# Cloning and Characterization of *iaaM* and *iaaH* from *Erwinia herbicola* pathovar *gypsophilae*

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## ABSTRACT

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*Erwinia herbicola* pv. *gypsophilae* induces galls on its host, *Gypsophila paniculata*. A 16-kb DNA fragment derived from a 78-Md native plasmid with homology to the *iaa* operon of *Pseudomonas syringae* pv. *savastanoi* was isolated from an EMBL3 library of *E. h. gypsophilae*, strain PD713, DNA. A 7.5-kb *Eco*RI fragment was subcloned into pUC118 to generate pEG101. *Escherichia coli* DH5α cells transformed with pEG101 produced indole-3-acetic acid (IAA) when cultured in medium supplemented with L-tryptophan (TRP). Permeabilized, transformed cells direct the synthesis of IAA from indole-3-acetamide (IAM). The IAA biosynthetic capability was localized to a 4.0-kb *Hin*dIII-*Eco*RI fragment through subcloning and insertional inactivation. The IAA biosynthetic genes of *E. h. gypsophilae* were designated *iaaM* and *iaaH* because of their structural and functional similarity to the *iaaM* and *iaaH* of *P. s. savastanoi*, which encode tryptophan-2-monooxygenase and indoleacetamide hydrolase, respectively. Insertional mutations were generated in *E. h. gypsophilae iaaM* and *iaaH*. Marker-exchange mutants of *E. h. gypsophilae*, generated using insertionally inactivated constructs, produced the same amount of IAA in culture as the wild type. The marker-exchange mutants, which exhibited either reduction or elimination of *iaaH* activity, induced smaller galls than did unmodified *E. h. gypsophilae*.

*Additional keywords*: hyperplasia, indoleacetic acid.

*Gypsophila paniculata* L. (baby's breath) is an ornamental used in commercial cut-flower production (35). *Erwinia herbicola* pv. *gypsophilae* induces gall formation on gypsophila plants (1,7,12). The host range of this pathogen is apparently restricted to gypsophila (40). Although another pathogenic strain of *E. herbicola* generates galls on table beets (*Beta vulgaris* L.) as well as gypsophila, *E. h. gypsophilae* induces galls only on gypsophila (2).

Indole-3-acetic acid biosynthesis is a virulence determinant for the gall-forming olive and oleander pathogen *Pseudomonas syringae* pv. *savastanoi* (5,6,17). In *P. s. savastanoi*, the conversion of L-tryptophan to indole-3-acetamide is catalyzed by tryptophan-2-monooxygenase, which *iaaM* encodes. Indole-3-acetamide (IAM) is hydrolyzed to indole-3-acetic acid (IAA) by indoleacetamide hydrolase, which *iaaH* encodes (6,15,43). *P. s. savastanoi iaaM* and *iaaH* are cotranscribed (27) and are constitutively expressed during bacterial growth in culture (11). In *Agrobacterium tumefaciens*, *iaaM* and *iaaH* occur in a monocistronic arrangement on the T-DNA and are expressed in transformed plant cells (25,29). Some *Agrobacterium rhizogenes* strains harbor IAA genes similar to those of *A. tumefaciens* (26,29,41). The nucleotide sequences and derived amino acid sequences of *iaaM* and *iaaH* of *P. s. savastanoi* share significant homology with *Agrobacterium iaaM* and *iaaH* (3,43). The *bam* gene of *Bradyrhizobium japonicum*, which encodes indoleacetamide hydrolase, shows nucleotide sequence homology to the other three sequenced *iaaH* genes (33). White and Ziegler (42) cloned an *iaa* operon from the bean pathogen *P. syringae* pv. *syringae*, which does not form galls, by utilizing its homology to the *P. s. savastanoi iaa* operon. Thus, the conversion of L-tryptophan to IAA via IAM is directed by genes occurring in several genera and species. The observed sequence homologies suggest a common evolutionary origin for this pathway (43).

Though the role of IAA biosynthesis as a virulence determinant has been established for *P. s. savastanoi* and *A. tumefaciens*, the function of this pathway in other bacteria is unknown (9,42). Moreover, many plant-associated bacteria, including pathogenic, saprophytic, and beneficial epiphytes, possess an IAA biosynthetic capacity apparently derived from a pathway, or pathways, in which IAM is not an intermediate (9,34). The role, if any, of IAA biosynthesis via alternate pathways in plant-bacteria interactions is also unknown. In addition, the IAA biosynthetic pathway of *P. s. savastanoi* and *A. tumefaciens* may serve multiple functions; it can detoxify several growth-inhibiting tryptophan analogues (16), and it may suppress the hypersensitive response of host plants during mixed infections that include incompatible bacterial species (30).

Previous studies indicated that the mode of hyperplasia production by *E. h. gypsophilae* resembles that of *P. s. savastanoi* (4). *E. h. gypsophilae* strains produce large amounts of IAA in culture. However, epiphytic *E. herbicola* isolates that do not generate galls on gypsophila are capable of producing similar amounts of IAA (22). The aim of the present work was to characterize IAA biosynthetic genes in *E. h. gypsophilae* and to establish the extent to which IAA production via the intermediate IAM determines virulence in this pathogen. A 7.5-kb fragment of *E. h. gypsophilae* DNA that harbors genes exhibiting structural and functional homology to *iaaM* and *iaaH* of *P. s. savastanoi* was cloned. The contribution of these genes to IAA production by *E. h. gypsophilae* in culture and to gall formation was examined.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** Sources and relevant characteristics of bacterial strains and plasmids are listed in Table 1. *E. h. gypsophilae* and *Escherichia coli* were cultured either in Luria-Bertani medium (LB) or in minimal A medium (24) supplemented with 0.2 mg/ml L-tryptophan. For *E. coli* cultures, minimal A was supplemented with 0.05 mM thiamine,

2.0 mM proline, and 0.3 mM leucine. The following antibiotic concentrations were used (in μg/ml): ampicillin 50, tetracycline 10, and streptomycin 20.

