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Copper as a Signal for Alginate Synthesis in *Pseudomonas syringae* pv. syringae

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Plant-associated pseudomonads are commonly exposed to copper bactericides, which are applied to reduce the disease incidence caused by these bacteria. Consequently, many of these bacteria have acquired resistance or tolerance to copper salts. We recently conducted a survey of 37 copper-resistant (Cu^r) *Pseudomonas* spp., including *P. cepacia*, *P. fluorescens*, *P. syringae*, and *P. viridiflava*, and found that a subset of the *P. syringae* strains showed a dramatic increase in exopolysaccharide (EPS) production on mannitol-glutamate medium containing $CuSO_4$ at 250 μg/ml. A modified carbazole assay indicated that the EPS produced on copper-amended media contained high levels of uronic acids, suggesting that the EPS was primarily alginic acid. Uronic acids extracted from selected strains were further confirmed to be alginate by demonstrating their sensitivity to alginate lyase and by descending paper chromatography following acid hydrolysis. Subinhibitory levels of arsenate, cobalt, lithium, rubidium, molybdenum, and mercury did not induce EPS production, indicating that alginate biosynthesis is not induced in *P. syringae* cells exposed to these heavy metals. A 200-kb plasmid designated pPSR12 conferred a stably mucoid phenotype to several *P. syringae* recipients and also increased their resistance to cobalt and arsenate. A cosmid clone constructed from pPSR12 which conferred a stably mucoid phenotype to several *P. syringae* strains but not to *Pseudomonas aeruginosa* was obtained. Results obtained in this study indicate that some of the signals and regulatory genes for alginate production in *P. syringae* differ from those described for alginate production in *P. aeruginosa*.

Phytopathogenic pseudomonads are pathogenic on a wide variety of plants and commonly invade susceptible host tissue through wounds or natural openings. Historically, chemical control of phytopathogenic pseudomonads has involved the application of bactericidal sprays containing copper or streptomycin as the active ingredient. However, the effectiveness of copper and streptomycin bactericides has generally decreased, and reports of resistance have become frequent (14). In *Pseudomonas syringae* pv. tomato, copper resistance (Cu^r) is mediated by copper-binding proteins located in the outer membrane and periplasm (11), a mechanism which is also conserved in Cu^r strains of *Escherichia coli* and *Xanthomonas campestris* (43, 56).

Exopolysaccharides (EPS) are known to chelate heavy metals and increase the tolerance of bacterial cells to toxic substances (7, 49, 66). In *Pseudomonas solanacearum*, causal agent of bacterial wilt on solanaceous plants, EPS has been implicated in virulence and contributes to wilting, the primary symptom induced by this pathogen (20, 39). Many plant-associated pseudomonads are known to produce two well-characterized EPS molecules: levan (a polymer of fructofuranan) and alginate, a copolymer of *O*-acetylated β-1,4-linked D-mannuronic acid and its C-5 epimer, L-guluronic acid (13, 27, 34). Fett et al. (26) have suggested that alginate may contribute to the water-soaking and blight symptoms observed in some diseases caused by *P. syringae.*

Alginate biosynthesis has been studied extensively in *Pseudomonas aeruginosa*, where it functions as a major virulence factor in strains infecting the lungs of cystic fibrosis patients (55). The alginate capsule surrounding *P. aeruginosa* provides a protective barrier against antibiotics and host immune defenses (47) and may also function to trap nutrients and water (15). Environmental signals for the induction of alginate biosynthesis in *P. aeruginosa* have been identified and include dehydration, high osmolarity, starvation, and a biofilm mode of growth (6, 18, 21, 22, 36). Singh et al. (57) recently showed that sodium chloride and ethanol significantly increased alginate production in a variety of fluorescent pseudomonads, suggesting that osmolarity and dehydration may be general signals for production of this polysaccharide.

Recent studies in our laboratory have focused on copper and streptomycin resistance in strains of *P. syringae* pv. syringae which cause a tip dieback and canker disease of woody plants (59–61). We noted that many Cu^r *P. syringae* strains isolated from woody ornamentals were heavily mucoid on media containing copper sulfate. In the present study, we determined that the EPS produced by these mucoid cells was alginic acid, and we conducted a survey to determine if copper was a general signal for alginate production in other pseudomonads. We also identified a 200-kb plasmid, pPSR12, which confers a stably mucoid phenotype to *P. syringae* recipients and resistance to copper, cobalt, and arsenate.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Tables 1 and 2 show the bacterial strains and plasmids used in the present study. *Pseudomonas* spp. and *E. coli* strains were routinely maintained on King's medium B (42) at 28°C and Luria-Bertani (LB) medium (48) at 37°C, respectively. *X. campestris* pv. vesicatoria was grown on nutrient agar (Difco Laboratories, Detroit, Mich.). Resistance to copper was determined with mannitol-glutamate (MG) medium (40) supplemented with 250 μg of cupric sulfate per ml (MGcu) (59). Resistance to other heavy metals was determined with MG medium supplemented with 0.25 g of

