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# Cloning of the Gene for Indoleacetic Acid-Lysine Synthetase from *Pseudomonas syringae* subsp. *savastanoi*

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**The phytopathogen *Pseudomonas syringae* subsp. *savastanoi* incites the production of galls on olive and oleander plants. Gall formation is dependent on bacterial production of the phytohormone indoleacetic acid (IAA). The genetic determinants for IAA synthesis are located on a plasmid (pIAA) and are organized in an operon in oleander strains of the bacterium. *P. syringae* subsp. *savastanoi* further converts IAA to an amino acid conjugate, 3-indole-acetyl-ε-L-lysine (IAA-lysine). The gene for IAA-lysine synthetase (*iaaL*) was found on the IAA plasmid by screening pIAA deletion mutants for the ability to convert IAA to IAA-lysine. The *iaaL* locus was then cloned in the vector pUC8 from a bank of *P. syringae* subsp. *savastanoi* EW2009 plasmid DNA to construct recombinant plasmid pLG87. The specific activity of IAA-lysine synthetase in *Escherichia coli* transformed with pLG87 was 47 times higher than that of the enzyme extract from *P. syringae* subsp. *savastanoi*. The direction of transcription of the *iaaL* gene was determined to be opposite to that of the IAA operon. The location of the *iaaL* gene on pIAA1 was mapped by Tn*5* insertion mutagenesis to a 2.5-kilobase-pair fragment 2 kilobase pairs from the IAA operon.**

*Pseudomonas syringae* subsp. *savastanoi* (Smith) Stevens incites a disease of oleander (*Nerium oleander* L.) and olive (*Olea europa* L.) that is characterized by tumorous outgrowths on the stems and leaves of infected plants (21, 26, 27). This development of galls is dependent on bacterial production of the phytohormone indoleacetic acid (IAA) (6, 23). The two enzymes involved in the conversion of tryptophan to IAA are tryptophan monooxygenase, which converts tryptophan to indoleacetamide, and indoleacetamide hydrolase, which catalyzes the conversion of indoleacetamide to IAA (16, 17). IAA can be further metabolized to an amino acid conjugate, 3-indole-acetyl-ε-L-lysine (IAA-lysine) (12, 13).

The genes for tryptophan monooxygenase, *iaaM*, and indoleacetamide hydrolase, *iaaH*, are organized in an operon in *P. syringae* subsp. *savastanoi* (7, 8, 28); the *iaaM* locus is the first gene transcribed. In strains isolated from oleander galls, the IAA operon is located on a plasmid (pIAA) (6, 9). Oleander gall isolates selected for resistance to the tryptophan analog α-methyl tryptophan are often incapable of synthesizing IAA and cannot incite the production of galls when inoculated onto oleander plants (23). These avirulent $IAA^-$ mutants fall into two classes when surveyed for their plasmid composition: those that lack the IAA operon, either by the loss of the IAA plasmid or by deletion of the IAA genes, and those that contain insertions in the *iaaM* gene (6, 8, 9).

Certain isolates of *P. syringae* subsp. *savastanoi* convert IAA to IAA-lysine (12, 13). The enzyme responsible for IAA-lysine synthesis is ATP dependent and catalyzes the formation of an amide bond between the carboxyl group of IAA and the epsilon amino group of lysine (13, 15). Previously, it was shown that variations in IAA pool size in *P. syringae* subsp. *savastanoi* affect gall development (23); therefore, factors affecting IAA pool sizes, such as the rate of synthesis of IAA and the rate of conversion of IAA to IAA-lysine, could alter virulence in *P. syringae* subsp. *savastanoi*. To facilitate an investigation into the role of IAA-lysine formation in gall development in infected plants, we examined the possibilities that (i) *iaaL*, the gene for IAA-lysine synthetase, was located on pIAA1 together with the IAA genes in *P. syringae* subsp. *savastanoi*; (ii) the *iaaL* gene was transcribed with the IAA operon; and (iii) variations occurred in the production of IAA-lysine in bacteria isolated from different hosts. Additionally, we describe the cloning of the *iaaL* gene and the determination of IAA-lysine synthetase activity in *Escherichia coli* transformed with a recombinant plasmid bearing the *iaaL* gene.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *P. syringae* subsp. *savastanoi* strains and plasmids used in this study are described in Table 1. *E. coli* 71-18 (25) and HB101 (3) were used for cloning work.

**Culturing of bacteria.** *P. syringae* subsp. *savastanoi* was cultured on either solid or liquid glucose-peptone medium (14) or minimal medium A (20) at 28°C. *E. coli* strains were cultured on L broth (20) or on minimal medium A amended with proline, leucine, and thiamine. When required for selection, antibiotics were added as follows: ampicillin (Sigma Chemical Co.), 100 μg/ml; kanamycin sulfate (Sigma), 30 μg/ml.

