Genetic Relationships among *Xanthomonas* Species and Pathovars Based on RFLP Analyses of PCR-amplified 16S, 23S rDNA and rDNA Internal Transcribed Spacer

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Abstract

Phylogenetic relationships among 43 bacterial strains of *Xanthomonas* and two strains of *Stenotrophomonas maltophilia* were clarified by PCR-restriction fragment length polymorphism (RFLP) analyses of three DNA fragments corresponding to 16S, 23S rDNA and the 16S-23S rRNA internal transcribed spacer region (ITS) using ten restriction enzymes. From the data of the 16S rDNA RFLPs, seven genotypes were detected, while eight genotypes were observed based on the data of the 23S rDNA RFLPs among *Xanthomonas* strains tested. When the data of the 16S and 23S rDNA RFLPs were combined, the *Xanthomonas* strains were divided into thirteen rDNA genotypes. Cluster analysis based on rDNA genotypes showed that they were divided into two groups designated as subgroups 1 and 2, which were distinguishable from the related genus *Stenotrophomonas*. On the basis of the ITS RFLPs, 20 types were observed, and two groups were formed. The members of each group were the same as those based on the rDNAs. The data suggests that this genus is phylogenetically grouped into at least two lineages.

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Key words : *Xanthomonas*, PCR-RFLP, *rrn* operon.

INTRODUCTION

The genus *Xanthomonas*, which mostly comprises phytopathogenic bacteria, is a member of the family Pseudomonadaceae. Until early 1990, *Xanthomonas* was composed of seven species, *Xanthomonas albilineans*, *X. axonopodis*, *X. campestris*, *X. fragariae*, *X. oryzae*, *X. populi* and *X. maltophilia*²⁸⁾. In addition, most of the members of *Xanthomonas* were classified as pathovars of species of *Xanthomonas campestris*. Accordingly, *Xanthomonas campestris* contained more than 140 pathovars. The taxonomic complexity in *Xanthomonas* resulted because the taxon is mainly based on host specificity ; most of them can not be distinguished phenotypically^{24,27,30)}. In 1993, Palleroni and Bradbury¹⁹⁾ proposed that *X. maltophilia* should be transferred to a separate new genus *Stenotrophomonas* because *X. maltophilia* is not a plant pathogen, although other members of *Xanthomonas* species cause diseases of diverse plants.

Currently, molecular techniques, such as RFLP analysis of genomic DNA^{1,3,4,17,22,32)} and PCR-amplified DNA^{13,18)}, DNA reassociation^{27,31)} and sequence analysis^{6,14)} have been used in genetic diversity, identification and the taxonomy of *Xanthomonas* species. Recently, the genus *Xanthomonas* was reclassified into 20 genospecies by DNA reassociation²⁷⁾, which is generally used

for species delineation³⁴⁾, and the 16S rDNA sequences of these species were analyzed⁸⁾. Thus, the phylogenetic relationships of the genus *Xanthomonas* are of interest.

At present, sequence analysis of ribosomal operons (called *rrn* operons) is the method of choice to determine phylogenetic relationships among organisms³⁵⁾. These operons contain three genes, which code the 16S rRNA gene (small subunit of ribosomal ; *rrs*), the 23S rRNA gene (large subunit of ribosomal ; *rrl*) and 5S rRNA gene. An internal transcribed spacer region called ITS (16S-23S spacer region) exists between *rrs* and *rrl*. PCR-RFLP based on *rrs* has been used by several authors^{11,15,16,18,25,26,33)}, because this method is useful and rapid for taxonomic and strain-typing purposes. In this study, therefore, PCR-amplified *rrs*, *rrl* and ITS region (*rrn* operon) of 43 strains from *Xanthomonas* species and two strains of *Stenotrophomonas maltophilia* were analyzed on the basis of their restriction fragment length polymorphisms (RFLPs) to determine phylogenetic relationships within the genus *Xanthomonas*.

MATERIALS AND METHODS

Bacterial strains Forty-three bacterial strains from *Xanthomonas* species, two strains of *Stenotrophomonas maltophilia* (previously, designated as *Xanthomonas maltophilia*) and four strains from *Pseudomonas*

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species were used in this study. Information on bacterial strains is shown in Table 1.