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
JM109	*recA supE hsdR gyrA relA*	52
HB101	*pro leu recA hsdR hsdM*	52
C600	*supE44 hsdR thi-1 thr-1 leuB6*	52
P. syringae pv. glycinea		
7a	Nonmucoid	64
PG4180	Nonmucoid	5
P. syringae pv. syringae		
FF5	Cu^s; no detectable plasmids	59
FF5.21	pPSR12; Cu^r; nonmucoid EMS mutant	This study
FF5.22	pPSR12; Cu^r; nonmucoid EMS mutant	This study
FF5.31	pPSR12; Cu^r Km^r; nonmucoid Tn*5* mutant	This study
FF5.32	pPSR12; Cu^r Km^r; nonmucoid Tn*5* mutant	This study
P. aeruginosa		
8830	alg^+ (stable mucoidy)	17
8821	alg^+ (unstable mucoidy)	17
8822	*alg* (nonmucoid revertant of 8821)	17
X. campestris pv. vesicatoria E3C5	Rif^r Cu^r	B. Staskawicz
Plasmids		
pUC119	Ap^r; ColE1 origin	65
pGS9	Cm^r Km^r; contains Tn*5*	54
pRK7813	Tc^r; cosmid vector	38
pRK2013	Km^r; helper plasmid	30
pCL8	*algL* gene in pUC118	8
pJG309	*algT* gene in pCP13	32
pCOP2	Tc^r; recombinant plasmid with Cu^r genes from *P. syringae* pv. tomato	4
pPSR12	Cu^r; 200 kb	61
pPSR4	Cu^r; 60 kb	60
pLAI 31	Tc^r; contains *repA* from *P. viridiflava*	44
pCLB9	Ap^r; 530-bp fragment of IS*50* in pUC119	C. Bender
pSK1	Tc^r; 50-kb insert in pRK7813; confers stably mucoid phenotype to nonmucoid *P. syringae* strains	This study

yeast extract per liter (MGY) and the additional metal compound. Ampicillin, tetracycline, and kanamycin were added to LB or MG medium at 40, 12.5, and 25 μg/ml, respectively.

Molecular genetic techniques. Genomic DNA was isolated from *P. syringae* by established procedures (58). Plasmids were isolated from *Pseudomonas* spp. by the plasmid isolation method described by Crosa and Falkow (16) with slight modifications (3). Restriction enzyme digests, agarose gel electrophoresis, Southern transfers, and isolation of DNA fragments from agarose gels were performed by standard protocols (52). The plasmid pCOP2, which contains copper resistance (Cu^r) genes from *P. syringae* pv. tomato PT23 (4), was mobilized into various copper-sensitive (Cu^s) *Pseudomonas* spp. by using a triparental mating procedure and the mobilizer plasmid pRK2013 (4). pPSR12, a self-transmissible 200-kb Cu^r plasmid, was transferred into selected strains of *P. syringae* pv. syringae by conjugation (59).

A cosmid library of pPSR12 was constructed in pRK7813 as described previously (2). The cosmid library was mobilized into the nonmucoid *P. syringae* pv. syringae B301D by use of pRK2013, and transconjugants were screened for EPS production, copper resistance, and tolerance to arsenate as described below.

Survey for induction of EPS in selected pseudomonads. Various plant-associated pseudomonads were examined for EPS production after a 72-h incubation on MG medium and MGcu at 28°C. When necessary, pCOP2 was introduced into Cu^s strains to obtain the Cu^r phenotype. Production of EPS was assessed visually, and strains were classified as heavily mucoid (Fig. 1A), nonmucoid (Fig. 1B), or slightly mucoid (cells were visibly mucoid but EPS production was substantially less than that shown in Fig. 1A).

Determination of MICs of various heavy metals. We also investigated whether EPS production in *P. syringae* was stimulated by metals other than copper and whether strains which produced EPS in the presence of copper showed elevated resistance to other heavy metals. Prior to testing for MICs, bacterial strains were grown on nutrient agar for 24 h; single colonies were then transferred to MGY agar supplemented with the metal(s) of interest. Stock solutions of 1 mM $HgCl_2$, 10 mM $CoCl_2$, 1 M $NaHAsO_4$, 1 M ammonium molybdate tetrahydrate $[(NH_4)_6Mo_7O_{24} \cdot 4H_2O]$, 1 M LiCl, and 1 M RuCl were prepared and filter sterilized. The MIC of each metal was defined as the concentration that inhibited confluent growth of the culture after a 72-h incubation at 28°C. The experiment was repeated to confirm the MICs.

Isolation and quantification of uronic acids. Selected pseudomonads were inoculated by dilution streaking onto MG medium containing 0, 150, and 300 μg of cupric sulfate per ml (three plates at each level) and incubated at 28°C for 72 h. Each plate was handled separately for quantification of uronic acids. Cells were washed from each plate and resuspended in 0.9% NaCl. Removal of cellular material from the mucoid growth and estimation of uronic acid content and total cellular protein were performed as described by May and Chakrabarty (46). Alginic acid from seaweed (*Macrocystis pyrifera*; Sigma Chemical Co., St. Louis, Mo.) was used as a standard in these experiments. The experiment was performed twice, and the means from both repetitions were expressed as uronic acids produced per milligram of protein.

