The Multi-Component Extracellular Polysaccharide of *Clavibacter michiganense* subsp. *insidiosum*

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ABSTRACT

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Extracellular polysaccharides (EPS) of many plant pathogenic bacteria are considered to be important virulence factors. A role in virulence expression for the EPS of *Clavibacter* (*Corynebacterium*) *michiganense* subsp. *insidiosum* has been proposed, but relatively little is known of the nature of this EPS beyond its basic repeating unit. We report here that the EPS of this pathogen consists of three different components, one EPS (component I) is an aggregate of another, 5 MDa EPS (component II). At least nine polypeptides are also associated with the component I aggregate. Component III (22 kDa) is different from the other two in its sugar composition and varies among strains and cultures from different media.

Additional key words: bacterial wilt of alfalfa, *Medicago sativa*.

Clavibacter (*Corynebacterium*) *michiganense* subsp. *insidiosum* is an important pathogen of alfalfa (*Medicago sativa* L.). It invades and colonizes the vessels of its host and induces water stress (3). Virulence of the bacterium is associated with the production of extracellular polysaccharide (EPS) (6). The exact role of EPS in virulence expression is not known, but one role is thought to be the induction of the observed plant water stress (20).

Characterization of a basic repeating unit of an EPS of *C. m.* subsp. *insidiosum* has been reported by Gorin et al (7). This repeating unit consists of 2 L-fucose: 1 D-galactose: 1 D-glucose: 1 pyruvate. Ries and Strobel (12) described a large glycopeptide produced in culture and in vivo by *C. m.* subsp. *insidiosum* that acted as a phytotoxin. The neutral sugar composition of this toxin is similar to that of the EPS described by Gorin et al (7), but pyruvate is not reported to be part of the toxin. Ries and Strobel (12) showed that the glycopeptide has a molecular mass of 5 MDa. In their study, Gorin et al (7) did not describe the size of the EPS molecule produced by *C. m.* subsp. *insidiosum*. It is not clear from these reports whether the glycopeptide described by Ries and Strobel (12) is the EPS of *C. m.* subsp. *insidiosum* described by Gorin et al (7). The similarity in neutral sugar composition of the two molecules suggests a relationship.

We (20) have found that molecular size is an important variable in the biological activity of wilt-inducing molecules such as the glycopeptide reported by Ries and Strobel (12). Using samples of the glycopeptide provided by them we were able to demonstrate that this molecule causes plant water stress by preventing xylem water movement (20). However, we were unable to confirm the reported (15) host-selective properties of this molecule (18).

Considering the accumulating evidence of the importance of EPS of bacterial wilt pathogens as a virulence factor (1,2,5), we felt it was important to reexamine the EPS of this bacterium. Because our studies have shown that any large polysaccharide produced by a vascular-inhabiting pathogen such as *C. m.* subsp. *insidiosum* can be phytotoxic, it was also the objective of this study to determine the size and nature of all macromolecules secreted in significant quantities into culture fluid by this bacterium.

MATERIALS AND METHODS

Media and growth conditions. The liquid medium (CH) used in most experiments consists of (per liter): 20 g of sucrose, 3.93 g of $K_2HPO_4 \cdot 3H_2O$, 0.3 g of $MgSO_4 \cdot 7H_2O$, 1.15 g of $NaH_2PO_4 \cdot 7H_2O$, 1 g of NH_4Cl, 2 g of Casamino acids (Difco Laboratories, Detroit, MI), 0.01 g of tryptophan, 1 ml of trace element solution consisting of (per 500 milliliters) 30 mg of H_3BO_3, 70 mg of $MnCl_2 \cdot 4H_2O$, 200 mg of $ZnCl_2$, 20 mg of $Na_2MoO_4 \cdot 2H_2O$, 50 mg of $FeCl_3 \cdot 5H_2O$, and 200 mg of $CuSO_4 \cdot 5H_2O$. After sterilization by autoclaving, the medium was allowed to cool and 10 ml of a filter-sterilized vitamin mix was added to each liter. The vitamin stock solution contained 50 μg/ml each of biotin, nicotinic acid, thiamine, pyridoxine, pantothenic acid, and riboflavin.

