Properties of the Extracellular Polysaccharides of *Clavibacter michiganense* subsp. *insidiosum* That May Affect Pathogenesis

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ABSTRACT

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The extracellular polysaccharide (EPS) of *Clavibacter* (*Corynebacterium*) *michiganense* subsp. *insidiosum* consists of three different sized components. Each of these EPS components reduced transpiration of alfalfa cuttings when introduced into the cut stem. The largest component accumulated in stems of cuttings and was unable to pass through leaf traces. This EPS component was too large to pass through pit membranes of alfalfa stems. The 5 MDa component likewise was unable to pass through leaf traces but is of a size that could allow it to pass through pit membranes of alfalfa stems. The smallest EPS component (22 kDa) passes freely through pit membranes and accumulates in leaves. EPS production was found to be regulated rather than constitutive, being produced primarily during stationary phase of growth. This implies that it may not be important in the initial events of host-pathogen interactions.

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

Vascular wilt pathogens were once thought to be the only group of microorganisms to inhabit the vascular system of plants. In recent years it has become evident that the vessels of many plants maintain populations of endophytic microorganisms (8). The existence of microorganisms that are able to live within plants without causing noticeable damage suggests that the damage caused by pathogens is not just the result of their growth or the extraction of nutrients from the plant. The biomass of such parasites is insignificant compared with that of the plant in most cases. It is also unlikely that the mere growth and presence of the parasite significantly impairs the ability of the plant to transport water because plants have excess water-conducting capacity (15). Thus, although vessels containing pathogens are probably nonfunctional, the pathogen would need to inhabit a significant proportion of a plant's vessels before water stress would result from its presence alone.

Endophytes and pathogens, while probably not differing in their abilities to grow within plants, differ in the consequence of their presence. Pathogens induce water stress of the plant. We have studied the question of how a vascular pathogen stresses its host using the system of the bacterial wilt pathogen, *Clavibacter* (*Corynebacterium*) *michiganense* subsp. *insidiosum* and its host, alfalfa (*Medicago sativa* L.). In previous work it has been shown that the water stress caused by the pathogen is the result of impaired water movement, particularly in the region between the petiole junction and the leaves (3). We have also found that the alfalfa plant is very susceptible to water stress caused by small numbers of large molecules that fall within specific size ranges

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(10,12). The extracellular polysaccharide (EPS) of *C. m.* subsp. *insidiosum* consists of molecules of three different sizes (11): the smallest (component III) is 22 kDa, component II is 5 MDa, and component I is a very large aggregrate of component II and at least nine polypeptides. All of these EPS components are of a size range that could enable them to cause water stress in alfalfa. It was the purpose of this study to determine if the EPS of this pathogen behaves within the alfalfa plant in a manner predicted from previous studies with model molecules (10,12).

MATERIALS AND METHODS

Cultures and growth conditions. Isolates of *C. m.* subsp. *insidiosum* were obtained from naturally infected alfalfa plants growing in Utah. The isolates were preserved by lyophilization; all experiments used cultures that were no more than three serial transfers from a lyophilized stock. Cultures were grown in CH medium (11) at 21 C with orbital shaking (50 rpm). Low nitrogen CH medium differed from CH medium by having only 0.02% (w/v) Casamino acids and the elimination of NH_4Cl.

Isolation of EPS. The three components of the EPS were isolated from culture fluid using previously described methods (11). To determine the time-course of EPS production in culture, Ut-1 isolate of *C. m.* subsp. *insidiosum* was grown in CH liquid medium. Time-course experiments were done with either 250 ml of media in 500-ml flasks or 500 ml of media in 1-L flasks. Aliquots were periodically removed for viable cell counts and determination of EPS composition. Bacterial concentrations were estimated from a standard curve of the relationship between colony-forming units (cfu) per millimeter and A_{550nm}. The amount of the EPS at each sampling time was determined from 5 ml of culture fluid. Bacterial cells were removed by centrifugation at 15,000 *g* for 30 min and the supernatant was added directly to a Fractogel TSK HW-75 F (MCB Manufacturing Chemists, Inc., Gibbstown, N.J.) gel filtration column (1.8 × 75 cm) equilibrated with distilled water. EPS component III was further purified by chromatography on BioGel P-60 (Bio-Rad Laboratories, Richmond, CA). Fractions were collected and dried under an air jet at 40 C. The residue in each tube was redissolved in 0.5 ml of 10 mM sodium acetate buffer, pH 5.0. Corresponding column fractions from duplicate culture flasks were combined to quantify carbohydrates by the anthrone method (9).