**Selection for α-methyl tryptophan-resistant mutants.** *P. syringae* subsp. *savastanoi* mutants resistant to α-methyl tryptophan were isolated by the procedure of Smidt and Kosuge (23). For further purification, resistant colonies were streaked out on plates of minimal medium A containing 200 μg of α-methyl tryptophan per ml. Individual colonies were then selected and screened for plasmid composition and IAA accumulation as previously described (6).

**Analysis of IAA-lysine.** [$^3$H]IAA ($1.4 \times 10^6$ dpm; specific activity, 21.6 Ci/mmol) or [$^{14}$C]IAA ($4.4 \times 10^5$ dpm; specific activity, 55 mCi/mmol) was added to 2-ml cultures of either *P. syringae* subsp. *savastanoi* or *E. coli*, which were then incubated with the radioactive material for up to 6 h at 28°C. The cultures were centrifuged at 3,000 × *g* for 10 min to pellet the cells; the supernatant fraction was acidified to pH 2.5 to 3.0 with 1 N HCl and extracted twice with equal

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TABLE 1. Production of IAA and IAA-lysine by *P. syringae* subsp. *savastanoi*

| *P. syringae* subsp. *savastanoi* strains | Location of IAA genes[a] | IAA production | IAA-lysine production | Reference(s) |
|---|---|---|---|---|
| Oleander isolates | | | | |
| EW2009 | pIAA1 | + | + | 6, 23 |
| EW2009-3[b] | | − | − | 6 |
| EW2009-5[b] | pIAA1::IS*51* | − | + | 8 |
| TK2015 | pIAA1 | + | + | 23 |
| TK2015-3[b] | pIAA1::IS*52* | − | + | 23 |
| TK2015-32[b] | | − | − | This report |
| PB213 | pIAA2 | + | + | 9 |
| PB213-3[b] | | − | − | This report |
| PB213-6[b] | ΔpIAA2 | − | − | This report |
| EW1017 | pIAA3 | + | + | 9 |
| EW1017-1[b] | | − | − | Lab strain |
| Olive isolates | | | | |
| EW1006 | Chromosomal | + | − | Lab strain |
| TK1050 | Chromosomal | + | − | Lab strain |
| TK1050-4[b,c] | | − | − | Lab strain |
| Privet isolate PB215 | Chromosomal | + | − | 9 |

[a] Lengths: pIAA1 is 52 kbp; pIAA1::IS*51* is 53.7 kbp; pIAA1::IS52 is 53.2 kbp; pIAA2 is 73 kbp; ΔpIAA2 is 59 kbp; pIAA3 is 72 kbp.

[b] Mutants were selected for resistance to α-methyl tryptophan and screened for the loss of IAA production, as described in the text. Loss of pIAA was confirmed by plasmid isolation and agarose gel electrophoresis as described in the text.

[c] Chromosomal deletion of IAA genes was characterized by Southern blot hybridization (L. Comai, unpublished results).

volumes of ethyl acetate to remove the unreacted IAA; IAA-lysine was retained in the aqueous phase. Radioactivity in the aqueous phase, determined by counting samples in a scintillation counter, was used to estimate the amount of IAA-lysine secreted by the bacteria.

**Growth curve versus IAA-lysine accumulation.** *P. syringae* subsp. *savastanoi* strains were inoculated into 50 ml of minimal medium A in Klett flasks. After cell density had reached $1 \times 10^8$ cells per ml, [$^3$H]IAA ($2.2 \times 10^6$ dpm; specific activity, 21.6 Ci/mmol) was added to the cultures. At appropriate time intervals, Klett readings for cell density were recorded, and 2-ml samples of the bacterial suspension were removed and analyzed for the presence of IAA-lysine as described above.

**Enzyme assay.** Overnight cell cultures (24 h) of *E. coli* or *P. syringae* subsp. *savastanoi* were centrifuged at $3,000 \times g$ for 10 min, and the resulting cell pellet was suspended in cold 50 mM Tris buffer (pH 7.8) with 10 mM β-mercaptoethanol. The cells were subjected to sonic disruption at 4°C, and the resulting suspension was centrifuged at $35,000 \times g$ at 4°C for 20 min. The pellet was discarded, and the crude cell lysate was assayed directly for IAA-lysine synthetase activity in a reaction mixture of [$^{14}$C]IAA (specific activity, 0.2 μCi/μmol), 2.4 μM ATP, 30 μM lysine, 1 μM $MnCl_2$, 1 μM $MgCl_2$, 50 μM potassium phosphate buffer (pH 7.2), 5 μM β-mercaptoethanol, and water to a final volume of 1.5 ml (13). Crude cell lysates that had been boiled for 10 min were used as negative controls and as determinants for background conversion levels. The reaction was allowed to proceed for 1 h at 23°C; the reaction mixture was acidified by the addition of 0.2 ml of 10% trichloroacetic acid and extracted twice with equal volumes of ethyl acetate. The radioactivity in samples from the organic and aqueous phases was measured in a scintillation counter and used to determine the percent conversion of [$^{14}$C]IAA to [$^{14}$C]IAA-lysine. A unit of IAA-lysine synthetase activity was defined as the amount of enzyme required to catalyze the conversion of 1 nmol of [$^{14}$C]IAA to [$^{14}$C]IAA-lysine per min. The specific activity of the enzyme was recorded as units per milligram of protein.

