A Comparison of the Quantities of Exopolysaccharide Produced by *Xanthomonas campestris* pv. *malvacearum* in Susceptible and Resistant Cotton Cotyledons During Early Stages of Infection

Margaret L. Pierce, Margaret Essenberg, and Andrew J. Mort

Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater 74078-0454.
Journal article J-6214 of the Oklahoma Agricultural Experiment Station, Oklahoma State University, Stillwater.
We thank Jean Winters for technical assistance and George Waller and Lori Thorne for performing the GLC-mass spectrometry.
Accepted for publication 22 October 1992.

ABSTRACT

Pierce, M. L., Essenberg, M., and Mort, A. J. 1993. A comparison of the quantities of exopolysaccharide produced by *Xanthomonas campestris* pv. *malvacearum* in susceptible and resistant cotton cotyledons during early stages of infection. Phytopathology 83:344-349.

Two pairs of susceptible and highly resistant lines of *Gossypium hirsutum* were compared for their influence on *Xanthomonas campestris* pv. *malvacearum* extracellular polysaccharide (EPS) production during the early stages of infection. Quantities of EPS in intercellular washing fluid from inoculated cotton cotyledons were measured and related to the bacterial population densities in those same cotyledons. The presence of pyruvylated mannose in EPS from *X. c. malvacearum*, as in xanthan gum from *X. c. campestris*, allowed accurate quantitation of EPS without interference from polysaccharides of plant origin. During the period when bacterial population densities were similar in susceptible and resistant cotyledons, the quantity of EPS present per bacterium was equal. Thus, no evidence was found for suppression by the resistant lines of EPS production by the bacteria. We conclude that perturbation in bacterial EPS production during the initial 48 h after infection is not required for the hypersensitive response of cotton foliar tissue to occur.

Additional keyword: bacterial blight.

Exopolysaccharide (EPS) biosynthesis of phytopathogenic bacteria is under complex regulation. In some cases, including *Xanthomonas campestris* pv. *campestris*, one of the regulatory systems appears to be a "pathogenicity regulon" also involving extracellular enzymes (7,9). Molecular genetic studies are providing evidence of multiple regulatory systems and the presence of both positive and negative regulation of the synthesis of individual EPSs (7). Complex effects of the environment on EPS production raise the question whether the apparent abundance of slime in compatible versus incompatible plant/bacteria interactions reflects mostly differences in numbers of bacteria or also differences in quantities of EPS per bacterium. Quantitative analysis of EPS from infected plants as a function of bacterial population is lacking. The purpose of this study was to determine quantities of EPS per bacterium in the cotton/*X. c. malvacearum* host/pathogen system, comparing for the same strain of *X. c. malvacearum* the effects of susceptible versus highly resistant cotton lines during the first 24–48 h of infection when the bacteria are growing logarithmically and differential resistance is being established.

Bacterial blight of cotton (*Gossypium hirsutum* L.), caused by *X. c. malvacearum*, is an angular leaf spot disease. Bacterial population densities in lesions from susceptible leaves exceed 10^8 colony-forming units (cfu) per square centimeter, and lesions appear as dark, water-soaked spots often limited by minor veins. In highly resistant cotton lines, a local hypersensitive response (HR) is accompanied by approximately 1–5% as much bacterial growth, and the infection sites do not become congested with fluid. The exocellular slimes produced by a number of *Xanthomonas* pathovars, including *malvacearum*, are composed of the same sugars as the slime of *X. c. campestris* (15,26,33), called xanthan gum, which has been well characterized. Xanthan consists of a celluloselike backbone of (1→4)-linked β-glucosyl residues, with a sidechain of mannosylglucuronosylmannose attached to every other glucosyl residue, creating a five-residue repeating unit (18,22). The mannosyl residue proximal to the backbone is acetylated, and the terminal mannose is frequently pyruvylated (18,22). Polysaccharide of the same sugar composition was extracted from plants infected with *X. c. campestris* (34).

Cytological methods for the estimation of EPS quantities have not been satisfactory, in part because the fibrillar material seen in electron micrographs of bacterial colonies growing in leaves possibly has multiple origins. In addition to bacterial EPS, the presence of pectic fragments within and around bacterial colonies in planta has been inferred (2,11). The possibility of variable hydration of fibrillar material within bacterial colonies also makes quantitative assessments of EPS based on micrographs uncertain.

Intercellular washing fluids (IWFs) efficiently extracted EPS during early stages of infection. The results of this study show that during the logarithmic growth phase of *X. c. malvacearum* in planta, equal quantities of EPS per bacterium are produced in resistant and susceptible cotton lines. Our results give no support to the hypothesis that EPS production prevents the HR from developing, since we observed no more EPS in compatible than in incompatible interactions.

MATERIALS AND METHODS

Plants. The cotton lines used were bacterial blight-susceptible cultivar Ac 44, which possesses no known major genes for resistance to *X. c. malvacearum* (3), a related highly resistant line OK 1.2 (30), and near-isogenic fully susceptible WbM (4.0) and highly resistant WbM (0.0) lines (13). Although the resistance gene content of the two resistant lines is not definitively known, each of these lines possesses more than one of the following determinants of resistance: major genes B_2, B_3, b_7, B_N, and the polygenic complex B_{Sm} (13,30). The bacterial strains used in this study possess avirulence genes that determine incompatibility toward each of these resistance genes, with the possible exception of B_3 (R. A. Samad and M. Essenberg, *unpublished results*).

Plants were grown in 15-cm-diameter clay pots in Jiffy Mix-Plus in a Conviron E15 growth chamber with a 14-h light/10-h dark cycle. The middle 10 h of the photoperiod was at the highest photosynthetic photon flux density, which averaged approximately 5×10^2 $\mu mol \cdot s^{-1} \cdot m^{-2}$ at plant height. Light intensity increased and decreased in stages (four stages for both the incandescent and fluorescent lamps) during the 2-h dawn and

dusk periods. Air temperature was 30 C during 8 h of the high-light period and 19 C during 8 h of the dark period. The temperature increased or decreased linearly during the 4-h transition periods. Cotyledons were inoculated as described previously (30) between 11 and 21 days after planting.

