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Characterization and Virulence Properties of *Erwinia chrysanthemi* Lipopolysaccharide-Defective, φEC2-Resistant Mutants

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Outer membrane alterations were characterized in spontaneous mutants of the *Erwinia chrysanthemi* 3937jRH, which were selected for resistance to bacteriophage φEC2. All but one of the mutants analyzed were affected in their lipopolysaccharide (LPS) structure, lacking the entire heterogeneous region of apparent high molecular weight present in the wild-type *E. chrysanthemi* LPS. At least two 3937jRH mutants, one selected as φEC2 resistant (RH6065) and the other previously selected (D. Expert and A. Toussaint, J. Bacteriol. 163:221–227, 1985) as bacteriocin resistant (R1456), were cross-resistant to bacteriophage Mu and had rough LPSs with an altered core structure. Two φEC2^r mutants (RH6053 and RH6065) were most severely affected in their outer membrane integrity and also lost their virulence on saintpaulia plants, although they still possessed normal extracellular levels of pectinolytic and cellulolytic activities. The two Mu^r mutants RH6065 and R1456 were also able to induce systemic resistance in the challenged plant. All the other φEC2^r mutants retained the virulence of 3937jRH.

Erwinia chrysanthemi, an enterobacterial phytopathogen, causes soft rot on many tropical and subtropical plant species. Unlike the other soft-rotting *Erwinia* species, these particular strains can infect only a limited number of hosts (10), suggesting a certain host specificity. Pectinolytic *Erwinia* spp., including *E. chrysanthemi*, induce rapid maceration and killing of parenchymatous tissues mainly by degrading the plant cell walls (2). Such a process involves bacterial extracellular depolymerizing activities (pectinases, cellulases, and proteases). Among the enzymes secreted, endopectic enzymes—up to five different pectate lyases in *E. chrysanthemi* (24)—appear to be essential for virulence (6). Furthermore, the endopectate lyases from *Erwinia* sp. can stimulate active plant defense reactions, in particular through the release from plant cell walls of unsaturated decagalacturonic acid that functions as an elicitor of phytoalexin accumulation (7, 8).

Expert and Toussaint (12) recently suggested that other determinants, such as an iron transport system, are important for the virulence of the *E. chrysanthemi* 3937jRH. Bacteriocin-resistant mutants affected in the outer membrane (OM) proteins normally induced under iron starvation, but still able to secrete the pectinases and cellulases in normal amounts, were all shown to produce a localized and necrotic response in the infected saintpaulia plants. These mutants were cross-resistant to the bacteriophage φEC2, and one of them (R1456) acquired, in addition, resistance to phage Mu (12). A similar necrotic response in inoculated saintpaulia plants can be detected after infections with incompatible strains, but in that case the response may involve some specificity mechanism(s) (M. Lemattre, personal communication).

Bacterial cell surface components such as the lipopolysaccharide (LPS) or proteins might also account for plant-bacterium recognition processes (11, 30, 37, 38). Purified LPS has also been reported to inhibit the responses normally induced by compatible and incompatible pathogens on tobacco plants and to induce this resistance systemically (31). Recently, an avirulent *galU* mutant (deficient in the synthesis of the core part of the LPS) was isolated from *Erwinia carotovora*, but its avirulence was claimed to result from the accumulation of galactose intermediates toxic for bacterial growth through the assimilation of plant cell components (18).

To further analyze the basic mechanisms involved in the successful infection by *E. chrysanthemi* 3937j of its natural host *Saintpaulia ionantha* (12), we studied new bacterial mutants altered in OM structure. From strain 3937jRH, we isolated a set of mutants resistant to the *E. chrysanthemi* transducing temperate phage φEC2 (23). We tested the composition of their OMs, as well as their pectinase and cellulase activities, their virulence, and their ability to trigger systemic resistance against further infection in *S. ionantha*.