Other media used were YDC and YSC, which consisted of 1.0% yeast extract (Difco), 0.5% calcium carbonate, and 1.5% glucose in YDC or 1.5% sucrose in YSC.

Inoculation of liquid media was standardized by washing cells from 5-day-old agar slants and adding 2 ml of the cell suspension containing approximately 1.6×10^9 colony-forming units (cfu) to 250 ml of medium. Cultures were incubated for 5 days at 21 C with

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gentle aeration provided by rotary shaking at 50 rpm. Strains Ut-1, Ut-3, and Ut-4 were isolated from infected plants obtained from three different locations in Utah. Other strains of *C. m.* subsp. *insidiosum* were obtained from F. L. Lukezic, Pennsylvania State University (strain 557) and G. A. Strobel, Montana State University [This strain is designated by us as MS-1. This is the strain used in the studies of Ries and Strobel (12,13).]. All strains were maintained by lyophilization. Cultures used in all experiments were no more than three serial transfers from a lyophilized stock.

EPS isolation. Cells were removed by centrifugation at 15,000 *g* for 30 min. The culture fluid was concentrated 10-fold by rotary evaporation at 40 C. Cold acetone (3 vol) was added to culture fluid before incubation overnight at −20 C. After centrifugation, the precipitate was resuspended in 10 mM sodium acetate buffer, pH 5.0 (acetate buffer), and dialyzed exhaustively against the same buffer at 4 C. Eighteen to 20 mg of the precipitate, as determined by the anthrone method (14), were applied to a carboxymethyl (CM) cellulose (Whatman CM-52, Whatman Chemical Separation, Inc., Clifton, NJ) cation exchange column, 2.5 × 20 cm, equilibrated with acetate buffer. After the effluent was concentrated and dialyzed, it was applied to a diethylaminoethyl- (DEAE) cellulose (Whatman DE-52) anion exchange column, 1.7 × 5 cm, equilibrated with acetate buffer. The effluent was collected and then the bound material was released by washing with 1 M sodium acetate buffer, pH 5.0. Both fractions were concentrated by rotary evaporation and dialyzed against 4 L of acetate buffer. Effluent and bound material were further purified by gel filtration on a Sepharose CL-2B (Pharmacia, Inc., Piscataway, NJ) column, 5 × 35 cm, equilibrated with acetate buffer containing 0.01% (w/v) sodium azide, or an Ultrogel ACA-34 (LKB, Rockville, MD) column, 2.8 × 40 cm, equilibrated with the same buffer. Fractions were monitored for hexose by the anthrone method (14) and polypeptide by absorbance at 280 nm. All peak fractions were pooled, concentrated by rotary evaporation, and dialyzed against acetate buffer. Component homogeneity was determined by gradient elution from a DEAE-cellulose column, 1.7 × 2.5 cm, equilibrated with acetate buffer. Elution was with a linear gradient formed using 10 mM and 500 mM sodium acetate buffer, pH 5.0. Eluted carbohydrate peaks, detected by the anthrone reaction (14), were split into three or four parts and analyzed for neutral sugar composition by gas-liquid chromatography (GLC) (10). For the EPS fraction that did not bind to DEAE cellulose, homogeneity was determined by reapplying that fraction to Ultrogel ACA-34 and splitting the resulting peak into leading, central, and trailing peak fractions.

