

Pseudomonas syringae pv. atropurpureaのコロナチン生合成に関わるpCOR1プラスミドDNAのジーンライブラリー

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Gene Library of pCOR1, Plasmid Involved in Coronatine Biosynthesis in *Pseudomonas* *syringae* pv. *atropurpurea*

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Key words: *Pseudomonas syringae* pv. *atropurpurea*, gene library, plasmid, coronatine.

Pseudomonas syringae pv. *atropurpurea*, the causal agent of the halo blight of Italian ryegrass (*Lolium multiflorum* Lam), produces coronatine which is essential for developing the symptoms^{1,2}). It has been proved that coronatine producing ability of the bacterium is associated with 58 Mdal plasmid, pCOR1. That is, all of pCOR1-cured strains of pv. *atropurpurea* lost their coronatine producing ability^{3,4}) and the cured strains restore coronatine production by re-introducing pCOR1 plasmid⁵). It has been reported that coronatine is also produced by other several *Pseudomonas syringae* pathovars including pv. *tomato*⁶), pv. *glycinea*⁷), pv. *mor-sprunorum*⁸) and pv. *maculicola*¹). Recently, Bender *et al.* (1989) reported that the plasmid pRT23A is involved in coronatine production in *P. syringae* pv. *tomato* and the plasmid DNA flanked Tn5 insertions which abolished coronatine production were isolated⁹). The DNA probe of two fragments from pRT23A hybridized strongly to pCOR1 plasmid of our strain pv. *atropurpurea* NIAES 1304, but not to total DNA of some *P. syringae* pathovars that do not produce coronatine (Bender, C.L., personal communication). It has been suggested that coronatine production in strains of *P. syringae* pv. *glycinea* is also associated with their indigenous plasmids¹⁰).

Thus, molecular genetics of coronatine production in *P. syringae* pathovars will open up evolution of pathogenicity. However, the genes involved in biosynthesis of coronatine has not been isolated from any pathovars of *P. syringae*. Here, we cloned the DNA fragments of pCOR1 with different restriction enzymes into pBR322 and pBR325 in *E. coli* and investigated their coronatine producing ability.

Restriction endonuclease digestion of pCOR1. pCOR1 plasmid of *P. syringae* pv. *atropurpurea* NIAES 1309 was isolated by the method of Birnboim and Doly (1979)¹¹) and

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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) Sato, M. (1986). *Ibid.* 52: 343-346. 5) Sato, M. (1988). *Ibid.* 54: 20-24. 6) Mitchell, R.E., Hale, C.N. and Shanks, J.C. (1983). Physiol. Plant Pathol. 23: 315-322. 7) Mitchell, R.E. and Young, H. (1978). Phytochemistry 17: 2028-2029. 8) Mitchell, R.E. (1982). Physiol. Plant Pathol. 20: 83-89. 9) Bender, C.L., Malvick, D.K. and Mitchell, R.E. (1989). J. Bacteriol. 177: 807-812. 10) Willis, J.W. and Leary, J.V. (1984). Phytopathology 74: 838. 11) Birnboim, H.C. and Doly, J. (1979). Nucleic Acids Res. 7: 1513-1523.

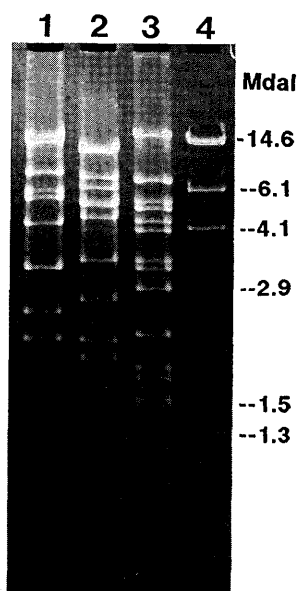


Fig. 1. Agarose gel electrophoresis after digestion of pCOR1 plasmid by restriction enzymes. Lane 1: *Bam*HI digestion, lane 2: *Eco*RI digestion, lane 3: *Hind*III digestion, lane 4: *Hind*III digestion of λ DNA. Molecular weight of each fragment is following: *Bam*HI; B1 (14.5 Mdal), B2 (13.0), B3 (7.5), B4 (5.8), B5 (5.4), B6 (4.4), B7 (3.2), B8 (2.4), B9 (1.9), B10 (1.3). *Eco*RI; E1 (11.3), E2 (7.3), E3 (6.5), E4 (5.6), E5 (5.4), E6 (4.5), E7 (3.5), E8 (2.8), E9 (2.2), E10 (2.0), E11 (1.8), E12 (1.6), E13 (1.3), E14 (1.1), E15 (0.9). *Hind*III; H1 (13.0), H2 (7.0), H3 (5.5), H4 (5.2), H5 (4.3), H6 (4.0), H7 (3.4), H8 (3.3), H9 (2.8), H10 (2.2), H11 (1.8), H12 (1.7), H13 (1.5), H14 (1.4), H15 (1.1), H16 (1.0).

