Alginate Production by Plant-Pathogenic Pseudomonads

Eighteen plant-pathogenic and three non-plant-pathogenic pseudomonads were tested for the ability to produce alginic acid as an exopolysaccharide in vitro. Alginate production was demonstrated for 10 of 13 fluorescent plant-pathogenic pseudomonads tested with glucose or gluconate as the carbon source, but not for all 5 nonfluorescent plant pathogens and all 3 non-plant pathogens tested. With sucrose as the carbon source, some strains produced alginate while others produced both polyfructan (levan) and alginate. Alginates ranged from <1 to 28% guluronic acid, were acetylated, and had number-average molecular weights of 11.3×10^3 to 47.1×10^3. Polyfructans and alginates were not elicitors of the soybean phytoalexin glyceollin when applied to wounded cotyledon surfaces and did not induce prolonged water soaking of soybean leaf tissues. All or most pseudomonads in rRNA-DNA homology group I may be capable of synthesizing alginate as an exopolysaccharide.

Exopolysaccharides (EPS) of plant-pathogenic bacteria are thought to play an important role in both the ecology and the pathogenicity of the bacteria. EPS may act as an adhesin on host leaf surfaces (43), and ability for copious EPS production has often been associated with increased virulence (12, 42). Infiltration of purified EPS into host tissues is reported to induce a variety of physiological host responses, including induced water soaking of leaf tissues (14) and ethylene biosynthesis (22).

Alginates are a group of structurally related polysaccharides composed of a linear backbone of 1,4-linked β-D-mannuronic acid and α-L-guluronic acid (26). Alginate is a major structural polysaccharide of brown seaweed, which is the source of alginate used for a variety of commercial applications. Alginate is also produced as an EPS of certain strains of *Pseudomonas aeruginosa* isolated almost exclusively from cystic fibrosis patients and of strains of *Azotobacter vinelandii* isolated from various natural habitats (26). Alginate production by *P. aeruginosa* is thought to be important for the survival of the organism in the lungs (40). Alginate-producing variants of *P. fluorescens*, *P. putida*, and *P. mendocina* have been reported (23, 25).

Some *P. aeruginosa* strains appear to be opportunistic plant pathogens (7, 9, 15, 30), but it is not known whether these strains also produce alginate. Gross and Rudolph (24) were the first to report that a plant-pathogenic pseudomonad, "*P. phaseolicola*" (the causal agent of halo blight of bean), produces alginate as an EPS. Gross (Proceedings of the Sixth International Conference on Plant Pathogenic Bacteria, in press) recently reported that several additional plant-pathogenic pseudomonads also produce alginate. Studies in our laboratory have independently confirmed alginate production by plant-pathogenic pseudomonads, and we have previously reported detailed studies concerning alginate production by "*P. glycinea*", the causal agent of bacterial blight of soybean (36). We now report the occurrence and characterization of alginate produced by additional plant-pathogenic pseudomonads.

(A preliminary report of part of this work has been presented [S. F. Osman and W. F. Fett, Proceedings of the Sixth International Conference on Plant Pathogenic Bacteria, in press].)

MATERIALS AND METHODS

Bacterial strains. *Pseudomonas* strains studied are listed in Table 1, along with the source and host of origin. "*P. phaseolicola*" NCPPB 1137 and 1139 were originally thought to be "*P. glycinea*", but this has been shown to be incorrect (35).

Growth media and culture conditions. Agar media used were King medium B (29); nutrient dextrose agar (nutrient agar [Difco Laboratories] plus 5 g of Difco yeast extract per liter and 10 g of Difco glucose per liter); *Pseudomonas* isolation agar (Difco); MacConkey agar (Difco); a semisynthetic medium (B-K) described by Bruegger and Keen (3), with potassium phosphate substituted for yeast extract, glucose (B-K+G) or sucrose (B-K+S) as primary carbon source (24 g per liter), and 15 g of agar per liter; and modified Vogel and Bonner medium (MVBM) (45), prepared as described by Chan et al. (6) and containing 3 mM Mg^{2+} and 214 mM gluconate as the sole carbon source. Liquid media used were B-K and MVBM, as described above, with agar omitted.

Liquid media were dispensed into 300-ml sidearm flasks (Bellco Glass, Inc.); 75 ml was used per flask. Suspensions of bacteria grown overnight on agar media were prepared in sterile water to give an optical density at 600 nm of 0.10, and 1 ml per flask was added with duplicate flasks per bacterial strain. Flasks were shaken at 200 rpm at room temperature, and growth was monitored by measuring the optical density at 600 nm.

