# Characterization of the Extracellular Polysaccharide Produced by *Clavibacter michiganensis* subsp. *michiganensis*

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## ABSTRACT

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An extracellular polysaccharide (EPS) produced by *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial canker in tomato, was isolated from culture filtrates by ultrafiltration. The components of EPS were shown to be L-fucose, D-galactose, D-glucose, pyruvate, succinate, and acetate in the approximate molar ratio of 2:1:1:1:0.5:1.5. Methylation analysis revealed that the tetrasaccharide-repeating unit consisted of one 1,3-linked glucosyl, one 1,4-linked fucosyl, one 1,3,4-linked fucosyl (branch unit) and one 1-linked galactosyl residue (terminal group). Pyruvate occupied the $O$-4 and $O$-6 position of the galactosyl residue, whereas the positions of the succinyl and $O$-acetyl substituents are, as yet, undetermined. These results indicate that the structure of the EPS, as far as the carbohydrate portion is concerned, is identical to that of the EPS of *C. m.* subsp. *insidiosus*, except for the presence of the succinyl and $O$-acetyl groups, which were not observed in the latter. Gel filtration chromatography demonstrated the molecular weight of the EPS to be variable, with sizes of about $10^6$ and $10^7$ Da. The high molecular weight of the EPS may be associated with the wilting symptoms shown by infected plants. Evidence is given that a high-molecular-weight EPS produced *in planta* is similar in composition to EPS produced in vitro.

Additional keywords: bacterial canker of tomato, *Lycopersicon esculentum*.

Extracellular polysaccharides (EPS) are produced by members of all species of phytopathogenic bacteria. These polysaccharides may be present as a discrete capsule or, more commonly, as extracellular slime, which generally is soluble and diffuses readily in liquid medium (21). EPS are composed of neutral hexoses (e.g., glucose, galactose, and mannose) and of 6-deoxyhexoses (e.g., fucose and rhamnose), with other possible constituents such as uronic acids. Amino sugars are less prevalent, but galactosamine is a major component of EPS produced by *Pseudomonas solanacearum* (34). Common substituent groups, esterified to the sugar residues, are pyruvate, succinate, and acetate. Most EPS molecules are heteropolysaccharides of high molecular weight, which are composed of repeating units. The chains formed by these repeating units may possess some degree of branching.

For several plant-pathogenic bacteria, similarity in the composition of EPS produced in vitro and of that produced in infected plants has been shown (1,2,6,11,26). It is likely that the production of EPS in the host plant is associated with pathogenesis. Multiple roles have been attributed to EPS, including the prevention of bacterial attachment to host cells (recognition) in order to suppress the hypersensitive reaction; the inhibition of bacterial agglutination with host agglutinins (immobilization of the bacterial cells); water retention in intercellular spaces, enabling bacterial establishment and multiplication; protection against inhibitory agents (e.g., bacteriostatic compounds); and induction of wilting by restriction of the water movement (4,20).

Wilt induction has been reported for EPS produced in vitro by *Clavibacter michiganensis* subsp. *michiganensis* (15–17,31), *C. m.* subsp. *insidiosus* (18,19,22,29,30), and *C. m.* subsp. *sepedonicus* (23–25). Minor amounts of protein were found to be associated with the EPS. The presence of EPS in infected plants was demonstrated for all three pathogens by serological assays. The composition and structure of the EPS produced by *C. m. insidiosus* is known (8–10). A structural analysis of the EPS of *C. m. michiganensis*, the cause of bacterial canker of tomato, has not been reported so far. Only the presence of four sugar residues (fucose, galactose, glucose, and mannose) was shown by paper chromatography (8,16).

This paper reports a partial structural analysis of the EPS produced by *C. m. michiganensis* in vitro and presents evidence for the production of a similar high-molecular-weight polysaccharide in infected plants.

## MATERIALS AND METHODS

**Bacteria and growth media.** *C. m. michiganensis* strain Cm 542 (NCPPB 1064, obtained from the National Collection of Plant Pathogenic Bacteria, Harpenden, UK) was used in this research. This strain was shown to be highly aggressive on tomato plants (31). Long-term storage was on special beads (Protect bacterial preservers, Technical Service Consultants Ltd., Bury, UK) at −80 C. Prior to use, the bacteria were cultured at 27 C on YPGA containing 0.5% yeast extract, 1% peptone, 0.5% glucose, and 1.5% agar. For EPS isolation, *C. m. michiganensis* cells were grown for 12 days at 27 C on a rotary shaker (150 rpm) in a liquid YGC medium consisting of 0.5% dialyzed yeast extract (dialyzed in a tube with a molecular weight cutoff of 12 kDa; the excluded fraction was used), 1.5% glucose, and 0.5% $CaCO_3$.

