Knockout of an Azorhizobial dTDP-L-Rhamnose Synthase Affects Lipopolysaccharide and Extracellular Polysaccharide Production and Disables Symbiosis with *Sesbania rostrata*

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Submitted 18 August 2000; Accepted 19 March 2001.

A nonpolar mutation was made in the *oac2* gene of *Azorhizobium caulinodans*. *oac2* is an ortholog of the *Salmonella typhimurium* *rfbD* gene that encodes a dTDP-L-rhamnose synthase. The knockout of *oac2* changed the lipopolysaccharide (LPS) pattern and affected the extracellular polysaccharide production but had no effect on bacterial hydrophobicity. Upon hot phenol extraction, the wild-type LPS partitioned in the phenol phase. The LPS fraction of ORS571-*oac2* partitioned in the water phase and had a reduced rhamnose content and truncated LPS molecules on the basis of faster migration in detergent gel electrophoresis. Strain ORS571-*oac2* induced ineffective nodule-like structures on *Sesbania rostrata*. There was no clear demarcation between central and peripheral tissues, and neither leghemoglobin nor bacteroids were present. Light and electron microscopy revealed that the mutant bacteria were retained in enlarged, thick-walled infection threads. Infection centers emitted a blue autofluorescence under UV light. The data indicate that rhamnose synthesis is important for the production of surface carbohydrates that are required to sustain the compatible interaction between *A. caulinodans* and *S. rostrata*.

From the rhizobial point of view, the establishment of a successful symbiosis with leguminous host plants depends on the production of Nod factor signals and exposure of the correct surface and/or extracellular polysaccharides (Niehaus and Becker 1998; Price 1999). Whereas the role of Nod factors as triggers of early plant responses is becoming quite well understood, the function of the bacterial envelope and the diverse types of surface polysaccharides (SPS) is far less defined. In several experimental systems, the importance of these classes of molecules for an efficient symbiosis has been demonstrated, but the molecular mechanisms or signaling pathways are not known. SPS have been implied in a multitude of processes such as internalization, suppression of defense responses, triggering of infection thread formation, and elicitat-

tion of feedback responses (Niehaus and Becker 1998; Price 1999). Few conclusive data about defined functions, however, are available.

In *Azorhizobium caulinodans* ORS571, a microsymbiont of *Sesbania rostrata*, random mutagenesis gave access to two essential nodulation loci, one for Nod factor synthesis, the other involved in SPS synthesis (Van den Eede et al. 1987). The latter locus, identified by a Tn5 insertion in the mutant strain ORS571-X15, is involved in the synthesis of activated monosaccharides, precursors for polysaccharide synthesis (Goethals et al. 1994), and corresponds to part of the *rfb* locus of *Salmonella typhimurium*. The mutation of ORS571-X15 is located in *oac3*, an ortholog of *rfbA* that encodes a dTDP-glucose synthase, which is implied in the synthesis of activated deoxyhexoses (Goethals et al. 1994). *oac3* is part of a cluster of four genes (*oac0*, *oac1*, *oac2*, and *oac3*) that encode enzymes for the synthesis of dTDP-L-rhamnose from glucose-1-phosphate (Goethals et al. 1994; W. D'Haeze and M. Holsters, *unpublished results*). The OAC3 enzyme catalyzes the first step in this pathway (Goethals et al. 1994). The dTDP-L-rhamnose synthase, which presumably catalyzes the last step, is encoded by the *oac2* gene. The transcriptional regulation of this region is not yet known. The mutation in the *oac3* gene caused a pleiotropic phenotype with rough colony morphology, clumping of bacteria in liquid media, altered O-antigen structure, and impaired nodulation. D'Haeze et al. (1998) studied the symbiotic behavior of this mutant in more detail and showed that bacterial invasion was arrested at the stage of intercellular infection pocket formation. The ORS571-X15 bacteria penetrated the host intercellularly through two to three cell layers and formed infection pockets in the outer cortex but could not induce the formation of inner cortical infection pockets and threads (D'Haeze et al. 1998).

To better study the symbiotic functions of azorhizobial surface carbohydrates, we characterized the *oac2* gene by creating a nonpolar-directed mutation and compared the mutant phenotype with the *oac3* mutation. Knockout of the rhamnose synthase gene caused a far less dramatic phenotype than that caused by the glucose synthase gene. dTPD-L-rhamnose synthesis seems to be important for a correct buildup of lipopolysaccharide (LPS) and extracellular polysaccharides (EPS), which play an essential role in the establishment of a successful symbiosis.

