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Biochemistry

Expression and fine structure of the gene encoding N^{ϵ}-(indole-3-acetyl)-L-lysine synthetase from *Pseudomonas savastanoi*

(plant hyperplasia/amino acid conjugate)

FRANCISCO F. ROBERTO*†, HARRY KLEE‡, FRANK WHITE§, RUSSELL NORDEEN*¶, AND TSUNE KOSUGE*||

‡Monsanto, Chesterfield, MO 63198; §Department of Plant Pathology, Kansas State University, Manhattan, KS 66506; and *Department of Plant Pathology, University of California, Davis, CA 95616

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ABSTRACT **The gene encoding N^{ϵ}-(indole-3-acetyl)-L-lysine synthetase, *iaaL*, from *Pseudomonas savastanoi* was localized within a 4.25-kilobase *Eco*RI fragment derived from pIAA1 of oleander strain EW 2009. Two open reading frames of 606 and 1188 nucleotides were identified upon sequencing, which directed the *in vitro* synthesis of M_r 21,000 and M_r 44,000 proteins. Expression of an open reading frame-2 subclone, pMON686, in *Escherichia coli* indicates that (indole-3-acetyl)-L-lysine synthetase is encoded solely by open reading frame-2. Hydrophobicity plots of the deduced open reading frame-1 protein suggest that it may be a membrane-bound protein, whereas the predicted *iaaL* gene product possesses considerable hydrophilic character, consistent with the demonstration of (indole-3-acetyl)-L-lysine synthetase activity in cell-free aqueous extracts. No nucleotide or protein homologies were found between *iaaL* and any sequences contained within the GenBank or National Biomedical Research Foundation data bases (April 13, 1989).**

Infection and colonization of olive and oleander plants by the plant pathogenic bacterium *Pseudomonas syringae* pv. *savastanoi* (*P. savastanoi*) is manifested by the appearance of hyperplasias termed "galls" (1–3). The production of indole-3-acetic acid (IAA) and cytokinins by the bacterium is necessary for the elicitation of this response in the plant, and therefore, these bacterially produced compounds are virulence factors in the disease interaction (4, 5). Further metabolism of IAA has been observed in *P. savastanoi* oleander isolates, and the amino acid conjugate, N^{ϵ}-(indole-3-acetyl)-L-lysine (IAA-lysine), and an acetylated derivative, N^{α}-acetyl-N^{ϵ}-(indole-3-acetyl)-L-lysine (*N*-acetyl-IAA-lysine) are found in culture filtrates of *P. savastanoi* (6–8).

The genes for synthesis of phytohormones reside on the 52-kilobase (kb) plasmid, pIAA1, in oleander strain EW2009 (9, 10). A 4.25-kb *Eco*RI fragment was found to confer on *Escherichia coli* the ability to synthesize IAA-lysine from IAA, and transposon mutations in the IAA-lysine synthetase locus in *P. savastanoi* were obtained by marker exchange (11, 12). The mutants showed an increase in IAA accumulation and were attenuated in virulence and in their ability to grow within the plant (12). Therefore, the IAA-lysine synthetase gene represents an additional bacterial locus that influences microbial–plant interactions.

The locus for IAA-lysine synthesis has not been fully characterized, and the number of genes involved is not known. We report here the determination of the nucleotide sequence** of the IAA-lysine synthetase gene *iaaL* and demonstrate that *iaaL* is sufficient for IAA-lysine production in *E. coli*.

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MATERIALS AND METHODS

Bacterial Strains, Media, and Plasmids. *E. coli* HB101 (pLG87) (11), HB101 (pLG87X), *E. coli* K38 (pMON686), K38 (pMON686ΔRV), K38 (pMON676), and pLG/SK were used for plasmid DNA isolations, DNA sequencing, and enzyme assays. Restriction enzyme sites for these plasmids derived from *P. savastanoi* pIAA1 and their orientation relative to one another are shown in Fig. 1. Bacteria were maintained on LB agar plates incubated at 37°C, and cells used for plasmid isolations were grown in LB liquid medium (broth). Plasmids were maintained on medium supplemented with ampicillin (50 μg/ml).