EPS isolation and purification. EPS was obtained either from liquid or agar cultures. Growth on agar media was scraped off with a bent glass rod and water. Stationary-phase cultures in liquid media were checked for purity by standard dilution plating techniques. In addition, the absence of *P. aeruginosa* as a contaminant was confirmed by plating onto KB agar and incubating at 42°C (lack of colonization by fluorescent bacteria indicated the absence of *P. aeruginosa*).

Bacteria were removed by centrifugation (16,300 × *g* for 30 to 60 min) followed by filtration of the supernatant fluid through 0.45-μm-pore-size filters. Highly viscous culture filtrates or agar plate washings were diluted with water

TABLE 1. Source and host of origin of pseudomonads

Bacterium	Strain	Source[a]	Host of origin
Phytopathogens			
"*P. aptata*"	NCPPB 2664	U. Mazzuchi	*Beta vulgaris*
P. avenae	VS-1	R. D. Gitaitis	*Paspalum urvillei*
P. caryophylli	CUCPB 1268	R. S. Dickey	*Dianthus caryophyllus*
P. cepacia	376	F. L. Lukezic	Not known
P. cichorii	P36	A. R. Chase	*Platycerium bifurcatum*
P. corrugata	NCPPB 2445	F. L. Lukezic	*Lycopersicon esculentum*
P. gladioli	P32	A. R. Chase	*Asplenium nidus*
"*P. glycinea*"	NCPPB 2159	NCPPB	*Glycine max*
	A-29-2	W. F. Fett	*Glycine max*
	J3-17-2	W. F. Fett	*Glycine max*
"*P. lachrymans*"	PL785	C. Leben	*Cucumis sativus*
"*P. morsprunorum*"	486	F. L. Lukezic	*Prunus avium*
"*P. papulans*"	17	T. J. Burr	*Malus sylvestris*
"*P. phaseolicola*"	Race 2	D. M. Webster	*Phaseolus vulgaris*
	At	D. M. Webster	*Phaseolus vulgaris*
	NCPPB 1137	NCPPB	*Neonotönia wightii*
	NCPPB 1139	NCPPB	*Neonotönia wightii*
"*P. pisi*"	Race 3	D. M. Webster	*Pisum sativum*
"*P. savastanoi*"	GTG 24	G. Surico	*Jasminum* sp.
P. syringae	Meyer	D. M. Webster	*Phaseolus vulgaris*
"*P. tabaci*"	Pt113	R. D. Durbin	*Nicotiana tobacum*
"*P. tagetis*"	Q1	R. D. Durbin	*Helianthus tuberosus*
"*P. tomato*"	84-86	R. D. Gitaitis	*Lycopersicon esculentum*
P. viridiflava	671	F. L. Lukezic	*Lycopersicon esculentum*
Nonphytopathogens:			
P. acidovorans	ATCC 15668	ATCC	
P. delafieldii	ATCC 17505	ATCC	
P. testosteroni	ATCC 11996	ATCC	

[a] NCPPB, National Collection of Plant Pathogenic Bacteria, Hatching Green, England. ATCC, American Type Culture Collection, Rockville, Md.

before centrifugation. Culture filtrates were dialyzed (3,500 molecular weight cutoff tubing) extensively against water at 4°C. Insoluble material was removed by centrifugation (16,300 × *g* for 30 min), and EPS was precipitated by the addition of 2 volumes of cold acetone (−20°C). After being kept overnight at −20°C the precipitated EPS was sedimented by centrifugation (16,300 × *g* for 1 h at 4°C). The supernatant fluid was taken to dryness under a stream of nitrogen. A small volume of water was added with stirring, and 2 volumes of cold acetone were again added with stirring. This procedure was repeated until no more EPS could be precipitated. Some EPS preparations were further purified by the method of Sutherland (41). The method involves a subsequent ultracentrifugation step (100,000 × *g* for 4 h) followed by redialysis against water and acetone reprecipitation.

Alginate preparations from *A. vinelandii* and *P. aeruginosa* Q60 were kindly supplied by I. W. Sutherland and A. Linker, respectively. Alginate, levan, and lipopolysaccharide (LPS)-protein preparations from "*P. glycinea*" were isolated previously in our laboratory (36).

