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Molecular and Physiological Characterization of *Pseudomonas syringae* pv. tomato and *Pseudomonas syringae* pv. maculicola Strains That Produce the Phytotoxin Coronatine

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The chlorosis-inducing phytotoxin coronatine is produced by several *Pseudomonas syringae* pathovars, including glycinea, morsprunorum, atropurpurea, and the closely related tomato and maculicola. To date, all coronatine-producing pv. glycinea, morsprunorum, and atropurpurea strains that have been examined carry the gene cluster that controls toxin production on a large plasmid. In the present study the genomic location of the coronatine gene cluster was determined for coronatine-producing strains of the pv. tomato-maculicola group by subjecting their genomic DNA to pulsed-field electrophoresis and Southern blot analysis with a hybridization probe from the coronatine gene cluster. The cluster was chromosomally borne in 10 of the 22 strains screened. These 10 strains infected both crucifers and tomatoes but could not use sorbitol as a sole source of carbon. The remaining 12 coronatine-producing strains had plasmid-borne toxin gene clusters and used sorbitol as a carbon source. Only one of these strains was pathogenic on both crucifers and tomatoes; the remainder infected just tomatoes. Restriction fragment length polymorphism analysis of the pv. tomato-maculicola coronatine gene clusters was performed with probes from *P. syringae* pv. tomato DC3000, a tomato and crucifer pathogen. Although the coronatine cluster appeared, in general, to be highly conserved across the pv. tomato-maculicola group, there were significant differences between plasmid-borne and chromosomally borne genes. The extensively studied coronatine cluster of pv. glycinea 4180 closely resembled the plasmid-borne clusters of the pv. tomato-maculicola group.

The tomato pathogen *Pseudomonas syringae* pv. tomato and the crucifer pathogen *P. syringae* pv. maculicola, along with several other *P. syringae* pathovars, produce coronatine, a phytotoxin that causes leaf chlorosis and plant stunting (26, 28, 41). Greenhouse studies with Tn*5*-induced Cor$^-$ mutants of *P. syringae* pv. tomato have shown that bacteria possessing this virulence factor achieve higher population levels and produce larger lesions on their host plants (3, 27). Toxin production thus appears to give this pathogen a selective advantage in its natural habitat. In a 1989 study, all 244 *P. syringae* pv. tomato strains isolated from Ontario tomato fields produced coronatine and reacted with TPR1, a DNA probe for coronatine producers (10). Despite the competitive edge it imparts to *P. syringae* pv. tomato strains, coronatine production is not a universal trait among field isolates of two other coronatine-producing *P. syringae* pathovars, glycinea and morsprunorum (15, 23, 39). Whether non-toxin-producing strains of *P. syringae* pv. maculicola also are common to host plant surfaces is unknown.

Recent studies have demonstrated that coronatine production is controlled by a gene cluster that is at least 30 kb in size (4, 24, 42). In *P. syringae* pv. tomato PT23.2 and *P. syringae* pv. glycinea PG4180, this cluster is located on a large plasmid (101 and 90 kb, respectively) (2, 4). Southern blot analysis using labeled fragments from the *cor* gene cluster of strain PT23.2 as probes indicated that the coronatine genes of 16 *P. syringae* pv. morsprunorum strains, several *P. syringae* pv. glycinea strains, and one *P. syringae* pv. atropurpurea strain also are carried on a plasmid (4, 23, 39). These studies confirmed earlier work which linked the presence of a large plasmid (87 kb) in *P. syringae* pv. atropurpurea to toxin production (33). To date, coronatine is the only bacterial phytotoxin that has been shown to be under the control of plasmid-borne genes (8, 21, 30). However, plasmid control of coronatine synthesis is not universal; there is one known *P. syringae* pv. tomato strain, DC3000, which carries the *cor* gene cluster on the chromosome (27). The genomic location of *P. syringae* pv. maculicola coronatine genes has not yet been determined.

Bacteria identified as *P. syringae* pv. tomato and those identified as *P. syringae* pv. maculicola are phenotypically very similar and may have overlapping host ranges (19, 35). The taxonomic and genetic relationships that exist within this group of bacteria, which also includes *P. syringae* pv. antirrhini and apii, are not well understood. Although classified as a *P. syringae* pv. tomato strain, DC3000 also infects members of the family *Cruciferae* (11, 19). In the present study, DC3000 was compared with coronatine-producing strains of pv. tomato and maculicola by using pathogenicity tests, carbon source utilization assays, the genomic location of the *cor* gene cluster, and restriction fragment length polymorphism (RFLP) analysis of the *cor* region. Although all the pv. maculicola strains were pathogenic on both crucifers and tomato, the only *Pseudomonas syringae* pv. tomato strains able to infect these two hosts were DC3000 and OH314. All the *P. syringae* pv. tomato strains, excluding DC3000 and OH314, carried their *cor* gene clusters on plasmids, while all but one of the *P. syringae* pv. maculicola strains had chromosome-borne toxin genes. Although RFLP analysis indicated that the *cor* regions of all the coronatine-producing strains we examined, including *P. syringae* pv. glycinea PG4180, were highly conserved, we found significant differences between plasmid-borne and chromosome-borne *cor* regions.

