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Role of Antibiotic Production by *Erwinia herbicola* Eh252 in Biological Control of *Erwinia amylovora*

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***Erwinia herbicola* Eh252 is a nonpathogenic epiphytic bacterium that reduces fire blight incidence when sprayed onto apple blossoms before inoculation with *Erwinia amylovora*, the causal agent of fire blight. Eh252 was found to produce on minimal medium an antibiotic that inhibited the growth of *E. amylovora*. This antibiotic was inactivated by histidine but not by Fe(II), was sensitive to proteolytic enzymes, and showed a narrow host range of activity. To determine the role of this antibiotic in the control of fire blight, two prototrophic Tn*5*-induced mutants, 10:12 and 17:12, that had lost their ability to inhibit *E. amylovora* on plates (Ant$^-$ mutants) were compared with the wild-type strain for their ability to suppress fire blight in immature pear fruits. The two mutants had single Tn*5* insertions in the chromosome; although they grew in immature pear fruits at a rate similar to that of the wild-type strain, neither of these mutants suppressed fire blight as well as Eh252 did. The Tn*5*-containing fragment isolated from 10:12 was used to mutagenize Eh252 by marker exchange. Derivatives that acquired the Tn*5*-containing fragment by homologous recombination lost the ability to inhibit *E. amylovora* on minimal medium. Furthermore, the three Ant$^-$ derivatives tested were also affected in their ability to inhibit *E. amylovora* in immature pear fruits. The results obtained suggest that antibiotic production is a determinant of the biological control of *E. amylovora* by Eh252, but that another mechanism(s) is involved.**

Erwinia herbicola (Lohnis) Dye is a species of the family *Enterobacteriaceae*. Strains belonging to this diverse group of bacteria are found in a variety of places: on plant surfaces as nonphytopathogenic epiphytes, in plant lesions as secondary invaders, and in humans and other animals as opportunistic pathogens. The strains that are pathogenic for humans and other animals are called *Enterobacter agglomerans* (47). Recently, based on total DNA homology and electrophoretic protein pattern similarities, some strains of *E. herbicola* and *E. agglomerans*, including the two type strains, were proposed to form a new genus called *Pantoea* (21).

Nonpathogenic yellow-pigmented bacteria, not always formally identified as *E. herbicola*, are often isolated from diseased plant tissues in association with the closely related bacterium *Erwinia amylovora* (17, 26, 38, 40, 64). *E. amylovora* causes fire blight, a typical necrotic disease that affects all plant species of the Pomoideae but is especially destructive to apple and pear trees. Some of the nonpathogenic strains isolated from fire blight lesions were reported to reduce the incidence of fire blight in the greenhouse and in the orchard. Control of fire blight has been achieved by spraying suspensions of the antagonistic strain onto apple (2, 22, 54, 64), pear (36, 40, 64), or hawthorn (58) blossoms before inoculating with *E. amylovora*. Isenbeck and Schulz also reported that injection of a suspension of *E. herbicola* in the stem of *Cotoneaster* sp. before inoculation with *E. amylovora* was as effective as injection with streptomycin in reducing fire blight infection (26).

Potential biological control agents for fire blight were identified among strains of *E. herbicola* isolated from shoots, leaves, or blossoms of apple trees by their ability to inhibit *E. amylovora* in immature pear fruits (1). The immature pear fruit assay was first developed to test in the laboratory the pathogenicity of suspected strains of *E. amylovora* (7). It was then modified to screen for potential biocontrol agents of fire blight (1). Inoculation of immature pear fruits with pathogenic strains of *E. amylovora* results typically in the production of exudate and necrosis (7), but if the fruits are first treated with bacteria that are antagonistic to *E. amylovora* no symptoms are observed (1). Furthermore, a correlation has been found between the ability of strains of *E. herbicola* to inhibit the development of *E. amylovora* in immature pear fruits and their ability to reduce the incidence of fire blight in apple orchards (1). *E. herbicola* Eh252, among other strains, was identified by this laboratory assay (3) and has since been shown to be effective in reducing fire blight in experimental apple orchards in New York (2, 4) and France (54).

Production of an inhibitory compound by Eh252 was first suggested by R. S. Wodzinski et al. after they noticed that a minimal medium broth in which Eh252 had grown could not support the growth of *E. amylovora* unless it was boiled under acidic conditions (60) or supplemented with Casamino Acids (63). Production of a substance inhibitory to *E. amylovora* in vitro was reported for several strains of *E. herbicola* and yellow epiphytic bacteria isolated from the fire blight lesions (2, 17, 27, 28). It has been suggested that these inhibitory substances are involved in the inhibition of *E. amylovora* in planta, but their role in the control of fire blight has never been demonstrated.