Analytical methods. Unless otherwise stated, all reagents were obtained from Sigma Chemical Co. Protein was determined by the colorimetric assay of Markwell et al. (33) with bovine plasma albumin (Bio-Rad Laboratories) as a standard, carbohydrate was determined by the colorimetric assay of Dubois et al. (13) with glucose as a standard, and uronic acid was determined by the colorimetric assay of Blumenkrantz and Asboe-Hansen (2) with D-glucuronic acid or D-mannurono-6,3-lactone as the standard. Acetyl content was determined by the colorimetric assay of McComb and McCready (34) with glucose pentaacetate as the standard. The colorimetric assay of Karkhanis et al. (27) was used to determine 3-deoxy-D-manno-2-octulosonic acid, and the colorimetric assay of Wright and Rebers (46) was used for heptose; commercial 3-deoxy-D-manno-2-octulosonic acid and sedoheptulose anhydride monohydrate, respectively, were used as standards.

Sugar composition. Reduced or unreduced EPS samples were hydrolyzed and characterized by gas-liquid chromatography (GC) and GC-mass spectrometry as the aldononitrile derivatives (44), as previously described (36).

Molecular weight determinations. Before determination of number-average molecular weight, alginate samples were passed through an ACA-202 gel permeation column (LKB Instruments, Inc.) with water or 0.1 M NaCl for elution. Fractions equal to the column void volume were collected. Number-average molecular weights were determined by a gel permeation–high-performance liquid chromatography method, as described previously (36).

Polyacrylamide gel electrophoresis. Selected alginate preparations were electrophoresed on polyacrylamide gels by the method of Bucke (4) with slight modifications. Instead of tube gels, slab gels containing 6% (wt/vol) acrylamide monomers and bisacrylamide at 2% of the monomer were used. The gel buffer was 0.25 M Tris hydrochloride (pH 8.3), and the running buffer was 0.1 M glycine-Tris (pH 9.0). A 15-μl sample of alginate preparation (1 mg/ml) was loaded, and small amounts of bromphenol blue (0.25%, vol/vol) were added as a marker dye. Samples were concentrated for 10 min at 20 V and then electrophoresed for 280 min at 220 V, at which time the marker dye had run 7.5 cm. Gels were stained overnight in Alcian blue (0.08%, wt/vol) in acetic acid (7%, vol/vol) and subsequently destained in 7% acetic acid.

Virulence and race determinations. The relative virulence

and physiologic race of the four strains of "*P. phaseolicola*" included in this study were determined by inoculation of bean (*Phaseolus vulgaris*) plants. The undersides of leaves of susceptible *Phaseolus vulgaris* cv. Red Kidney plants were forcibly sprayed with bacterial inocula containing approximately 5×10^5 or 5×10^7 CFU/ml until water soaking appeared. Inoculated plants were kept in a growth chamber at 24°C during the day and 20°C during the night and were observed for symptoms for up to 10 days. Droplets (10 μl) of inoculum containing approximately 5×10^5 or 5×10^7 CFU/ml were placed on detached pods, and then a 26-gauge 3/8-in. (0.95 cm) syringe needle was used to make a stab wound in the underlying pod tissues. Inoculated pods were kept under 100% relative humidity at room temperature and observed for symptoms for up to 6 days. Physiologic race was determined by inoculating leaves of *Phaseolus vulgaris* cv. Red Mexican UI 34 as described above.

Assays for biological activities. Sample preparations were tested for the ability to elicit accumulation of the soybean phytoalexin glyceollin by a cotyledon bioassay (21) as described previously (19). Levan and LPS-protein samples were prepared at 2 mg/ml in 0.1 M sodium phosphate buffer (pH 7.0). LPS-protein samples were gently heated immediately before use. Alginate samples obtained either from in vitro batch cultures of bacteria or from soybean leaves inoculated with compatible bacteria or a fungal mycelial elicitor preparation from *Phytophthora megasperma* f. sp. *glycinea* (19) were prepared in either 0.05 M sodium citrate buffer (pH 5.0), 0.1 M sodium phosphate buffer (pH 7.0), or 0.05 M glycine-NaOH buffer (pH 9.0) at 2 mg/ml for the alginates or 7.5 mg/ml for the fungal elicitor. Buffers alone were used as negative controls. All preparations were sterilized by heating at 121°C for 15 min. A 5-μl sample of the fungal elicitor and a 20-μl sample of the other preparations were assayed on wounded cotyledon surfaces. The presence of glyceollin in extracts from treated cotyledons was determined by a bioassay on thin-layer chromatography plates with a *Cladosporium* sp. as the bioassay organism (28), as described previously (17). This assay can detect as little as 0.5 μg of glyceollin (18).

