JOURNAL OF BACTERIOLOGY, July 1997, p. 4464–4472 Vol. 179, No. 14
0021-9193/97/$04.00+0

Characterization of the Alginate Biosynthetic Gene Cluster in *Pseudomonas syringae* pv. syringae

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Received 18 February 1997/Accepted 7 May 1997

Alginate, a copolymer of D-mannuronic acid and L-guluronic acid, is produced by a variety of pseudomonads, including *Pseudomonas syringae*. Alginate biosynthesis has been most extensively studied in *P. aeruginosa*, and a number of structural and regulatory genes from this species have been cloned and characterized. In the present study, an alginate-defective (Alg$^-$) mutant of *P. syringae* pv. syringae FF5 was shown to contain a Tn*5* insertion in *algL*, a gene encoding alginate lyase. A cosmid clone designated pSK2 restored alginate production to the *algL* mutant and was shown to contain homologs of *algD*, *alg8*, *alg44*, *algG*, *algX* (*alg60*), *algL*, *algF*, and *algA*. The order and arrangement of the structural gene cluster were virtually identical to those previously described for *P. aeruginosa*. Complementation analyses, however, indicated that the structural gene clusters in *P. aeruginosa* and *P. syringae* were not functionally interchangeable when expressed from their native promoters. A region upstream of the *algD* gene in *P. syringae* pv. syringae was shown to activate the transcription of a promoterless glucuronidase (*uidA*) gene and indicated that transcription initiated upstream of *algD* as described for *P. aeruginosa*. Transcription of the *algD* promoter from *P. syringae* FF5 was significantly higher at 32°C than at 18 or 26°C and was stimulated when copper sulfate or sodium chloride was added to the medium. Alginate gene expression was also stimulated by the addition of the nonionic solute sorbitol, indicating that osmolarity is a signal for *algD* expression in *P. syringae* FF5.

The phytopathogenic bacterium *Pseudomonas syringae* produces two well-characterized extracellular polysaccharide (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 (21, 29). Possible roles for the EPS molecules produced by *P. syringae* are varied and include avoidance of host plant cell recognition, resistance of bacterial cells to desiccation, and enhancement of epiphytic fitness (33, 41). Furthermore, alginate has been implicated in a symptom known as water soaking, where the intercellular tissues of infected plants become filled with water (20, 29). However, a role for alginate in the symptomology or virulence of *P. syringae* has not been proven.

Alginate biosynthesis has been extensively studied in *Pseudomonas aeruginosa*, where it functions as a major virulence factor in strains infecting the lungs of cystic fibrosis patients (56). In *P. aeruginosa*, genes that encode the biosynthesis and regulation of alginate map to four chromosomal locations. With the exception of *algC*, which is located at 10 min, most of the structural genes are located at 34 min. The regulatory genes map at 10 and 13 min, and the loci responsible for the genotypic switch to alginate production are located at 68 min (46). Most of the structural genes for alginate biosynthesis are clustered within an 18-kb region in the *P. aeruginosa* chromosome (16). Structural genes that have been characterized in this region include (see Fig. 1A) *algA*, which encodes a bifunctional enzyme that functions as a phosphomannose isomerase and a GDP-mannose pyrophosphorylase (57); *algG*, which encodes a C-5 epimerase (10); *algF*, *algI*, and *algJ*, which is involved in acetylation of the alginate polymer (26, 27, 57); and *algD*, which encodes a GDP-mannose dehydrogenase (17). This region also contains *algE*, which encodes a membrane protein with a putative role in polymer export (12), and *algL*, which encodes alginate lyase (8, 55). Several other genes, including *alg44*, *alg8*, and *algX* (*alg60*), also mapped within this region (43, 48, 65); however, the functional role of the proteins encoded by these genes remains unclear. Chitnis and Ohman (11) showed that the alginate biosynthetic gene cluster in *P. aeruginosa* is organized as a single operon with transcription initiating at the *algD* promoter.

Although *P. syringae* was previously shown to contain DNA homologous to *algA* and *algD* (22, 23), the physical location of these genes has not been determined. Furthermore, alginate-defective (Alg$^-$) mutants of *P. syringae* have been isolated but not previously characterized. In the present study, an Alg$^-$ mutant of *P. syringae* pv. syringae was shown to contain a Tn*5* insertion in the alginate structural gene cluster. The structural genes were cloned from *P. syringae*, and their organization was shown to be virtually identical to that of the structural gene cluster in *P. aeruginosa*. Complementation analysis and gene fusions were used to study the transcriptional organization and expression of the alginate structural gene cluster in *P. syringae.*

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Table 1 lists the bacterial strains and plasmids used in the present study. *Pseudomonas* spp. were routinely maintained on King's medium B (38), mannitol-glutamate medium (MG) (34), or MG supplemented with yeast extract at 0.25 g/liter (MGY) at 28°C; *Escherichia coli* strains were grown on Luria-Bertani LB medium (47) at 37°C. Antibiotics were added to media at the following concentrations (micrograms per milliliter): ampicillin, 40; tetracycline, 12.5; kanamycin, 25; spectinomycin, 25; streptomycin, 25; and piperacillin, 250.