Bacterial cultures. *X. c. malvacearum* strain 36 was used for preparing EPS from cultures. It is a spontaneous streptomycin-resistant mutant of high virulence derived from our race 3 field isolate (13). *X. c. malvacearum* strain 3631, a single-colony isolate from strain 36, was used for the experiments in which cotton plants were inoculated. It was maintained as previously described (30) and cultured in a 3-(N-morpholino)propanesulfonic acid (MOPS)-buffered salts medium. The MOPS medium was modified from that described (24) by omitting NaCl and supplementing with 0.2% Difco vitamin-free Casamino Acids as carbon source. Actively growing cultures were diluted with sterile water saturated with calcium carbonate to give the inoculum concentrations specified, except for the highest inoculum concentration of 2×10^8 colony-forming units (cfu) per milliliter, for which the bacteria were pelleted by centrifugation at 1,700 *g* for 10 min at room temperature and resuspended in calcium carbonate-saturated water.

Bacterial populations. Bacterial population densities were determined from homogenized leaf disks as previously described (23). For a set of cotyledons (three or five cotyledons per set) from which intercellular washing fluid (IWF) would be prepared, one 0.33-cm^2 leaf disk was taken from each cotyledon, and the set of leaf disks was homogenized together. Population densities were determined shortly after inoculation and again at the time of harvest. Initial population densities were not always taken from the same cotyledons that were harvested for IWF preparation. EPS quantities are expressed per bacterial population at the time of harvest. For the growth curves presented in Figure 2, individual disks from three cotyledons were combined for a determination of bacterial number, and two such determinations were averaged to provide each data point. Estimates of variability are standard error.

EPS from bacterial cultures. *X. c. malvacearum* strain 36 was cultured in Ornston-Stanier salts medium (27) plus 10 mM sucrose until early-stationary phase. EPS was prepared from culture supernatant fractions essentially by a previously described method (12). In brief, following removal of material that precipitated with cold 30% ethanol (final concentration), xanthan was precipitated three times with cold 75% ethanol. Metal ions were removed by passing the preparation over cation exchange resin (Dowex 50W-8X, H^+ form). Xanthan (grade II) (Sigma, St. Louis, MO) from *X. c. campestris* was dialyzed overnight against deionized water and lyophilized before being analyzed.

***X. c. malvacearum* EPS from cotyledonary IWF.** For every planting, a single inoculum of *X. c. malvacearum* was used for a resistant and susceptible pair of lines. Control cotyledons were prepared for each experiment; they were either not inoculated or they were mock-inoculated with $CaCO_3$-saturated water. Two IWF samples for each treatment/cultivar combination were independently prepared for each experiment. The method of Klement (19) for obtaining IWF was adapted for cotton cotyledons. Sets of three or five cotyledons were harvested; a 0.33-cm^2 disk was removed from each, pooled, and used to obtain fresh weight per square centimeter and bacterial population density as described above. After the midveins were removed and thin slivers parallel to the midveins were removed from the edges, the cotyledons were infiltrated with water, blotted carefully, placed cut edge down in a centrifuge tube above a grid, and centrifuged at 1,100 *g* for 15 min at 4 C. The cotyledons were then removed; IWF was withdrawn and its yield was determined by weighing. In most experiments a 0.2-ml rinse of the tube was added to the IWF. Bacteria were removed from the IWF by centrifugation at 12,000 *g* in a microcentrifuge for 10 min at 4 C. In some experiments, the IWF was boiled for 5 min and in some experiments a drop of toluene was added to kill remaining bacteria. The supernatant fractions were dialyzed overnight against deionized water at 4 C and stored frozen until aliquots were taken for sugar analysis.

***X. c. malvacearum* EPS from cotyledonary homogenates vs. IWF.** In an experiment designed to test the recovery of EPS in IWF compared to extracts of homogenized tissue, IWF was prepared a little differently from the method described above, to allow for paired samples of IWF and cotyledonary homogenate. For each pair of samples from a given treatment/cultivar combination, a set of four cotyledons was harvested. A pair of 5-cm^2 disks was cut from each cotyledon, and one set of four disks (one from each cotyledon) was used for IWF preparation, while the other set was homogenized. In this case, since the total leaf area used for IWF preparation was considerably smaller than in the method described above, the surface water remaining after infiltrating the disks was not blotted, and it provided enough liquid for adequate transfer of IWF from the centrifuge tube. Sets of disks for homogenizing were frozen and pulverized in liquid nitrogen. The samples were thawed in 1 ml of sterile water (25 C), containing 0.4% sodium dithionite to prevent oxidation of phenolics, and extracted by a further 1-min grinding with the pestle. Insoluble material was removed by centrifugation at 12,000 *g* in a microcentrifuge for 10 min at 4 C. The pellet was re-extracted with 0.5 ml of water; centrifugation was repeated and the supernatant fractions were combined, weighed, dialyzed, and frozen until aliquots were removed for sugar analysis.

EPS analysis. Aliquots of IWF or other samples containing from 30 to 300 μg of polysaccharide, plus 20 nmol inositol as internal standard, were placed in screw-capped glass vials (caps were Telfon-lined) and lyophilized. Methanolysis and derivatization to form the trimethylsilylated methyl glycosides were performed by a modification of the method of Chaplin (6) as described previously (20). Derivatized samples were dissolved in 30–300 μl of iso-octane, and a 1-μL aliquot was injected into a fused-silica capillary column (30 m $\times$ 0.25 mm i.d., Durabond-1 liquid phase, J & W Scientific, Inc., Rancho Cordova, CA) installed in a Varian 3300 gas-liquid chromatograph equipped with an on-column injector, helium carrier-gas, a flame-ionization detector, and a Varian 4290 integrator. The sample was injected at 105 C; after 1 min the temperature was raised at 10 degrees min^{-1} to 160 C and held for 4 min before being raised at 0.5 degrees min^{-1} to 170 C, then increased at 2 degrees min^{-1} to 204 C, then increased at 10 degrees min^{-1} to 230 C, where it was held for 20 min before being reset to 105 C.

