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Characterization of a DNA Region Required for Production of the Phytotoxin Coronatine by *Pseudomonas syringae* pv. *tomato*

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***Pseudomonas syringae* pv. *tomato* DC3000, a tomato pathogen, produces the chlorosis-inducing phytotoxin coronatine. A 30-kilobase fragment of chromosomal DNA containing genes that control coronatine synthesis (*cor*) was characterized using saturation mutagenesis with the transposons Tn*3*-Spice and Tn*phoA* and functional complementation analysis. The data indicated that the *cor* genes distributed across this 30-kilobase fragment are tightly clustered and fall into six distinct complementation groups, designated CorI to CorVI. Lack of alkaline phosphatase activity by Tn*phoA*-induced Cor$^-$ mutants suggested that the *cor* loci containing these insertion mutations do not code for secreted or membrane proteins. Cor$^-$ mutants induced by Tn*3*-Spice, which generates transcriptional gene fusions with the ice nucleation gene *inaZ*, expressed ice nucleation activity both *in vitro* and *in planta*. Growth on tomato plants increased expression of the gene(s) present in the CorII region 370-fold over that observed *in vitro*.**

Additional keywords: bacterial speck, *cor* gene cluster, plant-inducible.

Pseudomonas syringae pv. *tomato* (Okabe) Young *et al.*, causal agent of bacterial speck, produces necrotic lesions surrounded by chlorotic halos on tomato leaves; severely infected plants are stunted. Several lines of evidence suggest that chlorosis and stunting are due to the phytotoxin coronatine (Mitchell *et al.* 1983; Mitchell 1984). Coronatine also stimulates plant enzyme activities and causes hypertrophy of potato tuber tissue (Mino *et al.* 1980; Sakai *et al.* 1982; Volksch *et al.* 1989). This toxin, which is a secondary metabolite and is not host-specific, is produced by several other *P. syringae* pathovars, including *P. s.* pv. *glycinea* (Coerper) Young *et al.*, *P. s.* pv. *atropurpurea* (Reddy & Godkin) Young *et al.* (Nishiyama *et al.* 1976), and *P. s.* pv. *morsprunorum* (Wormald) Young *et al.* (Mitchell 1982). Although its synthesis requires the coupling of coronafacic acid by an amide bond to the atypical α-amino acid coronamic acid (Ichihara *et al.* 1977; Mitchell 1984; Parry and Mafoti 1986), the details of the pathway(s) involved have not been determined.

Little is known about the genes that control coronatine biosynthesis. Bender *et al.* (1989) isolated several Tn*5*-induced chlorosis-defective mutants of *P. s.* pv. *tomato* PT23.2 and mapped the Tn*5* inserts to the indigenous plasmid pPT23A. By contrast, genes involved in the production of coronatine by *P. s.* pv. *tomato* DC3000 are chromosomally located (Moore *et al.* 1989). Previously, we isolated a cosmid clone, pEC18, from a genomic library of *P. s.* pv. *tomato* DC3000 and showed that it carried genes involved in coronatine production. The object of this study is to characterize in detail the coronatine *cor* genes located on pEC18. Using transposon saturation mutagenesis and functional complementation analysis, we have shown that the *cor* genes of pEC18 are tightly clustered, span the entire 30-kilobase (kb) fragment of DC3000 DNA, and are organized into several complementation groups. Evidence is presented that some *cor* genes are constitutively expressed, while others are greatly induced upon exposure of the pathogen to the plant. The preliminary results of this work were presented at the 1989 American Phytopathological Society Annual Meeting, which was held August 20-24, in Richmond, VA.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study and their relevant characteristics are listed in Table 1. Strains of *P. s.* pv. *tomato* were grown routinely in nutrient broth-yeast extract-glucose (NBY) broth (Vidaver 1967) or on NBY agar at 25° C. Strains of *Escherichia coli* were grown on Luria-Bertani (LB) agar or in LB broth at 37° C unless otherwise indicated. The concentration of antibiotics used in agar for both *P. s.* pv. *tomato* and *E. coli* were as follows: carbenicillin, 200 μg/ml; chloramphenicol, 40 μg/ml; kanamycin, 50 μg/ml; spectinomycin, 100 μg/ml; streptomycin, 100 μg/ml; nalidixic acid, 100 μg/ml; or rifampicin, 100 μg/ml. In liquid media these antibiotics were used at half the above concentrations.

