Indigenous Plasmids of *Pseudomonas syringae* pv. *mori*, the Causal Agent of Bacterial Blight of Mulberry

Mamoru SATO*, Brian J. STASKAWICZ**
and Nickolas J. PANOPOULOS***

佐藤 守*・Brian J. STASKAWICZ**・Nickolas J. PANOPOULOS***;
クワ縮葉細菌病菌 *Pseudomonas syringae* pv. *mori* のプラスミド

Abstract

Strains of *Pseudomonas syringae* pv. *mori*, the causal agent of bacterial blight of mulberry, contain 2 to 4 indigenous plasmids ranging from approximately 4.5 to 70 mdal and were classified into 9 groups according to their characteristic plasmid patterns. A survey of 17 isolates did not reveal apparent correlations between plasmid pattern and toxigenicity, phage sensitivity or other phenotypes tested. All strains tested accepted freely the non-conjugative plasmid RSF1010 through mobilization by naturally occurring conjugative plasmid pBPW1 of *P. s.* pv. *tabaci* BR2, but re-transferred RSF1010 into *Escherichia coli* at very low frequency. Thus, it seems to be indicated that they possess an indigenous fertility system which is either naturally repressed or mobilizes RSF1010 with low efficiency.

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Key Words: *Pseudomonas syringae* pv. *mori*, plasmid.

Introduction

Indigenous plasmids have been reported in several plant pathogenic bacteria including a number of *Pseudomonas syringae* pathovars^{5,8,10,20}. Furthermore, it has been shown that plasmid pTi and pIAA 1 controls the pathogenicity of *Agrobacterium tumefaciens*^{18,19} and of *P. syringae* pv. *savastanoi*²¹, respectively. Although evidence for the involvement of plasmid in pathogenicities of other phytopathogenic bacteria is lacking⁹, the subject is of considerable interest in the genetic analyses of pathogenicities of these bacteria.

The presence of indigenous plasmids of *P. syringae* pv. *mori* (abbrev. *P. s. mori*), the causal agent of bacterial blight of mulberry, has not been reported previously. This report summarizes our preliminary results concerning the detection and characterization of plasmids in this bacterium.

* The Sericultural Experiment Station, Yatabe, Ibaraki 305, Japan 農林水産省蚕糸試験場

** International Plant Research Institute, San Carlos, CA 94070, U. S. A.

*** Department of Plant Pathology, University of California, Berkeley, CA 94720, U. S. A.

Materials and Methods

Media. King's B medium (KB)⁴⁾ was used to grow the bacteria and to perform plate matings. Genetic crosses were performed on KB or Minimal A-glucose agar medium⁷⁾ supplemented with streptomycin (15 to 20 μ g/ml) and/or rifampicin (100 μ g/ml). For *E. coli* recipients the selections were performed on KB agar containing the appropriate antibiotics by incubating at 37 C or 40 C (both temperature are nonpermissive for growth of *P. s. mori*).

Bacteria and Plasmids. The bacterial strains used in this study are listed in Table 1. All isolates of *P. s. mori* including the "halo blight" strain¹⁷⁾ were virulent

