Grouping *Pseudomonas syringae* Strains by Comparing DNA Homology at the *hrp* Gene Cluster and Its Neighboring Regions

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Abstract

We compared the structure and homology of the *hrp* gene cluster and its neighboring region for grouping *Pseudomonas syringae* pathovars. Cosmid clones selected from genomic libraries constructed for *P. syringae* pv. *glycinea*, pv. *maculicola* and pv. *tabaci* covered a genomic region of more than 80 kb that was homologous to *hrp* genes and neighboring regions in pv. *phaseolicola*. Comparison of restriction sites and homologies of this large genomic region revealed that the structures of the *hrp* gene clusters of pv. *glycinea*, pv. *phaseolicola* and pv. *tabaci* closely resemble each other, whereas that of pv. *maculicola* is distinct from those of the other three pathovars. Hybridization probes derived from pv. *phaseolicola* and pv. *maculicola* were used for grouping 32 pathovars of *P. syringae* by Southern hybridization analysis. Patterns of hybridization signal strength suggested that *P. syringae* pathovars can be classified into at least the following three groups: strains having strong homologies to probes from pv. *phaseolicola* (group I), strains having strong homologies to probes from pv. *maculicola* (group II) and strains having moderate to no homology to probes from either of the pathovars (group U).

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Key words: *Pseudomonas syringae*, grouping, DNA homology, *hrp* cluster and its neighboring regions.

INTRODUCTION

Pseudomonas syringae has a number of pathovars that are distinct in their host plants and/or disease symptoms. *P. syringae* is identified with several characteristics such as fluorescent pigment production, lack of soft rotting of potato and a positive hypersensitive reaction on tobacco plant, but each pathovar and strain has considerable phenotypic variations in its nutritional and physiological characteristics^{3,9,24,29}. In recent years, DNA-DNA hybridization and the polymerase chain reaction technique have been used to distinguish the strains or pathovars of the *P. syringae* group bacteria and to classify *P. syringae* into several groups^{7,8,10,14}.

Causing disease on host plants and eliciting the hypersensitive response (HR) on non-host plants are fundamental features controlled by the *hrp* genes of *P. syringae*. Originally describing the *hrp* genes in *P. s.* pv. *phaseolicola* (*Psp*) by Lindgren *et al.*¹⁶, indicated that Tn5-induced mutants of *Psp* had lost both the ability to induce halo-blight disease on host plants and the HR on non-host plants. Furthermore, these mutants were complemented by cosmid clones from a genomic library of

the wild-type strain, resulting in isolation of a cluster of *hrp* genes localized in about the 20 kb DNA region¹⁶. Thereafter, Lindgren *et al.*¹⁷ reported that sequences homologous to DNA segments derived from this region were detected in many pathovars of *P. syringae*, and that a Tn5-induced mutation of an *hrp* gene was transferred from the mutant clone of *Psp* to *P. s.* pv. *glycinea* (*Psg*) and *P. s.* pv. *tabaci* (*Pst*) strains by marker exchange mutagenesis but not to *P. s.* pv. *tomato*. These resulting progenies were phenotypically negative for pathogenicity and HR¹⁷. Since then, *hrp* genes were found in *P. s.* pv. *syringae*¹³, *Ralstonia* (*Pseudomonas*) *solanacearum*⁶, *Erwinia amylovora*²⁸ and some pathovars of *Xanthomonas campestris*^{1,5,30}. These *hrp* genes from different strains, species or genera hybridized to each other to varying degrees. In addition, amino acid sequences of several *hrp* gene loci have homology to secretion proteins of animal pathogens^{4,31}. Interestingly, nonphytopathogenic *P. fluorescens* share homology with *hrp* gene loci from *Pst*²¹. These reports let us infer that sequences similar to *hrp* genes are widely distributed in several bacteria and are differentiated to various functional genes at the evolutionary stage. On the other hand, a cluster of *avrE* genes from pv. *tomato*¹⁸, *avrPphE* gene

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from *Psp*²⁰⁾ and *hrmA* gene from pv. *syringae*¹¹⁾ which are adjacent to a cluster of *hrp* genes participating in the manifestation of pathogenicity. Pathogenicity to plants is one of the most important characters of *P.*

syringae. Therefore, this region including the *hrp* genes and their neighbors could be used as significant factors for characterizing *P. syringae*.