Transposon mutagenesis. Transposon Tn*3*-Spice (Lindgren *et al.* 1989) was used to generate random mutations in plasmid pEC18. *E. coli* HB101 (pTn*3*-Spice), which had been transformed with plasmid pEC18, was mated with *E. coli* C2110(*polA*) in the presence of the conjugational helper HB101(pRK2013). The mating mixture was plated on LB agar containing nalidixic acid, spectinomycin, and tetracycline to select for C2110(pEC18::Tn*3*-Spice) transconjugants. The position and orientation of each Tn*3*-Spice insertion were determined by restriction enzyme analysis

of the mutated plasmid. The restriction enzymes used, in both single and double digests, were *Bam*HI, *Sal*I, *Eco*RI, and *Xho*I.

Plasmid pEC18 also was subjected to Tn*phoA* mutagenesis (Manoi and Beckwith 1985) following the procedure of De Bruijn and Lupski (1984) using bacteriophage λ as a source of Tn*phoA*. The positions of the Tn*phoA* insertions were determined by restriction enzyme analysis with *Eco*RI, *Xho*I, and *Sal*I.

Once the location of the transposon insertion had been determined, each mutated plasmid was transferred by conjugation into *P. s.* pv. *tomato* DC3000. The insertion mutation was then incorporated into the host genome by the marker exchange technique described below.

Marker exchange mutagenesis. Plasmids pEC18::Tn*3*-Spice or pEC18::Tn*phoA* were transferred into strain DC3000 by conjugation. The resulting transconjugants were grown on NBY agar without antibiotics for several generations. Colonies that had undergone marker exchange were either spectinomycin-resistant and tetracycline-sensitive (Tn*3*-Spice) or kanamycin-resistant and tetracycline-sensitve (Tn*phoA*).

Table 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics[a]	Source or reference
Pseudomonas syringae pv. *tomato*		
DC3000	Rif^r	Cuppels 1986
Escherichia coli		
HB101	*hsdS20*, *recA13*, *ara14*, *lacY1*, *rps10*, *xyl5*, *mtl-1*	Boyer and Roulland-Dussoix 1969
DH5	*hsdR17*, *endA1*, *recA1*, *supE44*, *thi-1*, *gyrA96*	Bethesda Research Laboratories[b]
CC118	*recA*, *phoA*, *galK*, *rpsL*	Manoi and Beckwith 1985
C2110	Nal^r	Stachel *et al.* 1985
Plasmid		
pGS72	IncP1 (rep) Tc^r, Km^r, broad host range cloning vector, Mob$^+$	Selvaraj and Iyer 1985
pKT240	IncQ (rep), Ap^r, Km^r, broad host range cloning vector	Bagdasarian *et al.* 1983
pRK2013	ColE1 (rep) Km^r, Mob$^+$, Tra$^+$ (RP4 *tra*)	Ditta *et al.* 1980
pEC18	pLAFR1 containing a 30-kb fragment of DC3000 DNA	Moore *et al.* 1989
pEC23	pLAFR1 containing the 23-kb *Eco*RI fragment from pEC18	Moore *et al.* 1989
pEC7	pLAFR1 containing the 7-kb *Eco*RI fragment from pEC18	Moore *et al.* 1989
pEC3	3.8-kb *Xho*I fragment from pEC18 subcloned into pGS72	This study
pEC4	4.2-kb *Xho*I fragment cloned into pGS72	This study
pEC5	5.3-kb *Xho*I fragment cloned into pKT240	This study
pEC8	7-kb *Sal*I fragment cloned into pGS72	This study
pEC9	9-kb *Sal*I fragment cloned into pGS72	This study
pEC10	10-kb *Xho*I fragment cloned into pGS72	This study

[a] Rif, rifampicin; Nal, nalidixic acid; Tc, tetracycline; Km, kanamycin; Ap, ampicillin; ^{r}, resistant; rep, replicon; and kb, kilobase.
[b] Gaithersburg, MD.

