RESEARCH LETTER

# Integron variability in *Xanthomonas arboricola* pv. *juglandis* and *Xanthomonas arboricola* pv. *pruni* strains

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## Abstract

The integron platform and the gene cassette arrays of 34 *Xanthomonas arboricola* pv. *juglandis* and of 47 *Xanthomonas arboricola* pv. *pruni* strains isolated from different geographical areas were screened to check their variability. Genetic variability of the strains was also tested by means of BOX-PCR. For two representative strains of the two pathovars, the integrase gene *intI* and part of the flanking gene *ilvD* were also cloned and sequenced. Whereas *X. a.* pv. *pruni* strains did not show relevant variability, six *X. a.* pv. *juglandis* strains isolated in Australia showed some differences in the gene sequences. The ClustalW algorithm indicated that the majority of the *X. a.* pv. *juglandis* strains are closely related to *X. a.* pv. *pruni*, whereas the *X. a.* pv. *juglandis* strains isolated in Australia were more similar to *Xanthomonas hortorum* pv. *pelargonii*. Similarly, the gene cassette array pattern of the Australian strains, as well as that of the oldest strain maintained in culture, was different from the other strains. Also, three *X. a.* pv. *pruni* strains showed a different cassette array pattern when compared with the majority of other strains but no relationships with geographical area of isolation or host plant was revealed. This study confirmed that in addition to species, integrons may generate diversity also within two *X. arboricola* pathovars.

## Introduction

Integrons are defined as genetic elements that acquire and exchange exogenous DNA (i.e. the gene cassettes) by means of a site-specific recombination mechanism (Rowe-Magnus *et al.*, 2001). They may be located both on the chromosome and on plasmids and were found to be present in about 10% of bacteria that have been partially or completely sequenced (Boucher *et al.*, 2007). Integrons are usually known for their ability to assemble antibiotic resistance genes acquired by means of an integrase gene, *intI*, mediating recombination between a proximal primary recombination site, *attI*, and a secondary target, the *attC* site usually associated with a single ORF. The *attC*-ORF structure, a mobile element that can be laterally transferred between bacterial species, has been given the name 'gene cassette'. Transcription of integrated gene cassettes is driven by a promoter, $P_c$ (Rowe-Magnus *et al.*, 2001). The acquisition of one or more gene cassette(s) by sequential insertion at the *attI* site creates new integrons (Bennett, 1999).

Integrons have mainly been studied in human pathogens, i.e. *Vibrio* and *Treponema*, or in environmental species, i.e. *Pseudomonas*, *Methylobacillus* and *Geobacter* (Boucher *et al.*, 2007). Recently, Gillings *et al.* (2005) characterized the integrons of several bacterial species in the phytopathogenic genus *Xanthomonas*. The gene cassette arrays of different xanthomonads range from one to 22 and the integrase gene *intI* of the integron appears as frequently inactivated by the insertion of a transposon (Gillings *et al.*, 2005). Whereas *Pseudomonas* integrons show a remarkable variability in the composition of their cassette arrays when strains of a single species are compared (Holmes *et al.*, 2003), *Xanthomonas* integron cassette arrays showed a low degree of interstrain variability, possibly due to the loss of integrase activity. The inactivation of the integrase gene by the insertion of transposon-related, large deletions, frameshift and mutations to stop codon sequences has fixed the gene cassette array in particular lineages of the species. The inactivation of *intI* during the colonization of the different ecological niches, i.e. the host plants, is regarded as

fundamental for bacterial lineages to adapt to a particular host, thus becoming a pathovar (Gillings *et al*., 2005).

