

ASSESSMENT OF INTEGRON GENE CASSETTE ARRAYS IN STRAINS OF *XANTHOMONAS FRAGARIAE* AND *X. ARBORICOLA* pvs. *FRAGARIAE* AND *PRUNI*

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SUMMARY

A total of 54 *Xanthomonas* strains belonging to *X. fragariae* and to *X. arboricola* pathovars *fragariae* and *pruni* were screened for their integron cassette arrays banding pattern and for their repetitive-sequence PCR genomic pattern. Species and pathovars showed cassette array patterns that clearly differentiated them. Variability in the cassette array pattern was observed solely within strains of *X. a. pv. pruni*. No correlation between the two cassette array patterns shown by the strains of this pathovar and host plant or genomic pattern inferred by repetitive PCR was observed. The two *xanthomonads* pathogenic to strawberry, *X. fragariae* and *X. arboricola* pv. *fragariae*, showed different integron cassette arrays. In addition to species, integrons might generate diversity also within some *Xanthomonas* pathovars.

Keywords: integrons, mobile genetic elements, *Xanthomonas*, repetitive-sequence PCR, lateral gene transfer.

INTRODUCTION

Based on a polyphasic approach, Vauterin *et al.* (1995) revised the taxonomy of *Xanthomonas*, a genus containing almost exclusively plant pathogenic bacterial species. Twenty species were described and some pathovars, previously included in *X. campestris*, were elevated to species level. The new classification still contains many pathovars, a term that has no taxonomic standing in the International Code of Nomenclature of Bacteria (Sneath, 1992). However, since its proposal, the term was meant to be temporary and it was introduced mainly to differentiate, at infrasubspecific level, particular strains on the basis of distinctive pathogenicity to one or more plant hosts (Dye *et al.*, 1980). Restricted pathogenicity is a typical feature of *Xanthomonas* pathovars (Hayward, 1993). However, the genetic basis of this in

the different *Xanthomonas* pathovars and species is still not cleared and genetic mobile elements such as temperate phages, insertion sequences, transposons, and episomal plasmids may play an important role (Monteiro-Vitorello *et al.*, 2005).

Recently, a fundamental contribution for understanding the infrasubspecific genetic variation of *Xanthomonas* has been given by Gillings *et al.* (2005) who investigated in 32 strains the occurrence and the characteristics of another class of mobile genetic elements, the integrons. They pointed out that the acquisition of diverse gene cassettes by different lineages within *Xanthomonas* has contributed to the species-genome diversity of the genus. By following the traditional classification, they claimed to have investigated 12 pathovars of two *Xanthomonas* species. However, according to the list of strains they used, the species investigated should be three, namely *X. axonopodis*, *X. campestris* and *X. oryzae*. In addition, it is worth noting that by following the new classification of Vauterin *et al.* (1995), their study would concern seven or eight species, depending on the species assignments of the four *X. vesicatoria* strains they used. There would also be 12 or 13 pathovars, depending on whether the three *X. axonopodis* pv. *vitiens* strains they assessed belong to type A or B. Taking into consideration the new classification of Vauterin *et al.* (1995), the work of Gillings *et al.* (2005) appears even more significant, since integrons would seem to be involved in structuring the pathogenic specificity of many more *Xanthomonas* pathovars and species.

To further assess the presence of integrons and their possible role in the genetic diversity of *Xanthomonas*, we checked the presence and banding pattern of integron gene cassettes of 54 *Xanthomonas* strains belonging to *X. fragariae* and to *X. arboricola* pathovars *fragariae* and *pruni*. The strains used reflected a diversity of collection dates, locations and plant hosts. *X. fragariae* and *X. a. pv. fragariae* were not investigated by Gillings *et al.* (2005). In addition, they investigated only three *X. a. pv. pruni* strains. Similarly to Gillings *et al.* (2005), all strains were also assessed by repetitive-sequence PCR using BOXA1R primers to detect possible differences within and among the species and pathovars.

MATERIALS AND METHODS

Bacterial strains used in this study and their origin and host plant are listed in Table 1. Sixteen strains belonged to *Xanthomonas fragariae*, 10 to *X. arboricola* pv. *fragariae*, and 28 to *X. a.* pv. *pruni*. They were received from International Culture Collections or were maintained in the collection of our Institution. Some strains were previously assessed for their pathogenicity, and genetic diversity (Scortichini *et al.*, 1996; Scortichini and Rossi, 2003). Strains were grown on glucose-yeast extract-calcium carbonate medium (GYCA) (Van den Mooter *et al.*, 1987) at 27°C. Bacteria were transferred onto fresh plates at regular intervals.

