

Indoleacetic Acid Operon of *Pseudomonas syringae* subsp. *savastanoi*: Transcription Analysis and Promoter Identification†

THOMAS D. GAFFNEY,* OSWALDO DA COSTA E SILVA,§ TETSUJI YAMADA,||
AND TSUNE KOSUGE

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

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Expression of the indoleacetic acid (*iaa*) operon, which contributes to the virulence of the phytopathogenic bacterium *Pseudomonas syringae* subsp. *savastanoi*, was monitored by using broad-host-range *lacZ* reporter gene plasmids. A combination of translational (gene) fusions and transcriptional (operon) fusions of *P. syringae* subsp. *savastanoi* sequences to *lacZ* allowed localization of the *iaa* operon promoter. RNA recovered from *P. syringae* subsp. *savastanoi* strains was mapped with *iaa* operon-specific probes to precisely locate the transcription initiation site. When transcripts from an *iaaM::lacZ* fusion in *Escherichia coli* were analyzed, an identical transcription initiation site was observed. The DNA sequence of the *iaa* operon promoter closely resembled the consensus *E. coli* promoter sequence. We detected an active, constitutive level of indoleacetic acid biosynthetic gene expression during bacterial growth under a variety of conditions in the absence of host plant influence.

The plant pathogenic bacterium *Pseudomonas syringae* subsp. *savastanoi* incites the formation of tumorous galls on olive and oleander (40). Tumor formation requires bacterial production and secretion of compounds which act as plant growth hormones, including the auxin indoleacetic acid (IAA) and the cytokinin *trans*-zeatin (32, 36, 38). Genes required for IAA and cytokinin biosynthesis in *P. syringae* subsp. *savastanoi* have been isolated and sequenced, and homologies with genes present on *Agrobacterium tumefaciens* tumor-inducing plasmids have been demonstrated (31, 42). Virtually identical IAA biosynthetic genes are present in additional *P. syringae* subspecies (43). The capacity to synthesize IAA is, in fact, widespread among soil- and plant-associated bacteria (15, 25, 26). IAA producers include microbes with beneficial effects on plant growth (e.g., *Azospirillum* species) (2), as well as plant pathogens (15).

The IAA biosynthetic pathway of *P. syringae* subsp. *savastanoi* can serve multiple functions. In addition to its role in virulence, it can detoxify several tryptophan analogs capable of inhibiting bacterial growth (23). Furthermore, IAA itself can be converted by *P. syringae* subsp. *savastanoi* into additional compounds, including an IAA-lysine conjugate (16). *P. syringae* subsp. *savastanoi* synthesizes IAA from tryptophan in two steps (22, 42). Tryptophan 2-monooxygenase (EC 1.13.12.3), the *iaaM* gene product, converts L-tryptophan to indoleacetamide. Indoleacetamide hydrolase, the *iaaH* gene product, catalyzes the conversion of indoleacetamide to IAA. Both *iaaM* and *iaaH* are plasmid-borne in *P. syringae* subsp. *savastanoi* isolates from oleander hosts, while they have a chromosomal location in strains isolated from olive (6, 9). In each case examined, the

organization of the region including *iaaM* and *iaaH* has been conserved (29). Previous studies indicated that *iaaM* and *iaaH* are cotranscribed, with *iaaM* promoter proximal (8, 29).

To gain insight into the expression of *P. syringae* subsp. *savastanoi* *iaa* genes, the products of which participate in a secondary metabolic pathway required for full bacterial virulence, we utilized broad-host-range plasmids bearing *lacZ* reporter gene constructions (13). We created both operon and gene fusions, which allowed us to monitor expression of the IAA operon and to localize its promoter. The transcription initiation site was confirmed by a combination of S1 nuclease mapping and primer extension mapping, and the DNA sequence of the promoter was determined. Factors contributing to overall IAA production by *P. syringae* subsp. *savastanoi* are discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and the plasmid vectors used in this study are described in Table 1. A listing and description of the recombinant plasmids constructed in this study is provided in Fig. 1. *P. syringae* subsp. *savastanoi* was grown in either glucose-peptone medium (King's B) (21) or minimal A salts (28) supplemented with various carbon and nitrogen sources. Media were supplemented with 15 µg of tetracycline per ml for selection of broad-host-range plasmids. *Escherichia coli* was cultured in LB medium (3) or in minimal A salts supplemented with 0.2% (wt/vol) glucose. Concentrations of antibiotics used for selection of various *E. coli* plasmids were as follows: ampicillin, 50 µg/ml; tetracycline, 15 µg/ml.

Enzymes and reagents. Restriction endonucleases were purchased from New England BioLabs, Inc. (Beverly, Mass.) or Boehringer Mannheim Biochemicals (Indianapolis, Ind.); avian myeloblastosis virus reverse transcriptase was from Life Sciences, Inc. (St. Petersburg, Fla.); mung bean nuclease and T4 polynucleotide kinase were from New England BioLabs; RNasin was from Promega Biotec (Madison, Wis.); S1 nuclease was from International Biotechnologies, Inc. (New Haven, Conn.); and acrylamide and

* Corresponding author.

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

‡ Present address: CIBA-GEIGY Biotechnology, Box 12257, Research Triangle Park, NC 27709.

§ Present address: Max-Planck-Institut für Züchtungsforschung, Abt. Biochemie, 5000 Köln-30, Federal Republic of Germany.

|| Present address: Laboratory of Plant Pathology, College of Agriculture, Okayama University, Tsushima, Okayama, 700, Japan.

