Differential Involvement of Indole-3-Acetic Acid Biosynthetic Pathways in Pathogenicity and Epiphytic Fitness of *Erwinia herbicola* pv. *gypsophila*

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Erwinia herbicola pv. *gypsophila* (Ehg), which induces galls on *Gypsophila paniculata*, harbors two major pathways for indole-3-acetic acid (IAA) synthesis, the indole-3-acetamide (IAM) and indole-3-pyruvate (IPyA) routes, as well as cytokinin biosynthetic genes. Mutants were generated in which the various biosynthetic routes were disrupted separately or jointly in order to assess the contribution of IAA of various origins and cytokinins to pathogenicity and epiphytic fitness. Inactivation of the IAM pathway or cytokinin biosynthesis caused the largest reduction in gall size. Inactivation of the IPyA pathway caused a minor, nonsignificant decrease in pathogenicity. No further reduction in gall size was observed by the simultaneous inactivation of both IAA pathways only or in combination with that of cytokinin production. However, inactivation of the IPyA pathway caused a 14-fold reduction in the population of Ehg on bean plants. Inactivation of the IAM pathway or cytokinin production did not affect epiphytic fitness. While the apparent transcriptional activity of *iaaM-inaZ* fusion increased slightly in cells of Ehg on bean and gypsophila leaves, compared with that in culture, very high levels of induction were observed in cells injected into gypsophila stems. In contrast, moderate levels of induction of *ipdC-inaZ* in Ehg were observed on leaves of these plants and in gypsophila stems, when compared with that in culture. These results suggest that the IAM pathway is involved primarily in gall formation and support the main contribution of the IPyA pathway to the epiphytic fitness of this bacterial species.

The ability to produce the plant hormone indole-3-acetic acid (IAA) is widespread among soil, epiphytic, and tissue-colonizing bacteria (Costacurta and Vanderleyden 1995; Patten and Glick 1996). In several of these microorganisms, including *Agrobacterium tumefaciens*, *A. rhizogenes*, *Pseudomonas syringae* pv. *savastanoi*, *P. syringae* pv. *syringae*, and *Erwinia herbicola* pv. *gypsophila* (Ehg), IAA is involved in

pathogenesis (Clark et al. 1993; Fett et al. 1987; Morris 1986; White and Ziegler 1991; Mazzola and White 1994) while in others, such as members of the genera *Azospirillum*, *Rhizobium*, *Bradyrhizobium*, *Enterobacter*, *Xanthomonas*, and other *Pseudomonas* spp., it may benefit the bacterium by stimulating plant growth and increasing the release of plant metabolites that the bacteria can utilize (Bar and Okon 1993; Fett et al. 1987; Glick 1995; Sekine et al. 1989; Patten and Glick 1996). Other benefits may include suppression of plant defense, which facilitates bacterial invasion (Robinette and Matthysse 1990).

Several different IAA biosynthetic pathways are used by prokaryotes, and a single bacterial strain can contain more than one pathway. These metabolic routes, which are classified in terms of their intermediates, include the indole-3-acetamide (IAM), indole-3-pyruvate (IPyA), tryptamine, and indole-3-acetonitrile pathways (Costacurta and Vanderleyden 1995). The two major routes that appear to be most common in bacteria are the IAM and IPyA pathways. The IAM pathway occurs in all gall-forming bacteria (Manulis et al. 1991b; Morris 1986) and has also been described in *P. syringae* pv. *syringae* (White and Ziegler 1991), *Bradyrhizobium japonicum* and *Rhizobium fredii* (Sekine et al. 1989), *Azospirillum brasilense* (Bar and Okon 1993), and *Streptomyces* spp. (Manulis et al. 1994). Although the IAM pathway has been detected in higher plants (Kawaguchi et al. 1993; Satome et al. 1993), its presence there may be quite rare (Kawaguchi and Syono 1996). In contrast to the IAM pathway, which may be considered unique to bacteria, the IPyA pathway is generally assumed to be common in higher plants, although little data is available to support its function there (Kawaguchi and Syono 1996). Nevertheless, the IPyA route has been detected in most of the above mentioned bacteria and might be quite common in prokaryotes (Patten and Glick 1996). Genetic evidence for a key role of indolepyruvate decarboxylase in this pathway has been demonstrated in *Enterobacter cloacae* (Koga et al. 1991), *A. brasilense* (Costacurta et al. 1994), and recently in *E. herbicola* (Brandl and Lindow 1996).

E. herbicola is widespread in nature as an epiphyte on many plants (Starr 1981). However, some strains have evolved into plant pathogens that incite gall formation at the crown region of either *Gypsophila paniculata* (Volcani 1985) or table beet (Burr et al. 1991). Both pathovars contain an indigenous plasmid of about 150 kb (pPATH) that determines pathogenic-

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ity (Manulis et al. 1991a; I. Barash and S. Manulis, *unpublished*). *Ehg* possesses two major metabolic pathways for IAA biosynthesis: the IAM and IPyA pathways (Manulis et al. 1991b). The genes for the IAM pathway encoding tryptophan-2-monooxygenase (*iaaM*) and IAM hydrolase (*iaaH*) were cloned from the pPATH of *Ehg* and their role in pathogenicity was demonstrated (Clark et al. 1993). The gene encoding IPyA decarboxylase (*ipdC*), the key enzyme in the IPyA pathway, was characterized in a nonpathogenic strain of *E. herbicola* and its possible role in epiphytic fitness has been reported (Brandl and Lindow 1996, 1997). However, the role of the IPyA pathway in pathogenicity has not yet been assessed. The redundant pathways for IAA production in pathogenic *E. herbicola* strains raise the important question as to whether they both contribute to pathogenicity or are separately involved in different aspects of the life cycle of this species. Cytokinins are generally known to be involved in gall formation (Morris 1986). A gene for cytokinin biosynthesis (*etz*) was cloned from *Ehg* and sequenced (Lichter et al. 1995a; Lichter et al. 1995b). It is located downstream from another open reading frame (pre-*etz*) and both form an operon. The genes of the IAM pathway and the cytokinin genes are clustered on the pPATH of *Ehg* (Lichter et al. 1995a). The involvement of cytokinins in survival ability of bacteria on plant surfaces has not been investigated.