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TABLE 1. *P. syringae* strains used in this study

Pathovar and strain	Host	Source (geographic origin)[a]
Atropurpurea		
NK340	*Bromus* spp.	T. Denny (California)
1304	*Bromus* spp.	C. Bender (Japan)
Glycinea		
F111	Soybean	E. Ward (Ontario)
F117	Soybean	E. Ward (Ontario)
B3	Soybean	E. Ward (Ontario)
Maculicola		
438	Crucifer	T. Denny (California)
1820	Radish	NCPPB (California)
4981	Cauliflower	ICMP (Zimbabwe)
1766	Cauliflower	NCPPB (England)
2744	Mustard	ICMP (England)
795	Cauliflower	ICMP (New Zealand)
84-59	Cauliflower	W. Wiebe (California)
84-66	Cauliflower	W. Wiebe (California)
84-67	Cauliflower	W. Wiebe (California)
88-10	Cauliflower	W. Wiebe (California)
88-11	Cauliflower	W. Wiebe (California)
90-32	Cauliflower	W. Wiebe (California)
Tomato		
DC84-1	Tomato	D. Cuppels (Ontario)
DCT6D1	Tomato	D. Cuppels (Ontario)
DC89-4H	Tomato	D. Cuppels (Ontario)
188B (race 1)	Tomato	B. MacNeill (Ontario)
JL1035	Tomato	J. Lindemann (California)
SM78-1	Tomato	S. McCarter (Georgia)
AV80	Tomato	A. Vidaver (Nebraska)
1108	Tomato	NCPPB (England)
1318	Tomato	CNPB (Switzerland)
3357	Tomato	ICMP (New Zealand)
PST26L	Tomato	M. Hattingh (South Africa)
487	Tomato	A. Mavridis (Greece)
DC3000[b]	Tomato	D. Cuppels (England)
OH314	Nettle	D. Coplin (Ohio)
804s	Tomato	D. Coplin (Ohio)

[a] NCPPB, National Collection of Plant Pathogenic Bacteria, Hertfordshire, England; ICMP, International Collection of Microorganisms from Plants, Auckland, New Zealand; CNPB, Collection Nationale de Bactéries Phytopathogènes, Angers, France.

[b] DC3000 is a spontaneous Rif^r derivative of NCPPB1106.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *P. syringae* strains used in this study are described in Table 1. They routinely were grown in nutrient broth-yeast extract (NBY) medium at 25°C as previously described (9). *Escherichia coli* WA803(pGS9) (35), *E. coli* J5-3(pR1*drd*19) (5), *E. coli* J5-3(pRP1) (5), *E. coli* MM292(pRK2013) (14), and *E. coli* J5-3(pR40a) (5), whose plasmids were used as reference standards in determining the size of *P. syringae* plasmids, were grown at 37°C on Luria-Bertani medium (32) containing 50 μg of kanamycin per ml. *E. coli* HB101(pEC18), which contains the pLAFR1 clone of *P. syringae* pv. tomato DC3000 coronatine genes (27), and *E. coli* HB101(pSAY10), which contains the pLAFR3 clone of *P. syringae* pv. glycinea PG4180 coronatine genes (42), were grown on Luria-Bertani medium containing 12.5 μg of tetracycline per ml.

Isolation and manipulation of DNA. Procedures for the isolation and purification of *P. syringae* genomic and plasmid DNA have been described (9). Horizontal gel electrophoresis was performed with either 0.8 or 0.45% agarose and TBE buffer (89 mM Tris-HCl [pH 8.0], 89 mM boric acid, 2 mM EDTA) (32). Restriction endonucleases, purchased from Boehringer Mannheim (Montreal, Quebec, Canada) or Promega (Unionville, Ontario, Canada), were used according to the recommendations of the manufacturer.

PFGE. *P. syringae* chromosomal DNA was prepared for pulsed-field gel electrophoresis (PFGE) (6) by using a variation of the procedure described by Grothues and Tümmler (18). Bacteria were grown overnight at 25°C in 20 ml of NBY broth, harvested by centrifugation (5,000 × *g*) for 10 min at 4°C, washed once with TE buffer (10 mM Tris, 0.1 mM EDTA [pH 8.0]), and resuspended in TE to a final concentration of 5×10^9 CFU/ml. The bacterial suspension (1.5 ml) was mixed with 2.5 ml of melted 1% low-melting-point agarose (Bethesda Research Laboratories, Gaithersburg, Md.) (in 125 mM EDTA [pH 7.5]) and poured into plug molds supplied with the Beckman Geneline transverse alternating-field electrophoresis (TAFE) apparatus (Beckman Instruments, Palo Alto, Calif.). Once solidified, the plugs were removed from the mold and incubated overnight at 50°C in lysis buffer containing 10 mM Tris (pH 7.8), 500 mM EDTA, 1% sodium lauroyl sarcosinate, and 2 mg of proteinase K per ml (34). Lysis buffer was eluted from the plugs by incubating the plugs in TE buffer for 48 h with at least two buffer changes. The plugs were sealed into the wells of a 1% agarose gel with melted 1% agarose and subjected to TAFE in 1× TAFE buffer (10 mM Tris, 0.5 mM EDTA [free acid], 15 mM glacial acetic acid) for 6 h at 13°C and 250 mA (constant current) with a pulse time of 5 s and then for 16 h at 13°C and 175 mA (constant current) with a pulse time of 15 s. Gels were stained with ethidium bromide and photographed by standard procedures (32).

