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Phylogenetic Analysis of the pPT23A Plasmid Family of *Pseudomonas syringae*▽

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The pPT23A plasmid family of *Pseudomonas syringae* contains members that contribute to the ecological and pathogenic fitness of their *P. syringae* hosts. In an effort to understand the evolution of these plasmids and their hosts, we undertook a comparative analysis of the phylogeny of plasmid genes and that of conserved chromosomal genes from *P. syringae*. In total, comparative sequence and phylogenetic analyses were done utilizing 47 pPT23A family plasmids (PFPs) from 16 pathovars belonging to six genomospecies. Our results showed that the plasmid replication gene (*repA*), the only gene currently known to be distributed among all the PFPs, had a phylogeny that was distinct from that of the *P. syringae* hosts of these plasmids and from those of other individual genes on PFPs. The phylogenies of two housekeeping chromosomal genes, those for DNA gyrase B subunit (*gyrB*) and primary sigma factor (*rpoD*), however, were strongly associated with genomospecies of *P. syringae*. Based on the results from this study, we conclude that the pPT23A plasmid family represents a dynamic genome that is mobile among *P. syringae* pathovars.

Pseudomonas syringae is a fluorescent plant-pathogenic pseudomonad clustering within rRNA similarity group I of the genus *Pseudomonas* (23). The species is subdivided into at least 51 pathovars, which are mostly described on the basis of plant host range (52). Thus, although the *P. syringae* species as a whole causes plant diseases on a multitude of hosts, individual *P. syringae* pathovars typically have a limited host range of one to a few plant species. A comprehensive genetic analysis has indicated the existence of nine discrete genomospecies on the basis of total DNA-DNA homology and ribotyping (12). Individual genomospecies had DNA relatedness values between 66 and 100% with ΔT_m values of 0.0 to 4.5°C (12); these values are consistent with those used by others in delineating bacterial species (5, 27). Thus, the pathogen *P. syringae* is genetically diverse, presumably due to the adaptation of individual pathovars to their respective host plant environments.

Initial characterization of plasmids within the *P. syringae* species revealed that individual plasmids from different *P. syringae* pathovars could share extensive sequence homology (24, 47). Further understanding of the interpathovar relationship of *P. syringae* plasmids occurred following the subcloning of replication sequences from the 80-kb plasmid pOSU900 of *P. syringae* pv. syringae (19). A probe constructed from the pOSU900 replication sequences was later shown to hybridize to several native plasmids (50 to 80 kb) isolated from a distinct population of *P. syringae* pv. syringae (36, 37). Murillo and Keen (20) cloned the replication sequences from the 100-kb plasmid pPT23A of *P. syringae* pv. tomato PT23 and demonstrated that these sequences hybridized to additional plasmids within strain PT23 and to plasmids of a variety of sizes from a diversity of *P. syringae* pathovars. This important work was then followed by the sequencing of the replication gene (*repA*) from pPT23A (13) and the definition of the pPT23A plasmid family as a group comprised of plasmids mostly ranging from approximately 35 to 100 kb (with a few exceptions) that are believed to have originated from a common ancestor because they share a gene (*repA*) that encodes an essential replication protein (33). To date, pPT23A family plasmids (PFPs) have been confirmed in strains from 19 pathovars from four genomospecies (32, 41, 54); it should be noted that PFPs have been detected in all pathovars examined; thus, the current pathovar distribution is limited only in that additional pathovars have not been tested. The pPT23A plasmid family also appears to be unique in that a number of *P. syringae* strains harbor two to as many as six PFPs (32). While conventional wisdom would suggest that plasmids sharing highly similar replication sequences would not be capable of inhabiting the same bacterial cell, PFPs overcome this potential problem by an as-yet-unknown mechanism.

Genomic analyses have provided glimpses of the gene content of PFPs and similarities and differences among PFPs from various *P. syringae* pathovars. Eight complete PFP sequences are currently known including pFKN from *P. syringae* pv. maculicola (26), pDC3000A and pDC3000B from *P. syringae* pv. tomato (6), pPSR1 from *P. syringae* pv. syringae (40), pMA4326A and pPMA4326B from *P. syringae* pv. maculicola

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(34), and pPph1448A and pPph1448B from *P. syringae* pv. phaseolicola (17). These sequencing studies, combined with the results of many previous studies, indicate that genes encoding effector proteins of importance to host-pathogen interactions, other determinants involved in virulence and epiphytic fitness of *P. syringae*, determinants increasing strain survival on plants sprayed with agricultural bactericides, genes encoding plasmid replicative and transfer functions, and mobile genetic elements have been identified on PFPs from various pathovars of *P. syringae* (reviewed in reference 46). Notable examples include the *avrPphF* locus of pAV511 from *P. syringae* pv. phaseolicola, which contains two open reading frames that confer avirulence on bean cultivars carrying the *R1* resistance gene and contribute to virulence on other bean cultivars and on soybean (44), the biosynthetic gene cluster for the phytotoxin coronatine on pPG4180A from *P. syringae* pv. glycinea (1), and the *rulAB* locus on pPSR1 from *P. syringae* pv. syringae, which encodes tolerance to UV radiation (38, 39).

An understanding of the distribution of particular PFP-carried genes within and among pathovars should provide important clues in the evolutionary development of these plasmids. Determinants such as *rulAB* and *iaaL*, which are thought to be important to the survival and growth of *P. syringae* in the leaf surface or phyllosphere environment, are found on PFPs from at least 17 to 20 pathovars (14, 41). In contrast, genes encoding effectors, such as *avrPpiA1* and *avrRPM1*, are limited in distribution to only a few pathovars (54). A recent comparative genomic approach examining 31 PFPs revealed that the plasmids could be subdivided into major groups based on the conjugative transfer (type IV secretion) system encoded (54). Two distinct transfer systems were found, a type IVA (VirB-VirD4) system and a type IVB (Tra) system, and these systems were detected in 27 of 31 PFPs examined (54).

