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Isolation and Characterization of Extracellular Polysaccharide of *Clavibacter michiganensis* subsp. *sepedonicus*

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ABSTRACT

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The extracellular polysaccharide (EPS) of *Clavibacter michiganensis* subsp. *sepedonicus*, the causal organism of bacterial ring rot of potato, consists of four components (I–IV) that were separated on the basis of size and charge. These components were homogenous according to ion-exchange and/or gel permeation chromatography, to analysis of neutral sugar composition, and by reaction with polyclonal antisera. All components contained fucose, mannose, galactose, and glucose, although the ratios of these monosaccharides varied with strain and the culture medium in which the strains were grown. Components I and II appeared to be aggregates of component III, whereas component IV was of distinct composition. Protein associated with components I and III was not covalently bound and could be separated from the polysaccharides by DEAE-cellulose chromatography with an NaCl gradient. Spent culture fluid containing these EPS components induced wilting in potato cuttings. Nonfluidal colony morphology was correlated with lack of production of EPS components I and III for two strains but was not correlated with avirulence or attenuation of bacterial ring rot symptoms in eggplant or potato.

Clavibacter michiganensis subsp. *sepedonicus* is the causal agent of bacterial ring rot of potato, a disease that is of paramount importance to the North American seed potato industry (5). Bacterial ring rot is manifested in the foliar portions of infected plants by interveinal chlorosis and upward turning and necrosis of the leaf margins, followed by the eventual wilt and collapse of affected leaves and stems. Destruction of the vascular ring of afflicted tubers leads to the development of the characteristic "ring rot" symptom. Factors such as *C. m. sepedonicus* strain and host cultivar affect disease development (2).

Despite the importance of bacterial ring rot, very little is known about the mechanism by which the causal agent induces disease. Wilt has been reported to be induced by extracellular polysaccharide (EPS) produced in vitro by several phytopathogenic bacteria, including the related bacterium, *C. m. insidiosus* (9,16,21,22). *C. m. sepedonicus* produces copious amounts of EPS in culture (9,16), and isolation of a "toxic glycopeptide" from spent culture fluid and infected potato has been reported (17–19). However, except for indirect evidence that *C. m. sepedonicus* produces a multicomponent EPS (1), no systematic attempt has been made to isolate and characterize *C. m. sepedonicus* EPS. Multicomponent EPS has previously been demonstrated for *C. m. insidiosus* (21) and *C. m. michiganensis* (23). The individual EPS components produced by *C. m. insidiosus* have each been shown to be capable of wilting alfalfa cuttings (22). Bishop et al (1) have shown that loss of a 20-kDa polysaccharide reduced virulence in one *C. m. sepedonicus* strain.

Because of the accumulating evidence suggesting that EPS produced by *Clavibacter* spp. is involved in pathogenesis, characterization of *C. m. sepedonicus* is warranted. Such characterization is an essential step in determining the role of EPS in bacterial ring rot pathogenesis. The purpose of this study was to isolate and describe the EPS components produced by fluidal and nonfluidal strains of *C. m. sepedonicus*. The effect of differences in EPS production among strains on the infectivity and virulence of *C. m. sepedonicus* was also considered.

MATERIALS AND METHODS

Bacterial strains. *C. m. sepedonicus* strains SS43 (2), SS20 (13), SSNF50 (1), and SSNF68 (courtesy of S. De Boer) were isolated

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from infected potato plants or tubers. These strains are typical of *C. m. sepedonicus* in culture, gram reaction, and eggplant pathogenicity tests. Strains SSNF50 and SSNF68 are naturally occurring, nonfluidal variants of *C. m. sepedonicus*. Results reported herein apply to strain SS43 unless otherwise stated.

Bacterial culture. *C. m. sepedonicus* was cultured on nutrient broth-yeast extract agar (NBY) (24) at ambient temperature (about 23 C). Strains were renewed from lyophilized cultures after a maximum of five NBY transfers. Cultures were lyophilized in 7% (w/v) sucrose and 7% (w/v) peptone solution for long term storage after passage through eggplant (*Solanum melongena* L. 'Black Beauty') to ensure pathogenicity. We prepared cultures for EPS isolation by adding 5 ml of a 1.0×10^8 cfu/ml (OD_{600} = 0.1) suspension to 500 ml of a liquid growth medium in a 1-L Erlenmeyer flask on a rotary shaker. The suspension was grown for 7 days at 24 C and 150 rpm. Culture media used for EPS isolation included the following: a defined medium described by LaChance (12), amended with 0.2 g/ml each of alanine, asparagine, arginine, histidine, leucine, methionine, and proline (LM medium); LM with sucrose used in place of glucose as the main carbon source (LSM); and yeast extract-dextrose-calcium carbonate broth (YDC) (16).

Wilt assay. LM cultures of *C. m. sepedonicus* were prepared and cultured as described above. After 7 days of growth, the spent culture fluid was collected by centrifugation at 16,000 *g* for 30 min and passed through a 0.2-μ filter. An aliquot of the filtered culture fluid was autoclaved at 121 C for 20 min. Cuttings (10–15 cm) of greenhouse-grown potato (cv. Russet Burbank) were placed in Erlenmeyer flasks containing 75 ml of either LM medium (control), culture fluid, or autoclaved culture fluid. The flasks were then sealed with parafilm and held at ambient temperature under natural light for 5 days.