**EMBL3 library of plasmid DNA.** Plasmid DNA of *E. h. gypsophilae* was isolated by the procedure of Comai and Kosuge (5). Purified plasmid DNA was partially digested with *Sau*3A to generate fragment sizes ranging from 18 to 22 kb, was dephosphorylated, and was ligated into lambda vector EMBL3 according to the procedure described by Frischauf et al (10). The ligation mix was packaged using commercially available reagents (Gigapack; Stratagene, La Jolla, CA) and transfected into the P2 lysogen *E. coli* Q359 for amplification (13).

**Plaque hybridization and blots.** Transfer of plaques to nylon membranes (Hybond-N; Amersham, Arlington Heights, IL) was performed according to the manufacturer's instructions. Radiolabeled pCP3 DNA was used as a hybridization probe to detect sequences showing homology to the *iaaM* and/or *iaaH* genes of *P. s. savastanoi.*

Library screening and Southern blots utilizing heterologous probes were conducted using a low-stringency hybridization modification. The hybridization mix consisted of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 30% formamide, 10× Denhardt's solution, 50 mM phosphate buffer (pH 6.5), and 0.5 mg/ml denatured salmon sperm DNA. Hybridization was performed for 2 days at 37 C. The final wash was performed for 1 h at 65 C in 2× SSC containing 0.5% SDS.

Agarose gel electrophoresis, DNA transfer to membranes, high-stringency hybridizations, plasmid and lambda DNA preparations, radiolabeling of DNA probes, and other techniques were carried out according to standard procedures (21,36).

**Subcloning and insertional inactivation.** Lambda clone λSM5 was selected from the EMBL3 library of *E. h. gypsophilae* plasmid DNA based on its hybridization to radiolabeled pCP3 DNA under the conditions described above. A 7.5-kb *Eco*RI fragment that showed homology to pCP3 was subcloned from λSM5 into pUC118 in both orientations to generate pEG101 and pEG102. Based on the restriction map in Figure 1, several subclones were generated from pEG101 to further localize the IAA genes. pRB28 was generated by digesting pEG101 with *Bam*HI and recircularizing the plasmid with T4 DNA ligase, resulting in deletion of the fragment between the vector and insert *Bam*HI sites. Digestion of pRB28 with *Hin*dIII followed by ligation resulted in deletion of the fragment between the vector and insert *Hin*dIII sites, yielding construct pRH20 (Fig. 1). The 2.8-kb *Eco*RI-*Bam*HI fragment from pRB28 and the 2-kb *Hin*dIII-*Eco*RI fragment from pRH20 were subcloned into the multiple cloning site of pUC119 to generate pRB2819 and pRH2019, respectively (Fig. 1).

The 2.0-kb Ω interposon confers streptomycin resistance (28). Ω was inserted into the *Bam*HI site and into each of the three *Hin*dIII sites in the 7.5-kb *Eco*RI fragment in pEG101, and into the *Pst*I site present in the 2.8-kb *Eco*RI-*Bam*HI fragment in pRB28 as follows. pEG101 DNA was partially digested with either *Bam*HI or *Hin*dIII to linearize the DNA at a single site; samples were electrophoresed in low melting temperature agarose, and the 11-kb band was excised and extracted (a 6-kb band was isolated from electrophoresed pRB28 DNA that had been partially digested with *Pst*I). To generate pB5, the Ω interposon was excised from the cloning cassette construct pUC8Ω with *Bam*HI and ligated into pEG101 partially digested with *Bam*HI. To generate pH16, pH27, and pH32, Ω was excised from pUC8Ω by digestion

