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Involvement of a Plasmid in the Expression of Virulence in *Pseudomonas syringae* pv. *eriobotryae*

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Abstract

A representative strain of *Pseudomonas syringae* pv. *eriobotryae*, the causal agent of stem canker of loquat, was studied to determine whether plasmids are involved in its virulence. The strain NAE6 harbored three plasmids of approximately 25, 52, and 60 megadaltons (Mdal). The derivative strains which were cured of the 52 Mdal plasmid by culturing at a maximum growth temperature of 32°C were uniformly avirulent. The 52 Mdal plasmid DNA, which was isolated from agarose gels using agarase, was digested with *Bam*HI, ligated into the *Bam*HI cloning site of the broad host range cosmid pLAFR3, packaged into λ phage particles, and transduced into *Escherichia coli* HB101. By use of helper plasmid pRK2013, twenty-five recombinant plasmids were mobilized into an avirulent recipient *P. s.* pv. *eriobotryae* strain, which was cured of three plasmids. The transconjugants which received the recombinant plasmid pVIR6 containing *ca.* 23 kb insert DNA regained virulence. Southern hybridization analysis indicated that the 23 kb insert DNA originated from the 52 Mdal plasmid. These data demonstrate that the 52 Mdal plasmid of *P. s.* pv. *eriobotryae* is associated with virulence.

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Key words: virulence, 52 Mdal plasmid, *Pseudomonas syringae* pv. *eriobotryae*.

INTRODUCTION

Pseudomonas syringae pv. *eriobotryae* is a pathogenic bacterium causing stem canker of loquat. The canker bacterium has been classified into three groups (A, B, and C) on the basis of their pigment production and pathogenicity to loquat leaves[12]. The electrophoretic profiles of plasmid DNA obtained from group A, B, and C strains were all different. However, the plasmid profiles of strains belonging to the same group were similar[7]. All nine strains belonging to group A harbored three plasmids of 25, 52 and 60 megadaltons (Mdal). In previous papers[7,8], it was suggested that a 85 Mdal plasmid in group C strains was associated with virulence. However, further investigations were difficult because of its high molecular weight. In this paper, the relationship between plasmids with molecular weights below 85 Mdal in a group A strain and virulence is investigated, and it is demonstrated that the expression of virulence is associated with the 52 Mdal plasmid in the group A strain of *P. s.* pv. *eriobotryae*.

MATERIALS AND METHODS

Bacterial strains, plasmids and media. *P. s.* pv. *eriobotryae* NAE6 (Group A) was obtained from Dr. A. Morita, Nagasaki Fruit-Tree Experiment Station in Japan. *Escherichia coli* HB101 was used as a recipient in construction of a cosmid library. The broad host range cosmid pLAFR3[15] was used to construct the gene library of 52 Mdal plasmid DNA. The conjugative plasmid pRK2013[6] was used as a helper plasmid in matings. The plasmid RP4 (36 Mdal)[11], pAS8Tc^{s}repl::Tn7 (52 Mdal)[14], the large (124 Mdal) and small (29.8 Mdal) plasmid from *Agrobacterium radiobacter* 84[10], and *Hin*dIII digested λ DNA were used as markers for standard molecular weight.

Strains of *P. s.* pv. *eriobotryae* were cultured on PS agar[7], on YP agar[7], or in YP medium at 25°C. *E. coli* strains were cultured on YP agar or in YP medium at 37°C. Selective antibiotic concentrations were as follows: kanamycin (Kan), 50 μg/ml; nalidixic acid (Nal), 50 μg/ml; rifampicin (Rif), 50 μg/ml; tetracycline (Tet), 15 μg/ml.

Plasmid DNA isolation and electrophoresis. Large-scale isolations of the cosmid DNA were done using cesium chloride gradient centrifugation as described previously[9]. Small-scale isolations of plasmid DNA were done using two modified procedures of Kado and Liu[7,13].

