Evidence for Involvement of a Volatile Extracellular Factor in *Pseudomonas solanacearum* Virulence Gene Expression

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A complex regulatory network controls virulence genes of *Pseudomonas solanacearum*. Analysis of the transposon-generated mutant AW1-83 suggests that a new locus, designated *phcB*, may play a role in this network. AW1-83 (*phcB83*) produced at least 30-fold less than the wild type of extracellular polysaccharide (EPS I, encoded in part by *eps*) and at least seven extracellular proteins, but these traits were fully restored in response to one or more extracellular factors (EF) released by wild-type *P. solanacearum*. Presence of EF increased transcription of a genomic *eps*::*lacZ* fusion in a *phcB83* background more than 50-fold, restoring wild-type expression. The EF made by *P. solanacearum* was present in both the aqueous and the vapor phases. Millimolar levels of methanol (but not larger alcohols) and micromolar levels of C14- to C18-fatty acid methyl esters (but not larger or smaller methyl esters) also restored nearly wild-type expression of *eps*::*lacZ* in a *phcB83* background. The methoxy group was essential for this increase, since neither free fatty acids nor the ethyl or propyl esters were active. Growth with the C16-methyl ester restored normal production of EPS I and extracellular proteins by AW1-83. The wild-type *phcB* locus was subcloned on a 4-kb fragment and delimited to less than 2 kb by transposon inactivation and complementation studies. Genomic *phcB*::Tn*3*HoHo1 mutations appeared to eliminate EF production but did not uniformly reduce production of EPS I and extracellular proteins. Site-specific recombination of the *phcB83* allele into the genome of five other *P. solanacearum* strains revealed that they have a structurally and functionally conserved *phcB* locus. Although all 80 wild-type strains of *P. solanacearum* tested made some EF, out of seven genera of bacteria tested, only *Agrobacterium* produced an EF-like activity that stimulated visible EPS production by AW1-83. Our results suggest that the EF may be an extracellular signal molecule in *P. solanacearum* that is different from the acyl-homoserine lactone signal compounds produced by *Vibrio fischeri* and other Gram-negative bacteria.

Additional keywords: cell-cell signal, cell wall degrading enzymes.

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Bacterial pathogens often employ multiple virulence determinants that are coordinately regulated in response to the environment through complex signal transduction systems (Mekalanos 1992; Miller *et al.* 1989). Some human pathogens, such as enteropathogenic *Escherichia coli* and *Vibrio cholera*, respond to changes in the environment that, although not host specific, are associated with entry into host tissues. Among the diverse physical or chemical cues are iron, calcium, or oxygen concentrations; temperature; osmolarity; and pH (Meklanos 1992; Miller *et al.* 1989). Plant pathogens may also respond to altered osmolarity upon entry into host tissues by increasing expression of pathogenicity genes in the *hrp* cluster (Rahme *et al.* 1992). In addition, at least two phytopathogens (*Agrobacterium tumefaciens* and *Pseudomonas syringae* pv. *syringae*) activate virulence genes in response to host-specific molecules (Charles *et al.* 1992; Mo and Gross 1991). A better understanding of the conditions and signals that promote virulence should advance strategies for the prevention and treatment of bacterial diseases.

Recent work with *Pseudomonas solanacearum*, an economically important phytopathogenic bacterium, has revealed that it uses a multicomponent system to regulate virulence (Schell *et al.* 1993b). *P. solanacearum* causes a lethal wilting disease on many diverse species of plants (Hayward 1991), usually entering through wounds in the roots, penetrating the vascular system, and systemically colonizing the xylem (Buddenhagen and Kelman 1964; Wallis and Truter 1978). Two major virulence factors of *P. solanacearum* are the acidic, galactosamine-rich extracellular polysaccharide (EPS I) and an extracellular β-1,4-endoglucanase (EG) (Denny and Baek 1991; Denny *et al.* 1990; Orgambide *et al.* 1991), which are encoded by *eps* and *egl* genes, respectively (Schell *et al.* 1993a; Roberts *et al.* 1988). Part of the *P. solanacearum* virulence regulatory network modulates production of EPS I via two separate two-component systems (encoded by *vsrAD* and *vsrBC*) that positively control transcription of *eps* (Huang *et al.* 1993; Schell *et al.* 1994). Inactivation of either *vsr* system greatly reduces the ability of the pathogen to wilt tomato plants. The *vsr* systems also appear to positively and negatively regulate production of a number of extracellular proteins, some of which might be involved in pathogenicity.

Another component of the regulatory network is encoded by *phcA*, which directly or indirectly controls many virulence genes (Brumbley and Denny 1990; Brumbley *et al.* 1993; Schell *et al.* 1993b). Mutation of *phcA*, either spontaneously or by transposon insertion, dramatically changes the levels of most of the extracellular proteins produced by *P. solana-*

cearum (Schell *et al.* 1993b) including: a 50-fold reduction in EG production coincident with reduced transcription of *egl* (Brumbley and Denny 1990; Huang *et al.* 1989); a >15-fold decrease in pectin methylesterase (PME) activity (Schell *et al.* 1993b); and a 10-fold increase in endo-polygalacturonase activity (Brumbley and Denny 1990; Schell *et al.* 1993b; Huang *et al.* 1993). Other effects are a 30-fold decrease in EPS I coincident with reduced transcription of *eps* (Denny and Baek 1991; Brumbley and Denny 1990; Schell *et al.* 1993a) and an increase in motility (Brumbley and Denny 1990). The deduced amino acid sequence of PhcA (Brumbley *et al.* 1993) suggests that it is a member of the LysR family of transcriptional regulators (Schell 1993), implying that PhcA is a global regulator that transcriptionally controls a virulence regulon in *P. solanacearum*.

That the global regulation of virulence involves components in addition to *phcA* was suggested by the discovery of the transposon-generated mutant AW1-83 (Brumbley and Denny 1990). Like a *phcA* mutant, AW1-83 produces dramatically less EPS I and EG and has increased polygalacturonase and motility. However, AW1-83 has a wild-type *phcA* locus and is reversibly stimulated to make visible EPS slime when grown adjacent to wild-type *P. solanacearum* in the same petri plate (Brumbley and Denny 1990). These results suggested that AW1-83 produces EPS only in the presence of one or more compounds released from wild-type cells. Our objectives were to investigate the nature of this endogenous, extracellular factor (EF), to determine whether additional *phcA*-regulated virulence factors are affected by the EF, and to characterize the locus mutated in AW1-83. Our results indicate that production of the EF, which is water soluble and somewhat volatile, requires a new locus, designated *phcB*, that is involved in expression of several *phcA*-regulated virulence genes.

