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Detection and visualization of the major acidic exopolysaccharide of *Ralstonia solanacearum* and its role in tomato root infection and vascular colonization

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Abstract

Exopolysaccharides play an important role in the pathogenicity of *Ralstonia solanacearum*. We compared *in vitro* and *in planta* exopolysaccharide production of the pathogenic strain AW1 with that of three related mutant strains impaired in both their exopolysaccharide production and aggressiveness on tomato. The distinction between the two hexosamine-rich exopolysaccharides, namely the N-acetyl-glucosaminorhamnan and the major N-acetyl-galactosamine-containing acidic polymer was emphasized. The major acidic polymer was identified specifically by electron microscopy using glutaraldehyde/ruthenium red/uranyl acetate staining, by immunofluorescence using specific monoclonal antibodies and correlated to an appropriate biochemical analysis. The two mutant strains AW1-1 and AW-19A were totally devoid of any production of the major exopolysaccharide *in vitro* or *in planta* whatever the technique used. Infection and vascular colonization of tomato roots by the pathogenic strain were also compared to those of the mutant strains by light microscopy. Pathogenicity on tomato was assessed by root infection without any artificial injury. Light microscopy showed that the two mutant strains AW1-1 and AW-19A were poorly infective and unable to invade xylem vessels, while they induced defence mechanisms in root tissues and appeared aggregated or degenerated within cortical infection pockets. These two mutant strains were non-pathogenic or weakly aggressive, respectively. In contrast, the pathogenic strain AW1 and the hypoaggressive AW1-41 strains, which produce large amounts of the major acidic exopolysaccharide *in planta*, were both infective and invasive, and tomato root tissues exhibited only limited defence reactions. Thus, the major acidic exopolysaccharide produced by *Ralstonia solanacearum* is involved in root infection and vascular colonization, though its precise role is still unknown.

Abbreviations: CTAB – hexadecyltrimethylammonium bromide; GLC – gas liquid chromatography; GRU – glutaraldehyde/ruthenium red/uranyl acetate; mAbs – monoclonal antibodies; NMR – nuclear magnetic resonance.

Introduction

Ralstonia solanacearum, previously named *Burkholderia* (*Pseudomonas*) *solanacearum* (Yabuuchi et al., 1992; 1995), is the causative agent of bacterial wilt, which affects numerous host plants of economic importance world wide (Kelman, 1953; Buddenhagen and Kelman, 1964; Hayward, 1994). Abundant exopolysaccharide (EPS) or slime produced by pathogenic strains of *R. solanacearum* on agar medium and *in planta* has long been correlated with pathogenicity of *R. solanacearum* (Husain and Kelman, 1958; Wallis and Truter, 1978; Denny and Baek, 1991; Cook and Sequeira, 1991). In rich medium, the wild type strain GMI1000 of *R. solanacearum* produces three EPSs (Orgambide et al., 1991; Trigalet-Demery et al., 1993): the high-molecular-weight acidic polymer EPS1, which accounts for up to 90% of the dry

weight of the EPSs and two minor polymers, namely a N-acetyl-glucosaminorhamnan (EPS3) and a glucan (EPS4). The structure of the trisaccharidic repeating unit of EPS1, composed of N-acetyl-galactosamine, N-acetyl-galactosaminuronic acid and bacillosamine derivative in an equimolar ratio, has been established (Orgambide et al., 1991). The same repeating unit has been found in the EPS1 polymer produced by different pathogenic strains of *R. solanacearum* (Trigalet-Demery et al., 1993). Production of EPS is controlled by the *eps* and *ops* gene clusters (Denny and Baek, 1991; Cook and Sequeira, 1991; Kao et al., 1992). While the *ops* gene cluster is required for the biosynthesis of both the O-antigenic chain of lipopolysaccharide (LPS) and the hexosamine-rich EPS (Kao and Sequeira, 1991), the 18 kb *eps* gene cluster appears to be specifically involved in the production of the hexosamine-rich EPS (Schell et al., 1993). In addition, 5 kb downstream from the *eps* operon, a 3 kb long locus (*rgnII*) controls the conditional production of the hexosamine-rich EPS. Strains which have mutations in the *eps* or *rgnII* genes are classified as EPS^i (impaired) mutants (Denny et al., 1988; Denny and Baek, 1991; Schell et al., 1993). Regulation of the *eps* operon is complex and dependent on multiple environmental factors both *in vitro* and during the interaction with the host plant (Brumbley and Denny, 1990; Huang and Schell, 1995).

Until now, quantification of EPS has been carried out by colorimetric estimation of total hexosamines (Denny et al., 1988; Denny and Baek, 1991; Schell et al., 1993), but this quantification did not distinguish the N-acetyl-galactosamine-rich major EPS1 polymer from the N-acetyl-glucosaminorhamnan EPS3 and thus left the EPS^i denomination ambiguous. The distinction between EPS1 and EPS3 (structurally identical to LPS) is necessary since the O-antigen chain of LPS of *R. solanacearum* is composed of repeating units of rhamnose and N-acetyl-glucosamine residues (Akiyama et al., 1985; Kocharova et al., 1993; 1994) and also because LPS fractions have long been demonstrated to be biologically active in the interaction with the host plants (Whatley et al., 1980; Sequeira, 1982; Graham, 1983; Duvick and Sequeira, 1984).

Little is currently known about the role of the EPS produced *in planta* by *R. solanacearum*. This slime increases the viscosity of the sap and may thus block the vascular stream within xylem vessels (Husain and Kelman, 1958). Microscopic studies confirmed the accumulation of large amounts of bacterial EPS within infected vessels (Wallis and Truter, 1978), and demonstrated that a spontaneous EPS-deficient mutant strain of *R. solanacearum* was unable to invade protoxylem vessels (Vasse et al., 1995). The EPS may saturate lectin binding sites on cell walls (Sequeira and Graham, 1977) and thus prevent bacteria from being immobilized on mesophyll cell walls in tobacco leaves (Sequeira and Hill, 1974) where slimy pathogenic strains are not attached (Sequeira et al., 1977). In potato or tobacco cell walls, lectins have been shown to be specific for the internal N-acetyl-glucosamine in the core-LPS (Leach et al., 1982), and the major EPS has been demonstrated to be an effective inhibitor of bacterial agglutination by potato lectin (Duvick and Sequeira, 1984).

The present study was undertaken in order (i) to distinguish EPS1 from the EPS3 polymer microscopically, thus permitting a more precise description of the defect of EPS^i mutant strains and (ii) to address the role of EPS1 during the root infection process. Consequently, we compared the biochemical characterization and the microscopic appearance of the EPS1 polymer in some EPS^i mutants obtained by transposon insertion within the *eps* or *rgnII* genes and impaired in pathogenicity (Denny et al., 1988; Denny and Baek, 1991; Schell et al., 1993). In mutant strain AW 19A, Tn5 is located in the *eps*B gene (Clough et al., 1997); this gene encodes a transmembrane protein which is involved either in export of the trimeric repeat units across the inner membrane or in their polymerization. Mutant strain AW1-1 is in the left side of the *eps* gene cluster more than 17 kb away from mutant strain AW-19A, while AW1-41 is located within the *xps*R operon (Denny and Baek, 1991), though their precise locations are not fully characterized; additional work is still unpublished (Denny, personal communication). We also compared the infection of tomato roots and vascular colonization by these EPS^i mutant strains to that of a pathogenic strain.