For cloning experiments, the 25 and 52 Mdal plasmids of *P. s.* pv. *eriobotryae* were isolated from agarose gel using agarase. The plasmid DNA which was isolated by small-scale method from a derivative strain cured of 60 Mdal plasmid was electrophoresed on 0.6% low-melting

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agarose gel. Strips were cut off of both sides of the gel and stained with ethidium bromide (0.5 μg/ml). The strips were rinsed with water and aligned alongside the remainder of the gel slab. Using the stained strips as a guide, the bands of plasmid were cut out, and put into a centrifugation tube. Ten times the agarose volume of Bis Tris buffer (10 mM Bis Tris-HCl [pH 6.5], 1 mM EDTA) was added and shaked gently for 2-3 hr at room temperature. After removing the Bis Tris buffer, more buffer, in an amount equivalent to the amount of agarose, was added and melted by incubation at 65°C for 15 min. The melted agarose solution was cooled to 40°C. The agarase (New England Biolabs, Inc.) was added at a final concentration of 5 U/ml and incubated at 40°C for 15 hr. Sodium chloride was added to an agarase-treated solution at a final concentration of 0.5 M and chilled on ice for 15 min. After centrifugation (15,000 × *g*), the DNA in the supernatant was precipitated with 3 volumes of cold ethanol (−20°C) and resuspended in TES buffer (50 mM Tris, 5 mM EDTA, 50 mM NaCl, pH 8.0).

The electrophoresis of DNA samples was carried out in 0.7% or 1% agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.0) or TAE buffer (40 mM Tris, 1 mM EDTA, adjust to pH 8.0 with glacial acetic acid).

Curing of plasmid by heat treatment. *P. s.* pv. *eriobotryae* had an optimum growth temperature of 25-26°C. For curing of plasmid, strain NAE 6 was cultured on PS agar slants at a maximum growth temperature of 32°C. After one or two months, the cultures were streaked onto the PS agar medium and incubated at 25°C. Appeared colonies were subcultured for examination of their plasmid content.

Cosmid cloning. The 52 Mdal plasmid DNA (*ca.* 1 μg) of *P. s.* pv. *eriobotryae* was digested completely with *Bam*HI and treated with calf intestinal alkaline phosphatase according to the method of Bernard[2]. The solution was extracted with phenol / chloroform / isoamyl alcohol (25 : 24 : 1) and then with chloroform / isoamyl alcohol (24 : 1). DNA was precipitated from the aqueous phase with ethanol and resuspended in sterile distilled water.

The cosmid pLAFR3 DNA (*ca.* 5 μg) was divided into two aliquots. One was digested with *Eco*RI and the other with *Hin*dIII. Each DNA was treated with calf intestinal alkaline phosphatase and the reaction was terminated by incubating each sample at 70°C for 15 min. The dephosphorylated DNA samples were combined, extracted, and precipitated as described above. The *Eco*RI- and *Hin*dIII-digested DNA were then digested with *Bam*HI, extracted, and precipitated. The cosmid DNA was resuspended in sterile distilled water.

The treated insert DNA and cosmid DNA were mixed and ligated with T4 ligase. The ligated DNA was packed with a LAMBDA INN packaging kit (Nippon Gene Co., Ltd.) and introduced into *E. coli* HB101 according to the manufacturer's instructions. The recombinant plasmids were selected by plating on YP agar medium containing tetracycline. The pooled recombinant plasmids were mobilized from *E. coli* into *P. s.* pv. *eriobotryae* strains by employing the helper plasmid pRK2013 in matings.

The *Bam*HI-fragments from the 25 Mdal plasmid were also cloned into the *Bam*HI site of pLAFR3 using the same procedures, and used as a means of the 25Mdal plasmid elimination by incompatibility.

Plant inoculations. Bacteria, which were grown on PS or YP agar medium for 24 hr, were inoculated into one-year-old stems of loquat using a needle. The inoculated plants were covered with a polyethylene bag for 24 hr to keep the humidity high. Plants were maintained at glasshouse conditions of 20-28°C, and observed for the development of symptoms for up to 150 days after inoculation.