Molecular genetic techniques. Plasmids were isolated from *Pseudomonas* spp. by the plasmid isolation method described by Crosa and Falkow (13) with slight modifications (4). Restriction enzyme digests, agarose gel electrophoresis, Southern transfers, and isolation of DNA fragments from agarose gels were performed by standard methods (54). Genomic DNA was isolated from *P.*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. coli DH5α		54
P. syringae pv. syringae		
FF5	Cu^s, no detectable plasmids, nonmucoid	62
FF5	Cu^r, contains pPSR12, stably mucoid	37
FF5.21	Cu^r, contains pPSR12, nonmucoid EMS mutant	37
FF5.22	Cu^r, contains pPSR12, nonmucoid EMS mutant	37
FF5.31	Cu^r Km^r, contains pPSR12, nonmucoid, *algL*::Tn*5*	37
FF5.32	Cu^r Km^r, contains pPSR12, nonmucoid, *algR1*::Tn*5*	50
P. aeruginosa		
8830	Mucoid	14
8835	*algD* mutant of 8830	14
8853	*algA* mutant of 8830	14
Plasmids		
pBluescript SK+	Ap^r, ColE1 origin	Stratagene
pRK7813	Tc^r, cosmid vector	32
pRK2013	Km^r, helper plasmid	24
pRK415	Tc^r	35
pRG960sd	Sm^r Sp^r, contains promoterless *uidA* with start codon and Shine-Dalgarno sequence	64
pPSR12	Cu^r Sm^r, 200 kb, confers stable mucoidy to *P. syringae* pv. syringae FF5	37
pDS1	Ap^r, *algA* from *P. aeruginosa* in pUC119	A. Chakrabarty
pNZ15	Km^r, *algC* from *P. aeruginosa* in pRJD215	67
pSN11	Ap^r, *algD* from *P. aeruginosa* in pUC118	A. Chakrabarty
pPB76	Ap^r, *algF* from *P. aeruginosa* in pT7-6	A. Chakrabarty
pECL119	Ap^r, *algG* from *P. aeruginosa* in pUC119	A. Chakrabarty
pCA10	Ap^r, *algL* from *P. aeruginosa* in pUC119	8
pABR11	Ap^r, *algR1* from *P. aeruginosa* in pT7-7	A. Chakrabarty
pJG309	Tc^r, *algT* from *P. aeruginosa* in pCP13	28
pSK2021	Ap^r, *alg8 alg44* from *P. aeruginosa* in pMMB24	65
pAD501	Tc^r, alginate biosynthetic gene cluster from *P. aeruginosa* 8830 in pCP13	15
pSK2	Tc^r, alginate biosynthetic cluster from *P. syringae* pv. syringae FF5 in pRK7813	This study
pSK31	Tc^r Km^r, contains Tn*5*-inactivated alginate genes from FF5.31 in pRK7813	This study
pSK31.1	Ap^r, contains 3.6-kb *Bam*HI-*Eco*RI fragment containing 2.8 kb from Tn*5* and 0.8 kb of FF5.31 DNA in pBluescript SK+	This study
pAPE7.2	Ap^r, contains 7.2-kb *Eco*RI fragment from pSK2 in pBluescript SK+	This study
pAPE6.2	Ap^r, contains 6.2-kb *Eco*RI fragment from pSK2 in pBluescript SK+	This study
pAPE2.2	Ap^r, contains 2.2-kb *Eco*RI fragment from pSK2 in pBluescript SK+	This study
pAPE7.8	Ap^r, contains 7.8-kb *Eco*RI fragment from pSK2 in pBluescript SK+	This study
pSKK3.9	Ap^r, contains 3.9-kb *Kpn*I fragment from pSK2 in pBluescript SK+	This study
pSKK2.0	Ap^r, contains 2.0-kb *Kpn*I fragment from pSK2 in pBluescript SK+	This study
pSK3	Sm^r Sp^r, contains a 1.0-kb *Sma*I insert from pSKK2.0 in pRG960sd (*Sma*I-*Kpn*I-*uidA*)	This study
pSK4	Sm^r Sp^r, contains a 1.0-kb *Sma*I insert from pSKK2.0 in pRG960sd (*Kpn*I-*Sma*I-*uidA*)	This study
pAD4033	Ap^r, *algA* from *P. aeruginosa* under P*tac* control in pMMB22	8
pVD2X	Tc^r, contains *algD-xylE* transcriptional fusion	17

[a] Cu, copper; Km, kanamycin; Tc, tetracycline; Sm, streptomycin; Sp, spectinomycin; r, resistant; s, sensitive.

syringae by established procedures (61), and a total genomic library of FF5(pPSR12) was constructed in pRK7813 as previously described (1). Cosmid clones were mobilized into nonmucoid recipient strains by using a triparental mating procedure and the mobilizer plasmid pRK2013 (6).

Isolation and quantitation of alginate. Selected strains were inoculated by dilution streaking to MG agar (three plates per strain) and incubated at 28°C for 48 h. Each plate was handled separately for quantification of alginate. The cells were washed from each plate and resuspended in 0.9% NaCl. Removal of cellular material from the mucoid growth and estimation of alginate content and total cellular protein were performed as described by May and Chakrabarty (45). Alginic acid from seaweed (*Macrocystis pyrifera*; Sigma Chemical Co., St. Louis, Mo.) was used as a standard in these experiments. Each experiment was repeated, and the mean values are expressed as the amount of alginate produced per milligram of protein.

DNA hybridization experiments. DNA fragments used in Southern hybridization experiments were as follows: (i) a 2.0-kb *Sst*I fragment containing *algA* from pDS1; (ii) a 2.6-kb *Hin*dIII-*Sst*I fragment containing *algC* from pNZ15; (iii) a 1.1-kb *Sph*I-*Sst*I fragment containing *algD* from pSN11; (iv) a 1.1-kb *Pst*I-*Bam*HI fragment containing *algF* from pPB76; (v) a 0.6-kb *Hin*dIII fragment containing *algG* from pECL119; (vi) a 1.7-kb *Xba*I fragment containing *algL* from pCA10; (vii) a 1.0-kb *Nde*I-*Bam*HI fragment containing *algR1* in pABR11; (viii) a 3.3-kb *Xho*I-*Hin*dIII fragment containing *algT* in pJG309; (ix) a 1.1-kb *Sph*I-*Bgl*II fragment from pSK2021 containing *alg8*; and (x) a 1.4-kb *Bgl*II-*Kpn*I fragment containing *alg44* from pSK2021. DNA fragments were isolated from agarose gels by electroelution (54) and labelled with digoxigenin (Genius labelling and detection kit; Boehringer Mannheim, Indianapolis, Ind.) as described by the manufacturer. Hybridizations and posthybridization washes were conducted under the high-stringency conditions described previously (62).

DNA sequencing and analysis. Nucleotide sequencing reactions were performed by the dideoxynucleotide method (54) with AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, Calif.). Automated DNA sequencing was accomplished with an ABI 373A apparatus and the ABI PRISM Dye Primer cycle-sequencing kit (Perkin-Elmer). Automated sequencing was provided by the Oklahoma State University Recombinant DNA/Protein Resource Facility. The Tn*5* insertion in FF5.31 was localized by sequencing the DNA flanking the transposon by using the oligonucleotide 5′GGTTCCGTTCAGGACGCTAC,

which is derived from the border region of IS*50* (53). The location of *algF* was determined with an oligonucleotide primer designated P2 (5′CCCTGTTCCAGCAAA).