The ability of the various samples to induce persistent water soaking in soybean leaves was determined. All preparations were prepared at 2 mg/ml in water, and the LPS-protein preparations were gently heated before use. Trifoliate leaves of soybean cultivar Harosoy were infiltrated with the samples by using a syringe fitted with a short piece of plastic tubing at the end. Treated plants were left either uncovered or covered with a plastic bag on the laboratory bench at room temperature.

RESULTS

EPS production. Bacterial strains representing 18 plant-pathogenic and 3 non-plant-pathogenic pseudomonads were initially screened for EPS production during growth to stationary phase in B-K liquid medium containing 24 g of glucose per liter as the primary carbon source. Widely varying amounts of acetone-precipitable material were obtained (Table 2). The uronic acid content of the preparations ranged from 0 to 76% (Table 2), and the protein content was usually below 5 to 10%. No nucleic acid was detectable by UV absorption. When acetone-precipitable material of selected strains was further purified by ultracentrifugation (100,000 × *g* for 4 h), small whitish pellets were obtained which upon suspension in water at 1 mg/ml were opalescent, indicative of the presence of LPS. Subsequent acetone precipitation of the supernatant fluids gave precipitated material which produced clear, sometimes highly viscous, solutions when taken up in water at 1 mg/ml.

Selected strains of fluorescent plant-pathogenic pseudomonads ("*P. aptata*" NCPPB 2664, *P. cichorii* P36, "*P. phaseolicola*" NCPPB 1137, *P. syringae* Meyer, and "*P. tagetis*" Q1) which did not produce EPS high in uronic acid content when grown in B-K+G liquid medium were tested for production of acidic EPS in MVBM liquid medium with positive results (Table 2). *P. cichorii* P36, *P. viridiflava* 671, and the nonfluorescent plant pathogen *P. corrugata* 421 were also tested for acidic EPS production on MVBM agar, and "*P. papulans*" 17 was tested on B-K+S agar. Each strain was streaked on 20 culture dishes (100 by 15 mm) containing agar medium and incubated for 3 days at 28°C. *P. cichorii* P36, *P. viridiflava* 671, and "*P. papulans*" 17 exhibited mucoid growth and yielded 46.5, 58.5, and 102.9 mg of acetone-precipitable material with 40.4, 38.5, and 33.4% uronic acid, respectively. *P. corrugata* 421 did not give mucoid growth and was not examined further.

GC analysis of hydrolysates of reduced samples of acetone-precipitable material was done on samples which showed 10% or more uronic acid content by colorimetric assay. The analyses demonstrated the presence primarily of mannose with varying levels of gulose in the preparations. Guluronic acid ranged from less than 1% to 28%, and acetyl groups were present in the preparations examined (Table 3). Alginates used for molecular weight determinations often eluted from ACA-202 gel permeation columns in more than one fraction, but number-average molecular weights of the different fractions were similar. Values given in Table 3 are for the first alginate-containing fraction to elute from the column. For the preparations examined, number-average molecular weights ranged from 11.3×10^3 to 47.1×10^3.

Alginates produced in vitro by *P. aeruginosa* Q60, "*P. phaseolicola*" race 2, "*P. glycinea*" A-29-2, and "*P. glycinea*" NCPPB 2159 (number-average molecular weights, 47.1×10^3, 39.0×10^3, 4.5×10^3, and 3.3×10^3 [36; this study]), two alginates produced in vivo by "*P. glycinea*" A-29-2 (number-average molecular weights, 22.2×10^3 and 19.9×10^3 [36]), and one alginate produced in vivo by "*P. glycinea*" NCPPB 2159 (number-average molecular weight undetermined) were examined by polyacrylamide gel electrophoresis. The relatively high-molecular-weight alginates produced by *P. aeruginosa* and "*P. phaseolicola*" gave single diffuse areas of stained material confined to the upper one-quarter of the lanes. The low-molecular-weight alginates produced in vitro by "*P. glycinea*" A-29-2 and NCPPB 2159 both gave two narrow bands of stained material with one band running with the tracking dye and the other directly below. The three alginates produced by "*P. glycinea*" in leaves of compatible soybean cultivars were highly polydisperse, with faintly stained material running the length of the lanes.