Preparation of DNA Total bacterial DNA was prepared by using a CTAB extract method²⁹. Briefly, cultures were incubated for 16 hr at 28°C in 5 ml of LB medium (10 g Bacto-tryptone, 5 g Bacto-yeast extract and 5 g NaCl in 1 liter of distilled water). Cells were lysed by SDS, and the resulting lysate was treated with proteinase K. DNA was purified with a solution of CTAB/NaCl (10% cetyltrimethylammonium bromide in 0.7 M NaCl) followed by chloroform/isoamylalcohol (24 : 1) and phenol/chloroform/isoamylalcohol (25 : 24 : 1) extractions. DNA was recovered by isopropanol precipitation and washed in 70% ethanol. The pellet was resuspended in 100 μ l of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) with addition of 1 μ l of ribonuclease A (10 mg/ml) and stored at 4°C.

PCR amplification The PCR primers used in this study were designed from the conserved regions in the 16S rRNA and 23S rRNA genes by comparing *E. coli*⁵⁾ and *Pseudomonas*^{10,23)}. Primers 1F (5'-AGTTTGATCCTGGCTC-3' from position 10 to position 25 on *E. coli*) and 3R (5'-AAGGAGGTGATCCAGCC-3' from position 1525 to position 1541 on *E. coli*) amplified nearly the full length of the 16S rRNA gene. Primer 4F (5'-ACTAAGC-GTACAGGTGGAT-3' from position 10 to position 29 on *E. coli*) and 1401R (5'-AAGTACAGGAATAATAAC-3' from position 1389 to position 1406 on *E. coli*) amplified approximately half the length of the 23S rRNA gene. Primers 2F (5'-TCAAATGAATTGACGGGGC-3' from position 911 to position 930 on *E. coli*) and 5R (5'-AAGTCGCTGACCCATTATAC-3' from position 570 to position 589 on *E. coli*) amplified the ITS region containing the 3' end of 16S rRNA gene and the 5' end of 23S rRNA gene. PCR amplification was performed with a GeneAmp PCR System 2400 (Perkin-Elmer Corp.) using Expand High Fidelity PCR System (Boehringer Mannheim). The PCRs were carried out in 100 μ l reaction volume by mixing template DNA with reaction buffer containing 200 μ M of the deoxynucleotide triphosphate, 1 μ M of each primer, 2 mM MgCl₂ and 2.6 U of enzyme mix. Amplification followed the standard temperature profile: an initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 3 min, and a final extension at 72°C for 7 min. PCR products were analyzed by gel electrophoresis on 0.7% agarose (Agarose S; Nippon Gene) in 0.5 X TBE buffer (45 mM Tris-borate and 1 mM EDTA). Gels were stained with ethidium bromide and photographed under UV illumination with Polaroid type 667 film.

Restriction fragment analysis Aliquots (5–10 μ l) of PCR products were digested with 5 U of restriction endonuclease (Nippon Gene, Takara Shuzo, and Toyobo) in 15 μ l reaction volume with manufacturer's recommended buffer and temperature. The following ten enzymes were used: *Alu*I, *Dde*I, *Hae*III, *Hha*I, *Hinf*I, *Mbo*I, *Msp*I, *Mva*I, *Nci*I, and *Rsa*I. In some cases, an

additional nine restriction enzymes (*Aci*I, *Bfa*I, *Bst*UI, *Cfr*13I, *Mae*I, *Mae*III, *Nla*III, *Taq*I, and *Tsp*EI) were used. Digested DNA was analyzed by gel electrophoresis on 2.5 to 3% agarose (NuSieve GTG; FMC Bioproducts) in 0.5 X TBE buffer. Electrophoreses were carried out in 15-by 14-cm gels, and the gels were stained and photographed as described earlier.

RFLP data analysis RFLP bands of each strain were coded in binary form by scoring the presence or absence of each band. Pairwise distances for each RFLP data set were computed with the Dice similarity coefficient (S) by using the WinDist program³⁶⁾. A dendrogram was constructed from the distance matrix ($D = 1 - S$) data by UPGMA using the NEIGHBOR and DRAWGRAM program in PHYLIP package⁷⁾. The confidence limits of dendrograms were determined by bootstrap analysis with 1000 replications using the WinBoot program³⁶⁾.

RESULTS

PCR amplification

All 49 strains tested were amplified with the primers used. They produced a single band with each primer set, and these sizes corresponded to the expected sizes of the targeted 16S rRNA and 23S rRNA genes, respectively (data not shown). For the ITS region, they produced a band of approximately 1600 bp, and the resulting size of the ITS region ranged from 500 to 550 bp. It was the expected size, since we have recently determined the sequences of this region among *Xanthomonas* strains (unpublished data).

