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# Cotranscription of Genes Encoding Indoleacetic Acid Production in *Pseudomonas syringae* subsp. *savastanoi*†

CURTIS J. PALM,‡* THOMAS GAFFNEY,§ AND TSUNE KOSUGE

*Department of Plant Pathology, University of California, Davis, California 95616*

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**Indoleacetic acid (IAA) production by the plant pathogen *Pseudomonas syringae* subsp. *savastanoi* is essential for tumor formation on olive and oleander. The bacterium produces IAA from tryptophan in reactions catalyzed by tryptophan monooxygenase and indoleacetamide hydrolase. The genetic determinants are, respectively, *iaaM* and *iaaH*. In oleander isolates, the genes encoding the IAA biosynthetic enzymes are located on a plasmid; in olive isolates, the genes occur on the chromosome. The IAA genes from the oleander isolate strain EW2009 are located within a 4-kilobase (kb) segment of the 52-kb plasmid pIAA1. *Escherichia coli* strains harboring a recombinant plasmid, pCJP3, which contains this 4-kb fragment, excreted IAA into culture media, and crude cell extracts had both tryptophan monooxygenase and indoleacetamide hydrolase activity. In vitro coupled transcription-translation of pCJP3 demonstrated that this fragment coded for proteins of 62 and 47 kilodaltons which correspond to tryptophan monooxygenase and indoleacetamide hydrolase, respectively. Expression of these genes was dependent upon a vector promoter in pCJP3. However, in the absence of a vector promoter, *E. coli* containing recombinant plasmids with additional pIAA1 DNA in front of *iaaM* had high levels of tryptophan monooxygenase. Northern (RNA) hybridization experiments verified that *iaaM* and *iaaH* are cotranscribed as a portion of a ca. 4- to 5-kb transcript in vivo. Southern hybridization experiments with IAA plasmids from different oleander strains of *P. syringae* subsp. *savastanoi* revealed that all IAA plasmids contained a region of at least 10 kb of homology, with the IAA genes at one end. Repetitive DNA and a copy of IS51 were found at the end of this region of homology.**

Phytotumorigenicity of *Pseudomonas syringae* subsp. savastanoi (*P. savastanoi*) is dependent upon bacterial production of the plant hormone indoleacetic acid (IAA) in culture (14, 22, 23). IAA is produced from tryptophan by tryptophan 2-monooxygenase (EC 1.13.12.3), which catalyzes the conversion of tryptophan to indoleacetamide (IAM), and IAM hydrolase, which catalyzes the conversion IAM to IAA. Tryptophan monooxygenase has been purified to apparent homogeneity and found to be a 62,000-dalton protein requiring flavin adenine dinucleotide as a cofactor (9). The genetic determinants which encode the two proteins, *iaaM* for tryptophan monooxygenase and *iaaH* for IAM hydrolase, are adjacent (25) and located on plasmid DNA in oleander strains and on the chromosome in olive isolates. This difference in location suggests that at least once in their evolutionary history the genes for IAA production have been mobile and may have been, or still may be, part of a mobile genetic element.

Strains which have lost the ability to produce IAA are avirulent. In oleander strain EW2009, loss of pIAA1, a 52-kilobase (kb) plasmid, results in loss of IAA production; reintroduction of pIAA1 restores virulence and IAA production (4). The gene encoding tryptophan monooxygenase was cloned from pIAA1 on a 2.7-kb *Eco*RI restriction fragment in a recombinant plasmid designated pLUC1 (5). pLUC1 alone restored partial virulence in EW2009-3, a strain cured of pIAA1 (12). Sequence information indicated that *iaaM* and *iaaH* are adjacent (25). Moreover, insertions in *iaaM* have a polar effect on expression of *iaaH*, suggesting that the two genes exist in an operon with *iaaH* promoter distal. The aim of the present study was to provide evidence that *iaaM* and *iaaH* are organized as one transcriptional unit and to examine the organization of these genes in additional *P. syringae* subsp. *savastanoi* isolates. We demonstrate that *iaaM* and *iaaH* are cotranscribed and that although the location of these genes varies in different isolates, their organization is conserved.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The characteristics of the bacterial strains and plasmids used in this study are described in Tables 1 and 2. Bacteria were routinely grown in LB medium (16). Growth of *P. syringae* subsp. *savastanoi* for RNA isolation was on glucose-peptone medium (medium B; 4). Concentrations of antibiotics used in selection media are as follows (micrograms per milliliter): ampicillin, 25; chloramphenicol, 25; kanamycin, 25; and tetracycline, 15.

**DNA isolation.** Large-scale plasmid DNA isolations were carried out by the alkaline lysis method and purified by CsCl-ethidium bromide centrifugation as described by Maniatis et al. (15). Plasmid miniscreens were performed as described by Tait et al. (20). DNA restriction fragments were recovered from low-gelling-temperature agarose (FMC Corp., Marine Colloids Div., Rockland, Maine) and purified by phenol extraction as described by Weislander (21).

**Restriction digests and cloning.** Restriction enzymes (New England BioLabs, Inc., Beverly, Mass.; Bethesda Research Laboratories, Inc., Gaithersburg, Md.; and Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and T4 DNA ligase (New England BioLabs) were used according to the method of Maniatis et al. (15). Calf alkaline phosphatase (Boehringer Mannheim) was used to dephosphorylate DNA

* Corresponding author.

† Dedicated to the memory of Tsune Kosuge (deceased 13 March 1988).

‡ Present address: Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340.