EPS composition studies. Protein analysis was carried out using sodium dodecyl sulfate polyacrylamide gel electrophoresis as described by Takemoto and Kao (17). Slab gels, 0.75 mm thick, were silver stained using the Bio-Rad (Richmond, CA) method and reagents. Protein samples were prepared as a 1 μg/μl solution with solubilization buffer (17). Protein concentration was determined by either the Lowry (11) or Bio-Rad protein assay methods. Solubilization buffer, made fresh each time, consisted of 20% glycerol and 3% SDS added to a final Tris buffer concentration of 40 mM. A 10–15-μl sample was applied to sample wells. Molecular mass estimates were made by comparisons with protein standards (Bio-Rad) using 10, 12, and 15% (w/v) polyacrylamide gels.

Dissociation of component I was attempted by dialyzing the sample against 0.1% (w/v) SDS, 8 M urea, or 4 M NaCl dissolved in acetate buffer. After at least 1 hr at room temperature, the sample was assayed for dissociation of component I by gel filtration on a Sepharose CL-2B column, 2.8 × 25 cm, equilibrated with the appropriate dissociation reagent.

Ketoacids were detected by the procedure of Sutherland (16) and uronic acids by the procedure of Jones and Albersheim (10). Sugars were detected as their alditol acetate derivatives by GLC (10).

Formation of antibodies to EPS. To increase the antigenicity of EPS, bovine serum albumin (BSA) was conjugated to a component of the EPS from Ut-1 strain essentially as described by Gray (8). Component II (see Results) was purified as described above except that an additional gel chromatography step (Sepharose CL-2B) was added. A total of about 24 mg of component II (340 μg/ml in 10 mM phosphate buffer, pH 7.2) was treated with periodate (0.2 mg/ml). The reaction was at room temperature with continuous shaking for 45 min, and then stopped by the addition of 0.2 vol of 0.64 M ethylene glycol. After dialysis, BSA and cyanoborohydride were added to final concentrations of 2 mg/ml and 0.1 mg/ml, respectively. The solution was incubated at 40 C with shaking for 14 days. Successful conjugation was confirmed by chromatography.

Rabbits were injected using the methods and schedule of Himmelspach and Kleinhammer (9). The first injections were done intramuscularly using between 75 and 250 μg of the conjugate, followed by intraperitoneal injections of from 60 to 200 μg after 30 days and from 6 to 20 μg after another 15 days. We found that injections with antigen concentrations within these ranges resulted in equal titers.

RESULTS

Fractionation of extracellular macromolecules. Figure 1 reviews the procedures used in isolating the EPS of *C. m.* subsp. *insidiosum*, Ut-1 strain, and indicates the yields normally obtained