RESULTS

Extracellular complementation of mutant AW1-83 by an endogenous, extracellular factor present in the aqueous phase of wild-type cultures.

We previously reported that strain AW1-83, which is non-mucoid (visually lacking EPS) when cultured alone on a BGT agar plate, is reversibly stimulated to become very mucoid (copious EPS slime) when AW1 is grown adjacent to it (Brumbley and Denny 1990). These results suggested that AW1-83 is stimulated to produce EPS by an extracellular factor (EF) released by the wild type, but could also be explained by removal of an inhibitor or other environmental changes affected by the wild type.

To quantify EF-stimulated EPS production in AW1-83 (*phcB83*) and to determine whether EG activity (which is reduced in AW1-83 [Brumbley and Denny 1990]) is also increased by the EF, experiments were performed in liquid medium. Preliminary tests showed that co-culture of AW1-83 with AW1-2 (which makes <6% of wild-type galactosamine-rich EPS I and EG due to two insertion mutations in structural genes) resulted in AW1-83 producing wild-type levels of both molecules (not shown). Similarly, growth of AW1-83 in cell-free medium conditioned by AW1-2 restored EPS I and EG to wild-type levels; medium similarly conditioned by AW1-83 had no affect on either trait (Table 1). In contrast, growth of AW1-80 (*phcA80*::Tn*5*) in conditioned media did not increase its low levels of EPS I or EG (Table 1). Increased EPS I production by AW1-83 in response to EF was apparently due to enhanced transcription of *eps*, because the β-galactosidase activity in AW1-3 (*eps*::*lacZ*, *phcB83*) increased from 1.5 (± 0.5 SE) Miller units when grown in the absence of EF to a fully stimulated, wild-type level of 202 (± 27) units when grown in cell-free medium conditioned by AW1-2.

Table 1. Response of *phcB* and *phcA* mutants of *Pseudomonas solanacearum* to conditioned media

Test strain	Medium conditioned by[a]	EG[b]	EPS I[c]
AW1 (wild type)	Nothing	285 ±19	282 ± 17
AW1-83 (*phcB83*)	Nothing	24 ±8	6 ± 3
	AW1-83	9 ± 3	9 ± 2
	AW1-2	253 ± 25	221 ± 39
AW1-80 (*phcA80*)	Nothing	10 ± 4	28 ± 7
	AW1-83	20 ± 4	12 ± 3
	AW1-2	17 ± 4	23 ± 5

[a] Cell-free medium in which AW1-83 (*phcB83*) or AW1-2 (*eps-130*, *egl3*) had been grown previously was added to an equal volume of fresh EG medium.

[b] EG, endoglucanase activity in nmoles min^{-1} mg^{-1} cell protein. Values are mean ± SE for three or more experiments.

[c] EPS I, μg extracellular galactosamine polysaccharide mg^{-1} cell protein. Values are the mean ± SE for three or more experiments.

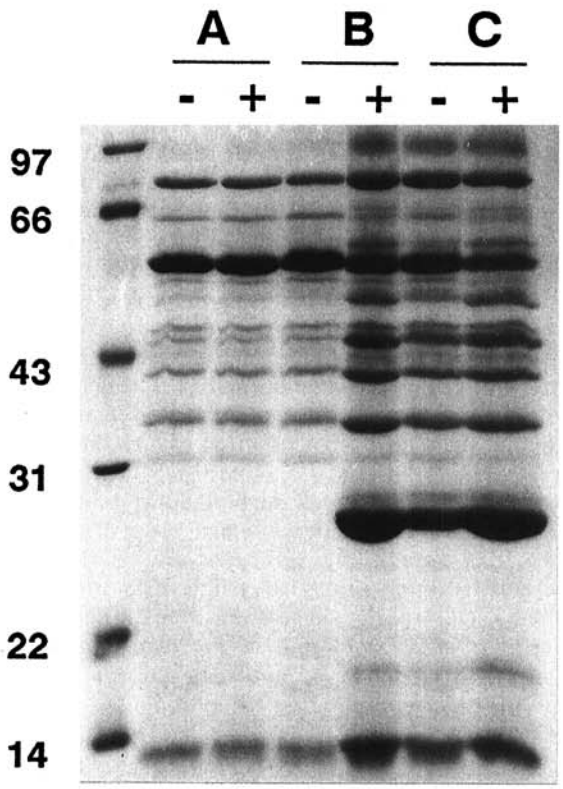

Fig. 1. Effect of C16-FAME on extracellular protein production by *Pseudomonas solanacearum*. Strains AW1-80 (A), AW1-83 (B), and AW1 (C) were grown in liquid medium in the absence (–) or presence (+) of 60 μM C16-FAME. SDS-polyacrylamide-gel electrophoresis was performed on dialyzed, concentrated supernatants of stationary-phase cultures as described previously (Schell 1987) and stained with Coomassie blue. The molecular weight standards run in the far left-hand lane are labeled in kilodaltons.

The simultaneous deficiency in EPS and EG production by AW1-83 is similar to that observed in *phcA* mutants (Table 1) (Brumbley and Denny 1990). Analysis of culture supernatants by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed that, comparable to a *phcA* mutant, AW1-83 produced dramatically less of at least seven extracellular proteins that are made by the wild-type parent (Fig. 1), including a >10-fold decrease in EG and PME production (Table 2). However, not all extracellular proteins were reduced because, like a *phcA* mutant, AW1-83 retained significant extracellular polygalacturonase activity (and not shown). Therefore, except for its ability to respond to the EF produced by wild-type *P. solanacearum*, AW1-83 is comparable to a *phcA* mutant in every trait examined.

Specific fatty acid methyl esters can substitute for the EF.

When a variety of pure compounds were tested for EF-like activity by application to the surface of a BGT plate previously streaked with AW1-83, we found that C16-, C17-, and C18-fatty acid methyl esters (FAMEs) stimulated visible EPS production, whereas C3-, C8-, C9-, C10-, C12-, and C13-FAMEs did not. To better characterize this size specificity, the EF-responsive strain AW1-3 was grown in BG liquid medium containing 0.1 or 1 mM of FAMEs with various chain lengths and then assayed for β-galactosidase (Table 3). Only the C14- to C18-FAMEs increased expression of the *eps*::*lacZ* fusion in AW1-3, with peak activity exhibited by the C16-FAME. Stimulation by C16- or C17-FAMEs was maximal at concentrations ≥25 μM, but decreased to about 5% of maximum (still 5- to 10-fold above levels without FAME) at 1.25 μM. C18-FAMEs with one to three double bonds in the carbon chain stimulated *eps*::*lacZ* in AW1-3 as well or better than the saturated C18-FAME (Table 3). However, neither the C16 or C18 free fatty acids nor ethyl or propyl esters of those fatty acids stimulated expression of *eps* even at 1 mM.

