Characterization of Exopolysaccharides Produced by Plant-Associated Fluorescent Pseudomonads

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A total of 214 strains of plant-associated fluorescent pseudomonads were screened for the ability to produce the acidic exopolysaccharide (EPS) alginate on various solid media. The fluorescent pseudomonads studied were saprophytic, saprophytic with known biocontrol potential, or plant pathogenic. Approximately 10% of these strains exhibited mucoid growth under the conditions used. The EPSs produced by 20 strains were isolated, purified, and characterized. Of the 20 strains examined, 6 produced acetylated alginate as an acidic EPS. These strains included a *Pseudomonas aeruginosa* strain reported to cause a dry rot of onion, a strain of *P. viridiflava* with soft-rotting ability, and four strains of *P. fluorescens*. However, 12 strains of *P. fluorescens* produced a novel acidic EPS (marginalan) composed of glucose and galactose (1:1 molar ratio) substituted with pyruvate and succinate. Three of these strains were soft-rotting agents. Two additional soft-rotting strains of *P. fluorescens* produced a third acidic novel EPS composed of rhamnose, mannose, and glucose (1:1:1 molar ratio) substituted with pyruvate and acetate. When sucrose was present as the primary carbon source, certain strains produced the neutral polymer levan (a fructan) rather than an acidic EPS. Levan was produced by most strains capable of synthesizing alginate or the novel acidic EPS containing rhamnose, mannose, and glucose but not by strains capable of marginalan production. It is now evident that the group of bacteria belonging to the fluorescent pseudomonads is capable of elaborating a diverse array of acidic EPSs rather than solely alginate.

Bacteria growing under natural conditions are often surrounded by a glycocalyx (5) which is usually composed primarily of high-molecular-weight acidic bacterial exopolysaccharide (EPS). Production of EPS is thought to play a variety of important roles in both the ecology of bacteria in general and in the pathogenicity of human, animal, and plant pathogens (5, 11). EPS has been reported (5) to act as an adhesin to inert and living surfaces, protect against antibacterial compounds, concentrate charged organic molecules and inorganic ions, and mediate biofilm and microcolony formation. With respect to bacterial interactions with plants, EPS has been reported to act as an adhesion to host leaf surfaces (30), induce ethylene biosynthesis (13), and act as a determinant of pathogenicity (11).

Previous studies in our laboratory (12, 25; W. F. Fett and M. F. Dunn, Plant Physiol., in press) as well as those by Gross and Rudolph (16–19) have demonstrated that leaf-spotting plant pathogenic fluorescent pseudomonads are capable of producing the acidic polymer alginic acid as an EPS both in vitro and in planta. When grown with sucrose as the primary carbon source, many of these strains are also capable of producing the neutral polymer levan, a fructan, as an EPS.

We have now extended this study to plant-associated fluorescent pseudomonads which are saprophytic, saprophytic with known biocontrol potential due to antifungal activity, or plant pathogenic.

(A preliminary report of this work has been presented [W. F. Fett, S. F. Osman, and M. F. Dunn, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, N-91, p. 259]).

MATERIALS AND METHODS

Bacterial strains. A total of 214 strains of plant-associated fluorescent pseudomonads originally isolated from root or fruit surfaces, seed, or soft-rot lesions were screened for the ability to produce mucoid growth on solid media. Mucoid strains selected for further study are listed in Table 1 along with their sources and original sites of isolation.

Biochemical and physiological tests. Oxidase reaction, production of arginine dihydrolase, ability to denitrify, hydrolysis of gelatin, growth at 41°C, and ability to induce the hypersensitive response on leaves of tobacco cv. Turk were tested for by standard methods (29). Porcine gelatin (Sigma Chemical Co.) was used in tests to determine gelatin hydrolysis. Strains were screened for production of nonfluorescent pigments on *Pseudomonas* agar F (PAF; Difco Laboratories), King's medium A agar (21), and yeast extract-glucose-$CaCO_3$ (30) agar media. Denitrification cultures were observed for growth and gas production for 5 days, and the presence of nitrite or nitrate was determined qualitatively after the incubation period as described by Smibert and Krieg (29a [method 2]). The ability to produce pectic enzymes was determined by using the crystal violet-pectate agar medium of Cuppels and Kelman (7). Strains of *P. fluorescens* were assigned to biovars on the basis of the ability to produce nonfluorescent pigments, the production of levan (see below), and the ability to denitrify (27).

