Genetic Diversity and Relationships of Two Pathovars of *Pseudomonas syringae*

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To determine genetic relationships within and between two pathovars of *Pseudomonas syringae*, strains typical of *P. syringae* pv. *tomato* (*P. s. tomato*) and selected strains of *P. syringae* pv. *syringae* (*P. s. syringae*) were characterized by three methods. DNA–DNA hybridization experiments showed that strains of *P. s. tomato* and *P. s. syringae* were, respectively, 86–100% and 37–47% homologous to DNA from a *P. s. tomato* reference strain when tested under stringent conditions. An analysis of electrophoretic variation in enzymes encoded by 26 loci placed 17 *P. s. tomato* strains studied in a group of four electrophoretic types, and these strains had a mean genetic diversity per locus of 0.076. Six *P. s. syringae* strains formed a second group of six electrophoretic types, which had a higher mean genetic diversity per locus of 0.479. The mean genetic distance separating *P. s. tomato* from *P. s. syringae* ($D = 0.94$) was unexpectedly large for strains of a single species. An analysis of restriction fragment length polymorphisms (RFLPs) with three cloned hybridization probes demonstrated that each of the *P. s. tomato* and *P. s. syringae* strains was unique. A method was developed to quantify the RFLP difference between pairs of strains, and cluster analysis revealed relationships among *P. s. tomato*, but not among *P. s. syringae*, that were similar to those based on enzyme polymorphisms. Implications of these findings for bacterial systematics and epidemiology are discussed.

INTRODUCTION

Phenotypic characters do not provide an adequate basis for classifying strains of the fluorescent phytopathogenic pseudomonads (Schroth *et al.*, 1981; Palleroni, 1984). Consequently, all oxidase-negative and arginine-dihydrolase-negative fluorescent bacteria currently are classified as *Pseudomonas syringae* van Hall (Palleroni, 1984), and infraspecific pathovars have been designated to meet the needs of plant pathologists concerned with differential pathogenicity of strains (Young *et al.*, 1978). Some pathovars of *P. syringae* can be distinguished by phenotypic characters (Schroth *et al.*, 1981; Hildebrand *et al.*, 1982; Denny, 1988), but in the absence of genetic analyses, the evolutionary relationships and taxonomic status of the pathovars have remained problematical.

The only reported genetic studies concerning the taxonomy of *P. syringae* are those of Palleroni *et al.* (1972) and Pecknold & Grogan (1973), who used DNA–DNA hybridization to subdivide *P. syringae* into three groups. However, only a small number of strains was studied and genetic diversity within the pathovars was not examined.

The objectives of the research reported here were to assess genetic diversity and evolutionary relationships among strains typical of *P. syringae* pv. *tomato* (Okabe) Young, Dye & Wilke (*P. s. tomato*) (Denny, 1988) and a selected sample of *P. syringae* pv. *syringae* van Hall (*P. s. syringae*).

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Abbreviations: RFLP, restriction fragment length polymorphism; ET, electrophoretic type.

The first of three methods of analysis used was DNA-DNA hybridization, which measures the relatedness of entire genomes and is relatively unaffected by point mutations or insertions and deletions of short DNA sequences (Schleifer & Stackebrandt, 1983; Johnson, 1984). This insensitivity to small changes makes DNA-DNA hybridization a poor choice for studying closely related strains. The second method used was multilocus enzyme electrophoresis, which relates polymorphic variation in the electrophoretic mobility of enzymes to allelic variation at the corresponding structural genes. Multilocus enzyme electrophoresis is more sensitive to small differences between strains than is DNA-DNA hybridization and has a number of other advantages that make it useful for studying variation within species (Selander *et al.*, 1986). The third method examined the restriction fragment length polymorphisms (RFLPs) revealed by digesting genomic DNA with restriction endonucleases. RFLP analysis is very sensitive to small changes in DNA organization (Nei & Li, 1979), and has the advantages that it may examine a larger part of the genome than does multilocus enzyme analysis and samples DNA segments without regard to protein coding capacity or expression.

All three methods indicated that the strains of *P. s. tomato* studied are more closely related to each other than to those of *P. s. syringae*, and that the two pathovars are genetically very distinct. These findings have implications for the systematics of these pathovars and provide insight regarding the genetic structure of *P. s. tomato* that will be useful in epidemiological research.

METHODS

Bacterial strains. The strains of *P. s. tomato* and *P. s. syringae* analysed and media used in culturing are described in the preceding paper (Denny, 1988). *Agrobacterium tumefaciens* strain A6 was received from A. Binns, Department of Biology, University of Pennsylvania, USA. *Escherichia coli* strain HB101 was as described in Maniatis *et al.* (1982).