Table 1. Tested bacterial isolates

Bacterial isolates	Properties ^{a)}	Source
<i>Pseudomonas syringae</i>		
pv. <i>mori</i>		
S 6801	Vir ⁺	Sato <i>et al.</i> ¹⁴⁾
S 6802	Vir ⁺	"
S 6803	Vir ⁺	"
S 6804	Vir ⁺	"
S 6805	Vir ⁺	"
S 6806	Vir ⁺	"
S 6807	Vir ⁺	"
S 6808	Vir ⁺	"
S 6809	Vir ⁺	"
S 6810	Vir ⁺	"
M 5	Vir ⁺	Sato ¹¹⁾
S 6914-1	Vir ⁺ Non-motile	Sato and Takahashi ¹²⁾
S 6801 rif	Vir ⁺ Rif ^r	Spontaneous mutant, this study
S 6802 rif	Vir ⁺ Rif ^r	"
S 6803 rif	Vir ⁺ Rif ^r	"
S 6804 rif	Vir ⁺ Rif ^r	"
S 6809 rif	Vir ⁺ Rif ^r	"
M 5 rif	Vir ⁺ Rif ^r	"
S 6914-1 rif	Vir ⁺ Rif ^r	"
pv. <i>mori</i>		
halo blight strain		
S 7133-2	Vir ⁺ (Halo)	Takahashi and Sato ¹⁷⁾
S 7133-3	Vir ⁺ (Halo)	"
S 7233-1	Vir ⁺ (Halo)	"
S 7233-2	Vir ⁺ (Halo)	"
S 7233-3	Vir ⁺ (Halo)	"
S 7133-2 rif	Vir ⁺ (Halo) Rif ^r	Spontaneous mutant, this study
S 7133-3 rif	Vir ⁺ (Halo) Rif ^r	"
S 7233-1 rif	Vir ⁺ (Halo) Rif ^r	"
S 7233-2 rif	Vir ⁺ (Halo) Rif ^r	"
S 7233-3 rif	Vir ⁺ (Halo) Rif ^r	"
pv. <i>tabaci</i>		
BR2 (RSF1010)	Sm ^r , Su ^r	Staskawicz <i>et al.</i> ¹⁶⁾
<i>Escherichia coli</i>		
SK 1592	<i>thi</i> ⁻ , hsdR ⁻ , hsdM ⁺	
V 517		Macrina <i>et al.</i> ⁶⁾

a) Vir, Rif, Sm and Su designate to virulence to mulberry, resistance to rifampicin, streptomycin and sulfonamide, respectively.
hsdM and hsdR designate host-controlled modification and restriction, respectively; *thi* stands for thiamine.

on mulberry. Spontaneous rifampicin resistant mutants were obtained by plating ca. 10^{10} cells on KB agar containing 100 $\mu\text{g/ml}$ rifampicin.

Detection of plasmids. Bacteria were grown overnight in liquid KB medium at 30 C and cleared lysate containing plasmid DNA was extracted according to the method of Birnboim and Doly (1979)¹⁾. Plasmid DNA were analysed by electrophoresis at 50 mA and 100 volts in 0.7 % agarose (Sigma, Type I, Low EEO) gel with Tris-borate buffer (89 mM Tris base, 2.5 mM disodium EDTA and 8.9 mM boric acid). The gels were stained with ethidium bromide (1 $\mu\text{g/ml}$) and photographed under short wave UV illumination. The sizes of the plasmids were estimated by comparison with eight bands of the plasmids in *E. coli* V 517 (Macrina *et al.*⁶⁾) in the same gel.

Plasmid transfers. Overnight cultures of donor and recipient cells were mixed in the ratio of 1:1 and collected on 0.45 μm Millipore filter. The filters were, then, incubated on KB agar at 30 C for 5 to 12 hr. After mating, cells were washed and resuspended in 1 ml of sterile distilled water and 0.1 ml of appropriate dilutions were plated on selective media. Donor and recipient cells were plated separately on the same selective media as the controls.

Results

Detection of indigenous plasmids in Pseudomonas syringae pv. mori

DNA extracted from 17 isolates of *P. s. mori* contained 2 to 4 indigenous plasmids whose size ranged from approximately 4.5 to 70 mdal (Fig. 1, Table 2). Although the detail relationships among the plasmids were not examined, 8 classes were distinguished based on their size. Thereby, *P. s. mori* tested here was classified into 9 groups based on their plasmid patterns (Table 2). Definite role of each plasmid determining some phenotypes such as toxigenicity of halo blight producing strains, phage sensitivity and carbohydrate (rhamnose or xylose) utilization could not be established. But all the halo

Fig. 1. Agarose gel (0.7%) electrophoresis of indigenous plasmid DNA from *Pseudomonas syringae pv. mori*.

Sample wells contained DNA from strains S6801(A), S6802(B), S6803(C), S6804(D), S6805(E), S6806(F), S6807(G), S6808(H), S6809(I), S6810(J), M5(K) and S6914-1(L), and halo blight strains S7133-2(M), S7133-3(N), S7233-1(O) S7233-2(P) and S7233-3(Q). The diffuse band present in all wells is chromosomal DNA.

