Plasmid DNA Detected in Pseudomonas *syringae* pv. *mori*, the Causal Agent of Bacterial Blight of Mulberry

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Indigenous plasmids have been detected in many species of phytopathogenic bacteria. Some of these plasmids coded an important gene, that is, pTi and pIAA1 (pIAA2) control the pathogenicity of *Agrobacterium tumefaciens* and *Pseudomonas syringae* pv. *savastanoi* respectively, or pDC250 of *Erwinia stewartii* and pBPW1 of *P. syringae* pv. *tabaci* have a conjugative function.[3,5,6] Therefore, the studies on indigenous plasmids are indispensable in the genetics of phytopathogenic bacteria. On the other hand, it is also important to introduce a transposable element (transposon) into the bacteria, because the element is usable as a variety of genetic manipulation in the bacteria.[2,6]

These subjects, however, have not been investigated in *Pseudomonas syringae* pv. *mori*, the causal agent of bacterial blight of mulberry, which is one of the most serious mulberry diseases in Japan. This report summarizes our recent results concerning the detection and characterization of indigenous plasmids in *P. syringae* pv. *mori* and the introduction of a transposon into the bacteria.

Plasmid pattern in *P. syringae* pv. *mori*[9,11]

A total of 76 isolates of *P. syringae* pv. *mori* isolated from various places of Japan was used in the experiment for detection of indigenous plasmids. Extract of plasmid DNA was performed according to the method described by Birnboim and Doly (1979).[1] Plasmid DNA was analysed by electrophoresis in 0.7% agarose gel with Tris-borate buffer. The gels were stained with ethidium bromide (1 μg/ml) and photographed under UV illumination. The sizes of the plasmids were estimated by comparison with standard plasmids in the same gel.

As the result, 56 isolates of *P. syringae* pv. *mori* contained 1 to 4 plasmids (Plate 1). Twenty isolates, however, harbored no plasmid. Although the detail relationships among the plasmids were not examined, the following six classes were distinguished based on their approximate molecular size: L1 (65–70 Mdal), L2 (60 Mdal), M1 (45–50 Mdal), M2 (35–40 Mdal), M3 (20–28 Mdal), and S (1–5 Mdal). Thereby, *P. syringae* pv. *mori* tested here were classified into 17 plasmid types based on their plasmid patterns (Table 1). The plasmid types were named A (no plasmid), B (one plasmid), C (two plasmids), D (three plasmids), and E (four plasmids) based on the number of plasmid contained. The distribution of the number of bacterial isolate in each plasmid type was unbalanced, showing that many strains belonged to the limited types such as A, B4, and D1.

Relationships between bacteriological properties and a specified plasmid[8,9,11]

In order to make clear whether a specified plasmid controls some property or not, an association between the plasmid and the following properties was investigated.

1) Halo-toxin productivity

P. syringae pv. *mori* is classified into two

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Table 1. Classification of *Pseudomonas syringae* pv. *mori* according to plasmid content

Type	Kind of plasmid (Mdal)						No. of strains
	L1 (65–70)	L2 (60)	M1 (45–50)	M2 (35–40)	M3 (20–28)	S (1–5)	
A	−	−	−	−	−	−	20
B1	+	−	−	−	−	−	2
B2	−	−	+	−	−	−	2
B3	−	−	−	+	−	−	3
B4	−	−	−	−	+	−	10
B5	−	−	−	−	−	+	3
C1	+	−	+	−	−	−	3
C2	+	−	−	+	−	−	1
C3	−	−	+	+	−	−	2
D1	+	−	+	−	+	−	15
D2	+	−	−	+	+	−	4
D3	−	+	+	−	+	−	5
D4	−	−	++	−	+	−	1
D5	−	−	+	+	+	−	2
D6	−	−	+	−	++	−	1
E1	+	+	−	−	−	++	1
E2	−	−	+	−	++	+	1

+ : Present, ++ : Two plasmids present, − : Absent

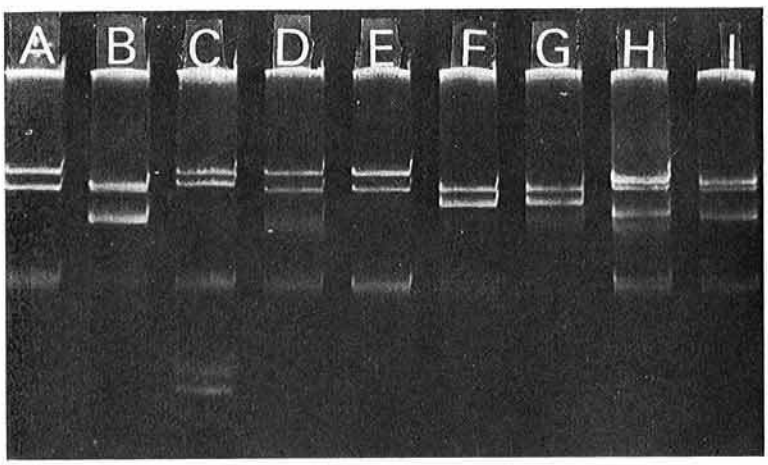

Plate 1. Agarose gel electrophoresis of plasmid DNA in *Pseudomonas syringae* pv. *mori*
Sample wells contained DNA from strains S6802∼S6810 (A∼I). The clear band is plasmid DNA and the diffuse one in all wells is chromosomal DNA.