DNA isolation. Total DNA was prepared from *P. s. tomato* and *P. s. syringae* strains by the method of Silhavy *et al.* (1984) with several modifications. Cells were grown overnight at 30 °C in 10 ml Luria broth (LB: tryptone, 10 g; yeast extract, 5 g; NaCl, 5 g; water, 1 l). The OD₆₀₀ of the culture was adjusted to 0.5 with sterile deionized water, and 40 ml of the resulting suspension was centrifuged at 2200 *g* for 20 min. The cell pellet was drained and suspended in 2.5 ml of 100 mM-Tris/HCl (pH 8.0), 100 mM-EDTA. Freshly prepared lysozyme solution (50 µl of a 5 mg ml⁻¹ solution in 250 mM-Tris/HCl, pH 8.0) was added, followed by 2.5 ml sterile deionized water to osmotically shock the cells and enhance enzyme infiltration. The reaction tube was placed on ice for 10 min, then 1.0 ml STEP buffer (0.5% w/v, SDS; 50 mM-Tris/HCl, pH 7.5; 0.4 M-EDTA; 1 mg fresh proteinase K ml⁻¹ was added with gentle mixing and the tube was incubated at 50 °C for 30 min. The lysate was extracted once with buffered phenol/chloroform (1:1, v/v) and once with chloroform, and the DNA was then precipitated with ethanol and spooled out. The DNA was dissolved in 5.0 ml 50 mM-Tris/HCl (pH 7.5), 1 mM-EDTA, 0.2 mg RNAase A ml⁻¹ by gentle overnight mixing at 4 °C. The DNA was again extracted, precipitated, and spooled out as above, dissolved in 2.0 ml TE buffer (10 mM-Tris/HCl, pH 8.0; 1 mM-EDTA), and stored at 4 °C.

DNA cloning. Total DNA from *P. s. tomato* strain B88 was partially digested with *Sau3A*I and size-selected on a linear 10–40% (w/v) sucrose gradient; fractions containing 20–35 kilobase fragments were identified by agarose gel electrophoresis (Maniatis *et al.*, 1982). The DNA was recovered from these fractions and ligated to pLAFR3 (Staskawicz *et al.*, 1987) DNA that previously had been digested with *Bam*HI and treated with bacterial alkaline phosphatase (Maniatis *et al.*, 1982). Ligation products were packaged *in vitro* (Silhavy *et al.*, 1984) and used to transfect *E. coli* strain JM107 (Yanisch-Perron *et al.*, 1985). Recombinants were selected on LB agar plates supplemented with 15 µg tetracycline ml⁻¹, 40 µg X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) ml⁻¹ and 40 µM-IPTG (isopropyl β-D-thiogalactopyranoside) and stored in glycerol at -80 °C (Silhavy *et al.*, 1984).

DNA-DNA hybridization. In preparation for dot-blot analysis of DNA homology, the DNA in a sample of solution was first sheared by forcing it rapidly through a 25 gauge hypodermic needle five or six times. The sheared DNA was then treated with proteinase K [0.1 mg enzyme ml⁻¹; 0.5% SDS; 5.0 mM-EDTA (final concentrations); 1 h, 37 °C], extracted once each with phenol, phenol/chloroform, and chloroform, precipitated with ethanol, and dissolved in 50 mM-sodium phosphate buffer, pH 7.0. The DNA concentrations were determined spectrophotometrically (Maniatis *et al.*, 1982), and the solutions were adjusted to contain 20 µg DNA ml⁻¹ and then frozen. Shortly before use, 5 µl of each DNA solution was treated with 95 µl 1.5 M-NaCl plus 0.1 M-NaOH for 20 min at room temperature and then held on ice. The samples were applied in duplicate to a nitrocellulose membrane in a dot-blot apparatus, and each well was washed with 0.4 ml 0.5 M-Tris/HCl (pH 7.5) plus 0.5 M-NaCl. The filter was removed from the apparatus, dried, and baked at 80 °C in a vacuum oven. Total DNA from *P. s. tomato* strain B88 was nick-translated to incorporate [³²P]dATP (Maniatis *et al.*, 1982) and hybridized under stringent conditions (Denny, 1988). After being washed, the membrane was autoradiographed to check for undesired spot spreading,

and the radioactivity in each spot was quantified by cutting the spot out and counting it in liquid scintillation fluid. Non-specific background was subtracted, and the data were expressed as percentages of the B88 probe hybridizing to itself, which was set at 100%.

RFLP analysis. Total DNA (2–3 µg), prepared as described above, was completely digested with either *Eco*RI or *Pst*I (4.0 mM-spermidine was included in the reaction mixture to facilitate digestion), electrophoresed on 0.6% (w/v) agarose gels, and blotted to nitrocellulose membranes (Smith & Summers, 1980). Selected cosmid clones of *P. s. tomato* DNA were labelled with [³²P]dATP and hybridized to membranes. After being washed, the membranes were autoradiographed to visualize the restriction fragments with homology to the cloned *P. s. tomato* DNA probe.