Conjugation of lysine to EPS. To obtain EPS components with high specific activity, uniformily labeled L-(^{14}C)-lysine (New England Nuclear, Boston, MA) was conjugated to each EPS component (6). Between 150 and 400 μg/ml of each component in 10 mM phosphate buffer, pH 7.2, was treated with 2 mg of periodate at room temperature for 1 hr with shaking. The reaction was stopped by addition of 0.5 ml of 0.6 M ethylene glycol. The reaction mixtures were then dialyzed, first against 10 mM phosphate buffer, pH 7.2, and then against 0.2 M phosphate buffer, pH 7.5. Seventeen μCi of L-(^{14}C)-lysine in 0.2 M phosphate buffer was added to each component. Cyanoborohydride was added at a concentration of 14 mg per milligram of carbohyrate from a 50-mg/ml stock solution in 0.2 M phosphate buffer, pH 7.5. The reaction mixture was incubated at 40 C for 2 wk. At the end of the incubation time, the unconjugated lysine was removed by gel filtration on BioGel P-10 eluted with water. Each component was repurified and checked for changes in molecular weight by gel filtration.

Autoradiography of EPS-treated alfalfa shoots. Two-month-old greenhouse grown seedlings of alfalfa cultivar DuPuit were used to assay EPS uptake and accumulation in alfalfa shoots. Approximately 20 seedlings of uniform size and shape were selected, cuttings were taken with a razor blade, recut under filtered-degassed water, and then placed in water in a growth chamber for 2 hr at 30 C as previously described (10). After this adaptation period, the eight most vigorously transpiring cuttings were selected and transferred to solutions containing 500 μg/ml of (^{14}C)-EPS components I, II, III, or a water control. The cuttings were again placed in the growth chamber and left for 2 hr. After this time, the seedlings were removed from the test solutions and placed in water for an additional hour in the growth chamber. Transpiration was determined by test solution weight loss. Amount of radiolabel taken up by the plant was determined by volume loss from the test solution. The (^{14}C)-EPS solutions into which the cuttings had been placed were tested by gel filtration to determine if the EPS components had altered in size during the test procedure.

The cut end of the stem was sealed with melted wax and the cutting was dried for 10 days in a plant press. Autoradiography of the cuttings was done by taping each dried cutting to a sheet of Kodak X-Omat AR X-ray film (Eastman Kodak Co, Rochester, NY), placing each sheet into a film holder, and then placing the film holders in a plant press.

Wilt bioassay of EPS components using alfalfa cuttings. The bioassay procedure used was essentially the same as described above (10). The EPS components were each tested at a concentration of 500 μg/ml in filtered, degassed water. Transpiration of each cutting in water for a 2-hr period was determined. The cutting was then transferred to the test solution. Transpiration over a 2-hr period was then compared with the transpiration of the same cutting in the previous water treatment. When 8-hr transpiration studies were done, the 2-hr period used for calculations was that between 6 and 8 hr after placing the cutting in the treatment solution.

Membrane filtration of EPS components. Membrane filters mimic pit membranes and other capillary pores in the water conducting pathways of plants. These filters have proven useful in predicting the effects of macromolecules on plant water movement. The EPS components were tested as previously described (12) using nitrocellulose filters of known pore diameters. The filters were held in a stirred cell holder (Millipore Corp., Bedford, MA) that was gas pressurized to 70 kPa. Both flow rates and amounts of each EPS component that passed through the filters were determined. Solutions of each component (500 μg/ml) in water were used for these experiments. Concentrations passing through the filters were determined using the anthrone method (9).

Detection of EPS in infected plants. Antibody to component II was obtained as previously described (11). Immunoglobulin G (IgG) from the antibody preparation was isolated and conjugated to horseradish peroxidase by the procedures of Farr and Nakane (5). Using standard enzyme-linked immunosorbent assay (ELISA) procedures (2), microtiter plates were coated with IgG, then various concentrations of the sample to be tested were added to the plates and finally the IgG-horseradish peroxidase conjugate was added. Plates were read using a spectrophotometer.