Southern blot analysis. After electrophoresis on horizontal agarose gels, DNA was transferred to Hybond N (Amersham, Oakville, Canada) nylon membranes by the standard capillary method (32). DNA electrophoresed on TAFE gels was transferred with the Beckman TAFE apparatus and Beckman DNA blotting electrodes. Preparation of ^{32}P-labeled DNA probes by nick translation, DNA-DNA hybridization, and autoradiography were carried out as described previously (9).

Conversion of plasmid DNA from supercoiled to open circular or linear form. Supercoiled plasmid DNA was converted to open circular or linear DNA by a modification of the method described by Levene and Zimm (22). Plasmid DNA was suspended in 48 μl of DNase I lysis buffer (20 mM Tris [pH 7.5], 2 mM $MgCl_2$, 5% glycerol) to a final concentration of 1.5 μg/μl and then treated in the dark at 37°C with ethidium bromide (0.15-μg/μl final concentration) for 15 min. DNase I (Sigma, St. Louis, Mo.) was added to a final concentration of 6 ng/μl, and the incubation at 37°C was continued for an additional 60 min. The preparation was extracted once with an equal volume of Tris-saturated phenol (pH 7.0) and once with an equal volume of chloroform-isoamyl alcohol (24:1). Traces of organic solvent were removed by incubating the tube for 1 h at 50°C in a fume hood. The aqueous phase (32 μl) was heated to 46°C and added to 63 μl of molten 1% low-melting-point agarose before being poured into a TAFE plug mold. One-half of the plug (36 μg of DNA) was subjected to PFGE as described above.

Plant pathogenicity tests. Bacteria grown overnight at 25°C on NBY agar were tested for pathogenicity on 2-week-old tomato seedlings (*Lycopersicon esculentum* Mill. 'Bonny Best') by a method described previously (9). Four-week-old cauliflower (*Brassica oleracea* var. botrytis cv. Early Snowball Super) and cabbage (*Brassica oleracea* var. capitata cv. Golden Acre) seedlings were inoculated by first gently rubbing the leaves with a water suspension of carborundum, rinsing off the carborundum with sterile distilled water, and then spraying the injured leaves with a water suspension of the bacteria (10^7 CFU/ml). If the bacterial strain was pathogenic, small dark brown lesions, sometimes surrounded by chlorotic halos, appeared within 4 days of inoculation on tomato seedlings and within 14 days of inoculation on crucifer seedlings.

Lesion size on infected tomato plants. *P. syringae* pv. tomato and *P. syringae* pv. maculicola strains were grown overnight in NBY broth at 25°C. The cells were collected by centrifugation (5,000 × *g* for 15 min at 4°C), washed once with 20 ml of sterile distilled water, and resuspended to an A_{600} of 0.2 in sterile distilled water. Three-week-old tomato plants were sprayed with the cell suspension and incubated in a growth chamber under conditions that have already been described (9). Eight days after inoculation, the diameters of 50 lesions (per strain) were measured with a Hastings hand lens; mean lesion size was calculated for each strain.

Tomato leaf bioassay for coronatine. A dried ethyl acetate extract of bacterial culture supernatant was redissolved in sterile, distilled water and applied to the surface of a tomato leaf. If the supernatant contained coronatine, the area around the injection site turned yellow within 5 days. This procedure has already been described in detail (27).

RESULTS AND DISCUSSION

Pathogenicity, coronatine production, and carbohydrate utilization. Fifteen *P. syringae* pv. tomato strains, twelve *P. syringae* pv. maculicola strains, three *P. syringae* pv. glycinea strains, and two *P. syringae* pv. atropurpurea strains were tested for pathogenicity on tomato and cauliflower plants (Table 2). Although all the *P. syringae* pv. maculicola strains formed lesions on both plants, only two of the *P. syringae* pv. tomato strains infected cauliflower as well as tomato plants. When cauliflower was replaced with another crucifer, cabbage, the results were the same (data not shown). Although the *P. syringae* pv. atropurpurea strains did not infect either host, *P. syringae* pv. glycinea, the soybean pathogen, formed one or two lesions per tomato plant. Our results confirmed those of Wiebe and Campbell, who tested 30 *P. syringae* pv. maculicola strains and

TABLE 2. Coronatine production, disease expression, and sorbitol and homoserine utilization by the *P. syringae* strains used in this study

Pathovar and strain(s)	Coronatine production[a]	Disease rating[b]		Utilization of sorbitol/ utilization of homoserine[c]
		Tomato	Cauliflower	
Tomato				
DC84-1, DCT6D1, 188B, DC89-4H, JL1035, 1318, SM78-1, PST26L, AV80, 3357, 487	+	++	−	+/−
DC3000, OH314	+	++	++	−/−
804s, 1108	−	N	−	+/−
Maculicola				
1820, 84-59, 438, 84-66, 84-67, 88-10, 88-11, 90-32	+	++	++	−/−
1766, 2744, 795	−	N	N	+/−
4981	+	++	++	+/−
Atropurpurea NK340, 1304	+	−	−	+/−
Glycinea F111, F117, B3	+	−/+	−	−/−

[a] Determined by tomato leaf bioassay (see text).

[b] Disease ratings: ++, highly virulent, causing chlorosis and necrosis; −/+, very weakly virulent, producing an occasional lesion; −, not pathogenic; N, virulent but not causing chlorosis.

[c] For the carbon source utilization assay, Vogel-Bonner minimal agar, supplemented with 0.3% filter-sterilized homoserine or sorbitol, was spot inoculated with 15-μl drops (8×10^5 CFU per drop) of a bacterial suspension and incubated at 25°C for 14 days (9).