GUS assays. Plasmid pRG960sd, which contains a promoterless glucuronidase (GUS) gene (*uidA*) downstream of a multiple-cloning site (64), was used to identify promoter sequences upstream of *algD* and to verify their transcriptional orientation. Subcloned DNA fragments were ligated into pRG960sd, and orientations were determined by restriction digests. These constructs were mobilized into *P. syringae* pv. syringae FF5 and promoter activities were initially screened by spotting bacterial suspensions (absorbance at 590 nm [A_{590}] = 0.05) on MGY agar medium amended with spectinomycin and 20 μg of X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide) per ml; the plates were then incubated at 28°C for 48 h.

Preliminary experiments indicated that even trace amounts of copper could significantly increase *algD* expression; therefore, additional precautions were taken to remove copper ions from glassware and media prior to quantitative analysis of glucuronidase activity. All glassware was rinsed twice with 2 N HCl, twice with 2 N nitric acid, once with 0.1 M EDTA, and once with copper-free water purified by ion-exchange chromatography. All media used in glucuronidase assays were treated with Chelex 100 to remove heavy metals by the batch method described by the manufacturer (Bio-Rad Laboratories, Richmond, Calif.).

Prior to the GUS assays, all strains were grown for 30 h on MGY agar containing spectinomycin. The bacterial concentration was adjusted to an A_{600} of 0.3 and incubated at 250 rpm for 10 h at 28°C in MGY broth amended with NaCl (0, 0.15, 0.3, or 0.4 M), sorbitol (0, 0.3, 0.6, or 0.8 M) or $CuSO_4$ (0 to 200 μM). The effect of temperature on *algD* transcription was also investigated by preparing bacterial suspensions as described above and incubating them at 18, 26, and 32°C. Promoter activity was determined by fluorometric analysis of GUS (66); fluorescence was monitored with a Fluoroscan II version 4.0 microplate reader (ICN Biomedicals, Inc., Costa Mesa, Calif.) in 96-well microtiter plates. The protein content in cell lysates was determined using the BioRad protein assay kit (Bio-Rad) as recommended by the manufacturer.

The kinetics of *algD* transcription in the presence and absence of copper was evaluated by initially cultivating strains on MGY agar as described above. The bacteria were then suspended in MGY or MGY containing 40 μM $CuSO_4$ (MGY-Cu) to an A_{600} of 0.1 and incubated at 250 rpm at 28°C. Aliquots of cells (three replicates per time point) were removed at 1, 5, 8, 12, 24, and 30 h and evaluated for growth by dilution plating and for transcriptional activity by glucuronidase assays.

Catechol 2,3-dioxygenase (CDO) activity. The *algD* promoter from *P. aeruginosa* 8830 was previously cloned as a transcriptional fusion to a promoterless *xylE* gene (17). In the present study, functional expression of the *algD-xylE* transcriptional fusion was detected by spraying plates with an aqueous solution of 0.5 M catechol as described previously (68). Colonies of cells expressing *xylE* turn yellow due to the conversion of catechol to 2-hydroxymuconic semialdehyde (68).

RESULTS

P. syringae pv. syringae FF5 was previously shown to be nonmucoid (37). However, when the 200-kb plasmid pPSR12 was introduced into strain FF5, the transconjugant exhibited a stable mucoid phenotype due to constitutive production of alginate at high levels (37). FF5(pPSR12) was previously mutagenized with both ethyl methanesulfonate (EMS) and Tn*5*, and several nonmucoid mutants were isolated (37). In an effort to further characterize the mutants, a genomic library of FF5(pPSR12) was constructed in pRK7813 and mobilized into the nonmucoid mutants FF5.21, FF5.22, and FF5.31. Approximately 200 transconjugants of each mutant were screened for mucoidy on MG containing tetracycline and ampicillin. Although FF5.21 and FF5.22 were not complemented for mucoidy by this approach, we did identify a single clone, pSK2, which restored mucoidy to the Tn*5* mutant designated FF5.31. FF5.31 containing pSK2 produced 743 μg alginate per mg of protein, a level comparable to that in the wild-type FF5(pPSR12) (37) and 52-fold higher than that produced by the nonmucoid mutant FF5.31. These results indicated that pSK2 restored alginate production to FF5.31.

DNA hybridizations. To determine whether pSK2 contained homologs of genes previously characterized from *P. aeruginosa*, probes were constructed from the following: (i) *algD*, a structural gene mapping at 34 min; (ii) *algR1*, a regulatory gene mapping at 10 min; (iii) *algC*, a structural gene mapping at 10 min; and (iv) *algT*, a gene involved in the genotypic transition to mucoidy which maps at 68 min. Although all four genes previously hybridized to total genomic DNA from *P. syringae* pv. syringae FF5 (36, 37), only *algD* showed hybridization to pSK2 (data not shown).

In *P. aeruginosa*, *algD* and *algA* are cotranscribed and the 14-kb area separating these two genes contains *algF*, *algL*, *algG*, *alg44*, and *alg8*, which are involved in the biosynthesis, modification, and processing of alginate (11, 46). All probes constructed from these loci hybridized to Southern blots containing pSK2 (data not shown), suggesting that the organization of the alginate biosynthetic gene cluster in *P. syringae* pv. syringae FF5 might be similar to that in *P. aeruginosa.*

