MPMI Vol. 8, No. 6, 1995, pp. 837–844.

Complementation Analyses of *Pseudomonas solanacearum* Extracellular Polysaccharide Mutants and Identification of Genes Responsive to EpsR

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Received 31 March 1995. Accepted 28 July 1995.

Many plant-pathogenic bacteria require extracellular polysaccharides (EPS) for successful infection. For example, mutations that prevent EPS production in the bacterial wilt pathogen, *Pseudomonas solanacearum*, will reduce the ability to wilt plants. While several *P. solanacearum* EPS genes have been identified and characterized, a systematic analysis of EPS mutants has not previously been performed. We have screened over 12,000 transposon-tagged mutants and categorized 66 EPS::*lacZ* mutants into nine complementation sets. Five of these are composed of previously characterized EPS structural and regulatory loci; four contain newly described EPS loci. One of the four novel complementation sets has been determined to be defective for both EPS and lipopolysaccharide production. We also used the EPS::*lacZ* mutants to examine the interaction between *P. solanacearum* EPS genes. Overexpression of the previously described regulator EpsR was found to down-regulate the expression of genes encoding production of the major acidic form of EPS.

Production of extracellular polysaccharide (EPS) is essential for normal infection by *Pseudomonas solanacearum*, an economically important pathogen of over 100 plant species in the Solanaceae and Musaceae plant families (for reviews, see Coplin and Cook 1990; Hayward 1991). Mutations that decrease the expression of EPS in planta consistently result in decreased virulence (Cook and Sequeira 1991; Denny et al. 1988; Denny and Baek 1991; Kao et al. 1992; Kelman 1954; Schell et al. 1987).

Several EPS genes and gene clusters have been identified in *P. solanacearum*. However, a thorough survey of EPS genes in *P. solanacearum* has been needed for identifying the approximate number of genes involved in EPS synthesis and for examination of the interaction between EPS genes. Complementation analyses with Tn*5* insertion mutants have defined three EPS structural gene clusters, named *ops* (for *o*utermembrane *p*oly*s*accharide; Kao and Sequeira 1991), *eps,* and *rgnII* (Denny and Baek 1991; Schell et al. 1993). The *ops* gene cluster contains at least seven complementation units, named *opsA* to *opsG*. These genes probably encode enzymes for nucleotide sugar synthesis. Some are also required for the synthesis of lipopolysaccharides (LPS) (Kao and Sequeira 1991). The *eps* cluster, probably coding for 11 proteins, is responsible for the synthesis of acidic EPS molecules (Orgambide et al. 1991; Schell et al. 1993). The *rgnII* cluster is not required for EPS production in plants, only in culture, which explains why mutations in *rgnII* do not affect virulence (Denny et al. 1988).

Since EPS plays a central role in *P. solanacearum* virulence, it is subject to a complex web of both positive and negative regulators (Huang et al. 1995; Kao et al. 1994). Among these, the *phcA* gene is a putative LysR-type transcriptional regulator (Brumbley and Denny 1990; Brumbley et al. 1993). The *vsrA* and *vsrD* genes appear to encode the sensor and activator of a two-component regulatory network (Huang et al. 1993; Schell et al. 1993; Huang et al. 1995; Huang and Schell 1995). The *vsrB* and *vsrC* genes encode the sensor and activator of a different two-component regulatory system (Huang et al. 1995). A gene involved in the production of a volatile extracellular factor named *phcB* has also been described (Clough et al. 1994). Finally, overexpression of the EpsR protein from multiple copy plasmids results in a decrease of EPS production and virulence and an increase in activity of some extracellular proteins such as polygalacturonase and tyrosinase (Huang and Sequeira 1990). The amino acid sequence translated from the open reading frame encoding EpsR bears striking resemblance to a class of bacterial regulatory proteins (reviewed in Gross et al. 1989). Mutation of the *epsR* gene demonstrated that it is not essential for either growth in culture or for production of EPS by visual inspection. However, the genes that are repressed by overproduction of EpsR have not been identified and the normal function of EpsR in the cell is unclear.

Here we report results from an extensive transposon-based mutagenesis of *P. solanacearum* EPS genes and functional grouping of EPS mutants by complementation analysis. This work also resulted in the identification and preliminary characterization of at least nine distinct complementation sets, at least four of which have not been previously described. In addition, analyses of the genetic interactions between complementation sets allowed us to identify the genes regulated by EpsR.

