A Small Plasmid Isolated from an Avirulent Mutant of *Pseudomonas solanacearum*

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Pseudomonas solanacearum E. F. Smith, a causal agent of bacterial wilt (mainly to solanaceous plants), is one of the most destructive plant pathogens in the world. Genes related to the virulence of *P. solanacearum* have been elucidated by several groups. Boucher *et al.*¹⁾ suggested that most virulent genes were clustered in a megaplasmid. Xu *et al.*²⁾ suggested that numerous genes were involved in virulence by transposon mutagenesis.

Although it is well known that *P. solanacearum* loses virulence spontaneously under certain laboratory conditions³⁾, genes governing and/or controlling avirulence have not been fully elucidated. Currier and Morgan⁴⁾, and Morales and Sequeira⁵⁾ reported that spontaneous avirulent mutants showed no detectable change in plasmid number and the size compared to the parental strains.

A spontaneous avirulent mutant, M4S, which was attempted for use in the biological control of bacterial wilt of tobacco, was isolated from still culture of virulent strain *P. solanacearum* U-7⁶⁾.

This paper describes the structure of the plasmid containing only in a spontaneous avirulent mutant, M4S, and discusses the origin of this plasmid.

P. solanacearum, M4S and U-7, described above were routinely grown on Kelman's TZC medium³⁾ at 30 °C for 3 days and stored in sterilized distilled water at room temperature.

Plasmids were extracted from the bacterial cells by the method as described by Comai and Kosuge⁷⁾ and fractionated by 0.7% agarose gel electrophoresis in TBE buffer (0.089 M Tris-base, 0.089 M boric acid, 0.002 M EDTA). The presence of plasmid DNA (*ca.* 6.6 kbp) was detected only in M4S (Fig. 1A, lane 2) but not in U-7 (data not shown). This plasmid was designated as pJTPS1 and purified by isopycnic centrifugation in CsCl/ethidium bromide as described⁸⁾. By this method, the megaplasmids, which were detected in several strains of *P. solanacearum* by the modified Eckhardt procedure^{9,10)}, were not detected.

The restriction map of pJTPS1 was determined by using various restriction endonucleases.

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Fig. 1. Restriction endonuclease cleavage analysis of plasmid pJTPS1 and the restriction map. (A) Restriction endonuclease cleavage analysis. Lane 1: lambda DNA digested with *Hind*III (size marker), lane 2: undigested pJTPS1, lane 3–8: pJTPS1 digested by restriction enzymes, with *Apa*I (lane 3), *Eco*RI (lane 4), *Hind*III (lane 5), *Pst*I (lane 6), *Stu*I (lane 7), and *Xho*I (lane 8), respectively. Position of closed circular (cc), linear (l), and open circular (oc) forms are indicated by arrows. (B) Restriction map of pJTPS1.

The plasmid pJTPS1 was digested with various restriction endonucleases and the reaction products were subjected to 0.7% agarose gel electrophoresis. It was found that the plasmid carries a single unique site for *Apa*I, *Eco*RI, *Eco*RV, *Hind*III, *Stu*I and *Xho*I (obtained from Nippon Gene Inc. and Takara Inc.) and quadruple sites for *Pst*I (Fig. 1A). Double and triple digestion of the plasmid with these restriction endonucleases were performed and the reaction products were fractionated by 0.7, 1.2, or 2.0% agarose gel electrophoresis (data not shown). We derived a restriction map of pJTPS1 as shown in Fig. 1B.