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RESULTS

Construction and phenotype of ORS571-oac2.

To create a nonpolar mutation, the *aphA-3* cassette (Ménard et al. 1993) was inserted into a *BsmI* site located in the 5' part of the *oac2* gene of *A. caulinodans* ORS571 (see below). The mutation was introduced into the ORS571 genome by exchange recombination with the suicide vector pJQ200uc1 (Table 1). For complementation analysis, the plasmid poacMG was constructed harboring *oac2* and two upstream open reading frames (W. D'Haeze and M. Holsters, *unpublished results*) cloned in the wide host-range vector pBBR1MCS-3 (Table 1).

The mutant strain ORS571-oac2 had normal growth behavior, indistinguishable from the wild-type strain on solid and liquid media (data not shown). Electrophoretic analysis of LPS preparations isolated by the hot phenol method (see below) revealed that the LPS from ORS571-oac2 partitioned in the water phase, similarly to the LPS from the mutant ORS571-X15 (Goethals et al. 1994). On the contrary, LPS from wild-type ORS571 and the complemented strain ORS571-oac2(poacMG) partitioned in the phenol phase. The *O* antigen of ORS571-oac2 LPS was altered and showed a lower degree of polymerization, comparable to the pattern of the ORS571-X15 LPS (Fig. 1, lanes 3 and 5). The band migration pattern of LPS, prepared from ORS571-oac2(poacMG), was similar to that of the wild type (Fig. 1, lanes 2 and 4). Analysis of the sugar composition of LPS preparations from the wild type and ORS571-oac2 showed the presence of 3-deoxy-D-manno-2-octulosonic acid (Kdo) and rhamnose in a molar ratio of 1:4.9 and 1:0.2, respectively. Additionally, the ratio of glucose to Kdo was changed in ORS571-oac2: 1:0.2 in the mutant versus 1:3.1 in wild-type ORS571.

The overall hydrophobicity of ORS571, ORS571-oac2, and ORS571-X15 was determined by a hydrocarbon adherence method (see below). ORS571-oac2 was slightly more hydrophobic than ORS571, but ORS571-X15 showed a strongly enhanced hydrophobicity (Fig. 2).

The *oac2* mutation causes arrest of nodule development.

Upon inoculation of *S. rostrata* stems or roots with strain ORS571-oac2, nodule initiation and the early nodulation stages were normal, but further nodule development was arrested, leading to severely defective organs in which no signs of nitrogen fixation could be observed (Fig. 3A). The nodule-like structures were green inside, indicating a significant reduction in the level of functional leghemoglobin (Fig. 3B). Complementation of the mutation by poacMG restored the ability to develop wild-type nodules and fix nitrogen (data not shown). Use of bacteria containing the β -glucuronidase reporter fused to the *nodA* promoter region provided the first indication of a lack of bacterial internalization and bacteroid formation in nodule-like structures (Fig. 3C). The plasmid pRG960SD-32 (Van den Eede et al. 1992) was introduced into ORS571 and ORS571-oac2, and approximately 50 stem nodules or nodule-like structures were harvested for each strain 3 weeks after inoculation. Hand-cut sections were stained with 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid and observed under a binocular (representative observations are shown in Fig. 3C). A section through nodules induced by ORS571(pRG960SD-32) shows central tissues colonized by bacteria. Remnants of infection pockets and infection threads can be seen in Figure 3C. A section through a defective nodule induced by ORS571-oac2(pRG960SD-32) shows infection centers with infection pockets and infection threads but no central tissue colonization (Fig. 3C).

Semi-thin sections (2.5 μ m) of 3-week-old nodules or nodule-like structures were stained with toluidine blue and viewed by bright-field optics (Fig. 4A to D). Wild-type nodules have a central tissue that is surrounded by a differentiated cortex, including an inner cortex made up of a few organized layers of flat cells that function as an O₂ diffusion barrier (James et al. 1998) (Fig. 3A). Sections of nodule-like structures induced by ORS571-oac2 showed abnormal tissue organization, without clear demarcation between central and peripheral tissues, specialized inner cortex, or any sign of