**Verification of IAA-lysine.** The product of IAA-lysine synthetase catalysis was isolated from the aqueous phase of the above reaction mixture by SEP-PAK C-18 (Waters Associates, Inc.) column chromatography. The column was first equilibrated with 10 mM ammonium acetate (pH 3.5); the aqueous fraction containing the reaction product was then passed through the column under $N_2$ pressure. The column was washed with 10 ml of $H_2O$, and the bound fraction was eluted with 3 ml of methanol to recover the reaction products. The methanol fraction was evaporated under $N_2$ at 30°C, and the concentrated material was applied to a silica thin-layer plate (Eastman Kodak 13181) with a fluorescent indicator. Standards of authentic IAA, IAA-lysine (synthesized by the method of Hutzinger and Kosuge [12]) and tryptophan were applied over the sample of reaction product. The thin-layer chromatographic plate was developed by two-dimensional chromatography by using the solvent systems ethyl acetate-isopropanol-$H_2O$ (6:2:1 [vol/vol/vol]) and isopropanol-ethyl acetate-concentrated ammonium hydroxide-$H_2O$ (9:7:2:2 [vol/vol/vol/vol]). After the chromatograms were developed, the fluorescent spots on the thin-layer chromatographic plates were marked, and the chromatograms were autoradiographed.

For further confirmation of the presence of IAA-lysine in the reaction mixtures, 10 μl of the methanolic samples from the C-18 column described above was chromatographed by high-pressure liquid chromatography (HPLC) with a reverse-phase Altex Ultrasphere-ODS 5-μm column (4.6 by 250 mm) as previously described for IAA analysis (1). The same procedure was used for the analysis of IAA-lysine in the supernatant fractions from cultures of *P. savastanoi* strains and *E. coli* transformants.

**Cloning of the *iaaL* locus.** *E. coli* 71-18 and the pUC8 vector were used in cloning the *iaaL* gene (25). The pUC8 vector has the M13mp8 muticloning site inserted into the β-galactosidase gene of *E. coli*. The insertion of foreign DNA into the cloning sites disrupts the β-galactosidase gene, so that *E. coli* 71-18 transformants containing inserts in the pUC8 vector can be chosen from on X-gal (International Biotechnologies, Inc.) indicator plates by choosing white colonies; blue colonies contain the unaltered vector. Procedures for the transformation of *E. coli* cells and for plasmid DNA isolation are described by Maniatis et al. (18). *P. syringae* subsp. *savastanoi* plasmid DNA isolation was performed as described by Comai and Kosuge (6).

The conditions for restriction endonuclease digestion and ligation of DNA by T4 ligase were specified by the manufacturers. The procedures for Southern blot hybridization and nick translation are described by Maniatis et al. (18).

**Tn*5* mutagenesis.** *E. coli* HB101 (3) and λ221 *rex*::Tn*5* *c*I857 *O*am8 *P*am29 were used for transposon mutagenesis of pLG87 to map the *iaaL* gene (18, 22).

**Synthesis of [$^3$H]IAA.** [$^3$H]IAA was synthesized from [$^3$H]tryptophan (specific activity, 29 Ci/mmol) with cell-free enzyme preparations from *P. syringae* subsp. *savastanoi*, by the procedure of Kosuge et al. (15).

**Source of biochemicals, radiochemicals, and restriction enzymes.** Biochemicals were obtained from Sigma, radiochemicals were purchased from Amersham Corp. and New England Nuclear Corp., and restriction enzymes and

T4 ligase were obtained from Bethesda Research Laboratories, Inc., and New England BioLabs, Inc.

## RESULTS

**Verification of IAA-lysine.** IAA-lysine was verified as the reaction product of in vitro enzyme assays of *P. syringae* subsp. *savastanoi* and *E. coli* clones by HPLC (Fig. 1) and by two-dimensional autoradiography with an authentic IAA-lysine standard (data not shown). In each case, identical chromatographic behavior was observed between the IAA-lysine standard and the product of the cell-free enzyme reaction.

