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Lipopolysaccharide-Defective Mutants of the Wilt Pathogen *Pseudomonas solanacearum*

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Lipopolysaccharide (LPS)-defective mutants of *Pseudomonas solanacearum* were used to test the hypothesis that differences in LPS structure are associated with the ability or inability of different strains to induce a hypersensitive response (HR) in tobacco. To obtain these mutants, LPS-specific bacteriophage of *P. solanacearum* were isolated and used to select phage-resistant mutants of the virulent, non-HR-inducing strain K60. The LPS of 24 of these mutants was purified and compared with that of K60 and its HR-inducing variant, B1. Upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis, LPS from K60 and other smooth strains separated into many evenly spaced bands that migrated slowly, whereas LPS from B1 and most phage-resistant strains separated into one to three bands that migrated rapidly. Carbohydrate analysis showed that the LPS of the phage-resistant strains lacked O-antigen sugars (rhamnose, xylose, and *N*-acetylglucosamine) and could be grouped into (i) those that had all core sugars (rhamnose, glucose, heptose, and 2-keto-3-deoxyoctonate), (ii) those that had no core rhamnose, and (iii) those that lacked all core sugars except for 2-keto-3-deoxyoctonate. The LPS composition of 10 of the rough, phage-resistant mutants was similar to that of the HR-inducing strain, B1, yet none of them induced the HR. Only 2 of 13 mutant strains tested caused wilting of tobacco, and these had rough LPS but produced large amounts of extracellular polysaccharide, unlike most LPS-defective mutants. The evidence did not support the hypothesis that the initial interaction between rough LPS and tobacco cell walls is the determining factor in HR initiation.

Specific interactions between cell surface components may play a critical role in the initial establishment of compatible or incompatible relationships between bacteria and plants (32). Surface carbohydrates, such as extracellular polysaccharide (EPS) and lipopolysaccharide (LPS), have been implicated as recognition and virulence factors for several species of phytopathogenic and symbiotic bacteria (5, 6, 9, 15, 24, 29).

The hypersensitive response (HR) induced in tobacco by incompatible strains of the wilt pathogen *Pseudomonas solanacearum* has been used extensively as a model system for host-parasite recognition (31). When incompatible strains of *P. solanacearum* (fluidal strains not pathogenic on tobacco, or certain afluidal, avirulent variants of pathogenic strains) are infiltrated into tobacco leaves, the infiltrated area collapses and desiccates within 24 h and bacterial populations decline. In contrast, pathogenic strains induce no response until 48 h after infection, when the infiltrated tissue becomes necrotic and the bacteria spread into the surrounding tissue. Other avirulent mutant strains induce no response other than slight chlorosis of the infiltrated area (25). Ultrastructural studies have shown that cells of an avirulent HR-inducing strain are attached to and enveloped by the host mesophyll wall, whereas compatible strains remain free in the intercellular spaces (33).

A hydroxyproline-rich glycoprotein that agglutinated only afluidal, avirulent mutants of *P. solanacearum* was isolated from potato and tobacco tissues (34). The agglutinin has been characterized chemically (22) and shown to be located on or in the cell wall (23). The agglutinin strongly precipitated LPS extracted from avirulent strains, but only weakly precipitated LPS from the virulent strain K60. The addition of EPS extracted from fluidal, virulent strains prevented both bacterial agglutination and the precipitation of LPS (34).

Whatley et al. (37) examined the LPS from a limited number of avirulent variants of strain K60 and found that those that induced the HR had incomplete (rough) LPS. They proposed that rough LPS mutants may be bound to the tobacco cell wall agglutinin through complementary linkages with the carbohydrates in the core region of the LPS. In strains with smooth LPS, these carbohydrates may be cryptic because of the presence of the O-antigen. A further extension of this hypothesis is that attachment of bacteria to the plant cell wall may be necessary for initiation of the metabolic processes that trigger the HR.

The goal of this research was to clarify the role of LPS in determining the ability of certain strains of *P. solanacearum* to induce the HR in tobacco. The specific objectives were (i) to use LPS-specific bacteriophage to select mutants of the virulent strain K60 that have defects in LPS structure; (ii) to compare the structure of the LPS of the phage-resistant mutants with that of the parent strain K60 and its avirulent, HR-inducing variant, B1; and (iii) to test the pathogenicity and HR-inducing ability of LPS- and EPS-deficient mutants. A preliminary report has been presented (C. Hendrick and L. Sequeira, Phytopathology **71**:880, 1981).

MATERIALS AND METHODS

Bacterial strains and culture conditions. Strains of *P. solanacearum* were grown at 28°C on an agar medium (TZC) containing (per liter): 1 g of Casamino Acids, 10 g of Bacto-Peptone, and 10 g of glucose, and 0.005% tetrazolium chloride (17) for 48 to 72 h, or in liquid culture in CPG broth (TZC medium without agar and tetrazolium chloride) for 24 to 48 h on a rotary shaker. Bacteria were stored in sterile distilled water in capped test tubes at room temperature (19).

