Combined organic phases were extracted back with H\(_2\)O and dried with a stream of N\(_2\). Samples were dissolved in a CHCl\(_3\)/IPA/H\(_2\)O mixture (5/3/0.25; v/v/v) and were then mixed with 1:1 (v/v) of 0.5 \(\mu\)M 2‘,4‘,6‘-trihydroxyacetophenone monomethyl glycoside in methanol, and 0.5 \(\mu\)l (1 \(\mu\)g) was spotted onto the \textit{S. meliloti} Lipid A VLCFA Biosynthesis

**Fluorescence Microscopy of Nodule Sections**—To detect the production of plant defense fluorescent polyphenolics in the root nodules, the nodules were fixed and analyzed as described previously (24). Competition assays were also performed as described before (8, 25). In brief, 3-day-old alfalfa seedlings were inoculated with the defined \textit{S. meliloti} strains, exactly as described previously (24). Competition assays were also performed as described before (8, 25). In brief, 3-day-old alfalfa seedlings were inoculated with a 1:1 ratio of \textit{Rm}1021 and the mutant strain. Four weeks after the inoculation, the bacteria were extracted and recovered on LB/MC Sm and LB/MC SmNm agar. Because both the parent and mutant strains grow on LB/MC Sm, whereas only the mutant strains will grow on LB/MC SmNm agar, the percentage occupancy of each strain could be determined.

**Salt Gradient Experiments**—The ability of the different strains to grow in the presence of a salt gradient was performed

---

**S. meliloti Lipid A VLCFA Biosynthesis**
as described previously (9), with slight modifications. Gradients were prepared in large square culture dishes (22.5 cm). A bottom layer was poured using 200 ml of LB NaCl agar and a top layer of 200 ml of LB agar supplemented with 0.5 M NaCl. Late exponential phase cultures were washed three times in LB NaCl and diluted to an A\textsubscript{600} of 0.2, and then 60 l was streaked across the plate. After 72 h at 30 °C, the minimum salt requirement for growth was determined assuming a linear gradient of NaCl across the plate.

Preparation of Monocytes and LPS Stimulation Studies—All studies were preapproved by the institutional ethics committee. Whole venous blood was collected from two healthy individuals who were not undergoing any drug therapy. The blood was diluted 1:1 in RPMI 1640 (Sigma-Aldrich), layered onto Histopaque-1077 (Sigma-Aldrich), and centrifuged at 800 g for 30 min. Peripheral blood mononuclear cells were removed from the interface and washed twice (10 min, 300 g) in RPMI before being counted and resuspended in growth media comprising RPMI 1640 supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin (Invitrogen), and 10% human AB serum (Sigma-Aldrich). Monocytes were plated at a density of $4 \times 10^5$ cells/well in 96-well plates and incubated for 90 min at 37 °C/5% CO\textsubscript{2}. Non-adherent cells were removed with two rinses of PBS. The effect of different S. meliloti LPS preparations were assessed by stimulating the monocytes for 6 h, and then the supernatants were collected. An E. coli commercial LPS preparation (0111:B4; Sigma catalog no. L4391) was used as a positive control. E. coli and S. meliloti LPS preparations used were 1 µg/ml in the monocyte stimulation experiments.

Assay of Human Cytokines in Culture Supernatants—The concentrations of TNF-α and IL-10 in culture supernatant were measured simultaneously using a human inflammatory cytokine cytometric bead array (BD Biosciences). Samples were analyzed on a multifluorescence BD FACSCaliburTM flow cytometer using BD CellQuestTM software and BD cytometric bead array software. The assay sensitivities for both cytokines were 10 pg/ml. The coefficients of variation of all cytokine assays were less than 10%.

Statistical Analysis—The statistical significance of the data was analyzed using Student’s t test in Microsoft Excel.

RESULTS

The acpXL-lpxXL Gene Cluster Is Organized into Two Operons—The acpXL-lpxXL gene cluster of rhizobia, including S. meliloti Rm1021 (Fig. 1A), is predicted to consist of three distinct transcriptional units (10, 12). To confirm the transcriptional organization of this gene cluster, RT-PCR was performed using RNA extracted from S. meliloti Rm1021. We obtained...
909- and 509-bp RT-PCR fragments using primer pairs internal to the acpXL and fabF2XL genes and fabF1XL and fabF1XL genes, respectively (Fig. 1, A and B), whereas in the absence of reverse transcriptase, no PCR products were observed with any primer pairs (Fig. 1B). In addition, no RT-PCR fragment was observed using S. meliloti RNA in combination with primers internal to the fabF1XL and adha2XL genes, indicating that these two genes are not co-transcribed (Fig. 1, A and B). Therefore, these findings show that in S. meliloti Rm1021, the acpXL, fabZXL, fabF2XL, and fabF1XL genes form part of the same transcriptional unit. Because the RT-PCR using primers internal to the adha2XL and lpxXL genes yielded a fragment of 477 bp, this confirmed that the adha2XL and lpxXL genes constitute the second transcriptional unit in the proposed lipid A VLCFA biosynthesis gene cluster (Fig. 1, A and B).

