

Production of Cytokinins by *Erwinia herbicola* pv. *gypsophilae* and Isolation of a Locus Conferring Cytokinin Biosynthesis

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Analysis of cytokinins produced in culture by the gall-forming bacterium *Erwinia herbicola* pv. *gypsophilae* indicated that pathogenic but not nonpathogenic strains secreted a significant amount of cytokinin. Chemical identification of cytokinins was performed by immunoaffinity chromatography followed by high-performance liquid chromatography and gas chromatographic mass spectrometry. The cytokinins were quantified by either radioimmunoassay or enzyme-linked immunosorbent assay using anti-cytokinin monoclonal antibodies. Zeatin, zeatin riboside, *iso*-pentenyladenine, and two immunoreactive zeatin-type compounds were the predominant cytokinins identified in the supernatant. A locus conferring cytokinin production was cloned from a cosmid library derived from plasmids of a pathogenic strain (Eh824-1). Expression was achieved following mobilization of the cosmid clones into a nonpathogenic strain (Eh3-1) or a deletion mutant (Eh3-106), but not in *Escherichia coli*. Eh3-106 contained a deletion in the pathogenicity-associated plasmid and lacked cytokinin production. A 3.9-kb DNA fragment from a cosmid clone was subcloned into a wide-host-range plasmid and found to restore production of all the previously detected cytokinins following its mobilization into nonpathogenic *E. herbicola* strains. This fragment overlapped the 3' end of the indole-3-acetic acid biosynthetic genes previously cloned from this plasmid, suggesting that indole-3-acetic acid and the locus specifying cytokinin biosynthesis are clustered on the pathogenicity-associated plasmid.

Additional keywords: *Gypsophila paniculata*, phytohormones, pLAFR3, plasmid deletion

Erwinia herbicola pv. *gypsophilae* induces gall formation in *Gypsophila paniculata* (baby's-breath) (Cooksey 1986; Manulis *et al.* 1991a), an ornamental used in commercial cut-flower production (Shillo 1985). Infection is initiated at wound sites, mainly during the development of rooted cuttings in nurseries. The host range is restricted to *gypsophila* (Volcani *et al.* 1985); gall-forming *E. herbicola* isolates from

beet (*Beta vulgaris* L.) can also incite galls on *gypsophila* (Burr *et al.* 1991).

The phytohormones indole-3-acetic acid (IAA) and cytokinins are considered to play a major role in the pathogenicity of gall-forming bacteria (Morris 1986). Previous studies have shown that *E. herbicola* possesses two major metabolic pathways for IAA biosynthesis. The indole-3-pyruvate route was present in all isolates examined, including nonpathogenic isolates, while the indole-3-acetamide (IAM) route was found only in pathogenic isolates (Manulis *et al.* 1991b). Further studies revealed that the genes for IAA biosynthesis via the IAM pathway reside on a native plasmid present only in pathogenic isolates (Manulis *et al.* 1991a). These genes are equivalent to the *iaaM* and *iaaH* genes reported for the IAM route in *Agrobacterium tumefaciens* and *Pseudomonas syringae* pv. *savastanoi* (Morris 1986). Marker exchange mutants of *E. h.* pv. *gypsophilae* generated by insertional inactivation of *iaaM* or *iaaH* induced smaller galls than the wild type did (Clark *et al.* 1993).

Gall-forming and other phytopathogenic bacteria are known to produce cytokinins (Morris 1986; Akiyoshi *et al.* 1987). The major route for cytokinin biosynthesis in bacteria is via the condensation of AMP and dimethylallyl pyrophosphate (DMAPP), catalyzed by a DMAPP:AMP transferase. The primary cytokinins isopentenyl adenine (iP), isopentenyl adenosine (iPA), or their phosphorylated derivatives are hydroxylated by a yet-undefined enzyme to form the highly biologically active *t*-zeatin (*Z*) derivatives (Letham and Palmi 1983; Barry *et al.* 1984; Heinemeyer *et al.* 1987). Genes encoding the DMAPP:AMP transferase have been isolated from *A. tumefaciens*, *A. rhizogenes*, *P. s.* pv. *savastanoi*, *P. solanacearum*, and *Rhodococcus fascians* (Morris 1986; Regier *et al.* 1989; Akiyoshi *et al.* 1989; Crespi *et al.* 1992). The *tzs* gene of *A. tumefaciens* was demonstrated to be under the control of *virA* and *virG* regulatory genes (Powell *et al.* 1988). Cytokinins may also be derived indirectly from tRNA in *A. tumefaciens* in which a DMAPP:tRNA transferase (*miaA*) was recently characterized (Gray *et al.* 1992). Mutation of the *miaA* gene results in loss of iP from the culture supernatant of a Ti-plasmid-lacking strain of *A. tumefaciens*, but the mechanism by which this iP is released from the tRNA is unknown (J. Gray and R. Morris, personal communication).

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The involvement of cytokinins in the pathogenicity of phytopathogenic bacteria has been studied (Morris 1986). However, with the exception of *A. tumefaciens* (Akiyoshi *et al.* 1983), the precise contribution of cytokinins to disease incitement has not yet been elucidated. In the present study we describe the identification of cytokinins produced by *E. herbicola* and the isolation of a plasmid-borne locus conferring cytokinin biosynthesis.

RESULTS

Cytokinin production by *E. herbicola*.