Seedlings of alfalfa cultivar DuPuit were inoculated with a suspension of strain Ut-1 (1.5–2.0 × 10^9 cfu per milliliter) by the root dip method. Seven-week-old seedlings were individually uprooted and dipped into either the bacterial suspension or 0.01 M phosphate-buffered saline, pH 7.2, for 30 min. Seedlings were then replanted in a soil:peat:vermiculite (1:1:1) mix. After about 2 mo seedlings were harvested, frozen, and ground in liquid nitrogen, boiled for 1 min in 10 mM sodium acetate, pH 4.0, and then the supernatant was collected by filtration through Whatman No. 1 filter paper (Whatman Chemical Separation, Inc., Clifton, NJ). From this step, the supernatant was treated the same as culture filtrate in the procedures for EPS purification (11).

RESULTS

Bioassay of EPS components. Each of the EPS components was able to reduce transpiration by cuttings of alfalfa (Table 1). The largest component (I) reduced transpiration the most, whereas the smallest, component III, reduced transpiration the least. These data confirm those reported previously (10), that molecules as small as 20 kDa are able to significantly reduce transpiration of cuttings. The effect of the two larger molecules on transpiration was evident after a short time of exposure. After 2 hr in solutions of EPS component I, transpiration of cuttings decreased by approximately 95%. After 8 hr of exposure to component III, transpiration was reduced to about the level caused by component II after 2 hr. The relative effects of the different EPS components

on transpiration are predictable on the basis of their size (10,12). Oxidation of the sugars of the EPS and conjugation to lysine did not alter the size or the activity of the EPS components in the bioassay. Size rather than molecular structure appears to be the characteristic important in determining the effect of the EPS components on transpiration.

Location of EPS accumulation in cuttings. When the radiolabeled EPS components were introduced into stem cuttings, using the same procedures described for the transpiration bioassay, components I and II accumulated in the stems of cuttings. These components did not pass through the leaf trace. Component III on the other hand accumulated in the leaf blades and buds of alfalfa and passed freely through all capillaries present in the xylem (Fig. 1). A small amount of radiolabel present in the leaf blades of alfalfa after treatment with components I and II was also detectable. Low molecular weight compounds were detected by gel filtration in the treatment solutions and could account for this low level of radiolabel in leaves. Efforts to remove these before introduction into the cuttings greatly reduced the amount present, but did not totally eliminate them as shown by the autoradiographs. We assume that these small molecules are unconjugated ^{14}C-lysine used to label the EPS molecules.

Passage of EPS molecules through pores of known size. Membrane filtration of macromolecules was used to estimate the size of pores the EPS components will pass through and how these molecules will affect water passage through the pores. In previous studies we estimated that pit membranes in stems have pores with diameters of about 0.3 μm, whereas pit membranes in the leaf traces have pores of about 0.1 μm diameter (10). Table 2 illustrates the effects of the three EPS components on flow rate of water through pores of sizes close to those found in alfalfa. This table also indicates how much of each component is able to pass through the various pores under a pressure differential of 70 kPa bars. This differential is much greater than expected in plants, but was found to be experimentally valid in studies with alfalfa stems (4). The data of Table 2 show that component I interferes with water movement through pores the size of pit membrane pores within alfalfa stems. Component I probably accumulates in the alfalfa stems because it does not readily pass through the pit membranes pores. Component II has less of an effect than component I on the flow rate of water through pores the sizes of alfalfa pit membranes and more of it is able to pass through pores of these sizes. Component III was able to pass, unrestricted, through all pore sizes tested and had little effect on flow rate. This unrestricted movement explains the ability of this component to pass through all capillaries of the xylem.

Time-course of EPS production in culture. When EPS accumulation in the culture fluid was measured over time it was found that production of component I lagged that of component II slightly. There was a peak, reproducible in four replications, in the accumulation of component II at 115 hr followed by a decline in total amount of this component that coincided with a peak in accumulation of component I at 145 hr. Because component II aggregates to form component I, such a pattern of accumulation of the two components is not surprising. All three components of the EPS began to accumulate in the culture fluid primarily at the beginning of the stationary phase of growth. The rapid increase in accumulation of the three components at between 50 and 100 hr corresponds to the end of log phase growth of the bacterium. Figure 2 describes the relationship between changes in colony-forming units with total EPS accumulation in the culture fluid. The EPS of *C. m.* subsp. *insidiosum* appears to be regulated, with EPS production beginning at the end of log phase growth.