The Cluster Mutants Lack High Molecular Weight LPS—The six genes in the S. meliloti acpXL-lpxXL gene cluster are hypothesized to encode proteins involved in the biosynthesis of the VLCFA-modified lipid A (10). To investigate whether the other four genes in the acpXL-lpxXL cluster are also involved in LPS biosynthesis, we constructed S. meliloti insertion mutants in the fabZXL, fabF2XL, fabF1XL, and adha2XL genes (Fig. 1A). Previously, an S. meliloti acpXL mutant was found to lack high molecular weight (HMW) LPS species (10). SDS lysis followed by SDS-PAGE/periodate-silver staining also determined that, unlike the parent strain, which produced both HMW and low molecular weight (LMW) LPS species, the cluster mutant strains only produced LMW LPS species (Fig. 1C). Hence, these analyses showed that all of the mutants in the acpXL-lpxXL gene cluster have LPS structural alterations relative to the parent strain and lack the ability to synthesize HMW LPS.

To confirm that the disrupted genes are responsible for the absence of HMW LPS in the mutant strains, the wild-type versions of these genes were cloned into the broad host range vector pRFT771 under control of the constitutively active trp promoter (18) and then mated into the appropriate mutant strain. The LPSs of these strains were then analyzed by SDS-lysis followed by SDS-PAGE/periodate-silver staining (Fig. 1C). We found that the complementing plasmids, but not the control plasmid (pRFT771), restored the ability of the mutant strains to produce HMW LPS (Fig. 1C), confirming that the disrupted genes alone were responsible for the LPS structural alterations in the mutant strains. Therefore, the fabZXL, fabF2XL, fabF1XL, and adha2XL gene products, like the acpXL and lpxXL gene products, are involved in S. meliloti LPS biosynthesis.

The Cluster Mutants Have Defects in Lipid A VLCFA Biosynthesis—To determine whether all the acpXL-lpxXL cluster gene products (Fig. 1A) are involved in the biosynthesis of VLCFA-modified lipid A, the LPS species from the four cluster mutants were isolated by hot water/phenol. The lipid A species in the aqueous phases were then purified from the rest of the LPS molecule by mild acid hydrolysis and analyzed by MALDI-TOF MS (Fig. 2). As controls, the lipid A species produced by the S. meliloti acpXL insertional mutant and parent strain were also analyzed (Fig. 2, A and B, respectively). We found that the S. meliloti parent strain produced four major VLCFA-modified lipid A species, two of which are penta-acylated (Figs. 2B and 3, structures A and B) and two of which are tetra-acylated (Figs. 2B and 3, structures A’ and B’). As observed previously (9), the S. meliloti acpXL mutant completely lacked VLCFA-modified lipid A species and produced a mixture of penta-, tetra-, and triacylated lipid A species (Figs. 2A and 3, structures C, D, and E, respectively). We found that the S. meliloti fabF2XL, fabF1XL, and adha2XL insertion mutants also completely lacked VLCFA-modified lipid A species (Fig. 2, C, D, and E, respectively) and produced the same three lipid A species (Fig. 3, structures D, C, and E) as the S. meliloti acpXL insertional mutant (Fig. 2A). In contrast, MALDI-TOF MS analysis of the S. meliloti fabZXL mutant lipid A sample showed that it contained a mixture of lipid A species with (Figs. 2F and 3, structures A, A’, B, and B’) and without the VLCFA modification (Figs. 2F and 3, structures C, D, and E). Therefore, this analysis showed that all of the mutants in the S. meliloti acpXL-lpxXL gene cluster have defects in the biosynthesis of VLCFA-modified lipid A.

To confirm that the disrupted genes are responsible for the absence/decrease of the VLCFA-modified lipid A in the cluster mutants, the lipid A species of the mutants with the respective wild-type copy of the disrupted gene cloned into pRFT771 were also analyzed by MALDI-TOF MS (Fig. 2, C–F). We found that, with the exception of the S. meliloti fabF2XL mutant (Fig. 2C), the lipid A alterations of the cluster mutants were restored by the presence of the respective complementing plasmids (Fig. 2, D–F). This showed that disruption of fabF1XL, fabZXL, or adha2XL in the mutant strains was responsible for their lipid A phenotypes. In the case of the S. meliloti fabF2XL mutant with pSmfabF2XL, we only found partial complementation of its lipid A alteration relative to the parent strain (Fig. 2, compare C and B). However, because partial complementation was observed, this finding confirmed that FabF2XL is involved in the biosynthesis of VLCFA-modified lipid A.