Growth media and culture conditions. All strains were tested for a mucoid phenotype on PAF medium. Selected strains were also screened for acidic EPS production on various additional media, including modified PAF containing either three times the normal concentration of glycerol in order to increase the carbon/nitrogen ratio or 214 mM gluconate as the carbon source, a modified Vogel and Bonner medium with 214 mM gluconate as the carbon source (3), *Pseudomonas* isolation agar, potato dextrose agar, MacConkey agar, desoxycholate citrate agar (all from Difco), yeast extract-glucose-$CaCO_3$ agar, Luria agar (23), and trypticase soy agar (BBL Microbiology Systems). The use of gluconate as a carbon source has been found to support

TABLE 1. Source and original site of isolation of fluorescent pseudomonads

Strain	Source	Isolated from:
P. aeruginosa		
DAR41360	E. J. Cother	Rotted onion
P. fluorescens		
W4F131	D. C. Gross	Potato rhizosphere
W4F1080	D. C. Gross	Potato rhizosphere
W4F1607	D. C. Gross	Potato rhizosphere
W4R720	D. C. Gross	Potato rhizosphere
Q72a-80	D. M. Weller	Wheat rhizosphere
R8z-80	D. M. Weller	Wheat rhizosphere
R4a-80	D. M. Weller	Wheat rhizosphere
AG8	C. H. Liao	Tomato fruit surface
87	J. M. Wells	Bell pepper fruit surface
88	J. M. Wells	Bell pepper fruit surface
ML5	C. Hepfer	Beet seed pericarp
W4F361	D. C. Gross	Potato rhizosphere
W4P5	D. C. Gross	Potato rhizosphere
55-1a	J. M. Wells	Rotted bell pepper
HT-04-1B	C. H. Liao	Rotted spinach
LU-04-1B	C. H. Liao	Rotted lettuce
PF-05-2	C. H. Liao	Rotted bell pepper
PM-LB-1	C. H. Liao	Rotted bell pepper
P. viridiflava		
SF-03-12B	C. H. Liao	Rotted zucchini squash

alginate production by fluorescent pseudomonads (3, 12). Cultures were grown at 4, 20, 28, or 37°C and observed for mucoid growth for up to 7 days.

Strains which exhibited a mucoid phenotype on one of the above agar media were also screened for the ability to produce levan by growing them on nutrient agar (Difco) plus sucrose (5%). Cultures were incubated at 28°C and observed for typical domed mucoid colonies for up to 7 days. For isolation of levan, putative levan-producing strains were grown on nutrient agar plus sucrose (5%) for 2 days at 28°C.

EPS isolation and purification. Before isolation of EPS from mucoid cultures, the presence of capsules was determined by using wet mounts with India ink (ink particle size had been reduced by sonication in the presence of glass beads to facilitate visualization of microcapsules). Mucoid growth was then scraped off by using a bent glass rod and water. After being stirred, bacterial cells were removed by centrifugation (23,000 × *g*, 45 min). When capsulated cells were present, a very loosely packed layer of cells covering a compact cell pellet resulted. This loose layer was collected, the capsular material was separated from the bacterial cells by homogenization by using a commercial blender, and the preparation was recentrifuged as above. Supernatant fluids were dialyzed extensively against water at 4°C; after dialysis, any insoluble material was removed by centrifugation (16,300 × *g*, 30 min), and samples were concentrated by lyophilization. Samples were taken up in water, $CaCl_2 \cdot 2H_2O$ was added to give a final concentration of 0.15%, and EPS was precipitated by the addition of ice-cold acetone (3 volumes). The precipitated EPS was collected by centrifugation (16,300 × *g*, 30 min), and the EPS-containing pellet was taken up in water. Precipitation with acetone was repeated one time as described above. Pelleted EPS was once again taken up in water and then subjected to ultracentrifugation (100,000 × *g*, 4 h). The supernatant fluid was dialyzed against water at 4°C and then lyophilized. Pelleted material was lyophilized without dialysis. After these purification steps, samples still containing greater than 5% protein were subjected to extraction with cold buffered phenol (20) to further reduce protein content.

Analytical methods. Unless otherwise stated, all reagents and standards were obtained from Sigma. Protein content was determined by the method of Bradford (2), with bovine serum albumin as the standard. Total neutral carbohydrate was determined by the method of Dubois et al. (8), with glucose as the standard, and uronic acid content was determined by the method of Blumenkrantz and Asboe-Hansen (1), with D-mannurono-3,6-lactone as the standard. Acetate and succinate contents were determined by the method of McComb and McCready (24), with glucose pentaacetate or mono-methylsuccinate, respectively, as the standards. Pyruvate content was determined either by an enzymatic assay utilizing lactose dehydrogenase (9) or by a high-performance liquid chromatography method (4).