Table 1. Bacterial strains and plasmids

Strains/plasmids	Relevant characteristics	Reference
<i>Azorhizobium caulinodans</i>		
ORS571	<i>A. caulinodans</i> wild-type strain (Cb) ^a	Dreyfus et al. 1988
ORS571-X15	ORS571 derivative carrying a Tn5 insertion in <i>oac3</i> (Cb, Km)	Goethals et al. 1994
ORS571-oac2	ORS571 derivative carrying the <i>aphA-3</i> cassette of pUC18K in <i>oac2</i> (Cb, Km)	This work
ORS571-oac2(poacMG)	ORS571-oac2 derivative carrying poacMG (Cb, Km, Tc)	This work
ORS571(pRG960SD-32)	ORS571 derivative carrying pRG960SD-32 (Cb, Sp, Sm)	Van den Eede et al. 1992
ORS571-oac2(pRG960SD-32)	ORS571-oac2 derivative carrying pRG960SD-32 (Cb, Km, Sp, Sm)	This work
Plasmids		
pRK2013	Helper plasmid used in triparental conjugations (Km)	Ditta et al. 1980
pBBR1MCS-3	Cloning vector (Tc)	Kovach et al. 1995
pUC19	Cloning vector (Cb)	Yanisch-Perron et al. 1985
pRG960SD-32	pRG960SD derivative carrying a <i>nodA-uidA</i> fusion (Sp, Sm)	Van den Eede et al. 1992
pRG290-6	pRK290 carrying a 6,300-bp <i>EcoRI</i> fragment that contains <i>oac2</i> (Cb)	Goethals et al. 1994
pUC19-oac3200ES	pUC19 derivative carrying a 3,200-bp <i>EcoRI-StuI</i> fragment of pRG290-6 (Cb)	This work
pUC18K	pUC18 derivative carrying the <i>aphA-3</i> cassette designed to make nonpolar mutations (Cb)	Ménard et al. 1993
pUC19-oac3200ES-K	pUC19-oac3200ES derivative carrying the <i>aphA-3</i> cassette in <i>oac2</i> (Cb, Km)	This work
pJQ200uc1	Selectable suicide vector containing <i>sacB</i> of <i>Bacillus subtilis</i> (Gm)	Quandt and Hynes 1993
pJQ-oac2-K	pJQ200uc1 derivative carrying <i>oac2</i> with the <i>aphA-3</i> cassette (Gm, Km)	This work
poacMG	pBBR1MCS-3 derivative carrying a 2,700-bp <i>EcoRI-PvuII</i> fragment of pUC19-oac3200ES that contains <i>oac0</i> , <i>oac1</i> , and <i>oac2</i> (Tc)	This work

^a Antibiotic resistances of strains and plasmids are mentioned. Cb, carbenicillin; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline.

bacterial internalization (Fig. 4B). A comparison of the “mature” central regions clearly showed the aberrant organization of the defective nodules induced by ORS571-oac2, without plant cell invasion and with a huge number of large infection threads (Fig. 4C and D).

Fig. 1. Deoxycholate-polyacrylamide gel electrophoresis band migration patterns of lipopolysaccharide (LPS). Lane 1: *Salmonella typhimurium*; lane 2: ORS571; lane 3: ORS571-oac2; lane 4: ORS571-oac2(poacMG); lane 5: ORS571-X15. LPS was prepared by the hot water-phenol method. Lanes 2 and 4: phenol phase; lanes 3 and 5: water phase. Gel was silver stained, as described in text. No LPS could be detected with this method in the water phase of ORS571 and ORS571-oac2(poacMG) and the phenol phase of ORS571-X15 and ORS571-oac2 (Goethals et al. 1994; data not shown).

Nodule penetration by ORS571-oac2 is blocked at the internalization stage and shows defense symptoms.

Cells adjacent to infection pockets induced by ORS571-oac2 were unusually large and their walls emitted a blue fluorescence under UV light, indicative of the presence of phenolic compounds (Fig. 5A and B). This strong autofluorescence had never been observed in the walls of cells adjacent to infection pockets formed by the wild-type strain (Fig. 5C and D).