MATERIALS AND METHODS

Strains and media. The bacterial strains and bacteriophages are listed in Table 1. *E. chrysanthemi* strains were usually grown at 30°C. Complete medium was Luria broth (L broth), and minimal medium was M63 supplemented with 2 g of the appropriate sugar per liter (19). Sodium polygalacturonate, methylated pectin, and carboxymethyl cellulose media used to assay pectinases and cellulases were as described previously (3). LA plates contained 12 g of Difco agar per liter. Iron-depleted culture conditions were those described earlier (12). Concentrations of cell surface-active agents were as follows: sodium deoxycholate (DOC), 0.1%; EDTA, 0.1%; sodium dodecyl sulfate (SDS), 0.1%; and Triton X-100, 1%.

Preparation of bacteriocin and bacteriophage lysates. Bacteriocin lysates were prepared and titrated as described by Expert and Toussaint (12). Mu lysates were prepared as described by Toussaint and Schoonejans (33). φEC2 c^+ and φEC2 *c*13 lysates were grown as described by Résibois et al. (23).

Bacteriophage and bacteriocin sensitivity. Sensitivity to bacteriocin and bacteriophages was determined by spot

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TABLE 1. Bacterial strains and phages

Strain	Description	Reference
Erwinia chrysanthemi		
3937jRH	Spontaneous derivative of strain 3937j; φEC2 and Mu sensitive; 3937j was isolated from *S. ionantha* by M. Lemattre	23
R1456	3937jRH mutant selected as resistant to the bacteriocin of strain 1456	12
RH6051 to RH6070	3937jRH φEC2^r mutants	This work
B374	Wild-type, φEC2 and Mu sensitive, isolated from *Pelargonium capitatum* by R. Dadant	14
B374 7.1	Mu^r mutant, lacks the terminal glucose of the LPS core region	27
1271, 1277, 1452, 1455, 1456, 1500, 1521, 1871, 1884	Wild-type bacteriocin-producing strains[a]	12
Erwinia carotovora 3912	Wild-type bacteriocin-producing strain	12
Erwinia uredovora 20D3	Wild-type bacteriocin-producing strain	32
Phages		
Mu *vir*3051	Virulent mutant of Mu	34
Mu *cts*62	Thermoinducible mutant of Mu	17
φEC2	Same as PhiEC2	23
φEC2 *c*13	Clear mutant, unable to lysogenize	23

[a] These strains were screened (12) from different collections for the production of bacteriocins active against three *E. chrysanthemi* strains: 3937jRH, B374, and 3665 (isolated from *Dieffenbachia* sp. by M. Lemattre). Their original sources are described in reference 12.

assays as described by Expert and Toussaint (12). Sensitivity to φEC2 was also tested routinely by spreading 0.2 ml of a high-titered (≥5 × 10^9 PFU/ml) φEC2 *c*13 lysate on LA plates. A 10-μl sample of the bacterial cultures (≈10^9 CFU/ml) to be tested was then spotted on these plates, and their growth was compared with the growth of the wild-type φEC2-sensitive or the mutant φEC2^r strains.

Isolation of φEC2^r mutants. High-titered (≥5 × 10^9 PFU/ml) φEC2 *c*13 lysates were used to select the resistant mutants from independent cultures of strains 3937jRH or B374. Undiluted phage solution was spotted onto soft-agar overlays seeded with 0.2 ml of the different bacterial cultures. The plates were incubated at 30°C for 24 h. Resistant colonies were then purified twice through single-colony isolation.

Bacteriophage and bacteriocin adsorption. Adsorption of the bacteriocins to bacterial cells was tested as described previously (12). Phage adsorption experiments were performed on 1 ml of L broth culture of exponentially growing cells with multiplicities of infection of 0.2 and 0.02. After 20 min of adsorption at 30°C, 5 ml of prewarmed L broth was added to 0.1 ml of the adsorption mixture, and the bacterial cells were killed by adding 0.05 volume $CHCl_3$ and removed by centrifugation. The supernatant was assayed for the remaining PFUs by spotting successive dilutions on the indicator strain. Sensitive strains left less than 5% free PFUs in the supernatant (usually ≈1%) and receptor mutants more than 95% free PFUs. Adsorption of Mu particles was tested in the presence or absence of 50 mM $CaCl_2$.

Tests for sensitivity to cell surface-active agents. Sensitivity of the mutants to the different cell surface-active agents was tested by the method of Expert and Toussaint (12). Increased sensitivity was scored when the efficiency of plating was lowered by a factor of 10^4 or more.