Southern hybridization. The recombinant plasmid DNA containing *ca.* 23 kb insert DNA was digested with *Bam*HI. The digested DNA was electrophoresed on 0.15% agarose gel in TAE buffer until the insert DNA was clearly separated from the vector. After staining with ethidium bromide, the bands of the insert DNA were cut out under ultraviolet light. The DNA to be used as a probe was purified from these gel pieces by employing an Easytrap kit (Takara Shuzo Co., Ltd.). The labelling of probe DNA with horseradish peroxidase, hybridization and detection by exposure on autoradiography film (Hyperfilm-ECL) were done using an ECL direct nucleic acid labelling and detection kit (Amersham), according to the manufacturer's instructions.

RESULTS

Correlation between the 52 Mdal plasmid and virulence

P. s. pv. *eriobotryae* NAE 6 harbored three plasmids approximately 25, 52 and 60 Mdal (Fig. 1A, lane 1). Curing of the 52 Mdal, 60 Mdal, or both plasmids in NAE 6 was observed after exposure of cells to elevated growth temperature (32°C) (Fig. 1A, lane 2-4). The derivative strains cured of 52 Mdal, 60 Mdal, or both plasmids were designated PE60, PE52, and PE25, respectively. The strain NAE 6 and its derivative strains were inoculated into the stems of loquat. Two or three weeks after a strain NAE 6 and derivative strain PE 52, which harbored 52 Mdal plasmid, were inoculated, a callus-like tissue developed at the inoculated part. In advanced stages, the protruded callus-like tissue dried and the symptom enlarged (Fig. 1B, 1 and 3). On the other hand, a callus-like tissue never formed on the loquat stem which was inoculated with derivative strain PE60 or PE25 cured of their 52 Mdal plasmid. Only the damage of the bark of the inoculated part was slightly noticeable (Fig. 1B, 2 and 4). These symptoms were similar to those on the stems which were inoculated with distilled water.

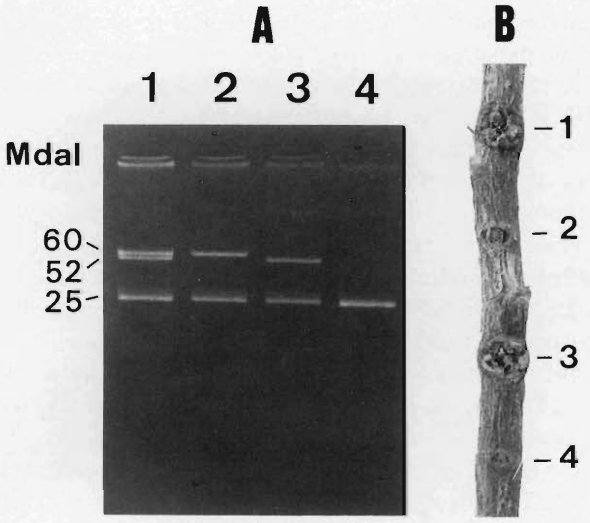

Fig. 1. Agarose gel electrophoresis of plasmids from *P. s.* pv. *eriobotryae* NAE6 strain and its derivative strains obtained by culturing at a maximum growth temperature of 32°C (A), and symptoms induced on stem of loquat by these four strains (B). A) Lane 1, NAE6; lane 2, derivative strain PE60; lane 3, derivative strain PE52; lane 4, derivative strain PE25. B) Symptoms 100 days after inoculation with NAE6 (1), PE60 (2), PE52 (3) and PE25 (4).