**Location of *iaaL* locus.** To determine whether the IAA-lysine synthetase locus (*iaaL*) or a locus that regulated the production of IAA-lysine was present on the IAA plasmid in oleander isolates, two classes of pIAA mutants were screened for the ability to convert [$^3$H]IAA to [$^3$H]IAA-lysine. The first class of mutant (represented by strains PB213-3 and TK2015-32) was cured of pIAA, and the second class (represented by strain PB213-6) contained large deletions in pIAA which included the IAA operon. When tested for the ability to convert [$^3$H]IAA to [$^3$H]IAA-lysine, both IAA$^-$ mutants were incapable of converting [$^3$H]IAA to [$^3$H]IAA-lysine (Table 1). This information indicated that *iaaL* or a locus that regulated the production of IAA-lysine in oleander isolates resided on the IAA plasmid.

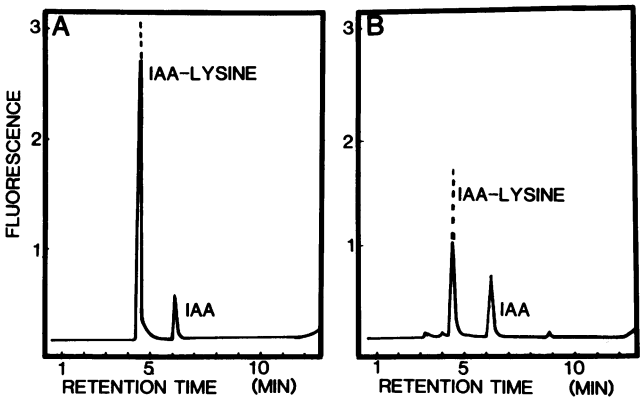


FIG. 1. Analysis by HPLC of the radioactive compounds isolated from IAA-lysine synthetase reaction mixtures; enzyme preparations were cell-free extracts of *E. coli* 71-18(pLG87) or *P. syringae* subsp. *savastanoi* EW2009. An authentic sample of IAA-lysine was used as a standard. HPLC was carried out as described in Materials and Methods. (A) Profiles of strain 71-18(pLG87) products. ——, Strain 71-18(pLG87) IAA-lysine synthetase products recovered by C-18 column chromatography. A 10-μl sample of the methanol eluate of the C-18 column was injected into the high-pressure liquid chromatograph. The assay for IAA-lysine synthetase was carried out as described in Materials and Methods; 0.3 mg of protein from the enzyme preparation from strain 71-18(pLG87) was added to the reaction mixture. – – –, Strain 71-18(pLG87) reaction products with 1 ng of authentic IAA-lysine standard added to the reaction mixture. (B) Profiles of strain EW2009 products. ——, Strain EW2009 IAA-lysine synthetase reaction products recovered by C-18 column chromatography. The sample used for HPLC was 10 μl of the methanol eluate of the C-18 column. The assay for IAA-lysine synthetase was carried out as described in the Materials and Methods; 1.6 mg of protein of the enzyme preparation from strain EW2009 was added to the reaction mixture. – – –, Strain EW2009 reaction products and 1 ng of authentic IAA-lysine standard. On a scale of 3 along the abscissa, 2 ng of IAA-lysine standard has a fluorescence of 1.5.