Because depletion of nitrogen from the culture medium could influence EPS production, we compared EPS production in high and low nitrogen media. At 48 hr, which is the beginning of stationary growth in CH medium, the total amount of EPS produced was about the same in high and low nitrogen media (Table 3). The amount of components I and II produced per cell, however, was greater at this time in low nitrogen medium than in high nitrogen medium. The data clearly show that EPS production in the low nitrogen medium, as in the high nitrogen medium, is regulated, with EPS production occurring primarily during stationary phase of growth.

Detection of EPS in infected plants. About 30 g of both infected and uninfected greenhouse grown seedlings that were not yet showing symptoms were extracted following procedures for EPS purification. The fractions from the 1 M sodium acetate washes of the DEAE-cellulose column were tested for antigenicity to component II antibody using ELISA. Comparison of the greatest dilutions that provided a positive reaction indicated that infected seedlings contained 10^5 more antigenic material than did uninfected seedlings. A positive reaction was considered any that was twice the absorbance of the blank values. When this preliminary experiment was repeated, a 10-fold difference between infected and uninfected seedlings was found.

TABLE 1. Transpiration bioassay of extracellular polysaccharide components

Sample	Assay time (hr)	
	2	8
Component I	4.2 ± 4.0[a]	nd[b]
Component II	18.4 ± 4.9	nd
Component III	59.7 ± 8.7	17.6 ± 3.1
Water	98.2 ± 8.7	76.6 ± 7.2

[a]Transpiration, percentage of same cutting in water ± S.E. All test solutions contained 500 μg/ml of extra cellular polysaccharide (EPS).
[b]nd indicates that measurements were not taken because previous studies have shown that transpiration of cuttings in solutions of large molecules does not change after about 1 hr (10).

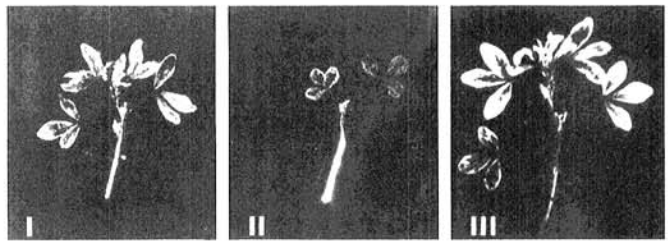

Fig. 1. Autoradiography of alfalfa cuttings after uptake by transpiration of EPS Components I, II, and III. Initial transpiration rates of cuttings in water were determined before placing the cuttings into solutions containing 500 μg/ml of each EPS component to which (^{14}C)-lysine had been conjugated. After 2 hr in EPS solutions, cuttings were placed in water again for 1 hr before drying and placing on X-ray film.

TABLE 2. Filtration of extracellular polysaccharide components through artificial membranes

EPS Tested	Filter pore dia, μm					
	Amount of EPS passing through filter[a] (%)			Flow rate % of water[b]		
	0.08	0.22	0.45	0.08	0.22	0.45
Component I	18.3 ± 2.2	48.3 ± 4.4	94.5 ± 10.6	21.0 ± 10.7	11.6 ± 1.9	54.1 ± 30.9
Component II	43.4 ± 10.2	81.0 ± 1.4	100.9 ± 0.7	23.7 ± 8.8	76.9 ± 7.4	90.5 ± 7.5
Component III	92.9 ± 2.0	87.0 ± 0.8	104.5 ± 4.2	75.9 ± 8.1	88.9 ± 2.8	80.5 ± 4.3

[a]Percentage of the 500 μg/ml of extracellular polysaccharide solution that passed through the filters was determined using the anthrone method (9). Filtration was done using a stirred cell holder pressurized to 70 kPa. Values are percentages ± S.E.
[b]Flow rate relative to that of water was determined by first measuring the flow rate of water through the filter. Values are percentages ± S.E.