Our long-term goal is to understand the molecular evolution of the pPT23A plasmid family, with one goal being to catalog the acquisition of genes by PFPs, track the reassortment of these genes at several levels within the *P. syringae* species (for example strains within populations, pathovars, and genomospecies), and identify and determine the functional significance of novel adapted alleles (mediated through base alterations). Here, we performed a comprehensive comparative sequence and phylogenetic analysis of the pPT23A plasmid family. Our experiments were designed to answer the following questions: (i) whether the pPT23A family plasmids have a phylogeny that is distinct from that of their *P. syringae* hosts; (ii) whether the individual genes on pPT23A family plasmids have a phylogeny that is distinct from that of *repA*; and (iii) in situations where strains harbor more than one PFP, whether the relationship of the *repA* genes of those plasmids suggests intrastrain duplication events or acquisition of new plasmids.

MATERIALS AND METHODS

Bacterial strains and plasmid isolation. The bacterial strains and plasmids utilized in this study are listed in Table 1. To extract genomic and plasmid DNA, *P. syringae* strains were grown at 28°C overnight with continuous shaking at 250 rpm in mannitol-glutamate broth supplemented with 0.1% yeast extract (MGY). Genomic DNA was extracted by using the DNeasy tissue kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions. Plasmids were extracted by a modified alkaline lysis method (18, 54). Briefly, bacterial cells were resuspended in E buffer (40 mM Tris-acetate, 2 mM EDTA, pH 7.9) and lysed by the addition of 2 volumes of lysing buffer (50 mM Tris, 3% sodium dodecyl sulfate, pH 12.6). Cell lysates were then incubated at 65°C for 40 min followed by extractions with phenol-chloroform and chloroform. Plasmid DNA in the supernatants was then precipitated using ethanol and resuspended in Tris-EDTA buffer. For purification, plasmids were separated in 0.7% agarose gels in Tris-acetate-EDTA buffer and then individual plasmids were isolated from the agarose gel using the QIAEX II agarose gel extraction kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions.

PCR amplifications and DNA sequencing. The chromosomal genes for DNA gyrase B subunit (*gyrB*) and primary sigma factor (*rpoD*) were amplified with the PCR primer pair gyrB-F–gyrB-R and rpoD-F–rpoD-R (Table 2), respectively (31). PCR amplifications were performed in a 50-μl reaction volume containing 1× PCR buffer, 2 mM $MgCl_2$, 0.75 mM of each deoxynucleoside triphosphate, 1 pmol of each primer, 1.0 U *Taq* polymerase, and 50 ng genomic DNA. PCR amplifications were carried out as follows: one cycle at 94°C for 5 min, 35 cycles at 94°C for 1 min, 60°C for the primer pair gyrB-F–gyrB-R or 55°C for the primer pair rpoD-F–rpoD-R for 1 min, 72°C for 1.5 min, and a final extension at 72°C for 10 min. PCR products were purified using the Gel Extraction kit (QIAGEN Inc., Valencia, CA), and the purified fragments were sequenced with the primers M13-40 and M13-R (Table 2) at the Genomics Technology Support Facility at Michigan State University.

The replication gene (*repA*) from individual PFPs was amplified by using the primer pair 532-F–1588-R (Table 2) (32), which flank a fragment of 1,399 bp containing 1,279 bp of the *repA* coding region plus 120 bp upstream of the putative start codon (13), and the remaining coding sequences of *repA* were amplified using a pair of degenerate primers, repA-F1–repA-R2 (Table 2). PCR amplifications were performed in a 50-μl reaction volume containing 1× PCR buffer, 4 mM $MgCl_2$, 0.75 mM of each deoxynucleoside triphosphate, 1 pmol of each primer, 1.0 U *Taq* polymerase, and 1 μl purified plasmid DNA. PCR amplifications were performed using the following parameters: one cycle at 94°C for 5 min, 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min, and a final extension at 72°C for 10 min. PCR amplifications using the primer pair repA-F1–repA-R2 were the same as those for the primer pair 532-F–1588-R except that 2 mM $MgCl_2$ and an annealing temperature of 50°C were used. Additionally, the conserved hypothetical protein gene (B0046), the GntR family transcriptional regulator gene (B0077), and the TraY protein gene (*traY*), which are located on PFPs (40), were amplified with primer pairs B0046-F–B0046-R, B0077-F–B0077-R, and traY-F–traY-R, respectively (Table 2). These primers amplified fragments consisting of nucleotides (nt) 8 to 450 of B0046, 6 to 405 of B0077, and 211 to 1109 of *traY*. PCR amplifications using these primer pairs were the same as those for the primer pair repA-F1–repA-R2 except that an annealing temperature of 57°C was used. PCR products were purified using the Gel Extraction kit, and purified fragments were cloned into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced using the primers SP6 and T7 at the Genomics Technology Support Facility at Michigan State University.

Phylogenetic analysis. Phylogenetic analyses were performed on individual gene sequences using PAUP*4.0b10 (42). Nucleotide sequences were aligned using Clustal W (43). Phylogenies were constructed in PAUP*4.0 using neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood methods. Neighbor joining trees were generated using the algorithm of Saitou and Nei (29) with Kimura two-parameter distances. Maximum parsimony and maximum likelihood analyses employed heuristic searches with 10 sequences of random taxon addition and tree bisection and reconnection (TBR) branch swapping. Appropriate evolutionary models for use in maximum likelihood analyses were determined for each data set using Modeltest version 3.7 (25). The NJ tree was specified as the starting tree for maximum likelihood searches. Support for groups obtained in phylogenetic analyses was assessed by bootstrap analysis using PAUP*4.0. We assessed congruence between phylogenetic trees obtained using different gene sequences by comparing the trees with respect to bootstrap support for alternate groupings. Support for alternate placements between the same taxa in different trees was considered to exist when the two different data sets both provided strong support for the competing relationships (50).