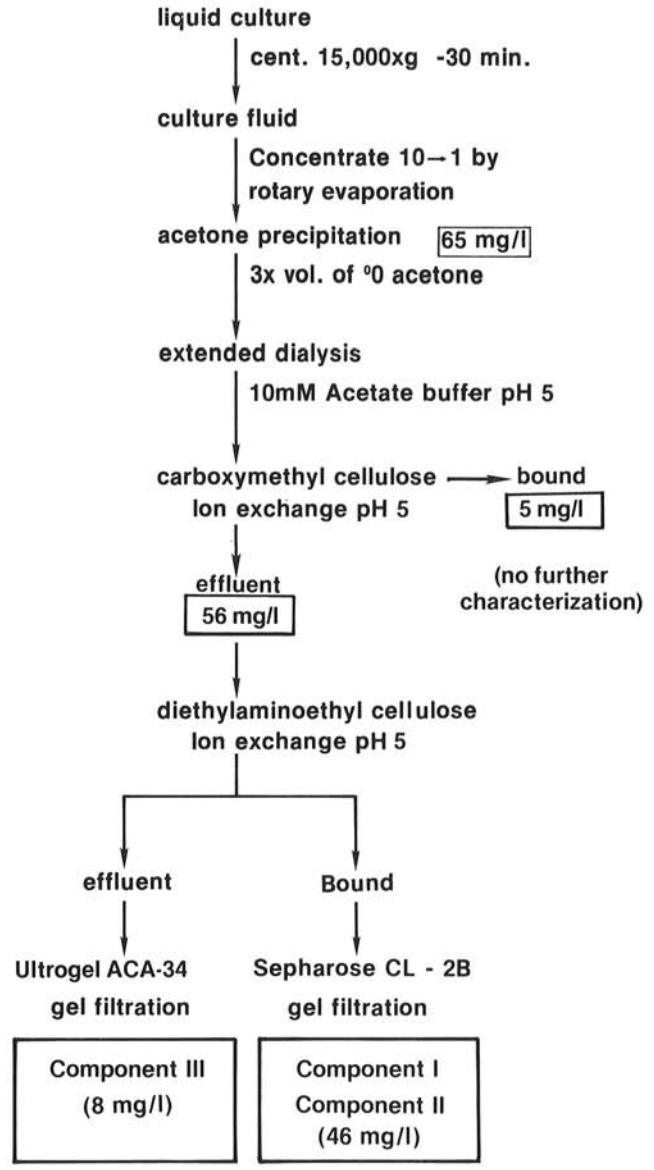


Fig. 1. Step-wise procedures used to purify extracellular polysaccharide of *Clavibacter michiganense* subsp. *insidiosum*. Yields of hexose equivalents per liter of culture fluid determined by the anthrone method (14) at various steps are indicated.

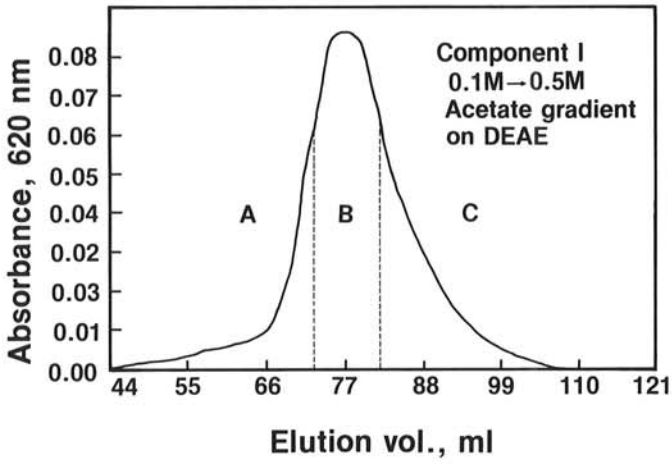
at each step. The yields are given as milligrams of hexose equivalents, determined by the anthrone procedure (14). The material that bound to the CM cellulose column was not homogeneous. It contained significant amounts of protein, determined by the Lowry method (11), as well as pigments. No further characterization of this fraction was done because all components were relatively low in molecular mass (less than 20,000 daltons). Most of the material that was applied to the DEAE-cellulose column was weakly bound. About 15% of this anthrone-positive material did not bind to the column under any conditions. Three distinct size classes of molecules were distinguishable on the gel filtration column. From the material bound by DEAE, one peak (I) eluted with the void volume of the Sepharose CL-2B column. This peak was also in the void volume of Bio-Gel A-150m (Bio-Rad) and Fractogel TSK-HW75(F) (MCB Manufacturing Chemists, Inc., Gibbstown, NJ). The second peak (II) from the DEAE-bound fraction was within the fractionating range of the Sepharose CL-2B gel. The third peak (III), which was present only in the DEAE-unbound fraction, was in the totally included volume of Sepharose CL-2B gel, but was within the fractionation range of Ultragel ACA-34.

Homogeneity and sizes of peaks. To check the homogeneity of the peaks, peaks I and II were separately reapplied to a DEAE-cellulose column then eluted with a linear acetate buffer gradient. The elution profiles from these columns are shown in Figures 2 and 3. The eluting peaks from each column were divided into three or four parts as shown, with each part being pooled and analyzed for neutral sugar content. The data shown are from one of the three times the experiment was done. The two peaks have essentially identical neutral sugar contents, and both peaks are homogeneous in neutral sugar content across the entire DEAE elution profiles. The two peaks do differ, however, in how tightly they are bound to the DEAE. Peak II is eluted at a much lower buffer molarity than is peak I.

