Molecular Microbiology (1994) **12**(6), 941-950

Two native plasmids of *Pseudomonas syringae* pathovar *tomato* strain PT23 share a large amount of repeated DNA, including replication sequences

Jesús Murillo[1*] and Noel T. Keen[2]
[1] Laboratorio de Patología Vegetal, Departamento de Producción Agraria, Universidad Pública de Navarra, 31006 Pamplona, Spain.
[2] Department of Plant Pathology, University of California, Riverside, CA 92521-0122, USA.

Received 17 December 1993; revised 8 March; accepted 22 March 1994. * For correspondence. E-mail jesus.murillo@unavarra.es; Tel. +34 948169133; Fax +34 948169732.

Summary

Strain PT23 of *Pseudomonas syringae* pv. tomato contains four native plasmids, designated as A, B, C and D. By DNA hybridization of genomic and plasmid DNA digests from the wild type and a plasmid-cured strain, we determined that *c*. 61 kb (*c*. 74%) of pPT23B is repeated in pPT23A and only *c*. 17 kb (*c*. 21%) is in single copy in strain PT23. pPT23B also contains DNA repeated in the chromosome that occurs in three DNA fragments of 0.6, 4.6, and 9.6 kb that might be transposable elements. Additionally, the 9.6 kb fragment also shares sequences with the three other plasmids of strain PT23. By DNA hybridization with the origin of replication from a native plasmid of *P*. *syringae* pv. syringae and *in vivo* replication tests, we identified the origins of replication of plasmids A, B, and D and showed that they cross-hybridize. The putative *par* region from pPT23A has also been identified and is not conserved in the other three native plasmids from strain PT23. By using the defined minimal origin of replication from pPT23A as a probe, we showed that it is highly conserved in 14 strains belonging to nine different pathovars of *P. syringae* and that as many as five different native plasmids with closely related origins of replication coexist in the same cell. The duplication and reorganization of plasmids might therefore occur at high frequency and could be responsible for the existence of large numbers of native plasmids in *P*. *syringae* strains.

Introduction

Plasmids are autonomous genetic elements that are present in a variety of organisms, including eukaryotes. Since they are usually stably maintained in different environmental conditions, it is generally assumed that plasmids confer a selective advantage to their host (Shaw, 1987; Coplin, 1989), although in most cases this role is obscure. The study of plasmids is of particular importance in the field of plant-microbe interactions since it is common to find plasmid-borne genes that are involved in the establishment or continuation of such interactions. Well-documented examples are the symbiotic plasmids (pSym) from *Rhizobium* (Martínez *et al.*, 1990) and the tumor-inducing (Ti and Ri) plasmids from *Agrobacterium* (Zambryski *et al.*, 1989).

Plasmids have been described in nearly all known genera of phytopathogenic bacteria (Coplin, 1989). In most cases native plasmids are cryptic, but as they are more intensively studied functions have been associated with them. Among pathovars of the phytopathogenic bacteria *Pseudomonas syringae,* native plasmids occur in varying numbers and sizes (Shaw, 1987; Coplin, 1989). In some cases, they have been shown to carry different avirulence genes (Tamaki *et al.*, 1988; Kobayashi *et al.*, 1990; Bavage *et al.*, 1991) or to be involved in the synthesis of indoleacetic acid (Comai and Kosuge, 1980), resistance to copper (Bender and Cooksey, 1987; Sundin *et al.*, 1989; Cooksey, 1990), resistance to streptomycin (Burr *et al.*, 1988), or synthesis of the phytotoxin coronatine (Bender *et al.*, 1991). Several authors have studied the molecular relationships between native plasmids in *P*. *syringae* pathovars by comparing plasmid size, restriction digestion profiles of total plasmid DNA, and DNA hybridization (reviewed in Shaw, 1987; Coplin, 1989). Different isolates within several pathovars

contain plasmids with varying degrees of sequence homology. Unfortunately, the presence of multiple plasmids in many strains and the lack of plasmid maps have hindered determination of the extent and location of homology.

Pseudomonas syringae pv. tomato strain PT23 contains four native plasmids (Bender and Cooksey, 1986) that are designated as pPT23A (100 kb), pPT23B (83 kb), pPT23C (65 kb), and pPT23D (35 kb). While plasmid C is cryptic, the other three have been related to pathogenicity or survival. pPT23A is a conjugative plasmid required for synthesis of the phytotoxin, coronatine, which is essential for the production of full disease symptoms in tomato plants (Bender *et al.*, 1987). Plasmid B contains avirulence gene D (*avrD)*, which confers avirulence to certain cultivars of soybean (Kobayashi *et al.*, 1990). Finally, pPT23D is involved in resistance to copper; its physical map has been established (Cooksey, 1987) and the copper resistance genes localized (Bender and Cooksey, 1987). We physically characterized plasmid B and showed that it contains a large quantity of sequences repeated in other native plasmids of strain PT23 (Murillo *et al.*, 1994). In this work, we studied repetitive DNA in the PT23 plasmids, which can play an important role in the generation of plasmid or chromosome rearrangements. The evidence obtained suggests that plasmids A and B may have arisen from a common ancestor. Our data also supports the idea that plasmid duplication, with subsequent modification of the resulting products to allow for their stable coexistence, is a common phenomenon among pathovars of *P. syringae*.

Results

DNA homology among the four native plasmids of P. syringae *pv.* tomato *PT23*

Previously, we reported the existence of DNA homology between pPT23B and other native plasmids in strain PT23 (Murillo *et al.*, 1994). To determine which plasmid(s) exhibited homology and to what extent, we performed a series of high-stringency Southern blot analyses using purified native plasmids A, B, C, and D as probes (Fig. 1). Plasmids A and B showed a large number of sequences in common (Fig. 1, panels B and C). By hybridization with individual restriction fragments from pPT23B (data not shown) it was determined that approximately 74% (*c*. 61 kb) of plasmid B was repeated in plasmid A (Fig. 2). The region of pPT23B carrying the *avrD* gene and downstream open reading frames (ORFs) (Kobayashi *et al.*, 1990) as well as the putative *par* region (Murillo *et al.*, 1994) were unique to this plasmid (Fig. 2). Plasmids A and B also hybridized to 6.6 kb and 5.2 kb *Eco*RI fragments from plasmids C and D, respectively (Fig. 1, panels B and C). This homology was exhibited by 10 kb and 11 kb *Eco*RI fragments from plasmids A and B, respectively, when plasmids C and D were used as hybridization probes (Fig. 1, panels D and E). Plasmids C and D contained only one *Eco*RI fragment in common, corresponding to the homologous fragments of plasmids A and B. The location in pPT23B and pPT23D of the fragment repeated in the four plasmids is shown in Fig. 2.