To establish a method for measuring *X. c. malvacearum* EPS, the sugar composition of its EPS prepared from cultures was compared with that of commercial EPS (xanthan gum) from *X. c. campestris*. A repeating unit from xanthan has the following molar composition (18,22): glc_2, $glcA_1$, man_2. The mannose residues contain substituents: The residue proximal to the backbone is acetylated, and the terminal mannose of the sidechain is frequently linked to pyruvate as an acetal. The gas-liquid chromatography (GLC) results showed the same peaks for the two polysaccharides (data not shown). The peaks for glucose, glucuronic acid, and mannose were identified by comparison of GLC retention times and relative integrated areas of the multiple methyl glycosides derived from one sugar to those of pure standards (Pfanstiehl Laboratories, Inc., Waukegan, IL). In addition, the chromatograms contained one more prominant peak, eluting between the peaks for mannose and glucose, which did not appear in chromatograms of a mixture of sugar standards. Upon subjecting a methanolyzed and trimethylsilylated sample of xanthan to gas-liquid chromatography mass spectrometry (LKB 2091), the unknown peak produced a fragment at m/z 363, diagnostic for a pyruvylated hexose (pyruvylated mannose in the case of xanthan). This designation was based on the study of a wide range of pyruvylated polysaccharides by Dudman and Lacey (10) in which they found that, unlike acid hydrolysis, methanolysis (as used here) leaves pyruvate acetals predominantly intact, thus releasing the methyl pyruvate esters of the pyruvylated methyl glycosides. All of the pyruvylated hexoses they tested after methanolysis and trimethylsilylation gave a characteristic fragment upon GLC mass spectrometry at m/z 363.

Integrated areas of the various peaks were converted to nanomoles of the particular sugar residues by reference to the

relative responses of sugar standards and the internal standard, except for pyruvylated mannose, for which only relative amounts contained in different samples are compared.

We corrected for the variation in efficiency of IWF recovery in the following way. Volumes of IWF yielded per square centimeter for the individual samples were normalized to the highest yield within an experiment. Before expressing the results of sugar analysis on a 'per square centimeter' basis, the sugar quantities were divided by the relative yield of IWF for the sample. The recovery of IWF was generally good, and averaged 9.3 ± 0.4 μl/cm^2. From tests in which cotyledons were weighed, then wetted, blotted, and weighed again, we estimate no more than 0.5 μl/cm^2 of the IWF came from residual surface water. An IWF yield of 9.3 μl/cm^2 compares favorably with an estimate of intercellular volume per square centimeter. This value is obtained by computing (initial cfu/cm^2)/(inoculated cfu/ml) from experiments in which leaf disks are harvested within a few minutes after bacterial inoculation. In one such experiment, WbM cotyledons averaged 8.9 ± 0.7 μl/cm^2, and in another, OK 1.2 and Ac 44 cotyledons averaged 11.4 ± 0.6 μl/cm^2.

Bacterial production of EPS was compared between samples by preparing IWF from a known amount of cotyledonary tissue, expressing results of sugar analysis on a per square centimeter basis, then subtracting background levels from uninfected cotyledons for glc, glcA, and man. Because cotton does not make pyruvylated mannose, the calculations based on this sugar did not require subtraction of background levels. The bacterial populations, determined by grinding tissue disks and plating out bacteria from the same cotyledons that yielded the IWF samples, were also expressed per square centimeter. The relative amounts of EPS per bacterium were then compared between samples. Estimates of variability are standard error unless otherwise stated.