Preliminary determination of cytokinins in culture filtrates from a pathogenic strain (Eh824-1) and a nonpathogenic strain (Eh3-1) of *E. herbicola* was performed by the cucumber cotyledon bioassay as described by Surico *et al.* (1985). The level of cytokinin-like activity in filtrates of the pathogenic strain Eh824-1 was a Z equivalent in the range of 200–300 ng/ml in three different experiments, whereas no significant activity could be detected in the nonpathogenic strain Eh3-1. In order to establish a correlation between cytokinin production and pathogenicity, 13 pathogenic and 11 nonpathogenic isolates of *E. herbicola* were examined for cytokinin production by enzyme-linked immunosorbent assay

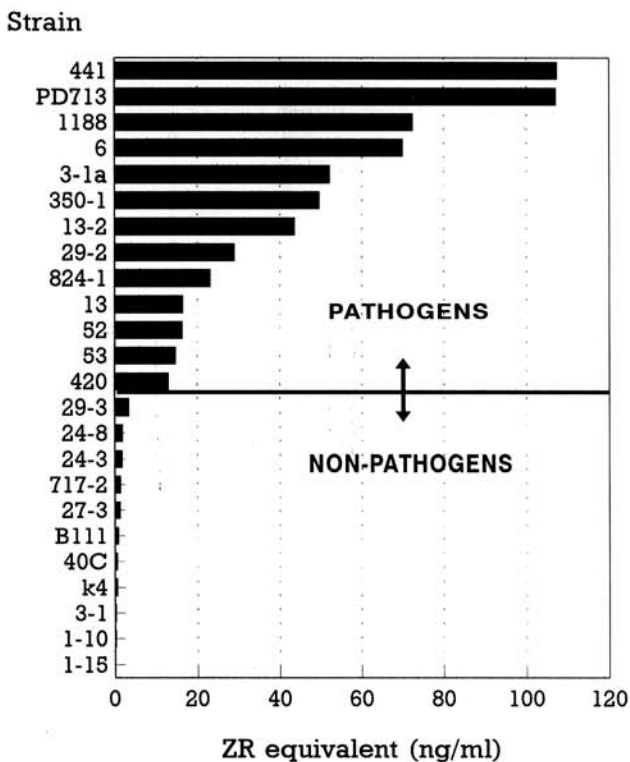


Fig. 1. Production of cytokinins by pathogenic and nonpathogenic isolates of *Erwinia herbicola*. Isolates were grown for 24 hr on modified minimal A medium, and the presence of cytokinins in the filtrates was assayed by enzyme-linked immunosorbent assay using anti-zeatin riboside (ZR) monoclonal antibodies. The assay was performed in triplicates, and the results presented are an average of two independent experiments. All pathogenic and nonpathogenic isolates except Eh1188 and Eh3-1 were isolated from gypsophila. Strain Eh1188, pathogenic on beet and gypsophila, was described by Burr *et al.* (1991). The nonpathogenic strain Eh3-1, a spontaneous derivative of Eh3-1a, has lost the pathogenicity-associated plasmid pPATH.

(ELISA) using anti-zeatin riboside (ZR) antibodies. The level of cytokinins in culture filtrates of pathogenic isolates was a ZR equivalent between 13 and 107 ng/ml (Fig. 1), in contrast to negligible amounts in nonpathogenic isolates.

Following the detection of cytokinins in the unfractinated culture media of pathogenic strains, we investigated the identity of the cytokinin or cytokinins produced by selected strains. It is known that quantification of phytohormone compounds in crude samples by ELISA can yield estimates much higher than those obtained following further purification (Cohen *et al.* 1987). Therefore immunopurification was first used to isolate cytokinins from culture supernatants. Immunopurified cytokinins were then fractionated by high-performance liquid chromatography (HPLC) and quantified by radioimmunoassay (RIA). The iPA contamination of the iPA3 clone (see Materials and Methods) did not permit determination of the presence of iPA in the bacterial supernatants. Our analysis indicated that Z, ZR, and iP were present at significant levels in the culture supernatants of the pathogenic strains Eh3-1a and Eh824-1 (Figs. 2A and 6A). Semiquantitative estimates of the amounts of cytokinins secreted are as follows (in nanograms per milliliter of supernatant): for strain Eh3-1a, Z, 4.3; ZR, 10.9; and iP, 1.9; whereas for strain Eh824-1, Z, 2.2; ZR, 1.4; and iP, 1.8. In contrast, no cyto-

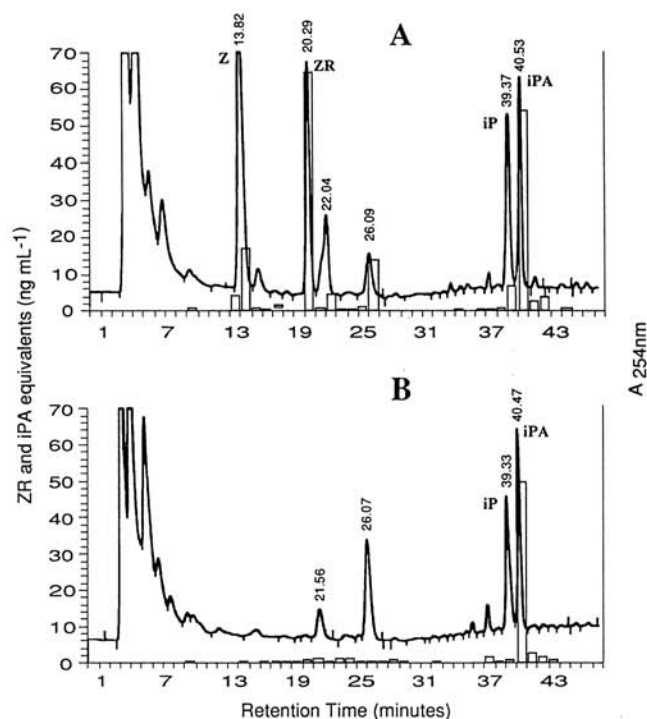


Fig. 2. High-performance liquid chromatography (HPLC) and radioimmunoassay (RIA) of cytokinin production by pathogenic and nonpathogenic strains of *Erwinia herbicola*. **A**, Eh 3-1a, wild-type pathogenic strain. **B**, Eh3-1, nonpathogenic mutant of Eh 3-1a. Z = t-zeatin. Fractions 10–30 and 30–45 were subjected to RIA using clone 16 anti-zeatin riboside (ZR) antibody and clone iPA3 anti-isopentenyl adenine (iP) antibody, respectively. Quantitative results of RIA are indicated for each 1-ml HPLC fraction; values of cytokinin levels in nanograms per milliliter of culture supernatant are provided in the Results section. Elution times for compounds of interest are indicated in minutes. The isopentenyl adenosine (iPA) peak is artifactual (see Materials and Methods). UV absorbance: continuous trace. RIA data: histogram.