Sugar composition. Sugars were identified by gas-liquid chromatography using a 15-m capillary column (SP-2330; Supelco). The column was temperature programmed from 125 to 225°C at 4°C/min. Before hydrolysis and derivatization, EPS samples containing uronic acid as measured by a colorimetic assay were reduced with sodium borohydride via the carbodiimide adduct as described previously (25). All samples, except those to be examined for the presence of levan, were hydrolyzed in 1 M H_2SO_4 at 100°C for 90 min. Putative levan samples were hydrolyzed in 1 M oxalic acid at 70°C for 90 min. After neutralization, sugars were characterized as their aldononitrile acetate derivatives (31) or, in the case of levan, as their acetate derivatives. Acetate derivatives were prepared by heating samples (2 mg) in pyridine (200 μl)–acetic anhydride (150 μl) at 70°C for 90 min.

Nuclear magnetic resonance. Carbon (^{13}C) nuclear magnetic resonance spectra were obtained on samples dissolved in ^2H_2O with tetrahydrofuran as the internal standard on a JEOL GX-400 instrument with wide-band decoupling. A pulse width of 13 μs, pulse delay of 5 s, acquisition time of 0.66 s, and probe temperature of 70°C were used. Typically, about 10,000 scans were accumulated.

RESULTS

Approximately 10% of the 214 strains of plant-associated fluorescent pseudomonads examined exhibited mucoid growth on agar media under the conditions tested. Of these, 20 were selected for further study. The taxonomic classification of the 20 strains was confirmed by the biochemical and physiological tests run. Of these 20 strains, only W4F1080 and W4F1607 consistently induced a hypersensitive response on tobacco leaves. Strain W4F131 gave variable results, while the other 17 strains were negative. Only the five strains of *P. fluorescens* isolated from rotted vegetables (Table 1) and the one strain of *P. viridiflava* exhibited pectolytic activity on crystal violet-pectate medium.

Initially, none of the five strains of *P. aeruginosa* pathogenic towards onion (6) gave mucoid growth on agar media. However, one strain did produce mucoid variants on *Pseudomonas* isolation agar after 5 days of incubation. One of these mucoid variants was purified three times by streaking for single colonies on PAF and had a stable mucoid phenotype. This strain was designated DAR41360M. Upon further testing, the mucoid variant was found to be indistinguishable from the parent strain. Both produced typical pigments on PAF and King's medium A agar and exhibited growth at 41°C.

The following incubation periods and culture conditions were found to be conducive for EPS production and were used to induce mucoid growth for EPS isolation and purification: 2 to 3 days on PAF at 28°C (strains DAR41360M,

TABLE 2. EPSs produced by fluorescent pseudomonads

Strain	Biovar	EPS produced			
		Levan	Alginate	Novel EPS	Marginalan
P. aeruginosa					
DAR41360M		−	+	−	−
P. fluorescens					
R8z-80	II	+	+	−	−
R4a-80	II	+	+	−	−
W4F1080	IV	+	+	−	−
W4F1607	IV	+	+	−	−
Q72a-80	V	−	−	−	+
AG8	V	−	−	−	+
W4R720	V	−	−	−	+
W4F131	V	−	−	−	+
87	V	−	−	−	+
88	V	−	−	−	+
ML-5	V	−	−	−	+
W4F361	V	−	−	−	+
W4P5	V	−	−	−	+
HT-04-1B	V	−	−	−	+
LU-04-1B	V	−	−	−	+
55-1a	V	−	−	−	+
PF-05-2	II	+	−	+	−
PM-LB-1	II	+	−	+	−
P. viridiflava					
SF-03-12B		−	+	−	−

55-1a, 87, 88, PF-05-2, PM-LB-1, and ML-5), 2 to 4 days on PAF at 20°C (strains R8z-80, R4a-80, Q72a-80, AG8, W4R720, W4F131, W4F361, and HT-04-1B), 1 day on PAF at 28°C followed by 2 days at 4°C (strain LU-04-1B), 3 days on the modified Vogel and Bonner agar at 20°C (strain SF-03-12B), 2 days on PAF with 214 mM gluconate at 28°C (strain W4P5), 5 to 6 days on yeast extract-glucose-$CaCO_3$ agar at 28°C (strains W4F1080 and W4F1607). Strains 55-1a, HT-04-1B, and ML-5 gave the most mucoid growth, with bacterial slime running onto the lids of inverted culture dishes during incubation.