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RESULTS

Mutagenesis using miniTn*5lacZ2*.

Usually between 1,000 and 2,000 kanamycin-resistant transformants of strain K60 were obtained per electroporation. Several electroporations were performed to generate more than 12,000 mutants. From this collection we obtained 120 EPS-impaired and EPS-deficient mutants. Of these, 51 were deficient in EPS production, as indicated by the characteristic dark red colony morphology when the cells were grown in the presence of tetrazolium chloride. These EPS-deficient mutants were used for subsequent analyses because they allowed easy visual scoring of complementations. Two mutants that were EPS-impaired (S28 and S71) were included in the analysis because they are also defective for LPS production. Since the miniTn*5lacZ2* contained a promoterless *lacZ* gene, the mutants were streaked on plates amended with the β-galactosidase indicator, X-gal, to determine if *lacZ* was in the same orientation as the EPS promoter. Twenty-six of the mutants were blue while 27 were colorless.

The EPS mutants could have transposons either in previously defined or in heretofore unidentified EPS genes. To distinguish between these possibilities, we further characterized all 53 strains and an additional 13 EPS-deficient EPS mutants previously isolated by Xu et al. (1988).

Novel *ops* mutations.

To identify whether the mutants possessed defective LPS, all 66 EPS mutants were assayed for the ability to propagate bacteriophage CH154 as previously described by Kao and Sequeira (1991). Wild-type *P. solanacearum* strain K60 is sensitive to CH154 while LPS$^-$ strains are resistant. Of the strains in Table 1, seven (KD500, KD600, S28, S50, S71, S98, S106) were resistant to CH154, and thus were candidates for being *ops* mutants. Five mutants (KD500, KD600, S50, S98, S106) were complemented to wild-type EPS production and sensitivity to CH154 by plasmid pL5001 containing *opsI* genes (Table 1). The remaining two, S28 and S71, likely have mutations in previously uncharacterized *ops* genes.

To determine more directly whether strains S28 and S71 are defective in LPS structure, we fractionated LPS on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and specifically stained resulting fractions with silver (Hitchcock and Brown 1983). Wild-type LPS molecules from strain K60 possess a ladder of higher molecular weight bands (Fig. 1, lane 1). Both S28 and S71 are missing most of these higher molecular weight bands, and these bands are not restored even after transformation with plasmids pL5001 or pL700A (Fig. 1, lanes 3 to 6). As a control we determined that the higher molecular weight bands are absent in strain KD500

Table 1. Complementation analyses of 66 extracellular polysaccharide (EPS) mutants

Strain	Cosmid										
	pL700A	pL5001	pL96	pGA93	pL90	pL97	pL41	pL112	pAW114	pL119	pL28
eps mutants:											
S15, S94, KD700	+	−	−	−	−	−	−	−		−	
S16	+	−	−		+	−	−		−	−	
S18, S19, S20, S21, S22, S26, S29, S31, S40, S68, S104	+	−	−		−						
S49	+	−	−	−	−		−		−		
S53, S66, S70, S113	+	−	−	−	−		−				
S89	+	−	−								
S92, S105, S118, KD710	+	−	−	−	+						
rgnII mutants											
S14	−	−	−		+			−			
S87, S95, S103, S107, S109, S111, S114, S115, KD711	−	−	−		+						
S90	−	−	−	−	+	−		−	−		
KD703, KD705	−	−	−		+	−	−				
phcA mutants											
S42, S80, S85, S86, S88, S91, S99, S102, S116, KD400, KD900	−	−	+	+	−	−	−	−		−	
S84, S96	−	−	+	+	−	−	−	−	−	−	
pL97 mutants											
KD704	−	−	−	−	−	+	−	−		−	
KD712	−	−	−	−	−	+	−	−	−	−	
S97	−	−	−		−	+		−	−	−	
pL41 mutant											
S41	−	−	−		−	−	+	−		−	−
vsrB/C mutants											
KD714	−	−	−	−	−	−	−	+	+	−	
S112	−	−	−	−	−	−	−	+	+		
pL119 mutant											
S119	−	−	−		−	−	−	−	−	+	
opsI mutants[a]											
S98, S106, KD500, KD600	−	+	−	−							
S50	−	+	−	−	−	−	−	−	−	−	−
opsII mutants[a]											
S28, S71	−	−	−	−	−	−	−	−	−	−	+
KD702	−	−	−	−	−	−	−	−	−	−	

[a] Resistant to bacteriophage CH154; all other mutants are sensitive to CH154.