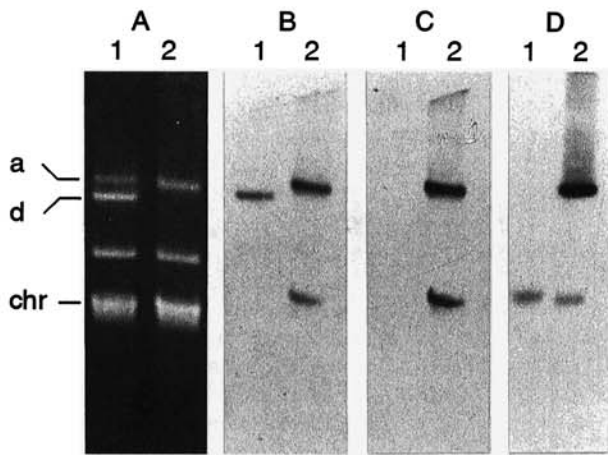


Fig. 3. Plasmids profile and Southern hybridization of *Erwinia herbicola* strain Eh824-1 and its Tn5 derivative, Eh3-106. **A**, Plasmid profile of the nonpathogenic strain Eh3-106 (lane 1) and the wild-type isolate Eh824-1 (lane 2); a and d refer to plasmid DNA. **B**, Southern hybridization of **A** using the deleted plasmid d as a probe. **C**, Southern hybridization of **A** using the 7.5-kb *EcoRI* plasmid fragment containing the indole-3-acetic acid biosynthetic genes as a probe (Clark *et al.* 1993). **D**, Southern hybridization of Eh3-106 plasmids (lane 1) and Eh4-38, a Tn5 nonpathogenic mutant of Eh824-1 bearing the Tn5 in a plasmid (lane 2), which was used as a hybridization control. Tn5 was used as a probe. Hybridization of Eh3-106 was confined to the chromosomal fraction (chr), in contrast with the plasmid location in the control Eh824-1 mutant (the chromosomal DNA fraction also contains linear plasmid fragments).

Transconjugants

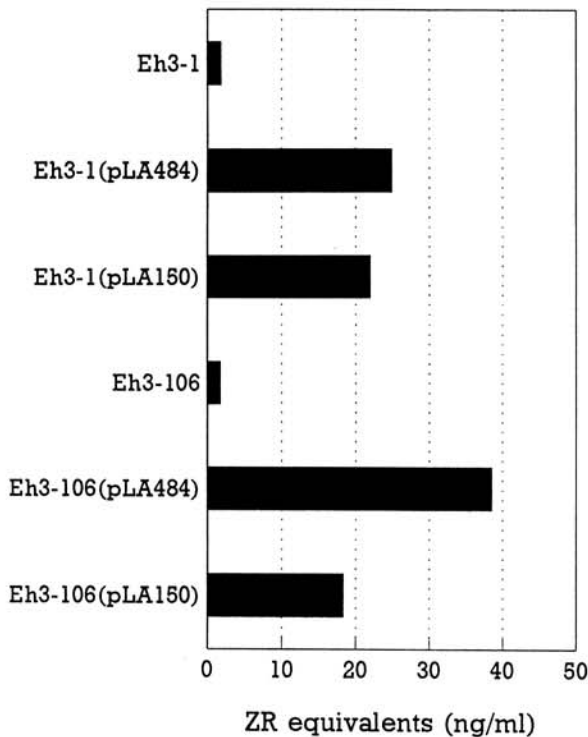


Fig. 4. Cytokinin expression by cosmid clones conjugated into nonpathogenic *Erwinia herbicola* strains. Transconjugates were obtained by triparental mating into the nonpathogenic strains Eh3-1 or Eh3-106, with pRK2073 used as a helper. Cytokinin expression in the supernatant of the transconjugates was determined by enzyme-linked immunosorbent assay, employing anti-zeatin riboside (ZR) monoclonal antibodies.

kinins could be identified in the nonpathogenic strain Eh3-1 (Fig. 2B). An additional immunoreactive (anti-ZR) compound with an HPLC retention time of 26.09 min was present in supernatants of Eh3-1a (Fig. 2A). This compound did not coelute with any available cytokinin standard, and its identity awaits further study.

Isolation of a deletion mutant of *E. h. pv. gypsophylae* lacking cytokinin production.

The plasmid pDG-S1 was employed for Tn5 mutagenesis of *E. h. pv. gypsophylae* (Eh824-1), and 1,200 mutants were screened for loss of pathogenicity on gypsophila as described by Manulis *et al.* (1991a). Among the 12 nonpathogenic mutants isolated, one mutant, designated Eh3-106, lacked cytokinin production, as revealed by ELISA of clarified supernatants (see below, Fig. 4). Analysis of immunopurified supernatants from the mutant strain Eh3-106 showed the lack of Z, ZR, and iP, which are produced by the wild-type strain Eh824-1 (see below, Fig. 6A and B). Eh3-106 contained a Tn5 transposon in its chromosomal DNA (Fig. 3D, lane 1) and had an altered plasmid profile, compared to the wild type (Fig. 3A and B). It has been previously shown that Eh824-1 carries two plasmids of similar size, in the range of 140–150 kb, one of which is associated with pathogenicity (Manulis *et al.* 1991a). The latter plasmid, which will henceforth be designated pPATH824-1, contained the genes for IAA biosynthesis (e.g., *iaaM* and *iaaH* [Manulis *et al.* 1991a; Clark *et al.* 1993]). Comparison of the plasmid profile of Eh824-1 with that of Eh3-106 indicates the appearance of a smaller plasmid of approximately 100 kb in the latter mutant (Fig. 3A). The size of this plasmid was calculated according to the *Erwinia stewartii* SW2 plasmid profile (Kado and Liu 1981). Southern