The S. meliloti fabZXL Mutant Is Substantially Reduced in Its Ability to Produce VLCFA-modified Lipid A—To determine the precise changes in the lipid A fatty acid content of the mutant strains relative to the parent strain, we used GC-MS (Table 3). Consistent with the MALDI-TOF MS findings, GC-MS analysis of the lipid A fatty acids showed that the S. meliloti fabF2XL, fabF1XL, and adha2XL insertion mutants completely lack 27OH-C28:0 and 29OH-C30:0. Therefore, the fabF2XL, fabF1XL, and adha2XL gene products play essential roles in the biosynthesis of the VLCFA-modified lipid A in S. meliloti. The VLCFA content of the S. meliloti fabF1XL and adha2XL mutant lipid A was also fully restored by the respective complementing plasmid (Table 3). In contrast, although the 29OH-C30:0 level was fully restored in the S. meliloti fabF2XL mutant by pSmfabF2XL, the 27OH-C28:0 level amount was only partially restored by this plasmid, and 25OH-C26:0 was detected in this sample (Table 3). This finding confirms that we were only partially able to complement the lipid A alteration in this mutant strain. We also found that the S. meliloti fabZXL insertion mutant lipid A contained substantially reduced amounts of 27OH-C28:0 and 29OH-C30:0 fatty acids relative to the parent strain (Table 3). Interestingly, the lipid A of this mutant strain contained increased amounts of 29OH-C30:0 compared
with 27OHC28:0 and also contained trace amounts of 25OH-C26:0, which was absent in the parent strain lipid A preparation (Table 3). Because the plasmid, pSmfabZXL, restored the lipid A VLCFA content of the S. meliloti fabZXL mutant to that of the parent strain, these data showed that the fabZXL gene product, although not essential, plays an important role in the biosynthesis of VLCFA-modified lipid A in S. meliloti.

The lipid A preparation of the S. meliloti parent strain contains mainly hydroxylated fatty acids, with only trace or minor amounts of non-hydroxylated fatty acids (Table 3). We showed previously that the lipid A of the S. meliloti acpXL mutant has elevated amounts of non-hydroxylated fatty acids because it can transfer a non-hydroxylated shorter chain fatty acid onto the lipid A in the absence of the VLCFA to create a penta-acylated lipid A species (9) (Fig. 3, structure D). Our analysis found that the lipid A samples from the S. meliloti fabF2XL, fabF1XL, fabZXL, and adhA2XL mutant strains contained elevated amounts of non-hydroxylated lipid A fatty acids relative

FIGURE 3. Proposed lipid A structures produced by the S. meliloti parent strain and acpXL-lpxXL cluster mutants. Structures A, A’, B, and B’ contain a VLCFA modification, whereas structures C–E lack VLCFAs. Structure D lacks the VLCFA modification but is still penta-acylated due to the addition of a non-hydroxylated fatty acid. The example shown contains C16:0, but based on our GC-MS analysis (Table 3), this could also be replaced by a C18:0 or C18:1.

FIGURE 2. The S. meliloti acpXL-lpxXL cluster mutants have alterations in their lipid A biosynthesis. Shown is MALDI-TOF MS of the lipid A species produced by each strain (acpXL (A), Rm1021 (B), fabF2XL without and with pSmfabF2XL (C), fabF1XL without and with pSmfabF1XL (D), adhA2XL without and with pSmadhA2XL (E), and fabZXL without and with pSmfabZXL (F)). The capital letters indicate the major lipid A species (see Fig. 3 for structures) in each sample (pSmacpXL, pSmfabZXL, pSmfabF2XL, pSmfabF1XL, pSmadhA2XL, and pSmplpxXL are wild-type versions of the disrupted genes in the mutant strains cloned into pRF771).
to the parent strain (Table 3). In all cases, the increase in non-
hydroxylated fatty acids in the mutant strains was reduced by
the respective complementing plasmid (Table 3). Conse-
quently, these findings confirmed our MALDI-TOF MS results
and showed that in the absence of either FabF2XL, FabF1XL,
FabZXL, or AdhA2XL, S. meliloti mutants can transfer a non-
hydroxylated shorter chain fatty acid onto the lipid A to cre-
ate a penta-acylated lipid A species (see Fig. 3, structure D).
In contrast to the non-hydroxylated fatty acids, we did not observe
any substantial differences in the amount of 3-hydroxylated
LPS species, did not have a significant difference in its
LPS is the complete LPS consisting of O-antigen, core, and lipid
A, whereas rough LPS lacks the O-antigen. It was shown previ-
ously that the
hydroxylated fatty acids
LPS (data not shown). This provides further support that the
acetate in the cluster mutant strains relative to the parent strain (Table 3).

