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

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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

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

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TABLE 1. Bacterial strains and plasmid cloning vectors

Strain or plasmid	Relevant characteristics	Source or reference
P. syringae subsp. *savastanoi*		
EW1006	Olive isolate; IAA$^+$; chromosomal *iaa* operon	9
EW2009	Oleander isolate; IAA$^+$; plasmid-borne *iaa* operon (52-kb pIAA1)	36
EW2009-3	IAA$^-$ derivative of EW2009; cured of pIAA1	36
PB213	Oleander isolate; IAA$^+$; plasmid-borne *iaa* operon (73-kb pIAA2)	9
TK800	Oleander isolate; IAA$^+$	S. Silverstone
P. syringae subsp. *syringae*	IAA$^+$	F. White (43)
P. syringae subsp. *pisi*	IAA$^+$	F. White (43)
E. coli		
HB101		27
DH5α		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 *lacZ*	13
pGD500	Tc^r; promoterless *lacZ*	13
pGD926	Tc^r; promoterless *lacZ* 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'-AATTCCATAGCGTGCGGGG-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 μg of total RNA isolated from either *P. syringae* subsp. *savastanoi* or *E. coli*. Pellets were suspended in a
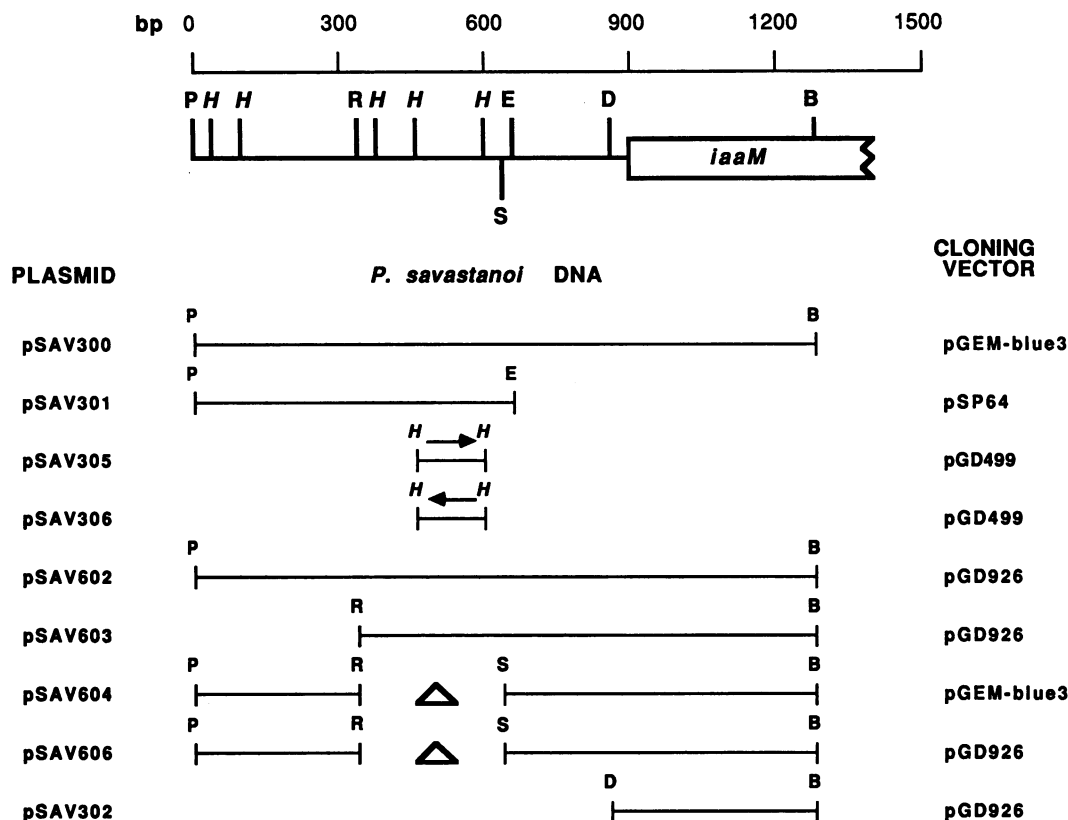
FIG. 1. Restriction endonuclease maps of *P. syringae* subsp. *savastanoi* DNA inserts in recombinant plasmids. Boundaries of *P. syringae* subsp. *savastanoi* DNA in the indicated plasmids are displayed below the restriction map of a portion of the *iaa* operon and 5' sequences (P, *Pst*I; R, *Eco*RV; S, *Sac*II; 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 (△) 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 µl) 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 µl with 0.3 M sodium acetate (pH 5.2), followed by the addition of 500 µl 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 µl of 2.5 mM deoxynucleoside triphosphates, 5 µl of 10× reverse transcriptase buffer (500 mM Tris [pH 8.0], 50 mM MgCl$_2$, 50 mM dithiothreitol, 500 mM KCl), 3.0 µl of RNasin, and 30 µl 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 µl of gel-loading buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.25% [wt/vol] bromphenol 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 µl of 10× S1 buffer (300 mM sodium acetate [pH 4.6], 500 mM NaCl, 10 mM ZnSO$_4$, 50% glycerol) and distilled water to a final volume of 300 µl. 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 µl of 3.0 M sodium acetate (pH 5.2), 2 µg of carrier tRNA, and 2 volumes of ethanol. Precipitates were suspended in 20 µl 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 µl 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.