strains of "halo blight strain" and "blight (non-halo) strain" by the presence of halo-toxin productivity.[17] Six halo blight strains were tested. All isolates contained indigenous plasmids which were divided into 6 classes based on the molecular size (Table 2). Although a specified plasmid was not detectable from all the halo strains, the strains from Shimane Prefecture always contained the 70 Mdal (L1) plasmid, showing there may be a correlation among the plasmid and toxin productivity in the strains.

The plasmid of Ym5–1 named pYM5 has a conjugative function as described later. In order to check the presence of plasmid control of toxin productivity, pYM5 was transferred to *P. syringae* pv. *mori* Ni27 (non-halo str.) by conjuga-

Table 2. Plasmid pattern in the halo blight strains of *Pseudomonas syringae* pv. *mori*

Isolate	Locality	Plasmid content (Mdal)					
		L1 (70)	M1 (50)	M2 (39)	(35)	M3 (28)	(25)
S7133-2	Shimane Prefecture	+	+	−	−	+	−
S7133-3	do.	+	−	−	+	−	+
S7233-1	do.	+	−	−	+	−	+
S7233-2	do.	+	−	−	+	−	+
S7233-3	do.	+	−	−	+	−	+
Ym5-1	Yamanashi Prefecture	−	−	+	−	−	−

+ : Present, − : Absent

tion. Then, the toxin productivity of the transconjugant Ni27 (pYM5) was examined by the bacterial inoculation to mulberry leaves. Any halo blight symptoms, however, did not appear. Moreover, the mutant of Ym5-1, which lost the toxin productivity, still harbored a whole pYM5 plasmid. The results strongly suggest that pYM5 did not code the gene(s) related to the toxin productivity in this bacterium.

2) *Phage sensitivity and the ability to decompose carbohydrates*

P. syringae pv. *mori* strains are classified into five lysotypes named A, B, C, D, and E according to their sensitivity to six phage strains.[12] And they are also classified by their ability to decompose rhamnose or xylose.[13]

Ten bacterial isolates including all lysotypes except D type, and all biochemical types were tested in the experiment for detecting their plasmid. Two to four plasmids, having a molecular size ranging from 4.5 Mdal to 70 Mdal, were isolated from all the tested bacteria. Each indigenous plasmid, however, did not have any relationships with the lysotypes or the biochemical types.

3) *Ice nucleation activity and pathogenicity*

Ice nucleation activity (INA) of a bacterium is considered one of the important factors inducing the frost damage of plants.[4] *P. syringae* pv. *mori* strains are divided into two groups of INA$^+$ and INA$^-$ by the presence of INA (ability to freeze the bacterial suspension at −5°C, in this case). Relationships between INA and plasmid patterns were examined using 34 INA$^+$ strains and 43 INA$^-$ strains. Most strains of INA$^+$ contained no plasmid (36.4%) or single plasmid (51.5%), whereas about 75% of INA$^-$ strains had 2 to 4 plasmids. The result suggests that the plasmids might concern the synthesis of some kind of protein related to INA.

It was also observed that some weakly virulent strains isolated from mulberry tree harbored no or single plasmid. This phenomenon suggests the possibility of the plasmid control of the pathogenicity to some extent.

Conjugative function of plasmids[8, 11, 15, 16]

Whether or not the plasmids of *P. syringae* pv. *mori* are conjugative was tested in an indirect way. A non-conjugative but mobilizable plasmid RSF1010 which encodes streptomycin(Sm)- and sulfonamide(Su)-resistance, was introduced into rifampicin(Rif) resistant mutant of *P. syringae* pv. *mori* by mating with *P. syringae* pv. *tabaci* BR2 (RSF1010). Then, *P. syringae* pv. *mori* (RSF1010) was mated with *E. coli* or *P. syringae* pv. *mori* in order to check the mobilization of RSF1010. Transmission of RSF1010 was confirmed by gel electrophoresis. Most strains (12/13 str.) tested were capable of donating RSF1010 in these crosses although at exceedingly low frequency(usually 10^{-8}–10^{-9}/recipient). The result in some isolates was shown in Table 3. This suggests that the strains tested possess an indigenous fertility system.