Identification of additional EPS genes.

The remaining 21 EPS mutants were classified after cloning out the complementing genes. The cosmid library of K60 DNA was electroporated into several of the EPS mutants. Five cosmids that complemented EPS production in S90, S97, S41, S112, and S119 were eventually found to complement all the unclassified EPS mutants except for KD702. We also identified the K60 homolog of *phcA* by complementing mutant S96. At least two independent complementing cosmids from the K60 cosmid library were isolated for each mutant strain complemented to wild-type. All of the complementing cosmids were found at a frequency of approximately 1 per 1,000 transformants. KD702 was unusual in that we were not able to isolate the complementing cosmid despite screening more than 10,000 colonies transformed with the K60 DNA library. It may contain either a dominant mutation or multiple mutations. When the cosmids were extracted from the original transformants and electroporated into the same strains, the transformed colonies regained the EPS^+ phenotype when visualized on plates. Furthermore, when one representative member of each complementation set was assayed for the abundance of hexosamines, the mutants have a greater than 20-fold decrease in hexosamines. Once complemented, however, these strains had hexosamine levels similar to wild-type strain K60 (Table 3). Each of the cosmids did not complement EPS production in representative members of the other five strains, indicating that they belong in distinct complementation sets. This observation argues against, but does not eliminate, the possibility that one or more of the cosmids may suppress the defect of another complementation set. The names of these complementing cosmids all reflect the strain from which they were derived. For example, pL90 was the cosmid that complemented S90.

Transforming the various cosmids containing EPS gene(s) into the 66 EPS mutants yielded the complementation sets shown in Table 1. pL96 and pGA93, which both contain homologs of the *phcA* gene, complemented the same 13 EPS^- mutants. pL90 complemented another 13 EPS mutants. pL97 complemented 3 EPS mutants. pL112 complemented two EPS mutants while pL41 and pL119 only complemented the mutant strain from which they were originally isolated.

The two sets represented by cosmids pL700A and pL90 contain DNA inserts that genetically overlap in the *P. solanacearum* chromosome (Table 1). Several strains, including KD710, S92, S105, and S118, were complemented to wild-type EPS production by both pL700A and pL90. In addition, restriction analyses demonstrate that pL700A and pL90 have restriction fragments in common (data not shown). These results are consistent with previous reports that *eps* and *rgnII* are physically located in adjacent regions of the *P. solanacearum* chromosome (Denny et al. 1988). A recently identified regulatory gene, *xpsR*, is located between the *eps* and *rgnII* clusters (Huang et al. 1995). It is presumably present within pL700 and/or pL90.

Several of the complementing sets could contain the recently identified *vsrA*, *vsrB*, *vsrC*, and *vsrD* genes, which are all part of a complex network that regulates *eps* expression (Huang et al. 1995). The *vsrA* and *vsrD* genes are linked, as

Table 3. Quantitation of hexosamine abundance in the extracellular polysaccharide complementation sets

Strain (gene affected)	Cosmid	Complementing cosmid	
		No	Yes
K60	–	4.2[a]	ND[b]
S49 (*eps*)	pL700A	<0.1	2.8
S96 (*phcA*)	pL96	<0.1	4.3
S97	pL97	<0.1	4.8
S41	pL41	<0.1	4.0
S112 (*vsrB/C*)	pL112	<0.1	3.9
S119	pL119	<0.1	4.0
S50 (*opsI*)	pL5001	<0.1	7.7
S28 (*opsII*)	pL28	<0.1	4.2

[a] Values in μg of hexosamine per ml per OD_{600} of cell culture. Measurements were made using Ehrlich's reagent as described in Huang et al. 1995. Each sample was assayed twice.
[b] Not determined.