In contrast to the above-mentioned findings of cassette arrays in *Xanthomonas*, strains of the pathovar *pruni* of *Xanthomonas arboricola* showed less homogeneity in integron gene cassette arrays (Barionovi & Scortichini, 2006). In fact, among the 28 strains tested, three showed a clearly different cassette array pattern. These findings prompted us to investigate if such variability is present in other pathovars of *X. arboricola* and in other *X. a*. pv. *pruni* strains isolated from different geographical areas and host plants. Furthermore, we wished to verify the sequence variability of the integrase gene and that of its flanking gene *ilvD*. Our study indicates that variability in integron cassette arrays exists in *Xanthomonas arboricola* pv. *juglandis*, the causal agent of walnut (*Juglans regia* L.) blight, and in *X. a*. pv. *pruni*, inciting the bacterial angular leaf spot of stone fruits. In the case of *X. a*. pv. *juglandis*, we also demonstrate an apparent relationship between the cassette arrays and the geographical origin of the strains studied. *Xanthomonas arboricola* pv. *juglandis* strains also showed differences in the sequence of the integrase gene, *intI*, and in the flanking gene *ilvD*.

## Materials and methods

### Bacterial strains

*Xanthomonas arboricola* pv. *juglandis* and *X. a*. pv. *pruni* strains used in this study are listed in Table 1. All strains were routinely cultured on glucose–yeast extract–calcium carbonate (GYCA) medium (Van den Mooter *et al*., 1987), at 25–27 °C.

### DNA preparation

For DNA preparation, a loopful of *c*. 3 mm in diameter was taken from single colonies grown for 24 h at 25–27 °C on GYCA, suspended in sterile saline (0.85% of NaCl in distilled water) and centrifuged at 12 000 ***g*** for 2 min. After the supernatant was discarded, the pellet was suspended in bidistilled, filtered water to an OD corresponding to $1–2 \times 10^7$ CFU mL$^{-1}$. The suspension was placed in boiling water for 10 min and then stored at − 20 °C.

### Integron gene cassette arrays

In order to detect cassette arrays and for tests for heterogeneity in the arrays, the proximal integron gene cassette region of *X. arboricola* strains was amplified using primers MRG17 (5′-GATACTYRGCGCAACACCGC) 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) and photographed with a Kodak Gel Logic 100 Imaging System apparatus. The runs were performed in triplicate.

### Cloning and DNA sequencing of the integrase gene

The PCR-amplified *ilvD*-integrase DNA fragments obtained using primers AJH95 (5′-GGCGGCCCCGGNATGCAR GARATG-3′) and MRG18 (5′-GCGGTGTTGCGCYRAG TATC-3′) of *X. a*. pv. *juglandis* DAR 76583 and *X. a*. pv. *juglandis* ISF B1 as well as *X. a*. pv. *pruni* ISF 465 and *X. a*. pv. *pruni* NCPPB 2587 were purified from 1.5% agarose gel with the Wizard SV Gel and PCR clean-up system, as described by the manufacturer (Promega) and ligated in pGem-T easy vector (Promega). Ligated plasmids were transformed into competent *Escherichia coli* strain TOP10 F′ cells (Invitrogen) and the bacteria spread onto Luria–Bertani agar plates containing 50 μg mL$^{-1}$ ampicillin, 0.5 mM isopropylthio-β-D-galactopyranoside and 80 μg mL$^{-1}$ X-gal. Plasmid DNA isolated from 15 white colonies was purified and digested according to Sambrook *et al*. (1989), and digested with EcoRI to verify the presence of the inserts. Plasmids were sequenced with primers M13F and M13R. DNA fragments were prepared for sequencing using a Qiagen Plasmid Midi kit according to the manufacturer's instructions (Qiagen). All the sequences were determined at the Primm sequencing service. The integron sequences of *X. a*. pvs. *juglandis* and *pruni* were aligned with other integron sequences of *Xanthomonas* species and pathovars obtained from NCBI GenBank. CLUSTALW (Higgins *et al*., 1994) was used to construct a dendrogram from aligned sequences based on the neighbour-joining algorithm. The confidence of genetic relationships was tested with 1000 bootstrap replications using MEGA version 3.1 (Kumar *et al*., 2004).

### Nucleotide accession numbers

The integron sequence data were submitted to the EMBL/GenBank database under the following accession numbers: *X. a*. pv. *juglandis* ISF B1, AM905244; *X. a*. pv. *juglandis* DAR 76583, AM905372; *X. a*. pv. *pruni* ISF 465, AM9053374; *X. a*. pv. *pruni* NCPPB 2587, AM905375.

