Diversity of the Fire Blight Pathogen in Production of Dihydrophenylalanine, a Virulence Factor of Some *Erwinia amylovora* Strains

Thomas Schwartz, Frank Bernhard, Richard Theiler, and Klaus Geider

Max-Planck-Institut für medizinische Forschung, Jahnstr. 29, D-6900 Heidelberg, Federal Republic of Germany.

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Correspondence: Dr. Klaus Geider, Max-Planck-Institut für medizinische Forschung, Jahnstr. 29, D-6900 Heidelberg, Federal Republic of Germany.

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ABSTRACT

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Various *Erwinia amylovora* strains from different origins were assayed for phytotoxin production. When cultivated pear cells were embedded in solidified plant medium and inoculated with bacteria, a zone of growth inhibition was observed for some of the strains. A membrane-diffusible toxic compound was identified as L-2,5-dihydrophenylalanine (DHP), an inhibitor of the shikimic acid pathway. In plants DHP caused a reduction of elicitor-induced synthesis of phytoalexins and killed cultivated pear cells within 1 day. Bacterial synthesis of DHP was prevented by aromatic amino acids in the growth medium and was increased when pear juice or malate was present. Pear cells also were killed by strains that did not produce DHP, indicating a second plant cell-damaging pathway.

Additional keywords: hypersensitive reaction, phenylalanine.

Erwinia amylovora is a gram-negative phytopathogenic bacterium that causes fire blight on pear and apple trees and other members of the family of Rosaceae. Characteristic symptoms of the disease are wilting and water soaking and eventual necrosis of affected tissues (36). Toxic substances have been reported to be involved in virulence of many plant pathogenic bacteria. Coronatine, phaseolotoxin, tabtoxin, and other toxins from *Pseudomonas syringae* pathovars have been biochemically and genetically characterized (30). Wild-type, toxin-negative strains of *P. syringae* grow normally on leaves, but do not cause typical disease symptoms (34). The described bacterial phytotoxins are nonhost specific and often inhibitors of plant enzyme activities. Toxin-defective mutants generated by transposon-mutagenesis showed reduced fitness of the pathovar in planta and were still pathogenic (4).

Low molecular weight compounds released from some *E. amylovora* strains have been reported to affect plants or cultivated plant cells (12,13,19). Feistner postulated L-2,5-dihydrophenylalanine (DHP) to protect *E. amylovora* against oxidative defense reactions of its host plants (12). He assumed DHP-mediated retardation of an otherwise early hypersensitive reaction (HR) of the plant until bacterial DHP synthesis would cease, thus causing recognition of the fire blight pathogen and induction of massive defense reactions. This would finally cause necrosis and plant cell death.

Other components from plant pathogenic bacteria may be not toxic per se for the bacterial host, but they are absolutely required to establish the pathogen in planta. Extracellular polysaccharides (EPS) are apparently a prerequisite for development of fire blight (1,3,5,35). Plugging of the vascular system by EPS seems to be an important factor for wilting of the plant (24,26). Protection of the invading pathogen against plant defense mechanisms like the HR may be the major task of EPS. In incompatible interactions, this general defense reaction localizes bacteria at the site of inoculation by a rapid collapse of surrounding plant cells and subsequent necrosis (20,22,27,28). Bacterial cell wall components may not only trigger HR, but also may be responsible for agglutination and immobilization of bacteria (40). Plant defense reactions include production of phytoalexins and other antimicrobial metabolites (20).

Analysis of the complex behavior of whole plants towards bacteria can be simplified by use of cultivated plant cells. Inoculations of plant cells with *Agrobacterium tumefaciens* and *Pseudomonas, Erwinia,* and *Xanthomonas* species have been studied this way (23). For our investigations, we used mainly cultivated pear cells. They were killed by DHP and by *E. amylovora* independent of its ability to produce DHP. This indicated that the fire blight pathogen has developed several strategies to damage its host plants. We show here that DHP also inhibits the shikimic acid pathway in plant cells and is only secreted by some virulent *E. amylovora* strains. Its role in pathogenesis of fire blight thus appears to be that of a virulence factor. Preliminary reports of this work have been included in the proceedings of several meetings (17,18).

MATERIALS AND METHODS

Plant and bacterial cells. Cultured pear (*Pyrus communis* 'Passe Crassane') cells (31) were obtained from B. Hess (Davis, CA). The plant cells were grown in the dark at 28 C on 0.8% B5 agar or in B5 medium (16), both with 1 μg of 2,4-dichlorophenoxyacetic acid per milliliter. Suspension cells were harvested by centrifugation at 50 *g,* and the pellet was suspended in B5 medium at a density of about 3×10^6 cells per milliliter. In an 8.5-cm plastic petri dish, 3 ml of this suspension was plated on the surface of B5 agar and excess liquid was withdrawn. To prepare agar-embedded cells, the plant cells were pelleted, disaggregated in a loosely fitting Teflon/glass homogenizer and added to a concentration of 20% (v/v) to B5 agar at 40 C, mixed and transferred into petri dishes. To facilitate contact of the cells with oxygen, the agar-layer in the dish did not exceed a height of 0.5 cm. Cell lawns and agar-embedded cells were inoculated with 10 μl of a freshly grown bacterial culture containing about 10^9 cells per milliliter. Viability of the plant cells was determined by staining with fluorescein diacetate (39). The virulent strains *E. amylovora* Ea7/74, Ea1/79, Ea273, E9 and the avirulent *E. amylovora* strains E8 and P66 have been described (11). *E. amylovora* strains Ea266, Ea273, CNPB1368 (Ea322), and E9 (Ea213) were provided by