Assays for alginic acid. Uronic acids were further characterized by sensitivity to alginate lyase (53) and by paper chromatography (46). Uronic acids were isolated from selected strains as described previously (46), dialyzed extensively in distilled H_2O at 4°C, precipitated with ethanol, washed with 70% ethanol, and dried by vacuum evaporation. Pellets were resuspended in 500 μl of 0.25 M Tris-HCl (pH 7.5)–0.06 M KCl and stored at −20°C until needed.

Cellular extracts containing alginate lyase activity were recovered from *E. coli* JM109 containing pCL8, a construct containing the *P. aeruginosa* alginate lyase gene (*algL*) under control of the *tac* promoter (8). JM109(pCL8) cells were grown to the mid-log phase in LB medium supplemented with ampicillin, induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and incubated for 4 h. Cells were harvested by centrifugation, washed with 0.9% NaCl, and disrupted by sonication in a solution containing 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, and 10 mM $MgCl_2$. This suspension was centrifuged at 16,000 × *g* for 30 min, and the supernatant fraction was concentrated by lyophilization. The lyophilized preparation, which contained alginate lyase activity, was incubated with uronic acids at room temperature (25°C) for various times, and the reactions were terminated by incubation at 100°C for 15 s. Alginate lyase activity was monitored at 235 nm (25) with a Hewlett-Packard model 8452A spectrophotometer.

Alginate lyase plate assays were performed as described by Gacesa and Wusteman (31) and Schiller et al. (53) with slight modifications. Seaweed alginate and uronic acid polymers isolated from selected bacterial strains were added to LB medium containing ampicillin to a final concentration of 0.3% (wt/vol). The pH of the medium was adjusted to 7.8, and agar was added to 0.6%. *E. coli* JM109(pUC119) and JM109(pCL8) were grown to the late log phase in LB medium and either induced with IPTG as described above or grown for an additional 4 h without induction. Ten-microliter aliquots of cells induced with IPTG or uninduced cells were inoculated onto plates containing either uronic

TABLE 2. Survey of plant-associated *Pseudomonas* spp. for EPS production when grown on MG medium and MGcu

Pseudomonas species and strain designation	Host plant	Mucoidy[a]		Source or reference[b]
		MG medium	MGcu	
P. cepacia				
945[c]	Onion	−	NG[d]	NCPPB
945(pCOP2)		−	−	This study
P. fluorescens				
Pf5[c] (biocontrol agent)		−	NG	C. Howell
Pf5(pCOP2)		−	−	This study
P. syringae pv. atropurpurea				
1304[c]	Rye grass	−	NG	M. Sato
1304(pCOP2)		−	−	This study
P. syringae pv. glycinea				
16a[c]	Soybean	−	NG	64
16a(pCOP2)		−	−	This study
44a[c]	Soybean	−	NG	64
44a(pCOP2)		−	−	This study
27a[c]	Soybean	++	NG	64
P. syringae pv. maculicola				
921[c]	Crucifers	−	NG	ICMP
921(pCOP2)		−	−	This study
438		+	NG	D. Cuppels
P. syringae pv. morsprunorum				
3714[c]	Cherry	−	NG	ICMP
3714(pCOP2)		−	−	This study
P. syringae pv. pisi				
1086-2[c]	Pea	−	NG	C. Bender
1086-2(pCOP2)		−	−	This study
P. syringae pv. syringae				
7C12	Ornamental pear	−	+	61
7D46	Ornamental pear	−	+	G. Sundin
8B48	Ornamental pear	−	+	61
8D44	Ornamental pear	−	+	G. Sundin
PSC1B	Corn	−	++	19
7A15	Ornamental pear	−	++	G. Sundin
7B44	Ornamental pear	−	++	61
7C6	Ornamental pear	−	++	G. Sundin
G1	Willow	+	++	59
7E50	Ornamental pear	+	+	G. Sundin
9A26	Ornamental pear	+	+	61
A2	Ornamental pear	++	++	59
H12	Willow	++	++	59
7B	Willow	++	++	K. Conway
7B12	Ornamental pear	++	++	61
7B22	Ornamental pear	++	++	G. Sundin
B48[c]	Peach	−	NG	19
B48(pCOP2)		−	−	This study
Cit7[c]	Citrus	−	NG	K. Willis
Cit7(pCOP2)		−	−	This study
Cit7(pPSR12)		−	++	This study
B301D[c]	Pear	−	NG	D. Gross
B301D(pCOP2)		−	−	This study
B301D(pPSR12)		−	++	This study
FF5[c]	Ornamental pear	−	−	59
FF5(pCOP2)		−	++	This study
FF5(pPSR4)		−	++	60
FF5(pPSR12)		+	++	This study
P. syringae pv. tabaci PTBR 2004	Tobacco	++	+	P. Shaw
P. syringae pv. tomato				
PT23	Tomato	−	−	3
4325[c]	Tomato	−	NG	ICMP
4325(pCOP2)		−	−	This study
DC3000[c]	Tomato	−	NG	D. Cuppels
DC3000(pCOP2)		−	−	This study
OK-1[c]	Tomato	−	NG	C. Bender
OK-1(pCOP2)		−	−	This study
P. viridiflava				
PV-1	Parsnip	−	−	J. Hunter
5787-3[c]	Tomato	−	NG	J. Jones
5787-3(pCOP2)		−	−	This study

[a] Strains were scored for production of EPS by the rating scale shown in Fig. 1. Symbols: −, nonmucoid; +, slightly mucoid; ++, moderately to highly mucoid.
[b] Abbreviations: NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, England; ICMP, International Collection of Microorganisms from Plants, Auckland, New Zealand.
[c] pCOP2, a clone containing the structural genes for copper resistance, was mobilized into these Cu^s strains to promote copper resistance.
[d] NG, no growth.