§ Present address: CIBA-GEIGY, Research Triangle Park, NC 27709.

TABLE 1. Bacterial strains and vectors

| Strain or vector | Relevant characteristic(s) | Reference or source |
|---|---|---|
| *E. coli* HB101 | | 15 |
| *P. savastanoi* | | |
| PB213 | Oleander isolate, pIAA2 | 7 |
| TK1006 | Olive isolate | 7 |
| EW1017 | Oleander isolate, pIAA3 | 7 |
| EW2009 | Oleander isolate, pIAA1 | 17 |
| EW2009-3 | Iaa$^-$, pIAA1 cured | 17 |
| Vectors | | |
| pBR328 | Ap$^r$ Cm$^r$ Tc$^r$ | 18 |
| pSa152 | Cm$^r$ Km$^r$ Spt$^r$ | 19 |
| pGX1515 | Ap$^r$ Tc$^r$ | Genex Inc. |

according to the directions of the manufacturer. *Escherichia coli* was transformed with plasmid DNA by the $CaCl_2$ procedure as described by Maniatis et al. (15).

**DNA hybridizations.** Southern hybridizations and nick translations were performed as described by Maniatis et al. (15), except that Nytran nylon membranes (Schleicher & Schuell, Inc., Keene, N.H.) were substituted for nitrocellulose filters.

**Determination of IAA and IAM production.** The Salkowski reagent for the detection of indoles in culture media and the procedures for extraction of indoles were previously described by Comai and Kosuge (4, 5). Thin-layer chromatography for separation of indoles was carried out on reverse-phase silica plates (Analtech, Newark, Del.) developed by a solvent of 30% methanol and 70% 20 mM acetic acid (vol/vol), pH 3.5. After development, plates were air dried, sprayed with dimethylaminobenzaldehyde, and heated to 65°C for 10 min to visualize indoles.

**Assay of tryptophan monooxygenase and IAM hydrolase.** Crude enzyme preparations from *E. coli* were prepared by the procedures of Comai and Kosuge (4). The tryptophan monooxygenase polarographic assay has been described previously (4, 9). IAM hydrolase activity was determined by a radioassay similar to that described by Kemper et al. (10). The substrate in the assays was [$^{14}$C]IAM (specific activity, 41 mCi/mmol; a gift from M. Sanger). Crude enzyme (1 to 5 μl) was incubated with 10 μM IAM (100,000 dpm) in 10 mM Tris (pH 8.3)–50 mM KCl in a final volume of 100 μl. Reactions were stopped by extractions (three times) with 100 μl of ethyl acetate. Radioactivity in the combined ethyl acetate fractions (which contained the IAM) and the aqueous phase (which contained the IAA) were determined in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

**Northern (RNA) hybridizations.** RNA was isolated from 100-ml bacterial cultures by the hot phenol procedure as modified by Aiba et al. (1). The RNA (10 μg) was fractionated by electrophoresis through formaldehyde gels (1.0% [wt/vol] agarose; 20 mM MOPS (morpholinepropanesulfonic acid), pH 7.0, 5 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde) and transferred to Nytran nylon membranes (Schleicher & Schuell). Transfer, hybridization, and washing conditions were those recommended by the membrane supplier, except that RNA-RNA hybridizations were carried out at 55°C. RNA probes were prepared by the in vitro transcription of internal portions of either *iaaM* or *iaaH* which had been cloned into the transcription vector pSP64 (Promega Biotec; Madison, Wis.). In vitro transcription with bacteriophage SP6 RNA polymerase was performed on linearized templates according to the instructions of the polymerase supplier (Promega Biotec). Transcripts were labeled through the incorporation of uridine 5′-[α-$^{32}$P]triphosphate (400 Ci/mmol; Amersham Corp., Arlington Heights, Ill.).

**Coupled transcription-translation system.** The proteins encoded by the determinants on the recombinant plasmids were labeled with [$^{35}$S]methionine (Amersham) by an *E. coli* coupled transcription-translation system (Amersham) (2). Radioactive proteins were subjected to electrophoresis in a vertical slab sodium dodecyl sulfate-polyacrylamide gel electrophoresis system as described by Laemmli (13). Gels were dried and exposed to Kodak X-ray film to determine the location of the labeled proteins.

## RESULTS

**Restriction mapping of pIAA1.** Studies of pIAA1 are complicated by the fact that all *P. syringae* subsp. *savastanoi* strains examined contained multiple cryptic plasmids in addition to pIAA. Because it has been difficult to cure any strain of all cryptic plasmids, a plasmid library was constructed from strain EW2009 plasmid DNA and clones were isolated which contained pIAA1 DNA.