TABLE 1. Bacterial strains, phages, and plasmids used in this study

| Designation | Relevant characteristics | Reference or source |
|---|---|---|
| *Esherichia coli* | | |
| DH5α | F⁻ *recA1 thi-1 endA1* ϕ 80d *lacZ*ΔM15 | GIBCO BRL, Gaithersburg, MD |
| Q359 | *hsdR supE tonA* (P2) | 13 |
| *Erwinia herbicola* pv. *gypsophilae* | | |
| PD713 | Wild-type strain | J. Janse |
| MXB5 | Sm$^r$ Sp$^r$ [a] | This study |
| MXH16 | Sm$^r$ Sp$^r$ | This study |
| MXH27 | Sm$^r$ Sp$^r$ | This study |
| MXP84 | Sm$^r$ Sp$^r$ | This study |
| MXH32 | Sm$^r$ Sp$^r$ | This study |
| Plasmids and phages | | |
| pUC118 | Ap$^r$; vector | 39 |
| pUC119 | Ap$^r$; vector | 39 |
| pLAFR3 | Tc$^r$; vector | 38 |
| pRK2013 | Km$^r$; Tra$^+$ Mob$^+$ ColE1 replicon | 8 |
| EMBL3 | λ replacement vector | 10 |
| pUC8Ω | Ap$^r$ Sm$^r$ Sp$^r$; Ω in pUC8 | N. Panopoulos |
| pUC1318 | Ap$^r$; vector | 14 |
| pCP3 | Ap$^r$; *iaaM*, *iaaH* of *P. savastanoi* | 43 |
| Plasmid[b] and phage constructs described in this study | | |
| λSM5 | 16-kb *E. h. gypsophilae* DNA in EMBL3 | |
| pUC1318Ω | Ap$^r$ Sm$^r$ Sp$^r$; Ω cassette in pUC1318 | |
| pEG101[c] | Ap$^r$; 7.5-kb *Eco*RI in pUC118 | Fig. 1 |
| pEG102 | Ap$^r$; 7.5-kb *Eco*RI in pUC118 | |
| pRB28 | Ap$^r$; 2.8-kb *Eco*RI-*Bam*HI in pUC118 | Fig. 1 |
| pRH20 | Ap$^r$; 2.0-kb *Hin*dIII-*Eco*RI in pUC118 | Fig. 1 |
| pRB2819 | Ap$^r$; 2.8-kb *Bam*HI-*Eco*RI in pUC119 | Fig. 1 |
| pRH2019 | Ap$^r$; 2.0-kb *Hin*dIII-*Eco*RI in pUC119 | Fig. 1 |
| pB5 | Ap$^r$ Sm$^r$ Sp$^r$; Ω in *Bam*HI site of pEG101 | Fig. 1 |
| pH16 | Ap$^r$ Sm$^r$ Sp$^r$; Ω in *Hin*dIII site of pEG101 | Fig. 1 |
| pH27 | Ap$^r$ Sm$^r$ Sp$^r$; Ω in *Hin*dIII site of pEG101 | Fig. 1 |
| pH32 | Ap$^r$ Sm$^r$ Sp$^r$; Ω in *Hin*dIII site of pEG101 | Fig. 1 |
| pP84 | Ap$^r$ Sm$^r$ Sp$^r$; Ω in *Pst*I site of pRB28 | Fig. 1 |

[a] Ap = ampicillin; Sm = streptomycin; Sp = spectinomycin; Tc = tetracycline; Km = kanamycin; and $^r$ = resistant.

[b] All subcloned or insertionally modified fragments are derived from the 7.5-kb *Eco*RI fragment of *E. h. gypsophilae* DNA that shows homology to the *Pseudomonas syringae savastanoi iaa* operon.

[c] pEG101 and pEG102 contain the 7.5-kb *Eco*RI fragment cloned in opposite orientations in pUC118.

with *Hin*dIII and was ligated with the *Hin*dIII partial digest of pEG101. Transformed *E. coli* DH5$\alpha$ cells resistant to ampicillin and streptomycin were screened by restriction mapping to identify plasmids containing Ω inserted into each of the desired sites.

To generate pP84, it was necessary to construct a new plasmid containing a modified Ω cassette, as pUC8Ω does not contain *Pst*I sites allowing excision of Ω. Ω was excised from pUC8Ω with *Hin*dIII and was ligated into pUC1318 digested with *Hin*dIII to generate an Ω cassette flanked by *Pst*I, *Xba*I, *Bam*HI, *Sma*I, *Sac*I, and *Eco*RI. This construct was designated pUC1318Ω; it lacks the flanking triple translational stops present in pUC8Ω. To generate pP84, Ω was excised from pUC1318Ω with *Pst*I and ligated into a *Pst*I partial digest of pRB28. Transformed cells were selected to grow on ampicillin and streptomycin and constructs were mapped.

**Marker exchange.** Site-directed replacement (31) of the native *E. h. gypsophilae* IAA genes with their insertionally inactivated counterparts was performed in a manner similar to that reported by Lindgren et al (19). The 9.5-kb *Eco*RI inserts from plasmids pB5, pH16, pH27, and pH32 were isolated and ligated into the *Eco*RI site of the wide host-range cosmid vector pLAFR3. The 5.0-kb *Eco*RI-*Bam*HI insert from pP84 was ligated into pLAFR3 digested with *Bam*HI and *Eco*RI. Each of these constructs was moved into *E. h. gypsophilae* PD713 via triparental mating using conjugational helper plasmid pRK2013 according to the procedure of Ditta et al (8). Tetracycline-resistant transconjugants were selected after 3–5 days growth at 30 C on minimal A medium amended with tetracycline and were tested for streptomycin resistance. Marker exchange was effected by culturing selected tetracycline- and streptomycin-resistant colonies to late log phase in LB without antibiotic selection on a gyrotory shaker at 220 rpm, diluting 1:1,000 in fresh LB, and repeating the growth cycle three times. Dilution series of cells from the final growth cycle were plated on LB containing streptomycin. Resistant colonies were replica-plated onto LB containing tetracycline. Genomic DNA preparations were made from tetracycline-sensitive, streptomycin-resistant colonies. *Eco*RI digests of DNA from each strain were electrophoresed, blotted, and probed with $^{32}$P-radiolabeled pEG101 DNA. The presence of a single hybridizing 9.5-kb *Eco*RI fragment on autoradiograms in conjunction with the absence of a 7.5-kb *Eco*RI fragment was accepted as evidence that a site-specific insertion of the 2-kb Ω interposon had occurred on the 78-Md native plasmid. Insertionally interrupted plasmid constructs pB5, pH16, pH27, pH32, and pP84 were used to generate *E. h. gypsophilae* PD713 marker-exchange derivatives MXB5, MXH16, MXH27, MXH32, and MXP84, respectively (Table 1).