TABLE 1. Bacterial strains and plasmid cloning vectors

Strain or plasmid	Relevant characteristics	Source or reference
<i>P. syringae</i> subsp. <i>savastanoi</i>		
EW1006	Olive isolate; IAA ⁺ ; chromosomal <i>iaa</i> operon	9
EW2009	Oleander isolate; IAA ⁺ ; plasmid-borne <i>iaa</i> operon (52-kb pIAA1)	36
EW2009-3	IAA ⁻ derivative of EW2009; cured of pIAA1	36
PB213	Oleander isolate; IAA ⁺ ; plasmid-borne <i>iaa</i> operon (73-kb pIAA2)	9
TK800	Oleander isolate; IAA ⁺	S. Silverstone
<i>P. syringae</i> subsp. <i>syringae</i>	IAA ⁺	F. White (43)
<i>P. syringae</i> subsp. <i>pisi</i>	IAA ⁺	F. White (43)
<i>E. coli</i>		
HB101		27
DH5 α		Bethesda Research Laboratories, Inc., Gaithersburg, Md.
Plasmid vectors		
pUC118	Ap ^r ; mp18 multicloning region	39
pSP64	Ap ^r ; M13 polylinker	Promega Biotec
pGEM-blue3	Ap ^r ; M13 polylinker	Promega Biotec
pGD499	Tc ^r Ap ^r <i>lacZ</i>	13
pGD500	Tc ^r ; promoterless <i>lacZ</i>	13
pGD926	Tc ^r ; promoterless <i>lacZ</i> missing ribosome-binding site and first seven codons	13

bisacrylamide were from Beckman Instruments, Inc. (Fullerton, Calif.). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.) and Fisher Scientific Co. (Santa Clara, Calif.). A synthetic oligonucleotide (19-mer) with the sequence 5'-AATTCCATAGCGTGC GGG-3' was obtained from New England BioLabs.

Plasmid DNA isolation. Plasmid DNA was isolated by the alkaline lysis procedure of Birnboim and Doly (4). Plasmid DNA obtained from large-scale alkaline lysis preparations was purified by cesium chloride-ethidium bromide density centrifugation (5).

RNA isolation. RNA was purified from 100-ml bacterial cultures by the hot-phenol procedure as modified by Aiba et al. (1).

Construction of plasmids containing transcriptional (operon) fusions. The 3.2-kilobase (kb) *Bam*HI-*Hind*III fragment of the broad-host-range promoter probe plasmid pGD499 was replaced with DNA fragments originally derived from the *iaa* operon-containing plasmid pIAA1 (6) to create operon fusions as described by Ditta et al. (13). pSAV305 and pSAV306 were identified among the plasmids constructed by the following strategy. The ca. 660-base-pair *Pst*I-*Eco*RI fragment of pSAV301 (which lacks internal *Bam*HI and *Hind*III sites) was electroeluted from a 1.0% agarose gel with an Elutrap apparatus (Schleicher & Schuell, Inc., Keene, N.H.) and digested with *Hae*III. The restriction fragments were ligated with *Hinc*II-digested pUC118. Since the *Hinc*II site of pUC118 is between *Bam*HI and *Hind*III sites, it was possible to excise the DNA inserts from pUC118 as *Bam*HI-*Hind*III fragments. These were ligated with pGD499 which also had been digested with *Bam*HI and *Hind*III.

Construction of plasmids containing translational (gene) fusions. To generate translational fusions between the tryptophan monooxygenase gene (*iaaM*) and *lacZ*, we replaced the 0.4-kb *Hind*III-*Bam*HI fragment of pGD926 (13) with DNA fragments terminating at a *Bam*HI site within *iaaM* such that codon 122 of *iaaM* was followed in frame by codon 8 of *lacZ*. The 1.3-kb *Pst*I-*Bam*HI fragment containing the 5' portion of *iaaM* was obtained from pCJP12 (29) and ligated with *Pst*I and *Bam*HI-digested pGEM-blue3 (Promega Biotec) to generate pSAV300. pSAV300 served as a starting point in the following gene fusion constructions.

The presence in pGEM-blue3 of a *Hind*III site proximal to

the *Pst*I site in the multicloning region allowed the excision of the 1.3-kb insert in pSAV300 as a *Hind*III-*Bam*HI fragment for ligation with *Hind*III- and *Bam*HI-digested pGD926. This pGD926 derivative was designated pSAV602.

To construct pSAV603, we ligated a 0.9-kb *Eco*RV-*Bam*HI fragment derived from pSAV300 with *Hinc*II- and *Bam*HI-digested pUC118. The insert was excised from pUC118 with *Hind*III and *Bam*HI (utilizing the *Hinc*II-proximal *Hind*III site of the pUC118 multicloning region) for ligation with pGD926 which likewise had been digested with *Hind*III and *Bam*HI.

For construction of pSAV606, sequences between the *Eco*RV and *Sac*II sites within the *P. syringae* subsp. *savastanoi* DNA 1.3-kb insert portion of pSAV300 were deleted by digesting pSAV300 with *Eco*RV and *Sac*II, removing the *Sac*II 3' overhang with mung bean nuclease, and religating. A plasmid exhibiting the desired ca. 300-base-pair deletion was designated pSAV604. The truncated (ca. 1.0-kb) insert fragment was removed from pSAV604 with *Hind*III and *Bam*HI for subcloning into pGD926.

Construction of pSAV302 entailed ligation of a 0.4-kb *Dra*I-*Bam*HI fragment derived from pSAV300 with *Hinc*II- and *Bam*HI-digested pUC118. This insert was removed from pUC118 as a *Hind*III-*Bam*HI fragment (utilizing the *Hind*III site of the pUC118 multicloning region) for subcloning into pGD926.

Bacterial conjugations. Broad-host-range plasmids were introduced by conjugation into *P. syringae* subsp. *savastanoi* recipients by the triparental mating system (14). Mating mixtures were incubated at 28°C for 8 to 12 h on LB agar before bacteria were removed to selective plates.

Enzyme assays. β -Galactosidase was assayed as described by Miller (28).