Electron microscopic observations showed striking differences between infection threads induced by wild-type or mutant bacteria (Fig. 6). Wild-type infection threads harbored few bacteria (Fig. 6A and E), which were embedded in an electron-dense matrix. The plant cells in the invasion zone had a large volume of cytosol and small vacuoles (Fig. 6A). Occasionally, infection thread wall degradation and bacterial internalization events were observed (Fig. 6A and C). In contrast, in nodule-like structures induced by ORS571-oac2, infection threads were wide and harbored many more bacteria that were irregular in shape and showed an electron-dense staining, which could be a sign of death (Fig. 6B, D, and E). Internalization events or internalized bacteria were not seen. The thread walls were extremely thick, consisting of many layers of a fibrous material (Fig. 6E). The plant cells in this tissue had a small volume of cytosol and large vacuoles (Fig. 6B). A higher magnification of an infection thread clearly shows the abnormal thread wall, which was tenfold thicker than in the wild-type nodules (Fig. 6E).

Quantification of SPS produced by ORS571, ORS571-oac2, and ORS571-X15.

The appearance of bacteria in infection pockets was observed by electron microscopy in an attempt to better understand the difference in the tissue-invasion capacity of ORS571-X15, which can only form infection pockets (D'Haeze et al. 1998), and ORS571-oac2, which can form infection pockets and threads (Fig. 6). Sections were made through a 4-day-old developing nodule and nodule-like structure induced by ORS571 and ORS571-oac2, respectively, and through a 7-day-old pseudonodule induced by ORS571-X15; similar infection pocket regions were studied. Wild-type bacteria were surrounded completely by a thick layer of low electron-dense material, suggesting the presence of a massive amount of what was most likely EPS (Fig. 7A and B). A similar layer also was present around ORS571-oac2, but in a lesser

Fig. 2. Hydrophobicity of ORS571, ORS571-oac2, and ORS571-X15. The hydrophobicity is expressed as the percentage of bacteria that remained in the inorganic phase after mixing with octane. The average of eight independent experiments is presented. Values that are significantly different ($p < 0.05$) are indicated by a, b, or c.

amount (Fig. 7C and D), in contrast to ORS571-X15, where it could never be detected (Fig. 7E and F).

In order to quantify EPS production, ORS571, ORS571-*oac2*, and ORS571-X15 were cultivated on plates containing MMO medium or LSO medium, with or without ammonium (see below). EPS were quantified by the anthrone method (Glucksmann et al. 1993; Reed et al. 1991). The results (Fig. 8) demonstrate that i) ORS571 produces a huge amount of EPS when grown on LSO medium, in contrast to cultivation on media supplied with ammonium; ii) ORS571-*oac2* produced more EPS on LSO medium than ORS571-X15, but approximately four to five times less than the wild-type strain; and iii) the presence of naringenin does not have a major influence on the production of EPS (Fig. 8). The sugar content of EPS prepared from ORS571, which was grown on LSO medium, was analyzed (see below). The high-pressure liquid chromatography (HPLC) data consisted of three peaks that eluted at 20.51, 23.39, and 25.64 min, in a relative ratio of 1:58:1.5, respectively (data not shown). Glucose, galactose, and arabinose–fucose standards were eluted at 20.30, 23.10, and 25.27 min, respectively.

DISCUSSION

The *oac2* gene of *A. caulinodans* is part of a cluster of four genes corresponding to the *rfb* locus of *S. typhimurium*. Previously, we characterized *oac3*, an *rfbA* ortholog, encoding a dTDP-L-glucose synthase (Goethals et al. 1994). dTDP-L-glucose is a precursor for dTDP-L-rhamnose synthesis. The OAC2 enzyme is a dTDP-L-rhamnose synthase that supposedly catalyzes the last step of this pathway. The knockout of *oac3* had dramatic consequences, causing a pleiotropic phenotype (Goethals et al. 1994). The LPS were affected, but most

likely other SPS were altered as well (see below). During its interaction with the host plant, the *oac3* mutant ORS571-X15 was arrested in outer cortical infection pockets, the primary colonization sites during the invasion process (D’Haeze et al. 1998).