EPS isolation. The EPS isolation procedure is outlined in Figure 1. Bacterial cells were removed from the culture fluid by centrifugation at 16,000 *g* for 30 min. The supernatant was then reduced to 10% of its original volume by rotary evaporation at 40 C. EPS was precipitated by the addition of 3 vol of cold (−20 C) acetone and overnight incubation at −20 C. Precipitated EPS was pelleted by centrifugation at 16,000 *g* for 30 min and then resuspended in 20 ml of 10 mM sodium acetate, pH 6.0 (acetate buffer). After removal of undissolved materials by centrifugation, the EPS solution was dialyzed at 4 C against acetate buffer and then passed over a 0.5- × 20-cm carboxyl-methyl (CM) cellulose column previously equilibrated with the same buffer. Effluent from the CM-cellulose column was collected and passed over a 2.5- × 13-cm column of diethylamino ethyl (DEAE) cellulose previously equilibrated with acetate buffer. Materials binding to CM- and DEAE-cellulose were released by washing the columns with 2 vol of 1 M sodium acetate, pH 6.0. The CM- and DEAE-bound fractions, as well as the DEAE effluent were then reduced in volume by rotary evaporation at 40 C, dialyzed extensively against deionized water, and stored in lyophilized form at −20 C for later analysis. Approximately 10 mg (dry weight) of the DEAE-bound fraction was dissolved in 5 ml of acetate buffer plus 0.02% sodium azide, pH 6.0, (acetate-azide buffer) and applied to a 2.5- × 37-cm column of Sepharose CL-2B (Pharmacia Inc., Piscataway, NJ) previously equilibrated with the same buffer. The CM-bound and DEAE effluent fractions were each dissolved in 10 ml of acetate-azide buffer and applied to a 2.5- × 46-cm column of Sephadex G-100 (Pharmacia Inc.), also equilibrated with acetate-azide buffer. Gel permeation column effluent was monitored by the phenol-sulfuric acid assay (7) for hexose and absorbance at 280 nm for protein. The proteinaceous nature of the materials absorbing at 280 nm was confirmed by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). Before further analysis, EPS components I and II and components III and IV were individually reapplied to and eluted from Sepharose CL-2B and Sephadex G-100 columns, respectively. Size of the individual EPS components was determined by calculation of K_{av} values and comparison with selectivity curves of neutral dextran standards.

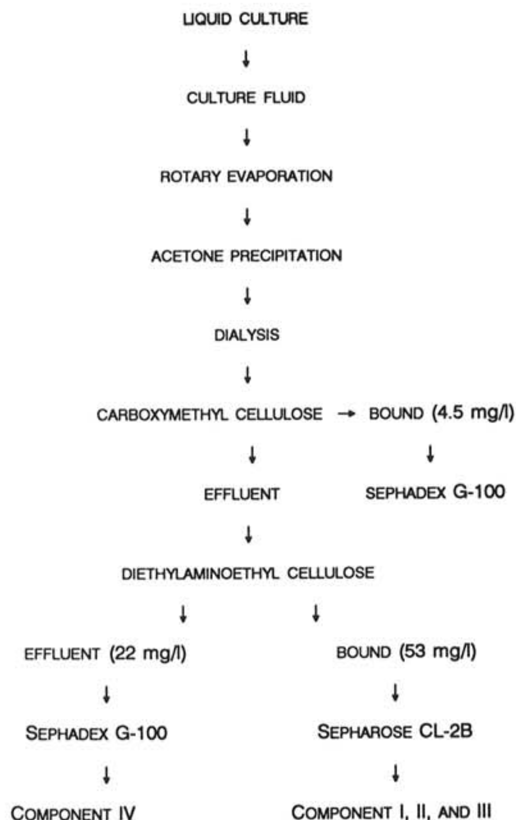

Fig. 1. Flowsheet of extracellular polysaccharide (EPS) purification procedure. Total yield of *Clavibacter michiganensis* subsp. *sepedonicus* strain SS43 extracellular product at various steps is given in milligrams per liter of culture fluid (dry weight).

Compositional analysis. Neutral sugars and uronic acids were analyzed by gas-liquid chromatography of alditol-acetate derivatives according to the method of Jones and Albersheim (11), modified by the omission of the enzymic treatment of the EPS and separation of the uronic acids by passage over Dowex-1 (acetate form) columns constructed with pasteur pipettes, rather than by the batch method described by the authors. The results of uronic acid analysis were confirmed by a colorimetric assay (4).

Examination of subunit structures of the individual EPS components was attempted by dissolution of the components in acetate buffer containing 0.1% sodium dodecyl sulfate (SDS). Dissociation was assayed by passage of the samples over a 2.5- × 37-cm column of Sepharose CL-2B previously equilibrated with the same buffer.