Previous work has shown that insertional inactivation of the IAM genes in *Ehg* did not affect the amount of IAA produced in culture but caused a significant reduction in gall size (Clark et al. 1993). Similarly, insertional mutagenesis of each of the two cytokinin biosynthetic genes resulted in smaller galls but did not eliminate gall formation (Lichter et al. 1995a). In ad-

dition, several studies have indicated that IAA production may contribute to the epiphytic survival of *P. syringae* pv. *savastanoi* and *E. herbicola* (Beattie and Lindow 1995; Silverstone et al. 1993; Brandl and Lindow 1996).

The present study was undertaken to gain insight into the involvement of the two pathways for IAA biosynthesis and the role of cytokinins in pathogenicity and epiphytic fitness of *Ehg*. This goal was achieved by inactivating one or more of these pathways in different combinations, using insertion or deletion mutagenesis. In addition, the transcriptional activity of the different genes involved in IAA biosynthesis was assessed in situ with ice nucleation reporter gene fusions in cells of *Ehg* that were either on the surface or within various plant species.

RESULTS

Contribution of different pathways for IAA and cytokinin biosynthesis to virulence of *Ehg*.

Marker exchange mutants deficient in either the IAM or IPyA pathways (Fig. 1) and cytokinin production as well as those lacking combinations of two or all three of the pathways were assayed for pathogenicity on gypsophila cuttings and 1-month-old gypsophila plants. Mutants of *Ehg* with blockage of either *iaaH*, abolishing IAA biosynthesis via the IAM pathway, or *etz*, abolishing cytokinin production, produced significantly smaller galls than the wild type (reduction of 37 to 41% in gall size) but still could induce gall formation (Table 1). In contrast, *Ehg*824-1MX119, a mutant with an insertion in *ipdC* abolishing IAA biosynthesis via the IPyA route, produced galls that were nearly as large as those incited by the parental strain (Table 1). Neither the inactivation of both pathways for

Fig. 1. Indole-3-acetic acid (IAA) biosynthetic pathways detected in *Erwinia herbicola* pv. *gypsophila*. *iaaM*, *iaaH*, and *ipdC* are genes encoding tryptophan-2-monooxygenase, indole-3-acetamide hydrolase, and indole-3-pyruvate decarboxylase, respectively.

IAA biosynthesis (IaaH⁻, IpdC⁻) nor both the IAM pathway and cytokinin biosynthesis (IaaH⁻, IaaM⁻, Etz⁻) nor all three pathways (IaaH⁻, IaaM⁻, IpdC⁻, Etz⁻) caused any further significant reduction in gall size greater than that of IaaH⁻ or Etz⁻ mutants alone (Table 1). The effect of the mutants on disease severity in whole plants generally was similar to that obtained with the gypsophila cuttings. However, the severity of disease conferred by mutants that lacked both cytokinin and IAA production via the IAM pathway (e.g., IaaH⁻, IaaM⁻, Etz⁻) appeared lower than that conferred by mutants deficient in either of the two pathways alone (Table 1). Moreover, a mutant lacking cytokinin production in the absence of both pathways for IAA biosynthesis (IaaH⁻, IaaM⁻, Etz⁻, IpdC⁻) caused slightly less galling than mutants deficient in cytokinin and IAA production via the IAM route alone.

Contribution of different IAA biosynthetic pathways and cytokinin production to epiphytic fitness of *Ehg*.

The population sizes of strain *Ehg*824-1, *Ehg*824-1MX54, and *Ehg*824-1MX119 were determined at various times after inoculation onto bean leaf surfaces. During the first 48 h after inoculation, in which the leaves were kept moist, the population size of all three strains increased an average of 437-fold (Fig. 2). However, a significant differential decrease in the population size of these strains was observed soon after leaves were allowed to dry. In contrast to *Ehg*824-1MX119 (IpdC⁻), which decreased about 795-fold by 72 h and 1,996-fold by 98 h after inoculation, the average decrease in the population sizes of both *Ehg*824-1MX54 (IaaH⁻) and *Ehg*824-1 was only 56.6-fold and 112-fold at these times, respectively (Fig. 2). Thus, the survival of the IpdC⁻ mutant was at least 14-fold lower than that of the parental strain and IaaH⁻ mutant under these harsh conditions. These results clearly suggest that IAA production via the IPyA pathway plays a significant role in the survival of *Ehg* on bean plants, as opposed to IAA production via the IAM pathway, while that via the IAM pathway did not significantly affect the survival of this species on bean leaves.

