Partial Characterization and Serological Specificity of the Lipopolysaccharide of *Erwinia chrysanthemi*

J. J. Bradshaw-Rouse, L. Sequeira, A. Kelman, and R. S. Dickey

Research specialist and professors, respectively, Department of Plant Pathology, University of Wisconsin-Madison, Madison 53706; and, last author, Department of Plant Pathology, Cornell University, Ithaca, NY 14853.

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ABSTRACT

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The sugar composition of the lipopolysaccharide (LPS) of 16 strains of *Erwinia chrysanthemi* (eight from corn, two from chrysanthemum, and six from other hosts) was analyzed by gas chromatography of the alditol acetate derivatives and by colorimetric assay. The LPS of all strains contained glucose, heptose, 3-deoxy-D-*manno*-octulosonic acid, glucosamine, and two unidentified sugars; other sugar components varied among different strains. Six groups, each containing one to six strains, were identified by cluster analysis of the mean molar ratios of rhamnose, fucose, mannose, galactose, and glucose. LPS composition was not correlated with virulence or original host of the strains. Agglutination of the strains by a corn seed agglutinin, possibly mediated by their LPS, was not correlated with ability to rot the corn hybrid 64A × W117. The antigenic reaction of the LPS from eight strains to antisera of *E. chrysanthemi* was correlated with sugar composition; the other LPS preparations did not react with any of the antisera.

A wide range of plant species are attacked by *Erwinia chrysanthemi* (6–9). Six phenotypic subdivisions (biovars) have been proposed by Dickey (6) and Dickey and Victoria (7) that contained strains isolated either from a specific host, closely related hosts, or several diverse hosts. From studies on DNA hybridization, Brenner et al (3) concluded that strains isolated from corn and grasses (except for one sugarcane strain) are distinctly different from those from other host plants. They recommended that strains of *Erwinia* (*Pectobacterium*) *chrysanthemi* from corn or grasses be designated *Pectobacterium chrysanthemi* pv. *zeae*. Soft-rotting *Erwinia* from corn also can be differentiated from other soft-rotting *Erwinia* strains that are nonpathogenic to corn on the basis of their resistance to DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one) (13).

It seemed possible that host-specificity of *E. chrysanthemi* could be associated with differences in components of the outer cell membrane. Lipopolysaccharides (LPS), for example, are thought to interact with specific receptor molecules (possibly lectins) in the host, thereby determining specificity of attachment and infection (15,17). With the crown gall pathogen, *Agrobacterium tumefaciens*, Whatley et al (22) showed that LPS and cell envelope preparations from site-binding, virulent, and avirulent strains of the bacterium inhibited tumor induction. Conversely, LPS from non-site-binding, avirulent strains was not inhibitory. Similarly, with the wilt-inducing pathogen, *Pseudomonas solanacearum*, agglutination of avirulent strains by proteins from either tobacco or potato cell walls was shown to be mediated by rough LPS, which lacks the O-antigen component (23). Avirulent or weakly virulent strains of *E. stewartii*, a wilt pathogen of corn, are agglutinated more strongly by a corn seed agglutinin than virulent strains (2). Although the sugar composition of the LPS of virulent strains was not distinguishable from that of avirulent ones, differences in agglutination were correlated with the amount of extracellular polysaccharide (EPS) produced by each strain.

Extracts of LPS have been used as antigens for serological studies of *E. chrysanthemi* (8,9). Five serovars were identified when 11 antisera, produced against Formalin-treated or glutaraldehyde-fixed strains, were used. The specificity of the antisera for *E. chrysanthemi* was established, but a definite relationship between original host or phenotypic properties and antigenic reaction was not demonstrated.