Analysis of the purified EPS preparations indicated that six strains produced alginate as an acidic EPS (Tables 2 and 3). Twelve strains produced an acidic EPS composed of glucose and galactose in a 1:1 molar ratio with pyruvate and succinate substituents (Tables 2 and 3). This EPS was first isolated in our laboratory from strain HT-04-1B and assigned the trivial name of marginalan (S. F. Osman and W. F. Fett, J. Bacteriol., in press). The determination of marginalan structure is the subject of a separate publication (Osman and Fett, in press). Two additional strains of *P. fluorescens* (PF-05-2 and PM-LB-1) produced a third novel acidic EPS whose structure has not yet been determined (Table 2). This EPS contains the neutral sugars rhamnose, mannose, and glucose (1:1:1 molar ratio) substituted with pyruvate and acetate. These two strains were the only ones to exhibit a significant percentage of cells with capsules capable of excluding India ink particles. Levan production on nutrient agar plus sucrose (5%) containing as the carbon source was found only for strains which produced either alginic acid or the EPS containing rhamnose, mannose, glucose, pyruvate, and acetate as acidic EPS (Table 2). Acidic EPS was not produced simultaneously with the neutral polymer levan under these conditions.

All bacterial alginates were acetylated, containing between 7 and 12% acetate, and all marginalan preparations contained pyruvate (11 to 22%) and succinate (15 to 26%) as determined by a colorimetric assay (Table 3). The presence of pyruvate, succinate, and acetate in EPS samples was confirmed by ^{13}C NMR.

DISCUSSION

Even though the oxidase- and arginine dihydrolase-positive group of fluorescent pseudomonads (primarily *P. aeruginosa*, *P. fluorescens*, and *P. putida*) is an important group of bacteria on the basis of ecological and pathological considerations, little work has been done on the nature of the EPSs produced by members of this group. Most studies in this area have concentrated on the slime produced by strains of *P. aeruginosa* which colonize the lungs of cystic fibrosis patients. This slime is composed of alginic acid and is thought to be a virulence determinant (for a review, see

TABLE 3. Composition of acidic exopolysaccharides produced by fluorescent pseudomonads

Strain	Alginate[a]		Marginalan[a]	
	% Guluronate[b]	% Acetate	% Pyruvate	% Succinate
P. aeruginosa DAR41360M	11.1	8.5		
P. fluorescens				
R8z-80	7.2 ± 0.6	7.7 ± 2.4		
R4a-80	4.7 ± 0.2	11.7 ± 1.1		
W4F1080	6.8	8.0		
W4F1607	3.9	8.0		
Q72a-80			11.2 ± 2.5	26.3 ± 4.2
AG8			17.8 ± 1.3	20.1 ± 3.8
W4R720			18.1 ± 2.2	20.3 ± 5.2
W4F131			16.7 ± 2.4	19.8 ± 2.5
87			17.4	25.0
88			15.8	20.0
ML-5			16.2	25.0
W4F361			21.8 ± 3.1	19.2 ± 7.1
W4P5			13.3	20.0
HT-04-1B			13.6 ± 0.6	25.4 ± 2.9
LU-04-1B			11.6	17.5
55-1a			13.6	15.0
P. viridiflava SF-03-12B	1.7	17.6		

[a] Values are for a single EPS preparation, except for values ± the standard deviation, which are mean values for two EPS preparations.
[b] Percent guluronic acid = 100 − percent mannuronic acid.

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P. viridiflava		
SF-03-12B	C. H. Liao	Rotted zucchini squash

alginate production by fluorescent pseudomonads (3, 12). Cultures were grown at 4, 20, 28, or 37°C and observed for mucoid growth for up to 7 days.

