

Pseudomonas syringae pv. atropurpurea におけるpCOR1プラスミドの植物内高頻度離脱

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High Frequency Exclusion of pCOR1 Plasmid from *Pseudomonas syringae* pv. *atropurpurea* in Plants

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Key words : *Pseudomonas syringae* pv. *atropurpurea*, plasmid, curing, phytotoxin.

Pseudomonas syringae pv. *atropurpurea* (abbrev. pv. *atropurpurea*) is the causal agent of halo blight of Italian ryegrass (*Lolium multiflorum* Lam). The bacterium produces a phytotoxin designated as coronatine responsible for developing symptoms of halo blight^{1,2)}. Coronatine producing ability of the bacterium is associated with 58 Mdal plasmid named pCOR1³⁾. Here, we report the experimental result that pCOR1 plasmid is excluded (curing) from the host bacterium pv. *atropurpurea* at a high frequency, accompanying with loss of bacterial virulence, when the bacterium was inoculated to the plants of tobacco or Italian ryegrass.

Bacterial strains used in this experiment were pv. *atropurpurea* NIAES 1309 wild type (furnished from Dr. K. Nishiyama, Natl. Inst. of Agro-Environmental Sciences) and NIAES 1309 (pBPW1::Tn7) obtained as transconjugants by mating with NIAES 1309 and bean halo strain *P. syringae* pv. *tabaci* BR2⁴⁾ (furnished from Dr. N. J. Panopoulos, Univ. of California, Berkeley, USA) harbouring a conjugative plasmid pBPW1::Tn7⁵⁾. Bacteria grown overnight in King's B medium⁶⁾ were inoculated to leaves of tobacco plant (*Nicotiana glauca* L. cv. Xanthi nc.), Italian ryegrass (cv. Mammoth A) and mulberry (*Morus bombycis* Koidz cv. Ichinose) by single prick method. One μ l of the bacterial suspension (10^4 to 10^5 colony forming unit) was placed on each needle puncture. Inoculated plants were kept in greenhouse at around 25 C for 7 days. Symptom was developed on the leaves of Italian ryegrass, while it did not on the leaves of tobacco and mulberry, non-host plants, even after 7 days. Six inoculated punctures were scissored from each plant, surface sterilized with 0.6% Antiformin (sodium hypochlorite solution), and homogenized in a small volume of sterile water. Viable bacteria in the homogenates were counted by the serial dilution plate method on King's B medium. Relative multiplication rate of bacteria in each plant was expressed by the following formula: Number of bacterial cells

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1) Nishiyama, K. (1981). Bull. Natl. Inst. Agr. Sci. Ser. C. 35 : 1-55. 2) Ichihara, A., Shiraishi, K., Sato, H., Sakamura, S., Nishiyama, K., Sakai, R., Furusaki, A. and Matsumoto, T. (1977). J. Am. Chem. Soc. 99 : 636-637. 3) Sato, M., Nishiyama, K., and Shirata, A. (1983). Ann. Phytopath. Soc. Japan 49 : 522-528. 4) Ribeiro, R. de L. D., Hagedorn, D. J., Durbin, R. D., and Uchytel, T. F. (1979). Phytopathology 69 : 208-212. 5) Sato, M., Nishiyama, K., and Panopoulos, N. J. (1985). Ann. Phytopath. Soc. Japan. 51 : 426-434. 6) King, E. O., Ward, M. K. and Raney, D. E. (1954). J. Lab. Clin. Med. 44 : 301-307.

estimated at 7 days after inoculation/ Number of bacterial cells inoculated. Experiment was repeated several times.