RFLP analysis of amplified 16S rRNA gene

Restriction fragments shorter than approximately 100 bp were not well-resolved by electrophoresis in 2.5–3% agarose. Three to nine restriction fragments were observed with each of the 10 restriction endonucleases in the amplified 16S rDNAs. Among strains of *Xanthomonas* and *S. maltophilia*, two to five distinct restriction patterns were detected with seven enzymes. Digestion with the following three enzymes, *Hha*I, *Hinf*I and *Rsa*I, did not produce any polymorphism among *Xanthomonas* and *S. maltophilia* strains used. Representative restriction patterns of 10 enzymes are shown in Fig. 1A.

The 16S genotypes which represent the combination of restriction patterns obtained with 10 restriction enzymes are shown in Table 2. The differences in patterns among the *Xanthomonas* and *S. maltophilia* strains tested were mainly caused by band shifts and, therefore, other band patterns were similar to each other (Fig. 1A). The restriction patterns of four strains of *Acidovorax avenae* (*Pseudomonas avenae*), *Burkholderia glumae* (*Pseudomonas glumae*), *Pseudomonas fuscovaginae* and *Pseudomonas syringae* pv. *oryzae* were distinctly different from those of *Xanthomonas* and *Stenotrophomonas* species (Fig. 1A). The combined results revealed that there were seven genotypes (1 to 7) in *Xanthomonas*, two genotypes (8 and 9) in *Stenotro-*

Table 1. RFLP types of *Xanthomonas*, *Stenotrophomonas* and *Pseudomonas* strains used in this study

Species or pathovars	Strain ^{a)}	16S rDNA genotype ^{b)}	23S rDNA genotype ^{c)}	rDNA genotype ^{d)}	subgroup ^{e)}	ITS type ^{f)}
<i>Xanthomonas albilineans</i>	ATCC 33915 ^T	5	5	11	2	19
<i>Xanthomonas axonopodis</i>	ATCC 19312 ^T	2	6	10	1	18
<i>Xanthomonas fragariae</i>	ATCC 33239 ^T	1	1	1	1	20
<i>Xanthomonas oryzae</i> pathovars						
pv. <i>oryzae</i>	ATCC 35933 ^T	1	1	1	1	2
pv. <i>orizicola</i>	NCPPB 1585*	1	1	1	1	3
<i>Xanthomonas campestris</i> pathovars						
pv. <i>alfalfae</i>	ATCC 11765	2	2	4	1	5
	MAFF 301090	2	2	4	1	
pv. <i>begoniae</i>	ATCC 8718	2	1	6	1	4
pv. <i>campestris</i>	ATCC 33913 ^T	1	1	1	1	3
	XCC 91-N1	1	1	1	1	2
pv. <i>cannabis</i>	MAFF 301606	1	2	2	1	
pv. <i>carotae</i>	ATCC 10547	1	1	1	1	6
	MAFF 301101	1	1	1	1	
pv. <i>cerealis</i>	NCPPB 1944*	3	3	3	2	7
pv. <i>citri</i>	MAFF 301077	1	2	2	1	
	NCPPB 409*	1	2	2	1	1
pv. <i>coriandri</i>	ATCC 17996*	2	2	4	1	8
pv. <i>cucurbitae</i>	ATCC 23378	1	1	1	1	9
	MAFF 301081	1	1	1	1	
pv. <i>dieffenbachiae</i>	ATCC 23379	4	4	7	1	10
	MAFF 301636	1	2	2	1	
	MAFF 301642	1	1	1	1	
pv. <i>glycines</i>	MAFF 301091	1	2	2	1	
	NCPPB 554*	1	2	2	1	1
pv. <i>graminis</i>	ATCC 29091*	3	3	3	2	11
pv. <i>hyacinthi</i>	ATCC 19314*	5	3	8	2	12
	MAFF 301390	5	3	8	2	
pv. <i>incanae</i>	ATCC 13462*	1	1	1	1	
	MAFF 210260	1	1	1	1	
pv. <i>malvacearum</i>	ATCC 9924	6	2	5	1	1
pv. <i>mangiferaeindicae</i>	ATCC 11637*	7	2	9	1	13
pv. <i>nigromaculans</i>	ATCC 23390*	1	1	1	1	14
pv. <i>phaseoli</i>	ATCC 9563*	1	2	2	1	4
pv. <i>physalidicola</i>	MAFF 301206*	2	7	12	1	
pv. <i>pruni</i>	ATCC 19316*	1	1	1	1	15
	MAFF 301420	1	1	1	1	
pv. <i>translucens</i>	ATCC 19319*	3	3	3	2	16
	MAFF 301035	3	3	3	2	
pv. <i>vesicatoria</i>	ATCC 35937	1	1	1	1	17
	MAFF 301256	1	8	13	1	
pv. <i>vitians</i>	ATCC 19320*	6	2	5	1	1
pv. <i>zantedeschiae</i>	MAFF 301639	1	1	1	1	
pv. <i>zinniae</i>	MAFF 302064	1	1	1	1	
<i>Stenotrophomonas maltophilia</i>	MAFF 301689	8	9	14		
	MAFF 520051	9	10	15		
<i>Pseudomonas fuscovaginae</i>	MAFF 301178	12	11	16		
<i>Pseudomonas syringae</i> pv. <i>oryzae</i>	MAFF 301530	13	12	17		
<i>Acidovorax avenae</i>	MAFF 301150	10				
<i>Burkholderia glumae</i>	MAFF 301169	11				