Total genomic DNA from strain WABCD1, derived from PT23 and cured of the four native plasmids, contained at least seven and five *Eco*RI fragments with homology to plasmids A and B, respectively (Fig. 1, panels B and C). Identical results were obtained with total genomic DNA from WABCD2 (data not shown), which

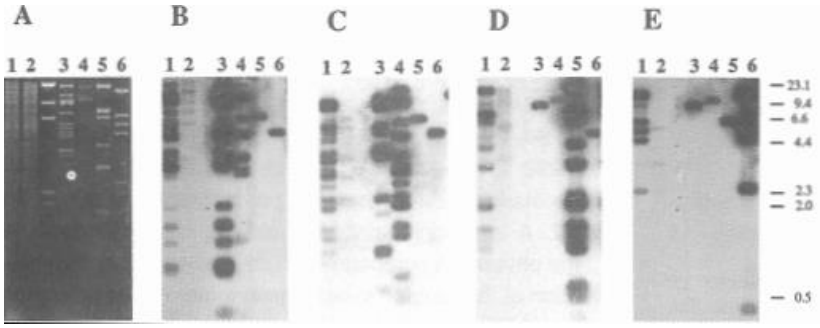

Fig. 1. Sequence conservation among native plasmids from *P. syringae* pv. tomato PT23. Genomic and plasmid DNA was digested with *Eco*RI, separated in 1 % agarose gels (A) and transferred to nylon filters. Filters were hybridized with ^{32}P-labelled pPT23A::Km (B), pPT23B(*avrD*::Tn*5*) (C), pPT23C (D) and pPT23D (E). Samples are: total genomic DNA from strains PT23 (lane 1) and WABCD1 (lane 2), and purified plasmid DNA from pPT23A::Km (lane 3), pPT23B(*avrD*::Tn*5*) (lane 4), pPT23C (lane 5), and pPT23D (lane 6). The positions of lambda *Hin*dIII fragments are indicated on the right.

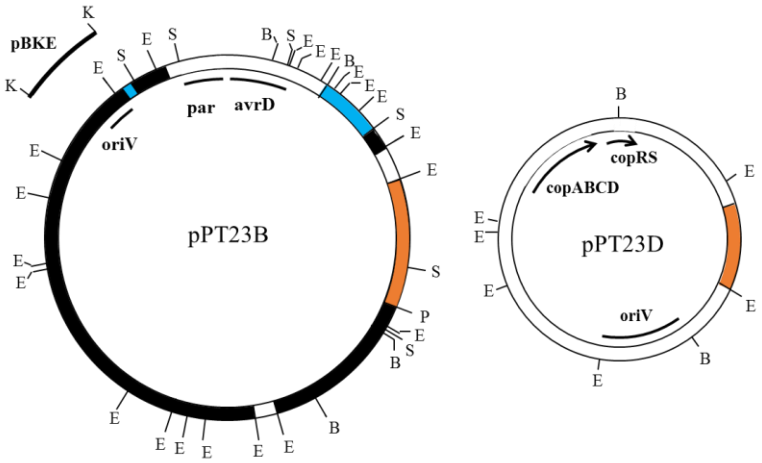

Fig. 2. Repetition of sequences in the plasmids of *P. syringae*. pv. tomato PT23. Restriction fragments from pPT23B that are repeated in pPT23A are shown in black and those repeated in the chromosome are shown in blue. The fragments repeated in all four native plasmids and the chromosome are shown in orange in pPT23B and pPT23D. The white areas in the maps indicate fragments not repeated elsewhere in the PT23 genome. The location in pPT23B of the 9.2 kb *Kpn*I insert of pBKE is indicated by a solid line outside the map. The approximate location of the origins of replication (oriV), *avrD* gene and four downstream ORFs (avrD), the putative *par* region, and the copper resistance genes (cop) are indicated by solid lines inside the circles. Plasmid maps are modified from Cooksey (1987), Kobayashi *et al.* (1990), Mills *et al.* (1993), and Murillo *et al.* (1994). B, *Bam*HI; E, *Eco*RI; P, *Pac*I; S, *Sac*II.

also lacks the four native plasmids. Consequently, the hybridizing bands probably represent DNA repeated in the chromosome rather than partial integration of the native plasmids. The fact that the pattern of hybridization was different with pPT23A than with pPT23B suggest that these plasmids may contain different sets of repeated elements. Two *Eco*RI fragments from total DNA of WABCD1 (3.5 and 5.8 kb) showed hybridization to all four of the native plasmids used as probes (Fig. 1, lane 2 in panels B to E). This suggests that the DNA common to all of the plasmids (see previous paragraph and Fig. 2) and the chromosome could be a transposable element.

When total DNA from WABCD1 was used as a probe, total plasmid DNA from strain PT23 showed 14 hybridizing bands of different intensity (Fig. 3, lane 1). Plasmids A and B each showed six homologous bands of different sizes. By hybridization of pPT23B subclones using the same DNA probe (data not shown), we showed that these bands in pPT23B were grouped in three non-contiguous areas of 0.6, 4.6, and 9.6 kb (Fig. 2) that could be transposable elements. The 0.6 kb fragment is located adjacent to *oriV* in an *Eco*RV-*Kpn*I fragment and the sequence of the 356 nucleotides closest to the *Kpn*I site in this fragment showed 94.1% identity with the right end of IS*801* from *P. s.* pv. phaseolicola (Fig. 4) (Romantschuk *et al.*, 1991). The 9.6 kb (*Eco*RI-*Pac*I) fragment contained the DNA from plasmid B exhibiting homology to the other three plasmids and the chromosome (Figs. 1 and 2). Taking these data into account, plasmid B may contain only *c*. 17 kb (*c*. 21%) of single-copy DNA (Fig. 2). This figure may, however, be an underestimate since the actual ends of the homologous regions cannot be precisely delimited with these assays and it is possible that non-homologous regions may be embedded in the repeated fragments. As expected, total DNA from WABCD1 hybridized to the 6.6 kb and the 5.2 kb *Eco*RI fragments from plasmids C and D, respectively (Fig. 3, lanes 5 and 6). This confirmed that the fragment common to the four native plasmids (Fig. 2) indeed hybridized to the 3.5 and 5.8 kb *Eco*RI fragments from WABCD1.

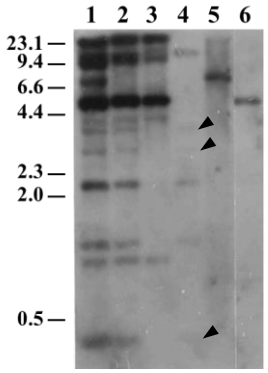

Fig. 3. Repetition of plasmid sequences in the PT23 chromosome. Southern blot hybridization of total plasmid DNA from strain PT23 (lane 1), PT23.2 (lane 2), which lacks plasmid C, and purified plasmid DNA from pPT23A::Km (lane 3), pPT23B(*avrD*::Tn*5*) (lane 4), pPT23C (lane 5), and pPT23D (lane 6) digested with *Eco*RI. Genomic DNA from strain WABCD1, cured of the four native plasmids, was used as a hybridization probe. Molecular size markers are shown in kb on the left.