Since several fluorescent plant-pathogenic pseudomonads are reported to be capable of producing the polyfructan levan when grown in sucrose-containing media (1, 8, 31, 32), we tested some of the alginate-producing strains for levan production. Bacteria were grown to stationary phase in B-K+S. After the initial acetone precipitation step, acetone-precipitable material was subjected to ultracentrifugation (100,000 × *g* for 4 h). Large clear pellets were obtained for "*P. morsprunorum*" 486, "*P. phaseolicola*" race 2, and "*P. tabaci*" Pt113, but only small white pellets were obtained for "*P. papulans*" 17 and "*P. savastanoi*" GTG 24 (Table 4). When taken up in water the large clear pellets gave clear

TABLE 2. Amount and uronic acid content of acetone-precipitable material produced in liquid media

Bacterium	Strain	Medium	Acetone-precipitable material (mg)	% Uronic acid
Nonfluorescent plant pathogens				
P. avenae	VS-1	B-K+G	0.5[a]	3.2
P. caryophylli	CUCPB 1268	B-K+G	1.5[a]	3.2
P. cepacia	376	B-K+G	4.5[a]	3.6
P. corrugata	421	B-K+G	1.0[a]	4.6
P. gladioli	P32	B-K+G	3.0[a]	4.8
Fluorescent plant pathogens				
"*P. aptata*"	NCPPB 2664	B-K+G	5.3[a]	0.4
		MVBM	6.8[b]	30.0
P. cichorii	P36	B-K+G	3.5[a]	13.0
		B-K+G	11.0[b]	6.8
		MVBM	10.0[a]	42.0
"*P. lachrymans*"	PL785	B-K+G	22.5[a]	5.6
"*P. morsprunorum*"	486	B-K+G	50.0[a]	16.0
"*P. papulans*"	17	B-K+G	120.0[a]	16.0
"*P. phaseolicola*"	Race 2	B-K+G	22.0[a]	76.0
		B-K+G	44.0[a]	20.0
	At	B-K+G	16.2[a]	20.0
	NCPPB 1137	B-K+G	1.0[a]	0
		MVBM	71.0[b]	61.4
	NCPPB 1139	B-K+G	7.0[a]	12.0
"*P. pisi*"	Race 3	B-K+G	7.0[a]	0
"*P. savastanoi*"	GTG 24	B-K+G	42.0[a]	30.0
P. syringae	Meyer	B-K+G	8.5[a]	4.0
		MVBM	24.5[b]	30.0
"*P. tabaci*"	Pt113	B-K+G	36.0[a]	13.0
"*P. tagetis*"	Q1	B-K+G	31.0[a]	2.2
		MVBM	27.4[b]	42.0
"*P. tomato*"	84-86	B-K+G	3.0[a]	4.0
P. viridiflava	671	B-K+G	2.5[a]	0
Non-plant pathogens				
P. acidovorans	ATCC 15668	B-K+G	2.0[a]	0
P. delafieldii	ATCC 17505	B-K+G	0	0
P. testosteroni	ATCC 11996	B-K+G	2.5[a]	0

[a] Material which precipitated after addition of 2 volumes of acetone. Not further purified.
[b] Acetone-precipitable material further purified by the method of Sutherland (41).

solutions and the small whitish pellets gave opalescent solutions. Pelleted material from four of the strains ranged from 1 to 4% in uronic acid, while that from *P. papulans* 17 contained 11% (Table 4). The supernatant fluids contained 14 to 38% uronic acid. Subsequent GC analysis of the pelleted material from "*P. morsprunorum*" 486, "*P. phaseolicola*" race 2, and "*P. tabaci*" Pt113 demonstrated the presence of only fructose. The pelleted material obtained from "*P. papulans*" 17 and "*P. savastanoi*" GTG 24 had a high protein content (24 to 33%). The pelleted material from *P. savastanoi* was also found to contain both 3-deoxy-D-manno-2-octulosonic acid (1.5%, wt/wt) and heptose (1.7%, wt/wt), indicating that the whitish pellets were composed primarily of protein and LPS. GC analysis of the reduced preparations obtained from the supernatant fluids indicated that they were composed of alginate containing various amounts of guluronate (Table 4).

Several agar media, including two (*Pseudomonas* isolation agar and MacConkey agar) which were reported to support the mucoid phenotype of clinical isolates of *P. aeruginosa* (5, 39), were tested for their ability to induce mucoid growth of alginate-producing plant-pathogenic pseudomonads. Also included in these studies were three strains of the "*P. glycinea*" which we previously found to synthesize alginate (36).

Only "*P. glycinea*" NCPPB 2159 was capable of growth on *Pseudomonas* isolation agar, and growth was nonmucoid (Table 5). All strains grew on MacConkey agar, but growth was nonmucoid. Agar media which supported mucoid growth of most strains were B-K+S and MVBM. The effect of incubation temperature on the growth of "*P. glycinea*" NCPPB 2159 on B-K+G agar was tested. No mucoid growth was observed during a 2-week incubation period at 20, 28, or 30°C.