4 *P. syringae* pv. tomato strains on several different plant species (41). On tomato leaves, 8-day-old lesions caused by *P. syringae* pv. tomato DCT6D1, JL1035, and 3357 (diameters, 0.59 ± 0.17 mm, 0.63 ± 0.17 mm, and 0.75 ± 0.18 mm, respectively [means ± standard deviations]) were significantly larger than those formed by *P. syringae* pv. maculicola 90-32, 1820, and 4981 (0.23 ± 0.1 mm, 0.23 ± 0.1 mm, and 0.39 ± 0.15 mm, respectively) and by *P. syringae* pv. tomato DC3000 and OH314 (0.40 ± 0.2 and 0.30 ± 0.1 mm, respectively).

The results of the tomato leaf bioassay for coronatine production are shown in Table 2. *P. syringae* pv. tomato 804s and 1108 and *P. syringae* pv. maculicola 1766, 2744, and 795 are old strains (18 years or more) and may have lost the ability to produce toxin since they were first isolated. Only 7 of the 25 *P. syringae* pv. maculicola strains screened by Wiebe and Campbell produced toxin (41). Likewise, our laboratory has noted that coronatine-producing *P. syringae* pv. tomato strains from Ontario fields must be kept frozen or lyophilized if they are to remain coronatine positive (9a). The same phenomenon has been observed with syringomycin, another *P. syringae* toxin (17).

P. syringae pv. tomato and *P. syringae* pv. maculicola have almost identical nutritional profiles and may represent a single genetic group (29). In fact, it has been suggested that they be combined into one species (38). Nevertheless, Hendson et al. were able to cluster the *P. syringae* pv. maculicola and pv. tomato strains included in their study into four subgroups on the basis of homoserine and sorbitol utilization and RFLP analysis with a rutin glycosidase gene probe (19). Group 1 grows only on sorbitol; group 3 grows on both sorbitol and homoserine; and groups 2 and 4 use neither compound. Groups 2 and 4 are distinguished by the RFLP patterns formed with the rutin glycosidase gene probe. Although we did not perform the RFLP analysis, all of the *P. syringae* pv. tomato strains included in our study, with the exception of DC3000 and OH314, appeared to be members of group 1 (Table 2). Conversely, the *P. syringae* pv. maculicola strains, with the exception of 1766, 2744, 795, and 4981, belonged to group 2 or 4. The results of the homoserine and sorbitol utilization assays for *P. syringae* pv. tomato DC3000 and *P. syringae* pv. maculicola 795, 2744, 4981, 84-67, and 1820 confirmed those obtained by Hendson et al. (19). Strain 1820 is the only known member of group 4. Our group 1 *P. syringae* pv. tomato strains differed from those of Hendson et al. in that they did not produce lesions on cabbage or cauliflower plants. However, it is the view of Hendson et al. that nutritional characterization forms a more reliable basis for identification than host-of-origin or pathogenicity tests.

On the basis of pathogenicity and carbon utilization assays, DC3000, which is a spontaneous Rif^r mutant of the pathotype strain for *P. syringae* pv. tomato (NCPPB1106) (9), more closely resembled strains identified as *P. syringae* pv. maculicola than other *P. syringae* pv. tomato strains. However, until the taxonomic status of the pv. tomato-maculicola group has been clarified, we suggest that DC3000 continue to be called *P. syringae* pv. tomato.

Plasmid analysis. Although several *P. syringae* pv. morsprunorum and glycinea strains carry large plasmids bearing coronatine genes, only one *P. syringae* pv. tomato strain was previously shown to have a plasmid-borne coronatine gene cluster (2, 23, 39). The majority of *P. syringae* pv. maculicola strains screened by Wiebe and Campbell have at least one plasmid, but it is not known whether any of these plasmids carry toxin genes (41). In the present study, we used PFGE to resolve large DNA molecules released from agarose-immobilized cells of the coronatine-producing *P. syringae* pv. tomato and *P. syringae* pv. maculicola strains listed in Table 2. This technique clearly separates plasmids from chromosomal DNA. Every coronatine-producing strain had at least one plasmid

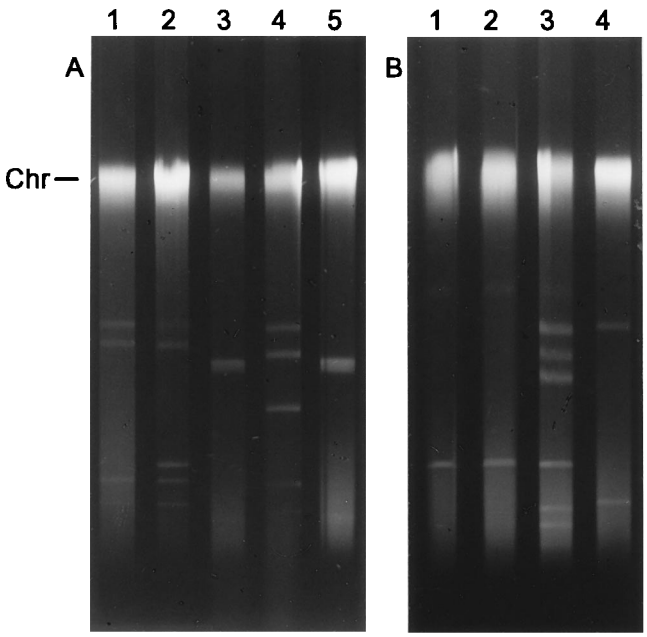

FIG. 1. Separation of *P. syringae* pv. tomato (A) and *P. syringae* pv. maculicola (B) plasmid and chromosomal DNA by pulsed-field electrophoresis. Each lane contains DNA from a different strain, as follows. (A) Lanes: 1, 188B; 2, AV80; 3, OH314; 4, DC84-1; 5, DC3000. (B) Lanes: 1, 84-66; 2, 84-67; 3, 84-59; 4, 88-11. Chr, chromosomal DNA.