Transcription initiation site mapping experiments. Primer extension (reverse transcriptase) mapping experiments and S1 nuclease mapping experiments were performed essentially as described by Débarbouillé and Raibaud (10) and Aiba et al. (1), respectively. For both types of mapping experiments, 5'-end-labeled DNA probes were recovered by elution from polyacrylamide gel slices. DNA (10 to 30 ng; usually 10,000 to 50,000 cpm) was mixed and lyophilized with 100 μ g of total RNA isolated from either *P. syringae* subsp. *savastanoi* or *E. coli*. Pellets were suspended in a

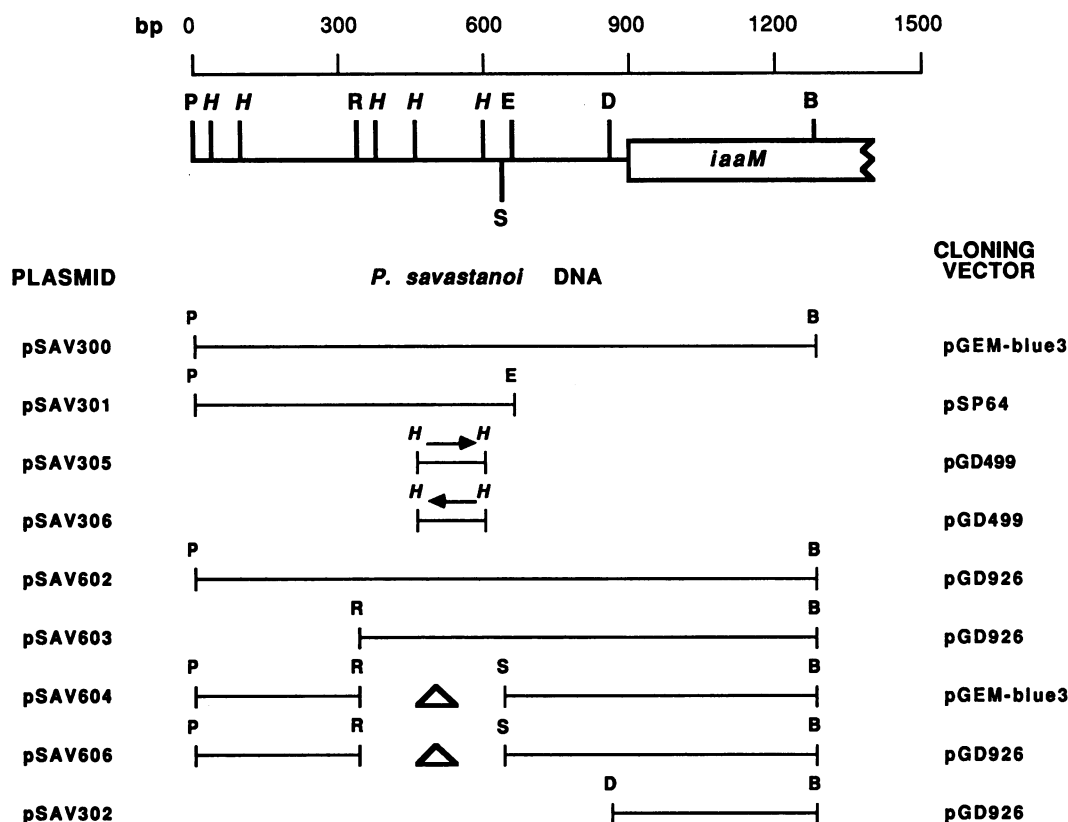


FIG. 1. Restriction endonuclease maps of *P. syringae* subsp. *savastanoi* DNA inserts in recombinant plasmids. Boundaries of *P. syringae* subsp. *savastanoi* DNA in the indicated plasmids are displayed below the restriction map of a portion of the *iaa* operon and 5' sequences (P, *Pst*I; R, *Eco*RV; E, *Eco*RI; D, *Dra*I; B, *Bam*HI; H, *Hae*III). Positions of *Hae*III sites are given only for the 660-base-pair (bp) region bounded by the *Pst*I and *Eco*RI sites. The triangles (Δ) indicate that sequences between the *Eco*RV and *Sac*II sites have been deleted in pSAV604 and pSAV606. The arrows indicate insert orientation relative to a *lacZ* reporter gene in pSAV305 and pSAV306. The jagged border on the boxed area representing the *iaaM* coding region signifies that only a 5' portion of *iaaM* is included in the map. The cloning vector used in the construction of each recombinant plasmid is listed at the right.

minimum volume of hybridization buffer (30 to 60 μ l) consisting of 80% formamide, 400 mM NaCl, and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 6.5), heated at 75°C for 10 min, and transferred immediately to a 50°C bath for a 3-h incubation.

For primer extension mapping, nucleic acids were precipitated after the 3-h incubation by bringing the total volume to 200 μ l with 0.3 M sodium acetate (pH 5.2), followed by the addition of 500 μ l of ethanol. The pellets were washed with 75% ethanol–25% 0.1 M sodium acetate (pH 5.2) and air dried. A reverse transcriptase reaction cocktail composed of 10 μ l of 2.5 mM deoxynucleoside triphosphates, 5 μ l of 10 \times reverse transcriptase buffer (500 mM Tris [pH 8.0], 50 mM MgCl₂, 50 mM dithiothreitol, 500 mM KCl), 3.0 μ l of RNasin, and 30 μ l of distilled water was used to suspend each pellet. Reverse transcriptase (50 U) was added to each sample. Incubation was at 42°C for 90 min. After phenol-chloroform (1:1) extraction and ammonium acetate precipitation, products were suspended in 20 μ l of gel-loading buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.25% [wt/vol] bromophenol blue, 0.25% [wt/vol] xylene cyanol), denatured by a 5-min incubation in a boiling water bath, and analyzed on a 7.0 M urea–8.0% polyacrylamide gel.

For S1 nuclease mapping, the 3-h, 50°C incubation was followed by the addition of 30 μ l of 10 \times S1 buffer (300 mM sodium acetate [pH 4.6], 500 mM NaCl, 10 mM ZnSO₄, 50% glycerol) and distilled water to a final volume of 300 μ l. S1

nuclease (150 U) was added to each sample, and incubation at 37°C was continued for 15 min. Reactions were stopped by phenol-chloroform (1:1) extraction. Nucleic acids were precipitated by the addition of 30 μ l of 3.0 M sodium acetate (pH 5.2), 2 μ g of carrier tRNA, and 2 volumes of ethanol. Precipitates were suspended in 20 μ l of gel-loading buffer, incubated for 5 min in boiling water, and loaded on a 7.0 M urea–8.0% polyacrylamide gel.