All strains of *P. solanacearum* were obtained from the culture collection maintained at the Department of Plant Pathology, University of Wisconsin-Madison. Strain K60,

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used in many experiments, is a race 1 strain originally isolated by A. Kelman from tomato in North Carolina. Strains B1 and Q are spontaneous avirulent mutants of K60. B1 induces the HR in tobacco, but Q does not (37).

Isolation of bacteriophage. Temperate bacteriophage were induced from lysogenic strains of *P. solanacearum* (strains 74, 87, 154, and 199) by adding mitomycin C (1 μg/ml) to 12- to 16-h cultures in CPG broth, then incubating at 30°C for 4 h (10). Chloroform was added to each culture (0.5 ml per 10 ml of culture), and the mixture was shaken vigorously and allowed to settle. Samples from the top layer were diluted and spotted on lawns of indicator bacteria; when the supernatant contained bacteriophage, zones of lysis or isolated plaques were visible after 12 to 18 h.

Phage also were isolated from soils infested with *P. solanacearum* and from tobacco stems infected with the pathogen. Infected tobacco stem tissue was comminuted in a Waring blender with 0.1 M potassium phosphate buffer (pH 7.0) containing 0.01 M cysteine. An early-log-phase broth culture (100 ml) of *P. solanacearum* K60 or B1 was mixed with 10 ml of the filter-sterilized tobacco extract or 20 g of soil and incubated for 12 to 16 h at 28°C. The culture was centrifuged (8,000 × *g* for 15 min) and filter sterilized, and the supernatant was tested for the presence of phage. The phage were purified by several serial single-plaque isolations and were propagated by the methods of Adams (2). Stock phage lysates were made in CPG broth and stored in capped test tubes over chloroform.

Inactivation of phage by purified LPS. The LPS from *P. solanacearum* K60 and B1, purified as described below, was used to inactivate the phage that were LPS specific. Equal volumes of phage (ca. 10^4 PFU/ml) and serial dilutions of LPS suspensions were mixed in wells of microtiter plates and incubated at 28°C for 90 min with occasional agitation. Controls consisted of phage incubated with buffer. Samples of the LPS-phage mixtures were then plated on lawns of strain K60 and, after 12 to 18 h, the number of plaques was compared with that in control plates.

Bacteriophage Psso 154 and Psso NCL. Two phage isolates that were inactivated by preincubation with LPS were selected for further study. Phage Psso 154 (formerly designated CH154) was a clear-plaque mutant of a phage that was lysogenic on *P. solanacearum* 154. Phage Psso NCL (formerly designated NC-L) was isolated from soil collected in a tobacco field in North Carolina. Phage designations were changed to correspond to the nomenclature recommended by Ackermann et al. (1).

High-titer lysates of these phages were prepared by infecting strain K60 (10^8 cells per ml) with bacteriophage at a ratio of 0.1 to 0.5 phage per bacterial cell in CPG broth at 28°C. After 24 h, the cultures were centrifuged (8,000 × *g*, 30 min) to remove bacterial debris and treated with chloroform (5 ml/liter). The phage were concentrated by the two-phase (dextran sulfate-polyethylene glycol) system described by Albertsson (4). The concentrated phage preparations were dialyzed against buffer (4 g of NaCl, 5 g of K_2SO_4, 3 g of Na_2HPO_4, 1.5 g of KH_2PO_4, 0.12 g of $MgSO_4$, and 0.015 g of $CaCl_2$ per liter, pH 7.0) and purified by equilibrium CsCl density gradient centrifugation (Beckman 60 Ti rotor, 40,000 rpm, 72 h). Purified phage preparations were negatively stained with a freshly prepared, saturated solution of uranyl formate in water. Equal volumes of phage and stain were mixed, and a drop of the mixture was placed on a carbon-coated collodion membrane on a copper grid. Excess fluid was removed with filter paper, and the preparation was examined with a JEM-7 electron microscope.

Isolation of phage-resistant mutants. Phage-resistant mutants of strain K60 were obtained by mixing 10^7 bacterial cells in soft agar overlays with enough phage (10^4 to 10^5 PFU) to form confluent plaques. Single colonies that grew in the zone of confluent lysis were streaked on TZC agar two or three times. Those mutants that were less fluidal than K60 were retested for phage sensitivity by spotting 10 μl of each phage stock on lawns of each mutant strain. Mutants resistant to phage Psso 154 were designated 154-1, 154-2, . . . etc; those resistant to phage Psso NCL were designated NCL1, NCL2, NCL3, . . . etc.

Spontaneous, nonfluidal (Eps^-) variants were obtained from stationary cultures of strain K60 and its phage-resistant mutants (18). After 7 days of stationary growth in CPG broth containing 0.75 g of agar per liter, bacteria were removed from the surface of the culture, streaked on TZC agar, and incubated for 48 h at 28°C. The small, butyrous, dark red colonies of the Eps^- variants were selected, and the strains were purified by repeated single-colony isolation.