To prepare total genomic DNA, loopfuls of single colonies of each strain grown for 3 days were suspended in sterile saline (0.85% of NaCl in distilled water) and centrifuged at $12,000 \times g$ for 2 min. The pellet was suspended in double-distilled, filtered, sterilized water to an optical density corresponding to $1-2 \times 10^8$ cfu/ml. The suspension was placed in boiling water for 10 min and then stored at -20°C.

In order to detect cassette arrays and test for heterogeneity in the arrays, the proximal integron gene cassette region of *Xanthomonas* strains was amplified using the primers MRG17 (5'-GATACTYRGCACCAACACCGC) and AJ60 (5'-CRRSKTCGGCTTGAAYGARTTG) (Gillings *et al.*, 2005). PCR mixtures and thermal cycling were those described by Gillings *et al.* (2005). The gels were stained with ethidium bromide visualized under a UV transilluminator (Spectroline, Westbury, New York, USA) and photographed with a Kodak Gel Logic 100 Imaging System apparatus. The

runs were performed in triplicate.

The repetitive-sequence PCR method used was that of Louws *et al.* (1994). BOXA1R primer (5'-CTACGGCAAGGCGACGCTGACG) synthesized by Eurogentec (Seraing Belgium) was used. PCRs were run on an Eppendorf Mastercycler programmable thermal controller (Eppendorf, Hamburg, Germany). The gels were stained and photographed as above. The runs were performed in triplicate.

RESULTS AND DISCUSSION

All 54 *Xanthomonas* strains generated complex banding patterns upon PCR amplification with primers AJH60 and MRG17. Representative gels of cassette array banding patterns are shown in Figs. 1 and 2. Cassette array PCRs were identical for all strains of *X. fragariae* and *X. arboricola* pv. *fragariae* but clearly different between the two xanthomonads. By contrast, two different banding patterns, A and B, were observed for strains belonging to *X. a.* pv. *pruni* (Fig. 2). In this case, the overall banding pattern among the 28 strains tested was very similar but one PCR product differentiated some strains from the others. However, no correlation between host plant and/or geographic area and cassette array pattern was found. In addition, we observed that strains within individual pathovars or species had identical or very similar cassettes and these exhibited no similarity to cassettes detected in the other pathovars or species.

Repetitive-sequence PCR genomic fingerprinting using the BOX primer showed differences within the three xanthomonads studied. Variability was clear in the

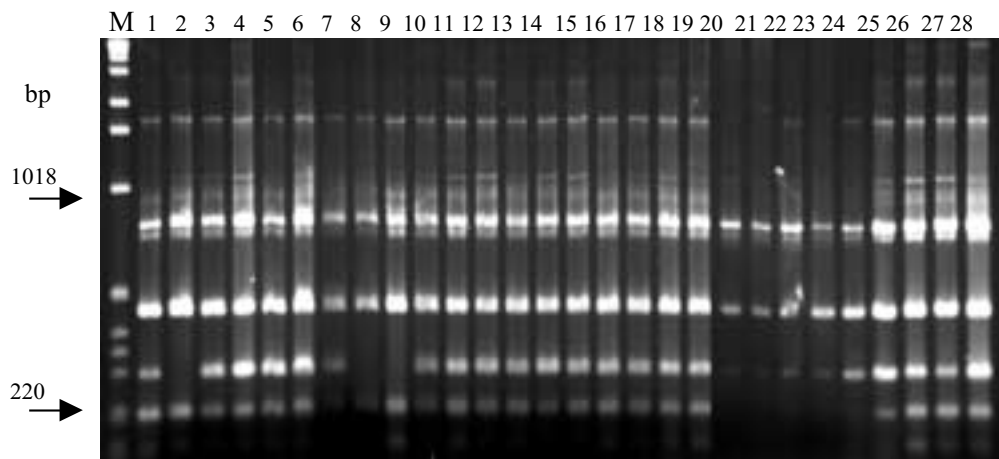


Fig. 1. PCR amplification of proximal integron gene cassettes in *Xanthomonas arboricola* pv. *pruni* strains by using primers MRG17 and AJH60. M: molecular size marker, 1-kb ladder (Gibco-BRL). Lane 1: NCPPB 3156; lane 2: ISF 55; lane 3: ISF 66; lane 4: ISF 461; lane 5: ISF 462; lane 6: ISF 463; lane 7: ISF 464; lane 8: ISF 465; lane 9: ISF 43; lane 10: ISF 45; lane 11: ISF FXP 12; lane 12: ISF NCPPB 1607; lane 13: NCPPB 2587; lane 14: NCPPB 2588; lane 15: ISF 514; lane 16: ISF 515; lane 17: ISF Sus 1; lane 18: ISF Sus 2; lane 19: ISF Sus 4; lane 20: ISF X Alb 2; lane 21: ISF X Alb 3; lane 22: ISF X1P; lane 23: ISF X2P; lane 24: ISF X Alb 4; lane 25: ISF X Alb 5; lane 26: ISF 444; lane 27: ISF Sus 5; lane 28: NCPPB 926.

