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# Involvement of Plasmid Deoxyribonucleic Acid in Indoleacetic Acid Synthesis in *Pseudomonas savastanoi*

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Olive (or oleander) knot is a plant disease incited by *Pseudomonas savastanoi*. Disease symptoms consist of tumorous outgrowths induced in the plant by bacterial production of indole-3-acetic acid (IAA). Synthesis of IAA occurs by the following reactions: L-typtophan → indoleacetamide → indoleacetic acid, catalyzed by tryptophan 2-monooxygenase and indoleacetamide hydrolase, respectively. Whereas the enzymology of IAA synthesis is well characterized, nothing is known about the genetics of the system. We devised a positive selection for the presence of tryptophan 2-monooxygenase based on its capacity to use as a substrate the toxic tryptophan analogue 5-methyltryptophan. Efficient curing of the bacterium of tryptophan 2-monoxygenase, indoleacetamide hydrolase, and IAA production was obtained by acridine orange treatment. Further, loss of capacity to produce IAA by curing was correlated with loss of a plasmid of 34 × $10^6$ molecular weight. This plasmid, here called pIAA1, when reintroduced into Iaa$^-$ mutants by transformation, restored tryptophan 2-monooxygenase and indoleacetamide hydrolase activities and production of IAA.

*Pseudomonas savastanoi* is a pathogen of olive and oleander plants inducing a disease known as "olive (or oleander) knot." The symptoms consist of tumor-like outgrowths called knots or galls appearing on infected twigs and leaves. Gall formation is a response of the plant to bacterial production of high concentrations of indoleacetic acid (IAA) (17). Synthesis of IAA from L-tryptophan is catalyzed in the pathogen by two enzymes: tryptophan 2-monooxygenase (EC 1.13.12.3 [Trp monooxygenase]), which decarboxylates Trp to produce indoleacetamide, and a hydrolase which cleaves indoleacetamide to ammonia and IAA (10) (Fig. 1).

In pseudomonads, the genetic information coding for degradation of a number of compounds is extrachromosomal (20). Therefore, it was of interest to determine whether enzymes involved in the production of the secondary metabolite IAA by *P. savastanoi* also were encoded by plasmid-borne genes. We present evidence which shows that (i) the capability of producing IAA is lost at high frequency upon treatment of the bacterial cells with acridine orange; (ii) the loss of IAA production is associated with the loss of a plasmid having a molecular weight of 34 × $10^6$; and (iii) the reintroduction by transformation of this plasmid into mutants lacking IAA production restores the capability of synthesizing IAA.

## MATERIALS AND METHODS

**Bacterial strains and plasmid content.** Strains of bacteria used and their plasmid contents are shown in Tables 1 and 2.

**Culture media.** Bacteria were routinely grown in a glucose-peptone (King B) medium (9). Selection of isolates for 5-methyl-Trp resistance was performed on minimal agar medium containing (in grams per liter): $K_2HPO_4$, 0.75; $KH_2PO_4$, 0.95; $MgSO_4 \cdot 7H_2O$, 0.4; glutamine, 5; and glucose, 15. After sterilization by autoclaving, the medium was cooled to 45°C; 5-methyl-Trp in 1 M NaOH was added to a final concentration of 200 μg/ml, and the pH was adjusted to 7.3 with 1 N HCl. Whenever media for streptomycin selection were needed, the antibiotic, in a final concentration of 200 μg/ml, was added to solid King medium B or the minimal agar medium after sterilization.

**Assay for Trp monooxygenase.** For measurements of Trp monooxygenase activity, $O_2$ consumption was determined by polarography in a reaction mixture of 16.6 mM Tris (pH 8.0), 66.6 mM KCl, 10 mM L-Trp, and 0.2 ml of extract in a final volume of 1.4 ml at 25°C (10). In substrate specificity studies, DL-5-methyl-Trp or α-methyl-Trp (20 mM) was substituted for L-Trp. To identify the reaction products, 1 ml of cell extract and 9.0 ml of the above reaction mixture were incubated at 23°C. After 30 min, the reaction mixture was acidified to pH 3 and shaken with an equal volume of ethyl acetate. The ethyl acetate fraction was removed, and the extraction was repeated twice. The ethyl acetate fractions were combined and evaporated under nitrogen. The residue was dissolved in a minimal volume of 95% ethanol. Five microliters of the sample in ethanol was applied to a thin-layer chromatography plate (silica gel 60; E. M. Laboratories, Elmsford, N.Y.), and the chromatographic plate was developed with either *n*-butanol-acetic acid-water (65:20:22 [vol/vol]) or *n*-propanol-methyl acetate-7 N $NH_4OH$ (45:35:20 [vol/vol]). Compounds on the chromatograms were located by spraying the plates with Ehrlich reagent (18).