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HT-04-1B	V	−	−	−	+
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55-1a	V	−	−	−	+
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SF-03-12B		−	+	−	−

55-1a, 87, 88, PF-05-2, PM-LB-1, and ML-5), 2 to 4 days on PAF at 20°C (strains R8z-80, R4a-80, Q72a-80, AG8, W4R720, W4F131, W4F361, and HT-04-1B), 1 day on PAF at 28°C followed by 2 days at 4°C (strain LU-04-1B), 3 days on the modified Vogel and Bonner agar at 20°C (strain SF-03-12B), 2 days on PAF with 214 mM gluconate at 28°C (strain W4P5), 5 to 6 days on yeast extract-glucose-$CaCO_3$ agar at 28°C (strains W4F1080 and W4F1607). Strains 55-1a, HT-04-1B, and ML-5 gave the most mucoid growth, with bacterial slime running onto the lids of inverted culture dishes during incubation.

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HT-04-1B			13.6 ± 0.6	25.4 ± 2.9
LU-04-1B			11.6	17.5
55-1a			13.6	15.0
P. viridiflava SF-03-12B	1.7	17.6		

[a] Values are for a single EPS preparation, except for values ± the standard deviation, which are mean values for two EPS preparations.
[b] Percent guluronic acid = 100 − percent mannuronic acid.

reference 15). *P. aeruginosa* is also considered to be an opportunistic plant pathogen. In this report, we have shown that *P. aeruginosa* which is capable of causing a dry rot of onion (6) also has the capability of producing alginate as an EPS. Similar to *P. aeruginosa* strains isolated from cystic fibrosis patients (3), the strains studied here produce alginate as a loosely held slime and not as a tightly held capsule.

An early study of the composition of EPS of *P. fluorescens* indicated that the EPS produced was a mannan (10). Two more recent studies indicated that mucoid variants of *P. fluorescens* and *P. putida* produce alginic acid as an EPS (14, 27). In a recent report (28), the EPS of single freshwater isolates of *P. fluorescens* and *P. putida* was characterized as containing equimolar amounts of glucose and galactose substituted with pyruvate and acetate, but no structural data was given. The composition of this EPS is very similar to that which we found for marginalan. Further comparative work is required to determine the exact relationship of these two polysaccharides. It appears that as a group, *P. fluorescens* and *P. putida* are capable of synthesizing a variety of acidic EPSs, but only a single acidic EPS is produced by a single strain. The particular acidic EPS synthesized by strains of *P. fluorescens* appears to be coupled to the ability to produce levan. All levan producers synthesized either alginate or the incompletely characterized novel EPS as an acidic EPS. All nonlevan producers synthesized marginalan as an acidic EPS. Thus, for *P. fluorescens*, the nature of the acidic EPS produced can be predicted by the ability to form levan. *P. aeruginosa*, *P. putida*, and *P. viridiflava* are not capable of levan production (26).

We previously reported that a single strain of *P. viridiflava* produced alginate (12), but the soft-rotting potential of this strain was not determined. In this report, we have shown that fluorescent pseudomonads corresponding to a strain of *P. viridiflava* and several strains of *P. fluorescens* with known soft-rotting ability (22; W. F. Fett, unpublished observation) also can produce acidic EPS. At this time, the taxonomic position of oxidase- and arginine dihydrolase-positive fluorescent pseudomonads with pectolytic activity is unsettled, with some authors suggesting the designation of *Pseudomonas marginalis* (7). As seen in this study and in that of Liao and Wells (22), soft-rotting strains do not always fit into biovar II of *P. fluorescens* as sometimes purported (27). The results of a recent market survey indicated that pectolytic fluorescent pseudomonads may be much more important soft rot agents of vegetables than previously thought (22). No relationship of the nature of the acidic EPS to the ability to cause soft rot was noted. The role of acidic EPS for pathogenicity of these soft-rotting strains is not yet known.

The nature of the acidic EPS produced by the strains used in this study was not determined by the ecological niche from which the strains were isolated. For instance, strains originally isolated from the potato rhizosphere produced either marginalan or alginate. Saprophytic fluorescent pseudomonads supplied to us as having biocontrol activity (strains W4F1080, W4F1607, W4F131, W4R720, W4P5, Q72a-80, R8z-80, and R4a-80) produced either alginate or marginalan as an acidic EPS. Possibly, mutants which overproduce acidic EPS may be better able to survive periods of drought under field conditions and afford more long-lasting biological control.

We conclude that the diverse group of bacteria assigned to *P. fluorescens* and *P. putida* is capable of elaborating a diverse array of acidic EPSs. Examination of additional strains may lead to the identification of other novel EPSs. The nature of the acidic EPS produced may be an additional useful taxonomic criterion for further division of this group of bacteria.

ACKNOWLEDGMENTS

We thank all of our colleagues who kindly supplied us with bacterial strains.

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