

Validity, sensitivity and resolution limit of the PCR-RFLP analysis of the *rrs* (16S rRNA gene) as a tool to identify soil-borne and plant-associated bacterial populations

Phil Oger^a, Yves Dessaux^{a*}, Annick Petit^a, Louis Gardan^b, Charles Manceau^b, Cécil Chomel^c, Xavier Nesme^c

^a Institut des sciences végétales, CNRS, avenue de la Terrasse, 91198 Gif-sur-Yvette cedex, France

^b Station de pathologie végétale, Inra, 42, rue Georges Morel, 49071 Beaucozéd cedex, France

^c Laboratoire d'écologie microbienne des sols, Université Claude Bernard-Lyon-1, UMR CNRS 5557, Inra, boulevard du 11 novembre, 69622 Villeurbanne cedex, France

Abstract – The value of the restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)-amplified *rrs* (16S ribosomal ribonucleic acid [rRNA] gene) for the rapid identification of bacteria isolated from soil or plants, and the limits of this technique, were evaluated using bacterial genera characteristic of the above-mentioned ecosystems. Results showed that up to two restriction site differences may occur between *rrs* operons within the same bacterial genome as well as between bacteria belonging to the same genospecies. In spite of these limited differences, members of the same genospecies yield very similar *rrs* RFLP patterns. The identification limit varies according to the analyzed taxa. Species can be differentiated amongst members of both the family *Rhizobiaceae* and the genus *Stenotrophomonas*, while the technique only allows grouping of closely related species amongst *Xanthomonas* spp. and amongst species related to *Pseudomonas syringae*. On the basis of their *rrs* RFLP profiles, all presently known species of *Agrobacterium* can be routinely identified using only the enzymes *Hpa*II (or *Msp*I), *Alu*I and *Hae*III. Moreover, the method was also found to be valuable in assessing the biodiversity of a bacterial community isolated from the rhizosphere. From the comparison of empiric *rrs* RFLPs, published sequences and deoxyribonucleic acid (DNA) pairing studies, we suggest that the occurrence of five different restriction sites within two *rrs* genes is

* Correspondence and reprints

the minimum difference required to clearly establish that two relevant bacteria belong to different genospecies. © Inra/Elsevier, Paris

PCR-RFLP / 16S / *rrs* / *Agrobacterium* / *Pseudomonas* / *Xanthomonas*

Résumé – Examen critique de l'analyse par RFLP du gène déterminant l'ARN 16S. L'intérêt et les limites de l'identification bactérienne basée sur l'analyse du gène codant pour l'ARN ribosomique 16S par PCR-RFLP ont été évalués dans plusieurs genres bactériens typiques des écosystèmes sol et plante. Les résultats montrent qu'il peut y avoir jusqu'à deux sites de restriction différents entre les gènes *rrs* de bactéries appartenant à la même espèce génomique ou entre différents opérons d'un même génome. Les résultats permettent cependant d'identifier les bactéries jusqu'au niveau de l'espèce chez les Rhizobiaceae et chez *Stenotrophomonas*, et jusqu'au niveau du groupe d'espèces apparentées d'un même genre chez *Xanthomonas* et dans le groupe apparenté à *P. syringae*. De plus, la méthode s'est révélée parfaitement adaptée pour identifier et classer les bactéries isolées d'un peuplement rhizosphérique, en permettant une identification approchée du genre, voire de l'espèce. La comparaison des données de PCR-RFLP avec les données de séquences et d'hybridations ADN-ADN suggèrent qu'un minimum de cinq sites de restriction polymorphes dans le gène 16S est nécessaire pour affirmer que des bactéries appartiennent à des espèces génomiques différentes. © Inra/Elsevier, Paris

PCR-RFLP / 16S *rrs* / *Agrobacterium* / *Pseudomonas* / *Xanthomonas*

1. INTRODUCTION

Evaluating the diversity and defining the structure and behaviour of microbial communities have been and still remain primary objectives in microbial ecology. This assertion is particularly valid when applied to soil-borne and plant-associated bacteria, which are involved in the major biochemical cycles or exercise known beneficial or detrimental effects such as biodepollution, symbiotic nitrogen fixation or induction of human, animal or plant diseases. Indeed, soil and plant microflora are probably amongst the main reservoirs of biodiversity. However, this microbial world remains largely unexplored and therefore poorly understood: Ward et al. (1990) estimated that no more than 10 % of soil bacteria have already been identified. This lack of data may be correlated with the shortage of tools to rapidly identify bacteria – and more crucially – to assess the behaviour of given bacterial population(s) amongst microbial communities of complex ecosystems.

Presently, the definition of bacterial species relies essentially on the results of deoxyribonucleic acid (DNA) pairing studies. For Wayne et al. (1987), two bacteria belong to the same species when their DNA-DNA relatedness is higher than 70 % (with $\Delta T_m < 5^\circ\text{C}$). Unfortunately, hybridisation-based techniques are generally tedious and time-consuming. The determination of the DNA pairing does not constitute an exception to this rule. Furthermore, it is solely a comparative method which always calls for the availability of DNA from reference strains. Consequently, this method is not appropriate to assess rapidly the phylogenic position of new isolates. The emergence of molecular techniques, particularly those dealing with the analysis of the sequence of parts of the bacterial ribosomal ribonucleic acid (rRNA) gene operon, have shed new light

on these questions (Woese, 1987; Normand et al., 1992; Stackebrandt et al., 1993). DNA sequencing of this part of the bacterial genome provides scientists with numerous data on the genetic distance separating bacteria, a feature which has led to a drastic revision of Aristotle's fixed image of the 'species'. Moreover, the availability of sequence data from a growing number of bacterial species ensures that any new strain may readily be positioned somewhere in the life tree without the absolute requirement of additional biochemical analysis. This is of primary importance when exploring a world where most inhabitants are still not cultivable.

At the beginning of this work, these techniques appeared as promising tools for the phylogenetic identification of the microorganisms, as exemplified by various studies performed on the sequence of the *rrs* gene, i.e. the 16S rRNA gene according to Riley's nomenclature (1993). Unfortunately, such a technique remains expensive, time-consuming and unavailable to field laboratories, even though the relevant sequences can be successfully obtained by polymerase chain reaction (PCR) amplification from numerous bacteria. It is also not appropriate to track soil-borne or plant-associated microbial communities. To solve these problems, we and others (Gurtler et al., 1991; Navarro et al., 1992; Vaneechoutte et al., 1992, 1993; Ponsonnet and Nesme, 1994a; Nesme et al., 1995; Laguerre et al., 1996; Latour et al., 1996) have proposed to evaluate the validity of the analysis of the restriction fragment length polymorphism (RFLP) of the 16S rRNA gene following its amplification by a PCR-based protocol. Compared to the sequencing technique, the PCR-RFLP analysis appears a priori faster, cheaper and accessible to field laboratories. However, its discriminating capacity has to be appreciated. To address this question, we evaluated the validity, sensitivity and resolution limits of the PCR-RFLP analysis of the 16S RNA gene with respect to the identification of a limited number of bacterial taxa including the genera *Agrobacterium* amongst the alpha subclass of *Proteobacteria*, and *Xanthomonas*, *Stenotrophomonas* and *Pseudomonas* amongst the gamma subclass of *Proteobacteria*. We also report data that showed the suitability of the technique in tracking the biodiversity of microbial communities from the rhizosphere.