Localization of the origins of replication of plasmids A, B, and D

Since pPT23A and pPT23B share a large number of sequences, we decided to determine

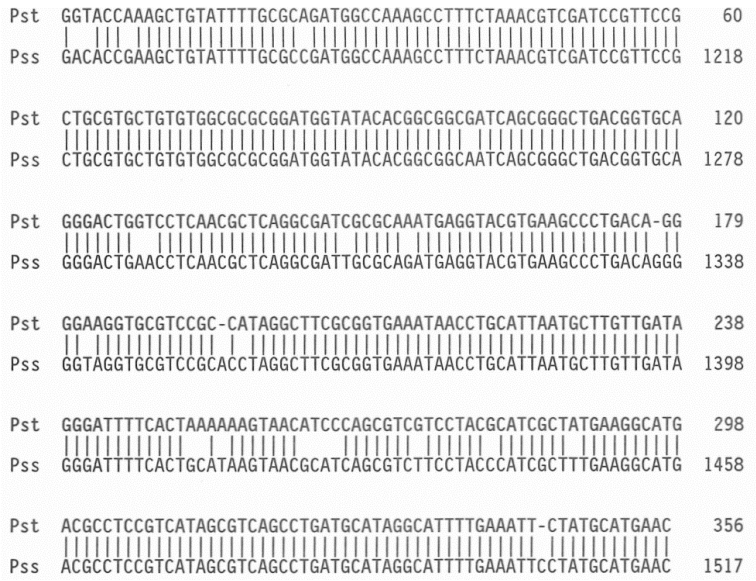

Fig. 4. Sequence alignment of IS*801* from plasmid pPT23B of *P. s.* pv. tomato PT23 (Pst) and from *P. s.* pv. syringae LR781 (Pss) (Romantschuk *et al.,* 1991). Identical nucleotides are connected by a bar. Dashes indicate spaces inserted to maximize alignment.

if they also shared DNA involved in replication. Plasmid pOSU22 (Fig. 5A), which contains the *oriV* and *par* regions from plasmid pOSU900 from *P. s.* pv. syringae J900 (Mukhopadhyay *et al.*, 1990), hybridized with total plasmid DNA from strain PT23 (C. Bender, personal communication). A 1.2 kb *Hin*dIII fragment from pOSU22 that contains part of the *oriV* was used as a hybridization probe against DNA from strain PT23 (Fig. 5). Total plasmid and genomic DNA showed the same hybridization pattern, consisting of five *Eco*RI fragments with homology to this probe (Fig. 5B). Two of these fragments (0.9 and 1.6 kb) were present in pPT23A. Hybridizing fragments of 3.4 and 6.2 kb occurred in pPT23B and a 7.5 kb hybridizing fragment was observed in pPT23D. Plasmid pPT23C did not show any homology to the 1.2 kb *Hin*dIII fragment (Fig. 5B, lane 5) or to an adjacent 0.9 kb *Eco*RI fragment that contained the remainder of the minimal origin of replication from pOSU22 (data not shown). These result suggested that plasmids A, B and D from PT23 have closely related origins of replication. Since this fact would normally preclude their coexistence in the same cell, we studied the functionality of these putative *oriV*.

The 0.9 and 1.6 kb *Eco*RI fragments from pPT23A homologous to the *oriV* from pOSU22 are contained in a 9.2 kb *Kpn*I fragment (Fig. 5A). When these three fragments were separately cloned in pK184, only plasmid pAKC, which contains the 9.2 kb *Kpn*I fragment, autonomously replicated in strain PT23 and concomitantly displaced pPT23A. Plasmid pAori1 and pAori2 (Fig. 6) were obtained by partially digesting pAKC with *Eco*RI and ligating fragments of *c*. 2.5 kb to pK184. pAori2 contained the 0.9 and 1.6 kb *Eco*RI fragments with homology to pOSU22 (Fig. 5) and an additional 0.3 kb *Eco*RI fragment. Plasmid pAori1 also contained the 0.9 and 1.6 kb fragments, but in the opposite orientation. These two plasmids were autonomously maintained in PT23 and caused the eviction of pPT23A, indicating that pPT23A probably contains only one functional origin of replication that is included in the 0.9 and 1.6 kb *Eco*RI fragments cloned in pAori1.

By hybridization with seven overlapping cosmid clones constituting pPT23B DNA (Murillo *et al.*, 1994), the 3.4 and 6.2 kb *Eco*RI fragments homologous to *oriV* from pOSU22 were localized adjacent to each other, spanning co-ordinates 68 to 77.6 on the physical map (Murillo *et al.*, 1994). Our repeated failure to obtain transformants of PT23 carrying any of these two fragments cloned in pK184 indicated that they cannot replicate autonomously and suggested that the putative *oriV* overlaps the *Eco*RI site that separates them. A 5 kb *Kpn*I fragment that overlaps the two *Eco*RI fragments (Figs 2 and 5) was accordingly cloned into pK184 in both orientations, resulting in pBKE and pBKE2. These two plasmids replicated autonomously in strain PT23, suggesting that the *Kpn*I fragment contains a functional *oriV*. However, after five consecutive transfers in KMB plus kanamycin, pBKE or pBKE2 only occasionally caused the eviction of plasmid