DNA isolation and manipulation. Plasmid DNA was isolated and purified from *E. coli* strains by the method of Clewell and Helinski (1969). The boiling method of Holmes and Quigley (1980) was used for plasmid screening.

Restriction enzyme digestion of DNA, agarose gel electrophoresis, isolation of restriction enzyme digest fragments using GeneClean (Bio-101, La Jolla, CA), Southern transfer, DNA-DNA hybridization, and autoradiography were performed as previously described (Moore *et al.* 1989).

Recombinant plasmids were constructed by the standard methods of Maniatis *et al.* (1982) and transformed into *E. coli* using the calcium shock method of Cohen *et al.* (1972).

Assay of ice nucleation activity. The ice nucleation activity of *P. s.* pv. *tomato* Tn*3*-Spice mutants was measured by the procedure of Lindgren *et al.* (1989) with a minor modification. The mutants were grown for 48 hr at 24° C in NBY broth containing spectinomycin. Bacteria were washed by centrifugation twice with 0.1 M potassium phosphate buffer, pH 7.2 (PM), and suspended in PM containing 0.1 L of Bacto peptone. The cell concentration of each suspension was estimated from turbidity measurements ($A_{600\ nm}$). Tenfold serial dilutions of each suspension were made in the same buffer, and each dilution was tested for ice nucleation activity at −7° C by the droplet-freezing technique (Lindgren *et al.* 1989).

P. s. pv. *tomato* Tn*3*-Spice mutants grown *in planta* also were tested for ice nucleation activity. Infected leaf tissue (0.25 cm^2) was ground in 5 ml of PM buffer, and the resulting suspension was serially diluted in the same buffer. The dilutions were then tested for ice nucleation activity as described above. The colony-forming units per gram fresh weight of tissue was determined by plating the dilutions on NBY agar containing spectinomycin.

Plant pathogenicity test and the bioassay for coronatine. Bacteria were tested for pathogenicity and screened for toxin production by methods previously described (Cuppels 1986; Moore *et al.* 1989).

RESULTS

Mutagenesis of the Cor region. The Tn*5* insertion sites for four of five *P. s.* pv. *tomato* DC3000 Cor$^-$ mutants map along a 30-kb segment of chromosomal DNA (Moore *et al.* 1989). To more precisely characterize the genetic organization of this region, we mutagenized pEC18, a pLAFR1 clone containing this DNA, with the transposons Tn*3*-Spice and Tn*phoA*. A total of 21 independent Tn*3*-Spice-induced and nine independent Tn*phoA*-induced mutants were isolated. The location of each insertion mutation was determined by restriction enzyme analysis (Fig. 1). Each mutated derivative of pEC18 was transferred by means of a triparental mating into the wild-type strain DC3000. The insertion mutation was recombined into the homologous site on the wild-type genome by marker ex-

change mutagenesis. Verification of marker exchange was conducted by Southern blot analysis (data not shown). Each marker-exchanged *P. s.* pv. *tomato* strain was tested for the ability to induce tomato leaf chlorosis. The Tn*3*-Spice-generated mutants 2082, 2084, and 2093, whose mutations are located on the right border of the pEC18 *P. s.* pv. *tomato* DNA insert, were the only mutants that caused yellowing.

