Detection and Identification of *Pseudomonas syringae* pv. *atropurpurea* by PCR Amplification of Specific Fragments from an Indigenous Plasmid

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ABSTRACT

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A highly sensitive and specific polymerase chain reaction (PCR) method was developed to detect pCOR1, a plasmid associated with pathogenicity to Italian ryegrass and synthesis of coronatine in *Pseudomonas syringae* pv. *atropurpurea*. Specific pCOR1 fragments were amplified from DNA extracts of *P. syringae* pv. *atropurpurea* with primers complementary to ends of fragments obtained from *Pst*I digestion of a 17-kb fragment, the largest from *Eco*RI digestion of pCOR1. One hundred twenty-seven bacterial strains, including 18 strains of *P. syringae* pv. *atropurpurea*, 7 Cor$^-$ mutants of *P. syringae* pv. *atropurpurea*, 52 strains of 38 different pathovars of *P. syringae*, 13 nonpathogenic strains of *P. syringae*, and 37 strains of different bacterial species, were tested by PCR. Though DNA sequences resembling parts of the 17-kb fragment were found in some strains of different pathovars of *P. syringae*, all the specific fragments of pCOR1 were amplified only from strains of *P. syringae* pv. *atropurpurea*. The results demonstrated that the distribution of pCOR1 is limited to strains of *P. syringae* pv. *atropurpurea*. It is suggested that pCOR1 may be the pathovar-specific plasmid and moreover that the confirmation of the presence of pCOR1 in the strain may enable a rapid and simple identification of *P. syringae* pv. *atropurpurea*.

Pseudomonas syringae pv. *atropurpurea*, the causal agent of halo blight of Italian ryegrass (*Lolium multiflorum* Lam.), produces the phytotoxin coronatine, the presence of which is related to symptom development (8,13). The coronatine-producing ability of *P. syringae* pv. *atropurpurea* strain NIAES1309 is associated with an 88-kb plasmid designated as pCOR1 (17,19). Strains without pCOR1 or with defective pCOR1 (60-kb) (obtained from NIAES1309 by treatment with acridine orange) (19) do not produce coronatine and are not pathogenic to Italian ryegrass. These strains do not induce any symptoms, though they multiply well in inoculated ryegrasses (16).

When pCOR1 was transferred into a strain lacking pCOR1, the strain became virulent (17), indicating that pCOR1 is related to the pathogenicity of the pathovar. All 32 strains of *P. syringae* pv. *atropurpurea* examined earlier (19) harbored a single plasmid with a similar size to that of pCOR1. It appeared that pCOR1 was distributed widely among the strains of *P. syringae* pv. *atropurpurea*.

Coronatine is also produced by several other pathovars of *P. syringae* (1,11,12), and epiphytic *Pseudomonas* sp. (18). Among these coronatine-producing bacteria that harbor plasmids with genes coding for coronatine biosynthesis, conserved DNA sequences were found in each plasmid including pCOR1 (2,3). Thus, a sensitive and reliable detection method is required to examine the distribution of pCOR1.

The polymerase chain reaction (PCR) method (14) has been used for the identification of some bacterial strains through the detection of strain-specific plasmids (4,7), because some indigenous plasmids provide a natural genetic marker (9). In this study, the sensitive and specific detection method for plasmid pCOR1 was established by using PCR amplification, and then the distribution of plasmid pCOR1 in bacteria was investigated, to enable a rapid and simple identification of *P. syringae* pv. *atropurpurea*.

MATERIALS AND METHODS

Bacterial strains and plasmids. Twenty-five strains of *P. syringae* pv. *atropurpurea* including 7 Cor$^-$ mutants of strain NIAES1309, 6 strains of *P. syringae* pv. *glycinea*, 1 strain of *P. syringae* pv. *maculicola*, and 13 strains of an unknown *P. syringae* pathovar isolated from Italian ryegrass (13) or from soybean (18) (Table 1) were analyzed for their pathogenicity, coronatine biosynthesis ability (13,19), and number of indigenous plasmids. Plasmids were isolated by the methods of Birnboim and Doly (5). Also, 45 strains of 36 pathovars of *P. syringae* (Table 2), 37 strains belonging to 24 diverse bacterial species (Table 3), and *Escherichia coli* HB101 with (clone No. A9) (20) or without a 17-kb fragment, the largest fragment from *Eco*RI-digests of pCOR1, inserted into pBR325, were analyzed by PCR amplification.

Subcloning and sequencing. Clone A9 of *E. coli* HB101 was grown in LB (Luria-Bertani: 1% bacto-tryptone, 0.5% bacto-yeast extract, and 1% NaCl) broth at 37°C with 10 µg of tetracycline and 50 µg of ampicillin per ml. Plasmid DNA was prepared by alkaline extraction (5) and purified on a sephadex-2B column. After agarose gel electrophoresis of *Eco*RI-digests of the plasmid, a 17-kb fragment was isolated by electroelution of the corresponding band. The eluted 17-kb *Eco*RI fragment was digested with the restriction endonuclease *Pst*I. The digested fragments were subcloned into Bluescript plasmid (Stratagene Cloning Systems, La Jolla, CA) in *E. coli* NM522. The clones were grown in LB broth at 37°C with 150 µg of ampicillin per ml. A one-step extraction method (6) was employed for the isolation of the plasmids from *E. coli* cells.