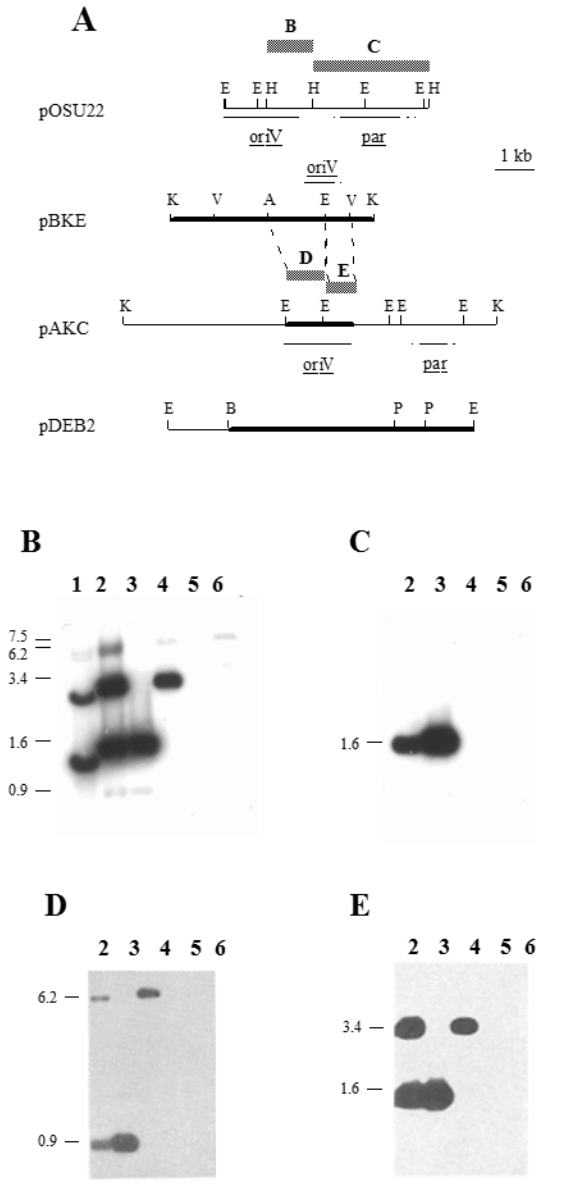

Fig. 5. Location and homology of the origins of replication from plasmids A, B and D and the *par* region from plasmid A. A. Horizontal lines represent the insert in plasmids pOSU22 (Mukhopadhyay *et al.*, 1990), pBKE, pAKC and pDEB2. Heavy lines indicate the minimum defined fragments that can replicate autonomously in strain PT23. Grey boxes denote the restriction fragments utilized as probes, and the letters above them indicate the panel that show the results of the hybridization. Probes were 1.2 kb *Hin*dIII (B) and 3 kb *Hin*dIII (C) from pOSU22; 0.9 kb *Eco*RI from pAori2 (D), and 0.7 kb *Hin*dIII from p2D924 (E). Broken lines delimit the region of homology between pBKE and pAKC using the probes indicated. Thick lines outside the maps show the approximate location of the origins of replication (*oriV*) and the *par* regions. The *Eco*RI and *Hin*dIII sites flanking the insert in pOSU22 belongs to the plasmid vector. A, *Acc*I; B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; V, *Eco*RV. Panels B to E: Southern blot hybridizations of genomic (lane 1) and total plasmid DNA (lane 2) from strain PT23 and purified plasmid DNA from pPT23A::Km (lane 3), pPT23B(*avrD*::Tn*5*) (lane 4), pPT23C (lane 5), and pPT23D (lane 6) digested with *Eco*RI. Numbers on the side of the panels show the size of the hybridizing fragments.

pPT23B and in most cases coexisted without being cointegrated into it.

Plasmid pDEB2 (Fig. 5A) contained a 7.5 kb *Eco*RI fragment from pPT23D (fragment *Eco*RI B, following the restriction fragment designation of Cooksey,1987) that showed homology to *oriV* from pOSU22. The 7.5 kb fragment was cloned in pK184 and oriented with the *Bam*HI site furthest away from the vector *lac* promoter. Plasmid pDBE was constructed by digesting pDEB2 with *Bam*HI and religating the large fragment, which resulted in the deletion of a 1.5 kb fragment. Upon electroporation into PT23, both pDEB2 and pDBE replicated autonomously and caused the eviction of pPT23D. To confirm the existence of only one *oriV* in pPT23D, we cloned several restriction fragments from this plasmid in pK184 and tested their ability to replicate in strain PT23. We repeatedly failed to recover any PT23 colonies on kanamycin-containing media following electroporation with recombinant plasmids containing fragments *Bam*HI B; *Pst*I A, B, D, and G; *Eco*RI C; and *Hin*dIII B, and E. However, we routinely obtained PT23 transformants by introducing plasmids containing fragments *Bam*HI A, *Eco*RI B, or *Hin*dIII A, all of which included the 6.0 kb *Eco*RI-*Bam*HI fragment with homology with *oriV*. Although the negative results with the other DNA fragments from pPT23D do not rule out the possibility of a second *oriV*, the data strongly suggest that pPT23D contains only one origin of replication, and that it is located in the 6.0 kb *Eco*RI-*Bam*HI fragment cloned in pDBE (Figs 2 and 5). Since plasmids pDBE, pAKC, pBKE and pBKE2 were lost with variable frequencies when PT23 cells were cultured in non-selective media, their respective inserts may not contain all of the information required for stable maintenance.

Definition of the minimum origin of replication of pPT23A

Different deletion derivatives of pAori1 and pAori2 were electroporated to strain PT23 to test their ability to replicate (Fig. 6). Deletions of as little as 10 nucleotides from the left border in pAori1 (e.g., p1D22) completely abolished its replicative capacity. On the other hand, only 0.6 to 0.7 kb of the 1.6 kb *Eco*RI fragment were essential for replication since plasmid p2D24, but not p2D914, could autonomously replicate and

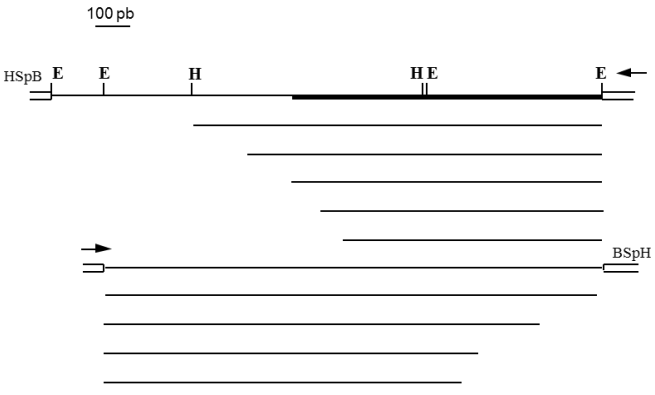

Fig. 6. Definition of the minimal origin of replication of pPT23A. Deletion derivatives of pAori2 and pAori1 were obtained by digestion with *Bam*HI-*Sph*I and treatment with exonuclease III, and are shown below each of these plasmids. Representative restriction sites are shown only in pAori2. The thick line in pAori2 shows the extent of the minimal origin of replication defined by pAori1 and p2D24. Open boxes represent pK184 DNA, and the arrows show the direction of transcription from the *lac* promoter in the vector. Replication: (+) can replicate in *Pst* PT23 and evict pPT23A, (-) does not replicate in *Pst* PT23; results are average of at least four independent electroporations. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Sp, *Sph*I.

evict pPT23A. Therefore, it was determined that the minimal region essential for replication of pPT23A was localized in a *c*. 1.6 kb fragment, defined by pAori1 and p2D24. Consistently, 100 to 1000 times more kanamycin-resistant colonies were recovered from PT23 electroporated with pAori2, p2D23, p2D511, or p2D24 than with pAori1 or pAKC. This suggested that a higher level of transcription of the 0.9 kb *Eco*RI fragment (owing to the *lac* promoter in the vector) could be increasing the stability of the plasmid, the capacity to evict pPT23A from PT23, or both.

Identification of the par region from pPT23A

A 1.6 kb *Eco*RI fragment from pPT23A (Fig. 5C) showed a strong hybridization signal when a 3.0 kb *Hin*dIII fragment from pOSU22 (Fig. 4A) that