### BOX-PCR

The repetitive-sequence PCR method and thermal cycling used was that of Louws *et al*. (1994). BOXA1R primer (5′-CTACGGCAAGGCGACGCTGACG) synthesized by Primm was used. PCRs were run on an Eppendorf Mastercycler programmable thermal controller. The gels were stained and photographed with a Kodak Gel Logic 100 Imaging System apparatus. The runs were performed in triplicate.

**Table 1.** List of *Xanthomonas arboricola* pv. *juglandis* and *X. arboricola* pv. *pruni* strains used in this study and integron gene cassette profile types

| Strain isolation | Host plant profile | Year | Country | Cassette |
|---|---|---|---|---|
| *X. arboricola* pv. *juglandis* | | | | |
| NCPPB 362 | *Juglans regia* | 1955 | UK | C |
| NCPPB 411$^T$ | *Juglans regia* | 1956 | New Zealand | A |
| NCPPB 412 | *Juglans regia* | 1956 | New Zealand | A |
| NCPPB 413 | *Juglans regia* | 1957 | New Zealand | A |
| NCPPB 1447 | *Juglans regia* | 1962 | Romania | A |
| CFBP 1022 | *Juglans regia* | 1967 | France | A |
| DAR 73873 | *Juglans regia* | 1999 | Australia | B |
| DAR 73874 | *Juglans regia* | 1999 | Australia | B |
| DAR 33423 | *Juglans regia* | NK | Australia | B |
| DAR 76582 | *Juglans regia* | NK | Australia | C |
| DAR 76583 | *Juglans regia* | NK | Australia | B |
| DAR 76584 | *Juglans regia* | NK | Australia | B |
| ISF B1 | *Juglans regia* | 2002 | Italy | A |
| ISF B2 | *Juglans regia* | 2002 | Italy | A |
| ISF B3 | *Juglans regia* | 2002 | Italy | A |
| ISF FR1 | *Juglans regia* | 2003 | Italy | A |
| ISF FR2 | *Juglans regia* | 2003 | Italy | A |
| ISF FR3 | *Juglans regia* | 2003 | Italy | A |
| ISF FR4 | *Juglans regia* | 2003 | Italy | A |
| ISF N15 | *Juglans regia* | 2004 | Italy | A |
| ISF N16 | *Juglans regia* | 2004 | Italy | A |
| ISF N17 | *Juglans regia* | 2004 | Italy | A |
| ISF N18 | *Juglan regias* | 2004 | Italy | A |
| ISF N19 | *Juglans regia* | 2004 | Italy | A |
| ISF N20 | *Juglans regia* | 2004 | Italy | A |
| ISF D1 | *Juglans regia* | 2005 | Italy | A |
| ISF D2 | *Juglans regia* | 2005 | Italy | A |
| ISF D3 | *Juglans regia* | 2005 | Italy | A |
| ISF G1.1 | *Juglans regia* | 2006 | Italy | A |
| ISF G1.2 | *Juglans regia* | 2006 | Italy | A |
| ISF G1.3 | *Juglans regia* | 2006 | Italy | A |
| ISF G1.4 | *Juglans regia* | 2006 | Italy | A |
| ISF G1.5 | *Juglans regia* | 2006 | Italy | A |
| ISF G1.6 | *Juglans regia* | 2006 | Italy | A |
| *X. arboricola* pv. *pruni* | | | | |
| NCPPB 2587 | *Prunus armeniaca* | 1973 | South Africa | A |
| DAR 56679 | *Prunus armeniaca* | 1987 | Australia | A |
| ISF XAlb 2 | *Prunus armeniaca* | 2005 | Italy | A |
| ISF XAlb 3 | *Prunus armeniaca* | 2005 | Italy | A |
| ISF XAlb 4 | *Prunus armeniaca* | 2005 | Italy | A |
| ISF XAlb 5 | *Prunus armeniaca* | 2005 | Italy | A |
| NCPPB 926 | *Prunus domestica* | 1960 | South Africa | A |
| DAR 61729 | *Prunus domestica* | 1988 | Australia | A |
| DAR 72009 | *Prunus dulcis* | 1996 | Australia | A |
| IVIA 3161-2-1 | *Prunus dulcis* | 2006 | Spain | A |
| IVIA 3181-3-1-8 | *Prunus dulcis* | 2006 | Spain | A |
| IVIA 3171-1-1-1 | *Prunus dulcis* | 2006 | Spain | A |
| IVIA 3162-4-5 | *Prunus dulcis* | 2006 | Spain | A |
| NCPPB 1607 | *Prunus persica* | 1964 | Australia | A |
| NCPPB 2588 | *Prunus persica* | 1973 | South Africa | A |
| NCPPB 3156 | *Prunus persica* | 1979 | Italy | A |
| DAR 33420 | *Prunus persica* | 1980 | Australia | A |
| DAR 41285 | *Prunus persica* | 1982 | Australia | A |
| DAR 41286 | *Prunus persica* | 1982 | Australia | A |
| DAR 41287 | *Prunus persica* | 1982 | Australia | A |
| ISF 45 | *Prunus persica* | 1993 | Italy | A |