Furthermore, conjugative function of pYM5 plasmid of *P. syringae* pv. *mori* Ym5-1 was examined by the method mentioned above. Ym 5-1^{rif} (RSF1010) was capable of donating RSF1010 to *E. coli* at a higher frequency (10^{-6}/recip.) than that of above mentioned strains. Moreover, RSF1010 was transferred to *P. syringae* pv. *mori* Ni27, which is non-halo strain and has no plasmid, at a frequency of 4.8×10^{-7}. In this case, selection was performed by culturing on minimal medium containing sodium tartrate because Ym5-1 was not able to use the carbon source. Then, the transconjugants were surveyed for detection of pYM5 plasmid co-transferred with RSF1010 by the agarose gel

Table 3. Conjugative mobilization of RSF1010 from *Pseudomonas syringae* pv. *mori* to recipient bacteria

P. syringae pv. *mori* (donor)	Recipient bacteria	Transfer frequency per recipient cell
Blight str.		
S6804^{rif} (RSF1010)	*E. coli* SK1592	1.8×10^{-8}
S6805 (RSF1010)	do.	3.1×10^{-8}
Halo str.		
S6914-1^{rif} (RSF1010)	do.	1.2×10^{-9}
S7133-2^{rif} (RSF1010)	do.	1.9×10^{-9}
Ym5-1^{rif} (RSF1010)	do.	4.2×10^{-6}
do.	*P. syringae* pv. *mori* Ni27	4.8×10^{-7}

electrophoresis. Among 107 colonies, five harbored the both plasmids of pYM5 and RSF1010. They were identified as Ni27(pYM5, RSF1010) according to their characterization such as colony types, use of tartrate and drug resistance. From the foregoing results, pYM5 evidently is a conjugative plasmid. This is one of a few examples of conjugative plasmid of phytopathogenic bacteria.

Introduction of a transposon[7,10,14]

Transposable drug resistance elements (transposon, Tn) are useful for a variety of genetic manipulation in bacteria: for example, 1) Tn insertion gives drug resistance marker to some replicon, 2) Tn induces a polar mutation on replicons, and so on. Their introduction into bacterial strains is generally accomplished by the use of a "carrier" replicon. There have not been any reports concerning Tn carrier suitable for plant pathogenic Pseudomonads. We selected the plasmid, pAS8 Tc^{s}Rep1 (RP4–ColE1, fused at their EcoR1 site) inserted by Tn7 as a carrier replicon. Since Pseudomonads are active recipients for RP4 but not ColEl, we reasoned that pAS8 Rep1 should not be able to replicate or maintain itself in these bacteria following conjugation, but Tn7 carried on RP4 might be rescued by selection for drug resistance.

E. coli AB2463(pAS8 Rep1::Tn7) was mated with different phytopathogenic Pseudomonads including *P. syringae* pv. *mori*, *P. syringae* pv. *tabaci* and *P. syringae* pv. *phaseolicola*. Selection was performed by temperature and drug resistance. As the result, transposable marker was transmitted to the recipient at frequencies of 10^{-5} to 10^{-7} (Table 4). Furthermore the transconjugants were characterized as follows: i) They contained no pAS8::Tn7 plasmid. ii) Some clones of pv. *tabaci* BR2 contained a "new" plasmid, pBPW1(indigenous plasmid) inserted by Tn7, judged from the analysis of Hind III restriction endonuclese. iii) Some clones of pBPW1::Tn7 lost their conjugative function by the polor mutation. iv) Tn7 was inserted into plasmids or chromosome of pv. *mori* as judged by the electrophoresis analysis. The results show that Tn7 was evidently present in the progeny of these bacteria.

Table 4. Transfer of transposable (Sm^r, Tp^r) and non-transposable (Km^r) markers of pAS8 Tc^s Rep1::Tn7 to *P. syringae* pathovars

Selection marker	Transfer frequency per recipient bacteria		
	pv. *mori* S6805	pv. *tabaci* BR2	pv. *phaseolicola* NPPH3007
Km	$<5\times10^{-7}$	$<10^{-7}$	$<10^{-7}$
Sm or Tp	3×10^{-5}	4×10^{-7}	1.6×10^{-6}

Conclusion

Many classes of plasmid pattern were found out in *P. syringae* pv. *mori*. The plasmid patterns showed no definite correlation with the phenotypes such as phage sensitivity and carbohydrate utilization. However, the involvement of these plasmids to some extent in toxin productivity, ice nucleation activity, and pathogenicity was suggested, although further studies will be required in order to make that clear.

The conjugative plasmid named pYM5 was obtained from the halo strain of *P. syringae* pv. *mori*. Besides this strain, many strains had a conjugative function. Our finding of a fertility system and the ability to obtain transposon insertions in *P. syringae* pv. *mori* will permit us to conduct further genetic studies of this bacterium.

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(Received for publication, April 11, 1983)