Materials and methods

Bacterial strains and growth conditions

Mutant strains AW1-41, AW1-1 and AW-19A of *R. solanacearum* were derived from the pathogenic strains AW1 or AW (Table 1). For microscopic study, mutant strains were constructed by insertion of plasmid pXLGD4 (Table 1), which contains the promoter region of the *Rhizobium meliloti* δ-amino levulinic acid synthetase gene and which constitutively expresses

Table 1. Bacterial strains and specific characteristics

Strains and plasmid	Relevant characteristics	Source	New denomination*
AW1	EPS^+, AW derivative, Nal^r	Denny et al., 1988	$EPS1^+$
AW1-41	EPS^i, AW1 derivative, *rgnII*::Tn5, Nal^r, Km^r	Denny et al., 1988	$EPS1^i$
AW-19A	EPS^i, AW derivative, *eps*::Tn5, Nal^r, Km^r	Schell et al., 1993	$EPS1^-$
AW1-1	EPS^i, AW1 derivative, *eps*::Tn5, Nal^r, Km^r	Denny et al., 1988	$EPS1^-$
AW1(pXLGD4)	EPS^+, β-Gal, Nal^r, Tc^r	this work	$EPS1^+$
AW1-41(pXLGD4)	EPS^i, β-Gal, Nal^r, Km^r, Tc^r	this work	$EPS1^i$
AW-19A(pXLGD4)	EPS^i, β-Gal, Nal^r, Km^r, Tc^r	this work	$EPS1^-$
AW1-1(pXLGD4)	EPS^i, β-Gal, Km^r, Nal^r, Tc^r	this work	$EPS1^-$
pXLGD4	*lacZ*, Tc^r	Ditter et al., 1985 Leong et al., 1985	

EPS^+: Wild type, mucoid colonies on agar medium.
EPS^i: Impaired production of exopolysaccharides, medium to very rough colony morphology on agar rich medium.
β-Gal: β-Galactosidase active is constitutively expressed.
*: This work.

β-galactosidase in this plasmid (Ditta et al., 1985; Leong et al., 1985). Bacterial strains were streaked out on triphenyltetrazolium chloride agar (Kelman, 1954) and grown at 30°C on a gyratory incubator shaker in rich liquid culture medium (Boucher et al., 1985) containing 0.5% (w/v) glucose or minimal liquid culture medium (Schell et al., 1988). Media were supplemented with tetracycline (10 mg ml^{-1}), kanamycin (50 μg ml^{-1}) and/or nalidixic acid (10 μg ml^{-1}), when required.

Plant cultures and inoculations

Tomato plant inoculations were performed with 20-h-old cultures grown in rich liquid medium.

For the study of EPS1 production or bacterial multiplication *in planta*, we used tomato plants of the susceptible cultivar Supermarmande grown in 8-cm-diameter peat pots containing friable horticultural humus. For EPS1 production analysis, four week-old plants were inoculated by pouring 100 ml of a bacterial suspension onto the roots as previously described (Trigalet and Trigalet-Demery, 1986) adjusted to 1×10^7 to 5×10^7 CFU ml^{-1} into each pot, unless otherwise indicated, without any artificial root injury. In order to determine generation time following an artificial inoculation, stem inoculations were carried out by injecting about 3×10^4 CFU into each plant by puncturing at the bottom of the stem. For microscopic analysis of tomato root infection, we used tomato plants of susceptible cultivar Supermarmande grown in axenic tubes (5 plants per tube) containing 0.46% Murashige and Skoog nutrient solution, as previously described (Vasse et al., 1995). Plants were inoculated by adding bacteria into the plant nutrient solution to give a final bacterial density of about 6×10^6 CFU ml^{-1}.

Electron microscopy of bacterial suspensions

Bacterial suspensions analyzed were (i) 24-h-old cultures in rich medium (end of the exponential growth phase), (ii) 48-h-old cultures in minimal medium (end of the exponential growth phase), and (iii) bacterial suspensions that oozed from infected plants. The glutaraldehyde/ruthenium red/uranyl acetate staining (GRU) procedure was carried out according to a slightly modified procedure of Mutaftschiev et al. (1982), using a 0.1% (w/v) uranyl acetate aqueous solution. Grids were examined with a Hitachi H 600 transmission electron microscope operating at 75 kV. Evaluating the presence of acidic EPS on the bacterial surface was carried out by random examination of an average of 600 bacteria in samples from culture in rich medium and stem exudates from infected tomato and of 150 bacteria in samples from culture in minimal medium.

Antibodies and immunofluorescence staining

Monoclonal antibodies (mAbs) were prepared by Biocytex Company (Marseille, France). Briefly, six-week old Balb/c mice were immunized intraperitoneally, monthly over four months, with 100 μg purified EPS1 from the pathogenic *R. solanacearum* strain GMI1000. Hybrids producing specific mAbs were selected by ELISA using the purified EPS1 antigen. Hybrid

X18F12 supernatant revealed the highest titer and was further used for immunofluorescence staining.

The specificity of the X18F12 supernatant was checked by ELISA against the various purified polysaccharides produced by *R. solanacearum*, namely the purified major acidic EPS1 antigen, the N-acetyl-glucosaminorhamnan EPS3, the glucan EPS4 and whole cells of the four strains under study. To avoid contamination of EPS1 with protein, EPS1 antigen was treated with proteinase K (Boehringer Mannheim) for 2 h at 37°C and further used as a control. Samples (50 µl) were deposited into the wells of a microtiter plate (Nunc-Immuno Plate Maxisorp TM) at a concentration of 2.5 µg polysaccharide per well or 10^7 bacteria per well, and allowed to evaporate overnight at 37°C. The wells were blocked with 3% (w/v) skimmed milk in phosphate buffered saline (PBS) 2 h at room temperature and rinsed three times with 1% (w/v) skimmed milk and 0.1% (v/v) Tween 20 in PBS. Undiluted X18F12 supernatant in PBS containing 1% (w/v) skimmed milk was added to the wells and incubated for 2 h at 37°C. After five rinses with PBS, goat anti-mouse, alkaline phosphatase-conjugated antibodies (TEBU) were added to the wells and incubated for 2 h at 37°C. After five washes with PBS the alkaline phosphatase substrate (Biorad) was added and incubated for 30 min at 37°C. The A_{405} was read with a spectrophotometer (Dynatech MR5000). Each value was measured in triplicate.

Immunofluorescence was carried out by an indirect procedure using an anti-mouse immunoglobulin conjugated with FITC (Boehringer Mannheim) as the secondary antibody. Immunofluorescence staining was observed with an epi-fluorescence microscope (Axiophot, Carl Zeiss, Germany) using an immersion Plan-Neofluar 100×/1.30 objective.

Purification and characterization of the hexosamine-containing polysaccharides

Cells were collected by centrifugation at the beginning of the stationary growth phase (24 h) in rich medium, unless otherwise indicated. The crude EPS produced *in planta* was prepared from bacterial suspensions obtained from material oozing from infected plants. Crude EPS was eluted with distilled water through a cation-exchange resin Dowex 50WX2-H^+ form (Fluka Co.), and the eluate was lyophilized as already described (Orgambide et al., 1991).