Analysis of *repA* nucleotide diversity. Sliding window analysis of *repA* nucleotide diversity π was conducted using the program DnaSP version 3.51 (28). The alignment consisted of 1,314 nucleotides of the *repA* coding region. Sites with gaps (positions 1120 to 1131 of *repA* nucleotide sequences) were excluded from the analysis. π is the average number of nucleotide differences per site between two sequences, and the sliding window method allows a value of π to be assigned at the midpoint of a window of defined length. In our analysis, we used a window of 100 nucleotides and measured π every 5 nucleotides.

TABLE 1. *Pseudomonas syringae* strains, plasmids, and sequences used in this study

Genomospecies	*P. syringae* pathovar	Strain	Host	*rpoD*	*gyrB*	Plasmid[b]	*repA*	*traY*	B0046	B0077	Reference/ source[d]
I	pisi	870A	Pea	AB016388	AB016387	pAV230	X[a]	–[c]	–	–	9
						pAV231	X	–	–	–	
	syringae	A2	Ornamental pear	X	X	pPSR1	X	–	–	–	40
		B86-17	Snap bean	X	X	pB86-17A	X	–	–	–	41
		HS191	Millet	X	X	pCG131	X	X	X		33
		4918	Butterfly pea	X	X	pPSS4918	X	–	–	–	AKV
II	aesculi	0893-23	Indian horse chestnut	X	X	pPA0893A	X	–	–	X	DAC
						pPA0893B	X	–	–	X	
						pPA0893C	X	–	–	X	
	ciccaronei	2342	Carob tree	X	X	p2342A	X	X	X	X	12
	glycinea	Race 6	Soybean	AB016324	AB016323	pREP601	X	–	–	–	32
						pREP603	X	–	–	–	
	mori	1642	Mulberry	X	X	p1642A	X	–	–	X	DAC
	phaseolicola	1390	Snap bean	X	X	p1390A	X	X	X	X	DAC
						p1390C	X	X	X	X	
		1302A	Snap bean			pAV505	X	–	–	–	15
		1449B	Snap bean			pAV511	X	–	–	–	15
						pAV512	X	–	–	–	
		1448A	Snap bean	AAZ36734	AAZ34879	pPh1448A	X	–	–	–	17
						pPh1448B	X	–	–	–	
	savastanoi	0485-9	Oleander	X	X	pPS0485A	X	–	–	X	JM
						pPS0485B	X	–	–	X	
						pPS0485C	X	–	–	X	
		0693-10	Oleander	X	X	pPS0693A	X	–	–	X	DAC
		1670	Olive	X	X	p1670A	X	–	–	X	DAC
						p1670B	X	–	–	X	
	tabaci	0893-29	Tobacco	X	X	pPSTA0893A	X	X	X	X	DAC
								–	–	–	
III	maculicola	M6	Crucifers	AY610898	AY610778	pFKN	X	–	–	–	26
		ES4326	Crucifers	AY610897	AY610777	pPMA4326A	X	–	–	–	34
						pPMA4326B	X	–	–	–	
		88.10	Cauliflower	X	X	pPSM8810	X	X	–	X	49
		90.32	Cauliflower	X	X	pPSM9032A	X	X	X	–	10
						pPSM9032B	X	X	X	X	
	persicae	1573	Peach	X	X	p1573C	X	X	X	X	12
	tomato	DC3000	Tomato	NC_004578	NC_004578	pDC3000A	X	X	–	–	6
						pDC3000B	X	–	X	X	
	tomato	UPN2A	Tomato	X	X	pPT23A	X	X	X	X	33
		UPN2B	Tomato			pPT23B	X	X	X	X	32
		OK-1	Tomato	X	X	pOK-1A	X	X	X	X	33
IV	garcae	2708	Coffee	X	X	pPG2708	X	X	X	X	NCPPB
		1634	Coffee	X	X	p1634A	X	X		X	13
						p1634B	X	X		X	
V	tremae	6111	*Trema orientalis*	X	X	p6111A	X	–	–	–	12
VIII	avellanae	4060	Hazelnut			p4060B	X	X	X	X	12
						p4060C	X	X	–	X	
	theae	2353	Tea	X	X	p2353B	X	X	X	X	12
Outgroup		LRB3W1		AB204720	AB204718						

[a] New sequences obtained in this study are designated by an X.
[b] GenBank accession numbers for complete plasmid sequences are as follows: pDC3000A, NC_004633; pDC3000B, NC_004632; pFKN, NC_002759; pPSR1, NC_005205; pPMA4326A, NC_005918; pPMA4326B, NC_005919; pPH1448A, NC_007274; pPH1448B, NC_007275.
[c] – indicates that the sequence was not recovered from the plasmid.
[d] Abbreviations: AKV, A. K. Vidaver; DAC, D. A. Cooksey; JM, J. Murillo; NCPPB, National Collection of Plant Pathogenic Bacteria, Sand Hutton, York, United Kingdom.

Nucleotide sequence accession numbers. GenBank accession numbers for sequences of the chromosomal genes *gyrB* and *rpoD* and the plasmid genes *repA*, B0046, B0077, and *traY* sequenced in this study were DQ072672 to DQ072693, DQ072694 to DQ072715, DQ072594 to DQ072607, DQ072608 to DQ072633, and DQ072634 to DQ072651, respectively.