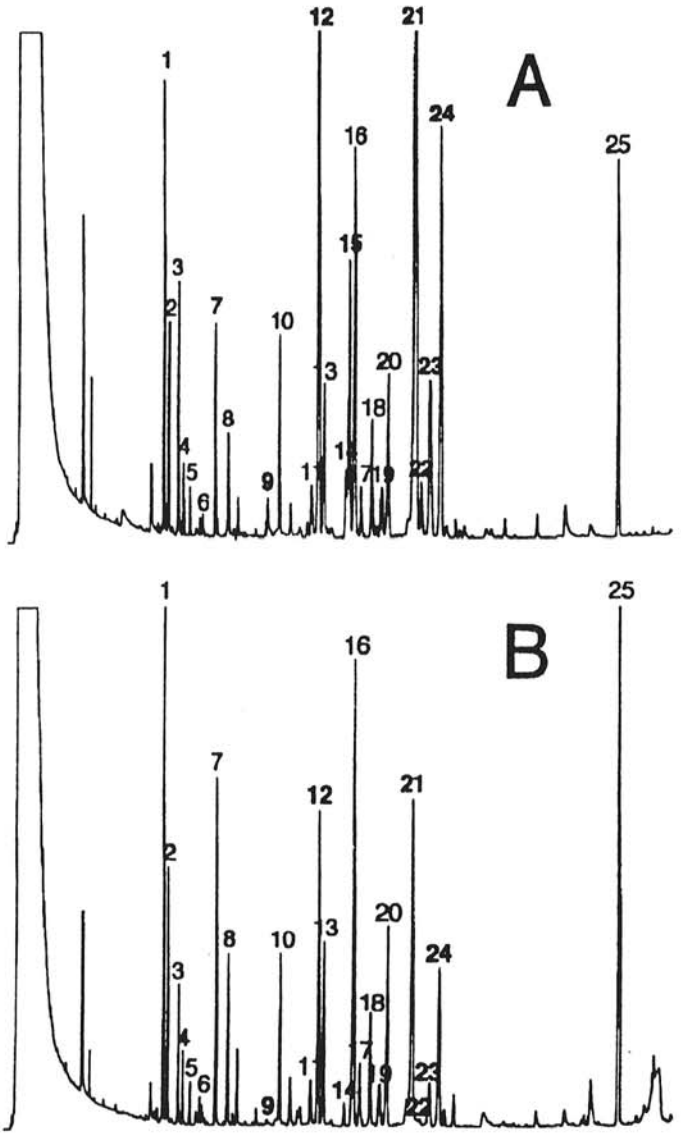

Fig. 1. Gas-liquid chromatogram tracings of the trimethylsilylated methyl glycosides obtained from dialyzed IWF prepared from **A,** inoculated and, **B,** mock-inoculated cotyledons of Ac 44. The inoculum concentration of *Xanthomonas campestris* pv. *malvacearum* was 1.9 × 10^8 cfu/ml; cotyledons were harvested for IWF preparation 23 h after inoculation. The aliquots of IWF taken for methanolysis represented 0.5 g fresh weight of cotyledon. For A, 0.5% of the sample was injected onto the column; for B, 1% of the sample was injected. Peaks for the sugars characteristic of *X. c. malvacearum* extracellular polysaccharide are numbered in boldface. Peaks are numbered as follows: Ara (1 and 2), Rha (3 and 4), Fuc (5 and 6), Xyl (7 and 8), GlcA (**9**, **22,** and **23**), GalA (10, 11, 18, and 19), Man (**12** and **14**), Gal (13, 16, 17, and 20), Pyr-Man (**15**), Glc (**21** and **24**), and I.S. (25).

RESULTS

Quantitative analysis of *X. c. malvacearum* EPS. Figure 1 shows chromatograms of IWF preparations from *X. c. malvacearum*-inoculated cotyledons and from control cotyledons. Peaks representing the sugars of EPS, mannose (peaks 12 and 14), pyruvylated mannose (peak 15), glucose (peaks 21 and 24), and glucuronic acid (peaks 9, 22, and 23), were more prominant in the chromatograms representing IWF from inoculated cotyledons than from control cotyledons. The peak from pyruvylated mannose (peak 15) was not present in the control. The integrated area of the internal standard (peak 25) in Figure 1A (inoculated) is 0.49 of that in Figure 1B (uninoculated). The chromatograms would represent the same amount of cotyledon if the peaks in 1A were divided by 0.49. Thus, the sugars of EPS were much more abundant in the sample from the inoculated cotyledons. Certain other sugars, e.g., rhamnose (peaks 3 and 4) and galacturonic acid (peaks 10, 11, 18, and 19) were also more abundant in the sample from the inoculated cotyledons.

Calculations of EPS quantities in IWF preparations were based on the peaks from all four of the sugars of EPS in order to maximize the accuracy of our determinations. Using pyruvylated mannose as a basis was valid because the degree of pyruvylation was the same in susceptible and resistant cotyledons. A sensitive indicator of degree of pyruvylation is the ratio of mannose to pyruvylated mannose because a decrease in one of those residues would be accompanied by a compensating increase in the other, since the sugar composition of bacterial EPS repeat units is normally invariant. We compared the integrated areas from mannose (after correcting for background levels) to pyruvylated mannose, and found for six independent determinations of each type, the ratio in the susceptible cotyledons was 2.38 ± 0.11, and in the resistant cotyledons it was 2.58 ± 0.19.

The susceptible and resistant lines did not differ in background (control) levels of glucose, glucuronic acid, and mannose. In nanomoles per square centimeter, the background levels were, in the susceptible versus the resistant lines, 2.1 ± 0.6 vs. 2.2 ± 0.5 for glucose, 0.37 ± 0.05 vs. 0.31 ± 0.02 for glucuronic acid, and 1.1 ± 0.3 vs. 1.2 ± 0.3 for mannose. On average, the background levels represented 22% of the levels in the inoculated samples.

To evaluate how well IWF rinsed EPS from the intercellular spaces, EPS was determined in paired samples of IWF vs. supernatant from tissue homogenates. The eight inoculated samples (four pairs) for this experiment were taken 3 days after inoculation with an inoculum concentration of 7 × 10^6 cfu/ml. On day three the population of *X. c. malvacearum* averaged 1.5 × 10^8 cfu/cm^2 in the susceptible and 0.2 × 10^8 cfu/cm^2 in the resistant line. After subtracting the appropriate background levels of sugars, the resulting quantities of EPS-derived sugars per square centimeter were compared between paired samples of IWF (numerator) and tissue homogenate (denominator). There were four ratios for each of the four sugars, and these 16 ratios averaged 1.1 ± 0.1. We concluded from this experiment that EPS was well extracted into IWF by our procedure.

Comparison of quantities of *X. c. malvacearum* EPS in susceptible and resistant cotyledons. For this comparison we considered what would be the best time after inoculation to prepare samples. The harvest should be late enough that sufficient bacterial growth and elaboration of EPS had occurred so that correction for plant-derived sugars would not be too large. Yet the harvest should be early enough to give a good yield of IWF. In preliminary experiments we had tried preparing IWF from advanced, water-soaked lesions, and found the yield of IWF was low. We settled on times when susceptible and resistant lines still had nearly equal populations of *X. c. malvacearum*, but as late as possible in the logarithmic phase of bacterial growth. At this time there would be no influence of growth phase per se on the comparison. In Figure 2, growth curves of *X. c. malvacearum* in WbM (4.0) (susceptible) and WbM(0.0) (resistant) are compared for an inoculum concentration of 4.9×10^6 cfu/ml. The times at which samples were harvested for IWF preparation (39–41 h after inoculation) and the bacterial populations present are indicated by circles. At the higher inoculum concentration of 1.9×10^8 cfu/ml, samples were prepared 20–23 h after inoculation. Bacterial populations in the cotyledons at the time of harvest are included in Table 1.