**Isolation of EPS from cultures.** Bacterial cells were removed by centrifugation (20 min at 15,000 $g$) and filtration of the supernatant fluid through a 0.2-$\mu$m filter. EPS was obtained by filtration of the culture filtrate through an ultrafilter with a nominal molecular weight limit of 10,000 (Minitan system, Millipore, Bedford, MA), followed by copious washing of the

retained fraction with purified water (Milli-Q water system, Millipore) and repeated ultrafiltration. The EPS fraction was lyophilized and stored at −20 C.

**Protein analysis and gel electrophoresis.** The protein content was determined with the single reagent Bio-Rad protein assay (Bio-Rad Laboratories, Munich), using plasma gamma globulin as a standard. EPS was dissolved in sample buffer consisting of 1% (w/v) sodium dodecyl sulfate (SDS), 8 M urea, 1 mM $Na_2EDTA$, 1 mM phenylmethylsulfonyl fluoride, 5% β-mercaptoethanol (v/v), 10 mM Tris-HCl (pH 7.8), and 0.01% bromophenol blue (w/v) and heated for 5 min in a boiling water bath. The samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 2.2–20% gradient gels using 25 mM Tris, 190 mM glycine, and 0.1% SDS (w/v) as electrophoresis buffer. The gels were subsequently stained for proteins with the Bio-Rad silver stain and for polysaccharides with Alcian blue reagent (33).

**Analytical methods.** Total hexoses and 6-deoxyhexoses were measured by the anthrone-sulfuric acid method (28) and the L-cysteine-sulfuric acid method (5) respectively. Uronic acid content was determined on nonhydrolyzed material by reaction with *m*-hydroxydiphenyl (3) with D-glucuronolactone as a standard. Pyruvate was measured spectrophotometrically as the 2,4-dinitrophenylhydrazone, after hydrolysis of the EPS with 1 N HCl for 3 h at 100 C, according to the method of Katsuki et al (14), with sodium pyruvate as the standard. Succinic acid was released by hydrolysis in 2 N trifluoroacetic acid for 6 h at 100 C, converted into methyl esters by methanolysis, and determined by gas-liquid chromatography (GLC) in the presence of glutaric acid as an internal standard (35). Acetate was measured colorimetrically as *O*-acetyl according to Hestrin (13), with glucose pentaacetate as the standard.

**Monosaccharide analysis.** To determine the sugar composition of the EPS, samples were hydrolyzed in 2 N trifluoroacetic acid at 121 C for 1 h, reduced with $NaBH_4$, and acetylated according to the procedure of Harris et al (12). The alditol acetates of the monosaccharides were separated by GLC on a Sil-43-CB column
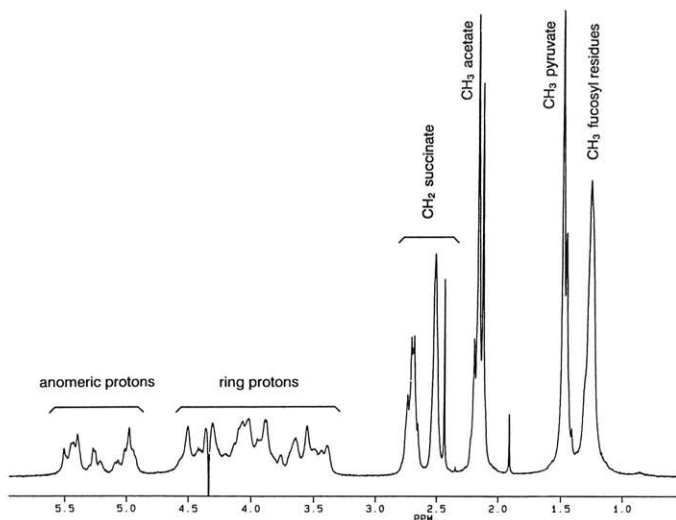
(Chrompack, Inc., Bridgewater, NJ) at 210 C using a Kipp Analytica system 8200 gas chromatograph (Kipp Analytica bv, Emmen, The Netherlands) equipped with a flame-ionization detector. Response factors relative to the internal standard myo-inositol were used to determine the weight percentages of the various monosaccharides.

