MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 1, No. 7, pp. 282-288, 1988

Acridine Orange Selects for Deletion of *hrp* Genes in All Races of *Pseudomonas solanacearum*

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Received 1 August 1988. Revised 28 September 1988. Accepted 29 September 1988.

Data previously established for *Pseudomonas solanacearum* strain GMI1000 concerning the structure of the genome in the vicinity of the cluster of *hrp* genes (Boucher *et al.* 1987, J. Bacteriol. 169:5626-5632) have been extended to 52 strains of *P. solanacearum* representing different races, biovars, and geographical origins. All pathogenic strains carry DNA sequences homologous to *hrp* genes present in strain GMI1000. For a majority of strains, as for strain GMI1000, acridine orange-resistant (Acr^r) derivatives are obtained following growth in the presence of the drug. The *hrp* region in these mutants was deleted, causing a simultaneous loss of pathogenicity toward host and of the ability to induce a hypersensitive response on nonhost plants. In addition, they were auxotrophic for methionine and excreted a brown pigment, two phenotypic traits that had previously been shown to be associated in Acr^r derivatives of strain GMI1000. These data collectively suggest a fairly similar organization of the *hrp* genes and the flanking DNA in this species. A restriction fragment length polymorphism study in the the *hrp* region and in three other randomly chosen regions shows the existence of a limited degree of polymorphism and suggests the coevolution of *hrp* genes with the rest of the genome.

Additional keywords: bacterial wilt, megaplasmid, host specificity, RFLP.

Bacterial wilt caused by *Pseudomonas solanacearum* is one of the most important and devastating bacterial diseases of plants (Buddenhagen and Kelman 1964; Buddenhagen 1986). Genetic approaches to pathogenicity determinants of this organism have been undertaken in different laboratories, and various Tn*5*-induced mutants altered in pathogenicity have been obtained (Staskawicz *et al.* 1983; Boucher *et al.* 1985; Xu *et al.* 1988; Roberts *et al.* 1988). We have recently shown that in *P. solanacearum* strain GMI1000, several *hrp* genes that control both pathogenicity on a compatible host and ability to induce the hypersensitive response (HR) on an incompatible plant (*hrp* genes) are clustered on the megaplasmid of this strain (Boucher *et al.* 1987). This region is deleted in acridine orange-resistant mutants (Acr^r) derived from the wild-type strain (Boucher *et al.* 1986). Also, Acr^r mutants exhibit auxotrophy for methionine (Met$^-$) and excrete a brown pigment during growth in rich culture medium (Message *et al.* 1978); they also have been shown to differ from the wild-type parent in the structure of their exopolysaccharide and lipopolysaccharide (Drigues *et al.* 1985). Therefore, in addition to *hrp* genes, the DNA region deleted from Acr^r mutants harbors: gene(s) conferring sensitivity to acridine; gene(s) governing methionine synthesis; gene(s) controlling colony morphology; and gene(s) that interfere with the production or release of the brown pigment. It does not harbor essential genes because it can be deleted without affecting the viability of the bacteria *ex planta* in rich media. Most of this work has been reviewed recently (Boucher *et al.* 1988).

Several studies, including serology (Digat and Cambra 1976; Harrison and Freeman 1961), phage typing (Okabe and Goto 1952; Okabe and Goto 1953), host range (Buddenhagen *et al.* 1962; Lozano and Sequeira 1970), and biochemical traits (Hayward 1964), have established the existence of a wide diversity of strains in this species. Hayward (1964) distinguished four biovars based on the ability of strains to use three hexose alcohols (mannitol, sorbitol, and dulcitol) and to produce acid from three disaccharides (cellobiose, maltose, and lactose). The classification of Buddenhagen *et al.* (1962) based on host specificity allows the distinction of three races or pathovars: race 1 isolates have a wide host range and can infect a large variety of Solanaceous and other plants, race 2 isolates specifically infect *Musa*, whereas races 3 isolates infect potato but not other Solanaceae. No strict correlation could be drawn between these two types of classification, although it is generally admitted (Hayward 1976; Buddenhagen 1986) that biovar 2 (as defined by Hayward 1964) corresponds to race 3 (as defined by Buddenhagen *et al.* 1962). More recently, a race 4 isolated from mulberry in China has been described (He *et al.* 1983).