Preparation of ³²P-end-labeled DNA fragments for transcript mapping. The plasmid pSAV301 (Fig. 1) was digested with either *Eco*RI (in both the generation of the 73-base reverse transcriptase primer and the 316-base S1 nuclease mapping probe) or *Rsa*I (in preparing the 279-base S1 nuclease mapping probe). Resulting linear DNA molecules were dephosphorylated with calf intestinal alkaline phosphatase (27) and 5' end labeled with [γ -³²P]ATP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) and T4 polynucleotide kinase (27). DNA fragments containing a single labeled end were generated by digestion with a second restriction endonuclease (*Eco*RV for both S1 mapping probes and *Hae*III for the reverse transcriptase primer). Labeled fragments were purified by electrophoresis through a 7.0 M urea–8.0% polyacrylamide gel, followed by incubation of the appropriate gel slices in 200 μ l of elution buffer (10 mM magnesium acetate, 500 mM ammonium acetate, 1 mM EDTA, 0.1% [wt/vol] sodium dodecyl sulfate) at 55°C for 12 h.

DNA sequencing. pUC118 derivatives containing *Hae*III fragments subcloned from the *Pst*I-*Eco*RI fragment of pSAV301 were sequenced by the dideoxy-chain termination method (34) with double-stranded DNA templates, [α - 35 S] dATP (600 Ci/mmol; Amersham), and a sequencing reagent kit obtained from International Biotechnologies.

Nucleotide sequence accession number. The GenBank accession number of the 133-base-pair *Hae*III fragment of pSAV305 is M35690.

RESULTS

Localization of IAA operon promoter. The genes responsible for IAA biosynthesis in *P. syringae* subsp. *savastanoi*, *iaaM* and *iaaH*, are adjacent and cotranscribed (29), with *iaaH* being promoter distal. We selected a *Bam*HI restriction site within *iaaM* (Fig. 2) as the junction in the creation of a series of gene fusions of *iaaM* to a *lacZ* reporter gene carried on the broad-host-range plasmid pGD926. Detection of β -galactosidase activity with these constructs relied on transcription of the *iaa* operon directed by *P. syringae* subsp. *savastanoi* sequences with promoter activity. Translation of the *iaaM::lacZ* fusion protein presumably required the *iaaM* ribosome-binding site (42), which was present in all the gene fusions. Comparison of reporter gene expression from the gene fusions located promoter activity within a 283-base-pair region between an *Eco*RV site and a *Sac*II site (Fig. 2). Plasmids which contained this region (pSAV602 and pSAV603) exhibited significant promoter activity, while deletion of the *Eco*RV-*Sac*II fragment in pSAV606 reduced expression of β -galactosidase to the background level observed with pSAV302. Data from additional operon fusions constructed in the broad-host-range plasmid pGD499, in which the promoterless *lacZ* reporter gene retained its own ribosome-binding site, supported this finding by identifying promoter activity within the ca. 660-base-pair *Pst*I-*Eco*RI fragment of pSAV301 (data not shown). The presence of an additional 8.0 kb of DNA 5' of the identified promoter region in one such operon fusion did not further influence promoter activity (data not shown).

The 660-base-pair *Pst*I-*Eco*RI fragment of pSAV301 (Fig. 1) was purified from agarose after electrophoresis through a 1.0% gel and digested with the restriction endonuclease *Hae*III. The *Hae*III fragments generated were cloned into the *Hinc*II site of pUC118 and subsequently excised from pUC118 as *Bam*HI-*Hind*III fragments for subcloning into the operon fusion vector pGD499. In pSAV305 (Fig. 1), a 133-base-pair *Hae*III fragment which mapped to the predicted portion of the original 660-base-pair fragment possessed significant promoter activity. When pSAV305 was mobilized into *P. syringae* subsp. *savastanoi* EW2009-3, it directed the expression of ca. 2,000 Miller units (28) of β -galactosidase. In contrast, 300 Miller units of β -galactosidase were detected when the 133-base-pair *Hae*III fragment was cloned in the opposite orientation in pSAV306. Likewise, pGD500, a derivative of pGD499 which serves as a promoterless *lacZ* plasmid in *E. coli* but which does exhibit various degrees of background promoter activity in some other gram-negative bacteria (13), expressed a high background of 300 Miller units of β -galactosidase in *P. syringae* subsp. *savastanoi*. None of the remaining *Hae*III fragments generated from the 660-base-pair *Pst*I-*Eco*RI fragment demonstrated promoter activity above this background level when subcloned as described above into pGD499. The DNA sequence of the 133-base-pair *Hae*III fragment of pSAV305 is presented in Fig. 3.