**Assay for indoleacetamide hydrolase.** For mea-

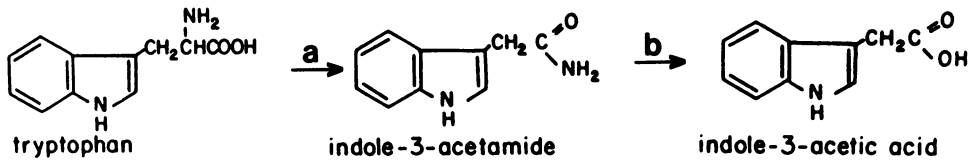


FIG. 1. *Pathway of IAA synthesis in P. savastanoi. In reaction a, catalyzed by Trp monooxygenase, molecular oxygen and Trp react to yield indoleacetamide, $CO_2$, and $H_2O$. In reaction b, catalyzed by indoleacetamide hydrolase, indoleacetamide is hydrolyzed to yield indole-3-acetic acid and ammonia.*

TABLE 1. *Strains of P. savastanoi*

| Strain | Phenotype | Plasmid content | Origin | Reference |
|---|---|---|---|---|
| 2009 | Wild-type, Iaa$^+$[a] | p38, pIAA1, p27, p22[b] | Field isolate | (17) |
| 2009-3 | Iaa$^-$ | p38, p27 | Spontaneous mutant of 2009 | (17) |
| 2009-6 | Iaa$^+$ | p38, pIAA1, p27 | Acridine orange-cured derivative of 2009 | This report |
| 2009-7 | Iaa$^+$ Str Su[c] | p38, pIAA1, p27, RSF1010 | Spontaneous derivative of 2009 | This report |

[a] Iaa$^+$, IAA production; Iaa$^-$, absence of IAA production.
[b] Plasmids are described in Table 2.
[c] Str, Streptomycin resistance; Su, sulfonamide resistance.

TABLE 2. *Characteristics of plasmid DNA*

| Plasmid | Phenotype | Mol wt ($10^6$) | Reference |
|---|---|---|---|
| RSF1010[a] | Str Su[b] | 5.5 | (5) |
| pIAA1 | Iaa$^+$[c] | 34 | This report |
| p38 | Cryptic | 38 | McGuire (unpublished data) |
| p27 | Cryptic | 27 | McGuire (unpublished data) |
| p22 | Cryptic | 22 | McGuire (unpublished data) |

[a] Purified RSF1010 plasmid DNA was a gift from N. J. Panopoulos, Department of Plant Pathology, University of California, Berkeley.
[b] Str, Streptomycin resistance; Su, sulfonamide resistance.
[c] Iaa$^+$, Indoleacetic acid production.

surement of indoleacetamide hydrolase activity, reaction mixtures contained 20 mM Tris (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 10 mM indoleacetamide, and enzyme preparation (10 mg of protein) in a 10-ml final volume. Procedures for incubation, extraction, and chromatography of indole compounds were identical to those used for Trp monooxygenase. Occurrence of indoleacetamide hydrolase activity was determined by the appearance of IAA on the chromatograms.

**Curing for detection of Iaa mutants.** Tubes containing 3 ml of King B broth and a specified concentration of curing agent (see Results) were inoculated with log-phase cells to give a final suspension of $10^3$ to $10^4$ colony-forming units per ml. Cultures were shaken at 28°C for 48 h, and then 0.1 ml of the cell suspension was further subcultured in 3.0 ml of King B broth. After at least 24 h at 28°C (see Results), bacteria were diluted in 0.85% NaCl and plated on solid King medium B. Single colonies were replica plated on minimal medium and on minimal medium plus 5-methyl-Trp. An Iaa$^-$ mutant and a wild-type isolate were added as controls. 5-Methyl-Trp-sensitive cells were checked for IAA production by culture in 1.5 ml of King medium B with shaking at 28°C. Tubes were capped in such a way as to allow aeration during growth. After a density of $5 \times 10^8$ cells per ml was reached, cultures were centrifuged at 5,000 relative centrifugal force (RCF), and an equal volume of Salkowski reagent was added to the supernatant fraction (4). A red color after 60 min indicated a positive reaction; samples that remained colorless or developed a barely detectable pink were scored as IAA negative.

**Plasmid isolation.** Highly purified covalently closed plasmid DNA was isolated by a modification of the technique described by Meyer et al. (14). Late log-phase cells from a 0.5-liter culture were chilled, washed once in TES buffer (50 mM NaCl, 5 mM EDTA, 30 mM Tris [pH 8.0]), and suspended in 20 ml of lytic mix (25% sucrose, 50 mM Tris [pH 8.0]). Eight milliliters of lysozyme (10 mg/ml in 0.25 M Tris [pH 8.0]) and 4 ml of 0.25 M EDTA (pH 8.0) were added, and the mixture was incubated on ice for 30 min. Thirty milliliters of Triton (0.2% Triton X-100, 50 mM EDTA, 30 mM Tris [pH 8.0]) were added with gentle agitation. The resulting lysate was denatured by the method of Currier and Nester (2) by addition of 3 M NaOH to pH 12.2. After 5 min, the pH of the lysate was brought back to 8.0 to 9.0 by addition of 2 M Tris (pH 7.0). CsCl (1 g/ml) and ethidium bromide (0.4 mg/ml) were added, and the mixture was clarified by centrifugation (20 min at $1.5 \times 10^4$ RCF), loaded in polyallomer ultracentrifuge tubes, and centrifuged to equilibrium in a Beckman Ti-60 rotor at $9 \times 10^4$ RCF. Fractions