In this paper we show that Acr^r mutants of a majority of *P. solanacearum* strains share most characteristics of Acr^r mutants of strain GMI1000 and particularly that they have lost DNA sequences corresponding to the *hrp* genes together with their pathogenic properties. This strongly supports a major role for *hrp* genes in pathogenic properties of *P. solanacearum* strains, whatever their race or biovar. However, we demonstrate the existence of some degree of variability in the distribution of restriction sites in the *hrp* region that is strain correlated with polymorphism in other genomic regions. This variability may be useful both for epidemiological purposes and for evolutionary studies in this species.

MATERIAL AND METHODS

Bacterial strains. *P. solanacearum* strains used in this study are listed in Table 1. They all show the wild-type

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Table 1. List of strains of *Pseudomonas solanacearum* used in this study and classifictions established according to biovars, races, and RFLP groups[a]

Strain	Alternative name	Source or reference	Isolated from	Geographical origin	Race	Biovar	RFLP groups according to			
							pVir2	pI1F4	pJ7D7	pAC5
GMI1000		Boucher *et al.* 1985	Tomato	Guyana	1*	4	1	1A		
UW143	S236	Lozano and Sequeira 1970	"	Australia-Qld.	1	3	1	1A	1A	1
031A		C. Hayward	Potato	Australia-Vict.	NP*	2	1	1A	1A	1
010		"	Tomato	Australia-Qld.	1	3	1	1A	1A	1
012A		"	*Rapistrum rugosum*	"	1	3	1	1A	1A	1
0170		"	Tobacco	"	1	3	1	1A	1A	1
0171		"	Eggplant	"	1	3	1	1A		
0192		"	*Xanthium pugens*	"	1	3	1	1A		
0234		"	*Pultanea*	"	1	3	1	1A		
0671		"	*Capsicum anuum*	"	1	3	1	1A		
007B		"	Ginger	"	1	4	1		1A	
043		"	Tomato	"	1	4	1	1A		
075		"	Ginger	"	1	4	1	1A	1A	
0211		"	"	"	1	4	1	1A	1A	
0224		"	"	"	1	4	1	1A	1A	
0263		"	"	"	1	4	1	1A		
UW130	S225	Lozano and Sequeira 1970	Tomato	Peru	1	3	1	1A	1B	1
UW152	S245	"	Potato	Australia	1	3	1	1A	1A	1
GT4		P. Prior	Tomato	Guadeloupe	1	4	1	1A	1B	1
GA2		"	Eggplant	"	1	3	1	1A	1D	
GA4		"	"	"	1	3	1	1A	1D	1
006		C. Hayward	*Xanthium pugens*	Australia-Qld.	1	3	1	1B	1A	1
UW74		L. Sequeira	Piper	Panama		4	1	1B	1A	1
009		C. Hayward	Ginger	Australia-Qld.	1	4	1	1B	1C	1
UW119	S213	Lozano and Sequeira 1970	Potato	Costa Rica	3(NP*)		0[b]	1B	1E	
0239		C. Hayward	*Capsicum*	Australia-Qld.	1	3	1	1B		
0333		"	*Solanum mauritianum*	"	1	3	1	1C		
0672C1		"	*Zinia*	"	1	3	1	1C		
003		"	Ginger	"	1	4	1	1C		
UW25	K60	Lozano and Sequeira 1970	Tomato	North Carolina	1*	1	2	2		
UW203		L. Sequeira	Tobacco	"	1	1	2	2	2A	0[b]
UW27	K105	Lozano and Sequeira 1970	"	Florida	1	4	2	2	2B	0[b]
UW154	S247	"	"	Columbia	1	1	2	2		
UW70	S210	"	Plantain	"	2	1	2	2		0[b]
UW134	S221	"	Potato	Kenya	1	1	2	2	2B	2
UW26	K74	"	Tomato	Georgia	1		2	2	2B	0[b]
UW80	S206	"	Potato	Columbia	3*	2	3	3A	3A	3A
UW81	S207	"	"	"	3	2	3	3A	3A	3A
UW85		L. Sequeira	Tomato	Ontario	1/2		3	3B	3B	3B
UW160	S253	Lozano and Sequeira 1970	Plantain	Peru	2	1	3	3B	3A	3C
UW82	S208	L. Sequeira	Potato	Columbia	3*	1	3	3B	3B	3C
UW127	S222	Lozano and Sequeira 1970	Plantain	Peru	2*	1	3	3B	3A	3D
UW128	S223	"	"	"	2*	1	3	3B	3A	3D
BA1		P. Frossard	Banana	Granada	2		4	4		
BA2		"	"	"	2		4	4	4	4
BA3		"	"	"	2		4	4		
BA4		"	"	"	2		4	4		
GT1		P. Prior	Tomato	Guadeloupe	1	1	5	5	5	
0731		C. Hayward	"	Australia-N.T.		2/3	6	6	6	6
0324		C. Hayward	*Salvia reflexa*	Australia-Qld.	1*	2	7	7A		7A
UW9	S147	Lozano and Sequeira 1970	*Heliconia*	Costa Rica	2(NP*)	1	7	7A	7	7B
UW135	S228	"	Banana	Honduras	2*	1	7	7B	7	7C

