Relation of Plasmid DNA to Indoleacetic Acid Production in Different Strains of *Pseudomonas syringae* pv. *savastanoi*

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(Received 25 November 1981; revised 25 February 1982)

Pseudomonas syringae pv. *savastanoi* is a bacterial pathogen of olive, oleander and privet, on which it induces tumorous overgrowths called knots or galls. Production of indoleacetic acid (IAA), a necessary factor for gall induction, is coded in oleander strain EW2009 by a 52 kb plasmid called pIAA1. To determine if this trait were commonly plasmid-borne in other strains of this pathogen, Iaa⁻ mutants were isolated from twelve different strains by selection for α -methyltryptophan resistance. Each mutant was compared in its plasmid pattern with the parent wild-type. In oleander strains PB205 and PB213 loss of IAA production was associated with loss of, or large deletions in, a 73 kb plasmid called pIAA2. In the remaining strains from olive and privet loss of IAA production was not accompanied by the loss of any plasmid. To prove that pIAA2 coded for the genes for IAA production, a radioactive probe containing the first gene of the IAA pathway (*iaaM*) was hybridized to the plasmid DNA of the twelve strains. Only pIAA2 showed homology. We concluded that of all strains studied only PB205 and PB213 harboured a plasmid coding for IAA production, while in the remaining strains the IAA genes were located on the chromosome.

INTRODUCTION

In plant-associated bacteria, plasmids carrying genes necessary for successful symbiosis or parasitism have been described in *Rhizobium* (Hirsch *et al.*, 1980), *Agrobacterium* (Watson *et al.*, 1975) and in the 'Oleander Knot' agent (Comai & Kosuge, 1980). Oleander Knot, caused by *Pseudomonas syringae* pv. *savastanoi* (Smith) Young, (henceforth referred to as pv. *savastanoi*) is a disease characterized by the development of galls on different organs of oleander plants. Galls are the result of hypertrophic and hyperplastic growth of the cells surrounding the infection site. A similar disease is induced on olive and privet. In contrast to crown gall, there is no evidence suggesting that plant cells infected by pv. *savastanoi* are transformed (E. E. Wilson, & T. Kosuge, unpublished results). Rather, knot formation appears to depend on bacterial production of phytohormones. Pathovar *savastanoi* produces both indoleacetic acid (IAA) (Beltra, 1959) and cytokinins (Surico *et al.*, 1975) in culture. A determining role for IAA in gall induction by oleander strains has been demonstrated (Smidt & Kosuge, 1978; L. Comai & T. Kosuge, unpublished results).

IAA is synthesized in two enzymic steps from L-tryptophan: conversion to indoleacetamide catalysed by tryptophan 2-monooxygenase (EC 1.13.12.3), and hydrolysis of indoleacetamide to IAA by indoleacetamide hydrolase (Kosuge *et al.*, 1966). The genetic loci for these two enzymes are designated *iaaM* and *iaaH* respectively and, in strain EW2009 (previously referred to as 2009), they are borne on a 52 kb plasmid called pIAA1 (Comai &

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Kosuge, 1980). These loci have been mapped on pIAA1 by molecular cloning and characterization of insertion mutations (Comai & Kosuge, 1982, and unpublished results).

The aim of this study was to determine if plasmid borne genes for IAA production were a characteristic common among *pv. savastanoi* strains. The pathogen is endemic in the Mediterranean region; thus, we chose to characterize a number of wild-type strains from southern Italy, which had been isolated from diseased olive, oleander and privet. According to the host from which they were isolated, these strains can usually be grouped in three subdivisions distinguishable on the basis of host range and other physiological characters (Bottalico & Ercolani, 1971; Wilson *et al.*, 1972).

Strains in which the IAA genes were plasmid borne were determined as follows: (i) isolation of spontaneous *Iaa*⁻ mutants (IAA-non-producers) from each strain; (ii) analysis of their plasmids to determine if loss of IAA production were associated with loss or rearrangement of extrachromosomal DNA; (iii) hybridization of a probe plasmid containing the cloned *iaaM* locus to the plasmid DNA of each strain by Southern blot hybridization (Southern, 1975).