a) Abbreviations : ATCC, American Type Culture Collection, Rockville, Md. ; MAFF, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan ; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, UK ; T, type strains and *, pathovar reference strains.

b) 16S rDNA genotypes are defined in footnote a in Table 2.

c) 23S rDNA genotypes are defined in footnote a in Table 2.

d) rDNA genotypes are defined by the combination of 16S and 23S rDNA types in Table 2.

e) Subgroups are defined by cluster analysis based on the combination of PCR-RFLP of 16S and 23S rDNA in Fig. 4.

f) ITS types are defined in footnote a in Table 2.

Fig. 1. Representative patterns of PCR-amplified 16S (A) and 23S rRNA (B) genes digested with ten endonucleases. The lane assignments (lanes A to G) are given in Table 2. Lane M, molecular weight marker (100 bp ladder, Gibco-BRL).

phomonas and four genotypes (10 to 13) in *Pseudomonas* (Table 2).

The genotypes of *S. maltophilia*, *A. avenae*, *B. glumae*, *P. fuscovaginae* and *P. syringae* pv. *oryzae* were different from those of *Xanthomonas* species. Thirty-eight strains of *Xanthomonas campestris* pathovars tested were divided into seven genotypes (Table 1). Genotype 1 was dominant and included three species, *X. campestris*, *X. fragariae* and *X. oryzae*. Genotype 2 was shared with two species, *X. axonopodis* and *X. campestris*. Similarly, genotype 5 was shared with two species, *X. albilineans* and *X. campestris*. Genotypes 3, 4, 6 and 7 were occupied with a single species *X. campestris*. Genotypes 4 and 7 were unique genotypes observed in *X. c.* pv. *dieffenbachiae* and *X. c.* pv. *mangiferaeindicae*, respectively. Intraspecies variation was observed in only one pathovar, *X. c.* pv. *dieffenbachiae* which was divided into two genotypes, 1 and 4. In addition, the digestion of

amplified 16S rDNA with *MspI* or *NciI* discriminated *Xanthomonas* from *Stenotrophomonas*.

RFLP analysis of amplified 23S rRNA gene

Based on the analysis of amplified 23S rDNAs, four to eight restriction fragments were observed with each of the 10 restriction endonucleases used. Similarly, two to five distinct restriction patterns were detected with all 10 enzymes among strains of *Xanthomonas* and *S. maltophilia* tested here. Representative restriction patterns are shown in Fig. 1B. The 23S genotypes, which represent the combination of restriction patterns obtained with 10 restriction enzymes are shown in Table 2. The differences in patterns among *Xanthomonas* and *S. maltophilia* strains tested were also caused by band shifts (Fig. 1B). Other band patterns were similar to each other. The restriction patterns of *P. fuscovaginae* and *P. syringae* pv. *oryzae* were different from those of *Xanthomonas* and *Stenotrophomonas* strains (Fig. 1B). The