Recombinant DNA Techniques. Restriction enzyme digestions, ligations, plasmid isolation, and purification were performed as described by Maniatis *et al.* (13).

Nucleotide Sequence Determination. Unidirectional exonuclease III deletions (14) were performed to produce a set of nested clones derived from pLG87, cloned in both orientations within pUC8. Except where noted, the DNA sequence was obtained from denatured double-stranded templates using pUC/M13 universal and reverse sequencing primers (New England Biolabs) and the cloned Klenow fragment of DNA polymerase I, as described (15). Other sequence information was obtained from single-stranded templates generated from pLG/SK (4.25-kb *Eco*RI fragment from pLG87 cloned in pBSSK+; Stratagene) and pMON676 using Sequenase (modified phage T7 DNA polymerase; United States Biochemical) according to the manufacturer's instructions. DNA sequence analysis was performed with the GCG software package (16) and protein homology searches were performed using the FASTA program of Pearson and Lipmann (17).

***In Vitro* Protein Synthesis.** DNA fragments spanning the *iaaL* locus were cloned into plasmid pT7-7, where they were placed under the control of the phage T7 promoter (18). These plasmids were introduced into *E. coli* K38 containing the plasmid pGP1-2, which directs the production of T7 RNA polymerase (18). Proteins were induced in the presence of [^{35}S]methionine and analyzed by electrophoresis on a 12.5% polyacrylamide gel containing SDS as described (18).

Enzyme Assay. Bacterial cells were grown overnight in 2 ml of LB broth supplemented with ampicillin and kanamycin

Abbreviations: IAA, indole-3-acetic acid; IAA-lysine, N^{ϵ}-(indole-3-acetyl)-L-lysine; *N*-acetyl-IAA-lysine, N^{α}-acetyl-N^{ϵ}-(indole-3-acetyl)-L-lysine.

†Present address: Biotechnology, Idaho National Engineering Laboratory, Idaho Falls, ID 83415-2203.

¶Present address: U.S. Department of Agriculture, Beltsville Agricultural Research Center West, Plant Molecular Biology Laboratory, Beltsville, MD 20705.

||Deceased, March 13, 1988.