containing the denser, satellite plasmid band were collected, pooled, and recentrifuged to equilibrium in the dye-cesium chloride gradient. Fractions containing the supercoiled DNA were collected, extracted four times with cesium chloride-saturated isopropanol to remove ethidium bromide, and dialyzed against TES buffer for 12 h at 4°C. Yeast tRNA was added to 10 μg/ml, and the solution was adjusted to 0.3 M sodium acetate. DNA was precipitated with 2 volumes of ethanol (−20°C) and sedimented by centrifugation at $1.2 \times 10^4$ RCF; the precipitate was then suspended in TES buffer, and the resulting preparation was stored at 4°C.

Plasmid DNA content was routinely screened by a modification of the Hansen and Olsen (6) technique. The cells in 25-ml cultures ($10^9$ colony-forming units per ml) were washed once in TES buffer, suspended in 2 ml of lytic mix, combined with 0.15 ml of lysozyme preparation (15 mg/ml in 0.25 M Tris [pH 8]) and 0.75 ml of 0.25 M EDTA (pH 8), and then incubated on ice for 15 min. Sodium dodecyl sulfate was added to a final concentration of 7.4%, and cells were allowed to lyse at 25°C with gentle agitation of the tubes. Heat pulsing for 1 min at 50°C was used to speed lysis. Then 0.7 ml of freshly prepared 3 M NaOH was added, and the suspension was mixed gently. After 2 min, the pH was lowered by addition of 1.4 ml of 2 M Tris (pH 7). Lysates were brought to 1 M NaCl and stored overnight at 4°C. After centrifugation for 20 min at 12,000 RCF (4°C), the supernatant fraction was collected, and DNA was precipitated by addition of 42% polyethylene glycol 6000 to a final concentration of 10%. After 4 h at 4°C, the tubes were centrifuged at 2,000 RCF for 10 min, and the pellets were dissolved in 1.5 ml of TES buffer. The resulting preparation was extracted with the same volume of TES buffer-saturated phenol and then centrifuged at 10,000 RCF for 10 min at 4°C; the aqueous phase was collected, and sodium acetate was added to a final concentration of 0.3 M. tRNA was added to a final concentration of 10 μg/ml, and nucleic acids were precipitated with addition of 2 volumes of cold 95% ethanol. Samples were stored at −20°C for at least 5 h and centrifuged at 12,000 RCF for 15 min. Pellets were partially dried with $N_2$ and suspended in 0.2 ml of sterile distilled water, and the solutions were stored at 4°C until used.

**Transformation procedures.** Transformation was carried out by the method of Chakrabarty et al. (1). Late-log-phase cells from a 20-ml culture were chilled, harvested by centrifugation, washed once with 10 ml of 10 mM NaCl, pelleted, and suspended in 10 ml of 0.1 M $CaCl_2$. The bacterial suspension was kept on ice for 20 min, and the cells were harvested by centrifugation; the precipitate of the cells was then suspended in 2 ml of a 0.1 M $CaCl_2$–15% glycerol solution. Samples (0.2 ml) of these competent cells were stored at − 70°C until needed. Alternatively, $CaCl_2$ was substituted throughout with 0.15 M $MgCl_2$ by the method of Mercer and Loutit (13). For transformation experiments, the stored suspension of competent cells was allowed to thaw on ice. A 0.1-ml amount of DNA solution in 0.1 M $CaCl_2$ or 0.15 M $MgCl_2$ was added, and the mixture was incubated for 60 min at 0°C, heat pulsed at 39°C for 2 min, chilled, and diluted with 20 volumes of King B broth. Transformation mixtures were incubated on a shaker for 6 h to allow expression of transformed DNA, and cells then were plated on selective medium.

**Restriction digestion of DNA.** Digestion with restriction endonuclease *Eco*RI (2 U/μg of DNA) was carried out in 100 mM Tris–5 mM $MgCl_2$–50 mM NaCl–0.02% Nonidet P-40 (Gallard Schlesinger Chemicals). The reaction mixture (30 μl) was incubated at 37°C for 30 min and stopped by addition of 10 μl of a urea-dye mixture (10 M urea, 0.02% bromophenol blue, 0.02% xylene cyanole FF).

**Agarose gel electrophoresis.** Plasmid DNA was subjected to electrophoresis in 0.5 to 0.7% agarose gels as described by Meyers et al. (15). Restriction digestion fragments were analyzed by electrophoresis in 0.7% agarose gels. Gels were run at 60 V until the bromophenol blue dye front reached the end of the slab and were stained by the procedures of Meyers et al. (15).