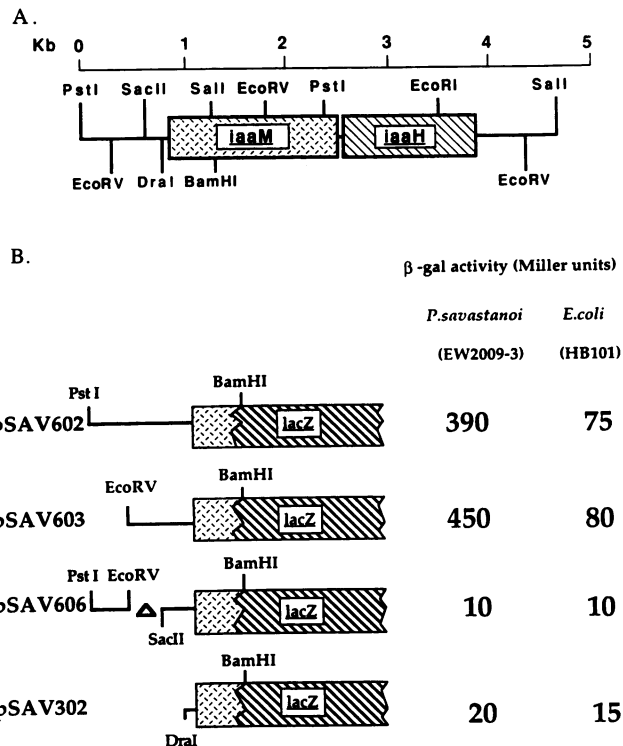
FIG. 2. β-Galactosidase expression from translational (gene) fusions of *iaaM* to a *lacZ* reporter gene. (A) The restriction endonuclease map of the *iaa* operon indicates the positions of *iaaM* and *iaaH*. The *Bam*HI site within *iaaM* was used to generate the translational fusion plasmids pSAV602, 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-
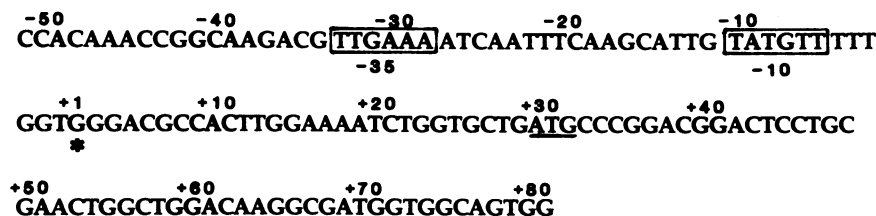
```
    -50            -40            -30          -20          -10
    CCACAAACCGGCAAGACG[TTGAAA]ATCAATTTCAAGCATTG[TATGTT]TTT
                               -35                          -10

    +1             +10            +20          +30          +40
    GGTGGGACGCCACTTGGAAAATCTGGTGCTGATGCCCGGACGGACTCCTGC
    *

    +50            +60            +70          +80
    GAACTGGCTGGACAAGGCGATGGTGGCAGTGG
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FIG. 3. DNA sequencing of the 133-base-pair HaeIII 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 EcoRI 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 ant 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 PstI-EcoRI 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. Analagous constitutive
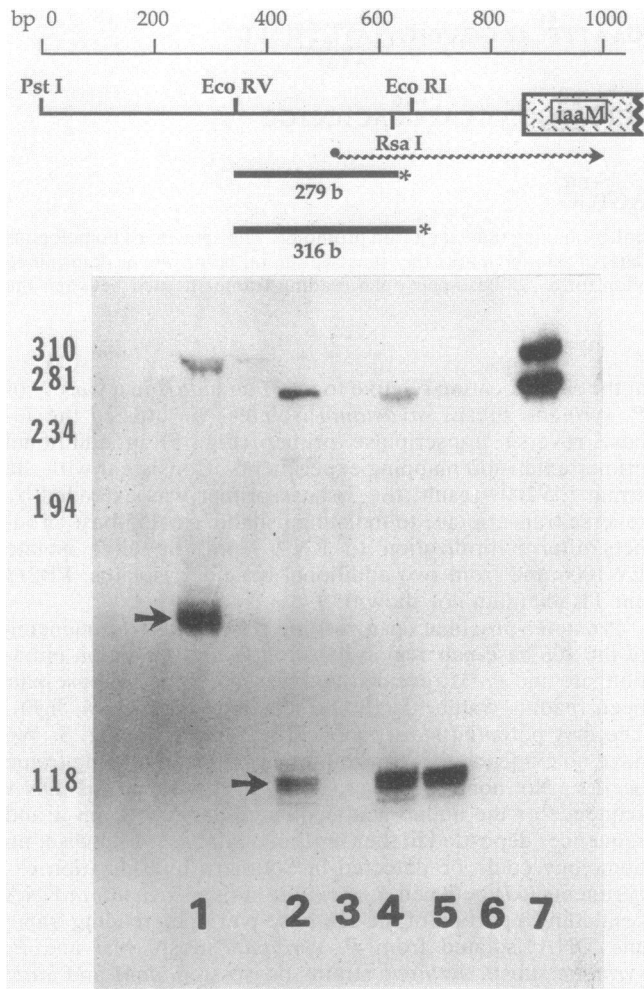