RESULTS

Phylogenies of chromosomal genes. The chromosomal genes *gyrB* and *rpoD* were adopted here to determine the course of *P. syringae* genome evolution since they are indispensable single-

TABLE 2. PCR primers used in this study

Primer	Sequence (5′–3′)	T_m (°C)	Target gene or description
532-F	GAACGGTGGACTTATGG	53	Replication gene (*repA*)
1588-R	CTCCAGCTTGCGGCCCC		
repA-F1	AGCTTCAAGAYCAGGGMAA	50	*repA*
repA-R2	ARRTCCATCARYCGGTCRAA		
B0046-F	ATTGGCGTGTTTTGATGCAC	57	Conserved hypothetical protein gene (B0046)
B0046-R	AAGGAAAGCCGCTCTACGG		
B0077-F	TAACCGCAAAACGCAGAAAC	57	GntR family transcriptional regulator gene (B0077)
B0077-R	ACCCAGGACACGCAGGATTT		
traY-F	ATTATCAATGGCGTTCTGGC	57	TraY protein gene (*traY*)
traY-R	TGCTCGCCCAGATTACTCAT		
gyrB-F	CGCCAGGGTTTTCCCAGTCACGACCMGGCGGYAAGTTCGATGACAAYTC	60	DNA gyrase B subunit (*gyrB*)
gyrB-R	TTTCACACAGGAAACAGCTATGACTRATBKCAGTCARACCTTCRCGSGC		
rpoD-F	CGCCAGGGTTTTCCCAGTCACGACAAGGCGARATCGAAATCGCCAAGCG	55	Primary sigma factor (*rpoD*)
rpoD-R	TTTCACACAGGAAACAGCTATGACGGAACWKGCGCAGGAAGTCGGCACG		
M13-40	CGCCAGGGTTTTCCCAGTCACGAC		Sequencing primers
M13-R	TTTCACACAGGAAACAGCTATGAC		

copy genes on which horizontal gene transfer seldom occurs and are widely accepted indices for phylogenetic analyses previously utilized for studies involving *P. syringae* and the *Pseudomonas* genus (30, 31, 51). Comparison of phylogenies constructed from sequences of plasmid genes and phylogenies constructed from sequences of conserved chromosomal genes is an effective method to evaluate the association of specific plasmid types with specific host chromosomal genotypes. In this study, we analyzed in both separate and simultaneous (combined) analyses (7) the partial sequences of *gyrB* (471 bp) and *rpoD* (462 bp) genes from 28 *P. syringae* strains, which belong to 16 pathovars of *P. syringae* and represent six genomospecies (Table 1).

The neighbor joining tree obtained via analysis of the combined (concatenated) *gyrB*/*rpoD* data set (Fig. 1) shows that the genomospecies of *P. syringae*, in general, cluster together very well (with the notable exception of *P. syringae* pv. maculicola ES4326, which has been noted previously as an "oddball" strain [30]). Maximum parsimony analysis of the *gyrB*/*rpoD* data set yielded four equally parsimonious trees (tree length, 418; consistency index, 0.565; retention index, 0.829), which were virtually identical to the NJ tree, except for the positions of taxa within two terminal clusters (*P. syringae* pv. savastanoi/ciccaronei and tomato/maculicola). Modeltest 3.7 was used to determine whether the Tamura-Nei DNA substitution model allowing invariant sites and applying a gamma rate parameter (TrN+I+G) was the appropriate model of DNA sequence evolution for the *gyrB*/*rpoD* data set. The maximum likelihood tree was identical to the NJ tree, with the exception that the positions of *P. syringae* pv. tabaci 0893 to 29 and the *P. syringae* pv. aesculi 0893 to 23/*P. syringae* pv. tremae 6111 cluster were reversed.

Four major clusters in the NJ tree were supported by bootstrap values of >95 in both the NJ analysis and the MP analysis (Fig. 1). First, the genomospecies I strains formed a coherent cluster. Second, there was a cluster consisting of strains belonging to genomospecies groups II and V (V has one strain in the data set, *P. syringae* pv. tremae). A cluster consisting of these first two major clusters was strongly supported by bootstrap analysis. Third, there was a cluster consisting of genomospecies IV strains. Finally, there was a cluster consisting of strains in genomospecies III and VIII, excluding *P. syringae* pv. maculicola ES4326.

We also analyzed the data from *gyrB* and *rpoD* separately. Trees resulting from these analyses each contained all four of the major clusters (described above) and differed only slightly from the combined tree with respect to the position of taxa within the four major clusters (data not shown).

Replication genes (*repA*) of the pPT23A family plasmids. Since the *repA* gene is essential for replication of the PFPs and is the only gene currently known to be distributed among all the plasmids, analysis of *repA* is appropriate in determining phylogenetic relationships among the PFPs. To analyze the phylogenetic relationship of PFPs, we cloned and sequenced the complete *repA* gene from 38 PFPs and included *repA* sequences from nine previously sequenced alleles (Table 1). Phylogenetic analysis of *repA* sequences showed that the 47 *repA* genes from *P. syringae* were clustered into four distinct groups with very high bootstrap support (Fig. 2). Analysis of plasmid host in conjunction with *repA* phylogeny indicated that the *repA* genes were not clustered based on the host strain of their plasmid or pathovar. Plasmids coexisting within single bacterial strains were either clustered into the same group (e.g., p1634A and p1634B in group B and pPMA4326A and pPMA4326B in group D) or into different groups (e.g., pPT23B and pPT23A in groups A and B, respectively). Many plasmids from different strains belonging to different pathovars shared high levels of *repA* nucleotide identity. These results indicated that *repA* from PFPs has a phylogeny that is distinct from that of the *P. syringae* hosts of these plasmids.