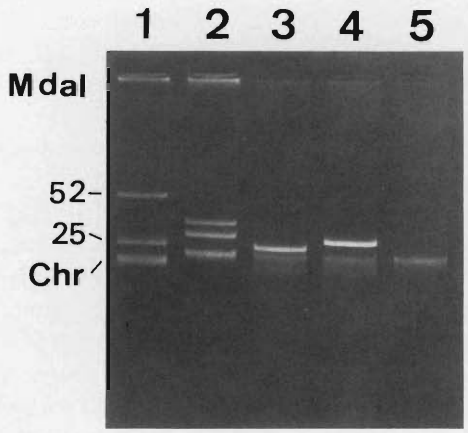

Fig. 2. Agarose gel electrophoresis of plasmids of donor, recipient, transconjugant, and cured strain. lane 1, derivative strain PE52; lane 2, donor strain, *E. coli* HB101 (Kan^r, Tet^r) which contained helper plasmid pRK2013 (upper band) and recombinant plasmid pCOS2 (second band from top); lane 3, recipient strain PE25 (Nal^r, Rif^r); lane 4, transconjugant strain (Nal^r, Rif^r, Tet^r) which contained pCOS2 and lost the 25 Mdal plasmid; lane 5, derivative strain PE0 (Nal^r, Rif^r, Tet^s) which was cured of pCOS2.

Cosmid clonig and selection for the cosmid clone carrying virulence gene(s)

The 52 Mdal plasmid DNA, which was isolated from agarose gel using agarase, was almost converted into the linear DNA. Such DNA sample was cleaved with *Bam*HI to yield five fragments (*ca.* 27.7, 23.1, 12.6, 12.0 and 4.9 kb). The 52 Mdal plasmid DNA digested with *Bam*HI was ligated into the *Bam*HI site of pLAFR3, packaged into λ phage particles, and transduced into *E. coli* HB101. Approximately 150 Tet^r colonies were obtained. These transductants contained five kinds of recombinant plasmids with different molecular sizes. The molecular sizes of these recombinant plasmids were larger than that of pLAFR3 (22 kb)[15].

By using of pRK2013, three kinds of recombinant plasmids (*ca.* 49, 35 and 27 kb) were successively introduced into the derivative strain PE25 (Nal^r, Rif^r) containing only 25 Mdal plasmid, but two (*ca.* 45 and 34 kb) were not introduced regardless of their molecular size. To introduce all kinds of recombinant plasmids into the recipient strain, the 25 Mdal plasmid, which could not be eliminated by exposure of cells to 32°C, was eliminated by using incompatibility response (Fig. 2). The helper plasmid pRK2013 was conjugally transferred into *E. coli* HB101 which harbored a recombinant plasmid, pCOS2, containing *ca.* 22 kb insert DNA derived from the 25 Mdal plasmid (Fig. 2, lane 2), and then the resulting strain was mated with PE25 (Fig. 2, lane 3). The 25 Mdal plasmid of PE25 transconjugant was displaced by the recombinant plasmid pCOS2 (Fig. 2, lane 4). The electrophoretic pattern of the introduced recombinant plasmid DNA digested with *Bam*HI was the same as that of the original recombinant plasmid pCOS2. Curing of the recombinant plasmid pCOS2 in PE25 transconjugant was done by exposure of cells to 32°C. This derivative strain which did not harbored a plasmid was still avirulent and designated PE0 (Fig. 2, lane 5).

Five kinds of the recombinant plasmids each containing *Bam*HI fragment from the 52 Mdal plasmid could be introduced into the avirulent strain PE0 by matings employing pRK2013 as the helper plasmid. A total of twenty-five transconjugants each containing a recombinant plasmid were inoculated into the stems of loquat. One recombinant plasmid containing a *ca.* 23 kb insert DNA, which was designated pVIR6, conferred a virulence phenotype (Fig. 3A, 1 and 3B, lane 1). The symptom of PE0 transconjugant containing pVIR6 was similar to those of NAE6 and PE52 (Fig. 3A, 1, 4 and 5). The inoculated strain PE0 (pVIR6) was reisolated from the symptom. The derivative strain PE25 containing pLAFR3 was avirulent (Fig. 3A, 3 and 3B, lane 3). This indicated that pLAFR3 had no effect on the virulence. Agarose gel electrophoresis of plasmids isolated from the strains which were used as inocula is shown in Fig. 3B.