[a] All the strains that share a common RFLP group number with a particular probe show an identical hybridication pattern with this particular probe. Information concerning biovar characteristics are given according to previously published information or according to personal information from the source. For a certain number of strains, the race identification was made or confirmed in our laboratory. These strain have an asterisk placed next to the race number. NP = nonpathogenic on any of the hosts tested.

[b] No homologous sequence.

fluidal colony morphology phenotype characteristic of virulent strains (Kelman 1954). They were isolated from a large variety of host plants and geographical origins (Table 1).

Plasmids. Plasmids pVir2, pI1F4, and pJ7D7 are from the genomic bank of strain GMI1000 made in the cosmid pLAFR3 (Lindgren *et al.* 1986; Boucher et al. 1987). They each carry about 25 kb of *P. solanacearum* DNA. The insert of pVir2 carries part of the *hrp* cluster previously described (Boucher *et al.* 1987). The inserts of pIIF4 and pJ7D7 contain genomic regions flanking the Tn*5* insertion sites of the previously described *dsp* mutants GMI1299 and GMI1314, respectively, which are nonpathogenic on homologous hosts but still able to induce an HR on tobacco (Boucher *et al.* 1985). Plasmid pJ7D7 will be described further elsewhere. It carries a sequence that is reiterated several times in the genome of strain GMI1000. Plasmid pAC5 is a derivative of pACYC184 (Chang and Cohen 1978) in which a randomly chosen *Hin*dIII fragment originating from strain UW25 has been inserted. None of the inserts of these hybrid plasmids shares homology with the three others.

Culture conditions. All strains were grown at 30° C. B broth, BGT agar, and mineral MP media have been previously described (Boucher *et al.* 1985; Boucher *et al.* 1986). When required, MP medium was supplemented with l-methionine (20 μg/ml).

Molecular biology techniques. Extraction of genomic DNA, digestions with *Eco*RI, gel electrophoresis, Southern transfers, and hybridizations under stringent conditions were conducted according to protocols previously described (Boucher *et al.* 1987).

Acridine orange treatment. Attempts to isolate Acr^r mutants were performed as previously described (Message *et al.* 1978). An overnight culture of cells grown in B broth was diluted a thousand-fold in B broth containing acridine orange (AO) (200μg/ml). Following incubation for 60 hr in the dark, a visible growth had occurred as seen by the increase of turbidity. One hundred microliters of a 10^{-4} dilution was then plated on BGT agar; this led to the growth of 100–200 colonies, which were then replica-plated on MP and MP + methionine agar plates to detect clones auxotrophic for methionine.