DISCUSSION

The EPS of *C. m.* subsp. *insidiosum* consists of three components, one (component I) of which is a very large aggregate of the 5 MDa component (II). When added to cuttings, these two large components both reduce transpiration of alfalfa cuttings enough to cause wilting of the cuttings. Both components (Fig. 1) accumulated in stems of the cuttings. The effective sizes of the molecules prevented their movement through the pit membrane pores of leaf traces of alfalfa (12) and resulted in the reduction of water movement through these pores (Figs. 1 and 3, Table 2A). It was not possible to determine by the method used whether EPS components I and II each accumulated in different locations within the stem. Some vessels in alfalfa stems do not end within the length of stem used (14), thus allowing any EPS component to freely move through the entire length of these vessels without passing through pit membranes. This may have resulted in both components I and II being found throughout the length of the stem. We would predict that on the basis of size, component II should be able to pass through pit membranes in the stem but not the leaf trace, whereas component I should not be able to pass through pit membranes of alfalfa stems. We cannot conclude from the autoradiographs (Fig. 1) whether this prediction is correct or not. The water stress induced by components I and II is probably due to their effect on water movement through capillary pores when introduced into the plant. Substantial alterations of the molecular structure of the EPS components by lysine conjugation, without significantly altering their sizes, did not affect their bioactivity. This suggests their size is the cause of their wilt-inducing abilities.

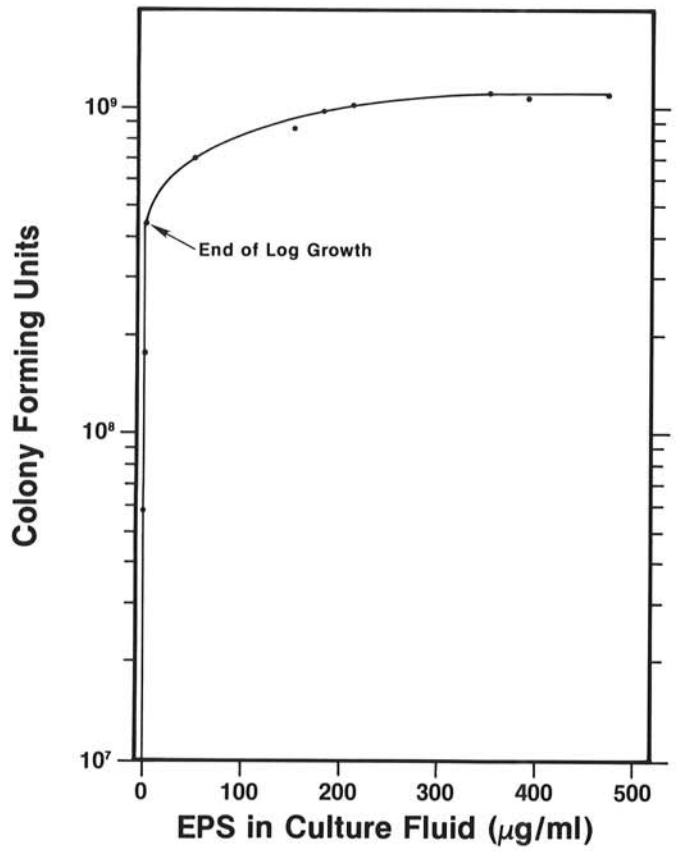

Fig. 2. Relationship between production of EPS components with numbers of colony forming units of *C. m.* subsp. *insidiosum*.

TABLE 3. Effect of nitrogen concentrations in culture media on production of extracellular polysaccharide by *Clavibacter michiganense* subsp. *insidiosum*

	High nitrogen[a]			Low nitrogen[b]		
Sampling[c] time (hr)	CFU/ml ×10^8	EPS Components[d] I & II	III	CFU/ml ×10	EPS Components I & II	III
0	0.3[e]	2.1	1.6	0.3	2.3	1.7
	0.3	2.4	1.3	0.3	2.3	1.0
48	...[f]	24.6	10.6	...	34.2	6.4
	3.0	20.9	7.8	2.2	26.7	3.6
65	3.5	35.1	10.8	2.7	50.0	7.1
	3.8	37.0	11.5	2.6	53.7	8.2
113	5.2	64.8	31.2	3.4	93.4	15.2
	5.6	76.9	30.5	3.4	93.5	13.7

[a] High nitrogen medium was the normal CH medium (11) in which the nitrogen sources are 0.2% (w/v) Casamino acids and 0.2% (w/v) NH_4Cl.
[b] Low nitrogen medium was modified CH medium in which Casamino acids were reduced to 0.02% (w/v) and NH_4Cl was eliminated.
[c] Time after start of culture. The 48-hr sampling time is at the end of log growth for cultures growing in CH medium.
[d] EPS components I and II were not separated. Numbers are μg/ml of hexose equivalents as determined by anthrone method (9).
[e] The two values for each sampling time were obtained from two separate culture flasks sampled and analyzed in parallel.
[f] Leader dots indicate that value was not determined.