Further fractionation of crude EPS was not achieved under dissociative conditions as previously described (Orgambide et al., 1991). The Dowex eluate was loaded on a Bio-Gel P-4 (Bio Rad lab.) column and eluted with 0.5% (v/v) acetic acid. The fraction excluded from the Bio-Gel P-4 column was further fractionated over an anion exchange DEAE Trisacryl M resin (I.B.F, Biotechnics) eluted successively with increasing concentrations of NaCl (0.08 M and 0.2 M) in a 0.01 M NH_4Cl (pH 8.3) solution. After dialysis, the fraction eluted with 0.2 M NaCl was concentrated and precipitated with a 2% (w/v) solution of CTAB (hexadecyltrimethylammonium bromide). The precipitate was recovered by centrifugation and was resuspended in a saturated solution of KCl. Then, CTAB was removed by extraction with chloroform. The aqueous phase was concentrated and dialyzed to remove KCl. A final purification step of EPS1 consisted of the extraction of traces of contaminating LPS from CTAB precipitated materials with a 1.5% (w/v) solution of Triton X114 (Adam et al., 1995). Traces of Triton X114 in aqueous phase were eliminated by extraction with chloroform.

Identification of the monosaccharides typifying the different polymers was carried out, from dialyzed and lyophilized extracts, by gas liquid chromatography (GLC) analyses as previously described (Orgambide et al., 1991) of the different fractions from each strain. ^{1}H-NMR spectra of the purified EPS1 from the different strains were performed at 80°C in ^2H_2O and recorded on a Bruker AM 250 instrument (Orgambide et al., 1991).

Isolation and characterization of cell-associated material

To obtain the cell-associated materials, bacterial pellets from 24-h-old cultures in rich medium were rinsed twice with a 0.5% (w/v) NaCl solution. After dialysis of the washes, the recovered materials were precipitated with a 2% (w/v) solution of CTAB. The presence of N-acetyl-galactosamine in the precipitated materials was assessed by GLC analyses of the trimethylsilyl derivatives of methyl glycosides.

Microscopic study of tomato root infection

Light microscopy was performed on whole roots and on root sections as previously described (Vasse et al., 1995). Whole roots of seedlings were processed 1, 2, and 3 weeks after inoculation, and root sections were obtained from root segments of interest dissected from whole roots already fixed and histochemically stained

to specifically reveal bacterial β-galactosidase activity. These segments were successively fixed again, dehydrated and progressively embedded in epoxy resin. Ultrastructural observations by transmission electron microscopy were performed on ultrathin sections of resin embedded root segments, as previously described (Vasse et al., 1995).

Pathogenicity, bacterial invasion and multiplication in tomato plants grown in pots

Pathogenicity was measured as percentages of wilting at different times after root inoculation without artificial injury. Bacterial invasion and quantification were done as described elsewhere (Frey et al., 1990; Etchebar et al., 1998) when 100% wilt was reached at the third and fourth days after inoculation for pathogenic strain AW1 and mutant strain AW1-41, respectively, or at the thirtheenth day for mutant strains AW1-1 and AW-19A.

The generation time *in planta* following artificial stem inoculation was determined by a daily sampling of 10 plants during five days. The 10 stems were surface sterilized with a 70% (v/v) ethanol solution and then crushed together into 35 ml of sterile distilled water with an electric crusher. Crushed material was filtered through a Whatman filter and the bacterial density of the solution obtained was determined. For each strain, generation times were calculated at intervals during five days.

Results

Microscopic examination of EPS1 production

Negative staining using ruthenium red (GRU staining) allowed us to rapidly observe the acidic extracellular materials which were associated with the cell surface or loose in extracellular medium. According to these observations, the *R. solanacearum* strain AW1 and related mutants (Table 1) could be separated into three groups according to the presence or the absence of such materials:

Group 1: Whatever the growth conditions, i.e. in rich and minimal media as well as in infected plant exudates, the pathogenic strain AW1 exhibited both regularly distributed surface-associated material in the form of knobs, and loose material further referred to as matrix (Figure 1a).

Group 2: In all growth conditions tested the mutant strains AW1-1 and AW-19A never exhibited either cell-associated knobs or matrix in the extracellular medium (Figure 1b).

Group 3: In all growth conditions tested, the mutant strain AW1-41 exhibited a variable ratio of cells harbouring knobs (Figure 1c). The presence of loose extracellular material was only observed in plant exudates (Figure 1d).

Whatever the growth conditions, the percentage of cells of the pathogenic strain AW1 exhibiting knobs was significantly higher than that of the mutant strains (Table 2).

By ELISA test, the X18F12 monoclonal antibodies (mAbs) bound to purified EPS1, purified EPS1 treated with proteinase K and whole cells of the strains AW1 and AW1-41, but did not bind to the N-acetyl-glucosaminorhamnan EPS3, the glucan EPS4 or whole cells of the strains AW1-1 or AW-19A. Similarly, immunofluorescence experiments using X18F12 mAbs gave positive labeling of smears from cultures of the strains AW1 and AW1-41 grown in rich medium whereas no labeling was observed with the mutant strains AW1-1 and AW-19A. Furthermore, smears of exudates from plants infected by the pathogenic strain AW1 or by the mutant strain AW1-41 were positively labeled by the X18F12 mAbs whereas exudates from plants infected by mutant strains AW1-1 or AW-19A were not (data not shown). Thus, microscopic study with GRU staining and mAbs showed that the mutant strains AW1-1 and AW-19A do not produce any detectable EPS1either *in vitro* or *in planta*.

Biochemical characterization of the major acidic EPS1 polymer

Following cation-exchange and gel permeation chromatography, one constituent (N-acetyl-galactosamine) of the acidic EPS1 polymer was identified by GLC in extracts from rich medium culture of both the pathogenic strain AW1 and the mutant strain AW1-41, as well as in exudates from tomato plants infected by either strain. No trace of N-acetyl-galactosamine derivative was detected in either mutant strain AW1-1 or AW-19A. Quantitative GLC analyses revealed that the Dowex eluate of the mutant strain AW1-41 contained about 20 times less N-acetyl-galactosamine derivative than that of the pathogenic strain AW1 when cultivated in rich medium, whereas the two strains produce comparable amounts of EPS1 *in planta*.