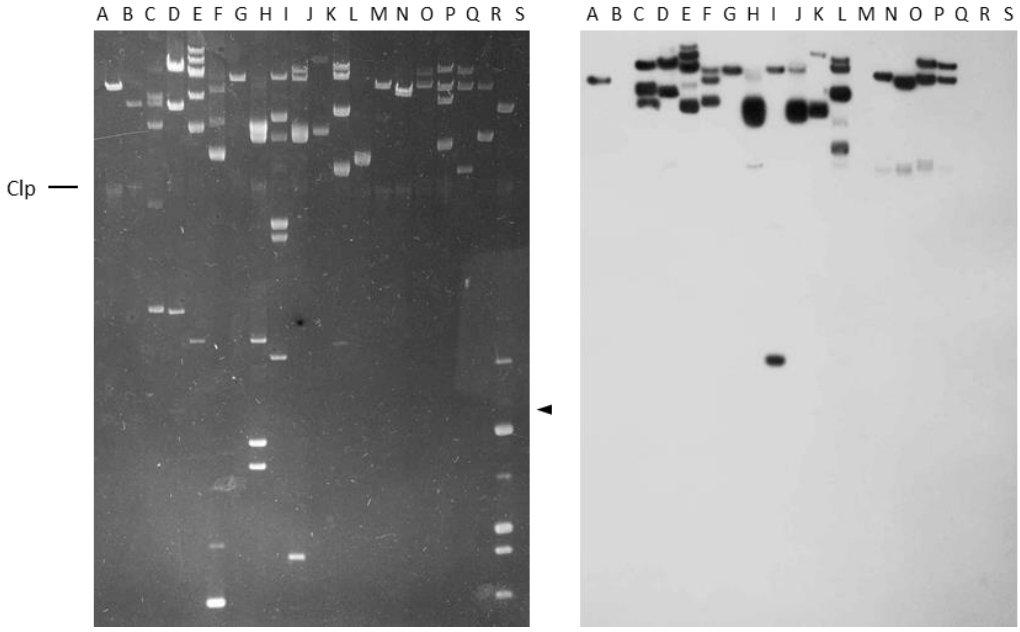

Fig. 7. Conservation of the pPT23A origin of replication in native plasmids from *Pseudomonas syringae* pathovars and *Xanthomonas campestris* pv. vesicatoria.
Left. Native plasmids were isolated by CsCl centrifugation and separated on a 0.6 % agarose gel.
Right. A gel similar to the one displayed in the left was transferred to nylon and hybridized using the 0.9 kb *Eco*RI fragment from pAori2 as a radioactive probe. Sources of DNA are *P. syringae* pathovars apii 1089-5 (A); atropurpurea 4451-75 (B); glycinea races 4 (C), 5 (D), and 6 (E); lachrymans (F); maculicola 0190-2 (G) and 4326 (H); mori 0782-30 (I); morsprunorum 0782-28 (J); phaseolicola 3121 (K); savastanoi 0485-9 (L); syringae PS61 (M); tomato B120 (N), DC3000 (O), PT23 (P) and PT30 (Q); *X. campestris* pv. vesicatoria 82BR (R), and *E*. *coli* V517 (S). The fifth and sixth bans from the top in lane F in panel 1 and fourth band from the top in lane L in panel 2 appeared spontaneously in DNA solutions stored for more than one month at 4°C. Clp indicates the position of chromosome and/or linear plasmid DNA. In lane H a closed circular plasmid is co-migrating with chromosomal DNA. The sizes of plasmids in lane S are *c*. 36, 4.8, 3.7 (arrowhead), 3.4, 2.6, 2.0, 1.8, and 1.4 kb (Macrina *et al.*, 1978)

contains the *par* region from pOSU900 (Mukhopadhyay *et al.*, 1990) was used as a hybridization probe. By hybridization of the same probe to different restriction fragments from pPT23A (data not shown), we localized this fragment to the 9.2 kb *Kpn*I fragment cloned in pAKC and adjacent to *oriV* (Fig. 5A). Although we have not shown that this putative *par* region is functional in pPT23A, homology with the *par* region from pOSU900 and its location close to pPT23A *oriV* indicate that it may function *in vivo*. Finally, we were not able to detect any homology between the 3.0 kb *Hin*dIII *par* probe or the putative *par* region from plasmid A (1.6 kb *Eco*RI fragment) and plasmids B, C, and D (Fig. 5C, and data not shown). This result suggests that the sequences involved in partitioning and plasmid number control in plasmid B, and perhaps in plasmid D, might have originated from plasmid(s) other than pPT23A.

Conservation of replication sequences from pPT23A

To determine the conservation of *oriV* from pPT23A, the 0.9 kb *Eco*RI fragment from pAori2 and the 0.7 kb *Hin*dIII fragment from p2D24 were used as radioactive probes in Southern blot experiments. The 0.9 kb fragment was homologous to a 6.2 kb *Eco*RI fragment from pPT23B (Fig. 5D) that is partially overlapped by the insert in pBKE (Fig. 2). The 0.7 *Hin*dIII fragment showed strong homology to a 3.4 kb *Eco*RI fragment from pPT23B (Fig. 4E). Using the same probe against different restriction digest of pBKE (data not shown) we determined that this homology is included in a 0.6 kb *Eco*RI-*Eco*RV fragment (Fig. 4A) that overlaps the previously defined origin of replication. The two probes also showed weak homology, only visible in overexposed autoradiograms, with the 7.5 kb *Eco*RI fragment containing *oriV* from pPT23D but no homology with pPT23C (Fig. 5) or total DNA from WABCD1 (data not shown), even after exposing the films for a week.

To check if plasmid repetition is a common situation among phytopathogenic pseudomonads, we analyzed plasmid DNA from *Xanthomonas campestris* pv. vesicatoria 82BR and 17 strains belonging to 11 pathovars of *P. syringae* using the 0.9 kb *Eco*RI fragment from pAori2 as a hybridization probe. All the strains examined contained 1 to 7 plasmids ranging from *c*. 1 to >100 kb (Fig. 7). Apart from *P. s.* pv. atropurpurea, pv. syringae PS61 and *X. campestris* pv. vesicatoria, all the strains contained at least one plasmid with homology to the probe used. Moreover, all the strains that contained more than one plasmid also showed hybridization with the *oriV* probe in more than one plasmid. The same results were obtained using the 0.7 kb *Hin*dIII fragment from p2D24 as a probe, except that the fourth plasmid from the top in pv. glycinea race 6 and in pv. savastanoi and the smaller plasmid from pv. mori did not show homology. In view of the fact that approximately equal quantities of plasmid DNA were loaded per lane in gels shown in Fig. 7 and the stringency of the hybridization solution (50% formamide, 5x SSC and 20 mM $NaPO_4$), the high intensity of the hybridizing bands indicates that these plasmids could share very closely related origins of replication.

Discussion

Around 74% (*c*. 61 kb) of the DNA from pPT23B, including the origin of replication, was repeated in pPT23A (Fig. 2). This strongly suggests that these plasmids resulted from the duplication and further rearrangement of a common plasmid ancestor, although both can coexist in the same cell in spite of their relatedness. The existence of repeated DNA, either as transposable elements (Berg and Howe, 1989) or other sequences (Koniine *et al.*, 1990; Martínez *et al.*, 1990; Simpson *et al.*, 1990; Hulton *et al.*, 1991; O'Donell and Williams, 1991), has been described in several species of bacteria, including *P. syringae* (Szabo and Mills, 1984; Coplin, 1989). To our knowledge, however, this is the first report of the stable coexistence of duplicated native plasmids in bacteria. The coexistence of two putatively incompatible plasmids suggests that they must provide a strong selective advantage to the cell. The production of coronatine, because of pPT23A, enables *P. s.* pv. *tomato* to reach and maintain high cell population numbers *in planta* (Bender *et al.*, 1987). However, bacteria devoid of pPT23B produce disease symptoms and reach cell populations that are indistinguishable from the wild type (Lorang *et al.*, 1994; Murillo *et al.*, 1994). It is possible that *avrD* or other genes

unique to pPT23B may increase the fitness of strain PT23, perhaps in another life-cycle stage.