The primary purpose of the present study was to determine whether sugar composition of LPS of strains of *E. chrysanthemi* from corn differed significantly from that of strains isolated from other host plants. Secondary objectives were to determine whether pathogenicity of these strains on corn was related to their agglutination with the corn seed agglutinin and whether any relationship existed between sugar composition of LPS and antigenic reactions.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains of *E. chrysanthemi* used in this study (Table 1) were maintained as suspensions in 5 ml of sterile distilled water in capped test tubes at

TABLE 1. Strains of *Erwinia chrysanthemi* included in this study

Strain	Original number[a]	Host	Location	Source
SR30	EC 16	*Chrysanthemum morifolium* Ramat.	New York	Burkholder
SR31	EC 17	*C. morifolium*	New York	Burkholder
SR58	C 8	*Zea mays* L.	North Carolina	Kelman
SR61	Wl-1	*Z. mays*	Wisconsin	Kelman
SR75		*Z. mays*	Wisconsin	Kelman
SR80	W3-20	*Z. mays*	Wisconsin	Kelman
SR90	I-7	*Z. mays*	India	Payak
SR120A	071-1230	*Z. mays*	Hawaii	Hayward
SR172	172	*Z. mays*	Colombia	Victoria
SR228	2	*Euphorbia pulcherrima* Willd.	Ohio	Hoitink
SR235	378	*Dieffenbachia amoena* Hort.	Honduras	Dickey
SR238	NCPPB 1849	*Parthenium argentatum* A. Gray	U.S.A.	Dickey
SR239	145	*Chrysanthemum maximum* Hort.	Italy	Mazzucchi
SR242	F-1	*Saintpaulia ionantha* H. Wendl.	France	Lamattre
SR245	A-15	*Ipomoea batatas (L.) Poir.*	U.S.A.	Schaad
SR261	CR 1	*Z. mays*	Costa Rica	Madrigal
SR297	SR 5	*Solanum tuberosum* L.	Peru	French

[a]NCPPB = National Collection of Plant Pathogenic Bacteria, Harpenden, England. Other numbers are those designated by the original source.

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ambient temperature (20–26 C). All strains were tested initially for pectolytic activity on a crystal violet pectate medium (4) and for phosphatase activity on 0.05% sodium phenolphthalein diphosphate agar (10). All strains gave positive reactions in both tests. Strains were grown on a Casamino acids-glucose-peptone agar medium (CPG) containing 0.005% triphenyltetrazolium chloride (TZC) for 48 hr at 28 C (12). When viewed under oblique lighting in this medium, colonies could be categorized as either fluidal (large and smooth) or butyrous (small and rough). For pathogenicity tests, cell suspensions in distilled water were streaked on TZC medium and, after 48 hr, single colonies were transferred to nutrient-dextrose agar (Difco) slants and incubated for 24 hr at 32 C. For extraction of LPS, each strain was grown in 1 L of CPG broth for 48 hr at 28 C on a rotary shaker. Bacterial cells were harvested by centrifuging at 16,300 *g* for 30 min. Cells were washed with deionized distilled water, recentrifuged, and extracted immediately or stored at −30 C.

Extraction and analysis of LPS. LPS was extracted from each of 16 strains of *E. chrysanthemi* by the standard hot phenol-water method (21) and lyophilized. The LPS was resuspended in deionized distilled water by heating to 50 C for 30 min and purified by repeated ultracentrifugation at 105,000 *g* for 4 hr until the supernatant reached $A_{280nm} = 0.01$ or less. Lyophilized samples (1–2 mg) of purified LPS were hydrolyzed with 2 N trifluoroacetic acid at 121 C for 1 hr and then alditol acetate derivatives were prepared by the method of Albersheim et al (1). Inositol was used as an internal standard. The sugar derivatives were extracted from the acetylation mixture with ethyl acetate, redissolved in acetone, and analyzed on a Varian 3740 gas chromatograph (Varian Instrument Group, Park Ridge, IL) fitted with a glass column (2 m × 6 mm) packed with 3% SP2340 on Supelcoport, 100–120 mesh. The content of 3-deoxy-D-*manno*-octulosonic acid (KDO) was determined colorimetrically by the procedure of Karkhanis et al (11).