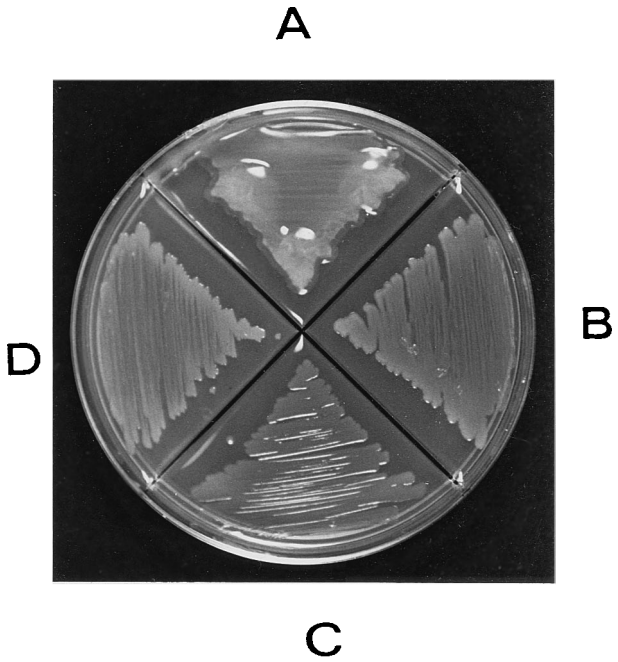

FIG. 1. Growth of selected pseudomonads on MG medium with and without $CuSO_4$. (A) *P. syringae* pv. syringae FF5(pPSR12) on MG medium showing a heavily mucoid phenotype; (B) *P. syringae* pv. syringae FF5 on MG medium exhibiting a nonmucoid phenotype; (C) *P. aeruginosa* 8822 on MG medium; (D) *P. aeruginosa* 8822 on MGcu.

acids or commercial alginate. Positive reactions were scored as visual depressions (≥1 cm) in the medium surrounding the *E. coli* colonies after a 24-h incubation at 37°C.

Uronic acid polymers from *P. syringae* pv. syringae FF5(pPSR4) and FF5(pPSR12) were hydrolyzed and further analyzed by descending paper chromatography (46). Samples containing polymeric uronic acids (2 mg/ml) were hydrolyzed overnight in 88% formic acid at 100°C, and the hydrolysate was dried under vacuum over NaOH pellets and redissolved in H_2O at 10 mg/ml. Hydrolysates (200 μg) were spotted onto Whatman no. 1 filter paper and separated by descending paper chromatography for 17 h in ethyl acetate-acetic acid-pyridine-water in a 5:1:5:3 (vol/vol) ratio (46). Mannuronic acid lactone (Sigma) and hydrolyzed seaweed alginate were used as controls. Chromatographs were dried and stained with silver nitrate, a specific stain for uronic acids (63).

DNA hybridization experiments. The DNA fragments used in Southern hybridization experiments were as follows: (i) a 3.2-kb *Xho*I-*Hin*dIII fragment containing *algT* from pJG309; (ii) a 2.8-kb *Eco*RI fragment containing *repA* from pLAI 31; and (iii) a 0.53-kb *Pst*I-*Hin*dIII fragment of IS*50* in pCLB9. DNA fragments were isolated from agarose gels by electroelution (52) and labelled with digoxigenin (Genius kit; Boehringer Mannheim, Indianapolis, Ind.) as described by the manufacturer. Hybridizations and posthybridization washes were conducted with the high-stringency conditions described previously (59).

Tn*5* and chemical mutagenesis. *P. syringae* pv. syringae FF5(pPSR12) was subjected to ethyl methanesulfonate (EMS) and Tn*5* mutagenesis to obtain nonmucoid mutants. Mutagenesis with EMS was performed by the method described by Eisenstadt et al. (24) with slight modifications. Bacterial cells were grown in King's medium B broth (5 ml) to 2×10^8 CFU/ml, pelleted at 8,000 × *g* for 10 min, and resuspended in 2.5 ml of MG broth. An equal volume of freshly prepared EMS (prewarmed to 28°C) was then added to give a final concentration of 10%, and cells were incubated with shaking at 280 rpm at 28°C for 2 h. The cells were washed twice in MG broth and plated on MG agar. After 2 days, individual colonies were transferred to MGcu, and the induction of EPS was scored as described above.