Figure 3 shows absolute amounts of EPS-derived sugars in nanomoles per 10^9 bacteria determined in one experiment with WbM(4.0) (susceptible) and WbM(0.0) (resistant) cotyledons inoculated with 5.5×10^6 cfu/ml. Pyruvylated mannose is not included here since its absolute amounts were not determined for the technical reason explained above. Sugar analysis of commercial xanthan gum gave an observed ratio of glucose/glucuronic acid of 1.7. In Figure 3, the ratio of glucose/glucuronic acid for the susceptible line is 1.77. The resistant line, however, contained relatively more glucuronic acid than was expected, suggesting that in inoculated resistant cotton, another glucuronic acid-containing polymer accumulates in addition to bacterial EPS. The relatively high levels of glucuronic acid in the resistant line were not just a feature of this experiment; they occurred, and to a similar extent, in all three of the experiments summarized in Table 1. Additional evidence from the chromatograms suggested accumulation of a glucuronoarabinogalactan-protein in the IWF from infected resistant cotyledons. We observed four- to fivefold increases above background levels in arabinose and galactose in samples from inoculated resistant cotton specifically. Arabinogalactan-proteins are known to contain glucuronic acid at variable, but significant, levels. For example, the arabinogalactan-proteins secreted by suspension-cultured rose cells contain glucuronic acid at 6–7 mole percent of the glycosyl residues (21). In Figure 3, the glucuronic acid in the resistant line in excess of that in the susceptible line was 4% of the combined increase in arabinose plus galactose.

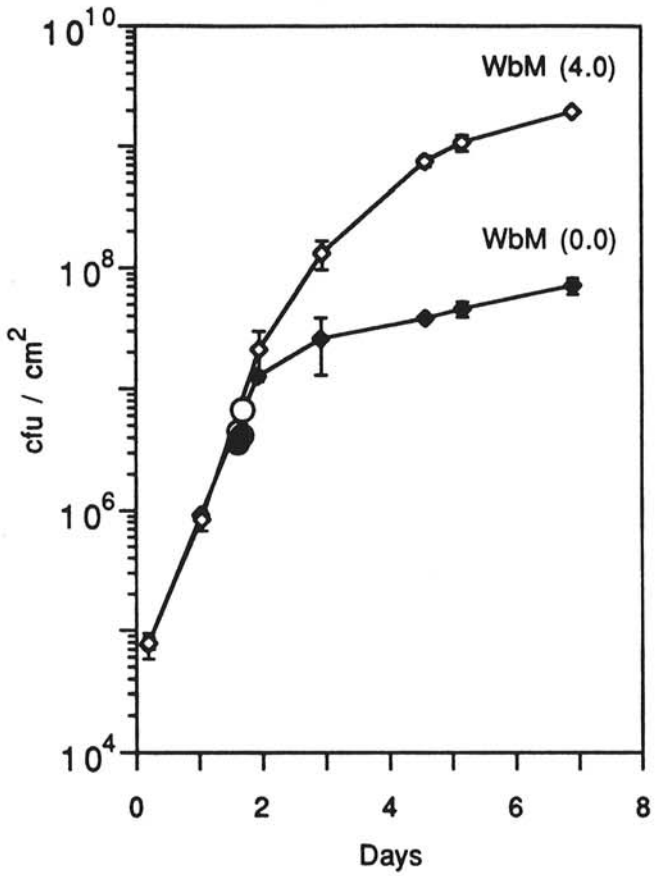

Fig. 2. Multiplication of *Xanthomonas campestris* pv. *malvacearum* in cotyledons of highly resistant WbM (0.0) (◆) and susceptible WbM (4.0) (◇) cotton lines. Inoculum concentration was 4.9×10^6 cfu/ml. For each curve, growth was followed in four cotyledons; each data point is the mean and standard error of determinations from two of the four cotyledons. The larger symbols, closed (WbM [0.0]) and open (WbM [4.0]) circles, show the bacterial populations and times of harvest of cotyledons from which IWF was prepared. The two IWF samples from each line were pooled for the analysis whose results are given in the middle experiment in Table 1.

The data in Figure 3 allow an estimation of the mass of EPS relative to bacterial mass. Based on the quantity of glucuronic acid in the susceptible line as an estimate of the nanomoles per 10^9 bacteria of the EPS repeating unit and the estimated weight of a repeating unit, the dry weight of xanthan gum was calculated to have been 380 μg per billion bacteria, an amount equal to 1.4 times the dry weight of a billion average *E. coli* cells (page 4 in [25]).

In Figure 3, the amounts of each sugar in the susceptible compared to the resistant line give a separate estimate of the relative amounts of EPS that the bacteria were producing in the two lines. *X. c. malvacearum* in the susceptible line was not producing more EPS than in the resistant line.

Table 1 summarizes the results from three experiments. Values based on pyruvylated mannose are probably the most accurate since its presence reflected only EPS, but each sugar provides