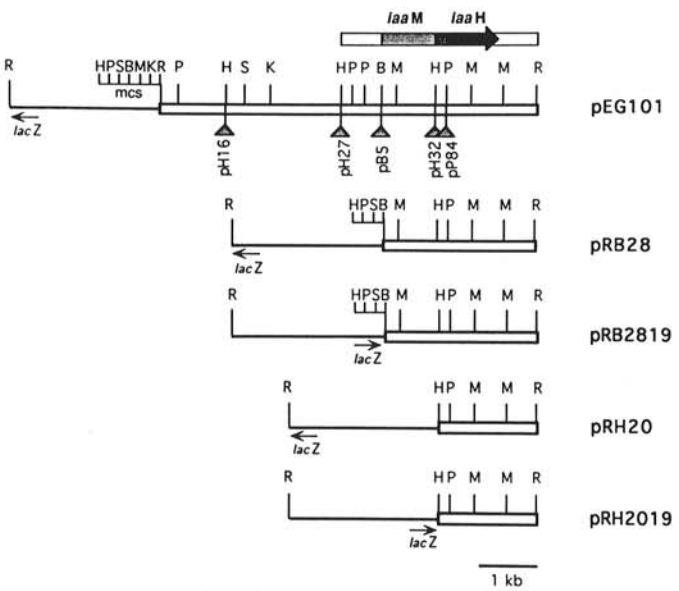


**Fig. 1.** Restriction endonuclease map of *Erwinia herbicola* pv. *gypsophilae* DNA cloned in pEG101 (R = *Eco*RI; P = *Pst*I; H = *Hin*dIII; S = *Sal*I; K = *Kpn*I; B = *Bam*HI; and M = *Sma*I). The pUC118 multiple-cloning site (mcs) is shown. Double lines denote *E. h.* pv. *gypsophilae* DNA fragments; single lines denote vector DNA. Arrows labeled "*lacZ*" indicate the direction of transcription from the *lacZ* promoter within either pUC118 or pUC119. The bar indicates DNA length in kilobase pairs. The location of the Ω interposon in the plasmids pH16, pH27, pB5, pH32, and pP84 is indicated on the pEG101 restriction map. The shaded boxes indicate the approximate boundaries and arrangement of *iaaM* and *iaaH*. Light shading shows the maximum boundaries of the fragment harboring the genes. Darker shading indicates the approximate location of *iaaM*, and the black arrow indicates the direction of transcription of *iaaH*.

**Determination of IAA biosynthesis.** In order to determine IAA content of culture supernatants, *E. coli* and *E. h. gypsophilae* strains were grown in minimal A medium amended with L-tryptophan and appropriate antibiotics (Table 1) and supplements. A 100-$\mu$l aliquot of cultured cells grown overnight in LB was inoculated into 100 ml of amended minimal A medium. Cultures were incubated in 500-ml flasks protected from light at 28 C for 48 h on a gyrotory shaker at 220 rpm. Cells were removed by pelleting for 30 min at 10,000 *g*; the supernatant was passed through a 0.45-$\mu$m filter. For each extraction, 40 ml of filtered supernatant was acidified to pH 3 with 5 N HCl and passed through an equilibrated $C_{18}$ cartidge (SEP-PAK $C_{18}$, Waters Associates, Milford, MA) according to the manufacturer's instructions. The retained fraction, containing IAA and other indoles, was eluted with 2.5 ml of methanol. Aliquots were passed through 0.2-$\mu$m filters (Microfilterfuge tubes; Rainin Instrument Co., Woburn, MA) and were analyzed by high-performance liquid chromatography (HPLC).

To test for the presence of indoleacetamide-hydrolase activity, *E. coli* or *E. h. gypsophilae* cells were grown under the culture conditions described above in a 1-L flask containing 300 ml of minimal A medium amended with appropriate supplements, antibiotics, and L-tryptophan. The permeabilized-cell assay for IaaH activity is similar to that described by Manulis et al (23). Cells were pelleted at 6,000 *g*, washed once in 10 mM potassium phosphate buffer (pH 7.2), and resuspended in Tricine-KOH buffer (0.5 M Tricine adjusted to pH 8.3 with KOH, 5 mM $MgSO_4$, and 1 mM 2-mercaptoethanol) at 1 g fresh weight of cells per 5 ml of buffer. To 2 ml of cell suspension was added 0.17 ml of 100 mM cetyltrimethylammonium bromide (CTAB), 0.06 ml of 10 mM $MgSO_4$, 0.6 ml of 10 mM potassium phosphate (pH 7.2), and 5 mg of IAM dissolved in 50 $\mu$l of methanol. The reaction mix was incubated for 16 h at 28 C with agitation, then centrifuged at 5,000 *g* for 15 min, and the pellet was discarded. The supernatant was adjusted to pH 3.0 with HCl, and a 0.5-ml aliquot was extracted three times with equal volumes of ethylacetate. The ethylacetate fractions were pooled and dried under a stream of nitrogen, and the residue was redissolved in 300 $\mu$l of methanol. The sample was filtered and analyzed by HPLC.

**HPLC and GC-MS analysis of IAA.** A Perkin Elmer Series 410 Bio LC pump equipped with a LC-95 spectrophotometric detector and LCI-100 computing integrator was used. Indoles were separated on a 25-cm $C_{18}$ reversed-phase column (4.6 mm ID, 5 $\mu$m particle size; Rainin Instrument Co.), preceded by a 5-cm $C_{18}$ guard column. The mobile phase was 26% methanol in 0.5% aqueous acetic acid (32). HPLC-grade reagents (Baxter, Burdick & Jackson Division, Muskegon, MI) were used. Flow rate was set at 1.0 ml/min, and absorbance was read at 280 nm. Filtered samples were injected onto the column in 25-$\mu$l aliquots. IAA was quantified by reference to a standard curve of peak area versus concentration obtained by using an authentic IAA (Sigma Chemical Co., St. Louis, MO) external standard.