Table 4. PhcA upregulates its own expression

phcA mutant	Plasmid	
	pLAFR3	pL96
S80	362 (10.4)[a]	728 (89.2)
S84	211 (81.8)	463 (78.4)
S88	186 (14.6)	595 (11.9)
S96	120 (17.8)	1,240 (89.3)
S102	138 (2.5)	2,230 (111.6)

[a] The first number is the average of β-galactosidase specific activity (O-nitrophyl galactoside [ONPG] hydrolyzed per mg of proteins per min.) measurements in *phcA* mutant strains harboring the plasmid noted. The number in parentheses is the value of standard deviation from three measurements.

Table 5. Effects of overproducing EpsR on the expression of EPS:*lacZ* fusions

Strain (gene affected)	Plasmid			
	$EpsR^-$		$EpsR^+$	
	pLAFR3	pepsRA	pGepsRA	pKL4
S112 (*vsrB/C*)	224 (12.4)[a]	333 (16.8)	261 (5.2)	320 (35)
S97 (pL97)	ND[b]	15.8 (0.9)[c]	16.4 (1.7)	ND
S84 (*phcA*)	218 (24)	287 (25.3)	239 (118)	205 (2.4)
S80 (*phcA*)	336.6 (10.2)	263 (18.9)	209.6 (38.6)	257.2 (36.2)
S87 (*rgnII*)	293	328 (69)	402 (69)	342 (74)
S90 (*rgnII*)	331	382 (69.2)	472 (57.9)	551 (127)
S50 (*opsI*)				
OD_{600} 0.6[d]	230	199	199	198
OD_{600} 1.0	128	154	104	173
S66 (*eps*)				
OD_{600} 0.6[d]	505	572	140	47
OD_{600} 4.0	1244	1396	217	206
S53 (*eps*)	ND	384	91.8	56.1
S31 (*eps*)				
Expt. 1	398	345	59.4	3.4
Expt. 2	378	ND	ND	34.1
S21 (*eps*)				
Expt. 1	ND	388	ND	20.6
Expt. 2	ND	489	ND	26.3

[a] The first number indicates the average β-galactosidase activity per mg of protein per min. The number in parentheses is the standard deviation of three independently assayed samples. Absence of standard deviations indicates that only one sample was tested.
[b] Not determined.
[c] Background level of β-galactosidase activity in this and all experiments is zero. These controls include K60 before transformation with Tn*5lacZ2*.
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are *vsrB* and *vsrC*. To identify the *vsrB/vsrC* mutants, several strains from each complementation set were transformed with plasmid pAW114 (kind gift of M. Schell), which contains the wild-type *vsrB/vsrC* genes. The set complemented by pL112 was found to be complemented by pAW114 (Table 1). To make a preliminary identification of *vsrA/vsrD* in the EPS complementation sets, we performed polymerase chain reactions on the nine EPS cosmids, using degenerate primers designed against the published *vsrA* amino acid sequence. Only the polymerase chain reactions using pL96 and pL112 as templates generated specific products of the expected size (data not shown).

Of the nine complementation groups, four are newly described. EPS genes within cosmids pL41, pL97, and pL119 do not appear to contain genes previously reported to be involved in EPS synthesis. pL28 is of note because it contains a locus distinct from *opsI* that is required for both EPS and LPS production.

Virulence level of each complementation unit.

A preliminary assessment of the virulence of several members of each of the nine complementation sets was determined using an eggplant seedling assay. This assay provides a suitable initial characterization of virulence level because it is convenient for assaying large number of mutant strains and is