Parts of *hrp* genes have been used to compare

Table 1. Bacterial strains

Species and pathovar	Strain		Host or origin
<i>Pseudomonas syringae</i>			
pv. <i>aceris</i>	kaede1-1	(# 440)	<i>Acer buergerianum</i>
<i>actinidiae</i>	KW11	(# 320) PT	kiwi
<i>aptata</i>	SB8601	(# 560)	sugar beet
<i>atropurpurea</i>	NIAES1309	(# 1678)	Italian ryegrass
<i>coronafaciens</i>	AVPCO8101	(# 196)	oat
<i>delphinii</i>	PDDCC529	PT	delphinium
<i>dendropanacis</i>	kakuremino-1	(# 453) PT	<i>Dendropanax trifidus</i>
<i>eriobotryae</i>	PERB8031	(# 930)	logquat
<i>glycinea</i>	r0		soybean
<i>japonica</i>	BPST802	(# 137)	barley
<i>lachrymans</i>	cucum-1	(# 105)	cucumber
<i>lapsea</i>	NCPBP2096	PT	corn
<i>maculicola</i>	ICMP3935	PT	cauliflower
	PMC8301		turnip
	R101		radish
	R1		radish
“ <i>magnoliae</i> ” ^{a)}	PMG8101	(# 183)	magnolia
<i>mori</i>	mori1	(# 131)	mulberry
<i>morsprunorum</i>	U7805	(# 408)	peach
<i>myricae</i>	yamamomo801	(# 166)	Japanese bay berry
<i>oryzae</i>	1-2	(# 541) PT	rice
<i>phaseolicola</i>	3121		bean (15)
	KZ2W	(# 191)	kudzu vine
<i>photinae</i>	photinia-1	(# 733)	<i>Photinia glabra</i>
<i>pisi</i>	pisum-1	(# 679)	pea
<i>porri</i>	ICMP8961		leak
<i>sesami</i>	PSES-1	(# 1472)	sesame
“ <i>spinaciae</i> ” ^{a)}	spin8605	(# 1449)	spinach
<i>striaefaciens</i>	avena2	(# 110)	oat
<i>syringae</i>	LOB2-1	(# 458)	lilac
<i>tabaci</i>	113A		tobacco
<i>theae</i>	tea632	(# 68)	tea
<i>tomato</i>	ICMP2844	PT	tomato
(<i>kiwi</i>) ^{b)}	KW741	(# 794)	kiwi
(<i>onion</i>) ^{c)}	ICMP3414		onion
<i>Pseudomonas</i>			
<i>cichorii</i>	TPS ash8101	(# 178)	tomato
<i>corrugata</i>	TSN8212	(# 385)	tomato
<i>ficuserectae</i>	L-7	(# 1391) T	<i>Ficus erecta</i>
<i>viridiflava</i>	hakusai801	(# 113)	chinese cabbage
<i>fluorescens</i>	IFO3507	(# 1023)	
<i>aeruginosa</i>	ZPB11	(# 572)	
<i>Eshelicia</i>			
<i>coli</i>	B		IAM1268 ^{e)}
	DH5 α ^{d)}		(24)

Numbers in parentheses correspond to collection number in the Shizuoka University Plant Pathology (SUPP) culture collection. PT, Pathotype strain. T, Type strain.

a) Pathovar names not validly published yet.

b) Unnamed pathovar from blossom rot of kiwi fruit.

c) Unnamed pathovar from bacterial leaf spot of onion.

d) This strain was used only for cloning.

e) This strain comes from the Institute of Applied Microbiology, the University of Tokyo.

Table 2. Vector plasmids and clones

	Relevant characteristics	Reference or source
Plasmid		
pGEM-7ZF(+)	cloning vector, Amp ^r	Promega
pLAFR3	cosmid vector, Tc ^r	(27)
pPL6	pLAFR3 containing <i>hrp</i> cluster of <i>P. syringae</i> pv. <i>phaseolicola</i> NPS3121	(16)
pDC541	pLAFR3 containing <i>hrp</i> and <i>avrE</i> cluster of <i>P. syringae</i> pv. <i>tomato</i> DC3000	(18)
Genomic library		
pSPlib.2	pLAFR3 cosmid library of <i>P. syringae</i> pv. <i>phaseolicola</i> NPS3121	(16)
pGlib.	pLAFR3 cosmid library of <i>P. syringae</i> pv. <i>glycinea</i> r0	This study
pMlib.	pLAFR3 cosmid library of <i>P. syringae</i> pv. <i>maculicola</i> PMC8301	This study
pTlib.	pLAFR3 cosmid library of <i>P. syringae</i> pv. <i>tabaci</i> 113A	This study