Pathogenicity tests. The pathogenicity of all strains of *E. chrysanthemi* was tested on 21-day-old seedlings of corn (*Zea mays* hybrid 64A × W117) grown as described by Lacy et al (13) and stem-inoculated as described by Victoria (20). The inoculum consisted of bacteria suspended in sterile saline at $A_{600nm} = 0.4$. Corn plants were each inoculated by injecting 0.2 ml of bacterial suspension (approximately 2×10^7 cfu/plant) into the center of the stalk with a hypodermic syringe fitted with a 25-gauge needle. Then the wound was sealed with sterile petrolatum. Control plants were injected with sterile saline. After inoculation, plants were maintained in a growth room at 32 C and a 12-hr photoperiod for 6 days. A completely randomized block design with 18 treatments (16 bacterial isolates plus controls) replicated four times was used. Corn plants were scored for severity of disease on the following scale: 0 = no visible symptoms; 1 = slight discoloration and necrosis at the point of inoculation; 2 = slight internal rotting; 3 = marked internal rotting, including rotting of the growing point, but no external symptoms; 4 = extensive rotting with external discoloration of the stalk; 5 = collapse of the plant. Because no significant differences were found between blocks ($F = 0.39$), replicates were pooled and data were analyzed by a one-way analysis of variance.

Extraction of corn agglutinin. Agglutinin was extracted from the seed meal of corn hybrid 64A × W117 by ammonium sulfate precipitation, as described by Bradshaw-Rouse et al (2). Protein concentration was assayed by the method of Lowry et al (14).

Agglutination assay. Agglutination assays were completed following the method of Sequeira and Graham (16). Individual colonies from cultures of *E. chrysanthemi* grown on TZC agar medium for 48 hr were suspended in 0.01 M sodium acetate buffer, pH 4.0, at $A_{600nm} = 0.4$ (approximately 1×10^8 cells per milliliter). Corn seed agglutinin, dialyzed against 0.01 M sodium acetate buffer, pH 4.0, was adjusted to 300 μg of protein per milliliter. Equal volumes (25 μl) of bacterial suspension and corn agglutinin were mixed and placed as drops on polystyrene petri plates. Plates were incubated at 24–26 C for 2 hr on a reciprocal shaker and then the drops were examined under a dissecting microscope. Agglutination was rated according to the following index: 0 = no agglutination; 1 = slight clumping; 2 = aggregation of cells in strands that converge towards the center; 3 = strong agglutination in the center of the drop, but many bacteria remaining in suspension; 4 = complete agglutination. Indices were calculated from a minimum of three replicates for each treatment.

Serological tests. The extracts of LPS were tested by the Ouchterlony double diffusion method. The preparation of the medium, design of well patterns, amounts of antigen and antisera, incubation of plates, and days of observation were as previously described (8,9).

RESULTS

Monosaccharide composition of the LPS. The lipopolysaccharide of *E. chrysanthemi* is a heteropolysaccharide. The LPS of all strains contained glucose, heptose, glucosamine, KDO, and two unidentified compounds, possibly sugars (Fig. 1 [a and b]). The KDO content varied from 12 to 41 μg/mg of LPS. The molar ratios (standardized to heptose = 1) of heptose, glucosamine, and KDO were similar in all strains. The occurrence and quantities of rhamnose, fucose, mannose, and galactose were variable. The base ratios of these sugars plus glucose were used to differentiate six groups of strains by cluster analysis based on shape difference coefficient (18,24) (Table 2).

Group 1 strains (SR31, SR228, SR235, SR242, and SR297) were characterized by LPS with a relatively high fucose content and little or no rhamnose. The LPS of SR80, the only strain in Group 2, contained relatively large quantities of both rhamnose and mannose. The LPS of group 3 strains (SR61 and SR75) contained rhamnose and fucose, and group 4 strains (SR261, SR90, SR172, SR120A, SR238, and SR239) contained only relatively low amounts of rhamnose. Group 5 included a single strain (SR3O) with LPS that contained galactose. Group 6 included only one strain (SR58), which was similar to group 3 except the LPS contained large amounts of glucose.