Table 6. Bacterial strains and plasmids used in this study

Strains and Plasmids	Relevant characteristics	Source
Escherichia coli		
DH5α	F-, *endA*1, *hrdR*17(rk⁻mk⁺) *recA*1	L. Snyder
Pseudomonas solanacearum		
K60	Wild-type virulent; EPS⁺;Tc^s	A. Kelman
KD700 series[a]	Tn*5* EPS^i CH154 sensitive	Xu et al. 1990
KD400, 500, 600, 900	Tn*5* EPS⁻ CH154 sensitive	Xu et al. 1988
S series	53 EPS mutants; Vir⁻; Kan^r	This work
Plasmids		
pLAFR3	Tc^r	Keen et al. 1988
pL700A	Cosmid isolate from K60 genomic library that hybridized to pKD700; Tc^r	Xu et al. 1990
pL5001	Cosmid subclone of *ops* complementation units;Tc^r	Cook and Sequeira 1991
pKL4	pLAFR5 containing a 20-kb *Sau*3A K60 chromosomal DNAfragment cloned into *Bam*H1 site with EpsR activity; Tc^r	Kao et al. 1994
pepsRA	Subclone in pLAFR3 of pKL4 containing *epsR* sequences from nucleotides 365 to 1,290.	Kao et al. 1994
pG-epsRA	Insert from pepsRA fused to the *opsG* promoter; pLAFR3	Kao et al. 1994
pT7-epsRA	Insert from pepsRA cloned in pET11 (Novagen Inc.)	Kao et al. 1994
pGA93	2.2-kb *Eco*RI-*Bgl*II fragment from pGA9 in pLAFR3; phcA+; Tc^r	T. Denny
pAW114	*vsrB* and *vsrC* genes in pLAFR3. Tcr	M. Schell
pL97	Cosmid isolate from K60 DNA library complementing S97; Tc^r; pLAFR3	This work
pL41	Cosmid from K60 DNA library. Originally complementing S41; Tc^r	This work
pL112	Cosmid from K60 DNA library. Originally complementing S112; Tc^r	This work
pL119	Cosmid from K60 DNA library. Originally complementing S119; Tc^r	This work
pL90	Cosmid from K60 DNA library. Originally complementing S90; Tc^r	This work
pL96	Cosmid from K60 DNA library. Originally complementing S96; Tc^r	This work
pL702	Cosmid from K60 DNA library. Originally complementing KD702; Tc^r	This work

[a] KD700 series includes: 700,702,703,704,705,710,711,712, and 714.

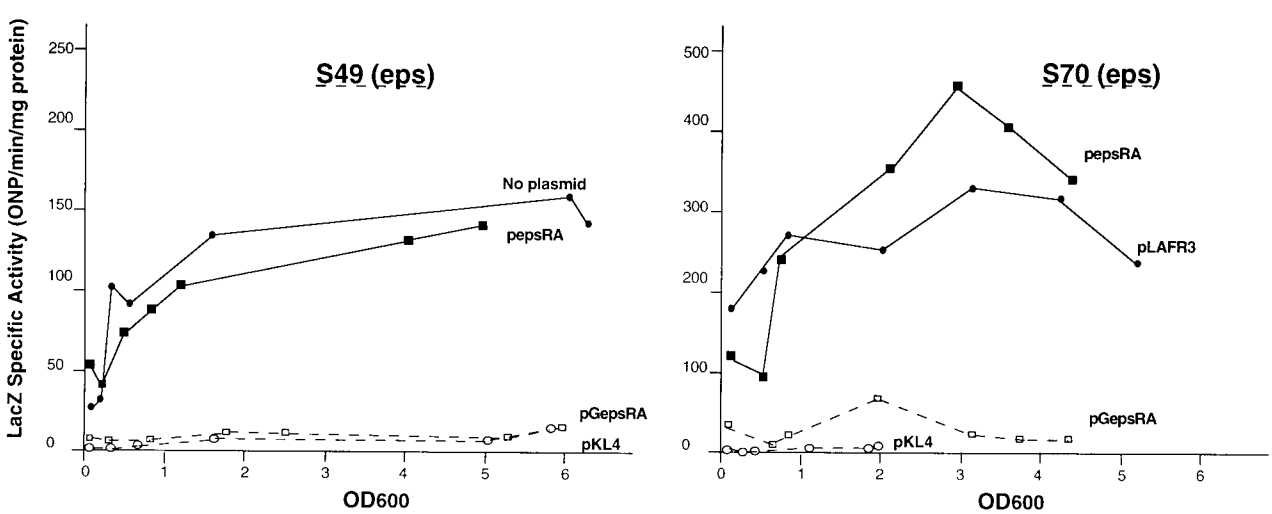

Fig. 2. EpsR shut-down of *eps* gene expression takes place throughout culture growth. Cells with mutations in the *eps* gene cluster were collected at different culture densities and frozen until all samples were ready for the β-galactosidase assay. β-galactosidase activity was measured as described in the Materials and Methods. S70 was transformed with each of four plasmids: pLAFR3, a broad host-range cloning vector (Keen et al. 1988), pepsRA, which contains a promoterless and inactive epsR gene, and pKL4 and pGepsRA, which both overexpress the EpsR protein (Kao et al. 1994). The four S49-derived strains harbor either no plasmid, pepsRA, pKL4, or pGepsRA.