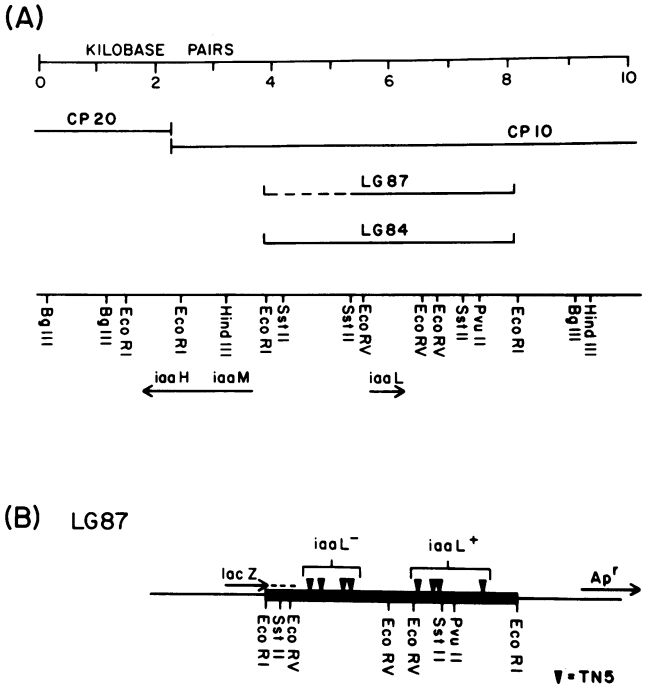


FIG. 2. (A) Partial restriction map of pIAA1 from strain EW2009. CP10 and CP20 are 18-kbp and 20-kbp fragments (C. P. Palm and T. Kosuge, unpublished results), respectively, of pIAA1 that are inserted into the *Kpn*I site of vector pSA152 (24). LG87 and LG84, 4.25-kbp and 6.2-kbp fragments, respectively, that were inserted into the *Eco*RI site of the vector pUC8(25). *Eco*RV and *Sst*II sites were mapped within fragment LG84 only. (B) Tn*5* insertion map of the *iaaL* locus in recombinant plasmid pLG87 (LG87 insert is enlarged from panel A). Tn*5* insertions in the regions designated *iaaL*$^-$ inactivated *iaaL*; those designated *iaaL*$^+$ did not affect production of IAA-lysine synthetase. *lacZ*, β-Galactosidase gene present in pUC8 (25); Ap$^r$, β-Lactamase gene present in pUC8, encoding resistance to ampicillin.

The tryptophan monooxygenase gene (*iaaM*) and the indoleacetamide hydrolase gene (*iaaH*) of *P. syringae* subsp. *savastanoi* are organized in an operon in oleander isolates (Fig. 2A). A third class of mutants with insertions in the *iaaM* gene inactivate the IAA operon and have undetectable tryptophan monooxygenase and indoleacetamide hydrolase activity (8). The nucleotide sequences of the *iaaM* and *iaaH* genes (28) and the results from a promoter probe analysis (7) indicate that the *iaaM* gene is the first gene transcribed in the operon. Thus, insertions in the *iaaM* gene would have a polar effect on the expression of genes distal to the IAA operon promoter. If the putative *iaaL* locus is also part of the IAA operon, mutants with insertions in the *iaaM* gene should not convert [$^3$H]IAA to [$^3$H]IAA-lysine. However, *iaaM* insertion mutants TK2015-3 and TK2009-5 both accumulated [$^3$H]IAA-lysine when administered [$^3$H]IAA; each result suggests that the *iaaL* locus (or a locus that regulated the production of IAA-lysine) was not promoter distal with respect to the *iaaM* gene.

When compared with the parental strain in its ability to produce IAA-lysine, the insertion mutant TK2015-3 acumulated [$^3$H]IAA-lysine in amounts comparable to those of TK2015 (Fig. 3). The conversion of [$^3$H]IAA to [$^3$H]IAA-lysine was detected in TK2015-3 immediately upon the addition of [$^3$H]IAA. By contrast, the pIAA$^-$ mutant

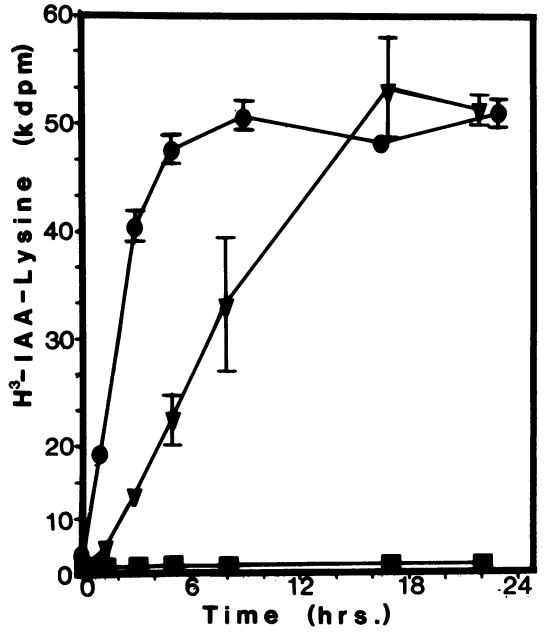


FIG. 3. Time course of accumulation of [$^3$H]IAA-lysine in culture over a 24-h growth period. [$^3$H]IAA was added to the cells at time zero, after which samples of culture fluid were monitored for [$^3$H]IAA-lysine accumulation at designated time points. Procedures for [$^3$H]IAA-lysine analysis are described in Materials and Methods. Symbols: ▼, TK2015, wild-type *P. syringae* subsp. *savastanoi*; ●, TK2015-3, spontaneous IAA$^-$ mutant of TK2015, in which IS*52* has insertionally inactivated the *iaaM* gene; ■, TK2015-32, spontaneous mutant of TK2015 lacking pIAA and incapable of converting tryptophan to IAA.

TK2015-32 did not accumulate [$^3$H]IAA-lysine during the 24-h incubation with [$^3$H]IAA.