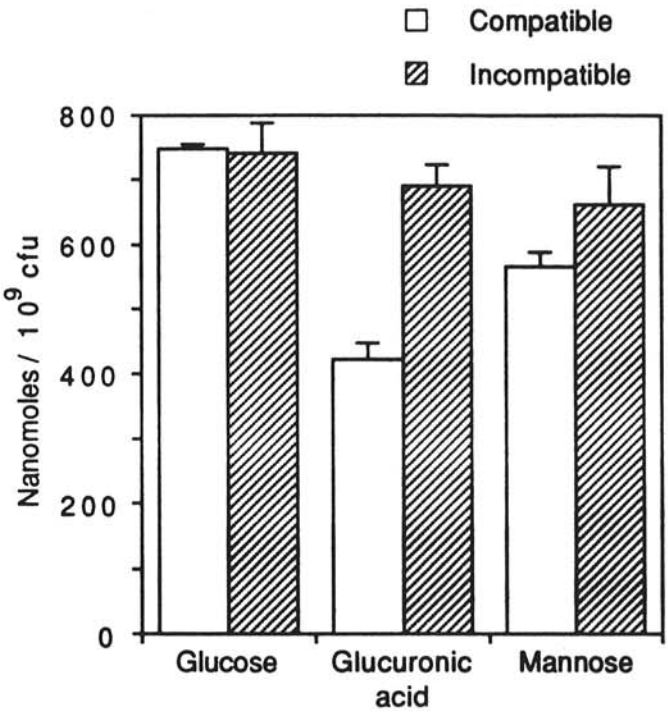

Fig. 3. A comparison between compatible and incompatible interactions in nanomoles of EPS-derived sugars in dialyzed IWF per billion *Xanthomonas campestris* pv. *malvacearum* present in the cotyledons at time of harvest. Plotted are the means and standard errors for two sets of three cotyledons for each line, WbM (4.0) (compatible, open bars) and WbM (0.0) (incompatible, shaded bars). Background levels of the sugars present in IWF from uninoculated controls were subtracted. Inoculum concentration was 5.5×10^6 cfu/ml, and harvest for IWF preparation was 44–50 h after inoculation.

an estimate of the relative amounts of EPS produced in the two different environments. A ratio of >1 would have indicated more EPS per bacterium in compatible (susceptible) than in incompatible (resistant) interactions. Obviously, that was not the case. A one-tailed t test did not support the hypothesis that the true ratio is greater than one ($P = 0.74$). In fact, the ratios are very close to one, especially those based on pyruvylated mannose. The average ratio from Table 1, excluding glucuronic acid values (for the reason described above), was 0.98 ± 0.03. We conclude that the amounts of EPS accumulated per bacterium in susceptible and resistant cotton cotyledons during the early stages of attack by *X. c. malvacearum* are the same.

DISCUSSION

This is the first report of bacterial EPS quantities from infected susceptible and resistant lines presented as a function of the bacterial population densities that were present. We can conclude that the early resistant response, i.e., initial recognition events, hypersensitive electrolyte leakage, and host cell collapse, did not interfere with *X. c. malvacearum*'s ability to produce EPS. Most likely, the environmental signals that differ between compatible and incompatible interactions do not affect bacterial production of EPS. Although positive and negative regulatory genes affecting EPS synthesis by *X. c. campestris* have been cloned recently (28,35), the external signals for these genes are not yet known.

We further conclude that bacterial EPS is probably not a factor in preventing resistant responses to *X. c. malvacearum*, since the HR occurs in the resistant lines in spite of quantities of EPS equal to those present in the susceptible lines. The EPS measured was essentially all produced in planta, since centrifugation of *X. c. malvacearum* to prepare the inoculum leaves the EPS in the supernatant fraction. It is unlikely that there was a difference in EPS/cfu at a stage in the interactions earlier than when we sampled, as that would necessitate different patterns of EPS accumulation as a function of time in compatible versus incompatible interactions, patterns that out of coincidence intersected at the times we sampled. Congruent with our results, Brown and Mansfield (4) found no differential contact between compatible and incompatible cells of *P. s. phaseolicola* and the host mesophyll cell walls and concluded that something other than physical contact per se was required for elicitation of resistant reactions.

At the beginning of this study we had thought that, if EPS/cfu were greater in compatible than in incompatible interactions, it could help explain the exceptional lack of close-fitting envelopment of compatible bacteria in cotton leaves found in earlier studies (1,5). The enveloping films are considered to result from condensation of fibrillar and/or amorphous material at the retreating air/water interface as the water in which bacteria were infiltrated into leaves is transpired (17). Our rationale was that more rapid production of EPS might maintain the air/water interface at a distance from compatible bacteria. Enveloping fibrillar material has not been shown to have a role in plant defense, but we were nevertheless curious about its apparent association with incompatible cotton/*X. c. malvacearum* interactions. From this study, EPS does not seem to be a factor controlling envelopment, and recently, work with plants grown in different growth chambers with higher humidity than in earlier studies has demonstrated a less-than-absolute difference in envelopment between compatible and incompatible interactions. Using several races of *X. c. malvacearum*, the frequency of envelopment of bacterial micro-colonies was compared between the highly resistant line AcIm and a group of other lines including fully susceptible Ac 44 plus three other congenic lines each containing only one resistance gene. In samples taken 4 h after inoculation, 36% of more than 200 bacterial micro-colonies observed in AcIm were enveloped, whereas in the other lines, few bacterial groups were enveloped in either compatible or incompatible interactions: 8% of more than 200 micro-colonies (B. Liu, P. Richardson, M. Essenberg, and M. Pierce, *unpublished observations*). Differences in probability of envelopment may be due to differences in activity of cell-wall degrading enzymes rather than any differences in production of EPS.

Considerable evidence supports a role for EPS in lesion development during the water-soaking phase of infection. Rudolph et al (31) summarized how EPS may contribute. Water-soaking is seen in compatible interactions after the logarithmic growth phase of *X. c. malvacearum* in planta, i.e., several days later than the sampling times in this project. In studies of advanced lesions induced by *Pseudomonas* pathovars, much lower quantities of EPS were obtained from incompatible compared to compatible interactions (29). Among compatible interactions, less EPS was detected in bean leaves by *P. syringae* pv. *syringae*, which did not produce water-soaking symptoms, compared to other pathovars in their hosts, in which water-soaking was produced (14). Quantities of *P. s. phaseolicola* EPS detected in infected bean leaves correlated with the development of water-soaking symptoms (16). Also, Rudolph and Mendgen (32) noted from an electron microscopic study that *P. s. phaseolicola* cells were closer together in colonies in resistant bean leaves than in susceptible ones, whether due to differences in degree of hydration of EPS and/or in amount of EPS per bacterium is unknown. Quantitation of EPS per bacterium at these later stages of infections would help us understand the water-soaking process.