The DNA from pPT23B repeated in pPT23A is organized in three parts that are separated by single-copy DNA (Fig. 2), which amounts to *c*. 17 kb (*c*. 21%). By summing the size of fragments from pPT23A that are not repeated in plasmid pPT23B or in the chromosome (Figs 1 and 3) a rough estimate indicates that at least 16 kb from this plasmid is single-copy DNA. This figure, however, is probably an underestimate of the actual amount since at least some of the genes involved in coronatine synthesis are also in single copy (Bender *et al.*, 1989). However, the fact that they contain repeated DNA (Bender *et al.*, 1991; C. Bender, personal communication) could make them appear to be repeated in hybridization experiments. It is possible that the putative plasmid ancestor underwent only a partial duplication and that the remaining single copy DNA was divided between plasmids A and B. Instead, part or all of the single-copy DNA could have been acquired from the chromosome or other plasmids. In support of the second alternative is the fact that the putative *par* region identified in pPT23B is homologous to plasmid RP4 DNA (Stayton *et al.*, 1991; Murillo *et al.*, 1994).

Repeated elements in the native plasmids of strain PT23 could provide regions of homology that enhance recombination between plasmids, and between plasmids and the chromosome. In this regard, Bender and Cooksey (1987) found that plasmid pPT23D, which confers copper resistance, was transferred by conjugation after forming a cointegrate with pPT23C. This cointegration probably took place through the repeated DNA contained by these two plasmids (see Fig. 2). Also, plasmid pMC7105 from *P. s.* pv. phaseolicola contains three different repeated sequences (Szabo and Mills, 1984). One of these sequences, designated as RS-II, is present in several copies and participates through homologous recombination in the integration and excision of pMC7105 in the chromosome. Another repeated sequence, RS-I, has been shown to be an insertion sequence (IS*801*; Romantschuk *et al.*, 1991) that is present in several *P. syringae* pathovars (Romantschuk *et al.*, 1990). Plasmid B also included three DNA fragments (repeated in the chromosome) that could be transposable elements. One of these fragments (*c*. 0.6 kb) is located close to *oriV* and part of it shows 94.1% nucleotide identity with IS*801* (Figs 2 and 4). Since IS*801* is 1.5 kb long, pPT23B probably contains only a portion of a previous copy of this element that was partially deleted during a reorganization event. It has been reported that insertion sequences similar to IS*801* could be involved in plasmid rearrangements in *P. s.* pv. tomato (Coplin, 1989). Another repeated region, contained in a 9.6 kb *Eco*RI-*Pac*I fragment (Fig. 2), is also present in the other three native plasmids and in the chromosome. This suggests that this putative mobile element might have a high frequency of transposition.

Since they strongly hybridized to each other (Fig. 5), the origins of replication from pPT23A and pPT23B must have suffered only minor modifications during the duplication process to allow for their stable coexistence. Additionally, plasmids pBKE and pBKE2, which contain *oriV* from pPT23B, were generally compatible with pPT23B and only occasionally caused its eviction after repeated subculturing in media with kanamycin. Although we cannot entirely discard the possibility that pPT23B may contain another functional origin of replication, these results suggest that the region(s) involved in incompatibility in plasmid B may have evolved in a manner that facilitates its coexistence with related plasmids in the same cell. Other sequences involved in the maintenance of plasmids A and B do not seem to have been conserved. In this respect, a fragment containing the putative *par* region from plasmid A did not show hybridization with any of the other native plasmids (Fig. 5). The adjacent location of the putative *par* region and *oriV* in pPT23A resembles the situation in pOSU900 (Fig. 5) (Mukhopadhyay *et al.*, 1990), and suggests that gene organization in pPT23A might be closer to the organization of the putative plasmid ancestor that produced plasmids A and B. Additionally, the putative *par* region from pPT23B is also in single copy (Fig. 2) and shows homology to the *par* region of plasmid RP4 (Stayton *et al.*, 1991; Murillo *et al.*, 1994), supporting the idea that part of the replication DNA was incorporated into pPT23B from an incoming plasmid.

Based on DNA hybridization data of plasmid profile gels, the origin of replication from pPT23A is well conserved among 14 strains belonging to 9 pathovars of *P. syringae*. Since several strains showed more than one hybridizing plasmid it can

be argued that the homology observed could be due to some other repeated sequence, like a transposable element, that is located close to *oriV*. However, two different and non-cross-hybridizing DNA probes spanning the minimum *oriV* from pPT23A showed a similar pattern of hybridization with plasmid profile gels (Fig. 7 and data not shown). The results strongly support the idea that the sequences involved in replication are indeed repeated in these native plasmids and therefore that they originated from a common plasmid ancestor. The existence of more than two hybridizing plasmids of different sizes in some of the strains examined (e.g., four in pv. glycinea R4 and pv. savastanoi and five in pv. glycinea R6) indicates that the duplication and reorganization of plasmids, including deletions and probably acquisition of new DNA, might occur with a high frequency and could be the responsible for the existence of large numbers of native plasmids in phytopathogenic pseudomonads (Coplin, 1989). Since we examined only one strain of pathovars atropurpurea and syringae, it would be necessary to examine more strains in the future to determine the degree of conservation of plasmid DNA sequences in these pathovars. The availability of highly conserved origins of replication from *P. syringae* could help in the future to obtain strains cured of native plasmids. The origins of replication cloned in this work could serve to evict native plasmids by incompatibility in other *P. syringae* strains or to identify other *oriV* by hybridization. Cloning of the identified *oriV* and its reintroduction in the parent strain could lead, in one step, to curing of the native plasmid from which it originated. Undoubtedly, further study of the mechanisms underlying native plasmid duplication, their contribution to genetic flux in the bacterial host as well as its occurrence in other bacterial species will shed light on our understanding of mechanisms for genome evolution.

Experimental procedures

Strains, media and plasmids

Escherichia coli DH5α (Gibco BRL) was used for cloning procedures and S17.1 (Simon *et al.*, 1983) for conjugation. *E. coli* V517 (Macrina *et al.*, 1978) and *P. syringae* pathovars syringae PS51 and PS61 (Bender and Cooksey, 1986); tomato PT23, PT30 (Bender and Cooksey, 1986), PT23.2 (Bender and Cooksey, 1987), B120; apii 1089-5; maculicola 0190-2; mori 0782-30; morsprunorum 0782-28, and savastanoi 0485-9 were obtained from D. Cooksey (U. C. Riverside). *P. s.* pv. atropurpurea 4451-75 was obtained from S. Roberts (U. C. Riverside), pv. phaseolicola 3121 from N. J. Panopoulos (U. C. Berkeley) and pv. maculicola 4326 from K. Davis (Ohio State University). *X. campestris* pv. vesicatoria 82BR was received from B. Staskawicz (U. C. Berkeley). *P. s.* pv. tomato WB, WABC1, WABD, WABCD1, HWBC (Murillo *et al.*, 1994), WABCD2 (this work), and DC3000 (Cuppels, 1986); pv. lachrymans, pv. glycinea races 4, 6 (Kobayashi *et al.*, 1990) and 5, were maintained in this laboratory. Strain WABCD2 is a PT23 derivative cured of the four native plasmids and was obtained by curing plasmids pPT23A and pPT23D from strain HWBC by electroporation with pAori2 and pDBE (this work). These latter plasmids were then segregated from WABCD2 after several transfers in non-selective media. *E. coli* was cultured in LB media (Sambrook *et al.*, 1989) at 37°C. *Pseudomonas* and *Xanthomonas* strains were grown at 28°C in KMB (King *et al.*, 1954) or MGY (Bender and Cooksey, 1986) media. When appropriate, media were supplemented with antibacterial compounds at the following concentrations (in μg ml^{-1}): ampicillin, 50; kanamycin (Km), 50; cupric sulfate, 200 (0.8 mM); streptomycin, 25; tetracycline, 15 or 45 when used in combination with cupric sulfate in MGY plates.