Table 1. The effect of inactivation of the pathways for indole-3-acetic acid (IAA) and cytokinin biosynthesis on pathogenicity of *Erwinia herbicola* pv. *gypsophila* (*Ehg*824-1)

Strain	Characteristics	Pathogenicity test	
		Cuttings (gall size) ^x	Whole plants (dis. sev.) ^y
<i>Ehg</i> 824-1	Wild type	100 a ^z	3
<i>Ehg</i> 824-1MX119	IpdC ⁻	84 a	2 to 3
<i>Ehg</i> 824-1MX54	IaaH ⁻	63 b	2
<i>Ehg</i> 824-1MX16	IaaH ⁻ , IpdC ⁻	59 b	2
<i>Ehg</i> 824-1MX37	Etz ⁻	53 b	2
<i>Ehg</i> 824-1MX211	IaaM ⁻ , IaaH ⁻ , Etz ⁻	53 b	1 to 2
<i>Ehg</i> 824-1MX132	IaaM ⁻ , IaaH ⁻ , Etz ⁻ , IpdC ⁻	49 b	1

^x Mean gall size was obtained by measuring the fresh weight of hyperplastic tissue 14 days after inoculation of cuttings in three experiments as described in Materials and Methods. Mean gall fresh weight of *Ehg*824-1 was 273 mg. Gall sizes are presented as percentages of size measured for wild-type strain.

^y dis. sev. = disease severity, rated by visual estimation of the symptoms as follows: 0, no symptoms; 1, slight swelling of the inoculation site; 2, large swelling of the inoculation site and appearance of gall; 3, appearance of large galls, split abscission and dehydration; 4, plant death. Results shown were taken from at least three different experiments.

^z Means followed by the same letter do not differ significantly according to Duncan's new multiple range test ($P < 0.05$).

In order to substantiate the above results and to investigate the effect of the interactions between cytokinin production and the different pathways for IAA biosynthesis on survival of *Ehg*, the epiphytic growth and survival of additional mutants was investigated (Table 2). Based on the dynamics of bacterial population sizes described in Figure 2, epiphytic populations of the various mutants were determined 48 and 72 h after inoculation (shortly before and 24 h after leaves were allowed to dry). Simultaneous disruption of both IAA pathways (IaaH⁻, IpdC⁻ mutants) did not significantly further reduce the survival of *Ehg*, compared with the disruption of the IPyA pathway (IpdC⁻) alone. Furthermore, mutants having disruptions of genes involved in cytokinin production and the IAM pathway (Etz⁻, IaaM⁻, IaaH⁻) survived similarly to the wild-type strain whereas a mutant in which cytokinin production and IAA biosynthesis in both pathways were inactivated (Etz⁻, IaaM⁻, IaaH⁻, IpdC⁻) exhibited the same population level as the IpdC⁻ mutant. These results indicate that neither cyto-

Fig. 2. Population size of parental strain *Ehg*824-1 of *Erwinia herbicola* pv. *gypsophila* (circles), strain *Ehg*824-1MX119, which is a marker exchange mutant disrupted in the indole-3-pyruvate (IPyA) pathway (IpdC⁻) (squares), and strain *Ehg*824-1MX54, which is a marker exchange mutant disrupted in the indole-3-acetamide (IAM) pathway (IaaH⁻) (triangles) on bean leaves. The leaves were kept moist for the first 48 h after inoculation and then kept dry for the rest of the experiment. The vertical bars represent the standard error of the mean log-transformed bacterial population size per gram of leaf tissue.

Table 2. The effect of inactivation of the pathways for indole-3-acetic acid (IAA) and cytokinin biosynthesis on the epiphytic fitness of *Ehg*824-1^y

Strain	Characteristics	Bacterial population (log CFU per g fresh wt) ^z	
		48 h	72 h
<i>Ehg</i> 824-1	Wild type	6.43 a	5.15 a
<i>Ehg</i> 824-1MX119	IpdC ⁻	6.23 a	4.15 b
<i>Ehg</i> 824-1MX54	IaaH ⁻	6.39 a	5.00 a
<i>Ehg</i> 824-1MX16	IaaH ⁻ , IpdC ⁻	6.14 a	4.43 b
<i>Ehg</i> 824-1MX211	Etz ⁻ , IaaM ⁻ , IaaH ⁻	6.61 a	5.15 a
<i>Ehg</i> 824-1MX132	Etz ⁻ , IaaM ⁻ , IaaH ⁻ , IpdC ⁻	6.47 a	4.46 b

^y Bean plants were kept moist for 48 h after inoculation and then kept dry for 24 h.

^z For each column, means followed by the same letter do not differ according to Duncan's new multiple range test ($P < 0.05$).

kinins nor IAM produced via the IAM pathway have any measurable effect on epiphytic fitness of *Ehg* on bean leaves.

Transcriptional activity of *ipdC* and *iaaM* in cells on plants.

The transcriptional activity of the *ipdC* genes from both *Ehg* and *E. herbicola* strain 299R as well as the *iaaM* gene from *Ehg* was measured while cells were on bean or gypsophila leaves or after injection into gypsophila stems, by measuring the ice nucleation activity of cells harboring appropriate *inaZ* gene fusions. The ice nucleation activity of cells harboring fusions of *inaZ* to *ipdC* from both *Ehg* and strain 299R in their chromosome was very low in culture but increased about 100,000-fold within 1 day after cells were applied to bean or gypsophila leaves (Fig. 3A and B). Little further increase in ice nucleation activity was observed with further incubation time on leaves. The ice nucleation activity of the two different strains harboring *ipdC-inaZ* gene fusions was similar to each other at a given sampling time on leaves of a given plant species, and the activity of a given strain on bean and gypsophila was also very similar. In contrast, cells harboring the plasmid-borne *iaaM-inaZ* fusions expressed moderate levels of ice nucleation activity in culture, which increased only about 10- to 100-fold after cells were inoculated onto bean or gypsophila leaves (Fig. 3A and B). While the ice nucleation activity of cells harboring *ipdC-inaZ* gene fusions that were injected into gypsophila stems was similar to that of cells carrying the same gene fusion while on leaves, cells harboring the *iaaM-inaZ* fusion exhibited very high levels of ice nucleation activity while in gypsophila stems (Fig. 3C). The ice nucleation activity of cells harboring *iaaM-inaZ* fusions was over 100,000-fold higher when injected into gypsophila stems than in culture and was over 1,000-fold higher than that of the same strain on bean or gypsophila leaves.