To investigate the origin of pJTPS1, Southern blot hybridization analysis of the total DNA isolated from U-7 with pJTPS1 was performed. pJTPS1 digested with *Hind*III was used as a hybridization probe. The total genomic DNA of U-7 was isolated by the procedure described by Xu *et al.*²⁾ except that isopycnic centrifugation in CsCl/ethidium bromide was omitted. After treatment with RNase I (Sigma), 60 µg of the total DNA was digested with *Eco*RI, *Hind*III and *Pst*I, respectively, and subjected to electrophoresis in a 0.7% agarose gel in TBE buffer. For DNA labeling, blotting and DNA hybridization, ECLTM gene detection system (Amersham Inc.) was used according to the manufacturer's specification. At least five *Pst*I fragments hybridizing to the labeled probe were observed (Fig. 2, lane B-2) and three bands positioned at 3.8, 1.1 and 0.6 kbp, respectively, were identical to the *Pst*I fragments of pJTPS1. Two bands hybridizing at the positions about 6.2 and 4.9 kbp with *Eco*RI digestion and two bands hybridizing at the position larger than 20 kbp with *Hind*III digestion were observed (Fig. 2, lane B-4, B-3, respectively). These results suggest that pJTPS1 is genome or megaplasmid born and possibly integrated into a genome or megaplasmid linearly with a cut at a certain position of 1.2 kbp *Pst*I fragment including *Apa*I site (see Fig. 1B). By the comparison of *Pst*I fragment hybridized to the labeled probe in U-7's DNA with the given amounts of pJTPS1 (Fig. 2, lane 2, 5, 6, 7 and 8), and by the assumption that the size of genomic DNA of *P. solanacearum* is approximately 5×10^6 bp, the homologous gene(s) to pJTPS1 existing in U-7 was estimated to be a single copy.

Currier and Morgan⁴⁾ and Morales and Sequeira⁵⁾ reported that spontaneous avirulent

Fig. 2. Southern blot hybridization analysis of the total DNA isolated from U-7 probed with *Hind*III-digests of pJTPS1. (A) The total DNA was electrophoresed in 0.7% agarose and stained with ethidium bromide. (A) Lane 1: lambda DNA digested with *Hind*III (size marker), lane 2–4: total DNA isolated from U-7 digested with restriction enzymes, lane 2: *Pst*I, lane 3: *Hind*III and lane 4: *Eco*RI, respectively. Lane 5–8: threshold amount of pJTPS1 digested with *Pst*I, lane 5: 500 ng, lane 6: 50 ng, lane 7: 5 ng and lane 8: 0.5 ng. (B) Hybridization of the total DNA with ECLTM (Amersham, Inc.)-labeled pJTPS1. Lanes correspond to (A).

mutants of *P. solanacearum* harbored the same plasmid(s) as the parental strain without detectable changes in size. Furthermore, a small plasmid, like pJTPS1, was not detected in an avirulent mutant strain of *P. solanacearum* OM2¹¹⁾ or its parental strain Ps48 by this method (data not shown).

The fact that pJTPS1 exists only in an avirulent mutant, M4S, but not in the parental virulent strain of *P. solanacearum*, U-7, may represent a unique finding in plant pathogenic bacteria, although there are some reports that certain plasmid-like DNA or double-stranded RNA are possibly involved in the hypovirulence of the fungal plant pathogens¹²⁾. Furthermore, it appears that pJTPS1 may be derived from genome or megaplasmid.

It is not clear whether the presence of pJTPS1 is correlated to the mutation from virulence to avirulence. Studies are under way to determine whether pJTPS1 is involved in the loss of virulence of *P. solanacearum* by direct transformation of pJTPS1 into the virulent strain, U-7.

和 文 摘 要

根岸秀明・山田哲治・白石友紀・奥 八郎・田中 博: *Pseudomonas solanacearum* の非病原性突然変異株から分離されたプラスミド

Pseudomonas solanacearum の自然発生の非病原性突然変異株, M4S から約 6.6 kbp の環状 2 重鎖のプラスミド DNA, pJTPS1 を分離した。pJTPS1 は M4S の親株である病原性菌, U-7 からは検出されなかった。

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このプラスミドは, *Apa*I, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I, *Stu*I, *Xho*I などの制限酵素認識部位をもっていた。U-7 の全 DNA とのハイブリダイゼーション分析の結果から, pJTPS1 はゲノムまたはメガプラスミド由来であろうと推測された。

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