Pathogenicity tests. These were conducted on potato (cv. Sirtema), tomato (cv. Supermarmande), eggplant (cv. Giniac), and diploid *Musa* sp. Inoculations were performed by stabbing a micropipette tip containing 10^8 cells in a volume of 100 μl of water into the lower part of 3-wk-old potato shoots grown from tubers or in the stem of 10-cm-high tomato or eggplant seedlings. *Musa* seedlings (10 cm in height) were inoculated by stabbing the micropipette tip into the pseudo-stem. Disease symptoms were recorded following incubation of inoculated plants for 4 wk in a growth chamber (30° C, 18 hr light). Strains that wilted potato, tomato, and eggplant were considered as race 1 isolates; strains that exclusively wilted potato were considered as race 3 isolates, whereas strains that wilted *Musa* but not the Solanaceous hosts were considered as race 2. For pathogenicity tests on tobacco (cv. Bottom special), bacterial suspensions, prepared in water and adjusted to 10^8 cells per milliliter, were infiltrated into leaf parenchyma. Inoculated plants were kept for 48 hr in the laboratory at room temperature. Because *P. solanacearum* is not naturally present in France, all the inoculation experiments were conducted in P3 laboratory facilities.

RESULTS

Isolation of Acr^r mutants. We had previously shown that deletion mutants for *hrp* genes in strain GMI1000 could easily be obtained by selection for Acr^r mutants. We tested 21 other strains for their ability to generate Acr^r mutants following growth in the presence of AO. Although all of the strains tested were sensitive to the drug, in all the cases growth of the culture was visible on the third day following incubation in the presence of the drug. However, this experiment allowed us to distinguish two groups of strains based on the ability to yield methionine auxotrophs (Met$^-$) (Table 2).

The 15 strains of the first group yielded Met$^-$ derivatives under these conditions. For all but one, Met$^-$ mutants represent more than 50–100% of the bacterial population grown in the presence of the drug. The frequency of such mutants for strain UW27 never exceeded a few percent. In all cases these mutants had simultaneously acquired the ability to excrete a large amount of a brown diffusible pigment into the culture medium. We will show below that they shared other of the properties of the previously described Acr^r mutants of strain GMI1000 (Message *et al.* 1978), and they will be further referred to as Acr^r mutants. On the contrary, the eight strains of the second group, although they poorly grew in the presence of AO, did not yield Met$^-$ mutants at a

Table 2. Classification of strains of *P. solanacearum* based on their ability to generate Acr^r mutants, and comparison of parental strains and Acr^r or AOT derivatives for their ability to induce symptoms on tobacco and for the presence of sequences homologous to the insert of plasmid pVir2

	Tobacco response[a]		Presence of pVir2 sequences[b]	
Strains	**Wild type**	**Derivative**	**Wild type**	**Derivative**
Strains that segregate Acr^r mutants				
GMI1000	HR	–	+	–
UW143	HR	–	+	–
UW74	HR	–	+	–
006	D	–	+	nt
010	HR	–	+	nt
012A	nt	nt	+	nt
UW27	D	D	+	+
UW154	D	–	+	nt
UW82	HR	–	+	–
UW85	HR	–	+	–
BA1	nt	nt	+	–
BA2	nt	nt	+	–
UW119	–	–	+	nt
0324	–	–	+	nt
UW9	HR	–	+	nt
Strains that do not segregate Acr^r mutants				
UW25	D	D	+	+
UW203	nt	nt	+	nt
UW152	HR	HR	+	+
UW70	HR	HR	+	nt
UW160	HR	HR	+	+
731	D	D	+	+
UW134	D	D	+	+
UW81	D	D	+	nt

[a] HR = hypersensitive response; D = disease; nt = not tested.
[b] + = homologous sequences present; – = no homologous sequences; nt = not tested.

detectable frequency (less than 10^{-2}). However, the question was raised as to whether for strains of this second group clones obtained following growth in the presence of the drug could be further distinguished from the parental strains on the basis of additional traits associated with the Acr^r phenotype. Therefore, for each strain that did not generate Met$^-$ auxotrophs, a clone resulting from growth in the presence of AO and designated AOT (acridine orange tolerant), was purified and was further compared with the parental strain. Similar characterizations were also conducted on Acr^r mutants and parental strains. In some cases, prototrophic AOT clones derived from strains that did generate Acr^r mutants were also included in these assays.