FIG. 4. S1 nuclease protection experiment for mapping the *iaa* operon transcription initiation site. Restriction map positions of the 279-base *Rsa*I-*Eco*RV and 316-base *Eco*RI-*Eco*RV hybridization probes are indicated. An asterisk denotes the ^{32}P-labeled end of each probe. The wavy arrow represents 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 ϕX174 *Hae*III 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
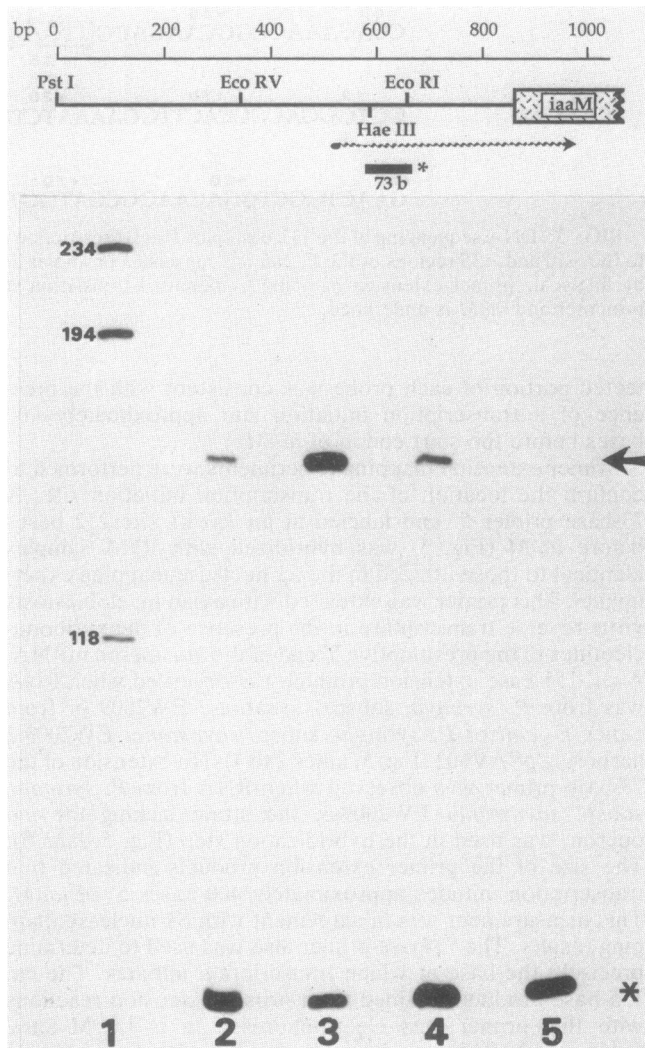
FIG. 5. Reverse transcriptase primer extension mapping of the *iaa* operon transcription initiation site. The restriction map position of the 73-base *Eco*RI-*Hae*III hybridization probe is indicated. A small asterisk adjacent to the 73-base probe denotes its ^{32}P-labeled end. The wavy arrow represents 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, ϕX174 *Hae*III 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 μg/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 HaeIII 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 μM 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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LITERATURE CITED

1. **Aiba, H., S. Adhya, and B. deCrombrugghe.** 1981. Evidence for two functional gal promoters in intact Escherichia coli cells. J. Biol. Chem. **256:**11905–11910.