A physical map of pSK2, constructed with *Eco*RI and *Kpn*I, was used to further localize the *P. syringae* alginate biosynthetic cluster (Fig. 1B). To facilitate the mapping of the alginate biosynthetic genes encoded by pSK2, *Eco*RI fragments 1, 2, 3, and 4 were subcloned in pBluescript SK+. The T3 and T7 primers were then used to sequence 200 to 500 nucleotides of insert DNA adjacent to each *Eco*RI site (Fig. 1C). By using this approach and the alginate gene probes cited above, we showed that *algA*, *algX*, and *algG* spanned *Eco*RI fragments 1 and 2, 2 and 3, and 3 and 4, respectively, whereas *algL* mapped within *Eco*RI fragment 2 as indicated (Fig. 1B). To localize *alg44*, *alg8*, and *algD*, *Kpn*I fragment 5 was subcloned in pBluescript SK+ as pSKK3.9 (Fig. 1B); sequence analysis indicated that *alg44* spanned *Kpn*I fragments 4 and 5 and showed that *algD* mapped within the right border of *Kpn*I fragment 5 (Fig. 1B). Hybridization analysis indicated that *alg8* was flanked by *alg44* and *algD* as indicated (Fig. 1B). Since *algA* and *algF* are separated by approximately 200 bp in *P. aeruginosa* (58), we investigated whether a similar arrangement existed in *P. syringae*. The P2 primer was derived from the *algA* homolog in *P. syringae* and corresponded to nucleotides 140 to 154 in *algA* from *P. aeruginosa* (58). When P2 was used to generate the sequence 5′ to *algA* in *P. syringae*, the results showed that *algF* was located 194 bp upstream of *algA* (Fig. 1B). These results indicated that the spatial organization of *algA* and *algF* is virtually identical in the two species. Table 2 contains a complete summary of the sequence analyses conducted in the present study.

Location of Tn*5* insertion in FF5.31. A genomic library of FF5.31 was constructed in pRK7813, and a clone containing the Tn*5* insertion from FF5.31 was recovered and designated pSK31 (Fig. 1D). The internal *Bam*HI site in Tn*5* and 0.8 kb of flanking DNA were cloned in pBluescript SK+ as pSK31.1 (Fig. 1D). A primer specific for the border region of IS*50* indicated that the Tn*5* insertion was located in *algL* at nt 1135 of the corresponding *P. aeruginosa* sequence (Fig. 1B). A 600-nucleotide stretch downstream of the Tn*5* insertion was sequenced in pSK31.1 and showed 71% identity to *algL* from *P. aeruginosa.*

Complementation experiments. Our results indicated that the structural genes encoding alginate biosynthesis were arranged similarly in *P. syringae* and *P. aeruginosa*; therefore, we investigated whether pAD501, which contains the structural genes from *P. aeruginosa* 8830, could restore the mucoid phenotype to *P. syringae* FF5.31. *P. aeruginosa* 8853 and 8835, which contain defects in *algA* and *algD*, respectively, could be complemented for alginate production by pAD501; however, pAD501 did not confer alginate production to FF5.31 (data not shown). Similarly, the introduction of pSK2, which has an insert size of 25 kb harboring the *P. syringae* alginate gene cluster, restored mucoidy to *P. syringae* pv. syringae FF5.31 but not to *P. aeruginosa* 8853 or 8835. These results, in addition to other observations (37), suggest that the regulation and transcriptional activation of alginate biosynthesis differ in *P. syringae* and *P. aeruginosa.*