TABLE 2. Plasmids

| Plasmid | Cloned fragment | Relevant characteristic | Vector and cloning site[b] | Reference or source of insert |
|---|---|---|---|---|
| pLUC2 | 2.8-kb *Eco*RI | Ap$^r$ Tc$^r$ *iaaM*$^+$ | pBR328, *Eco*RI (Cm) | 5 |
| pCJP1 | 3.4-kb *Sal*I | Ap$^r$ Cm$^r$ | pBR328, *Sal*I (Tc) | pIAA1 |
| pCJP3 | 4.0-kb *Eco*RI-*Sal*I | Ap$^r$ *iaaM*$^+$ *iaaH*$^+$ | pBR328, *Eco*RI (Tc)-*Sal*I (Cm) | pLUC2/pCJP1 |
| pCJP4 | 6.2-kb *Eco*RI-*Sal*I | Ap$^r$ *iaaM*$^+$ *iaaH*$^+$ | pBR328, *Eco*RI (Tc)-*Sal*(Cm) | pCJP3/pIAA1 |
| pCJP5 | 1.1-kb *Hin*dIII | Ap$^r$ Km$^r$ *iaaH*$^+$ | pBR328, *Eco*RI (Tc)-*Sal*I (Cm) | pCJP4[c] |
| pCJP6 | 1.1-kb *Hin*dIII | Ap$^r$ Km$^r$ | pBR328, *Eco*RI (Tc)-*Sal*I (Cm) | pCJP4[c] |
| pCJP7 | 3.4-kb *Sal*I | Ap$^r$ | pGX1515, *Sal*I | pCJP1 |
| pCJP8 | 4.0-kb *Eco*RI-*Sal*I | Ap$^r$ | pGX1515, *Eco*RI-*Sal*I | pCJP7/pLUC2 |
| pCJP9 | 10.2-kb *Eco*RI-*Sal*I | Ap$^r$ *iaaM*$^+$ *iaaH*$^+$ | pGX1515, *Eco*RI-*Sal*I | pCJP7/pCJP12 |
| pCJP10 | 20-kb *Kpn*I | Cm$^r$ *iaaM*$^+$ | pSa152, *Kpn*I (Km) | pIAA1 |
| pCJP12[d] | 12-kb *Bgl*II/*Kpn*I | Cm$^r$ *iaaM*$^+$ | pSa152, *Kpn*I (Km) | pCJP10 |
| pCJP20 | 20-kb *Kpn*I | Cm$^r$ | pSa152, *Kpn*I (Km) | pIAA1 |

[a] Phenotype as expressed in *E. coli*.

[b] In all inserts with pBR328 as vector, the inserted *iaa* genes are in the same orientation as the vector promoter. In the clones in the pGS1515 vectors there is no promoter reading into the cloned DNA. The antibiotic resistance gene inactivated is indicated in parentheses.

[c] pCJP5 and pCJP6 have a 1.1-kb kanamycin resistance gene (3) inserted (in opposite orientations) in the *Hin*dIII site of pCJP4.

[d] A *Bgl*II-*Kpn*I deletion of pCJP10.

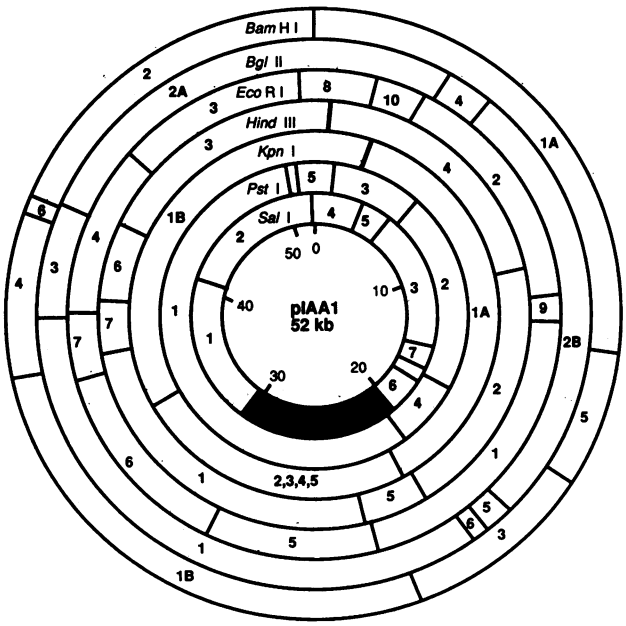


FIG. 1. Restriction map of pIAA1. The map was constructed as described in the text. The shaded region indicates the area where fragment sizes and order have not been determined. The sizes are shown in kilobases. Coordinate 0 was arbitrarily chosen as the *Sal*I site in the *iaaM* gene.

To construct this library, purified plasmid DNA from strain EW2009 was treated with restriction endonuclease *Kpn*I and the resulting fragments were ligated into the *Kpn*I site of the vector plasmid pSa152. This ligated DNA was used to transform *E. coli* HB101. Two recombinant plasmids, pCJP10 and pCJP20, were isolated which collectively contained 40 kb of pIAA1 including the regions with *iaaM* and *iaaH*. Each of these plasmids contained a different *Kpn*I fragment of 20 kb. These two plasmids were further digested with restriction nucleases *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Pst*I and *Sal*I singly and in combinations to determine the order of the restriction sites on pIAA1. In addition, strain EW2009 plasmid DNA was separated in low-gelling-temperature agarose, and the pIAA1 band was excised from the gel. pIAA1 DNA was then directly digested with the above enzymes, and the products were separated by electrophoresis in agarose gels to determine the number and size of fragments generated by the digests. To map the region of the plasmid not covered by the two *Kpn*I clones, Southern blots of EW2009 plasmid DNA were performed. The probes were small fragments from the ends of the cloned *Kpn*I fragments. This allowed the ordering of fragments which overlap with the two large *Kpn*I fragments. The restriction map of pIAA1 is shown in Fig. 1.

**Cloning of *iaaM* and *iaaH*.** Polar effects of naturally occurring insertion events suggested that *iaaM* and *iaaH* are organized in an operon and *iaaH* is promoter distal with respect to *iaaM* (6). We constructed a number of chimeric plasmids bearing different restriction fragments of pIAA1 (Fig. 2 and Table 2). These were introduced into *E. coli* HB101 to determine expression of *iaaM* and *iaaH* by measuring enzyme activities and indole production.