Transposon mutagenesis of strain FF5(pPSR12) was performed with *E. coli* C600 containing the Tn*5* suicide vector pGS9 as described previously (3). Transposon mutants of FF5(pPSR12) were selected on MG agar containing kanamycin (selective marker for Tn*5*) and ampicillin (counterselective antibiotic; inhibits *E. coli*). Transposon mutants which appeared after a 48-h incubation at 28°C were restreaked onto MG medium supplemented with ampicillin and kanamycin and evaluated for EPS production as described above.

RESULTS

Survey for EPS production. Thirty-seven plant-associated pseudomonads were examined visually for EPS production on MG medium (Table 2); 11 *P. syringae* strains, belonging to four pathovars, were stably mucoid on MG medium. These included *P. syringae* pv. glycinea 27a; *P. syringae* pv. maculicola 438; *P. syringae* pv. syringae G1, 7E50, 9A26, A2, H12, 7B, 7B12, and 7B22; and *P. syringae* pv. tabaci PTBR 2004. Of the remaining 26 pseudomonads, eight Cu^r *P. syringae* pv. syringae strains (7C12, 7D46, 8B48, 8D44, PSC1B, 7A15, 7B44, and 7C6) grew on MGcu and were visibly more mucoid when copper was added to the medium (Table 2). With the exception of strain PSC1B, all of these strains were isolated from woody ornamentals exposed to bactericidal sprays containing copper as an active ingredient.

To determine whether copper functioned as a signal for EPS induction in the 16 nonmucoid Cu^s strains, pCOP2, a recombinant plasmid containing the Cu^r genes from *P. syringae* pv. tomato PT23, was mobilized into each of these (Table 2). Although all transconjugants grew on MGcu, only *P. syringae* pv. syringae FF5(pCOP2) produced EPS in response to copper, indicating that copper is not a general stimulus for EPS biosynthesis in *Pseudomonas* spp.

With the exception of PSC1B, all *P. syringae* pv. syringae strains which became mucoid on MGcu were previously shown to contain Cu^r plasmids of different sizes. To determine whether Cu^r and the ability to produce EPS in response to copper could be encoded by a common plasmid, pPSR12, a 200-kb plasmid present in many Cu^r strains (61), was introduced into several Cu^s recipients. *P. syringae* pv. syringae FF5, B301D, and Cit7 containing pPSR12 each became heavily mucoid on MGcu. B301D and Cit7 transconjugants containing pCOP2, a clone containing structural genes for Cu^r, were nonmucoid on MGcu. Therefore, these results indicate that pPSR12 encodes an additional trait enabling these strains to produce EPS in response to copper because transconjugants of B301D and Cit7 containing pCOP2 were nonmucoid on MGcu.

P. aeruginosa 8821 and 8822 were also screened for EPS production in response to copper. Although the MIC of copper sulfate for both strains was 500 μg/ml, neither strain became any more mucoid on MG medium containing $CuSO_4$, regardless of the level included in the medium (Fig. 1C and D). Efforts to transfer pPSR12 into *P. aeruginosa* 8821 and 8822 failed, presumably because pPSR12 is a narrow-host-range plasmid. To determine whether the copper-responsive trait encoded by pPSR12 could function in *P. aeruginosa*, a cosmid library of pPSR12 was constructed in pRK7813. Cosmid pSK1, which contains a 50-kb insert from pPSR12, conferred a stably mucoid phenotype to *P. syringae* pv. syringae B301D, regardless of whether copper was present in the medium. pSK1 also conferred stable EPS production to the nonmucoid *P. syringae* pv. syringae FF5 and *P. syringae* pv. glycinea 7a, 16a, and PG4180 but did not confer mucoidy to *P. aeruginosa* 8822. These data indicate that pPSR12 and pSK1 encode one or more genes which result in increased alginate production in the presence of copper and which enable selected recipients to produce alginate in the absence of copper, resulting in a stably mucoid phenotype.

Production of uronic acids in selected *P. syringae* strains. Although both strains were inhibited on MG medium plus 300 μg of $CuSO_4$ per ml, *P. syringae* pv. syringae PSC1B and FF5 produced significantly larger quantities of uronic acids on MG medium supplemented with 150 μg of $CuSO_4$ per ml than on MG medium without copper (Table 3). For FF5, a strain lack-

TABLE 3. Uronic acids produced by selected strains of *P. syringae* on MG medium containing various levels of copper sulfate

Strain	Uronic acids produced (μg/mg of protein)[a] on:		
	MG medium	MGcu150[b]	MGcu300[b]
PT23	121 a	212 a	185 a
PSC1B	151 a	693 b	—[c]
FF5	58 a	300 b	—
FF5(pPSR4)	56 a	840 b	480 b
FF5(pPSR12)	1,196 a	5,047 b	5,728 b
FF5.21	86 a	272 b	254 b
FF5.22	82 a	58 a	106 a
FF5.31	90 a	324 b	362 b
FF5.32	54 a	116 b	116 b

[a] Means in the same row followed by the same letter are not significantly different at $P = 0.05$.

[b] MGcu150, MG medium plus 150 μg of $CuSO_4$ per ml. MGcu300, MG medium plus 300 μg of $CuSO_4$ per ml.

