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AFLP ANALYSIS OF *XANTHOMONAS AXONOPODIS* AND *X. ARBORICOLA* STRAINS USED IN XANTHAN PRODUCTION STUDIES REVEAL HIGH LEVELS OF POLYMORPHISM

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ABSTRACT

Amplified fragment length polymorphism (AFLP) was used to analyze the genetic diversity of 14 strains of *Xanthomonas arboricola* pv. *pruni* and seven strains of *X. axonopodis* pv. *phaseoli*, which are used in xanthan production studies. Relationships identified by the AFLP profiles were assessed for xanthan production capacity, geographical location and host plant. Strains were isolated from 10 different geographic regions in South and Southeast States in Brazil. Data were analyzed for genetic similarity using the Dice coefficient and subjected to UPGMA cluster analysis. A total of 128 AFLP fragments were generated from four primer combinations: *Eco*RI+C/*Mse*I+0, *Eco*RI+A/*Mse*I+0, *Eco*RI+G/*Mse*I+T and *Eco*RI+G/*Mse*I+A. Of these, 96.1% were polymorphic. *X. axonopodis* pv. *phaseoli* (S_D = 0.27) was shown to be more polymorphic than *X. arboricola* pv. *pruni* (S_D = 0.58). All 14 pathovar *pruni* strains were included in a single main group (S_D = 0.58), while the pathovar *phaseoli* strains were divided into three separate groups, with one group containing five strains (S_D = 0.38) and two isolated groups (S_D = 0.31 and 0.27) composed of only one strain each. Species were distinguished by three and eight specific AFLP markers present in the pathovar *phaseoli* and the pathovar *pruni*, respectively. For the unique strain without xanthan production capacity (*X. axonopodis* pv. *phaseoli* str. 48), nine specific AFLP bands were found. There was no evidence that geographic area or host plant influenced genetic heterogeneity. Correlations between AFLP patterns and xanthan production capacity were found in some strains, but were not consistent enough to establish a relationship.

Key words: DNA fingerprints; genomic diversity; pathovar *phaseoli*; pathovar *pruni*.

INTRODUCTION

The genus *Xanthomonas* is composed of phytopathogenic bacterial species that cause diseases in different crop plants, resulting in significant crop losses worldwide. Each species infects specific hosts (18). In addition to causing diseases in crops, most of the species produce xanthan gum via an aerobic fermentation process. Xanthan gum is an important biopolymer and is used in the food, oil and cosmetics industries. For industrial production of xanthan gum, *X. campestris* pv. *campestris* strain NRRL B-1459 is normally used. However, other *Xanthomonas* species have been shown to be capable of xanthan production, including strains with considerable xanthan yields such as the *X. arboricola pv. pruni* strains isolated from Brazil (2, 4, 20).

X. arboricola pv. *pruni* (19) is the causal agent of Prunus

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Bacterial Spot disease (PBS), which infects cultivated *Prunus* species and their hybrids. In southern Brazil, hundreds of strains have been isolated from peach and plum trees at the Centro de Pesquisa Agropecuária de Clima Temperado. From this collection, diverse studies have been conducted, mainly investigating xanthan yields obtained by fermentation processes, including their rheological properties and chemical compositions (2, 3, 13, 17, 20). *X. axonopodis* pv. *phaseoli* (14, 19) infects mainly *Phaseolus vulgaris* and causes Common Bacterial Blight, but other legume species are also naturally infected. Xanthan production studies have also been reported for this strain (11, 12).

Many studies have used molecular biology techniques with diverse *Xanthomonas* species to show high levels of genetic diversity (polymorphism) in the genus as well as within species (1, 8, 9, 21, 22, 25). On the other hand, pathovars from different species have shown strong genetic similarities, resulting in a total reclassification of the *Xanthomonas* genus (19). Amplified fragment length polymorphism (AFLP) has been used successfully to study genetic diversity in *Xanthomonas* (22), allowing for the identification of pathovars and allowing strains with a high degree of genetic similarity to be distinguished (23).

The purpose of the present study was to investigate the genomic variability of the *X. arboricola* pv. *pruni* and *X. axonopodis* pv. *phaseoli* strains used in xanthan production studies. For the AFLP analyses, strains with different xanthan production capacities in MPII medium were chosen.

MATERIAL AND METHODS

Bacterial strains

A total of 21 *Xanthomonas* strains were used in this study, including 7 *X. axonopodis* pv. *phaseoli* and 14 *X. arboricola* pv. *pruni* strains (Table 1), isolated from 10 different geographic regions in South and Southeast States in Brazil (Fig. 1).