**Table 1.** Continued.

| Strain isolation | Host plant profile | Year | Country | Cassette |
|---|---|---|---|---|
| DAR 69849 | *Prunus persica* | 1994 | Australia | A |
| ISF 461 | *Prunus persica* | 1996 | Italy | A |
| ISF 462 | *Prunus persica* | 1996 | Italy | A |
| ISF 463 | *Prunus persica* | 1996 | Italy | A |
| ISF 464 | *Prunus persica* | 1996 | Italy | A |
| ISF 465 | *Prunus persica* | 1996 | Italy | B |
| ISF 514 | *Prunus persica* | 1997 | Italy | A |
| ISF 515 | *Prunus persica* | 1997 | Italy | A |
| IVIA 2826-2 | *Prunus persica* | 2003 | Italy | A |
| IVIA 2835-1 | *Prunus persica* | 2003 | Spain | A |
| IVIA 2863-1 | *Prunus persica* | 2004 | Spain | A |
| ISF X1P | *Prunus persica* | 2005 | Italy | A |
| ISF X2P | *Prunus persica* | 2005 | Italy | A |
| DAR 33337 | *Prunus salicina* | 1978 | Australia | A |
| DAR 56680 | *Prunus salicina* | 1987 | Australia | A |
| ISF 55 | *Prunus salicina* | 1991 | Italy | B |
| ISF 66 | *Prunus salicina* | 1991 | Italy | A |
| ISF 43 | *Prunus salicina* | 1993 | Italy | B |
| ISF 444 | *Prunus salicina* | 1998 | Italy | A |
| ISF XP12 | *Prunus salicina* | 2000 | Italy | A |
| ISF Sus 1 | *Prunus salicina* | 2002 | Italy | A |
| ISF Sus 2 | *Prunus salicina* | 2002 | Italy | A |
| ISF Sus 4 | *Prunus salicina* | 2002 | Italy | A |
| IVIA 2647-1-3 | *Prunus salicina* | 2002 | Spain | A |
| IVIA 2649-1 | *Prunus salicina* | 2002 | Spain | A |
| ISF Sus 5 | *Prunus salicina* | 2003 | Italy | A |

T, Pathotype strain; NK, not known.

CFBP, Collection Française de Bactéries Phytopathogènes, Angers, France; DAR, Australian Collection of Plant Pathogenic Bacteria, Rydalmere, Australia; ISF, Culture Collection of C.R.A.-Centro di Ricerca per la Frutticoltura, Roma, Italy; IVIA, Culture Collection of Instituto Valenciano de Investigaciones Agrarias, Moncada-Valencia, Spain; NCPPB, National Collection of Plant Pathogenic Bacteria, York, UK.