The two sets of autoradiographs from the pathovars were analysed separately. The first step was to assign, in ascending order, a unique number to each band (i.e. a continuous set of numbered fragments was generated from the six different digest/probe combinations in each set of autoradiographs). Each band was considered a separate character, and the fragment profiles were recorded as a string of ones and zeros, indicating, respectively, the presence or absence of a fragment. The proportion of mismatched fragments between pairs of strains (*D*) was calculated by the equation

$$D = 1 - [2n_{xy}/(n_x + n_y)]$$

where n_{xy} is the number of fragments shared by the two strains and n_x and n_y are the numbers of fragments in strain *x* and strain *y*, respectively. A matrix was constructed with the *D* values for all pair-wise combinations of strains, and cluster analysis was done by the unweighted pair-group method with averages (UPGMA).

Multilocus enzyme electrophoresis. Techniques for preparing soluble extracts of bacterial enzymes, their electrophoresis on starch gels, and the demonstration of enzyme activity by selective staining are described in detail by Selander *et al.* (1986). In addition to buffer systems A (Tris/citrate, pH 8.0), C (Poulík, pH 8.7), D (lithium hydroxide, pH 8.2), E (Tris/malate, pH 7.4), G (phosphate, pH 7.0), and I (Tris/EDTA/borate, pH 8.7), two additional buffer systems were used: J, which uses the electrode buffer of G and a 1:20 dilution of the electrode buffer for the gel (pH 6.7); and K, which is buffer system I modified to contain 2 parts of G plus 78 parts of I for the electrode buffer and 0.78 mM-KH₂PO₄ and 0.12 mM-citric acid in the system I gel buffer, with the pH adjusted to 8.2. The buffer systems used for the 26 enzymes assayed were as follows: buffer A, malate dehydrogenase (MDH) and malic enzyme (MAE); C, phenylalanyl leucine peptidase (PE4); D, hexokinase (HEX), phenylalanyl leucine peptidase (PE3) and α-naphthyl propionate esterase (ES1); E, phosphoglucose isomerase (PGI), glucose-6-phosphate dehydrogenase (G6P), peroxidase (PER), phosphoglucomutases (PG1 and PG2), leucylalanine peptidase (PE1), phenylalanyl leucine peptidase (PE2) and alkaline phosphatase (ALP); G, NADP-dependent glucose dehydrogenase (GDH), adenylate kinase (ADK), nucleoside phosphorylases (NP1 and NP2) and catalases (CT1 and CT2); I, isocitrate dehydrogenase (IDH), indophenol oxidase (IPO) and α-naphthyl acetate esterase (ES2); J, xanthine dehydrogenase (XDH) and NAD-dependent glyceraldehyde phosphate dehydrogenase (GPI); and K, guanine deaminase (GDA). Peptidases were differentiated by mobilities and specificities for four peptidase substrates, but for convenience, three of the peptidases were assayed with phenylalanyl leucine.

Electromorphs of an enzyme were equated with alleles at the corresponding structural gene locus, and electromorph profiles for the set of 26 enzymes assayed were equated with multilocus genotypes. Genetic diversity (*h*) for a locus was calculated as

$$h = \left(1 - \sum x_i^2\right) \cdot [n/(n-1)]$$

where x_i is the frequency of the *i*th allele and *n* is the number of strains or ETs (Selander *et al.*, 1985, 1986). Genetic distance between pairs of strains was calculated as the proportion of mismatched loci, and cluster analysis was done as indicated above.

RESULTS

DNA–DNA hybridization

The *P. s. tomato* strains were from 86 to 131% (= 100%) homologous to ³²P-labelled reference DNA from *P. s. tomato* strain B88, whereas the *P. s. syringae* strains were from 37 to 47% homologous to this *P. s. tomato* DNA (Table 1). The occurrence of values much larger than 100% among the *P. s. tomato* strains indicates that much of the observed variation can be attributed to experimental error. Direct binding assays are inherently variable because of inconsistent binding of DNA to the membrane (Johnson, 1984), and the variation observed among experiments was in line with expectations (see Selander *et al.*, 1985). It is also possible that variation in the quality of the DNA preparations affected the capacity of the bound DNAs to hybridize.

Table 1. *Homology of P. syringae pv. tomato and P. syringae pv. syringae to strain B88 total DNA*

Hybridization of the ^{32}P -labelled B88 DNA to itself was set at 100% in each experiment. Hybridization of the other DNAs to this probe are reported as relative percentages $\pm$ SD based on results from 2 to 5 independent experiments (see Methods). As a test of non-specific binding, *E. coli* strain HB101 was $4.9 \pm 1.0\%$ homologous, and *Agrobacterium tumefaciens* strain A6 was $1.0 \pm 0.7\%$ homologous. Abbreviations are: ATCC, American Type Culture Collection, Rockville, Md., USA; CNBP, Collection National de Bactéries Phytopathogènes, Angers, France; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, UK; PDDCC, Plant Diseases Division Culture Collection, Auckland, NZ.