Table 2. Classification of *Pseudomonas syringae* pv. *mori* strains according to plasmid content and an association between plasmid and some phenotypes

Bacterial isolates	Plasmid content ^{a)}								No. of plasmid	Grouping ^{b)}	Halo toxin	Lysotype ^{c)}	Decomposition ^{d)} of	
	A	B	C	D	E	F	G	H					Rhamnose	Xylose
S6801	-	+	+	-	-	+	-	-	3	I	-	E	+	+
S6802	+	-	+	-	-	-	-	-	2	II	-	C	-	+
S6803	-	-	++ ^{e)}	-	-	+	-	-	3	III	-	C	-	-
S6804	+	+	-	-	-	-	+	+	4	IV	-	B	+	+
S6805	+	-	+	-	-	-	-	-	2	II	-	Un-determined	-	+
S6806	+	-	+	-	-	-	-	-	2	II	-	C	-	+
S6807	-	-	+	+	-	-	-	-	2	V	-	A	+	+
S6808	-	-	+	+	-	-	-	-	2	V	-	E	+	+
S6809	-	+	+	-	-	+	-	-	3	I	-	B	+	+
S6810	-	+	+	-	-	+	-	-	3	I	-	B	+	+
M5	+	-	+	-	-	+	-	-	3	VI	-	NT ^{f)}	NT	NT
S6914-1	-	+	+	-	-	+	-	-	3	VII	-	A	NT	NT
S7133-2	+	-	+	-	+	-	-	-	3	VIII	+	NT	NT	NT
S7133-3	+	-	-	+	-	+	-	-	3	IX	+	NT	NT	NT
S7233-1	+	-	-	+	-	+	-	-	3	IX	+	NT	NT	NT
S7233-2	+	-	-	+	-	+	-	-	3	IX	+	NT	NT	NT
S7233-3	+	-	-	+	-	+	-	-	3	IX	+	NT	NT	NT

a) Molecular weight of plasmids determined by coelectrophoresis with a DNA standard of *E. coli* V517⁹⁾ is following: A; 70 mdal, B; 60. C; 50, D; 35, E; 28, F; 25, G; 4.8, H; 4.5.

b) Grouping by plasmid pattern.

c) Lysotype has been determined by the drop method using 6 strains of virulent phage in *P. s. mori*¹³⁾.

d) That has been examined by production of acid on Ayers' medium with each carbohydrate¹⁴⁾.

e) Two plasmids, very similar in size.

f) Not tested.

blight-causing strains contained the largest plasmid designated A, although some non-halo producing strains also had this plasmid.

Conjugative mobilization of RSF1010 from *Pseudomonas syringae* pv. *mori* to *Escherichia coli*

The large size of several of the *P. s. mori* plasmids suggested that they may be conjugative. This was tested in an indirect way: A non-conjugative but mobilizable plasmid RSF1010, which encodes for streptomycin- and sulfonamide-resistance, was introduced into rifampicin resistant mutant of *P. s. mori* by mating with *P. syringae* pv. *tabaci* BR2(RSF1010). BR2 contains the indigenous conjugative plasmid pBPW1 which mobilizes RSF1010 readily to a number of bacteria. After mating, RSF1010⁺ transconjugants were selected by plating on KB agar with rifampicin (100 µg/ml) and streptomycin (20 µg/ml). The presence of RSF1010 and the absence of pBPW1 in the transconjugants were confirmed by observing the corresponding bands of DNA extracts on agarose gel electrophoresis. RSF1010 was transferred in this way to all the isolates of *P. s. mori* examined at frequencies of 1.2×10^{-1} to 1.8×10^{-5} per recipient cell.

Transconjugants containing RSF1010 but not pBPW1 were subsequently mated with *E. coli* SK1592 selecting for transfer of streptomycin resistance. Transmission of RSF 1010 was again confirmed by gel electrophoresis. All isolates except S6809 were

Table 3. Conjugative mobilization of RSF1010 from *Pseudomonas syringae* pv. *mori* to *Escherichia coli* SK1592