Extracted plasmids were digested with *Bam*HI, *Bst*XI, *Eco*RI, *Eco*RV, *Hin*cII, and *Pst*I (Toyobo Co., Ltd., Osaka, Japan) to determine the sizes and directions of inserted *Pst*I fragments in Bluescript plasmid. The DNA sequences of each end of the *Pst*I fragments were determined by the dideoxy chain termination with an automated DNA sequencer (370A; Applied Biosystems Inc., Foster City, CA), using fluorescent primers (Applied Biosystems) and *Taq* DNA polymerase (Promega Corporation, Madison, WI) (15).

PCR amplification. Based on the DNA sequence data, 11 primers (Fig. 1) were synthesized by using an automatic synthesizer (Model 394; Applied Biosystems) (Table 4).

Amplifications were performed in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). A 100-µl reaction mixture consisting of 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl, 0.01% gelatin (wt/vol), 0.1% Triton X-100 with 0. 2 mM each dNTP, 40 pmol of each primer, and

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1.25 U of *Taq* DNA polymerase (Promega) was mixed with the template. The PCR was cycled 30 to 50 times at 94°C for 1 min, at 55°C for 2 min, and at 72°C for 3 min. Each of the subcloned *Pst*I fragments, the 17-kb fragment of pCOR1, pCOR1, and plasmids isolated from *P. syringae* pathovars and epiphytic strains of *P. syringae* (Table 1) were used as template DNA. Template DNA from a bacterial suspension of NIAES1309 was also prepared by heat treatment: one loop from a 24-h culture grown on a slant containing LB broth and 1.5% bacto-agar was suspended in 100 µl of distilled water (approximately 10^9 CFU/ml), boiled for 5 min, and then 1/10 volume of the supernatant was subjected to PCR amplification. Heat treatment was used for 82 other bacterial strains (Tables 2 and 3).

For the direct detection of pCOR1 from infected leaves of Italian ryegrass on which the strain NIAES1309 had been inoculated, each lesion was dissected and macerated in 100 µl of water, and then allowed to stand for 15 min (10). One, 5, and 10 µl of the resultant suspension was used as template DNA in PCR amplification.

Five microliters of the PCR products was electrophoresed in 1% agarose gel in TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA, pH 8.0) and the gel was photographed after staining with ethidium bromide. To confirm the specificity of the PCR amplifications with each primer set, PCR products were cleaved by the enzymes shown in Figure 1, and were analyzed for restriction fragment length polymorphisms (RFLPs) on 1% agarose gels. Locations of *Pst*I fragments in the 17-kb *Eco*RI fragment of pCOR1 were also determined by the results of PCR amplifications by using combinations of each primer set (Fig. 1).

Hybridization. After electrophoresis, DNA was transferred to a nylon membrane (Hybond-N^+, Amersham) by semidry electroblotting (NA-1512, Nihon Eido, Toyko, Japan) at 160 mA for 30 min. Direct nucleic acid labeling and detection system through enhanced chemiluminescence (ECL, Amersham International, Bucks, U.K.) was used for the hybridization. The 17-kb *Eco*RI fragment and amplified fragment B (Fig. 1) were labeled as DNA probes with horseradish peroxidase.

RESULTS

Mapping of 17-kb *Eco*RI fragment of pCOR1. Restriction endonuclease cutting sites in the 17-kb *Eco*RI fragment of pCOR1 were mapped (Fig. 1). Based on the nucleotide sequence at the ends of the subcloned fragments (A, 0.6-kb; B, 1.6-kb; C, 2.0-kb; D, 3.6-kb; and E, 9.2-kb), 11 primers (P1 to P11) were prepared (Fig. 1 and Table 4). When primer sets P1-P2, P3-P4, P3-P6, P1-P4, P5-P8, or P7-P8 were used with pCOR1 as template, a single band of the predicted size was obtained with each primer set. However, primer set P9-P10 did not amplify DNA of pCOR1, but a band of the predicted size was amplified when the cloned fragment D was used as template. When fragment D was digested with *Eco*RI, it was cleaved into two fragments, designated as D1 and D2. These results indicate that fragments D1 and D2 are located on each end of the 17-kb fragment (Fig. 1) and that fragment D, which was a ligation product of D1 and D2 at their *Eco*RI cutting site, had inserted into pBluescript.

Sensitivity of PCR detection for pCOR1. To evaluate the sensitivity of the PCR method, fragment A was amplified by using primers P1 and P2 from varying amounts of pBluescript subcloned with fragment A. The electrophoresis of the PCR products showed the amplification of a fragment corresponding to fragment A even from 0.1 fg of the plasmid (equivalent to approximately 300 copies).