Table 1. Bacterial isolates used in this study, their plant host and geographical origin.

Species	Strain	Host isolation	Origin[a]	Xanthan production capacity (g L^{-1})[b]
X. axonopodis pv. *phaseoli*	32	*Phaseolus vulgaris*	A	2.4
	48	*Phaseolus vulgaris*	B	0.0
	29	*Phaseolus vulgaris*	C	2.2
	2	*Phaseolus vulgaris*	D	5.7
	12	*Phaseolus vulgaris*	J	2.4
	14	*Phaseolus vulgaris*	J	1.9
	16	*Phaseolus vulgaris*	J	2.3
X. arboricola pv. *pruni*	42	*Prunus pruni*	E	5.0
	61	*Prunus persica*	F	4.0
	19	*Prunus pruni*	G	4.1
	30	*Prunus pruni*	G	6.6
	26	*Prunus persica*	G	7.0
	81	*Prunus persica*	H	5.0
	106	*Prunus persica*	I	6.8
	108	*Prunus persica*	I	5.2
	109	*Prunus persica*	I	4.8
	112	*Prunus persica*	I	4.7
	115	*Prunus persica*	I	5.5
	55	*Prunus pruni*	J	7.4
	101	*Prunus persica*	J	6.1
	103	*Prunus persica*	J	7.8

[a] See Fig. 1.

[b] In MPII medium at 28ºC after 72 h (xanthan production of *X. axonopodis* pv. *phaseoli* strains in these conditions was previously determined by Mayer (11); xanthan production of *X. arboricola* pv. *pruni* strains in these conditions was previously determined by Borges (3)).

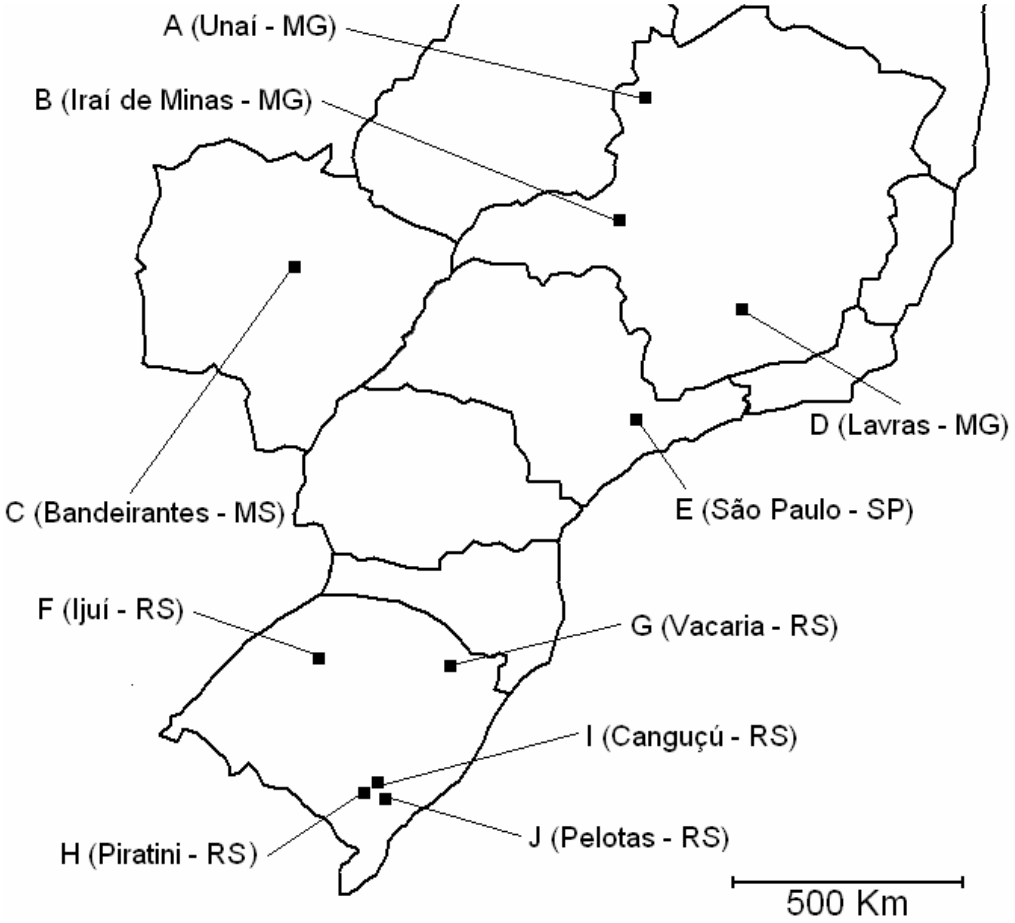

Figure 1. Detailed map of the geographic locations (in Brazil) where the 21 *Xanthomonas* strains used in this study were isolated.