Dr. S. Beer. For cocultivation of bacteria and plant cells, 200 μl of bacterial culture was spread on a B5 agar plate. A sterile cellophane membrane then was placed on the plate, and pear cells were plated on it. The cellophane did not become permeable for bacteria, even after coincubation of plant cells and bacteria for 14 days.

Chromatographic procedures for identification of DHP. *E. amylovora* strains were grown in minimal medium (MM: 3.0 g of K_2HPO_4, 1.0 g of NaH_2PO_4, 1.0 g of NH_4Cl, 0.15 g of KCl, 1 mM $MgSO_4$, 0.1 mM CaCl, 25 mg of nicotinic acid, and 10 g of sucrose per liter) to stationary phase. After centrifugation, activated charcoal (3%, w/v) was added to the supernatant. Adsorbed compounds were eluted with methanol/0.5 N formic acid (4:1). The solvent was evaporated and residual material dissolved in water (10 ml for 500 ml of starting culture). Further purification was by ion-exchange chromatography according to Feistner (12). Samples of 10 μl were analyzed with a high-pressure liquid chromatograph (HPLC) fitted with a Beckman ODS column (length, 25 cm). The column was run in water/methanol (80:20) at 1.5 ml/min. Peak detection was by adsorbance at 210 nm. The retardation times for phenylalanine and DHP were 3.77 and 4.77 min, respectively. The peaks were further identified by addition of DHP as a reference (from Dr. G. Feistner, Stanford University). Thin-layer chromatography (TLC) was performed on an aluminum foil coated with silica gel in 2-butanol/acetic acid/water (4:1:1). The plate was dried, sprayed with ninhydrin reagent, and exposed to 100 C for 3 min. DHP produces a characteristic orange spot in contrast to bluish spots of most other amino acids. The R_f-value for DHP was 0.65. The sensitivity of DHP detection by HPLC and TLC was similar.

Determination of organic acids and phytoalexins. Pear cells (1 g of tissue or washed suspension cells) were extracted for 3 min in 20 ml of boiling methanol. The tissue then was homogenized in a mortar and extracted again with boiling methanol. The combined fractions were cleared from insoluble matter by filtration through Celite. The solvent then was evaporated and the residue dissolved in 20 ml of water and loaded on a cation-exchange column (Dowex 1-X8, Cl^--form) followed by a anion-exchange column (Dowex AG50W X8, H^+-form). This column was eluted with 6 N formic acid. Fractions of the eluate were evaporated, and the residue dissolved in 1.5 ml of water and analyzed by HPLC (Bio-Rad Aminex HPX-87H, 300 × 7.8 mm: Bio-Rad, Richmond, CA) with a UV-detector at 210 nm. To determine phytoalexin induction, we used a parsley cell line established by Dr. K. Hahlbrock and kindly supplied by Dr. H. Kauss. The cells were grown in B5 medium. Commercially available chitosan (Sigma) was purified according to Young et al (41). Induction of phytoalexin synthesis (29) by chitosan (9) was determined in 20-ml parsley cell cultures after 20 h of incubation at 26 C and found to be optimal at 30 μg chitosan per milliliter. When DHP was applied, it was added to the culture at the beginning of the elicitor treatment. The supernatant of suspension cells was extracted twice with dichloromethane, the organic phase evaporated and dissolved in 1 ml of methanol. The absorbance of phytoalexin was measured at 320 nm after dilution in methanol (10).

RESULTS

Growth inhibition of pear cells by various *E. amylovora* strains. When agar-embedded pear cells were inoculated with *E. amylovora,* some strains caused a significant growth inhibition zone of the surrounding pear cells (Fig. 1). A large zone of growth inhibition of pear cells was visible around colonies of the virulent strain Ea273 and the avirulent *E. amylovora* strain E8, but not around strains Ea7/74 and Ea1/79. For *E. amylovora* strains Ea273 and E8, killing of the pear cells presumably occurred early after inoculation, because no cell proliferation was seen in the inhibition zone. The size of this zone was variable and seemed to be dependent on the time after plating the pear cells. The inhibition zone was decreased by about half on pear cells inoculated 2 days after plating, whereas a change of the amount of plant cells added did not give a significant effect in the range of 10–20% homogenized plant cells. When a concentration of 30% plant cells was used, the bacteria spread across the plate. This resulted in a lack of the inhibition zone and the plant cells were slowly killed by the bacteria, as described below for plant cell lawns.