For further evaluation of the sensitivity, approximately 10^9 CFU of NIAES1309 per ml was serially diluted in 10-fold steps in 200 µl of water. Half of each diluted suspension was cultured on 1.5% bacto-agar

Table 1. Detection of pCOR1 in 45 strains of coronatine-producing or -nonproducing bacteria by polymerase chain reaction (PCR) amplification with pCOR1-specific primer sets

Bacterial strain[u]	Pathogenicity[v]	Coronatine biosynthesis[w]	No. of indigenous plasmids[x]	PCR signal with primers and RFLP pattern[y] P1-P2 (*Bst*XI)	P3-P4 (*Eco*RV)	P1-P4 (*Pst*I)	P5-P8 (*Hinc*II)	P7-P8 (*Hinc*II)	Strain source[z]
Pseudomonas syringae pv. *atropurpurea*									
1309	+	+	1 (pCOR1)	0. 6 (a)	1. 6 (a)	2.2 (a)	2. 0 (a)	2. 0 (a)	NIAES
#60	–	–	0	–	–	–	–	–	M. Sato
#59, #85, #86, #133, #311	–	–	1 (pCOR1-d)	–	–	–	–	–	M. Sato
301313	–	–	0	–	–	–	–	–	MAFF
301302, 301303, 301305, 301306	+	+	1	0.6 (a)	1.6 (a)	2.2 (a)	2.0 (a)	2.0 (a)	MAFF
301307, 301310, 301311, 301312	+	+	1	0.6 (a)	1.6 (a)	2.2 (a)	2.0 (a)	2.0 (a)	MAFF
1304	+	+	1	0.6 (a)	1.6 (a)	2.2 (a)	2.0 (a)	2.0 (a)	NIAES
N089-1	+	+	1	0.6 (a)	1.6 (a)	2.2 (a)	2.0 (a)	2.0 (a)	K. Nishiyama
301017 (PT), 301018, 301019	+	+	1	0.6 (a)	1.6 (a)	2.2 (a)	2.0 (a)	2.0 (a)	MAFF
M5, M16, M25, M33	+	+	1	0.6 (a)	1.6 (a)	2.2 (a)	2.0 (a)	2.0 (a)	H. Miyagawa
P. syringae from Italian ryegrass									
1308	–	–	1	–	1.6 (b)	–	–	–	NIAES
N016-1, N019-1, N020-1, N023-1	–	–	0	–	–	–	–	–	K. Nishiyama
N062-2, N063-1, N065-1, N067-1	–	–	0	–	–	–	–	–	K. Nishiyama
N068-1, N069-1	–	–	0	–	–	–	–	–	K. Nishiyama
P. syringae pv. *glycinea*									
KN35, KN41, KN44, KN45, KN130	+	+	4	–	1.6 (c)	–	–	2.0 (b)	K. Nishiyama
KN168	+	+	4	–	1.6 (c)	–	–	2.0 (b)	K. Nishiyama
P. syringae from soybean									
S-1, S-2	–	+	5	–	1.6 (c)	–	–	2.0 (b)	M. Sato
P. syringae pv. *maculicola*									
H330	+	+	4	–	1.6 (c)	–	–	–	M. Sato

[u] (PT): type strain of pathovar. #: deletion mutants obtained from strain NIAES1309 by acridine orange treatment. 301313: spontaneous mutant obtained from strain NIAES1309.

[v] Strains induced (+) or did not induce (–) symptoms on inoculated host plants.

[w] Result of bioassay with fragments of potato tuber (13,19). Coronatine-producing (+)and -nonproducing (–) strains.

[x] pCOR1-d: deficient pCOR1 (19). Number of plasmids was detected by electrophoresis in 0.7% agarose gel and staining with ethidium bromide.

[y] Restriction fragment length polymorphism pattern. Numbers indicate size (kb) of amplified fragment of PCR product with each primer set. (–) = no amplified fragment in PCR product. PCR products were digested with each restriction endonuclease (RE), then the digestion patterns on 1% agarose gels were compared with those of standard fragments of pCOR1, respectively. Same letters in parentheses in each column indicate identical digestion pattern with each RE.

[z] NIAES: National Institute of Agro-Environmental Sciences, Japan. MAFF: Ministry of Agriculture, Forestry and Fisheries, Japan.

in LB broth in petri dishes for 2 days at 30° C, then the number of CFU in each suspension was calculated from the numbers of colonies in each petri dish. The remaining diluted bacterial suspensions were boiled. A 10-µl supernatant of each boiled suspension was subjected to PCR directly with primers P1 and P2. Based on the electrophoresis of the PCR products, the fragment A of pCOR1 was amplified even from 1/10 volume of the suspension that formed one colony on the petri dish. The result indicated the PCR amplification detected pCOR1 from 0.1 to 1.0 CFU of NIAES 1309. Thus, heating of bacterial suspensions was a rapid and simple method for the extraction of template DNA.