The data on the consequences of an *oac2* knockout throw light on the role in symbiosis of the synthesis of dTPD-L-rhamnose for the production of LPS and EPS in *A. caulinodans*. With use of the hot water–phenol method, LPS from ORS571-*oac2* and ORS571-X15 partitioned in the water phase and therefore had different physicochemical properties than the LPS of the wild-type strain and complemented mutants (Goethals et al. 1994; this work) that partitioned in the phenol phase. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis and silver staining, which are considered suitable tools for an initial characterization of LPS (Forsberg and Carlson 1998; Hitchcock and Brown 1983), showed that the band migration patterns of LPS from the *oac2* and the *oac3* mutants were similar to each other and differed from the patterns of the wild type and the complemented mutant by a shift toward lower molecular weight. The presumed function of the *oac2* gene was supported by the presence of reduced rhamnose–Kdo levels in the LPS fraction of ORS571-*oac2*. Rhamnose incorporation in the O antigen of LPS synthesized by *Rhizobium* sp. CFN42 (Kannenberg and Brewin 1994) and *R. trifolii* 4s (Wang and Hollingsworth 1994) has been observed. In a variety of bacteria such as *S. typhimurium* strain LT2 (Jiang et al. 1991), *Yersinia enterocolitica* serotype O:3 (Zhang et al. 1993), and *S. enterica* (Xiang et al. 1993), the *rfbABCD* genes have been demonstrated to be involved in the synthesis of dTDP-rhamnose that is used to synthesize the O antigen. The same feature may be true for *A. caulinodans* ORS571 because ORS571-*oac2* produced truncated LPS

Fig. 3. Stem nodulation by wild-type ORS571 and ORS571-*oac2* mutant. **A**, *Sesbania rostrata* plants, the roots of which were inoculated with ORS571 (left) and ORS571-*oac2* (right), were photographed 3 weeks after inoculation. **B**, Stems inoculated with ORS571 (left) and ORS571-*oac2* (right). Nodules were cut by hand 20 days after inoculation. Orange-brown is the characteristic color for leghemoglobin. **C**, GUS-stained, hand-cut sections of 3-week-old nodules induced by ORS571(pRG960SD-32) (top) and ORS571-*oac2*(pRG960SD-32) (bottom). Bars = 1 mm.

molecules, on the basis of faster migration in detergent gel electrophoresis. The rhamnose levels of LPS from ORS571-*oac2* were reduced, although not to zero, indicating that yet another rhamnose synthase is present in *A. caulinodans*. Two pathways for rhamnose synthesis occur in some *Salmonella* spp. serovars (Schnaitman and Klena 1993).

Rhamnose also is a component of a nodule polysaccharide produced by *Bradyrhizobium japonicum* (An et al. 1995) and has been found in the EPS of some *Bradyrhizobium* spp. strains (Niehaus and Becker 1998). During growth in infection pockets, ORS571 produced a thick, low, electron-dense layer, consisting presumably of EPS. ORS571-*oac2* was surrounded by a much thinner layer of low, electron-dense material, which could be either capsular polysaccharides (CPS) or a truncated form of EPS, whereas ORS571-X15 bacteria were completely bare. This observation suggests that wild-type EPS may contain glucose and rhamnose. Conditions were obtained, under which EPS production could be quantified and characterized. Free-living, wild-type bacteria produced massive amounts of EPS when cultivated on LSO medium. ORS571-*oac2* and ORS571-X15 produced fivefold and tenfold less EPS than ORS571, respectively. Sugar determination of wild-type EPS revealed two minor and one major products with elution times corresponding to those of glucose, arabinose-fucose, and galactose, respectively. The major product is most likely a glu-

cose derivative rather than a galactose, as suggested by preliminary cellulose thin-layer chromatography analyses (W. D'Haeze and M. Holsters, *unpublished results*). Unexpectedly, no rhamnose could be detected. More likely, rhamnose was converted into another compound, although it cannot be excluded that the free-living conditions did not resemble exactly the in situ conditions, thereby affecting the synthesis of rhamnose, or that the method used was not sensitive enough to detect rhamnose in these samples. It is obvious that a more thorough characterization of EPS is required in a follow-up study.

In hydrophobicity assays in which EPS is washed away, wild type and *oac2* behaved similarly, although their LPS are very different, thus suggesting a third actor in the play, possibly CPS, which could be responsible for the thin slime layer surrounding ORS571-*oac2* in infection pockets. In ORS571-X15, all SPS molecules containing either rhamnose or glucose derivatives are absent, and the result is an arrest in the outer cortex. The formation of outer cortical infection pockets is a process that depends on Nod factors (D'Haeze et al. 1998). The CPS of *A. caulinodans* may be required for the release from outer infection pockets and the formation of infection threads, whereas EPS-LPS may be important for the release from infection threads and the subsequent internalization into plant cells. Additionally, LPS and CPS produced by ORS571, ORS571-*oac2*, and ORS571-

Fig. 4. Morphology of nodules and nodule-like structures. Light micrographs of longitudinal sections through **A and B**, peripheral and **C and D**, central regions of **A and C**, wild-type stem nodules and **B and D**, nodule-like structures induced by ORS571-*oac2*. Double arrowhead indicates flattened inner cortex cells. i = infected cell; it = infection thread; u = uninfected cell; vb = vascular bundle. Bars = 50 μ m.