The goal of this study was to determine the role of the antibiotic produced by Eh252 in the biological control of fire blight. We isolated Tn*5*-induced mutants of Eh252 that did not inhibit *E. amylovora* on a petri dish, and we compared their abilities to inhibit *E. amylovora* in immature pear fruits with that of the wild-type strain. The two mutants tested

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TABLE 1. Bacterial strains, plasmids, and bacteriophage

Strain, plasmid, or phage	Characteristics[a]	Source or reference
E. herbicola		
Eh252	Wild-type strain isolated from *Malus pumila* (New York)	CUCPB[b]
10:12, 17:22, 42:6	Tn*5*-induced mutants of Eh252, do not produce antibiotic in vitro	This work
Eh112Y-C	Derivative of Eh112Y that carries a Tn*5* insertion in a ca. 144-kb plasmid	20
E. amylovora Ea273	Wild-type strain isolated from *M. pumila* (New York)	CUCPB
E. coli		
HB101	*hsdR20 recA13 ara-14 proA2 lacY1 galK2 prs-120 xyl-5 leu-1 supE44*	9
ECG18	HB101 Δ*lamB*	34
SCS1	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1*	Stratagene Inc.
LE392	*hsdR514 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55*	39
DH5α	*endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*argF-lacZYA*)U169ϕ80d *lac* ZD M15)	Bethesda Research Laboratories
Plasmids		
pTROY9	pLAFRB *malK*::IS*3*, *lamB*, Tet^r	15
pRK2013	IncP Km^r TraRk2$^+$ Δ*repRK2 repE1*$^+$	19
pRZ102	ColE1 Tn*5*	29
pBR325	Ap^r Tc^r Cm^r	8
pCPP710	*Eco*RI Tn*5*-containing fragment isolated from 10:12 cloned into pBR325; Ap^r Tc^r Km^r	This work
Bacteriophage λ Tn5	*b*221 *Oam Pam rex*::Tn*5 c*I857	6

[a] Ap^r, Tc^r, Cm^r, and Km^r indicate resistance to ampicillin, tetracycline, chloramphenicol, and kanamycin, respectively. Ant$^-$, non-antibiotic-producing mutant.
[b] CUCPB, Cornell University Collection of Phytopathogenic Bacteria.

were not as effective as Eh252 in suppressing fire blight. Furthermore, non-antibiotic-producing derivatives of Eh252 obtained by marker exchange mutagenesis with the Tn*5*-containing fragment isolated from one of the mutants previously tested were also impaired in their ability to inhibit *E. amylovora* in immature pear fruits. These results suggest that antibiotic production by Eh252 is a determinant of the biological control of fire blight.

(Preliminary accounts of some of this work have appeared previously [51–53].)

MATERIALS AND METHODS

Strains, plasmids, and media. The bacterial strains, plasmids, and bacteriophage used in this study are described in Table 1. Except when otherwise stated, strains of *E. herbicola* and *Escherichia coli* were incubated at 37°C and strains of *E. amylovora* were incubated at 28°C. The complete medium was Luria broth (L broth) (37). The minimal medium was M9 (37); it contained 2 g of glucose per liter as the carbon source and was supplemented with nicotinic acid (50 mg/liter) to support the growth of *E. amylovora*. Production of an inhibitory compound by *E. herbicola* was tested on a specific minimal medium called GA (61), which contained 20 g of glucose, 0.3 g of L-asparagine, 11.5 g of K_2HPO_4, 4.5 g of KH_2PO_4, 0.12 g of $MgSO_4$, and 50 mg of nicotinic acid per liter. Two selective media, MS and Sasser medium, were used for growth of *E. herbicola* recovered from immature pear fruits. MS medium was as described by Miller and Schroth (38), except that mannitol was used as the carbon source. Sasser medium contained 0.1 g of $MgSO_4$, 2.0 g of D-(+)-trehalose, 0.2 g of 2,6-diaminopurine, 15.0 g of NaCl, 0.8 g of K_2HPO_4, 0.8 g of KH_2PO_4, and 0.01 g of yeast extract (44); after autoclaving for 5 min at 120°C, the pH was adjusted to 4.9 with 1 N HCl. Colonies on Sasser medium were counted after 2 days of incubation. For marker exchange experiments, the low-phosphate medium of Roeder and Collmer was used (41). Iron-deficient medium was obtained by adding 50 to 200 μg of ethylenediamine-*N*,*N*′-bis(2-hydroxyphenylacetic acid) (Fluka A. G. Chemical Co., Neu Ulm, Germany) per ml. Ethylenediamine-*N*,*N*′-bis(2-hydroxyphenylacetic acid) stock solutions were deferrated by the procedure of Rogers (42). When required, the media were solidified with Bacto-Agar (12 g/liter for plates and 7 g/liter for overlays; Difco Laboratories, Detroit, Mich.). When needed, supplements were added at the following concentrations: kanamycin, 25 mg/liter; chloramphenicol, 20 mg/liter; tetracycline, 10 mg/liter; amino acids, purines, or pyrimidines, 50 to 100 μg/liter. Auxotrophic requirements were characterized by the nutritional pool system of Davis et al. (14).

Antibiotic production assay. Colonies of *E. herbicola* to be tested for antibiotic production were spread off-center of a GA-medium plate in a spot 0.5 to 1 cm in diameter. The plates were then incubated at 37°C for 48 h. *E. amylovora* Ea273, used as a negative control, was incubated at 28°C. After 48 h of incubation, the bulk of the bacterial growth was scraped off the plates before exposure to chloroform vapors. Plates were then overlaid with GA medium seeded with *E. amylovora* Ea273 (about 6×10^5 CFU/ml). After 24 to 36 h of additional incubation at 28°C, a zone of inhibited growth of the indicator strain was visible surrounding the spot where Eh252 had grown. No inhibition was detected when Ea273 was used instead of Eh252.