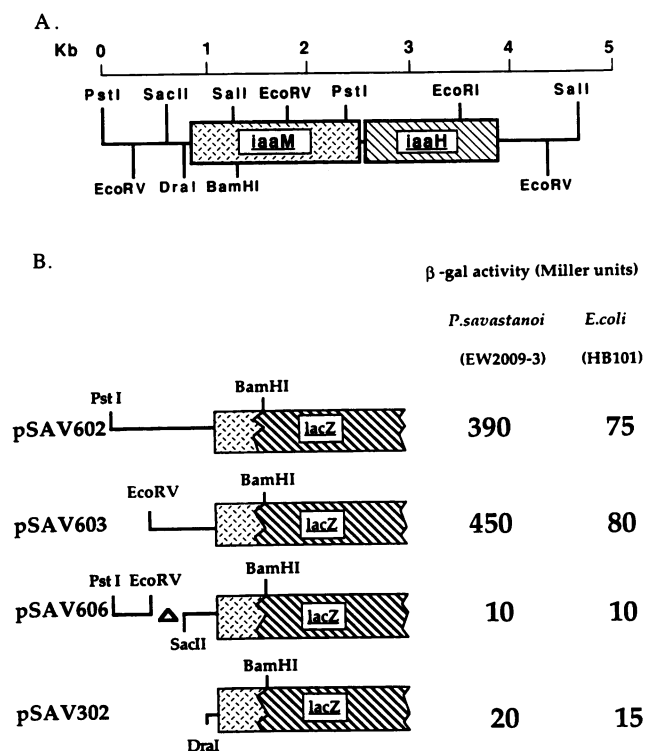


FIG. 2. β -Galactosidase expression from translational (gene) fusions of *iaaM* to a *lacZ* reporter gene. (A) The restriction endonuclease map of the *iaa* operon indicates the positions of *iaaM* and *iaaH*. The *Bam*HI site within *iaaM* was used to generate the translational fusion plasmids pSAV602, pSAV603, pSAV606, and pSAV302 as described in Materials and Methods. (B) Partial maps of the translational fusion plasmids depicting *P. syringae* subsp. *savastanoi* sequences 5' of the fusion site are presented adjacent to β -galactosidase levels detected when each plasmid was introduced into either *P. syringae* subsp. *savastanoi* EW2009-3 or *E. coli* HB101. β -Galactosidase activity is expressed in Miller units (28) and was measured in these experiments when bacterial cultures reached an optical density at 600 nm of between 0.6 and 1.0. The triangle (Δ) in the map of pSAV606 indicates that sequences between the *Eco*RV and *Sac*II sites were deleted.

Mapping of *iaa* operon transcription initiation site. Two mRNA-mapping techniques, S1 nuclease protection mapping and primer extension mapping (1, 10), were utilized to determine the transcription initiation site of the *iaa* operon. In S1 nuclease mapping experiments, two single-stranded DNA probes, one 5' end labeled at an *Rsa*I site 293 bases before *iaaM* and one 5' end labeled at an *Eco*RI site 252 bases before *iaaM* (Fig. 4), were hybridized with RNA from wild-type *P. syringae* subsp. *savastanoi* EW2009 and with RNA from both *E. coli* and *P. syringae* subsp. *savastanoi* EW2009-3 harboring the *iaaM::lacZ* gene fusion construct pSAV602. Each labeled probe had one terminus at an *Eco*RV site 571 bases before *iaaM* (Fig. 4). A ca. 115-base portion of the 279-base *Rsa*I-*Eco*RV probe was protected from S1 nuclease digestion owing to its hybridization with *iaa* operon transcripts (Fig. 4; lanes 2, 4, and 5). RNA from *P. syringae* subsp. *savastanoi* EW2009-3, a strain lacking the *iaa* operon owing to loss of the native plasmid pIAA1, failed to protect the *Rsa*I-*Eco*RV probe from digestion (Fig. 4, lane 3). A ca. 155-base portion of the 316-base *Eco*RI-*Eco*RV probe was protected from S1 nuclease digestion by *iaa* operon transcripts (Fig. 4, lane 1). The length of the pro-

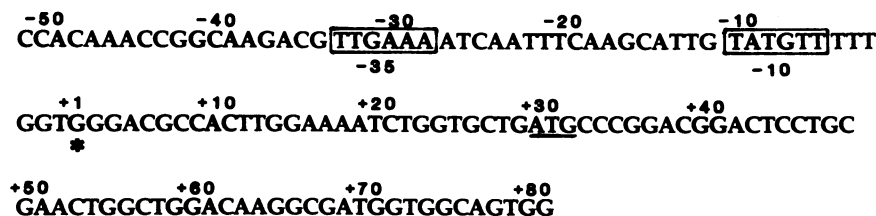


FIG. 3. DNA sequencing of the 133-base-pair *Hae*III restriction fragment containing the *iaa* operon promoter. The sequences homologous to the -10 and -35 regions of the *E. coli* σ^{70} consensus promoter are boxed. An asterisk marks the transcription initiation site as determined by fine-scale primer extension mapping. A potential translation start codon for a 228-base-pair open reading frame situated between the promoter and *iaaM* is underlined.

tected portion of each probe was consistent with the presence of a transcription initiation site approximately 400 bases before the start codon of *iaaM*.

Primer extension mapping experiments were performed to confirm the location of the transcription initiation site. A 73-base primer 5' end labeled at an *Eco*RI site 252 bases before *iaaM* (Fig. 5) was hybridized with RNA samples identical to those utilized in the S1 nuclease mapping experiments. This primer was extended with avian myeloblastosis virus reverse transcriptase in the presence of deoxyribonucleotides to the presumptive 5' end of the *iaa* operon mRNA. A ca. 155-base extension product was observed when RNA was from *P. syringae* subsp. *savastanoi* EW2009 or from either *E. coli* or *P. syringae* subsp. *savastanoi* EW2009-3 harboring pSAV602 (Fig. 5, lanes 2 to 4). No extension of the 73-base primer was observed when RNA from *P. syringae* subsp. *savastanoi* EW2009-3, the strain lacking the *iaa* operon, was used in the hybridization step (Fig. 5, lane 5). The size of the primer extension products indicated that transcription initiates approximately 400 bases 5' of *iaaM*. This measurement was in agreement with S1 nuclease mapping results. The 73-base primer also was used to determine precisely the base at which transcription initiates. The ca. 155-base product obtained from primer extension reactions with this primer was electrophoresed in a 7.0 M-8.0% polyacrylamide sequencing gel adjacent to DNA sequencing reactions primed with a synthetic oligonucleotide (19-mer). The 19-mer had a sequence identical to the first 19 bases of the 73-base primer. The residue at which transcription initiates (indicated by an asterisk in Fig. 3) was determined by identifying the product of the sequencing reactions which comigrated with the primer extension product (data not shown).