**Proton nuclear magnetic resonance (NMR) spectroscopy.** For proton ($^1$H) NMR spectroscopy, lyophilized samples were dissolved in $^2H_2O$. Spectra were obtained on a Bruker AM-600 instrument (Bruker Analytische Messtechnik, Karlsruhe, FRG) at a measuring temperature of 60 C. The water peak (about 4.3 ppm) was eliminated by means of presaturation. The external chemical shift standard was sodium trimethylsilyl-propionate.

**Methylation analysis.** Polysaccharides were methylated by the method of Harris et al (12). The methylated derivatives were hydrolyzed and the resulting methylated sugars converted into their alditol acetates. The partially methylated alditol acetates were separated by GLC on a J&W DB225 column (30 m × 0.25 mm; J&W Scientific Inc., Folsom, CA), using a temperature program from 140 to 240 C at 2°C/min, and identified by mass spectrometry. Mass spectra were recorded with a VG Micromass 7070F mass spectrometer (VG Instruments, Altrincham, U.K.) working in the electron impact ionization mode at 70 eV. The relative areas of the peaks resulting from the GLC analysis were converted into relative mole percentages by using the effective-carbon-response factors of the derivatives (27).

**Gel filtration chromatography.** EPS preparations were fractionated by gel filtration on a Sephacryl S-1000 column (Pharmacia, Inc., Piscataway, NJ) with a bed size of 1.6 × 50 cm. This gel filtration medium has a fractionation range for dextrans of $5 × 10^5$ to at least $10^8$ Da. EPS was dissolved in a buffer (pH 6.8) consisting of 0.05 M phosphate and 0.1 M $Na_2SO_4$, at a concentration of approximately 2 mg/ml, and 1-ml samples were applied onto the column, which was eluted with the same buffer. Fractions of 3 ml were collected, and the carbohydrate content of the fractions was determined with the anthrone-sulfuric acid method.

**Isolation of EPS from infected plants.** Three-week-old plants of tomato cultivars Moneymaker and Irat L-3 were inoculated with *C. m. michiganensis* as described before (31). Three weeks after the inoculation the leaves were detached and the stem dissected into 10-cm segments. The segments were vacuum-infiltrated with 0.05 M phosphate buffer, pH 6.8, and placed vertically in centrifuge tubes equipped with a lower compartment accessible only to fluids. Fluid of the vascular system was collected by centrifugation (swing-out rotor, 15 min at 400 *g*), and bacterial cells and plant debris were eliminated by centrifugation (20 min at 15,000 *g*) and 0.2-μm filtration. The vascular washing fluid thus obtained was dialyzed against purified water, lyophilized, dissolved in 1 ml of a 0.05 M phosphate buffer, pH 6.8, supplemented with 0.1 M $Na_2SO_4$, and subjected to gel filtration chromatography on Sephacryl S-1000. The effluent was monitored by measuring the refractive index. The fractions of high molecular weight were combined, dialyzed against purified water, lyophilized, redissolved in 0.25 ml of water, and analyzed for monosaccharide composition. The same procedure was followed for uninoculated plants.

## RESULTS

**Isolation of EPS.** The yield of EPS isolated from cell-free culture filtrate was approximately 0.7 g (dry weight) per liter. Small amounts of protein (0.4%) were still present after ultrafiltration.



**Fig. 1.** Proton nuclear magnetic resonance spectrum of extracellular polysaccharide isolated from culture filtrate of *Clavibacter michiganensis* subsp. *michiganensis*. The signal of hemideuterated water (4.3 ppm) was suppressed.

TABLE 1. Composition of the extracellular polysaccharides produced by *Clavibacter michiganensis* subsp. *michiganensis* in vitro

|  | D-Glucose | D-Galactose | L-Fucose | Pyruvate | Succinate | Acetate |
|---|---|---|---|---|---|---|
| Weight percent | 18.1 | 18.8 | 34.5 | 9.2 | 6.4 | 7.0 |
| Moles | 1.01 | 1.04 | 2.10 | 1.05 | 0.54 | 1.61 |
| Molar ratio[a] | 0.97 | 1.00 | 2.02 | 1.01 | 0.52 | 1.55 |

[a] Based on the amount of D-galactose (in moles) set to 1.0.