**The sequence reported in this paper has been deposited in the GenBank data base (accession no. M35373).

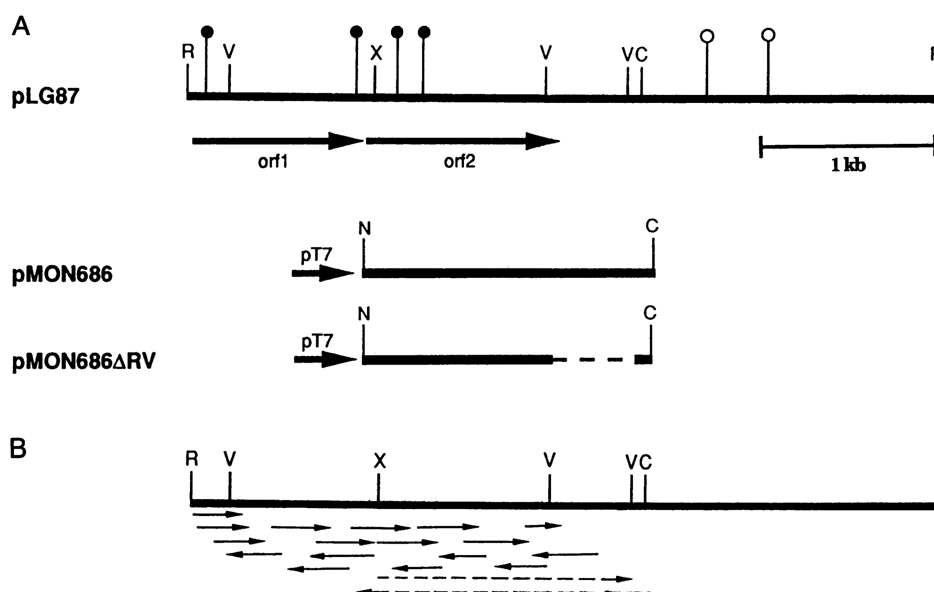

FIG. 1. Maps of clones used, Tn5 insertions, and sequencing strategy. (A) pLG87 is shown. R, *Eco*RI; V, *Eco*RV; X, *Xba* I; C, *Cla* I; N, *Nde* I. Other subclones are shown oriented to pLG87. Inserts in pMON686 and pMON686ΔRV are under the control of the bacteriophage T7 promoter. Tn5 insertions are depicted above the map. Solid circles indicate that the Tn5 insertion inactivates *iaaL*. Open circles indicate that the Tn5 insertion has no effect on *iaaL*. (B) DNA sequencing strategy. Sequence information obtained from double-stranded templates (pLG87 deletions) is depicted by solid arrows, with arrowheads indicating the direction and extent of the sequencing reaction away from primer; dashed arrows indicate sequences obtained from single-stranded templates (generated from pLG/SK) and oligonucleotide primers.

(required for maintenance of pGP1-2) at 28°C. A 0.4-ml sample from the overnight culture was inoculated into 40 ml of LB broth containing antibiotics and incubated for 5 hr at 28°C in 125-ml flasks. The cultures were heated to 42°C in a water bath with gentle shaking and then incubated at 37°C for 1 hr. The bacteria were harvested by centrifugation and resuspended in 10 ml of 0.05 M Tris·HCl/0.02 M 2-mercaptoethanol, pH 8.0. The samples were chilled on ice for 15 min and lysed by sonication. Sonication was performed for two 30-sec pulses with a 1-min interval at 100 W by using the standard probe on a Braun Sonic 2000 sonicator. Each lysate was centrifuged for 20 min at 17,000 rpm in a Ti 70 (Beckman Instruments) rotor at 4°C. The lysate was kept on ice and a sample was removed for measurement of IAA-lysine synthetase activity.

Activity was assayed in a total reaction volume of 0.6 ml for each lysate in a 1.5-ml Eppendorf tube. The reaction mixture consisted of 28 mM K_2HPO_4 (pH 7.8), 0.5 mM $MnCl_2$, 0.5 mM $MgCl_2$, 16.7 mM L-lysine, 1.3 mM ATP, 55 μM IAA, to which 4 μl of [^{3}H]IAA (specific activity, 21.7 Ci/mmol, 1 Ci/ml; 1 Ci = 37 GBq; Amersham) and 0.28 ml of lysate were added. The reaction mixtures were incubated for 2 hr at 28°C at which time 0.3 ml of 10% (wt/vol) trichloroacetic acid was added. Denatured protein was pelleted by centrifugation for 3 min in an Eppendorf microcentrifuge. The supernatant (0.5 ml) was extracted twice with an equal volume of ethyl acetate. Radioactivity in samples of extract (50 μl) was measured in 5 ml of Scintiverse-BD solution (Fisher) in a United Technologies Packard Tri-Carb series 4000 scintillation counter.

Total protein of each lysate was measured by the method of Bradford (19) using a commercial kit according to the supplier's recommendations (Bio-Rad).

Each bacterial strain was assayed three times and the radioactivity was normalized to the total protein and averaged.