Pathogenicity of Acr^r or AOT derivatives. Pathogenicity was first assayed by infiltration of bacterial suspensions into tobacco leaves parenchyma. Under these conditions all the wild-type strains induced some kind of reaction within 48 hr (Table 2). With certain strains this was a typical HR occurring within 24 hr. With other strains the reaction, which only became visible after 24 hr and led to an expanding necrotic lesion, most probably reflected disease development. In the same conditions all AOT strains (whether they originated from strains that yield Acr^r mutants or not) had retained the parental phenotype following infiltration into tobacco leaves. In contrast, all but one (UW27 Acr^r) of the Acr^r derivatives had lost the ability to induce any visible reaction on tobacco. For the Acr^r derivatives of strains UW143 and UW85, this loss of pathogenicity was further confirmed because these mutants did not induce any response following inoculation of potato stems, whereas the wild-type parents induced typical wiltings within 2 wk. It should be noted that in the tobacco leaves infiltration assay, several wild-type strains did not behave as previously reported by Lozano and Sequeira (1970). In particular, we did not find a correlation between the type of response obtained in this assay and the grouping of strains according to races. Although we agree that all the race 2 isolates induced a HR, we found that this reaction was equally induced by all race 3 isolates tested and by five out of 10 race 1 isolates tested. It is not clear presently whether this discrepancy between our results and previously published data could reflect differences in the experimental conditions used.

Presence of a deletion in Acr^r mutants. We had previously shown that Acr^r derivatives of strain GMI1000 carried a large deletion of the megaplasmid (Boucher *et al.* 1986) and that this deletion extended over the cluster of *hrp* genes already described (Boucher *et al.* 1987). We assayed a limited number of strains to see if such a deletion was present in Acr^r and AOT derivatives of other strains. This was done by hybridization of *Eco*RI genomic blots with a pVir2 probe that covers part of the *hrp* cluster. The results of these experiments are shown in Table 2 and Figure 1. They establish that: all the wild-type strains tested shared sequences common to pVir2; these sequences are deleted from all Acr^r mutants except UW27Acr^r, which had previously been shown to have retained pathogenicity; and all AOT derivatives retained the corresponding genes.

Together, these data show that AOT strains could not be distinguished from their parental strains by any of the characters assayed and therefore suggest that these strains are identical to their original parent. They probably result from residual growth of the wild-type strain in the presence of the drug.

Although the pVir2 region is present in all the wild-type strains tested, the distribution of *Eco*RI restriction sites in this region differs between strains. Based on restriction fragment length polymorphism (RFLP) analysis, this should allow the investigation of the degree of evolution of *hrp* genes in the species. This was done in the following experiments.

Evaluation of RFLP among *P. solanacearum* strains. The RFLP variations in the *hrp* region was first investigated for the 52 *P. solanacearum* strains listed in Table 1. This was done by hybridizing the *Eco*RI genomic blots with a pVir2 probe. The results are presented in Figure 2A and Table 1 and confirm the presence of a region homologous to the pVir2 region in all but one (UW119) of the strains tested. In addition, it established that they were patterns of *Eco*RI restriction sites in this region among the different strains allowing the classification of these strains in seven clearly distinct RFLP groups (Table 1). Strains sharing identical hybridizing bands were given a single RFLP group number and were considered to belong to the same RFLP group. It is clear that different RFLP groups may, in some cases, share a degree of similarity, as exemplified by the presence of a 4.2-kb fragment in strains of groups 2, 3, and 7 and by the presence of an additional 8-kb fragment common to strains of groups 2 and 3.