The enzyme activities found in bacteria containing the different clones are shown in Table 3. Culture filtrates from cells which had both tryptophan monooxygenase and IAM hydrolase activity contained 0.35 μmol of IAA per mg (dry weight) of cells after 1 day of growth. Culture filtrates from cells which had only tryptophan monooxygenase activity contained only IAM, while filtrates from cells containing only IAM hydrolase or neither enzyme activity had no detectable amounts of IAA or IAM. pCJP3 is the plasmid with the smallest insert which confers both enzyme activities upon *E. coli*. Therefore, both *iaaM* and *iaaH* must be contained on the 4.0-kb *Eco*RI-*Sal*I fragment contained in this clone.

No tryptophan monooxygenase activity was found in transformants with pCJP5 and pCJP6 (Table 3 and Fig. 2). This was expected, since sequence analysis and restriction mapping revealed that the *Hin*dIII site, the site of insertion of the kanamycin resistance gene cartridge used in these constructs (Fig. 2), occurs within *iaaM*. Depending upon the orientation of the kanamycin cartridge, different effects on IAM hydrolase activity in these two clones were observed. Since there is no transcription terminator in the kanamycin gene cartridge, genes 3′ to the site of insertion may be expressed. There was a significant level of IAM hydrolase activity in transformants with pCJP5, presumably due to transcription directed from the kanamycin resistance gene promoter. The level of IAM hydrolase activity observed with pCJP5 was still only 15% of that found in transformants containing pCJP4, the parental plasmid lacking the kanamycin cartridge (Fig. 2). In pCJP6, the kanamycin gene is in the opposite orientation of that in pCJP5 (Fig. 2). *E. coli* transformed with pCJP6 exhibited 200-fold reduction in IAM hydrolase activity compared with levels in cells transformed with pCJP4. This reduction in activity occurred even though *iaaH* remained intact. The polar effect of this insertion demonstrates that *iaaH* is transcribed on the same RNA as *iaaM* in *E. coli*.

**In vitro protein synthesis.** Purified plasmid DNA was used in an *E. coli* in vitro coupled transcription-translation system to determine the size of proteins encoded by the cloned *P. syringae* subsp. *savastanoi* DNA. Two proteins, 62 and 47 kilodaltons (kDa), were encoded by the cloned *P. syringae* subsp. *savastanoi* determinants contained in these clones (Fig. 3). The two proteins are the sizes predicted by the open reading frames found by nucleotide sequence analysis of *iaaM* and *iaaH* (25), and the 62-kDa protein is the same size as purified tryptophan monooxygenase (9). When the proteins produced by each plasmid and the enzyme activities in *E. coli* extracts containing the plasmids are compared (Table 3), the same polar effect is seen with the proteins produced by the plasmids as with the enzyme activities. The 62-kDa protein was present only in protein synthesis reactions driven by plasmids which conferred tryptophan monooxygenase activity to *E. coli* extracts (pLUC2, pCJP3, and pCJP4). The 62-kDa protein, tryptophan monooxygenase, was not present in in vitro reactions driven by pCJP5 or pCJP6, and there was no tryptophan monooxygenase activity in extracts from cells containing pCJP5 or pCJP6 DNA.

The 47-kDa protein was present in reactions driven by plasmids which confer IAM hydrolase activity in cell extracts and was absent in reactions with plasmids which did not confer this activity (Table 3 and Fig. 3). pCJP6 had the kanamycin gene inserted in the tryptophan monooxygenase gene, and the polar effect of the insertion was revealed by the pattern of proteins produced in the in vitro transcription-translation reactions. Thus, in Fig. 3, the reaction mixture with pCJP6 DNA shows the absence of tryptophan monooxygenase protein and the greatly reduced amounts of IAM hydrolase protein. This corresponds to no tryptophan monooxygenase activity and over 200-fold reduction in IAM