less subjective than assays using numerical disease indices. Several previous works had used this assay to determine virulence, including those by Xu et al. 1988, Cook and Sequeira 1991, Kao and Sequeira 1991, and Kao et al. 1994. At least eight independent plants were inoculated by each *P. solanacearum* strain and the results of two independently performed virulence assays are presented in Table 2. Wild-type strain K60 usually kills between 80 and 100% of the inoculated plants after a 10-day incubation period while an avirulent strain will kill less than 10% of the plants in the same time. Except for mutants complemented by pL90 (*rgnII*), there is a consistent correlation between reduction in virulence and loss of EPS production in culture. None of the EPS-deficient mutants was able to kill more than 50% of the eggplants. Furthermore, virulence was increased almost to wild-type level when EPS production was restored. Since pL90 contains both *eps* and *rgnII* genes, mutants complemented by pL90 had two virulence patterns. Several, such as strains S14 and S115, are virulent even before complementation by pL90 while strains S95 and S90 are defective in virulence (Table 2). S14 and S115 presumably are mutant within *rgnII* while S90 and S95 are mutant within *eps*.

Interaction between EPS genes.

Sorting of the EPS:*lacZ* mutants into complementation sets permits analysis of the effects of regulatory genes. To ensure that interactions can be observed when low copy cosmids are transformed into the mutant strains, we first analyzed the effects of introducing *phcA* from cosmid pL96. Brumbley et al., (1993) previously reported that PhcA positively regulates its own expression. In our collection of *phcA* mutants, five (S80, S84, S88, S96, and S102) have the *lacZ* reporter gene in register with the *phcA* promoter, as judged by colonies turning blue in the presence of X-gal. All five strains had an increase in β-galactosidase activity of twofold to 12-fold when harboring pL96 in comparison to the control, pLAFR3 (Table 4). The variation in the effect of *phcA* on its own expression may be due to the location of the transposon within the *phcA* gene. These observations confirm the observations of Brumbley et al. (1993) and validate the use of our EPS mutants for the examination of interaction between EPS genes.

Since *opsII* is functionally distinct from *opsI*, we examined whether the expression of *opsI* is affected by multiple copies of *opsII* and *eps*. Separate cultures of the *opsI* mutant S50 were transformed with pLAFR3, pL28 (*opsII*), pL5001 (*opsI*), and pL700 (*eps*). The cells were harvested at several densities and assayed for β-galactosidase activity. No significant differences in β-galactosidase activity were observed in repeated trials (data not shown).

Regulation of *eps* gene expression.

Overproduction of EpsR has been shown to decrease both EPS production and virulence (Huang and Sequeira 1990). The effect of EpsR on the expression of several EPS genes is presented in Table 5. Sets represented by pL28, pL41, and pL119 were not tested since these sets do not have transcriptionally active *lacZ* fusions.

To assay the effects of EpsR, strains were individually transformed with two or more of the following four plasmids: pLAFR3, pepsRA, pGepsRA, and pKL4. The first two plasmids do not express the EpsR protein while the latter two can shut off EPS production in culture (Kao et al. 1994). We note that pKL4 contains a wild-type copy of the *epsR* gene, while pGepsRA expresses the EpsR protein from the heterologous *opsG* promoter (Table 6). Usually the four derivatives of each strain were grown in CPG medium amended with kanamycin and tetracycline. Cultures were harvested at different densities and assayed for LacZ activity. EpsR had no significant effect on *lacZ* expression of EPS sets other than *eps* (Table 5). However, plasmids expressing EpsR reduced β-galactosidase activity in all six of the *eps* mutants tested (Table 5; Fig. 2). The decrease was between fourfold (in strain S53) and 30-fold. The variation in different *eps* strains was reproducible and may be due to the position of *lacZ* insertions in the eps gene cluster.