Determination of homogeneity. EPS components I and II were individually reapplied to a 2.5- × 13-cm column of DEAE-cellulose previously equilibrated with acetate buffer and eluted with a linear gradient of 10–500 mM sodium acetate, pH 6.0. The resulting peaks were divided into lead, middle, and tail portions, and the neutral sugar composition was determined as described above. Component IV was passed over a 2.5- × 46-cm column of Sephadex G-100 and analyzed as described for components I and II.

Ouchterlony double diffusion tests were performed with 0.7% agarose in 0.05 M Tris-HCl, pH 7.4, plus 0.85% NaCl (TBS). EPS samples were prepared as 1-mg/ml (w/v) solutions in TBS. A heavy suspension of *C. m. sepedonicus* cells in TBS was vortexed for 2 min in 1 ml of glass beads (0.17–0.18 mm diameter) to disrupt the cells (14) and used as a control. Samples were tested with goat polyclonal antisera prepared against whole, glutaraldehyde-fixed *C. m. sepedonicus* cells according to the procedure of Slack et al (13).

Infectivity titrations. Infectivity titrations were performed in eggplant (cv. Black Beauty) as described by Bishop et al (1) and in potato plantlets (cv. Russet Burbank) grown from nodal cuttings of virus-free in vitro plantlets tested free from known

potato pathogens (15). Inoculum was grown to midlog phase (about 70 h) in 50 ml of filter-sterilized LM. Eggplants and potato plantlets were stem-inoculated, via a 27.5-gauge needle, with 10 μl of a bacterial suspension containing 0, 10^2, 10^5, or 10^8 cfu of *C. m. sepedonicus*. The number of days from inoculation to symptom expression and the proportion of symptomatic plants was recorded. Potato plantlets were assayed for infection by the latex agglutination procedure (14).

Statistical analysis. Analysis of variance of compositional data and analysis of covariance of infectivity titration data were performed with PROC GLM in PC-SAS (SAS Institute, Cary, NC).

RESULTS

Wilting assay. Cuttings placed in autoclaved and nonautoclaved culture filtrate displayed wilting and chlorosis symptoms approximately 72 h after treatment (Fig. 2). These symptoms were characteristic of those expressed by naturally infected plants. Cuttings placed in LM medium remained healthy for the duration of the experiment.

EPS isolation. Four EPS components were identified in the ion-exchange fractions of the fluidal strains. The bound CM-cellulose fraction contained approximately 4.5 mg/L (dry weight) of carbohydrate that eluted in the included volume of Sephadex G-100. Because of its small size, this material was not further characterized. Three EPS components, designated I, II, and III, bound to DEAE-cellulose and were separated by use of the gel-filtration medium, Sepharose CL-2B (Fig. 3A). Component I eluted in the void volume of the Sepharose CL-2B column and consequently is of high molecular weight (>20 MDa). Component II, by comparison with neutral dextrans, was approximately 4.5 MDa ($K_{av} = 0.308$). The third component, component III, eluted below the fractionation range of the Sepharose column. Comparison of component III with neutral dextrans on Sephadex G-100 showed it to be approximately 2.1 kDa ($K_{av} = 0.252$). The neutral DEAE-cellulose fraction contained small amounts of components I and II as determined by passage over Sepharose CL-2B and compositional analysis, as well as an additional polysaccharide, designated component IV (Fig. 3B). Component IV ($K_{av} = 0.257$) has a molecular weight of approximately 2.0 kDa. All four components were produced by fluidal strains of *C. m. sepedonicus* regardless of culture medium. There was no significant difference in the amounts of EPS components recovered from the fluidal strains that we examined (by analysis of variance [ANOVA]: $R^2 = 0.042$, $P = 0.741$; $R^2 = 0.217$, $P = 0.43$; $R^2 = 0.131$, $P = 0.079$; and $R^2 = 0.11$, $P = 0.133$ for components I, II, III, and IV, respectively) (Table 1). The nonfluidal strains, SSNF50 and SSNF68, did not produce detectable amounts of components I and II but were not significantly different from fluidal strains in production of components III and IV ($R^2 = 0.324$, $P = 0.546$; and $R^2 = 0.319$, $P = 0.554$, respectively).

Fig. 2. Wilt induced in potato cuttings by *Clavibacter michiganensis* subsp. *sepedonicus* culture filtrates. Left to right: control (LM medium, a defined medium [12] amended with 0.2 g/ml each of alanine, asparagine, arginine, histidine, leucine, methionine, and proline), LM culture filtrate, autoclaved LM culture filtrate.