homology and restriction fragment length polymorphism for grouping of *P. syringae*^{15,17,25}). However, a structural comparison of this entire large genomic region including the *hrp* gene cluster has not been performed. Such a comparison might give us information on the structural similarity of the genomes of these organisms. This information is never obtained through single gene analysis and may reflect more precisely the history of organisms than single gene analysis.

In this study, we describe the utility of a comparison of large genomic regions including *hrp* and its neighboring regions for grouping of *P. syringae* pathovars.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and culture conditions Bacterial strains and plasmids are shown in Tables 1 and 2. *Pseudomonas* strains were grown in YP medium (yeast extract 5 g, peptone 10 g, water 1 liter, pH 6.8) with or without agar at 27°C. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium¹⁹) with or without agar at 37°C. Antibiotics were used at 50 µg/ml for ampicillin and 30 µg/ml for tetracycline.

DNA preparation DNA was prepared as described by Ausubel *et al.*²⁾. Genomic DNAs for Southern hybridization were extracted from pathovars of *P. syringae* and *Pseudomonas* spp. using the CTAB procedure. Plasmid DNA was isolated from *E. coli* using the boiling and alkaline lysis methods¹⁹⁾. Total DNA for construction of genomic libraries was prepared using the SDS-lysis method and purified by CsCl/EtBr gradient centrifugation. Restriction fragments for subcloning and probes for Southern hybridization were isolated from agarose using GeneClean II kit (Bio101 Inc.) according to the manufacturer's instruction.

Cloning procedures Restriction fragments were cloned into pLAFR3²⁷⁾ or pGEM7f+. Ligation was performed with T4 DNA ligase as described by Ausubel *et al.*²⁾. Packagene kit (Promega Biotech. Inc.) was used for *in vitro* packaging as recommended by the supplier.

Southern hybridization Southern hybridization was performed with a Genius kit (Boehringer Mann-

heim). Template DNAs for probes (1–2 µg) were labeled by random primed incorporation of digoxigenin-labeled dUTP. After DNA samples were digested with appropriate restriction endonucleases, 0.5 or 5 µg DNA were electrophoresed in 0.7% agarose gels and transferred onto a nylon membrane. Hybridization was performed as recommended by the supplier for 16 hr at 68°C. The membranes were washed twice in wash buffer 1 (2× SSC, 0.1% SDS) at room temperature for 5 min and then in wash buffer 2 (0.5× SSC, 0.1% SDS) at 68°C for 15 min before immunological detection.

Screening of clones including regions homologous to *hrp* gene and neighbor region The 17.0 kb *EcoRI* fragment from pPL6 containing *hrp* genes of *Psp*¹⁶⁾ was used as the hybridization probe for screening clones from each genomic library. Selected clones and subclones were digested with appropriate restriction endonucleases and used as hybridization probes for genome walking. The cosmid clone pDC541 containing *hrp* and *avrE* genes of pv. *tomato*¹⁸⁾ was used to detect *avrE* genes from pv. *maculicola* (*Psm*).

RESULTS

Comparison of restriction sites and homologous regions in four pathovars of *P. syringae* using selected clones

Cosmid clones selected from the genomic library of *Psm* covered a genomic region of over 80 kb. Also, the clones from *Psg*, *Psp* and *Pst* covered over 100 kb of the region containing the *hrp* genes of each pathovar. Genomic libraries of *Psm* did not yield any clones that linked *hrpD* to *hrpE* but they did hybridize with all *hrp* genes probes from *Psp*. These clones were digested with appropriate restriction endonucleases, and restriction site maps were constructed (Fig. 1). Several DNA fragments from *Psp* and *Psm* were used as Southern hybridization probes (Fig. 2). Comparison of restriction sites the large genomic region revealed that the latter three pathovars closely resembled each other, whereas *Psm* was quite different from the three pathovars (Fig. 1). The map right outside the *hrp* homologous region of *Psm* closely resembled that of the *avrE* locus of pv.