Pathogenicity and induced systemic resistance tests. The pathogenicity test on whole living saintpaulia plants (*S. ionantha* cv. Blue rhapsody) was performed as previously described (12) on at least three plants with two inoculated leaves for each mutant to be tested. Symptoms were scored every 2 days and for at least 3 wk. Induced systemic resistance was tested by inoculating one leaf of the plant with the mutant to be tested and by challenging the plant on another leaf with strain 3937j 0, 12, 24, 72, or 192 h later. Symptoms were then scored as for the pathogenicity test, and induced systemic resistance was scored by the absence of soft-rot symptoms on the leaf inoculated with the challenging strain 3937j. Plant tissue maceration assays were performed with avirulent mutants on leaves cut off from young saintpaulia plants as described previously (12).

Pectate lyase, pectin methyl esterase, and endo-β-glucanase activities. The detection of the activities of pectate lyase, pectin methyl esterase, and endo-β-glucanase in culture supernatants or cell lysates by the cup-plate technique and the detection of pectate lyase isozymes by electrofocusing in ultrathin polyacrylamide gels were described earlier (1, 3).

OM preparation and LPS miniscale extraction. OM for protein analysis was prepared as miniscale Triton-insoluble extracts (12). The following modifications were designed for LPS miniscale preparations. The cells from a 5-ml culture were suspended before the sonication step (on ice) in 1 ml of 25 mM Tris (pH 7.5)–0.25 mM EDTA. The lysis mixture was centrifuged for 30 s at 8,000 × *g*. The supernatant was treated with 110 μl of a Triton X-100 (20%) solution and recentrifuged (in a microcentrifuge) for 90 min at 4°C. The supernatant was discarded, the Eppendorf tube was drained, and the pellet was suspended in 100 μl of sample buffer (12.5% [vol/vol] glycerol, 2% SDS, 125 mM Tris [pH 6.8], 5 mM EDTA; bromophenol blue was added as the tracking dye). Pronase was added at a final concentration of 50 μg/ml, and the mixture was incubated for 90 to 120 min at 37°C and then overnight at 4°C. The suspension was incubated for 5 to 10 min at 100°C, and pronase was again added at a total concentration of 100 μg/ml. The preparation was then incubated for another hour at 37°C before being loaded (usually 3 to 5 μl) on the gel for analysis or stored at −20°C. When the gels were stained with Coomassie blue, these extracts showed no protein material.

LPS purification and electrophoresis. LPS (yield, ≈10 mg) was extracted by the phenol-water method (36) from strains 3937jRH and B374 (≈4.5 g [wet weight]) grown exponen-

tially in 1 liter of M63 medium supplemented with glucose. It was purified either by three successive ultracentrifugation steps (12 h, 110,000 × *g*) or by DNase and RNase digestion as described by Carlson et al. (5) followed by overnight dialysis against water and ultracentrifugation. To check for contamination by peptidoglycan, LPS was submitted to lysozyme digestion (15,000 U/ml for 2 h at 37°C) in 25 mM Tris (pH 7)–5 mM EDTA–0.05% (wt/vol) N_3Na and subsequently diluted to obtain the final concentrations of the sample buffer, incubated for 10 min at 100°C, and then submitted to pronase digestion as described for the miniscale extraction. Smooth-type LPS from *Escherichia coli* serotype O26:B6 was purchased from Sigma Chemical Co. (St. Louis, Mo.).

Samples (3 μg) of LPS were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (15) on 13.5% gels (0.2% SDS) with Tris-glycine buffer (22). The gels were stained either by the silver-staining method (15) or by the periodate-Schiff method (29). Wild-type LPS purified through ultracentrifugation steps, by DNase and RNase digestion, or by lysozyme digestion showed the same pattern as the LPS obtained from miniscale extractions (Fig. 1), irrespective of the staining technique used. This suggested that there was no contamination by other silver or periodate-Schiff cross-reacting material (nucleic acids or peptidoglycan) in our miniscale preparations.

OM protein electrophoresis. OM proteins were analyzed by SDS-PAGE as described earlier (12).

Phage inactivation experiments. Inactivation experiments were performed as described previously (28), but at 30°C instead of 37°C.