EPS composition. All EPS components contained fucose, mannose, galactose, and glucose. Components I, II, and III have similar neutral sugar composition, approximately 1:7:2:2 (fucose/mannose/galactose/glucose). Component IV was essentially mannan in nature. ANOVA showed that neutral sugar composition varied significantly between strains (Table 1) and growth media (Table 2) (in both cases, $R^2 > 0.8$, $P = 0.0001$ for each component). There was no evidence for a uronic acid constituent of any of the EPS components. An undetermined amount of protein was eluted from Sepharose CL-2B in the same fractions as components I and III (Fig. 3A). These proteins appear to be contaminants because they did not precisely co-elute with the EPS components, and similar protein peaks were observed in the acidic ion-exchange fractions of isolates SSNF50 and SSNF68, which do not produce component I. Further, treatment of components I and II with SDS caused dissociation of these components into a subunit of size and composition similar to that of component III and also caused the protein peak associated with component I to elute in the totally included volume of Sepharose CL-2B. The protein associated with component III and with the subunit of components I and II had a lower elution volume on Sephadex G-100 than did the polysaccharide, although we could not separate these substances completely. Finally, no protein peak was observed when component I was eluted from DEAE-cellulose with a 0–500 mM NaCl gradient (Fig. 4A).

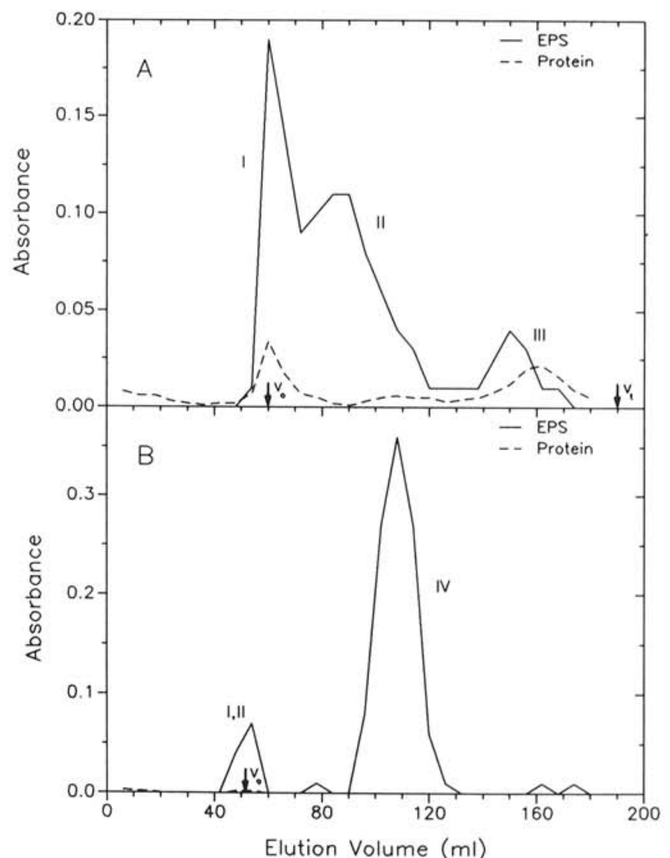

Fig. 3. Elution of ion exchange fractions of extracellular polysaccharide (EPS) of *Clavibacter michiganensis* subsp. *sepedonicus* strain SS43 on gel permeation media equilibrated with acetate buffer. The column effluent was monitored by phenol-sulfuric acid assay (490 nm) for hexose (solid line) and by absorbance at 280 nm for protein (broken line). **A,** DEAE-bound fraction on Sepharose CL-2B. **B,** DEAE effluent fraction on Sephadex G-100. V_t for the Sephadex G-100 column was 250 ml. Unlabeled polysaccharide peaks were not repeatable and are thought to be artifacts of the assay method.

Homogeneity. Single, symmetrical peaks were observed for both components I (Fig. 4A) and II (Fig. 4B) after elution from DEAE-cellulose with a linear acetate gradient, and for the elution of component IV from Sephadex G-100 (Fig. 4C). Elution of component I from the DEAE column required a higher salt concentration than did elution of component II. Results of gas-liquid chromatography analysis of the lead, middle, and tail portions of components I, II, and IV peaks are shown in Table 3. The sugar composition of components II and IV was independent of peak position (by ANOVA: $P = 0.9506$ and 0.975, respectively), but not for component I ($P = 0.006$), which contained more fucose in the middle portion of the peak. Ouchterlony double diffusion tests on individual EPS components with goat polyclonal antisera specific for whole *C. m. sepedonicus* cells produced single precipitin bands for each component (I, II, and III; Fig. 5). Component IV did not yield a detectable reaction in this test. Multiple precipitin bands were not observed for individual components, and gel migration rates differed according to component size. Smaller components migrated more rapidly than the larger components. This test also showed that components I, II, and III share common antigenic sites.

Infectivity titrations. All strains were pathogenic on eggplant and potato plantlets, and symptoms typical of bacterial ring rot were produced on each host species. Colony morphology of bacterial strains upon reisolation from test plants was typical of the original strains. A significant dose effect was observed on the number of plants displaying symptoms in both eggplant and potato plantlets and the number of potato plantlets becoming infected ($P = 0.0014$, 0.0001, and 0.0035, respectively), but these relationships were not significantly affected by either strain ($P = 0.6284$, 0.2455, 0.1932) or dose × strain interaction ($P = 0.5865$, 0.5175, 0.6303) (Fig. 6). No statistically significant differences in the latent periods of infection were observed between any of the strains in either eggplant ($P = 0.288$) or potato ($P = 0.851$) at inoculum levels of 10^5 cfu/ml or higher (Table 4). Latent period of infection of treatments given lower initial inoculum levels were not amenable to statistical analysis because of inconsistent symptom expression by both host species.