SDS-PAGE analysis of culture supernatants showed that C16-FAME stimulated AW1-83 (but not AW1-80) to produce wild-type levels of the extracellular proteins missing from untreated cultures (Fig. 1). Moreover, EG activity produced by AW1-83 increased about 10-fold (to 220 ± 35 nmoles min^{-1} mg^{-1} cell protein) and PME activity increased 60-fold (to 59 ± 1.8 units mg^{-1} cell protein) when 30 μM of the C16-FAME was added to the medium. Testing the same samples for production of EPS I showed a greater than 30-fold increase (to 285 ± 15 μg hexosamine mg^{-1} cell protein) in response to the C16-FAME. Therefore, the C16-FAME in the aqueous phase restored wild-type level production of all the gene products affected in AW1-83.

The *P. solanacearum* EF is also present in the vapor phase.

We were surprised to find that AW1-83 also became visibly mucoid when grown separated from wild-type AW1 by the plastic barrier in a "split plate" (not shown), suggesting that the EF is volatile enough to enter the vapor phase. When the possibility of a gaseous EF was examined using lid-agar plates (see Methods), we found that AW1-83 became extremely mucoid when grown above lawns of nonmucoid AW1-1 (either on BGT or MM lid-agar layers), but was unaffected by a lawn of AW1-83 growing on the lid-agar (Fig. 2A–C). Nonmucoid AW1-1, which tests showed produced EF in amounts equal to AW1, was used as the EF source in this and subsequent assays to ensure that any EPS observed in the lid-agar plates was produced by AW1-83. After 2 days of growth, as few as 100 colonies of AW1-1 on the lid-agar were sufficient to make AW1-83 mucoid over the entire plate, and single colonies stimulated mucoid growth of AW1-83 within about a 2-cm circular zone centered above each colony (Fig. 3). These observations demonstrate that EF is present in the vapor phase

Table 2. Effect of transposon insertions in and flanking the *phcB* locus on production of EF and virulence factors by *Pseudomonas solanacearum*

Strain[a]	EF[b]	Virulence factors[c]		
		EPS I	EG	PME
Wild type				
AW1	+	295 ±12	239 ± 18	55 ± 5
Mutants				
AW1-83	–	9 ± 2	20 ± 5	1 ± 0.3
AW1-160	–	115 ± 5	67 ± 9	13 ± 0.4
AW1-161	–	50 ± 13	55 ± 6	10 ± 3
AW1-162	–	43 ± 4	53 ± 3	9 ± 3
AW1-164	–	113 ± 5	87 ± 6	19 ± 1
AW1-165	–	27 ± 9	40 ± 2	3 ± 1
AW1-166	+	186 ± 11	196 ± 35	40 ± 4
AW1-168	+	228 ± 12	210 ± 32	45 ± 3
Mutants + pLMO4				
AW1-83	+	279 ± 19	139 ± 14	39 ± 3
AW1-161	+	191 ± 18	126 ± 5	19 ± 1
AW1-162	+	177 ± 14	130 ± 8	18 ± 1
AW1-164	+	170 ± 10	134 ± 11	17 ± 1
AW1-165	+	148 ± 8	122 ± 8	17 ± 1

[a] Strains AW1-160, AW1-166, and AW1-168 have insertions outside of *phcB* as defined by complementation analysis.
[b] Production of EF was assessed using lid-agar plates with AW1-83 or AW1-3 as the EF-responsive strain. +, EF produced; –, no EF detected.
[c] EPS I and EG are as defined in Table 1. PME, pectin methylesterase activity in units mg^{-1} cell protein. Values are means ± SE for four or more replications.

Table 3. Induction of *eps*::*lacZ* expression in strain AW1-3 by fatty acid methyl esters[a]

FAME[b]	LacZ activity[c]
None or C8 to C13	1.3 ± 0.1
C14	26 ± 5
C15	57 ± 8
C16	184 ± 4
C17	126 ± 11
C18	41 ± 15
C18:1 *cis*-9	54 ± 20
trans-9	61 ± 14
C18:2 cis-9, 12	52 ± 17
C18:3 cis-9, 12, 15	36 ± 12

[a] These saturated and unsaturated compounds did not stimulate β-galactosidase activity in AW1-3 above background levels even at 1 mM: fatty acids of C16, C18, C18:1 (*cis*-9 and *trans*-9), C18:2 (*cis*-9, 12), C18:3 (*cis*-9, 12, 15); FAMEs of C3, C4, C6, C8, C10, C12, C13, C19, C20, C22, C24; fatty acid ethyl and propyl esters of C16 and C18.
[b] Fatty acid methyl esters (C# = chain length).
[c] AW1-3 (*phcB83*::Tn*5*$^+$,*eps-130*::*lacZ*) was grown from 10^4 cells ml^{-1} to 10^9 cells ml^{-1} in BG broth amended with indicated FAMEs at 100 μM initial concentration and β-galactosidase activity (LacZ activity in Miller units) was determined. Values are the averages of three experiments ± SE. β-galactosidase activity of AW1-130 (*eps-130*::*lacZ*) cultured in the absence of any compound was 161 ± 8 units.

above wild-type *P. solanacearum* growing on agar medium, but that in the lid-agar plates the gaseous EF is effective within a limited distance from its point of origin.

To assess whether the volatile EF regulated EPS production via transcription of the *eps* gene cluster, lid-agar plates were made with AW1-3 as the EF-responsive strain and AW1-1 as the EF-producer. After 2 days of incubation, β-galactosidase activity of AW1-3 cells was determined after they were washed off the agar. Expression of *eps*::*lacZ* was increased about 40-fold by growth over a lawn of AW1-1, whereas a lawn of AW1-83 had no effect (Fig. 2). Thus, coincident with EPS production, the EF stimulated transcription of *eps*.