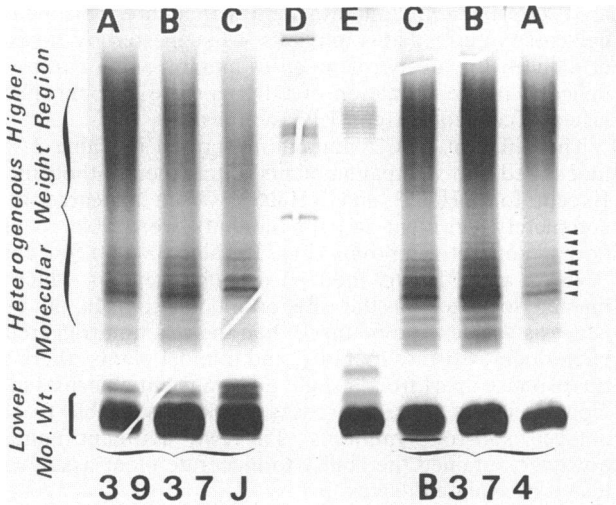

FIG. 1. Silver-stained SDS-PAGE (13.5% acrylamide) analysis of LPS from the two wild-type strains 3937jRH and B374. Each lane was loaded with 3 μg of LPS. Purified water-phenol extracts were analyzed before (lane A) and after (lane B) lysozyme digestion and compared with the Triton-insoluble miniscale extracts (lane C). The same profiles were obtained by periodate-Schiff staining. Arrows indicate bands separated from one another by a small and constant interval reminiscent of the smooth LPS of enteric bacteria, such as *E. coli* serotype O26:B6 (lane E). The apparent molecular weights (mol. wt.) of standard proteins (purchased from Bio-Rad Laboratories, Richmond, Calif.) are (lane D, from top to bottom): phosphorylase B, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,400.

TABLE 2. Phenotype of the mutants

Strain	Resistance to[a]:			Sensitivity to:		LPS phenotype[b]
	Bacteriocins	Mu	φEC2	DOC	SDS	
Wild type	S	S	S	R	R	S
RH6051	S	S	R	R	R	S
RH6068	S	S	R	R	R	R
RH6069	S	S	R	R	R	R
RH6061	I	S	R	R	R	R
RH6064	I	S	R	R	R	R
RH6066	I	S	R	R	R	R
RH6070	R	S	R	R	R	R
RH6053	R	S	R	S	R	RR_1
RH6065	R	R	R	S	S	RR_3
R1456	R	R	R	(S)[c]	S	RR_2

[a] The mutants were tested for their resistance to all the bacteriocins and to the phages (Table 1). R, Resistance (receptor mutants) to all the bacteriocins or to the phages; I, partial insensitivity to some bacteriocins, but still able to adsorb them; S, sensitivity.

[b] Obtained by SDS-PAGE from LPS miniextractions (Fig. 2). S, smooth type; R, semirough or rough type, lacking the higher-molecular-weight region; RR, rough type, lacking the higher-molecular-weight region and altered in the electrophoretic mobility of the low-molecular-weight band(s). The subscripts 1, 2, and 3 indicate the extent of the electrophoretic mobility change.

[c] (S), Partial sensitivity; efficiency of plating, 10^{-3}.

RESULTS

Spontaneous φEC2^r mutants. φEC2 *c*13 was used to select for spontaneous resistant mutants of 3937jRH. Twenty survivors of phage infection were recovered at frequencies ranging from 10^{-6} to 10^{-7}. They all failed to adsorb the phage. A set of 10 independent isolates resistant to the phage was further analyzed.

All the mutants previously isolated (12) for resistance to bacteriocins produced by different *Erwinia* strains were also φEC2^r. One of these mutants (strain R1456) was also resistant to phage Mu, which grows on 3937jRH (13). Therefore, the 10 φEC2^r mutants were tested for their sensitivity to these bacteriocins and to Mu (Table 2). Three mutants (RH6053, RH6065, and RH6070) were resistant to the bacteriocins tested, lacking receptor function for all of them. Three other mutants (RH6061, RH6064, and RH6066) showed a decreased sensitivity to several bacteriocins, and the others remained bacteriocin sensitive. Only one of the 10 isolates (RH6065) was also resistant to Mu.