SDS-PAGE of EPS samples followed by silver staining yielded four distinct protein bands with molecular weights ranging from 25,000 to approximately 70,000. However, these proteins were not covalently bound to the carbohydrate portion of the EPS, since Alcian blue staining revealed all carbohydrates to be present in one diffuse spot in the high molecular weight section of the gel (>260 kDa). Thus the small percentage of protein was to be considered as contamination.

**Components of EPS.** GLC analysis of the alditol acetate derivatives of the monosaccharides in EPS showed fucose, galactose, and glucose to be present in a ratio (of weight percent) of 1.83:1.00:0.96 (average values of several determinations performed on two different EPS preparations). In addition, a trace amount of mannose (<2%) was detected.

Determination of the hexose and 6-deoxyhexose content showed that the EPS contained approximately 370 $\mu$g hexoses and 345 $\mu$g deoxyhexoses per mg. A small amount of uronic acid residues, less than 1% (w/w), was detected. Since monosaccharides (e.g., glucose) also give a slight, unspecific reaction with $m$-hydroxydiphenyl, this low percentage presumably is not significant.

Analysis of the EPS preparation by $^1$H-NMR spectroscopy (Fig. 1; see 7 and 10 for signal assignments) showed the presence of a peak at 1.25 ppm, assigned to the methyl protons of L-fucosyl residues. The peaks at 1.46 and 2.15 ppm represent the methyl protons of pyruvate and acetate, respectively. The occurrence of more peaks in a doublet or triplet (as for the acetyl groups) suggests that more binding positions to the sugar residues exist. Triplets at 2.48 and 2.69 ppm correspond to the methylene protons of succinyl groups. The presence of protons of $\beta$-anomeric linkages is shown by the peak at 4.9 ppm, and the peaks around 5.4 ppm are assigned to protons of $\alpha$-anomeric linkages. The complex region from 3.4 to 4.5 ppm represents the ring protons of the sugar residues.

Chemical determinations confirmed the presence of succinate (64 $\mu$g per milligram of EPS) and $O$-acetyl (69.5 $\mu$g per milligram of EPS). An analysis for ketoacids showed pyruvate to be present in an average amount of 92 $\mu$g per milligram of EPS. From all data obtained, as summarized in Table 1, it can be concluded that the EPS consists of L-fucose, D-galactose, D-glucose, pyruvate, succinate, and acetate in molar ratios of approximately 2:1:1:1:0.5:1.5. The total of the weight percentages recovered is 94%. The remaining 6% can be accounted for by water and cations.

**Structure of EPS.** Methylation analysis of EPS (Table 2) resulted in two partially methylated fucosyl residues, one partially methylated glucosyl residue, and one partially methylated galactosyl residue in approximately equimolar amounts, in accordance with the monosaccharide composition. Based on the methylated sugar pattern, EPS consists of the following structural elements: (1→4)-linked L-fucosyl residues, (1→3)-linked D-glucosyl residues, branched L-fucosyl residues linked through $O$-1,3,4, and D-galactosyl terminal groups, with pyruvate occupying the $O$-4 and $O$-6 positions by acetal linkages. The succinyl and acetyl substituents are esterified to the sugar residues at, as yet, undetermined positions.

**Homogeneity of EPS.** The homogeneity of the EPS was checked by application of Sephacryl gel filtration chromatography. Only small amounts of EPS (2 mg) were loaded onto the column to avoid viscosity problems. Two major peaks (I and II) were apparent in the elution profile of the EPS (Fig. 2). The calculated $K_{av}$ values for peaks I and II are 0.45 and 0.70, respectively. Sizes of approximately $10^7$ and $10^6$ Da were estimated for the components of peaks I and II, based on the selectivity curve ($K_{av}$ versus log MW) of dextran standards in aqueous buffer (Pharmacia handbook "Gel filtration: Theory and practice"). Fractions of peak I (eluted volume from 48 to 69 ml) as well as fractions of peak II (75 to 96 ml) were combined and, after dialysis and lyophilization, analyzed for monosaccharide