Research on a set of chromosomal transposon mutants in the cluster of genes responsible for EPS production in *Erwinia stewartii* has shown that, although these mutants caused varying degrees of water-soaking in maize seedlings, all were impaired relative to the parental strain (8). The lesions induced by the mutants were fewer and smaller, and became necrotic sooner than normal, which is what one would expect if EPS contributes significantly to the fluid-holding capacity of the lesion and thereby to the growth of the bacteria (8). Fluid absorption in water-soaked lesions may be by both EPS and loosened host cell wall polymers. Benhamou (2) showed convincingly, with specific gold-conjugated probes, that for *Clavibacter michiganensis* subsp. *michiganensis* in tomato leaves, pectinlike polymers and hydroxyproline-rich glycoproteins were located around bacterial colonies and between

TABLE 1. *Xanthomonas campestris* pv. *malvacearum* exopolysaccharide (EPS)-derived sugars (nanomoles per billion bacteria) in dialyzed intercellular washing fluids from inoculated cotton cotyledons, expressed as ratios of compatible over incompatible levels

Lines	Phenotype[a]	Harvest time (h after inoculation)	Population density of *X. c. malvacearum* at harvest (cfu/cm^2)	EPS per bacterium in S/EPS per bacterium in R			
				Glucose	Glucuronic acid	Mannose	Pyruvylated mannose
Ac 44[b]	S	21–23	$(1.5 \pm 0.3) \times 10^7$	0.97	0.67	1.18	0.99
OK 1.2[b]	R	20–22	$(1.2 \pm 0.1) \times 10^7$				
WbM (4.0)[c]	S	39–41	$(5.6 \pm 1.1) \times 10^6$	0.93	0.59	0.82	1.05
WbM (0.0)[c]	R	39–41	$(3.9 \pm 0.3) \times 10^6$				
WbM (4.0)[d]	S	46–50	$(2.4 \pm 1.1) \times 10^7$	1.01	0.61	0.86	0.98
WbM (0.0)[d]	R	44–49	$(0.44 \pm 0.05) \times 10^7$				

[a] S = susceptible; R = resistant.
[b] Inoculum concentration of *X. c. malvacearum* was 1.9×10^8 cfu/ml.
[c] Inoculum concentration was 4.9×10^6 cfu/ml; these samples are from the experiment presented in Figure 2.
[d] Inoculum concentration was 5.5×10^6 cfu/ml; these samples are from the experiment presented in Figure 3.

bacterial cells in the intercellular spaces. In contrast, the EPS, visualized by labeling its *N*-acetylgalactosamine residues with a gold-conjugated lectin, was confined to a smaller radius surrounding the bacterial cells.

Our measurements of EPS in IWFs appear to have been quantitative when compared to measurements in extracts from cotyledonary homogenates and had the advantage of less interference from plant-derived polysaccharides. The background levels of glucose, mannose, and glucuronic acid were about eightfold higher in extracts from homogenates compared to IWFs. Our IWF method was appropriate to the early stages of infection but not for the water-soaking stage due to the increased viscosity of the fluid in the lesions. At the water-soaking stage, leaf homogenates would be appropriate for analysis of EPS. Comparative analysis based on pyruvylated mannose, as used in this study, would increase the feasibility. Fortuitously, *X. c. malvacearum* EPS contains a relatively high degree of pyruvylation. Orentas et al (26) showed that EPS from *X. c. malvacearum* contained one of the highest levels of pyruvate of the xanthomonads surveyed.