Inactivation of the antibiotic activity by proteolytic enzymes, histidine, or Fe(II). A single spot of Eh252 was grown for 48 h in the center of a GA plate, removed, and killed with chloroform vapors as described above. Holes were punched into the agar around the spot where Eh252 had grown. One of the following solutions was then added to each hole: 10 μl of a 20-mg/ml solution of pronase or proteinase K, 5 μl of an 8-mg/ml solution of histidine, 5 μl of a 10 mM $FeCl_3$ solution

made in 10 mM HCl, or 10 μl of sterile distilled water as a negative control. The plates were then incubated for 2 h at 37°C before an overlay of GA medium seeded with Ea273 was made. Inactivation of the antibiotic by these different substances was determined after an additional 24 h of incubation at 28°C. Inactivation of the antibiotic activity by Fe(II) was also tested by incorporating Fe(II) (50 μM) in the bottom layer and the overlay.

Determining the host range of antibiotic activity. A single spot of Eh252 was grown for 48 h in the center of a GA plate, removed, and killed with chloroform vapors as described above for the antibiotic production assay. The strains to be tested for their sensitivity to Eh252 were grown overnight in L broth. A sterile cotton swab dipped into a 0.1 dilution of each bacterial suspension (made in 10 mM $MgSO_4$ solution) was streaked from the edge of the spot where Eh252 had grown to the edge of the plate. The sensitivity of the strains was recorded after incubation at 28°C for an additional 24 h. On each plate, 8 to 12 strains were assayed, including Ea273 and Eh252 as positive and negative controls, respectively.

Detection of siderophore activity. Siderophore activity was detected by the chemical assay devised by Schwyn and Neilands (45). The blue medium they developed contains a complex of chrome Azurol S-iron III-hexadecyltrimethylammonium bromide that gives a blue color to the medium but turns orange when the iron is removed from the complex. Thus, when grown on this medium, colonies of a strain that produces and excretes a siderophore are surrounded by an orange halo.

Preparation of bacteriophage lysates. Lambda bacteriophage stocks were prepared in SM buffer (57) from plate lysates as described by Maniatis et al. (35). Bacteriophage λ Tn*5* was propagated on *E. coli* LE 392; stocks of this bacteriophage were also titered on LE 392.

Construction of a λ-sensitive derivative of Eh252. Plasmid pTROY9, which carries and expresses constitutively the *lamB* gene from *E. coli*, was mobilized into Eh252 by triparental mating with pRK2013 as the helper plasmid. The mating was carried out as described by Chatterjee (10). Mid-log-phase cultures of HB101(pRK2013), ECG18(pTROY9), and Eh252 were mixed and collected on a nitrocellulose membrane filter (0.22-μm pore size, 25-mm diameter; Millipore Corp., Bedford, Mass.) that was incubated on a fresh L-agar plate at 37°C for 8 h. The culture was then resuspended and diluted in 10 mM $MgSO_4$ and spread onto selective medium. Both *E. coli* strains were counterselected on minimal medium, and the Eh252(pTROY9) transconjugants were selected by the addition of tetracycline. The physical presence of the pTROY9 in the transconjugants was checked by agarose gel electrophoresis after extraction of plasmid DNA by the procedure of Kado and Liu (30).

Isolation of Tn*5*-induced mutants of Eh252. The strain of *E. herbicola* to be infected with λ Tn*5* was grown at 37°C to 10^8 cells per ml in L broth containing tetracycline to ensure maintenance of the pTROY9. The cells were washed twice in 1 M NaCl and resuspended in half the initial volume of culture in SM buffer in which the gelatin was omitted. Different volumes of λ Tn*5* stocks containing about 2×10^{10} to 3×10^{10} PFU/ml were added to the cells. Phages were then allowed to adsorb at 37°C for 45 min. The infected cells were then diluted 1/10 in L broth and incubated with shaking at 37°C for 30 min to allow phenotypic expression. Selection of putative Tn*5*-induced mutants of Eh252 was made on complete medium containing kanamycin. The frequency of kanamycin-resistant derivatives was calculated as the number of bacteria that acquired antibiotic resistance divided by the total number of bacteria infected.

Identification of Ant$^-$ mutants of Eh252. To identify mutants that did not inhibit *E. amylovora*, fresh colonies of kanamycin-resistant derivatives of Eh252 were transferred onto a fresh overlay of GA medium seeded with *E. amylovora* Ea273. The kanamycin-resistant derivatives of Eh252 were grown for 16 h at 37°C in grids of 24 on L plates with kanamycin. They were then transferred individually onto an overlay seeded with Ea273 by using a replicator with metal prongs. After 24 h of incubation at 28°C, most of the colonies were each surrounded by a zone of inhibition indicating antibiotic production. Derivatives that were not surrounded by a zone of inhibition were then retested with the antibiotic production assay described previously. When no zone of inhibition was detected with this latter assay, the strain was tested again at least twice.

Inhibition of *E. amylovora* in immature pear fruits. Inhibition of *E. amylovora* by *E. herbicola* in immature pear fruits was tested as previously described (1). Immature pear fruits (*Pyrus communis* cultivar Bartlett) kept in the dark at 0 to 4°C with moderate aeration, were surface disinfected with 70% ethanol. The fruits were cut in half longitudinally and placed in plastic boxes on sterile paper towels previously moistened with sterile water. Then 50 μl of a suspension of *E. herbicola* made in 5 mM phosphate buffer pH 6.5 was introduced into a well (approximately 5 mm deep) bored in the cheek of each pear half with a sterile no. 1 cork borer. When this suspension was totally absorbed by the fruit (approximately 2 h), 50 μl of a suspension of *E. amylovora* made in 5 mM phosphate buffer (pH 6.5) was introduced into the same well. The fruits were incubated at 27°C and scored daily for evidence of infection. A fruit was considered infected when drops of bacterial exudate and/or necrosis was detected in and around the well. All control fruits, in which 5 mM phosphate buffer (pH 6.5) was used in place of *E. herbicola*, were infected after 3 days of incubation. When the bacterial suspension of *E. amylovora* was replaced by buffer, no sign of infection was detected after 7 days of incubation. In every experiment, each treatment was applied to 6 or 12 pear halves from different fruits.