Plasmids pUC118/119 (Vieria and Messing, 1987), pUC128/129, pRK415 (Keen *et al.*, 1988), and pMTL24 (Chambers *et al.*, 1988) were used for cloning purposes. Plasmid pK184 (Km^R) (Jobling and Holmes, 1990), which does not replicate in *Pseudomonas*, was utilized for testing the functionality of putative origins of replication. Plasmids pPT23B(*avrD*::Tn*5*) (Murillo *et al.*, 1994) and pPT23A::Km (this work) were purified from cultures of *P. syringae* pv. syringae PS51 transformants and pPT23C and pPT23D were extracted from strains WABD and WABC1 (Murillo *et al.*, 1994), respectively. These plasmids were used as sources for the cloning of restriction fragments for testing origins of replication.

Construction of pPT23A::Km

Total plasmid DNA from strain WB (cured of plasmid B) (Murillo *et al.*, 1994) was digested with *Bam*HI and a *c*. 5.2 kb fragment was cloned into the same site of pUC118, resulting in pA52. The 5.2 kb fragment was not present in *Bam*HI digests of total plasmid DNA from a strain cured of plasmids A and B and it was accordingly assumed to have originated from pPT23A. This DNA fragment also gave an identical restriction pattern to a previously cloned 5.2 kb *Bam*HI fragment from pPT23A occurring in the coronatine biosynthetic region (Bender *et al.*, 1989). The Km^R cassette from pMKm (Murillo *et al.*, 1994) was cloned as a *Bam*HI fragment into the unique *Bgl*II site occurring approximately in the middle of the insert of pA52. The resulting construct was digested with *Bam*HI and the large fragment, containing the 5.2 kb fragment plus the Km^R gene, was cloned into the same site of pRK415, resulting in pAKA1. Strain 23KA was then constructed by conjugating pAKA1 into PT23 and homogenotizing the Km^R marker into pPT23A. Total plasmid DNA extracted from 23KA was electroporated into *P. s.* pv syringae PS51. One of the resulting Km^R clones contained a plasmid with the same overall restriction pattern as pPT23A and was retained for further research and designated pPT23A::Km.

Genetic and molecular biological techniques

Plasmids were introduced into *Pseudomonas* by electroporation or conjugation with *E*. *coli* S17.1 as described (Keen *et al.*, 1992). Standard molecular biology techniques (Sambrook *et al.*, 1989) were followed in general. Plasmid DNA was isolated according to a modified alkaline lysis method and plasmid profile gels were made by separating the plasmids by electrophoresis on 0.6% agarose gels at *c*. 3.8 V cm^{-1} at 4°C (Murillo *et al.*, 1994). Plasmid size was estimated by comparison with the migration distance of plasmids from *E. coli* V517 (Macrina *et al.*, 1978). Native plasmid DNA purified by CsCl centrifugation from some of the *P. syringae* pathovars, such as lachrymans and savastanoi, degraded or generated lower molecular weight forms upon storage at 4°C. Consequently, native plasmids DNA solutions were generally kept at -20°C for long-term storage. Subcloning of DNA was done essentially as described (Crouse *et al.*, 1983). Exonuclease III deletions of plasmids pAori1 and pAori2 digested with *Bam*HI and *Sph*I were performed using the Erase-a-Base system (Promega). Deletion products were analysed on 1.4% agarose gels and selected clones were sequenced to map the exact end-points. Fragments used as radioactive probes were separately subcloned, excised from the vectors and electrophoresed in low-melting point agarose gels. The DNA bands to be used as probes were excised from the gel in a minimal quantity of agarose and melted for 10 min at 65°C, then 15 µl of the slurry were labelled with [α-^{32}P]-ATP or [α-^{32}P]-CTP (3000 Ci $mmol^{-1}$; ICN Biomedicals, Inc.) by random primer essentially as described (Sambrook *et al.*, 1989). After digestion with restriction enzymes and separation in agarose gels, DNA was transferred to nylon membranes (MSI, Micron Separations, Inc.) and hybridized overnight at 42°C in a solution containing 50% formamide, 5x SSC (1x SSC is 0.15 M NaCl, 0.015 M sodium citrate), 20 mM $NaPO_4$ pH 6.7, 1x Denhardt's solution, and 0.1 mg of herring sperm DNA ml^{-1} (Kobayashi *et al.*, 1990). After hybridization blots were washed twice (15 min each) at 42˚C in 2x SSC-0.1% SDS and exposed damp to Kodak Ortho-G film for 3 to 48 h at -70°C with intensifying screens. When necessary, blots were used once again after removing the first probe according to the manufacturer's recommendations.

Acknowledgements

J.M. was supported, in part, by grants 93-258 (DGICYT) and PF90-6984578 from the Ministerio de Educación y Ciencia, Spain. This work was supported by National Science Foundation Grant MCB-9005388-02. We thank D. Cooksey, K. Davis, S. Roberts, N. J. Panopoulos, and B. Staskawicz for providing strains, and to D. Mills and C. Bender for pOSU22. We are also grateful to C. Bender, D. Cooksey and R. Rodriguez for helpful comments, and to C. Boyd for excellent technical assistance.

References

Bavage, A.D., Vivian, A., Atherton, G.T., Taylor, J.D., and Malik, A.N. (1991) Molecular genetics of *Pseudomonas syringae* pathovar *pisi*: plasmid involvement in cultivar-specific incompatibility. *J Gen Microbiol* **137**: 2231-2239.

Bender, C., and Cooksey, D. (1986) Indigenous plasmids in *Pseudomonas syringae* pv. *tomato*: conjugative transfer and role in copper resistance. *J Bacteriol* **165**: 534-541.

Bender, C.L., and Cooksey, D.A. (1987) Molecular cloning of copper resistance genes from *Pseudomonas syringae* pv. *tomato*. *J Bacteriol* **169**: 470-474.

Bender, C.L., Stone, H.E., Sims, J.J., and Cooksey, D.A. (1987) Reduced pathogen fitness of *Pseudomonas syringae* pv. *tomato* Tn*5* mutants defective in coronatine production. *Physiol Mol Plant Pathol* **30**: 273-283.

Bender, C.L., Malvick, D.K., and Mitchell, R.E. (1989) Plasmid-mediated production of the phytotoxin coronatine in *Pseudomonas syringae* pv. *tomato*. *J Bacteriol* **171**: 807-812.

Bender, C.L., Young, S.A., and Mitchell, R.E. (1991) Conservation of plasmid DNA sequences in coronatine-producing pathovars of *Pseudomonas syringae*. *Appl Environ Microbiol* **57**: 993-999.

Berg, D.E., and Howe, M.M., (eds) (1989) *Mobile DNA.* Washington, D. C.: American Society for Microbiology.