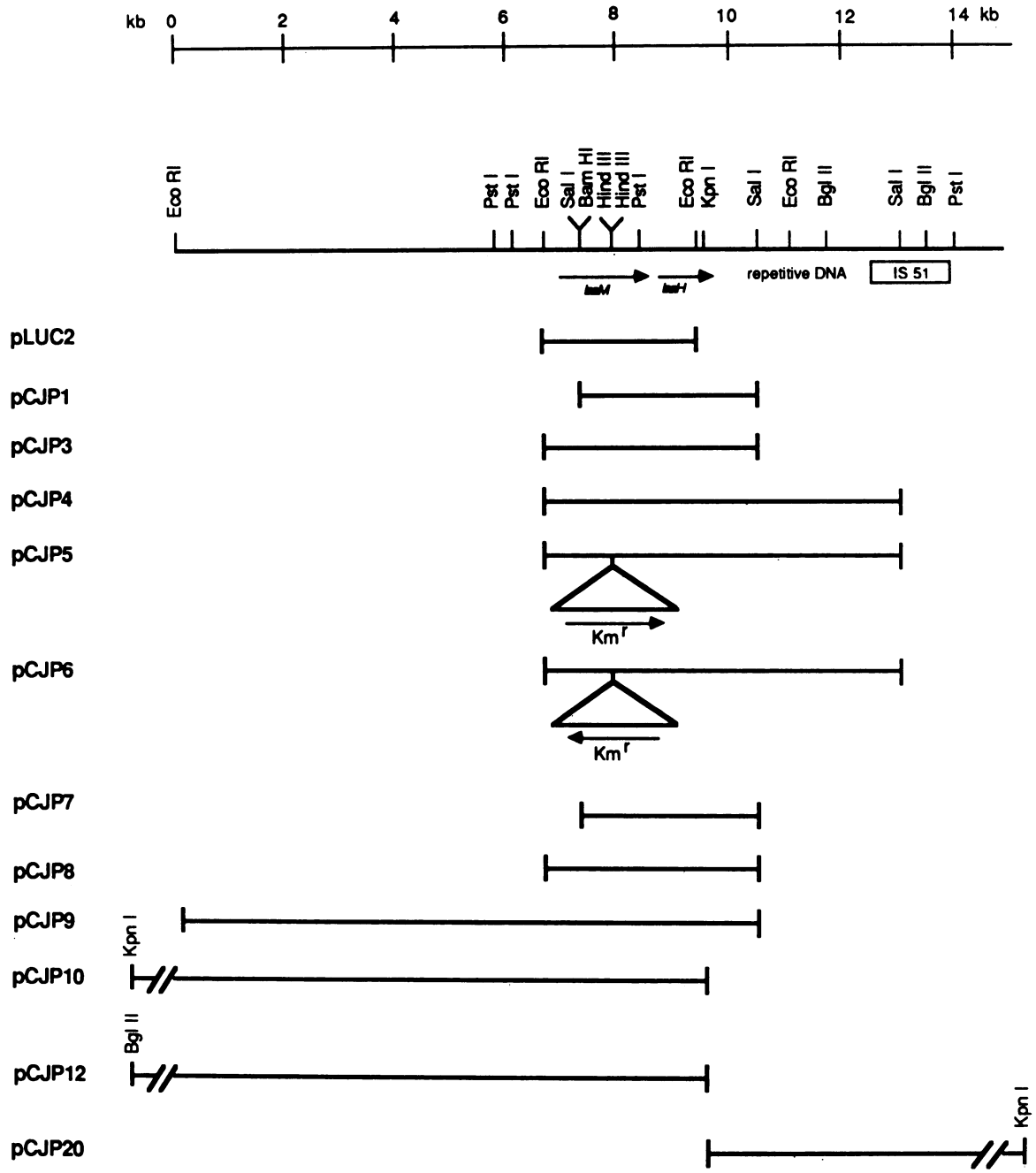


FIG. 2. Restriction endonuclease map of recombinant plasmids containing pIAA1 DNA. The figure shows the restriction map and sizes of DNA inserts in the plasmids constructed in this study. The lines directly below the map indicate the locations of open reading frames as determined by sequence analysis of pCJP3 DNA (25). The vector for the first six plasmids is pBR328, that for the next three is pGX1515, and that for the last three is pSa152. The length of cloned DNA is shown above the map in kilobase pairs.

hydrolase activity in extracts of cells containing this plasmid. No new proteins were produced in the transcription-translation reaction when pCJP3 was replaced with pCJP4 DNA which contained additional DNA distal to *iaaH*.

**Construction of recombinant plasmids containing IAA promoter.** The plasmids conferred the following tryptophan monooxygenase activities (nanomoles of $O_2$ uptake per minute per milligram of protein): pLUC11, 0.5; pCJP3, 50; pCJP8, 0.4; pCJP9, 20; and pCJP12, 10. As in previous studies, we found no or very low promoter activity present on *Eco*RI fragment 8 in *E. coli* (4); *E. coli* transformed with plasmid pLUC11, which contained the fragment in a promoter probe vector, had very low tryptophan monooxygenase activity. In contrast, *E. coli* transformed with a larger fragment of pIAA1 DNA (pCJP12) had high levels of tryptophan monooxygenase activity; as noted in Fig. 2, this fragment contained pIAA1 DNA that extended further upstream from *iaaM*. These regions recently have been shown to contain sequences resembling *Pseudomonas* and *E. coli* promoters (O. da Costa e Silva, T. Gaffney, and T. Kosuge, J. Cell. Biochem. **12C**(Suppl.):251), suggesting that the promoter for the IAA operon occurs upstream from sequences

TABLE 3. Enzyme assays

| Strain and plasmid | Production of[a]: | |
|---|---|---|
| | Tryptophan monooxygenase | IAM hydrolase |
| *E. coli* | 0.0 | 0.0 |
| *E. coli*(pLUC2) | 150 | 0.0 |
| *E. coli*(pCJP3) | 140 | 3.8 |
| *E. coli*(pCJP4) | 150 | 4.2 |
| *E. coli*(pCJP5) | 0.0 | 0.65 |
| *E. coli*(pCJP6) | 0.0 | 0.0016 |

[a] Tryptophan monooxygenase activity was measured as nanomoles of $O_2$ uptake per minute per milligram of protein. IAM hydrolase activity was measured as nanomoles of IAA produced per minute per milligram of protein. 0.0, No detectable activity.

found in *Eco*RI fragment 8. To further define the location of the IAA promoter, recombinant plasmids were constructed in the vector pGX1515 (Table 2 and Fig. 2). The soluble fraction of *E. coli* cell lysates transformed with pCJP8 had very low tryptophan monooxygenase activity. This low level of activity is comparable to that shown by *E. coli* cells containing pLUC11 and is close to background levels. In contrast, *E. coli* transformed with pCJP9 had 50-fold-higher tryptophan monooxygenase activity compared with the activity resulting from transformation with pCJP8. The increased activity may be due to promoter activity associated with the *Eco*RI fragment (Fig. 2) which occurred in pCJP9 but not in PCJP8. Having shown that promoter activity resided in a region upstream from *iaaM*, we constructed probes from different regions within and outside regions associated with *iaaM* and *iaaH* for use in Northern hybridization experiments.