Cluster Mutants Do Not Have Alterations in Their LPS Sugar
Composition—In bacterial species lacking VLCFA-modified
LPS, the LMW and HMW LPS bands observed by SDS-PAGE
are defined as rough and smooth LPS, respectively (26). Smooth
LPS is the complete LPS consisting of O-antigen subunit.
Therefore, it was proposed that in S. meliloti, HMW LPS species are polymerized LPS mol-
ecules containing the VLCFA modification. To confirm that
this was also the case with other cluster mutants, we analyzed the
sugar composition of the purified aequous phase LPS extracted from the S. meliloti ipxXL and adhA2XL insertional
mutants by GC-MS. However, we found no significant differ-
ence in the sugar composition of the S. meliloti ipxXL and adhA2XL insertional mutant strains relative to the parent strain
LPS (data not shown). This provides further support that the
absence of HMW LPS in the S. meliloti cluster mutants is not
due to the absence of the O-antigen subunit.
The Cluster Mutants Have Reduced Alfalfa Competitiveness
and a Minimum Salt Requirement for Growth—S. meliloti
acpXL and ipxXL insertional mutants are symbiotically compe-
tent with alfalfa but have a reduced competitiveness in infecting
legume nodules relative to the parent strain (9, 10). We also
found that all of the other S. meliloti acpXL-ipxXL cluster
mutants were able to nodulate alfalfa and support plant growth
to a similar extent as the parent strain after a 4-week growth

| Fatty acyl residue | acpXL | fabZXL | fabF2XL | fabF1XL | adhA2XL |
|-------------------|-------|--------|---------|---------|---------|
| VLCPA             | 25.6  | ND     | 25.2    | ND      | 32.9    |
| 27OH-C28:0        | 5.4   | ND     | 7.3     | ND      | 6.0     |
| 29OH-C30:0        | ND    | ND     | ND      | ND      | ND      |
| 250OH-C26:0       | ND    | ND     | ND      | ND      | ND      |

Non-hydroxylated fatty acids

| Cluster mutants | Lipid A VLCFA Biosynthesis | AcpXL fabZXL | FabF2XL | FabF1XL | AdhA2XL |
|----------------|-----------------------------|--------------|---------|---------|---------|
| C18:1          | TR                           | 17.5         | 13.3    | 3.1     | 19.9    |
| C16:0          | ND                           | 4.0          | 2.0     | ND      | 3.7     |
| C18:0          | ND                           | 4.8          | 6.7     | TR      | 7.5     |

Hydroxylated fatty acids

| FabF1XL | acpXL | fabF2XL | fabF1XL | adhA2XL |
|---------|-------|---------|---------|---------|
| 3OH-C14:0 | 21.7 | 34.7   | 32.9    | 22.2    |
| 3OH-C16:0 | 11.3 | 6.8    | 6.6     | 5.5     |
| 3OH-C18:0 | 28.8 | 28.8   | 33.2    | 30.9    |

Non-hydroxylated fatty acids

| C18:1          | TR       | 17.5     | 13.3    | 3.1     | 19.9    |
| C16:0          | ND       | 4.0      | 2.0     | ND      | 3.7     |
| C18:0          | ND       | 4.8      | 6.7     | TR      | 7.5     |

TABLE 4

Competitiveness of the mutants in the acpXL-ipxXL cluster

| S. meliloti mutant | Amount of each mutant strain within the nodules (percentage of total bacteria within the nodules)
|-------------------|---------------------------------------|
| Strain used in the competition assay | Class I nodules | Class II nodules |
| acpXL | 3.3 ± 8.5 (n = 16) | 53.3 (n = 1) |
| fabZXL | 5.9 ± 9.6 (n = 22) | 92.9 ± 10.1 (n = 2) |
| fabF2XL | 3.9 ± 9.0 (n = 18) | 85.2 ± 15.5 (n = 6) |
| fabF1XL | 6.1 ± 11.4 (n = 21) | 94.1 ± 10.3 (n = 3) |
| adhA2XL | 3.1 ± 9.1 (n = 21) | 90.3 ± 13.1 (n = 3) |

The mutant and parent strains were inoculated onto alfalfa seedlings in a 1:1 ratio.

The data sets shown are average values from the defined number of nodules (n).
acpXL-lpxXL cluster mutant strains have slight differences in their alfalfa competitiveness.

S. meliloti mutants lacking the lipid A VLCFA are known to be sensitive to high osmolarity relative to the parent strain (9). We also found that the S. meliloti fabF2XL, fabF1XL, fabZXL, and adha2XL mutants have slightly increased sensitivities to hyperosmotic salt stress relative to the parent strain (data not shown). However, more significantly, we found that unlike the parent strain, all of the cluster mutants have a minimum salt requirement for growth and that this, with the exception of the S. meliloti fabF2XL mutant with pSmfabF2XL, is overcome by the presence of the respective complementing plasmid (Fig. 4A). Consistent with our biochemical characterization of the lipid A, we found that pSmfabF2XL only partially complemented the minimum salt requirement growth phenotype of the S. meliloti fabF2XL mutant (Fig. 4A). We also determined that the S. meliloti lpxXL mutant has the highest salt requirement for growth among the cluster mutants (Fig. 4A).