Promoter DNA sequence. In appropriate positions (boxed in Fig. 3) relative to the transcription initiation site are sequences homologous to the -10 and -35 regions of the consensus (σ^{70}) *E. coli* promoter sequence (17, 33). These regions are separated in the *iaa* operon promoter by 17 base pairs, a distance considered to be optimal in *E. coli* promoters (17, 33). This promoter sequence is notably rich in A · T base pairs (22 of the 29 base pairs bordered by the -10 and -35 regions). Transcript mapping experiments with RNA isolated from *E. coli* HB101 harboring pSAV602 (Fig. 4, lane 5; Fig. 5, lane 4) confirmed that transcription of the *P. syringae* subsp. *savastanoi* *iaa* operon in *E. coli* initiated at a location identical to that observed in *P. syringae* subsp. *savastanoi*.

Transcription of *iaa* genes in various *P. syringae* subsp. *savastanoi* isolates. *iaaM* and *iaaH* map at different locations in various *P. syringae* subsp. *savastanoi* isolates (9, 29), but in each case examined the organization of the *iaa* genes was conserved (29). To determine whether transcription initiated

at the same location relative to *iaaM* and *iaaH* in a variety of *P. syringae* subsp. *savastanoi* isolates, we utilized the 73-base reverse transcriptase primer (Fig. 5) in additional primer extension mapping experiments. Consistent with the strain EW2009 result, the 73-base primer was extended by reverse transcriptase to indistinguishable ca. 155-base products after hybridization to RNA from the olive isolate EW1006 and from two additional oleander isolates, PB213 and TK800 (data not shown).

Promoter-proximal open reading frame. DNA sequencing of the 406-base-pair region between the transcription initiation site and *iaaM* revealed the presence of a 228-base-pair open reading frame (GenBank accession number M35690). The first potential start codon is underlined in Fig. 3. We have no evidence that translation of this open reading frame occurs. No homology was detected between this DNA sequence or the amino acid sequence deduced from it and sequences deposited in the GenBank database. Likewise, no homology could be detected in Southern hybridization experiments (37) between *P. syringae* subsp. *savastanoi* DNA containing a portion of the 228-base-pair open reading frame and DNA isolated from *P. syringae* subsp. *pisi* and *P. syringae* subsp. *syringae* strains possessing *iaaM* and *iaaH* homologs. The *Pst*I-*Eco*RI restriction fragment of pSAV301, which contains the *P. syringae* subsp. *savastanoi* *iaa* operon promoter region and a 122-base-pair portion of the open reading frame, did not hybridize with the *P. syringae* subsp. *pisi* or the *P. syringae* subsp. *syringae* DNA under conditions less stringent than those which did allow hybridization of a *P. syringae* subsp. *savastanoi* *iaaM* probe with DNA from these bacteria (data not shown).

Expression of *P. syringae* subsp. *savastanoi* *iaa* operon. The plasmid pSAV602, in which expression of a gene fusion between *iaaM* and *lacZ* is directed by the *iaa* operon promoter, was mobilized into *P. syringae* subsp. *savastanoi* EW2009-3, a IAA⁻ derivative lacking the native plasmid pIAA1. pSAV602 and the additional broad-host-range constructions utilized in this study are low-copy-number plasmids maintained in *P. syringae* subsp. *savastanoi* at a level similar to that of native *iaa* operon-containing plasmids (based on intensities of respective plasmid DNA bands in agarose gels when DNA is recovered from strains containing both native and recombinant plasmids). β -Galactosidase activity due to expression of the *iaaM*::*lacZ* fusion protein was monitored as a measure of *iaa* operon expression. Expression of the fusion protein did not fluctuate over more than a twofold range regardless of the growth medium utilized (Table 2). Since it was possible that the loss of pIAA1 in EW2009-3 might have removed some component required for transcriptional regulation of the *iaa* operon, pSAV602 also was mobilized into an IAA⁺ strain, *P. syringae* subsp. *savastanoi* PB213. Analogous constitutive