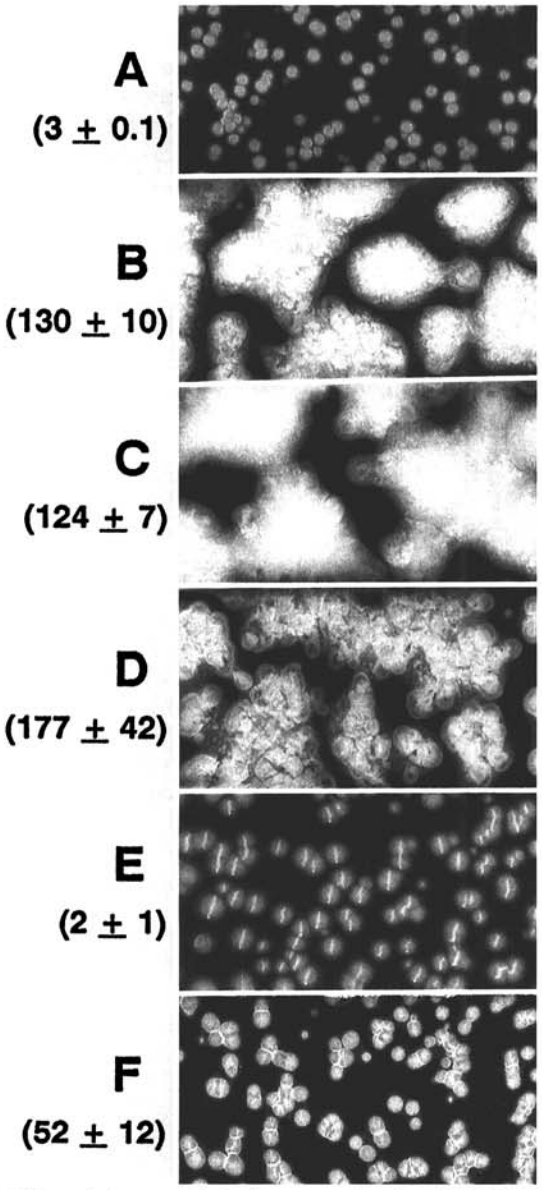

Fig. 2. Effect of the extracellular factors, methanol, and C16-FAME in the vapor phase on extracellular polysaccharide production and *eps*::*lacZ* activity. Panels show colonies of AW1-83 on BG agar from the lid-agar plates incubated 2.5 days at 30° C. The lids of the plates (not shown) had (A) a lawn of AW1-83 on BG agar, (B) a lawn of AW1-1 on BG agar, (C) a lawn of AW1-1 on MM agar, (D) 10 µl of methanol, (E) 10 µl of hexane, or (F) 10 µl of 1.0 M C16-FAME in hexane. Numbers in parentheses are the β-galactosidase activities for duplicate plates that had AW1-3 (*eps-130*::*lacZ phcB83*) instead of AW1-83 on the agar layer in the bottom of the plate; values are the average Miller units for three experiments ± SE. When grown on BG agar under standard conditions, β-galactosidase activity in AW1-130 (*eps-130*::*lacZ*) was 158 ± 3 Miller units.

Effect of volatile compounds on EPS production.

When spotted on the lid of inverted petri plates at the onset of incubation, 10 µl of methanesulfonic acid methyl ester (not shown) or methanol stimulated AW1-83 growing on BG agar to be come mucoid, and AW1-3 to increase expression of the *eps*::*lacZ* fusion (Fig. 2D). However, the effect of methanol vapors on EPS production by AW1-83 was not identical to that of the EF, because the EPS slime was more transparent; methanol did not have this effect on the wild type. Methanol also stimulated *eps*::*lacZ* expression in AW1-3 in liquid culture, but a concentration of 2.5 mM (100-fold higher than with C16-FAME) was required to achieve wild-type levels of *eps* expression. Ten microliters of pure hexane added to the petri plate lid had little affect on AW1-83 (Fig. 2E), but 10 µl of 1.0 M C16-FAME in hexane stimulated production of a relatively small amount of transparent EPS slime and a >15 fold increase in β-galactosidase activity from the *eps*::*lacZ* fusion in AW1-3 (Fig. 2F). Thus, the C16-FAME is capable of stimulating *eps*::*lacZ* via the vapor phase, but apparently was insufficient to restore wild-type EPS production.

Vapors released from ripe tomato fruit and processed tomato-fruit products (e.g., canned tomatoes, tomato paste,

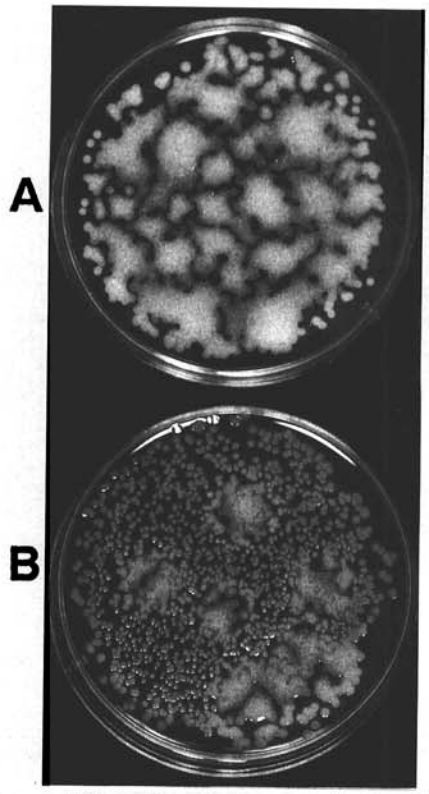

Fig. 3. Effect of AW1-1 colony density on production of extracellular polysaccharide by AW1-83 in lid-agar plates. Shown are the agar layers from the bottom of lid-agar plates that were spread with 10^3 cfu of AW1-83 and incubated for 2 days over (A) 107 cfu or (B) 8 CFU of AW1-1 that were growing on lid-agar layers.

tomato juice) stimulated AW1-83 to become very mucoid, but chopped stems of tomato plants did not (not shown). Ripe tomatoes release both methanol and numerous volatile, methylated compounds (Petró-Turza 1987) that could account for the observed EF-like activity. However, the active compound was not ethylene, a volatile regulator of several plant functions, because concentrations of the pure gas between 0.1 and 10,000 ppm did not stimulate AW1-83 to produce visible EPS. Other compounds without detectable activity included: acetone, acetylaldehyde, chloroform, ethanol, formaldehyde, formic acid, hydroxybutyric acid, isoamyl alcohol, and jasmonic acid methyl ester (a volatile signal compound in plants [Farmer and Ryan 1990]).

Isolation and mapping of the *phcB* locus.