2. MATERIALS AND METHODS

2.1. Bacterial strains

The bacteria investigated by the PCR-RFLP method belonged to readily defined genospecies of *Agrobacterium*, *Pseudomonas* (of the so-called *P. syringae* group), *Xanthomonas* and *Stenotrophomonas* characterised by biochemical traits and DNA pairing studies. Strains were from the collection of our laboratories, from the Collection française des bactéries phytopathogènes (CFBP, Inra-Angers, France) and from the Laboratorium voor Microbiologie, Gent Culture Collection (LMG, Gent, Belgium).

Strains were also collected from the root system of the plants by washing the roots in sterile distilled water (10 mL of water g⁻¹ of root) with vigorous shaking for 1 min. Washed roots were eliminated and dilution series of the bacterial suspensions were performed. Aliquots were plated onto modified LB (LBm) solid medium (Vaudequin-Dransart et al., 1995) supplemented with

400 $\mu\text{g}\cdot\text{mL}^{-1}$ cycloheximide. Plates were incubated for 1 week at 28 °C and inspected every day for the appearance of bacterial colonies. Colonies were purified on the same medium to homogeneity.

2.2. Amplification of the 16S gene

For *Agrobacterium* and *Xanthomonas*, the 16S rRNA gene was amplified exactly as described by Nesme et al. (1996), by using genomic DNA isolated by the protocol of Marmur (1961), and the prokaryote specific primers used for PCR-FGPS6 (5'-GGA GAG TTA GAT CTT GGC TCA G-3') and FGPS1509' (5'-AAG GAG GGG ATC CAG CCG CA-3'), designed from the conserved regions of the 16S and allowing amplification of 99.5 % of the gene. For *Pseudomonas* strains, PCR was performed on frozen bacterial pellets processed for 4 min at 94 °C (*ca.* 10^7 cells per amplification reaction) using the following PCR reaction mixture: Tris-HCl, pH 9.0, 10 mM; KCl 50 mM; MgCl_2 1.5 mM; dNTP 200 μM (each); primers 1 μM (each); and *Taq* DNA polymerase 0.5 U (Appligène, Illkirch, France). Amplification cycles were as follows: 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C completed by a 3-min elongation at 72 °C. Primers were rD1 (19-mer) and fD1 (20-mer) as described by Weisburg et al. (1991). The 16S rRNA gene from the root-associated bacteria were amplified by adding 4 μL (*ca.* 10^5 cells per amplification reaction) of a fresh bacterial cell suspension to 100 μL of the reaction mixture described later. Cycles were as follows: bacterial cell lysis and DNA denaturation were performed for 5 min at 95 °C, followed by five amplification cycles (30 s at 95 °C; 45 s at 60 °C; 2 min at 72 °C) and 30 cycles (1 min at 95 °C, 1 min at 60 °C and 2 min at 72 °C). Reactions were terminated by a 10-min incubation at 72 °C. The amplification was performed using the set of primers FGPS6 and FGP1509' described earlier. Typically, the PCR reaction mixture consisted of Tris-HCl, pH 9.0, 10 mM; KCl 50 mM; MgCl_2 1.5 mM; gelatine 0.2 mg/mL; dNTP 200 μM (each); primers 0.1 μM (each); and *Taq* DNA polymerase 0.4 U (Appligène, Illkirch, France).

2.3. RFLP analysis

Restriction digests were performed using different endonucleases following standard procedures and the instructions of the enzyme manufacturers. Reactions were performed in a final volume of 10 to 50 μL , using 1 to 30 μg of PCR products and the relevant amount of restriction enzyme. Restriction fragments were separated in 1.5 to 3 % standard agarose gels. The sizes of the fragments were analysed by comparison with the 123 bp molecular weight marker from Gibco BRL (Gaithersburg, USA) or with a $\Phi\text{X174}/\text{HaeIII}$ molecular marker (Eurogentec, Seraing, Belgium). Sizes were deduced either by visual inspection or by computer analysis using the software Taxotron (Institut Pasteur, Paris, France), Bioprofil and Biogene (Vilbert-Lourmat, France). The restriction maps of *Xanthomonas* and *Stenotrophomonas* were determined from the observed RFLP by comparison with those inferred from published *rrs* sequences of reference strains. Restriction site differences were analysed by the Wagner parsimony method using the MIX software of the PHYLIP package (Felsenstein, 1993). The number of nucleotide substitutions per nucleotidic site was estimated according to Nei and Li (1979). The distance matrix was used

to construct dendrograms using methods of Fitch and Margoliash, UPGMA, neighbour-joining and maximum-likelihood as described by Felsenstein (1992). Numerical analysis based on presence/absence of restriction fragments were performed by using the Jaccard coefficient and UPGMA (Sneath and Sokal, 1973).

2.4. Ribotyping

Standard ribotyping was performed essentially as previously described by Grimont and Grimont (1986). Bacterial DNA was prepared as described by Glickmann et al. (unpublished report) for Gram-negative bacteria and by the method of Dubnau and Cirigliano (1972) for those exhibiting a variable Gram reaction. Genomic DNA preparations of the clones were submitted to endonuclease digestion using either *EcoRI*, *BamHI* or *HindIII* restriction enzymes. DNA fragments were separated in standard agarose gels. Gels were blotted onto an Amersham Hybond N membrane using a standard protocol. Hybridisation was carried out essentially as described by Dessaux et al. (1995) using a nonradioactive probe which encompassed the entire 16S rRNA gene sequence of *Agrobacterium* strain C58. Alternatively, the ribotyping was performed by RFLP analysis of PCR-amplified intergenic spacers lying between the 16S (*rrs*) and the 23S rRNA (*rrl*) genes as previously described (Ponsonnet and Nesme, 1994a, 1994b).