DISCUSSION

The effect of exogenous auxin of bacterial origin in the outcome of the interaction between a plant and bacterium partly depends on the amount of hormone that is available to the plant cells. This may be affected by the physical relationship between the two organisms. Bacteria associated with plants can be located either inside the plant or on plant surfaces. The first case is generally representative of biotrophic phytopathogenic bacteria that multiply within the apoplast whereas the second case is typical of bacteria colonizing the phyllosphere or the rhizosphere (Alfano and Collmer 1996). As indicated earlier, *E. herbicola* is a predominant epiphyte on many plants (Starr 1981). We have previously shown that the pathogenicity of *Ehg* is dependent on the presence of pPATH, which harbors numerous genes encoding virulence or pathogenicity factors such as IAA and cytokinin biosynthesis, Hrp proteins, or host specificity determinants (Clark et al. 1993; Lichter et al. 1995a; Manulis et al. 1997; Nizan et al. 1997). Furthermore, at least six copies of the insertion element IS1327 were identified on the pPATH, which could imply a recent horizontal transfer of DNA (Lichter et al. 1996). These findings have led us to postulate that *Ehg* is a recently evolved pathogen and traits conferring pathogenicity on gypsophila are superimposed on those conferring epiphytic fitness. Therefore, *Ehg* should be well adapted for living both on plant surfaces and in the apoplast of its host plant.

Fig. 3. Ice nucleation activity of *Erwinia herbicola* pv. *gypsophiae* strain *Ehg*824-1MX119 (circles), *Ehg*824-1/pVSI-ice (triangles), and *E. herbicola* strain 299RMX149 (squares) harboring fusions of *inaZ* to *ipdC*, *iaaM*, and *ipdC*, respectively. Ice nucleation activity was measured in bacterial cells recovered from (A) bean or (B) gypsophila leaves, or from (C) injected gypsophila stems at various times after inoculation. The vertical bars represent the standard error of the mean log-transformed ice nuclei per cell from 10 samples at each time.

A major finding of this study is the demonstration of differential contribution of the IAM and IPyA pathways to both pathogenicity and epiphytic fitness. While inactivation of the IAM pathway reduced gall formation by approximately 40%, little effect was observed when the IPyA pathway was inactivated (Table 1). In contrast, the epiphytic bacterial population size was about 14-fold lower than the wild-type strain when the IPyA pathway was disrupted, while no change in epiphytic fitness was observed when the IAM pathway was disrupted (Fig. 2, Table 2). These results can be best explained in the light of the hypothesis that the IPyA and IAM pathways are adapted to be expressed in two different microenvironments, namely, plant surfaces and apoplast, respectively. Strong support for this assertion was obtained in this study.

While *iaaM* was apparently very highly transcribed in cells of *Ehg* within wounded gypsophila stems, its transcription was much lower in cells on either bean or gypsophila leaves. In contrast, *ipdC* transcription was much higher on both bean and gypsophila leaves as well as in gypsophila stems than in culture. In addition, the level of transcription of *iaaM* within stems was apparently much higher than that of *ipdC* in cells of *Ehg* in any habitat. Despite the fact that *iaaM-inaZ* gene fusions were located on pVSP61, a plasmid with perhaps as many as 15 copies per cell, the difference in ice nucleation activity of cells harboring *iaaM-inaZ* and *ipdC-inaZ* fusions was over 100,000-fold in stems (Fig. 3). Because ice nucleation activity increases with approximately the square of ice protein abundance (Lindgren et al. 1989), this difference in copy number of the reporter gene fusions would have been expected to yield at most a 225-fold difference in ice nucleation activity. Clearly the difference in activity of cells harboring these two reporter gene fusions was much greater than this in gypsophila stems. It might be expected that relatively high amounts of IAA would be needed for the incitation of galls. Hence, the apparently lower levels of IAA that might be produced via the IPyA pathway, due to the relatively low level of transcription of *ipdC* in stems, would be expected to contribute relatively little to gall formation, as observed here (Table 1). In contrast, the requirements for IAA production to enhance the epiphytic fitness of *Ehg* while on leaf surfaces may be more modest, and thus that contributed via the IPyA pathway may be sufficient to meet this need. While *iaaM* is apparently transcribed in cells of *Ehg* while on leaf surfaces (Fig. 3), its level of transcription is much lower than that inside stems and only slightly higher than that observed in culture. While it is difficult to project how much IAA is produced by *Ehg* using the IAM pathway on leaf surfaces, we would speculate that it is lower than that from the IPyA pathway due to the relatively much lower rate of transcription of *iaaM* as opposed to *ipdC* on leaves compared with the rates within stems. If we can assume that the production of IAA by *Ehg* is directly related to the level of transcription of genes encoding enzymes for its synthesis, then we can make some conjecture as to levels of in situ production. That is, the apparent level of transcription of *iaaM* in cells on leaves is at most only about 3% of that in cells in stems since the difference in ice nucleation of cells in stems and on leaves was approximately 1,000-fold; ($1,000^{0.5} = 32$). It seems likely that 3% of the amount of IAA that is produced inside plants may not be biologically significant if produced on leaf surfaces. In contrast, there is apparently at least a small effect of IAA from the IPyA path-

way on gall formation, and the transcription of *ipdC* is similar in *Ehg* both within and on plants (Fig. 3), suggesting that IAA from the IPyA pathway is more abundant on leaves than that from the IAM pathway.