Pear cells can be cultivated on a cellophane membrane placed on top of B5 agar without strong interference with cell growth. When the plate was inoculated with *E. amylovora* strain E9, the pear cell lawn above the bacteria did not grow (Fig. 2). This inhibitory effect was not observed for the virulent strain Ea7/74. In this case the pear cells grew as well as in the control without bacteria.

Identification of the plant cell toxin released by *E. amylovora* strains. We subsequently attempted to purify and characterize the compound(s) secreted by *E. amylovora* strains E8, E9, and Ea273, which seemed to be responsible for growth inhibition of pear cells. In the course of this work we learned from G. Feistner (12) that strain E8 secretes DHP when grown in minimal medium with vigorous aeration. We examined various *E. amylovora* strains for their production of DHP. DHP was identified from bacterial culture supernatants in methanol/charcoal eluates by HPLC (Fig. 3) and by TLC as an orange spot after ninhydrin treatment. Phenylalanine eluted before DHP in reverse-phase chromatography and was visible as a blue spot in TLC below the orange DHP spot. The secretion of DHP was correlated with the inhibitory effects of strains Ea273, E8, and E9 on agar-embedded pear cells (Table 1). Strains that did not produce a significant

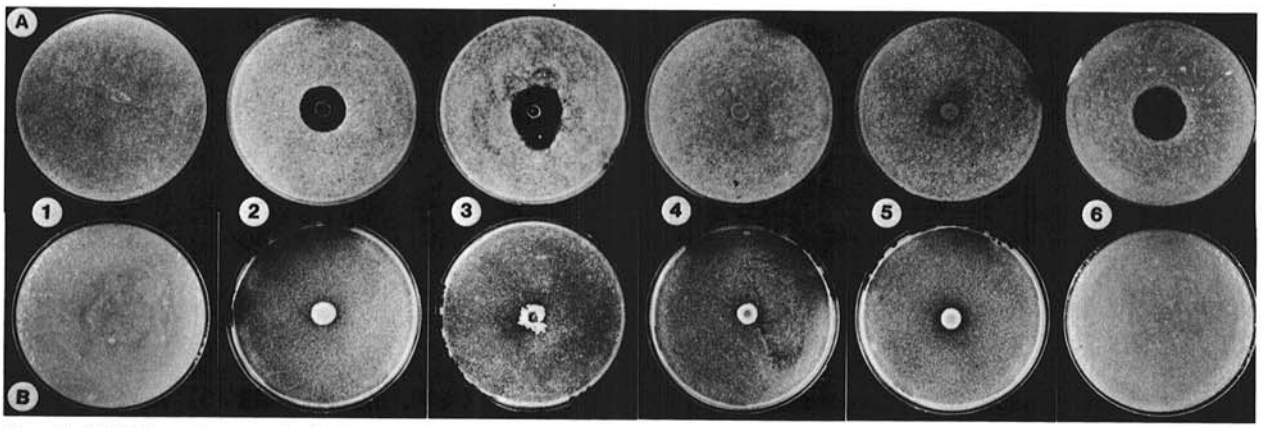

Fig. 1. Growth inhibition of agar-embedded pear cells by various *Erwinia amylovora* strains and by dihydrophenylalanine (DHP) in the presence and absence of phenylalanine. The cells were inoculated with 10 μl of bacteria in the late log-phase or with DHP (3 mg/ml). **A:** Plant cells in B5 agar; **B:** as in row A, but phenylalanine was added to the agar (0.2 mg/ml). Panel 1, control; 2, strain E8; 3, strain Ea273; 4, strain Ea7/74; 5, Ea1/79; 6, DHP. The plates were photographed at day 10 after inoculation.

zone of growth inhibition also did not synthesize DHP under conditions optimal for DHP production. Partially purified culture supernatants were applied after the ion-exchange column to agar-embedded pear cells. Only samples from supernatants of strains Ea273, E8, and E9, but not from Ea7/74, Ea1/79, or Ea11/88, produced zones of growth inhibition.

Factors influencing bacterial DHP synthesis. DHP was first isolated from cultures of *Streptomyces* (14). Its inhibitory effect to bacteria was explained by feedback inhibition, where DHP competes for phenylalanine in the shikimic acid pathway (15). When pear slices were inoculated with virulent *E. amylovora* strains and ooze was assayed for its DHP-content by TLC, we found the toxin production by strain Ea273, but not by strain Ea1/79. These results show that the DHP phenotype of *E. amylovora* strains was not changed in planta. *E. amylovora* strain E8 did not produce DHP in rich medium. By adding phenylalanine, tryptophane, or tyrosine to minimal medium, synthesis of DHP was also abolished (data not shown). Other amino acids, such as asparagine, aspartic acid, cysteine, or glycine, did not change the bacterial capacity to synthesize DHP. Addition of shikimic acid to medium with aromatic amino acids restored DHP production. These results indicate that synthesis of DHP in the shikimic acid pathway occurs after the synthesis of shikimic acid.

The addition of pear cell extract to minimal medium increased DHP synthesis by strain E8 more than fivefold. In searching for an active ingredient, we found malate, but not citric acid, to stimulate DHP production to a similar extent. The content of both organic acids was measured in different pear tissues (Table 2). Pear cells in suspension culture contained high levels of malate. The concentrations of malate and citric acid were low in tissue of mature pears. Immature pears, best suited for virulence assays, had a high content of both organic acids.