Monitoring the rate of growth of *E. herbicola* in immature pear fruits. Preparation and treatment of the immature pear fruits were as described above for the inhibition assay, except that suspensions of *E. amylovora* were omitted. For each strain of *E. herbicola* monitored, three pear halves were used per time point. The halves were sliced into pieces approximately 1.5 cm thick and placed in a sterile polyethylene bag containing 30 ml of 5 mM phosphate buffer (pH 6.5). Bacteria growing in the pear tissues were then expelled by crushing the pear slices for 5 min with a model 400 Stomacher (Seward Laboratory, London, England). The suspension containing crushed pear tissue was allowed to settle before serial 0.1 dilutions were made in 5 mM phosphate buffer (pH 6.5) and spread on MS, Sasser medium, and L plates containing kanamycin when appropriate. As a control for each time point, three pear halves that received only buffer were treated like those inoculated with *E. herbicola*. The numbers of colonies that grew on the different media were recorded after 24 or 48 h of incubation at 37°C.

General DNA manipulations. Plasmid DNA was extracted by the procedure of Kado and Liu (30). Total DNA from *E. herbicola* was isolated as described by Klötz and Zimm (32) and purified by ethidium bromide-cesium chloride centrifugation (35). When used as cloning vector or as probe, plasmid DNA was isolated from overnight cultures by a

cleared lysate procedure (12) and further purified by ethidium bromide-cesium chloride centrifugation (35). Restriction endonucleases, T4 DNA ligase, and bacterial alkaline phosphatase were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., and used as specified by the manufacturer. When used as a probe, DNA was labeled by the random primer labeling method of Feinberg and Vogelstein (18) with 50 μCi of [α-^{32}P]dGTP per reaction or digoxigenin-labeled dUTP as part of a nonradioactive DNA detection kit from Boehringer Mannheim. DNA was transferred to Gene Screen Plus nylon membranes (Dupont, NEN Research Products, Boston, Mass.) or Hybond-N nylon membranes (Amersham Co., Arlington Heights, Ill.). DNA transfers and hybridizations were performed as recommended by the suppliers. Autoradiography was carried out at −80°C using XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) and Cronex Lightning Plus intensifying screens (E.I. Du Pont de Nemours and Co., Wilmington, Del.). The enzymatic reactions necessary to detect the hybridizing bands with the nonradioactive detection kit were carried out as recommended by the supplier.

Cloning of the Tn*5*-containing fragment from strain 10:12. Total DNA from the Ant$^-$ mutant 10:12 was digested to completion with *Eco*RI and size fractionated on a sucrose gradient (35). Fragments over 10 kb long were collected, purified, and ligated into the plasmid vector pBR325, which had been previously linearized by *Eco*RI and dephosphorylated. The DNA was then transformed into *E. coli* SCS1 as recommended by the supplier (Stratagene, La Jolla, Calif.). Transformants were selected on complete medium containing kanamycin. We checked to make sure that the selected plasmids had lost the chloramphenicol resistance, as expected because of insertional inactivation, but retained ampicillin and tetracycline resistance. One of these plasmids, pCPP710, was used for marker exchange mutagenesis.

Marker exchange mutagenesis. Plasmid pCPP710 was introduced into Eh252 by transformation by the calcium chloride method of Maniatis et al. (35). Exchange recombination of the mutagenized gene carried on the plasmid into the Eh252 chromosome was then achieved by cultivating Eh252 (pCPP710) at 37°C in a phosphate-limited medium containing kanamycin as described by Roeder and Collmer (41). The cultures were then diluted in 10 mM $MgSO_4$ solution and spread onto L-kanamycin plates. Putative mutants were screened for sensitivity to tetracycline, indicating loss of the plasmid vector, and tested for their ability to inhibit growth of *E. amylovora* on GA medium.

RESULTS

Detection and characterization of an antibiotic activity by Eh252. When *E. herbicola* Eh252 was grown for 48 h on a plate of GA medium, the subsequent growth of *E. amylovora* was inhibited in a zone surrounding the spot where Eh252 had grown (Fig. 1). Since Eh252 could grow under iron-limited conditions and produced a siderophore-type molecule (55), we investigated whether inhibition of *E. amylovora* might be due to restriction of iron. The addition of Fe(II) at a high concentration (50 μM) to GA medium did not prevent the inhibition of *E. amylovora* by Eh252 (Fig. 2A), indicating that this inhibition was not due to a siderophore produced by *E. herbicola*.

When a complete medium such as L medium was used, no inhibition of *E. amylovora* by Eh252 was observed. This is in agreement with results from R. S. Wodzinski et al., who found that the addition of Casamino Acids or even only histidine to a minimal medium broth in which Eh252 had grown could prevent the inhibition of *E. amylovora* (63). Indeed, when histidine alone was added to the GA medium, the inhibition of *E. amylovora* was prevented (Fig. 2A). The inhibition of *E. amylovora* was also prevented by the addition of pronase or proteinase K (Fig. 2A). The host range of the antibiotic produced by Eh252 was determined only for the strains that were able to grow on GA medium, thus excluding all the xanthomonads and gram-positive strains in our collection. Among the strains that were tested, none of the 52 plant pathogenic *Pseudomonas* strains, the strain of *Agrobacterium radiobacter*, or the strain of *Klebsiella pneumoniae* was sensitive to Eh252. In contrast, the growth of 62 of the 67 *Erwinia* strains tested was inhibited by Eh252 (Table 2, Fig. 2B).