Direct detection of pCOR1 from infected plant. Specific fragments of pCOR1 were amplified from only 1 µl of the macerated suspension (approximately 10^6 CFU/ml). When 5 or 10 µl of the suspension was added to the PCR mixture, no fragment was amplified with any of the primer sets, indicating that the sap of ryegrass contains some inhibitors to the PCR reaction.

Detection of pCOR1 from bacterial strains. Plasmids isolated from each of the 34 bacterial strains were subjected to PCR by using primer set P1-P2, P3-P4, P1-P4, P5-P8, or P7-P8 (Table 1). Eighteen strains of *P. syringae* pv. *atropurpurea* gave products with predicted size for the five primer sets. No PCR product was yielded by using primer sets with seven Cor⁻ mutants obtained from NIAES1309, of which five lacked pCOR1 (Table 1). When the primer set P3-P4 was used for the amplification, products of 1.6 kb corresponding to fragment B were obtained from the plasmids of 28 strains (Table 1 and Fig. 2A). When these PCR products were digested with *Eco*RV to compare their RFLPs, three different patterns were observed (Table 1). Three bands corresponding to *Eco*RV digests of fragment B were obtained for strains of *P. syringae* pv. *atropurpurea* (Fig. 2C), while two bands were obtained for other bacteria. When the primer set P7-P8 was used for the amplification, a product corresponding to fragment C was obtained from the plasmids of 26 of the strains tested (Table 1). When these PCR products were digested with *Hin*cII (Table 1), two bands were obtained for strains of *P. syringae* pv. *atropurpurea* but no cleaved band was obtained for the other bacteria.

Indigenous plasmids from 17 strains of *P. syringae* pv. *atropurpurea* showed the same size and RFLPs as pCOR1. Based on the results of the PCR and RFLPs of the PCR products, the plasmids from the strains of *P. syringae* pv. *atropurpurea* were identified as pCOR1. Furthermore, based on the results shown in Table 1, three primer sets, P1-P2, P1-P4, and P5-P8, seemed to be specific for pCOR1 detection. These primer sets were used for pCOR1 detection from 45 strains of 36 different pathovars of *P. syringae* (Table 2). Though it was not obvious that these strains harbored indigenous plasmids and that fragments in the PCR products were amplified from their plasmids, fragments with the same size as that of pCOR1 fragments were amplified from some strains. However, RFLPs of these fragments were different from those of pCOR1 fragments (Table 2). Based on these results, it was considered that the strains of *P. syringae* tested did not harbor a pCOR1 plasmid. Furthermore, 37 strains belonging to 24 bacterial species were tested with the three primer sets. No pCOR1 fragments were amplified from these strains (Table 3). Based on the results shown in Tables 1, 2, and 3, only strains of *P. syringae* pv. *atropurpurea* and clone A9 gave products of pCOR1 fragments, indicating that pCOR1 is limited to strains of *P. syringae* pv. *atropurpurea*.

Hybridization. pCOR1 and five plasmids extracted from the five deletion mutants were digested with *Eco*RI. Southern blotting of pCOR1 digests after agarose gel electrophoresis showed hybridization with the 17-kb fragment used as the probe. However, the 17-kb fragment did not hybridize with digests of the plasmids from the deletion mutants.

Table 2. Detection of pCOR1 in 36 pathovars of *Pseudomonas syringae* by polymerase chain reaction (PCR) amplification with pCOR1-specific primer sets