Phenylalanine suppresses growth inhibition by DHP on plant cells. Whereas phenylalanine prevented the formation of growth inhibition zones observed on agar-embedded pear cells (Fig. 1, bottom row), the addition of purified DHP to agar-embedded pear cells caused a zone of growth inhibition (Fig. 1, A6). No zone of growth inhibition was formed after application of DHP to plant cells embedded in agar with phenylalanine (Fig. 1, B6). This implies that phenylalanine suppresses growth inhibition by DHP not only in bacteria (15) but also in plant cells. The addition of shikimic acid to the agar could also relieve the inhibitory effect of DHP to plant cells. When 0.2 mg was added per milliliter of agar, no inhibition was seen for strain E8 or for a droplet containing DHP (3 mg/ml). The lack of the growth inhibition zone around colonies of strains Ea273 and E8 in the presence of phenylalanine (Fig. 1, B2, B3) should be predominantly due to abolition of bacterial DHP-synthesis by the aromatic amino acid.

Inhibition of phytoalexin synthesis in plant cells by DHP. Phytoalexin synthesis of cultured parsley cells is a convenient system to measure plant defense reactions (29). We induced plant cells with chitosan, an abiotic elicitor, and measured the influence of DHP on phytoalexin-synthesis (Fig. 4). Increasing amounts of DHP reduced the formation of furano-coumarins in elicitor-induced cells down to the background level of the assay. As coumarins and flavonoids depend on a functional shikimic acid pathway, the inhibition of aromatic amino acid synthesis by DHP seems to affect phytoalexin production.

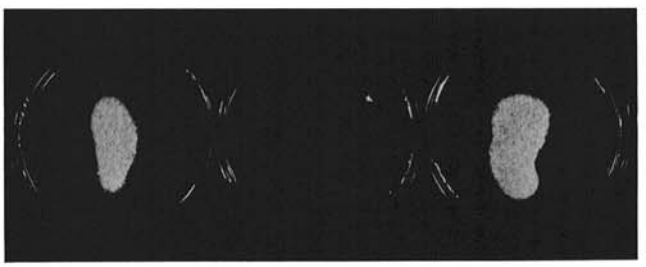

Fig. 2. Growth inhibition of a lawn of pear cells separated from *Erwinia amylovora* by a cellophane membrane. The plant cells were cultured in liquid medium and plated on a large cellophane disk, which was layered on B5 agar with or without bacteria. Left, control; center, *E. amylovora* E9; right, *E. amylovora* Ea7/74. The photograph was taken 8 days after plating of the pear cells.

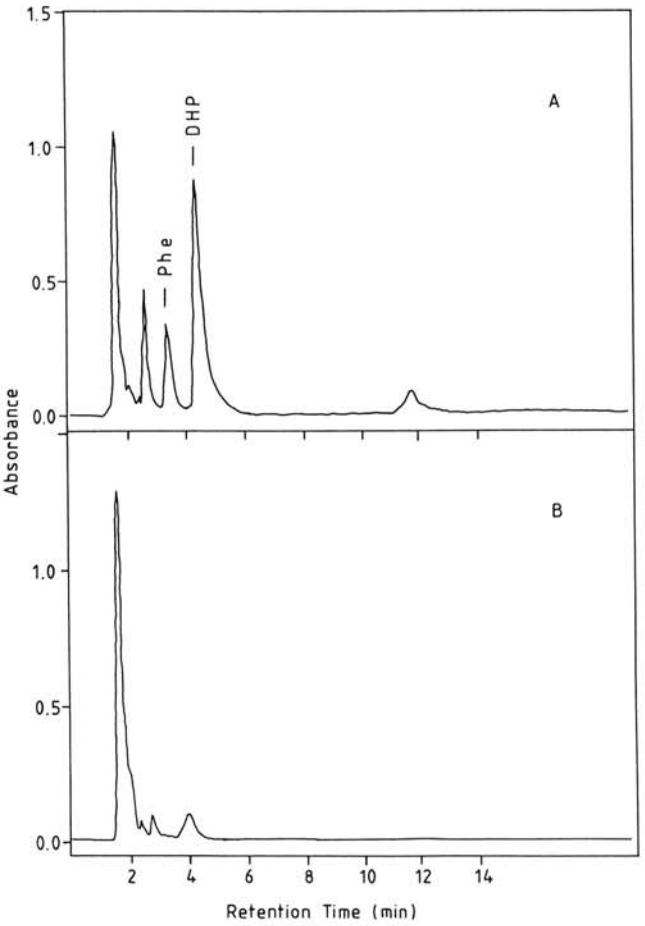

Fig. 3. Analysis of partially purified culture supernatants of strains E8 and Ea7/74 by high-pressure liquid chromatography. The samples were separated on a ODS-column (25 cm) with a flow rate of 1.5 ml/min. Peak detection was at 210 nm. The elution times of DHP and phenylalanine (Phe) are indicated. **A,** Sample from strain E8; **B,** sample from strain Ea7/74.