The finding of the relative importance of IAA from the IPyA pathway in the epiphytic fitness of *Ehg* is consistent with the pattern of regulation of *ipdC* in situ. It is noteworthy that the *ipdC* genes in *Ehg* and in the epiphytic *E. herbicola* strain 299R are both strongly induced on leaf surfaces, compared with that in culture (Fig. 3). Thus, the *ipdC* from *Ehg* and strain 299R are apparently regulated similarly. Previously, we have shown that *ipdC* encoding IPyA decarboxylase, regarded as the key enzyme catalyzing the rate-limiting step of IAA synthesis (Koga et al. 1991), was induced both on wet and dry plants and by low water availability (Brandl and Lindow 1997), suggesting that this gene is responsive to conditions encountered by *Ehg* on leaves. The physical conditions experienced by *Ehg* within plant apoplasts are likely to be much different from those experienced on leaf surfaces. Thus, it is significant that the *ipdC* gene of *Ehg* is induced in epiphytic cells, in addition to those within plants. Such a pattern of expression suggests that the presence of *ipdC* in *Ehg* preceded that of *iaaM* and *iaaH* and confers a benefit to the cell while the bacterial cell is living on plants.

Although the IAM pathway has been occasionally reported in plants (Satome et al. 1993; Kawaguchi et al. 1993), it is generally considered to be primarily a microbial IAA biosynthetic route (Patten and Glick 1996). It has been previously postulated that the IPyA pathway, which is commonly distributed in higher plants, is under stringent regulation to control IAA supply (Manulis et al. 1991b). By utilizing the IAM pathway, which presumably is not regulated by the plant, the pathogen accumulates the large amounts of IAA necessary for gall development.

It has been previously reported that inactivation of either cytokinin production or IAA biosynthesis via the IAM pathway caused a reduction in gall size (Clark et al. 1993; Lichter et al. 1995a). On the other hand, overproduction of cytokinins by *Ehg* resulted in a significant enlargement of gall size (Lichter et al. 1995a) and a similar effect may be assumed for excessive production of IAA, as reported for *P. syringae* pv. *savastanoi* (Glass and Kosuge 1986). It is noteworthy that simultaneous inactivation of the biosynthetic pathways of both phytohormones, including the IAM and IPyA routes, did not result in any further reduction in gall size (Table 1). Although the possibility of the presence of additional routes for IAA biosynthesis in *Ehg* cannot be excluded, it appears that exogenous IAA and cytokinins are not necessary for gall formation but both are required to obtain optimal gall development. Recent study suggests that gall formation in *Ehg* is dependent on the presence of *hrp* genes (Nizan et al. 1997).

Our results are the first to indicate that genes involved in IAA production via the IAM pathway are induced in wounded plants. All tumorigenic bacteria possess this pathway (Morris 1986; Manulis et al. 1991b) and this route is strongly linked to pathogenicity in other species in addition to *Ehg*. In all cases of bacterial hyperplasia, wounding of plant tissues is required for gall formation. Even though gall-forming bacteria such as *P. syringae* pv. *savastanoi* and *Ehg* commonly occur as epiphytes on plants, galling occurs only after plants are wounded. In addition, *P. syringae* pv. *savastanoi* has been shown to oc-

cur as an endophyte within healthy trees (Azad and Cooksey 1995). The level of ice nucleation activity measured in *Ehg* harboring *iaaM-inaZ* gene fusions was much higher (>1,000 ice nuclei per cell) than that seen in other studies of plant-inducible genes (Lindgren et al. 1989; Rahme et al. 1992). The detection of more ice nuclei than viable cells suggests that some cells containing ice nuclei were nonviable, or that ice nuclei were released from cells as membranous vesicles when ice nuclei were highly expressed, as in other studies (Phelps et al. 1986). Since the population size of *Ehg* harboring *iaaM-inaZ* gene fusions was similar to that of the parental strain, the latter explanation appears more likely. Nonetheless, the level of transcription of *iaaM* within gypsophila stems is apparently very high.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The bacterial strains, plasmids, and cosmids used in this work are described in Table 3. *Ehg824-1* and derivatives of the nonpathogenic strain *Eh299R* were grown routinely at 28°C in Luria-Bertani (LB) broth or on LB agar plates. *Escherichia coli* strains were cultured at 37°C in LB. Antibiotics were used at the following concentrations (µg per ml): ampicillin (Amp), 150; rifampicin (Rif), 150; streptomycin (Sm), 50; spectinomycin (Sp), 50; tetracycline (Tc), 12; and kanamycin (Km), 30.

DNA manipulations.

Isolation of plasmid DNA from *E. coli* and *E. herbicola*, agarose gel electrophoresis, restriction mapping, subcloning, transformations, conjugative transfer of plasmids to *E. coli* or *E. herbicola*, and polymerase chain reaction (PCR) were carried out according to standard procedures (Sambrook et al. 1989) or as recommended by the supplier.