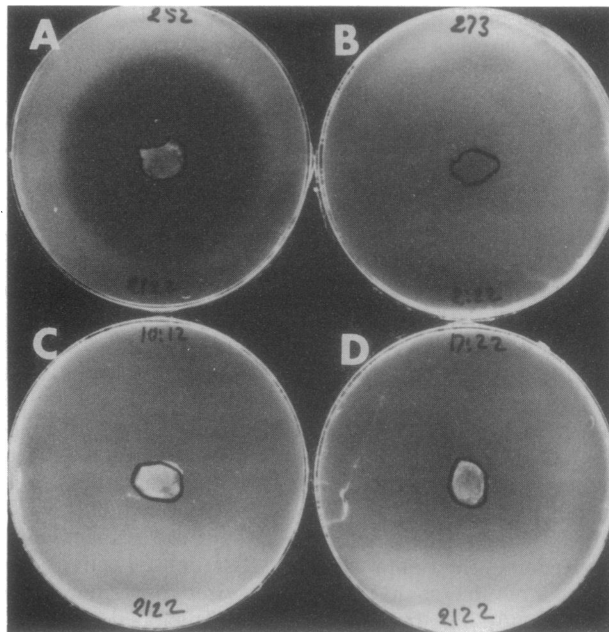

FIG. 1. Inhibition on GA medium of *E. amylovora* Ea273 by *E. herbicola* Eh252 (A) or by the non-antibiotic-producing mutants 10:12 (C) and 17:22 (D). Strain Ea273 (B) was used as a negative control. The experiment was carried out as described in Materials and Methods.

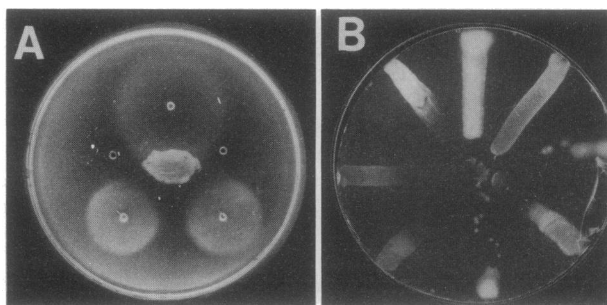

FIG. 2. (A) Inactivation of the antibiotic activity of *E. herbicola* Eh252 by the following (clockwise from the top): histidine, Fe(II), pronase, proteinase K, and sterile distilled water used as a negative control. (B) *Erwinia* strains sensitive to the antibiotic produced by Eh252 (clockwise from the top): Eh252, Eh330, Eh147, Eh314, Eh112Y, Ea322, Ea321, and Ea273. The experiments were carried out as described in Materials and Methods.

TABLE 2. *Erwinia* strains sensitive to the antibiotic produced by *E. herbicola* Eh252[a]

Species	No. of strains	
	Tested	Sensitive
E. amylovora	12	12
E. carotovora subsp. *atroseptica*	9	9
E. carotovora subsp. *betavasculorum*	2	1
E. carotovora subsp. *carotovora*	9	9
E. chrysanthemi	27	26
E. herbicola	5	3
E. rhapontici	2	2
E. stewartii	1	0

[a] Strains were tested as described in the text.

Isolation of Tn*5*-induced mutants of strain Eh252. Infection with a λ Tn*5* lysate of a derivative of Eh252 harboring pTROY9, a plasmid that confers λ-binding ability, gave rise to kanamycin-resistant derivatives at a frequency of about 10^{-5} with multiplicities of infection of about 30. In the absence of antibiotic selection, the plasmid pTROY9 was very unstable, as indicated by sensitivity to tetracycline. However, this rapid loss of pTROY9 was probably not due to incompatibility between this plasmid and the indigenous plasmids of Eh252, since no difference in the plasmid content could be detected between Eh252 and derivatives that had carried pTROY9 (data not shown). The percentage of kanamycin-resistant derivatives carrying an auxotrophic mutation (1.4%) and analysis of 20 of these auxotrophic mutants indicated that in Eh252 Tn*5* insertions were mostly random, stable, and unique and were the cause of the mutant phenotypes (data not shown).

Among the 1,500 kanamycin-resistant derivatives isolated, three prototrophic mutants 10:12, 17:22, and 42:6, did not inhibit the growth of *E. amylovora* on GA plates (Fig. 1). These mutants were probably affected in antibiotic production and were called Ant$^-$ for non-antibiotic-producing mutants. Other mutant phenotypes, including mutants that consistently produced zones of inhibition much smaller than that of Eh252 (data not shown), were also observed. This mutant phenotype could be due to numerous factors only indirectly linked to antibiotic production; thus these mutants were not studied further. The rates of growth of the three Ant$^-$ mutants in L broth and in GA liquid medium were similar to that of the wild-type strain. When plated onto an iron-deficient medium, L or M9 medium containing 100 μg of ethylenediamine-*N,N'*-bis(2-hydroxyphenylacetic acid) per ml, the two mutants tested, 10:12 and 17:22, grew as well as Eh252. In addition, when streaked onto the blue medium of Schwyn and Neilands, unless it was supplemented with Fe(II), colonies of 10:12 and 17:22 were surrounded by an orange halo equivalent to that of Eh252. These results suggest that these two mutants were not impaired in siderophore production, which is in agreement with the fact that the addition of Fe(II) to GA medium did not prevent the inhibition of *E. amylovora*.