Extraction of LPS. For extraction of smooth-type LPS, the hot phenol-water (PW) method of Westphal and Jann (36) was used. For extraction of rough-type LPS, a modification of the phenol-chloroform-petroleum ether (PCP) method of Galanos et al. (12) was used. The modification consisted of precipitating the LPS from the phenol phase with two volumes of cold acetone rather than by the dropwise addition of distilled water. This modification was especially useful if the LPS was extremely rough or if the LPS-containing phenol phase was very viscous. The LPS obtained by either of these procedures was dried under vacuum, suspended in distilled water, and purified further by repeated ultracentrifugation (105,000 × *g*, 4 h) until the optical density of the supernatant at 260 nm was less than 0.1. The LPS pellet was resuspended in distilled water, lyophilized, and stored at −20°C. In some experiments, the LPS from several smooth strains was extracted first by the PCP method, then by the PW method. The LPS extracted by either procedure contained low levels (less than 0.5%) of contaminating sugars such as ribose (from nucleic acid), galactosamine (from EPS), and arabinose.

Different LPS molecules were compared on the basis of their electrophoretic mobility by polyacrylamide gel electrophoresis (PAGE), using the method of Laemmli (21). Samples of LPS (2 mg/ml) were suspended in sample buffer (0.5 M Tris-hydrochloride, 3% sodium dodecyl sulfate [SDS], 10% glycerol, 0.0001% bromphenol blue, pH 6.8) and heated for 5 min at 100°C. The resolving gel contained 12.5% polyacrylamide in 1.5 M Tris-hydrochloride buffer (pH 8.8) containing 0.4% SDS. The stacking gel contained 3% polyacrylamide in 0.5 M Tris-hydrochloride buffer (pH 6.8) containing 0.4% SDS (21). Electrophoresis was carried out in Tris-glycine buffer containing 0.1% SDS (pH 8.8) for 1 h at 30 mA, then for 4 h at 45 mA. Gels were fixed overnight in 25% isopropanol and stained either by the periodate-Schiff method (11) or with the ammoniacal silver stain of Tsai and Frasch (35).

Carbohydrate analysis. Lyophilized LPS samples (200 μg to 2 mg) were hydrolyzed at 120°C for 90 min with 2 N trifluoroacetic acid (1 ml) containing inositol (100 μg/ml) as an internal standard. Then alditol acetate derivatives were prepared (3) and analyzed with a Varian model 3740 gas chromatograph (Varian Instruments Division, Palo Alto, Calif.) equipped with a flame ionization detector and glass columns packed with 3% SP-2340 on Supelcoport. The column temperature was increased from 145 to 245°C at 2°C/min. Derivatives of glucosamine (GlcN) and galactosamine

were separated on a column packed with 3% OV-275 on Gas-Chrom Q. The column temperature was increased from 180 to 250°C at 2°C/min. Peak areas were quantitated with Varian CDS 111 or Hewlett-Packard 3390A integrators. 2-Keto-3-deoxyoctonate was determined by the method of Karkhanis et al. (16). Sugar standards were obtained from Sigma Chemical Co., St. Louis, Mo.

For comparison of different LPS preparations, normalized amounts (relative molar ratios) of the LPS sugars were calculated by determining the amount of each sugar per milligram of LPS, then assigning glucose a molar value of 2.0 and calculating the relative molar amounts of the other sugars with respect to glucose.

HR. The ability of each strain of *P. solanacearum* to induce the HR in tobacco leaves was assayed by the method of Klement (20). Tobacco plants were grown as described previously (33). For use as inoculum, bacteria were grown on TZC for 48 h, then suspended in distilled water at an optical density at 600 nm of 0.1 (ca. 10^8 cells per ml).

Pathogenicity tests. To test their pathogenicity, bacteria were grown on TZC for 48 h and then were suspended in 0.01 M potassium phosphate buffer (pH 7.0) at an optical density at 600 nm of 1.0 (ca. 10^9 cells per ml). Tobacco seedlings (*Nicotiana tabacum* L. 'Bottom Special') were grown singly in silica sand in 10-cm pots, watered daily with Hoagland solution, and maintained in a controlled environment chamber at 28°C and a 12-h photoperiod. Seedlings were stem inoculated when approximately 15 cm tall by inserting a micropipette containing 20 μl of inoculum into the axil of the fourth or fifth fully expanded leaf (7). After 4 to 12 h, the micropipettes were removed and the inoculation sites were covered with petrolatum. Five plants were inoculated with each bacterial strain. Disease ratings were made every 3 or 4 days for 35 to 40 days according to the following scale: 1, no symptoms; 2, epinasty and distortion of young leaves; 3, one or two leaves wilted; 4, three or four leaves wilted; 5, most leaves wilted; 6, plant dead. After 35 to 40 days, bacteria were reisolated from plants and tested for phage resistance.

RESULTS

Isolation and characterization of bacteriophage. Phage selected after enrichment on strain K60 could not infect strain B1, but phage selected after enrichment on B1 could also infect K60, although with lower efficiency. No B1-specific phage were isolated. A range of strains of *P. solanacearum* was tested for sensitivity to a series of 15 phage isolates. No correlation was found between phage sensitivity and race of the bacterial strain or its ability to induce the HR (data not shown).