Fig. 1. Restriction enzyme map of *Pseudomonas syringae* pathovars. Filled patterns indicate homology region. Narrow lines mean getting clones. Dotted line is representation of a gap. Vertical lines and alphabet on the line mean the cutting site: B, *Bam*HI; E, *Eco*RI; H, *Hind*III.

Fig. 2. Hybridization probes to compare homology in *Pseudomonas syringae*. Letters under filled patterns correspond to letters for probes in Tables 3 and 4. Letters on the vertical lines indicate restriction enzyme sites: H, *Hind*III; K, *Kpn*I.

*tomato*¹⁸⁾. Hereafter, we tentatively refer to the “right or left” of the *hrp* region, devoting the direction containing *hrpR* or *hrpL*, respectively. The cosmid clone pDC541 from pv. *tomato* strain DC3000¹⁸⁾ involving *avrE* locus was hybridized to *Psm* clones (Fig. 2; l, m and n). Further, parts of the DNA sequences in fragment l and m correspond to *hrpS* and transcription unit II of *avrE*. These results suggested that this region was equivalent to *avrE*. Furthermore, this region had homology to the other three pathovars in exactly the same position. The next right side of the deduced *avrE* regions of *Psm* (Fig. 2, o) had no homology to the clones from the other pathovars. For the *hrp* gene cluster and its left outside, homologies were detected reciprocally by all probes used but hybridization signals varied in their patterns and strength. Fragments j and l from *Psm* were detected

weakly, indicating homology signals against other pathovars.

Comparison of homology in *hrp* and its neighboring region among *P. syringae*

All hybridization probes from *Psp* and *Psm* were used to detect homologies among the various *P. syringae* pathovars listed on Table 3. Some probes (a, b and i), which are remotely located from the *hrp* gene cluster, hybridized to all bacterial species tested. Probes corresponding to the *hrp* region (c, d, e and K) hybridized to all *P. syringae* strains with relatively homogeneous strength. Other probes (f, g, h, j, l, m, n and o) hybridized to a wide variety of strains belonging to *P. syringae* pathovars with significant variations in homology signal strength.

Patterns of hybridization signal strength grouped *P.*

Table 3. Summary of hybridization results

	Probes from pv. <i>phaseolicola</i>								Probes from pv. <i>maculicola</i>						
	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o
<i>P.s. phaseolicola</i> ^{a)}	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.glycinea</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.tabaci</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.lachrymans</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.mori</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.dendropanacis</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.eriobotryae</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.myricae</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.photinae</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.sesami</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.?(kiwi)</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.ficuserectae</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.maculicola</i> (R1)	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.maculicola</i> ^{b)}	+	+	+	+	+	+	+	—	+	+	+	+	+	+	+
<i>P.s.tomato</i>	+	+	+	+	+	+	+	—	+	+	+	+	+	+	+
<i>P.s.theae</i>	+	+	+	+	+	+	+	—	+	+	+	+	+	+	+
<i>P.s.delphinii</i>	+	+	+	+	+	+	+	—	+	+	+	+	+	+	+
<i>P.s.magnoliae</i>	+	+	+	+	+	+	+	—	+	+	+	+	+	+	+
<i>P.s.actinidiae</i>	+	+	+	+	+	+	+	—	+	+	+	+	+	+	+
<i>P.s.spinaciae</i>	+	+	+	+	+	+	+	—	+	+	+	+	+	+	+
<i>P.s.syringae</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.lapsa</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.japonica</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.apitata</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.aceris</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.pisi</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.coronafaciens</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.striaefaciens</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.?(onion)</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.atropurpurea</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.oryzae</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.porri</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.s.morsprunorum</i>	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—
<i>P.viridiflava</i>	+	+	—	—	+	—	—	—	+	—	—	—	—	—	—
<i>P.cichorii</i>	+	+	—	—	+	—	—	—	+	—	—	—	—	—	—
<i>P.corrugata</i>	+	+	W	+	W	—	—	+	+	+	—	—	—	—	—
<i>P.fluorescens</i>	+	+	W	+	W	—	—	+	+	+	—	—	—	—	—
<i>P.aeruginosa</i>	+	+	—	—	W	—	—	—	+	—	—	—	—	—	—
<i>E.coli</i>	+	+	—	—	—	—	—	—	+	—	—	—	—	—	—