No Tn*3*-Spice insertion mutations occurred within the central region of the *cor* gene cluster of pEC18. In a second attempt to introduce insertion mutations into this region, we cloned the 10-kb central *Xho*I fragment into the 13-kb IncP plasmid vector pGS72 and subjected the resulting recombinant plasmid, pEC10, to Tn*3*-Spice mutagenesis. Although we obtained a few insertions into the *Xho*I fragment, none could be recombined into the homologous site on the wild-type genome, even when we used a method based on plasmid incompatibility (Coplin and Majerczak 1990).

Detection of ice nucleation activity by Tn*3*-Spice mutants. Tn*3*-Spice contains a promoterless ice nucleation gene (*inaZ*) originating from a strain of *P. s.* pv. *syringae* van Hall (Lindgren *et al.* 1989). When Tn*3*-Spice inserts into a gene in the appropriate orientation, a transcriptional fusion with ice nucleation activity results. Since *P. s.* pv. *tomato* DC3000 is unable to form ice nuclei on its own (S.-W. Ma and D. A. Cuppels, unpublished data), any ice nucleation activity in the Cor::Tn*3*-Spice mutants must be due to promoter activity originating from the *cor* target gene. To avoid interference from vector promoters, we assayed only those mutants that resulted from marker exchanges between the wild-type genome and the mutagenized pEC18 (Fig. 1). The ice nucleation activity of each mutant strain was determined both *in vitro* and *in planta* (Table 2). Strains 2003, 2006, 2014, 2022, 2024, 2025, 2026, 2027, 2037, 2038, and 2083 were the only mutants in which the *inaZ* gene had the same transcriptional orientation as the *cor* gene into which Tn*3*-Spice had inserted. Although these strains formed ice nuclei both *in vitro* and *in planta*, only mutant strain 2083 expressed significantly greater ice nucleation activity *in planta*. The ice nucleation gene had the opposite orientation in mutant strains 2007, 2008, 2017, 2021, 2023, and 2028.

Complementation analysis of Cor mutants using Tn*3*-Spice-mutated derivatives of pEC18 and pEC18 subclones. To more precisely define the organization of *cor* genes on pEC18, we conducted a complementation analysis of selected Cor$^-$ mutants. Tn*3*-Spice-containing derivatives of plasmid pEC18, prefixed with the letter "L" to distinguish them from the homogenotized Tn*3*-Spice-mutated strains, were introduced into seven of the Cor$^-$ mutants that had been generated by Tn*5* or Tn*phoA* mutagenesis. Each of the resulting merodiploid strains was tested for the ability to induce chlorosis on tomato seedlings (Table 3). To ensure that the restoration of chlorosis to these mutants was due to complementation and not to the cointegration of the plasmid into the genome, we isolated plasmid DNA from each strain and subjected it to restriction enzyme analysis. In all cases, the restriction pattern was identical to that of the original Tn*3*-Spice-containing pEC18 derivative.