Sliding window analysis of nucleotide divergence of the *repA* coding region showed that high levels of variability were observed from nucleotide positions 1000 to 1314 (Fig. 3). This result is in agreement with a previous report that the RepA proteins from PFPs were poorly conserved from residues 374 to 437 at their C-terminal ends but conserved from residues 1 to 373 at their N-terminal ends (33). Additionally, a 12-nt deletion located at 11 nt before the start codon ATG of *repA* was found in 10 plasmids, which were distributed among different *P. syringae* pathovars (data not shown). The 10 plasmids

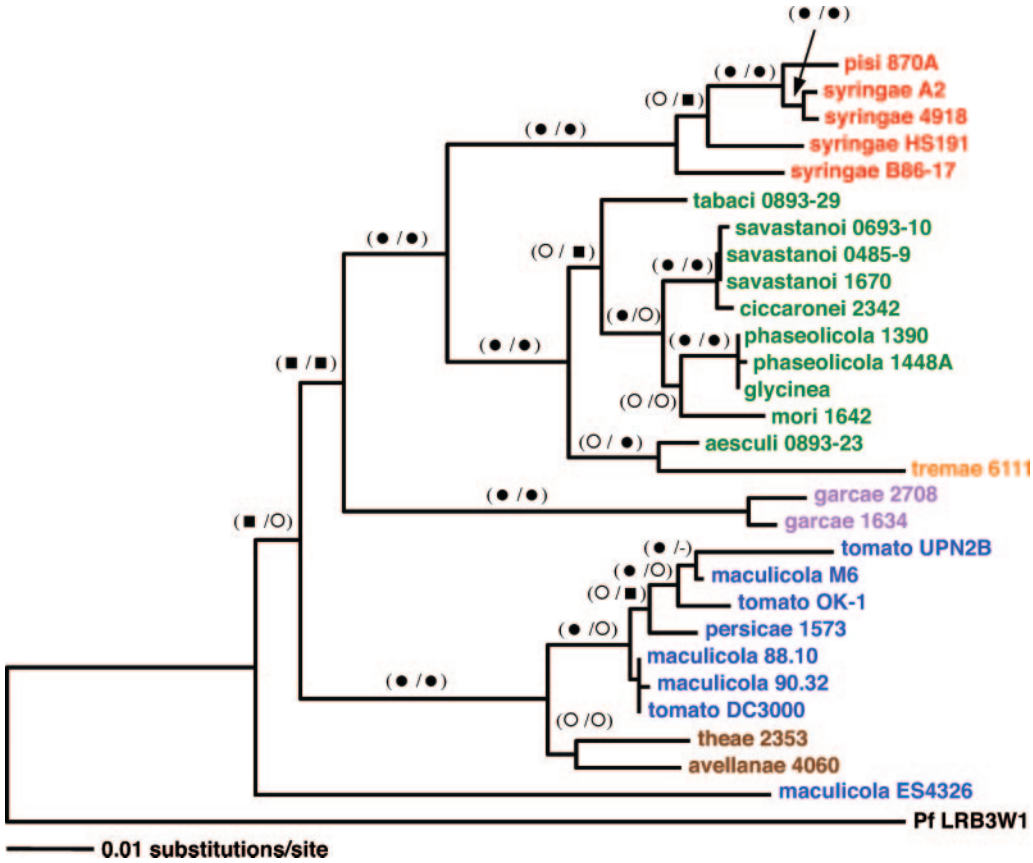

FIG. 1. Phylogenetic analysis of the concatenated *gyrB-rpoD* (genomic) data set from 28 *Pseudomonas syringae* strains with *Pseudomonas fluorescens* LRB3W1 as the outgroup. The tree shown was obtained by NJ using Kimura two-parameter distances. Symbols on branches show bootstrap values with both the NJ (1,000 replicates) and MP (1,000 replicates) bootstrap values shown in parentheses (NJ/MP). Symbols for bootstrap values: ●, >90; ○, 70 to 90; ■, 50 to 70; -, <50. Taxa are color coded by genomospecies (13) as follows: red, genomospecies I; green, genomospecies II; blue, genomospecies III; purple, genomospecies IV; orange, genomospecies V; brown, genomospecies VIII.

having the 12-nt deletion were clustered into different groups based on nucleotide sequences of the *repA* coding region (pPSS4918 and pPSM9032A in group A; pPG2708 and pOK-1A in group B; and the remaining six plasmids pPS0485A/B/C, p1670B, p2342A, and p6111A in group C) (Fig. 2). These results indicated that the 12-nt deletion upstream of *repA* is not associated with nucleotide sequences of the *repA* coding region.

Phylogenies of other genes on PFPs. Except for *repA*, no other gene is known to be distributed among all the PFPs examined (54). The conserved hypothetical protein gene (B0046), the GntR family transcriptional regulator gene (B0077), and the TraY protein gene (*traY*) were selected for phylogenetic analyses of genes other than *repA* on PFPs because these genes were found to be distributed among a large number of PFPs and on PFPs from different groups based on the type IV secretion system encoded (54). Since the plasmid pPG2708 had all three genes examined, it was used as an outgroup for the phylogenetic trees of B0077, B0046, and *traY*. Phylogenetic analysis of DNA sequences of B0046, B0077, and *traY* showed that individual genes on PFPs had a phylogeny that was distinct from that of *repA* (Fig. 2 and 4). Thus, groups supported well by bootstrap analysis in the B0077, *traY*, and B0046 trees are in different places compared to the *repA* tree. For example, based on nucleotide sequences of *repA*, the plasmids p4060B and p2342A from *P. syringae* pv. avellanae and ciccaronei, respectively, are grouped together (Fig. 2); however, the B0077 and *traY* sequences from these plasmids are placed in different groups (Fig. 4). Conversely, both the *traY* and B0046 sequences from pOK-1A and pPT23B and from p1390A and p1390C were similar; however, the *repA* genes from these two sets of plasmids were phylogenetically distinct (Fig. 2 and 4).

Although phylogenies of three genes on PFPs were different from each other (Fig. 4), we observed that the plasmids pPSM8810 and p1390C isolated from genomospecies III and II, respectively, had nearly identical sequences of B0077. Similarly, the plasmids pPSM8810, pDC3000A, pPSM9032A, and pPSM9032B from genomospecies III and the plasmid pCG131 from genomospecies I also had nearly identical sequences of *traY*. These results indicated that interplasmid transfer of genes might have occurred among PFPs in strains of different *P. syringae* genomospecies.