Confirmation of the identity of IAA was obtained by gas chromatography-mass spectrometry (GC-MS). Peaks were collected off the HPLC column and were extracted three times with diethyl ether. Pooled ether fractions were evaporated under nitrogen and the residue was dissolved in 100 $\mu$l of acetonitrile. The trimethylsilyl derivative (IAA-TMS) was generated following the addition of 50 $\mu$l of bis(trimethylsilyl) trifluoroacetamide (Pierce, Rockford, IL) by heating the mixture at 70 C for 15 min. IAA-TMS was analyzed on a Hewlett-Packard 5970 MSD gas chromatograph-mass spectrometer equipped with a 30-m DB-

5 fused-silica column set in the temperature programming mode (90–280 C at 7 C per minute). Mass spectra of derivatized samples were compared to a mass spectrum obtained from a TMS-derivatized authentic IAA standard.

**Pathogenicity tests.** *E. h. gypsophilae* PD713 and the five marker-exchange derivatives listed in Table 2 were tested for their capacity to elicit gall formation on gypsophila cuttings as described by Manulis et al (22) in three separate experiments. From 6 to 20 replicate plants per strain were inoculated in each experiment. As a negative control, cuttings were dipped in sterile phosphate buffer (pH 6.5) and were potted and incubated in the same manner as inoculated cuttings. Hyperplastic tissue growth was excised from the inoculation site of each plantlet and was weighed.

**Statistical analyses.** Statistical computations were made using software provided by Statistical Analysis Systems (SAS) (release 6.01; SAS Institute, Inc., Cary, NC). The SAS general linear models procedure was used to perform analysis of variance on weighed galls and on log-transformed measurements of IAA concentrations produced by cultured cells. Mean comparisons of these data were made with Fisher's least significant difference test. This test controls the comparison-error rate.

## RESULTS

**Cloning and expression of an IAA biosynthetic pathway.** *E. h. gypsophilae* PD713 contains three large, cryptic plasmids (22). Sequences harboring the *iaa* operon of *P. s. savastanoi* hybridize at low stringency to a 7.5-kb *Eco*RI fragment on blots of restricted, electrophoresed *E. h. gypsophilae* PD713 plasmid DNA. Plasmid pCP3 is composed of a 4.0-kb *Eco*RI-*Sal*I fragment containing the *P. s. savastanoi iaa* genes cloned into plasmid vector pBR328. Radiolabeled pCP3 DNA was used to isolate λSM5 from the *E. h. gypsophilae* PD713 library by plaque hybridization under low stringency conditions. λSM5 contains a 16-kb fragment of *E. h. gypsophilae* PD713 plasmid DNA and was previously localized to the 78-Md native plasmid by Southern blot hybridization (22). Radiolabeled λSM5 DNA was subsequently hybridized to pCP3 DNA digested with either *Pst*I and *Sal*I or *Sph*I and *Sal*I. These enzymes were selected because they generate fragments entirely internal to the *P. s. savastanoi iaaM* and *iaaH* coding frames, based on the published map and sequence (43). Our analysis showed that λSM5 contains sequences homologous to both the *iaaM* and the *iaaH* genes of *P. s. savastanoi* (data not shown).

The *E. h. gypsophilae* sequences showing homology to the *P. s. savastanoi iaa* operon were further characterized by subcloning and insertional inactivation. All sequences on λSM5 showing homology to pCP3 are located on a 7.5-kb *Eco*RI fragment. The 7.5-kb *Eco*RI fragment was subcloned from λSM5 into pUC118 in both orientations to generate pEG101 (Fig. 1) and pEG102. Both constructs direct the synthesis of IAA in culture when transformed into *E. coli* DH5α; however, the level of expression was low (0.81 μg/ml for pEG101; Table 3). In contrast, pCP3, which contains the *iaa* operon of *P. s. savastanoi* driven by a vector promoter, can generate 120 μg/ml IAA in culture under these conditions. Both pEG101 and pEG102 also direct the synthesis of IAA from IAM in the assay for IaaH activity. The expression of the *E. h. gypsophilae* PD713 IAA genes by the subcloned fragment positioned in either orientation with respect to the *lac*Z promoter of pUC118 suggests that they are driven by a promoter from *E. h. gypsophilae* PD713 located on the 7.5-kb *Eco*RI fragment rather than being driven by the *lac*Z promoter.

Subclones were generated from pEG101 to further localize the IAA genes. The construct pair pRH20 and pRH2019 was used to test whether reversing the orientation of these fragments with respect to the *lac*Z promoter of the vector results in increased gene activity, by fortuitously placing either or both of the *E. h. gypsophilae* PD713 IAA genes under the control of the *lac*Z promoter (Fig. 1). Construct pRH2019 generated over 22-fold

TABLE 2. Comparisons of mean gall size for *Erwinia herbicola* pv. *gypsophilae* PD713 and marker-exchange derivatives

| Strain | Mean gall size (mg fresh wt)[a] | | | | | |
|---|---|---|---|---|---|---|
| | Experiment 1[b] | | Experiment 2 | | Experiment 3 | |
| PD713 | 575 | A | 180 | A | 133 | A |
| MXB5 | 367 | B | 120 | B | 36 | B |
| MXP84 | 192 | C | 104 | B | 40 | B |
| MXH16 | NT | | NT | | 59 | B |
| MXH32 | NT | | NT | | 39 | B |
| MXH27 | NT | | NT | | 38 | B |

[a] Mean gall size was obtained by measuring the fresh weight of hyperplastic tissue in milligrams 14 days after inoculation of cuttings, in three experiments. For each column, means followed by the same letter do not differ according to Fisher's least significant difference test. NT = not tested.