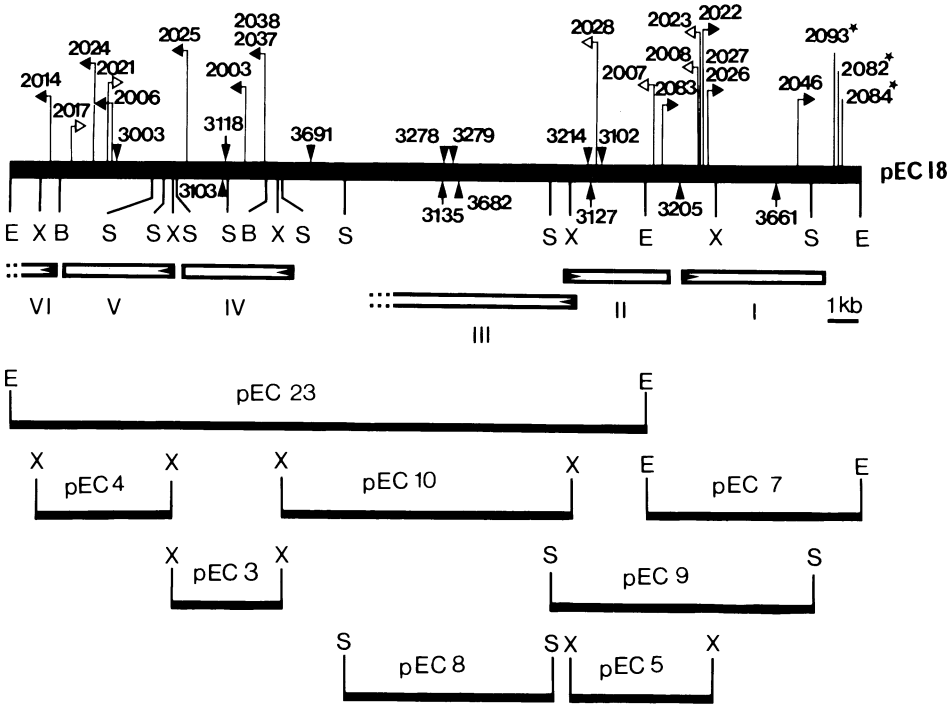

Fig. 1. Physical and genetic map of the Cor region of *Pseudomonas syringae* pv. *tomato* cloned in pEC18 and subclones carrying portions of the Cor region. The position and orientation of transposon Tn*3*-Spice insertions (▶, positive ice nucleation phenotype; ▷, negative ice nucleation phenotype) and the positions of Tn*5* (↓) and Tn*phoA* (▼) insertions are indicated. The proposed genetic organization of the *cor* genes is shown by open bars, and the inside arrows indicate the transcriptional direction of the *cor* gene groups. (★) signifies a positive toxin phenotype. The dotted line represents the possible position(s) of the transcriptional termination site. Restriction sites are abbreviated as follows: B, *Bam*HI; E, *Eco*RI; S, *Sal*I; and X, *Xho*I.

Surprisingly, mutant 3003 was not complemented by pL2007 and pL2083. Complementation tests between homogenotized Tn*3*-Spice, Tn*5*, or Tn*phoA* mutants and subclones of pEC18 (Table 1 and Fig. 1) also were performed (Table 4). Based on the results of these complementation analyses, the Tn*3*-Spice, Tn*5*, and Tn*phoA* mutants fell into six distinct complementation groups, CorI to CorVI (Fig. 1). Because a limited number of insertion mutations were analyzed, the lengths of the DNA regions defined by these groups are only approximate.

DISCUSSION

The data presented in this study, together with previous observations (Moore *et al.* 1989), indicate that several of the *P. s.* pv. *tomato* genes required for synthesis of the polyketide coronatine are tightly clustered and are distributed across a region of at least 30 kb. *Streptomyces* genes involved in the biosynthesis of polyketide antibiotics also are clustered (Malpartida and Hopwood 1986; Stanzk *et al.* 1986). Since ours is the first report concerning the organization of coronatine genes, whether or not the arrangement seen in *P. s.* pv. *tomato* DC3000 is representative of all coronatine-producing bacteria is not known. C. L. Bender and S. A. Young (Physical characterization of mutations in plasmid pPT23A which results in the coronatine-defective phenotype, Fifth International Fallen Leaf Lake Conference, Molecular Biology of Plant Pathogenic Bacteria, Fallen Leaf Lake, South Lake Tahoe, CA, September 14, 1989) proposed that genes required for coronatine synthesis in *P. s.* pv. *tomato* PT23.2 were organized into two noncontiguous DNA regions (7.2 and 20 kb, respectively) on the plasmid pPT23A. In an earlier study we showed that a 5.2-kb *Xho*I fragment from the coronatine gene cluster of strain DC3000 hybridized strongly with the same two *Eco*RI fragments of DNA from a *P. s.* pv. *tomato* strain, two *P. s.* pv. *glycinea* strains, and a *P. s.* pv. *atropurpurea* strain (Moore *et al.* 1989). Thus, even if the genetic maps are not identical, the genes of toxin-producing strains may be highly conserved.