3. RESULTS AND DISCUSSION

3.1. Reliability of the method

With all the assayed bacteria, the experimental conditions for PCR allowed the amplification of a single band of the expected size. In particular, this was true for the unidentified bacteria isolated from the root system of the plants (data not shown). Digestion of PCR products generally gave unambiguous patterns from which positions of restriction sites could be inferred from the published sequences. In rare instances such as with *Stenotrophomonas* strains LMG 11002 and LMG 10853, complex patterns were obtained indicating the occurrence of *AluI* and *HpaII* dimorphic sites within a single genome (Nesme et al., 1995). This confirms the occurrence of RFLP detectable differences within *rrs* operons of a given genome as reported in other bacterial species (Mevarech et al., 1989; Gurtler et al., 1991). On the other hand, bacteria belonging to the same genospecies gave identical *rrs* patterns except for strain LMG 852 of *X. arboricola* pv. *pruni*, which differed from other members of the species by one restriction site (*table I*).

3.2. Validity of the method

Results confirm that PCR amplification of *rrs* is a versatile method that can be used to obtain large copy numbers of this gene from various known and unknown soil-borne and plant-associated bacteria. The analysis of the polymorphism of the restriction fragments of *rrs* of various organisms led, however, to variable conclusions according to the considered taxa. These are detailed here.

Table I. Discriminating capability of *rrs* RFLPs amongst *Xanthomonas* and *Stenotrophomonas* genospecies.

Name ^a	Strain no. ^b	<i>rrs</i> RFLP ^c	Site differences with pattern A
<i>X. campestris</i>	LMG 568 ^T	A	
<i>X. bromi</i>	LMG 947 ^T	A	
<i>X. cucurbitae</i>	LMG 8662	A	
<i>X. pisi</i>	LMG 847 ^T	A	
<i>X. vasicola</i>	LMG 736 ^T	A	
<i>X. cassavae</i>	LMG 673 ^T	A	
<i>X. vesicatoria</i>	LMG 911 ^T	A	
<i>X. arboricola</i>	LMG 689 ^T	A	
	LMG 8047	A	
	LMG 5403*	A	
	LMG 12141*	A	
	LMG 8680	A	
	LMG 852*	B	<i>AluI</i> (165 + 42 - > 207) ^d
<i>X. codiae</i>	LMG 8678 ^T	H	<i>HpaII</i> (660 - > 33 + 450)
<i>X. melonis</i>	LMG 8672	C	<i>NdeII</i> (317 - > 57 + 260)
<i>X. azonopodis</i>	LMG 538 ^T	C	<i>NdeII</i> (317 - > 57 + 260)
	LMG 7455*	A	
	LMG 5743 ^T	D	
<i>X. populi</i>	CFBP 2112*	A	<i>CfoI</i> (220 - > 380)
	LMG 708 ^T	Af	<i>BsaJI</i> (273 - > 90 + 188)
<i>X. fragariae</i>	LMG 5047 ^T	Ao	<i>BsoFI</i> (282 - > 50 + 110 + 122)
<i>X. oryzae</i>	LMG 471 ^T	E	<i>SacFI</i> (59 + 58 - > 117)
<i>X. sacchari</i>			<i>Tsp509I</i> (78 + 166 - > 244)
			<i>BsaJI</i> (75 + 97 - > 172) <i>BstUI</i> (276 - > 572)
<i>X. albilineans</i>	LMG 494 ^T	F	<i>SacFI</i> (59 + 58 - > 117) <i>Tsp509I</i> (78 + 166 - > 244)
			<i>BsaJI</i> (75 + 97 - > 172) <i>BsaJI</i> (273 - > 90 + 183)
			<i>AluI</i> (83 + 165 - > 248)

Table I. Continued.

Name ^a	Strain no. ^b	<i>rrs</i> RFLP ^c	Site differences with pattern A
<i>X. hyacinthii</i>	LMG 742	F	<i>Scr</i> FI (59 + 58- > 117) <i>Tsp</i> 509I (78 + 166- > 244) <i>Bsa</i> II (75 + 97- > 172) <i>Bsa</i> II (273- > 90 + 183) <i>Alu</i> I (83 + 165- > 248)
<i>X. translucens</i>	LMG 726*	G	<i>Scr</i> FI (59 + 58- > 117) <i>Tsp</i> 509I (78 + 166- > 244) <i>Bsa</i> II (75 + 97- > 172) <i>Bsa</i> II (273- > 90 + 183) <i>Alu</i> I (83 + 165- > 248) <i>Alu</i> I (376- > 181 + 195)
<i>S. maltophilia</i> M1b	LMG 958 ^T	Q	14-0 ^e
<i>S. maltophilia</i> M1g	LMG 11002	P	15-1
<i>S. maltophilia</i> M1a	LMG 10996	O	14-2
<i>S. maltophilia</i> M1?	LMG 10853	M	13-3
<i>S. maltophilia</i> M1h	LMG 10882	K	14-4
<i>S. maltophilia</i> M2	LMG 11087	N	12-5
<i>S. maltophilia</i> M8	LMG 10857	I	8-8
<i>S. maltophilia</i> M5	LMG 11108	J	7-9
<i>S. maltophilia</i> M3	LMG 11114	L	16-14

^a According to Vauterin et al., 1995; in spite of their name, most '*S. maltophilia*' strains belonged to different genospecies (Nesme et al., 1995). ^b LMG: Laboratorium voor Microbiologie Gent Culture Collection, Gent, Belgium; CFBP: Collection française de bactéries phytopathogènes, Angers, France; T: type strain; *: pathovar reference strain. ^c As defined after digestion with 14 four-base endonucleases (Nesme et al., 1995). ^d For each site, the length of the fragments differing from that of pattern A are given. ^e First and second values indicate the number of restriction site differences with patterns A and Q, respectively.

3.3. *Xanthomonas* and *Stenotrophomonas*

Genera *Xanthomonas* and *Stenotrophomonas* belong to the gamma subclass of the *Proteobacteria* (Woese, 1987). *Xanthomonas* spp. contains species causing diseases on various plants, while bacteria belonging to the related genus *Stenotrophomonas* (formerly *Xanthomonas maltophilia*) are not plant pathogens in spite of their frequent isolation from plant material. Twenty-one genospecies have been delineated among the former genus *Xanthomonas* (Vauterin et al., 1990, 1995), amongst which 20 were retained in the genus; the others were transferred to the new genus *Stenotrophomonas* (Palleroni and Bradbury, 1993).