Strain	Percentage homology	Strain	Percentage homology	Strain	Percentage homology
<i>P. s. tomato</i>		<i>P. s. tomato</i>		<i>P. s. syringae</i>	
B88	100.0	JL1053	106.8 ± 9.2	B48	37.6 ± 2.2
B19	89.5 ± 8.3	JL1060	97.0 ± 2.47	B61	42.2 ± 5.3
B117	105.0 ± 2.6	JL1075	88.9 ± 6.3	B64	37.7 ± 3.1
B118	94.9 ± 4.5	JL1105	91.2 ± 8.3	B76	42.3 ± 2.9
B120	86.9 ± 13.8	JL1120	97.7 ± 6.8	B78	47.1 ± 5.4
B121	104.6 ± 8.0	RG4	105.0 ± 2.83	B84	42.8 ± 4.8
B122	116.5 ± 4.9	NCPPB 2424	121.5 ± 5.0	PSC1B	45.5 ± 2.9
B125	124.5 ± 7.8	NCPPB 880	92.4 ± 3.2		
B181	86.4 ± 13.6	CNBP 1323	108.0 ± 9.9		
B191	92.9 ± 9.0	10862	101.2 ± 11.0		
T4B1	113.0 ± 7.1	30555	119.0 ± 11.3		
AV80	101.2 ± 11.0	31861	131.5 ± 9.2		
JL1031	88.9 ± 10.6	PDDCC 3357	96.7 ± 7.5		

Multilocus enzyme electrophoresis

On the basis of results of phenotypic tests (preceding paper: Denny, 1988), a total of 23 strains of *P. s. tomato* and *P. s. syringae* were selected, assigned code numbers, and analysed in a blind experiment. Within this group, 10 electrophoretic types (ETs) were identified by the electromorph profile for 26 polymorphic enzymes (Table 2). The strains could be divided into two groups: group A contained all 17 *P. s. tomato* strains in the sample and group B all the *P. s. syringae* strains. Most of the *P. s. tomato* strains were identical in multilocus genotype (ET 1), with only four strains representing the other three ETs. In contrast, each *P. s. syringae* strain was distinctive.

Mean genetic diversity (H), which is a measure of the allelic frequency at each locus, was 0.076 for the *P. s. tomato* strains (group A) and 0.479 for the *P. s. syringae* strains (group B) (Table 3). When all the strains were combined (group A plus group B), the mean genetic diversity of 0.444 was lower than that of *P. s. syringae* alone because of the large number of less variant *P. s. tomato* strains. When mean genetic diversity was calculated for ETs, the values for *P. s. tomato* and the combined group increased.

Cluster analysis of genetic distances based on enzyme polymorphisms confirmed the separation of the *P. s. tomato* and *P. s. syringae* strains tested (Fig. 1). On average, pairs of isolates of these two pathovars differed at more than 90% of their loci. All four ETs of *P. s. tomato* were closely related, forming a cluster at a genetic distance of about 0.23. The 13 strains in ET 1 were most closely related to the single strain in ET 2, with differences occurring only at the XDH and CT2 loci. The remaining two ETs in group A differed only at the ES1 locus. ET 3 was represented by the second oldest strain (NCPBP 880 from Yugoslavia) and a strain recently isolated in California (JL1053), and ET 4 consisted of the oldest strain in the collection (ATCC 10862).

The depth of genetic divergence among the six ETs of *P. s. syringae* was greater than that of the four ETs of *P. s. tomato*. The most divergent strain, B61 from wheat (ET 10), was separated from the main cluster that contained the remaining five strains in two subclusters. One subcluster had a second wheat strain (B64, ET 5) and a peach strain (B48, ET 6); the other subcluster had a corn strain (PSC1B, ET 7) and two tomato strains (B76, ET 8; B78, ET 9). It is

Table 2. Allele profiles at 26 enzyme loci for 10 electrophoretic types (ETs) represented by 17 strains of *P. syringae* pv. *tomato* (group A) and 6 strains of *P. syringae* pv. *syringae* (group B)