TABLE 1. Synthesis of dihydrophenylalanine (DHP) by various *Erwinia amylovora* strains and its effect on agar-embedded pear cells

Strain	Origin	Phenotype	DHP production[a]	Growth inhibition zone with pear cells
CNPB1368	France	Virulent	−	−
E8[b]	United States	Avirulent	+	+
E9	United States	Virulent	+	+
Ea1/79	Germany	Virulent	−	−
Ea11/88	Germany	Virulent	−	−
Ea266	Canada	Virulent	−	−
Ea273	New York	Virulent	+	+
Ea5/84	Egypt	Virulent	−	−
Ea7/74	Germany	Virulent	−	−
Ea8439	New Zealand	Virulent	−	−
Eam7	California	Virulent	−	−
P66	England	Avirulent	−	−

[a] About 20 mg of DHP was determined per liter culture supernatant for positive strains. Production was confirmed by high-pressure liquid chromatography and thin-layer chromatography.

[b] Spontaneous extracellular polysaccharides-deficient mutant of strain E9 (25).

Involvement of DHP in killing of pear cells by *E. amylovora* and crater formation on pear tissue. A dense lawn of pear cells was inoculated with bacteria from several *E. amylovora* strains, and the cell viability was measured at various times after inoculation by staining with fluorescein diacetate (Table 3). The strains used spread across the pear cell lawn and were monitored by bioluminescence (R. Theiler, N. Jahn, and K. Geider, *unpublished*) or by plating samples from the rim of the plant agar plates. Two weeks after inoculation the pear control cells were essentially unchanged in viability, but most plates inoculated with *E. amylovora* did not contain any viable plant cells. This killing of pear cells was not correlated to the ability of the *E. amylovora* strains to synthesize DHP. The avirulent strains P66 and E8 damaged the plant cells to a different extent. The strong impact of strain E8, which may rely on its defect in EPS-synthesis (25) in addition to its DHP production, resulted in the fastest pear cell damage of the strains investigated (Table 3). The appearance of the pear cell lawn surface was brown for E8 compared with the lighter color in case of inoculation with other strains. Strain P66 carries a deficiency for HR-induction on nonhost plants (37) and was found to damage the pear cells more slowly than the other strains applied. To exclude the possibility that non-DHP producing strains still synthesize low quantities of the compound that cannot be detected easily by our assay procedures, we added phenylalanine to the plant agar. As mentioned above, phenylalanine prevents *E. amylovora* from synthesizing DHP because it inhibits the bacterial shikimic acid pathway. The presence of phenylalanine had no effect on killing of pear cells by any of the *E. amylovora* strains applied (Table 3). This demonstrates the ability of *E. amylovora* to kill plant cells also in the absence of DHP synthesis. On the other hand, when pear cells in suspension were grown in the presence of DHP, concentrations above 25 μg/ml killed the plant cells (data not shown). DHP secretion could therefore be a factor in increasing plant cell damage by the fire blight pathogen.

Pear slices were inoculated with strains Ea7/74, Ea1/79, Ea273, and E9 and were compared for their reaction with the pathogen. All strains produced ooze, whereas only the latter two strains, which synthesize DHP, caused crater formation and necrotic symptoms at the inoculation site. Inoculation of DHP-pretreated slices with the DHP-negative strain Ea1/79 gave rise to ooze production in a much shorter time than inoculation of untreated pear slices (data not shown). Impairment of plant defense reactions and damage of cells at the surface of the tissue by DHP could be responsible for creating an environment that enables the fire blight pathogen to colonize this pear tissue faster than normal pear slices.

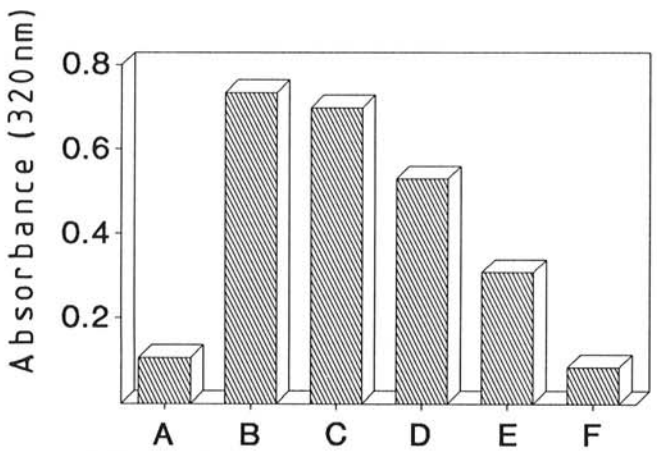

Fig. 4. Inhibition by dihydrophenylalanine of phytoalexin-induction in parsley cells. Phytoalexin synthesis was induced with chitosan. **A,** Control without chitosan and without DHP. **B–F,** Induction with 30 μg of chitosan per milliliter of parsley cell suspension. **B,** Without DHP. **C,** With 5 μg of DHP per milliliter. **D,** With 10 μg of DHP per milliliter. **E,** With 25 μg of DHP per milliliter. **F,** With 50 μg of DHP per milliliter. The units refer to the extinction of a 1:10 dilution of the methanol extract.