Seven K60-specific phage were tested for their ability to bind to, and thus be inactivated by, purified K60 LPS. Two of these phage isolates, Psso 154 and Psso NCL, were partially inactivated by 90 min of preincubation with 300 μg of K60 LPS per ml, but preincubation with 300 μg of B1 LPS per ml had no effect. These two isolates were chosen for further study.

Phage Psso 154 had a polygonal head (49 ± 2.8 nm wide) and a long contractile tail (110 ± 6.5 nm long) with short tail fibers (Fig. 1A); its buoyant density was 1.4610 g/ml. Phage Psso NCL had a polygonal head (57 ± 1.1 nm wide) and a short tail (10 to 20 nm) that was poorly resolved by electron microscopy (Fig. 1B); its buoyant density was 1.4980 g/ml. Psso 154 had a morphology characteristic of group A in Bradley's classification scheme (8), and Psso NCL belonged to group C.

Isolation of phage-resistant mutants. Twenty-seven phage-resistant mutants of strain K60 were isolated; most of the strains formed colonies that were smaller and less fluidal than those of the parent strain, although two strains, 154-7 and NCL9, had colony morphology identical to that of K60. Spontaneous, nonfluidal (Eps^-) variants of strain K60 and its phage-resistant mutants were selected after growth in still culture. The Eps^- variants formed small, butyrous, dark red colonies on TZC agar that were similar to those of strain B1.

Electrophoresis of LPS from phage-resistant mutants. Preparations of LPS from strain K60 and its phage-resistant mutants were compared by SDS-PAGE. The LPSs from all strains were resolved into many bands of various mobilities (Fig. 2–4). With LPS from K60 and two of its spontaneous Eps^- derivatives, many evenly spaced, slow-moving bands, as well as a fast-moving band, were resolved in 7.5% acrylamide gels. LPS from B1 gave only one fast-moving band (Fig. 2). With few exceptions, LPS from the phage-resistant strains was resolved into one to three fast-moving bands in 12.5% gels (Fig. 3 and 4). In some cases, silver staining allowed detection of very faint, slow-moving bands in LPS from these strains (Fig. 4). The LPS from some of the

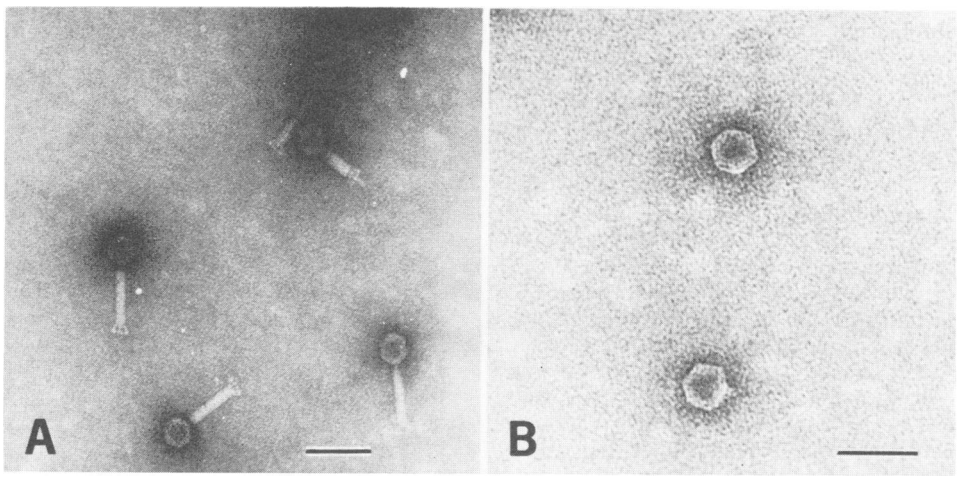

FIG. 1. Electron micrographs of bacteriophage of *P. solanacearum*. (A) Psso 154; (B) Psso NCL. Bars, 100 nm.

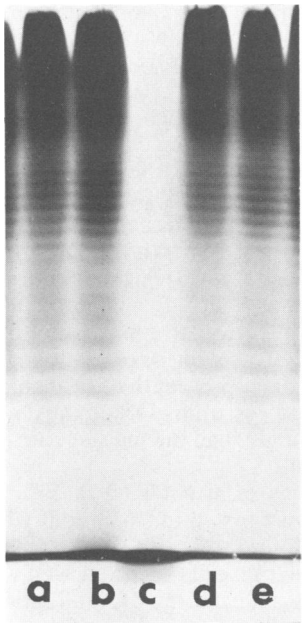

FIG. 2. SDS-PAGE (7.5% acrylamide, 0.1% SDS, pH 8.8) of LPS (100 μg per well) from strains of *P. solanacearum*. (a) K60 LPS, PW-extracted; (b) K60 LPS, PCP-extracted; (c) B1 LPS, PCP-extracted; (d) 25-3 (K60 Eps$^-$), PW-extracted; (e) 25-6 (K60 Eps$^-$), PW-extracted. The gel was stained with periodic acid-Schiff reagent (11).