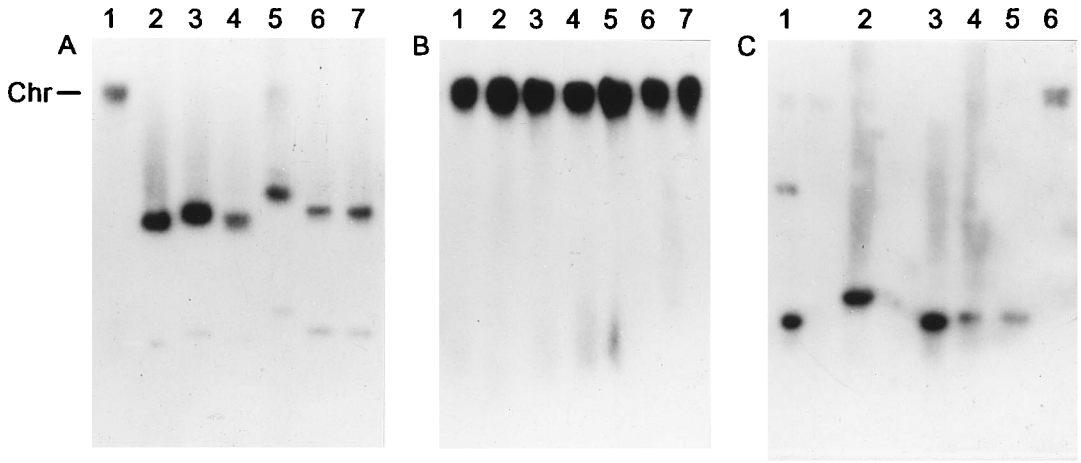

FIG. 2. Southern blot analysis of genomic DNA from coronatine-producing *P. syringae* strains that has been resolved by pulsed-field electrophoresis. The probe was a ^{32}P-labeled 4.5-kb *Eco*RI-*Xho*I fragment of the *P. syringae* pv. tomato DC3000 coronatine gene cluster. Each lane contains DNA from a different strain, as follows. (A) Lanes: 1, pv. tomato DC3000; 2, pv. maculicola 4981; 3, pv. tomato DCT6D1; 4, pv. tomato 487; 5, pv. tomato 3357; 6, pv. tomato SM78-1; 7, pv. tomato JL1035. (B) Lanes: 1, pv. maculicola 84-67; 2, pv. maculicola 84-66; 3, pv. maculicola 84-59; 4, pv. maculicola 88-10; 5, pv. maculicola 88-11; 6, pv. maculicola 90-32; 7, pv. maculicola 1820. (C) Lanes: 1, pv. glycinea F117; 2, pv. glycinea F111; 3, pv. glycinea B3; 4, pv. atropurpurea NK340; 5, pv. atropurpurea 1304; 6, pv. maculicola 438. Chr, chromosomal DNA.

band. PFGE results for selected strains are shown in Fig. 1. Southern blot analysis, using a ^{32}P-labeled 4.5-kb *Eco*RI-*Xho*I fragment from the coronatine gene cluster of strain DC3000 as the probe, indicated that the toxin genes of the *P. syringae* pv. glycinea strains, the *P. syringae* pv. atropurpurea strains, and all the *P. syringae* pv. tomato strains, with the exception of DC3000 and OH314, were plasmid borne (results for 20 of the 27 strains tested are shown in Fig. 2). The toxin genes of the group 2 *P. syringae* pv. maculicola strains were chromosomal, while those of the only group 1 *P. syringae* pv. maculicola strain tested, 4981, were on a plasmid. To confirm these results, we subjected purified and *Eco*RI-cut plasmids from each pv. maculicola strain to Southern blot analysis with the same 4.5-kb *Eco*RI-*Xho*I fragment from the coronatine gene cluster as the hybridization probe. The only pv. maculicola strain to hybridize with the probe was strain 4981 (data not shown). Thus, Southern blot analysis of genomic DNA that has been released from agarose-immobilized bacterial cells and then subjected to PFGE appears to be an effective way of determining whether or not a particular gene or DNA sequence is plasmid or chromosome borne. This procedure is particularly useful for dealing with large plasmids that are difficult to isolate.

Several of the strains whose results are shown in Fig. 2A had two probe-positive plasmid bands, one toward the middle of the gel and one toward the bottom of the gel. To determine whether these two bands represent two different plasmids or two different forms of the same plasmid molecule, we digested purified supercoiled PST26L plasmid DNA with DNase I for 0, 15, 30, or 60 min according to the procedure Levene and Zimm developed for generating open circular DNA (22) and then subjected the DNA to PFGE. The upper bands, which were clearly visible at 0 min, completely disappeared by 60 min; the reverse was true for the lower bands (data not shown). Thus, the lower bands seen in lanes 2 through 7 of Fig. 2A and in lanes 1 through 5 of Fig. 2C appear to be open circular or linear versions of the coronatine plasmid of these six strains. When subjected to conventional horizontal agarose gel electrophoresis, supercoiled plasmid DNA has a greater mobility than nicked plasmid DNA, which becomes entrapped in the gel matrix (22). Simske and Scherer hypothesized that the pulse-induced backward diffusion which occurs in a pulsed-field gel frees open circular forms from the gel matrix and thus significantly accelerates their migration towards the positive electrode (36).