The eight strains of *E. chrysanthemi* originally isolated from corn belonged to LPS groups 2, 3, 4, and 6; the eight strains isolated from other host plants belonged to LPS groups 1, 4, and 5. Thus, the LPS sugar composition of these strains is not correlated with the host from which the strain originally was isolated.

LPS and culture age. When the LPS of *E. chrysanthemi* strain SR261 was extracted from cultures grown for periods varying from 24–72 hr, no significant variation ($F = 5.08$ at $P = 0.05$) was

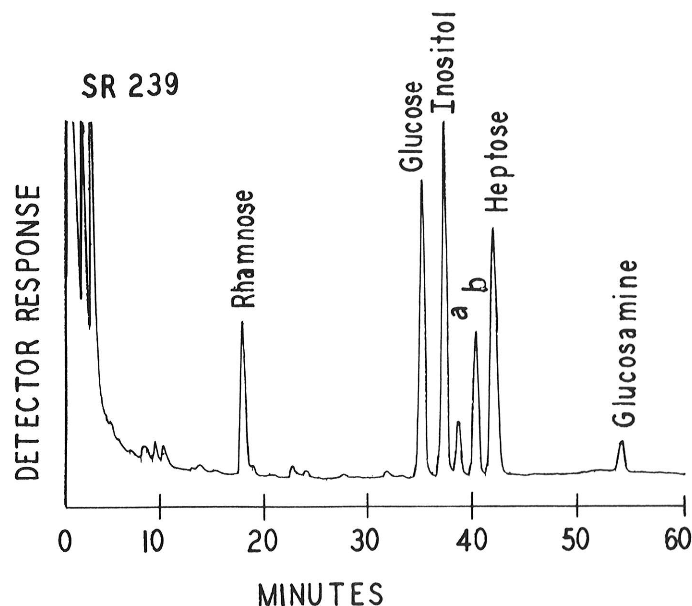

Fig. 1. Typical chromatogram from alditol acetate sugar analysis of lipopolysaccharide of *E. chrysanthemi* strain SR 239. The two unidentified sugars are labeled a and b. Column temp 145–250 C for 10 min. Flow rate: 30 ml/min, nitrogen.

TABLE 2. Monosaccharide composition of the lipopolysaccharide of different strains of *Erwinia chrysanthemi*

Strain	LPS[a] Group	Serovar[b] Group	Molar Ratio[c] RHA[d] (mean ± SD)	FUC (mean ± SD)	MAN (mean ± SD)	GAL (mean ± SD)	GLC (mean ± SD)
SR31	1	I	0.0	3.2 ± 0.8	0.0	0.0	0.8 ± 0.3
SR228	1	I	0.3 ± 0.3	2.9 ± 0.4	0.0	0.0	0.5 ± 0.01
SR235	1	I	0.0	3.1 ± 0.1	0.0	0.0	0.8 ± 0.02
SR242	1	I	0.0	3.4 ± 1.0	0.0	0.0	0.3 ± 0.01
SR297	1	I	0.0	3.4 ± 0.8	0.0	0.0	0.5 ± 0.01
SR80	2	U	4.5 ± 0.5	0.0	3.2 ± 0.2	0.0	1.0 ± 0.9
SR61	3	II	2.8 ± 0.3	2.1 ± 0.8	0.0	0.0	0.5 ± 0.01
SR75	3	II	3.0 ± 0.7	2.2 ± 0.6	0.0	0.0	0.3 ± 0.01
SR261	4	U	0.5 ± 0.3	0.0	0.0	0.0	1.8 ± 0.8
SR90	4	U	0.3 ± 0.04	0.0	0.0	0.0	0.4 ± 0.2
SR172	4	U	0.4 ± 0.03	0.0	0.0	0.0	0.5 ± 0.2
SR120A	4	U	0.6 ± 0.01	0.0	0.0	0.0	0.7 ± 0.4
SR238	4	U	0.5 ± 0.01	0.0	0.0	0.0	0.4 ± 0.1
SR239	4	U	0.4 ± 0.1	0.0	0.0	0.0	0.5 ± 0.1
SR30	5	U	0.5 ± 0.2	0.0	0.0	0.8 ± 0.1	0.9 ± 0.2
SR58	6	II	3.1 ± 0.4	2.8 ± 0.2	0.0	0.0	6.3 ± 0.3