Table 2. Summary of RFLP types

Genotype ^{a)}	No. of strains (16S/23S/ITS)	Restriction patterns ^{b)} of amplified 16S, 23S rDNA genes and ITS digested with:		
		16S	23S	ITS
		ADHHHMMNR	ADHHHMMNR	ADHHHMMNR
1	26/19/4	AAAAAAAAAA	AAAAAAAAAA	AABAAABAAA
2	6/13/3	AAAAABAAAA	AAAAABABBB	AAAABAAAAA
3	4/6/2	CBBAABABAA	BABBBABAC	BAAABAAAAA
4	1/1/2	ACAAABAAAA	ABAAAABABB	BDDAABBAAB
5	3/1/1	BBBAAAABAA	BABBBABBBB	DCBAABDAAA
6	2/1/1	BAAAAAAAAA	ACAAAAAAAAA	AAAABAAAAC
7	1/1/1	AAAAACAAAA	CAAAAABABB	CECBCCBBBD
8	1/1/1	DACAAABABA	AAAAAAAAACA	BCBAABBAAB
9	1/1/1	EADAAACCCA	AACCBACCDE	BFAABDAAAE
10	1/1/1	FDEBDDDDDB	BDBDBBCDDD	BGBAAEBAAA
11	1/1/1	GEFCCEEEEC	DEDECCEEEF	CHCCCCBBB
12	1/1/1	HFGDDFFFFD	DFDECCEEFG	EI CBCCBBB
13	1/-/1	HFGDEGFFFD		ABBAAABAAA
14	-/-/1			BAAABDAAAA
15	-/-/1			ABAADDAAAA
16	-/-/1			CECCCCBBB
17	-/-/1			ABAABDAAAF
18	-/-/1			BJ BAAABAAA
19	-/-/1			FKEDEFECCE
20	-/-/1			GLAEFBDAAA

a) Genotype represents the combination of restriction patterns obtained with the 10 restriction enzymes used in 16S, 23S rDNA and ITS PCR-RFLP analyses.

b) A to L denote the types of restriction patterns for the restriction enzyme as follows: The ADHHHMMNR column indicates the restriction patterns (Figs. 1 and 2) obtained, respectively, with the endonucleases *Aha*I, *Dde*I, *Hae*III, *Hha*I, *Hin*II, *Mbo*I, *Msp*I, *Mva*I, *Nci*I and *Rsa*I used for PCR-RFLP analysis.

combined results showed that there were eight genotypes (1 to 8) in *Xanthomonas*, two (9 and 10) in *Stenotrophomonas* and two (11 and 12) in *Pseudomonas* (Table 2). None of these genotypes, except for genotype 1, were shared with the two *Xanthomonas* species (Table 1). The thirty-eight tested strains of *Xanthomonas campestris* pathovars were divided into six genotypes (Tables 1; genotypes 1, 2, 3, 4, 7, 8). Genotype 1, containing 19 strains, was dominant and shared with three species, *X. campestris*, *X. fragariae* and *X. oryzae*. Genotypes 4, 5 and 6 were specific to genotypes observed in *X. c. pv. dieffenbachiae* (ATCC 23379), *X. albilineans* and *X. axonopodis*, respectively. Intraspecific variation was also observed in *X. c. pv. dieffenbachiae*, which was divided into three genotypes 1, 2 and 4, respectively. In contrast with 16S rDNA analysis, digestion of amplified 23S rDNA with four enzymes *Hha*I, *Msp*I, *Mva*I or *Nci*I allowed to discriminate *Xanthomonas* from *Stenotrophomonas*.

rDNA genotype

Based on the combined data of 16S and 23S rDNA analyses, thirteen rDNA genotypes (1 to 13) were observed in 43 tested strains of *Xanthomonas* and were distinguishable from those (14 and 15) of the *Stenotrophomonas* strains (Table 1). None of these genotypes, except for rDNA genotype 1, were shared with two *Xanthomonas* species. The combined results of the 16S and 23S rDNA analyses also revealed that the same

genotype was shared with different species or pathovars. For example, genotype 1 was the dominant type and included 10 pathovars of *X. campestris*, and two pathovars of *X. oryzae* and *X. fragariae*.

RFLP analysis of amplified ITS region

Analysis of the ITS regions containing the 3' end of 16S rDNA and the 5' end of 23S rDNA using 27 strains of *Xanthomonas* showed 3 to 12 different patterns with each of the 10 restriction endonucleases used. Partial results are shown in Fig. 2. The combined results using 10 enzymes indicated that there were 20 different types in the ITS region (Table 2). None of these ITS types were common to two species, although *X. oryzae. pv. oryzae* and *X. campestris pv. campestris* (XCC 91-N1) in type 2 and *X. o. pv. oryzicola* and *X. c. pv. campestris* (ATCC 33913) in type 3 were exceptional cases (Table 1). Twenty-two strains of *Xanthomonas campestris* pathovars tested were divided into 17 ITS types. These results showed that the ITS region was highly variable, although same ITS types were also shared with two (type 4) to four (type 1) pathovars. Most of the *Xanthomonas* strains tested could be discriminated at the level pathovar or species based on these three PCR-RFLP analyses. However, some strains could not be distinguished from *X. campestris pv. campestris*, *X. c. pv. citri*, *X. c. pv. glycines*, *X. c. pv. malvacearum*, *X. c. pv. vitians*, *X. oryzae pv. oryzae* and *X. o. pv. oryzicola*.