X15 will be purified and characterized in a follow-up study in order to better correlate differences in infection behavior with differences in cell envelope properties.

The invasion of ORS571-*oac2* was accompanied by strong symptoms of defense at the level of infection threads and surrounding plant cells such as thick walls, autofluorescence, and possibly loss of bacterial viability. Somewhat similar observations have been made in root nodules of *Medicago truncatula* (Niehaus et al. 1998), which were induced by the *Sinorhizobium meliloti* LPS mutant Rm6963 and in pea nodules by *Rhizobium leguminosarum* LPS mutant 3841 (Perotto et al. 1994). In those systems, however, internalization of bacteria did take place, whereas in the interaction between ORS571-*oac2* and *S. rostrata*, bacterial exit from infection threads was blocked completely. It seems logical to presume that the arrested nodule development is the consequence of arrested bacterial invasion and the elicitation of defense responses. Perhaps nodule development stops because a conditional effect of the *oac2* mutation is lethal for the bacterial viability in infection threads, which is caused by a lack of suppression of defense, or because the truncated SPS induce a defense reaction. Finally, it is possible that LPS or EPS are active signaling molecules for the release of bacteria from infection threads. Indeed, Verma and Hong (1996) proposed that a group of rhizobial signal molecules, other than Nod factors, may trigger endocytosis. These molecules were defined as "endocytosis factors" (Verma and Hong 1996).

Hence, the *oac2* knockout renders the interaction between *A. caulinodans* and *S. rostrata* incompatible at the bacterial internalization stage. The molecular mechanisms that mediate the switch from a compatible to an incompatible interaction remain to be described, but a controlling role of rhamnose-containing LPS and EPS is extremely probable.

Fig. 5. Epifluorescent micrographs of nodules and nodule-like structures. Infection threads viewed by normal (left) and fluorescence (right) microscopy in **A** and **B**, 3-week-old nodule-like structure induced by ORS571-*oac2* and in **C** and **D**, nodules induced by ORS571. Bars = 1 mm (**A** and **C**) and 100 µm (**B** and **D**).

MATERIALS AND METHODS

Bacterial strains and plasmids.

Bacterial strains and plasmids are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani medium (Miller 1972). *A. caulinodans* ORS571 strains were grown on either YEB (Geremia et al. 1994), MMO (Goethals et al. 1989), or LSO medium, with or without 0.1% (NH₄)₂SO₄ (Elmerich et al. 1982). If applicable, naringenin was added at a final concentration of 20 µM. Antibiotics were added as described by D'Haeze et al. (1998).

Molecular biology techniques.

Standard molecular techniques were performed as described by Sambrook et al. (1989). To construct the nonpolar mutant ORS571-*oac2*, a filled-in, 850-bp *EcoRI*-*HincII* fragment from plasmid pUC18K (Ménard et al. 1993) containing the kanamycin resistance gene cassette (*aphA-3*) was inserted into a T4 DNA polymerase-treated *BsmI* site of *oac2* in plasmid pUC19-*oac3200ES*, yielding pUC19-*oac3200ES-K* (Table 1). In this construct, the ATG start codon, located downstream from the kanamycin resistance gene, is in-frame with the TAA stop codon of the *oac2* gene, as confirmed by sequencing.

A filled-in, 4,195-bp *EcoRI*-*HindIII* fragment from pUC19-*oac3200ES-K* containing the mutated *oac2* gene was cloned in the *SmaI* site of the suicide vector pJQ200uc1 (Quandt and Hynes 1993). The resulting plasmid was mobilized into the wild-type strain ORS571 by triparental mating (Van den Eede et al. 1992). Cointegrates were identified by selection for gentamicin (Gm) resistance and screening for sensitivity to 15% (wt/vol) sucrose, both markers of pJQ200uc1. Candidate double recombinants were selected from cointegrates by growth in the presence of 15% sucrose and kanamycin and subsequent screening for Gm sensitivity. The ORS571-*oac2* mutant was confirmed by DNA gel blot analysis. To construct a complementing plasmid, poacMG, a 2.7-kb *EcoRI*-*PvuII* fragment containing *oac2*, was isolated from the plasmid pUC19-*oac3200ES* and cloned in the wide host-range vector pBBR1MCS-3 (Table 1).