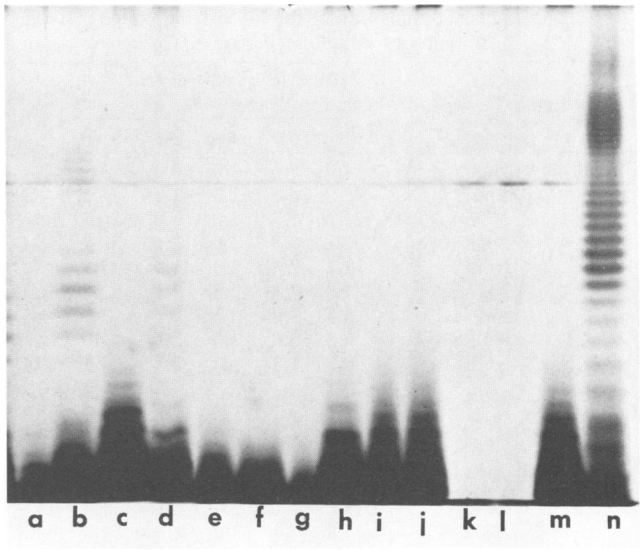

FIG. 4. SDS-PAGE (12.5% acrylamide, 0.1% SDS, pH 8.8) of LPS (10 μg per well) from phage-resistant mutants of *P. solanacearum* K60. Lanes: a, B1; b, 154-1; c, 154-3; d, 154-5; e, 154-8; f, 154-10; g, 154-13; h, NCL1; i, NCL2; j, NCL3; k, NCL7; l, NCL10; m, NCL11; n, smooth-type LPS from *E. coli* (obtained commercially). The gel was stained with ammoniacal silver stain (35).

phage-resistant strains (154-2, 154-7, 154-11, NCL4, and NCL9), however, resembled that of K60.

Sugar composition of LPS from strains K60 and B1. Analysis of the neutral sugars in LPS from K60 by gas-liquid chromatography indicated that rhamnose (rha), xylose (xyl), glucose (glc), mannoheptose (hep), and *N*-acetylglucosamine (GlcNAc) were present (Table 1). The identities of these sugars (except for hep) were confirmed by mass spectroscopy by C. J. Baker, U.S. Department of Agriculture, Beltsville, Md. (unpublished data). All major alditol acetate peaks in the chromatograms were identified. By our procedures, GlcN and GlcNAc could not be distinguished. Uronic acids were not detected by the carbazole colorimetric assay (J. Duvick, unpublished data). The LPS from B1 lacked xyl and had relatively less rha and GlcNAc and more hep and glc than did K60 LPS.

The sugar composition of B1 LPS and the results from the PAGE analysis are consistent with the previous conclusion that B1 had rough LPS, that is, it lacks the O-antigen region (37). The O-antigen in K60 LPS appears to contain rha, xyl, and GlcNAc. The composition of the O-antigen region could be inferred by subtracting the relative molar values for sugars found in B1 from those found in K60. This assumes that B1 contains a complete core and no O-antigen. The relative molar ratios of the O-antigen sugars are approximately 4 rha:1 xyl:1 GlcNAc, with an estimated average chain length of seven O-antigen repeating units. This O-antigen sugar ratio was confirmed directly by analysis of oligosaccharides obtained by bacteriophage-mediated hy-

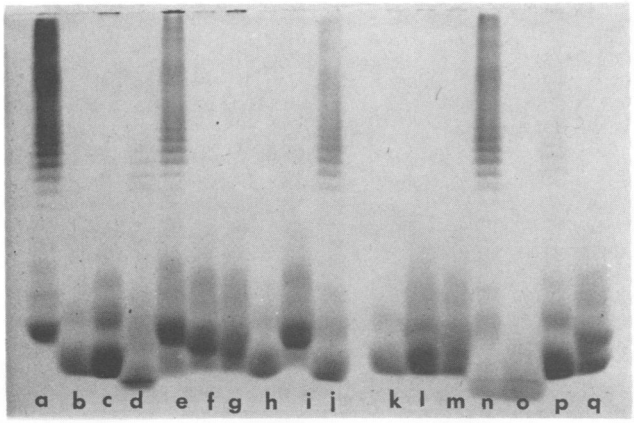

FIG. 3. SDS-PAGE (12.5% acrylamide, 0.1% SDS, pH 8.8) of LPS (40 μg per well) from phage-resistant mutants of *P. solanacearum* K60. Lanes: a, K60; b, B1; c, Q; d, 154-1; e, 154-2; f, 154-3; g, 154-4; h, 154-5; i, 154-6; j, 154-11; k, NCL1; l, NCL2; m, NCL3; n, NCL4; o, NCL7; p, NCL8; q, NCL11. The gel was stained with periodic acid-Schiff reagent (11).

TABLE 1. Sugar composition of LPS from *P. solanacearum* K60 and B1 and from Eps$^-$ variants of K60

Strain	Relative molar ratio of the following LPS sugar[a]:					KDO (%)[b]
	rha	xyl	glc	hep	GlcN	
K60[c]	33.0	7.2	2.0	2.5	10.4	1.8
B1	5.6	0.0	2.0	2.4	2.0	3.9
(O-antigen)[d]	27.4	7.2	0.0	0.1	8.4	
B1-DC (K60 Eps$^-$)	41.2	10.3	2.0	2.1	17.6	2.2
PDA 5B (K60 Eps$^-$)	25.3	6.6	2.0	2.3	9.2	1.3

[a] Normalized amounts of LPS sugars, calculated assuming that glc = 2.0.
[b] Estimated by the method of Karkhanis et al. (16). KDO, 2-Keto-3-deoxyoctonate.
[c] Average of 10 independent determinations.
[d] Calculated by subtracting the B1 value from the K60 value.