We tested the sensitivity of the mutants to different cell surface-active agents (EDTA, DOC, SDS, and Triton X-100) (Table 2). RH6053 showed an increased sensitivity to DOC, as did strain RH6065; the latter was in addition more sensitive to SDS. RH6065 thus behaved toward DOC and SDS very much like the previously isolated strain R1456. None of the φEC2^r mutants had an increased sensitivity to EDTA or Triton X-100.

OM LPS analysis. Sandulache et al. (27) have shown that in the Mu^r mutant B374 7.1, the terminal glucose residue of the LPS core region is missing, suggesting that in *E. chrysanthemi* the Mu receptor resides in the LPS core part, as it is the case in other Mu-sensitive bacteria (27, 28). Therefore, it seemed likely that at least strain RH6065 was altered in its LPS, and we investigated the possibility that φEC2^r mutants were all affected in their LPS, a structure which often functions as a phage receptor (4).

Profiles of purified LPS or OM prepared from parental strains and φEC2^r mutants and treated with pronase were compared by SDS-PAGE. Similar analysis of smooth *E. coli* and *Salmonella* strains have shown that the banding region

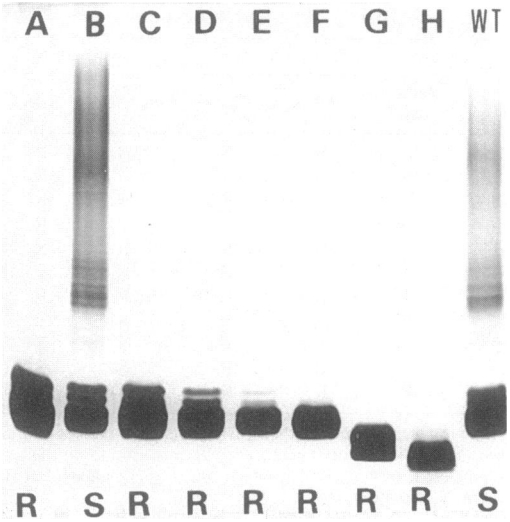

FIG. 2. Silver-stained SDS-PAGE (13.5% acrylamide) analysis of LPS from 3937jRH mutants. LPS was obtained by the pronase-digested miniscale extraction from mutants RH6064 (A), RH6051 (B), RH6068 (C), RH6069 (D), RH6070 (E), RH6053 (F), R1456 (G), and RH6065 (H). The wild-type 3937jRH LPS is shown in lane WT. The S and R phenotypes noted at the bottom are described in Table 2. Migration was from top to bottom.

of apparent high molecular weight consists of LPS molecules which differ from one another by one O-antigen side chain repeat unit, while the low-molecular-weight bands correspond to incomplete LPS molecules in which the core is substituted with an average length of one O-antigen repeat unit (16, 21). In *E. chrysanthemi* B374, the structure of the core region was found to be similar to that of *E. coli* B (27) and was proposed to be Glc-β1,6-Glc-α1,3-(Hep-α1,7-)Hep-α1,3-Hep-, where Glc is glucose and Hep is L-glycero-D-mannoheptose.

LPS of both of the wild-type *E. chrysanthemi* strains analyzed (3937jRH and B374) showed distinct banding regions (Fig. 1): (i) a low-molecular-weight region characterized by a few bands and separated by a blank region from a (ii) higher-molecular-weight heterogeneous region containing many bands. Several of these bands were separated by a small and apparently constant interval (arrows). The heterogeneous profile obtained in the upper part of the gel might reflect a great heterogeneity of the LPS molecules, a tendency of LPS molecules to aggregate, or both.

All but one (RH6051) of the φEC2^r mutants lacked at least the putative O-antigenic side chains (Fig. 2; Table 2). RH6065 and RH6053 are, in addition, affected in the electrophoretic mobility of the low-molecular-weight bands (Fig. 2; Table 2). RH6065, the most affected mutant, displayed an LPS profile very similar, albeit not identical, to that of strain B374 7.1 (data not shown), a rough mutant with a proposed "Rc" chemotype (27). Such changes in the electrophoretic mobility of LPS molecules are reminiscent of the different chemotypes identified in rough mutants of *Salmonella* species in which the LPS has an altered core structure and in which the extent of the missing part of the core is correlated with the extent of the change in electrophoretic mobility of the low-molecular-weight bands (15). As did strain RH6065, strain R1456 seemed to have a rough LPS with an altered core structure, which was consistent with their Mu^r phenotype (Fig. 2; Table 2).