Thin Layer Chromatography. Lysate (0.45 ml) was passed twice over a Sep-Pak C_{18} column (Waters) equilibrated with 10 mM ammonium acetate (pH 3.5). The column was washed twice with 5 ml of deionized H_2O and the bound IAA-lysine was eluted with two 1.5-ml volumes of methanol. The eluate was dried in a vacuum centrifuge and resuspended in 0.05 ml of distilled H_2O. A 6-μl sample was applied to a precoated silica gel 60 plate (HPTLC; EM Science). [^{3}H]IAA (4 μl at 10 μCi/ml) and IAA-lysine (6 μl at 10 mg/ml) were applied as standards. The chromatogram was developed in butanol/acetic acid/water, 4:1:4 (vol/vol). After drying, the chromatogram was sprayed with 0.2% ninhydrin. After visualizing the amino acid-positive spots, the treated plate was fluorographed.

RESULTS

DNA Sequence Determination. A previous study (11) described Tn5 insertions in the left half of pLG87 that prevented expression of IAA-lysine synthetase, whereas two insertions farther downstream had no effect (Fig. 1A). Exonuclease III-generated deletion subclones were produced from pLG87 and sequenced through the second *Eco*RV site (solid arrows, Fig. 1B) revealing two open reading frames (ORFs). The first ORF (ORF1) contained 606 nucleotides and is predicted to encode a polypeptide containing 202 amino acids with a molecular weight of 21,000 (Fig. 2). ORF2 contained 1188 nucleotides and is predicted to encode a protein containing 396 amino acids with a molecular weight of 44,000. A potential ribosome binding site, -UGGU-, for ORF1 was observed 15 base pairs (bp) upstream of the methionine initiation codon, and 6 bp upstream of the ORF2 translation initiation site the sequence -AGGA- was observed, which agrees quite well with the consensus Shine–Delgarno sequence. No sequences resembling the consensus *E. coli* promoter motif were observed on the 5′ side of either ORF1 or ORF2.

Hydrophobicity plots of ORF1 and ORF2 revealed considerable hydrophobic character for the ORF1-predicted protein, extending virtually the entire length of the polypeptide, with the exception of the amino and carboxyl termini. On the other hand, ORF2 appeared to encode a fairly hydrophilic protein (Fig. 3). No extended nucleotide or amino acid homologies have been found upon comparison of ORF1