LPS preparation and analysis.

Bacteria were grown in YEB medium until stationary phase and harvested by centrifugation. LPS was extracted from the bacterial pellets by the hot-water method (Goethals et al. 1994). The water phases were washed additionally with phenol to remove traces of phenol-soluble material. Water and phenol phases were dialyzed (molecular weight cut off at 12,000 to 14,000; Spectra/Por, Spectrum, Laguna Hill, CA, U.S.A.), and treated with 20 µg of proteinase K per ml at 37°C overnight. The samples were dialyzed overnight against distilled water and concentrated in vacuo. Prior to electrophoresis on a 12.5% polyacrylamide gel with deoxycholate as detergent (Carlson et al. 1995), LPS fractions were ultracentrifuged at 100,000 g for 4 h. The LPS pellet was resuspended in water, and gels were silver stained to detect LPS (Tsai and Frasch 1982). LPS from *S. typhimurium* was used as control (Sigma-Aldrich, St. Louis, MO, U.S.A.). To check for the presence of other Kdo-rich polymers, LPS preparations were electrophoresed, and the gel was silver stained after treatment with Alcian blue (Reuhs et al. 1993). No evidence for the presence of other major Kdo polymers was obtained (data not shown).

To determine monosaccharides, LPS were hydrolyzed in 1% acetic acid at 100°C for 90 min, cooled to room temperature, and centrifuged ($1000 \times g$) for 5 min to remove lipid A (Dazzo et al. 1991). The supernatant was washed, and lipid-free polysaccharides were dried and hydrolyzed to monosaccharides,

according to Stevenson et al. (1994). Monosaccharides were converted to their corresponding trimethylsilylethers of methyl glycosides and analyzed by gas chromatography–mass spectrometry, as described (Wolucka and de Hoffmann 1995). Glucose and rhamnose were identified by their retention times and

Fig. 6. Electron micrographs of central tissues and infection threads in stem nodule and nodule-like structures induced by ORS571 and ORS571-oac2, respectively. **A**, Wild-type central tissue. Arrow indicates a bacterium releasing from an infection thread. **B**, Central tissue of a nodule-like structure induced by ORS571-oac2. **C**, Enlargement of **A**, showing rod-shaped bacteria in infection threads. **D**, Enlargement of **B**, showing aberrantly shaped bacteria. **E**, Closeup of the wall of abnormal threads in ORS571-oac2-induced nodule-like structures (inset: wild-type infection thread wall for comparison). b = bacteria; it = infection thread; itw = infection thread wall. Bars = 10 μ m (**A** and **B**) and 2 μ m (**C–E**).

mass spectrometry spectra. Kdo was quantified by a colorimetric method (Strominger et al. 1959).

Hydrophobicity assay.

Bacteria were grown in MMO medium until early stationary phase. The hydrophobicity was determined as described previously (Goethals et al. 1994). A statistical analysis was performed with use of the Student's *t* test.

Quantification and sugar analysis of EPS.

EPS were quantified by the anthrone method. Briefly, bacteria were grown on plates for 3 to 4 days to avoid contaminations of sugars in liquid media. Bacteria with EPS were removed from the plate, transferred to a recipient, resuspended in 300 μ l of λ buffer (10 mM Tris-HCl; 10 mM MgSO₄, pH 7.2), and centrifuged for 5 min at 14,000 rpm. The supernatant was put aside, and the pellet was washed with an additional 300 μ l of λ buffer, centrifuged again, and added to the first supernatant. The bacterial pellet was resuspended in 500 μ l of

λ buffer to measure the optical density (OD) at 600 nm. Of the combined supernatant, 50 to 300 μ l were diluted with distilled water to a final volume of 500 μ l. One milliliter of 0.2% anthrone (Sigma-Aldrich) in concentrated H₂SO₄ was then added carefully, and the mixture was vortexed for 15 s. Subsequently, the OD₆₂₀ was measured. D-glucose was used as a standard to calculate the glucose equivalents in the EPS samples, which were divided by the respective OD₆₀₀ in order to normalize influences of different bacterial densities.