Results of *rrs* PCR-RFLP analysis with 14 endonucleases showed that the 16S genes of the bacteria belonging to the genera *Xanthomonas*, *Stenotrophomonas* and *Xylella* shared 69 common restriction sites. This is much more than the number of sites shared with any other genus of the same subclass (not shown). This result, therefore, confirms the close relatedness of *Xylella* and *Xanthomonas* (*sensu lato*) already shown by Wells et al. (1987). However, eight restriction sites were found almost exclusively in *Xanthomonas* and *Stenotrophomonas* spp. and ten exclusively in *Xylella*, allowing the clear differentiation of the latter genus (Nesme et al., 1995). Numerous genospecies of *Xanthomonas* could not be differentiated on the basis of their *rrs* RFLP profiles, and several exhibited only one or two restriction site differences. These results clearly show that the PCR-RFLP method allows only the identification of groups of related species rather than that of well-defined species of *Xanthomonas* spp. Conversely, all nine strains of *Stenotrophomonas* yielded distinguishable patterns (table I). Data were precise enough to provide unambiguous trees of pattern relationships with the parsimony method as well as with distance-based methods, Fitch-Margoliash, neighbour-joining, UPGMA and maximum-likelihood. Furthermore, phylogenetic distances calculated from *rrs* PCR-RFLP results strongly suggested that several genospecies may exist within the so-called species *S. maltophilia*, a result which exemplifies the discriminative power of the method within this taxon (Nesme et al., 1995).

3.4. The *Pseudomonas syringae* group of species

Results similar to those obtained with *Xanthomonas* spp. were found for *Pseudomonas syringae*, a group of oxidase negative species that also belong to the gamma subclass of *Proteobacteria* (Woese, 1987). The *P. syringae* group gathers essentially plant pathogenic bacteria. The current classification of this species was originally based on the nature of the host plants infected by the bacteria (Schaad, 1982; Young et al., 1992), a criterion which allowed pathologists to sort isolates into pathovars (strains of the same pathovar were able to infect the same host plants). This classification has recently been re-examined in the light of molecular data. Young et al. (1996) list over 50 pathovars of *P. syringae*, three pathovars of *P. savastanoi* and four related species (*P. amygdalii*, *P. caricapapayae*, *P. ficuserectae* and *P. meliae*). DNA pairing studies indicate that these pathovars and related species indeed fall within nine genospecies (Gardan et al., 1997).

RFLP of PCR-amplified *rrs* was analysed by using 13 endonucleases. Eleven enzymes generated near or identical patterns, and only *HmaI* and *AhaI* generated more variable patterns. Analysis of 42 strains belonging to the nine genospecies revealed that strains belonging to one genospecies may cluster with strains of other genospecies and may even share identical patterns (figure 1). Surprisingly, however, the *rrs*-based phenogram is not congruent with the DNA pairing studies, as exemplified by the pv. *pisi* strain (genospecies 1), which gathers with most members of genospecies 3 in one cluster, and by the pv. *persicae* strain (genospecies 3), which gathers with most members of the genospecies 1 in a remote cluster. This apparent lack of congruency may be caused by horizontal transfers of *rrs* from one genospecies to the other after their speciation, or by a high degree of reverse mutations in this gene. Neither hypothesis fits with our view of bacterial evolution. Furthermore, if true, our hypothesis would strongly argue against the application of *rrs* to ground bacterial phylogeny as proposed by Woese (1987). However, in our opinion, the apparent incongruency of the present results should be considered very cautiously because differences between patterns were not analysed in terms of polymorphic restriction sites (since relevant *rrs* sequences are lacking) but in terms of restriction fragment length using an automated device. Furthermore, the UPGMA method used to construct dendrograms is not fully appropriate to phylogenetic studies. The above results, however, are consistent with the detection of a very small number of polymorphic restriction sites in *rrs* and in *rriI* in the analysed species (Manceau and Horvais, 1997). With these reservations, results obtained with the genospecies related to *P. syringae* are identical to those described earlier with *Xanthomonas* spp. For both taxa, the *rrs* PCR-RFLP method can be used only to identify a group of closely related genospecies and not a given genospecies.

That the genospecies of *P. syringae* and related species defined on the basis of DNA pairing studies cannot be differentiated on the basis of distinctive phenotypic characters creates another discrepancy in the classification of this group of bacteria. Taken together, these data and the results generated by *rrs* PCR-RFLP analysis may impede the recognition of the genospecies defined previously as *bona fide* new species. However, the current division in nine genospecies remains in agreement with the results obtained by classical ribotype analysis (Gardan et al., unpublished data) and by PCR-RFLP analysis of the intergenic spacer located between *rrs* and *rriI* (Manceau and Horvais, 1997). It is tempting to speculate that, in contrast to the slow evolving *rrs* or *rriI* genes, noncoding sequences in the vicinity of these genes may constitute 'fast-running molecular clocks' suitable for analysis of the relatednesses of closely related genospecies.

Finally, the identification of six *P. fluorescens* and three *P. putida* biovars (Laguerre et al., 1996; Latour et al., 1996) provides additional information on the method in the genus *Pseudomonas*. These authors showed that *rrs* PCR-RFLP analysis and biovar classification is coherent. This suggests that, contrary to what we found with *P. syringae*, a strong polymorphism can be evidenced between members of *P. fluorescens* and *P. putida*. However, the present classification of the two latter species is based upon biochemical traits and remains uncertain. Thus, in our hands, DNA pairing studies showed that most biovars belong to different genospecies with a rate of DNA pairing