**Chemicals and enzymes.** *Eco*RI restriction endonuclease was purchased from Miles Biochemicals, Elkhart, Ind.; agarose was purchased from Seakem (Marine Colloids, Rockland, Maine); ethidium bromide and acridine orange were purchased from Calbiochem, La Jolla, Calif.; CsCl (technical grade) was purchased from KBI, Revere, Pa.; xylene cyanole and bromophenol blue were purchased from Bio-Rad Laboratories, Richmond, Calif., and nutrient media were purchased from Difco Laboratories, Detroit, Mich. All other chemicals were purchased from Sigma Chemical Co., St. Louis, Mo.

## RESULTS

**Differential sensitivity to 5-methyl-Trp.** To provide evidence for the role of Trp monooxygenase in conferring resistance to the Trp analogue 5-methyl-Trp, we examined the substrate specificity of this enzyme. Monooxygenase activity for 5-methyl-Trp was close to that found with Trp; α-methyl-Trp, a growth inhibitor of wild-type *P. savastanoi* (17), was not oxidized (Table 3). Further, 5-methyl-Trp was converted into a product, presumably 5-methylindoleacetamide, since it chromatographed closely with indoleacetamide in two different solvent systems. No reaction product was found when α-methyl-Trp was incubated with the enzyme. Oxidation of 5-methyl-Trp by Trp monooxygenase effectively detoxifies the compound since neither

TABLE 3. *Substrate specificity of Trp monooxygenase*

| Substrate | Concn (mM) | Relative activity[a] |
|---|---|---|
| L-Trp | 10 | 1 |
| DL-5-Methyl-Trp | 20 | 0.89 |
| DL-α-Methyl-Trp | 20 | ND[b] |

[a] Relative activity is that obtained with L-Trp as a substrate. Specific activity was 0.75 U/mg of protein. One unit of activity is defined as the amount of enzyme that catalyzes the uptake of 1 μmol of $O_2$ per minute.

[b] ND, Not detectable.

5-methylindoleacetamide nor 5-methyl-IAA inhibited growth of *P. savastanoi*. Possession of Trp monooxygenase activity, therefore, confers resistance to 5-methyl-Trp. Mutants such as 2009-3, which lack the enzyme, are inhibited by the Trp analogue. This differential sensitivity allows selection for loss of Trp monooxygenase by replica plating on 5-methyl-Trp-supplemented minimal medium. An example of this technique is shown in Fig. 2.

Conversely, the reversion of Iaa mutants to wild-type phenotype could be detected by direct plating on 5-methyl-Trp medium. However, this assay was limited by a high background of mutation to 5-methyl-Trp resistance apparently mediated by a mechanism other than production of Trp monooxygenase. True Iaa$^+$ revertants could not be detected among 5-methyl-Trp-resistant mutants (M. L. Smidt, unpublished data). Nevertheless, this selection is useful for screening transformants for IAA production if cotransformation with an R factor is included.

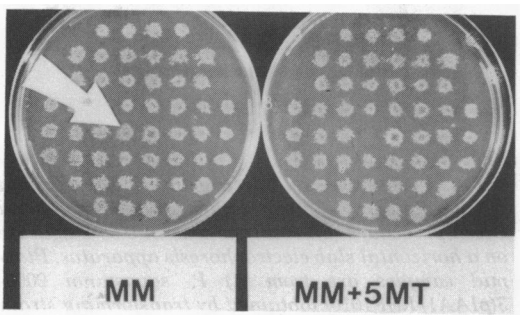


FIG. 2. *Selective growth inhibition of Iaa$^-$ mutants by 5-methyl-Trp. Abbreviations: MM, minimal medium; MM + 5 MT, minimal medium plus 5-methyl-Trp. The arrow points to an Iaa$^-$ mutant colony incapable of synthesizing IAA. Remaining colonies are Iaa$^+$. Bacteria were replica plated from a master plate on the two media.*

**Curing.** Various treatments were tested for their curing efficiency on isolate 2009. Statisfactory results were obtained only when acridine orange-treated cells were subcultured in King B medium (Table 4). This step was necessary because most cells in acridine orange grow in chains of two to eight cells due to impaired ability of daughter cells to undergo complete fission. If segregants with an altered plasmid composition appeared during growth in acridine orange, their detection based on failure to grow on 5-methyl-Trp would be masked by the heterogeneity of colonies arising from chains of cells. Further, our results suggest that segregation or loss of plasmid-coded determinants occurred only during growth in acridine orange since increased subculturing (treatment 6, Table 4) did not affect curing frequency.