Boyd et al. (8) previously generated a mutation in the *P.*

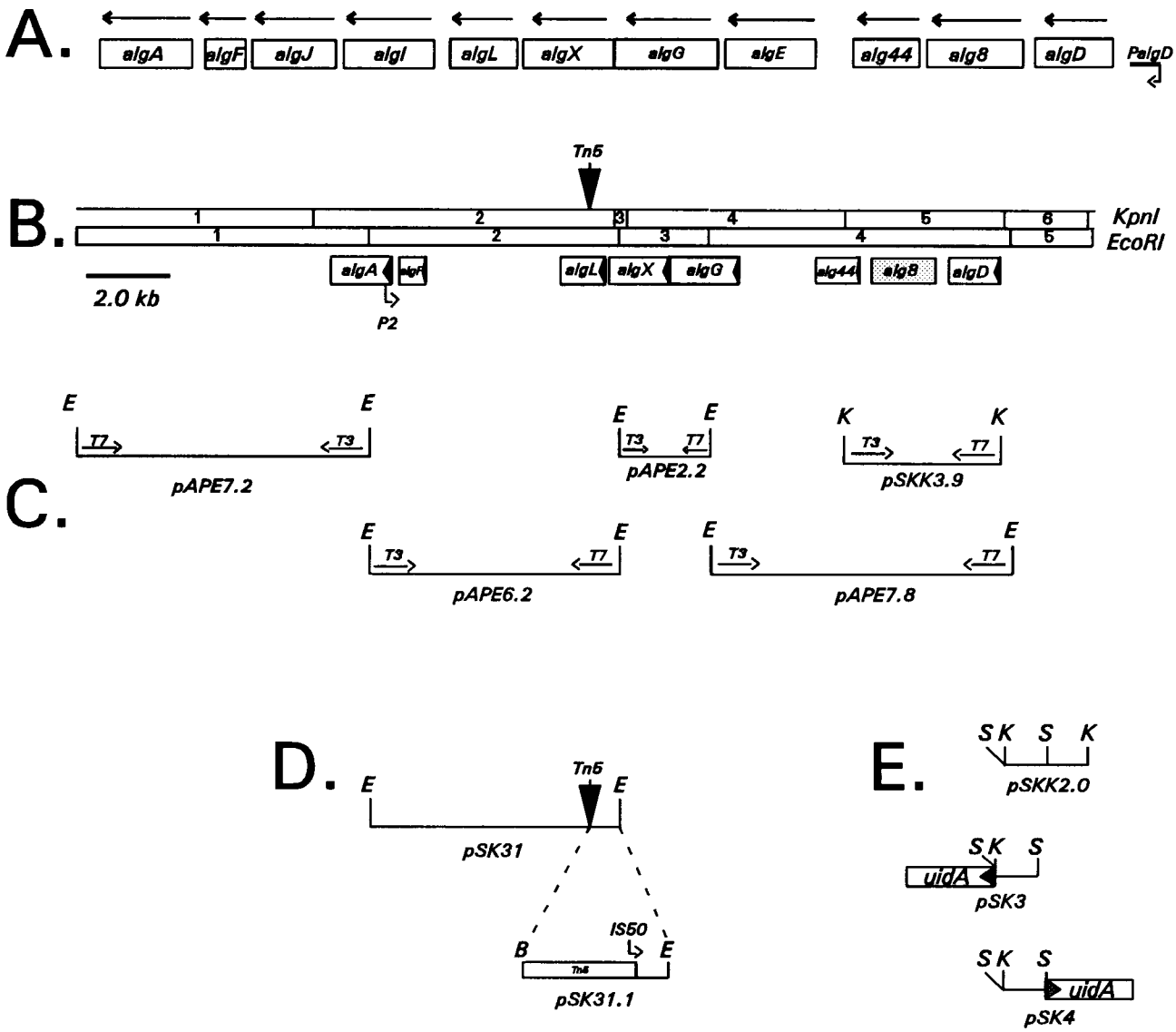

FIG. 1. (A) Organization of the alginate structural gene cluster in *P. aeruginosa*. The size of each gene and its physical location are based on previously published maps (27, 46). Arrows above the genes indicate the direction of transcription. The location and orientation of the *algD* promoter in *P. aeruginosa* (*PalgD*) are also indicated. (B) Physical and functional map of the alginate structural gene cluster in pSK2, a cosmid clone isolated from *P. syringae* pv. syringae FF5. The arrow above the physical map shows the location of the Tn*5* insertion in the alginate-defective mutant FF5.31. With the exception of *alg8*, which mapped to *Kpn*I fragment 5 based on Southern hybridization and restriction mapping, all the remaining genes were initially localized by Southern hybridization and confirmed by sequence analysis. The direction of translation is indicated by the arrows within each open reading frame and was based on nucleotide sequence analysis. The size of each gene was based on the sequenced *P. aeruginosa* homolog as follows: *algD*, 1,310 bp (17); *alg8*, 1,493 bp (43); *alg44*, 950 bp (43); *algG*, 1,631 bp (25); *algX*, 1,424 bp (48); *algL*, 1,106 bp (8); *algF*, 650 bp (58); and *algA*, 1,440 bp (15). The location of the P2 primer which was used to localize *algF* is also indicated. (C) Cloned fragments used to sequence selected regions of the *P. syringae* alginate gene cluster. The T7 and T3 primers were used to sequence 200 to 500 nucleotides in pAPE7.2, pAPE6.2, pAPE2.2, pAPE7.8, and pSKK3.9 in the directions indicated. (D) pSK31 and pSK31.1, the clones used to localize the Tn*5* insertion in FF5.31. The location of the IS*50* primer which was used to localize the Tn*5* insertion in FF5.31 is indicated. (E) pSK3 and pSK4, which contain a 1-kb region upstream of *algD* in pRG960sd and were used to establish the direction of transcription. Abbreviations: E, *Eco*RI; K, *Kpn*I; S, *Sma*I.

aeruginosa algL gene which resulted in a nonmucoid phenotype due to polar effects on the downstream *algA*; the latter gene is essential for alginate biosynthesis since it encodes phosphomannose isomerase and GDP-mannose pyrophosphorylase, the first and third enzymes, respectively, in the alginate pathway. Alginate production was restored to the *algL* mutant when *algA* was supplied in *trans* and expressed from the P*tac* promoter (8). In the present study, pAD4033, a construct containing *P. aeruginosa algA* under P*tac* control, complemented FF5.31 for alginate production. FF5.31(pAD4033) produced 703 μg of alginate/mg of protein, a level significantly higher than that produced by the mutant FF5.31 (Table 3), and equivalent to that in FF5.31(pSK2), which produced 743 μg of uronic acid polymers/mg of protein. Furthermore, pAD4033 did not confer alginate production to FF5.32 (Table 3), a nonmucoid strain containing a Tn5 insertion in *algR1* (50).

Transcriptional organization of the alginate biosynthetic gene cluster in *P. syringae*. In *P. aeruginosa*, transcription of the alginate biosynthetic gene cluster begins upstream of *algD* and proceeds through *algA* (11). Complementation of the polar

TABLE 2. Partial sequence analysis of selected genes in the *P. syringae* alginate biosynthetic gene cluster and relatedness to the corresponding genes in *P. aeruginosa*

Gene	Nucleotides sequenced[a]	% Identity[b]	Reference
algA	40–889	74	15
algD	512–709	78	17
algF	824–1141	67	58
algG	203–735	65	25
algL	1–255	61	8
	535–1135	71	8
algX	2412–2940	68	48
alg44	2018–2367	65	43

[a] The nucleotides cited correspond to those published for *P. aeruginosa*. For example, 849 bp of *algA* was sequenced in *P. syringae* and corresponded to nucleotides 40 to 889 in the sequence reported for *algA* in *P. aeruginosa* (15). With the exception of *algF* and *algL*, all sequences were generated with the T3 and T7 primers as described in the text. The *algF* sequence was generated with the P2 primer, and nucleotides 535 to 1135 of *algL* were obtained with the IS*50* primer (see the text).

[b] The percent nucleotide identity when the *P. syringae* sequence was compared to the sequence published for *P. aeruginosa*.

mutation in FF5.31 by pAD4033 indicated that *algA* was transcribed downstream of *algL* in *P. syringae*. Since this result suggested that the transcriptional organization of the alginate structural genes might be conserved in the two species, we investigated whether a promoter region could be located upstream of *algD* in pSK2. In *P. aeruginosa*, the transcriptional start site for the alginate biosynthetic gene cluster and the *algD* promoter were localized 367 and 477 bp, respectively, upstream of the *algD* translational start site (17, 39). Assuming that this arrangement is conserved in *P. syringae*, we might expect to find the *algD* promoter region within the 2.0-kb *Kpn*I fragment (fragment 6, Fig. 1B) which maps 380 bp upstream of the *algD* translational start in *P. syringae*. To test this hypothesis, this fragment was subcloned in pBluescript SK+ to generate pSKK2.0 (Fig. 1E). Digestion with various restriction enzymes indicated that a *Sma*I site was located in the middle of the *Kpn*I fragment, providing a convenient site for subcloning into pRG960sd, a vector containing a promoterless GUS gene (64). The 1-kb *Kpn*I-*Sma*I fragment adjacent to *algD* was subcloned in both orientations in pRG960sd, resulting in two constructs designated pSK3 and pSK4 (Fig. 1E).

pSK3 and pSK4 were introduced into FF5, and the transconjugants were examined for GUS activity. The mean GUS activity in FF5(pSK3) was 36 U/mg of protein, a level approximately ninefold higher than the levels produced by FF5(pRG960sd) and FF5(pSK4). This result indicated that pSK3 contained a functional promoter region and that transcription proceeded from *algD* into the structural gene cluster as predicted.