Component III is much smaller than components I and II but was still able to cause water stress in alfalfa cuttings (11). This component of the EPS was able to move through all of the capillary pores of the xylem and accumulated in the leaf blades (Fig. 1). The cell wall capillaries between the vessels and evaporative surfaces of the leaf mesophyll may be where this EPS component accumulates, thereby causing water stress. Carpita et al (1) found that dextrans of about 7 kDa are unable pass through the capillaries in parenchyma cell walls. They tested five different plant species and found that the pores in the cell walls were similar in diameter. It is assumed that those of alfalfa would be similar to those tested and that a carbohydrate of 22 kDa will plug these capillaries. The effect on transpiration of plugging mesophyll capillaries would be the same as that of plugging capillaries in the xylem: reduced transpiration. This is the most likely mode of action of component III in reducing transpiration.

EPS production appears to be regulated in *C. m.* subsp. *insidiosum*. EPS is detectable in culture only with the onset of stationary phase (Figs. 2 and 3). This appears to represent

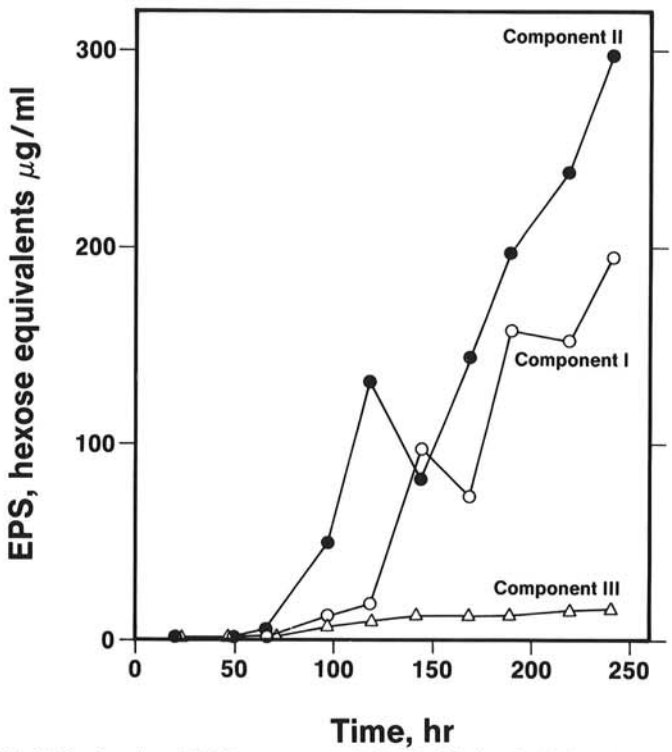

Fig. 3. Production of EPS components I, II, III with time in shake cultures of *C. m.* subsp. *insidiosum*. The EPS components were separated by gel filtration and quantitated by the anthrone method (9). Data presented are from one of four replications.

developmental regulation rather than a response to nutrient changes in the culture fluid, i.e., major increases in the ratio of carbon to nitrogen (Table 3). If EPS induction of plant water stress or protection from agglutination is important in the colonization of the plant by the pathogen, we would expect that the EPS production would be constitutive rather than produced primarily during stationary phase. Perhaps conditions in plants induce EPS production before stationary phase. On the other hand, EPS in plants may be produced only during stationary phase, as in culture, and any role it may play in host-pathogen interactions may be secondary to its role in bacterial dormancy.

Our current evidence that EPS is produced within the plant is limited. Ries and Strobel (7) reported the isolation of a molecule from diseased plants that is similar in size and composition to that of component II. We have provided preliminary evidence that antibody to component II reacts more to extracts from infected plants, isolated after the EPS purification procedure, than to extracts from uninfected plants. Clearly, additional evidence is needed that EPS components of *C. m.* subsp. *insidiosum* are present within infected plants in locations and quantities to cause the observed symptoms. The evidence that the EPS is the cause of the observed water stress in infected plants (3) is currently circumstantial. Our evidence is limited to a demonstration that the bacterium produces EPS molecules in culture that are able to reproduce both the symptoms and the mechanism of the water stress caused by the pathogen in naturally infected alfalfa (11,13).

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