Single colony isolation was carried out for each isolate obtained from diseased plants. Coronatine productivity of these isolates was examined by the potato tuber assay described by Nishiyama¹⁾. The test was repeated 3 times when negative reaction was observed. As shown in Table 1, coronatine minus (Cor^-) mutants were obtained at high frequencies of 6.7% and 19.9% from Italian ryegrass and tobacco plant, respectively, inoculated with NIAES 1309 wild type strain. A higher frequency of appearance of Cor^- mutants was observed in the progenies of NIAES 1309 (pBPW1::Tn7) isolated from these plants, suggesting pBPW1::Tn7 plasmid enhance to exclude pCOR1 plasmid to some extent. However when both strains were separately inoculated to mulberry leaves, their progenies lost the coronatine productivity at low frequencies (1.3% to 2.3%). In contrast, all of the progenies of NIAES 1309 isolated from hypertrophic tissues of infected potato tuber and those subcultured on King's B plates, tested as controls, were still capable of producing coronatine.

Thirty five isolates of Cor^- mutants thus obtained were picked up at random and used for testing plasmid content. Plasmid isolation was performed according to the procedures described by Birnboim and Doly⁷⁾. Plasmid DNA was analyzed by electrophoresis at 18 mA and 100 volts in 0.8 % agarose gel with Tris-borate buffer described previously³⁾. As a result, none of Cor^- mutants tested harboured pCOR1 plasmid. In the case of Cor^- mutants originated from NIAES 1309 (pBPW1::Tn7), the pBPW1::Tn7 plasmid alone was always detected as an autonomous replicon (Fig.1). The pBPW1::Tn7 in Cor^- mutants was further confirmed by endonuclease Hind III digestion (data not shown). In contrast, all of Cor^+ progenies of both strains always harboured pCOR1 plasmid in agreement with previous studies³⁾. Since no bacteria was isolated from uninoculated plants, it was considered that Cor^- mutants were not originated from contaminated bacteria.

Nishiyama and Ezuka have described that avirulent strains of *pv. atropurpurea* collected from fields or those obtained during subculture still maintained their ability of multiplication in Italian ryegrass⁸⁾. Similar results were obtained in the present study

Table 1. Exclusion frequency of pCOR1 plasmid in *Pseudomonas syringae* pv. *atropurpurea* NIAES 1309 in plants

Inoculated plants	Bacterial strain	
	NIAES 1309 wild type	NIAES 1309 (pBPW1::Tn7)
Italian ryegrass (leaves)	8/119 (6.7%) ^{a)}	26/108 (24.1%)
Tobacco plant (leaves)	39/196 (19.9%)	39/127 (30.7%)
Mulberry (leaves)	1/75 (1.3%)	2/ 87 (2.3%)
Potato (tubers)	0/125 (0%)	Not tested
King's B medium	0/100 (0%)	0/ 84 (0%)

a) No. of pCOR1⁻isolates/No. of isolates tested.

7) Birnboim, H. C. and Doly, J. (1979). Nucl. Acids Res. 7 : 1513-1523.

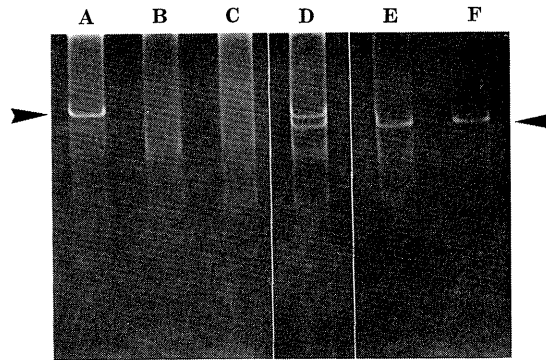


Fig. 1. Agarose gel electrophoresis of plasmid DNA in coronatine minus mutants (Cor^-) isolated from plants infected with *Pseudomonas syringae* pv. *atropurpurea* NIAES 1309 wild type (WT) or NIAES 1309 (pBPW1::Tn7). Lane A; 1309 WT, B; Cor^- from WT-infected tobacco, C; Cor^- from WT-infected Italian ryegrass ($\text{Cor}^- \#33$), D; 1309 (pBPW1::Tn 7), E; Cor^- from 1309 (pBPW1::Tn7) -infected tobacco, F; Cor^- from 1309 (pBPW1::Tn7)-infected Italian ryegrass. Upper band (the left arrow) is pCOR1 plasmid (58 Mdal) and lower one (the right arrow) is pBPW1::Tn7 plasmid (39 Mdal).