DISCUSSION

Virulence of pathogens and development of disease symptoms on infected plants often rely on secretion of bacterial toxins. *P. syringae* pathovars produce low molecular weight compounds causing chlorosis in the host plant, although they are not required for establishment of the pathogen (30). This points to a helper function of the phytotoxin for bacterial propagation in planta. Toxins are therefore not causative agents for establishment of a bacteriosis, but rather virulence factors. This interpretation was supported by data obtained with a Tn*5* mutant of *P. syringae* (4). The mutant had lost its ability to synthesize toxin, but still was virulent on its host plant. Release of toxic substances was postulated for *E. amylovora* (7). The toxic effect of those culture supernatants was subsequently correlated to an enrichment of salts during purification (2).

Feistner (12,13) identified two reducing low molecular weight substances from *E. amylovora* that interfered with vitality of plant cells as 6-thioguanine and DHP. He could show synthesis of DHP by *E. amylovora* strain E9 and its avirulent mutant E8. The substance was found to be toxic on suspension culture cells from pear and not produced by the avirulent strain P66. We observed a growth inhibition zone on agar-embedded pear cells when the plates were inoculated with some strains of *E. amylovora.* Furthermore, the inhibiting substance was diffusible through a cellophane disk, which was impermeable to substances above a molecular weight of 15,000 Da (6). Growth of plant cells layered on top of the membrane was drastically inhibited by toxin-producing bacteria on the agar plate. The toxin was identified as DHP by HPLC and TLC analysis. The reducing capacity of its carbon ring could neutralize oxidative defense reactions of the host plants (12). DHP also may be incorporated into proteins as an analog of phenylalanine. In mammalian cells DHP inhibits hydroxylation of aromatic rings (38). We could suppress growth inhibition with DHP by adding phenylalanine or shikimic acid to the plant agar. This indicates an inhibitory function of DHP in the shikimic acid pathway. These results are therefore in contrast to the assumption of Feistner (12) that DHP could interfere with plant defense as an antioxidant. In bacteria DHP acts as a false feedback

TABLE 2. Content of citrate and malate in pear tissues

Tissue	Content (mg/g dry wt)		Symptom production (strain Ea1/79)
	Malate	Citrate	
Cultivated pear cells	89	0.3	Ooze
Immature pear	28	15	+
Mature pear	7	0.4	−

TABLE 3. Viability of plated pear cells inoculated with *Erwinia amylovora*[a]

Strain	Viable cells (%) at		
	Phenylalanine	Day 7	Day 14
Ea7/74	−	30	0
	+	30	0
Ea1/79	−	20	0
	+	10	0
E9	−	20	0
	+	20	0
E8	−	0	0
	+	0	0
P66	−	30	10
	+	30	5
Control	−	99	80
	+	100	70

[a] The plant cells were plated on B5 agar without and with phenylalanine (0.2 mg/ml) and inoculated with 10 μl of bacteria. Pear cells were removed from the plates at a distance of 2.5 cm from the center and stained with fluorescein diacetate. At least 100 cells were counted, and the fraction of the positively stained cells was determined.

inhibitor of the condensation reaction of erythrose-4-phosphate and phosphoenolpyruvate (15). It also has an inhibitory effect on prephenic acid dehydratase. This results in a deficiency of synthesis of phenylalanine and presumably also of tyrosine and tryptophane.

Another disturbance of plant cells could arise from the involvement of chorismic acid in the biosynthesis of vitamins and in modification of tRNA, which was originally observed in bacteria (21). Aromatic amino acids also are precursors of growth hormones and in steps involved in plant defense reactions. Phenylalanine ammonium lyase (PAL), which catalyzes the conversion of phenylalanine to cinnamic acid (42), is a key enzyme in the phenylpropanoid pathway. Many elicitors induce its synthesis in plant cells. We used chitosan from crab shells for PAL induction in parsley and pear cells. This elicitor also induces steps of defense reactions as the formation of phytoalexins, which can be conveniently measured in parsley cells (29), whereas their induction in pear cells has not been described. The addition of DHP together with chitosan reduced the amount of phytoalexin elicited in parsley cells. Most likely, in the presence of DHP the shortage in the pool of aromatic amino acids results in weak defense responses and may also be responsible for a decrease of auxin synthesis.

Fast-growing bacteria were not affected by DHP (15). Their high phenylalanine pool was suggested for this behavior, although other steps in the complex shikimic acid pathway may also contribute. Bacterial synthesis of DHP is inhibited by aromatic amino acids. Production of DHP was resumed after the addition of shikimic acid, suggesting its synthesis in a step after shikimic acid as assumed for *Streptomyces arenae* (33). When pear cell extracts were added to minimal medium, DHP synthesis could be increased. From many candidates for a stimulatory effect, malate but not citric acid was found to be stimulatory in bacterial DHP production. Malate is a constituent of immature pears, which are highly sensitive for induction of fire blight symptoms. It also was found to be an attractant in chemotaxis of *E. amylovora* (32). A role in general stimulation of the cellular metabolism of *E. amylovora* seems to be possible.