***algD* activity in response to selected environmental and nutritional factors.** GUS activity in FF5(pSK3) is stimulated by sodium chloride; for example, it was 10-fold higher when the cells were grown in media containing 0.3 M NaCl (Fig. 2A). Controls consisting of FF5 with the vector only (pRG960sd) and with *algD* in the transcriptionally inactive orientation (pSK4) showed no response to the addition of sodium chloride (Fig. 2A). To determine whether the effect of added NaCl was ionic or osmotic, the effect of sorbitol (a nonionic, nonmetabolizable solute) was examined for its effect on alginate gene expression. Sorbitol was added to MGY at 0.3, 0.6, and 0.8 M, concentrations which are osmotically equivalent to 0.15, 0.3, and 0.4 M NaCl, respectively. The results obtained with sorbitol were very similar to those shown for NaCl; for example, GUS activity was highest for FF5(pSK3) incubated in MGY containing 0.6 M sorbitol (Fig. 2B). Therefore, the stimulation of alginate gene expression by the addition of NaCl is due to increased osmolarity rather than to an ionic effect.

When *P. syringae* FF5(pSK3) was incubated at three different temperatures, the activity of the *algD* promoter was highest when it was grown at 32°C (Fig. 3). GUS activity in the control strains remained low, regardless of temperature (Fig. 3).

P. syringae pv. syringae FF5 previously showed enhanced alginate production in response to copper (37). In the present study, we investigated whether the *algD* promoter was transcriptionally activated in response to copper. The transcriptional activity of the *algD* promoter in pSK3 increased steadily as the copper concentration increased from 16 to 80 μM and then began to decline (Fig. 4). GUS activity in the control strains remained low regardless of the copper content (Fig. 4). When *P. aeruginosa* 8821(pVD2X) was monitored for CDO activity on MGY agar containing 0, 100, 200, or 300 μM $CuSO_4$, the addition of copper did not stimulate CDO activity at any level. However, the addition of 0.15, 0.3, and 0.4 M NaCl to MGY stimulated CDO activity in 8821(pVD2X) as reported previously (7). These results suggest that copper functions as a transcriptional signal for the *algD* promoter in *P. syringae* but not in *P. aeruginosa*.

Kinetics of *algD* transcription. In the absence of copper, transcription of the *algD* promoter in pSK3 remained moderately low, increasing from 50 U at 1 h to 200 U at 30 h (Fig. 5A). However, when FF5(pSK3) was cultivated in MGY-Cu, *algD* activation increased exponentially from 70 U (1 h) to 700 U (8 h) (Fig. 5B). The increase and subsequent decrease in *algD* activity in MGY-Cu were positively correlated with bacterial growth (Fig. 5B), suggesting that *algD* is activated very soon after exposure to copper. The GUS activity in the control strains, FF5(pSK4) and FF5(pRG960sd), remained low (25 to 50 U) throughout the sampling period (data not shown).

DISCUSSION

To our knowledge, this is the first report documenting the cloning of an alginate structural gene cluster from a pseudomonad other than *P. aeruginosa*. In a preliminary report, Morea et al. (49) described the isolation of the cosmid clone pAM21 from *P. fluorescens* and showed that pAM21 contained homologs of *algD*, *algG*, and *algA*; however, the localization of other structural genes and the transcriptional organization were not discussed. *algA*, *algD*, and *algL* have been characterized in *Azotobacter vinelandii*, and genes involved in the epimerization of mannuronate to guluronate and the export of alginate have also been identified (9, 18, 19, 42, 51, 52). The

TABLE 3. Alginate production by derivatives of *P. syringae* pv. syringae FF5

Strain[a]	Alginate production (μg/mg of protein)[b]
FF5(pPSR12)	1,333a
FF5.31	109c
FF5.31(pAD4033)	703b
FF5.32	102c
FF5.32(pAD4033)	111c

[a] Strains were assayed for uronic acid polymer production on MGY containing the following antibiotics: streptomycin, FF5(pPSR12); kanamycin, FF5.31 and FF5.32; and kanamycin, piperacillin, and 1 mm isopropyl-β-D-thiogalactopyranoside, FF5.31(pAD4033) and FF5.32(pAD4033).

[b] Mean values followed by the same letter are not significantly different at $P = 0.01$ by the Student-Newman-Keuls test.