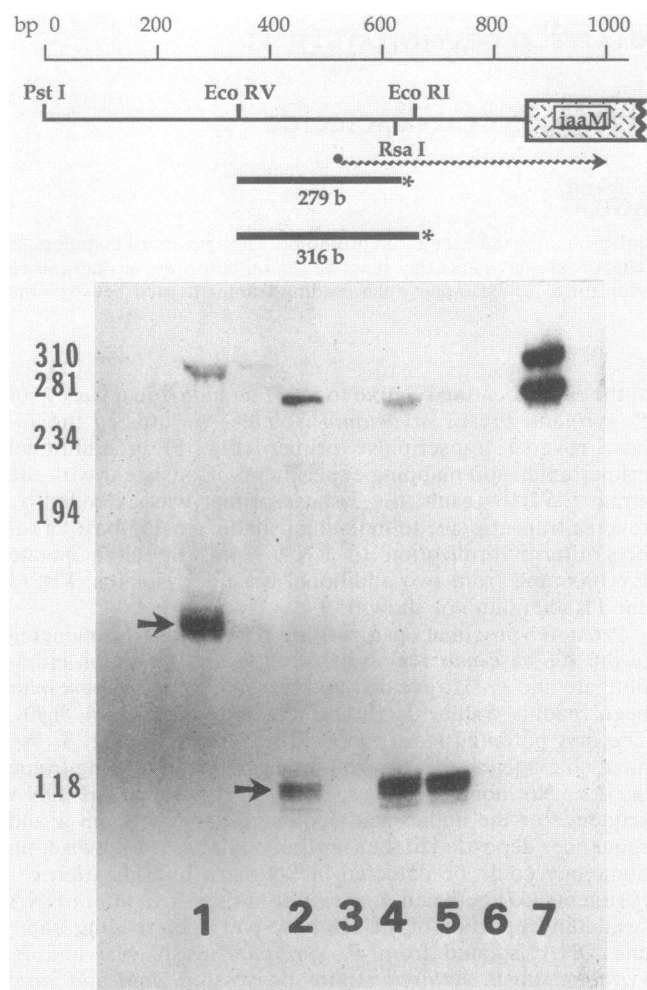


FIG. 4. S1 nuclease protection experiment for mapping the *iaa* operon transcription initiation site. Restriction map positions of the 279-base *RsaI*-*EcoRV* and 316-base *EcoRI*-*EcoRV* hybridization probes are indicated. An asterisk denotes the ^{32}P -labeled end of each probe. The wavy arrow represents the direction of transcription of the *iaa* operon. RNA recovered from the strains designated below was hybridized under conditions favoring RNA-DNA hybridization with the indicated probes and digested with 150 U of S1 nuclease as described in Materials and Methods. Lane 1, *P. syringae* subsp. *savastanoi* EW2009-3(pSAV602) RNA; 316-base probe. Lane 2, *P. syringae* subsp. *savastanoi* EW2009 RNA; 279-base probe. Lane 3, *P. syringae* subsp. *savastanoi* EW2009-3 RNA; 279-base probe. Lane 4, *P. syringae* subsp. *savastanoi* EW2009-3(pSAV602) RNA; 279-base probe. Lane 5, *E. coli* HB101(pSAV602) RNA; 279-base probe. Lane 6, No RNA added (control); 279- and 316-base probes. Lane 7, No S1 nuclease added (control); *P. syringae* subsp. *savastanoi* EW2009 RNA; 279- and 316-base probes. Arrows indicate the positions of the fragments protected from S1 nuclease digestion. Bacteriophage ϕ X174 *HaeIII* restriction fragment size markers (base) (not shown) migrated to the positions indicated at the left of lane 1.

expression was observed (data not shown). Measurements of β -galactosidase activity taken at different time points along the bacterial growth curve also displayed little variability (data not shown).

DISCUSSION

The virulence of *P. syringae* subsp. *savastanoi* toward its plant hosts is dependent in part on the synthesis of a

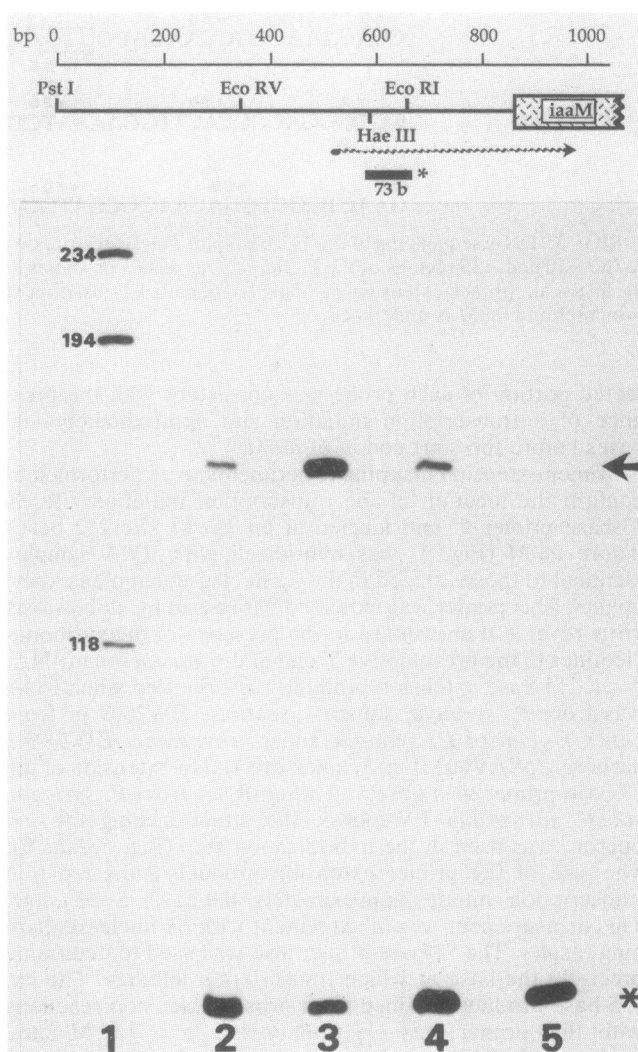


FIG. 5. Reverse transcriptase primer extension mapping of the *iaa* operon transcription initiation site. The restriction map position of the 73-base *EcoRI*-*HaeIII* hybridization probe is indicated. A small asterisk adjacent to the 73-base probe denotes its ^{32}P -labeled end. The wavy arrow represents the direction of transcription of the operon. Hybridization of the 73-base probe with RNA recovered from the strains indicated below served to prime avian myeloblastosis virus reverse transcriptase-directed DNA polymerization as described in Materials and Methods. The arrow on the autoradiograph identifies the position of the ca. 155-base extended products. The asterisk at the bottom of the autoradiograph marks the position of residual unextended 73-base primer. Lane 1, ϕ X174 *HaeIII* restriction fragment size markers; lane 2, *P. syringae* subsp. *savastanoi* EW2009 RNA; lane 3, *P. syringae* subsp. *savastanoi* EW2009-3(pSAV602) RNA; lane 4, *E. coli* HB101(pSAV602) RNA; lane 5, *P. syringae* subsp. *savastanoi* EW2009-3 (IAA⁻) RNA. Numbers on left show size in bases. bp, Base pairs.

secondary metabolite, the plant growth hormone IAA, from the precursor tryptophan. Using *lacZ* as a reporter gene, we identified the promoter which directs transcription of the *iaa* operon and measured transcription of the operon under a variety of growth conditions. Our results indicate that transcription of the *iaa* operon proceeds in a constitutive manner for all culture conditions examined. The failure of medium composition to significantly influence extractable *P. syringae* subsp. *savastanoi* tryptophan monooxygenase activity

TABLE 2. β -Galactosidase activity from *iaaM::lacZ* fusion in *P. syringae* subsp. *savastanoi* EW2009-3(pSAV602) with various growth media

Carbon source ^a (+ supplement)	Nitrogen source ^b	β -Galactosidase activity (U) ^c
Glucose	Glutamine	610
Glucose (+ tryptophan)	Glutamine	670
Glucose	Ammonium sulfate	590
Glucose (+ tryptophan)	Ammonium sulfate	690
Glycerol	Glutamine	620
Fructose	Ammonium sulfate	900
Mannitol	Ammonium sulfate	800
Citric acid	Ammonium sulfate	650
Succinic acid	Ammonium sulfate	750
Complex media		
King's B		560
LB		650

^a Individual carbon sources were added to minimal A salts at 0.4% (wt/vol), except for mannitol, which was added at 0.2% (wt/vol). L-Tryptophan was supplemented at 250 μ g/ml where indicated.