TABLE 2. Sugar composition of the LPSs from phage-resistant mutants of *P. solanacearum* K60[a]

Type of LPS	Strain	Relative molar ratio of the following LPS sugar[b]:					KDO (%)[c]
		rha	xyl	glc	hep	GlcN	
Smooth	154-2	14.5	2.6	2.0	2.4	4.9	2.3
	154-7	29.9	6.6	2.0	2.2	16.3	ND[d]
	NCL4	22.2	5.3	2.0	3.0	6.7	4.2
	NCL4Eps$^-$	30.1	5.5	2.0	2.8	9.4	ND
	NCL9	33.1	7.6	2.0	1.9	11.9	ND
	NCL9Eps$^-$	36.8	7.3	2.0	2.1	12.2	ND
	154-11	5.5	2.0	2.0	2.1	3.2	ND
	NCL8	6.8	0.6	2.0	2.3	2.0	3.3
	NCL8Eps$^-$	6.7	1.3	2.0	2.3	2.1	ND
Rough							
Group I	B1	5.6	—[e]	2.0	2.4	2.0	3.9
	Q	6.1	—	2.0	2.7	1.2	ND
	154-1	5.2	—	2.0	5.1	3.1	3.9
	154-3	3.8	—	2.0	2.1	1.7	3.9
	154-4	4.8	—	2.0	2.5	1.8	3.8
	154-5	4.9	—	2.0	2.4	2.2	3.5
	154-6	5.2	—	2.0	2.2	2.1	3.7
	NCL1	5.3	—	2.0	2.0	1.1	3.2
	NCL2	6.8	—	2.0	2.2	1.7	3.3
	NCL3	5.4	—	2.0	2.2	1.6	2.9
	NCL11	7.3	—	2.0	2.3	2.0	3.1
	NCL11Eps$^-$	6.1	—	2.0	2.3	2.1	ND
Group II	154-8	—	—	2.0	1.9	1.5	ND
	154-10	—	—	2.0	2.1	1.8	ND
	154-13	—	—	2.0	2.3	2.7	5.2
Group III	NCL7	—	—	Tr	Tr	2.0	6.3
	NCL10	—	—	Tr	Tr	2.0	ND

[a] Strains are grouped according to pattern of LPS migration in SDS-polyacrylamide gels.
[b] Calculated assuming that glc = 2.0, except for group III LPS, in which GlcN = 2.0.
[c] Calculated by the method of Karkhanis et al. (16). KDO, 2-Keto-3-deoxyoctonate.
[d] ND, Not determined.
[e] —, Not present in detectable amounts.

drolysis of the K60 O-antigen (C. Hendrick and L. Sequeira, Phytopathology **73**:808, 1983).

When the polysaccharide from B1 LPS obtained after weak acid hydrolysis (1% acetic acid, 90 min at 100°C) and removal of the insoluble lipid A was analyzed by gas-liquid chromatography, rha, glc, hep, and only trace amounts of GlcN were detected (data not shown). Therefore, for comparative purposes, GlcN was assigned a relative molar value of 2.0 for intact B1 LPS on the assumptions that GlcN is present only in the lipid A region and that the lipid A of *P. solanacearum* LPS is similar to that of other bacteria in having a backbone of two GlcN units per molecule (38). Normalized values for the other sugars were then calculated. The results indicated that the neutral core sugars were present in a proportion of 6 rha:2 glc:2 hep (Table 1).

Sugar composition of LPS from phage-resistant mutants. The sugar composition of the LPS from 24 phage-resistant mutants and two K60 Eps$^-$ variants was determined by gas-liquid chromatography (Tables 1 and 2). The composition was correlated with the electrophoretic mobility of LPS of each strain. Those strains that had smooth-type LPS on gels contained O-antigen sugars in ratios similar to those of K60 LPS. Those strains that had rough-type LPS on gels lacked O-antigen sugars (with the exception of rha, which is also present in the core) and were similar to B1 in LPS composition. Three strains (154-11, NCL8, and NCL8 Eps$^-$) had lower levels of rha and GlcNAc than other smooth strains and apparently had a preponderance of very short O-antigen chains.

The strains that had rough-type LPS on gels could be subdivided into three groups corresponding to mutations extending progressively into the core region of the LPS. The strains in group I included B1, Q, and 10 of the phage-resistant strains; their LPS lacked xyl and contained rha, glc, hep, and GlcN. The LPS from the three strains in group II (154-8, 154-10, and 154-13) lacked both rha and xyl but retained glc, hep, and GlcN in an approximate ratio of 2:2:2. The LPS from the two strains in group III (NCL7 and NCL10) had only trace amounts of glc and hep; GlcN was the only neutral sugar present in quantifiable amounts. Thus, the LPS of these two strains lacked most of the core sugars and, correspondingly, had the highest electrophoretic mobility (Fig. 3 and 4).