This study confirmed that a DNA region of more than 30-kb is involved in the synthesis of coronatine by *P. s.* pv. *tomato*. The biosynthetic pathway for this toxin must be very complex, requiring several enzymes. Production of another bacterial polyketide, the antibiotic erythromycin, involves at least 30 enzymatic steps (Martin and Liras 1989). Parry and Mafoti (1986) have shown that the coronafacoyl moiety of coronatine is derived from a branched polyketide with pyruvate as the starter unit. The mechanism of cyclization and reduction to give the bicyclic ring is unknown. How coronamic acid, which is derived from isoleucine, is coupled to coronafacic acid and subsequently converted to the cyclopropane by an enzyme(s)-

Table 2. Ice nucleation activity of *in vitro* and *in planta* cultures of homogenotized Tn*3*-Spice-induced Cor⁻ mutants of *Pseudomonas syringae* pv. *tomato* DC3000

Fusion plasmid	Mean log ice nuclei/cell (−7° C)		Ratio of activities[b]
	In vitro	*In planta*[a]	
pEC2003	−4.74[c]	−5.00	0.55
pEC2006	−4.80	−5.05	0.56
pEC2008	nd[d]	nd	···
pEC2014	−2.40	−3.25	0.14
pEC2017	nd	nd	···
pEC2021	nd	nd	···
pEC2022	−3.62	−3.68	0.88
pEC2023	nd	nd	···
pEC2024	−2.60	−3.54	0.12
pEC2025	−4.92	−3.87	11.3
pEC2026	−3.62	−3.68	0.88
pEC2027	−3.62	−3.68	0.88
pEC2028	nd	nd	···
pEC2038(2037)	−1.44	−2.54	0.08
pEC2083	−5.16	−2.59	371.00
pEC2007	nd	nd	···

[a]Bacteria were eluted from leaf lesions and immediately tested for ice nucleation activity.
[b]Ratio of activities = *in planta* activity per *in vitro* activity.
[c]Results are expressed as the mean of two separate assays.
[d]nd, not detected.

Table 3. Complementation analysis of homogenotized Tn*5*- or Tn*phoA*-induced Cor⁻ mutants of *Pseudomonas syringae* pv. *tomato* DC3000 by the introduction of Tn*3*-Spice-containing derivatives of pEC18[a]

Plasmids	Cor⁻ mutants						
	3003	3102	3103	3214	3135	3661	3682
pL2008	+[b]	+	+	+	+	−	+
pL2027	+	+	+	+	+	−	+
pL2046	+	+	+	+	+	−	+
pL2007	−	−	+	−	+	+	+
pL2083	−	−	+	−	+	+	+
pL2025	+	+	−	+	+	nt	nt
pL2037	+	+	−	+	nt	+	nt
pL2038	+	+	−	nt	nt	+	nt
pL2017	−	+	+	+	+	+	+
pL2021	−	+	nt	+	+	+	+
pL2024	−	+	+	nt	+	+	+
pL2014	+	+	+	+	+	+	+

[a]Tn*3*-Spice-containing derivatives of pEC18 were triparentally mated into each of the Tn*5* or Tn*phoA* mutants of *P. s.* pv. *tomato* DC3000 in the presence of helper plasmid pRK2013. Transconjugants were selected on nutrient broth-yeast extract-glucose plates supplemented with rifampicin and spectinomycin, purified, and tested for restoration of the ability to induce chlorosis on tomato leaves.
[b]+, complementation; −, no complementation; and nt, not tested.