DISCUSSION

The pPT23A plasmid family of *P. syringae* is defined as a group of plasmids presumed to share a common ancestor with all members containing the conserved essential replication

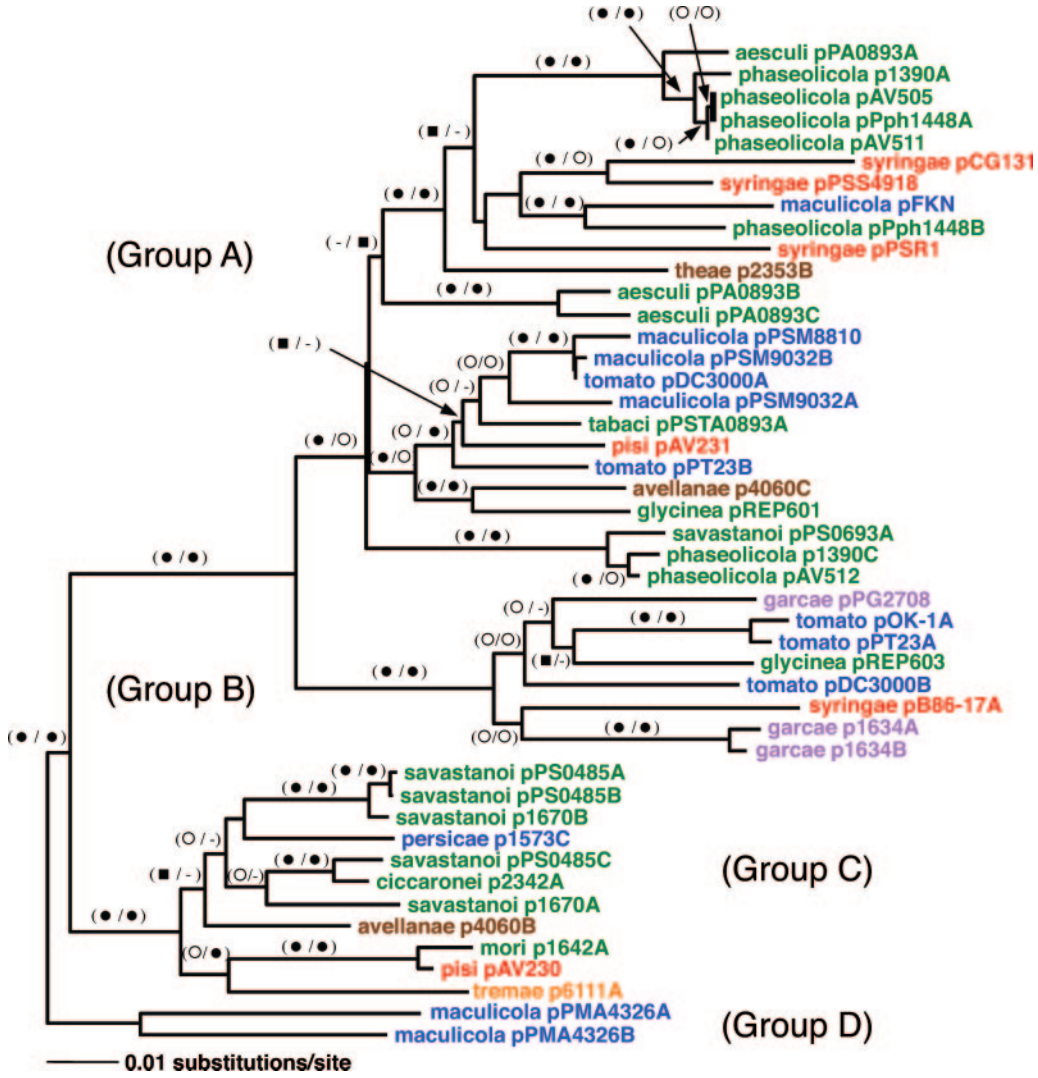

FIG. 2. Phylogenetic analysis of full nucleotide sequences of *repA* of pPT23A family plasmids from *Pseudomonas syringae*, using the *repA* sequences of *P. syringae* pv. maculicola pPMA4326A and pPMA4326B as the outgroup. The tree shown was obtained by neighbor joining using Kimura two-parameter distances. Symbols on branches show bootstrap values with both the NJ (1,000 replicates) and MP (1,000 replicates) bootstrap values shown in parentheses (NJ/MP). Symbols for bootstrap values: ●, >90; ○, 70 to 90; ■, 50 to 70; -, <50. Plasmids are color coded by the genomospecies (13) of the pathovars from which they were isolated as follows: red, genomospecies I; green, genomospecies II; blue, genomospecies III; purple, genomospecies IV; orange, genomospecies V; brown, genomospecies VIII.

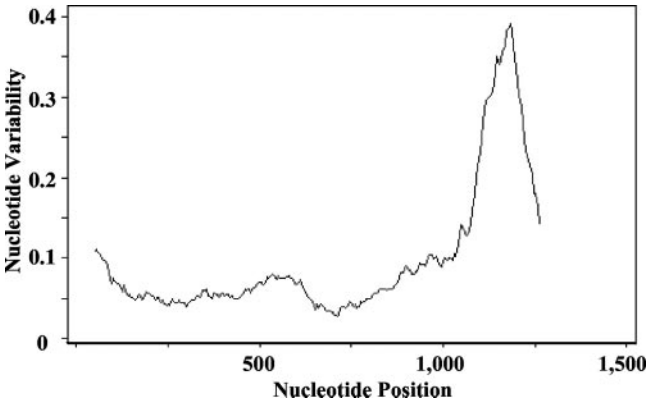

FIG. 3. Sliding window analysis of *repA* nucleotide diversity (π) using the computer program DnaSP. The alignment consisted of 1,314 nucleotides. Sites with gaps (positions 1120 to 1131 of *repA* nucleotide sequences) were excluded from the sliding window analysis.