AW1-83 has both Tn*5* and IS*50* insertions as well as the chloramphenicol resistance gene of the original Tn*5* suicide plasmid (Brumbley 1992; Brumbley and Denny 1990). Transformation using AW1-83 genomic DNA and selection for associated antibiotic resistance markers reproduced the phenotype of the original AW1-83 mutant (see creation of AW1-3 and below; see also Brumbley and Denny 1990). Besides demonstrating that the inserted DNA is linked to the phenotype of AW1-83, these results indicate that possible unlinked mutations do not contribute to the phenotype. To determine the location of the insertion elements in the genome, Southern blots of restriction endonuclease-digested genomic DNA (*Bam*HI, *Eco*RI, *Hin*dIII, and all pair-wise combinations) from AW1-83 and cosmid clones carrying the Tn*5* insertion and flanking DNA from AW1-83 (Clough 1991) were probed separately with pHB9, pBR325 (the vector for the Tn*5*-donor plasmid), and an internal fragment from Tn*5*. pHB9 was used because it contains *phcA* and flanking DNA that detected a restriction fragment length polymorphism associated with the Tn*5* insertion in AW1-83 (Brumbley 1992). The results showed that Tn*5*, pBR325, and IS*50* form a single, contiguous insertion (designated Tn*5*$^+$) approximately 14 kb downstream from *phcA* (Fig. 4).

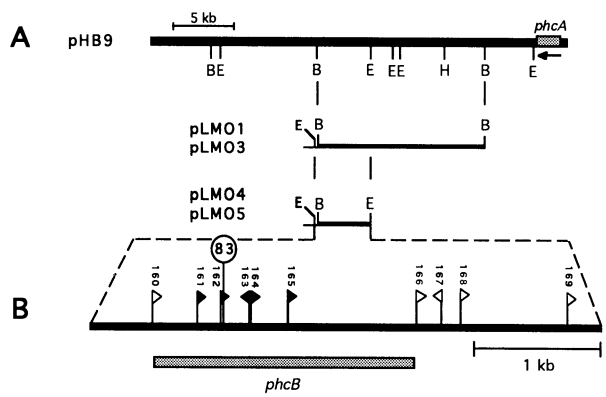

Fig. 4. The *phcA-phcB* region in *Pseudomonas solanacearum*. (A) Maps of the cosmid pHB9 and subclones containing 12.3- or 4-kb fragments that complement AW1-83. B, E, and H indicate restriction sites for *Bam*HI, *Eco*RI, and *Hin*dIII, respectively. The left-most *Eco*RI site in the pLMO subclones is from the multicloning site in the vector. (B) The 4-kb *Bam*HI-*Eco*RI region containing *phcB* showing positions of the genomic Tn*5*$^+$ insertion in AW1-83 (circle) and the Tn*3*HoHo1 insertions (triangular flags). The flags point in the direction of *lacZ* transcription within Tn*3*HoHo1; filled flags denote insertions in pLMO4 that eliminate complementation of AW1-83. The maximum size of the *phcB* locus as defined by the complementation data is indicated by the hatched bar.

We found that AW1-83 carrying pHB9 in *trans* was fully mucoid, presumably because it contains a wild-type copy of the region affected by the Tn*5*$^+$ insertion. The *phcB* locus, defined as the region that complements AW1-83, was subcloned from pHB9 into the broad host vector pLAFR3, first on a 12.3-kb *Bam*HI fragment in pLMO3 and then on a 4-kb *Eco*RI fragment in pLMO4 (Fig. 4). When introduced into AW1-83, pLMO3 and pLMO4 fully restored EPS slime production on BGT plates regardless of the orientation of the cloned fragment (not shown), suggesting that the locus is expressed from its own promoter. More extensive analysis showed that pLMO4 restored EF, EPS I, EG, and PME production to near wild-type levels (Table 2), indicating that it carries a functional *phcB* locus. Because neither subclone restored *phcA* mutants (not shown) and cloned *phcA* does not complement AW1-83 (Brumbley and Denny 1990), these two loci are physically and functionally distinct.

pLMO4 was mutagenized with Tn*3*HoHo1 and the position and orientation of each insertion was mapped. The mutated plasmids were transferred into AW1-83 by conjugation, and the resulting merodiploids were examined visually for enhanced production of EPS slime on BGT plates. Plasmids with mutant alleles 160 and 166 to 169 complemented AW1-83, whereas alleles 161 to 165 did not, indicating that the *phcB* locus spans at most the 2 kb between insertions 160 and 166 (Fig. 4). Each of the Tn*3*HoHo1 insertions was recombined into the genome of AW1 by allelic replacement to create strains AW1-160 to AW1-169, and the fidelity of the insertions was confirmed on a Southern blot of *Eco*RI-*Bam*HI digested genomic DNA that was probed with pLMO4 (not shown).

When grown on BGT plates, *P. solanacearum* mutants with Tn*3*HoHo1 insertions outside of *phcB* were mucoid, although they produced less visible EPS that was more transparent than in the wild type. Unexpectedly, the five mutants with insertions inside *phcB* (AW1-161 to AW1-165) were slightly to moderately mucoid, unlike the nonmucoid phenotype of AW1-83. The mutants were subsequently tested for EF production on lid-agar plates with AW1-83 or AW1-3 as the EF-responsive strains. Mutants AW1-160 to AW1-165 did not stimulate any visible EPS production by AW1-83 or increase expression of the *eps*::*lacZ* fusion in AW1-3, and are considered to be EF-negative (Table 2). In contrast, mutants AW1-166 to AW1-169 were EF-positive in these assays, although they appeared to make less EF than the wild type. Therefore, except for AW1-160, loss of EF activity in the mutants coincided with inability of the corresponding plasmid-borne allele to restore EPS slime production in AW1-83 merodiploids. The Tn*3*HoHo1 insertion in AW1-160 may be in a second complementation unit that is necessary for EF production. Alternatively, the insertion in AW1-160 might have a secondary effect on *phcB* that is masked by multiple copies of the plasmid-borne allele.