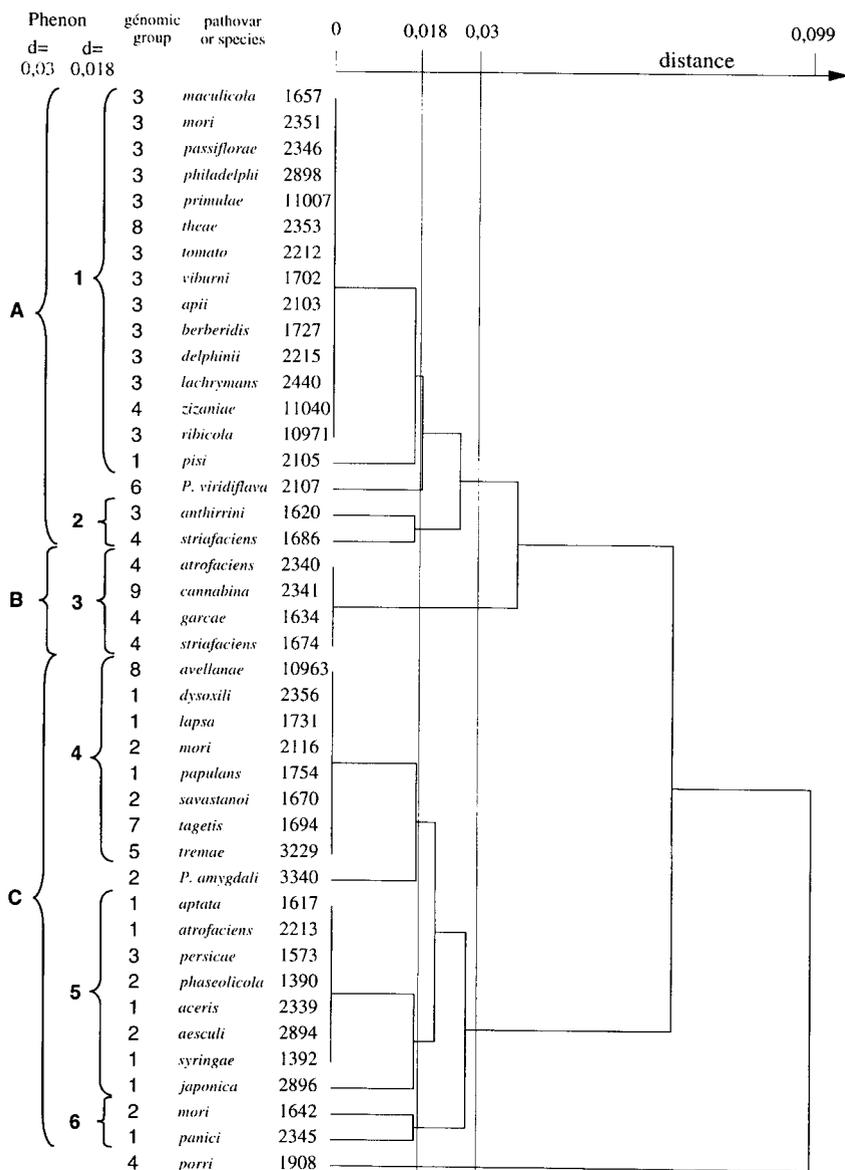


Figure 1. Dendrogram of relatedness of 42 strains of the *P. syringae* group of species.

as low as 10% within the biovar IV alone (unpublished data). Thus, we assume that the resolution of the method is probably similar whatever the species of *Pseudomonas* and that inconsistencies merely originated from species definitions, which are based upon DNA pairing with *P. syringae* and related species, and upon biochemical traits with *P. fluorescens* and *P. putida*.

3.5. *Agrobacterium*

The genus *Agrobacterium* is a member of the *Rhizobiaceae* family (Kerstens and De Ley, 1984), which has been included in the alpha subclass of *Proteobacteria* on the basis of ribosomal characteristics (Woese et al., 1984a). Several members of the genus induce hyperplastic disorders in many kinds of plants, which are known as crown gall, hairy root or cane gall diseases. The pathogenicity is almost entirely encoded by transferable Ti or Ri plasmids (Ream, 1989), and the delineation of species was, until recently, based on pathogenic traits (Kerstens and De Ley, 1984) encoded by plasmids that are transferable between strains (Van Larebeke et al., 1975). Independently of the plasmid content of the strains, several biovars have been described on the basis of chromosomal (and therefore more stable) characters (Keane et al., 1970; Kersters et al., 1973; Kerr and Panagopoulos, 1977; Holmes and Roberts, 1981). DNA pairing studies showed that different biovars belong to different genospecies (Heberlein et al., 1967; Kersters and De Ley, 1984; Popoff et al., 1984; Ophel and Kerr, 1990). This observation led to a revised nomenclature of the genus (Ophel and Kerr, 1990; Sawada et al., 1993; Bouzar, 1994), in which biovar 1 is now designated *A. tumefaciens*, biovar 2 *A. rhizogenes* and biovar 3 *A. vitis*. A fourth species consisting of only three strains is known as *A. rubi*. In addition, some strains (like NCPPB1650, and AF3.10) can be separated from other agrobacteria to constitute a nuclei around which future *Agrobacterium* isolates may cluster (Kerstens et al., 1973; Bouzar et al., 1995). Nevertheless, the genospecies status of the biovar 1 (characterised by the production of 3-ketolactose) is more complex than generally considered in recent taxonomic revisions. For instance, biovar 1 includes strains like TT111, which is genetically only 50 % homologous with the reference strain B6 (Kerstens et al., 1973). The occurrence of nine genospecies within biovar 1 was unequivocally established by Popoff et al. (1984). DNA pairing studies performed using nuclease S1 and ΔTm analysis made it possible for these authors to show that strain B6 and bacteria related to TT111 indeed belong to different genospecies. The phylogenetic relationships of members of the genus *Agrobacterium* were studied by *rrs* sequencing in several research groups (Sawada et al., 1993; Willems and Collins, 1993; Yanagi and Yamasato, 1993; Bouzar et al., 1995). All identified genospecies of *Agrobacterium* could be distinguished on the basis of their *rrs* sequences. Results also showed that the two closely related genospecies defined by strains B6 and TT111 differed by ten nucleotides in their *rrs* sequences (table II). A similar distance has been detected between a representative strain of *A. rubi* and strain NCPPB1650, which probably belong to different species as judged by the analysis of various phenotypical traits (Kerstens et al., 1973). This assertion, however, remains to be confirmed by DNA pairing studies.

Interestingly, that *A. tumefaciens*, *A. rhizogenes* and *A. vitis* are readily identifiable using *rrs* PCR-RFLP was demonstrated prior to the availability of the corresponding *rrs* sequences (Ponsonnet and Nesme, 1994a). Furthermore, several *rrs* patterns were obtained within members of biovar 1, confirming the heterogeneity of the taxon. Those empirical results can now be re-evaluated in light of the analysis of the *rrs* sequences. For this purpose, we performed a computer simulation of *rrs* restriction with 22 endonucleases available from various manufacturers, recognising a 4-bp sequence. The simulation showed

Table II. Discriminating capability of the *rrs* PCR-RFLP method amongst genospecies of *Agrobacterium*.