No 5-methyl-Trp-sensitive clones produced IAA. Neither Trp monooxygenase nor indoleacetamide could be detected in extracts of these strains. Ten of the cured isolates and ten randomly selected wild-type isolates from treatments 5 and 6 (Table 4) were subsequently analyzed for plasmid composition. All Iaa$^-$ mutants had lost the second largest plasmid (molecular weight = $34 \times 10^6$) (track 2, Fig. 3; track 3, Fig. 4). The same plasmid was present in all Iaa$^+$ clones. The smallest plasmid was lost in one of the Iaa$^-$ (track 2, Fig. 4) and two of the Iaa$^+$ clones (data not shown). These latter Iaa$^+$ isolates were designated 2009-6. Henceforth, the $34 \times 10^6$-molecular-weight plasmid was designated

TABLE 4. *Efficiency of various treatments for curing Trp monooxygenase activity in P. savastanoi*

| Treatment[a] | Concn (μg/ml) | No. of colonies | | Curing frequency |
|---|---|---|---|---|
| | | Screened | Iaa$^-$ | |
| 1. Acridine orange | 50 | 274 | 0 | $<3.6 \times 10^{-3}$ |
| 2. Acridine orange | 75 | 336 | 1 | $3 \times 10^{-3}$ |
| 3. Nalidixic acid | 2 | 196 | 0 | $<5 \times 10^{-3}$ |
| 4. Mitomycin C | 0.16 | 236 | 0 | $<4.2 \times 10^{-3}$ |
| 5. Acridine orange + 10 generations in King B broth | 75 | 384 | 10 | $2.6 \times 10^{-2}$ |
| 6. Acridine orange + 30 generations in King B broth | 75 | 192 | 4 | $2.1 \times 10^{-2}$ |

[a] All treatments with the exception of number 1 employed sublethal doses of the curing agent. These were determined by inoculating with $10^5$ colony-forming units a series of 3-ml King B broth tubes containing increasing concentrations of the agent. Subculturing in King B broth, described in the text, was not used in treatments 1 through 4.

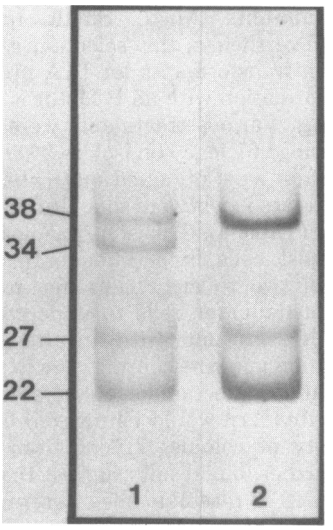


FIG. 3. *Agarose gel electrophoresis of supercoiled DNA isolated by dye-buoyant density centrifugation from (1) P. savastanoi 2009, IAA producer, and (2) P. savastanoi 2009, non-IAA producer, obtained by acridine orange treatment. Analysis was in 0.7% agarose on a vertical slab electrophoresis apparatus (see the text).*

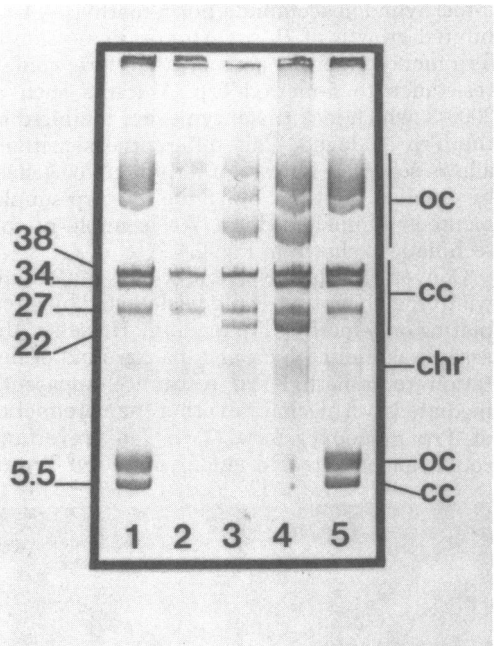


FIG. 4. *Agarose gel electrophoresis of plasmid DNA isolated by a modification of the Hansen and Olsen procedure (6). Analysis was in 0.55% agarose on a horizontal slab electrophoresis apparatus. Plasmid samples are from (1) P. savastanoi 2009-3(pIAA1/RSF1010) (obtained by transforming strain 2009-3 with a mixture of pIAA1 and RSF1010 as described in the text; Trp monooxygenase activity and IAA production were restored concomitantly), (2) 2009-3, Iaa⁻ spontaneous mutant, (3) 2009, Iaa⁻ mutant isolated after acridine orange treatment, (4) 2009, wild type, and (5) same as 1. Abbreviations: cc, covalently closed DNA; oc, open circular DNA; chr, chromosomal DNA.*

pIAA1 in reference to its putative association with IAA production.