When the mutants were examined quantitatively for EPS I, EG, and PME production, AW1-160 and the *phcB* mutants produced less of all three molecules than AW1-166 and AW1-168, which have insertions flanking *phcB* (Table 2). However, in none of the *phcB* mutants were EPS I, EF, and PME reduced to the low levels found in AW1-83. The pres-

ence of pLMO4 in *trans* increased production of EF, EPS I, EG, and PME by the *phcB* mutants, but none became fully wild-type (Table 2). The addition of 30 μM C16-FAME during growth of AW1-165 increased EPS I production fourfold (to 110 ± 16 μg hexosamine mg^{-1} cell protein), EG production twofold (to 71 ± nmoles min^{-1} mg^{-1} cell protein), and PME production fivefold (to 17 ± 1 units mg^{-1} cell protein). Production of these extracellular factors was not significantly increased when C16-FAME was added to the other Tn*3*HoHo1 mutants (not shown). Thus, although the *phcB* mutants were uniformly negative for EF production, there was unexpected variation among the mutants for production of the *phcA*-regulated traits examined and for response to C16-FAME.

Conservation of the *phcB* locus in *P. solanacearum*.

A transformation approach was used to determine whether the *phcB* locus was present in other *P. solanacearum* strains. AW1 and nine additional strains were transformed with genomic DNA from AW1-83 followed by selection for kanamycin resistance. If the *phcB83*::Tn*5*+ can replace the wild-type *phcB* in the genome by allelic recombination, then numerous transformants with properties similar to AW1-83 (e.g., reduced EPS production) should be recovered. Five of the nine strains gave nonmucoid, kanamycin-resistant colonies that had a morphology like AW1-83, and representative colonies of each transformant became mucoid in response to the EF produced by AW1-1 in lid-agar plates. Thus, these five strains (K60, UW130, UW150, GMI1000, and 82N (Cook *et al.* 1989; Boucher *et al.* 1985; Denny *et al.* 1988) appear to have a functional *phcB*-like locus that is sufficiently conserved to allow recombinational exchange with *phcB83*::Tn*5*$^+$.

A diverse collection of bacteria (see Methods) were assayed for EF activity by testing their ability to stimulate AW1-83 to become mucoid. Eighty wild-type strains of *P. solanacearum* tested in BGT split plates all stimulated AW1-83 to produce increased amounts of EPS; 34 were roughly comparable to AW1, 31 were distinctly less effective than AW1, and 15 barely induced EPS production. Of the 24 additional species of bacteria (in seven genera) streaked adjacent to AW1-83 on standard BGT plates, only the three *Agrobacterium* species had any effect, and that effect was comparable to the weakest EF-producing strains of *P. solanacearum*. The EF-like activity produced by *Agrobacterium* was independent of the pTi or pRi plasmids, because some strains of *A. radiobacter* (e.g., A12/91, which by definition lacks pTi or pRi) stimulated EPS production in AW1-83, whereas some strains of *A. tumefaciens* (e.g., C58 and B6) and *A. rhizogenes* (e.g., K599) were negative. The six positive strains of *Agrobacterium* also produced volatile EF-like activity when tested in lid-agar plates.

DISCUSSION

We found an unusual mutant of *P. solanacearum* that is the result of a Tn*5*$^+$ insertion near *phcA*, but in a new, independent locus designated *phcB*. Similar to inactivation of *phcA*, the *phcB83* mutant allele in AW1-83 reduced virulence and production of at least seven PhcA-regulated extracellular factors. Unlike a *phcA* mutant, however, AW1-83 produced wild-type amounts of EPS I, EG, PME, and other extracellular proteins when grown with either culture supernatants containing EF made by *P. solanacearum* or C16-FAME. In addition, virulence of AW1-83 on tomato plants was restored when it was co-inoculated with the low-virulence, EF-producer strain AW1-2 (Clough 1991), suggesting that all important virulence factors were restored to normal by the EF.

Wild-type strains of *P. solanacearum* that represent the genetic and geographical diversity of this pathogen (Cook *et al.* 1989, 1991) all made a volatile EF, suggesting that this is a fundamental property of the species. However, the EF is not a common bacterial metabolite, because few of the other bacteria tested produced detectable activity. That *P. solanacearum* produced EF when growing on a minimal medium indicated that it is synthesized de novo and is not a by-product of the complex medium. EF activity was present in both the aqueous and the vapor phases of cultures, so the active compound(s) are both water soluble and volatile. However, because the volatile EF did not diffuse very far in the lid-agar petri plate cultures it may 1) be only slightly volatile at room temperature, 2) rapidly partition into the aqueous phase, or 3) rapidly decompose when in the vapor phase. It is likely that the EF in the aqueous and vapor phases is the same compound, but this remains to be confirmed.

The purpose and function of the EF is unclear, but it could serve as an extracellular signal molecule involved in cell-cell communication. Several prokaryotes have been reported to produce extracellular molecules that serve as signals for their behavior or development (Stephens 1986; Kaiser and Losick 1993; Kim *et al.* 1992; Clewell 1993; Horinouchi and Beppu 1992). The most pertinent example is regulation of bioluminescence in marine *Vibrio* species by acyl-homoserine lactone autoinducers, which appear to serve as a signal for bacterial cell density (Meighen 1991). Recent reports show that related homoserine lactones are produced by a variety of bacteria to regulate processes such as synthesis of carbapenem antibiotics and extracellular enzymes in *Erwinia carotovora* (Bainton *et al.* 1992; Pirhonen *et al.* 1993; Jones *et al.* 1993), production of elastase in *Pseudomonas aeruginosa* (Jones *et al.* 1993; Passador *et al.* 1993), and conjugation in *A. tumefaciens* (Zhang *et al.* 1993). All of these bacteria have related genes involved in producing or sensing the homoserine lactone signal compounds (Piper *et al.* 1993; Passador *et al.* 1993; Pirhonen *et al.* 1993; Jones *et al.* 1993). Although preliminary data suggest that *P. solanacearum* makes a homoserine lactone (unpublished), it appears that the EF is distinctly different because 1) it is volatile and acyl-homoserine lactones are not (Eberhard *et al.* 1981), 2) neither *E. carotovora* nor *P. aeruginosa* produced EF-like activity even though both produce homoserine lactones, and 3) some *A. radiobacter* strains produced EF-like activity even though they presumably lack the pTi-encoded homoserine lactone.

Several results support the possibility that the EF made by *P. solanacearum* acts as a volatile signal molecule in AW1-83 to regulate virulence genes. First, the concentration at which C16-FAME was active (10^{-6} M) is similar to that at which the homoserine lactone autoinducer acts as a cell-cell signal to induce expression of bioluminescence genes in *V. fischeri* (Eberhard *et al.* 1981). Second, the presence of such small amounts of the EF simultaneously affected multiple traits, at least two of which (EPS I and EG) are regulated by *phcA* at the transcriptional level. EF may be affecting activity or synthesis of a transcriptional regulatory protein in AW1-83. One

possibility is PhcA, because loss of either the EF or PhcA affects the same set of virulence genes and, as a member of the LysR family (Brumbley and Denny 1993), PhcA probably requires a low molecular weight signal molecule for activity (Schell 1993).