Table 1. *Xanthomonas* strains screened for the presence of integron cassette arrays.

Strain	Host plant	Year of isolation	Country
<i>Xanthomonas fragariae</i>			
NCPPB 1469	<i>Fragaria x ananassa</i>	1960	USA
NCPPB 1822	"	1966	USA
NCPPB 2473	"	1972	Italy
NCPPB 3028	"	1977	Brazil
NCPPB 3743	"	1990	Brazil
NCPPB 4055	"	1999	Greece
ISF 819.1	"	2003	Italy
ISF 819.2	"	2003	Italy
ISF 819.3	"	2003	Italy
ISF 821.1	"	2003	Italy
ISF 821.2	"	2003	Italy
ISF 821.3	"	2003	Italy
ISF 821.5	"	2003	Italy
ISF 821.6	"	2003	Italy
ISF 824.1	"	2003	Italy
ISF 824.2	"	2003	Italy
<i>X. arboricola</i> pv. <i>fragariae</i>			
PD 2780	<i>Fragaria x ananassa</i>	1993	Italy
PD 2782	"	1993	Italy
ISF 23	"	1999	Italy
ISF 99	"	1999	Italy
ISF 113	"	2000	Italy
ISF 124	"	2000	Italy
ISF 128	"	2000	Italy
ISF 129	"	2000	Italy
ISF 137	"	2000	Italy
ISF 139	"	2000	Italy
<i>X. arboricola</i> pv. <i>pruni</i>			
NCPPB 2587	<i>Prunus armeniaca</i>	1973	South Africa
ISF XAlb 2	"	2005	Italy
ISF XAlb 3	"	2005	Italy
ISF XAlb 4	"	2005	Italy
ISF XAlb 5	"	2005	Italy
NCPPB 926	<i>Prunus domestica</i>	1960	South Africa
NCPPB 1607	<i>Prunus persica</i>	1964	Australia
NCPPB 2588	"	1973	South Africa
NCPPB 3156	"	1979	Italy
ISF 45	"	1993	Italy
ISF 461	"	1996	Italy
ISF 462	"	1996	Italy
ISF 463	"	1996	Italy
ISF 464	"	1996	Italy
ISF 465	"	1996	Italy
ISF 514	"	1997	Italy
ISF 515	"	1997	Italy
ISF X1P	"	2005	Italy
ISF X2P	"	2005	Italy
ISF 55	<i>Prunus salicina</i>	1991	Italy
ISF 66	"	1991	Italy
ISF 43	"	1993	Italy
ISF 444	"	1998	Italy
ISF XP12	"	2000	Italy
ISF Sus 1	"	2002	Italy
ISF Sus 2	"	2002	Italy
ISF Sus 4	"	2002	Italy
ISF Sus 5	"	2003	Italy

ISF: Culture Collection of C.R.A.-Istituto Sperimentale per la Frutticoltura, Roma, Italy