Construction of marker exchange mutants of *Ehg824-1* deficient in IAA and cytokinins.

Inactivation of the IPyA pathway in *Ehg824-1* (Fig. 1) was achieved by insertional mutagenesis of the IPyA decarboxylase gene (*ipdC*). pMB149 (Brandl and Lindow 1996), containing *ipdC* with an insertion of Tn3-Spice in the same orientation as transcription of *ipdC*, was introduced from *E. coli* DH5α into *Ehg824-1* and *Eh299R* via triparental matings with the *E. coli* helper strain HB101 (pRK2013). The resulting transconjugants were cultured overnight at 28°C in LB agar without antibiotic selection. Cells were then plated on LB containing Rif, Tc, and Sp. Curing of pLAFR3 from transconjugants was carried out by subculturing in the absence of antibiotic selection for about 10 generations, as previously described (Clark et al. 1993). Colonies from dilution plates of these cultures were then replica plated onto LB agar containing either Rif, Tc, and Sp, or Rif and Sp. Colonies resistant to Rif and Sp and sensitive to Tc were selected. *Bam*HI-*Eco*RI digests of total DNA from the wild-type strains and their marker exchange mutants were examined by Southern blot hybridization to confirm the presence of an insertion in the *ipdC* mutants. A 1.2-kb *Sac*I-*Eco*RV internal fragment of *ipdC* obtained from pUB1.2 (Brandl and Lindow 1996) was used as a probe. A 9-kb fragment hybridized to this probe in *Ehg824-1* in contrast to a 4.8-kb fragment in *Eh299R* (Brandl and Lindow 1996); this result arises from a polymorphism in this region in these two strains. Southern hybridization of *Eh299R* marker exchange mutants exhibited two expected bands of 3.5 and 3 kb, whereas a different pattern of hybridizing fragments (6.9 and 3.9 kb) appeared in marker exchange mutants of *Ehg824-1*. Tryptophol is a major mandatory byproduct of the IPyA pathway (Fig. 1). Therefore, marker exchange mutants were tested for the production of tryptophol by thin layer chromatography as previously described (Manulis et al. 1991b). Tryptophol was detected in *Ehg824-1* and *Eh299R* but

Table 3. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
<i>Escherichia coli</i>		
DH5α		Sambrook et al. 1989
HB101		Sambrook et al. 1989
<i>Erwinia herbicola</i>		
<i>Ehg824-1</i>	Rif ^r , wild-type pathogenic strain of gypsophila	Manulis et al. 1991a
<i>Eh299R</i>	Rif ^r , wild-type nonpathogenic strain	Brandl and Lindow 1996
<i>Ehg824-1MX119</i>	Rif ^r , Sp ^r , Sm ^r , IpdC ⁻ , marker exchange mutant of <i>Ehg824-1</i>	This study
<i>Ehg824-1MX54</i>	Rif ^r , Km ^r , IaaH ⁻ , marker exchange mutant of <i>Ehg824-1</i>	This study
<i>Ehg824-1MX16</i>	Rif ^r , Sp ^r , Sm ^r , IaaH ⁻ , IpdC ⁻ , marker exchange mutant of <i>Ehg824-1MX119</i>	This study
<i>Ehg824-1MX37</i>	Rif ^r , Km ^r , Etz ⁻ , Km gene in <i>Eco</i> RI site of <i>etz</i>	Lichter et al. 1995b
<i>Ehg824-1MX211</i>	Rif ^r , Km ^r , IaaM ⁻ , IaaH ⁻ , Etz ⁻ , marker exchange mutant of <i>Ehg824-1</i>	This study
<i>Ehg824-1MX132</i>	Rif ^r , Km ^r , Sp ^r , Sm ^r , IaaM ⁻ , IaaH ⁻ , Etz ⁻ , IpdC ⁻ , marker exchange mutant of <i>Ehg824-1MX119</i>	This study
Plasmids		
pBS	Amp ^r , cloning vector, pBluescript KS+	Stratagene, La Jolla, CA
pUC4K	Km ^r , Amp ^r , Km resistance gene used for gene disruption	Pharmacia, Piscataway, NJ
pLAFR3	Tc ^r , cosmid vector	Staskawicz et al. 1987
pTn3-Spice	Tc ^r , Sp ^r , clone containing the <i>inaZ</i> reporter gene in Tn3	Lindgren et al. 1989
pRK2013	Km ^r , Tra ⁺ , Mob ⁺ ColE1 replicon	Ditta et al. 1980
pEG101	Amp ^r , 7.5-kb <i>Eco</i> RI fragment from pPATH of <i>Ehg713</i> cloned in pUC118, containing <i>iaaM</i> and <i>iaaH</i>	Clark et al. 1993
pMBL-23B	Amp ^r , 0.6-kb <i>Eco</i> RI- <i>Alu</i> I fragment cloned from pMBL-2B	Lichter et al. 1995a
pSM1	Km ^r , Tc ^r , pLAFR3 containing 7.5-kb <i>Eco</i> RI fragment + 1.3-kb Km ^r cassette	This study
pSM2	Km ^r , Tc ^r , pLAFR3 containing Δ <i>iaaM</i> (0.9 kb)-Km ^r cassette (1.3 kb)- Δ <i>etz</i> (0.7 kb)	This study
pVSP61	Km ^r , pVS1 replicon	Loper and Lindow 1994
pVSI-ice	Km ^r , pVSP61 containig <i>iaaM</i> promoter fused into <i>inaZ</i>	This study

was absent in all marker exchange mutants derived from these strains. The marker exchange mutant *Ehg824-1MX119* (IpdC⁻) was selected for further studies.