[b] In experiment 1, the fresh weight of galls in milligrams was determined for 10 galls per bacterial strain. In experiment 2, 20 galls were measured per strain. In experiment 3, 6–10 galls were measured per strain. Thirty-five cuttings were dipped in sterile phosphate buffer and utilized as negative controls.

TABLE 3. Comparisons of IAA content of culture supernatants and comparisons of relative IaaH activities of permeabilized *Escherichia coli* DH5α cells containing plasmid constructs

| Plasmid in *E. coli* DH5α | IAA content of culture supernatant (μg/ml) | | IAA produced from IAM (μg/[g. f. wt. cells]) | |
|---|---|---|---|---|
| | mean | | mean | |
| pEG101 | 0.81 | A | 957 | AB |
| pH16 | 0.72 | AB | 511 | BC |
| pEG102 | 0.56 | AB | 484 | BC |
| pH27 | 0.47 | B | 381 | C |
| pB5 | 0.028 | C | 263 | C |
| pRB2819 | 0.069 | C | 1,460 | A |
| pRB28 | 0.055 | C | 497 | BC |
| pRH2019 | 0.077 | C | 1,190 | AB |
| pRH20 | 0.010 | C | 53 | D |
| pH32 | 0.092 | C | 24 | D |
| pP84 | 0.032 | C | 0.2 | E |
| pUC118 | 0.070 | C | 0.4 | E |

Data were obtained by high-performance liquid chromatography (HPLC) analysis. *E. coli* DH5α(pUC118) served as a negative control in these experiments. A minor peak showing a retention time equal to that of IAA was obtained in all samples, including the negative control, and was categorized as "IAA", although positive identification was not made. Gas chromatography-mass spectrometry (GC-MS) was used to confirm the identity of IAA in culture supernatant of *E. coli* DH5α(pEG101). Values are the means of two experiments. For each column, means followed by the same letter do not vary according to Fisher's least significant difference test. All values are uncorrected for recovery efficiencies.

TABLE 4. Comparisons of IAA contents of culture supernatants of *Erwinia herbicola* pv. *gypsophilae* PD713 and its marker-exchange derivatives and comparisons of relative IaaH activities of permeabilized cells

| *E. h.* pv. *gypsophilae* strain or derivative | IAA content of culture supernatant (μg/ml) | | IAA produced from IAM (μg/[g. f. wt. cells]) | |
|---|---|---|---|---|
| | mean | | mean | |
| PD713 | 7.3 | A | 100 | A |
| MXH16 | 8.0 | A | 27 | B |
| MXB5 | 4.9 | A | 8.1 | C |
| MXH27 | 6.5 | A | 6.0 | CD |
| MXH32 | 7.5 | A | 2.7 | D |
| MXP84 | 5.4 | A | 0.83 | E |

Data were obtained by high-performance liquid chromatography (HPLC) analysis. Gas chromatography-mass spectrometry (GC-MS) was used to confirm the identity of IAA in culture supernatant of *E. h. gypsophilae* PD713. Values are the means of two experiments. For each column, means followed by the same letter do not vary according to Fisher's least significant difference test. All values are uncorrected for recovery efficiencies.

more IAA from IAM in the IaaH assay compared to pRH20 (Table 3). Neither construct produced detectable quantities of IAA from TRP in culture supernatants. These results suggest that the direction of transcription of *iaaH* is toward the *lac*Z promoter-proximal end of the 7.5-kb *Eco*RI fragment in pEG101, and that *iaaH* is contained in its entirety on the subcloned 2-kb *Hin*dIII-*Eco*RI fragment.

Insertional mutants were generated by ligating the Ω interposon into restriction sites along the length of the 7.5-kb *Eco*RI fragment to generate constructs pH16, pH27, pB5, pH32, and pP84. Ω insertion into the *Hin*dIII site distal to the *lac*Z promoter in pEG101 (pH16) did not significantly alter the IAA content of culture supernatant compared to pEG101, indicating that the site lies outside the IAA genes (Fig. 1, Table 3). The Ω insertion in pH27 decreased, but did not eliminate, conversion of TRP to IAA and IaaH activity in transformed *E. coli*. Insertions into either the *Bam*HI site (pB5) or the *lac*Z promoter-proximal *Hin*dIII site (pH32) eliminated the conversion of TRP to IAA but not the conversion of IAM to IAA; insertions are probably located within the *iaaM* structural gene. Insertion of Ω into the *lac*Z promoter-proximal *Pst*I site (pP84) eliminates IaaH activity in transformed *E. coli*, indicating that the *Pst*I site lies within the *iaaH* structural gene.

**Insertional inactivation and marker exchange.** The Ω interposon was used to generate five constructs containing insertional mutations spanning the 7.5-kb *Eco*RI fragment in pEG101 (Fig. 1). Two of these constructs, pB5 and pH32, contain Ω within the *iaaM* gene; pP84 contains Ω within *iaaH*, based on results obtained by examining the expression of the IAA genes in *E. coli*. Site-directed replacement of the native genes or sequences with the insert-containing constructs was effected by homologous recombination in *E. h. gypsophilae* PD713. The capacity for IAA production in culture of *E. h. gypsophilae* PD713 and its marker-exchange derivatives was measured, and a determination of the relative levels of IaaH activity in these strains was made (Table 4). Inactivation of either *iaaM* (MXB5, MXH32) or *iaaH* (MXP84) in *E. h. gypsophilae* had no significant effect on the ability of the marker-exchange strains to generate IAA from TRP in culture.