During an experiment to test for binding of the fluorescent β-linked polysaccharide stain Cellufluor (Calcofluor white M2R; Polysciences, Inc.) to alginate, it was noted that addition of Cellufluor at 0.02% (wt/vol) to MVBM agar induced slightly mucoid growth by "*P. aptata*" NCPPB 2664, mucoid growth by "*P. phaseolicola*" NCPPB 1137, and enhanced mucoid growth of "*P. glycinea*" A-29-2, but not mucoid growth by "*P. glycinea*" 2159.

Relative virulence and physiologic race of "*P. phaseolicola*." Tests to compare the relative virulence of strains of "*P. phaseolicola*" on bean leaves and pods of the susceptible cultivar Red Kidney showed strains At and race 2 to be of highest virulence, NCPPB 1139 to be of intermediate virulence, and NCPPB 1137 to be of lowest virulence. Physiologic race was determined by inoculation of leaves of cultivar Red Mexican UI 34. Strains NCPPB 1137 and

TABLE 3. Composition and molecular weight of alginates

Bacterium	Strain	Medium[a]	% Guluronic acid[b]	% Acetate	$\overline{M}_n(10^3)$[c]
A. vinelandii			37.0	ND[d]	39.0
"*P. aptata*"	NCPPB 2664	MVBM	4.7	ND	ND
P. cichorii	P36	MVBM	<1	ND	ND
		MVBM agar	18.0	ND	ND
"*P. morsprunorum*"	486	B-K+G	26.9	3.0	ND
"*P. papulans*"	17	B-K+G	<1	11.6	47.1
		B-K+S agar	<1	ND	ND
"*P. phaseolicola*"	Race 2	B-K+G	4.0	ND	39.0
		B-K+G	20.2	6.6	27.9
	At	B-K+G	3.3	ND	11.3
	NCPPB 1137	MVBM	<1	ND	ND
	NCPPB 1139	B-K+G	5.6	ND	ND
"*P. savastanoi*"	GTG 24	B-K+G	12.0	ND	ND
P. syringae	Meyer	MVBM	1.1	ND	ND
"*P. tabaci*"	Pt113	B-K+G	27.2	5.8	ND
"*P. tagetis*"	Q1	MVBM	2.3	ND	ND
P. viridiflava	671	MVBM agar	6.5	ND	ND

[a] All liquid media except when noted.
[b] Percent guluronic acid = 100 − percent mannuronic acid and was determined by the GC method of Vadas et al. (44).
[c] $\overline{M}_n$, Number-average molecular weight.
[d] ND, Not determined.

NCPPB 1139 were not pathogenic on cultivar Red Mexican UI 34 (typical of race 1), while strains race 2 and At were pathogenic toward this cultivar (typical of race 2).

Biological activity. In cotyledon bioassays designed to test for the ability to elicit accumulation of the soybean phytoalexin glyceollin, only the fungal elicitor preparation from mycelia of *Phytophthora megasperma* f. sp. *glycinea* caused a reddish-brown discoloration of the wounded cotyledon surfaces with accompanying glyceollin accumulation. The pH of the fungal elicitor preparation did not appear to affect the level of browning or glyceollin accumulation. The polyfructan, alginate, and LPS-protein preparations from "*P. glycinea*" 2159 and A-29-2 grown in batch culture, an alginate sample prepared from leaves of soybean cultivar Harosoy infected with the compatible "*P. glycinea*" strain A-29-2, and the buffer controls were all inactive.

The same preparations were used in assays designed to test for induction of persistent water soaking in leaves of cultivar Harosoy which is susceptible to "*P. glycinea*" A-29-2 (race 4) but resistant to "*P. glycinea*" NCPPB 2159 (race 1) (20). All visible water soaking of treated areas disappeared by 30 min after infiltration if the plants were left uncovered. Results of experiments in which treated plants were kept under high humidity by being covered with plastic bags were highly variable and sometimes difficult to interpret owing to the presence of water-soaked areas on untreated areas of the leaves as a result of the high humidity. Overall, no conclusive evidence for induction of persistent water soaking by any of the preparations was found.