METHODS

Bacterial isolates. Fourteen isolates of *pv. savastanoi* were used. Eleven were from the collection of the Istituto di Patologia Vegetale at the University of Bari, Italy and had been isolated between 1965 and 1976 from knots of oleander (PB204, PB205, PB213), olive (PB202, PB207, PB230, PB231) and privet (PB209, PB215, PB217, PB218). The remaining three used as references or for some preliminary experiments, were olive strains EW1006 and TK1050 and oleander strain EW2009 from the collection of the Department of Plant Pathology, University of California, Davis. Plasmids from olive strain PB207 were used as molecular weight standards in Fig. 1. This strain, together with others, differs from traditional *pv. savastanoi* isolates in the amount of IAA produced, pathogenicity and physiological characters. Thus, its characterization was not included in this work and will be the object of further studies. Bacteria were purified on solid medium containing (g l⁻¹): beef extract, 3; peptone, 5; sucrose, 50; agar, 15; and were stored at 4 °C on a similar medium in which glycerol (20 g) was substituted for sucrose.

Plasmid isolation, restriction endonuclease digestion and electrophoresis. The protocols of plasmid isolation, restriction endonuclease digestion, and electrophoresis have been described (Comai & Kosuge, 1982).

Isolation of *Iaa*⁻ mutants. To isolate spontaneous *Iaa*⁻ mutants bacteria were grown in glucose/peptone broth (Comai & Kosuge, 1982) to late exponential phase, washed in 10 mM-saline, and serially diluted on minimal medium A (Miller, 1972) to which α -methyltryptophan (150 μ g ml⁻¹) had been added after autoclaving. The plates were incubated at 23 °C for 5 d at which time two colonies per strain were picked, purified twice and stored on agar slants at 4 °C for later characterization.

Determination of IAA production. This followed the procedure of Comai & Kosuge (1980).

In vitro labelling of DNA and Southern blot hybridization. DNA was labelled *in vitro* by 'nick translation' with *Escherichia coli* DNA polymerase I according to Rigby *et al.* (1977). Southern blot hybridization (Southern, 1975) followed the protocol of Davis *et al.* (1980) and used nitrocellulose filters (BA85) from Schleicher and Schuell, Keene, New Hampshire. Hybridization was carried out in 50% (v/v) formamide at 42 °C.

Biochemicals. Agarose HGT was purchased from Marine Colloids, Rockland, Maine; restriction endonucleases from Miles Biochemicals, Eckhart, Indiana, and New England Biolabs, Beverly, Massachusetts; dNTPs and DNAase I from Boehringer Mannheim; *Escherichia coli* DNA polymerase I from Bethesda Research Lab, Bethesda, Maryland; all other chemicals from Sigma and Calbiochem-Behring.

RESULTS

Plasmids in *pv. savastanoi* strains. Plasmid DNA was isolated from each strain of *pv. savastanoi* and characterized by agarose gel electrophoresis. Figure. 1(a) shows the electrophoretic pattern of plasmid DNA. Four to seven plasmid species were resolved in each strain ranging in size from 7 to 100 kb. All Italian isolates were conspicuous for the number of plasmid species when compared with Californian strains EW1006 and EW2009 which are representative of many Californian isolates.

Relation between α -methyltryptophan resistance and IAA production. To isolate *Iaa*⁻ mutants we used selection for α -methyltryptophan resistance as described by Smidt &

Fig. 1. (a) Agarose gel electrophoresis of plasmid DNA purified from *pv. savastanoi* strains. Olive strains: A, EW1006; B, PB207; C, PB202; D, PB230; E, PB231. Oleander strains: F, PB205; G, PB204; H, PB213; I, EW2009. Privet strains: J, PB209; K, PB215; L, PB217; M, PB218. On the right are marked the estimated sizes (kb) of the plasmids from PB207. These were calculated from their electrophoretic mobilities in relation to the following standards: RSF1010 (8.3 kb), RSF1010::Tn1 (13.3 kb), pLUC1::Tn1 (16 kb) and RK2 (57 kb).