**Transformation.** To overcome the problem of selection against a high background mutation for 5-methyl-Trp resistance, we used a double selection for streptomycin and 5-methyl-Trp resistance to isolate RSF1010/pIAA1 double transformants. The streptomycin resistance factor RSF1010 (5 μg of DNA in 0.3 ml of transformation mixture) was taken up by isolate 2009 with a frequency of $1 \times 10^{-7}$ cells. The presence of RSF1010 in the transformants was demonstrated by agarose gel electrophoresis. Although the majority of such transformants retained the four plasmids of the wild-type 2009 (track 1, Fig. 3; track 4, Fig. 4), a few, such as the one used here (designated 2009-7), lost the cryptic plasmid p22 while retaining the three largest plasmids.

To demonstrate transformation, plasmid samples from strains 2009-6 and 2009-7 containing pIAA1 and RSF1010 were incubated with cells of Iaa⁻ mutant 2009-3. Different proportions of the two plasmids also were employed to determine frequency of cotransformation. Transformed cells were allowed to express their phenotypes in King B broth (10 volumes) for 6 h under moderate shaking. Cells were then pelleted by centrifugation at 5,000 RCF, suspended in 0.85% NaCl, and plated on selective medium with streptomycin alone or on streptomycin plus 5-methyl-Trp. Streptomycin-resistant colonies were visible after 2 days of incubation at 25°C. Colonies resistant to both streptomycin and 5-methyl-Trp appeared after 5 or 6 days (Table 5).

All clones resistant to both 5-methyl-Trp and streptomycin produced IAA when assayed with Salkowski reagent. Two randomly selected clones examined for plasmid composition had acquired, together with RSF1010, a plasmid exhibiting the mobility of pIAA1 (tracks 1 and 5, Fig. 4). The identity of this plasmid as pIAA1 was conclusively determined by *Eco*RI digestion (Fig. 5). Fragments generated by endonuclease digestion of pIAA1 can be recognized by comparing plasmid digests of the wild-type 2009 (track 4, Fig. 5) with those of a cured mutant of 2009 lacking pIAA1 (track 3, Fig. 5). Those same fragments occurred in plasmid digests of the transformant (pIAA1/RSF1010), (track 5, Fig. 5) but were missing in digests of Iaa⁻ mutant

TABLE 5. *Transformation of P. savastanoi 2009-3 by pIAA1 and RSF1010 DNAs*[a]

| pIAA1/ RSF1010 ratio[b] | pIAA1 (μg)[c] | Frequency of cotransformation[d] |
|---|---|---|
| 0.23 | 10 | $1.0 \times 10^{-3}$ |
| 0.77 | 3 | $6.6 \times 10^{-3}$ |
| 4.0 | 9 | $1.8 \times 10^{-2}$ |

[a] The concentration of each plasmid was estimated from the band density of agarose gels. The molar ratio of pIAA1 to RSF1010 was calculated from densitometric tracings of photographic negatives.

[b] Molar ratio of pIAA1 to RSF1010. The plasmid ratios were obtained by mixing appropriate amounts of plasmid preparations from transformant 2009-7 (RSF1010) with isolate 2009-6 preparations.

[c] Micrograms of pIAA1 per 0.3 ml of transforming solution.

[d] Frequency was obtained by dividing the number of 5-methyl-Trp- and streptomycin-resistant colonies by the total number of streptomycin-resistant colonies. Frequency of the RSF1010 transformants was approximately $10^{-7}$ when the technique of Chakrabarty et al. (1) was used. It was at least fivefold higher when $MgCl_2$ was used in place of $CaCl_2$.

2009-3 (track 2, Fig. 5). This further indicates that the plasmid introduced into strain 2009-3 by transformation is pIAA1.

The transformant 2009-3(RSF1010)/pIAA1) was assayed for Trp monooxygenase and indoleacetamide hydrolase activities. In keeping with resistance to 5-methyl-Trp and capacity for IAA production in culture, Trp monooxygenase and hydrolase activities of the transformant were comparable to those found in parent 2009; preparations from mutant 2009-3 had no detectable Trp monooxygenase and indoleacetamide hydrolase activities.