[c] —, these strains did not grow on plates containing this level of copper sulfate.

ing indigenous plasmids, this indicated that the ability to produce EPS in response to copper is chromosomally encoded in this strain. When FF5 contained the Cu^r plasmid pPSR4, uronic acid production by this transconjugant was 15-fold higher on MG medium plus 150 μg of $CuSO_4$ per ml than on nonamended MG medium. When FF5 contained pPSR12, cells were stably mucoid on MG medium lacking copper and uronic acid production was approximately 20 times higher than that of the wild-type FF5 on nonamended medium (Table 3). Uronic acid production by the Cu^r strain *P. syringae* pv. tomato PT23 (Table 3) and by *P. aeruginosa* 8821 and 8822 was not significantly impacted by the addition of $CuSO_4$ to MG medium.

Assays for alginic acid. A plate assay was used to determine whether uronic acid polymers could be degraded by alginate lyase. LB medium was amended with uronic acid polymers isolated from selected strains and then inoculated with *E. coli* JM109(pCL8) cells overexpressing *algL*. A depression approximately 1 cm in diameter surrounding colonies of JM109 (pCL8) cells was visible when these were incubated with uronic acid polymers isolated from *P. aeruginosa* 8830 and *P. syringae* pv. syringae FF5 (Table 4). Since alginate lyase specifically degrades alginate, these results indicated that the uronic acids produced by *P. syringae* pv. syringae FF5 were likely to be alginic acid. Uronic acid polymers isolated from the alginate nonproducer *X. campestris* pv. vesicatoria E3C5 were not degraded by alginate lyase (Table 4).

Alginate lyase acts specifically on polymannuronic acids con-

TABLE 4. Plate assay for degradation of uronic acids by alginate lyase

Source of uronic acids	Degradation[a]	
	JM109(pUC119)[b]	JM109(pCL8)[b]
P. aeruginosa 8830	−	+
P. syringae pv. syringae		
FF5(pPSR4)	−	+
FF5(pPSR12)	−	+
X. campestris pv. vesicatoria E3C5	−	−

[a] Symbols: −, no visible degradation was observed; +, a depression of ≥1 cm was apparent around the *E. coli* cells overexpressing *algL*.

[b] Indicator strains. *E. coli* JM109(pCL8) cells were overexpressing the cloned alginate lyase gene (*algL*); JM109(pUC119) cells were included as a negative control.

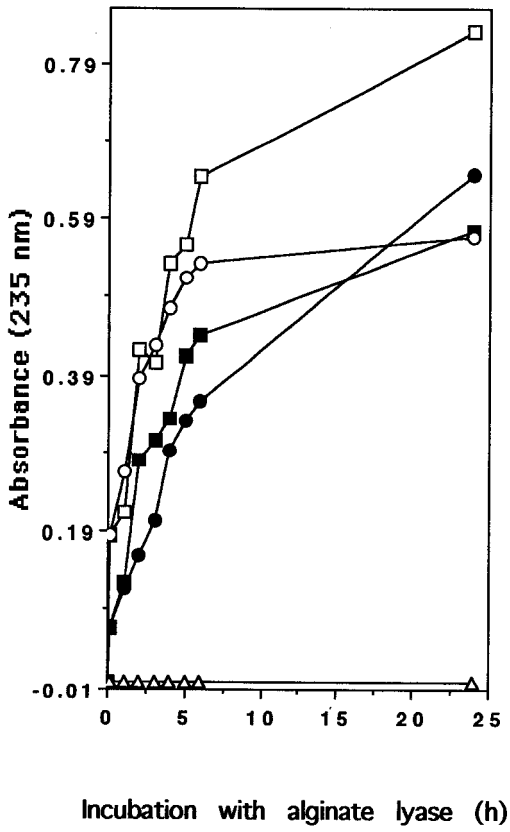

FIG. 2. Digestion of uronic acids extracted from *P. syringae* pv. syringae FF5(pPSR4) (○) and FF5(pPSR12) (●) and from *P. aeruginosa* 8830 (■) with alginate lyase. Seaweed alginate (□) and EPS from *X. campestris* pv. vesicatoria (△) were also included as positive and negative controls, respectively.

taining β-1,4 linkages, and the residues which result from lytic cleavage show an increased A_{235} (25). Figure 2 shows the results of incubating uronic acid polymers from various sources with alginate lyase and monitoring the reaction products for A_{235}. When uronic acids from *P. syringae* pv. syringae FF5 were incubated with alginate lyase, the increase in A_{235} was similar to that obtained with uronic acid polymers from *P. aeruginosa* 8830 and seaweed alginate (Fig. 2), thereby providing additional evidence that the uronic acids produced by FF5 were alginic acid.

When hydrolyzed uronic acids from FF5(pPSR4) and FF5(pPSR12) were analyzed by paper chromatography, a single spot which cochromatographed with mannuronic acid lactone (data not shown) was observed. This was also the major fraction visualized in seaweed alginate. These results indicate that mannuronic acid is the primary component of the alginate produced by FF5, which is consistent with results obtained for other pathovars of *P. syringae* (35, 50).