LITERATURE CITED

1. Al-Mousawi, A. H., Richardson, P. E., Essenberg, M., and Johnson, W. M. 1982. Ultrastructural studies of a compatible interaction between *Xanthomonas campestris* pv. *malvacearum* and cotton. Phytopathology 72:1222-1230.
2. Benhamou, N. 1991. Cell surface interactions between tomato and *Clavibacter michiganense* subsp. *michiganense*: Localization of some polysaccharides and hydroxyproline-rich glycoproteins in infected host leaf tissues. Physiol. Mol. Plant Pathol. 38:15-38.
3. Brinkerhoff, L. A. 1963. Variability of *Xanthomonas malvacearum*: The cotton bacterial blight pathogen. Okla. Agric. Exp. Stn. Tech. Bull. T 98:1-95.
4. Brown, I. R., and Mansfield, J. W. 1988. An ultrastructural study, including cytochemistry and quantitative analyses, of the interactions between pseudomonads and leaves of *Phaseolus vulgaris* L. Physiol. Mol. Plant Pathol. 33:351-376.
5. Cason, E. T., Jr., Richardson, P. E., Essenberg, M. K., Brinkerhoff, L. A., Johnson, W. M., and Venere, R. J. 1978. Ultrastructural cell wall alterations in immune cotton leaves inoculated with *Xanthomonas malvacearum*. Phytopathology 68:1015-1021.
6. Chaplin, M. F. 1982. A rapid and sensitive method for the analysis of carbohydrate components in glycoproteins using gas-liquid chromatography. Anal. Biochem. 125:336-341.
7. Coplin, D. L., and Cook, D. 1990. Molecular genetics of extracellular polysaccharide biosynthesis in vascular phytopathogenic bacteria. Mol. Plant-Microbe Interact. 3:271-279.
8. Coplin, D. L., and Majerczak, D. R. 1990. Extracellular polysaccharide genes in *Erwinia stewartii*: Directed mutagenesis and complementation analysis. Mol. Plant-Microbe Interact. 3:286-292.
9. Daniels, M. J., Osbourn, A. E., and Tang, J.-L. 1989. Regulation in *Xanthomonas*-plant interactions. Pages 189-196 in: Signal Molecules in Plants and Plant-Microbe Interactions. NATO ASI Series, Vol. H36. B. J. J. Lugtenberg, ed. Springer-Verlag, Berlin.
10. Dudman, W. F., and Lacey, M. J. 1986. Identification of pyruvated monosaccharides in polysaccharides by gas-liquid chromatography-mass spectrometry. Carbohydr. Res. 145:175-191.
11. Ebrahim-Nesbat, F., and Slusarenko, A. J. 1983. Ultrastructure of the interaction of cells of *Pseudomonas phaseolicola* with cell walls of a resistant and susceptible bean cultivar. Phytopathol. Z. 108:148-159.
12. El-Banoby, F. E., and Rudolph, K. 1979. Induction of water-soaking in plant leaves by extracellular polysaccharides from phytopathogenic pseudomonads and xanthomonads. Physiol. Plant Pathol. 15:341-349.
13. Essenberg, M., Doherty, M. d'A., Hamilton, B. K., Henning, V. T., Cover, E. C., McFaul, S. J., and Johnson, W. M. 1982. Identification and effects on *Xanthomonas campestris* pv. *malvacearum* of two phytoalexins from leaves and cotyledons of resistant cotton. Phytopathology 72:1349-1356.
14. Fett, W. F., and Dunn, M. F. 1989. Exopolysaccharides produced by phytopathogenic *Pseudomonas syringae* pathovars in infected leaves of susceptible hosts. Plant Physiol. (Bethesda) 89:5-9.
15. Fett, W. F., and Osman, S. F. 1985. Purification and characterization of *Xanthomonas campestris* pv. *glycines* exopolysaccharide. Plant Sci. (Shannon) 40:99-104.
16. Gross, M., and Rudolph, K. 1987. Demonstration of levan and alginate in bean plants (*Phaseolus vulgaris*) infected by *Pseudomonas syringae* pv. *phaseolicola*. J. Phytopathol. (Berl.) 120:9-19.
17. Hildebrand, D. C., Alosi, M. C., and Schroth, M. N. 1980. Physical entrapment of pseudomonads in bean leaves by films formed at air-water interfaces. Phytopathology 70:98-109.
18. Jansson, P.-E., Kenne, L., and Lindberg, B. 1975. Structure of the extracellular polysaccharide from *Xanthomonas campestris*. Carbohydr. Res. 45:275-282.
19. Klement, Z. 1965. Method of obtaining fluid from the intercellular spaces of foliage and the fluid's merit as substrate for phytobacterial pathogens. Phytopathology 55:1033-1034.
20. Komalavilas, P., and Mort, A. J. 1989. The acetylation at O-3 of galacturonic acid in the rhamnose-rich portion of pectins. Carbohydr. Res. 189:261-272.
21. Komalavilas, P., Zhu, J.-K., and Nothnagel, E. A. 1991. Arabinogalactan-proteins from the suspension culture medium and plasma membrane of rose cells. J. Biol. Chem. 266:15956-15965.
22. Melton, L. D., Mindt, L., Rees, D. A., and Sanderson, G. R. 1976. Covalent structure of the extracellular polysaccharide from *Xanthomonas campestris*: Evidence from partial hydrolysis studies. Carbohydr. Res. 46:245-257.
23. Morgham, A. T., Richardson, P. E., Essenberg, M., and Cover, E. C. 1988. Effects of continuous dark upon ultrastructure, bacterial populations and accumulation of phytoalexins during interactions between *Xanthomonas campestris* pv. *malvacearum* and bacterial blight susceptible and resistant cotton. Physiol. Mol. Plant Pathol. 32:141-162.
24. Neidhardt, F. C., Bloch, P. L., and Smith, D. F. 1974. Culture medium for enterobacteria. J. Bacteriol. 119:736-747.
25. Neidhardt, F. C., Ingraham, J. L., and Schaechter, M. 1990. Physiology of the Bacterial Cell: A Molecular Approach. Sinauer, Sunderland, MA. 506 pp.
26. Orentas, D. G., Sloneker, J. H., and Jeanes, A. 1963. Pyruvic acid content and constituent sugars of exocellular polysaccharides from different species of the genus *Xanthomonas*. Can. J. Microbiol. 9:427-430.
27. Ornston, L. N., and Stanier, R. Y. 1966. The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. J. Biol. Chem. 241:3776-3786.
28. Osbourn, A. E., Clarke, B. R., Stevens, B. J. H., and Daniels, M. J. 1990. Use of oligonucleotide probes to identify members of two-component regulatory systems in *Xanthomonas campestris* pathovar *campestris*. Mol. Gen. Genet. 222:145-151.
29. Osman, S. F., Fett, W. F., and Fishman, M. L. 1986. Exopolysaccharides of the phytopathogen *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 166:66-71.
30. Pierce, M., and Essenberg, M. 1987. Localization of phytoalexins in fluorescent mesophyll cells isolated from bacterial blight-infected cotton cotyledons and separated from other cells by fluorescence-activated cell sorting. Physiol. Mol. Plant Pathol. 31:273-290.
31. Rudolph, K. W. E., Gross, M., Neugebauer, M., Hokawat, S., Zachowski, A., Wydra, K., and Klement, Z. 1989. Extracellular polysaccharides as determinants of leaf spot diseases caused by pseudomonads and xanthomonads. Pages 177-218 in: Phytotoxins and Plant Pathogenesis. NATO ASI Series, Vol. H27. A. Graniti, R. D. Durbin, and A. Ballio, eds. Springer-Verlag, Berlin.
32. Rudolph, K., and Mendgen, K. 1985. Multiplication of *Pseudomonas syringae* pv. *phaseolicola* "in planta" II. Characterization of susceptible and resistant reactions by light and electron microscopy compared with bacterial countings. Phytopathol. Z. 113:200-212.
33. Sutherland, I. W. 1981. *Xanthomonas* polysaccharides—improved methods for their comparison. Carbohydr. Polym. 1:107-115.
34. Sutton, J. C., and Williams, P. H. 1970. Comparison of extracellular polysaccharide of *Xanthomonas campestris* from culture and from infected cabbage leaves. Can. J. Bot. 48:645-651.
35. Tang, J.-L., Gough, C. L., and Daniels, M. J. 1990. Cloning of genes involved in negative regulation of production of extracellular enzymes and polysaccharide of *Xanthomonas campestris* pathovar *campestris*. Mol. Gen. Genet. 222:157-160.