Bacterial species	Strain designation(s)[x]	PCR signal with primers and its RFLP pattern[y] P1-P2 (*Bst*XI)	P1-P4 (*Pst*I, *Eco*RV)	P5-P8 (*Hin*cII)	Strain source[z]
Pseudomonas syringae					
pv. *aceris*	302273 (PT)	–	1.9 (d) (d)	–	MAFF
pv. *aesculi*	302251 (PT)	–	–	–	MAFF
pv. *antirrhini*	302252 (PT)	–	–	–	MAFF
pv. *apii*	302274 (PT)	–	–	–	MAFF
pv. *aptata*	302253 (PT)	–	1.9 (d) (e)	–	MAFF
pv. *atrofaciens*	302254 (PT)	–	–	–	MAFF
pv. *berberidis*	302255 (PT)	–	–	–	MAFF
pv. *cannabina*	302256 (PT)	–	–	–	MAFF
pv. *ciccaronei*	302275	–	–	–	MAFF
pv. *coronafaciens*	302257 (PT)	0.6 (b)	2.2 (e) (c)	1.0 (d)	MAFF
	301016, 301061, 301314	–	–	1.9 (c)	MAFF
pv. *delphinii*	302258 (PT)	–	–	–	MAFF
pv. *dysoxyli*	302276 (PT)	–	–	–	MAFF
pv. *eriobotryae*	301062	–	–	2.0 (a)	MAFF
pv. *helianthi*	302261 (PT)	–	2.2 (c) (c)	–	MAFF
pv. *lachrymans*	302278 (PT)	–	–	–	MAFF
pv. *lapsa*	302263 (PT)	–	–	–	MAFF
pv. *mori*	S6801	–	–	2.0 (a)	M. Sato
	S6805, S8347, S8349-4	–	–	–	M. Sato
pv. *morsprunorum*	302280 (PT)	–	–	–	MAFF
pv. *panici*	302281 (PT)	–	1.9 (d) (d)	2.0 (a)	MAFF
pv. *papulans*	302265 (PT)	–	–	–	MAFF
pv. *passiflorae*	302266 (PT)	–	–	–	MAFF
pv. *perisicae*	302267 (PT)	–	1.9 (d) (d)	2.0 (a)	MAFF
pv. *phaseolicola*	Kuz1, Kuz2, Kuz3	–	–	–	M. Sato
pv. *philadelphi*	302268 (PT)	–	–	–	MAFF
pv. *pisi*	302269 (PT)	–	–	2.0 (a)	MAFF
pv. *primulae*	302294 (PT)	–	–	–	MAFF
pv. *ribicola*	302295 (PT)	–	–	2.0 (b)	MAFF
pv. *savastanoi*	302283 (PT)	–	2.2 (c) (c)	–	MAFF
pv. *sesami*	302284 (PT)	–	–	–	MAFF
pv. *striafaciens*	301032	–	–	–	MAFF
pv. *syringae*	302155 (T)	–	–	–	MAFF
pv. *tabaci*	301075	–	–	–	MAFF
	BR2	–	–	–	M. Sato
pv. *tagetis*	302271 (PT)	–	1.9 (b) (b)	–	MAFF
pv. *tomato*	302272 (PT)	–	–	–	MAFF
pv. *ulmi*	302286 (PT)	–	–	–	MAFF
pv. *viburni*	302287 (PT)	–	–	–	MAFF
Escherichia coli	HB101	–	–	–	M. Sato
	HB101/A9	0.6 (a)	2.2 (a) (a)	2.0 (a)	M. Sato

[x] (T): type strain of bacterial species. (PT): type strain of pathovar.

[y] Restriction fragment length polymorphism pattern. Numbers indicate size (kb) of amplified fragment of PCR product with each primer set. (–) = no amplified fragment in PCR product. Size of the fragments amplified from strain HB101/A9 corresponds to that of standard fragments of pCOR1. PCR products were digested by restriction endonucleases (RE) in each column, then the digestion patterns on 1% agarose gels were compared with those of standard fragments of pCOR1, respectively. Same letters in parentheses in each column indicate identical digestion pattern with each RE.

[z] MAFF: Ministry of Agriculture, Forestry and Fisheries, Japan.

Table 3. Detection of pCOR1 in 24 bacterial species by polymerase chain reaction (PCR) amplification with pCOR1-specific primer sets

Bacterial species	Strain designation(s)[x]	PCR signal with primers[y] P1-P2	P1-P4	P5-P8	Strain source[z]
Alcaligenes faecalis	1474	–	–	–	JCM
Bacillus cereus	2152	–	–	–	JCM
B. pumilus	2508	–	–	–	JCM
B. subtilis	1465	–	–	–	JCM
Bradyrhizobium japonicum	U110	–	–	–	T. Yokoyama
Clostridium butyricum	J1391	–	–	–	M. Sato
Erwinia ananas	301718, 301720, 301922	–	–	–	MAFF
	Mei7, TM2	–	–	–	M. Sato
E. herbicola pv. *milletiae*	301747	–	–	–	MAFF
E. herbicola	Mei3, DW1	–	–	–	M. Sato
Micrococcus sp.	Tob11	–	–	–	M. Sato
Pseudomonas andropogonis	301154	–	–	–	MAFF
P. avenae	K1, K2, K3	–	–	–	I. Matsuda
	H8514	–	–	–	I. Kadota
P. caryophylli	301192	–	–	–	MAFF
P. cichorii	NR7634	–	–	–	I. Matsuda
P. fluorescens	13525	–	–	1.9	ATCC
	LRB3W1	–	–	–	K. Tsuchiya
P. gladioli pv. *gladioli*	NR8810	–	–	–	I. Matsuda
P. glumae	NR8902	–	–	–	I. Matsuda
P. marginalis	SHIZU	–	–	–	I. Matsuda
P. plantarii	301723 (T)	–	–	–	MAFF
P. putida	39168	–	–	–	ATCC
	301684, 301685	–	–	–	MAFF
P. viridiflava	Ko8	–	–	–	M. Sato
Serratia marcescens	F-1-1	–	–	–	M. Sato
Staphylococcus sp.	Tob30	–	–	–	M. Sato
Xanthomonas campestris					
pv. *campestris*	301151	–	–	–	MAFF
pv. *citri*	301079	–	–	–	MAFF
pv. *oryzae*	301225	–	–	–	MAFF
Escherichia coli	HB101	–	–	–	M. Sato
	HB101/A9	0.6	2.2	2.0	M. Sato

[x] (T): type strain of bacterial species.