A number of *P. syringae* subsp. *savastanoi* strains isolated from olive, oleander, or privet were screened for the ability to convert [$^3$H]IAA to [$^3$H]IAA-lysine (Table 1). Previously, it was shown that oleander isolates bear the IAA genes on a plasmid, although the size of the IAA plasmid may vary among strains (9). In all cases, the ability of oleander isolates to form IAA-lysine was correlated with the presence of pIAA; concomitant with the loss of this plasmid was the loss of the ability of the bacterium to form IAA-lysine. In olive and privet isolates, which contain the IAA genes on the chromosome or on a megaplasmid (9), the conversion of [$^3$H]IAA to [$^3$H]IAA-lysine was undetectable.

**Cloning and expression of *iaaL* in *E. coli*.** Evidence from the previous experiments indicates that the *iaaL* gene, or a locus that regulated the production of IAA-lysine, resided on the IAA plasmid because the conversion of [$^3$H]IAA to [$^3$H]IAA-lysine was undetectable in *P. syringae* subsp. *savastanoi* mutants lacking pIAA. To determine the location of the *iaaL* locus, we cloned the gene from pIAA1 and examined its expression in *E. coli*. The conversion of [$^3$H]IAA to [$^3$H]IAA-lysine in the *E. coli* clones indicates that the *iaaL* gene had been cloned, rather than a locus that regulated the production of IAA-lysine; neither an IAA biosynthetic pathway nor an enzyme converting IAA to IAA-lysine has been reported to occur in *E. coli*. *E. coli* cells bearing constructs with *Kpn*I fragments CP10 and CP20 inserted in plasmid vector pSA152 (24) were screened for the accumulation of [$^3$H]IAA-lysine in the media after incubation with [$^3$H]IAA. CP10 and CP20 (C. P. Palm and T. Kosuge, unpublished results) are contiguous fragments which span 40 kilobase pairs (kbp) of the 52-kb pIAA1 plasmid and include the IAA operon (Fig. 2A). Although a low level of accumulation of [$^3$H]IAA-lysine (3% of the [$^3$H]IAA added, 4-h incubation) was detected in transformants containing pCP10, no accumulation of [$^3$H]IAA-lysine was detected in *E. coli*(pCP20). To further delineate the location of *iaaL* within fragment CP10, fragments of CP10 were generated by *Eco*RI digestion and ligated into the *Eco*RI site of pUC8, and the resulting plasmids were transformed into *E. coli* 71-18. Transformants were screened for the ability to accumulate [$^3$H]IAA-lysine in the media. One of these transformants, *E. coli*(pLG84), containing a 6.25-kbp *Eco*RI fragment of pIAA1, accumulated [$^3$H]IAA-lysine (10% conversion of [$^3$H]IAA to [$^3$H]IAA-lysine). This fragment was further trimmed down to 4.25 kbp by partial digestion with *Sst*II, and the resulting fragment was religated into pUC8 to construct recombinant plasmid pLG87. When tested for the formation of IAA-lysine, *E. coli*(pLG87) accumulated 8% of the administered [$^3$H]IAA as [$^3$H]IAA-lysine after a 4-h incubation with [$^3$H]IAA. This suggests that the putative *iaaL* locus resided in a 6.25-kbp *Eco*RI fragment adjacent to an *Eco*RI-*Sal*I fragment that contained the IAA operon (Fig. 2A).

The optimal expression of IAA-lysine synthetase in *E. coli* would facilitate characterization of the enzyme activity. However, *E. coli* whole cells containing the cloned *iaaL* locus accumulated only low levels of [$^3$H]IAA-lysine when incubated with [$^3$H]IAA. In parallel experiments, as much as 70% of the [$^3$H]IAA added to *P. syringae* subsp. *savastanoi* whole cells was converted to [$^3$H]IAA-lysine. *P. syringae* subsp. *savastanoi* has an active transport system for IAA (19) and presumably a secretion system for IAA-lysine, whereas a similar system has not been described in *E. coli*. Thus, to avoid limitations imposed by the absence of an IAA transport or a IAA-lysine secretion system or both, the conversion of IAA to IAA-lysine was examined in cell-free preparations of *E. coli*(pLG87) and *P. syringae* subsp. *savastanoi*; it was apparent that *E. coli*(pLG87) contained a much higher level (47-fold) of IAA-lysine synthetase activity than did *P. syringae* subsp. *savastanoi* (Table 2). As expected, the conversion of [$^{14}$C]IAA to [$^{14}$C]IAA-lysine was not detected in cell-free preparations of either strain

TABLE 2. Expression of IAA-lysine synthetase

| Strain | Plasmid | Sp act[a] (U/mg of protein) |
|---|---|---|
| *P. syringae* subsp. *savastanoi* | | |
| EW2009 | pIAA1 | 0.251[b] |
| TK2009-3 | No pIAA1 | 0.0[c] |
| *E. coli* | | |
| 71-18(pLG87) | 4.25-kbp *P. syringae* subsp. *savastanoi* DNA in pUC8 | 11.7[b] |
| 71-18(pLG90) | Orientation isomer of pLG87 | 0.085[b] |
| 71-18(pLG24) | Tn*5* Insertion in pLG87 | 0.0[c] |
| 71-18(pUC8) | Vector plasmid | 0.0[c] |

[a] One unit is defined as the amount of enzyme required to catalyze the conversion of 1 nmol of [$^{14}$C]IAA to [$^{14}$C]IAA-lysine per min under standard assay conditions.