Genomic DNA extraction

The strains were incubated in 20-ml tubes containing 5 ml YM medium (0.3% malt extract, 0.3% yeast extract, 1% glucose, 0.5% peptone) for 24 h at 300 rpm and 28ºC. Aliquots of 2 ml of culture ($O.D._{580}$ = 1) were collected for extraction. The cells were washed twice with water and subsequently centrifuged to remove the xanthan gum. The cells were lysed using 500 µl of 5% SDS solution at 60ºC for 40 min. Genomic DNA was purified using phenol/chloroform extraction, precipitated with ice-cold ethanol and resuspended in 30 µl TE buffer. The concentration was determined with a spectrophotometer measuring A_{260} (1 absorbance unit = 50 µg ml^{-1}). DNA quality was checked by measuring A_{280} and DNA integrity was confirmed on a 1% agarose gel. The DNA samples were adjusted to 100 ng μl^{-1} and stored at -20ºC.

AFLP protocols

The procedures were performed as described by the commercial AFLP kit for microorganisms from Invitrogen™ Life Technologies (Carlsbad, CA, USA), with minor modifications. The protocol involved the following steps: 1) digestion of total genomic DNA with two restriction endonucleases and ligation of oligonucleotide adapters; 2) preselective amplification and selective amplification of a set of restriction fragments by PCR; and 3) separation of amplified DNA fragments with denaturing polyacrylamide gels.

DNA digestion and adapter ligation

DNA digestion was performed for 4 h at 37ºC. Reactions were carried out in 10 µl volumes containing 200 ng of sample DNA, 0.65 U each of *Eco*RI and *Mse*I enzymes and 2.5 µl of

5X reaction buffer (50 mM Tris-HCl (pH 7.5), 50 mM Mg-acetate, 250 mM K-acetate). Digestion was confirmed on a 1% agarose gel. Endonucleases were denaturated at 70ºC for 15 min. DNA ligation was performed by adding 9.6 µl of adapter solution (*Eco*RI/*Mse*I adapters, 0.4 mM ATP, 10 mM Tris-HCl (pH 7.5), 10 mM Mg-acetate, 50 mM K-acetate) and 0.4 U of T_4 DNA ligase. Reactions were subsequently incubated at 20ºC for 5 h.

DNA amplification

Preselective amplification assays were carried out in 12 µl volumes containing 7.6 µl of ligation mixture as template (not diluted), 1 U of *Taq* DNA polymerase, 16 ng of *Eco*RI+0 primer, 16 ng of *Mse*I+0 primer containing dNTPs and 1.2 µl of 10X buffer PCR plus Mg [100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 500 mM KCl). The thermal profile consisted of an initial denaturation step at 94ºC (2 min), followed by 20 cycles at 94ºC (30 s), 56ºC (1 min) and 72ºC (1 min), with a final extension for 5 min at 72ºC.

Selective amplifications contained 2.5 µl of DNA (preamplified and diluted 20-fold diluted), 7 ng of *Eco*RI primers, 15 ng of *Mse*I primers containing dNTPs and 1 µl of 10X buffer PCR plus Mg [100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 500 mM KCl). The thermal profile consisted of an initial denaturation step at 94ºC (3 min), followed by 30 cycles at 94ºC (1 min), 65ºC (decreasing 1ºC per cycle during the first 10 cycles) (1 min) and 72ºC (1.5 min), with a final extension for 5 min at 72ºC. Seven primer combinations were tested: *Eco*RI+C/*Mse*I+0, *Eco*RI+A/*Mse*I+0, *Eco*RI+T/*Mse*I+0, *Eco*RI+G/*Mse*I+T, *Eco*RI+A/*Mse*I+C, *Eco*RI+G/*Mse*I+A and *Eco*RI+AC/*Mse*I+G. PCR reactions were performed in a Peltier PTC-100 thermocycler.