DISCUSSION

Filtrates of spent *C. m. sepedonicus* culture fluid are able to induce symptoms typical of bacterial ring rot, including chlorosis and wilting in potato cuttings. The biological activity of these filtrates is not destroyed by autoclaving, which indicates that the causal factor is thermostable. EPS produced by *C. m. sepedonicus* has been implicated as a phytotoxic compound that meets this criterion (9,16–19). This study indicates that high molecular weight polysaccharides make up the majority of the macromolecules present in *C. m. sepedonicus* culture fluid and that this EPS consists of four components. Components I, II, and III are acidic EPS that have similar composition (approximately 1:7:2:2;

TABLE 1. Amounts recovered and composition of extracellular polysaccharides isolated from several strains of *Clavibacter michiganensis* subsp. *sepedonicus* grown in LM medium

Component	Strain	Amount[a]	Percentage of neutral sugar[b]			
			Fucose	Mannose	Galactose	Glucose
I	SS43	3.5 ± 2.0	9.7 ± 2.3	61.7 ± 5.2	15.5 ± 1.9	13.1 ± 1.6
	SS20	3.0 ± 0.6	4.9 ± 3.9	5.5 ± 5.5	68.8 ± 16.2	21.5 ± 5.8
	SSNF50	0	...	...	...	...
	SSNF68	0	...	...	...	...
II	SS43	5.3 ± 3.2	9.9 ± 1.5	57.8 ± 2.4	14.5 ± 1.6	19.1 ± 2.8
	SS20	3.1 ± 0.6	9.3 ± 4.8	2.8 ± 1.8	55.5 ± 3.3	32.9 ± 0.7
	SSNF50	0	...	...	...	...
	SSNF68	0	...	...	...	...
III	SS43	1.6 ± 1.2	6.5 ± 1.9	64.8 ± 1.3	13.9 ± 1.2	15.0 ± 1.9
	SS20	1.2 ± 0.2	17.7 ± 7.1	6.8 ± 6.8	59.8 ± 16.3	16.4 ± 15.3
	SSNF50	0.8 ± 1.0	3.8 ± 2.8	66.1 ± 7.9	8.1 ± 7.1	23.0 ± 3.0
	SSNF68	0.3 ± 0.2	7.8 ± 6.8	25.6 ± 3.1	30.6 ± 0.4	36.5 ± 3.7
IV	SS43	2.0 ± 2.3	3.8 ± 1.0	84.4 ± 4.9	6.2 ± 2.5	6.2 ± 1.5
	SS20	1.0 ± 0.1	7.1 ± 6.1	29.2 ± 5.9	36.4 ± 2.3	29.7 ± 1.5
	SSNF50	0.8 ± 0.1	42.8 ± 27.2	20.8 ± 19.8	28.1 ± 1.9	9.3 ± 8.3
	SSNF68	0.2 ± 0.1	6.3 ± 15.3	1.0 ± 0.0	62.1 ± 6.2	22.5 ± 21.5

[a] Milligrams per liter of glucose equivalents. Average of two replicates plus or minus standard deviation is given.
[b] Sugar composition determined by analysis of alditol-acetate derivatives and expressed as a percentage of total sugars. Average of two replicates plus or minus standard deviation is given.

TABLE 2. Composition of *Clavibacter michiganensis* subsp. *sependonicus* strain SS43 extracellular polysaccharide after growth on different media

Component	Medium[b]	Percentage of neutral sugar[a]			
		Fucose	Mannose	Galactose	Glucose
I	LM	9.7 ± 2.3	61.7 ± 5.2	15.5 ± 1.9	13.1 ± 1.6
	LSM	16.9 ± 1.8	13.4 ± 1.0	44.3 ± 2.0	25.4 ± 0.8
	YDC	15.2 ± 2.0	21.8 ± 0.7	44.7 ± 1.4	18.3 ± 1.0
II	LM	9.9 ± 1.5	57.8 ± 2.4	14.5 ± 1.6	19.1 ± 2.8
	LSM	18.8 ± 0.9	11.4 ± 0.5	43.1 ± 2.0	26.6 ± 0.3
	YDC	19.0 ± 1.0	15.5 ± 6.0	39.9 ± 1.3	25.8 ± 8.2
III	LM	6.5 ± 1.9	64.8 ± 1.3	13.9 ± 1.2	15.0 ± 1.9
	LSM	13.4 ± 4.0	23.5 ± 7.9	31.7 ± 9.1	31.4 ± 5.2
	YDC	17.5 ± 2.8	19.2 ± 0.9	41.3 ± 3.0	20.3 ± 0.7
IV	LM	3.8 ± 1.0	84.4 ± 4.9	6.2 ± 2.5	6.2 ± 1.5
	LSM	Trace	36.4 ± 3.1	17.9 ± 16.9	46.2 ± 14.3
	YDC	10.8 ± 9.8	11.9 ± 10.0	14.6 ± 13.6	65.3 ± 14.8

[a] Sugar composition determined by analysis of alditol-acetate derivatives and expressed as a percentage of total sugars. Average of two replicates plus or minus standard deviation is given.
[b] LM, a defined medium (12) amended with 0.2 g/ml each of alanine, asparagine, arginine, histidine, leucine, methionine, and proline; LSM, LM with sucrose as the main carbon source; YDC, yeast extract-dextrose-calcium carbonate broth.