(b) Plasmid DNA from the gel in Fig. 1 (a) was transferred to a nitrocellulose filter after breakage by depurination in 0.25 M-HCl and hybridized to ^{32} P-labelled plasmid pLUC1 using the conditions specified in Methods. Lane designations as in (a). Lanes A to D are not shown since no components hybridized with the probe.

Kosuge (1978). To test the feasibility of this technique for the isolation of *Iaa*⁻ mutants from different strains, we tested fifty α -methyltryptophan resistant mutants obtained from each of strains EW2009, EW2015 and TK1050. All those derived from strains EW2009 and TK1050 were *Iaa*⁻ mutants; derivatives of EW2009 all had lost plasmid pIAA1, while no changes were seen in the plasmids of *Iaa*⁻ strains derived from TK1050. In the latter strain, genes for IAA synthesis are chromosomal (L. Comai, unpublished results). In contrast to derivatives of strains EW2009 and TK1050, most of the α -methyltryptophan resistant mutants of strain EW2015 were still IAA producers. EW2009 and EW2015 are closely related oleander strains since they harbour identical plasmids. However, EW2015 is non-fluorescent on glucose/peptone medium. The production of a water-soluble fluorescent pigment is typical of members of the *P. syringae* group (Leisinger & Margraff, 1979).

Further, we tested ten *Iaa*⁻ mutants, which had been isolated other than by α -methyltryptophan resistance (Comai & Kosuge, 1980) and all these were α -methyltryptophan resistant. In addition, when plasmid pIAA1 was reintroduced by transformation, sensitivity to α -methyltryptophan was regained. We concluded that in strain EW2009, and other fluorescing strains, loss of IAA production always resulted in increased resistance to α -methyltryptophan.

Isolation of *Iaa*⁻ mutants. α -Methyltryptophan resistant mutants were isolated from each of the Italian strains, with the exception of PB204 which was already resistant to this analogue. Mutants appeared at frequencies ranging from 10^{-5} to 10^{-6} . When tested for IAA production all the mutants and PB204 were negative. When inoculated in olive plants, *Iaa*⁻ mutants did not induce galls, while the wild-types, with the exception of PB204, produced the knot disease (G. Surico & L. Comai, unpublished observations).

Fig. 2. Agarose gel electrophoretic analysis of plasmid DNA from wild-type strain PB213 (A) and its *Iaa*⁻ derivatives PB213-1 (B) and PB213-2 (C).

Analysis of plasmid DNA in Iaa⁻ *mutants.* Plasmid DNA was isolated from each of two mutants and compared with that of the wild-type by agarose gel electrophoresis. Of the eleven strains studied, two, PB205 and PB213, lost a plasmid, or part of it, concurrently with IAA production. Figure 2 shows the plasmid pattern of wild-type strain PB213, and of two *Iaa*⁻ derivatives. Both lacked the largest plasmid, 73 kb. One *Iaa*⁻ mutant, however, showed a new plasmid species, 50 kb. Identical events were observed in *Iaa*⁻ mutants derived from PB205. Since we postulated that the 73 kb plasmid coded for IAA production, we called it pIAA2.

No changes from the respective wild types were detected in the plasmids of the *Iaa*⁻ mutants isolated from the remaining strains.

Identification of the plasmid coding IAA production by hybridization. To confirm the results from plasmid analysis we determined which plasmids in the eleven Italian and two Californian strains contained sequences homologous to the *iaaM* locus. *In vitro* ³²P-labelled plasmid pLUC1, which contains fragment *EcoRI*-M of pIAA1 coding *iaaM*, was hybridized to the plasmids shown in Fig. 1(a) by Southern blot hybridization. Strains PB205, PB213, and, as expected, EW2009 exhibited pLUC1 homologous sequences (Fig. 1b). The difference in bound radioactivity between pIAA1 and pIAA2 is due to the different amount of plasmid DNA. Two additional bands visible in the tracks of PB205 and EW2009 do not correspond to any of the other plasmids and may represent linear forms of pIAA1 and pIAA2. By longer exposures a similar band was also detected in the track of PB213.