Electrophoresis of PCR products

The final amplified products were denatured at 94ºC and immediately placed on ice. Samples (6 µl) were loaded and separated on a 6% polyacrylamide denaturing gel at a constant power of 60 W for 2 h at 50ºC in 1X TBE buffer. Finally, the gels were silver stained according to Creste *et al.* (6).

Data analysis

AFLP markers (100 to 450 bp) obtained were manually scored for either present [1] or absent [0] bands in individual lanes. Only strong bands were scored; faint bands were discarded. The binary matrix was used for calculation of genetic diversity among isolates on Dice's coefficient. Cluster analysis and dendrogram construction were done using the unweighted pair group method with an arithmetic mean (UPGMA) in the NTSYS-PC 2.1 software (Applied Biostatistics Inc). The bootstrap values were generated using Winboot software (24).

RESULTS

Selection of AFLP primers

Primer combination assays were performed to determine which AFLP primers had the optimal number of selective nucleotides to generate an average of 30 bands per sample. After screening seven primer combinations, four were chosen for use in this study (Table 2). The primer combinations *Eco*RI+T/*Mse*I+0 and *Eco*RI+A/*Mse*I+C did not generate clear AFLP patterns and the number of bands of the primer combination *Eco*RI+AC/*Mse*I+G was very low. The reproducibility of the AFLP profiles was determined using two isolates of *X. arboricola* pv. *pruni* and two isolates of *X. axonopodis* pv. *Phaseoli*, which were each processed three times. No differences were observed between experimental replicates.

Polymorphism level and cluster analysis

The sizes of bands scored in all gels were in the range of 100-450 bp. In total, 128 amplification products were produced, of which 123 were polymorphic (Table 2). Consequently, only five bands were shared among all strains, showing a high polymorphism level (91.9% to 100%), depending on the primer combination used. Within species, the polymorphic level was 94.7 and 87.2% in pathovar *phaseoli* and *pruni*, respectively.

Table 2. Primer combination, groups formed, number of AFLP bands and percentage of polymorphic bands resulting from AFLP analysis of *X. axonopodis* pv. *phaseoli* and *X. arboricola* pv. *pruni.*

No.	Primer combination	No. of AFLP bands	No. of Polymorphic bands	Polymorphism (%)
1	*Eco*RI+C/*Mse*I+0	33	32	97
2	*Eco*RI+A/*Mse*I+0	37	34	91.9
3	*Eco*RI+G/*Mse*I+T	30	29	96.7
4	*Eco*RI+G/*Mse*I+A	28	28	100
Total		**128**	**123**	**96.1**
	Group I	65	63	96.9
	Group II	28	28	100
	Group III	25	25	100
	Group IV	80	75	93.7

Size range: 100-450 bp.

The 123 AFLP polymorphic fragments were used to construct the dendrogram and to estimate the genetic diversity among the *Xanthomonas* strains. The cophenetic correlation coefficient (r), calculated between the binary matrix data and the dendrogram generated, was 0.97. Based on a cut-off point of 0.33 (calculated by NTSYS-PC 2.1 software) in Dice's similarity coefficient scale, the 21 strains were divided into four groups (Fig. 2). All 14 strains of *X. arboricola* pv. *pruni* were grouped into a single main cluster (S_D = 0.58), named Group IV. Additionally, in this group, two subgroups were identified, named A (S_D = 0.78) and B (S_D = 0.82). *X. axonopodis* pv. *phaseoli* strains were more polymorphic, with strains 2, 12, 16, 29 and 32 in Group I (S_D = 0.38) and the other two strains (48 and 14) inserted in the isolated Group II (S_D = 0.27) and Group III (S_D = 0.31), respectively. The presence of three exclusive AFLP bands in all *X. axonopodis* pv. *phaseoli* strains and eight in all *X. arboricola* pv. *pruni* strains have distinguished these pathovars.