<i>P. syringae</i> pv. <i>mori</i> (donor)	Transfer frequency per recipient cell	Characterization of the transconjugants ^{a)}		
		Su	Sm	RSF1010
S 6801 rif (RSF1010)	1.6×10^{-5}	+	+	+
S 6802 rif (RSF1010)	5.9×10^{-8}	+	+	+
S 6803 rif (RSF1010)	1.5×10^{-9}	+	+	+
S 6804 rif (RSF1010)	1.8×10^{-8}	+	+	+
S 6805 (RSF1010) ^{b)}	3.1×10^{-8}	+	+	+
S 6809 rif (RSF1010)	ND ^{c)}			
M 5 rif (RSF1010)	2.0×10^{-8}	+	+	+
S 6914-1 rif (RSF1010)	1.2×10^{-9}	+	+	+
S 7133-2 rif (RSF1010)	1.9×10^{-9}	+	+	+
S 7133-3 rif (RSF1010)	5.5×10^{-9}	+	+	+
S 7233-1 rif (RSF1010)	2.0×10^{-9}	+	+	+
S 7233-2 rif (RSF1010)	6.3×10^{-9}	+	+	+
S 7233-3 rif (RSF1010)	4.1×10^{-9}	+	+	+

a) Su: Resistant to sulfonamide, Sm: Resistant to streptomycin. Presense of RSF 1010 was confirmed by agarose gel electrophoresis.

b) S6805 (RSF1010) was obtained by the transformation with RSF1010 from *Pseudomonas putida* AC808. All other strains were constructed by mating with *P. s. tabaci* BR2(RSF1010), but none of them contained the pBPW1 plasmid indigenous to this strain.

c) Not detectable.

capable of donating RSF1010 in these crosses although at exceedingly low frequency (Table 3). This suggests that the strains tested possess an indigenous fertility system. However, this system either is hardly utilized to mobilize RSF1010 or is naturally repressed.

Discussion

Our studies showed that plasmids are ubiquitous among *P. s. mori* strains as in other plant pathogenic bacteria. At present, their functions are unknown and no apparent correlations between these plasmids and phage sensitivity, carbohydrate utilization, pathogenicity or fertility properties were noted in our studies. However, there may be a correlation among plasmid A and toxin production, because all "halo" strains have this plasmid. In order to clarify the functions of plasmid A and of the others, however, further studies and detailed molecular characterization of them are necessary. Unlike some plasmids in other plant pathogenic bacteria^{3,16)}, *P. s. mori* plasmids do not appear to have derepressed fertility functions, as judged by their inability to mobilize RSF1010. This conclusion is further supported by the inability to demonstrate the transmission of the transposable element Tn7 from *P. s. mori* strains to *E. coli* and the other bacteria even in the presence of conjugative plasmid RPl, while transmission of this element can be clearly demonstrated in *P. s. tabaci* BR 2 carrying the pBPW1 plasmid, whether the transposon is inserted either into pBPW1 itself or into the chromosome of this bacterium (Sato *et al.*¹⁵⁾ and our unpublished data). At least some *P. s. mori* strains tested were

ineffective donors of plasmid RPl even though they accepted the plasmid freely by conjugation. We speculate that these strains may be "fertility sinks" for transmissible plasmids. However, they clearly have good recipient ability for the pBPW1 mating system and for RPl too as well. Our findings of the ability to obtain transposon insertions¹⁵⁾ and of the ability of RSF1010, useful cloning vector, to replicate stably in *P. s. mori* strains will permit us further genetic studies of this bacterium.

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和 文 摘 要

クワ縮葉細菌病菌 *Pseudomonas syringae* pv. *mori* のプラスミド

佐藤 守・Brian J. STASKAWICZ・Nickolas J. PANOPOULOS

クワ縮葉細菌病菌 *Pseudomonas syringae* pv. *mori* の各菌株は、分子量約4.5～70メガダルトンのプラスミドを、それぞれ2～4個、保有していた。供試17菌株は、そのプラスミドパターンから9群に分けることができた。これらプラスミドパターンとハロー毒素産生能、ファージ感受性あるいは他の性状との間には明らかな関係を認められなかった。

供試したすべての菌株は、*P. syringae* pv. *tabaci* BR2 の伝達性プラスミド pBPW1 によって非伝達性プラスミド RSF1010 を高頻度に受容し、かつ、それを大腸菌（受容菌）に、非常に低率ではあるが、再伝達した。このことは、本菌のプラスミドが抑制された状態で存在するか、あるいは RSF1010 を低率で可動化できる固有の接合システムをもっていることを示している。