Because peak III does not bind to DEAE, we tested for homogeneity by fractionation on a gel filtration column of the proper gel size. Figure 4 shows the elution profile of peak III on Ultragel ACA-34. This elution profile shows peak III elutes as a single peak. This peak was divided into three parts and analyzed for neutral sugar content (Fig. 4). Peak III is homogeneous across the elution profile for all components except glucose, of which there is more in the trailing portion of the peak than in the leading and center portions. Peak III differs from peaks I and II because it contains rhamnose and mannose in significant quantities. In the remainder of this paper, the three peaks will be considered as three separate components of EPS, labeled as component I, II, and III, corresponding, respectively, to peaks I, II, and III.

The size of component I cannot be accurately determined by gel filtration because it elutes in the void volume of all available gels. The size of component II was estimated by gel filtration chromatography using as references blue dextran and a glycopeptide found by Ries and Strobel (12) to be 5 MDa by light scattering measurements (gift of G. A. Strobel, Montana State University, Bozeman). Component II elutes at the same volume as this molecule. Using dextrans (Pharmacia) as standards, component III has an estimated molecular mass of 21,800 daltons by gel filtration.

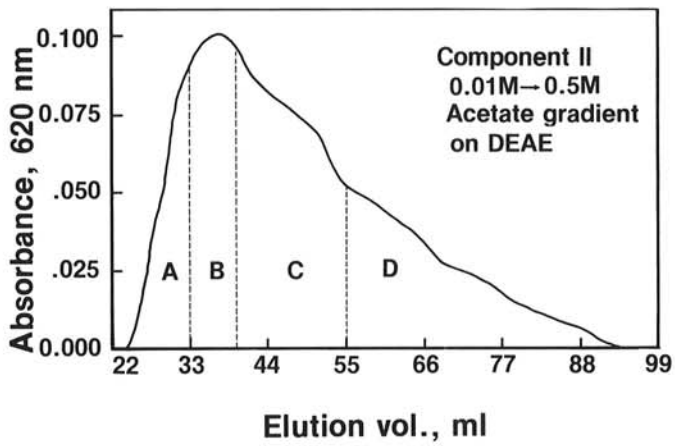
Composition of EPS components. The three components of EPS were analyzed for neutral sugar, protein, ketoacid, and uronic acid content. Figures 2–4 show the neutral sugar content of each component. Components I and II have similar carbohydrate composition. They both have a residue ratio of approximately 2 fucose:1 galactose:1 glucose. The composition of component III is substantially different, with residue ratios of 3 galactose:1



GLC analysis of Component I Gradient (% total)

Sugars	A	B	C
Rha	-	-	-
Fuc	39.9	41.8	39.5
Rib	-	-	-
Ara	T	T	T
Xyl	T	T	T
Man	T	T	T
Gal	27.2	27.2	26.9
Glu	31.0	31.7	30.4

Fig. 2. Analysis for homogeneity of component I of extracellular polysaccharide of strain Ut-1 after gradient elution from DEAE-cellulose column. Fractions were pooled as indicated to be analyzed for sugar composition. Sugar composition determined as alditol acetate derivatives by gas-liquid chromatography, expressed as percentages of total sugars detected. T indicates that only a trace amount was detected.