## Results

All 81 *X. arboricola* strains generated complex banding patterns upon PCR amplification with primers AJH60 and MRG17, enabling the amplification of the integron gene cassette arrays. The overall patterns of the two pathovars was clearly different. In addition, within each pathovar, some strains showed a different banding pattern. The integron gene cassette arrays of the majority of *X. a.* pv. *juglandis* strains showed the same pattern (group A). However, six strains isolated in Australia and the oldest *X. a.* pv. *juglandis* strain maintained in an international culture collection, namely NCPPB 362, isolated in 1955 in the United Kingdom, showed a distinct profile. In fact, five of the six Australian strains clustered within group B, whereas NCPPB 362 and DAR 76582 clustered within group C (Fig. 1a). Concerning *X. a.* pv. *pruni*, three of 47 strains tested (i.e. ISF 43 and ISF 465, isolated from *Prunus persica*, and ISF 55, isolated from *Prunus salicina*) showed a different pattern with one PCR product differentiating the three strains (Fig. 1b). In this case, no relationships between the geographical area of strain isolation or host plant from which the strain was isolated could be inferred.

BOX-PCR fingerprinting confirmed the genetic diversity of the *X. a.* pv. *juglandis* strains isolated in Australia. In fact, these strains showed differences in the banding pattern when compared with the other *X. a.* pv. *juglandis* strains. However, the genetic variability of this pathovar is relevant, as pointed out by means of other techniques and strains, different fingerprinting being observed (see Discussion). The three *X. a.* pv. *pruni* strains showing a different cassette array pattern did not show relevant differences with the other strains in their BOX-PCR profiles (data not shown).

Primers AJH95 and MRG18 enabled PCR amplification of part of the *ilvD* gene and of the integrase gene *intI*. All the *X. a.* pv. *juglandis* strains isolated in Australia showed a slightly different PCR product from the other 32 *X. a.* pv. *juglandis* and 47 *X. a.* pv. *pruni* strains tested. For comparative purposes, the PCR product of *X. a.* pv. *juglandis* DAR 76583 and ISF B1, as well as of *X. a.* pv. *pruni* ISF 654 and NCPPB 2587, was cloned and sequenced. ClustalW analysis revealed that *X. a.* pv. *juglandis* ISF B1, representing the

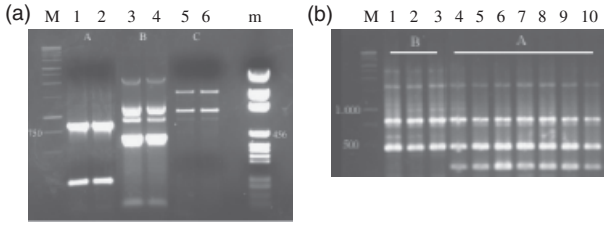


**Fig. 1.** (a) PCR amplification of the proximal integron gene cassette in *Xanthomonas arboricola* pv. *juglandis* strains using primers MRG17 and AJH60. M, molecular size marker, 1-kb ladder (Promega); m, molecular size marker, pGem (Promega). Lane 1, NCPPB 411; lane 2, ISF N17; lane 3, DAR 76583; lane 4, DAR 76584; lane 5, NCPPB 362; and lane 6, DAR 76582. A, B and C indicate the three groups of *X. a*. pv. *juglandis* strains revealed by integron gene cassette analysis. The majority of strains are included into the group A. (b) PCR amplification of the proximal integron gene cassette in *Xanthomonas arboricola* pv. *pruni* strains using primers MRG17 and AJH60. M, molecular size marker, 1-kb ladder (Promega). Lane 1, ISF 55; lane 2, ISF 43; lane 3, ISF 465; lane 4, ISF 464; lane 6, ISF 463; lane 7, ISF 515; lane 8, IVIA 2826-2; lane 9, IVIA 2849-1; and lane 10, ISF 444. A, B, the two groups of *X. a*. pv. *pruni* strains revealed by integron gene cassette analysis. The majority of strains are included within group A.