Twenty-three strains were examined. Multiple strains were observed for ET 1 (strains B19, B88, B118, B120, B121, T4B1, AV80, RG4, JL1120, 30555, NCPPB 2424, CNBP 1323 and PDDCC 3357) and ET 3 (strains JL1053 and NCPPB 880). For these ETs, representative strains are listed. Distinctive mobility variants, designated as electromorphs, were numbered in order of decreasing rate of anodal migration. Electromorphs were equated with alleles at the corresponding gene locus. Absence of enzyme activity attributed to a null allele was designated as 0. Enzyme abbreviations: IPO, indophenol oxidase; IDH, isocitrate dehydrogenase; PG1, PG2, phosphoglucomutases; ES1, α -naphthyl propionate esterase; ES2, α -naphthyl acetate esterase; ALP, alkaline phosphatase; PGI, phosphoglucose isomerase; G6P, glucose-6-phosphate dehydrogenase; ADK, adenylate kinase; GDH, NADP-dependent glucose dehydrogenase; NPI, NP2, nucleoside phosphorylases; MAE, malic enzyme; MDH, malate dehydrogenase; HEX, hexokinase; GDA, guanine deaminase; PE1, leucylalanine peptidase; PE2, PE3, PE4, phenylalanyl leucine peptidases; PER, peroxidase; GPI, NAD-dependent glyceraldehyde phosphate dehydrogenase; XDH, xanthine dehydrogenase; CT1, CT2, catalases.

Reference ET isolate	n	Allele at indicated enzyme locus																									
		IPO	IDH	PG1	PG2	ES1	ES2	ALP	PGI	G6P	ADK	GDH	NP1	NP2	MAE	MDH	HEX	GDA	PE1	PE2	PE3	PE4	PER	GP1	XDH	CT1	CT2
<i>Group A (P. s. tomato)</i>																											
1 AV80	13	1	3	2	4	2	2	1	1	2	2	3	1	2	1	2	2	3	1	5	4	2	1	1	5	1	5
2 B181	1	2	3	2	4	2	1	1	2	2	2	3	1	2	1	2	2	3	1	5	4	2	1	1	0	1	0
3 JL1053	2	1	2	3	2	4	2	1	1	2	2	1	1	2	1	2	2	3	2	4	1	2	1	1	5	1	5
4 10862	1	1	2	3	2	1	2	1	1	2	2	1	1	2	1	2	2	3	2	4	1	2	1	1	5	1	5
<i>Group B (P. s. syringae)</i>																											
5 B64	1	2	4	1	3	2	0	2	2	1	3	2	2	1	2	1	1	1	2	5	2	0	5	2	3	0	4
6 B48	1	2	4	2	1	3	0	2	2	1	1	2	1	1	2	1	3	1	1	5	5	3	2	2	4	0	1
7 PSC1B	1	2	4	1	3	2	0	2	2	1	3	4	1	3	2	1	1	2	1	3	3	1	3	2	2	0	2
8 B76	1	2	5	4	3	3	1	2	2	1	4	4	1	2	1	2	1	2	2	4	2	3	2	3	2	1	0
9 B78	1	2	5	4	3	2	0	2	2	1	4	4	1	1	2	1	1	2	2	1	5	1	3	2	2	0	2
10 B61	1	2	3	1	4	2	0	2	2	1	3	2	2	3	2	3	1	2	3	6	0	3	4	2	1	0	3

Fig. 1. Genetic relationships among 10 ETs of *P. s. tomato* (ETs 1 to 4) and *P. s. syringae* (ETs 5 to 10). The dendrogram was constructed as described in the text based on electromorphs at 26 enzyme loci. See Table 2 for a listing of the strains in each ET.

Table 3. Genetic diversity at 26 enzyme loci among pathovars of *P. syringae*

Mean genetic diversity (H) was calculated by the formula $H = h/n$. The calculations for *P. s. tomato* and *P. s. syringae* used the allele frequencies within each of these two pathovars, whereas the total values were calculated independently from the allele frequencies in the combined sample of strains or ETs.

	No. of :		Percentage polymorphic loci	Mean no. of alleles	Mean genetic diversity	
	Isolates	ETs			Isolates	ETs
<i>P. s. pv. tomato</i>	17	4	30.8	1.3	0.076	0.179
<i>P. s. pv. syringae</i>	6	6	73.1	2.5	0.479	0.479
Total	23	10	100.0	3.5	0.444	0.683

interesting to note that the two tomato strains are so different, since both were isolated during the same year in south Georgia.

RFLP analysis

For this study, three random segments of the genome of *P. s. tomato* strain B88 were cloned in the cosmids pB88-4, pB88-6 and pB88-9; each segment contained approximately 20 kilobases of *P. s. tomato* DNA (about 0.5% of the total genome). These clones were selected because they had 4–10 evenly distributed *Eco*RI or *Pst*I restriction fragments, the same enzymes used to digest the genomic DNA. There was no cross-hybridization among these three probes (data not shown), an important consideration when the data are to be used in constructing genetic distance dendrograms (Lawson *et al.*, 1986). When used as hybridization probes, these clones detected genomic fragments that ranged in size from approximately 1–20 kilobases. Because the resolution of DNA fragments of this size range on standard agarose gels decreases rapidly with increasing size (Maniatis *et al.*, 1982), only 'major' changes in fragment size were detected in this study. Vector DNA did not hybridize to *P. s. tomato* or *P. s. syringae* DNA under the conditions used.