^b Glutamine replaced ammonium sulfate where indicated as a nitrogen source in the minimal A medium. Glutamine was added at 0.2% (wt/vol).

^c Levels of β -galactosidase activity expressed in Miller units (28) represent the average of values obtained from a minimum of two experiments with a minimum of two repetitions per experiment.

in previous studies (24, 36) supports this conclusion. Our experiments did not address whether expression of *iaa* genes differ when *P. syringae* subsp. *savastanoi* is associated with host plants. However, it is clear that active transcription of these genes proceeds in the absence of a host plant.

A *Pseudomonas putida* consensus sequence for constitutive promoters associated with genes involved in the catabolism of several aromatic compounds has been proposed (19). Since IAA biosynthesis by *P. syringae* subsp. *savastanoi* also could be viewed as incomplete catabolism of L-tryptophan, we had considered the possibility that the *iaa* operon promoter has some similarity to this consensus sequence. In contrast, another class of *Pseudomonas* promoters among those studied to date resembles the *E. coli* consensus promoter (11, 20), and several members of this group are constitutively expressed (12). The DNA sequence determined for the *iaa* operon promoter (Fig. 3) places it in the *E. coli* consensus class. Our data also indicate that the *iaa* operon promoter is functional in *E. coli* (Fig. 2, 4, and 5). Full promoter activity was detected in a 133-base-pair *Hae*III restriction fragment containing only 20 additional base pairs before the -35 region of the promoter, indicating both that sequences further upstream of the *E. coli*-type promoter are not required for expression and that the identified 5' end of the *iaa* operon mRNA is indeed the transcription initiation site and not a processing site.

The *iaa* operon promoter is situated over 400 base pairs 5' of *iaaM*, the gene encoding tryptophan monooxygenase. While the region between the promoter and *iaaM* contains a 228-base open reading frame, it is not known at present whether a corresponding gene product is synthesized. It is unlikely that such a product would be required for the virulence of *P. syringae* subsp. *savastanoi* since a cloned portion of the *iaa* operon lacking the intact 228-base open reading frame was capable of restoring virulence to an IAA⁻ *P. syringae* subsp. *savastanoi* mutant (7).

Isolates of the *P. syringae* subsp. *pisi* and *syringae* which contain *iaaM* and *iaaH* genes nearly identical in DNA sequence and arrangement to those of *P. syringae* subsp. *savastanoi* (43) lack DNA sequences which hybridize with a

probe containing the *P. syringae* subsp. *savastanoi* *iaa* operon promoter and a portion of the 228-base open reading frame. Homology among the *iaa* regions of the three subspecies ends at a point less than 200 base pairs 5' of the *iaaM* start codon, suggesting that this position represents one endpoint of whatever genetic exchange event(s) may have led to distribution of the *iaa* genes (43). It has been suggested that *iaaM* and *iaaH* are, or once were, part of a mobile DNA element (29, 41). Conceivably, the promoter directing transcription of the *iaa* genes in *P. syringae* subsp. *savastanoi*, which is situated over 200 base pairs further upstream than the site where homology with *iaa* region sequences from the other *P. syringae* subspecies end, was not originally associated with these genes. It will be of interest to identify the regulatory sequences controlling transcription of *iaaM* and *iaaH* in *P. syringae* subsp. *pisi* and *P. syringae* subsp. *syringae*.

Despite the constitutive expression of *iaaM* and *iaaH* in culture, several factors contribute to the secretion of various levels of IAA by *P. syringae* subsp. *savastanoi*. Tryptophan monooxygenase has a K_m of 50 μ M for its substrate tryptophan, high enough to ensure that available tryptophan under limiting conditions preferentially services protein synthesis requirements (18). This enzyme is sensitive to feedback inhibition by both indoleacetamide and IAA (18). The availability of exogenous tryptophan can dramatically increase the yield of IAA from a *P. syringae* subsp. *savastanoi* culture without significantly altering tryptophan monooxygenase activity (24) and without requiring an increase in transcription of *iaaM* and *iaaH*. The conversion of IAA to an amino acid conjugate, IAA-lysine, in some *P. syringae* subsp. *savastanoi* isolates further influences IAA accumulation. It is not known whether host plants supply *P. syringae* subsp. *savastanoi* with significant levels of exogenous tryptophan for IAA production. Leaves and stems of tobacco infected with *Pseudomonas solanacearum* accumulate levels of tryptophan substantially higher than those observed in healthy tissues (30). If *P. syringae* subsp. *savastanoi* hosts respond to infection in a similar manner, bacterial IAA production might be elevated, again without a requirement for increased *iaa* operon transcription.

It has been suggested that auxin and cytokinin concentrations in plant tissues invaded by pathogens can affect levels of hydrolytic enzymes such as chitinases and β -1,3-glucanases which are thought to be involved in plant defense responses (35). In addition to its role in tumorigenesis and its potential role in the detoxification of tryptophan analogs, expression of the IAA operon of *P. syringae* subsp. *savastanoi* in conjunction with bacterial cytokinin biosynthesis might alter the expression of certain plant defense enzymes. It remains to be determined whether the expression of genes specifying IAA biosynthesis in various plant-associated bacteria, including several pathogens such as *P. syringae* subsp. *pisi* and *P. syringae* subsp. *syringae* in which the function of such genes is unknown, allows these bacteria to influence aspects of the plant defense response as a step toward optimizing their interaction with host plants.

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