Out of 12 *E. amylovora* strains from various countries, only three were found to produce DHP, whereas nine others turned out to be nonproducers. Strain E8 is actually a spontaneous mutant derived from strain E9 (25). Thus, fewer than one quarter of the assayed isolates produce the toxin. A DHP-deficient mutant of strain Ea273 was created by Tn*5*-mutagenesis and showed no significant changes in the development of ooze on pear slices or of disease symptoms on pear seedlings (T. Schwartz and K. Geider, *unpublished*).

Together, these data suggest that DHP is a virulence factor of *E. amylovora,* which supports producing strains to attack the plant cells in the bacterial environment by suppressing plant defense reactions. As for *P. syringae* pathovars, the production of a plant toxin in *E. amylovora* strains is not a prerequisite for pathogenicity. Many virulent *E. amylovora* strains did not secrete any DHP into synthetic medium. They did not produce a significant growth inhibition zone of pear cells soon after inoculation and did not inhibit growth of pear cells separated by cellophane disk. Nevertheless, these strains had a high capacity to kill cultivated pear cells in direct contact with the plant cells. This suggests the release of additional components from the bacteria causing phytotoxic effects. Plant cells may be affected by oligosaccharides with signal functions (8). Some of those compounds could be produced in plants by *E. amylovora* or could occur in the bacterial cell walls and be released by the pathogen into its environment.

LITERATURE CITED

1. Ayers, A. R., Ayers, S. B., and Goodman, R. N. 1979. Extracellular polysaccharide of *Erwinia amylovora:* A correlation with virulence. Appl. Environ. Microbiol. 38:659-666.
2. Bauer, D. W., and Beer, S. V. 1985. Evidence that a putative necrotoxin of *Erwinia amylovora* is an artifact caused by the activity of inorganic salts. Physiol. Plant Pathol. 27:289-298.
3. Bellemann, P., Jahn, N., Theiler, R., and Geider, K. 1990. Transposon mutagenesis of *Erwinia amylovora.* Acta Hortic. 273:233-237.
4. Bender, C. L., Stone, H. E., and Cooksey, D. A. 1987. Reduced pathogen fitness of *Pseudomonas syringae* pv. *tomato* Tn*5* mutants defective in coronatine production. Physiol. Mol. Plant Pathol. 30:273-283.
5. Bennett, R. A., and Billing, E. 1980. Origin of the polysaccharide component of ooze from plants infected with *Erwinia amylovora.* J. Gen. Microbiol. 116:341-349.
6. Bopp, M., and Knoop, B. 1964. Culture methods for bryophytes. Pages 96-105 in: Cell culture and somatic cell genetics of plants, Vol. 1. Academic Press, New York.
7. Buchanan, G. E., and Starr, M. P. 1980. Phytotoxic material from associations between *Erwinia amylovora* and pear tissue culture: Possible role in necrotic symptomatology of fire blight disease. Curr. Microbiol. 4:63-68.
8. Bucheli, P., Doares, S. H., Albersheim, P., and Darvill, A. 1990. Host pathogen interactions XXXVI. Partial purification and characterization of heat-labile molecules secreted by the rice blast pathogen that solubilize plant-cell wall fragments that kill plant cells. Physiol. Mol. Plant Pathol. 36:159-173.
9. Conrath, U., Domard, A., and Kauss, H. 1989. Chitosan-elicited synthesis of callose and of coumarine derivatives in parsley cell suspension cultures. Plant Cell Rep. 8:152-155.
10. Davis, K. R., and Hahlbrock, K. 1987. Induction of defense responses in cultured parsley cells by plant cell wall fragments. Plant Physiol. 84:1286-1290.
11. Falkenstein, H., Bellemann, P., Walter, S., Zeller, W., and Geider, K. 1988. Identification of *Erwinia amylovora,* the fireblight pathogen, by colony hybridization with DNA from plasmid pEA29. Appl. Environ. Microbiol. 54:2798-2802.
12. Feistner, G. J. 1988. (L)-2,5-Dihydrophenylalanine from the fire blight pathogen *Erwinia amylovora.* Phytochemistry 27:3417-3422.
13. Feistner, G., and Staub, C. M. 1986. 6-Thioguanine from *Erwinia amylovora.* Curr. Microbiol. 13:95-101.
14. Fickenscher, U., Keller-Schierlein, W., and Zähner, H. 1971. Stoffwechselprodukte von Mikroorganismen. 87. Mitteilung. L-2,5-Dihydrophenylalanin. Arch. Mikrobiol. 75:346-352.
15. Fickenscher, U., and Zähner, H. 1971. Stoffwechselprodukte von Mikroorganismen. 88. Mitteilung. Zur Wirkungsweise des L-2,5-Dihydrophenylalanins-eines Phenylalaninantagonisten. Arch. Mikrobiol. 76:28-46.
16. Gamborg, O. L., Miller, R. A., and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50:151-158.
17. Geider, K., Falkenstein, H., Bellemann, P., Jahn, N., Schwartz, T., Theiler, R., and Bernhard, F. 1990. Virulence factors of *Erwinia amylovora.* Acta Hortic. 273:227-231.
18. Geider, K., Falkenstein, H., Bellemann, P., Jahn, N., Schwartz, T., Theiler, R., and Bernhard, F. 1990. Virulence factors of *Erwinia amylovora* influencing development of fireblight symptoms. Pages 669-673 in: Proc. Int. Conf. Plant Pathog. Bact., 7th. Z. Klement, ed. Akadémiai Kiadó, Budapest.
19. Goodman, R. N., Chun, W., and Feistner, G. 1990. Bioassay for necrogenic response of DHP and virulence of *Erwinia amylovora.* Acta Hortic. 273:221-226.
20. Goodman, R. N., Kiraly, Z., and Wood, K. R. 1986. Pages 347-415 in: The Biochemistry and Physiology of Plant Disease. University of Missouri Press, Columbia.
21. Hagervall, T. G., Jönsson, Y. H., Edmonds, C. G., McCloskey, J. A., and Björk, G. R. 1990. Chorismic acid, a key metabolite in modification of tRNA. J. Bacteriol. 172:252-259.
22. Hahlbrock, K., and Scheel, D. 1987. Biochemical responses of plants to pathogens. Pages 229-254 in: Innovative Approaches to Plant Disease Control. I. Chet, ed. John Wiley & Sons, New York.
23. Helgeson, J. P., and Deverall, B. J. 1983. Use of Tissue Culture and Protoplasts in Plant Pathology. Academic Press, New York.
24. Hsu, S. T., and Goodman, R. N. 1978. Production of a host-specific, wilt-inducing toxin in apple cell suspension cultures inoculated with *Erwinia amylovora.* Phytopathology 68:351-354.
25. Huang, P.-Y., Huang, J.-S., and Goodman, R. N. 1975. Resistance mechanisms of apple shoots to an avirulent strain of *Erwinia amylovora.* Physiol. Plant Pathol. 6:283-287.
26. Husain, A., and Kelman, A. 1958. Relation of slime production to mechanism of wilting and pathogenicity of *Pseudomonas solanacearum.* Phytopathology 48:155-165.
27. Klement, Z. 1982. Hypersensitivity. Pages 150-177 in: Phytopathogenic Prokaryotes, Vol. 2. M. S. Mount and G. H. Lacy, eds. Academic Press, New York.