TABLE 2. Methylation analysis of extracellular polysaccharides of *Clavibacter michiganensis* subsp. *michiganensis*

| Methylation products | T[a] | Mole (%) |
|---|---|---|
| 2,3-Di-$O$-methyl-fucose | 1.04 | 25.5 |
| 2-$O$-Methyl-fucose | 1.18 | 30.8 |
| 2,4,6-Tri-$O$-methyl-glucose | 1.27 | 23.5 |
| 2,3-Di-$O$-methyl-galactose | 1.67 | 20.2 |

[a] Retention time (T) of the corresponding alditol acetates relative to 1,5-di-$O$-acetyl-2,3,4,6-tetra-$O$-methyl-D-glucitol.
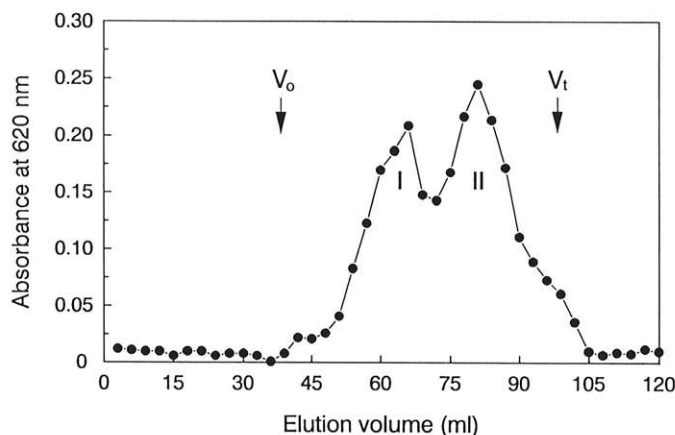


Fig. 2. Elution profile of *Clavibacter michiganensis* subsp. *michiganensis* extracellular polysaccharide after Sephacryl S-1000 gel filtration chromatography. The column was eluted with a buffer (pH 6.8) consisting of 0.05 M phosphate and 0.1 M Na$_2$SO$_4$. Fractions of 3 ml were collected and assayed for carbohydrates by the anthrone-H$_2$SO$_4$ method (A$_{620}$). The arrows mark the void volume (V$_o$) and total volume (V$_t$) of the column.
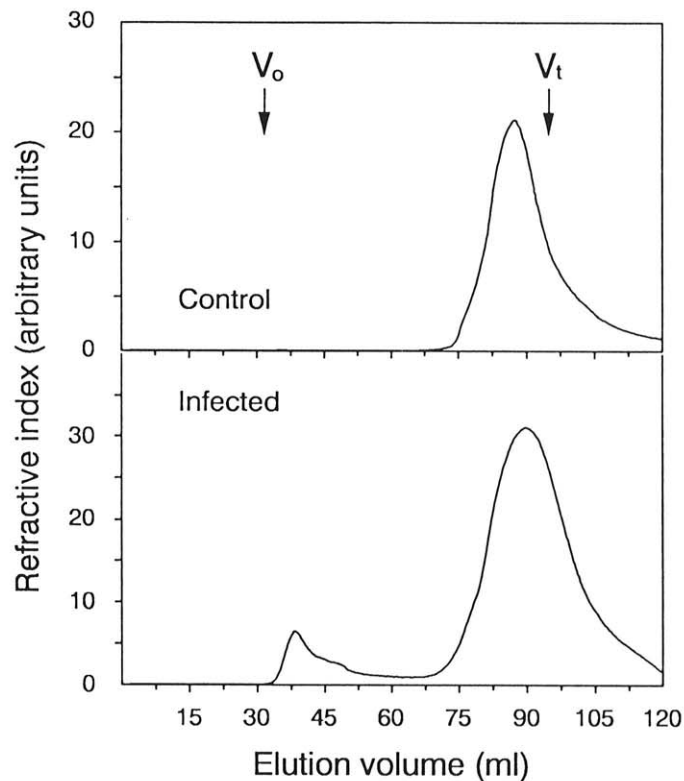


Fig. 3. Sephacryl S-1000 elution profiles of cell-free material isolated from the vascular system of control (uninfected) plants and plants infected with *Clavibacter michiganensis* subsp. *michiganensis*. The column was eluted with a buffer (pH 6.8) consisting of 0.05 M phosphate and 0.1 M Na$_2$SO$_4$, and the effluent was monitored for sugars by measuring the refractive index. The arrows mark the void volume (V$_o$) and total volume (V$_t$) of the column.

composition. Fucose, galactose, and glucose were present in weight percentages of 55.4, 21.2, and 23.4 (peak I), and 52.8, 22.9, and 24.2 (peak II), respectively. Hence, the ratio and the monosaccharide composition are similar for both peaks.