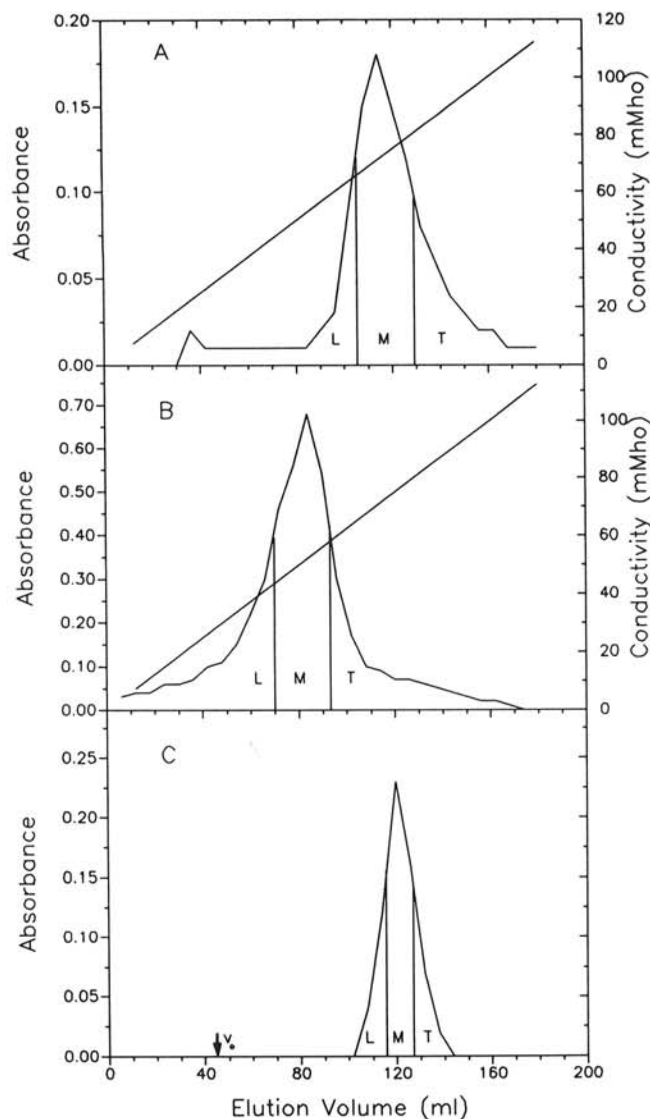

Fig. 4. Homogeneity determinations for components I, II, and IV. Components **A,** I and **B,** II were bound to DEAE-cellulose equilibrated with 10 mM acetate buffer and then eluted with a linear 10–500 mM acetate gradient. Conductivity data are indicative for this gradient rather than absolute. V_t for the Sephadex G-100 column was 250 ml. **C,** Component IV was applied to Sephadex G-100 and eluted with acetate-azide buffer. Column effluent was monitored with the phenol-sulfuric acid method. The lead, middle, and tail portions of the polysaccharide peaks are indicated by the letters L, M, and T, respectively.

fucose/mannose/galactose/glucose) and react similarly with polyclonal antisera made against whole *C. m. sepedonicus* cells. Components I and II are large (≥5 MDa) and appear to be aggregates of component III. Components I, II, and III are compositionally and serologically distinct from a fourth polysaccharide, component IV, which is composed primarily of mannose and did not react in Ouchterlony double diffusion tests. Although component III is of a similar size and neutral sugar composition to the glycopeptide described by Strobel (17–19), it is not a glycopeptide. Small amounts of protein are associated with components I and III (Fig. 3), but this protein is not covalently bound to either of these components, because it could be removed by treatment of these components with SDS or by elution of these components from DEAE-cellulose with an NaCl gradient.

Multicomponent EPS have previously been described for *C. m. insidiosus* (21) and *C. m. michiganensis* (23). Van Alfen et al (21) have shown that *C. m. insidiosus* produces EPS consisting of three components: a 22-kDa polysaccharide, a 5-MDa polysaccharide, and a large polysaccharide that is an aggregate of the 5-MDa component. Van den Bulk et al (23) reported that *C. m. michiganensis* produces 6- and 7-MDa EPS components, but did not provide evidence for aggregates of these polysaccharides. A relationship between in vitro and in planta EPS production for *C. m. michiganensis* has been shown (23), and presumptive evidence has been provided for the production of EPS in the host by *C. m. insidiosus* (9,21) and for *C. m. sepedonicus* (16,19). Bishop et al (1) have demonstrated that EPS fractions of greater than 100 kDa and approximately 20 kDa correspond to antigens from potato infected with *C. m. sepedonicus* in Ouchterlony double diffusion serology.