Burr, T.J., Norelli, J.L., Katz, B., Wilcox, W.F., and Hoying, S.A. (1988) Streptomycin resistance of *Pseudomonas syringae* pv. *papulans* in apple orchards and its association with a conjugative plasmid. *Phytopathology* **78**: 410-413.

Comai, L., and Kosuge, T. (1980) Involvement of plasmid deoxyribonucleic acid in indoleacetic acid synthesis in *Pseudomonas savastanoi*. *J Bacteriol* **143**: 950-957.

Cooksey, D.A. (1987) Characterization of a copper resistance plasmid conserved in copper-resistant strains of *Pseudomonas syringae* pv. *tomato*. *Appl Environ Microbiol* **53**: 454-456.

Cooksey, D.A. (1990) Plasmid-determined copper resistance in *Pseudomonas syringae* from impatiens. *Appl Environ Microbiol* **56**: 13-16.

Coplin, D.L. (1989) Plasmids and their role in the evolution of plant pathogenic bacteria. *Annu Rev Phytopathol* **27**: 187-212.

Crouse, G.F., Frischauf, A., and Lehrach, H. (1983) An integrated and simplified approach for cloning into plasmids and single-stranded phages. *Methods Enzymol* **101**: 78-89.

Cuppels, D.A. (1986) Generation and characterization of Tn*5* insertion mutations in *Pseudomonas syringae* pv. *tomato*. *Appl Environ Microbiol* **51**: 323-327.

Chambers, S.P., Prior, S.E., Barstow, D.A., and Minton, N.P. (1988) The pMTL nic-cloning vectors. I. Improved pUC polylinker regions to facilitate the use of sonicated DNA for nucleotide sequencing. *Gene* **68**: 139-149.

Hulton, C.S.J., Higgins, C.F., and Sharp, P.M. (1991) ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli*, *Samonella typhimurium* and other enteric bacteria. *Mol Microbiol* **5**: 825-834.

Jobling, M.G., and Holmes, R.K. (1990) Construction of vectors with the p15a replicon, kanamycin resistance, inducible *lacZ*α and pUC18 or pUC19 multiple cloning sites. *Nucleic Acids Res* **18**: 5315-5316.

Keen, N.T., Tamaki, S., Kobayashi, D., and Trollinger, D. (1988) Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. *Gene* **70**: 191-197.

Keen, N.T., Shen, H., and Cooksey, D.A. (1992) Introduction of DNA into plant pathogenic bacteria. In *Molecular Plant Pathology. A Practical Approach*. Vol. I. Gurr, S.J., McPherson, M.J. and Bowles, D.J. (eds). Oxford: IRL Press, pp. 45-50.

King, E.O., Ward, N.K., and Raney, D.E. (1954) Two simple media for the demonstration of pyocyanin and fluorescein. *J Lab Clin Med* **44**: 301-307.

Kobayashi, D.Y., Tamaki, S.J., and Keen, N.T. (1990) Molecular characterization of avirulence gene D from *Pseudomonas syringae* pv. *tomato*. *Mol Plant-Microbe Interact* **3**: 94-102.

Koniine, Y., Adachi, T., Inokuchi, H., and Ozeki, H. (1990) Genomic organization and physical mapping of the transfer RNA genes in *Escherichia coli*. *J Mol Biol* **212**: 579-598.

Lorang, J.M., Shen, H., Kobayashi, D., Cooksey, D., and Keen, N.T. (1994) *AvrA* and *avrE* in *Pseudomonas syringae* pv. *tomato* PT23 play a role in virulence on tomato plants. *Mol Plant-Microbe Interact* **7**: 508-515.

Macrina, F.L., Kopecko, D.J., Jones, K.R., Ayers, D.J., and McCowen, S.M. (1978) A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. *Plasmid* **1**: 417-420.

Martínez, E., Romero, D., and Palacios, R. (1990) The *Rhizobium* genome. *Crit Rev Plant Sci* **9**: 59-93.

Mills, S.D., Jasalavich, C.A., and Cooksey, D.A. (1993) A two-component regulatory system required for copper-inducible expression of the copper resistance operon of *Pseudomonas syringae*. *J Bacteriol* **175**: 1656-1664.

Mukhopadhyay, P., Mukhopadhyay, M., and Mills, D. (1990) Construction of a stable shuttle vector for high-frequency transformation in *Pseudomonas syringae* pv. *syringae*. *J Bacteriol* **172**: 477-480.

Murillo, J., Shen, H., Gerhold, D., Sharma, A.K., Cooksey, D.A., and Keen, N.T. (1994) Characterization of pPT23B, the plasmid involved in syringolide production by *Pseudomonas syringae* pv. *tomato* PT23. *Plasmid* **31**: 275-287.

O'Donell, K.J., and Williams, P.A. (1991) Duplication of both *xyl* operons on TOL plasmid pWW15. *J Gen Microbiol* **137**: 2831-2838.

Romantschuk, M., Zhao, Y., McCluskey, K., Williams, J., and Mills, D. (1990) Repeated sequences in *Pseudomonas syringae* pv. *phaseolicola*; distribution and possible function as insertion sequences. *Symbiosis* **8**: 21-31.

Romantschuk, M., Richter, G.Y., Mukhopadhyay, P., and Mills, D. (1991) IS*801*, an insertion sequence element isolated from *Pseudomonas syringae* pathovar *phaseolicola*. *Mol Microbiol* **5**: 617-622.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Shaw, P.D. (1987) Plasmid ecology. In *Plant-Microbe interactions: molecular and genetic perspectives*. Vol. 2. Kosuge, T. and Nester, E.W. (eds). New York: Macmillan, pp. 3-39.

Simon, R., Priefer, U., and Pühler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Bio/Technology* **1**: 784-790.

Simpson, W.J., Garon, C.F., and Schwan, T.G. (1990) *Borrelia burgdorferi* contains repeated DNA sequences that are species specific and plasmid associated. *Infect Immun* **58**: 847-853.

Stayton, M., Hanekamp, T., Kobayashi, D., Hayes, S., Tamaki, S., and Keen, N. (1991) A putative DNA invertase found adjacent to the avirulence gene D in *Pseudomonas syringae* pv. *tomato*. *Phytopathology* **81**: 1145 (abstr.).

Sundin, G.W., Jones, A.L., and Fulbright, D.W. (1989) Copper resistance in *Pseudomonas syringae* pv. *syringae* from cherry orchards and its associated transfer in vitro and in planta with a plasmid. *Phytopathology* **79**: 861-865.

Szabo, L.J., and Mills, D. (1984) Integration and excision of pMC7105 in *Pseudomonas syringae* pv. *phaseolicola*: involvement of repetitive sequences. *J Bacteriol* **157**: 821-827.

Tamaki, S., Dahlbeck, D., Staskawicz, B., and Keen, N.T. (1988) Characterization and expression of two avirulence genes cloned from *Pseudomonas syringae* pv. *glycinea*. *J Bacteriol* **170**: 4846-4854.

Vieria, J., and Messing, J. (1987) Production of single stranded plasmid DNA. *Methods Enzymol* **153**: 3-11.

Zambryski, P., Tempé, J., and Schell, J. (1989) Transfer and function of T-DNA genes from *Agrobacterium* Ti and Ri plasmids in plants. *Cell* **56**: 193-201.