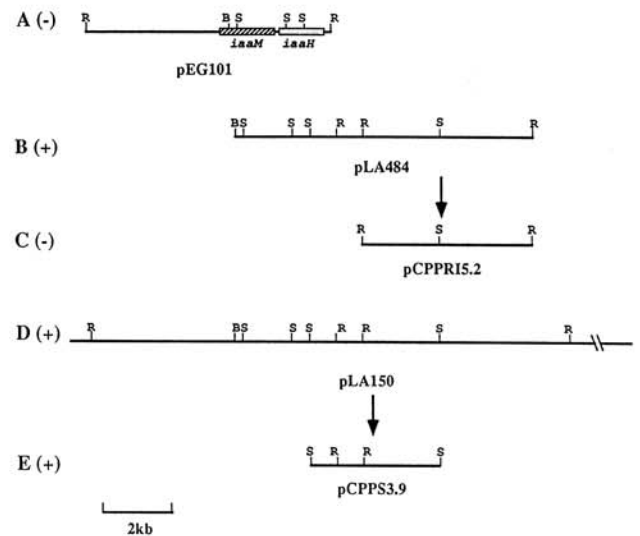


Fig. 5. Partial restriction map of the cosmid clones expressing cytokinin production and their plasmid subclones. Restriction mapping was carried out with *EcoRI* (R), *BamHI* (B), and *SmaI* (S). **A**, pEG101 is a 7.5-kb *EcoRI* fragment containing the indole-3-acetic acid biosynthetic genes and cloned into pUC118 (Clark *et al.* 1993). **B**, pLA484 is a cosmid clone isolated from the pLAFR3 cosmid library of Eh824-1 plasmids. **C**, pCPPR15.2 is a 5.2-kb *EcoRI* fragment subcloned from pLA484 into pCPP50. **D**, pLA150 is a cosmid clone isolated from the pLAFR3 cosmid library of Eh824-1 plasmids. **E**, pCPPS3.9 is a 3.9-kb *SmaI* fragment subcloned from pLA150 into pCPP50. Cytokinin production in Eh3-106 is symbolized by +, and absence of cytokinins by -.

hybridization of the plasmids, using this smaller plasmid as a probe, confirmed that it originated from one of the two larger plasmids (Fig. 3B). Plasmid fragments in the chromosomal fraction are responsible for the hybridization with this fraction in lane 2 of Figure 3B. The fact that no such hybridization was observed with the Eh3-106 chromosomal fraction (Fig. 3B, lane 1) is attributed to the expected lower fragility of the smaller plasmid, compared to that of the full-size plasmid. Hybridization of the plasmids using the 7.5-kb *EcoRI* fragment containing the IAA biosynthetic genes as a probe demonstrated its absence in Eh3-106 (Fig. 3C). pPATH was defined by its hybridization with the 7.5-kb *EcoRI* fragment. Therefore the negative hybridization with Eh3-106 (Fig. 3C, lane 1) provides indirect evidence that the newly appeared smaller plasmid is a deletion derivative of pPATH824-1 and lacks the IAA biosynthetic genes and probably the cytokinin biosynthetic locus. Additional direct evidence is provided in the next section. It is unclear whether the deletion was a direct consequence of the transposon mutagenesis process or whether it was due to a subsequent independent event.

Cloning and expression of the Eh824-1 locus conferring cytokinin biosynthesis.

Since all the pathogenic isolates of *E. herbicola* carry a pathogenicity-associated plasmid which is lacking in the non-

pathogenic isolates (Manulis *et al.* 1991a), it was suspected that the gene or genes responsible for cytokinin production might reside on this plasmid. Therefore, in order to isolate and delineate the genetic locus responsible for cytokinin biosynthesis, a cosmid library was constructed from the plasmids of Eh824-1. This library was screened for cytokinin production by ELISA with anti-t-ZR monoclonal antibodies. Attempts to express the cosmid clones in *Escherichia coli* were unsuccessful, and therefore they were mobilized by tri-parental mating into either Eh3-1 or Eh3-106. Two transconjugate clones containing pLA484 and pLA150 showed significant cytokinin production in both strains of *E. herbicola* (Fig. 4). The two isolated cosmid clones hybridized with the 7.5-kb *EcoRI* plasmid fragment (Fig. 5A) described above (results not shown). A partial restriction map of pLA484 (~9 kb) and pLA150 (~25 kb) is shown in Figure 5B and D, in alignment with the 7.5-kb *EcoRI* fragment (Fig. 5A). The transconjugated cosmid clones in Eh3-106 (Fig. 4) did not restore pathogenicity to Eh3-106.

A 5.2-kb *EcoRI* fragment from pLA484 was subcloned into the *EcoRI* site of the wide-host-range plasmid pCPP50, to generate pCPPRI5.2 (Fig. 5C). The resulting subclone in two orientations did not express cytokinin production either in *E. coli* or in *E. herbicola* Eh3-106. The next step included subcloning a 3.9-kb *SmaI* fragment from pLA150 into the *SmaI* site of pCPP50, to generate pCPPS3.9 (Fig. 5E). The 5' end of the 3.9-kb *SmaI* fragment overlaps the 3' region of *iaaH*, whereas its 3' end overlaps the 5' region of pCPPRI5.2 (Fig. 5C). Cytokinin production by pCPPS3.9 could be detected only following transconjugation of this subclone into *E. herbicola* Eh3-106 (Fig. 6C) and in two orientations of the subclone.