+, Hybridization signal was detected; —, Hybridization signal was not detected; W, Not clear but broad and weak hybridization signal observed.

a) Containing two strains.

b) Containing three strains.

syringae strains into at least three types (Table 4). The first group contained strains of pvs. *phaseolicola*, *glycinea*, *tabaci*, *lachrymans*, *mori*, *dendropanacis*, *eriobotryae*, *myricae*, *photinae*, *sesami*, an undetermined pathovar causing kiwifruit blossom rot disease and *P.ficuserectae*. This group (hereafter referred to as group I) showed strong homology to probes from *Psp* (f, g and h), and low homology to probes from *Psm* (j, l, m, n and o). The second group contained strains of pvs. *maculicola*, *tomato*, *theae*, *delphinii*, *actinidiae*, “*spinaciae*” and “*magnoliae*”. In contrast to group I, this group (group II) showed strong homology to probes from *Psm* and

moderate or no homology to probes from *Psp*. The third group is negatively defined as strains showing moderate or low homology to probes of either *Psp* or *Psm*. Therefore, this group will be referred to as group U (U for “undetermined”).

DISCUSSION

Comparison of homology and restriction sites of over 100 kb large genomic region demonstrated that *Psp*, *Psg* and *Pst* are much alike not only in the *hrp* gene cluster but also in its neighboring region. Previous reports

Table 4. Summary of hybridization results

		Probes from pv. <i>phaseolicola</i>			Probes from pv. <i>maculicola</i>				
		f	g	h	j	l	m	n	o
1,2	<i>P.s.phaseolicola</i> ^{a)}	+++	+++	+++	++	+	++	++	+
3	<i>P.s.glycinea</i>	+++	+++	+++	++	+	++	++	+
4	<i>P.s.tabaci</i>	+++	+++	+++	++	+	++	++	+
5	<i>P.s.lachrymans</i>	+++	+++	+++	++	+	++	++	+
6	<i>P.s.mori</i>	+++	+++	+++	++	+	++	++	+
7	<i>P.s.dendropanacis</i>	+++	+++	+++	++	+	++	++	+
8	<i>P.s.eriobotryae</i>	+++	+++	+++	++	+	++	++	+
9	<i>P.s.myricae</i>	+++	+++	+++	++	+	++	++	+
10	<i>P.s.photinia</i>	+++	+++	+++	++	+	++	++	+
11	<i>P.s.sesami</i>	+++	+++	+++	++	+	++	++	+
12	<i>P.s.?(kiwi)</i>	+++	+++	+++	++	+	++	++	+
13	<i>P.ficuserectae</i>	+++	+++	+++	++	+	++	++	+
14	<i>P.s.maculicola</i> (R1)	++	++	++	++	+	++	++	+
15,16,17	<i>P.s.maculicola</i> ^{b)}	++	++	—	+++	+++	+++	+++	+++
18	<i>P.s.tomato</i>	++	++	—	+++	+++	+++	+++	+++
19	<i>P.s.theae</i>	++	++	—	+++	+++	+++	+++	++
20	<i>P.s.delphinii</i>	++	++	—	+++	+++	+++	+++	++
21	<i>P.s.magnoliae</i>	++	++	—	+++	+++	+++	+++	+++
22	<i>P.s.actinidiae</i>	++	++	—	+++	+++	+++	+++	+++
23	<i>P.s.spinaciae</i>	++	++	—	+++	+++	+++	+++	+++
24	<i>P.s.syringae</i>	++	++	++	++	+	++	++	+
25	<i>P.s.lapsa</i>	++	++	++	++	+	++	++	+
26	<i>P.s.japonica</i>	++	++	++	++	+	++	++	+
27	<i>P.s.apitata</i>	++	++	++	++	+	++	++	+
28	<i>P.s.aceris</i>	++	++	++	++	+	++	++	+
29	<i>P.s.pisi</i>	++	++	++	++	+	++	++	+
30	<i>P.s.coronafaciens</i>	++	++	++	++	+	++	++	+
31	<i>P.s.striaefaciens</i>	++	++	++	++	+	++	++	+
32	<i>P.s.?(onion)</i>	++	++	++	++	+	++	++	+
33	<i>P.s.atropurpurea</i>	++	++	++	++	+	++	++	+
34	<i>P.s.oryzae</i>	++	++	++	++	+	++	++	+
35	<i>P.s.porri</i>	++	++	++	++	+	++	++	+
36	<i>P.s.morsprunorum</i>	++	++	++	++	+	++	++	+
37	<i>P.viridiflava</i>	—	—	—	—	—	—	—	—
38	<i>P.cichorii</i>	—	—	—	—	—	—	—	—
39	<i>P.corrugata</i>	—	—	+	+	—	—	—	—
40	<i>P.fluorescens</i>	—	—	+	+	—	—	—	—
41	<i>P.aeruginosa</i>	—	—	—	—	—	—	—	—
42	<i>E.coli</i>	—	—	—	—	—	—	—	—