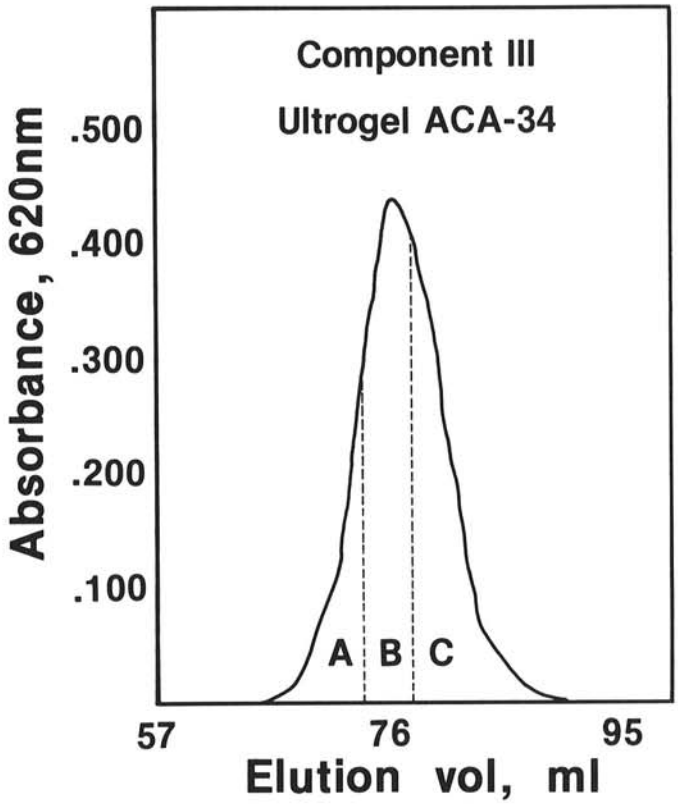
GLC analysis of Component II Gradient (%total)

Sugars	A	B	C	D
Rha	-	-	-	-
Fuc	42.4	45.7	42.3	43.2
Rib	-	-	-	-
Ara	T	T	-	-
Xyl	-	T	-	-
Man	-	-		-
Gal	26.7	25.0	25.7	25.9
Glu	30.8	29.3	32.0	30.8

Fig. 3. Analysis for homogeneity of component II of extracellular polysaccharide of strain Ut-1 after gradient elution from DEAE-cellulose column. Fractions were pooled as indicated to be analyzed for sugar composition. Sugar composition determined as alditol acetate derivatives by gas-liquid chromatography, expressed as percentages of total sugars detected. T indicates that only a trace amount was detected.

rhamnose:1 fucose and lower amounts of glucose and mannose. Uronic acid residues were not detected in any of the three components. Components I and II were found to be positive for ketoacids each having 2.0 ± 0.1 μg of ketoacid per 10 μg of carbohydrate (by authorized method). Gorin et al (7) have previously reported the ketosugar to be pyruvate. Only component I has detectable amounts of protein associated with the carbohydrate. By Lowry assay there was 13% protein.

Peptides associated with component I. Lowry positive and 280 nm absorbing material was found associated with component I. Because component I voids all gel filtration columns, it was necessary to bind it to a DEAE-cellulose column and elute with a linear buffer gradient in order to assure that the peptides were not merely contaminants. When this procedure was followed, 280 nm absorbing material eluting from the DEAE cellulose column came off as a peak that coincided with that of the carbohydrate.



GLC analysis of Component III (% total)

Sugars	A	B	C
Rha	17.5	18.1	17.6
Fuc	21.0	20.8	20.3
Rib	-	-	-
Ara	-	-	-
Xyl	-	-	-
Man	3.8	4.0	4.0
Gal	53.9	54.8	50.8
Glu	3.8	2.3	9.1

Fig. 4. Analysis for homogeneity of component III of extracellular polysaccharide of strain Ut-1 after gel-filtration chromatography on Ultragel ACA-34. Fractions were pooled as indicated to be analyzed for sugar composition. Sugar composition determined as alditol acetate derivatives by gas-liquid chromatography, expressed as percentages of total sugars detected.

Purified component I was treated with 0.1% SDS, 8 M urea, or 4 M NaCl or boiled for 30 min in attempts to dissociate the protein-carbohydrate complex. The two salts had no effect on removal of the proteinaceous material as judged by chromatography on Sepharose CL-2B. SDS and boiling, however, separated the peptides from the carbohydrate moiety. Under these conditions the carbohydrate portion of component I dissociated into a lower molecular weight molecule that cochromatographed with component II and was identical in neutral sugar content. The 280 nm absorbing material was detected as a diffuse peak within the included volume.