We asked if the divergences observed for the *hrp* region could be correlated with potential divergences in other parts of the genome. This could reflect basic structural differences of the entire genomes, indicating the coevolution of housekeeping and *hrp* genes. Therefore, additional hybridizations were performed by using three other probes. Two of them (pI1F4 and pJ7D7) carried fractions of strain GMI1000 genome, whereas the last one (pAC5) carried a DNA fragment originating from strain UW25. The results shown in Table 1 and in Figure 2B–2D basically led to the same grouping of the strains, although these additional

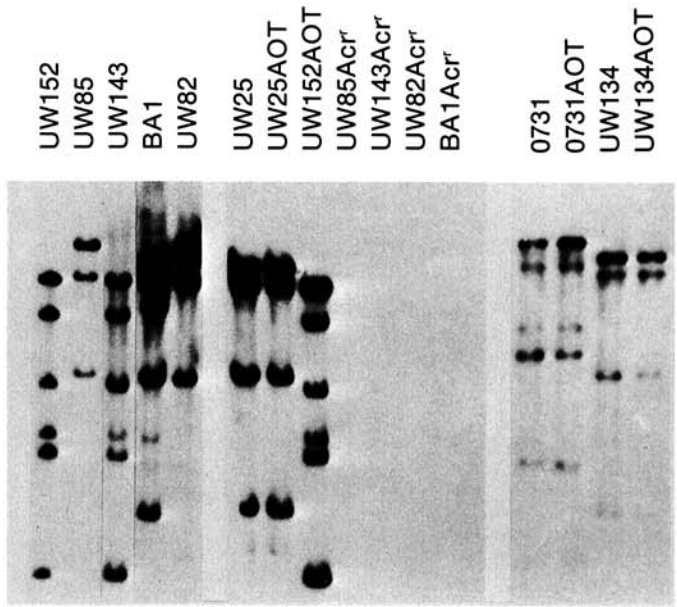

Fig. 1. Hybridization of *Eco*RI genomic blots of various wild-type strains and of their respective Acr^r or AOT derivatives with probe pVir2.