The results of this study indicate that growth medium and strain influence the molar ratio of the neutral sugars that compose the individual EPS components but do not alter the EPS components produced by individual strains of *C. m. sepedonicus*. Bishop and Slack (3) have shown that infection with *C. m. sepedonicus* results in reduced transpiration in potato plants and that the reduction of transpiration and associated wilting appear to be the result of reduced xylem function. Variability in the composition of these components and reduced xylem function are consistent with a physical mode of action, such as the vascular occlusion model proposed by Van Alfen for *C. m. insidiosus* (6,20,22). Van Alfen has shown that molecules with a molecular weight greater than 20 kDa are capable of inducing wilt (6) and that such wilting may be the result of increased resistance to xylem flow because of the accumulation of these macromolecules in pit membranes (20). The individual EPS components produced by *C. m. insidiosus*, which are similar in size to those produced by *C. m. sepedonicus*, have been shown by Van Alfen (22) to be capable of vascular occlusion and inducing water stress in alfalfa cuttings. The high molecular weight polysaccharides were shown to accumulate in different areas of the stem, whereas the 22-kDa component accumulated in the leaf blades. These results suggest that the individual EPS components may cause physical blockage at

TABLE 3. Neutral sugar composition of extracellular polysaccharides of *Clavibacter michiganensis* subsp. *sepedonicus* strain SS43 eluted from ion-exchange and gel permeation columns

Component	Peak fraction	Percentage of neutral sugar[a]			
		Fucose	Mannose	Galactose	Glucose
I	Lead	8.2 ± 0.8	61.3 ± 1.2	16.1 ± 1.1	14.3 ± 0.8
	Middle	12.9 ± 0.2	55.5 ± 2.0	17.5 ± 1.8	14.1 ± 0.4
	Tail	8.5 ± 1.5	66.5 ± 12.2	13.5 ± 0.3	11.5 ± 0.4
II	Lead	10.0 ± 0.3	56.9 ± 9.3	16.6 ± 1.3	17.6 ± 5.6
	Middle	11.7 ± 10.7	55.3 ± 13.2	12.6 ± 11.6	23.0 ± 8.5
	Tail	8.0 ± 0.1	61.1 ± 4.4	14.1 ± 0.4	16.8 ± 4.1
IV	Lead	2.4 ± 1.4	91.1 ± 8.9	2.7 ± 2.7	4.8 ± 3.8
	Middle	4.5 ± 4.5	79.4 ± 20.5	7.9 ± 7.9	8.2 ± 8.2
	Tail	4.6 ± 3.6	82.8 ± 17.2	8.0 ± 8.0	5.6 ± 4.6

[a] Cultures grown on LM medium (a defined medium [12] amended with 0.2 g/ml each of alanine, asparagine, arginine, histidine, leucine, methionine, and proline). Sugar composition determined by analysis of alditol-acetate derivatives and expressed as a percentage of total sugars. Average of two replicates plus or minus standard deviation is given.

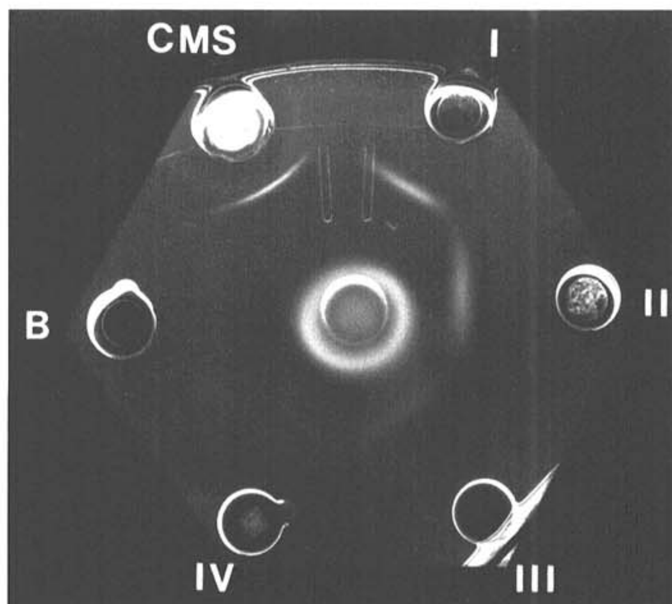

Fig. 5. Ouchterlony double diffusion test of purified extracellular polysaccharide (EPS) components with goat polyclonal antisera specific for whole *Clavibacter michiganensis* subsp. *sepedonicus* strain SS43 cells. Roman numerals indicate EPS component, CMS indicates a sample of whole *C. m. sepedonicus* cells grown on nutrient broth-yeast extract agar, and B indicates a buffer control.

TABLE 4. Latent period of infection of several *Clavibacter michiganensis* subsp. *sepedonicus* strains

Strain	Eggplant[a]	Potato[b]
SS43	17.0 ± 3.4	23.0 ± 1.6
SS20	17.2 ± 3.0	35.8 ± 7.8
SSNF50	14.8 ± 1.8	30.0 ± 9.2
SSNF68	15.8 ± 2.9	25.0 ± 8.8

[a] Mean number of days (average of five plants) from inoculation to symptom expression at 10^5 cfu/ml plus or minus standard deviation.