The other previously isolated bacteriocin-resistant mutants (12), cross-resistant to φEC2, were also analyzed and found to have an altered LPS structure, lacking at least the O-antigenic side chains (data not shown). Analysis of the LPS patterns of φEC2^r mutants derived from B374 again showed that most of them lack the heterogeneous region of high molecular weight (data not shown).

Analysis of OM proteins. We looked for possible alterations in the OM proteins of the φEC2^r mutants by SDS-PAGE for strains grown in L broth or minimal medium with glucose or glycerol as the carbon sources. No significant qualitative change could be observed in the profiles (data not shown). Comparison of OM protein patterns obtained from strains grown under iron starvation showed that the three inducible polypeptides migrating in the 80-kilodalton range in the wild-type strain remained inducible in all the φEC2^r mutants with an altered LPS. In strain RH6051, the three iron-regulated proteins could also be detected under high iron conditions, as shown for one mutant previously isolated for its resistance to the bacteriocin 20D3 (12; data not shown).

Taken together, these data suggest that φEC2 binds to the LPS molecule as part of the cell surface receptor. Inactivation experiments indeed confirmed that LPS from wild-type 3937jRH and B374 strains inactivated the phage. The concentration of LPS that caused a 50% decrease in phage titer was ≈10 μg of LPS per ml.

Pathogenicity of φEC2^r mutants. Before testing the virulence of the φEC2^r mutants, we demonstrated that their other relevant properties (production of extracellular pectate lyases, pectin methyl esterase, and endoglucanases assayed by cup plate assay or isoelectrofocusing) were normal (data not shown). The generation time of strain RH6065 in L broth or glucose minimal medium, however, was 1.7 times longer than that of the wild-type strain. Strains RH6053, RH6065, and R1456 were Gal$^+$, eliminating the possibility that they are *galE* or *galU* mutants and hence are poisoned by galactose intermediates in plants, as suggested by Jayaswal et al. (18) for an avirulent *galU* mutant of *E. carotovora* deficient in the formation of UDP glucose-pyrophosphorylase and consequently in LPS synthesis.

The different φEC2^r mutants described in Table 2 were inoculated into saintpaulia plants to test their pathogenicity. Except for RH6053 and RH6065, which appeared to be completely avirulent, all the mutants were able to elicit normal soft-rot symptoms (Fig. 3; Table 3). In a few cases, RH6053 and RH6065 induced a slight necrosis which remained localized at the site of inoculation. In all cases necrosis was less pronounced than the reaction triggered by bacteriocin-resistant mutants, and in most plants, there was no response apart from a slight and transient chlorosis in the inoculated leaf. In a few cases RH6053 was able to elicit delayed soft-rot symptoms. The two avirulent mutants, however, retained the ability to macerate plant tissue when tested on isolated leaves.

To characterize the behavior of the avirulent mutants in planta, we tested them on saintpaulia plants for the ability to induce resistance against further infection. A leaf was inoculated with a potential inducing strain, and this inoculation was followed at different times by the inoculation of the virulent strain in the same or in another leaf. Preliminary data indicated that, of the mutants tested, only strains RH6065 and R1456 induced systemic resistance against further infection with the wild-type strain. A lag period was necessary to obtain the protection (24 h or less) between inducing and challenging inoculations. The protection was transient since it was not detected when the lag period between the two inoculations exceeded 72 h. In a few cases