Further RFLP analysis using nine restriction enzymes

Fig. 2. Restriction patterns of PCR-amplified ITS digested with *Hae*III (A), *Hinf*I (B), *Mbo*I (C), and *Msp*I (D). Lanes: 1, ATCC 35933; 2, NCPPB 1585; 3, ATCC 33913; 4, XCC 91-N1; 5, ATCC 11765; 6, ATCC 8718; 7, ATCC 10547; 8, NCPPB 409; 9, NCPPB 1944; 10, ATCC17996; 11, ATCC 23378; 12, ATCC 23379; 13, NCPPB 554; 14, ATCC 29091; 15, ATCC 19314; 16, ATCC 13462; 17, ATCC 9924; 18, ATCC 11637; 19, ATCC 23390; 20, ATCC 9563; 21, ATCC 19316; 22, ATCC 19319; 23, ATCC 35937; 24, ATCC 19320; 25, ATCC 19312; 26, ATCC 33915; 27, ATCC 33239; M, molecular weight marker (100 bp ladder, Gibco-BRL).

Table 3. Detailed ITS types and restriction patterns with nine other enzymes

Species or pathovars	Strain	Detailed type	Restriction patterns ^{a)} of amplified ITS digested with nine other enzymes	Initial type ^{b)}	rDNA genotype
			ABBCMMNTT		
<i>X. oryzae</i> pv. <i>oryzae</i>	ATCC 35933	2A	1 1 1 1 1 2 1 1	2	1
<i>X. campestris</i> pv. <i>campestris</i>	XCC 91-N1	2B	1 1 1 1 1 3 1 1	2	1
<i>X. oryzae</i> pv. <i>oryzicola</i>	NCPPB 1585	3A	1 1 1 1 1 2 1 1	3	1
<i>X. campestris</i> pv. <i>campestris</i>	ATCC 33913	3B	1 1 1 1 1 2 3 1 1	3	1
<i>X. campestris</i> pv. <i>citri</i>	NCPPB 409	1A	1 1 1 1 1 1 1 1 1	1	2
pv. <i>glycines</i>	NCPPB 554	1A	1 1 1 1 1 1 1 1 1	1	2
pv. <i>malvacearum</i>	ATCC 9924	1A	1 1 1 1 1 1 1 1 1	1	2
pv. <i>vitians</i>	ATCC 19320	1A	1 1 1 1 1 1 1 1 1	1	5

a) 1 to 3 denote the types of restriction patterns for the restriction enzyme as follows. The ABBCMMNTT column indicates the restriction patterns obtained, respectively, with the endonucleases *Aci*I, *Bfa*I, *Bst*UI, *Cfr*131, *Mae*I, *Mae*III, *Nla*III, *Taq*I and *Tsp*EI used for PCR-RFLP analysis.

b) Initial type corresponds to ITS types defined in footnote a in Table 2.

for the ITS region showed that strains belonging to the rDNA genotype 1, *X. c.* pv. *campestris*, *X. o.* pv. *oryzae* and *X. o.* pv. *oryzicola* were distinguishable from each other (Table 3). But *X. c.* pv. *citri* and pv. *glycines* of rDNA genotype 2, and *X. c.* pv. *malvacearum* and pv. *vitians* of rDNA genotype 5 were not distinguishable by

this analysis (Table 3). Therefore, these four pathovars appeared to be genetically closely related to each other.

Cluster analysis of combined 16S and 23S rDNA RFLPs

To estimate the relationships between the 17 rDNA genotypes, cluster analysis was performed based on the

Fig. 3. Dendrogram constructed with UPGMA on the basis of the combined data set of 16S and 23S rDNA restriction patterns. The rDNA genotypes (1 to 17) are defined in Table 1. The numbers shown next to the nodes indicate percent bootstrap values of the 1000 replicates.