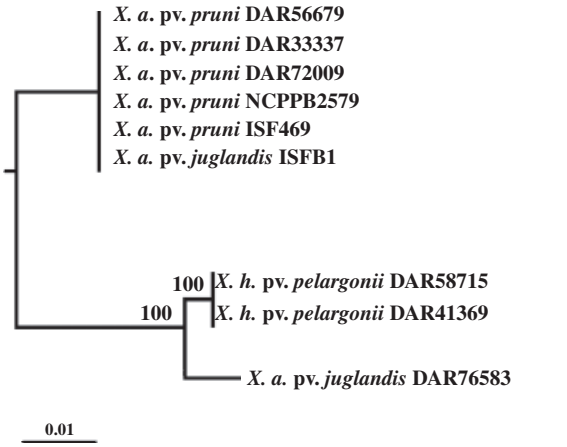


**Fig. 2.** Dendrogram of relationships between *Xanthomonas arboricola* pvs. *juglandis* and *pruni* strains obtained with CLUSTALW and the neighbour-joining algorithm. The dendrogram was obtained by comparing part of the *ilvD* gene and part of the integrase *intI* gene.

majority of the strains, was closely related to the *X. a*. pv. *pruni* strains, whereas *X. a*. pv. *juglandis* DAR 76583 was closely related to *Xanthomonas hortorum* pv. *pelargonii* (Fig. 2), thus confirming the difference of this strain, and, probably, of all Australian strains that showed a very similar PCR product.

## Discussion

This study has shown that variability can exist in the integron gene cassette arrays of strains belonging to *X. a*. pvs. *juglandis* or *pruni*. This variability appears to be more relevant in pv. *juglandis*, which showed three different cassette array profiles. Interestingly, all the six strains isolated in Australia tested here were clearly shown to be different from the majority of the strains obtained from other areas. However, the oldest *X. a*. pv. *juglandis* strain maintained in an international culture collection, NCPPB 362, isolated in the United Kingdom in 1955, showed a different pattern to that of the Australian strains. The distinctiveness of these strains was also confirmed by BOX-PCR, revealing a different fingerprinting pattern. These findings confirm that the genetic variability of *X. a*. pv. *juglandis* is relevant, as indicated previously using amplified fragment length polymorphism and repetitive-sequence PCR analysis (Loreti *et al*., 2001; Scortichini *et al*., 2001). In addition, three of the 47 *X. a*. pv. *pruni* strains tested showed a distinct banding pattern for their cassette arrays. No correlation between geographical area of isolation or host plant was found. All the additional 19 strains not studied previously (Barionovi & Scortichini, 2006) showed the same profile as the majority of the strains, including those isolated in Australia. By contrast, BOX-PCR fingerprinting did not reveal differences for the three strains showing a different cassette array pattern from those of the other strains. Whereas *X. a*. pv. *juglandis* was not studied by Gillings *et al*. (2005), these authors reported for one of the three *X. a*. pv. *pruni* strains investigated, namely DAR 33337, a different gene cassette array.

Gillings *et al*. (2005) stressed that inactivation of the integrase gene in particular lineages has fixed the gene cassette arrays in the different pathovars and has highlighted the possible link between such a fixation of cassette arrays in different *Xanthomonas* species and pathovars and the events that lead to the host-specific pathogenesis in such lineages. They also note that, in general, strains within individual pathovars have identical cassettes. However, the present study revealed that strains of the same pathovar may have different cassette arrays. This could mean that adaptation to particular environments encountered by some lineages of certain pathovars can be putatively linked to different gene cassette arrays even though the function of these genetic elements in xanthomonads is largely unknown.

Sequencing of part of the *ilvD* gene and of the integrase gene *intI* of the integron platform revealed that some *X. a*. pv. *juglandis* strains differ from the majority of the strains. In fact, DAR 76583, representative of the six strains isolated in Australia that showed a different PCR product, appears to be more closely related to *X. h*. pv. *pelargonii* than to the strains of pv. *pruni* as observed for the majority of the strains. Gillings *et al*. (2005) found that large deletions are present along the integrase gene of some *Xanthomonas* species. *Xanthomonas arboricola* pv. *juglandis* was not

included in their study but the relevant difference found here between DAR 76583 and the other *X. a.* pv. *juglandis* strains could be explained by such possible deletion in the integron gene in some *X. a.* pv. *juglandis* lineages or by relevant mutations in the sequence itself, leading these strains to show more homology with other *Xanthomonas* species. However, the ecological role(s) played by such different integron platforms within the pathovar *juglandis* is currently unknown.

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