The expression of the *eps* genes has been reported to increase with cell density (Denny et al. 1988). In the strains assayed, there was a reproducible three- to fivefold increase in LacZ activity when the cultures grew from an optical density of 0.2 to beyond 3.0. The expression of EpsR resulted in a marked decrease in LacZ activity during all growth phases tested (Fig. 2). Strains harboring pKL4 were reproducibly more repressed in *lacZ* expression than strains harboring pGepsR, which contains a functional *opsG* promoter fused to the open reading frame of the *epsR* gene. This result may be due to a difference in the strength of the *epsR* and *opsG* promoters. Western blots using antiserum directed at the EpsR protein (Kao et al. 1994) revealed that pKL4 produces approximately two- to threefold more EpsR than strains containing the pGepsR plasmid (data not shown).

DISCUSSION

In this communication, we have completed a large-scale genetic identification of EPS genes in *P. solanacearum*. Preliminary complementation analyses using cosmids containing wild-type DNA revealed nine distinct sets of EPS genes from a total of 66 mutant strains tested. The incidence of identified mutants within each group ranged from 25 members in the *eps* group to one mutant apiece within three independent sets. Virulence assays of members within these nine sets indicates that all except one are required for efficient killing of plants. It is of interest to note that while EPS is known to be a heterogeneous mix of polymers that are synthesized and regulated by a vast network of genes (Orgambide et al. 1991; Huang et al. 1995), mutations in different complementation sets result in the loss of EPS production. This suggests either that different EPS genes contribute to a common pathway for EPS production or that the regulation of separate pathways is linked. Our complementation system will provide an organizational framework for future analyses of the function of EPS genes, their regulation, and their interaction with one another. In this vein, we have already determined that the overexpression of the putative EPS-repressor, EpsR, decreases the expression of only the *eps* genes but not genes in the other sets.

The EPS complementation sets.

Of the nine EPS sets, several may be physically linked. The mutation in KD710 is known to reside in the *eps* gene cluster (Kao et al. 1992). KD710 and several other strains with defects in the *eps* region were complemented back to wild-type EPS production and virulence with plasmid pL90. Denny et

al. (1988) reported that *rgnII*, which is located near *eps*, is required for EPS expression only in culture and not in planta. At least a portion of *rgnII* may be harbored in pL90. We also found that some of the mutants complemented by pL90 were not affected in virulence in eggplant seedling assays.

In addition to *eps* and *rgnII*, some of the EPS mutations may be also closely linked in the *P. solanacearum* chromosome. Preliminary random amplified polymorphism DNA analysis indicates that these cosmids pL112 and pL96 produce bands in common, which are not found in the plasmid vector, pLAFR3 (R. McWilliams and C. C. Kao, unpublished result). In addition, oligonucleotides directed against *vsrA* sequence yielded bands of the expected size from both cosmids. Analysis of the physical linkage of various cosmids is in progress.

The *phcA* gene is believed to be present in one functional copy in the *P. solanacearum* genome, but our screen yielded 13 mutants. This unexpectedly high incidence of mutants for a single gene may be due to two possibilities: (i) *phcA* can restore EPS expression from strains that have mutations in other unlinked loci; or (ii) the *phcA* gene resides in an area that is hypermutable. We note that of the 13 mutations complemented by *phcA*, none was complemented by cosmids other than pL96 or by subclone pGA93, which contains a 2.2-kb wild-type *phcA* gene from *P. solanacaerum* strain AW-1. This result suggests that all 13 strains have mutations in the *phcA* gene, not in unlinked genes, and that the *phcA* gene is hypermutable.

It is possible that other EPS genes in addition to the nine sets described in this report may exist. However, our screen is thorough in both the number of transposon mutants screened (>12,000), and in that we uncovered multiple alleles of all the previously characterized EPS mutants (*eps*, *rgnII*, *opsA-G*, *vsrB/C*, *vsrA/D*, and *phcA*). However, our screen is biased against selection of mutants that are impaired in but not devoid of EPS production. This would explain the relatively low frequency of *opsI* mutants in our screen since *opsC-*, *opsD-*, and *opsE-* strains are only impaired for EPS production (Cook and Sequeira 1991).

EpsR.

Regulation by EpsR is highly specific. Of the six complementation groups analyzed for effect of overexpressing EpsR, only the expression of *eps* was affected. The repression of *eps* genes would explain the shut-off of EPS production by EpsR. These results demonstrate that, when overexpressed, EpsR may provide another layer of an increasingly complex network that regulates *eps* genes. Regulation of *eps* genes by EpsR does not extend to the *rgnII* genes.