NCPPB: National Collection of Plant Pathogenic Bacteria, York, UK

PD: Culture Collection of Plant Protection Service, Wageningen, The Netherlands

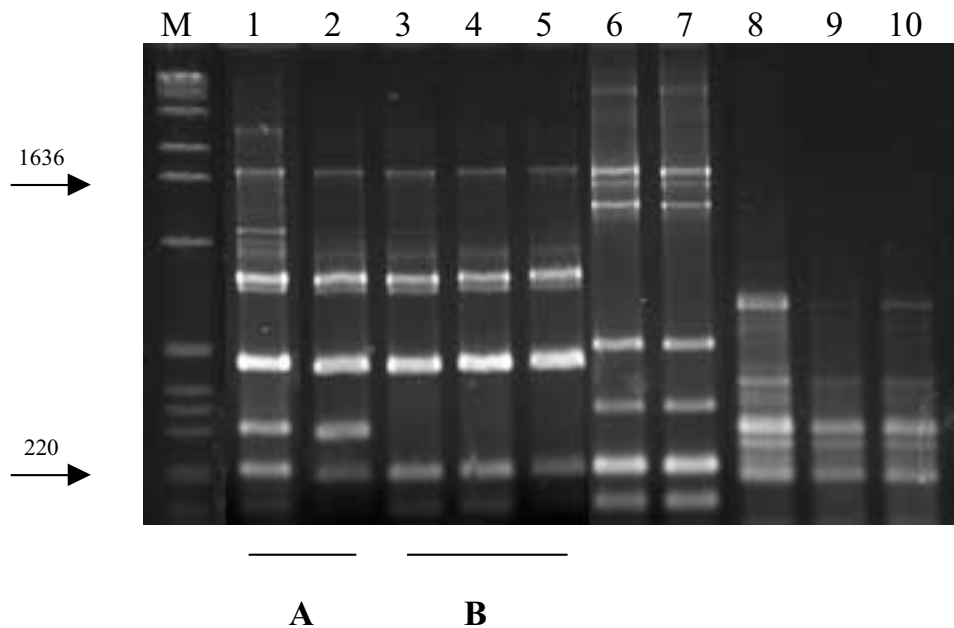


Fig. 2. PCR amplification of proximal integron gene cassettes in representative strains of *Xanthomonas fragariae* and *Xanthomonas arboricola* pathovars *fragariae* and *pruni* strains by using primers MRG17 and AJH60. M: molecular size marker, 1-kb ladder (Gibco-BRL). Lane 1: *X. arboricola* pv. *pruni* NCPPB 2588; lane 2: *X. a.* pv. *pruni* ISF 464; lane 3: *X. a.* pv. *pruni* ISF 55; lane 4: *X. a.* pv. *pruni* ISF 43; lane 5: *X. a.* pv. *pruni* ISF 465; lane 6: *X. a.* pv. *fragariae* PD 2780; lane 7: *X. a.* pv. *fragariae* PD 2782; lane 8: *X. fragariae* NCPPB 2473; lane 9: *X. fragariae* ISF 821.1; lane 10: *X. fragariae* ISF 821.2. *X. arboricola* pv. *pruni* showed two distinct banding patterns, A (lanes 1 and 2) and B (lanes 3, 4 and 5).

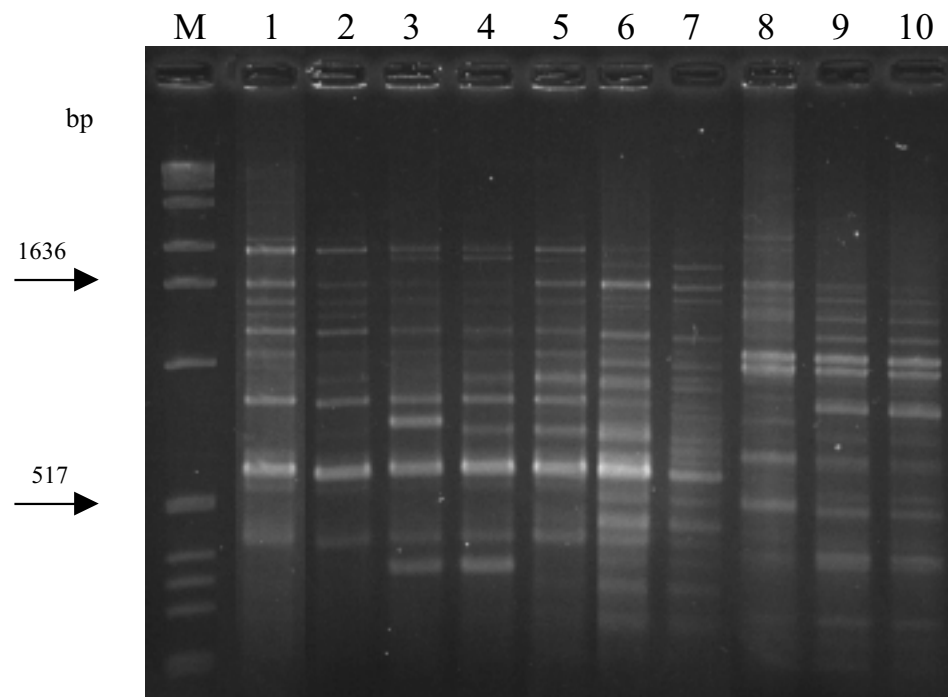


Fig. 3. Repetitive-sequence PCR genomic fingerprintings using BOXA1R primer of representative strains of *Xanthomonas fragariae* and *Xanthomonas arboricola* pathovars *fragariae* and *pruni* strains. M: molecular size marker, 1-kb ladder (Gibco-BRL). Lane 1: *X. arboricola* pv. *pruni* NCPPB 2588; lane 2: *X. a.* pv. *pruni* ISF 464; lane 3: *X. a.* pv. *pruni* ISF 55; lane 4: *X. a.* pv. *pruni* ISF 43; lane 5: *X. a.* pv. *pruni* ISF 465; lane 6: *X. a.* pv. *fragariae* PD 2780; lane 7: *X. a.* pv. *fragariae* PD 2782; lane 8: *X. fragariae* NCPPB 2473; lane 9: *X. fragariae* ISF 821.1; lane 10: *X. fragariae* ISF 821.2.