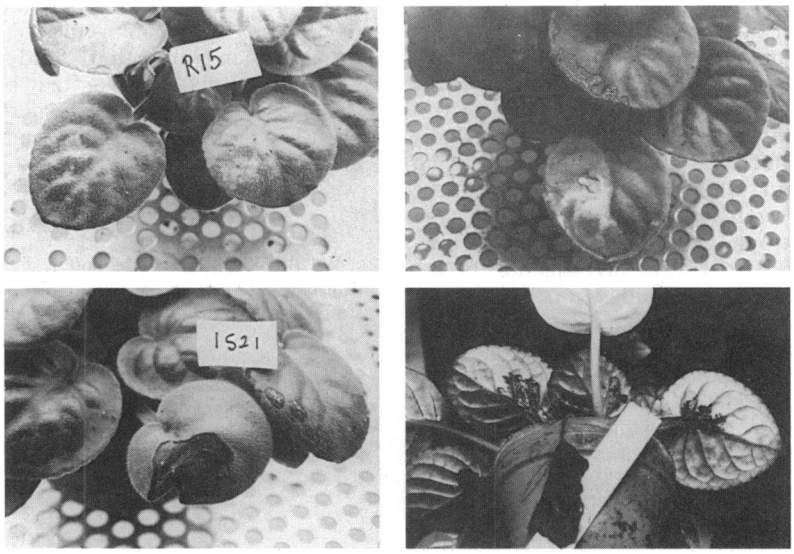

FIG. 3. Pathogenicity phenotypes of the mutants. Leaves of the infected saintpaulia plants are shown after 4 weeks of phenotype development. Pathogenicity tests were performed by infiltrating the leaf parenchyma with 100 μl of a 3×10^8-CFU/ml suspension of the following 3937jRH derivatives: (top left) RH6065, showing the absence of soft-rot symptoms; (top right) RH6053, showing a slight necrosis at the site of injection; and (bottom left) R1521, showing the typical necrosis induced by the bacteriocin-resistant mutants. For comparison, the invasive stage of the soft rot disease developed by 3937j is also shown (bottom right).

a challenging inoculation of strain 3937jRH caused maceration which remained localized in the challenged leaf, while in most plants it did not result in any symptom. This result shows that the two mutants are able to initiate a host-defensive response extending to the whole plant.

DISCUSSION

To study the OM structures involved in the successful infection of *E. chrysanthemi*, we isolated a set of φEC2^r mutants derived from strain 3937jRH. All but one were altered in their LPS, and none of the mutants altered in the LPS had significant changes in either OM proteins or production of extracellular pectinases and cellulases.

TABLE 3. Pathogenicity of the mutants

Strain	No. of tests[a]	Phenotype on plant[b]			
		Chlorosis	Necrosis	Maceration	Soft rot
Wild type	26	0	(5)	+	+
RH6051	6	0	0	+	+
RH6068	6	0	0	+	+
RH6069	6	0	0	+	+
RH6061	6	0	0	+	+
RH6064	6	(2)	0	+	+
RH6066	6	0	0	+	+
RH6070	6	(1)	0	+	+
RH6053	16	6	5[c]	5	−
RH6065	26	16	10[c]	0	−
R1456	26	0	+	0	−
Control[d]		+			−

[a] The tests were performed as described in the text. Each test corresponds to one of the two inoculated leaves per plant.

[b] The phenotypes were scored after 4 to 8 weeks. +, More than two-thirds of the responses; the numbers indicated for each phenotype on the plant correspond to the number of tests giving the corresponding response; the numbers in parentheses indicate exceptional responses. Chlorosis, necrosis, and maceration, See Fig. 3; Soft rot is a global response.

[c] The necrosis induced by the RH6053 andRH6065 mutants is reduced as compared with the necrosis induced by the bacteriocin-resistanct mutants (Fig. 3).

[d] A 100-μl sample of sterile 150 mM NaCl or distilled water was infiltrated in the leaf parenchyma as a control.

A now widely used simple technique for analyzing LPS (15, 16) revealed that all the available mutants except one (RH6051) had lost the higher-molecular-weight heterogeneous region detected in the parental strain. The majority of the φEC2^r mutants isolated from strain B374 showed the same LPS defect. The wild-type laddered LPS pattern strongly suggests that this region corresponds to a structure analogous to the O-antigenic side chains of smooth enteric LPS (16). Smooth LPS might be common among *Erwinia* spp. and be partly responsible for the different O-serotypes described (9, 26).