HR. Derivatives of strain K60 that lacked EPS or had defective LPS were tested for their ability to induce the HR when infiltrated into tobacco leaves. Approximately 75 spontaneous Eps$^-$ variants of strain K60 were tested; none of these variants induced the HR, although many induced some necrosis in all or part of the infiltrated tissue after 72 h. Forty-seven mutants of K60 resistant to the LPS-specific phage Psso 154 and Psso NCL, and their Eps$^-$ derivatives, also were tested. None of these strains induced the HR (Table 3). Some of the Eps$^+$ phage-resistant strains induced necrosis in all or part of the infiltrated tissue after 72 h; none

TABLE 3. HR induced in tobacco leaves by phage-resistant mutants of *P. solanacearum*[a]

Strain	Response		Strain	Response	
	24 h[b]	72 h[c]		24 h	72 h
K60	−	n,sp	NCL1	−	n
B1	+	n	NCL1Eps$^-$	−	c
154-1	−	c	NCL2	−	n+c
154-1Eps$^-$	−	c	NCL2Eps$^-$	−	c
154-2	−	c	NCL3	−	n
154-2Eps$^-$	−	c	NCL3Eps$^-$	−	c
154-3	−	n	NCL4	−	n+c
154-3Eps$^-$	−	c	NCL4Eps$^-$	−	c
154-4	−	n	NCL5	−	c
154-4Eps$^-$	−	c	NCL6	−	c
154-5	−	n	NCL7	−	c
154-5Eps$^-$	−	c	NCL7Eps$^-$	−	c
154-6	−	n	NCL8	−	n
154-7	−	n	NCL8Eps$^-$	−	c
154-8	−	c	NCL9	−	n
154-10	−	c	NCL9Eps$^-$	−	c
154-13	−	c	NCL10	−	c
154-20	−	c	NCL11	−	n+c
154-20Eps$^-$	−	c	NCL11Eps$^-$	−	c
154-21	−	c	NCL20	−	n
154-21Eps$^-$	−	c	NCL20Eps$^-$	−	c
154-22	−	c	NCL21	−	c
			NCL21Eps$^-$	−	c
			NCL23	−	n
			NCL23Eps$^-$	−	c

[a] Bacteria (ca. 10^8 CFU/ml) were infiltrated into the intercellular spaces of tobacco leaf intercostal tissue. Plants were incubated at 28°C in the light for 6 to 7 h and evaluated after 24 and 72 h.
[b] +, Watersoaking or necrosis or both after 24 h; −, no response after 24 h.
[c] Abbreviations: n, necrosis; c, chlorosis; n,sp, necrosis spreading from infiltration panel.

TABLE 4. Pathogenicity to tobacco of LPS-defective mutants of *P. solanacearum*[a]

Group[b]	Strain	Disease index[c]
I	NCL1	3.5[d]
	154-5	2.4[d,e]
	154-4	1.2[e]
	154-1	1.0[e]
	154-3	1.0[e]
	NCL2	1.0[e]
	NCL3	1.0[e]
	NCL11	1.0[e]
	B1	1.0[e]
II	154-8	1.0[e]
	154-10	1.4[e]
	154-13	1.4[e]
III	NCL7	1.0[e]
	NCL10	1.0[e]
Control	K60	3.2[d]

[a] Tobacco seedlings were stem inoculated with 20 μl of bacterial suspensions (10^9 per ml).

[b] For identification of groups, see Table 3.

[c] Average disease index of five plants after 35 to 40 days. Disease index was rated on a scale from 1.0 to 6.0 as described in the text.

[d,e] Values with the same superscript are not significantly different ($P = 0.05$).

of the Eps$^-$ phage-resistant strains induced a host response other than slight chlorosis.

Pathogenicity tests. Thirteen LPS-defective mutants of strain K60 were tested for pathogenicity on tobacco seedlings. Two of the mutant strains (NCL1 and 154-5) caused wilting of tobacco; the other strains were nonpathogenic (Table 4). Bacteria were reisolated from plants showing wilt symptoms after inoculation with either NCL1 or 154-5; the reisolated bacteria had the same colony morphologies on TZC medium and the same phage sensitivities as the original isolates. Purified LPS from the reisolated strain NCL1 had the same sugar composition and electrophoretic mobility as the LPS purified from the original isolate. Both NCL1 and 154-5 had rough LPS but produced considerable amounts of EPS in culture.

DISCUSSION

The LPS-specific phage Psso 154 and Psso NCL were useful for selection of LPS-defective mutants of *P. solanacearum* K60. When LPS from these mutants was analyzed by SDS-PAGE, the patterns observed were similar to those reported for *Escherichia coli* and *Salmonella* LPS (13, 14, 27, 28). The multiple, evenly spaced bands may represent LPS molecules with different numbers of O-antigen units per molecule (13, 28). It is not clear, however, to what extent charged groups in the O-antigen or core region and heterogeneity in lipid A substituent groups (26, 30) influence the mobility of LPS on gels.