EpsR is of special interest because of it can reduce EPS production in wild-type *P. solanacearum* strains. We have previously reported that knockouts of *epsR* do not affect EPS production when colonies were visually inspected (Kao et al. 1994). The availability of strains containing *eps*::*lacZ* fusions will now permit a more quantitative analysis of the effects of EpsR and the mechanism of gene regulation by EpsR. This work is now in progress.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Bacterial strains and plasmids used are listed in Table 6.

Growth and maintenance of bacterial strains.

Strains of *P. solanacearum* were routinely cultured in CPG medium (per liter: 10 g of tryptone, 5 g of glucose, 1 g of casamino acids, 1 g of yeast extract) at 30°C. The strains were maintained on CPG medium containing 1.5% agar. In cultures of both *Escherichia coli* and *P. solanacearum*, antibiotics were used, when appropriate, at the following concentrations: kanamycin, 50 μg/ml; tetracycline, 15 μg/ml; ampicillin, 50 μg/ml. To aid in the identification of EPS mutants on plates, tetrazolium chloride was added to 0.004%. X-gal (5-bromo-4 chloro-3-indolyl-β-D-galactopyranoside, Sigma Inc., St. Louis, MO) was added to 20 μg/ml.

Molecular techniques.

Transposon mutagenesis was performed with plasmids harboring mini-Tn*5lacZ*2 (deLorenzo et al. 1990). Usually, 10-ml cultures of wild-type *P. solanacearum* K60 strain were collected at an optical density at 600 nm (OD_{600}) of approximately 1.0, washed three times with sterile water, and finally resuspended in 100 μl of water. The cells were then electroporated along with 5 μg of mini-Tn*5lacZ*2 plasmid using a Gene Pulser (BioRad, Hercules, CA) set at 25 μFD with a field strength of 6,000 volts/cm. After electroporation, cells were allowed to recover for 3 h in CPG broth before plating on selective medium. Large scale preparations of plasmids from transformed *E. coli* and *P. solanacearum* were made using Qiagen columns (Qiagen, Inc., Chatsworth, CA). Southern hybridizations were performed as previously described (Kao et al. 1994). Electrophoresis and staining of lipopolysaccharides were performed as described by Hitchcock and Brown, (1993).

Quantitation of EPS production.

Cells were grown in minimal medium amended with 1 g of yeast extract per liter and 2 g of casamino acids per liter. Culture supernatant were collected at OD_{600} of 1.0 and quantitated for hexosamine as previously described by Huang et al. (1993); N-acetyl galactosamine purchased from Sigma was used as standard.

β-galactosidase assay.

Cultures were grown to the desired optical density in the appropriate selective medium, washed once with water, and then suspended in Z-buffer (Sambrook et al. 1989). Cell lysates were prepared with a Model 50 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) set at a 30% output for 10 to 12 s per sample. The samples were centrifuged at 14,000 × *g* for 5 min to remove cell debris, and the supernatants were used directly for both β-galactosidase assays and protein determinations by the Bradford assay (Bradford 1976). Bovine serum albumin (Sigma, St. Louis, MO) was used as the protein concentration standard. Specific activities of the β-galactosidase protein were calculated as the μM of O-nitrophenyl galactoside hydrolyzed per min per mg of protein.

Virulence assay.

Eggplant (cv. Black Beauty) seeds were surface sterilized and assayed for killing by *P. solanacearum* as previously described (Xu et al. 1988). The seeds were germinated in the dark at room temperature and then transferred to a growth

chamber. Plants between 7 and 8 days old were inoculated with bacteria (at an OD_{600} of 0.1) as previously described (Xu et al. 1988). The number of dead plants was recorded at between 7 and 10 days after inoculation.

ACKNOWLEDGMENTS

We thank Laura Kao and Erin O'Reilly for critiques of this manuscript, Yves Brun for making available the miniTn*5lacZ2* transposon, Tim Denny for plasmid pGA93, Mark Schell for plasmid pAW114, and Caitilyn Allen for a cosmid library of K60 DNA. This work was partially funded by a start-up grant from the National Science Foundation.

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