**Transcription of the IAA operon.** To verify that *iaaM* and *iaaH* are cotranscribed in vivo, we performed Northern hybridizations with RNA isolated from *P. syringae* subsp. *savastanoi* EW2009, EW2009-3, EW2009-5, and PB205-1L. Probes specific for *iaaM* and *iaaH* each hybridized with a mRNA population from the wild-type strain EW2009 in the 4- to 5-kb range (Fig. 4, lanes 1), as did a probe corresponding to a region 250 to 900 base pairs (bp) upstream of *iaaM* (ca. 650-bp *Pst*I-*Eco*RI fragment) (data not shown). Probes corresponding to the region 900 to 1,500 bp upstream of *iaaM* (600-bp *Pst*I-*Pst*I fragment) and 1,200 to 3,200 bp downstream of *iaaH* (2,000-bp *Eco*RI-*Sal*I fragment) failed to hybridize with the 4- to 5-kb transcript (data not shown). Hybridization of either *iaaM* or *iaaH* probes with the full-length transcripts did not occur when RNA was isolated from the insertion mutants EW2009-5 and PB205-1L (Fig. 4, lanes 2 and 3). In EW2009-5, *iaaM* is interrupted by IS51, and in PB205-1L, *iaaM* is interrupted by IS52 (6). The *iaaM* probe, which includes sequences upstream of the insertion sites in these mutants, did hybridize with truncated transcripts (lanes A2 and A3). The *iaaH* probe, which consists entirely of sequence downstream of the insertion sites, did not hybridize with mRNA from the insertion mutants (lanes B2 and B3). Presumably, transcription of the IAA operon in strains EW2009-5 and PB205-1L terminates either within the IS element or shortly beyond. Strain EW2009-3, an EW2009 derivative cured of pIAA1, failed to synthesize IAA operon transcripts (lanes 4).

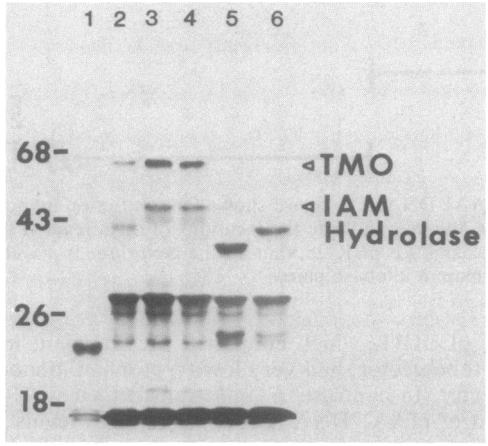


FIG. 3. In vitro coupled transcription-translation assays. An autoradiogram of polyacrylamide gel of in vitro protein synthesis reactions is shown. Lanes: 1, pBR328; 2, pLUC2; 3, pCJP3; 4, pCJP4; 5, pCJP5; and 6, pCJP6. The numbers on the left of the gel are size markers (kilodaltons). The arrows indicate the locations where tryptophan monooxygenase (TMO) and IAM hydrolase migrate.

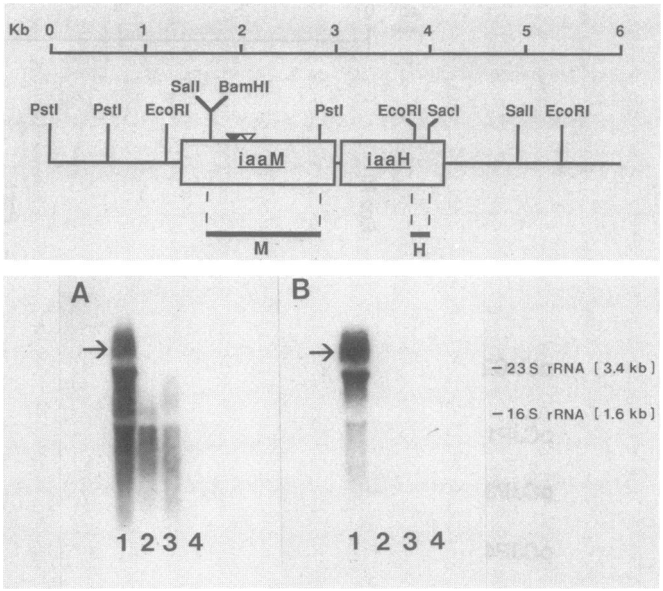


FIG. 4. Northern hybridization analysis of the *iaa* genes. Total RNA from *P. savastanoi* EW2009 (lanes 1), EW2009-5 (lanes 2), PB205-1L (lanes 3), and EW2009-3 (lanes 4) was electrophoresed through formaldehyde gels and transferred to nylon membranes. Radiolabeled RNA corresponding to the region of *iaaM* designated by the bar labeled M was used as the probe in panel A. Radiolabeled RNA corresponding to the region of *iaaH* designated by the bar labeled H was used as the probe in panel B. The largest population of *iaa* gene transcripts (ca. 4 to 5 kb) is indicated by an arrow in each panel, and the migration positions of the two largest classes of rRNA are indicated to the right of panel B. Symbols: ▼, site of insertion of IS52 within *iaaM* in strain PB205-1L; ▽, site of insertion of IS51 within *iaaM* in strain EW2009-5.