Inactivation of the IAM pathway in *Ehg824-1* and *Ehg824-1MX119* was performed by manipulation of pEG101 (Clark et al. 1993). pEG101, which harbors the determinants of the IAM pathway, contains a 7.5-kb *EcoRI* insert from the pPATH of *Ehg*. A 1.3-kb Km resistance cassette was obtained by digesting pU4K with *Bam*HI and inserting it into the *Bgl*III site located in the *iaaH* gene in pEG101 (Fig. 4A). This plasmid was then cut with *EcoRI* and a 8.8-kb DNA fragment containing the Km resistance gene (7.5-kb *EcoRI* fragment + 1.3-kb Km gene cassette) was isolated and inserted into the *EcoRI* site of pLAFR3 to produce pSM1, and transformed into *E. coli* DH5 α . pSM1 was then introduced into *Ehg824-1* and *Ehg824-1MX119* by triparental mating and colonies resistant to Rif, Km, and Tc were selected. After cells were cured of pSM1, colonies resistant to Rif and Km but sensitive to Tc were selected. Verification of the marker exchange and plasmid curing in the mutants of *Ehg824-1* and *Ehg824-1MX119* was performed by PCR. A downstream primer (5'-TCCGTGA

TGGCGATGCAC-3') and an upstream primer (5'-CCGACCACAGGTCGTTGGG-3') of the *Bgl*III site in *iaaH* were constructed according to the *iaaH* sequence of *Ehg* (accession number L33866). The insertion of the 1.3-kb *Bam*HI fragment harboring the Km resistance gene yielded a 1.7-kb fragment instead of the 0.4-kb native product between the two primers. The marker exchange mutants *Ehg824-1MX54* (*IaaH*⁻), derived from *Ehg824-1*, and *Ehg824-1MX16* (*IaaH*⁻, *IpdC*⁻), derived from *Ehg824-1MX119*, were chosen for further study. Both mutants were unable to convert IAM into IAA, as opposed to the wild type, when assayed with concentrated bacterial cells as previously described (Manulis et al. 1991b).

In order to obtain a simultaneous inactivation of the IAM pathway and cytokinin biosynthesis, advantage was taken of the fact that the genes encoding for these phytohormones form a cluster on pPATH in *Ehg* (Fig. 4B). pMBL-23B was employed for constructing a marker exchange mutant of *Ehg824-1*. This plasmid is composed of a 0.6-kb *EcoRI*-*AluI* insert cloned into pBS. The insert contains 370 bp from the 3' end of the *etz* gene and a small downstream segment of the pPATH (Lichter et al. 1995a). To this plasmid a 0.9-kb *Bam*HI-*Fsp*I insert removed

Fig. 4. Physical map of constructs used for generation of marker exchange mutants. **A**, pEG101: a 7.5-*EcoRI* fragment of pPATH containing the genes encoding enzymes of the indoleacetamide pathway and cloned into pUC118. *iaaM*: tryptophan-2-monooxygenase; *iaaH*: indole-3-acetamide hydrolase. **B**, pLA150: a cosmid clone of pPATH in pLAFR3 containing the cluster of genes encoding for cytokinin and indole-3-acetic acid (IAA) via the indoleacetamide pathway. pre-*etz* and *etz*-genes encoding for cytokinin. Km = kanamycin resistance gene cassette. Restriction enzymes: R = *EcoRI*; B = *Bam*HI; F = *Fsp*I; Bgl = *Bgl*III; A = *AluI*; S = *Stu*I.

from *iaaM* of pEG101 was ligated into the *Bam*HI-*Sma*I sites of pMBL-23B. A *Km^r* gene was then ligated into the *Pst*I site of pBS located between *iaaM* and *etz*, resulting in a 2.9-kb construct composed of *iaaM* (0.9 kb)–*Km^r* (1.3 kb)–*etz* (0.7 kb) (Fig. 4B). This 2.9-kb fragment was then removed from pBS by digestion with *Bam*HI and partial digestion with *Hind*III and ligated into pLAFR3, which was previously cut with *Bam*HI and *Hind*III to produce pSM2. Following transformation into *E. coli* (HB101), pSM2 was conjugated into *Ehg824-1* and *Ehg824-1MX119*, respectively, to produce *Ehg824-1MX211* (*IaaM⁻*, *IaaH⁻*, *Etz⁻*) and *Ehg824-1MX132* (*IaaM⁻*, *IaaH⁻*, *Etz⁻*, *IpdC⁻*). Molecular verification of the insertional mutants was obtained by comparison of Southern blots of the DNA from the mutants with their parental strains after digestion with *Hind*III and hybridization with an *Fsp*I-*Stu*I fragment internal to the insert. Biochemical assays for production of IAM and IPyA (Manulis et al. 1991b) or cytokinin (Lichter et al. 1995b) confirmed the genotype of the different mutants.

Plasmid pVSI-ice containing *iaaM* promoter from *Ehg* fused into *inaZ* was constructed as follows. A 230-bp fragment containing the putative *iaaM* promoter was amplified by PCR with the primers 5'-GGGAATTCAGCAGCAAGACTAATACG-3' and 5'-CCGAATTCCTTCCAGTTACGTTTCG-3' (accession no. L33867). The amplified fragment was digested by *Eco*RI and ligated into the *Eco*RI site of the plasmid pVSP61 (Loper and Lindow 1994). A promoterless *inaZ* gene was then cloned into the *Bam*HI-cleaved pVSP61 containing the *iaaM* promoter to yield pVSI-ice. This plasmid was introduced by triparental mating into strain *Ehg824-1*, in which it was stably maintained, to produce *Ehg824-1/pVSI-ice*.