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gaattccatagcgtgcggggcttggaggagcgccgcggcctgagtatctgtggctaacccttgcggcttcggtgctggtc   80
gctgtcgagcagctatgcgtgcagatcctgcgcagtgcaggcttcggcaaacaggcgatgtggaccctgctgacggggac  160
                  M  R  A  D  P  A  Q  C  R  L  R  Q  T  G  D  V  D  P  A  D  G  D
ggccgccgttgccatcgcagatcccctgcttattgtggcgttcgatctgggcctggtgggtgccggcatcgctacctgat  240
G  R  R  C  H  R  R  S  P  A  Y  C  G  V  R  S  G  P  G  G  C  R  H .R  Y  L  I
atcgagcctggtatcggcctgtctggggttttactacgttcaccgagtcgcccatctgacctgtcgggtcagcctgaaga  320
 S  S  L  V  S  A  C  L  G  F  Y  Y  V  H  R  V  A  H  L  T  C  R  V  S  L  K  N
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 L  S  G  D  I  R  N  I  G  R  T  A  L  P  A  V  I  G  N  L  A  T  P  V  G  M
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A  Y  V  M  A  A  M  A  P  F  G  S  Q  A  L  A  T  I  G  V  I  D  R  V  I  Q  V
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 A  F  C  V  V  F  A  L  P  G  A  L  I  P  I  L  G  Q  N  L  G  A  M  N  T  A  R
gcgtgtctcaagccataaagatgacgtacggattgttgatcggctacggctcagtgacctcgctgttactcattctgctc  640
 V  S  Q  A  I  K  M  T  Y  G  L  L  I  G  Y  G  S  V  T  S  L  L  L  I  L  L
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A  E  P  L  A  S  L  F  H  L  A  A  E  R  Q  V  V  F  F  A  F  C  R  W  G  G  A
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 L  D  A  H  R  A  A  I  H  C  H  L  S  L  P  Q  Y  G  A  T  G  V  R  H  T  V  R
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 L  V  P  R  H  L  G  T  M  P  F  V  W  Y  G  A  H  K  F  G  S  V  G  V  M  L
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 R  D  P  F  N  R  E  P  I  P  P  Q  E  *
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                                                         M  T  A  Y  D  M  E
aaggaatggagcagaatttccaccactgccgctaaaatccaccagaacaacgattttgaaggattcacttatcaggactt 1200
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 F  S  A  L  N  N  T  I  T  M  M  A  G  N  C  G  A  H  V  V  S  V  G  D  I  T  L
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 V  T  K  S  H  F  E  A  L  N  S  I  K  L  N  V  L  L  G  V  P  S  T  I  L  Q
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F  I  N  A  M  Q  H  N  G  V  H  I  N  I  E  K  V  V  F  T  G  E  S  L  K  T  F
ccagaagaaaatcatcaggcaagcctttggcgaacaagtctccatcgtcggtgtgtatggcagttccgagggcggcattc 1760
 Q  K  K  I  I  R  Q  A  F  G  E  Q  V  S  I  V  G  V  Y  G  S  S  E  G  G  I  L
tcggtttcaccaacagcccttgccacactgaatacgagtttctgtccgacaagtatttcatcgaaaaagaaggcgacagc 1840
 G  F  T  N  S  P  C  H  T  E  Y  E  F  L  S  D  K  Y  F  I  E  K  E  G  D  S
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I  L  I  T  S  L  T  R  E  N  F  T  P  L  L  R  Y  R  L  G  D  T  A  T  L  S  M
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 K  G  D  K  L  Y  L  T  D  I  Q  R  E  D  M  S  F  N  F  M  G  N  L  I  G  L  G
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 S  E  R  G  K  M  L  Y  I  I  D  R  R  N  *
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gcggattcgcatcaccgccgtcattcaccacgatgaggagccgcct  2766
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FIG. 2. Nucleotide sequences and predicted amino acid sequences of ORF1 (nucleotides 174–999) and ORF2 (nucleotides 1179–2278). The amino acid sequence is located below the corresponding DNA sequences. *, Termination codons.

and ORF2 sequences with the GenBank or National Biomedical Research Foundation protein data bases (April 13, 1989).

Subcloning of ORF2 and Expression of IAA-Lysine Synthetase. To determine which ORF encoded IAA-lysine synthetase, the *Xba* I site 157 bp within ORF2 was disrupted. This site was filled-in with the large fragment of DNA polymerase I (Klenow enzyme) and the blunt ends were ligated with T4 DNA ligase. This derivative of pLG87, designated pLG87X, was unable to express a functional IAA-lysine synthetase (Table 1). The loss of activity indicated that ORF2 either alone or in concert with ORF1 was required for IAA-lysine synthesis. To establish that expression of ORF2 alone was sufficient for the conversion of IAA to IAA-lysine, a subclone of ORF2 was constructed in pT7-7. An *Nde* I–*Cla* I fragment containing ORF2 was excised from pLG87 and ligated into the vector to produce pMON686 (Table 1). A derivative of this plasmid, pMON686ΔRV, was then created by deleting an *Eco*RV fragment. The effect of the deletion was to remove the carboxyl-terminal 41 amino acid residues of the ORF2 protein. Both plasmids were introduced into *E. coli* K38 containing the plasmid pGP1-2, which expresses the T7 RNA polymerase when the culture is grown at 37°C (18). The plasmid pMON686 directed the production of [^{3}H]IAA-lysine from [^{3}H]IAA by extracts of *E. coli*, but deletion of 123 nucleotides on the 3′ side of ORF2 (pMON686ΔRV) abolished IAA conversion (Table 1). The ATP dependence of the synthetase was also demonstrated by the dramatic reduction in activity when ATP was omitted from the reaction mixture (Table 1).