Restriction endonuclease analysis of plasmids in oleander isolates. The results with strains PB205 and PB213 indicate that plasmid pIAA2 bears the genes coding for IAA production. A plasmid of similar size was present in the IAA-non-producing strain PB204, but did not hybridize to the probe plasmid pLUC1. To determine if pIAA2 had any relationship with pIAA1 and with the cryptic plasmid in strain PB204, plasmid DNA from strain PB213, from its two *Iaa*⁻ derivatives, PB213-1 and PB213-2 (Fig. 2), and from strains PB204 and

Fig. 3

Fig. 4

Fig. 3. Restriction endonuclease *Eco*RI-generated fragments of plasmid DNA from strain EW2009 (A) and PB213 (C) were separated by agarose gel electrophoresis and transferred to a nitrocellulose filter (B and D, respectively) and hybridized to ^{32} P-labelled plasmid pLUC1 using the conditions specified in Methods.

Fig. 4. Agarose gel electrophoresis of *Eco*RI restriction endonuclease-treated plasmid DNA from the wild-type, *Iaa*⁺, strain EW2009 (A), *Iaa*⁻ mutants PB213-2 (B) and PB213-1 (C), wild-type, *Iaa*⁺, PB213 (D), and the *Iaa*⁻ strain PB204 (E). The lines on the left of track (A) mark the fragments of DNA belonging to pIAA1 (Comai & Kosuge, 1980); the arrow indicates the position of the fragment which binds to pLUC1.

EW2009 (Fig. 1), was treated with restriction endonuclease *Eco*RI. The resulting fragments were separated by agarose gel electrophoresis and transferred to a nitrocellulose filter for hybridization to pLUC1. It was found that pLUC1 hybridized to the homologous fragment of pIAA1 and to a fragment of PB213 plasmid DNA having the same molecular weight (Fig. 3). That segment was part of pIAA2 since it was missing in the digests of PB213-1 and PB213-2, the two derivatives lacking pIAA2 (Fig. 4). The restriction fragments generated by digestion of pIAA2 can be identified by comparing the patterns of the wild-type, PB213 with those of the pIAA2-less derivative PB213-1. PB213-2 contains some of the fragments of pIAA2 and thus harbours a deletion derivative of pIAA2, here called pIAA2 Δ 1. The fragments of pIAA2 constituting pIAA2 Δ 1 can be identified by comparing the three patterns. PB204 harboured a plasmid closely related to pIAA2, as demonstrated by the presence in PB204 of most *Eco*RI fragments belonging to pIAA2, but lacking homology to *Eco*RI-M since no

hybridization with pLUC1 was evident (data not shown); it was called pIAA2 Δ 2 on the assumption that it is a deletion derivative of pIAA2. The segments of pIAA1 formed by *Eco*RI restriction digestion have been previously identified (Comai & Kosuge, 1980) and are marked on Fig. 4. From the *Eco*RI digestion pattern of pIAA2 it can be deduced that most of the homology with pIAA1 is confined to the proximity of the IAA genes, the remainder being apparently unrelated to pIAA1.

DISCUSSION

By the use of combined genetic and physical techniques we have screened a number of *pv. savastanoi* strains for the presence of plasmid DNA bearing determinants for IAA production. Spontaneously occurring *Iaa*⁻ mutants can be isolated from a wild-type population by their increased resistance to the tryptophan analogue α -methyltryptophan. Although the mechanism of resistance is not known, it is probably due to the larger tryptophan pools exhibited by *Iaa*⁻ mutants (M. Smidt & T. Kosuge, unpublished results). The reason this relationship is not observed in the non-fluorescent strain EW2015 is unknown. Using strain EW2009, which harbours pIAA1, we determined that loss of IAA production is always associated with loss of the plasmid or insertional inactivation of the IAA genes (L. Comai & T. Kosuge, unpublished results); also, α -methyltryptophan resistance is associated with all *Iaa*⁻ mutants, since *Iaa*⁻ mutants isolated by other techniques (Comai & Kosuge, 1980) are all resistant to α -methyltryptophan. Point mutations leading to loss of IAA production were never found in strain EW2009. On the basis of these observations we speculate that in any fluorescent strain of *pv. savastanoi* the plasmid coding for IAA production could be identified by its absence or rearrangement in the corresponding *Iaa*⁻ mutants.