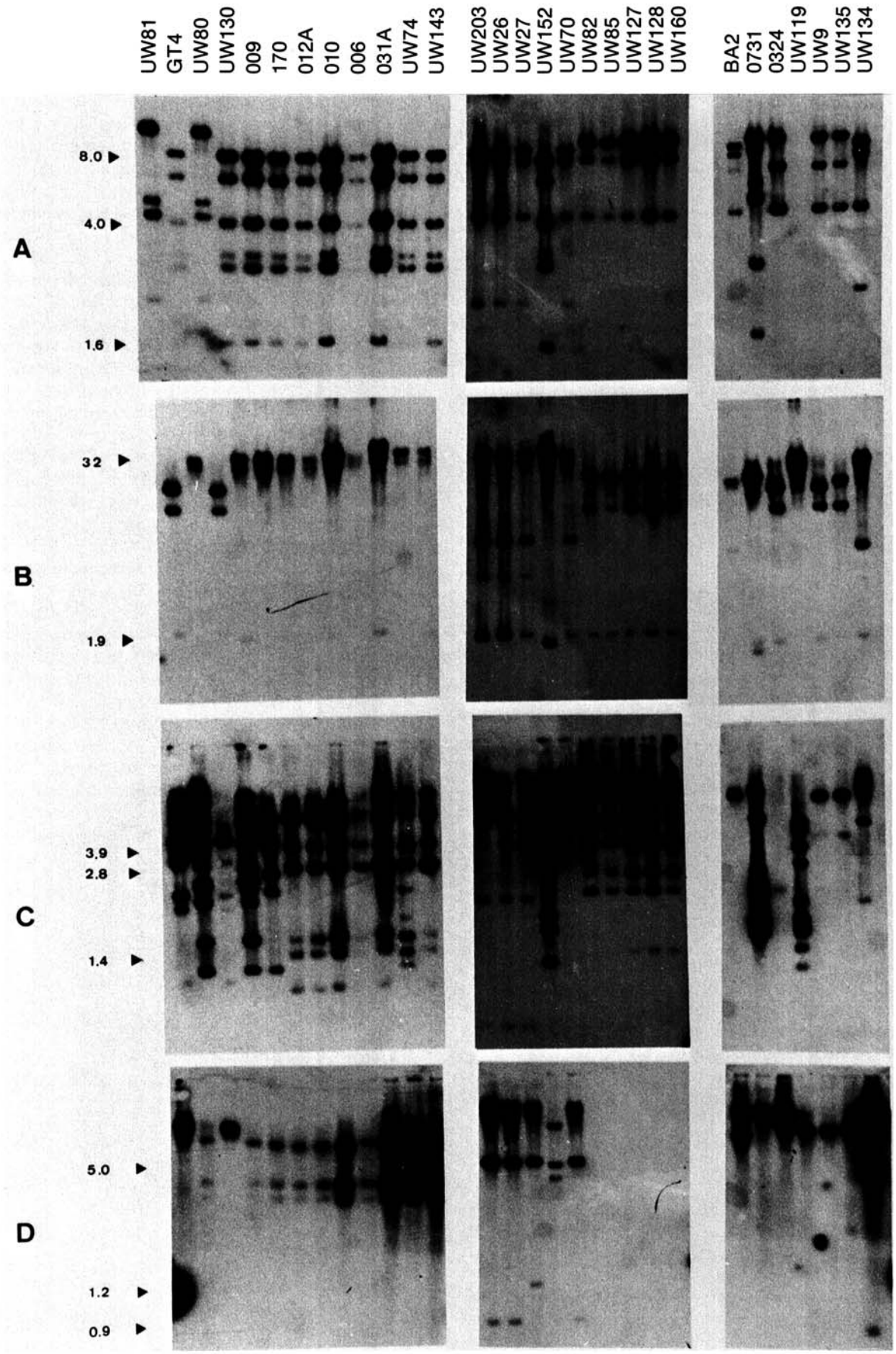

Fig. 2. Hybridization patterns of *Eco*RI genomic blots of various wild-type strains with probes pVir2 (**A**), pI1F4 (**B**), pJ7D7 (**C**), and pAC5 (**D**).

probes were usually more discriminatory than pVir2, leading to the splitting of the original pVir2 groups from one to five subgroups. These results therefore suggest that strains of the same RFLP group as defined by pVir2 probably share a high degree of resemblance in the distribution of restriction sites along the entire genome and that they are clearly distinct from strains of a different RFLP group. This conclusion is further substantiated by comparison of the ethidium bromide stainings of the electrophoregrams of *Eco*RI digests of genomic DNAs, which show similarity between strains of the same RFLP group but differ between strains of different groups (Fig. 3).

Correlation between RFLP grouping and races grouping. The data presented above suggested a correlation between RFLP grouping and races; however, for a limited number of strains, this correlation was not established. Therefore, we investigated if this was due to previous error in the identification of races. To test this, 11 of the strains listed in Table 1 were chosen for inoculation on a set of four plants (tomato, eggplant, potato, and *Musa*). For nine of them the race identification was confirmed, but two were not pathogenic on any of the plants tested.

DISCUSSION

In this paper we have shown that DNA sequences homologous to the pVir2 insert are present in all but one of the *P. solanacearum* strains studied. With a DNA fragment internal to the *hrp* gene cluster of pVir2, we have demonstrated that this homology includes the *hrp* genes (data not shown). These genes are probably required for pathogenicity in all the strains because all the Acr^r mutants that had deletion in this region had also lost the ability to induce disease or HR on tobacco. In addition, the only strain that did not show homology with the pVir2 probe (UW119) was found to be nonpathogenic on all of the plants tested. On the other hand, UW27Acr^r, the only Acr^r mutant that had retained pathogenicity, also retained *hrp* DNA sequences.