The identity of the radiolabeled metabolite of IAA as IAA-lysine was verified by thin-layer chromatography of the aqueous reaction products that had been concentrated on a small reverse-phase silica column (Fig. 4). Authentic IAA-lysine was run as a standard (Fig. 4*A*, lane 2) and, after development of the chromatogram, detected by reaction with ninhydrin spray reagent. A ninhydrin-reacting compound from a lysate of *E. coli* (pMON686) was found to comigrate with the standard (Fig. 4*A*, lane 4), and upon fluorography the

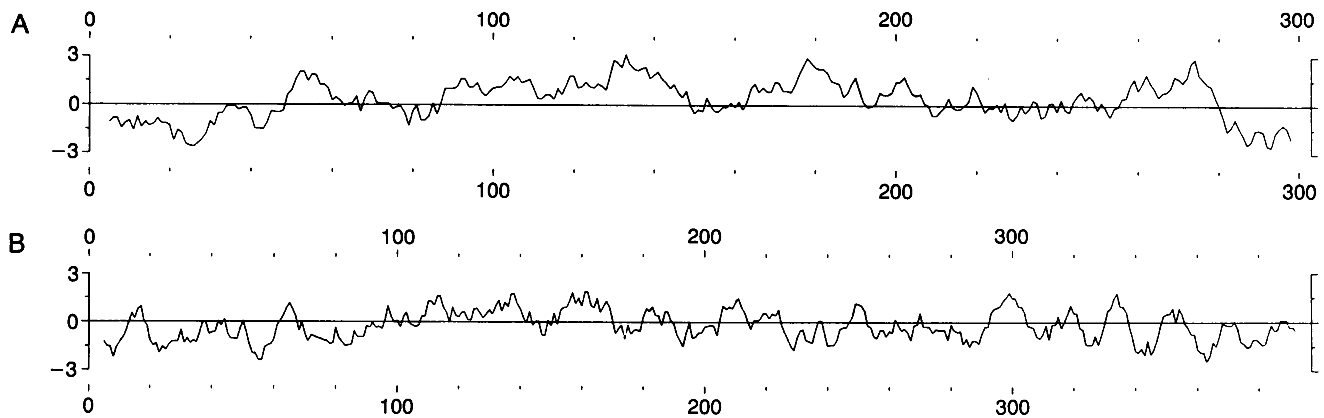

FIG. 3. Hydropathy plots for ORF1 and ORF2 peptides. Analysis was performed using the Wisconsin Genetics Computer Group computer program (16), according to the rules established by Kyte and Doolittle (20). When the line is in the upper half of the frame, it indicates a hydrophobic region, and when it is in the lower half, it indicates a hydrophilic region.

radioactivity was determined to be found only in this compound (Fig. 4*B*, lane 4). Radiolabeled IAA was also run as a fluorography standard on the same thin-layer plate to rule out the radioactivity as being contributed by IAA, which might have been carried over during the ethyl acetate extraction procedure. As can be seen from the fluorogram, IAA (Fig. 4*B*, lane 1) has a much greater mobility in the selected solvent system than does IAA-lysine (lane 4).

***In Vitro* Protein Expression.** Proteins expressed in *E. coli* from pMON686 were labeled with [^{35}S]methionine and separated by SDS/PAGE. A fluorogram showing the electrophoretic separation of these proteins is shown in Fig. 5. The estimated molecular weight of the protein expressed by *iaaL* (ORF2) is ≈44,000, which agrees with the predicted molecular weight of 44,605. The doublet observed is likely due to posttranslational modification. Radiolabeled proteins encoded by pLG87 were synthesized in a DNA-directed *in vitro* transcription/translation system where two proteins of M_r 21,000 and M_r 41,000 were observed upon fluorography (data not shown).