28. Klement, Z., and Goodman, R. N. 1967. The hypersensitive reaction to inoculation by bacterial plant pathogens. Annu. Rev. Phytopathol. 5:17-44.
29. Kombrink, E., and Hahlbrock, K. 1986. Responses of cultured parsley cells to elicitors from phytopathogenic fungi. Plant Physiol. 81:216-221.
30. Mitchell, R. E. 1984. The relevance of non host-specific toxins in the expression of virulence by pathogens. Annu. Rev. Phytopathol. 22:215-245.
31. Pech, J. C., and Romani, R. J. 1979. Senescence of pear fruit cells cultured in a continuously renewed, auxin-deprived medium. Plant Physiol. 63:814-817.
32. Raymundo, A. K., and Ries, S. M. 1980. Chemotaxis of *Erwinia amylovora.* Phytopathology 70:1066-1069.
33. Shimada, K., Hook, D. J., and Floss, H. G. 1978. Biosynthesis of the antibiotic 2,5-dihydrophenylalanine by *Streptomyces arenae.* Biochemistry 17:3054-3058.
34. Staskawicz, B. J., and Panopoulos, N. J. 1979. A rapid and sensitive microbiological assay for phaseolotoxin. Phytopathology 69:663-666.
35. Steinberger, E. M., and Beer, S. V. 1988. Creation and complementation of pathogenicity mutants of *Erwinia amylovora.* Mol. Plant-Microbe Interact. 1:135-144.
36. Van der Zwet, T., and Keil, H. L. 1979. Fire Blight: A bacterial disease of rosaceous plants. U.S. Dep. Agric. Agric. Handb. 510.
37. Walters, K., Maroofi, A., Hitchin, E., and Mansfield, J. 1990. Gene for pathogenicity and ability to cause the hypersensitive reaction cloned from *Erwinia amylovora.* Physiol. Mol. Plant Pathol. 36:509-521.
38. Watanabe, Y., Fujiwara, M., and Hayaish, O. 1978. 2,5-Dihydrophenylalanine: A competitive inhibitor of indoleamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase. Biochem. Biophys. Res. Commun. 85:273-279.
39. Widholm, J. M. 1972. The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol. 47:189-194.
40. Yoshikawa, M. 1983. Macromolecules, recognition, and the triggering of resistance. Pages 267-299 in: Biochemical Plant Pathology. John Wiley & Sons, Chichester.
41. Young, D. H., Köhle, H., and Kauss, H. 1982. Effect of chitosan on membrane permeability of suspension-cultured *Glycine max* and *Phaseolus vulgaris* cells. Plant Physiol. 70:1449-1454.
42. Zimmermann, A., and Hahlbrock, K. 1975. Light-induced changes of enzyme activities in parsley suspension cultures. Arch. Biochem. Biophys. 166:54-62.