The results clearly show that the LPS contains at least part of the φEC2 receptor and suggest that the receptor includes the O-antigen-like structure of the LPS molecule in both strains 3937jRH and B374. However, some observations suggest that this structure, although necessary, may not be the sole component of the phage receptor, as is the case for some coliphages or bacteriocins (4). (i) Inactivation of the phage required relatively high amounts of purified LPS, indicating either the presence of a low number of efficient binding sites or the reversibility of the binding step of the phage on the external part of the LPS; (ii) at least one of the mutants scored as φEC2^r did not display any visible change in its LPS pattern, which, of course, does not rule out the possibility of a minor modification not detectable by the method used.

Strain 3937jRH φEC2^r mutants were tested for their pathogenicity on *S. ionantha*. Two mutants (RH6053 and RH6065) lost their virulence, being unable to induce a phenotypic response on saintpaulia plants except for an occasional slight necrosis. One of these (RH6065) and the previously isolated bacteriocin-resistant mutant R1456 acquired the capacity to induce systemic resistance in the

plants. The avirulent mutants could still macerate plant tissues. All other φEC2^r isolates remained virulent.

In addition to the loss of the O-antigen-like structure, the two avirulent mutants RH6065 and RH6053 were possibly altered deeper in the LPS molecule as seen by the electrophoretic mobility change of the low-molecular-weight bands. RH6065 may be deeply affected in the core structure since it was resistant to phage Mu, which is known to bind to the core region in the *E. chrysanthemi* LPS (27), and displayed the most affected electrophoretic mobility of the low-molecular-weight bands. Biochemical investigation of the LPS composition of the mutants will allow a better characterization of their chemotypes. Moreover, both mutants exhibited increased sensitivity to cell surface-active agents (DOC, SDS), as do some *E. coli* or *Salmonella* rough or deep rough mutants (20). Strain RH6053, however, is still Mu^s.

These results emphasize the possible existence in *E. chrysanthemi* of a correlation between the loss of pathogenicity and severe alterations of the OM integrity. The putative O-polysaccharidic side chains of the LPS seemed to be dispensable for the establishment of a compatible association between *E. chrysanthemi* 3937jRH and its host since they could be lost without affecting the virulence, whereas the core might be directly or indirectly implicated in this process. The loss of virulence of some mutants could result either from the disappearance of a structure (LPS) protecting the bacterium from potential defense mechanism or allowing the initial stage of the disease, or from the appearance of a structure (LPS) able to elicit plant defense response. In the first context, some rough mutants, like the two avirulent mutants, might be more susceptible to antimicrobial agents (20) such as phytoalexins (25, 35) or might be enhanced in the release of some signal able to induce such host defense mechanisms (7). Alternatively, exposure of a specific portion of the LPS might allow it to serve as bacterial receptor (or signal) for some plant factor(s) (as lectins for instance), resulting then in drastic bacterial agglutination (30). Avirulent mutants affected in such a manner have been described in the literature, but the role of the LPS still remains largely obscure (11, 31).

An active role of some *E. chrysanthemi* mutants in triggering plant defense mechanisms could account for the preliminary assays of cross-protection. Indeed, only the two most affected LPS mutants (RH6065 and R1456) were able to induce systemic resistance in the plants. Some hypothetical signal released through leakiness of the mutant cells or the exposure of LPS site(s) otherwise protected might trigger the appropriate defense mechanism for induced systemic resistance, i.e., the de novo production of a diffusible product in the plant.

This study revealed that previously isolated 3937jRH bacteriocin-resistant mutants, all of which were cross-resistant to φEC2, were all affected in their LPS (they had lost the O-antigen-like structure). All were also avirulent and were affected in 80-kilodalton-range OM proteins induced by iron starvation (12). On the other hand, several mutants selected for their resistance to phage φEC2 were cross-resistant to the bacteriocins, and those had alterations either in the O-antigen-like structure and then were still virulent (RH6070 for instance) or probably deeper and then were avirulent, although they had no alterations in the 80-kilodalton proteins. This suggests that the loss of virulence in bacteriocin-resistant mutants was not due to their loss of the O-chains, although the high complexity of a gram-negative bacterial OM renders the analysis of the function of each isolated component very difficult. Recently, new mutants of strain 3937j defective in iron transport were shown to be affected in pathogenicity (C. Enard and D. Expert, manuscript in preparation). Study of these mutants, together with our results, suggests that both the LPS and the iron transport system of *E. chrysanthemi* are important in the plant-bacterium interactions leading to successful invasive infection.

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