DISCUSSION

Until recently only *P. aeruginosa* strains isolated almost exclusively from cystic fibrosis patients and *A. vinelandii* strains isolated from their natural habitats were known to produce alginic acid as an EPS (26). The fluorescent bacteria *P. fluorescens*, *P. mendocina*, and *P. putida* were subsequently found to produce alginic acid, but only under conditions of stress (bacteriocin, bacteriophage, antibiotic) (23, 25). Several nonfluorescent pseudomonads could not be

TABLE 4. EPS production in a semisynthetic liquid medium with sucrose as primary carbon source

Bacterium	Strain	Preparation[a]	Dry wt (mg)	% Uronic acid	Identity	% Guluronate[b]
"*P. morsprunorum*"	486	Pellet	300	2.0	Polyfructan	
		Supernatant	50	13.6	Alginate	8.0
"*P. papulans*"	17	Pellet	4	11.4	LPS-protein	
		Supernatant	113	38.0	Alginate	<1
"*P. phaseolicola*"	Race 2	Pellet	120	3.6	Polyfructan	
		Supernatant	98	35.0	Alginate	<1
"*P. savastanoi*"	GTG 24	Pellet	37	1.0	LPS-protein	
		Supernatant	10	19.4	Alginate	28.0
"*P. tabaci*"	Pt113	Pellet	150	2.4	Polyfructan	
		Supernatant	45	25.6	Alginate	<1

[a] Culture filtrates were subjected to acetone precipitation, and then the precipitated material was subjected to ultracentifugation (100,000 × *g*, for 4 h).
[b] Percent Guluronate = 100 − percent mannuronate.

TABLE 5. Ability of various agar media to support mucoid growth of alginate-producing plant-pathogenic pseudomonads

Bacterium	Strain	Growth[a] on following medium[b]						
		KB	NDA	B-K+G	B-K+S	MVBM	PIA	Mac
"*P. aptata*"	NCPPB 2664	−	−	−	+	−	NG	−
P. cichori	P36	−	±	−	−	+	NG	−
"*P. glycinea*"	NCPPB 2159	−	−	−	+	−	−	−
	A-29-2	−	−	−	+	+	NG	−
	J3-17-2	−	−	−	+	+	NG	−
"*P. morsprunorum*"	486	−	−	−	+	+	NG	−
"*P. papulans*"	17	+	+	+	+	+	NG	−
"*P. phaseolicola*"	Race 2	−	−	−	+	+	NG	−
	At	−	−	−	+	+	NG	−
	NCPPB 1137	−	−	−	−	−	NG	−
	NCPPB 1139	−	−	−	+	+	NG	−
"*P. savastanoi*"	GTG 24	−	+	−	−	+	NG	−
P. syringae	Meyer	−	+	−	+	+	NG	−
"*P. tabaci*"	Pt113	+	+	−	+	+	NG	−
"*P. tagetis*"	Q1	−	−	−	+	+	NG	−
P. viridiflava	671	±	+	−	+	+	NG	±

[a] +, Mucoid growth; ±, slightly mucoid growth; −, nonmucoid growth; NG, no growth. The samples were observed for up to 7 days at 28°C.
[b] For descriptions of the various media, see materials and methods. Symbols: NDA, nutrient dextrose agar; PIA, *Pseudomonas* isolation agar; Mac, MacConkey agar.

induced to produce alginate by antibiotic stress or mutagenesis (23).

Our previous study (36), the present study, and those of Gross (in press) and Gross and Rudolph (24) indicate that at least 14 fluorescent plant-pathogenic pseudomonads also are capable of alginate production under appropriate conditions in vitro and in vivo. Three species of fluorescent plant-pathogenic bacteria ("*P. lachrymans*", "*P. pisi*", and "*P. tomato*"), which did not produce alginate when grown in B-K liquid media with glucose, were reported by Gross (in press) to be capable of alginate synthesis. We did not examine our strains further, but the differing results were most probably due to different culture conditions. The combined results to date strongly indicate that alginate biosynthesis may be a common feature for the majority of pseudomonads in rRNA-DNA homology group I (11, 37), which includes all the fluorescent and a few nonfluorescent pseudomonads. All alginate-producing bacteria, including *A. vinelandii*, may be restricted to a single rRNA superfamily (10).

Alginate production by *P. aeruginosa* is known to be highly variable (5). Such variability also appears to exist for alginate production by plant-pathogenic pseudomonads. For instance, *P. viridiflava* 671 produced alginate on MVBM agar but not in B-K liquid medium with glucose. Alginate was produced by "*P. aptata*" NCPPB 2664, "*P. phaseolicola*" NCPPB 1137, *P. syringae* Meyer, and "*P. tagetis*" Q1 in MVBM liquid but not B-K+G liquid medium (Table 2). Several strains which produced alginate in B-K+G liquid medium gave nonmucoid growth on B-K+G agar media. Alginate production by *P. aeruginosa* is often rapidly lost upon culturing on agar media (26). This appears not to be the case for the plant-pathogenic pseudomonads studied in our laboratory, since many have been in culture for several years. This stability should facilitate their use in genetic studies.