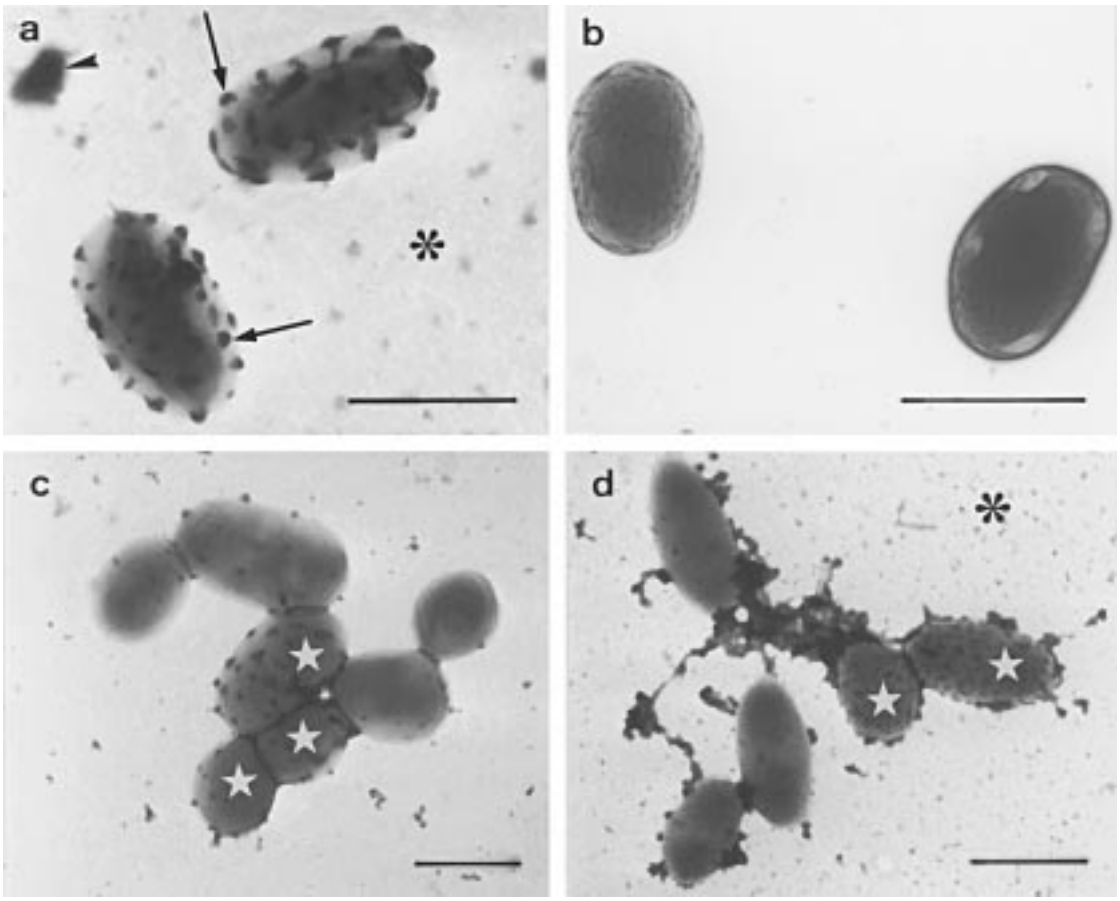

Figure 1. GRU staining of bacterial suspensions of the wild type AW1 strain and related mutant strains (AW1-41 and AW-19A) from 24-h-old cultures in rich medium (a–c) and plant exudates (d). All pictures were overexposed to show the knobs on bacterial surface in order to tell the strains apart. (a) Most cells of the wild type AW1 strain exhibited numerous knobs regularly distributed on their surface (arrows) and loose extracellular material (matrix) was observed (asterisk). Large particles of extracellular material were observed (upper left corner, arrowhead). (b) The surface of bacterial cells of the mutant strain AW-19A was completely devoid of any knob and no extracellular matrix was visualized. (c) Some bacteria of the mutant strain AW1-41 harbored knobs (stars), while others did not. (d) As in (c) some bacteria (stars) of the mutant strain AW1-41 originating from stem exudate of an infected plant exhibited numerous knobs on their surface and an extracellular matrix was clearly observed (asterisk). Scale bars = 1 μm.

Small amounts of a N-acetyl-glucosamine (EPS3 marker) derivative were detected in Dowex and/or Bio-Gel P-4 eluates from cultures of the AW1, AW1-41, AW1-1 and AW-19A strains grown in rich medium and from plant exudates of the AW1 and AW1-41 strains.

Purified EPS1 polymers produced in rich medium or *in planta* by the strains AW1 and AW1-41 exhibited ^{1}H-NMR spectra identical to the reference spectrum of the EPS1 produced by the wild type strain GMI1000 (Orgambide et al., 1991).

The presence of EPS1 polymer in the cell-associated knobby materials revealed by the GRU staining was assessed. Centrifugation of bacteria of the wild type strain AW1 or the mutant strain AW1-41 grown in rich medium was not sufficient to wash off the knobs. Two washes of AW1 bacteria with NaCl solution (0.5% w/v), removed most of the knobs, whereas two NaCl (0.5% w/v) washes of bacteria from a culture of the strain AW1-41 stripped off all of the knobs (not shown). As expected, GLC analysis showed that

Table 2. Percentages of bacterial cells exhibiting knobs, by the GRU method, in different growth conditions. Results are expressed as percentages of cells exhibiting knobs, sampled during *in vitro* culture, in rich (24 h) or minimal medium (48 h), or from infected plants. An average of 600 cells were counted for each sample from either rich medium or infected plant exudates; 150 cells were counted for samples from minimal medium

Growth conditions	Strains			
	AW1	AW1-41	AW1-1	AW-19A
Rich medium	97[a]	42[b]	0	0
Minimal medium	95[a]	42[b]	0	0
In planta	95[a]	85[c]	0	0

[a,b,c] Data followed by the same letter are not significantly different according to the 95% confidence limits of frequencies.

the cell-associated materials recovered from the strain AW1 or AW1-41 contained EPS1 polymer whereas no N-acetyl-galactosamine was ever detected in washes of the strains AW1-1 and AW-19A.

These results demonstrate that the knobby materials, which are associated to the bacterial surface and visualized by the GRU staining, contained the acidic EPS1 polymer. Moreover, the three distinct groups of strains observed by microscopic observation of knobs corresponded to their respective biochemical EPS1 phenotypes and reinforced the distinction between the wild type strain AW1 ($EPS1^+$), the hypoaggressive mutant strain AW1-41 ($EPS1^i$, inducible *in planta*) and the two non-pathogenic mutant strains AW1-1 and AW-19A ($EPS1^-$).

Root infection and vascular colonization by the wild type $EPS1^+$ strain

The interaction between tomato roots and strain AW1(pXLGD4) was studied at different stages after inoculation using whole axenic root systems sampled at different times. Bacteria were observed in plant tissues by microscopy after histochemical staining for bacterial β-galactosidase activity. One week after inoculation, bacteria had (i) colonized the surface of some root parts, and (ii) intercellularly infected crack spaces at the axils of some developing secondary roots and some root extremities. At this stage bacteria had progressed intercellularly into the inner cortex along the vascular cylinder (Figure 2a). Root xylem invasion occurred during the second week after inoculation and was observed mainly at the axils of developed secondary roots (Figure 2b). Large infection pockets had developed intercellularly in the cortex and in the central cylinder and invasion of xylem vessels had occurred (Figure 2c). Three weeks after inoculation, vascular colonization was detected in most of the plants. Thus, the pathogenic strain AW1(pXLGD4) is as infective and invasive as the reference pathogenic strain GMI1000 (Vasse et al., 1995).