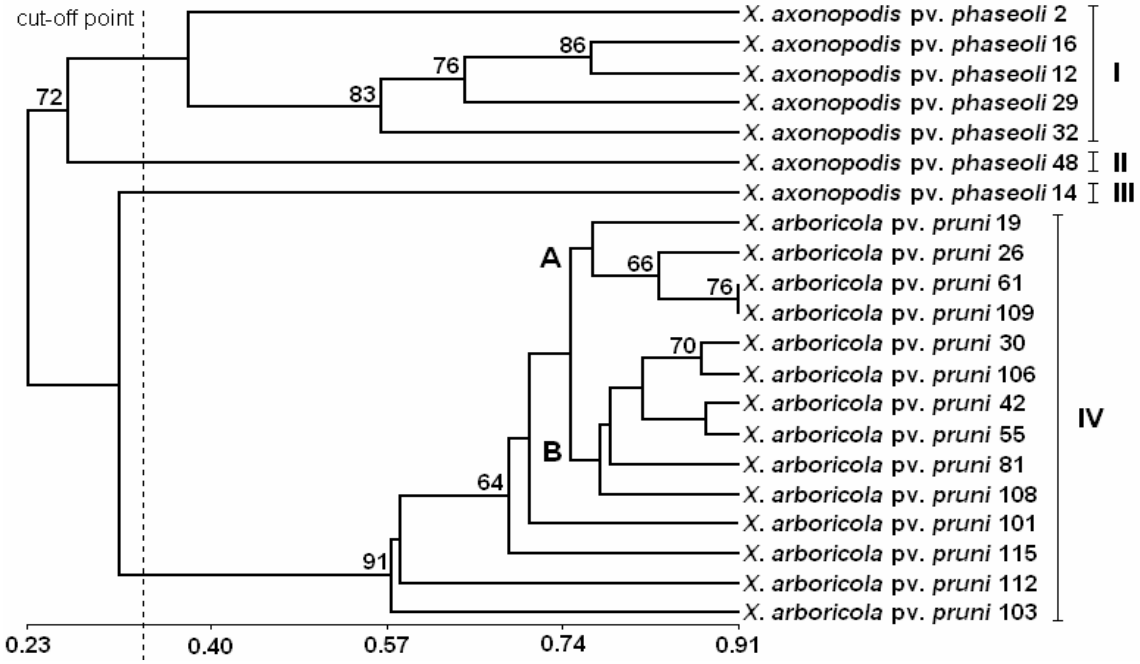

Figure 2. Dendrogram obtained by UPGMA method using Dice's similarity coefficient from 21 *Xanthomonas* strains based on 123 polymorphic AFLP markers. Four groups were generated (I, II, III and IV). Subgroups in group IV are indicated as "A" and "B". Bootstrap values larger than 50% (obtained from 1000 replicates) are also displayed.

Genetic heterogeneity was not influenced by the geographical location of the strains. Different patterns were found within one site, and similar molecular patterns were found in strains collected at different sites. In pathovar *pruni*, subgroups A and B contain strains isolated from different sites and the two most genetically-related strains (*X. arboricola* pv. *pruni* str. 61 and 109; S_D = 0.91) were isolated 400 km away from each other. Additionally, this pathovar no showed differences in AFLP profiles based on the host isolation (*Prunus persica* and *P. pruni*). In the pathovar *phaseoli*, three strains were isolated from the same place, but only two (*X. axonopodis* pv. *phaseoli* str. 12 and 16) showed the highest genetic similarity (S_D = 0.77) in comparison with the other strains of the pathovar.

Robust relationships between genetic similarity and xanthan production capacity with these strains were not found. However, similar xanthan yields were observed for *X. arboricola* pv. *pruni* str. 61 and 109 (S_D = 0.91; xanthan production: 4.1 and 4.8 g L^{-1}, respectively) and for *X. arboricola* pv. *pruni* str. 30 and 106 (S_D = 0.87; xanthan production: 6.6 and 6.8 g L^{-1}, respectively). The two most polymorphic strains had the lowest xanthan production capacity (*X. axonopodis* pv. *phaseoli* str. 14) or no production capacity (*X. axonopodis* pv. *phaseoli* str. 48). Moreover, for the unique strain with no xanthan production capacity, nine specific AFLP markers were identified.

DISCUSSION

Typically, 30 to 80 restriction fragments are amplified in each AFLP reaction and detected by denaturing gel electrophoresis. This number is mainly influenced by three factors: number of selective nucleotides used in each primer combination, recognition sites of the restriction enzymes and C+G content of the genome analyzed (21). The relatively low number of bands obtained with each primer combination (approximately 32) was partly due to the fact that the restriction enzymes *Eco*RI (G↓AATTC) and *Mse*I (T↓TAA) recognize T+A-rich sequences, but *Xanthomonas* genomes have low T+A contents, with about 29-37% T+A. On the other hand, complex fingerprints (with a large number of bands) can be difficult to analyze manually. Thus, the quantity of AFLP fragments obtained in each primer combination was considered to be satisfactory.

The cophenetic correlation coefficient value found indicates high co-linearity between the matrix data and the dedrogram generated. Based on this value, the UPGMA method was used to cluster the genotypes. High bootstrap values showed that this clustering was well supported and that the dendrogram generated was robust.