[y] Numbers indicate size (kb) of amplified fragment of PCR product with each primer set. (–) = no amplified fragment in PCR product. Size of the fragment amplified from HB101/A9 corresponds to the size of a standard fragment of pCOR1.

[z] ATCC: American Type Culture Collection, U.S. JCM: Japanese Collection of Microorganisms, Riken, Japan. MAFF: Ministry of Agriculture, Forestry and Fisheries, Japan.

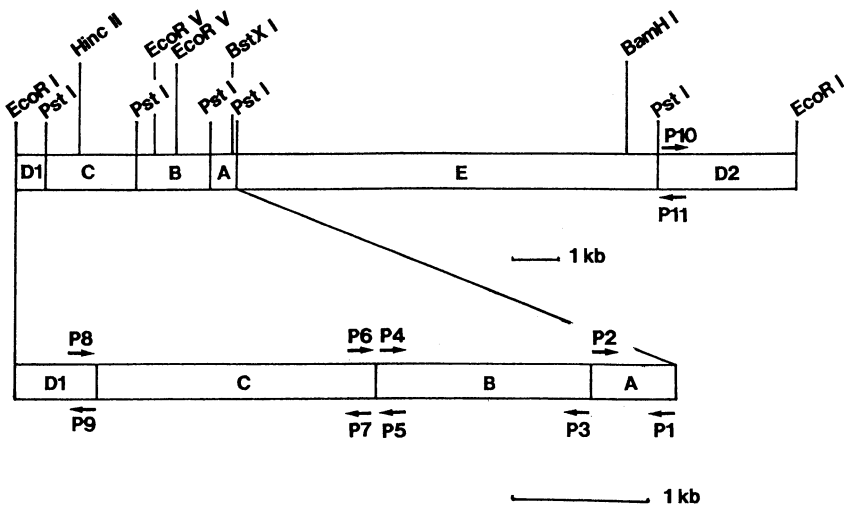

Fig. 1. Restriction enzyme map of 17-kb fragment of pCOR1 plasmid. P1 to P11 indicate locations of nucleotide sequences used as primers with directions (5′ to 3′) indicated by arrows.

When fragment B was used as the DNA probe, the probe hybridized with all the PCR products that were amplified with pCOR1 of NIAES1309 or plasmids of three other strains of *P. syringae* pv. *atropurpurea*, one strain of pv. *maculicola*, six strains of pv. *glycinea*, and two epiphytic strains of *P. syringae* with primer set P3-P4 (Fig. 2B). The probe also hybridized with *Eco*RV-digests of those PCR products.

DISCUSSION

The coronatine-producing ability of strain NIAES1309 is associated with pCOR1 (19), which harbors genes for coronatine biosynthesis. Based on the observation that defective pCOR1 (size approximately 60 kb) obtained from five deletion mutants lacking the ability to produce coronatine (19) did not act as template in PCR and did not hybridize with the 17-kb fragment of pCOR1, it may be concluded that the sequence corresponding to the 17-kb fragment was missing in those defective plasmids. As a result, it is suggested that the 17-kb fragment is involved in coronatine biosynthesis. This assumption is supported by the hybridization results of Bender et al. (2), who observed that a DNA probe of plasmid pPT23A from *P. syringae* pv. *tomato*, which is involved in coronatine production, hybridized with 17-kb and 8.1-kb *Eco*RI fragments of the indigenous plasmid (pCOR1) from our strain *P. syringae* pv. *atropurpurea* NIAES 1304. The results of PCR with primer sets P3-P4 and P7-P8 (Table 1 and Fig. 2A) and the results of hybridization shown in Figure 2B indicate that there are common DNA sequences between the 17-kb fragment of pCOR1 and other plasmids extracted from other coronatine-producing bacteria tested. Bereswill et al. (3) identified a *cfl* gene, which encodes the coupling of coronafacic acid and coronamic acid, as common DNA sequences between plasmids in coronatine-producing strains of *P. syringae* pv. *glycinea*, *P. syringae* pv. *atropurpurea*, and other three pathovars of *P. syringae*. The *cfl* gene was not present on the 17-kb fragment, though it was found on other fragments of pCOR1 (21).