The VLCFA Modification Does Not Suppress the Immunogenicity of S. meliloti LPS or Affect the Production of Fluorescent Plant Defense Polyphenolics—The LPS of rhizobia, including S. meliloti, are substantially reduced in their ability to induce cytokine production from human monocytes relative to E. coli LPS (27). Because E. coli LPS lacks VLCFA modifications (6), one possibility is that the VLCFA modification is important to prevent S. meliloti LPS-induced activation of human monocytes. Likewise, it was previously hypothesized that the VLCFA modification is important to suppress the induction of the plant

**FIGURE 4.** The S. meliloti acpXL-lpxXL mutants have a minimum salt requirement for growth, and their LPS is less immunogenic than the parent strain LPS. A, the S. meliloti parent strain with the control plasmid (pRF771) or the mutant strains with either pRF771 or the respective complementing plasmid were streaked onto a gradient of LB-NaCl supplemented with NaCl ranging from 0 to 500 mM. The minimum salt concentration for growth was determined assuming a linear salt gradient across the agar. The absence of a bar indicates that the strain grows in the absence of sodium chloride. B and C, human monocytes from two individuals were exposed to 1 μg/ml purified LPS from the defined strains, and then the amount of TNF-α (B) and IL-10 (C) in the supernatant was determined after 6 h of incubation. The cytokine amounts shown are expressed as a percentage of the cytokine induction by E. coli LPS, which induced TNF-α and IL-10 levels from human monocytes of 973.2 ± 73.0 and 228.1 ± 34.3 pg/ml for individual 1 (filled bars) and 2241.3 ± 216.4 and 291.6 ± 27.5 pg/ml for individual 2 (open bars), respectively. The significance values (***, p < 0.001; **, p < 0.01; *, p < 0.05) represent comparisons of the individual LPS responses induced by the mutant strains relative to the S. meliloti parent strain. D, the autofluorescence of plant defense polyphenolics in alfalfa nodules infected with the designated strains was determined using fluorescent microscopy. The nodules from five different alfalfa plants for each strain were analyzed, and representative nodule images are shown. Scale bars, Rm1021, acpXL, and lpxXL (2 mm) and msbA2 (1 mm). Error bars, S.D.
S. meliloti Lipid A VLCFA Biosynthesis
defense response during S. meliloti infection of the legume root nodules (9, 10). To investigate the effect of the VLCFA modification on the immunogenicity of S. meliloti LPS, we analyzed the ability of purified LPS from the S. meliloti acpXL, adhA2XL, and lpxXL mutant strains to induce human monocytes to produce cytokines relative to purified LPS from the S. meliloti parent strain and an E. coli commercial LPS preparation (Fig. 4, B and C). We found that the S. meliloti parent strain LPS was substantially reduced in its ability to induce both TNF-α and IL-10 cytokines from human monocytes relative to E. coli LPS (Fig. 4, B and C). However, we determined that the LPS species produced by the VLCFA biosynthesis cluster mutants, which completely lack the VLCFA modification, were even more reduced in their ability to induce cytokine production from human monocytes relative to the parent strain (Fig. 4, B and C). In fact, no cytokine induction was observed with LPS from the lpxXL mutant. Consequently, these findings show that the lipid A VLCFA modification is not suppressing the activation of human monocytes by S. meliloti LPS but is required for the activation process.

As an initial means to investigate the effect of the lipid A VLCFA on the plant defense response, we analyzed the production of autofluorescent plant defense polyphenolics produced in the root nodules after infection with different S. meliloti strains using microscopy (25). Using this methodology, we observed a clear induction of root nodule autofluorescent defense molecules with an S. meliloti msbA2 insertion mutant (Fig. 4D), which was previously shown to induce this response (25). In contrast, we did not observe any significant reproducible difference in root nodule autofluorescence after infection with either the S. meliloti acpXL and lpxXL insertion mutants relative to the parent strain (Fig. 4D). Therefore, the S. meliloti acpXL and lpxXL genes are not required to suppress the induction of root nodule autofluorescent defense molecules in alfalfa.