In the assay described above for determining the host range of the antibiotic, none of the three Ant$^-$ mutants was sensitive to Eh252. This result suggests either that the immunity system was not affected by the Tn*5* insertion in these mutants or that Eh252 is resistant to its own antibiotic by a mechanism that is independent of antibiotic production.

Genomic analysis of the Ant$^-$ mutants. We ensured the presence of a unique Tn*5* in the genome of the Ant$^-$ mutants

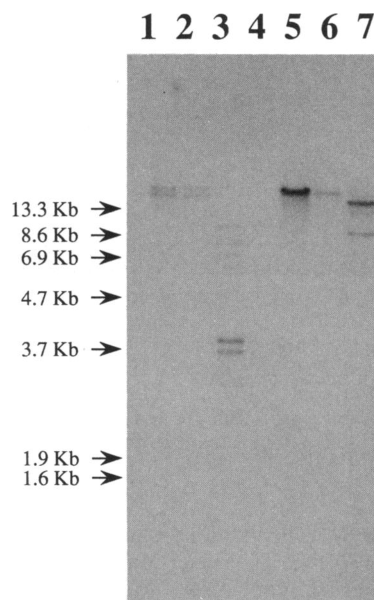

FIG. 3. Southern blot analysis of genomic DNA isolated from the Ant$^-$ mutants 10:12 (lanes 1 and 5), 17:22 (lanes 2 and 6), and 42:6 (lanes 3 and 7) and from the wild-type strain Eh252 (lane 4) with plasmid pRZ102 as a probe. The DNA was cut to completion with either *Bam*HI (lanes 1 to 3) or *Eco*RI (lanes 4 to 7). The arrows indicate the sizes of DNA fragments.

by DNA-DNA hybridization analysis with the Tn*5*-containing plasmid pRZ102 as a probe. Genomic DNA from the Ant$^-$ mutants was digested either with *Bam*HI, which cleaves within Tn*5*, or with *Eco*RI, which does not. Thus, a unique Tn*5* insertion was revealed by two bands when the DNA was cut with *Bam*HI and by one band when the DNA was cut with *Eco*RI. A single Tn*5* insertion was shown for mutants 10:12 and 17:22, whereas three Tn*5* insertions were detected in three distinct *Eco*RI fragments of 13.5, 11, and 7.8 kb for mutant 42:6 (Fig. 3). For 10:12 and 17:22, the Tn*5* insertion was found in an *Eco*RI fragment of the same apparent size (ca. 16 kb). Furthermore, when total DNA from 10:12 was cut by *Bam*HI, the two bands detected were equivalent in size to the two bands detected for 17:22 (Fig. 3). These data suggest that the Tn*5* insertions in 10:12 and 17:22 are probably in the same fragments of DNA and possibly at the same locations.

With the exception of one microcin, all protein antibiotics produced by enterobacterial strains are plasmid encoded (33). Thus, we compared the plasmid content of Eh252 with that of the Ant$^-$ mutants and looked for possible insertion of Tn*5* in one of the plasmids of the Ant$^-$ mutants. When plasmid DNA was extracted from Eh252 by the procedure of Kado and Liu (30), three plasmids of ca. 100, 200, and 280 kb could be detected on agarose gel electrophoresis (data not shown). Three plasmids of similar sizes were also found for the three Ant$^-$ mutants (data not shown). To determine whether a Tn*5* was inserted in one of the plasmids harbored by the Ant$^-$ mutants, blots prepared from agarose gel electrophoresis of plasmid DNA extracted from these mutants were hybridized with plasmid pRZ102 as a probe. As positive and negative controls, respectively, we used plasmid DNA isolated from Eh252 and from a derivative of *E. herbicola* Eh112Y that carries a Tn*5* insertion in a ca. 144-kb plasmid (20). None of the plasmids isolated from Eh252,

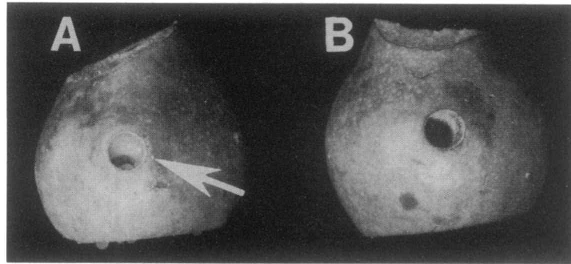

FIG. 4. (A) Immature pear fruit infected with *E. amylovora*. The arrow points to bacterial exudate produced in the well of the fruit due to growth of the pathogen. Fruits were considered infected only when there was production of bacterial exudate in or around the well where both bacteria (*E. herbicola* and *E. amylovora*) were introduced. (B) Immature pear fruit treated with buffer instead of *E. amylovora* was used as a negative control.

10:12, or 17:22 hybridized with the probe, indicating that for 10:12 and 17:22 the Tn*5* insertion was probably in the chromosome. For mutant 42:6, the largest plasmid hybridized with the probe, indicating that at least one copy of Tn*5* was in this ca. 280-kb plasmid (data not shown). Several attempts to transfer by mating the kanamycin resistance from 42:6 to different strains of *E. coli* failed, which could indicate that this plasmid is not conjugative or transfers only at a low frequency. The Tn*5* insertions in 42:6 were found in different *Eco*RI fragments than were those in 10:12 and 17:22. However, because it had several Tn*5* insertions, this mutant could not be readily compared with Eh252 for control of fire blight on immature pear fruits. Thus, we focused our study on the two other mutants.