To examine the effects of the insertional mutations on the expression of *iaaH* in *E. h. gypsophilae*, each of the strains was tested for IaaH activity (Table 4). Replacement of the native *iaaH* gene by the insertionally inactivated gene contained in pP84 resulted in virtual elimination of IaaH activity in *E. h. gypsophilae* MXP84; a 100-fold reduction occurred in the capacity of MXP84 to generate IAA from IAM relative to the parental PD713. Comparison of the IaaH activities of the five marker-exchange derivatives parallels the results obtained from the expression of *iaaH* in insertionally inactivated constructs in *E. coli* (Table 3).

**HPLC and GC-MS analyses of culture supernatant.** The IAA biosynthetic pathway, encoded by the 7.5-kb *Eco*RI fragment contained in pEG101, directs low levels of IAA biosynthesis in *E. coli* DH5α. Levels detectable by the extraction protocol and HPLC analytical system used in this study were obtained by culturing *E. coli* DH5α containing plasmid constructs in minimal medium amended with 0.2 g/L of TRP. Several HPLC mobile phases were tested for their capacity to resolve IAA from tryptophan, indole-3-acetamide, and indole-3-ethanol, which were identified as major indole compounds extracted from culture supernatants of the strains examined in this study. The mobile phase utilized, 26% methanol in 0.5% aqueous acetic acid, resolved all major components. However, no mobile phase tested eliminated the occurrence of a minor peak, observed in analyses of the culture supernatant of *E. coli* DH5α(pUC118), that exhibited a retention time indistinguishable from that of the authentic IAA standard. *E. coli* DH5α(pUC118) was used as a negative control in these experiments. If it is assumed that this minor peak is composed of IAA, based on its retention time, then it is equivalent to, on average, 0.07 μg per milliliter of IAA in the culture supernatant. However, IAA production has not been reported previously in *E. coli*, and it is possible that this peak represents a compound other than IAA. The occurrence of this peak established the lower limit for detection of IAA production in this system at approximately 0.07 μg per milliliter of IAA in culture supernatant. We included this peak in the statistical analysis of IAA content for all constructs and interpreted the capability of each plasmid construct to direct the synthesis of IAA from either TRP or IAM based on whether the value obtained was statistically different from the negative control, *E. coli* DH5α(pUC118). All values for IAA content in Table 3 are interpreted as being below the limit for detection of IAA production in this system if they do not differ statistically from the negative control. Using this argument, only constructs pEG101, pEG102, pH16, and pH27 direct the synthesis of IAA from TRP in *E. coli* DH5α, and only pP84 lacks the ability to produce IAA from IAM.

GC-MS was used to ascertain the identity of IAA in culture supernatants of *E. coli* DH5α(pEG101) and *E. h. gypsophilae* PD713. Ion profiles of samples extracted from peaks isolated by HPLC were identical to those of the trimethylsilylated IAA standard (m/z 202 = 100%; m/z 304 = 4%; m/z 319 [M+] = 19%).

**Effect of insertional inactivation of *iaaM* and *iaaH* on pathogenicity.** Insertion of Ω into five sites in marker-exchange derivatives of *E. h. gypsophilae* PD713 did not affect IAA production in cultured cells, but in all cases significantly reduced or eliminated the IaaH activity measured in permeabilized cells (Table 4). Galls formed by all marker-exchange strains were smaller than those formed by parental *E. h. gypsophilae* PD713 (Table 2). However, hyperplastic tissue growth was obtained at the site of inoculation in each case; fresh weight of excised tissue ranged from 30 to 60% of that obtained from cuttings inoculated with PD713. Hyperplasias were not observed on negative control cuttings treated with sterile phosphate buffer.

## DISCUSSION

*Erwinia herbicola* is a heterogeneous taxon composed of bacteria that may develop pathogenic or epiphytic associations with plants (37). Classified within the Enterobacteriaceae, *E. herbicola* is relatively distant taxonomically from the other bacteria in which IAA biosynthesis has been examined (i.e., Pseudomonadaceae and Rhizobiaceae). Like *Erwinia*, however, *Agrobacterium, Pseudomonas,* and *Bradyrhizobium* are components of epiphytic or rhizosphere populations and therefore may be temporally juxtaposed to *E. herbicola* in a variety of habitats and conditions, which provides the opportunity for genetic exchange. Sequence homology has been established between the cloned IAA genes of *P. s. savastanoi, A. tumefaciens, A. rhizogenes,* and *B. japonicum* (3,33,43). That sequences encoding the IAA genes of *P. s. savastanoi* and *E. h. gypsophilae* exhibit homology suggests a common origin for the *E. h. gypsophilae* genes as well. The IAA genes reside on plasmid DNA in *A. tumefaciens*, in oleander strains of *P. s. savastanoi*, and in *E. h. gypsophilae*. It is possible that plasmid transfer between a member of the Pseudomonadaceae and *E. herbicola* resulted in transfer of the IAA genes. For example, Lacy et al (18) demonstrated transfer of an oxytetracycline resistance plasmid from *P. syringae* pv. *syringae* to *Erwinia amylovora* on pear blossoms, showing that transfer of wide host-range plasmids can occur under field conditions.