DISCUSSION

Why Is the Cluster FabZXL Gene Not Essential for Lipid A VLCFA Biosynthesis?—In this study, we identified three novel genes, fabF1XL, fabZXL, and adhA2XL, which are involved in the biosynthesis of VLCFA-modified lipid A in S. meliloti. In addition, we determined that that the fabZ gene product is important but not essential for this process. Another putative fabZ gene is annotated in the S. meliloti Rm1021 genome (28). We determined that this putative fabZ encodes a protein with 47% similarity (26% identity) to the cluster FabZXL protein. The fabZ gene is located on the S. meliloti chromosome in a separate gene region predicted to be involved in lipid A biosynthesis (6, 28). This region also contains the gene encoding LpxA, which is one of the most conserved genes in lipid A biosynthesis (6, 28). Therefore, the possibility exists that this second putative FabZ protein is able to partially compensate for the absence of the cluster FabZXL protein in the biosynthesis of VLCFA-modified lipid A. E. coli FabZ is involved in the dehydration of short-chain hydroxyacyl-acyl carrier proteins (hydroxyacyl-ACPs) (29). However, the VLCFA-containing lipid A in the fabZXL mutant contains greater levels of 29OH-C30:0 than 27OH-C28:0 and also detectable levels of 25OH-C26:0. Therefore, this suggests that the second potential FabZ dehydratase may have broader chain length specificity and dehydrate both short- and long-chain lipid A fatty acids. The appearance of trace amounts of 25OH-C26:0 in the S. meliloti fabZXL mutant but not in the parent strain also suggests that the second potential FabZ protein is less effective at dehydrating longer chain hydroxyacyl-ACPs than the cluster FabZXL protein, leading to the accumulation of this slightly shorter VLCFA.

Why Did We Only Observe Partial Complementation of the S. meliloti fabZXL Mutant?—We found that, as in R. leguminosarum (12), the S. meliloti FabF2XL protein is essential for the biosynthesis of VLCFA-modified lipid A in S. meliloti. However, we were unable to fully complement the lipid A alteration of this mutant strain. Because the fabF2XL gene is the third gene in a four-gene operon (Fig. 1A), the plasmid insertion may be exerting a polar effect on the downstream fabF1XL gene. Therefore, full complementation of this mutant strain might require both the plasmid-encoded fabF2XL and fabF1XL genes. However, because we did not experience complementation problems with any of the other cluster insertion mutants, this suggests that a promoter on the plasmid used for the insertion mutagenesis can drive transcription of the downstream genes. Another possibility to account for our inability to fully complement the lipid A alteration of the S. meliloti fabZXL might be that the level of FabF2XL is critical for the proper biosynthesis of the VLCFA-modified lipid A and that the plasmid-encoded fabF2XL gene did not enable the correct expression level of this protein. Both the FabF1XL and FabF2XL proteins are predicted to encode 3-oxo-ACP synthases, which catalyze the reaction of the acyl-ACP with malonyl-ACP to produce a 3-oxoacyl-ACP (10). Therefore, if both of these proteins have preference differences for the chain length of the acyl-ACP, changing the levels of FabF2XL might affect the biosynthesis of VLCFA-modified lipid A. However, we found that the plasmid-encoded fabF1XL gene fully complemented the lipid A alteration of the S. meliloti fabF1XL mutant, suggesting this was not the case with FabF1XL. As observed in the S. meliloti fabZXL mutant, we also detected small amounts of 25OH-C26:0 in the S. meliloti fabZXL mutant with pSmfabF2XL. Because this fatty acid is not normally found in the parent strain LPS, this suggests that too much FabF2XL might also lead to an imbalance in synthesis, leading to the accumulation of 25OH-C26:0.

Why Is the VLCFA Biosynthesis Cluster Important for Nodule Colonization?—We determined that the fabF2XL, fabF1XL, fabZXL, and adhA2XL gene products enhanced the competitiveness of S. meliloti within alfalfa nodules. Although we did observe a small number of nodules where the mutant strains out-competed the parent strain, it is likely that this is due to variability in the plant response because the alfalfa seedlings are not clonal. Upon entry into the root nodules, S. meliloti encounters a number of different stresses within the symbiosome compartment, including low oxygen, acidic pH, osmotic changes, and nodule-specific cytokine-rich peptides (1). Based on our salt growth assay, we discovered that the VLCFA-modified lipid A is critical for growth of S. meliloti in the absence of sodium chloride. Because low levels of cations can help stabilize interactions between different LPS species (6), these findings provide further evidence that the VLCFA modification plays a...
key role in outer membrane stability, enabling S. meliloti to grow in the absence of salt-LPS stabilizing conditions. We found previously that, unlike the other cluster mutants, the S. meliloti lpxXL mutant is unable to synthesize penta-acylated lipid A (9). Because the S. meliloti lpxXL mutant has the greatest salt requirement for growth of the cluster mutants, this suggests that the inability of this mutant to synthesize penta-acylated lipid A dramatically decreases the stability of its outer membrane relative to the other mutants. Therefore, the reduced competitiveness of the cluster mutants in legume colonization might just arise through an increased sensitivity to symbiosome-encountered stresses relative to the parent strain due to weakened outer membranes.