Root infection and vascular colonization by the $EPS1^i$ mutant strain

One week after inoculation, a limited number of root tips, sites of emergence and axils of secondary roots were surface-colonized by the strain AW1-41(pXLGD4). Small infection pockets were observed only at few axils of secondary roots (Figure 2d). The number of axil infection sites did not increase during the second week. However, vascular colonization of the primary roots of some plants was observed (Figure 2e and f). This colonization was manifested as infection of small intercellular spaces along the vascular cylinder of secondary roots which may lead to xylem vessel invasion (not shown). Thus, the mutant strain AW1-41(pXLGD4) is both infective and invasive, though to a lesser extent than the pathogenic strain AW1(pXLGD4).

Root infection by the $EPS1^-$ mutant strains

During the first week after inoculation of mutant strain AW1-1(pXLGD4) bacteria only colonized the outer part of some secondary root axils, but cortical infection could not be detected (data not shown). At this stage, the bases of secondary roots with surface-colonized axils had begun to thicken and to develop numerous long root hairs. Two weeks after inoculation, the bases of secondary roots with colonized axils were even thicker and the number of long root hairs even greater. On primary roots and in the vicinity of these colonized areas, browning of some outer cortical cells was observed (Figure 3a). At the same time, parts of some secondary roots were heavily infected (Figure 3b). Transverse semithin sections of such root parts revealed an infection of most of the intercellular spaces of the inner cortex proximal to the endodermis (Figure 3c). The infection process did not evolve any further and mutant strain AW1-1(pXLGD4) was never detected either in central cylinder or in xylem vessels, even three weeks after inoculation. Thus mutant strain AW1-1(pXLGD4) is weakly infective but not invasive.

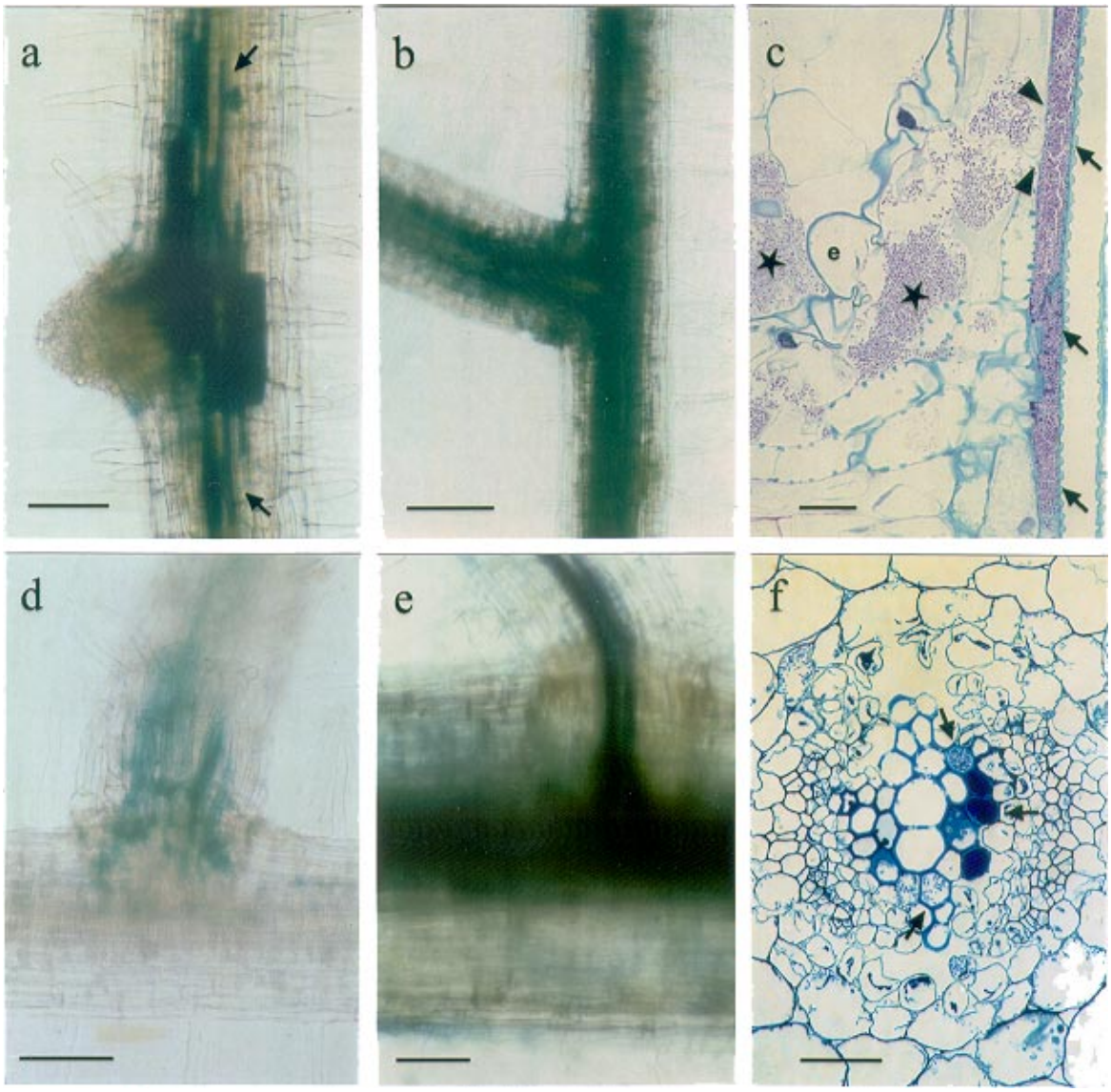

Figure 2. Light microscopy of tomato root infection and invasion by *R. solanacearum* strains AW1(pXKGD4) (a–c) or AW1-41(pXLGD4) (d–f). Bacteria were localized by histochemical staining of the bacterial β-galactosidase activity in whole roots (a, b, d and e) while semithin sections were post-stained with toluidine blue (c and f). (a) Intercellular infection of emergence site of secondary root observed one week after inoculation. Bacteria have progressed along the central cylinder (arrows). (b) Intercellular infection and vascular colonization observed at the axil of a secondary root, two weeks after inoculation. (c) Longitudinal section of the axil of a secondary root, two weeks after inoculation. Infected intercellular spaces (stars) were observed on each side of the endodermis (e). Sites where bacteria penetrated into an outer xylem vessel are indicated (arrowheads) as well as the vascular colonization of primary root (arrows). (d) Weak cortical infection (i.e. small infection pockets) of a secondary root axil observed one week after inoculation. (e) Vascular colonization of a primary and a secondary root observed at a root axil, two weeks after inoculation. (f) Transverse section of primary root, two weeks after inoculation. Colonization of several xylem vessels was observed (arrows). Scale bars = 100 μm (a, b, d and e), 25 μm (c and f).