[b] Each numerical value is the mean of three trials.

[c] Numerical values obtained were less than those for boiled enzyme controls.

TK2009-3, an isolate of *P. syringae* subsp. *savastanoi* lacking pIAA1, or *E. coli* 71-18(pUC8).

An additional clone (pLG90) was generated in which the 4.25-kbp *Eco*RI *P. syringae* subsp. *savastanoi* DNA fragment was inserted in pUC8 in an orientation opposite to that of pLG87. Enzyme assays showed that transformants with this construct converted low levels of [$^{14}$C]IAA to [$^{14}$C]IAA-lysine (Table 2), indicating that the transcription of the *iaaL* gene by *E. coli* is controlled by the β-galactosidase promoter in pUC8. This information also indicates that the direction of transcription of the *iaaL* gene was opposite to that of the IAA operon.

**Tn*5* mutagenesis of the *iaaL* locus.** To determine the location of the *iaaL* gene on the cloned fragment of pIAA1, *E. coli*(pLG87) was subjected to Tn*5* insertion mutagenesis. Tn*5* insertions into the *iaaL* gene inactivate the gene, and *E. coli* cells bearing these constructs have undetectable IAA-lysine synthetase activity in cell-free preparations. Plasmid DNA from *E. coli* transformants resistant to kanamycin and ampicillin was digested with various restriction endonucleases to map the sites of Tn*5* insertion in fragment LG87 (Fig. 2B). In the 32 colonies examined, eight different Tn*5* insertions were located in the 4.25-kbp *P. syringae* subsp. *savastanoi* DNA fragment. When assayed for the conversion of [$^{14}$C]IAA to [$^{14}$C]IAA-lysine in a cell-free enzyme assay, four of the eight Tn*5* insertion mutants lacked IAA-lysine synthetase activity (Table 2, pLG24). The pLG87-Tn*5* insertion sites in these mutants mapped within a 1.8-kbp *Eco*RV fragment, as determined by restriction endonuclease digestion and Southern blot hybridization (Fig. 4). The 1.8-kbp *Eco*RV fragment of pLG87 (lane 2) was enlarged to 7.5 kbp by the insertion of the 5.7-kbp Tn*5* DNA (lane 3). The insertion event was confirmed by Southern blot analysis by using the 1.8-kbp *Eco*RIV fragment as a probe, which hybridized to the larger 7.5-kbp fragment (lane 5). Insertions 4 to 6.25 kbp upstream from the IAA operon did not affect the ability of *E. coli*(pLG87) to convert IAA to IAA-lysine. We conclude that insertions within an 1.8-kbp *Eco*RV fragment inactivated the cloned *iaaL* locus in *E. coli* and that the *iaaL* gene in *P. syringae* subsp. *savastanoi* was located within a 2.5-kbp fragment 2 kbp from the IAA operon on the IAA1 plasmid (Fig. 2).

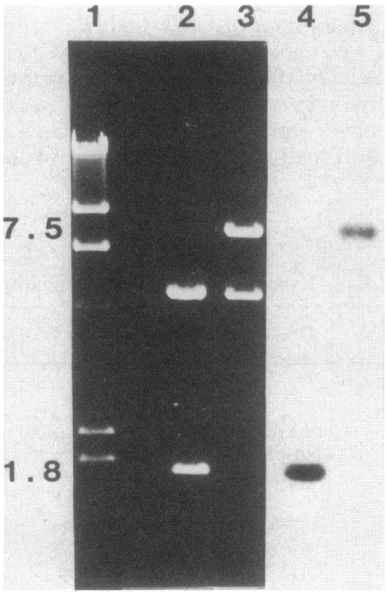


FIG. 4. Restriction endonuclease cleavage analysis of Tn*5* insertions in pLG87. Lanes: 1, coliphage lambda digested with *Hin*dIII; 2, pLG87 digested with *Eco*RV; 3, pLG87::Tn*5* (inactivated *iaaL* gene) digested with *Eco*RV; 4, Southern blot analysis of lane 2 with 1.8-kbp *Eco*RV fragment from pLG87 used as the probe; 5, Southern blot analysis of lane 3 with 1.8-kbp *Eco*RV fragment from pLG87.