2. **Barbieri, P., A. Bernardi, E. Galli, and G. Zanetti.** 1988. Effects of inoculation with different strains of Azospirillum brasiliense on wheat roots development, p. 181–188. In W. Klingmuller (ed.), Azospirillum IV: genetics, physiology, ecology. Springer-Verlag KG, Berlin.
3. **Bertani, G.** 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic Escherichia coli. J. Bacteriol. **62:**293–300.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
5. **Clewell, D. B., and D. R. Helinski.** 1969. Supercoiled circular DNA-protein complex in Escherichia coli: purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. USA **62:**1159–1166.
6. **Comai, L., and T. Kosuge.** 1980. Involvement of plasmid deoxyribonucleic acid in indoleacetic acid synthesis in Pseudomonas savastanoi. J. Bacteriol. **142:**950–957.
7. **Comai, L., and T. Kosuge.** 1982. Cloning and characterization of iaaM, a virulence determinant of Pseudomonas savastanoi. J. Bacteriol. **149:**40–46.
8. **Comai, L., and T. Kosuge.** 1983. Transposable element that causes mutations in a plant pathogenic Pseudomonas sp. J. Bacteriol. **154:**1162–1167.
9. **Comai, L., G. Surico, and T. Kosuge.** 1982. Relation of plasmid DNA to indoleacetic acid production in different strains of Pseudomonas syringae pv. savastanoi. J. Gen. Microbiol. **128:**2157–2163.
10. **Débarbouillé, M., and O. Raibaud.** 1983. Expression of the Escherichia coli malPQ operon remains unaffected after drastic alteration of its promoter. J. Bacteriol. **153:**1221–1227.
11. **Deretic, V., J. F. Gill, and A. M. Chakrabarty.** 1987. Alginate biosynthesis: a model system for gene regulation and function in Pseudomonas. Bio/Technology **5:**469–477.
12. **Deretic, V., W. M. Konyescni, C. D. Mohr, D. W. Martin, and N. S. Hibler.** 1989. Common denominators of promoter control in Pseudomonas and other bacteria. Bio/Technology **7:**1249–1254.
13. **Ditta, G., T. Schmidhauser, E. Yakobson, P. Lu, X.-W. Liang, D. R. Finlay, D. Guiney, and D. R. Helinski.** 1985. Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. Plasmid **13:**149–153.
14. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range cloning system for gram-negative bacteria: construction of a gene bank of Rhizobium meliloti. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
15. **Fett, W. F., S. F. Osman, and M. F. Dunn.** 1987. Auxin production by plant-pathogenic pseudomonads and xanthomonads. Appl. Environ. Microbiol. **53:**1839–1845.
16. **Glass, N. L., and T. Kosuge.** 1986. Cloning of the gene for indoleacetic acid-lysine synthetase from Pseudomonas syringae subsp. savastanoi. J. Bacteriol. **166:**598–603.
17. **Hawley, D. K., and W. R. McClure.** 1983. Compilation and analysis of Escherichia coli promoter DNA sequences. Nucleic Acids Res. **11:**2237–2255.
18. **Hutcheson, S. W., and T. Kosuge.** 1985. Regulation of 3-indoleacetic acid production in Pseudomonas syringae pv. savastanoi. J. Biol. Chem. **260:**6281–6287.
19. **Inouye, S., Y. Asai, A. Nakazawa, and T. Nakazawa.** 1986. Nucleotide sequence of a DNA segment promoting transcription in Pseudomonas putida. J. Bacteriol. **166:**739–745.
20. **Itoh, Y., L. Soldati, V. Stalon, P. Falmagne, Y. Terawaki, T.**
21. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. **44:**301–307.