If the search for a pattern between genetic similarity and geographical isolation includes both pathovars, partial relationships can be observed. However, more detailed observations show that differences in the AFLP profiles are caused by the pathovar genetic profile, not by the geographic area of each strain. These results are in accordance with Zaccardelli *et al*. (25), who analyzed 109 *X. arboricola* pv. *pruni* strains from different geographical locations and also concluded that the AFLP technique did not have sufficient resolution to distinguish this pathovar on the basis of geographic area or host plant. Additionally, in a study using AFLP with 66 isolates of *X. arboricola* pv. *juglandis* from different countries, Loreti *et al*. (9) concluded that geographic location could be only partly responsible for genomic heterogeneity.

The high degree of genetic diversity found in the pathovar *phaseoli* in this study is consistent with the results of Alavi *et al*. (1), who first reported a large degree of genetic diversity in this pathovar using AFLP assays from a worldwide collection. These authors identified three distinct genetic lineages, but the lineages were not geographically linked. The absence of a pattern between genetic similarity and geographic location with the pathovar *phaseoli* was also found by Manceau *et al*. (10), who studied polymorphisms in the ISXax1 insertion sequence. The dissemination of *X. axonopodis* pv. *phaseoli* through contaminated seeds is the probable reason for the lack of a geographically structured genetic profile (10).

Environmental microorganism dissemination is normally

influenced by wind and rain events over time. Thus, the presence of a high level of genetic heterogeneity among pathovar *pruni* strains isolated from the three places (H, I and J) that have relatively small distances between them (< 100 km) is strong evidence that the pathovar *pruni* population from this geographical region is composed of different genetic groups. This evidence is strengthened by the hundreds of strain isolates from this region (20), indicating a very rich *Xanthomonas* microbiota. Nevertheless, to strengthen this evidence, a genetic diversity study that includes a larger number of strains will be necessary. In the pathovar *phaseoli*, it is possible that geographic area is partly responsible for the high levels of polymorphism found. However, to prove this, it will be necessary to study more than one strain from each location.

Genetic polymorphism is also influenced by IS elements, which play an important role in bacterial evolution. These mobile genetic elements are an important source of genomic variability in prokaryotes, mainly by promoting chromosomal rearrangements and genetic exchanges between bacterial populations (10, 16). Pathovar *pruni* were shown to be missing a considerable number of IS elements in the pathovar *pruni* genome compared to the reference strains *X. campestris* pv. *campestris* B100 and *X. campestris* pv. *vesicatoria* 85-10 (determined by microarray comparative genomic hybridization (unpublished data)). Interestingly, Manceau *et al.* (10) identified a new insertion sequence (ISXax1) in pathovar *phaseoli* and suggested that it may contribute to genetic diversity.

The absence of clear relationships between genetic similarity, xanthan production capacity and plant host isolation can be explained by the small DNA regions that are responsible for these features. Host specificity to phytopathogenic bacteria is usually established by specific molecular receptors present on both surfaces. In these microorganisms, the molecular receptors are encoded by a small number of genes, representing a small part of the genome. The same is true of the xanthan biosynthetic process, which is encoded by a 16-kb operon of 12 genes (*gum*B to *gum*M), as well as some other related genes, representing less than 1% of the *Xanthomonas* genome (about 5 Mb). Thus, the polymorphisms present in a single gene or operon represents only a small part of the polymorphisms in the entire genome, and establishing relationships based on these specific features can be very difficult with techniques such as AFLP assays, which explore genetic polymorphisms at the genome level.

In this study, AFLP analysis proved to be reproducible and sufficiently sensitive to determine genetic diversity in the pathovars of the strains studied. Moreover, the influence of SNPs (single nucleotide polymorphisms) in the AFLP analysis makes this technique a powerful tool to estimate genetic diversity among very similar genomes, something that is difficult to determine with other techniques, such as RAPD (Random Amplification of Polymorphic DNA).

In conclusion, we report a high level of polymorphism among the 21 *Xanthomonas* tested. The *X. axonopodis* pv. *phaseoli* genome was shown to be more polymorphic in comparison with *X. arboricola* pv. *pruni* and the strains were differentiated by specific AFLP markers present in each pathovar. AFLP-based genetic profiles and xanthan production capacity relationships were not consistent enough to establish a pattern and we did not find evidence that geographic area or host plant influences genetic heterogeneity.

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