EPS samples were hydrolyzed in 2 M trifluoroacetic acid at 121°C for 2 h. The sugar analysis was performed as described by Honda et al. (1989). Briefly, approximately 20 μ g of glucose equivalents were taken and mixed with 50 μ l of 0.5 M methanolic solution of 3-methyl-1-phenyl-2-pyrazolin-5-on (Sigma-Aldrich) and 50 μ l of 0.3 M NaOH. This mixture was incubated at 70°C for 30 min, cooled, and neutralized with 150 μ l of 0.1 M HCl. The solution was evaporated until dry. The pellet was resuspended in 200 μ l of water and 200 μ l of chloroform, and the mixture was shaken vigorously and centrifuged for 5 min at 14,000 rpm. The water phase was dried, and the pellet was resuspended in 100 μ l of 18% acetonitrile in 0.1 M phosphate buffer (pH 7.0). A fraction was analyzed by HPLC (Honda et al. 1989).

Nodulation assays.

S. rostrata seeds were surface sterilized and germinated (Goormachtig et al. 1995). For root nodulation assays, 2-day-old seedlings were transferred to tubes as described (Fernández-López et al. 1998). For stem nodulation assays, plants were grown in pots. Stems were inoculated by painting with a bacterial suspension. Plants were cultured at 28°C, with a light period of 16 h. Reisolation of ORS571-oac2 from stem nodules was performed as described by D'Haeze et al. (1998).

GUS staining of stem nodule sections.

Nodules induced by ORS571 derivatives carrying the *nodA-uidA* reporter plasmid were harvested 3 weeks after inoculation. Hand-cut sections were washed with 90% acetone and GUS stained, according to D'Haeze et al. (1998), with

Fig. 7. Electron micrographs of infection pockets in **A and B**, a 4-day-old developing nodule induced by ORS571, **C and D**, a 4-day-old nodule-like structure induced by ORS571-oac2, and **E and F**, a 7-day-old pseudonodule induced by ORS571-X15. b = bacteria; cw = thickened cell wall of a neighboring cell; s = extracellular polysaccharides. Bars = 10 μ m (**A**, **C**, and **E**) and 1 μ m (**B**, **D**, and **F**).

Fig. 8. Extracellular polysaccharides (EPS) quantification. ORS571, ORS571-oac2, and ORS571-X15 were cultivated on MMO medium (dotted bars) and nitrogen fixation medium without ammonium (black bars) or with 0.1% (NH₄)₂SO₄ (white bars) in the presence (+) or absence (–) of naringenin. The equivalent mass of EPS per optical density at 600 nm for ORS571 grown on nitrogen fixation medium in the absence of naringenin was set at 100%. An average of two independent quantifications is presented.

0.5 mg of 5-bromo-4-chloro-3-indolyl- β -D-glucuronide per ml. Stained sections were observed with a binocular (Zeiss, Jena, Germany).

Microscopy.

Nodules harvested 4 days and 3 weeks after inoculation were treated for light and electron microscopy, as described by D'Haeze et al. (1998). Nodules for light microscopy were fixed by 2.5% glutaraldehyde, embedded in Technovit 7100 (Kulzer Histo-Technik, Wehrheim, Germany), and cut into 2.5- μ m sections. The sections were stained with 0.5% toluidine blue, mounted with Depex (Sigma-Aldrich), and examined under the bright field of a Diaplan microscope (Leitz, Wetzlar, Germany). Nodules for electron microscopy were fixed by a mixture of 2.5% formaldehyde and 2.5% glutaraldehyde in 0.1 M sodium-cacodylate buffer, dehydrated, and embedded in LR White hard grade (London Resin, Basingstoke, U.K.). Sections of 2 μ m were viewed by phase contrast with a light microscope. Once infection threads were located, thin sections of 60 nm were cut, stained with 2% uranyl acetate for 12 min followed by 1% lead citrate for 5 min, and then examined by transmission electron microscopy (Elmiskop 101; Siemens, Karlsruhe, Germany). For fluorescence microscopy an Axioskop microscope (Zeiss) was used. The blue autofluorescence was observed under UV light.

ACKNOWLEDGMENTS

We are grateful to R. Rozenberg and E. de Hoffmann (Department of Chemistry, University of Louvain-la-Neuve, Belgium) for the opportunity to perform GC-MS and to help interpret the data; C. Verplanck for growing *Sesbania rostrata* plants; F. Van Gijsegem for providing pUC18K and for critical reading of the manuscript; M. De Cock for help in preparing the manuscript; and K. Spruyt, R. Verbanck, and S. Debruyne for photographs and artwork.

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