Plasmids purified from coronatine-producing *P. syringae* pv. tomato strains were subjected to electrophoresis on conventional 0.45% horizontal agarose gels (Fig. 3). Each strain carried a minimum of two plasmids, which ranged in size from 120 to 2 kb (Table 3). There were eight different plasmid profiles among the 13 strains examined. Denny also observed a wide range of plasmid sizes and profiles among the *P. syringae* pv. tomato strains included in his study (12). The one pv. maculicola strain with plasmid-borne coronatine genes, strain 4981, had one plasmid in each of the size groups C, E, and F (Fig. 3; data for the group F plasmid not shown). The group A plasmid of strain 3357 was estimated to be 115 kb by comparing it with reference plasmids (Fig. 3). This estimate was in good agree-

TABLE 3. Plasmid profiles of coronatine-producing strains of *P. syringae* pv. tomato

Strain	No. of plasmids in size group (size [kb])[a]						
	A (120–110)	B (100–90)	C (89–80)	D (79–65)	E (45–30)	F (20–5)	G (<5)
3357	1						1
SM78-1		1		2			
188B		1		2			
PST26L		1		1	1		
DC84-1		1		1	1		
DCT6D1		1	1				
1318		1	1		1		
AV80		1	1				
JL1035		1	1		1		
DC89-4H		1		1			
487			1	2	1		
3000				2			
OH314				2			

[a] Plasmid size was determined by subjecting purified plasmid DNA to agarose gel electrophoresis using plasmids of known molecular sizes as reference standards (pR40a [146 kb], pR1*drd*19 [93 kb], pRP1 [57 kb], pRK2013 [48 kb], and pGS9 [30 kb]).

TABLE 4. RFLP analysis of the coronatine gene cluster in *P. syringae* pv. tomato and *P. syringae* pv. maculicola strains[a]

Pathovar and strain	Size(s) (kb) of fragment(s) hybridizing with probe:											
	A				B				C			
	*Eco*RI	*Sst*I	*Bam*HI	*Xho*I	*Eco*RI	*Sst*I	*Bam*HI	*Xho*I	*Eco*RI	*Sst*I	*Bam*HI	*Xho*I
Tomato												
DCT6D1	6.9	6.2	8.5	14	22	8.8	7, 8.5	4.5, 0.3	22	3.2	15	13
3357	6.9	6.2	8.5	14	22	8.8	7, 8.5	4.5, 0.3	22	3.2	11.5	>21
PST26L	6.9	6.2	8.5	14	22	8.8	7, 8.5	4.5, 0.3	22	3.2	11.5	>21
JL1035	6.9	6.2	8.5	14	22	8.8	7, 8.5	4.5, 0.3	22	3.2	11.5	>21
DC3000[b]	6.9	6.2	12	5.8	22	8.8	7, 12	4.8	22	3.2	>21	11.5
OH314[b]	6.9	6.2	12	5.8	22	8.8	7, 12	4.8	22	3.2	>21	11.5
Maculicola												
4981	6.9	6.2	8.5	14	22	8.8	7, 8.5	4.5, 0.3	22	3.2	8.7	>21
438[b]	6.9	6.2	12	5.8	22	8.8	7, 12	4.8	22	3.2	11.5	11.5
1820[b]	6.9	6.2	12	5.8	22	8.8	7, 12	4.8	22	3.2	11.5	11.5
84-59[b]	6.9	6.2	12	5.8	22	8.8	7, 12	4.8	22	3.2	>21	11.5

[a] DNA was isolated from the various strains, digested with *Eco*RI, *Sst*I, *Bam*HI, or *Xho*I, and then subjected to Southern blot analysis with ^{32}P-labeled hybridization probes derived from the coronatine gene cluster: probe A, a 4.5-kb *Eco*RI-*Xho*I fragment; probe B, a 4.8-kb *Xho*I fragment; probe C, a 3.2-kb *Sst*I fragment (Fig. 4).
[b] Strain with chromosomally borne coronatine gene cluster. Genomic DNA was used for the analyses of these strains, while plasmid DNA purified by cesium chloride-ethidium bromide centrifugation was used for all the others.

ment with the totalled sizes of the *Sst*I or *Bam*HI restriction fragments derived from this plasmid (120 ± 4.5 kb) (data not shown). Southern analysis of TAFE gel blots, using the ^{32}P-labeled 4.5-kb *Eco*RI-*Xho*I fragment from the DC3000 coronatine gene cluster as the probe, demonstrated that for every strain examined, including *P. syringae* pv. maculicola 4981, the toxin gene cluster was on the largest plasmid present. Therefore, the toxin plasmid of most strains was in the group B (100- to 90-kb) size range; the exceptions were *P. syringae* pv. tomato 3357, which had a group A toxin plasmid, and *P. syringae* pv. tomato 487 and *P. syringae* pv. maculicola 4981, which carried group C coronatine plasmids. Similar size ranges were reported for the coronatine plasmids present in *P. syringae* pv. tomato PT23.2, *P. syringae* pv. glycinea PG4180, *P. syringae* pv. atropurpurea PA1304, and several field isolates of *P. syringae* pv. morsprunorum and glycinea (2, 4, 23, 39).