**EPS *in planta*.** The elution profile of the preparation isolated from the vascular system of healthy control plants showed the presence of one major peak with molecules of low molecular weight after Sephacryl S-1000 gel filtration chromatography (Fig. 3). Gel filtration chromatography of the preparation isolated from infected plants yielded an additional peak consisting of components of high molecular weight. These components eluted at a smaller volume (close to the void volume) than both components of EPS produced in culture (Fig. 2), suggesting that their size is larger. The isolated high molecular weight components contained hexoses and deoxyhexoses in a ratio of approximately 1:1 and about 20–25% protein. About 1 mg of polysaccharide was obtained from the vascular washing fluid (~10 ml) of four infected plants. SDS-PAGE, followed by staining for protein and polysaccharides, showed that these constituents were not covalently bound. Apparently, the proteins that coeluted with the high-molecular-weight polysaccharides were aspecifically bound to these molecules.

The high-molecular-weight fractions were pooled, dialyzed, lyophilized, and deproteinized, and the monosaccharide composition was determined. GLC analysis of the resulting alditol acetates (Table 3) showed a monosaccharide composition of fucose, galactose, and glucose, with an average molar ratio of 2.07:1.00:0.97 for material isolated from both infected Moneymaker and Irat L-3 plants. Hence, EPS is also produced *in planta* and is similar in composition to EPS produced in culture.

## DISCUSSION

The technique used to isolate EPS from culture filtrate, based on ultrafiltration, is a simple method to obtain pure preparations with only small amounts of protein contamination. Because this protein fraction was not covalently bound to the polysaccharides, the EPS isolated from cultures of *C. m. michiganensis* is not a glycoprotein. This is in contrast with the suggestion made by Rai and Strobel (16) and Krämer (15) that the polysaccharide material they had obtained from the same bacterium was a glycopeptide. However, they only showed the presence of polysaccharides and amino acids, without investigating whether these two constituents were covalently linked to each other.

The EPS produced by the strain of *C. m. michiganensis* used throughout this study contained the neutral monosaccharides glucose, galactose, and fucose, and acidic pyruvyl, succinyl, and *O*-acetyl groups. The positions of succinyl and *O*-acetyl groups were not determined, but for *O*-acetyl the proton NMR spectrum suggests that several binding positions are possible. This is strengthened by the molar ratio of the EPS components, which shows that repeating units with more than one acetyl group are present. From the structure of the tetrasaccharide repeating unit

it appears that EPS molecules are linear polymers with side groups. The composition and structure of this repeating unit of *C. m. michiganensis* EPS is likely identical to that of *C. m. insidiosus* EPS proposed by Gorin et al (10). Identical monosaccharides and glycosidic linkages and a similar acetal formed by pyruvate and galactose were reported by these authors. They deduced the sequence of sugar residues in the chain by analyzing the oligosaccharide fragments obtained from partial acid hydrolysis (8,9) and the anomeric configurations of the glycosidic bonds by complementary studies with NMR (10). These anomeric configurations, one $\beta$-anomeric linkage for D-glucose and $\alpha$-anomeric linkages for the D-galactose and both L-fucose residues, are presumably the same for the repeating unit of *C. m. michiganensis*, since both $\alpha$- and $\beta$-anomeric linkages were detected with proton NMR analysis. However, *O*-acetyl and succinyl substituent groups were not found for the EPS of *C. m. insidiosus*. The proposed structure of the tetrasaccharide repeating unit of the *C. m. michiganensis* EPS is shown in Figure 4. Confirmation of this structure awaits further studies.