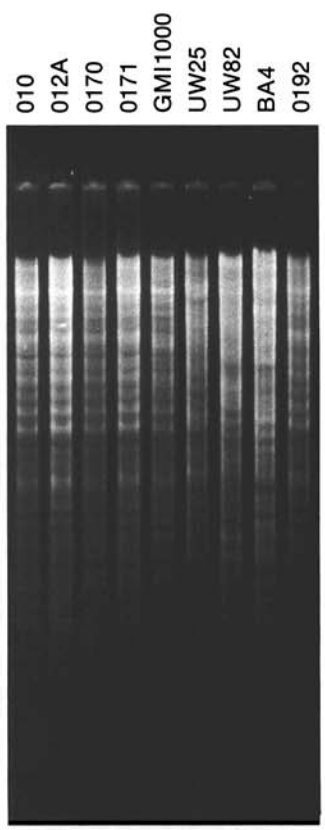

Fig. 3. Ethidium bromide-stained profiles of *Eco*RI genomic digests of various strains belonging to RFLP group 1 (010, 012A, 0170, 0171, GMI1000, and 0192) compared with profile of a group 2 strain (UW25), a group 3 strain (UW82), and a group 4 strain (BA4).

We established that for a majority (if not all) of *P. solanacearum* strains, these *hrp* genes are linked to genes involved in the metabolism of methionine, in sensitivity to AO, and in the release of a brown pigment. Whether these mutants are also affected in the structure of the lipopolysaccharide and exopolysaccharide has not been tested in this work. We also show that in a majority of strains, the region in which these genes are located is unstable and that following growth in the presence of AO, deleted mutants are easily obtained, although the frequency of such mutant in the initial population is unknown. It is not clear whether the drug only acts as a selective agent or if it may be involved in the induction of the deletion event. Although the resistance to AO was not established for strain UW119, the fact that this strain was found to be deleted of the *hrp* cluster and auxotrophic for methionine suggests that such deletion events might spontaneously have occurred during storage in the absence of the drug. Therefore, AO might not be absolutely required for the induction of deletions in the *hrp* region. A certain number of strains do not yield Acr^r mutants. This could reflect a different genomic organization, but such strains are not distinct from Acr^r generating strains in terms of RFLP. It therefore appears that this inability to obtain such mutants could be due to minor differences that would require different experimental conditions to select Acr^r mutants. However, increasing the concentration of AO up to 500 μg/ml resulted in total inhibition of growth of strain UW25, but no Acr^r mutants could be obtained (data not shown).

In this paper we show the existence of a polymorphism of the *Eco*RI restriction fragments in the species *P. solanacearum*. Although still preliminary, this RFLP work shows that the grouping obtained is largely independent of the probe used.

From the evolutionary point of view, this is an indication that the region encoding *hrp* functions has evolved concomitantly with the rest of the genome. This suggests that present strains resulted from evolution of a common ancestor in which *hrp* genes were present, rather than from recent transfer of *hrp* genes into nonpathogenic organisms.

The data presented in this study establish the existence of a certain correlation between RFLP grouping and race grouping. For example, 33 out of 34 race 1 isolates tested fall into RFLP groups 2 and 5. This classification also tends to distinguish strains of biovar 1 from strains of biovars 3 and 4 among race 1 isolates. This is in agreement with Cook *et al.* (unpublished data). However, these correlations are not perfect; for example, it is clear that RFLP group 3 comprises strains pertaining to races 2 (UW127, UW128), 3 (UW80, UW82), and the unusually wide host range strain UW85.

Therefore, although a particular RFLP group of bacteria usually seems to be associated with a particular group of plants, subtle differences, not detected with the

hybridizations performed, might result in a change in host specificity. These differences could be the result of mutations or of genetic transfer between strains, and would suggest a relatively simple genetic determinism for host specificity in this bacterium. This has recently received experimental support because transfer of a 13-kb DNA fragment originating from a peanut isolate into a narrow host range race 3 isolate resulted in extension of the host range of the recipient race 3 strain to peanut (Ma *et al.* 1988).

Finally, RFLP grouping offers a new means of strain identification that might be useful for epidemiological studies.

ACKNOWLEDGMENTS

We thank S. Tsuyumu for stimulating discussion, and L. Sequeira, C. Hayward, P. Frossard, and P. Prior for providing us with *P. solanacearum* strains. We acknowledge J. Dénarié and P. Boistard for critical reading of the manuscript.

This work was supported by grant RECH/8700787 from Conseil Régional Midi Pyrénées.

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