Southern hybridization analysis

To determine the origin of the 23 kb insert DNA of the recombinant plasmid pVIR6, hybridization analysis was carried out. The labeled 23 kb insert DNA hybridized to the 52 Mdal plasmid in PE52, not to the 25 Mdal plasmid (Fig. 4B, lane 1). The probe also hybridized to itself (Fig. 4B, lane 3) and to pVIR6 (Fig. 4B, lane 2), not

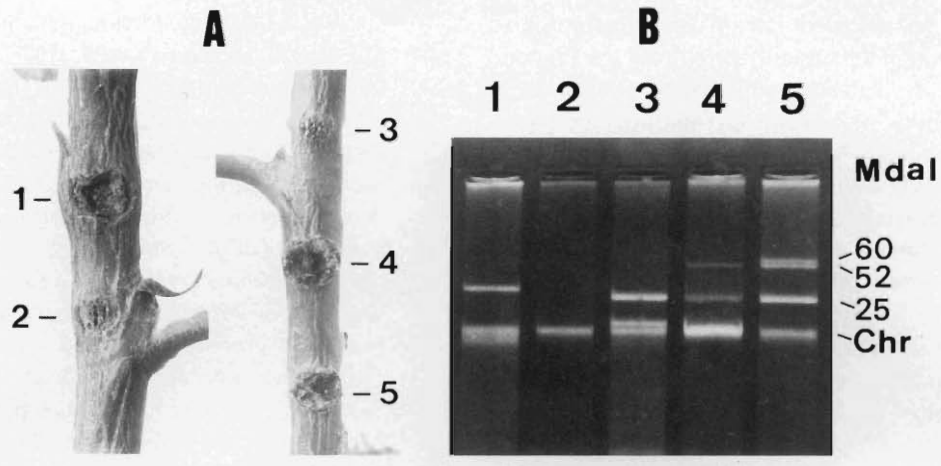

Fig. 3. Symptoms induced on stems of loquat by the strains of *P. s.* pv. *eriobotryae* (A), and agarose gel electrophoresis of plasmids from the strains which were used as inocula (B). A) Symptoms 40 days after inoculation with PE0 containing recombinant plasmid pVIR6 (1), PE0 (2), PE25 containing pLAFR3 (3), PE52 (4), and NAE6 (5). B) lane 1, PE0 containing pVIR6; lane 2, PE0; lane 3, PE25 containing pLAFR3 (second band from top); lane 4, PE52; lane 5, NAE6.

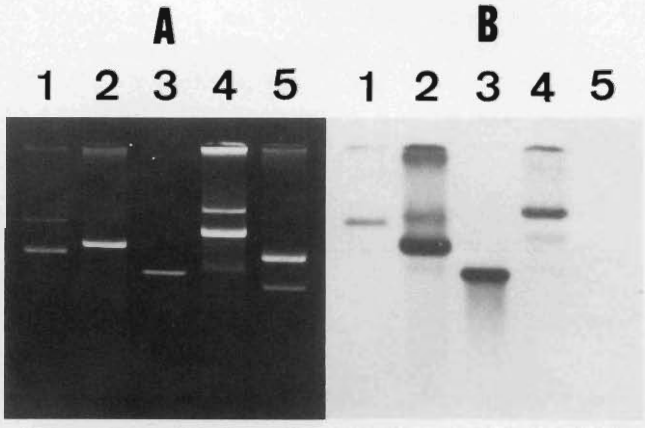

Fig. 4. Hybridization of the 23 kb insert DNA of pVIR6 with plasmids from strains of *P. s.* pv. *eriobotryae*. A) Ethidium bromide-stained gel. lane 1, PE52; lane 2, PE0 containing pVIR6; lane 3, the 23 kb insert DNA; lane 4, NAE57-D (group C strain); lane 5, PE25 containing pLAFR3. B) Southern blot of the gel in panel A probed with labeled pVIR6 insert DNA.

to pLAFR3 (Fig. 4B, lane 5). This indicated that the 23 kb insert DNA originated from the 52 MdaI plasmid. Furthermore, hybridization occured with the 85 MdaI plasmid in the group C strain NAE57-D (Fig. 4B, lane 4).