combined data sets of the 16S and 23S rDNA restriction patterns by using UPGMA (Fig. 3). The obtained dendrogram showed that all the *Xanthomonas* strains tested here were grouped into the same cluster (cluster 1). The bootstrap results are indicated on the dendrogram in Fig. 3. The bootstrap value for the branch of clusters 1 and 2 was 100%, indicating that these two clusters are phylogenetically separated, although these clusters are more closely related than cluster 3 (*Pseudomonas* cluster). Cluster 1 was composed of two groups designated as subgroups 1 and 2. The bootstrap values for the internal branch of subgroups 1 and 2 were 99.9 and 100%, respectively, indicating that these were very strong subclusters, although the bootstrap value for the branch of subgroups 1 and 2 was relatively low (88.7%). Members of subgroup 1 were composed of rDNA genotypes 1, 2, 5, 6, 7, 9, 10, 12 and 13 defined in Table 1. This group included 21 pathovars of *X. campestris* and two pathovars each of *X. oryzae*, *X. axonopodis* and *X. fragariae*. Members of subgroup 2 were composed of rDNA genotypes 3, 8 and 11 defined in Table 1. Strains within this group were *X. albilineans* and four pathovars of *X. campestris*. The four pathovars of *X. campestris* belonging to subgroup 2 were pathovars *cerealis*, *graminis*, *hyacinthi* and *translucens*.

Cluster analysis of ITS region RFLPs

Based on the analysis of the ITS region, the obtained dendrogram indicated that the genetic distance was much greater than that by analysis of rDNA (Fig. 4). *Xanthomonas* strains tested here were divided into two groups designated as clusters 1 and 2. Clusters 1 fell into the subgroups 1 of the analysis of combined 16S and 23S

Fig. 4. Dendrogram constructed with UPGMA on the basis of ITS restriction patterns. The ITS types (1 to 20) are defined in Table 2. The numbers shown next to the nodes indicate percent bootstrap values of the 1000 replicates.

rDNA RFLPs, while cluster 2 fell into subgroup 2. The bootstrap results were illustrated on the dendrogram in Fig. 4. The bootstrap value of cluster 1 was 100%,

indicating that it was a very strong cluster. The bootstrap values for internal branches of cluster 1 were less than 94%, indicating that the interrelationships among the strains within cluster 1 were not clear. The bootstrap value of cluster 2 was unexpectedly low (62.1%). Within this cluster, ITS types 7, 11, 12 and 16 were more closely related to each other, with a bootstrap value of 100%.

DISCUSSION

We showed that the PCR-RFLP analysis of the *rrn* operon is a simple and rapid technique to evaluate phylogenetic relationships among strains of *Xanthomonas* and the related genus *Stenotrophomonas*. On the basis of the combination of PCR-RFLP of 16S and 23S rDNA, *Xanthomonas* strains tested here fell into the same cluster, which is distinguishable from the cluster of *Stenotrophomonas*. The bootstrap value for this branch is 100%, indicating that the genera *Xanthomonas* and *Stenotrophomonas* are phylogenetically separable. It agreed with the previous report demonstrating that the PCR-RFLP of the amplified the 16S rRNA gene could distinguish the genus *Stenotrophomonas* from the genus *Xanthomonas*¹⁸⁾. Comparative RFLP analysis of the two genera with those of the *Pseudomonas* strains tested here, however, showed that *Xanthomonas* and *Stenotrophomonas* were relatively closely related. Hauben *et al.*⁹⁾ reported that the two genera exhibited a mean sequence similarity value of 96.6% in the 16S rDNA. They also raised the question whether the differences between their 16S rDNA sequences (2.9 to 4.8%) were significant enough to classify them into two separate genera. Additional information on the sequences of the 16S-23S rRNA internal transcribed spacers (ITS) and the 23S rRNA gene may clarify this question. Comparison between *Xanthomonas* and *Stenotrophomonas* based on the partial sequence of the 5' end of the 23S rRNA gene indicated that the level of homology between their 23S rDNA sequences was approximately 92.8 to 95.9% (unpublished data). The homology value is relatively lower than that of 16 rDNA sequence.

Based on cluster analysis of the rDNA (combination of 16S and 23S rDNA), the *Xanthomonas* strains were divided into two groups designated as subgroups 1 and 2. The bootstrap values for the internal branch of the two subgroups were 99.9 and 100%, respectively, indicating these two lineages are strong clusters. The obtained results suggest that the *Xanthomonas* strains tested here are phylogenetically grouped into two lineages. Subgroup 1 consisted of *Xanthomonas axonopodis*, *X. campestris*, *X. fragariae* and *X. oryzae*, while subgroup 2 comprised *Xanthomonas albilineans* and *X. campestris*. Unexpectedly, strains of *X. campestris* fell into both subgroups. Strains within subgroup 1 are pathovars *alfalfae*, *begoniae*, *campestris*, *cannabis*, *carotae*, *citri*, *coriandri*, *cucurbitae*, *dieffenbachiae*, *glycines*, *incanae*, *malvacearum*, *mangiferaeindicae*, *nigromaculans*,