	<i>A. sp.</i> AF3.10	<i>A. sp.</i> 1650	<i>A. rubi</i>	<i>A. tum.</i> B6	<i>A. tum.</i> TT111	<i>A. vitis</i>	<i>A. rhiz.</i>
<i>A. sp.</i> AF3.10		8 (26)	8 (28)	7 (30)	9 (40)	15 (72)	17 (90)
<i>A. sp.</i> NCPPI650	<i>HaeIII</i> (1)		4 (10)	6 (21)	3 (21)	9 (67)	17 (86)
	<i>HpaII</i> (2)						
<i>A. rubi</i>	<i>HaeIII</i> (1)	<i>HpaII</i> (1)		8 (24)	5 (23)	10 (72)	17 (88)
	<i>HpaII</i> (1)						
<i>A. tumefaciens</i> B6	<i>AbaI</i> (1)	<i>AbaI</i> (1)	<i>AbaI</i> (1)		3 (10)	14 (70)	18 (75)
	<i>HpaII</i> (2)	<i>HaeIII</i> (1)	<i>HpaII</i> (1)				
<i>A. tumefaciens</i> TT111	<i>AbaI</i> (1)	<i>AbaI</i> (1)	<i>AbaI</i> (1)	<i>HaeIII</i> (1)		14 (63)	17 (77)
	<i>HaeIII</i> (1)		<i>HpaII</i> (1)				
<i>A. vitis</i>	<i>HaeIII</i> (3)	<i>HaeIII</i> (2)	<i>HaeIII</i> (2)	<i>AbaI</i> (1)	<i>AbaI</i> (1)		19 (87)
	<i>HpaII</i> (1)	<i>HpaII</i> (1)	<i>HpaII</i> (2)	<i>HaeIII</i> (3)	<i>HaeIII</i> (2)		
<i>A. rhizogenes</i>				<i>HpaII</i> (1)	<i>HpaII</i> (1)		
	<i>AbaI</i> (4)	<i>AbaI</i> (4)	<i>AbaI</i> (4)	<i>AbaI</i> (3)	<i>AbaI</i> (3)	<i>AbaI</i> (4)	
	<i>HaeIII</i> (2)	<i>HaeIII</i> (1)	<i>HaeIII</i> (1)	<i>HaeIII</i> (2)	<i>HaeIII</i> (1)	<i>HaeIII</i> (1)	
	<i>HpaII</i> (2)	<i>HpaII</i> (1)	<i>HpaII</i> (1)	<i>HpaII</i> (1)	<i>HpaII</i> (1)	<i>HpaII</i> (2)	

Values in the upper right part of the table indicate the number of discriminating enzymes (out of 22 used in computer simulations) followed by the number of nucleotide differences calculated after *rrs* sequence alignments (in parentheses). Data on the lower left indicate discriminating enzymes amongst the minimal set of three selected after simulation. Values in parentheses indicate the number of polymorphic sites.

that all the tested sequences can be distinguished by means of *rrs* PCR-RFLP, even when the number of nucleotide substitutions is as low as ten (e.g. as observed between closely related B6 and T111 genospecies or between strain NCPPB1650 and *A. rubi*; table II). The use of 22 enzymes is not possible for the purpose of routine identification. Thus, considering both the resolution properties of agarose gels (fragments smaller than 75 bp cannot be easily seen in gels) as well as the cost of endonucleases, we found that *HpaII* (or *MspI*), *AluI* and *HaeIII* would be the smallest and cheapest set of four-base endonucleases required to identify all the species *Agrobacterium* on the basis of the RFLP profiles of their *rrs* gene (table II). With such a limited set of enzymes, the average price for species identification is about 4 ECU per isolate, which is cheaper than every conventional gallery method. Results of the simulation were confirmed with all tested strains (data not shown), and this method is now of routine use in many laboratories studying wild isolates of *Agrobacterium* spp. In addition, the PCR-RFLP analysis of the ribosomal region consisting of the *rrs* gene plus the intergenic spacer (IGS, located between *rrs* and *rrl*, i.e. the 23S rRNA gene) allowed us to obtain both a clear identification of the species of *Agrobacterium* – by the mean of *rrs* fragments – and a very accurate typing of the isolates at the strain level – by means of IGS fragments (Ponsonnet and Nesme, 1994b). This PCR-RFLP equivalent of the classical ribotyping coupled to PCR-RFLP analysis of pTi regions now constitutes a powerful tool for epidemiological studies on crown gall dissemination (Pionnat et al., 1996).

Thus, for *Agrobacterium* spp., the results clearly showed that this method allows the discrimination of strains belonging to different genospecies. The method should also permit the identification of strains of the new *Agrobacterium* sp., whose original representatives were isolated from galled fig trees in Florida (Bouzar et al., 1995; Vaudequin-Dransart et al., 1995). The sequencing of the *rrs* of one of these 'fig strains' indeed revealed that the rate of nucleotide substitutions ranged from 1.8 to 2.7 % when the 16S rRNA gene of the fig strain was compared to the corresponding genes of *A. tumefaciens* and *A. rubi*, whereas the rate of nucleotide substitutions of the *rrs* genes of these two closely related species only reached 1.5 % (Bouzar et al., 1995). This result is of interest since fig strains and *A. tumefaciens* strains were not easily distinguishable on the basis of other morpho-biochemical characters such as total fatty acid composition. The validity of the PCR-RFLP method was also exemplified by the analysis of the *Agrobacterium rrs* sequences available from the data base. Amongst those, the digestion in vitro and in silico revealed that two of these sequences were obtained from misidentified *Agrobacterium* strains, originally identified as *A. tumefaciens*. Using a selected set of 12 enzymes, these two isolates were unambiguously associated with other strains of the *A. rhizogenes* species to which they indeed belong (Oger et al., unpublished data).

Laguerre et al. (1994b) extended the *rrs* PCR-RFLP analysis to most members of the *Rhizobiaceae* by examining 48 strains representing the eight recognised *Rhizobium* species, two new Phaseolus bean *Rhizobium*, *Bradyrhizobium* spp., *Agrobacterium* spp. and unclassified rhizobia from various host plants. Twenty-one composite patterns were obtained from the combined data generated using nine endonucleases. Species assignments were in full agreement with the established taxonomic classification, and the *rrs* PCR-RFLP method now provides a rapid tool for the identification of root nodule isolates and the

detection of new data. It can thus be concluded that *rrs* PCR-RFLP allows the delineation of most species among *Rhizobiaceae* members.

3.6. Plant-associated bacteria

The previously mentioned studies exclusively involved known bacterial species and therefore yielded data on the validity, sensitivity and resolution limit of the PCR-RFLP technique with respect to these species only. Soil-borne and plant-associated bacteria being extremely diverse, we feel that there is a need for a more general evaluation of the validity of the PCR-RFLP technique. To address this question, we took advantage of previous studies performed on bacteria associated with the root system of the legume plant *Lotus corniculatus* (Oger et al., 1997). These bacteria were first identified using morphological and biochemical criteria. Five morpho-biochemical (MB) groups were evidenced but their precise identification met with limited success. We used this material to investigate whether the PCR-RFLP technique could provide us with more accurate data and whether these would be in agreement with the results of other molecular, high-resolution identification techniques. Restriction enzymes enabling the efficient discrimination of isolates of unrelated species were selected by a computer simulation of the digestion of *rrs* of four bacteria belonging to the species *Escherichia coli*, *Pseudomonas aeruginosa*, *Frankia* sp. and *Agrobacterium tumefaciens*. Twelve inexpensive enzymes were retained because they appear to generate quite different restriction patterns. As described earlier, this set of enzymes also allowed the differentiation of four related species of *Agrobacterium*: *A. tumefaciens*, *A. rubi*, *A. rhizogenes* and *A. vitis*, both in silico and in vitro (Oger et al., unpublished data).