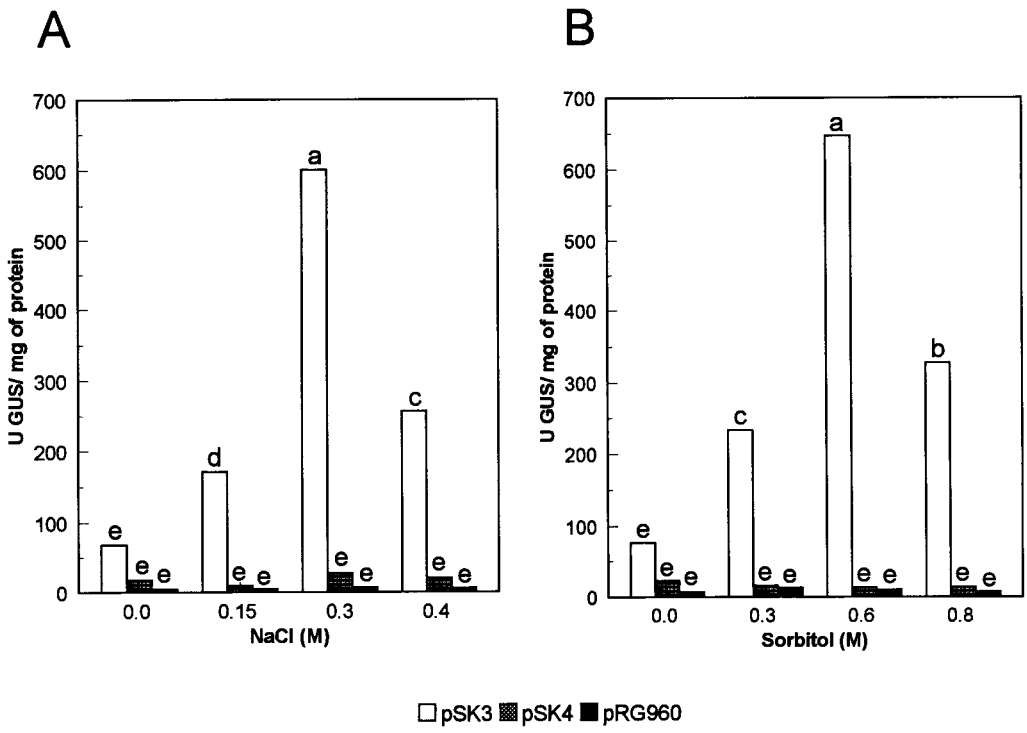

FIG. 2. GUS activity in *P. syringae* pv. syringae FF5 derivatives cultivated in MGY containing sodium chloride (A) or sorbitol (B). Prior to the GUS assays, all strains were grown for 30 h on MGY agar containing spectinomycin. The bacterial concentration was adjusted to an A_{600} of 0.3, and the bacteria were incubated at 250 rpm for 10 h at 28°C in MGY broth amended with NaCl (0, 0.15, 0.3, or 0.4 M) or sorbitol (0, 0.3, 0.6, or 0.8 M). pSK3 contains the *algD* promoter in the transcriptionally active orientation, whereas pSK4 contains the *algD* promoter in the opposite orientation. pRG960sd contains a promoterless glucuronidase gene. Values are the means from one experiment of three replicates each; the experiment was repeated with similar results. Treatments accompanied by the same lowercase letter were not significantly different at $P = 0.01$ by the Student-Newman-Keuls test.

order of the structural genes in *A. vinelandii* is similar to that conserved in *P. aeruginosa* and *P. syringae* (51), but the transcriptional organization is quite different and is organized into at least four operons (60).

In general, *P. syringae* strains produce low levels of alginate in vitro and appear nonmucoid. However, we previously demonstrated that the introduction of the naturally occurring Cu^r plasmid pPSR12 into several *P. syringae* strains resulted in mucoid strains due to increased alginate production (37). The nature of the pPSR12-encoded factor(s) that confers constitutive alginate production to *P. syringae* has not yet been identified. However, the introduction of pPSR12 into nonmucoid strains has made it possible to recover stably mucoid strains, and these were indispensable in the isolation of Alg^- mutants.

P. syringae pv. syringae FF5 was originally isolated from ornamental pear trees showing the extensive necrosis associated with bacterial blight symptoms (62). Efforts to reproduce these symptoms on pear seedlings were completely unsuccessful (3). However, when pear plantlets grown in tissue culture were inoculated with this pathogen, extensive necrosis was obtained (5). Several alginate-defective mutants were screened for the development of symptoms on pear trees grown in tissue culture, but all caused necrosis, and a role for alginate in virulence or pathogenicity was not apparent from these inoculations (5). Experiments are under way to generate Alg^- mutants of *P. syringae* pathovars which are more amenable to in planta studies; the results should indicate whether alginate plays a role in symptom development or virulence.

The *algD* promoter region from *P. syringae* pv. syringae FF5 (Ps*algD*) was transcriptionally activated in response to both NaCl and sorbitol with optimal transcription at 0.3 and 0.6 M, respectively. The activation of Ps*algD* in response to increased osmolarity is consistent with the results of Singh et al. (59), who showed that alginate production by *P. syringae* pv. glycinea

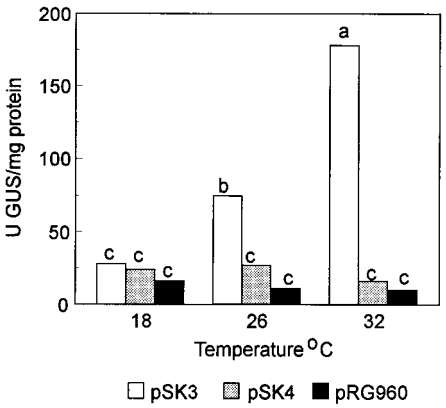

FIG. 3. GUS activity in *P. syringae* pv. syringae FF5 derivatives grown in MGY at different temperatures. All strains were initially grown for 30 h on MGY agar containing spectinomycin. The bacterial concentration was adjusted to an A_{600} of 0.3, and the bacteria were incubated at 250 rpm in MGY broth for 10 h at 18, 26, or 32°C. pSK3, pSK4, and pRG960sd are defined in the legend to Fig. 2. Values are the means from one experiment of three replicates each; the experiment was repeated with similar results. Treatments accompanied by the same lowercase letter are not significantly different at $P = 0.01$ by the Student-Newman-Keuls test.

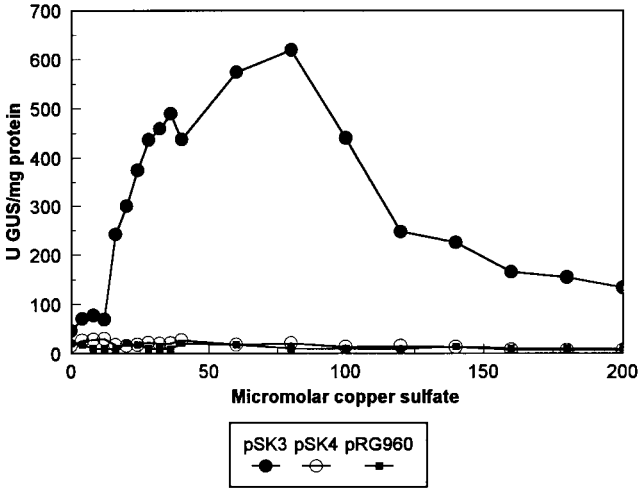

FIG. 4. GUS activity in *P. syringae* pv. syringae FF5 derivatives grown at 28°C in MGY containing different concentrations of copper. The strains were initially grown for 30 h on MGY agar containing spectinomycin. The bacterial concentration was adjusted to an A_{600} of 0.3, and the bacteria were incubated at 250 rpm for 10 h at 28°C in MGY broth amended with $CuSO_4$ (0 to 200 μM). pSK3, pSK4, and pRG960sd are defined in Fig. 2. Values are the means from one experiment of three replicates each; the experiment was repeated with similar results.