Table 4. Complementation analysis of homogenotized Tn*5*-, Tn*phoA*-, or Tn*3*-Spice-induced Cor⁻ mutants of *Pseudomonas syringae* pv. *tomato* by the introduction of pEC18 and various subclones of pEC18

Cor⁻ mutants	Subclones				
	pEC18	pEC23	pEC9	pEC7	pEC10[a]
2014	−[b]	−	−	−	−
2017	+	+	−	−	−
2021	+	+	−	−	−
2006	+	+	−	−	−
2025	+	+	−	−	−
2038	+	+	−	−	−
3135	+	+	−	−	−
3682	+	+	−	−	−
3127	+	−	+	−	−
2046	+	nt	−	+	−
2008	+	nt	−	+	−
3661	+	−	−	+	−

[a]The results obtained with pEC10 were also obtained with pEC3, pEC4, pEC5, and pEC8.
[b]As given in Table 3.

mediated oxidative-cyclization step also is unclear (Mitchell 1989). Since none of the Tn*phoA* Cor$^-$ mutants, which were scattered across the entire 30-kb *cor* gene cluster, produced a functional alkaline phosphatase, the *cor* genes containing these insertions do not code for secreted or membrane proteins. Thus, these genes may code for cytoplasmic enzymes involved in the biosynthetic pathway or for the regulatory molecules that control these enzymes.

Our results show that the *cor* genes are organized into at least six distinct complementation groups. Since mutants 2046, 2008, and 3661 were complemented by pEC7 but not pEC9, CorI genes extend beyond the *Sal*I restriction site but stop before the Tn*3*-Spice insertion site of the toxin-positive strain 2093. Because pEC9 but not pEC5 restores toxin production to mutants 3127, 3102, and 3214, transcription of CorII probably starts at a position between the *Xho*I and *Sal*I sites. Mutants 3135 and 3682 were complemented by pL mutants from regions CorI, II, IV, and V and thus clearly identified a CorIII region. The lack of Tn*3*-Spice mutations in CorIII may be due to transposition immunity or a related phenomenon (Kleckner 1981). Kleckner has suggested that Tn*3*, which transposes with high specificity into plasmid DNA, is highly sensitive to the state of the target DNA. Since pEC23 but not pEC8 or pEC10 restored toxin production to strains 3135 and 3682, CorIII is transcribed in a direction opposite to that of regions CorI and CorII, and its transcriptional start site is located to the right of the *Xho*I restriction site. Thus, although further analysis is required, it appears that CorII and CorIII overlap. No mutants of CorIV were corrected by the introduction of subclone pEC3, which consists of a 3.8-kb *Xho*I fragment cloned into pGS72. Thus, CorIV must extend to the right of the *Xho*I restriction site. Since plasmid pEC4, a pGS72-derivative carrying a 4.2-kb *Xho*I fragment, did not complement the CorV mutants 2021, 2006, and 2017, the transcription initiation site for CorV must be to the right of the *Xho*I restriction site. Because 2014, the mutant which defines CorVI, was not complemented by any of the pEC18 subclones, only a portion of this region was present on pEC18.

Although all six complementation groups were expressed during growth *in vivo* and *in planta*, only CorII was plant-inducible. Several other plant-associated bacteria also have genes that are induced by plant material (Peters and Verma 1990). In *Rhizobium*, most *nod* (nodulation) genes are derepressed upon exposure to a special class of plant phenolics called flavonoids (Long 1989). In the presence of these compounds, the protein binds to the upstream consensus sequence of other *nod* operons, thus allowing the initiation of transcription. The *vir* (virulence) genes of *Agrobacterium tumefaciens* (Smith & Townsend) Conn, which are required for DNA transfer from the bacterium to its host plant, are induced by a number of low molecular weight plant phenolic compounds (Halverson and Stacey 1986). These compounds, normally not present in healthy plants, appear when the plant has been wounded. Exposure of the pathogen to plant cells also induces one of the *hrp* (hypersensitive response and pathogenicity) gene clusters of *P. solanacearum* (Smith) Smith (Huang *et al.* 1990) and a region in the *hrp* gene cluster of *P. s.* pv. *phaseolicola* (Burkholder) Young *et al.* (Lindgren *et al.* 1989).

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