It was hypothesized previously that the VLCFA might affect the ability of rhizobia to suppress the plant defense response upon entry into the plant cell (9, 10, 30, 31). However, we observed no substantial increase in the production of autofluorescent plant defense polyphenolics in the root nodules of alfalfa after infection with either the S. meliloti acpXL or lpxXL mutants. Because we showed previously that LpxXL is essential for the biosynthesis of S. meliloti bacteroid VLCFA-modified lipid A (5), these findings provide evidence that the lipid A VLCFA is not essential to suppress the plant defense response upon infection of S. meliloti. This finding is consistent with a previous study whereby the purified LPS from an S. meliloti acpXL mutant was as effective as the parent strain LPS in suppressing an artificially induced oxidative burst in Medicago truncatula and alfalfa tissue culture systems (32). However, we cannot rule out at this stage the possibility that the altered lipid A structures in our VLCFA biosynthesis mutants have more subtle effects on the legume defense response relative to the parent strain, which could not be detected using this experimental approach.

Why Is the Cluster Mutant LPS Less Immunogenic than the Parent Strain LPS?—It is well established that the VLCFA-modified LPS species produced by rhizobia are less immunogenic than E. coli LPS, which lack these modifications (27, 33). The VLCFA-modified LPS of Rhizobium sin-I also antagonizes the ability of E. coli LPS to induce monocytes to produce cytokines, and using a chemical synthesis approach, the VLCFA modification was shown to be important for the antagonist activity of synthetic lipid A (33, 34). In this study, we found that purified S. meliloti LPS from the cluster mutants, which completely lacks the VLCFA, was reduced or unable to induce human monocytes to produce cytokines relative to the VLCFA-modified LPS of the parent strain. Therefore, this finding demonstrates that the low immunogenicity of S. meliloti LPS relative to E. coli LPS is not due to the presence of VLCFAs. The bacterial lipid A component of the LPS is recognized by monocytes via Toll-like receptor 4 (TLR4) (35). E. coli lipid A is hexa-acylated whereas S. meliloti LPS is penta- and tetra-acylated (6). Consequently, the differences in the ability of S. meliloti and E. coli LPS to induce cytokine production from monocytes might be due to differences in the degree of acylation of their LPS affecting their interaction with TLR4. This could also account for why the LPS species of the cluster mutants are less immunogenic than the S. meliloti parent strain LPS because a proportion of their LPS species are only triacylated. This could also explain the results obtained with LPS from the lpxXL mutant because, unlike the LPS from the other mutant strains, it does not contain any penta-acylated lipid A species (9). In addition, the LPS from the cluster mutants might be less effective than the parent strain LPS because the VLCFA modification may aid the interaction with the Toll-like receptors. Because we found that the cluster mutant LPS lacks HMW species, HMW LPS might also be better at inducing monocyte cytokine production relative to LMW LPS. However, further studies would need to be performed to determine how the different lipid A species are recognized by the human monocyte receptors.

In conclusion, this study, combined with previous work (9, 10), has found that all of the genes in the S. meliloti acpXL-lpxXL cluster are involved in the biosynthesis of VLCFA-modified LPS. Other rhizobia and Brucella species, which form prolonged mammalian infections, also produce VLCFA-modified LPS and have an acpXL-lpxXL gene cluster (7). Therefore, these studies will provide important insights into the role of this unusual bacterial lipid modification in both plant and mammalian infections.

REFERENCES

1. Gibson, K. E., Kobayashi, H., and Walker, G. C. (2008) Annu. Rev. Genet. 42, 413–441
2. Jones, K. M., Kobayashi, H., Davies, B. W., Taga, M. E., and Walker, G. C. (2007) Nat. Rev. Microbiol. 5, 619–633
3. Mergaert, P., Uchiumi, T., Alunni, B., Evanno, G., Cheron, A., Catrice, O., Mausset, A. E., Barloy-Hubler, F., Calibert, F., Kondorosi, A., and Kondorosi, E. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 5230–5235
4. Campbell, G. R., Reuhs, B. L., and Walker, G. C. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 3938–3943
5. Haag, A. F., Wehmeier, S., Beck, S., Marlow, V. L., Fletcher, V., James, E. K., and Ferguson, G. P. (2009) J. Bacteriol. 191, 4681–4686
6. Raetz, C. R., and Whitfield, C. (2002) Annu. Rev. Biochem. 71, 635–700
7. Bhat, U. R., Carlson, R. W., Busch, M., and Mayer, H. (1991) Int. J. Syst. Bacteriol. 41, 213–217
8. Ferguson, G. P., Datta, A., Baumgartner, I., Roop, R. M., 2nd, Carlson, R. W., and Walker, G. C. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 5012–5017
9. Ferguson, G. P., Datta, A., Carlson, R. W., and Walker, G. C. (2005) Mol. Microbiol. 56, 68–80
10. Sharypova, L. A., Niehaus, K., Holst, O., and Becker, A. (2003) J. Biol. Chem. 278, 12946–12954
11. Ardissonne, S., Kobayashi, H., Kambara, K., Rummel, C., Noel, K. D., Walker, G. C., Broughton, W. J., and Deakin, W. J. (2011) J. Bacteriol., in press
12. Vanderlinde, E. M., Muszynski, A., Harrison, J. J., Koval, S. F., Foreman, D. L., Ceri, H., Kannenberg, E. L., Carlson, R. W., and Yost, C. K. (2009) Microbiology 155, 3055–3069
13. Sambrook, J., Fritsch, E. E., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., p. A.1, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
14. Serrania, J., Vorho¨lter, F. J., Niehaus, K., Pu¨hler, A., and Becker, A. (2008) J. Biol. Chem. 283, 7828–7831
15. Katzen, F., Becker, A., Jelmini, M. V., Oddo, C. G., and Ielpi, L. (1999) Appl. Environ. Microbiol. 65, 278–282
16. Finan, T. M., Kunkel, B., De Vos, G. F., and Signer, E. R. (1986) J. Bacteriol. 167, 66–72
17. Wells, D. H., and Long, S. R. (2002) Mol. Microbiol. 43, 1115–1127
18. Reuhs, B. L., Kim, J. S., Badgett, A., and Carlson, R. W. (1994) MPMI 7, 240–247
S. meliloti Lipid A VLCFA Biosynthesis