DISCUSSION

The naturally occurring auxin IAA is critical to plant growth and development. IAA appears to control the processes of apical dominance, cell elongation, and xylem differentiation (21). Regeneration of plants *in vitro* has been demonstrated to depend on the ratio of auxin to cytokinin in growth medium (22). Modified forms of IAA are well known in plants, where they are believed to confer transport, storage, and detoxification properties on the auxin. Glycosyl conjugates are frequently found in seed tissues, where they are believed to provide storage and/or transport forms of IAA that can be utilized during germination and early development (23). Aspartyl and glutamyl conjugates of IAA are also readily isolated from plant tissues and have been postulated to represent inactive forms of the hormone (23).

We describe here the nucleotide sequence of a bacterial enzyme involved in the further metabolism of IAA. Two ORFs have been found within a 4.25-kb *Eco*RI fragment from the plant-pathogenic bacterium, *P. savastanoi*, that have been shown to direct production of IAA-lysine (11). ORF1 is predicted to encode a protein of M_r 21,000 and ORF2 is predicted to encode a protein of M_r 44,000. The estimated molecular weights of proteins synthesized *in vitro* from these genes agree with those predicted from the DNA sequence. A subclone containing only ORF2 (pMON686) was sufficient to produce a protein of M_r 44,000 and to allow conversion of IAA to IAA-lysine in cell-free assays. We thus conclude that ORF2 alone comprises *iaaL*, the gene encoding IAA-lysine synthetase.

ORF1 and ORF2 are probably members of an operon since transposon insertions at the 5′ end of pLG87 and not in ORF2 affected IAA-lysine synthetase expression. A sequence resembling *Pseudomonas putida* promoters (24) is located 30 bp upstream of the ATG start of ORF1 but has only 9-bp spacing rather than the proposed consensus TGGC-(10 bp)-TGCT. Promoters so far identified in *P. savastanoi* appear to resemble the *E. coli* consensus promoter (ref. 25; T. Gaffney, and T.K., unpublished results; J. Kelly and T.K., unpublished results). Neither class of sequences has been identified

Table 1. Production of IAA-lysine by *E. coli*

Strain	Plasmid	IAA-lysine, cpm
HB101	pLG87	30,954 ± 3250
HB101	pLG87X	961 ± 17
K38	pMON686	38,768 ± 2004
K38	pMON686ΔRV	977 ± 128
K38	pMON686 (minus ATP)*	3,346 ± 330
K38	pMON686 (boiled)†	649 ± 96
K38	No plasmid	753 ± 44

All K38 strains contained pGP1-2, which contains the T7 RNA polymerase gene. The cpm reported were the mean (± SEM) of three assays normalized to the amount of total protein within the assay.
*ATP was omitted from the assay.
†Extract was boiled for 5 min prior to assay.

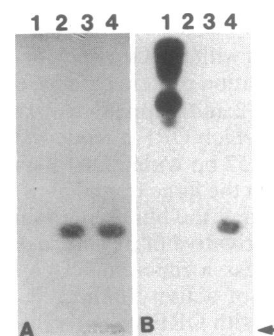

FIG. 4. IAA-lysine production directed by *iaaL*/ORF2. Samples from lysates were loaded in each lane. Lanes: 1, [^{3}H]IAA; 2, IAA-lysine (10 mg/ml); 3, K38 (pGP1-2, pMON686ΔRV); 4, K38 (pGP1-2, pMON686). A 6-μl sample was applied at the origin (arrow) and dried. Chromatogram was developed in butanol/acetic acid/water, 4:1:4 (vol/vol), stained with 0.2% ninhydrin, and photographed (*A*). The chromatogram was then autoradiographed for 3 days (*B*).