RFLP analysis of the *cor* gene cluster in *P. syringae* pv. tomato and *P. syringae* pv. maculicola. RFLP analysis is a sensitive, cost-effective means of determining sequence variations and has been used to assess genetic diversity among *P. syringae* pv. tomato strains (12). DNA from the *P. syringae* pv. tomato and *P. syringae* pv. maculicola strains listed in Table 4 was digested with *Eco*RI, *Sst*I, *Bam*HI, or *Xho*I and subjected to Southern blot analysis. The hybridization probes used in this study were three fragments from pEC18, a pLAFR1 clone containing genes of the DC3000 coronatine gene cluster (Fig. 4) (27). When the DNA was digested with *Eco*RI or *Sst*I, the RFLP patterns were the same for all 10 strains. In earlier work, *Eco*RI-cut DNA from the coronatine producers *P. syringae* pv. morsprunorum PM567 and PM3714, pv. glycinea PG4180, and pv. atropurpurea PA1304 also had almost identical patterns when hybridized with three *Sst*I fragments from the coronatine gene cluster of *P. syringae* pv. tomato PT23.2 (4). The strains listed in Table 4 came from several different regions of the world and had isolation dates spanning nearly 25 years. Because the coronatine gene clusters of this diverse group of strains had many of the same restriction sites, the overall gene arrangement in this portion of the cluster appears to be highly conserved in the pv. tomato-maculicola group. However, significant differences between the chromosomally borne and plasmid-borne toxin gene clusters did appear when the three probes were hybridized to the *Xho*I and *Bam*HI digests (Table 4). The plasmid-borne genes had an extra *Xho*I site in the probe B region but lacked the *Xho*I site forming the right-hand border of probe A. They also had an extra *Bam*HI site approximately 1.5 kb from the right-hand border of probe A. There was no single pattern associated with plasmid-borne or with chromosomally borne toxin gene clusters when *Bam*HI digests were hybridized to probe C. Because this probe is located on the border of the coronatine cluster (1) and because the *Bam*HI fragments are relatively large, some polymorphism is to be expected. When Ullrich et al. hybridized labeled pSAY1,

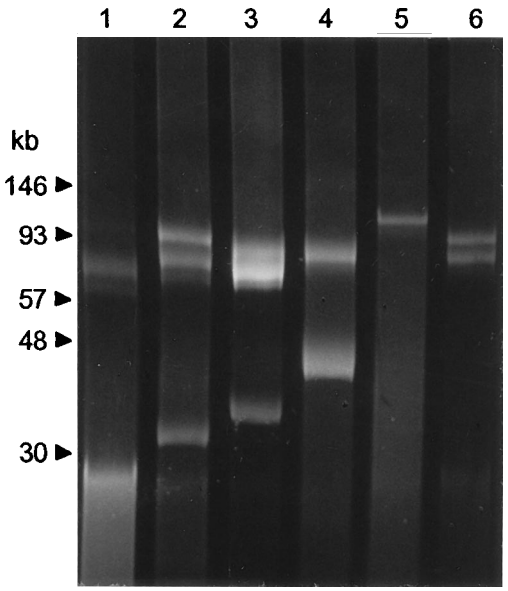

FIG. 3. Plasmid profiles of selected strains of *P. syringae* pv. tomato and *P. syringae* pv. maculicola. Plasmids purified by cesium chloride centrifugation were subjected to horizontal agarose gel electrophoresis in 0.45% agarose gels at approximately 3.3 V/cm for 19 h. Sizes and mobilities of five reference plasmids (pR40a [146 kb], pR1*drd*19 [93 kb], pRP1 [57 kb], pRK2013 [48 kb], and pGS9 [30 kb]) are indicated on the left. Lane 1, pv. tomato 1108; lane 2, pv. tomato DC84-1; lane 3, pv. tomato 487; lane 4, pv. maculicola 4981; lane 5, pv. tomato 3357; lane 6, pv. tomato DC89-4H.

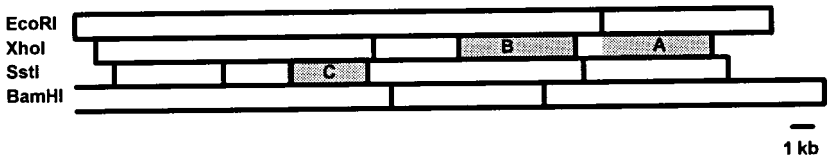

FIG. 4. Restriction map of the *P. syringae* pv. tomato DC3000 DNA cloned into pEC18. The various restriction fragments are represented by rectangular boxes, with the shaded areas denoting fragments used as probes in RFLP analysis of DNA from coronatine-producing strains of *P. syringae* pv. tomato and *P. syringae* pv. maculicola.

which contains 30 kb of the *P. syringae* pv. tomato PT23.2 coronatine gene cluster (4), to *Bam*HI digests of plasmid DNA from 13 field isolates of *P. syringae* pv. glycinea, they also noted variability in the size of the fragments flanking the cluster (39).