Because the 280 nm absorbing material was positive by Lowry and 'Bio-Rad' Bradford dye binding procedures, it was assumed to be primarily polypeptides. To determine how many different polypeptides were present, purified component I was run on SDS-polyacrylamide gels. Silver staining revealed peptides of various sizes associated with component I. Gels of 10, 12, and 15% polyacrylamide were run with appropriate standards to estimate molecular masses. The molecular masses of the polypeptides obtained from the best of multiple gels were 75,750, 70,050, 59,800, 21,650, 18,775, 16,650, 15,700, 14,850, and 12,750 daltons.

Association of EPS with bacterial cells. Using the India ink method (4), we have failed to detect a capsule around cells of *C. m.*

TABLE 1. Extracellular polysaccharide composition of different strains of *Clavibacter michiganense* subsp. *insidiosum* grown on YSC medium

Sugars[a]	Component I	Component II	Component III
Ut-1 Strain			
Rha	...	...	T[b]
Fuc	40.2	43.3	T
Rib	...	...	...
Ara	T	T	T
Xyl	T	...	...
Man	T	...	52.7
Gal	27.2	25.8	33.3
Glu	31.1	30.9	12.8
Ut-3 Strain			
Rha	...	...	1.3
Fuc	45.2	40.5	1.9
Rib	...	T	...
Ara	2.3	T	T
Xyl	T	T	...
Man	7.9	8.4	88.3
Gal	22.2	17.7	3.9
Glu	21.5	31.7	3.8
Ut-4 Strain			
Rha	...	...	...
Fuc	47.8	47.7	2.2
Rib	T	...	T
Ara	T	T	T
Xyl	T	T	T
Man	6.4	7.6	88.1
Gal	18.9	20.3	3.3
Glu	25.0	22.0	5.5
MS-1 Strain			
Rha	...	...	...
Fuc	43.0	42.0	1.5
Rib	...	...	...
Ara	1.1	T	T
Xyl	T	T	T
Man	6.8	10.5	92.1
Gal	24.8	22.7	1.5
Glu	24.3	23.9	4.3
557 Strain			
Rha	...	...	...
Fuc	40.0	46.3	...
Rib	...	T	...
Ara	T	T	...
Xyl	T	...	...
Man	2.6	2.5	...
Gal	23.2	24.6	...
Glu	33.1	26.4	...

[a] Sugar composition determined as alditol acetate derivatives by gas-liquid chromatography, expressed as percentage of total sugars detected.
[b] T indicates a trace amount was detected.

TABLE 2. Extracellular polysaccharide composition of strain Ut-1 of *Clavibacter michiganense* subsp. *insidiosum* as affected by different media

	YDC		YSC			CH		
	Component I + II	Component III	Component I	Component II	Component III	Component I	Component II	Component III
Rha[a]		2.7	...	...	T[b]	...	...	20.4
Fuc	47.6[c]	12.2	40.2	43.3	T	39.5	43.0	23.1
Rib	...	...	...	...	...	...	...	...
Ara	...	T	T	T	T	T	T	...
Xyl	...	T	T	...	...	T	...	...
Man	...	48.9	T	...	52.7	T	...	3.4
Gal	24.1	28.3	27.2	25.8	33.3	27.6	25.6	52.4
Glu	28.3	6.9	31.1	30.9	12.8	31.0	31.3	0.8

[a] Sugar composition determined as alditol acetate derivatives by gas-liquid chromatography, expressed as percentage of total sugars detected.
[b] T indicates only a trace amount was detected.
[c] Components I and II were not separated, so the values are composite of Components I and II.

subsp. *insidiosum*. Antibody to component II was used to determine if bacterial cells that were washed once by centrifugation were antigenic. Antibody used in this experiment showed reciprocal titers of 1,024 to component I and II and a titer of 512 to component III, but it showed a titer of only 32 to bacterial cells. This antibody did not cross-react with EPS from *Leuconostoc dextran*, *Pseudomonas syringae* pv. *syringae* or *P. s.* pv. *phaseolicola*, nor to any of the individual sugars of the EPS of *C. m.* subsp. *insidiosum*.