Inhibition of *E. amylovora* in immature pear fruits treated with Eh252 or one of the Ant$^-$ mutants. We first ensured that differences in disease protection between the Ant$^-$ mutants and Eh252 were not due to different growth rates in immature pear fruits. In three independent experiments, the growth rate of 10:12 and 17:22 in pear tissue was found to be similar to that of Eh252 (data not shown). The role of antibiotic production in the control of fire blight could now be assessed by comparing the ability to inhibit *E. amylovora* development in immature pear fruits of Ant$^-$ mutants with that of Eh252. The ability to control the disease was compared by counting for each treatment (Ant$^-$ mutant or Eh252) the number of fruits inoculated with *E. amylovora* that showed signs of fire blight infection (Fig. 4).

We first compared the number of *E. amylovora*-infected fruits treated with Eh252 with the number of those treated with 10:12. The concentrations of *E. herbicola* used in these experiments ranged from 5×10^3 to 5×10^6 CFU/ml (Fig. 5). The number of infected fruits that were treated with the Ant$^-$ mutant was always greater than that with the wild-type strain, indicating that 10:12 did not protect the fruits from fire blight as well as did Eh252. The difference between 10:12 and Eh252 in disease protection was best observed when populations of these two strains were 5×10^4 and 5×10^5 CFU/ml (Fig. 5B and C, respectively). When higher populations of Eh252 or 10:12 were used, the number of fruits that were infected decreased. Indeed, when the population of *E. herbicola* was 5×10^6 CFU/ml (Fig. 5D), only half of the pears treated with 10:12 were infected 5 days after inoculation. When higher concentrations of 10:12 were used, no sign of infection could be detected 7 days after inoculation (data not shown). With the lowest concentration of *E. herbicola* (5×10^3 CFU/ml), the number of fruits that were infected was the greatest; yet not all of the fruits treated with 10:12 were infected after 3 days of incubation, whereas all of the fruits treated with buffer instead of *E. herbicola* were infected within 3 days (Fig. 5A).

These results suggest that antibiotic production by Eh252 is involved in the biological control of fire blight in immature pear fruits. However, they could also reflect a unique characteristic of 10:12 rather than a consequence of the loss of antibiotic production. Thus, we also studied the effectiveness of 17:22 in reducing fire blight development in immature pear fruits. Six pear halves were used for each of the three concentrations of Eh252, 10:12, or 17:22 (Table 3). The number of infected fruits treated with 17:22 was always equivalent to that treated with 10:12 and always greater than that treated with Eh252. As found previously, the greater the concentration of *E. herbicola* used, the greater the level of disease protection. Although no difference in disease protection between the three strains of *E. herbicola* could be detected 2 days after inoculation with 4×10^3 CFU/ml, an important difference between Eh252 and the Ant$^-$ mutants was still detected up to 4 days after inoculation when 4×10^4 and 4×10^5 CFU/ml were used. Probably because of the high level of susceptibility of the pears used in this test, almost all of the fruits treated with 17:22 or 10:12 were infected after 3 days of incubation, even when the highest concentration of *E. herbicola* was used (10^5 CFU/ml). However, as noted earlier for 10:12, other tests showed that 17:22 at a high concentration still protected immature pear fruits from fire bight infection (see Table 5). The data obtained with 10:12 and 17:22 suggest that production of an antibiotic by Eh252 is a major determinant of the inhibition of *E. amylovora* in immature pear fruits but that its role is masked by other mechanisms when large populations of *E. herbicola* are used.

Marker exchange mutagenesis. To determine whether loss of antibiotic production and decreased efficiency in controlling fire blight are linked and induced by the Tn*5* insertion in 10:12, we analyzed in immature pear fruits non-antibiotic-producing derivatives of Eh252 obtained by marker exchange mutagenesis with the Tn*5*-containing fragment isolated from 10:12. The plasmid pBR325 was used to clone the Tn*5*-induced mutation carried by 10:12 because, as previously shown for pBR322 derivatives introduced into *Erwinia chrysanthemi* strains (41), this plasmid was unstable in Eh252 when the culture was grown in phosphate-limited medium. Since no *Eco*RI site is present in Tn*5*, plasmid pCPP710 was constructed by cloning the genomic DNA isolated from 10:12 into the *Eco*RI site of pBR325 and selecting for kanamycin resistance. The resulting plasmid was 18.9 kb long. The Tn*5* was flanked by 1.7 and 5.4 kb of genomic DNA, making pCPP710 suitable to use for marker exchange mutagenesis.