DISCUSSION

The 60 and 52 MdaI plasmids in *P. s.* pv. *eriobotryae* were cured by culturing at an elevated temperature for one or two months. Loss of the 52 MdaI plasmid coincided with loss of virulence. Since virulence is not a useful selection marker for further investigation, an attempt was made to detect another phenotypic marker encoded on the 52 MdaI plasmid in the same manner as described earlier[7]. However, no phenotypic traits was detected. In the second place, construction of a gene libraly of the 52 MdaI plasmid DNA in cosmid vector pLAFR3 was attempted. Since the derivative strain which harbored only the 52 MdaI plasmid could not be obtained, several attempts were made to isolate the 52 MdaI plasmid DNA from the agarose gel. Isolating the 52 MdaI plasmid DNA from the agarose gel by electroelution, by using DEAE paper, or by using powdered glass was difficult because of the plasmid's high molecular weight. Recovery of the 52 MdaI plasmid DNA from low-melting agarose gel was also difficult. The isolation of the 52 MdaI plasmid was finally performed using agarase.

The recombinant plasmid pVIR6 containing *ca.* 23 kb insert DNA derived from the 52 MdaI plasmid was introduced into the derivative strain PE0 which was cured of three plasmids, but not into PE25 containing the 25 MdaI plasmid. The 23 kb insert DNA had no detectable homology with the 25 MdaI plasmid in Southern hybridization experiment. pLAFR3 could also coexist with the 25 MdaI plasmid. The reason why pVIR6 could not coexist with the 25 MdaI plasmid was not discovered.

When the recombinant plasmid pVIR6 was introduced into the derivative strain PE0 lacking three plasmids, the avirulent recipient strain PE0 regained virulence. Furthermore, it was confirmed by hybridization analysis that the insert DNA originated from the 52 MdaI plasmid. The present study demonstrates that the 52 MdaI plasmid in *P. s.* pv. *eriobotryae* NAE 6 is associated with virulence.

The result of the hybridization experiment shows the existence of structural homology of the 23 kb insert DNA with a 85 MdaI plasmid in the group C strain. The loss of the 85 MdaI plasmid resulted in a loss of virulence[7]. However, the 85 MdaI plasmid was not

reintroduced to an avirulent strain in an attempt to restore virulence, and as a result the role of the plasmid in the expressin of virulence could not be confirmed.

Virulence plasmids in several phytopathogenic bacteria have been reported[1,3-5,14,16]. It is known that these virulence plasmids contain the genes for phytohormone biosynthesis, phytotoxin biosynthesis, or aggressiveness against plants. This study indicates that the 52 Mdal plasmid is a virulence plasmid in a broad sense. However, the virulence function of the 52 Mdal plasmid was not discovered. Further work is in progress to investigate the function and structure of virulence gene(s) on the 52 Mdal plasmid in *P. s.* pv. *eriobotryae*.

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和 文 摘 要

上運天博：ビワがんしゅ病菌の病原性に関与するプラスミド

ビワがんしゅ病菌 NAE6 は約 25, 52 および 60 メガダルトン (Mdal)の 3 つのプラスミドを有しており，それらプラスミドと病原性との関連を検討した。NAE6 を生育最高温度の 32°C で培養することにより得られた 52 Mdal プラスミド欠落株は常に病原性を失っていた。アガラーゼ処理によりアガロースゲルから分離した 52 Mdal プラスミド DNA を *Bam*HI で消化し，広宿主範囲を示すコスミドベクター pLAFR3 の *Bam*HI サイトに連結し，パッケージング後，大腸菌に導入した。DNA 断片が挿入された 25 の pLAFR3 をヘルパープラスミド pRK2013 を用いて，3 つのプラスミドが欠落し病原性を失ったビワがんしゅ病菌に導入し，ビワ茎に接種した。その結果，約 23 kb の DNA 断片が挿入された pVIR6 を導入した菌株は病原性を回復していた。また，ハイブリダイゼーションの結果は 23 kb の DNA 断片が 52 Mdal プラスミド由来であることを示していた。以上の結果より，52 Mdal プラスミドは病原性と関連することが明らかとなった。