20. Gudlavalleti, S. K., and Forsberg, L. S. (2003) *J. Biol. Chem.* **278**, 3957–3968

21. Muszynski, A., Laus, M., Kijne, J. W., and Carlson, R. W. (2011) *Glycobiology* **21**, 55–68

22. Bhat, U. R., Forsberg, L. S., and Carlson, R. W. (1994) *J. Biol. Chem.* **269**, 14402–14410

23. York, W. S., Darvill, A. G., Mcneil, M., Stevenson, T. T., and Albersheim, P. (1986) *Methods Enzymol.* **118**, 3–40

24. Leigh, J. A., Signer, E. R., and Walker, G. C. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6231–6235

25. Beck, S., Marlow, V. L., Woodall, K., Doerrler, W. T., James, E. K., and Ferguson, G. P. (2008) *Microbiology* **154**, 1258–1270

26. Schild, S., Lamprecht, A. K., and Reidl, J. (2005) *J. Biol. Chem.* **280**, 25936–25947

27. Tsukushi, Y., Kido, N., Saeki, K., Sugiyama, T., Koide, N., Mori, I., Yoshida, T., and Yokochi, T. (2004) *J. Endotoxin Res.* **10**, 25–31

28. Galibert, F., Finan, T. M., Long, S. R., Puhler, A., Abola, P., Ampe, F., Barloy-Hubler, F., Barnett, M. J., Becker, A., Boistard, P., Bothe, G., Boutry, M., Bowser, L., Buhrmester, J., Cadieu, E., Capela, D., Chain, P., Cowie, A., Davis, R. W., Dreano, S., Federspiel, N. A., Fisher, R. F., Gloux, S., Godrie, T., Goffeau, A., Golding, B., Grouzy, J., Gurjal, M., Hernandez-Lucas, L., Hong, A., Huizar, L., Hyman, R. W., Jones, T., Kahn, D., Kahn, M. L., Kalman, S., Keating, D. H., Kiss, E., Komp, C., Lelaure, V., Masuy, D., Palm, C., Pock, M. C., Pohl, T. M., Portetelle, D., Purnelle, B., Ramsperger, U., Surzycki, R., Thebault, P., Vandenbol, M., Vorholter, F. J., Weidner, S., Wells, D. H., Wong, K., Yeh, K. C., and Batut, J. (2001) *Science* **293**, 668–672

29. Mohan, S., Kelly, T. M., Eveland, S. S., Raetz, C. R., and Anderson, M. S. (1994) *J. Biol. Chem.* **269**, 32896–32903

30. Vedam, V., Haynes, J. G., Kannenberg, E. L., Carlson, R. W., and Sherrier, D. J. (2004) *MPMI* **17**, 283–291

31. Vedam, V., Kannenberg, E., Datta, A., Brown, D., Haynes-Gann, J. G., Sherrier, D. J., and Carlson, R. W. (2006) *J. Bacteriol.* **188**, 2126–2133

32. Scheidle, H., Gross, A., and Niehaus, K. (2005) *New Phytol.* **165**, 559–565

33. Vandenplas, M. L., Carlson, R. W., Jeyaretnam, B. S., McNeill, B., Barton, M. H., Norton, N., Murray, T. F., and Moore, J. N. (2002) *J. Biol. Chem.* **277**, 41811–41816

34. Zhang, Y., Wolfert, M. A., and Boons, G. J. (2007) *Bioorg. Med. Chem.* **15**, 4800–4812

35. Hold, G. L., and Bryant, C. E. (2011) in *Bacterial Lipopolysaccharides* (Knirel, Y. A., and Valvano, M. A., eds) 1st Ed., Springer, Berlin

36. Meade, H. M., Long, S. R., Ruvkun, G. B., Brown, S. E., and Ausubel, F. M. (1982) *J. Bacteriol.* **149**, 114–122