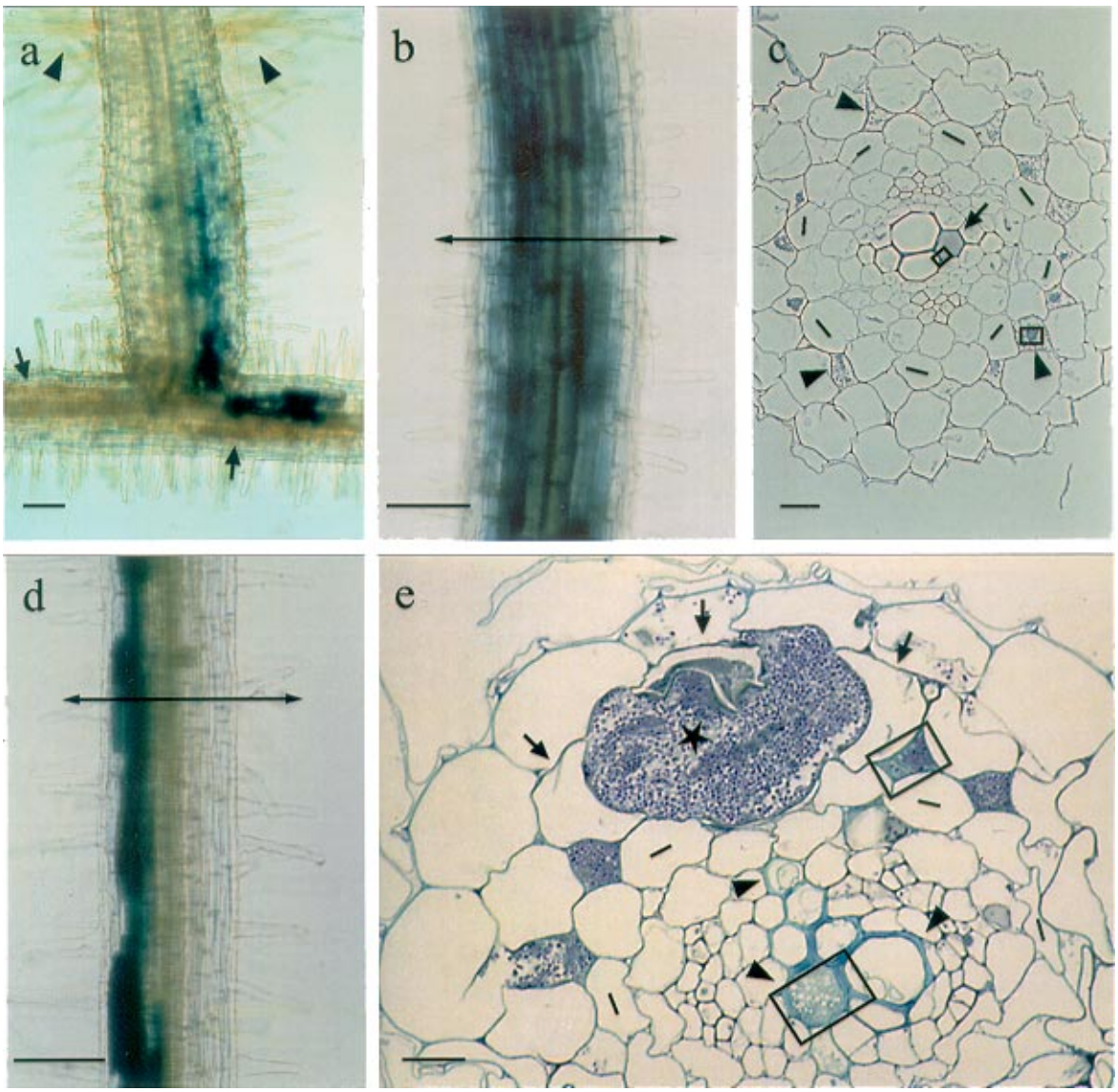

Figure 3. Light microscopy of tomato root infection by the *R. solanacearum* EPS1$^-$ strains AW1-1(pXLGD4) (a–c) or AW-19A(pXLGD4) (d and e). Bacteria were visualized by histochemical staining of the bacterial β-galactosidase activity in whole roots (a, b and d) while semithin sections were post-stained with toluidine blue (c and e). (a) Infected secondary root axil observed two weeks after inoculation. The base of the infected secondary root was twice as thick as the primary root and exhibited abnormally numerous long root hairs (arrowheads). Brown cells (arrows) were observed at cortical level of the primary root. (b) Part of a secondary root with intercellular infection along the vascular cylinder, two weeks after inoculation. (c) Section of an infected secondary root-part as indicated in (b). Bacteria were observed in most of the cortical intercellular spaces (arrowheads) proximal to the endodermis (dashes). One of the xylem vessels appeared to be filled with a homogeneous material (arrow). (d) Infection was observed only in parts of some secondary roots, two weeks after inoculation. Bacteria were localized along one side of the vascular cylinder. (e) Transverse section of a secondary root as indicated in (d). The infected part has been enlarged to show that bacterial development in one of the intercellular spaces of the inner cortex resulted in the formation of an infection pocket (star). Such a development led to the deformation and the degeneration of neighboring cortical cells (arrows). The presence of vesicular material was observed in several xylem vessels in the vascular bundle facing the infection pocket (arrowheads). Scale bars = 100 μm (a, b and d), 10 μm (c and e).