Most of the phage-resistant strains had rough LPS, although two of these mutants (154-7 and NCL 9) were fluidal and had smooth LPS. Other mutants (e.g., 154-2, 154-11, NCL4, and NCL8) had a preponderance of smooth-type LPS with various amounts of O-antigen sugars. These strains may be phage resistant because of lysogeny, because of small alterations in the O-antigen, or because only a small proportion of the LPS molecules were of the appropriate length for phage attachment.

On the basis of LPS sugar composition, the strains with rough LPS could be separated into three groups with increasingly defective LPS (Fig. 5). The LPS from group I strains was similar to that of B1 in sugar composition; all of the strains lacked xyl and contained rha, but there was a high degree of variability in the sugar ratios within the group. This variability suggests that group I LPS strains lack the O-antigen, but that some have a complete core and others have reduced amounts of rha or glc in the core. Some of the group I strains were identical to B1 in electrophoretic mobility, containing one fast-moving band; other strains had LPS with two or more fast-moving bands. It is likely that charge differences within the LPS population account for the different bands, since no consistent alteration in sugar ratios was associated with strains with more than one band. For some group I strains, faint slow-moving bands were evident after electrophoresis, indicating that the LPS mutations in these strains may be leaky.

The LPS of strains in groups II and III was more defective in core sugars than that of strains in group I, in that it lacked rha (group II) or rha, glc, and hep (group III). The LPS from group II strains was similar to B1 LPS in electrophoretic mobility; group III LPS had the highest mobility, running ahead of the dye front. The group III strains are analogous to the "heptoseless" mutants of *E. coli* or *Salmonella* spp. (38). All three types of LPS-deficient mutants of *P. solanacearum* should prove useful for studies of the structure and biosynthesis of the LPS core region.

All of the phage-resistant mutants produced EPS, although they were less fluidal than the parent, K60. Variants that did not produce visible EPS were selected by plating from stationary broth cultures. Strain B1 and phage-resistant mutants that have rough LPS and are Eps$^-$ can be distinguished from strains with smooth LPS (either Eps$^+$ or Eps$^-$) by their ability to autoagglutinate in saline (E. Barlow and H. Mino, unpublished data). In preliminary experiments, an agglutinin isolated from potato tubers (22) agglutinated all phage-resistant strains with rough-type LPS to the same extent that it agglutinated B1, but strain K60 (smooth LPS) was agglutinated only weakly. These results suggest that some of the LPS- and EPS-deficient strains have surface properties similar to those of the HR-inducing strain B1, since they behave similarly in agglutination tests.

Many of the phage-resistant mutants and their Eps$^-$ derivatives were similar to strain B1 in LPS composition and electrophoretic mobility, as well as in agglutination properties, yet none of these mutants induced the HR. Furthermore, none of the 75 K60 Eps$^-$ spontaneous variants tested induced the HR. It seems likely, therefore, that neither loss of EPS nor loss of the O-antigen portion of LPS is sufficient to convert K60 into an HR-inducing strain. The HR-inducing properties of strain B1 must represent alterations in factors other than, or in addition to, those involved in EPS and LPS synthesis.

Pathogenicity tests confirmed the observation of Kelman (17) that EPS is a critical determinant of pathogenicity. Of the 13 LPS-defective mutants tested, two (NCL1 and 154-5)

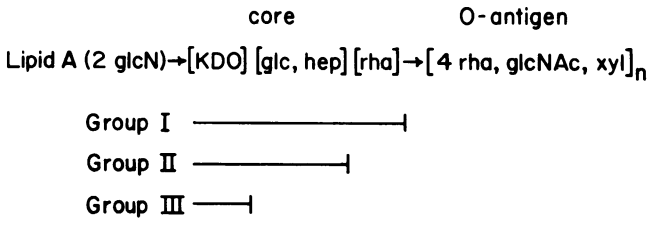

FIG. 5. Diagram of the LPS-defective mutants of *P. solanacearum*.

caused disease symptoms in tobacco seedlings. These two mutants had rough LPS similar to that of B1 but produced EPS in culture. Apparently, some strains with rough LPS can multiply in the host and cause wilting if they can produce sufficient EPS. Other phage-resistant strains that produce only low levels of EPS caused partial necrosis of tobacco leaves 48 to 72 h after infiltration, but did not spread systemically or cause disease symptoms in tobacco seedlings.

Our results do not support the hypothesis that a sugar-specific interaction between rough LPS of *P. solanacearum* and a receptor in tobacco cell walls is the determining factor in HR initiation. Differences in HR-inducing ability could be associated with minor differences in LPS structure that were undetectable by the methods used in this study. Furthermore, we have not eliminated the possibility that our LPS mutants differed from strain B1 in having defects in other functions required for HR induction. For example, since defects in O-antigen synthesis have been reported to cause changes in other cell wall constituents (26), it is possible that LPS-defective strains that induce the HR differ from noninducing strains in the amount or type of outer membrane proteins expressed. Further research will be needed to clarify this point. At the same time, it is clear that recognition in this system is not simply a matter of loss of EPS and the O-polysaccharide portion of LPS of *P. solanacearum* and that other unknown factors are the important determinants of incompatibility.

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