Involvement of Plasmid DNA in the Productivity of Coronatine by *Pseudomonas syringae* pv. *atropurpurea*

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佐藤 守*・西山幸司**・白田 昭** : *Pseudomonas syringae* pv. *atropurpurea*
のコロナチン生産性とプラスミド DNA との関係

Abstract

Pseudomonas syringae pv. *atropurpurea*, a causal agent of halo blight of Italian ryegrass, produces coronatine (Cor) which is a toxin inducing halo blight lesions on the leaves. The relationship between Cor productivity and a specified plasmid DNA in this bacterium was studied. The plasmid of approximately 58 Mdal named as pCOR1 was detected in all of the 34 Cor⁺ wild strains tested including NIAS 1309. On the contrary, none of pCOR1 was detected in all of the Cor⁻ strains obtained as spontaneous mutants from NIAS 1309 or isolated from the host plants. Furthermore, all of the 38 Cor⁻ mutants obtained from NIAS 1309 by the treatment with acridine orange (75 to 200 ppm) simultaneously lost pCOR1 plasmid completely. Among them, however, 29 mutants still harboured a smaller plasmid (about 40 Mdal) seemed to be a defective pCOR1. These results strongly suggest that the plasmid pCOR1 codes Cor productivity of the bacterium.

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Key Words : *Pseudomonas syringae* pv. *atropurpurea*, coronatine, plasmid.

Introduction

Pseudomonas syringae pv. *atropurpurea* (Reddy and Godkin 1923) Young, Dye and Wilkie 1978 (abbrev. pv. *atropurpurea*) is a causal agent of halo blight of Italian ryegrass (*Loium multiflorum* Lam.)¹⁶⁾. The bacterium produces a physiologically active substance named as coronatine, known to be indispensable for developing symptoms of halo blight^{7,10,11,14)}. Coronatine (Cor) productivity of the bacterium is detectable by activity of inducing hypertrophic growth of potato tuber tissues after inoculation of the pathogen^{10,11)}. Junior author obtained some avirulent mutants from a virulent strain of pv. *atropurpurea* during its subculturing. Many avirulent strains were also isolated from diseased plants. He found that the bacteriological properties of these avirulent strains were much the same as those of the virulent strains of pv. *atropurpurea* except virulence¹²⁾. The phenomena mentioned above suggest that the gene(s) involved to Cor productivity might be encoded on the indigenous plasmid of the bacterium.

A definite evidence of plasmid-coded determinants of pathogenicity in plant pathogenic bacteria has been shown in *Agrobacterium tumefaciens*^{19,20)} and *Pseudomonas*

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syringae pv. *savastanoi*^{2,3)}. The existence of plasmid controlling pathogenicity has been suggested with *Pseudomonas syringae* pv. *syringae*⁵⁾ and some other bacteria. Since the coronatine productivity is indispensable for pathogenicity of pv. *atropurpurea*, we attempted to make clear whether the coronatine productivity of this bacterium is encoded on some plasmid or not.

Materials and Methods

Bacterial strains and plasmids. The main strains of pv. *atropurpurea* used in this experiment and their phenotype are shown in Table 1. All of the Cor⁺ strains of pv. *atropurpurea* are pathogenic, while Cor⁻ strains are non-pathogenic. Besides these strains, 32 of the virulent and 37 of the avirulent strains isolated from the diseased ryegrass leaves were also used (Table 3).

Some plasmids, such as RSF 1010 (5.5 Mdal), pBPW1 (30 Mdal), pBPW1::Tn 7 (39 Mdal) and pAS8Tc^rrepl::Tn 7 (52 Mdal) were used as markers for standard molecular weight. They were kindly supplied by Dr. N. J. Panopoulos^{15,17)}.

Detection of plasmids. Bacteria were grown overnight in liquid King's B (KB) medium⁸⁾ at 28°C and plasmid DNA was extracted according to the method described by Birnboim and Doly¹⁾. Plasmid DNA was analysed by electrophoresis at 18 mA and 100 volts in 0.7% agarose (Sigma, Type 1, Low EEO) gel with Tris-borate buffer (89 mM Tris base, 2.5 mM disodium EDTA and 8.9 mM boric acid). The gels were stained with ethidium bromide (1 µg/ml) and photographed under UV illumination after the electrophoresis. The molecular size of the plasmid was estimated in comparison with that of the standard plasmid DNA in the same gel.

Curing method. Curing test was performed by a modification of the technique described by Comai and Kosuge²⁾. Tubes containing 5 ml of KB broth supplemented with a specified concentration of acridine orange were inoculated with bacterial cells of log-phase to give a final concentration of 10⁶⁻⁷/ml. After the cultures were shaken at 28°C for 48 hr, 0.1 ml of the cell suspension was further subcultured in 5 ml of KB broth. After 24 or 48 hr of shake culture at 28°C, the bacteria were diluted appropriately in distilled water, 0.2 ml of the bacterial suspension was spread on solid KB, and the plates were incubated at 28°C for 48-72 hr. The colonies thus obtained were subcultured on KB slants or KB plates, and their Cor productivity was examined.