phaseoli, *physalidicola*, *pruni*, *vesicatoria*, *vitians*, *zantedschiae* and *zinniae*. Strains within subgroup 2 are pathovars *cerealis*, *graminis*, *hyacinthi* and *translucens*. Our results are similar to those of Manceau and Horvais¹⁵⁾, who have demonstrated that *Pseudomonas syringae* pathovars were distributed into three clusters based on PCR-RFLP of *rrn* operon. Extensive studies based on protein profiles²⁹⁾, fatty acid profiles³¹⁾, DNA hybridization^{9,20,30,31)}, RFLP of genomic DNA¹²⁾, and rRNAs³⁾ indicated that pathovars of *X. campestris* were heterogeneous. These facts were also supported by the obtained results here. Currently, molecular approaches are used in taxonomic studies; DNA-DNA hybridization is generally used for species delineation. Recently, Vauterin *et al.*²⁷⁾ reclassified the genus *Xanthomonas* into 20 genomic species based on DNA-DNA hybridization study. Although not all the results of this study were in accordance with theirs, they were supported by those of Stackebrandt and Goebel²¹⁾, who reported that rDNA analysis should not replace delineation of species based on DNA-DNA hybridization.

On the basis of the 16S-23S rRNA internal transcribed spacers (ITS), a large polymorphism was detected by RFLPs, although the length of this region is shorter than that of the 16S or 23S rDNA. When the data obtained by ITS and rDNA analyses were combined, each *X. campestris* pathovar was discriminated. However, the exceptions were between *X. campestris* pv. *citri* and pv. *glycines*, and *X. c.* pv. *malvacearum* and pv. *vitians*. Cluster analysis showed that *Xanthomonas* strains tested were also divided into two groups, and members of each group were the same as those based on rDNAs. The presumption that *Xanthomonas* strains are phylogenetically grouped into at least two lineages was confirmed by this ITS analysis. Although *X. albilineans* of subgroup 2 by rDNA analyses corresponded to subgroup 2 by cluster analysis using ITS data, genetic distance between the species and other members was very great and the bootstrap value of the branch was low. This fact suggests that *X. albilineans* may be a third group designated as subgroup 3, though further study is required to clarify it.

Considering the differences between strains of subgroups 1 and 2, the former is mainly pathogenic to dicotyledonous plants, while the latter is composed of strains pathogenic only to monocotyledonous plants. In addition, members of subgroup 1 have an average G+C content of 65 to 66 mol%, but those of subgroup 2 have an average G+C content of 69 mol%⁸⁾. *X. albilineans* is an exception in subgroup 2, and its G+C content is 64.2%⁸⁾. However, other common features to distinguish the two groups are difficult to find.

The PCR-RFLP analysis of the *rrn* operon in this study appears to be a rapid, useful tool for evaluating the genetic relatedness of the genus *Xanthomonas*. Based on the results of this study, this method allows us to demonstrate that phylogenetically, the *Xanthomonas* strains tested here are grouped into at least two line-

ages. Further research is needed to clarify the relationships such as the comparative analyses of pathogenicity related genes.

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和 文 摘 要

落合弘和・加来久敏：16S, 23S rDNA および rDNA スペーサーの PCR-RFLP 解析に基づく *Xanthomonas* 属 pathovar 間の遺伝的関係

Xanthomonas 属細菌各 pathovar と *Stenotrophomonas maltophilia* の系統関係を明らかにするために、PCR で増幅した3種類の DNA 断片、16S rDNA、23S rDNA と rDNA スペーサー (ITS) についてそれぞれ10種類の制限酵素による PCR-RFLP 解析を行った。その結果、供試した *Xanthomonas* 属細菌は、それぞれ16S rDNA の多型解析では7つのタイプ、23S rDNA の多型解析では8タイプが検出された。これら2つの結果を総合すると、*Xanthomonas* 属細菌は13種類の rDNA 型に分類された。この rDNA 型に基づいたクラスター解析の結果、供試した *Xanthomonas* 属細菌は subgroup 1, 2 と命名した2つのグループに類別され、さらにそのクラスターは近縁属である *Stenotrophomonas* とは区別された。一方、ITS に基づく多型解析では、20のタイプが見いだされ、rDNA と同様に2つのグループに類別された。また、おのおののグループに属する菌株群は、rDNA の解析に基づくものと一致した。以上の結果から、*Xanthomonas* 属細菌は、系統的には少なくとも2つのグループで構成されていることが示唆された。