22. **Kosuge, T., M. G. Heskett, and E. E. Wilson.** 1966. Microbial synthesis and degradation of indole-3-acetic acid. I. The conversion of L-tryptophan to indole-3-acetamide by an enzyme from Pseudomonas savastanoi. J. Biol. Chem. **241:**3738–3744.
23. **Kosuge, T., C. J. Palm, S. W. Hutcheson, N. L. Glass, and T. Yamada.** 1985. pIAA1, a virulence plasmid in Pseudomonas savastanoi, p. 807–813. In D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson, and A. Hollaender (ed.), Plasmids in bacteria. Plenum Publishing Corp., New York.
24. **Kuo, T. T., and T. Kosuge.** 1969. Factors influencing the production and further metabolism of indole-3-acetic acid by Pseudomonas savastanoi. J. Gen. Appl. Microbiol. **15:**51–63.
25. **Libbert, E., S. Wichner, E. Duerst, W. Kaiser, R. Kunert, A. Manicki, R. Manteuffel, E. Riecke, and R. Schroder.** 1968. Auxin content and auxin synthesis in sterile and non-sterile plants, with special regard to the influence of epiphytic bacteria, p. 213–230. In F. Wightman and G. Setterfield (ed.), The biochemistry and physiology of plant growth substances. Runge Press, Ottawa, Canada.
26. **Libbert, E., S. Wichner, U. Schiewer, H. Risch, and W. Kaiser.** 1966. The influence of epiphytic bacteria on auxin metabolism. Planta **68:**327–334.
27. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Palm, C. J., T. Gaffney, and T. Kosuge.** 1989. Cotranscription of genes encoding indoleacetic acid production in Pseudomonas savastanoi. J. Bacteriol. **171:**1002–1009.
30. **Pegg, G. F., and L. Sequeira.** 1968. Stimulation of aromatic biosynthesis in tobacco plants infected by Pseudomonas solanacearum. Phytopathology **58:**476–483.
31. **Powell, G. K., and R. O. Morris.** 1986. Nucleotide sequence and expression of a Pseudomonas savastanoi cytokinin biosynthetic gene: homology with Agrobacterium tumefaciens tmr and tzs loci. Nucleic Acids Res. **14:**2555–2565.
32. **Roberto, F. F., and T. Kosuge.** 1987. Phytohormone metabolism in Pseudomonas syringae subsp. savastanoi, p. 371–380. In J. E. Fox and M. Jacobs (ed.), Molecular biology of plant growth control. Alan R. Liss, Inc., New York.
33. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13:**319–353.
34. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
35. **Shinshi, H., D. Mohnen, and F. Meins, Jr.** 1987. Regulation of a plant pathogenesis-related enzyme: inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. Proc. Natl. Acad. Sci. USA **84:**89–93.
36. **Smidt, M., and T. Kosuge.** 1978. The role of indole-3-acetic acid accumulation by alpha methyl tryptophan-resistant mutants of Pseudomonas savastanoi in gall formation on oleanders. Physiol. Plant Pathol. **13:**203–214.
37. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
38. **Surico, G., L. Comai, and T. Kosuge.** 1984. Pathogenicity of strains of Pseudomonas syringae pv. savastanoi and their indoleacetic acid-deficient mutants on olive and oleander. Phytopathology **74:**490–493.
39. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153D:**3–11.
40. **Wilson, E. E., and A. R. Magie.** 1963. Physiological, serological,

and pathological evidence that *Pseudomonas tonelliana* is identical with *Pseudomonas savastanoi*. Phytopathology **53**:653–659.

41. **Yamada, T., P.-D. Lee, and T. Kosuge.** 1986. Insertion sequence elements of *Pseudomonas savastanoi*: nucleotide sequence and homology with *Agrobacterium tumefaciens* transfer DNA. Proc. Natl. Acad. Sci. USA **83**:8263–8267.

42. **Yamada, T., C. J. Palm, B. Brooks, and T. Kosuge.** 1985.

Nucleotide sequences of the *Pseudomonas savastanoi* indoleacetic acid genes show homology with *Agrobacterium tumefaciens* T-DNA. Proc. Natl. Acad. Sci. USA **82**:6522–6526.

43. **Ziegler, S. F., F. F. White, and E. W. Nester.** 1987. Genes involved in indole acetic acid production in plant pathogenic bacteria, p. 18–25. *In* E. L. Civerolo, A. Collmer, R. E. Davis, and A. G. Gillaspie (ed.), Plant pathogenic bacteria. Martinus Nijhoff Publishers, Dodrecht, The Netherlands.