[a] LPS groups derived from cluster analysis using shape difference coefficient = 0.01 (18).
[b] LPS used as antigen to determine serovar (9). U = untyped, no reaction with any of the antisera.
[c] $\text{Molar ratio} = \frac{\mu\text{g monosaccharide/mg LPS}}{\text{MW of monosaccharide}} \times \frac{\text{MW heptose}}{\mu\text{g heptose/mg LPS}}$
[d] Abbreviations: RHA = rhamnose, FUC = fucose, MAN = mannose, GAL = galactose, and GLC = glucose.

TABLE 3. Monosaccharide composition of lipopolysaccharide extracted from *Erwinia chrysanthemi* strain SR261 at different incubation times

Incubation time (hours)	Molar ratio[y] RHA[z] (mean ± SD)	GLC (mean ± SD)	HEP	GLC-A (mean ± SD)
24	0.36 ± 0.02	17.7 ± 0.43 a[y]	1.0	0.37 ± 0.16
32	0.31 ± 0.02	9.31 ± 0.30 b	1.0	0.27 ± 0.05
40	0.30 ± 0.02	2.75 ± 0.16 c	1.0	0.30 ± 0.12
48	0.38 ± 0.04	2.40 ± 0.19 cd	1.0	0.31 ± 0.11
56	0.34 ± 0.05	2.08 ± 0.14 d	1.0	0.39 ± 0.26
64	0.34 ± 0.02	1.10 ± 0.20 e	1.0	0.26 ± 0.05
72	0.38 ± 0.01	1.17 ± 0.01 e	1.0	0.35 ± 0.04

[y] Glucose molar ratios followed by the same letter are not significantly different at the 95% confidence level (pooled standard deviation = 0.23).
[z] Abbreviations: RHA = rhamnose, GLC = glucose, HEP = heptose, GLC-A = glucosamine, SD = standard deviation.

observed in the molar ratios of rhamnose and glucosamine in the LPS (Table 3). The glucose content, however, decreased significantly as incubation time increased (F = 3,029.65 at P = 0.05).

Relationship between agglutination and virulence. As a group, the ability of strains of *E. chrysanthemi* to cause disease in corn (hybrid 64A × W117) was poorly correlated (r^2 = 0.075) with agglutination by the corn seed agglutinin (Table 4). However, the mean agglutination index for strains of *E. chrysanthemi* originally isolated from corn (1.1) was lower than that for strains from other host plants (3.2). Furthermore, disease index was negatively correlated ($r^2 = -0.23$) with agglutination index for corn isolates, thus, the higher the agglutination index the lower the disease index. Disease index was correlated positively (r^2 = 0.68) with agglutination index for strains of *E. chrysanthemi* isolated from host plants other than corn.

Serological relationships. There was a correlation between LPS sugar composition and reaction to 11 *E. chrysanthemi* antisera. The LPS of all five strains of group 1 that contained fucose and glucose (Table 2) produced precipitin bands with serovar I antisera. Cross reactions with antisera of the other serovars did not occur. The two strains of group 3 (SR61, SR75) and the single strain of group 6 (SR58), which produced LPS that contained rhamnose, fucose, and glucose, only reacted with the antisera of serovar II. The LPS of the remaining eight strains in groups 2, 4, and 5, which primarily contained rhamnose and glucose, did not react with any of the 11 antisera.