Selected portions of the autoradiographs of the *Eco*RI-digested DNAs probed with the three cosmids are shown in Fig. 2. Cosmid pB88-4, which probed the most variable genomic location,

Fig. 2. Selected RFLPs detected by hybridization of ^{32}P -labelled cosmids of cloned *P. s. tomato* DNA to *Eco*RI-digested *P. s. tomato* genomic DNAs bound to a nitrocellulose filter. (a), (b) and (c), cosmids pB88-4, pB88-6 and pB88-9, respectively, used as the hybridization probes. Genomic DNAs: lane 1, 10862; lane 2, CNBP1323; lane 3, NCPPB 880; lane 4, NCPPB 2424; lane 5, PDDCC 3357; lane 6, AV80; lane 7, RG4; lane 8, T4B1; lane 9, B88. Pointers designate the position of *Eco*RI fragments from the cosmid clones; the largest fragment is the 21.6 kilobase pLAFR3 vector plus the *Eco*RI-*Sau*3AI *P. s. tomato* DNA fragment.

hybridized strongly to a few bands and weakly to many bands (more clearly seen in other exposures that are not shown). Most of the strong bands in the genomic lanes were aligned with the restriction fragments generated by digestion of the cloned probes. One band in the genomic digests will never be in alignment, however, since the *Eco*RI-*Sau*3AI fragment of *P. s. tomato* DNA that remains attached to the vector will hybridize to a genomic fragment of different size. Weakly hybridizing bands were presumed to represent a sequence in the probe that was partially homologous to sequences repeated elsewhere in the genome. Cosmid pB88-6 detected only one polymorphic fragment with two alternative states. No polymorphisms were detected with cosmid pB88-9, indicating a completely conserved region of the genome. These cosmids hybridized weakly to a number of bands in *P. s. syringae* genomic DNA (data not shown).

In analysing the data, we assumed that the regions of the genome examined with the hybridization probes are representative of the entire genome. Because the probes were uncharacterized, however, we could not assume the absence of insertion or deletion mutations; consequently, established methods of analysis could not be applied to the data (Whittam *et al.*, 1986; Nei, 1987). Instead, we analysed the data by a method similar to that used for protein polymorphisms. In this case, the presence or absence of each band was recorded for all the strains, and the proportion of mismatched bands (*D*) calculated as the measure of genetic distance. There were 104 different bands from the *P. s. tomato* strains and 88 different bands from the *P. s. syringae* strains (counting both strongly and weakly hybridizing bands), but no one strain had more than 55% or 36% of these bands, respectively.

An example of the distance values for the comparison of strain B88 to all other *P. s. tomato* strains is given in Table 4. For the purpose of this example only, values were calculated for the individual probes and the different digests; it is the combined values in the last column that were normally calculated and used as part of the distance matrix. This analysis confirmed the conclusions based on visual inspection of Fig. 2: cosmid pB88-4 detected the most variation, cosmid pB88-6 detected relatively little variation, and cosmid pB88-9 detected no variation.

Table 4. *Pairwise comparison of the RFLP patterns of strain B88 with those of selected P. syringae pv. tomato strains*

Each probe (pB88-4, pB88-6 and pB88-9) was hybridized to genomic DNA digested with either *EcoRI* or *PstI* restriction endonucleases. The number of dissimilar restriction fragments in each pairwise combination with strain B88 was determined and distance values were calculated using the formula given in the text. The values in parentheses are the number of restriction fragments that hybridized to a given probe. The number of dissimilar bands in each combination can be calculated by adding the number of bands for strain B88 and the other strain in each pair and multiplying by the distance. When the RFLP pattern of B88 and the paired strain are identical, the distance is zero and the number of fragments was omitted from the Table.