purified by CsCl-ethidium bromide density gradient centrifugation. Purified plasmid DNA was digested with the restriction enzyme such as *Eco*RI, *Hind*III, *Bam*HI and *Sal*I. Digested DNAs were subjected to agarose gel electrophoresis (Fig. 1). The average sizes of digested DNA fragments were estimated by comparison with those of phage λ by *Eco*RI and *Hind*III digestion. Tests were repeated 5 times. DNA fragments obtained by *Eco*RI, *Bam*HI, *Hind*III and *Sal*I were designated E1–E15, B1–B10, H1–H10 and S1–S15, respectively. A total molecular weight (MW) of these fragments was about 58 Mdal (MW of each fragment except *Sal*I digestion is indicated in Fig. 1). *Sal*I fragments are following: S1 (11.2 Mdal), S2 (7.9), S3 (7.6), S4 (5.8), S5 (5.3), S6 (4.0), S7 (3.3), S8 (2.5), S9 (2.0), S10 (1.8), S11 (1.6), S12 (1.5), S13 (1.5), S14 (1.1), S15 (1.0).

Cloning of DNA fragments from pCOR1 plasmid in *E. coli*. About 600 ng of *Eco*RI- and *Bam*HI-digested pCOR1 DNA and ca. 200 ng of *Eco*RI-digested pBR325 and *Bam*HI-digested pBR322 were ligated with T4 ligase after treatment of the vectors with alkaline phosphatase. Transformation was performed according to Mandel and Higa (1970)¹²⁾. *E. coli* HB101 was used as recipient cells. In the case of ligation mixture, *Eco*RI-digested pCOR1 and pBR325, transformed cells were selected for tetracycline (10 μ g/ml). About 90% of 200 transformants obtained were resistant to tetracycline and ampicillin (50 μ g/ml) which indicates the insertion of pCOR1 DNA fragments into *Eco*RI site of pBR325. In the case of ligation mixture of *Bam*HI-digested pCOR1 and pBR322, ampicillin-resistant and tetracycline-sensitive (insert site) clones were selected. About 100 clones were isolated.

Plasmid DNAs in *E. coli* transformants were isolated by the method of Holmes and Quigley (1981)¹³. Then, plasmid DNAs digested by *Eco*RI or *Bam*HI were subjected to agarose gel electrophoresis. As shown in Fig. 2, most DNA fragments of pCOR1 digested by *Eco*RI, except E9 (2.2 Mdal) and E12 (1.6 Mdal), were cloned into pBR325 in *E. coli* HB101. Some of these clones, such as clone numbers of A'55, A'53, A'16, A21, A10 (Fig. 2), contained the recombinant plasmid inserted plural fragments due to partial digestion of pCOR1. On the other hand, most DNA fragments of pCOR1 digested by *Bam*HI, except B8 fragment (2.4 Mdal), were cloned into pBR322.

Assay of coronatine productivity in *E. coli* transformants. The presence of coronatine producing ability of these *E. coli* transformants was investigated by a bioassay using potato pieces described by Nishiyama (1981)¹¹. Coronatine extraction was conducted according to following method. The bacterial strains were inoculated to 50 ml of a minimal glucose medium (K_2HPO_4 7 g, KH_2PO_4 1.5 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $(NH_4)_2SO_4$ 1 g, glucose 10 g) supplemented with proline (40 μ g/ml), leucine (40 μ g/ml) and thiamine (1 μ g/ml), and cultured by shaking at 25 C for 4–5 days. Culture filtrates were adjusted at pH 3.0 and subjected to extraction with 50 ml ethyl acetate twice. The ethyl acetate extracts were dehydrated by adding a small amount of anhydrous Na_2SO_4 , filtered and rotary-evaporated. Each residues were dissolved in 500 μ l acetone. Twenty or fifty μ l of the samples was dropped on a potato disk and dried. The disk was put on potato tuber piece in a petri dish. The presence of coronatine producing ability was judged by appearance of a hypertrophic growth on potato which was observed 3 to 5 days after inoculation¹¹. Fifteen clones of *Eco*RI fragments (clones of Fig. 2 and others) and 13 clones of *Bam*HI fragments were assayed for the production of coronatine. As a result, any strains of *E. coli* transformants did not indicate a distinct coronatine producing ability, whereas same extracts from culture filtrate of *pv. atropurpurea* NIAES 1309 induced