Whether alginate or polyfructan (levan) alone or alginate in combination with polyfructan are produced as EPS by plant-pathogenic bacteria appears to depend both on bacterial strain and the available carbon source. Gross and Rudolph (24) reported that "*P. phaseolicola*" produced both levan (75%) and alginate (24%) as EPS when sucrose was the sole carbon source during growth in vitro. Gross (in press) reported that "*P. glycinea*", "*P. lachrymans*", "*P. pisi*," and "*P. tomato*" were also capable of producing both levan and alginate from sucrose. In the present study "*P. papulans*" 17 and "*P. savastanoi*" GTG 24 produced alginate alone, and "*P. morsprunorum*" 486, "*P. phaseolicola*" race 2, and "*P. tabaci*" Pt113 produced both levan and alginate in the approximate ratios 6:1, 1.2:1, and 3.3:1, respectively (Table 4), when grown with sucrose as the carbon source. In our previous study (36) we found that "*P. glycinea*" A-29-2 and NCPPB 2159 produced only levan from sucrose. Thus, the ability to produce alginate from sucrose may be strain dependent for "*P. glycinea*".

The ability to produce levan as determined by mucoid growth on sucrose-containing agar media is a widely used taxonomic criterion for the separation of fluorescent pseudomonads (31, 37). A potential problem with this method for rapid screening for levan production is demonstrated by our results with "*P. papulans*" 17. This strain gave mucoid growth on B-K+S agar medium as a result of production of alginate, not levan. Mucoid growth on sucrose-containing agar medium is often indicative of levan production, but this should be confirmed by more stringent methodologies.

In common with other bacterial alginates (26), those produced by plant-pathogenic pseudomonads are acetylated (Table 3). The percent guluronic acid is quite variable between strains and between different preparations from the same bacterial strain. The number-average molecular weights as determined by gel-permeation high-performance liquid chromatography are also quite variable, ranging from 3.8×10^3 for "*P. glycinea*" alginates produced in vitro (36) to 47.1×10^3 for "*P. papulans*" 17 alginate produced in vitro. Evans and Linker (16) determined a range of molecular weights for *P. aeruginosa* alginates of 9×10^4 to 48×10^4 and 12×10^3 for a single *A. vinelandii* alginate by viscosimetry. Pier et al. (38) estimated the molecular weights of *P. aeruginosa* alginates to be greater than 100,000 on the basis of gel permeation chromatography. It is not clear whether such a range in molecular weights truly exists or whether this is due in part to the different methodologies used.

Polyacrylamide gel electrophoresis demonstrated that the relatively high-molecular-weight alginates produced by *P. aeruginosa* Q60 (47.1×10^3) and "*P. phaseolicola*" race 2 (39.0×10^3) and the low-molecular-weight (3×10^3 to 5×10^3) alginates produced by "*P. glycinea*" in vitro are of low dispersity, while the intermediate-molecular-weight alginates produced in vivo by "*P. glycinea*" are highly polydisperse. Buckmire (5) reported *P. aeruginosa* alginate to be of high or low dispersity dependent on in vitro growth conditions.

A role for alginic acid as a determinant of host specificity appears unlikely owing to its production as an EPS by several plant-pathogenic pseudomonads of differing host range and physiologic race. In our tests, alginic acid did not elicit a hypersensitive response (rapid host cell necrosis accompanied by phytoalexin accumulation). El-Banoby and Rudolph (14) reported that EPS of several plant-pathogenic pseudomonads and xanthomonads is capable of preferentially inducing water soaking of compatible leaf tissues, but we could not confirm this for alginic acid.

We believe that alginic acid is a virulence factor, however. Gross (in press) reported that alginic acid but not levan is produced by "*P. phaseolicola*" in susceptible bean leaves. We also previously found (36) that "*P. glycinea*" produces only alginate in leaves of susceptible soybean cultivars but does not produce alginate or produces it at much lower levels in resistant soybean cultivars. In the present study, relative virulence of four strains of "*P. phaseolicola*" showed a positive correlation with the level of alginate production in vitro in B-K+G liquid medium. At the early stages of infection, alginic acid may afford a protective environment owing to its hygroscopic and ionic nature, enabling the bacteria to produce various extracellular enzymes and other bacterial products which alter the host to favor bacterial multiplication. Proof that alginic acid is a virulence factor awaits further study.

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