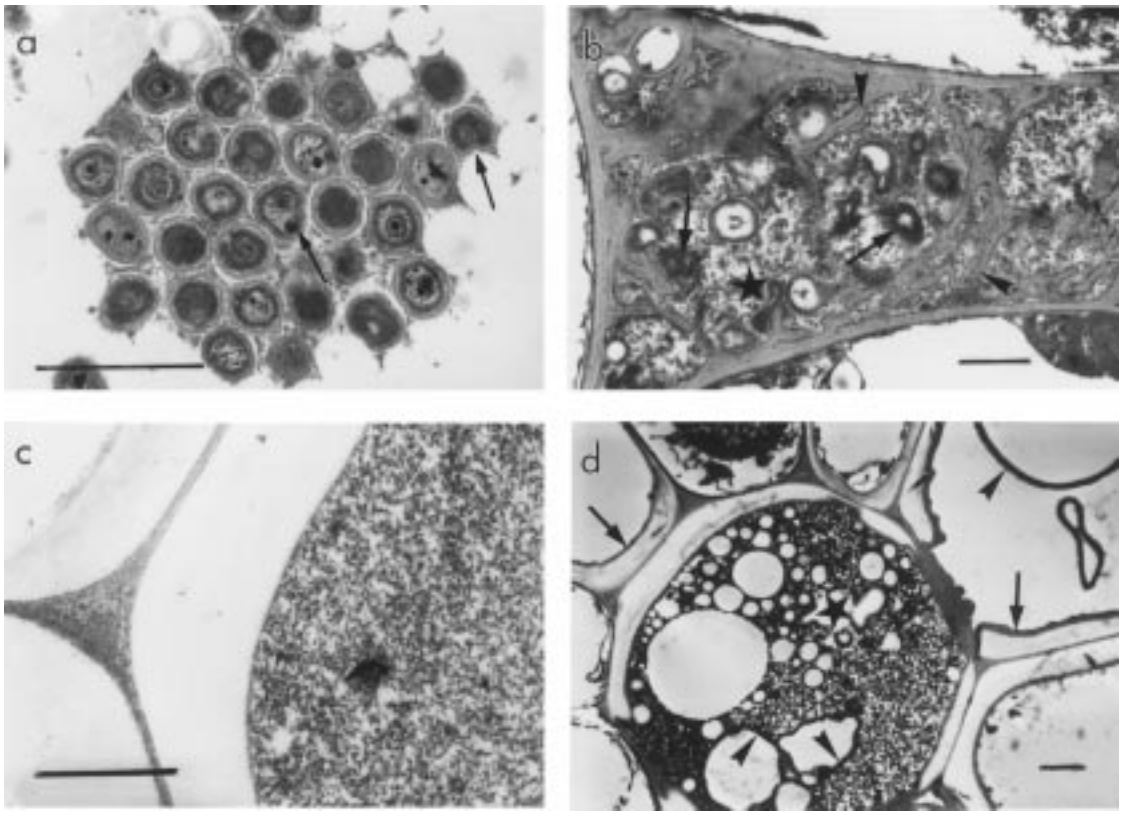

Figure 4. Electron microscopy observation of the framed parts shown in Figure 3c (a and c) and Figure 3e (b and d). (a) In this infected intercellular space, bacteria of strain GMI1562 (AW1-1 derivative) appeared to be agglutinated and some of them degenerated (arrows). (b) In an infected intercellular space located next to the large infection pocket, bacteria of strain GMI1564 (AW-19A derivative) appeared degenerated (arrows) and embedded in a material of granular appearance (star) between numerous cell wall outgrowths (arrowheads). (c) The material filling a metaxylem vessel and which seems to be homogeneous in light microscopy appeared as granular when observed by TEM. (d) Vascular coating material appeared as deposit on inner face of xylem vessel cell walls (arrows) or as bubbles in vessels lumen (arrowheads). The large production of such a material filled one of the metaxylem vessel (star). Scale bars = 1 μm.

Infection by mutant strain AW-19A(pXLGD4), although comparable on the whole to that of mutant strain AW1-1(pXLGD4), demonstrated some significant differences. No sign of any infection could be detected for one week following inoculation. During the second week after inoculation, large infection pockets developed in files on one side of the cortex in parts of some secondary roots (Figure 3d). Transverse semithin sections revealed great numbers of bacteria in some intercellular spaces of the cortex. These intercellular spaces looked abnormally large and were surrounded by compressed and degenerated cortical cells (Figure 3e). These cortical infection pockets developed far away from the sites of secondary root emergence. Mutant strain AW-19A(pXLGD4) was detected in xylem vessels in only one out of twenty plants examined three weeks after inoculation. Thus, mutant strain AW-19A(pXLGD4) is weakly infective and very poorly invasive.

Examination by transmission electron microscopy showed bacterial cells of mutant strain AW1-1(pXLGD4) aggregated and degenerated in most of the infected intercellular spaces in cortex (Figure 4a). Degenerated cells of mutant strain AW-19A(pXLGD4) and cell wall outgrowths were observed in infected intercellular spaces (Figure 4b). Granular material filling xylem vessels and bubbles of vascular coating material were observed in roots infected by either strain (Figure 4c and d). Thus, root infection by the two EPS1$^-$ mutant strains induces production of uncharacterized material within xylem vessels and intercellular spaces and degenerating bacteria are noticed in infected

Table 3. Relative aggressiveness and penetration of the strains in tomato plants

Bacterial strains	% Wilted plants (days post inoculation)	% Colonized at hypocotyl level	% Height of stem colonized[a]	Bacterial density at hypocotyl level[a] (CFU g^{-1} of dry weight)	
				Wilted	Symptomless
AW1	100 (3)	100	98 (3,9)	1.8×10^{11} (0.5×10^{11})	no plant
AW1-41	100 (4)	100	98 (3,3)	1.9×10^{11} (0.5×10^{11})	no plant
AW-19A	13 (13)	100	66 (18)	7×10^{10} [b]	2×10^{9} (3.6×10^{9})
AW1-1	0 (13)	80	58 (24)	no plant	1.9×10^{9} (1.9×10^{9})

Root inoculated tomato plants of cultivar Supermarmande were assessed for bacterial presence at the hypocotyl level and all along the stem and bacterial density in the hypocotyl was assessed. Twenty plants were analyzed for the strains AW1 and AW1-41 after 3 and 4 days post-inoculation (d.p.i.), respectively. Sixteen plants for the strains AW1-1 and AW-19A were assessed at 13 d.p.i.
[a] Mean value and standard deviation (in parenthesis).
[b] This value results from the analysis of two plants only.

cortical intercellular spaces. Such an abundance of these materials was never observed with pathogenic strain AW1(pXLGD4) or with mutant strain AW1-41(pXLGD4), both $EPS1^+$ *in planta*, which otherwise infected crack spaces intercellularly and invaded xylem vessels.

Bacterial multiplication in the tomato stem

Following root inoculation of plants grown in pots, the invasiveness of pathogenic strain AW1 and EPS^i mutant strain AW1-41 were quite comparable, i.e. they induced a total and rapid wilting of all plants (within 3–4 days), colonized the whole stem and multiplied up to more than 10^{11} CFU g^{-1} of dry weight at hypocotyl level (Table 3). On the contrary, the two $EPS1^-$ mutant strains AW-19A and AW1-1 were hypoaggressive and significantly less invasive than the two former strains. Even 13 days after inoculation, these two $EPS1^-$ strains were not able to colonize the whole stem. In addition, the majority of plants which remained symptomless harbored bacteria at levels 100 fold less dense than in wilted plants, in the region of the hypocotyl.

Following inoculation by puncturing with a 3×10^4 CFU $stem^{-1}$ inoculum, the multiplication rates of the four strains were almost identical during the first 48 h (exponential growth phase). The average generation time was about 3.5 h, and within 48 h roughly 16 generations were achieved by the strains AW1 and AW1-41, 15 and 13 generations by the mutant strains AW1-1 and AW-19A, respectively. Five days after inoculation, all four strains reached an average density of 4×10^9 CFU $stem^{-1}$, and 70% and 60% of plants inoculated by the strains AW1 and AW1-41 were wilted, respectively, whereas no wilting was observed on plants inoculated by either strains AW1-1 or AW-19A. Thus, following artificial introduction by stem puncturing, the ability to multiply within host plant tissues by the two $EPS1^-$ mutants was comparable to that of the two *in planta* $EPS1^+$ strains; although these two strain groups expressed different aggressiveness.

Discussion

This study was carried out to distinguish between the production of the two different hexosamine-containing polymers produced by mutant strains impaired in EPS production and to examine the effect of these mutations on root infection, plant colonization and disease. Results from microscopic and biochemical analyses revealed that the three mutant strains formerly referred to as EPS^i (Denny et al., 1988; Denny and Baek, 1991; Schell et al., 1993) can be separated into two different groups according to their characteristically different production of the EPS1 polymer. In fact, our data demonstrate that a biochemical purification scheme that combines gel permeation and ion exchange chromatography allows the detection of minute amounts of N-acetyl-galactosamine, a specific marker for EPS1, and that the 0.2 M NaCl elution over DEAE is a decisive step in the biochemical analysis of the EPS1 production by mutant strains. This new purification procedure can advantageously replace the previous procedure which was based upon dissociative conditions (Orgambide et al., 1991). Biochemical analyses revealed the presence of N-acetyl-glucosamine derivative, the specific marker of EPS3 (LPS), in culture supernatants of all of the strains under study. GRU staining provides a rapid, easily reproducible