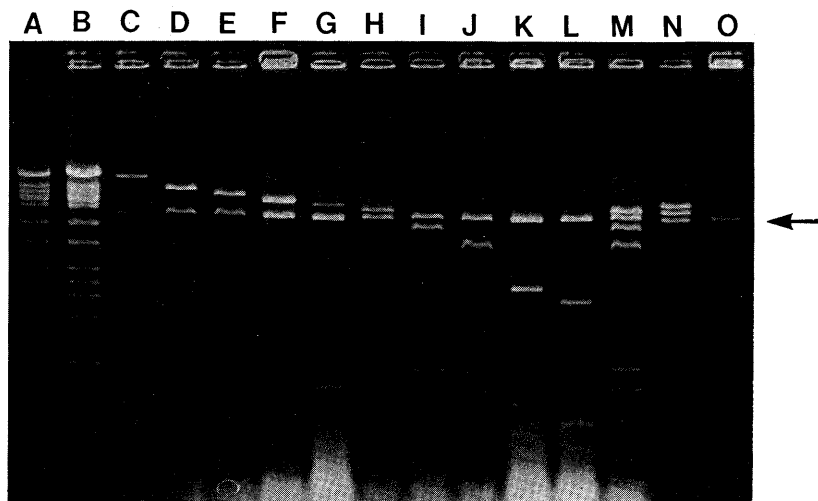


Fig. 2. Agarose gel electrophoresis of *Eco*RI digestion of recombinant plasmids from *E. coli* transformants. Lane A, B: pCOR1 (E1–E15 fragments), lane C: clone No. A9 (E1 and pBR325), lane D: A'5 (E2/pBR325), lane E: A'20 (E3/pBR325), lane F: A24 (E4/pBR325), lane G: A'55 (E5/E14/pBR325), lane H: A'12 (E6/pBR325), lane I: A'53 (E7/E13/pBR325), lane J: A'3 (E8/pBR325), lane K: A'43 (E10/pBR325), lane L: A'16 (E11/E15/pBR325), lane M: A21 (E6,7,8,13,14/pBR325), lane N: A10 (E5/E6/pBR325), lane O: HB101 (pBR325). Arrow shows pBR325 plasmid.

13) Holmes, D.S. and Quigley, M. (1981). Anal. Biochem. 114: 193.

hypertrophic outgrowths of potato. Moreover, any coronatine-producing clone was not detected too by the potato assay of inoculating directly young bacterial cultures.

This failure to express genes involved in coronatine production in *E. coli* HB101 may be due to the following reasons. 1) Although complete set of the genes of coronatine biosynthesis were cloned, the genes did not express in *E. coli*, as it had been observed in phaseolotoxin-genes and *hrp* genes of *Pseudomonas syringae* pv. *phaseolicola* by unknown reasons^{14,15}. It is impossible to check gene expression of these recombinant plasmids in *P. syringae* pv. *atropurpurea*, since they do not replicate in this bacterium (data not shown). 2) The genes are not clustered in pCOR1 plasmid and a part of genes located on the chromosome. 3) The coding region of genes is larger than that of the cloned inserts, or the genes are cleaved by the both restriction enzymes.

Although much additional work is needed to clone the gene involved in coronatine biosynthesis of *P. syringae* pv. *atropurpurea*, the results obtained in the present study will be used to facilitate the future cloning and expression of the genes producing coronatine in *P. syringae* pathovars. Moreover, this gene library will be useful for cloning of other important genes involved mobilization, "plasmid stability" and toxin immunity, the gene of which is expected to located on pCOR1 plasmid.

和 文 摘 要

佐藤 守・佐藤姚子・加藤 明・西山幸司・酒井富久美：*Pseudomonas syringae* pv. *atropurpurea* のコロナチン生合成に関わる pCOR1 プラスミド DNA のジーンライブラリー

Pseudomonas syringae pv. *atropurpurea* のコロナチン生合成に関与することが示されている pCOR1 プラスミドの各種制限酵素切断による解析を行い、かつ、*Eco*RI, *Bam*HI 断片を、pBR325, pBR322 をベクターとして *E. coli* HB101 株にクローニングを行った。その結果、同プラスミドのほぼ全域をクローニングすることができ、各種遺伝子研究に有用なジーンライブラリーが作成された。一方、これら *E. coli* の形質転換体は、いずれもコロナチン産生能を示さず、HB101 株を宿主としてのコロナチン生合成遺伝子の単離はできなかった。

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- 14) Peet, R.C., Lindgram, P.B., Willis, D.K. and Panopoulos, N.J. (1986). J. Bacteriol. 166: 1096-1105.
15) Lindgren, P.B., Peet, R.C. and Panopoulos, N.J. (1986). Ibid. 168: 512-522.