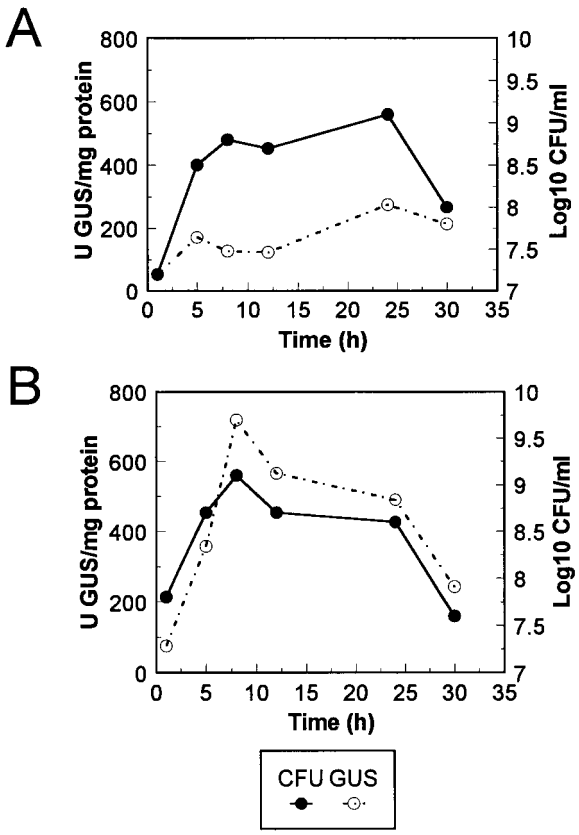

FIG. 5. Kinetics of *algD* activity in *P. syringae* pv. syringae FF5(pSK3). The bacteria were initially grown for 30 h on MGY agar containing spectinomycin. The cells were then suspended in MGY or MGY-Cu to an A_{600} of 0.1 and incubated at 250 rpm at 28°C. Aliquots of cells (three replicates per time point) were removed at 1, 5, 8, 12, 24, and 30 h and evaluated for growth by dilution plating and for transcriptional activity by GUS assays. (A) Bacterial growth and GUS activity in MGY broth containing no copper; (B) the same parameters in MGY broth containing 40 μM $CuSO_4$. Values are the means from one experiment of three replicates each; the experiment was repeated with similar results.

increased when NaCl or sorbitol was added to the growth medium. Similar results were obtained for the transcriptional activation of *algD* in *P. aeruginosa* (7), and it was concluded that high osmolarity was a stimulus for enhanced *algD* transcription. Phytobacteria may be exposed to high osmolarities on the leaf surface (2), and the increased synthesis of EPS molecules like alginate may be critical to epiphytic survival during conditions of osmotic stress.

Transcription of *algD* in *P. syringae* pv. syringae FF5 was temperature sensitive, with optimal expression occurring at 32°C. The increased production of alginate in response to high temperatures could be advantageous since the alginate capsule would provide some protection from the dehydration and desiccation which develop during heat stress. Interestingly, Leitão et al. (40) reported that activation of the *P. aeruginosa algD* promoter was highest at 35°C. In *P. aeruginosa*, transcription of *algD* is dependent on *algT* (*algU*), which encodes an alternative sigma factor related to σ^E (30, 44, 56). *algT* (*algU*) has been shown to increase the tolerance of *P. aeruginosa* to heat stress (44). Although the mechanism for increased *algD* transcription in *P. syringae* is unknown, it is tempting to speculate that the *algT* homolog in *P. syringae* might activate *algD* during heat stress.

Our results indicated that expression of the Ps*algD* promoter was significantly enhanced by fairly low levels of copper (16 to 80 μM) (Fig. 4). Furthermore, Ps*algD* expression in the presence of copper showed the greatest stimulation during log phase (Fig. 5B). The biological rationale for Cu-mediated stimulation of alginate production is not known. Although the strong affinity of alginate for copper ions (Cu^{2+}) has been well documented (31), we have been unable to establish that alginate production significantly enhances the tolerance of *P. syringae* to copper (37). However, Cu^{2+} is known to generate free radicals and other toxic molecules (63), and the increased production of alginate in response to copper could be stimulated by oxidative stress.

Although pAD501 and pSK2 contain functional alginate gene clusters from *P. aeruginosa* and *P. syringae*, respectively, complementation experiments indicated that these genes were not functionally interchangeable when expressed from their native promoters. However, FF5.31 could be complemented for alginate production by pAD4033, a construct containing the *P. aeruginosa algA* homolog under P*tac* control. This result indicates that *algA* is functionally interchangeable in the two species when expressed from the P*tac* promoter. Although sequence analysis of the 5′ region of the *algD* homolog in *P. syringae* revealed 78% identity with *P. aeruginosa*, the relatedness between the two species upstream of the *algD* translational start site was less than 40% at the nucleotide level (50). This divergence may reflect the adaptation of the two species to distinctly different ecological niches. Furthermore, it is clear that signals for alginate induction differ in the two species since copper ions stimulated alginate production in selected *P. syringae* strains but not in *P. aeruginosa*. *P. syringae* colonizes plant surfaces and is often exposed to intensive bactericidal sprays containing copper; however, exposure of clinical strains of *P. aeruginosa* to $CuSO_4$ is probably minimal. Future studies aimed at the regulation of alginate biosynthesis in *P. syringae* may provide additional insight into the role of this EPS in phytopathogenic pseudomonads.

ACKNOWLEDGMENTS

C.B. acknowledges support for this project by the Oklahoma Agricultural Experiment Station. A.M.C. acknowledges support by Public Health Service grants AI 16790-17 and AI 31546-03 from the National Institutes of Health.

We thank Mohamed Fakhr for technical assistance.

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