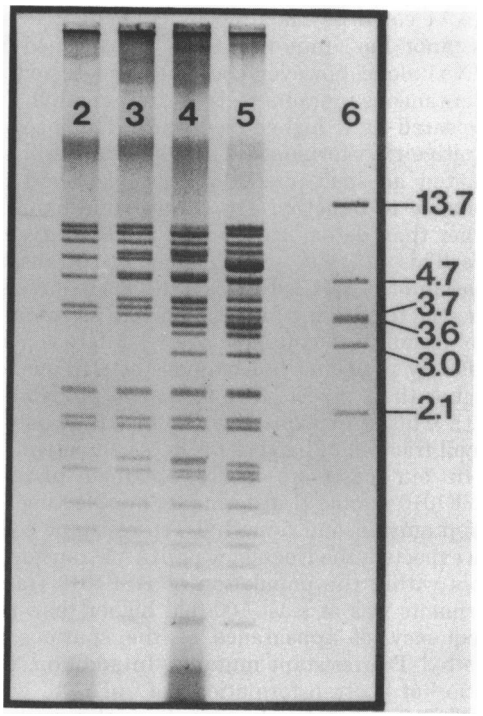


FIG. 5. *Agarose gel electrophoresis of endonuclease EcoRI-treated plasmid DNA. DNA was isolated by a modification of the Hansen and Olsen procedure (6). Analysis was in 0.7% agarose on a horizontal slab gel apparatus. Samples are from (2) P. savastanoi 2009-3, Iaa⁻, spontaneous mutant, (3) 2009, Iaa⁻ mutant isolated after acridine orange treatment, (4) 2009, wild type, (5) 2009-3(pIAA1/RSF1010) (obtained by transforming strain 2009-3 with a mixture of pIAA1 and RSF1010 plasmid DNA), and (6) digests of lambda phage (7). Gels 2 through 5 correspond to plasmid samples 2 through 5 in Fig. 5.*

## DISCUSSION

Many unusual metabolic activities of *Pseudomonas* species are plasmid-coded functions. In this report, evidence is presented supporting the extrachromosomal nature of genes involved in IAA synthesis. An assay based on the capability of Trp monooxygenase to detoxify 5-methyl-Trp to 5-methylindoleacetamide was developed as a basis for screening cells for the loss or acquisition of Trp monooxygenase activity. Iaa$^+$ cells, the wild-type phenotype, can grow in the presence of 5-methyl-Trp, whereas Iaa$^-$ mutants are inhibited. 5-Methyl-Trp was shown to be a potent inhibitor of anthranilate synthase in *Escherichia coli* (16). By replica plating single colonies on 5-methyl-Trp medium, clones lacking Trp monooxygenase activity can be detected by their failure to grow. With this assay, we tested various chemicals that had been shown by others to interfere with the maintenance of extrachromosomal DNA. Mitomycin C, probably the most common curing agent used by *Pseudomonas* geneticists, and nalidixic acid were ineffective under the conditions used. Acridine orange alone yielded a very low and erratic curing frequency. However, when acridine orange-treated cells were transferred to King B broth before plating on a selection medium, higher frequencies for curing were achieved. Growth in acridine orange seems to prevent complete scission of dividing cells and results in production of chains of cells. Therefore, it is conceivable that direct plating of cells from such cultures results in single colonies originating from more than one cell, thereby masking detection of mutant phenotypes.

Iaa$^-$ mutants lack plasmid pIAA1; loss of the plasmid was selective since pIAA1 was present in Iaa$^+$ cells. Further, we were able to show that

pIAA1 could be taken up by competent P. savastanoi Iaa$^-$ mutants. Cells transformed by pIAA1 alone, however, could not be detected, as spontaneous mutants resistant to 5-methyl-Trp appeared at a higher frequency than pIAA1-positive transformants. These mutants still exhibited an Iaa$^-$ phenotype but displayed resistance to 5-methyl-Trp through mechanisms other than detoxification by Trp monooxygenase. This difficulty was overcome by cotransformation of pIAA1 with RSF1010, a small resistance factor, coding for resistance to streptomycin. Simultaneous uptake of two different plasmids by the same cell happens at a frequency higher than expected by statistical probability (11). This is explained by the fact that only a small fraction of bacterial cells is competent.

In our case, we easily obtained pIAA1/RSF1010 double transformants by selecting for streptomycin and 5-methyl-Trp-resistant cells. As expected, the frequency of pIAA1 transformants within the population of RSF1010 transformants was at least 100-fold higher than the frequency of appearance of the spurious 5-methyl-Trp-resistant mutants. In addition, the ratio of cotransformation of pIAA1 with RSF1010 was a function of the molar ratio of these two plasmids in the transformation mixture. Trp monooxygenase and indoleacetamide hydrolase activities were restored concomitantly with pIAA1 uptake. Thus, the transformed 2009-3(pIAA1) strain had regained the whole enzymatic system that converts Trp into IAA. Further, the identity of the plasmid species taken up by the transformants was confirmed by *Eco*RI restriction-digest pattern, thereby conclusively establishing the relationship between pIAA1 and synthesis of IAA.

One more gene product is known to be involved in the IAA metabolic pathway—an ATP-dependent transferase that catalyzes the conjugation of IAA to lysine (8), whose relationship to the pIAA1 plasmid was not studied in the present investigation. It is conceivable that this system might also be plasmid coded with Trp monooxygenase and indoleacetamide hydrolase. Future studies will investigate the possibility that these genes might also be organized together in an IAA operon.

IAA is a determinant of pathogenicity in the interaction between *P. savastanoi* and the host plant. Mutants that fail to produce IAA do not produce galls (17). Gall formation may represent a selective advantage to *P. savastanoi*, providing a favorable environment in which the pathogen can multiply and successfully withstand periods of extreme weather.