using one of the Cor^- mutants designated as $\text{Cor}^- \#33$, isolated from Italian ryegrass leaves inoculated with NIAES 1309 wild type. Although the strain was not capable of inducing any symptom on Italian ryegrass, it multiplied well in the leaves indicating the values (6.7–20.4) of relative multiplication index higher than those (3.3–14.5) of NIAES 1309 wild type at 7 days after inoculation. The values of multiplication index of NIAES 1309 wild type in tobacco plants and in mulberry leaves were much smaller as compared to that in Italian ryegrass, showing 0.1 to 0.4 and 0.001 to 0.003, respectively (Table 2). These facts indicate that the population of pv. *atropurpurea* is maintained in tobacco leaves but decreased drastically in mulberry leaves for one week after inoculation. A low frequency of plasmid exclusion in mulberry leaves may be attributed to this phenomenon. From the foregoing results, it is considered that the exclusion of pCOR1 plasmid in this bacterium, resulting loss of coronatine productivity, was induced by some products of such plants as tobacco and Italian ryegrass. In our preliminary data, the plasmid exclusion also occurred by co-cultivation of bacteria and growing tobacco plant cells derived from protoplasts, suggesting that plant cells produce some inducers.

The present study further supports our previous results³⁾ related on the plasmid control of coronatine productivity in pv. *atropurpurea*. In the field, avirulent strains of pv. *atropurpurea* are sometimes isolated from Italian ryegrass leaves^{1,8)}. And none of these avirulent strains harbours pCOR1 plasmid³⁾. It seems likely that the virulence loss by pCOR1 exclusion is a common phenomenon in this bacterium in the field and these avirulent mutants may survive on plant surface as a plant epiphyte.

8) Nishiyama, K. and Ezuka, A. (1979). Ann. Phytopath. Soc. Japan. 45: 566 (Abstr.).

Table 2. Multiplication of *Pseudomonas syringae* pv. *atropurpurea* NIAES 1309 and its coronatine minus (Cor⁻) mutants in some plants and their pathogenicity

Bacterial strain	Plant	Relative multiplication index ^{a)} (average)	Symptom
NIAES 1309	Ryegrass	3.3-14.5 (7.8)	Necrotic spots with halo
	Tobacco	0.1- 0.4 (0.3)	No symptom
	Mulberry	0.001-0.003 (0.0026)	No symptom
Cor-#33	Ryegrass	6.7-20.4 (13.6)	No symptom

a) Expressed in the text.

Recently, it has been reported that some chemically uncharacterized plant products regulate the virulence or survival of plant pathogenic bacteria by stimulating its DNA. For instance, some plant products induced the multiplication of prophage accompanied with bacterial lysis in *Pseudomonas syringae* pv. *mori*^{9,10)}, activated the virulence promoter of Ti plasmid in *Agrobacterium tumefaciens*¹¹⁾, and produced pectin lyase in *Erwinia* spp.¹²⁾. The present data, which seem to be the first evidence for exclusion of pathogenicity-related plasmid in plant pathogenic pseudomonads, also may be an example of "virulence regulation" in plant pathogenic bacteria by plant products.

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和 文 摘 要

佐藤 守: *Pseudomonas syringae* pv. *atropurpurea* における pCOR1 プラスミドの植物内高頻度離脱

Pseudomonas syringae pv. *atropurpurea* NIAES 1309 株をイタリアンライグラスあるいはタバコ葉に接種し、1週間後に再分離すると、pCOR1 プラスミドを離脱し、コロナチン生産能を喪失した菌株が高頻度で得られた。植物体中にプラスミド離脱の誘導物質の存在が推定された。pCOR1 を除去された菌株は、イタリアンライグラス葉に病徴を示さないが、組織内では野生型と同様によく増殖した。

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