Strain	Distance from strain B88 using:						Combined
	Probe 4		Probe 6		Probe 9		
	<i>EcoRI</i>	<i>PstI</i>	<i>EcoRI</i>	<i>PstI</i>	<i>EcoRI</i>	<i>PstI</i>	
B88	0 (13)	0 (16)	0 (7)	0 (10)	0 (6)	0 (5)	0 (57)
B19	ND	0.385 (10)	ND	0.0	ND	0.0	0.1786
B118	0.200 (12)	0.214 (12)	0.0	0.0	0.0	0.0	0.1009
B120	0.120 (12)	0.133 (14)	0.0	0.0	0.0	0.0	0.0631
B121	0.071 (15)	0.267 (14)	0.0	0.0	0.0	0.0	0.0877
B181	0.037 (14)	0.0 (16)	0.0	0.0	0.0	0.0	0.0087
T4B1	0.111 (14)	ND	0.0	ND	0.0	ND	0.0566
AV80	0.071 (15)	0.267 (14)	0.0	0.0	0.0	0.0	0.0877
JL1053	0.500 (7)	0.259 (11)	0.333 (8)	0.100 (10)	0.0	0.0	0.2115
JL1120	0.154 (13)	0.200 (14)	0.0	0.0	0.0	0.0	0.0893
RG4	0.524 (8)	0.520 (9)	0.143 (7)	0.100 (10)	0.0	0.0	0.2745
NCPPB 2424	0.304 (10)	0.448 (13)	0.143 (7)	0.100 (10)	0.0	0.0	0.2222
NCPPB 880	0.875 (3)	0.790 (3)	0.200 (8)	0.182 (12)	0.0	0.0	0.3830
CNBP 1323	0.259 (14)	0.200 (14)	0.0	0.0	0.0	0.0	0.1150
10862	0.900 (7)	0.826 (7)	0.200 (8)	0.182 (12)	0.0	0.0	0.4314
30555	0.072 (15)	0.226 (15)	0.0	0.0	0.0	0.0	0.0783
PDDCC 3357	0.333 (8)	0.600 (9)	0.0	0.0	0.0	0.0	0.2157

ND, No data due to poor digestion of the genomic DNA.

This analysis showed that NCPPB 880 and ATCC 10862 were the most atypical strains. The results from the *EcoRI*-digested DNAs were very similar to those from the *PstI*-digested DNAs, which suggests that increasing the number of probes to examine a larger percentage of the genome should give more useful information than examining one region of genomic DNA digested with many different restriction enzymes.

The RFLP distance matrices were analysed to produce the dendrograms in Fig. 3. Within the *P. s. tomato* group (Fig. 3a), strains NCPPB 880 and ATCC 10862 fell in a separate cluster and were clearly different from the other strains. In the other cluster, all the other *P. s. tomato* strains were slightly different, but strain JL1053 was the most unusual. The dendrogram for the *P. s. syringae* group (Fig. 3b) is presented separately since there were no shared hybridization bands that could be used to determine the relationship between the *P. s. syringae* and *P. s. tomato* strains. This limitation of RFLP analysis is the trade-off that must be accepted for the method's enhanced sensitivity to small differences.

There were two major clusters of *P. s. syringae* strains. One cluster contained the corn and the two wheat strains (PSC1B, B64 and B61), and the second cluster consisted of the peach strain and the two tomato strains (B48, B76 and B78). This analysis separated the strains that are pathogenic on monocots from those that are pathogenic on dicots. Except for the clustering of the two tomato strains, the relationships among the *P. s. syringae* strains from the RFLP analysis were different from those based on enzyme polymorphisms. However, the results based on RFLPs should be considered preliminary, because the low homology between the *P. s. tomato* probes and *P. s. syringae* genomic DNAs resulted in a number of weakly hybridizing bands being used in the analysis.

Fig. 3. Genetic relationships within the two *P. syringae* pathovars. The dendrograms were constructed based on RFLP analysis as described in the text for: (a) 17 strains of *P. s. tomato*; (b) 6 strains of *P. s. syringae*.

DISCUSSION

Our ability to measure genetic diversity with the DNA-DNA hybridization assay was limited by the variability inherent in the procedure. Little could be concluded regarding the extent of diversity within the two pathovars, but the low degree of homology between the *P. s. syringae* DNAs and the *P. s. tomato* reference DNA clearly distinguished between the two pathovars. Our 42% average homology between pathovars is less than the 58 to 66% homology values reported by Palleroni *et al.* (1972) and Pecknold & Grogan (1973), presumably because we used more stringent hybridization conditions ($T_m - 20^\circ\text{C}$) and a different washing procedure ($T_m - 15^\circ\text{C}$). Recent interpretations of DNA hybridization data have suggested that bacteria should be considered separate species if they are less than 60 to 70% homologous under optimal conditions (Schleifer & Stackebrandt, 1983; Brenner, 1981; Johnson, 1984). If this criterion is applied to *P. syringae* pathovars, then *P. s. tomato* should be considered specifically distinct from *P. s. syringae*.

Multilocus enzyme electrophoresis strongly supported the conclusion that *P. s. tomato* and *P. s. syringae* are distinct species. The genetic distance between the pathovars ($D > 90\%$) was unusually high for strains of a single species (Selander, 1985). Furthermore, although the sample size is too small to warrant firm conclusions, the rather large mean genetic diversity among strains of *P. s. syringae* ($H = 0.479$) suggested that further study will lead to the taxonomic subdivision of this pathovar. The level of genetic diversity in *P. s. syringae* appeared to support the hypothesis that variation was higher among strains from diverse habitats (e.g. wide host range *P. s. syringae*) than among those from a uniform habitat (e.g. *P. s. tomato* from tomato) (Hildebrand *et al.*, 1982).