Gel filtration chromatography showed that EPS molecules are not homogeneous in molecular weight. However, based on the similar monosaccharide composition of peak I and peak II, we assume that only one type of repeating unit is present and that the difference in molecular weight is due to variation in chain length. Aggregation of EPS molecules can be excluded, since gel filtration under denaturing conditions (i.e., elution with 8 M urea) resulted in a similar elution profile (data not shown). The molecular weights of the EPS molecules were estimated to be approximately $10^6$ and $10^7$. Such high molecular weights were also found for the EPS of *C. m. insidiosus* (29,30). Van Alfen and associates showed the presence of 3 components: a small component of 22 kDa, a 5 MDa component, and a larger component that voided all sieving columns. The latter two components had the same composition as described by Gorin et al (10). The largest component was not able to pass through pit membranes of alfalfa stems, and the 5 MDa component was unable to pass through pit membranes of leaves, as demonstrated by the location of radiolabeled EPS components in the plant and filtration experiments using membranes with known pore diameters. Each component was able to reduce the transpiration of alfalfa cuttings when introduced in the cut stem, causing wilting of the cuttings. Similar results were also obtained with *C. m. michiganensis* EPS introduced into tomato cuttings (31). Although the EPS of *C. m. michiganensis* produced in culture is of high molecular weight and is also able to reproduce the wilting symptoms in tomato, the mechanism through which wilting is induced is not known. Restriction of water movement by blocking the vessels may be a part of the action of EPS, but an effect at the cellular level
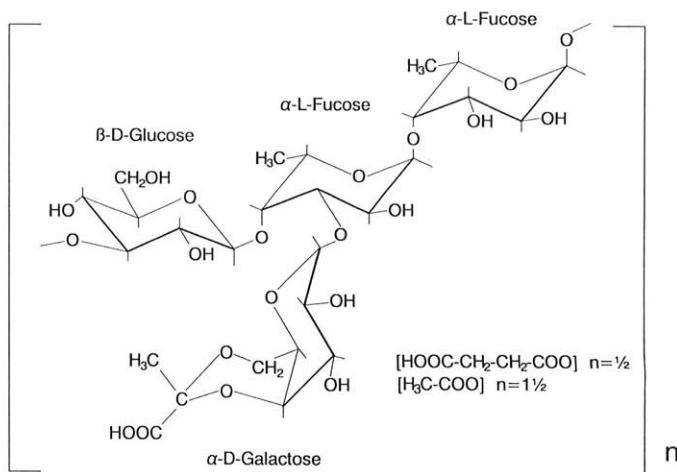
TABLE 3. Monosaccharide composition of extracellular polysaccharides (EPS) produced by *Clavibacter michiganensis* subsp. *michiganensis in planta* and in culture

| Components | EPS *in planta* cv. Moneymaker[b] | EPS *in planta* cv. Irat L-3 | EPS in culture |
|---|---|---|---|
| Fucose | 45.3 | 49.8 | 48.3 |
| Arabinose | T[c] | ··· | ··· |
| Xylose | T | ··· | ··· |
| Mannose | T | ··· | T |
| Galactose | 27.7 | 25.5 | 26.4 |
| Glucose | 27.0 | 24.7 | 25.3 |

[a] Percentage of total amount of monosaccharides detected.
[b] Samples isolated from healthy control plants showed traces of arabinose, xylose, mannose, and glucose after 10-fold concentration.
[c] Trace.



**Fig. 4.** Proposed structure of the tetrasaccharide-repeating unit of *Clavibacter michiganensis* subsp. *michiganensis* extracellular polysaccharide. The positions of the succinyl and acetyl groups are not known. The sequence of sugar residues and the anomeric configurations have to be confirmed by further studies.

may not be excluded, as shown by an inhibiting effect of the EPS on callus formation from *Lycopersicon peruvianum* protoplasts (32).

This study showed preliminary evidence that a high-molecular-weight EPS is also produced *in planta* and is similar in composition to that produced in vitro. This has also been shown for several other plant pathogenic bacteria, such as *Xanthomonas campestris* (26), *X. oryzae* (1), *Erwinia amylovora* (2), and *Pseudomonas syringae* pathovars (6,11). The results indicated that the EPS produced *in planta* is of higher molecular weight than the EPS produced in culture. This difference in size might be due to the formation of aggregates between EPS molecules and associated proteins. Van Alfen et al (29) suggested that the largest protein-containing EPS component isolated from culture filtrate of *C. m. insidiosus* was an aggregate of smaller, 5 MDa EPS components with polypeptides. In the EPS fraction isolated from culture filtrate of the strain of *C. m. michiganensis* used in this study, hardly any protein was present, and aggregates did not occur. However, the EPS fraction isolated from infected tomato plants had a considerably higher protein content, possibly resulting in the formation of aggregates with EPS molecules. The specificity of the association between protein and EPS *in planta* needs further study, as does the possible involvement of this interaction in the wilting phenomena caused by EPS.

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