Iaa⁻ mutants were isolated from each strain and their plasmid DNA analysed. In two strains, PB205 and PB213, a plasmid was lost concurrently with IAA production. When the plasmids from all strains were tested for sequences homologous to the IAA genes, only the 73 kb plasmid (pIAA2) present in PB205 and PB213, showed homology. The other oleander strain PB204, which had lost the capacity for IAA production during repeated subculture, harboured a plasmid closely related to pIAA2, but which had a deletion in the IAA region. Thus, all virulent oleander strains so far tested bear their IAA genes on a plasmid. All olive and privet strains tested do not, although the possibility that some of these strains harbour a very large plasmid which was not isolated by the technique used, cannot be excluded.

The relationship between pIAA1 and pIAA2 is not clear. Although the regions associated with IAA production are very similar in both plasmids as shown by *Eco*RI endonuclease hydrolysis and blot hybridization, the remainder of the plasmids show different *Eco*RI restriction patterns. This problem is currently being investigated together with the organization of the IAA genes on the chromosome (or large plasmids) of the other strains.

This work is an additional step toward the understanding of the organization of genes for virulence in phytopathogenic pseudomonads. This group of parasitic bacteria conceivably evolved from saprophytic pseudomonads, substituting catabolic versatility for capacity to parasitize plants (Misaghi & Grogan, 1969). Production of toxins, indoleacetic acid and probably cytokinins, contribute to virulence and ultimately to parasitic fitness. The evolution of virulence is a matter of conjecture; however, there is a remarkable similarity between the pathway of catabolism of L-lysine by *Pseudomonas fluorescens* (Takeda & Hayaishi, 1966) and synthesis of IAA from L-tryptophan by *pv. savastanoi* (Kosuge *et al.*, 1966). Whatever the origin of the genes for IAA production among strains of *pv. savastanoi*, there is a considerable diversity as to where these genes are located. This heterogeneity may reflect the nature of these genes. Although *Iaa*⁻ mutants can grow in the host (Smidt & Kosuge, 1978), they cannot stimulate formation of galls, which otherwise could provide an ideal environment for the multiplication of the parasite. Thus, the IAA genes could confer a marked selective

advantage and in this capacity resemble genes for antibiotic resistance. The capacity of these genes to become associated with different replicons would help transmission of an evolutionary advantageous trait among the members of a population. The molecular events leading to the presence of the IAA genes on plasmids and chromosome could be similar to F' and Hfr formation in *E. coli* or to transposition of genetic elements. Studies are now in progress to characterize the system.

Preliminary pathogenicity studies have shown that in olive and privet strains IAA production is necessary for gall development (G. Surico, unpublished results) as has been reported for oleander strains (Smidt & Kosuge, 1978). So far, in isolates from California and southern Italy only strains isolated from oleander harbour a plasmid coding for IAA production. In olive and privet isolates thus far examined, the genes for IAA production are chromosomal.

In summary, a variety and number of plasmid species were observed in strains of *pv. savastanoi*, from southern Italy. In the oleander strains a plasmid coding for IAA production was identified by analysing *iaa*⁻ mutants and by the presence of sequences homologous to the *iaaM* locus. Plasmids in the remaining strains from olive and privet exhibited neither rearrangements upon loss of IAA production, nor hybridization to the probe DNA. The new IAA plasmid, called pIAA2, did not resemble pIAA1 in its *EcoRI* endonuclease fragments except in the region bearing the IAA genes.

Supported in part by Grant 59-2063-1-1-643-0 from the Competitive Research Grants Office of the United States Department of Agriculture, Science and Education Administration.

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