For *P. s. tomato*, the genetic relationships detected by RFLP analysis were similar to those obtained by multilocus enzyme electrophoresis, except that RFLPs discriminated among all the *P. s. tomato* strains. The greater sensitivity of the RFLP analysis is illustrated by the relationship

between strains B88 and B181, for which a mismatch in one of 58 bands resulted in a distance of 1%. It is possible, however, that the RFLP analysis detected an uncharacteristic amount of variation among the *P. s. tomato* strains because of the apparent repeated sequence on the cloned DNA in cosmid pB88-4. If such a repeated sequence is part of a mobile genetic element, then this cosmid probe might have examined unusually variable, non-representative regions of the genome. Seven of the *P. s. tomato* strains are identical when only the bands that hybridize strongly to cosmid pB88-4 are used in the RFLP analysis, which is more like the results from the enzyme analysis. Examining more of the genome with additional cloned probes would resolve this uncertainty.

An alternative method for using RFLPs to study variation in bacteria is to examine the electrophoretic profiles of DNA digests immediately after staining agarose gels with ethidium bromide (Glynn *et al.*, 1985; Hartung & Civerolo, 1987). These DNA 'fingerprints' offer the advantages of simplicity and speed, but several disadvantages of this method should be considered. First, the large number of DNA bands on the gel means that only marked differences will be observable, whereas differences between closely related strains such as those detected with cosmid pB88-6 will likely go unnoticed. Second, without hybridization probes to detect homology, the relationships between strains with different fingerprints must be established by other means. For example, the fact that differences between the DNA fingerprints of *P. s. tomato* strains ATCC 10862 and B88 (unpublished data from DNA gels used to produce the Southern blots for this research) were almost as large as those observed when a *P. s. syringae* strain was compared to B88 might lead to the erroneous conclusion that the two *P. s. tomato* strains were not related if only the DNA fingerprints were considered. The combination of these factors would make it impossible to quantify relationships, as was done in this study.

In work similar to ours, Lazo *et al.* (1987) recently reported using RFLP analysis to distinguish pathovars of *Xanthomonas campestris*. They found some probes that would differentiate all 26 pathovars, but in contrast to our results with *P. s. tomato* and *P. s. syringae*, some regions of the *X. campestris* genome were highly conserved among all the pathovars examined (e.g. at the species level). Lawson *et al.* (1986) used RFLP analysis in a preliminary study to demonstrate genotypic diversity among some *P. syringae* pathovars and rhizosphere-associated fluorescent pseudomonads. They also used a method of data analysis similar to ours, but did not combine data from multiple probes to produce a functional dendrogram. Even more sophisticated use of RFLPs than what we describe is possible with probes that have been restriction mapped. With well-characterized probes, it would be possible to calculate the proportion of base pair substitution between pairs of strains (Nei, 1987).

There are many ways that enzyme electrophoresis and RFLP analyses may aid in the identification of bacteria. These methods could be used to characterize strains of bacteria found in agro-ecosystems (Lawson *et al.*, 1986). Besides providing information on the genetic structures of natural populations, the ability to identify clonally related individuals would be especially useful in epidemiological research. It is also likely that specific hybridization probes could be developed to rapidly differentiate related strains of bacteria. The poor hybridization of *P. s. tomato* clones to *P. s. syringae* DNA is a good indication of the feasibility of this approach, and a probe to identify *X. campestris* pv. *phaseolicola* has been reported (Roth & Johnson, 1985). These procedures might also be developed to the point where they could confirm the identity of non-pathogenic variants and simplify the identification of pathogens isolated from unusual environments (e.g. non-host or non-plant associations), which at the present time can be quite difficult (Schroth *et al.*, 1981; Palleroni, 1984).

The recovery of closely related strains of *P. s. tomato* from around the world suggests that this bacterium was recently disseminated. The most likely mechanism of dissemination is by contaminated seed (McCarter *et al.*, 1983) and infected plants (Bonn *et al.*, 1985). The requirements of exploiting a narrow ecological niche (the tomato plant), coupled with the purging of variation through stochastic extinction and periodic selection of clonal cell lines, could explain the low genetic diversity observed within *P. s. tomato* (Hildebrand *et al.*, 1982; Selander, 1985). The relatively high genetic diversity within *P. s. syringae* may reflect one of several situations. If some strains of *P. s. syringae* have strong host preferences, the same

processes that affect *P. s. tomato*, operating in conjunction with multiple hosts, could result in genetically diverse clusters. A generally low degree of host specificity, however, would also result in a high degree of diversity, but clusters would not correlate with the hosts from which the strains were isolated. The results from the enzyme polymorphism analysis suggest that this latter possibility is more likely, but many more strains must be examined before reaching a firm conclusion.

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