DNA hybridizations. We suspected that pPSR12 might contain a regulatory gene which somehow confers a stably mucoid phenotype to selected recipients. In *P. aeruginosa*, the *algT* gene encodes σ^E, an alternative sigma factor which is involved in the transcriptional activation of the alginate biosynthetic pathway (23). Previously, *algT* was demonstrated to allow transition of various nonmucoid pseudomonads to mucoidy when present as part of a multicopy plasmid (32). When genomic DNA from strain FF5 and plasmid pPSR12 were probed with the *algT* gene, hybridization to a 7-kb *Bam*HI fragment in *P.*

TABLE 5. MICs of selected metals

Metal[a]	MG medium	MIC (mM) for strain:		
		FF5	FF5(pPSR4)	FF5(pPSR12)
Mercury	Without Cu	0.075	0.075	0.10
	With Cu[b]	—[c]	0.0125	0.075
Cobalt	Without Cu	0.25	0.25	1.50
	With Cu	—	0.25	1.50
Arsenic	Without Cu	10	10	100
	With Cu	—	5	100
Molybdenum	Without Cu	40	40	40
	With Cu	—	40	40
Lithium	Without Cu	20	50	50
	With Cu	—	30	75
Rubidium	Without Cu	100	150	150
	With Cu	—	150	200

[a] Metals included the following: $HgCl_2$, $CoCl_2$, $NaHAsO_4$, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, LiCl, and RuCl.
[b] $CuSO_4 \cdot 5H_2O$ was added at 250 μg/ml.
[c] —, no growth.

syringae pv. syringae FF5 occurred. However, no homology was observed between pPSR12 and *algT* (data not shown). *repA* is a regulatory gene which was shown previously to restore alginate production to nonmucoid mutants of *Pseudomonas viridiflava* (44). However, pSK1, the cosmid constructed from pPSR12 which confers stable mucoidy to nonmucoid *P. syringae* strains, showed no homology to *repA*. These results suggest that the gene encoded on pPSR12 and pSK1 is not homologous to *algT* or *repA*, which are both capable of converting nonmucoid strains to the mucoid phenotype.

Response of *P. syringae* strains to selected heavy metals. Because copper was shown to induce alginate production in *P. syringae* pv. syringae FF5 (Table 3), we investigated whether mercury, cobalt, arsenate, molybdenum, lithium, or rubidium could stimulate EPS production. The concentrations of metals tested for EPS induction were 0.001 to 0.5 mM $HgCl_2$, 0.04 to 0.5 mM $CoCl_2$, 10 to 250 mM $NaHAsO_4$, 10 to 200 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 10 to 500 mM LiCl, and 10 to 500 mM RuCl. None of these metals induced EPS synthesis, indicating that production of alginate is not a generalized response to heavy metal exposure in *P. syringae* FF5.

One function of exopolysaccharides is to protect bacteria from toxic substances; consequently, we wanted to assess whether the EPS produced in response to copper significantly increased tolerance to other metals. When *P. syringae* pv. syringae FF5(pPSR4) was cultivated on MGY supplemented with 250 μg of $CuSO_4$ per ml and containing Hg, Co, As, Mo, Li, or Ru, tolerance to these metals was not enhanced (Table 5), even though FF5(pPSR4) cells were heavily mucoid in response to copper.

To determine whether pPSR4 or pPSR12 conferred resistance to other heavy metals, FF5, FF5(pPSR4), and FF5 (pPSR12) were evaluated for tolerance to the six metals listed in Table 5. Interestingly, we noted that FF5(pPSR12) showed more tolerance to cobalt and arsenate than FF5 and FF5 (pPSR4), suggesting that pPSR12 confers some protection from these two metals.

Recovery and analysis of alginate-defective mutants. Approximately 1,500 colonies of FF5(pPSR12) derived from EMS and Tn*5* mutagenesis were inspected visually for mucoidy on MG medium. Two nonmucoid EMS-derived mutants, FF5.21 and FF5.22, and two nonmucoid Tn*5* mutants, FF5.31 and FF5.32, were analyzed further. Alginate production by the four mutants was approximately 13- to 22-fold less than the amount produced by the wild-type FF5(pPSR12) on MG medium (Table 3). With the exception of FF5.22, the remaining three nonmucoid mutants showed significant increases in uronic acid production when copper sulfate was added to the medium. Southern hybridization with a probe constructed from IS*50* indicated that the Tn*5* insertions in FF5.31 and FF5.32 were located in the chromosome (data not shown).

All four nonmucoid mutants were screened for tolerance to the six metals listed in Table 5. In all experiments, the MICs of the metals for the nonmucoid mutants were not significantly different from that for FF5(pPSR12) on media containing arsenate, cobalt, lithium, rubidium, molybdenum, and mercury. These results suggest that EPS production by FF5(pPSR12) does not significantly increase the tolerance of these cells to various heavy metals. The MIC of $CuSO_4$ for FF5.22, FF5.31, and FF5.32 was 800 μg/ml and equivalent to that of the wild-type FF5(pPSR12); the MIC for FF5.21, however, was slightly lower (600 μg of $CuSO_4$ per ml).