Based on the results shown in Table 1, the indigenous plasmids of 18 strains of *P. syringae* pv. *atropurpurea* tested were identified as pCOR1, though they were collected not only from Italian ryegrass but also from perennial ryegrass, mountain brome grass, and smooth brome grass at different locations of six prefectures in Japan. Apparently, pCOR1 was limited to *P. syringae* pv. *atropurpurea* (Tables 1, 2, and 3; Fig. 2B). This observation suggests that pCOR1 is distributed in all the strains of *P. syringae* pv. *atropurpurea* at least in Japan, though further tests on strains collected from different locations outside of Japan are needed to obtain conclusive results on

Table 4. Nucleotide sequences of primers used in polymerase chain reaction and their location

Primer	Sequence	Corresponding location
P1	5′-GGGCTGCAGGAGAGTCCCAATGGA-3′	Fragment A
P2	5′-TTCCTGCAGAGCTATGGCCACTTG-3′	Fragment A
P3	5′-TTCCTGCAGCAAAGTCAGACCGCG-3′	Fragment B
P4	5′-GGGCTGCAGGCGCTTGGGTGCGCC-3′	Fragment B
P5	5′-TCCCCCACCGGCGCACCCAAGCG-3′	Fragment B
P6	5′-TTCTGAAGGTAATCATGTGTTGCT-3′	Fragment C
P7	5′-CTGCAGGAGCAACACATGATTACC-3′	Fragment C
P8	5′-GCGGGTCTTGCACGTACTTGGCGA-3′	Fragment D (D1)
P9	5′-AGAGGGTCGCCAAGTACGTGCAAGACCCGC-3′	Fragment D (D1)
P10	5′-AGGACCTCCTTGATTGCATCCGCTGTGCTT-3′	Fragment D (D2)
P11	5′-AAGCACAGCGGATGCAATCAAGGA-3′	Fragment D (D2)

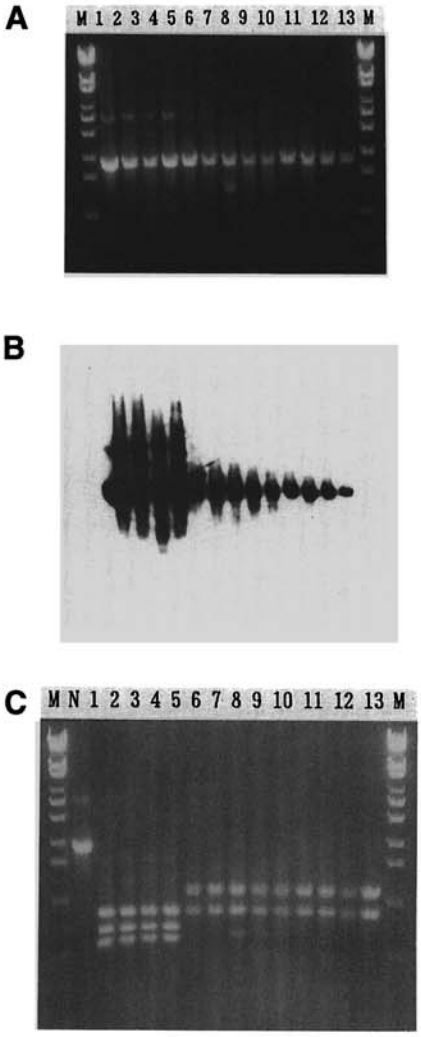

Fig. 2. Polymerase chain reaction (PCR) products with primers P3 and P4 were electrophoresed in 1% agarose gel and stained with ethidium bromide (**A**), then transferred to a nylon membrane for hybridization with the probe of fragment B (**B**). PCR products were digested with *Eco*RV, then electrophoresed (**C**). Template DNAs used were extracted from *Pseudomonas syringae* pv. *atropurpurea* NIAES1309, NIAES1304, NIAES1305 and NIAES1017 (lanes 1 to 4); *P. syringae* pv. *maculicola* H-330 (lane 5); *P. syringae* pv. *glycinea* KN35, KN41, KN44, KN45, KN130, and KN168 (lanes 6 to 11); and epiphytic bacteria S-1 and S-2 (lanes 12, 13). Lane M: *Eco*T14I-digested λ-phage as DNA marker. Lane N: nondigested PCR product from NIAES1309.

the horizontal distribution of pCOR1. PCR analysis showed that none of the 102 different strains tested harbored plasmid pCOR1 except for *P. syringae* pv. *atropurpurea* (Tables 1, 2, and 3). The distribution of pCOR1 restricted to *P. syringae* pv. *atropurpurea* and correlation with the pathogenicity of *P. syringae* pv. *atropurpurea* indicate that pCOR1 is specific to the pathovar and has been associated with the bacterial strains since they were differentiated as *P. syringae* pv. *atropurpurea*.

The bacterium *P. syringae* pv. *atropurpurea* easily lost pCOR1 when inoculated to tobacco or Italian ryegrass plants, though the bacterium hardly lost pCOR1 when subculturing in culture medium (16). The 11 strains isolated from diseased Italian ryegrass, which show the same properties as those of *P. syringae* pv. *atropurpurea* but lack pathogenicity and the ability to produce coronatine, did not harbor a whole pCOR1 (Table 1). We referred to these avirulent strains as "*Pseudomonas syringae* from Italian ryegrass" in this study, because a pathovar should be named after the respective *pathogenic* bacteria. We consider that these avirulent strains may become pathogenic strains when they acquire the pCOR1 plasmid. Consequently, the confirmation of the presence of pCOR1 in a strain will be an important criterion for rapid and simple identification of *P. syringae* pv. *atropurpurea*.