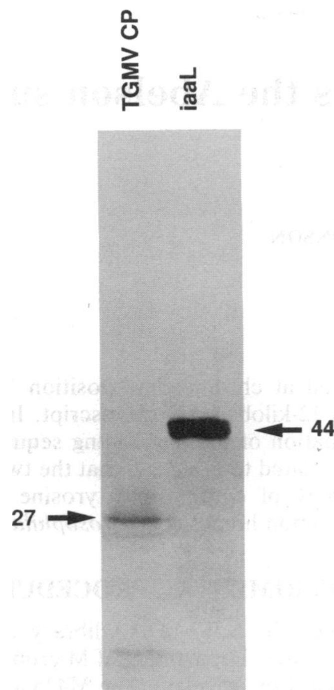

FIG. 5. *In vitro* synthesis of *iaaL*. Plasmid pMON686 was introduced into *E. coli* K38 also containing pGP1-2 (directs production of bacteriophage T7 RNA polymerase). Proteins were labeled with [^{35}S]methionine. A plasmid encoding tobacco golden mosaic virus coat protein (TGMV CP) was electrophoresed in parallel to provide a fluorography standard. The *iaaL* gene product was estimated to have a molecular weight of 44,000, based on the known M_r 27,000 of TGMV CP. Molecular weights ($\times 10^{-3}$) are shown.

upstream of ORF2. A 2-kb *Sac* II fragment 5′ to ORF1 (56 bp upstream) was deleted in the construction of pLG87 (11). Therefore, the promoter and additional ORFs may exist upstream from the characterized region.

IAA-lysine is modified to form *N*-acetyl-IAA-lysine in Italian oleander isolates of *P. savastanoi* (8). Hutzinger and Kosuge (6) noted the formation of an unknown metabolite of IAA-lysine in strain EW 2009, which could be identical to *N*-acetyl-IAA-lysine. *N*-Acetyl-IAA-lysine is difficult to separate from IAA-lysine in the solvent system used in this study (data not shown), and the acetylated compound does not react with ninhydrin (8). Perhaps ORF1 is a component of the acetylation mechanism for IAA-lysine. Alternatively, Glass and Kosuge (11) reported that exogenous IAA was not readily converted to IAA-lysine by whole cells of *E. coli*, whereas *P. savastanoi* whole cells convert greater than 70% of added IAA to IAA-lysine. ORF1 might therefore constitute part of an IAA transport mechanism, which has been reported for *P. savastanoi* (26). The hydrophobicity plot of ORF1 suggests a hydrophobic possibly membrane-associated protein that might be consistent with a role in transport of IAA or IAA-lysine.

The secretion of plant growth regulators, including IAA and cytokinins, is necessary for expression of disease by *P. savastanoi*. The production of IAA-lysine suggests that such conjugates might play a role in the disease interaction. *P. savastanoi* oleander strains that were mutagenized by transposon insertion at the *iaaL* locus showed reduced virulence and ability to grow within host tissues (12). The bacterium may produce IAA-lysine to regulate the levels of IAA seen by the plant. IAA-lysine is a much less potent (20–40% as active) growth stimulator, when compared to free IAA in *Avena* coleoptile bioassays (6). Inactivation of the *iaaL* locus may lead to an increase in IAA concentration that could trigger unfavorable conditions for bacterial growth in the host plant.

A number of plant pathogenic bacteria possess genes directing the production of plant growth regulators (7, 10, 27–30). *Agrobacterium tumefaciens* and *P. savastanoi* have been shown to possess IAA biosynthetic genes with considerable homology to one another (30). A study of the *rolC* locus of *Agrobacterium rhizogenes* suggests that the gene product may reduce levels or decrease sensitivity of host tissues to IAA (31). A homolog to *iaaL* could be expected to provide *rolC*-like effects. However, no significant nucleotide homologies were observed with *rolC* or other sequences contained in the GenBank or National Biomedical Research Foundation data bases.

Our report describes the sequence of a bacterial gene encoding an enzyme that metabolizes the plant growth hormone IAA to a less-active product, and we anticipate that *iaaL* will contribute to further understanding of plant development.

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