PCR products ranging from 100 to 2,000 bp (Fig. 3). It is interesting to note that *X. a. pv. pruni* ISF 43 and ISF 55 showed a similar but not identical pattern upon repetitive PCR. By contrast, these strains exhibited the same cassette array banding pattern. On the other hand, *X. a. pv. pruni* ISF 464 and ISF 465 proved identical when assessed with repetitive PCR but were different in their cassette array patterns.

This study confirms that integrons might play an important role in structuring the genetic diversity of *Xanthomonas* species and pathovars. All 54 strains tested generated complex banding patterns corresponding to four distinct cassette arrays, and each species and pathovar showed a distinct and recognizable pattern. Variability in cassette arrays was observed solely within strains of *X. arboricola* pv. *Pruni*, which showed two distinct patterns. These patterns, however, did not seem associated with a particular host plant or a geographic area from which the strains were originally isolated. Gillings *et al.* (2005) did not find differences in the cassette array banding pattern of the three *X. a. pv. pruni* strains they tested, although they pointed out differences in the number of gene cassettes for one of the strains tested.

Our study indicates that integrons, in addition to species (Gillings *et al.*, 2005), might generate diversity also within the pathovars, as we have noted for *X. a. pv. pruni*. The variability found in the cassette arrays of *X. a. pv. pruni* could be explained by recombinations in which integrons acquire diverse genes that cause the loss and rearrangements of the cassette arrays (Gillings *et al.*, 2005). However, why such diversity is present in this pathovar and not in others has not been investigated. Repetitive-sequence PCR using the BOXA1R primer clearly differentiated the different *Xanthomonas* strains.

Variability was observed within *X. a. pv. fragariae* and *X. a. pv. pruni* as previously pointed out (Louws *et al.*, 2001; Scortichini and Rossi, 2003). Also in this case no correlation between strains and host plants or geographic area was observed. As in the present study, Gillings *et al.* (2005) found variability among the three *X. a. pv. pruni* strains they tested with repetitive PCR and the BOXA1R primer. *X. fragariae* strains also showed variability upon repetitive PCR as previously ascertained by using other techniques (Roberts *et al.*, 1998; Janse *et al.*, 2001).

It is worth noting that despite the genetic variability shown by *X. fragariae* and *X. a. pv. fragariae* upon repetitive PCR, their respective integron cassette array patterns did not reveal any difference among the strains tested.

Also some *X. a. pv. pruni* strains showing the same cassette array banding pattern did not always display the same repetitive PCR fingerprint. This could indicate that, despite the strong uniformity of cassette arrays, different genetic lineages are present in these xan-

thomonads. This would confirm the hypothesis of Gillings *et al.* (2005) that integrons might be strictly linked to host-specific pathogenicity and that local environmental factors can further modulate the adaptability of the different species and pathovars, generating different lineages.

Integrons may be important for incorporating laterally transferred genes into the chromosome, and the phylloplane is thought to be an important niche for generating lateral gene transfer among bacteria (Lindow and Leveau, 2002; Gillings *et al.*, 2005). *X. fragariae* as well as *X. arboricola* pathovar *fragariae* and *pruni* are leaf-associated pathogens. However, two different *Xanthomonas* species pathogenic to strawberry, namely *X. fragariae* and *X. arboricola* pv. *fragariae* showed clearly different integron cassette arrays and both are characterized by a restricted pathogenicity. This could mean that besides integrons other groups of genes play a role in defining the host specificity of plant pathogenic xanthomonads or that different gene cassette arrays can be acquired via lateral gene transfers by different xanthomonads living in the same environment. For *Xanthomonas*, host specialization might occur in genetically different lineages (i.e. species) of this genus strictly associated with the plants. Gillings *et al.* (2005) argued that the selection pressures exerted by agricultural monocultures can favour particular strains having the capability able to invade and proliferate in the biomass of the crop. Then, the appearance of pathovars may be the result of the selection pressure of human activity. We fully agree with such an evolutionary scenario (Scortichini, 2005).

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