A regulatory function of chromosomal genes on IAA production cannot be excluded in our studies. However, involvement of plasmid-coded genes in plant pathogenesis by bacteria was conclusively demonstrated in the crown gall disease caused by *Agrobacterium tumefaciens* (19). Virulent strains of this plant pathogen harbor a large plasmid (Ti), a section of which (the T region) is inserted in plant cells, where it induces tumorous growth. *A. tumefaciens* also produces IAA, and a gene(s) necessary for its production is apparently carried on the Ti plasmid (12). It has been suggested that this gene(s) might be located on the T region and that production of IAA in plant cells, mediated by bacterial genetic material, is directly responsible for the induction of tumorous growth. However, little is known about the IAA synthesis pathway in *A. tumefaciens*, and its involvement in oncogenicity remains to be established. Nevertheless, it is intriguing that in the plant parasitic bacteria *A. tumefaciens* and *P. savastanoi* genes for pathogenicity are located on extrachromosomal elements. Plasmid-coded determinants of pathogenicity in *Pseudomonas* sp. have been previously suggested (3); we provide definite evidence for such a case in the *P. savastanoi*-olive (or oleander) interaction.

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## LITERATURE CITED

1. **Chakrabarty, A. M., J. R. Mylroie, D. A. Friello, and J. G. Vacca.** 1975. Transformation of *Pseudomonas putida* and *Escherichia coli* with plasmid-linked drug-resistance factor DNA. Proc. Natl. Acad. Sci. U.S.A. **72**:3647-3651.
2. **Currier, T. C., and E. W. Nester.** 1976. Isolation of covalently closed circular DNA of high molecular weight from bacteria. Anal. Biochem. **76**:431-441.
3. **Gonzales, C. F., and A. K. Vidaver.** 1978. Analysis of plasmids of syringomycin producing strains of *Pseudomonas syringae*. Proc. Int. Conf. Plant Pathog. Bact. **4**: 31-38.
4. **Gordon, S. A., and R. P. Weber.** 1951. Colorimetric estimation of indoleacetic acid. Plant Physiol. **26**:192-195.
5. **Guerry, P., J. Van Embden, and S. Falkow.** 1974. Molecular nature of two nonconjugative plasmids carrying drug resistance genes. J. Bacteriol. **117**:619-630.
6. **Hansen, J. B., and R. H. Olsen.** 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. **135**:227-238.
7. **Helling, R. B., H. M. Goodman, and H. W. Boyer.** 1974. Analysis of endonuclease R·*Eco*RI fragments of DNA from lambdoid bacteriophages and other viruses by agarose-gel electrophoresis. J. Virol. **14**:1235-1244.
8. **Hutzinger, O., and T. Kosuge.** 1968. Microbial synthesis

and degradation of indole-3-acetic acid. III. The isolation and characterization of indole-3-acetyl-ε-L-lysine. Biochemistry **7**:601–605.

9. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. **44**:301–307.
10. **Kosuge, T., M. G. Heskett, and E. E. Wilson.** 1966. Microbial synthesis and degradation of indole-3-acetic acid. I. The conversion of L-tryptophan to indole-3-acetamide by an enzyme from *Pseudomonas savastanoi*. J. Biol. Chem. **241**:3738–3744.
11. **Kretschmer, P. J., A. C. Y. Chang, and S. N. Cohen.** 1975. Indirect selection of bacterial plasmids lacking identifiable phenotypic properties. J. Bacteriol. **124**: 225–231.
12. **Liu, S. T., and C. I. Kado.** 1979. Indoleacetic acid production: a plasmid function of *Agrobacterium tumefaciens* C58. Biochem. Biophys. Res. Commun. **90**:171–178.
13. **Mercer, A. A., and J. S. Loutit.** 1979. Transformation and transfection of *Pseudomonas aeruginosa*: effects of metal ions. J. Bacteriol. **140**:37–42.
14. **Meyer, R., D. Figurski, and D. R. Helinski.** 1977. Physical and genetic studies with restriction endonucleases on the broad host-range plasmid RK2. Mol. Gen. Genet. **152**:129–135.
15. **Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow.** 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529–1537.
16. **Moyed, H. S.** 1960. False feedback inhibition: inhibition of tryptophan biosynthesis by 5-methyltryptophan. J. Biol. Chem. **235**:1098–1102.
17. **Smidt, M., and T. Kosuge.** 1978. The role of indole-3-acetic acid accumulation by alpha methyl tryptophan-resistant mutants of *Pseudomonas savastanoi* in gall formation on oleanders. Physiol. Plant Pathol. **13**:203–214.
18. **Sprince, H.** 1960. A modified Ehrlich benzaldehyde reagent for detection of indoles on paper chromatograms. J. Chromatogr. **3**:97–98.
19. **Watson, B., T. C. Currier, M. P. Gordon, M. Chilton, and E. W. Nester.** 1975. Plasmid required for virulence of *Agrobacterium tumefaciens*. J. Bacteriol. **123**:255–264.
20. **Wheelis, M. L.** 1975. The genetics of dissimilarity pathways in *Pseudomonas*. Annu. Rev. Microbiol. **29**:505–524.