It is noteworthy that pLA150 and pCPPRI5.2 (Fig. 5D and C) but not pCPPS3.9 or the previously mentioned 7.5-kb *EcoRI* fragment (Fig. 5E and A) hybridized with Eh3-106 (results not shown). These results further define the deleted plasmid of Eh3-106 as a derivative of pPATH. Accordingly, the downstream border of the deletion should lie between the right *SmaI* and *EcoRI* sites (Fig. 5B and C).

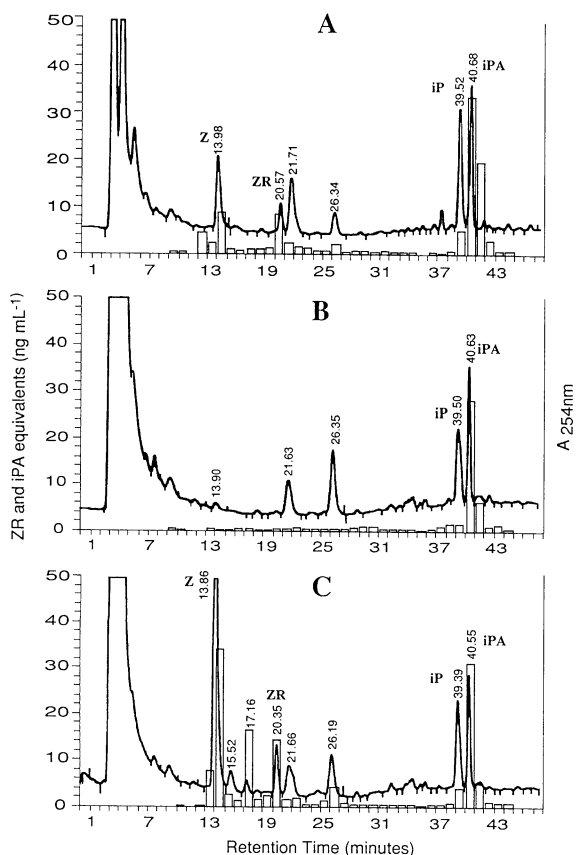


Fig. 6. Comparative analysis of cytokinins produced by *Erwinia herbicola* pv. *gypsophylae* strain Eh824-1 and strain Eh3-106 with and without pCPPS3.9. Analyses were performed by high-performance liquid chromatography and radioimmunoassay as described in Figure 2. **A**, Eh824-1. **B**, Eh3-106. **C**, Eh3-106(pCPPS3.9). The iPA peak is artificial (see Materials and Methods).

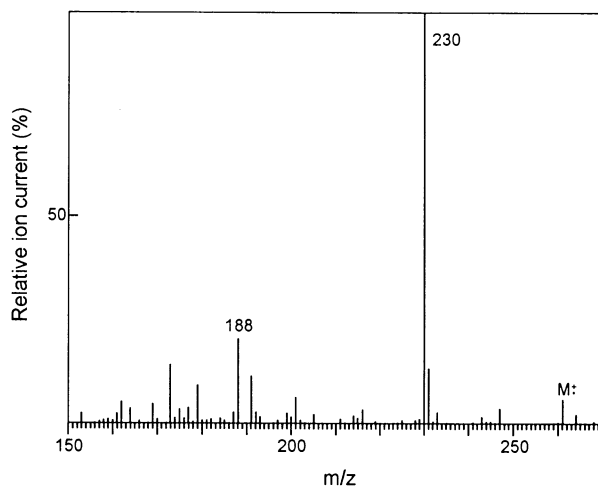


Fig. 7. Mass spectrum of permethylated zeatin. Zeatin was purified from culture supernatant of Eh3-106(pCPPS3.9) (the peak eluting at 13.86 min as shown in Fig. 6C). The spectrum is identical with that of authentic zeatin.

Analysis of the culture medium of Eh3-106(pCPPS3.9) revealed the presence of Z, ZR, and iP (Fig. 6C). No cytokinins were detected for the uncomplemented strain Eh3-106 (Fig. 6B) other than iPA, which was artifactual (see Materials and Methods). Semiquantitative estimates of the amounts of cytokinins produced by Eh3-106(pCPPS3.9) are as follows (in nanograms per milliliter of supernatant): Z, 10.1; ZR, 2.7; and iP, 1.4. The amounts of Z and ZR secreted by this strain appear higher than those secreted by the wild-type Eh824-1 (Fig. 6A). Confirmation of the chemical identity of Z was obtained by gas chromatographic (GC) mass spectrometry (Fig. 7). The spectrum has, as expected, a molecular ion at $m/z = 261$ and a base peak at $m/z = 230$ and was identical to that obtained from a Me-Z standard. Other unidentified immunoreactive compounds were eluted at HPLC retention times of 17.16 min and 26.19 min (Fig. 6C), but insufficient amounts of these putative cytokinins were available for GC mass spectrometry confirmation.