**Homology with other pIAA plasmids.** The IAA genes characterized in this study were cloned from pIAA1 of strain EW2009. In other oleander strains of *P. syringae* subsp. *savastanoi*, two other IAA plasmids, pIAA2 and pIAA3, have been identified. In addition, in olive strains of the bacteria, the IAA genes are located on chromosomal DNA. In all strains examined, *Eco*RI fragment 8 is conserved (7). To determine how far beyond this fragment homology exists

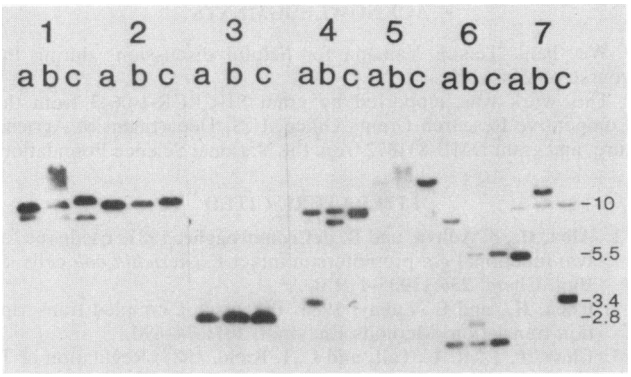


FIG. 5. Southern blot analysis of IAA plasmids using probes from different regions of pIAA1. Plasmid DNA from strain EW2009 (pIAA1), PB213 (pIAA2), and EW1017 (pIAA3) was digested with different restriction enzymes and separated by electrophoresis. The probe used in this analysis was radiolabeled pIAA1 *Eco*RI fragment 8. Lanes: a, PB213; b, 1017; c, EW2009; 1, *Bam*HI; 2, *Bgl*II; 3, *Eco*RI; 4, *Hin*dIII; 5, *Kpn*I; 6, *Pst*I; 7, *Sal*I. The numbers on the right of the autoradiogram are fragment sizes in kilobase pairs. (Multiple bands in lanes 1b and 6b are due to partial digests of DNA.)

on the different IAA plasmids, hybridization experiments were performed. Southern blots were prepared from plasmid DNA from strain EW2009 (pIAA1), PB213 (pIAA2), and EW1017 (pIAA3), which was digested with several different restriction enzymes and separated by agarose gel electrophoresis. With a probe of *Eco*RI fragment 8 (Fig. 5), we determined that restriction enzyme fragments *Bam*HI 2, *Hin*dIII 3, *Pst*I 5, and *Sal*I 2 of pIAA1 were conserved in the two other plasmids. Such results indicate that the three IAA plasmids have identical restriction maps for at least 10 kb upstream of *iaaM*. Additional Southern hybridizations indicate that the homology ends approximately 20 kb upstream of *iaaM* (data not shown). Also in Fig. 5 it can be seen that the three IAA plasmids differ in sequences immediately downstream from *iaaH*, since not even the shortest fragment which overlaps *Eco*8 in pIAA1, *Sal*4, was present in the other two IAA plasmids. None of the fragments which extend beyond *iaaH* in pIAA1 was present in the other IAA plasmids. In addition pIAA2 and pIAA3 did not have any fragments of common size between them which mapped to this area. Southern hybridization experiments indicate that a region of not more than 7 kb was present on the chromosome of olive strains; the region has an identical restriction map to that of the plasmid-borne IAA genes (data not shown).

## DISCUSSION

IAA production by *P. syringae* subsp. *savastanoi* is necessary for tumor development on olive and oleander. Oleander strains of the bacteria which have lost the IAA plasmid are avirulent when inoculated on the host plant. However, as shown previously, the Iaa$^-$ bacteria are able to grow as well as wild type both in culture and in plant tissue (17). This demonstrates that IAA production by *P. syringae* subsp. *savastanoi* is a secondary metabolic pathway not required for growth of the pathogen and also separates virulence expression from growth of the pathogen. In order to have enough tryptophan for the essential requirements of the cell, mechanisms must exist in *P. syringae* subsp. *savastanoi* to control flow of the primary metabolite tryptophan into the IAA pathway (11). To better understand the mechanisms controlling IAA production in *P. savastanoi*, we have cloned the *iaa* genes on small recombinant plasmids and characterized the organization of these genes. The goal of this study was to characterize the genes of IAA biosynthesis.