Table 1. Strains of *Pseudomonas syringae* pv. *atropurpurea* used in this experiment

Strains	Phenotype ^{a)}	Origin
NIAS 1304 ^{b)}	WT Cor ⁺	Diseased Italian ryegrass
NIAS 1308	WT Cor ⁻	ditto
NIAS 1309	WT Cor ⁺	ditto
NIAS 1313	Cor ⁻	Spontaneous mutant of NIAS 1309

a) WT: Wild type, Cor⁺: Positive in coronatine productivity, Cor⁻: Negative in coronatine productivity

b) List of cultures in the Plant Pathology Division of National Institute of Agricultural Sciences (Misc. Publ. Natl. Inst. Agric. Sci., Ser. C, No. 3, by Nishiyama *et al.*)

Bioassay of coronatine production. Cor production of the bacterium was assayed by the method described previously¹⁰⁾. Young bacterial culture was inoculated to the small pieces (about $2 \times 2 \times 0.6$ cm) of potato tuber placed on the moistened filter paper in a Petri dish. Cor production was checked by the hypertrophic growth of inoculated tuber pieces after 5 days incubation at 23 C. The test was repeated 3 times when negative reaction was observed.

Results

Relationship between coronatine productivity and an indigenous plasmid in pseudomonas syringae pv. atropurpurea

Relation between pathogenicity-related properties, such as Cor productivity and multiplication ability in Italian ryegrass, and the presence of a specified plasmid in *pv. atropurpurea* was studied. The experiments for detecting indigenous plasmids in four strains of *pv. atropurpurea* (Table 1) were carried out by the agarose gel electrophoresis. A plasmid of approximately 58 Mdal molecular weight was detected from two strains

Fig. 1. Agarose gel electrophoresis of plasmid DNA isolated from *Pseudomonas syringae* *pv. atropurpurea*.

A; NIAS 1304 (Cor⁺), B; NIAS 1308 (Cor⁻)
C; NIAS 1309 (Cor⁺), D; NIAS 1313 (Cor⁻)
Arrows show pCOR1 plasmid.

(virulent and Cor⁺), whereas both avirulent strains (Cor⁻) of NIAS 1313 (a spontaneous mutant from NIAS 1309) and NIAS 1308 (an isolate from the diseased plant) did not show the 58 Mdal plasmid DNA (Table 2 and Fig. 1). These results suggest that a close relationship exists between the 58 Mdal plasmid and Cor productivity of the bacterium. However, the plasmid did not show any relationship with the ability of multiplication in the host plant. The plasmid was, therefore, named as pCOR1 here.

Further experiments were performed using 32 virulent strains and 37 avirulent strains of *pv. atropurpurea* isolated from halo blight lesions on the leaves of Italian ryegrass. As the results, plasmid DNA of about 58 Mdal corresponding to pCOR1 was detected from all virulent isolates of *pv. atropurpurea* tested, whereas it was

Table 2. Relationship between the properties related pathogenicity on Italian ryegrass and existence of pCOR1 plasmid in *Pseudomonas syringae* *pv. atropurpurea*

Strains	Coronatine production	Multiplication ability	Pathogenicity	Existence of pCOR1
NIAS 1304	+	+	+	+
NIAS 1308	-	+	-	-
NIAS 1309	+	+	+	+
NIAS 1313	-	+	-	-

Table 3. Detection of pCOR1 plasmid in the virulent and avirulent strains of *Pseudomonas syringae* pv. *atropurpurea*

pv. <i>atropurpurea</i> strain	Coronatine production	No. of strains tested (A)	No. of pCOR1 ⁺ strains (B)	B/A(%)
Virulent strain	+	32	32	32/32(100%)
Avirulent strain	—	37	0	0/37(0%)

not detected in all avirulent strains (Table 3).

Curing of coronatine productivity in *Pseudomonas syringae* pv. *atropurpurea* by acridine orange

Young culture of pv. *atropurpurea* NIAS 1309 was treated with various concentrations of acridine orange (AO). Then, many colonies obtained were screened to search for Cor⁻ mutants by the bioassay of coronatine production. As shown in Table 4 and Fig. 2, Cor⁻ mutants were obtained by the AO treatment at a concentration higher than 100 ppm. The frequency of the mutation was especially high (10.7%) when the bacteria were treated at 200 ppm. The rough colony mutants were also obtained at a frequency ranging from 4.4 to 18.8% by the AO treatment.