TABLE 4. Agglutination index and virulence of 16 strains of *Erwinia chrysanthemi* on *Zea mays* (64A × W117)

Strain no.	Colony type[x]	Agglutination (mean ± SD)[y]	Disease index (mean ± SD)[z]
Corn isolates			
SR61	B	0.0	5.0 ± 0.0 c
SR75	B	1.1 ± 0.3	3.0 ± 1.4 b
SR80	B	0.0	1.7 ± 1.5 ab
SR90	B	0.0	4.6 ± 1.4 c
SR120A	B	2.9 ± 0.4	4.8 ± 0.6 c
SR120A	F	0.0	5.0 ± 0.0 c
SR172	B	1.0 ± 0.0	2.4 ± 0.7 b
SR261	B	4.0 ± 0.0	2.4 ± 1.8 b
Non-corn isolates			
SR30	B	1.0 ± 0.0	0.6 ± 0.5 a
SR31	B	1.0 ± 0.0	1.0 ± 0.5 a
SR228	B	2.3 ± 0.5	3.1 ± 1.4 b
SR235	F	0.3 ± 0.3	2.5 ± 0.9 b
SR238	B	2.7 ± 0.5	4.5 ± 1.2 c
SR239	B	2.0 ± 0.0	3.2 ± 1.3 b
SR242	B	3.5 ± 0.5	3.0 ± 1.8 b
SR245	B	4.0 ± 0.0	4.6 ± 0.9 c
SR297	B	3.0 ± 0.0	2.2 ± 0.9 b

[x] B = butyrous, F = fluidal.
[y] Agglutination index: 0 = no agglutination, and 4 = complete agglutination. Average of at least three replicates.
[z] Disease index: 0 = no disease, and 5 = complete collapse of plant. Significant differences between treatments: F = 25.81 (95% confidence level). Means followed by the same letter are not significantly different (pooled standard deviation = 1.05). Mean of three replicates with four observations per replicate.

DISCUSSION

Our results indicate that the lipopolysaccharide of different strains of *E. chrysanthemi* is a variable heteropolysaccharide. Heterogeneity in the LPS sugar composition is common in other Enterobacteriaceae, including *Escherichia coli*, *Salmonella typhimurium* (19), and *Erwinia carotovora* subsp. *carotovora* (5). The sugar composition of LPS of strains of *E. chrysanthemi* and *E. carotovora* subsp. *carotovora* appear to be similar. In both of these bacteria, the presence and quantity of rhamnose, fucose, mannose, and galactose in their LPS was variable (5). A correlation between LPS sugar composition and original host of the strains has not been demonstrated for either bacterium.

It has been suggested that the sugar components of the LPS of *Erwinia carotovora* subsp. *carotovora* possibly are antigenic determinants of serological specificity (5). Our serological results support the same suggestion for strains of *E. chrysanthemi*. Based on the LPS sugar analysis of *E. chrysanthemi* strains (Table 2), it is probable that additional serovars would be designated if the appropriate strains were used for production of antisera. As previously reported (8,9,25), a definite correlation between serological reactions and original host of strains of *E. chrysanthemi* was not demonstrated.

Sugar composition of LPS may also be of taxonomic value for characterization of specific strains, but would be most useful when linked to cluster analysis, which is based primarily on presence or absence of specific sugar components. Although culture age does not appear to affect the qualitative sugar composition of LPS, caution must be exercised because glucose content varied with growth period of the culture, as shown for strain R261.

In contrast to other host-pathogen systems (16,22), there appears to be no correlation between virulence and LPS structure in the corn-*E. chrysanthemi* system. Similarly, pathogenicity of strains of *E. chrysanthemi* to corn did not appear to bear any relationship to agglutination with the corn seed agglutinin. Although the average agglutination index of corn strains was lower than that for non-corn strains, in some instances individual corn strains had agglutination values that were as high as those from some non-corn strains. In addition, for the subset of non-corn strains, agglutination was positively correlated ($r^2 = 0.68$) with pathogenicity on corn, which is contrary to expectation; i.e., strong agglutination of weak or nonpathogenic strains. With *E. stewartii*, on the other hand, there is a clear relationship between extracellular polysaccharide (EPS) production, pathogenicity, and agglutination (2). EPS is the major component of the bacterial slime that gives a fluid appearance to colonies grown on solid media. Based on the simple comparison of colony type (presence or absence of fluidity) with agglutination and pathogenicity (Table 4), however, it does not seem likely that EPS is a critical factor determining host specificity of *E. chrysanthemi*.

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