Comparison of pEC18 and pSAY10. Extensive genetic analysis of the plasmid-borne 33-kb coronatine gene cluster of *P. syringae* pv. glycinea PG4180 has been performed (1, 40, 42). *Eco*RI, *Bam*HI, *Sst*I, and *Xho*I digests of pEC18 and pSAY10, a pLAFR3 clone containing 30 kb of the PG4180 *cor* cluster, were hybridized with probes A, B, and C; the *Sst*I and *Xho*I digests also were probed with the 3.6-kb *Xho*I fragment to the left of probe B, the 2.9- and 4.4-kb *Sst*I fragments to the left of probe C, and the 8.8- and 6.2-kb *Sst*I fragments (no. 6 and 5) of pSAY10 (42). The RFLP patterns obtained from this analysis indicated that the two clones overlapped from the left border of probe C to the right border of the 6.9-kb *Eco*RI fragment of pEC18 (Table 5). The *Eco*RI, *Sst*I, *Bam*HI, and *Xho*I RFLP patterns of the PG4180 DNA in this overlap region were identical to those of *P. syringae* pv. tomato JL1035, PST26, and 3357 (Tables 4 and 5). The *cor* gene cluster of *P. syringae* pv. glycinea PG4180 extends from 2 kb to the left of probe C to 13.4 kb beyond the right border of probe A (1). Whether or not the DC3000 cluster has the same boundaries is unknown. The *Sst*I fragment adjacent to the left border of probe C in DC3000 is 2.9 kb, while the corresponding fragment in PG4180 is 5.1 kb (1). The existence of several strain-related differences in this region indicates that it may indeed be the boundary for many pv. tomato-maculicola coronatine gene clusters.

In summary, all group 1 strains of *P. syringae* pv. tomato, as well as 4981, the only coronatine-producing group 1 *P. syringae* pv. maculicola strain assayed, carried the coronatine gene cluster on a large (80- to 120-kb) plasmid. Conversely, the coronatine-producing group 2 *P. syringae* pv. maculicola and tomato strains had chromosome-borne toxin genes. RFLP analysis demonstrated that portions of the chromosomal *cor* gene cluster differ significantly from those of the plasmid-borne cluster. Coronatine is not the only phytobacterial virulence factor that is controlled by plasmid genes in some strains but not in others. *P. syringae* pv. savastanoi genes for indoleacetic acid production can be chromosomal or plasmid borne depending upon whether the strain came from olive or oleander plants (7). Likewise, the endocellulase gene of the tomato pathogen *Clavibacter michiganensis* subsp. *michiganensis* is present on a plasmid in strain NCPPB382 but appears to be chromosomal in other strains that have been screened (25). Although the other well-studied *P. syringae* toxins (tabtoxin, syringomycin, and phaseolotoxin) appear to be under chromosomal control, there is no evidence to rule out the possibility that one or more of the genes required for their synthesis may also reside on plasmids in some *P. syringae* strains (16). Why a bacterial virulence factor is encoded by a plasmid in some strains but not in others is unclear (8, 13). Because the copy numbers of some plasmids may increase in response to fluctuations in the environment, Coplin hypothesized that plasmid-carried genes may be more rapidly and highly expressed than those found on the chromosome (8). Differences in expression between plasmid-borne and chromosome-borne *cor* genes have not yet been reported. A plasmid location also may mean that a gene is more readily lost or gained during changes in environmental conditions and thus may be able to confer an increased fitness on the host bacteria (8). Genes whose products confer an advantage in all potential habitats of the bacterium tend to be chromosomal (13). Integration of plasmids or portions of plasmids into the chromosome has been described for a number of *Pseudomonas* species (20). The plasmid pMMC7105 is thought to be inserted into (and excised from) the *P. syringae* pv. phaseolicola chromosome by means of the

TABLE 5. RFLP analysis of the coronatine gene clusters of pEC18 and pSAY10[a]

Probe	Size(s) (kb) of fragment(s) hybridizing to probe[b]							
	pEC18				pSAY10			
	*Eco*RI	*Sst*I	*Bam*HI	*Xho*I	*Eco*RI	*Sst*I	*Bam*HI	*Xho*I
A	6.9	6.2	>21	5.8	6.9	6.2	8.5	14
B	22	8.8	7, >21	4.8	>21	8.8	7, 8.5	4.5, 0.3
C	22	3.2	>21	11.6	>21	3.2	4	>21
3.6 kb *Xho*I	ND	8.8	ND	3.6	ND	8.8	ND	3.6
2.9 kb *Sst*I	ND	2.9	ND	11.6	ND	NH	ND	NH
4.4 kb *Sst*I	ND	4.4	ND	11.6	ND	NH	ND	NH
8.8 kb *Sst*I	ND	4.8, 3.6	ND	8.8	ND	8.8	ND	4.5, 3.6, 0.3
6.2 kb *Sst*I	ND	6.2	ND	5.8, >21	ND	6.2	ND	14

[a] Plasmid DNA was digested with *Eco*RI, *Sst*I, *Bam*HI, or *Xho*I and then subjected to Southern blot analysis with ^{32}P-labeled hybridization probes derived from either pEC18 or pSAY10. The pEC18-derived probes were A, B, and C and the 3.6-kb *Xho*I and the 2.9- and 4.4-kb *Sst*I fragments, while the pSAY10 probes were the 8.8- and the 6.2-kb *Sst*I fragments (Fig. 4).

[b] ND, not determined; NH, no homology to the probe.

repetitive sequences RSI, RSII, and RSIII (37). These elements, which are found in a number of other *P. syringae* pathovars, including pv. tomato (31), may have had a role in the present-day arrangements and locations of the *cor* gene clusters of *P. syringae* pv. tomato and *P. syringae* pv. maculicola.

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