DISCUSSION

The results presented indicate that only pathogenic strains of *E. herbicola* secrete significant amounts of cytokinin into the culture medium. This finding was confirmed by both immunological and biological assays, suggesting that the chemically identified cytokinins may be partly responsible for the pathogenic phenotype. The level of cytokinins accumulated in culture by different pathogenic strains varied considerably (Fig. 1). A significant variation in cytokinin quantitation among different strains of *Agrobacterium* spp. and *Pseudomonas* spp. has also been reported (Akiyoshi *et al.* 1987). The predominant cytokinins identified in *E. h. pv. gypsophylae* were Z, ZR, and iP (Figs. 2 and 6). An additional immunoreactive compound was also observed and may represent another unidentified cytokinin. The production of iPA could not be monitored, owing to the contamination of the iPA3 clone with this compound. Therefore, the possibility that iPA may be present in either pathogenic or nonpathogenic strains remains to be determined. The amounts of the individual cytokinins detected in cultures of *E. herbicola* were substantially lower than those in *P. s. pv. savastanoi* but appear to be in the range reported for some strains of *P. solanacearum* (MacDonald *et al.* 1986; Akiyoshi *et al.* 1987). In comparison to *Agrobacterium* containing the *tzs* gene, *E. herbicola* secretes more cytokinins than uninduced cultures but less than induced cultures of the former (Powell *et al.* 1988). Since the nature of the regulation of the cytokinin biosynthetic gene in *E. herbicola* is unknown, it is difficult to make a meaningful comparison between the two bacteria.

Cytokinin production was exclusive to pathogenic strains of *E. herbicola*, which are distinguished by the presence of the pPATH plasmid (Manulis *et al.* 1991a). Therefore, it was initially suggested that the cytokinin biosynthetic gene may reside on this plasmid, as previously demonstrated for the IAA biosynthetic genes (Clark *et al.* 1993). This premise was corroborated by a deletion mutant of Eh824-1 (Eh3-106) which lacks the DNA fragment specifying the IAA biosynthetic genes as well as cytokinin production (Fig. 3). The production of cytokinins by strains containing pCPPS3.9, which overlaps the 3' end of *iaaH* (Fig. 5), provides evidence for the clustering of the IAA genes and the locus responsible

for cytokinin biosynthesis. The cloned locus conferred the ability to produce all the cytokinins detected for the wild types. The higher concentration of cytokinins in the pCPPS3.9-complemented strain may be attributed to a higher copy number of the plasmid vector.

It is of interest to note that mobilization of neither of the cosmid clones pLA150 and pLA484 into the deletion mutant Eh3-106 could restore pathogenicity to this strain. Since pLA150 overlaps only 11–14 kb of the deleted portion of pPATH in Eh3-106, it can be presumed that additional genes responsible for pathogenicity reside in the deletion.

Attempts to isolate the cytokinin gene by heterologous probes from *A. tumefaciens* and *P. s. pv. savastanoi* (Powell and Morris 1986) or by degenerate polymerase chain reaction primers have given negative results (A. Lichter, unpublished results). This could imply a low homology of the *E. herbicola* cytokinin gene to the previously characterized biosynthetic genes. Alternatively, it is possible that the cloned locus contains a regulatory gene necessary for cytokinin biosynthetic gene expression. In addition, no cytokinin production could be detected in *E. coli* bearing the cloned locus, in contrast to the high expression of cytokinin following mobilization into nonpathogenic *E. herbicola* strains (Fig. 4). The foregoing phenomenon may support the possibility that the isolated locus is a regulatory gene. However the possibility cannot be excluded that this locus constitutes a biosynthetic gene that cannot be expressed in *E. coli* because of various factors.

Among the gram-negative phytopathogenic bacteria, cytokinin secretion has previously been reported in *Pseudomonas* and *Agrobacterium* species, which are classified in the *Pseudomonadaceae* and *Rhizobiaceae*, respectively (Akiyoshi *et al.* 1987; Star 1981). The present study is the first report on the production of cytokinins by an *Erwinia* species, a member of the *Enterobacteriaceae*, which is relatively distant, taxonomically, from the other two families (Star 1981). However, production of cytokinin and isolation of a corresponding biosynthetic gene has also been reported in *Rhodococcus fasciens*, a nocardioform, gram-positive phytopathogenic bacterium (Crespi *et al.* 1992). Thus, the cytokinin-specifying genes seem to be distributed among disparate pathogenic bacteria. Current research is aimed at characterization of the locus specifying cytokinin biosynthesis and elucidating its role in the pathogenicity of *E. h. pv. gypsophylae*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

Sources and relevant characteristics of bacterial strains, cosmids and plasmids are listed in Table 1. Among the isolates of *E. herbicola* listed in Figure 1, pathogenic strains 441, PD713, 6, 13-2, 350-1, 3-1a, 29-2, 824-1, 13, 52, 420, and 53 and nonpathogenic strains 29-3, 24-3, 27-3, 717-2, B111, 40C, 1-10, 24-8, 1-15, and k4 were isolated from gypsophila. All these isolates were described by Manulis *et al.* (1991a). Strain Eh1188, pathogenic on beet as well as gypsophila, was described by Burr *et al.* (1991). Strain Eh3-1 is a nonpathogenic spontaneous derivative of Eh3-1a and lacks the pathogenicity-associated plasmid.

E. h. pv. gypsophylae and *E. coli* strains were grown at 28°C and 37°C, respectively, either in Luria-Bertani medium (LB) or in a modified minimal A medium (Miller 1972). The latter

contained 1% glycerol (w/v), 0.1% casamino acids (w/v), and adenine (10 µg/ml) where specified. For *E. coli* cultures, minimal A medium was amended with 0.05 mM thiamine for strain DH5α and 0.3 mM leucine for strain DH10B. The following antibiotic concentrations were used (in micrograms per milliliter): tetracycline (Tc), 12; ampicillin, 100; kanamycin (Km), 50; rifampicin, 100; and spectinomycin, 100.

For analysis of extracellular cytokinin production, starter cultures (3 ml) were grown overnight in LB broth. Minimal A medium (200 ml) in 1-L silanized flasks was inoculated with 1 ml of the starter, and growth was monitored by measurement of A_{600} . Cultures were grown to stationary phase, and cell densities and viable cell counts were determined at 24–27 hr. Aliquots (1 ml) were withdrawn for approximate determination of cytokinin content by ELISA, and 25-ml aliquots were withdrawn for complete cytokinin identification. Prior to analysis, all aliquots were centrifuged for 20 min at 8,000 rpm; the clarified supernatants were transferred to new tubes, frozen in liquid nitrogen, lyophilized, and stored at 4° C.