The set of 12 enzymes was used to sort 57 clones isolated from the rhizosphere of *Lotus* plants. Seventeen RFLP groups were obtained. Most of them exhibited very distinct patterns, whereas others were distinguished only by a single enzyme. In this latter case, the 'molecular proximity' of these bacteria was always correlated with their morpho-biochemical 'proximity' (table III). To validate the classification obtained by the PCR-RFLP technique, we sorted the 57 rhizospheric isolates on the basis of classical ribotyping, using the enzymes *EcoRI* and *HindIII*. Results were compared to those generated by the PCR-RFLP method. Remarkably, a given ribotype was always associated with a single PCR-RFLP group and was never shared by bacteria exhibiting different *rrs* patterns. This was also true for the closely related RFLP groups whose delineation relies on the profiles generated by a single restriction enzyme: a given ribotype was clearly associated with each of these closely related PCR-RFLP groups. The reverse proposition, however, was not true. Isolates clustered in a given PCR-RFLP group may exhibit one or more ribotypes. This situation is in full agreement with the results described earlier with *Pseudomonas* spp.

Some root-associated isolates were identified as *Rhizobiaceae* and *Corynebacterium* on the basis of their respective morpho-biochemical characters. Interestingly, an excellent correlation was obtained between the computerised and experimental data for the isolate of the family *Rhizobiaceae*, which appears to belong to the genus *Agrobacterium*. Conversely, the *rrs* PCR-RFLP of the coryneform isolate was completely unrelated to those generated by computer simulation based on the published 16S rRNA gene sequence of this genus.

Table III. Comparison of the classification generated by morpho-biochemical analysis, RFLP of the 16S rRNA gene and ribotyping amongst 57 root-associated bacteria.

MB groups	16S groups	Ribogroups (R groups)	
		<i>EcoRI</i>	<i>HindIII</i>
MB1	G3	nt.	R'3
MB2	G6a	nt.	R'5
	G6b	R6	nt.
	G6c	R7	R'6, R'7
	G6d	R8	nt.
	G6e	R9	nt.
MB3	G10	R12	R'10
	G11	nt.	R'11
MB4	G7	R10	R'8
MB5	G1	R1 to R3	R'1, R'2
	G2	nt.	nt.
	G4	R4	R'4
	G5	nt.	R'5
	G8	R7	R'9
	G9	R11	R'4
	G12	nt.	nt.
	G13	R13	R12

This table shows the relationships existing between the groups defined by the various methods used in this paper. MB refers to the different morphological/biochemical groups, 16S groups to those obtained by PCR-RFLP analysis of the 16S rRNA gene, R and R' groups to the *EcoRI* and *HindIII* ribogroups, respectively. Group numbers were arbitrarily given and do not reflect the possible relatedness of the various profiles except for groups G6a to G6e, which are closely related. nt.: not tested.

Within this taxon, however, our experimental patterns appeared related but still dissimilar to those obtained from members of the genus *Clavibacter*. Interestingly, our isolate and most members of the genus *Clavibacter* share some morpho-biochemical characters (such as Gram variable staining) and the property of being plant-associated bacteria (Oger et al., unpublished data).

Thus, the PCR-RFLP technique proved to be very useful in sorting unknown plant-associated bacteria. Contrary to what was observed with the *P. syringae* strains, the results obtained by the PCR-RFLP analysis of the 16S gene were in excellent agreement with both morpho-biochemical data and ribotyping, though the PCR-RFLP method appeared less discriminate than the ribotyping under our experimental conditions.

Results obtained in this part of the study do not permit us to determine precisely the resolution of the PCR-RFLP technique with bacteria isolated from a community. This question is under further investigation and will be answered only once the relevant microorganisms have been identified (by the analysis of the *rrs* sequence of a representative member of each 16S group). Nevertheless, this shows that the PCR-RFLP technique may be used to discriminate unknown bacterial isolates, a feature which indicates that precise

identification of bacteria is not always a prerequisite in the assessment of the evolution of microbial communities.

3.7. Resolution limits of the method

Clearly, these results showed that differences in *rrs* are great enough to permit the identification of species of *Rhizobiaceae*, but only of groups of species closely related to *Pseudomonas syringae* and *Xanthomonas* sp. A question then arises: do these results indicate that the *rrs* molecular clock runs faster in *Rhizobiaceae* than in Xanthomonadaceae? Perhaps and probably not. Genospecies of *Stenotrophomonas* and *Rhizobiaceae* are efficiently distinguishable by the method and both *Stenotrophomonas* and *Rhizobiaceae* are isolated from soil or from material which somehow 'trapped' the bacteria. The ecology of *Agrobacterium* (and that of other *Rhizobiaceae*), which involves frequent contacts with the soil microflora, would favour the diversity emphasised by the *rrs* PCR-RFLP method and by ribotyping, a case identical to that of the fluorescent *Pseudomonas* isolated from soils which exhibit very dissimilar *rrs* PCR-RFLPs (Laguerre et al., 1996a; Latour, 1996). On the other hand, *P. syringae* and related species as well as pathogenic xanthomonads are very often isolated from plant aerial lesions caused by clonal dissemination of the bacteria but not by trapping from the rich soil reservoir. This latter situation could lead to the frequent isolation of identical or closely related bacterial clones as deduced from the occurrence of bacteria of identical ribotype in different biotopes. In our opinion, the various levels of resolution observed between taxa should be attributed to sampling procedures and bacterial origins rather than to molecular clock differences between taxa. It is therefore tempting to conclude that the resolution limit of the *rrs* PCR-RFLP method lies at the group of related species. These results might be extended in the near future to the *Rhizobiaceae* in light of the DNA pairing studies performed by Popoff et al. (1984) on agrobacteria belonging to the biovar 1.

3.8. Sensitivity of the method

The sensitivity of the method could be evaluated by the minimum number of polymorphic restriction sites required to ensure that two bacteria belong to different genospecies. Ideally, this should not depend upon the taxa considered and should be based upon the fact that bacteria of the same genospecies harbour very similar *rrs*. Some conclusions can be drawn from our empirical results. First, the presence of two polymorphic sites within the rRNA operons of a single bacterial genome clearly showed that a two restriction site difference between the *rrs* of two bacteria is not sufficient to demonstrate that they belong to different species. The role of the *rrs* (or *rrs* product) similarities in taxonomy was clarified by Stackebrandt and Goebel (1994). Their comparative studies indicated that DNA-DNA association studies still remain the superior method that needs to be performed when *rrs* similarity is 97 % or higher. DNA pairing studies are therefore not required when the *rrs* sequences differ by 44 nucleotides or more. This also means that at least nine polymorphic restriction sites are required to discriminate unambiguously sequences differing by 44 bases (*table IV, figure 2*). The proposition of Stackebrandt and Goebel is certainly

Table IV. Comparison of *rrs* differences amongst genera *Xanthomonas* and *Stenotrophomonas* obtained either from PCR-RFLP analysis or from published sequences.