The chemical nature of the EF produced by *P. solanacearum* is currently under investigation in our labs. However, a common feature of all the pure compounds which induced AW1-83 to produce EPS is an OCH_3 group. Although the EF is volatile, it does not have to be a very small compound like methanol, since we demonstrated that the C16-FAME was also able to stimulate *eps*::*lacZ* via the vapor phase. That FAMEs as large as C16 are volatile is not surprising when one considers the volatile insect pheromones (e.g., pentadecane, hexadecadinenol, and octadecenyl acetate) that have greater than 15 carbons (Law and Regnier 1971; Mori 1989). Stimulation of EPS production by FAMEs was moderately chain-length specific, which argues against spontaneous hydrolytic release of methanol from these compounds as an explanation for their activity. It could be that relative FAME activity reflects biological availability (e.g., as micelles) or their partition coefficient with bacterial membranes. It also is possible that the FAMEs (and indeed the EF) are not the ultimate inducer, but rather that substrate-size-specific esterases may continually release methanol from certain FAMEs (or the EF) within or near *P. solanacearum*.

A potential *in planta* source of methanol to induce virulence genes in *P. solanacearum* is pectin, the methyl ester of polygalacturonic acid, which is a major component of plant primary cell walls. *P. solanacearum* produces a PME that may release enough methanol from hydrolysis of pectin to activate virulence genes. In support of this, we found that when *E. coli* carrying a cloned *pme* gene from *P. solanacearum* on a multi-copy plasmid was grown on pectin-supplemented medium in lid-agar plates it stimulated visible EPS production by AW1-83 via the vapor phase (unpublished).

The wild-type *phcB* locus was subcloned and transposon mutagenesis used to identify a <2-kb region required for both production of EF and complementation of *phcB83* in AW1-83. All the *phcB* mutants had reduced production of EPS I, EG, and PME, but the magnitude of the reduction was not uniform. The extent to which these traits were reduced was not strictly related to the location of the Tn*3*HoHo1 insertions. Only AW1-165, with an insertion 0.4 kb away from the Tn*5*$^+$ site in AW1-83, had a phenotype comparable to AW1-83, whereas mutants with closer insertions did not. Preliminary DNA sequence analysis (unpublished) suggests that there is a 1,404-nucleotide open reading frame that is consistent with the *phcB* locus, but did not reveal motifs or homologs in the databases that provided an obvious explanation for our results. However, sequence analysis of a Tn*5*-*lacZ* mutation that causes a phenotype indistinguishable from that of AW1-83 found that it had inserted 14 bases away from the site of *phcB83*::Tn*5*$^+$. Thus, although there are unexplained *phcB* allele-specific effects, the *phcB83* allele is not unique in its ability to affect *phcA*-regulated traits. It could be that some of the *phcB* mutants have sufficient residual EF-production to stimulate themselves, but too little for us to detect using lid-agar plates. Alternatively, the *phcB* alleles may indirectly affect components of the PhcA regulon. Considerable additional research will be required to comprehend the role of *phcB* and the significance of the EF and other potential signal molecules for *P. solanacearum*, but greater understanding of these processes has the potential to dramatically alter our concepts of the biology and ecology of this important phytopathogen.

MATERIALS AND METHODS

Strains, plasmids, and culture methods.

The recombinant bacteria and plasmids used are listed in Table 4. Bacteria tested for an EF that stimulates EPS pro-

Table 4. Recombinant strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source
Pseudomonas solanacearum		
AW1	Wild-type Nx^r	Denny *et al.* 1988
AW1-1	*eps-1*::Tn*5*, EPS$^-$ Nx^r Km^r	Denny *et al.* 1988
AW1-2	*eps-1*::Tn*5*, *egl3*::Tn*3*HoHo1, EPS$^-$ EG$^-$ Nx^r Km^r Ap^r	Denny *et al.* 1990
AW1-3	*eps-130*::Tn*3*HoHo1, *phcB83*::Tn*5*+, EPS$^-$ Nx^r Km^r Ap^r Cm^r, LacZ$^+$ in response to EF	This work
AW1-80	*phcA80*::Tn*5*, Nx^r Km^r, not stimulated by EF	Denny *et al.* 1988
AW1-83	*phcB83*::Tn*5*+, Nx^r Km^r Ap^r Cm^r, resembles AW1-80 but is stimulated by EF to wild type	This work
AW1-130	*eps-130*::Tn*3*HoHo1, EPS$^-$ Nx^r Ap^r	Denny and Baek 1991
AW1-160 to AW1-169	AW1::Tn*3*HoHo1, Nx^r Ap^r Tc^s	This work
Plasmids		
pUC9	ColE1, Ap^r	Vieira and Messing 1982
pLAFR3	Broad-host-range cosmid vector, Tc^r	Staskawicz *et al.* 1987
pHB9	Cosmid clone containing *phcA* and *phcB* from AW1, in pLAFR3	Brumbley and Denny 1990
pLMO1, pLMO3	12.3-kb *Bam*HI fragment from pHB9 containing *phcB*, in pUC9 and pLAFR3, respectively	This work
pLMO4, pLMO5	4-kb *Eco*RI fragment from pLMO1 containing *phcB*, in pLAFR3 and pUC9, respectively	This work

[a] Ap^r, Cm^r, Km^r, Nx^r and Tc^r denote ampicillin, chloramphenicol, kanamycin, nalidixic acid, and tetracycline resistance, respectively; Tc^s, tetracycline sensitive; EPS; extracellular polysaccharide; LacZ+, β-galactosidase positive. See text for a full description of the *phcA* mutant phenotype. AW1-83 was generated by a transposition event where Tn*5*, the vector (pBR325), and IS*50* inserted as a contiguous element, designated Tn*5*$^+$. *phcA80*::Tn*5* was previously designated as *phcA0*::Tn*5* (Brumbley and Denny 1990).

duction by AW1-83 included: 80 wild-type *P. solanacearum* strains (39 from a study of genetic diversity (Cook *et al.* 1989), representing the two major restriction fragment length polymorphism (RFLP) divisions within the species, 20 of 28 RFLP groups, and four of the five races), and *Agrobacterium radiobacter* (four strains), *A. rhizogenes* (three strains), *A. tumefaciens* (16 strains), *Bacillus cereus*, *B. mycoides*, *B. subtilis*, *Clavibacter michiganensis*, *Erwinia amylovora*, *E. carotovora*, *E. chrysanthemi*, *E. stewartii*, *E. uredovora*, *E. coli*, *Pseudomonas aeruginosa*, *P. avenae* (syn. *Acidovorax avenae* subsp. *avenae*), *P. cattleyae* (syn. *A. avenae* subsp. *cattleyae*), *P. cepacia*, *P. cichorii*, *P. corrugata*, *P. fluorescens*, *P. gladioli*, *P. syringae* (22 pathovars; see Denny 1988), *P. viridiflava*, *Xanthomonas campestris* (two pathovars).