The PCR method used in these experiments was highly sensitive and enabled the detection and identification of the plasmid pCOR1 in bacteria. The PCR method allowed for the investigation of horizontal and vertical distributions of the pathogenicity-related plasmid pCOR1 in bacteria. Furthermore, the method is a useful tool to confirm plasmid transmission in nature. The method also could be used for the diagnosis of diseases caused by strains of *P. syringae* pv. *atropurpurea*.

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LITERATURE CITED

1. Bender, C. L., Stone, H. E., Sims, J. J., and Cooksey, D. A. 1987. Reduced pathogen fitness of *Pseudomonas syringae* pv. *tomato* Tn*5* mutants defective in coronatine production. Physiol. Mol. Plant Pathol. 30:273-283.
2. Bender, C. L., Young, S. A., and Mitchell, R. E. 1991. Conservation of plasmid DNA sequences in coronatine-producing pathovars of *Pseudomonas syringae*. Appl. Environ. Microbiol. 57:993-999.
3. Bereswill, S., Bugert, P., Volksch, B., Ullrich, M., Bender, C. L., and Geider, K. 1994. Identification and relatedness of coronatine-producing *Pseudomonas syringae* pathovars by PCR analysis and sequence determination of the amplification products. Appl. Environ. Microbiol. 60:2924-2930.
4. Bereswill, S., Pahl, A., Bellemann, P., Zeller, W., and Geider, K. 1992. Sensitive and species-specific detection of *Erwinia amylovora* by polymerase chain reaction analysis. Appl. Environ. Microbiol. 58:3522-3526.
5. Birnboim, H. C., and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513-1523.
6. Choudhury, K. 1991. One step 'miniprep' method for the isolation of plasmid DNA. Nucleic Acids Res. 19:2792.
7. Firrao, G., and Locci, R. 1994. Identification of *Clavibacter michiganensis* subsp. *sepedonicus* using the polymerase chain reaction. Can. J. Microbiol. 40:148-151.
8. Ichihara, A., Shiraishi, K., Sato, H., Sakamura, S., Nishiyama, K., Sakai, R. Furusaki, A., and Matsumoto, T. 1977. The structure of coronatine. J. Am. Chem. Soc. 99:636-637.
9. Lazo, G. R., and Gabriel, D. W. 1987. Conservation of plasmid DNA sequences and pathovar identification of strains of *Xanthomonas campestris*. Phytopathology 77:448-453.
10. Louws, F. J., Fulbright, D. W., Stephens, C. T., and de Bruijn, F. J. 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. Appl. Environ. Microbiol. 60:2286-2295.
11. Mitchell, R. E. 1982. Coronatine production by some phytopathogenic pseudomonads. Physiol. Plant Pathol. 20:83-89.
12. Mitchell, R. E., and Yong, H. 1978. Identification of a chlorosis-inducing toxin of *Pseudomonas glycinea* as coronatine. Phytochemistry 17:2028-2029.
13. Nishiyama, K. 1981. Studies of the pathogenicity-related substances in *Pseudomonas coronafaciens* var. *atropurpurea*. Bull. Natl. Inst. Agric. Sci. Ser. C. 35:1-55.
14. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R. G., Horn, G. T., Mullis, K. B., and Erlich, H. A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487-491.
15. Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463-5467.
16. Sato, M. 1986. High frequency exclusion of pCOR1 plasmid from *Pseudomonas syringae* pv. *atropurpurea* in plants. Ann. Phytopathol. Soc. Jpn. 52:343-346.
17. Sato, M. 1988. *In planta* transfer of the gene(s) for virulence between isolates of *Pseudomonas syringae* pv. *atropurpurea*. Ann. Phytopathol. Soc. Jpn. 54:20-24.
18. Sato, M., and Nishiyama, K. 1992. Epiphytic bacteria producing coronatine-like substance

and epiphytic bacteria having the ability of *in planta* plasmid-transfer. (Abstr.) Ann. Phytopathol. Soc. Jpn. 58:600.
19. Sato, M., Nishiyama, K., and Shirata, A. 1983. Involvement of plasmid DNA in the productivity of coronatine by *Pseudomonas syringae* pv. *atropurpurea*. Ann. Phytopathol. Soc. Jpn. 49:522-528.
20. Sato, M., Sato, Y., Nishiyama, K., and Sakai, F. 1989. Gene library of pCOR1, plasmid involved in coronatine biosynthesis in *Pseudomonas syringae* pv. *atropurpurea*. Ann. Phytopathol. Soc. Jpn. 55:653-656.
21. Sato, M., Takahashi, Y., and Zhu, Y. 1995. Location site of coronatine-biosynthetic gene regions *cfl* in pCOR1 plasmid of *Pseudomonas syringae* pv. *atropurpurea*. (Abstr.) Ann. Phytopathol. Soc. Jpn. 61:263.