gene *repA*. With the current known distribution of PFPs in *P. syringae* now expanded to 28 of 51 known pathovars from six of nine known genomospecies, we hypothesize that PFPs are universally distributed among pathovars of this species. Our data indicated that the phylogeny of *repA* is distinct from that of the *P. syringae* host, suggesting that the intrapathovar and interpathovar transfer of PFPs among strains has affected the observed distribution of these plasmids today. pPT23A family plasmids are maintained within *P. syringae* populations through at least three mechanisms: (i) vertical transmission through replication and partitioning of plasmid copies to bacterial daughter cells during cell division, (ii) horizontal transmission via transfer mechanisms such as conjugation, and (iii) integration of plasmid DNA into the host chromosome. The maintenance of PFPs in *P. syringae* strains is also apparently facilitated through the contribution of PFP-carried genes to the

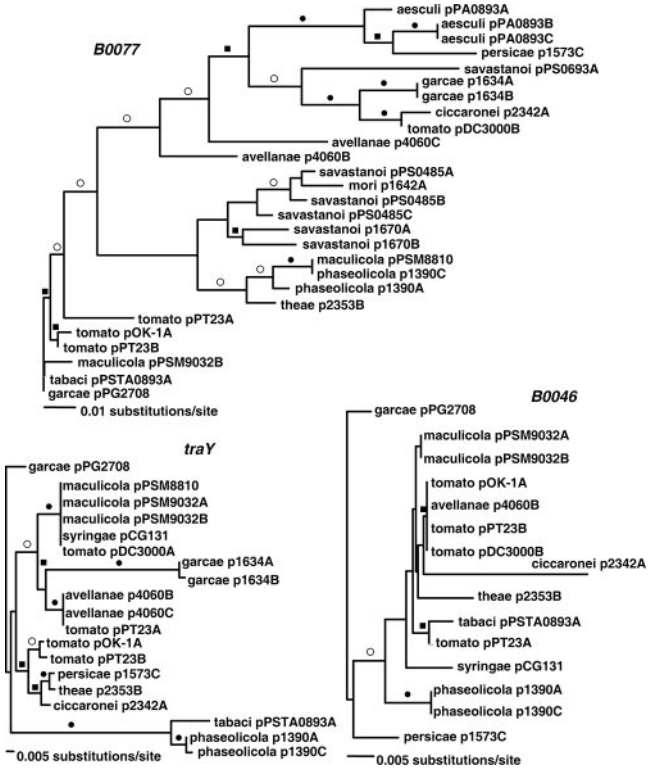

FIG. 4. Neighbor joining analyses using Kimura two-parameter distances of partial nucleotide sequences of plasmid-carried genes from pPT23A family plasmids, using sequences from the *P. syringae* pv. garcae plasmid pPG2708 as the outgroup to allow comparisons across the three genes: B0077 (238 bp), *traY* (294 bp), and B0046 (285 bp). Symbols on branches show NJ bootstrap values (1,000 replicates). Symbols for bootstrap values: ●, >90; ○, 70 to 90; ■, 50 to 70.

ecological fitness and/or virulence of host cells. Thus, an understanding of the evolution of the pPT23A plasmid family in *P. syringae* requires knowledge of plasmid distribution and the distribution of plasmid-specific genes within the divergent pathovars of this species and delineation of the phylogenetic relationships of these genetic determinants. Phylogenetic studies of plasmids must also be framed with the realization that horizontal transfer contributes to plasmid evolution both by providing new bacterial hosts to which the plasmid may be better adapted and by providing opportunities for the acquisition of novel genes.

Conjugation of PFPs has been demonstrated previously, typically involving intrapathovar transfer of plasmids containing bactericide resistance markers (8, 40). Recently, two distinct putative conjugative transfer systems were uncovered in genome sequencing projects (6, 35). Both of these type IV secretion systems were similar to those found in other plant-associated bacteria (54), implying that horizontal plasmid transfer is an important factor in the evolution of plant-pathogenic bacteria. Indeed, although the rhizosphere represents an environmental hot spot for plasmid transfer, conjugation can readily occur in the phyllosphere, a location where most *P. syringae* strains can establish relatively large populations (4, 11, 45).

Horizontal transfer of PFPs is the best explanation for our observation of the phylogenetic incongruence between the *repA* gene of many PFPs and their current bacterial (*P. syringae* pathovar) host. The frequency of occurrence of plasmid transfer between strains remains unknown as is knowledge of the partners in potential interpathovar mating events. In addition, the transfer of PFPs between *P. syringae* strains could yield unpredictable results in that almost all *P. syringae* strains surveyed already contain at least one PFP. We highlight three possible scenarios involving PFP transfer in *P. syringae*. (i) The donor plasmid is compatible with the preexisting PFP in the recipient. Both plasmids can be maintained as separate entities; however, if the plasmids contain homologous sequences, recombination can occur, leading to gene reassortment among plasmids. Gene reassortments would result in phylogenetic incongruence between individual genes and the PFP *repA* gene, as shown in our data (Fig. 4). (ii) The donor plasmid is incompatible with the preexisting PFP in the recipient. During the eventual eviction of one of the plasmids, mobile elements present on the lost plasmid are transposed to the plasmid maintained in the cell. The transposition of the streptomycin resistance transposon Tn*5393* to a novel PFP was demonstrated to occur in this manner (36). A large number of mobile elements, including several insertion sequences, have been detected on PFPs, and the current pathovar distribution of these elements may be reflected in various plasmid transfer events. (iii) The donor plasmid is compatible with the preexisting PFP in the recipient and contains homologous sequences resulting in a recombination event that also involves an extensive duplication. The existence of coresident PFPs containing an extensive amount of duplicated DNA has been shown in at least two *P. syringae* pv. tomato strains (6, 20).