The presence of *iaaH* on pEG101 was demonstrated by results obtained from the permeabilized cell assay for IaaH activity (Table 3). Evidence for the existence of a functional *iaaM* gene on pEG101 is outlined in the following argument. IaaH activity was detected in permeabilized *E. coli* cells containing an intact *iaaH* gene, even in cases in which no IAA biosynthesis was detected in cultured cells. IaaH activity measured in permeabilized cells transformed with the subclone pRB2819 was higher than activity measured in cells transformed with pEG101 (Fig. 1, Table 3). However, while pEG101 directed the synthesis of detectable levels of IAA from TRP in *E. coli* DH5α(pEG101), no IAA production was measured in *E. coli* DH5α(pRB2819). These results suggest that the expression of *iaaH* alone is insufficient to direct IAA biosynthesis from TRP in cultured *E. coli* cells, and that a gene upstream from *iaaH* on pEG101 directs the synthesis of IAM. The presence

of an IaaM-like activity associated with pEG101 implies that the corresponding gene is functionally equivalent to *iaaM* of *P. s. savastanoi*. Further structural evidence that an *iaaM* gene is located in this region was derived from DNA hybridization data that demonstrated the presence of sequences on pEG101 homologous to DNA fragments containing sequences specific to *iaaM* of *P. s. savastanoi* (data not shown).

Insertions of Ω into restriction sites spanning the 7.5-kb *Eco*RI fragment of pEG101 were generated to define the boundaries of the IAA genes and to produce marker-exchange mutants of *E. h. gypsophilae* PD713 in which *iaaM* or *iaaH* had been insertionally inactivated (Fig. 1). *iaaM* and *iaaH* are located on a 4.0-kb *Hin*dIII-*Eco*RI fragment, as are four of the Ω insertion sites. However, insertion of Ω into a *Hin*dIII site (pH16) 2.4 kb upstream from the boundary of this fragment also resulted in significant reduction of IaaH activity in the corresponding marker-exchange strain, *E. h. gypsophilae* MXH16 (Table 4). This effect may be due to an Ω-generated polar effect or an insertional interruption of sequences that have a regulatory function in *trans*. All marker-exchange strains showed a reduction in IaaH activity, and all showed significant decreases in virulence, as defined by a decrease in measured gall size (Table 2). Unless the presence of the Ω element somehow interferes with virulence expression via another mechanism, this result indicates that reduction or elimination of IaaH activity in *E. h. gypsophilae* PD713 is associated with a decrease in virulence. Because no direct measure of IaaM activity was made, no correlation was established between IaaM activity and virulence.

IAA production by *E. coli* DH5α harboring pEG101 is low relative to the IAA biosynthetic capability of cells transformed with pCP3, which contains the *P. s. savastanoi iaa* operon driven by a vector promoter (43). The results of this study suggest that the *E. h. gypsophilae* IAA biosynthetic pathway encoded by pEG101 is not driven by a vector promoter. IAA production by *E. coli* DH5α(pEG101) may be low as a result of low expression of either or both of the IAA genes under the control of the *E. h. gypsophilae* promoter in this construct. Inactivation of *iaaH* in *E. h. gypsophilae* MXP84 did not result in a significant decrease in IAA production by cultured cells. The low level of IAA biosynthesis directed by *iaaM* and *iaaH* in *E. coli* DH5α(pEG101) may reflect a low level of expression of these genes in cultured *E. h. gypsophilae* cells as well. It has been suggested that the *iaa* operon of *P. s. savastanoi* is part of a mobile genetic element (27,43), and that the constitutively expressed promoter driving this operon was not originally associated with it (11). It is possible that the genetic exchange that led to the incorporation of *iaaM* and *iaaH* into the *E. h. gypsophilae* genome placed these genes under the control of a promoter unrelated to the *P. s. savastanoi iaa* promoter.

In *P. s. savastanoi* and *A. tumefaciens*, the IAA genes are virulence determinants essential to the ability of the bacteria to form galls on their hosts. In oleander strains of *P. s. savastanoi*, loss of a 52-kb native plasmid harboring the *iaa* operon results in virtual elimination of IAA biosynthesis in culture as well as loss of pathogenicity (5). Expression of *iaaM* and *iaaH* in transformed plants is required for tumor formation by *A. tumefaciens* (20). In *E. h. gypsophilae*, the correlation between expression of *iaaM* and *iaaH*, production of IAA in culture, and pathogenicity differs from that observed in other gall-forming pathogens. Manulis et al (22) used Southern blot hybridizations to test for homology between the 7.5-kb *Eco*RI fragment in pEG101 and DNA isolated from 50 pathogenic and nonpathogenic *E. herbicola* field isolates. Hybridization was observed only to plasmid DNA from pathogenic strains; no hybridization was observed to the nonpathogenic strains tested. This result suggested that, as in other gall-forming pathogens, *iaaM* and *iaaH* may be required for virulence in *E. h. gypsophilae*. However, the results obtained in the present study indicate that, while expression of *iaaH* contributes to virulence, insertional inactivation does not severely attenuate virulence in a manner analogous to that observed in other pathogens. Moreover, *E. h. gypsophilae* strains containing insertionally interrupted *iaaM* and *iaaH* produce the same amounts of IAA from TRP in culture as the unmodified parental strain. IAA biosynthesis via an alternate pathway (23) apparently contributes to IAA production in *E. h. gypsophilae* in culture. Manulis et al (22) observed that many nonpathogenic field isolates of *E. herbicola* lacking the *iaaM* and *iaaH* sequences produce as much IAA in culture as is produced by *E. h. gypsophilae* strains.

While IAA biosynthesis probably is required for gall induction by *E. h. gypsophilae*, this species is apparently unique in having two alternate pathways by which significant amounts of IAA can be produced. The relative contribution of these two pathways to IAA production may differ in various environments because inactivation of *iaaH* reduces gall size but does not reduce IAA production in culture media containing tryptophan. The presence in *E. h. gypsophilae* of two pathways producing a single secondary metabolite implicated in pathogenicity may necessitate the coordinate regulation of the two pathways. Further work is needed to define all the genetic determinants of the alternate IAA biosynthetic pathway and its regulation in this pathogen.

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