## DISCUSSION

We have cloned the *iaaL* locus into pUC8 and detected a high expression of IAA-lysine synthetase in *E. coli* transformants. The specific activity of the enzyme in cell-free preparations from *E. coli*(pLG87) was significantly higher than that in preparations from wild-type *P. syringae* subsp. *savastanoi*. When the cloned insert was oriented in the opposite direction in the vector, a low specific activity of IAA-lysine synthetase was detected in *E. coli* transformants. *P. syringae* subsp. *savastanoi* promoters are weakly recognized in *E. coli*; the efficient expression of a *P. syringae* subsp. *savastanoi* gene in *E. coli* requires the presence of an *E. coli* promoter (7, 11, 28). Therefore, the expression of IAA-lysine synthetase in *E. coli* is dependent on the *lacZ* promoter in the pUC8 vector. In addition, the pUC8 vector is a pBR322 derivative and is maintained at between 20 to 30 copies per cell. Both of these factors could contribute to the high specific activity of IAA-lysine synthetase detected in *E. coli* extracts. The high expression of the cloned IAA-lysine synthetase gene in *E. coli* could facilitate the purification and characterization of the enzyme; the strategy of using a high expression of cloned genes in *E. coli* has facilitated the purification of *P. syringae* subsp. *savastanoi* tryptophan monooxygenase (11).

The two genes, *iaaM* and *iaaH*, coding for the synthesis of IAA, occur on a plasmid in oleander isolates of *P. syringae* subsp. *savastanoi* and are organized in an operon (6, 8). We determined that the *iaaL* locus was on the same plasmid as the IAA genes but was not part of the IAA operon, because insertion mutations in the *iaaM* gene had no effect on the ability of the bacterium to synthesize IAA-lysine. However, the *iaaL* locus was within 2 to 3 kbp of the IAA operon but was transcribed in the opposite direction (Fig. 2). It is unknown whether any functions relating to IAA metabolism are located within the 2- to 3-kbp region between the *iaaL* gene and the IAA operon. Different sizes of the IAA plasmid and various plasmid compositions occur among isolates of *P. syringae* subsp. *savastanoi* (9). Loss of pIAA is always correlated with loss of the ability to synthesize IAA-lysine, indicating that the *iaaL* locus is on the same plasmid as the IAA operon in a variety of oleander isolates. Olive and privet isolates, however, did not accumulate IAA-lysine to an appreciable extent. It is unknown at this time whether the inability to form IAA-lysine in these isolates plays a significant role in the virulence of the bacterium.

The production by *P. syringae* subsp. *savastanoi* of a secondary metabolite, IAA, helps confer tumorigenicity on olive and oleander plants. Loss of the capacity to synthesize IAA results in the inability of the bacterium to incite galls, although the mutant will multiply as well as its parent in host tissue for up to 16 days after inoculation (23). Thus, IAA as a metabolite is not necessary for the *P. syringae* subsp. *savastanoi* to grow within host tissue, but is required for the expression of symptoms of the disease.

Bacterial mutants with elevated levels of IAA in culture will incite the formation of larger galls on oleander plants than will the wild-type *P. syringae* subsp. *savastanoi* (23).

Therefore, the amount of IAA accumulated in culture can be related to virulence on olive and oleander as assayed by gall size. The IAA pool size in the bacterium is regulated by the rate of synthesis of IAA and the rate of conversion of IAA to additional compounds such as IAA-lysine (15). Thus, perturbations in the conversion of IAA to IAA-lysine are expected to affect gall development.

IAA-lysine is one-third as active as IAA in growth-promoting activity as assayed by the *Avena* coleoptile growth curvature test (13) and may also be less active than IAA in promoting gall formation in olive and oleander plants. It is presumed that IAA is freed in plant tissue by the hydrolysis of IAA-lysine and that IAA is the active form promoting tumor formation. However, free IAA would be susceptible to degradation by host plant peroxidases and IAA oxidases, while IAA conjugates are resistant to degradation. Conjugates are regarded as storage forms of IAA in plants (4). In addition, plants contain the necessary enzymes that hydrolyze IAA amino acid conjugates and release the biologically active IAA (2, 10). Therefore, it is possible that IAA-lysine produced and secreted into host tissue by *P. syringae* subsp. *savastanoi* may also function as a storage form of IAA in gall tissue. Although *P. syringae* subsp. *savastanoi* is the only bacterium described that produces an IAA conjugate, such compounds are ubiquitous in plants and account for 98% of the IAA sequestered in inactive forms (4, 5). IAA conjugates are thought to be slow-release forms of IAA that can be hydrolyzed when IAA is needed by the plant. By studying the role of IAA-lysine in gall development on olive and oleander, concepts concerning the role of IAA conjugates in plants can be revealed.

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