It is generally accepted that plasmids that have related sequences functioning in replication or partition cannot be stably maintained within single cells due to incompatibility effects (3, 21). In this study, however, we observed that some PFPs having closely related *repA* sequences could coexist within single bacterial strains. It was previously postulated that those plasmids might contain another functional origin of replication (32). However, recent sequencing analyses have shown that the coexisting plasmids pDC3000A and pDC3000B in *P. syringae* pv. tomato DC3000 and pPMA4326A and pPMA4326B coexisting in *P. syringae* pv. maculicola ES4326 carried only a single replication gene (6, 34), which suggested that these PFPs might have evolved a mechanism allowing them to escape incompatibility. For example, although the RepA proteins of pPMA4326A and -B shared 96% amino acid identity, they differed in the sequence of a 19-bp putative stem-loop structure located 221 bp upstream of *repA*, which might be complementary to the replication protein C terminus (34), thereby enabling these two plasmids to coexist in one strain.

Our sequencing data for *repA* genes from coexisting PFPs from 14 *P. syringae* strains further suggest that two mechanisms influence the cooccurrence of multiple PFPs in individual strains. The RepA sequences from coexisting plasmids from six strains (*P. syringae* pv. phaseolicola 1448A, *P. syringae* pv. savastanoi 0485-9 and 1670, *P. syringae* pv. maculicola 90-32 and ES4326, and *P. syringae* pv. garcae 1634) were highly similar (Fig. 2), indicating a possibility that microevolutionary mutational alterations resulted in the evolution and stable coexistence of two distinct plasmids. We hypothesize that mutations affecting the RepA amino acid sequence in conjunction with changes affecting the DNA origin of replication target site would optimize this compatible arrange-

TABLE 3. Distribution of selected genes carried on pPT23A family plasmids among *Pseudomonas syringae* pathovars and genomospecies[a]

Gene[b]	Gene product	Function	Genomic location(s)	No. of pvs., gnms	Reference(s)
avrD	Effector	V	P	19, 5	53
avrPphD	Type III effector	V	P	4, 3	54
cor	Coronatine toxin synthesis	V	P, C	5, 3	1
efe	Ethylene synthesis	V	P	5, 3	48
iaaM	Tryptophan 2-monooxygenase	V	P, C	12, 4	14
virPphA	Type III effector	V	P, C	7, 2	15, 16, 54
iaaL	Indole 3-acetic acid–lysine synthetase	Ep	P, C	38, 7	14
lsc	Levansucrase	Ep	P	5, 3	54
mcp	Chemotaxis transducer	Ep	P	3, 3	54
rulAB	UV radiation tolerance	Ep	P, C	17, 4	41, 54
repA	Plasmid replication	Pl	P	28, 6	This study
traY	Conjugation	Pl	P	7, 3	54
virB4	Conjugation	Pl	P	8, 4	54
IS*801*	Insertion sequence	MGE	P, C	10, 4	1, 54
B0046	Conserved hypothetical protein	U	P	9, 5	This study
B0077	GntR family regulator	R	P	13, 4	This study

[a] Abbreviations: C, chromosome; Ep, epiphytic fitness; gnms., genomospecies; MGE, mobile genetic element; P, plasmid; pvs., pathovars; Pl, plasmid related; R, regulatory; U, unknown; V, virulence.
[b] New designations for effector genes: *avrD* = *avrD1*; *avrPphD* = *hopD1*; *virPphA* = *hopAB1*.

ment through rendering each RepA protein specific for its cognate origin sequence. The RepA sequences from coexisting plasmids from seven other strains (*P. syringae* pv. pisi 870A, *P. syringae* pv. glycinea Race 6, *P. syringae* pv. phaseolicola 1390 and 1449B, *P. syringae* pv. tomato DC3000 and PT23, and *P. avellanae* 11144) were distinct and, in most cases, separated into different phylogenetic groups (Fig. 2). In these situations, it is most likely that at least one of the multiple coexisting PFPs was acquired by horizontal transfer. The *P. syringae* pv. aesculi strain 0893-23 was unique in that the strain contained two plasmids with highly similar RepA sequences (pPA0893B and pPA0893C) and a third plasmid (pPA0893A) with a distinct RepA sequence (Fig. 2).

Thus, the pPT23A plasmid family represents a dynamic genome subject to recombination, gene acquisition, gene exchange, and gene delivery to new *P. syringae* hosts. These plasmids represent genetic mosaics, structures that have been observed with many bacterial plasmids (22), and in addition to encoding type III effectors, individual PFPs have been found to carry genes directing the production of the phytotoxin coronatine, epiphytic fitness determinants, and plasmid-specific functions and genes with known homologs whose significance for *P. syringae* fitness remains unknown (1, 2, 6, 14, 15, 16, 34, 40, 46). An analysis of the pathovar distribution of individual genes known to be carried on PFPs illustrates alternative broad and narrow pathovar ranges of specific genes (Table 3). The genomospecies distribution of these genes suggests that many of these sequences are highly mobile within the *P. syringae* species. Genes encoding proteins with plasmid-specific functions or functions predicted to determine traits of general ecological importance to the *P. syringae* species are the most widely distributed; in contrast, certain type III effector genes and other virulence determinants are only rarely observed in the *P. syringae* species (Table 3). In addition, some genes such as *iaaL* and *iaaM* are chromosomally located in a number of *P. syringae* pathovars, further highlighting their mobility (Table 3). Our previous data indicated that PFPs from diverse pathovars could be categorized by the presence of a particular type IV secretion system (type IVA or type IVB) and that certain fitness-enhancing genes were usually associated with plasmids carrying one or the other type IV secretion system (54). The evidence for interplasmid transfer of genes obtained in this study signifies that attempts to reconstruct the evolution of individual PFPs would be difficult.

In summary, our study has demonstrated key tenets in the evolution of the pPT23A plasmid family, namely, that PFPs have been readily transferred among *P. syringae* pathovars and between genomospecies and that individual genes can move from plasmid to plasmid. Because of the importance of horizontal transfer in the evolution of virulence in *P. syringae*, it is likely that PFPs play a critical role in gene acquisition and deployment among strains and pathovars. We are currently pursuing a mechanistic analysis of the molecular evolution of PFPs, with a goal of understanding how these mosaic structures are generated and understanding the selective forces driving maintenance or reorganization of plasmid sequences.

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