Genospecies	LMG N°	<i>rfs</i> RFLP	<i>X fr</i>	<i>X po</i>	<i>X ar</i>	<i>X ca</i>	<i>X co</i>	<i>X br</i>	<i>X cu</i>	<i>X az</i>	<i>X or</i>	<i>X va</i>	<i>X pi</i>	<i>X me</i>	<i>X ve</i>	<i>X ca</i>	<i>X tr</i>	<i>X hy</i>	<i>X sa</i>	<i>X al</i>	<i>S ma</i>
<i>X. fragariae</i>	708	Af	D	A	A	H	A	A	C	Ao	A	A	A	C	A	A	G	F	E	F	Q
<i>X. populi</i>	5743	D	8	1	1	3	1	1	2	3	1	1	1	2	1	1	5	6	5	6	15
<i>X. arboricola</i>	747	A	7	1	0	3	1	1	2	3	1	1	1	2	1	1	7	6	5	6	15
<i>X. cassavae</i>	673	A	7	3	2	2	0	0	1	2	0	0	0	1	0	0	6	5	4	5	14
<i>X. codiae</i>	8678	H	12	8	7	5	2	0	1	2	2	2	2	3	2	2	8	7	6	7	14
<i>X. bromi</i>	947	A	7	3	2	7	2	2	3	4	2	2	2	3	2	2	8	7	6	7	14
<i>X. cucurbitae</i>	690	A	6	2	1	6	1	1	1	2	0	0	0	1	0	0	6	5	4	5	15
<i>X. axonopodis</i>	538	C	16	12	11	11	16	11	1	3	1	1	1	0	1	0	7	6	5	6	15
<i>X. oryzae</i>	5047	Ao	8	4	3	8	3	3	14	3	2	2	2	3	2	2	8	7	6	7	16
<i>X. vasicola</i>	736	A	7	3	2	7	0	1	11	3	1	1	1	1	1	1	7	6	5	6	14
<i>X. pisi</i>	847	A	6	2	1	6	1	0	12	2	1	1	0	1	0	0	6	5	4	5	14
<i>X. melonis</i>	8670	C	6	2	1	8	3	2	10	4	3	2	2	1	1	1	7	6	5	6	15
<i>X. vesicatoria</i>	911	A	6	2	1	6	1	0	12	2	1	0	0	2	1	0	6	5	4	5	14
<i>X. campestris</i>	568	A	7	1	0	7	2	1	11	3	2	2	1	1	1	6	5	4	5	14	
<i>X. translucens</i>	876	G	41	35	34	34	39	34	33	36	34	34	35	35	35	34	6	5	4	1	20
<i>X. hyacinthi</i>	739	F	42	36	35	40	35	36	32	37	35	36	36	36	36	35	4	3	0	19	
<i>X. sacchari</i>	471	E	24	18	17	21	19	18	24	19	18	18	18	18	18	17	27	28	3	15	
<i>X. albilineans</i>	494	F	40	34	33	33	38	35	34	32	35	35	34	34	34	33	7	7	26	19	
<i>S. maltophilia</i>	958	Q	48	44	43	45	47	45	53	46	45	45	43	43	44	43	69	72	45	70	

Values on the upper right are the number of restriction site differences obtained after digestion with 14 enzymes (table I). Data on the lower left indicate the number of nucleotide site differences (Hauben et al., 1997).

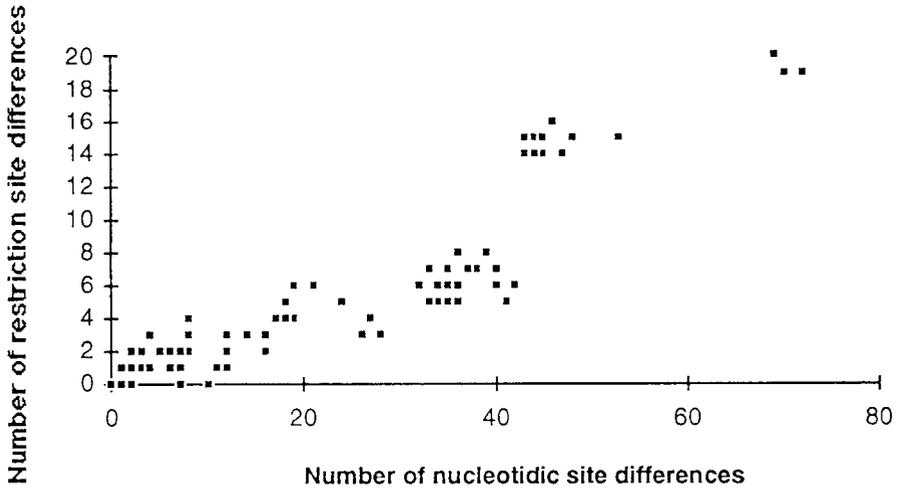


Figure 2. Relation between nucleotide site and restriction site differences in *rrs* amongst genospecies of *Xanthomonas* and *Stenotrophomonas*. The *rrs* (ca. 1 485 nucleotides long) were digested after polymerase chain reaction amplification using 14 four-base endonucleases (from data of table IV).

extremely conservative since, in our hands, nine restriction site differences could be obtained between strains of *Xanthomonas* and *Stenotrophomonas* or between strains of remote species of *Agrobacterium* (table II). A less conservative view could be based on our results (Nesme et al., 1995) as well as those of Stackebrandt and Goebel (1994), which both show that *rrs* similarity lower than 99.3 % (equivalent to at least ten nucleotide substitutions) only occurs between members of different genospecies. From our empirical results obtained with 14 enzymes, five restriction site differences at least are necessary to ensure that two *rrs* differ by at least ten nucleotides (figure 1). Thus, we propose that five restriction site differences should be sufficient to establish by PCR-RFLP of the 16S rRNA gene(s) that two bacteria belong to different genospecies. Overall, our results strongly support the view that the analysis of the restriction profile of the 16S rRNA gene is a rapid and versatile method to analyse known and unknown bacterial populations. The *rrs* PCR-RFLP study is simpler and faster than sequencing and is suitable for use in almost any laboratory. If sufficient enzymes are used and the positions of the recognition sites are known, the technique should also allow the accurate description of phylogenetic relatedness.

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