Immunoaffinity purification of cytokinins.

Lyophilized samples of culture supernatant (25 ml) were dissolved in dimethyl sulfoxide (400 µl) and diluted gradually to 40 ml with ammonium acetate buffer (40 mM, pH 7.0).

Approximately 1.4 kBq (50,000 cpm) each of [³H]-iP tri-alcohol and [³H]-ZR tri-alcohol were added to allow correction for extraction losses. Samples were passed through DEAE cellulose columns (10-ml bed volume) connected in tandem to immunoaffinity columns, each containing 2.5 ml of microcrystalline cellulose to which clone 16 (anti-ZR) and clone iPA3 (anti-iPA) monoclonal antibodies (Trione *et al.* 1985, 1987) had been conjugated (MacDonald and Morris 1985). The columns were equilibrated with ammonium acetate buffer (40 mM, pH 7.0, 120 ml) and, after sample application, were washed with a further 200 ml. Cytokinins were eluted from the immunoaffinity columns with 5 ml of methanol. Triethylamine (25 µl) was added to each eluate, which was then dried *in vacuo*.

Clone iPA3 was used to purify and identify iP and iPA. However, a test of a control column (to which no cytokinins were added) revealed that significant quantities of iPA were bound to the immunoaffinity material prior to sample addition. Presumably this originated in the mouse ascites fluid. As a result, the antibody contributed a significant background of iPA to the samples, and therefore it could not be used to determine iPA levels in bacterial culture fluids. It did not, however, contain any iP, Z, or ZR and did not interfere with the analysis of these cytokinins.

Table 1. Bacterial strains, cosmids, and plasmids used in this study

	Relevant characteristics ^a	Reference or source
<i>Escherichia coli</i>		
DH5α	<i>leu</i>	Hanahan 1983
DH10B	<i>thi-1</i>	Bethesda Research Laboratories, Gaithersburg, MD
<i>Erwinia herbicola</i> pv. <i>gypsophilae</i>		
Eh824-1	Wild-type pathogenic strain	Manulis <i>et al.</i> 1991a
Eh3-1a	Wild-type pathogenic strain	This study
Eh3-1	Nonpathogenic strain derived from 3-1a	Manulis <i>et al.</i> 1991a
Eh3-1 Rif ^R	Rif ^R	This study
Eh3-106	Eh824-1::Tn5, Km ^R , nonpathogenic, a 40- to 50-kb deletion in pPATH824-1	This study
Eh4-38	Eh824-1::Tn5, Km ^R , nonpathogenic, Tn5 in one of the large plasmids	This study
Transconjugants of <i>E. h.</i> pv. <i>gypsophilae</i>		
Eh3-1(pLAFR3)	Rif ^R Tc ^R , cosmid vector	This study
Eh3-1(pLA484)	Rif ^R Tc ^R , cosmid clone	This study
Eh3-1(pLA150)	Rif ^R Tc ^R , cosmid clone	This study
Eh3-106(pLA484)	Km ^R Tc ^R , cosmid clone	This study
Eh3-106(pLA150)	Km ^R Tc ^R , cosmid clone	This study
Eh3-106(pCPP50)	Km ^R Ap ^R , plasmid vector	This study
Eh3-106(pCPPR5.2-1)	Km ^R Ap ^R , plasmid subclone	This study
Eh3-106(pCPPR5.2-10)	Km ^R Ap ^R , plasmid subclone	This study
Eh3-106(pCPPS3.9-1)	Km ^R Ap ^R , plasmid subclone	This study
Eh3-106(pCPPS3.9-10)	Km ^R Ap ^R , plasmid subclone	This study
Cosmids		
pLAFR3	Tc ^R , broad-host-range vector <i>IncP-1 rlx RK2⁺ lacZα</i> Tra ⁻ Mob ⁺ cos	Staskawicz <i>et al.</i> 1987
pDG-S1	Tc ^R Km ^R , pLAFR3::Tn5 with a levan-sucrase gene from <i>Bacillus</i>	D. Gurian-Sherman, E. Clark, and S. E. Lindow
pLA484	Tc ^R , a 9-kb cosmid clone tandem vector of pLAFR3	This study
pLA150	Tc ^R , a 25-kb cosmid clone in pLAFR3	This study
Plasmids		
pRK2073	Sp ^R Sm ^R IncP Tra RK2 ⁺ rep RK2 repEI ⁺	Ditta <i>et al.</i> 1980
pCPP50	Ap ^R , IncP RK2 rep wide-host-range plasmid vector of 7.3 kb with <i>lpp</i> promoter, <i>lacI</i> , <i>lacO</i> , and multiple cloning site	D. Bower, Cornell University
pCPPS3.9-1	Ap ^R , a 3.9-kb <i>SmaI</i> subclone of pLA150 in pCPP50	This study
pCPPS3.9-10	Ap ^R , orientation opposite that of pCPPS3.9-1	This study
pCPPRI5.2-1	Ap ^R , a 5.2-kb <i>EcoRI</i> subclone of pLA484 in pCPP50	This study
pCPPRI5.2-10	Ap ^R , orientation opposite that of pCPP5.2-1	This study

^a R = resistance to ampicillin (Ap^R), kanamycin (Km^R), rifampicin (Rif^R), streptomycin (Sm^R), spectinomycin (Sp^R), or tetracycline (Tc^R).

HPLC fractionation, RIA, and ELISA of cytokinins.