Pathogenicity tests.

Pathogenicity tests were carried out on young, excised *Gypsophila paniculata* var. *perfecta* cuttings essentially as described previously (Lichter et al. 1995a; Manulis et al. 1991a). Following the removal of a 2- to 3-mm section from the bottom of the stem, the cutting was inoculated by touching it to a 1-day-old bacterial colony, placed in a well of a plastic tray filled with vermiculite, and incubated in a growth chamber that was maintained at 26 to 29°C, and with high humidity generated by computer-controlled mist sprinklers (every 10 min for 10 s during the first 48 h and later at 25-min intervals for 10 s). Pathogenicity was scored 15 to 20 days after inoculation by using five replicates per strain. The galls were removed flush from the edge of the stem, washed with tap water, gently dried with a paper towel, and weighed.

Pathogenicity tests were also performed on 5-week-old gypsophila seedlings grown in peat-sand (1:1) mixture and placed under a shade net (Lichter et al. 1995a). Inoculation was carried out by injecting 0.1 ml of a bacterial suspension (10^7 cells per ml) prepared from a culture grown overnight in LB broth. The bacterial suspension was injected through the apex of the seedling into the stem. Symptoms were evaluated 1 month after inoculation according to an arbitrary scale described in Table 1.

Epiphytic fitness test.

Determination of epiphytic fitness was carried out essentially as described elsewhere (Lindow 1993). Bean seedlings (*Phaseolus vulgaris* cv. Bush Blue Lake 274) were grown for 2 weeks on a greenhouse bench. Wetting of leaves during

daily watering was minimized in order to achieve a low population of contaminating bacteria on the surface of the seedlings at the time of inoculation. Plants were inoculated by immersing the leaves in 2 liters of bacterial suspension in sterile containers. Bacterial suspensions were prepared from cells grown on rifampicin-containing LB agar plates incubated overnight at 28°C. Cells were suspended in sterile 10 mM potassium phosphate buffer (pH 7.0) and diluted with 1 mM potassium phosphate buffer to a final concentration of 10^5 cells per ml. Ten pots of bean seedlings (each pot containing 10 plants with two primary leaves each) were immersed in a suspension of a given bacterial strain for about 3 s and were then placed upright for about 5 s to drain excess liquid from the leaves. A plastic bag was then placed over the plants and was sealed loosely at the base of the pot. The pots were then placed in a growth chamber maintained at 25°C under previously described conditions (Lindow 1993). The plastic bags were removed after 48 h and the plants were then incubated under dry conditions on a laboratory bench. To prevent wetting of the leaves, the plants were drip irrigated. Thirty individual leaves were collected per each bacterial strain at various time intervals as indicated in the results. Each leaf was immersed in 20 ml of washing buffer (0.1 M potassium phosphate buffer, pH 7.0, containing 0.1% peptone) in large (25 mm) test tubes that were immersed in an ultrasonic bath and sonicated for 7 min. The test tubes were then vortexed for 15 s and 10-fold serial dilutions were plated onto LB agar containing 100 mg of rifampicin, 100 mg of cycloheximide, and 25 mg of benomyl per ml. Bacterial populations were estimated by counting, with a laser counter (model 500A, Spiral Systems, Cincinnati, OH), the number of colonies arising on appropriate portions of plates after a continuous dilution of bacterial suspensions was applied with a spiral plater/diluter (Spiral Systems model D). Because our epiphytic bacterial populations were described by a log-normal distribution, population sizes were log-transformed before calculating mean population sizes for a given strain at a given sampling time.

Measurements of ice nucleation activity.

Strains *Ehg824-1MX119*, *Ehg824-1/pvsi-ice*, and *E. herbi-*
cola strain 299RMX149 (Brandl and Lindow 1997) were either sprayed onto bean or gypsophila leaves at a concentration of 10^6 cells per ml and incubated under moist conditions as noted above or were injected into gypsophila stems at a concentration of 10^8 cells per ml as described above. Ten individual leaves from plants inoculated with a given strain were harvested at various times and placed individually in 25-mm test tubes containing 20 ml of washing buffer, and sonicated for 7 min. For assay of injected stems, the leaves were excised from the gypsophila plants flush with the stem, and 10 2-cm stem segments excised such that the site of injection was centered on the stem segment. The excised stem segments were then surface sterilized by immersing them in 0.5% sodium hypochlorite for 10 min and then rinsing them three times in sterile, distilled water. Each stem segment was then macerated with a mortar and pestle with 2 ml of washing buffer. For all samples, the concentration of ice nuclei was determined by placing 40 10- μ l drops of appropriate 10-fold dilutions of these leaf washings of stem macerates on a paraffin-coated aluminum foil sheet cooled to -9°C; the concentration of ice nuclei was calculated from the fraction of drops that froze, as

in previous studies (Lindgren et al. 1989). The number of cells of each strain was determined by plating appropriate dilutions of leaf washings or stem macerates onto King's medium B containing 100 µg ml⁻¹ rifampicin and 100 µg ml⁻¹ cycloheximide as described above. The number of ice nuclei measured in each sample was normalized for the number of rifampicin-resistant bacterial cells recovered from that sample.

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