Detection of pCOR1 from the 38 isolates of Cor⁻ mutant was performed by the agarose gel electrophoresis. As shown in Table 5 and Fig. 3, the plasmid DNA of 58 Mdal was not found from any of the Cor⁻ isolates. Twenty nine out of 38 isolates, however, still harboured a plasmid of approximately 40 Mdal, suggesting that DNA fragment of 18 Mdal was deleted from pCOR1 (Fig. 2). On the contrary, all of the 182 Cor⁺ isolates including 8 rough colony type mutants treated with AO (75 to 200

Table 4. Curing of coronatine productivity in *Pseudomonas syringae* pv. *atropurpurea* NIAS 1309 by treatment with acridine orange (AO)

Concentration of AO (μ g/ml)	No. of colonies		Curing frequency (%)
	Screened	Cor ⁻	
75	31	0	0
100	400	1	0.25
150	336	1	0.30
200	336	36	10.7
Total	1103	38	3.4

Table 5. Detection of pCOR1 plasmid in the coronatine producing wild types and non-producing mutants isolated from *Pseudomonas syringae* pv. *atropurpurea* NIAS 1309 treated with acridine orange

Coronatine production	No. of isolates		Frequency of pCOR1 ⁺ isolates (%)
	Screened	pCOR1 ⁺ (pCOR1-d ^{a)})	
—	38	0 (29)	0
+	182 ^{b)}	182 (0)	100

a) The plasmid of about 40 Mdal (probably a deficient pCOR1).

b) Eight isolates of rough colony mutant were included.

Fig. 2. Bioassay of coronatine production using the pieces of potato tuber.
A, B; Wild type strain NIAS 1309
C; Cor^- mutant of NIAS 1309 by treatment with acridine orange (100 ppm)

Fig. 3. Agarose gel electrophoretic analysis of plasmid DNA from wild type strain NIAS 1309 (A) and its Cor^- mutants (B, C^a, D^a) obtained by treatment with acridine orange.

a) Molecular size of plasmid was approximately 40 Mdal. Arrows show pCORI plasmid.

ppm) still contained a whole pCORI plasmid. Slight change in colony morphology was observed in all of the cured Cor^- isolates after 2 to 3 days when they were subcultured.

Discussion

Cor productivity is an indispensable factor for the exhibition of halo blight lesions on the leaves by pv. *atropurpurea*^{7,10,11,14}. We concluded that Cor productivity was controlled by pCORI plasmid from the following reasons; 1) pCORI was always detected from all virulent (Cor^+) strains of pv. *atropurpurea*, 2) the plasmid was not detectable from all Cor^- mutants which lost Cor productivity spontaneously or by the treatment with AO, 3) it was also not detectable from avirulent strains of pv. *atropurpurea* isolated from Italian ryegrass.

A total of 29 isolates among 38 Cor^- mutants obtained by the AO treatment contained a plasmid of about 40 Mdal, which was suspected as a deficient pCORI. Although it is suggested that Cor^+ gene(s) is encoded on the DNA fragment deleted from pCORI, further studies including restriction digestion analysis of pCORI or deleted DNA will be required in order to prove the hypothesis.

It has been reported that Cor is produced by various pathovars belonging to *Pseudomonas syringae* such as pv. *glycinea*⁹, pv. *maculicola*^{10,11}, pv. *morsprunorum*^{10,11}. Some strains of these pathovars contain the same plasmid in molecular size as pCORI (Sato *et al.*, unpublished data), suggesting that the Cor productivity in the pathovars other than pv. *atropurpurea* is also coded on the plasmid pCORI.

Avirulent strains which lost both of the Cor productivity and pCORI still maintain the ability of multiplication in the host plant. This fact suggests that genes concerning "multiplication" were not coded on pCORI but probably on chromosome.

In this study, a slight alteration of colony morphology was induced by curing pCORI.

Similar phenomenon was observed in *Pseudomonas syringae* pv. *phaseolicola* when the indigenous plasmid of the bacterium was cured⁴⁾. Certain cell surface components^{5,6)} and periplasmic protein¹⁶⁾ have also been shown to be encoded by plasmid-borne genes in some phytopathogenic bacteria. Accordingly, besides Cor productivity, the synthesis of some kinds of protein related to colony morphology might also be encoded by pCOR1 in pv. *atropurpurea*.

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和文摘要

Pseudomonas syringae pv. *atropurpurea* のコロナチン
生産性とプラスミド DNA との関係

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ライグラス類かさ枯病細菌 *Pseudomonas syringae* pv. *atropurpurea* の病原性発現には、本菌のコロナチン (Cor) 生産が不可欠である。この Cor 生産性と特定のプラスミドとの関係を調べた。野生株 NIAS 1309 を含む34株の Cor 生産株 (Cor⁺) からプラスミドの検出を行った結果、すべての菌株から約 58 Mdal のプラスミド (pCORI と命名) が検出された。一方 NIAS 1309 から得られた Cor⁻ 変異株および多数の野外分離菌の Cor⁻ 株からは、pCORI が検出されなかった。さらに、NIAS 1309 をアクリジン処理をし、得られた Cor⁻ 変異株38株は、いずれも同時に pCORI を失っていたが、それらのうち29菌株は pCORI の一部の DNA が欠失したと思われる約 40 Mdal のプラスミドを所持していた。以上の結果から、本菌のコロナチン生産性はプラスミド支配による可能性が極めて強いものと結論した。