Previous experiments showing that insertions in *iaaM* have a polar effect on *iaaH* expression suggested that the two genes are organized as an operon, with *iaaH* being promoter distal. Our data support this hypothesis. Initially, it was thought that the IAA operon contained only two genes, *iaaM* and *iaaH*, and these were the genes that were first cloned and characterized. Both *iaaM* and *iaaH* are contained within a 4-kb DNA fragment from pIAA1, and *iaaH* is located immediately after *iaaM*. A recombinant plasmid, pCJP3, containing this 4-kb fragment conferred upon *E. coli* the ability to produce IAA in culture media. Enzyme activities of both tryptophan monooxygenase and IAM hydrolase were found in crude lysates of *E. coli* transformed with pCJP3. These activities correlate to proteins of 62,000 and 47,000 daltons, respectively, as shown by in vitro transcription-translation experiments. It was shown previously that for pLUC2 (*Eco*RI fragment 8) in *E. coli*, efficient *iaaM* expression is due to the vector promoter (5). pCJP3 is identical to pLUC2 in the region 5′ of *iaaM*, so efficient transcription in pCJP3 is also due to a vector promoter. Since *iaaH* was efficiently expressed in *E. coli* containing pCJP3, it is likely that there is no transcriptional stop signal between *iaaM* and *iaaH* and that the two genes are cotranscribed on the same mRNA. Also supporting this idea is the marked polar effect seen in *iaaH* expression with pCJP6. *iaaH* expression, as measured by IAM hydrolase activity, decreased over 200-fold when *iaaM* was disrupted by the kanamycin resistance gene cartridge insertion. This polar effect was also seen in the protein synthesis experiments, in which the amount of IAM hydrolase protein produced in vitro was greatly reduced by this insertion in *iaaM*. Northern hybridizations indicated that an in vivo message of between 4 and 5 kb hybridized to probes derived from both *iaaM* and *iaaH*. This full-length transcript was not detected in two Iaa$^-$ insertion mutants when RNA from these strains was hybridized with either probe. The *iaaM* probe, which included sequences upstream of the insertion sites, hybridized to truncated transcripts from the insertion mutants, while the *iaaH* probe, which contained sequences downstream from the insertion sites, did not. These findings are consistent with the idea that the polar effect of the insertions upon *iaaH* expression is a result of premature transcription termination of the IAA operon mRNA. These data taken together show that *iaaM* and *iaaH* are transcribed together as a polycistronic message and that *iaaH* is promoter distal to *iaaM*.

Experiments indicate that *iaaH* is the last gene in the operon. In vitro protein synthesis experiments show no difference in proteins made between pCJP3 and the longer clone pCJP4. The three different IAA plasmids and the chromosomal *iaa* genes examined all differ in their restriction enzyme patterns just beyond the end of *iaaH*. The end of the IAA mRNA was mapped to be just beyond *iaaH* by S1 nuclease mapping experiments and a terminatorlike stem-and-loop structure followed by several consecutive thymidines is found in the nucleotide sequence directly after *iaaH* (T. Yamada, Ph.D. dissertation, University of California, Davis, 1985).

It was originally thought that the promoter for the IAA operon was directly in front of *iaaM* and that the reason the genes were not expressed well in *E. coli* was that *Pseudomonas* promoters were recognized inefficiently by *E. coli*

RNA polymerase. However, *E. coli* transformed with a recombinant plasmid containing *Eco*RI fragments 3 and 8 had significant tryptophan monooxygenase activity. Since plasmid pCJP9 had no vector promoter at the cloning site, expression was due to sequences contained within the cloned DNA. With the 6.7-kb *Eco*RI fragment 3 deleted (pCJP8), there was very low tryptophan monooxygenase activity, even though the *iaaM* gene itself was intact. The polar effect of this deletion is evidence that sequences within *Eco*RI fragment 3 are required for expression of the IAA operon in *E. coli*. In addition, Northern blot experiments with RNA isolated from *P. syringae* subsp. *savastanoi* indicated that the mRNA for the IAA operon was at least 4 kb in size and that sequences greater than 250 bp upstream of *iaaM* hybridized to this mRNA. Also, promoter-probe studies indicate that the promoter for the operon was contained within the ca. 650-bp *Pst*I-*Eco*RI fragment which is immediately before *Eco*RI fragment 8 (T. Gaffney and T. Kosuge, unpublished data). Additional experiments are in progress to characterize the promoter for the IAA operon and to determine whether an additional gene exists between this promoter and *iaaM*.

The *iaa* genes are located on plasmid DNA, although on plasmids of different sizes, in all oleander strains of the bacteria that have been examined; in contrast, they are present on the chromosome of all olive strains examined. Three different IAA plasmids have been identified in strains from oleander. The extent of homology between these plasmids has not been fully examined, but a region of between 10 and 20 kb ending with the IAA genes is homologous on all three plasmids. Another gene of IAA metabolism has been mapped to this region of homology. The gene for IAA-lysine conjugate synthetase has been characterized (8) and found to be in the same relative location to *iaaM* and *iaaH* on all three IAA plasmids.

The *iaa* genes are present in different locations in different strains of *P. syringae* subsp. *savastanoi*. IAA biosynthesis genes are also present in other bacterial plant pathogens. Sequence analysis of *iaaM* and *iaaH* revealed significant homology with *tms-1* and *tms-2* of *Agrobacterium* transfer DNA (T-DNA) (25), although *tms-1* and *tms-2* are not cotranscribed. In addition, recent studies indicated that sequences highly homologous to *iaaM* and *iaaH* are present in several other *P. syringae* pathovars (26). The organization of the two genes appears to be conserved, although the level of IAA production is much lower for these pathovars. Since the homology with the *P. syringae* subsp. *savastanoi iaa* genes ends abruptly just 5′ to *iaaM*, it is possible that the promoter differences in these other pathovars account for the difference in expression. Such results indicate that the genes for IAA production in bacterial phytopathogens had a common origin and that they moved between *Pseudomonas syringae* and *Agrobacterium tumefaciens*. It is also noteworthy that a 530-bp sequence of IS51 is conserved almost intact in the center region of octopine T-DNA (24). IS51 is also located adjacent to *iaaH* on pIAA1 (C. J. Palm and T. Kosuge, unpublished data). The mechanism by which the *iaa* genes have been able to transfer from plasmid to chromosome and between species is unknown, although it can be speculated that IS51 plays a role in this transfer. The cloned DNA from pIAA1 will be a useful tool in investigating the relationship between the chromosomal and plasmid-borne IAA operons and further investigating the mechanisms which control IAA synthesis in *P. savastanoi*.

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