P. solanacearum strains were routinely grown at 30° C in BG medium (1% Bacto peptone, 0.1% Casamino Acids, 0.1% yeast extract, and 0.5% glucose) or BGT agar (BG plus 1.6% agar and 0.005% tetrazolium chloride). *E. coli* strains were grown at 37° C in Luria-Bertani medium (Maniatis *et al.* 1982). Minimal-medium agar (MM agar) contained (per liter): 1.75 g of K_2HPO_4, 0.75 g of KH_2PO_4, 0.15 g of Na citrate, 0.25 g of $MgSO_4 \cdot 7H_2O$, 1.25 g of $(NH_4)_2SO_4$, and 15 g of agar (autoclaved separately). After autoclaving, filter-sterilized glucose was added to a final concentration of 0.5% (w/v).

Unless otherwise specified, cell-free conditioned medium was prepared by growing AW1-83 or AW1-2 in EG medium (Brumbley and Denny 1990) to an OD_{600nm} of 0.2–1.2, removing the cells by centrifugation, filtering the supernatant through 0.25 or 0.45 μm nitrocellulose, and mixing the filtrate 1:1 with fresh EG medium. When screening for substitutes for the EF, <1 mg amounts of pure compounds were applied to a <1-cm^2 area on agar plates previously streaked with strain AW1-83. Otherwise, compounds were dissolved in hexane (reagent grade; J. T. Baker Chemical Co., Phillipsburg, NJ) and then diluted at least 1,000-fold into liquid media; controls received the same volume of pure hexane. Antibiotic concentrations were: ampicillin (100 μg/ml for *E. coli*, 10 μg/ml for *P. solanacearum)*, chloramphenicol (50 μg/ml), kanamycin (50 μg/ml), nalidixic acid (20 μg/ml), and tetracycline (15 μg/ml). Fatty acids, fatty acid methyl esters (FAMEs), and other fatty acids esters, all of which were 99% pure, were purchased from Sigma Chemical Corp. (St. Louis, Mo.).

Plate assays for volatile EF activity.

The "split-plate" assay for the EF in the vapor phase was conducted by growing AW1-83 and a test strain on agar media on opposite sides of a plastic barrier molded into the bottom of a petri plate (Falcon #1003, Becton Dickson and Co., NJ). The "lid-agar" plate assay, was performed as follows. An EF-responsive strain (either AW1-83 or AW1-3) and a potential EF-producer strain were spread on layers of agar medium in separate 100-mm diameter petri plates. Then a ring of agar about 5-mm wide was removed from the circumference of the EF-producer plate and the remaining agar plug (about 75 mm in diameter) repositioned so that it adhered to the lid with the inoculated side up. This agar-bearing lid was substituted for the ordinary lid of a plate with an EF-responsive strain, and the resulting lid-agar plate incubated in an inverted position. In this way, the EF-responsive strain was held about 5 mm above the potential EF-producer strain, but the agar plug on the lid did not seal the air gap between the petri plate's lid and bottom.

Assays for EPS I and enzyme activity.

For quantification of EG and galactosamine-containing EPS I, cells were grown in EG medium and assays performed as previously described (Brumbley and Denny 1990). PME activity was assayed at 37° C by mixing 5–50 μl of culture supernatant with 300 μl of 0.5% (w/v) citrus pectin (Sigma P-9135; previously neutralized with NaOH), 0.005% (w/v) phenol red, 2-mM Tris-HCl (pH 6.7), and 2 mM $CaCl_2$ and incubating until the pH decreased to 5.2, as indicated by the red-to-yellow color change of the phenol red. One unit of PME activity is defined as the amount of enzyme that released 1 nmole of H^+ per min. To determine β-galactosidase activity we used the method of Miller (Miller 1972). Cells were grown in BG to an OD_{600} of about 1.0, harvested by centrifugation, suspended in one-third volume 10-mM Tris (pH 7.5) buffer, permeabilized using chloroform and sodium dodecyl sulfate, and assayed using o-nitrophenyl-β-D-galactopyranoside at a final concentration of 0.8 mg/ml.

Genetic methods, including plasmid and strain construction.

Plasmid isolation, matings, and other genetic methods were performed as described (Brumbley and Denny 1990; Carney and Denny 1990; Maniatis *et al.* 1982). Plasmids pLMO1 and pLMO3 were constructed by ligating the 12.3-kb *Bam*HI fragment of the cosmid pHB9 into the *Bam*HI site of pUC9 and pLAFR3, respectively (Fig. 4). Plasmids pLMO4 and pLMO5 were constructed by ligating the 4-kb *Eco*RI fragment of pLMO1 into the *Eco*RI site of pLAFR3 and pUC9, respectively (Fig. 4). In each case, clones were recovered that contained the fragment in both orientations with respect to the *lacZ* promoter in the vector. Tn*3*HoHo1 transposon mutagenesis of pLMO4, which creates transcriptional or translational *lacZ* fusions, and subsequent allelic replacement of the insertions into the genome of AW1 were performed as previously described (Carney and Denny 1990), except that MM agar with the appropriate antibiotics was used to counterselect the auxotrophic *E. coli* donor strains.

Isolation of and transformation with genomic DNA from *P. solanacearum* has been described (Boucher *et al.* 1985; Carney and Denny 1990). *P. solanacearum* strain AW1-3 was created by transforming AW1-130 (*eps-130*::Tn*3*HoHo1) with genomic DNA from AW1-83 and selecting for resistance to kanamycin, ampicillin, and chloramphenicol. Representative colonies were tested for the ability to respond to EF by whether they changed from the flat, dark red-colored colony type typical of phcA and phcB83 mutants to the slightly domed, less red colony type typical of eps mutants (Denny et al. 1988); one such colony was selected and designated AW1-3.

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