+++ , Strong hybridization signal was always detected; ++ , Hybridization signal was detected but not always at 0.5 μ g blotting; + , Weak hybridization signal was detected but not always at 5 μ g blotting and was not detected at 0.5 μ g blotting; — , No hybridization signal.

a) Containing two strains.

b) Containing three strains.

described that *hrp* genes of *Psg* and *Pst* can be mutated by marker exchange mutagenesis with the *hrp* gene clone of *Psp* harboring a Tn5 insertion in it, and that these mutants can be restored to a wild-type phenotype upon introduction of clones carrying the corresponding wild-type locus from *Psp*¹⁷⁾. At the left outside of *hrpL* region of *Psp* exists *avrPphE*²⁰⁾. This region was detected on *Psp* and *Pst* but not on *Psg*. On the contrary, our studies detected homology to this region in *Psg* and showed that restriction sites in this region are conserved. These results suggest that these three patho-

vars are genetically closely related. On the other hand, *Psm* has a distinct restriction site map and different homology pattern from the other three pathovars. This result predicts that *Psm* may have differentiated from other pathovars belonging to the same *P. syringae* at an early time in evolution.

The restriction site map, DNA homology and DNA sequence led to the conclusion that the right end and right outside of the *hrp* homologous region from *Psm* corresponded to *hrpZ*, *hrpS* and *avrE* region of pv. *tomato*. *Psm* and pv. *tomato* closely resemble each other

Fig. 3. Southern blot analysis of *Eco*RI-digested 5 μ g (upper row) or 0.5 μ g (lower row) of genomic DNA of *Pseudomonas syringae* strains using probe h (A) and probe o (B). Probe names are shown in Fig. 2. Lane numbers correspond to numbers of Table 4.

in several phenotypic characteristics²⁹). These results suggested that *Psm* and *pv. tomato* have a genetically close relationship. This genomic region of *Psm* is considered to be an *avrE* gene.

The 2.3 kb *Hind*III fragment containing the deduced *hrpS* to *hrpZ* genes from *Psm* (Fig. 2, 1) showed weak homology signals to *Psp*, *Psg* and *Pst*. The *hrpZ* gene and its neighboring region encode harpin and secretion

protein²³). The *hrpZ* from *pv. tomato* has high homology to *hrpZ* from *pv. syringae* and *Psg*, but ORF1 (called *hrpA* but not identical to *hrpA* of *Psp*) has little homology to others. Therefore, this fragment showed weak homology signals to other strains.

At the right outside of the *avrE* region, homology signals were not continuously detected between *Psm* and the other three strains. However, clones containing loci

homologous to this region of *Psp* but not continuing to the *avrE* region were isolated from a genomic library of *Psm*. These results suggest that gene rearrangement might occur in this region.

When parts of *hrp* and its neighboring region from *Psp* and *Psm* were used as hybridization probes, the strains of *P. syringae* classified into three groups named group I, group II and group U. Bacterial strains used in this study are the representatives of each pathovar or species. Some are pathotype or type strains as shown in Table 1, and others have been identified in our laboratory as typical through bacteriological and pathological properties. Therefore, we can speculate that this grouping may reflect relationships between each pathovar.