Variation of EPS among different strains. Five different strains of *C. m.* subsp. *insidiosum* were tested for size and neutral sugar composition of their EPS (Table 1). Components I and II of the EPS were present in all strains with neutral sugar ratios that varied only slightly. Mannose content showed the greatest variation from strain to strain. Component III was lacking from one of the strains (557) and was quite variable in composition in the other strains, particularly with respect to their mannose:galactose ratios. The MS-1 strain is the same as used by Ries and Strobel (12) in their studies. The EPS components produced by this strain are the same as those of Ut-3 and Ut-4.

Variation of EPS composition in different culture media. One strain (Ut-1) was grown on three different media and EPS composition compared. Components I and II varied only slightly in the different media (Table 2). Minor changes in medium composition affected the sugar ratios of component III. Fucose was lacking in component III of cells grown in YSC, but was present when grown on YDC and CH. CH medium has sucrose as the carbon source, so presence or absence of sucrose alone is not the factor affecting sugar composition of component III.

DISCUSSION

The EPS produced by *C. m.* subsp. *insidiosum* consists of three different components, one of which corresponds in size to the glycopeptide reported as a phytotoxin by Ries and Strobel (13), but this component (II) is not a glycopeptide, and it has the same composition as that described by Gorin et al (7). We have found that the EPS of Strobel's strain (MS-1) is indistinguishable from that of strains Ut-3 and Ut-4 (Table 1).

An unknown number of component II molecules appear to aggregate along with at least nine polypeptides to form component I. This component is very large, voiding all molecular sieving columns. The aggregate is stable under high salt conditions, but dissociates in SDS and when boiled. Neither it nor component II are artifacts of the isolation procedure because both can be detected in culture fluids that have not been processed by the purification procedure. Components I and II can cause water stress if introduced into plant cuttings. Wilting results from plugging pit membranes (19).

The number and sizes of polypeptides associated with component I are constant under various isolation procedures. To assure that the polypeptides are not contaminants, component I, after purification, was rebound to a DEAE-cellulose column and eluted with a linear gradient before isolation of the polypeptides. Although this procedure does not totally exclude the possibility that some polypeptides associated with component I are contaminants, it greatly reduces this possibility. The fact that high salt solutions do not remove these polypeptides also suggests that there are specific interactions between them and component II to form component I. Dissociation by SDS and boiling, however, indicates that the polypeptides are not covalently bound to the polysaccharide.

The third component (III) is much smaller and of different composition from the other two. Component III is variable in composition in different strains (Table 1) and its composition is influenced by culture media (Table 2). This molecule is free of ketoacids and contains no detectable amino acids. Clearly, the composition of this group of similarly sized small carbohydrates is not as tightly regulated by *C. m.* subsp. *insidiosum* as is the composition of components I and II.

The material used by Gorin et al (7) for linkage studies was an unfractionated total EPS preparation. Our studies suggest that the neutral sugars detected by these workers represent 85% (by anthrone analysis) of the total sugars of the three components. The ratios of the sugars they reported match the ratios detected for the sugars of components I and II. The amount of ketoacid detected by us was about twofold greater than that accounted for by the repeating unit (7).

The three components of the EPS of *C. m.* subsp. *insidiosum* are each able to cause alfalfa cuttings to wilt (19). Such wilt-inducing ability does not necessarily mean that these compounds are each important as virulence factors for this pathogen. It has been reported that mutants of this bacterium that are unable to produce EPS are avirulent (6). These studies were based on colony appearance, so a more quantitative confirmation of these observations is needed.

The following paper (19) describes efforts to understand how these EPS components could be involved in host-pathogen relations of bacterial wilt of alfalfa.

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