[b] Mean number of days (average of five plants) from inoculation to symptom expression at 10^8 cfu/ml plus or minus standard deviation.

different locations of the xylem and, thus, may play differential roles in the wilting process.

In previous studies of other systems (8,10), nonfluidal colony morphology has been correlated with reduced EPS production and avirulence. Our results demonstrate a relationship between nonfluidal colony morphology and lack of in vitro production of EPS components I and II for *C. m. sepedonicus* strains SSNF50 and SSNF68. However, loss of these components affects neither the ability of *C. m. sepedonicus* to infect a susceptible host nor does it affect the development of bacterial ring rot foliar symptoms as determined with fluidal and nonfluidal strains in eggplant and potato plantlet bioassays. This result was suggested by Strobel (17), who reported that *C. m. sepedonicus* EPS voiding Sephadex G-200 (>200 kda) did not possess physiological activity. Bishop et al (1), on the other hand, have reported the isolation of a spontaneous nonfluidal mutant of *C. m. sepedonicus* strain SS20 (SSNF201) that did not produce polysaccharide corresponding to low molecular weight EPS (component III and/or IV) in vitro and displayed attenuated virulence in potatoes grown from tuber seed pieces, but not in eggplant seedlings (1). Although these data may suggest that high molecular weight EPS components are not necessary for wilt development, they do not preclude that these components contribute to water stress. In dye transport studies of potato plants infected with *C. m. sepedonicus* strain SS43, Bishop et al (3) showed that a significant proportion of xylem vessels remain functional in plants exhibiting depressed transpiration and wilt. Thus, blockage in stems may require addi-

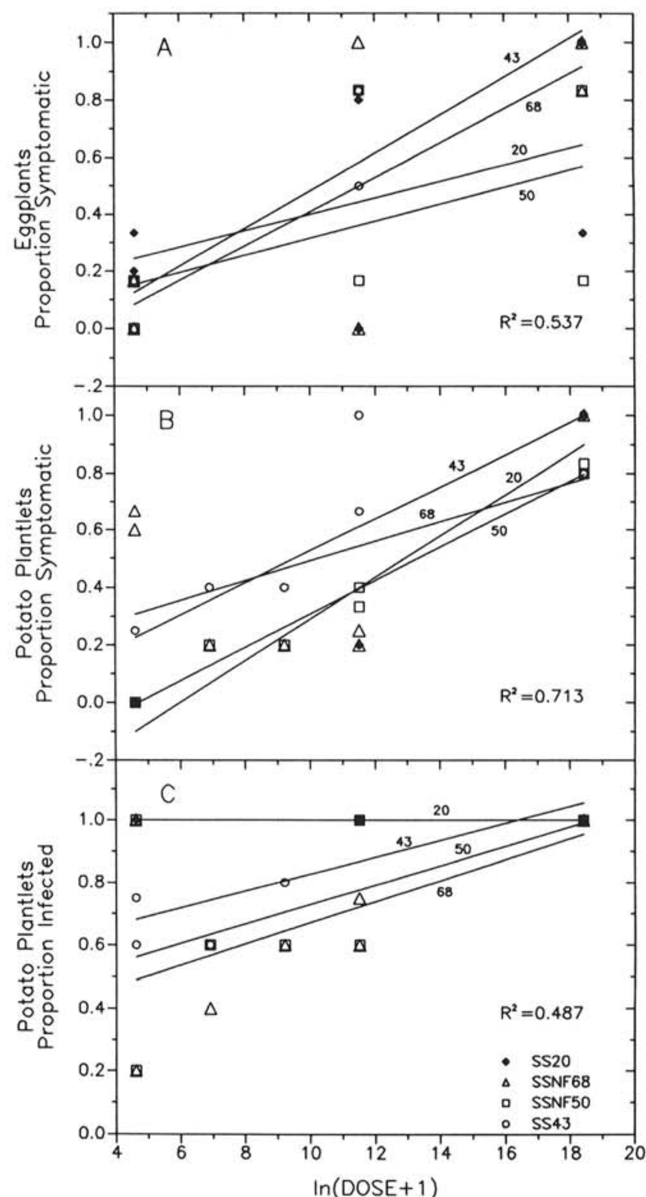

Fig. 6. Infectivity titration results for fluidal and nonfluidal strains of *Clavibacter michiganensis* subsp. *sepedonicus*. **A,** Proportion of eggplants developing symptoms. **B,** Proportion of potato plantlets developing symptoms. **C,** Proportion of potato plantlets infected. Data shown are the combined results of two separate experiments.

tional stress (e.g., temperature or moisture extremes) for symptom onset, whereas blockage in leaves may be sufficient to induce wilting. Functionally, eggplant seedlings respond similarly to potato leaves in assays. Additional studies utilizing appropriate EPS mutants of *C. m. sepedonicus* should further elucidate the specific roles of these polysaccharides in bacterial ring rot pathogenesis.

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