The immunoaffinity-purified cytokinins were fractionated by HPLC on an octadecylsilica column (Beckman Ultrasphere ODS, 5 μ m, 4.6 \times 250 mm) equilibrated in triethylammonium acetate buffer (40 mM, pH 4.5) and eluted at a flow rate of 1.0 ml/min with a linearly increasing gradient of acetonitrile (from 10 to 15% over 20 min, 20 to 30% over 20 min, and 30 to 100% over 1 min). For complete separation of iP from iPA, this gradient was modified to increase linearly in acetonitrile (from 18 to 21% over 40 min). Fractions (1 ml) were collected and dried to completion *in vacuo* after the addition of triethylamine (25 μ l). Ten percent of the fractions bracketing the retention times of the internal standards [³H]-iP trialcohol and [³H]-ZR trialcohol were counted in 3 ml of scintillation cocktail (DuPont, Formula 963) to determine their recoveries. Cytokinins in individual HPLC fractions were quantified in triplicate by RIA as described by MacDonald *et al.* (1981) and Macdonald and Morris (1985). ELISA was performed as described by Jameson and Morris (1989).

Gas chromatography and mass spectroscopy.

Cytokinins, detected by RIA, were definitively identified by GC mass spectrometry. Active fractions were permethylated (Morris 1977), dissolved in dichloromethane (5 μ l), and injected into a Varian gas chromatograph fitted with an Alltech SE54 capillary column (10 m \times 0.25 mm i.d., film thickness 0.25 μ m), with a helium carrier (40 cm/sec). A deactivated gooseneck Varian injection liner was employed to minimize sample loss. Electron impact mass spectra were acquired on a Finnigan 4023 quadrupole spectrometer operating at 70 eV. Injector and transfer lines were held at 270° C, and the column at 50° C. After injection, the column temperature was raised from 50 to 175° C at 40° C/min to remove the bulk of the solvent; permethylated cytokinins were eluted by increasing the temperature from 175 to 300° C at 20° C/min and holding the column at 300° C for 10 min.

Recombinant DNA techniques.

Restriction enzyme digestions, subcloning procedures, Southern blot analysis, and colony hybridization were carried out as described by Sambrook *et al.* (1989). Nonradioactive DNA probes were labeled with a DIG luminescent kit purchased from Boehringer GmbH (Mannheim, Germany). Cosmid DNA isolation was performed with Qiagen midi columns (Qiagen Inc., Düsseldorf, Germany) according to the manufacturer's instructions. A GeneClean II kit (Bio 101, Inc., La Jolla, CA) was used for DNA isolation from agarose gels. Isolation of plasmids from *E. herbicola* for profile studies and Southern blots was conducted by the method of Kado and Liu (1981) from 10-ml overnight cultures. Triparental mating was performed by the plate mating procedure of Ditta *et al.* (1980), with *E. coli* strain containing pRK2073 used as helper plasmid. Subcloning was performed with the wide-host-range plasmid pCPP50, which was constructed and kindly provided by D. Bower, Department of Plant Pathology, Cornell University.

Transposon mutagenesis.

The procedure for transposon mutagenesis of *E. h. pv. gypsophylae* strain Eh824-1 with the Tn5 vector pDG-S1 was as described by Lubin (1991). pDG-S1 is composed of the

pLAFR3 cosmid vector (Staskawicz *et al.* 1987), containing Tn5 and a levan-sucrase gene, which increases its instability at high sucrose concentration. It was constructed and kindly provided by D. Gurian-Sherman, E. Clark, and S. E. Lindow, of the University of California, Berkeley. pDG-S1 (5 ng of DNA) was transformed by electroporation into Eh824-1 rifampicin-resistant competent cells (4 \times 10⁹ cells per 40 μ l) at 2,500 V. Transformants were selected on LB agar containing Km. pDG-S1 was cured by six successive replica transfers of the transformants on LB agar amended with 5% sucrose and testing for Km resistance and Tc sensitivity.

pLAFR3 library of plasmid DNA.

Plasmid DNA of *E. h. pv. gypsophylae* (Eh 824-1) was isolated by the procedure of Comai and Kosuge (1982). The plasmids were further purified by CsCl/EtBr gradient centrifugation for 16 hr at 45,000 rpm in a Contron ultracentrifuge with a Vti65 Beckman rotor. Purified plasmid DNA was partially digested with *Sau*3A, but no fractionation of DNA fragments was performed prior to ligation to the cosmid vector arms. The strategy used to construct the plasmid library in pLAFR3 was according to Staskawicz *et al.* (1987). *In vitro* packaging of DNA was carried out with a kit purchased from Boehringer. The packaged pLAFR3 mixture was transduced into *E. coli* DH5 α , and colonies were selected on LB agar plates containing Tc and X-Gal. Cosmid clones (525) were stored at -80° C in 96-well culture plates (Nunc, Roskilde, Denmark).

Screening of the library for cytokinin production.

Cosmid clones were recovered from the frozen stocks by replica plating into 96-well tissue culture plates containing 150 μ l of LB amended with Tc per well. The plates were incubated on a shaker (150 rpm) at 37° C for 24 hr. A sample (50 μ l) of each cosmid clone was used to inoculate a 50-ml polypropylene tube containing 10 ml of minimal medium A amended with thiamine and Tc. The cultures were incubated at 37° C for 24 hr on a rotary shaker at 220 rpm. Following centrifugation the supernatants were transferred into 15-ml polypropylene tubes and kept at 4° C for up to 48 hr until they were analyzed for cytokinins by ELISA.

Since the expression of cytokinins in *E. coli* was ambiguous, the cosmid clones were mobilized into a deletion mutant of *E. h. pv. gypsophylae*, Eh 3-106, which lacks cytokinin production. The mobilization was carried out by triparental mating, with pRK2073 used as a helper. The transconjugates were selected on LB amended with Km and Tc. Each transconjugate was grown in 10 ml of minimal A broth amended with Km and Tc at 28° C, and the supernatant was analyzed for cytokinins by ELISA.

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