We had expected that the grouping of *P. syringae* pathovars based on the similarity of *hrp* and neighboring genomic regions might reflect to some extent their pathogenicity, *i.e.* symptom and host range, and that the grouping might be contradictory, in some point to reported groupings based on phenotypic traits or total DNA homology. The result was unexpected as each of our three groups contained various types of pathovars having distinct host ranges and symptoms. Rather, our grouping agreed with those based on phenotypic traits and total DNA homology. For example, pvs. *phaseolicola*, *glycinea*, *mori*, *lachrymans* and *tabaci* are reported to form one group and pv. *tomato*, *maculicola* and *delphinii* are also suggested to form another group based on phenotypic traits and total DNA homologies^{12,22,26,29}. Our result coincided with those groupings and further assigned several more pathovars to each groups.

Concerning the pathogenic traits, one distinct feature can be identified. All tested strains causing knots or galls on trees are grouped in group I together with *Psp* and *Psg*. Another well-known gall-forming bacterium *P. s. pv. savastanoi* was not used in this study. Gardan *et al.*⁸) reported by result of DNA-DNA hybridization that *Psp* and *Psg* were related to pv. *savastanoi*, which may support our hypothesis that *P. syringae* pathovars causing knot or galls are close allies. We are now preparing to investigate directly whether some strains of pv. *savastanoi* really belong to our group I.

Group I strains had similar RFLP patterns when digested with *EcoRI*, showing small number of restriction site variations; however, group II and group U had large variations in terms of RFLP patterns.

We used four strains of *Psm* which correspond to four groups of *Psm*. Takikawa *et al.*²⁹) reported that *Psm* is classified in four groups based on phenotypic and pathogenetic properties. In this study, one of them, strain R1 (group IV of *Psm*, corresponding to radish leaf-spot-forming strains), was shown to be distinct on the basis of homology pattern of the *hrp* and its neighboring chromosome. Our unpublished data also suggested that the strains of the phenotypic group IV of *Psm* are distinct genetically, phenotypically and pathologically, showing the heterogeneity of *Psm* strains.

Three DNA probes from *Psm*, *i.e.* the right outside of

the *avrE* region, the 2.3 kb *HindIII* fragments containing *hrpS* through *hrpZ* and the left outside of *hrpL*, specifically hybridized to the seven strains of group II, whereas the probe from *Psp* containing the right outside of the *avrE* region specifically hybridized to group I strains. These probes may be useful for discriminating homology groups, because they reflect not quantitative but qualitative differences.

Our study left fourteen strains as group U, *i.e.* unclassified. They are clearly heterogeneous, although some closely resembled each other. Among them, pv. *syringae* and pv. *coronafaciens* represent two more distinct groups, as suggested by our preliminary studies. Preparation of probes from these bacteria and a comparison of the homology of these groups are under way.

Our study may lead not only to developing rapid detection and identification methods but also to understanding the genetic structure and evolution of *P. syringae* group bacteria. In particular, the relationship between the evolution of *hrp* genes and the evolution of the total genome is expected to be resolved.

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和 文 摘 要

井上康宏・瀧川雄一： *hrp* 遺伝子群およびその周辺領域の相同性の比較による *Pseudomonas syringae* 群細菌の類別

P. syringae 群細菌の病原型 (pathovar) 間の遺伝子レベルでの相違を調査するために, *P. s.* pv. *glycinea* (*Psg*), pv. *maculicola* (*Psm*), pv. *phaseolicola* (*Psp*), pv. *tabaci* (*Pst*) の遺伝子ライブラリーより *hrp* 遺伝子とその周辺領域のクローンを選抜し, DNA の相同性と制限酵素地図の比較を行った。*Psg*, *Psp*, *Pst* は調査した全領域において相同性が高く, 制限酵素地図がよく一致していたが, *Psm* は相同性は認められるもの

の制限酵素切断部位は全く異なっていた。*hrpRS* の右外側領域はすべての菌株において *P. s. pv. tomato* から分離報告されている *avrE* 領域と相同性を示した。*avrE* 相同領域の外側では *Psm* と他の菌株との間に相同性が検出できなかった。他の *P. syringae* 群細菌の病原型間において *Psp* と *Psm* より作成したプローブを用いてゲノミックサザンを行った結果、*hrp* 領域の

周辺においては、比較的均一で高い相同性が確認された。*avrE* 相同領域およびその外側領域では *Psp* 由来のプローブと高い相同性を示すグループ (group I)、*Psm* 由来のプローブと高い相同性を示すグループ (group II)、いずれに対しても相同性を検出できないか低い相同性のみを示すグループ (group U) に類別された。