alternative method that involves small volumes of bacterial suspensions. Ruthenium red contributes to EPS1 visualization at the ultrastructural level because it is a heavy metallic element. EPS1 is probably condensed because of the combined action of ruthenium red, a polycation, and glutaraldehyde, a fixative that binds to amino groups. This condensation probably impairs any further immunolocalization. However, this method allowed us to determine that part of EPS1 polymer produced is cell-associated and cannot be washed off by a simple centrifugation. Thus, the classical quantification method of EPS1 in supernatants clearly underestimates the total amount of the polymer produced. The GRU staining has also permitted separation of the strains, in good agreement with the three biochemical groups, namely (i) the AW1 strain which abundantly produces EPS1 and exhibits knobs and matrix, (ii) the AW1-1 and AW-19A mutant strains which are devoid of any biochemically, immunologically or microscopically detectable EPS1, (iii) the AW1-41 mutant strain whose EPS1 production depends on growth conditions. This distinction into three groups is also reinforced by the results of the labeling with specific mAbs directed against EPS1. Thus, biochemical analysis demonstrated that GRU staining is strictly correlated with the quantification or the visualization of the EPS1 polymer and does not correspond to EPS3. GRU staining and immunofluorescence microscopy allow specific identification of the EPS1 polymer produced by *R. solanacearum in vitro* and *in planta*. These methods can be used instead of both the colorimetric estimation of total hexosamines, which is easy but indiscriminate, and the complete purification scheme which is precise but time consuming. This specific study of the EPS1 production led us to assign the AW1-1 and AW-19A mutant strains as strictly $EPS1^-$ instead of the former, ambiguous EPS^i denomination (Denny and Baek, 1991; Schell et al., 1993).

Structural analyses show that the EPS1 polymer produced by the pathogenic strains AW1 (tomato, Alabama), related mutant strain AW1-41 and GMI1000 (tomato, French Guyana) strains are identical. Microscopic observations and structural identity of the polymer produced by the K60 pathogenic strain (tobacco, North Carolina) and the GMI1000 strain (Vasse, unpublished observations; Trigalet-Demery et al., 1993) strongly suggest that the structure of the repeating unit is not strain-specific.

Immunofluorescence microscopy has already been used to demonstrate the presence of pathogenic strain AW1 and mutant strain AW1-1 within primary root cortex only hours after soil infestation (Saile et al., 1997). Our microscopic analysis describes in more detail the different stages in the root infection process by mechanism responses by EPS1-deficient mutants of *R. solanacearum* on axenic tomato plants and defense responses by the host-plant. Differences between the four strains under study were observed. Our data indicated that the root infection process by the pathogenic strain AW1 was identical to that of the pathogenic strain GMI1000 (Vasse et al., 1995). Although the time courses of infection and vascular colonization on axenic plants of the pathogenic type AW1(pXLGD4) and mutant strain AW1-41(pXLGD4) were comparable, the latter $EPS1^i$ mutant was less invasive, producing fewer and smaller infection pockets and less colonization of vascular bundles as compared to the wild type strain AW1(pXLGD4). In contrast, the two $EPS1^-$ mutant strains infected cortical intercellular spaces of parts of secondary roots, more slowly than the two $EPS1^+$ strains, but did not invade AW1-1(pXLGD4) or weakly colonize AW-19A(pXLGD4) xylem vessels.

In tomato plants grown in pots, even though strain AW1-41 was slightly hypoaggressive, both strains AW1 and AW1-41 invaded the whole stem and induced rapid wilting of all plants. In contrast, even if most plants were colonized at the hypocotyl level, strains AW1-1 and AW-19A induced no or weak wilting and could be considered as non-pathogenic and very hypoaggressive, respectively. These results on plants grown in pots agree with and complement those previously reported (Denny et al., 1988; Denny and Baek, 1991; Saile et al., 1997). It is not surprising that the two $EPS1^-$ strains are more invasive in pots than in test tubes because plants grown in pots have an abundant root system, much more developed than the root system of plants grown in test tubes, with an increased number of potential infection sites which render such a root system more susceptible to infection. Moreover, following infection by either of the $EPS1^-$ mutants, tomato root tissues produced electron-dense materials in the lumen and on the inner face of vessel cell walls. Such vascular coating materials have already been described in other vascular pathogen–plant interactions and have been associated with non-specific defense mechanisms of the plant (Robb et al., 1991; Shi et al., 1992). These defense mechanisms could be responsible for the limited invasiveness of the $EPS1^-$ mutant strains, as shown in case of *Verticillium* wilt (Robb et al., 1987), and

were not generally observed after inoculation with the wild type strain AW1 or the EPS1^{i} mutant strain AW1-41. However, following inoculation of tomato plants grown in pots, these mechanisms may be less efficient because both EPS1^{-} mutants could be isolated at the hypocotyl level of symptomless plants.

The acidic EPS of *Rhizobium* has long been considered necessary for infection of the legume host-plant (Leigh and Walker, 1994). Otherwise, it is noteworthy that some mutants of *Rhizobium meliloti* or *Bradyrhizobium japonicum*, which were deficient in the production of the acidic EPS, induced defense reactions and were not fully symbiotic (Niehaus et al., 1993; Parniske et al., 1994). Our results revealed that the acidic EPS1 of *R. solanacearum* is also necessary for an efficient infection and that some EPS1^{-} mutants induced defense reactions in susceptible tomato roots.

Growth rate study after artificial inoculation into stem revealed on the one hand that the two EPS1^{-} mutant strains exhibit the same multiplication rates as the two EPS1^{+} strains *in planta* (AW1 and AW1-41). On the other hand, it indicated that at similar bacterial densities *in planta* only EPS1^{+} strains induced wilting. These results clearly suggest that EPS1 is implicated in wilting of colonized plants.

The EPS of *R. solanacearum* may act as a protective coating that prevents bacteria from agglutination by divalent cations in intercellular spaces or fixation onto plant cell walls through the binding between LPS and lectins, after leaf infiltration (Sequeira et al., 1977; Sequeira and Graham, 1977; Sequeira, 1982). Since the EPS1^{-} mutant strain AW1-1 is strongly agglutinated within intercellular spaces in the root cortex and since both of the EPS1^{-} mutant strains, AW1-1 and AW-19A, degenerate within intercellular spaces whereas the *in planta* EPS1^{+} strains are neither agglutinated nor degenerated, it is possible that EPS1 also prevents these phenomena during the root infection process. Attachment of EPS1^{-} bacteria to plant cell walls could trigger defense mechanisms, that could lead to the death of bacteria in intercellular spaces and xylem vessels. The fact that EPS1^{-} mutant strains are still able to multiply to some extent may be accounted for a dose-phenomenon, i.e. agglutination and/or immobilization factors would be saturated in the presence of an excess of bacteria. Thus, defense mechanisms of the host plant may be overcome following stem puncturing or invasion through wounded roots because an excess of EPS1^{-} bacteria are artificially introduced.

Thus, after assessing the EPS1 production defect with methods specific for the EPS1 polymer and comparing the root invasion process of the strains under study, we propose that the EPS1 polymer produced by *R. solanacearum* is necessary for efficient tomato root intercellular infection and vascular colonization. We still have to elucidate the mechanism by which this polymer favors infection and vascular colonization of tomato roots.

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