DISCUSSION

A number of *P. syringae* pv. syringae strains identified in the present study were stably mucoid or mucoid in the presence of $CuSO_4$. With the exception of *P. syringae* pv. syringae PSC1B, which was isolated from corn (19), these strains originated from woody ornamentals exposed to copper bactericides in Oklahoma nurseries (61). Most of these strains could be differentiated on the basis of size and phenotype of resident indigenous plasmids and were assigned to different clusters when analyzed by arbitrarily primed PCR. Therefore, the mucoid strains identified in the present study are part of a genetically diverse population of *P. syringae* strains which reside in a habitat where exposure to copper bactericides is frequent.

Our results indicate that copper is not a general signal for alginate production in *P. syringae*. However, a number of *P. syringae* pv. syringae strains utilized in the present study produced significantly higher levels of alginate in response to copper. The mechanism involved in the induction of alginate by copper is presently unknown but could involve the transcriptional activation of alginate genes in response to copper. *P. syringae* was shown previously to contain DNA homologous to structural (*algA*, *algC*, and *algD*) and regulatory (*algR1*) genes for alginate production in *P. aeruginosa* (28, 29, 41). Results obtained in the present study suggest that *P. syringae* may also contain an *algT* homolog. The isolation of structural and regulatory genes for alginate production in *P. syringae* and their cognate upstream sequences will help clarify whether transcriptional activation occurs in response to copper.

In the present study, we identified a plasmid, pPSR12, which contains a gene(s) conferring a stably mucoid phenotype to several *P. syringae* recipients. Goldberg et al. (32) showed previously that a plasmid containing the *algT* gene from *P. aeruginosa* activated alginate production in a variety of *Pseudomonas* spp. and resulted in conversion from a nonmucoid state to a mucoid phenotype. More recently, DeVries and Ohman (23) showed that *algT* (*algU*) encodes σ^E, an alternative sigma factor required for transcriptional activation of *algD* (45). In the present study, we found evidence for an *algT* homolog in the chromosomal DNA of *P. syringae* pv. syringae FF5; however, the function of the pPSR12-encoded gene which confers stable mucoidy to *P. syringae* remains obscure. Furthermore, no homology could be demonstrated between *repA* and the pPSR12-encoded gene; *repA* was shown previously to be related to *lemA* and was required for alginate production in *P. viridiflava* (44). In *P. solanacearum*, phenotype conversion and the subsequent loss of mucoidy were shown to be caused by insertions and

deletions in *phcA*, a transcriptional regulator in the LysR family (9). Thus, the possibility that pPSR12 encodes a regulatory gene similar to *phcA* remains.

Several *P. syringae* strains which showed stable EPS production in vitro were identified in the present study; these strains will be extremely useful in future genetic and biochemical studies. In vitro production of alginate in *Pseudomonas* spp. is highly variable and strongly influenced by culture conditions (10, 12, 27). We found that MG agar medium is an excellent substrate to use for screening alginate production; however, we observed very little production of alginate when strains were cultivated in MG broth. Similarly, Darzins and Chakrabarty (17) found that *P. aeruginosa* 8830 produced alginate on solid medium in 24 h but remained incapable of synthesizing alginate in liquid medium, regardless of the carbon source. Elevated EPS production on solid medium may reflect the stimulation of alginate biosynthesis via cell-to-cell contact, a density-dependent phenomenon.

Production of EPS in response to toxic compounds has been associated with increased tolerance to the offending substance. For example, mucoid variants of *P. aeruginosa* show enhanced resistance to carbenicillin (33). With one exception (FF5.21), the alginate-defective mutants isolated in the present study were not affected in tolerance to copper, cobalt, or arsenate when compared with the tolerance of the mucoid wild-type FF5(pPSR12). These results suggest that alginate production does not significantly increase heavy metal tolerance in vitro. However, in situ studies have shown that bacteria which form biofilms can sometimes withstand many times the dosage of antibiotic sufficient to eradicate planktonic organisms (1). Although biofilm formation by *P. syringae* on plant surfaces has not been reported, these bacteria can colonize the intercellular spaces of host plants as a solid bacterial mass (51). Consequently, it is possible that biofilm formation by *P. syringae* occurs and consequently increases tolerance to toxic compounds as it does in *P. aeruginosa* (62).

The results of the present study suggest that some of the signals and regulatory genes for alginate production in *P. syringae* differ from those described in *P. aeruginosa*. We found that copper stimulated alginate production in a subset of *P. syringae* pv. syringae strains, and we identified a plasmid-encoded gene(s) which confers stable mucoidy to *P. syringae* but not to *P. aeruginosa*. These results may reflect the different ecological niches inhabited by the two organisms. For example, *P. syringae* colonizes plant surfaces and is exposed to intensive bactericidal sprays containing copper; however, exposure of clinical strains of *P. aeruginosa* to $CuSO_4$ may be minimal. To our knowledge, the specific induction of alginate biosynthesis by copper has not been demonstrated previously. EPS production in response to copper can be an industrially important problem because EPS have been associated with biocorrosion and material failure (37). Although the mechanisms and rationale for EPS induction by copper remain obscure, the phenomenon could be widespread and warrants further study.

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