Four independent Eh252(pCPP710) transformants, called A, B, C, and D, were retained for marker exchange mutagenesis. These derivatives were grown independently in low-phosphate medium containing kanamycin, and aliquots were spread on L-kanamycin plates after 16 and 26 h of incubation. From each sample, isolated colonies randomly chosen were individually tested for sensitivity to tetracycline and inhibition of *E. amylovora* on GA plates. Some colonies were sensitive to tetracycline, presumably indicating the loss of the plasmid vector, but retained the ability to inhibit *E. amylovora*, suggesting that insertion of the Tn*5* in these mutants occurred by transposition rather than by marker exchange recombination (Table 4). To determine whether the Ant$^+$ phenotype of some kanamycin-resistant tetracy-

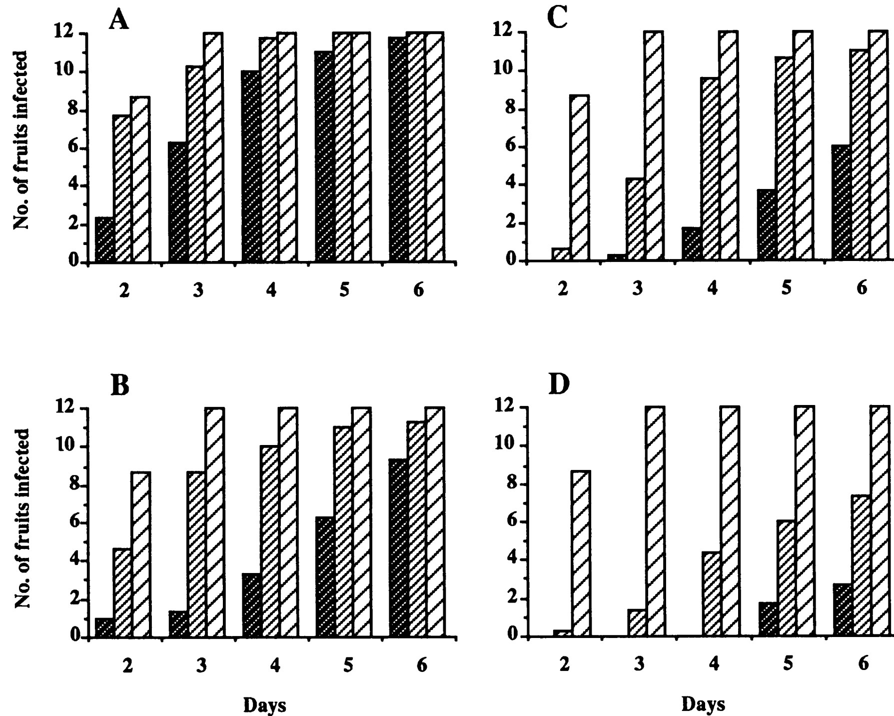

FIG. 5. Number of immature pear fruits infected that were treated with *E. herbicola* Eh252 (▨), with the Ant$^-$ mutant 10:12 (▨), or with buffer (▨) and inoculated with *E. amylovora* Ea273. The bars represent the average numbers of fruits infected from three independent experiments. Concentrations of *E. herbicola*: A, 5×10^3 CFU/ml; B, 5×10^4 CFU/ml; C, 5×10^5 CFU/ml; D, 5×10^6 CFU/ml. The concentration of *E. amylovora* used in these experiments was 3×10^5 to 4×10^5 CFU/ml.

cline-sensitive derivatives could be attributed to random Tn*5* insertion and to identify Ant$^-$ derivatives in which only marker exchange recombination had taken place, 23 kanamycin-resistant tetracycline-sensitive derivatives isolated after 16 h of incubation in low-phosphate medium were selected. They were called, according to the strain from which they were isolated, A_1, A_2, B_1 to B_3, C_1 to C_9, and D_1 to D_9. Only strains C_3, D_3, D_6, and D_7 were Ant$^+$. The numbers of Tn*5* insertions in these derivatives and their origins (transposition or homologous recombination) were determined by Southern hybridization analysis. Total DNA from the 23 derivatives was isolated, digested with *Eco*RI or *Bam*HI, and probed with plasmid pRZ102. The number and sizes of hybridizing bands obtained for each derivative were then compared with those of 10:12 (Fig. 6). Only the non-antibiotic-producing derivatives, and all of them except one, C5, had bands similar in size to those of 10:12. This strong correlation indicates that Tn*5* insertion in 10:12 was responsible for the loss of antibiotic production. The derivative C_5 might have lost the ability to produce antibiotic by spontaneous mutation or by Tn*5* insertion in another gene necessary for antibiotic production. Since none of the four antibi-

TABLE 3. Infection of immature pear fruits treated with *E. herbicola* Eh252, 10:12, or 17:22 and inoculated with *E. amylovora* Ea273[a]

Treatment (CFU/ml)	No. of infected fruits on the following day after inoculation:			
	2	3	4	5
Eh252 (4×10^5)	0	1	3	5
10:12 (4×10^5)	1	5	6	6
17:22 (4×10^5)	4	6	6	6
Eh252 (4×10^4)	1	1	3	5
10:12 (4×10^4)	4	6	6	6
17:22 (4×10^4)	5	5	5	5
Eh252 (4×10^3)	2	5	6	6
10:12 (4×10^3)	4	5	6	6
17:22 (4×10^3)	6	6	6	6
Buffer	6	6	6	6

[a] The concentration of *E. amylovora* used in these experiments was 2×10^6 CFU/ml. Samples containing 50-μl suspensions of *E. herbicola* and then of *E. amylovora* were used per fruit. Six halves of immature pear fruits were used per treatment.

TABLE 4. Antibiotic production and sensitivity to tetracycline of Eh252(pCPP710) derivatives grown in low-phosphate medium[a]

Eh252(pCPP710) derivative	Time of growth (h)	No. of Tc^s colonies/no. tested	No. of Tc^s Ant$^-$ colonies/no. of Tc^s colonies tested
A	16	42/48	9/9
B	16	46/48	9/9
C	16	29/48	8/9
D	16	32/48	6/9
A	26	48/48	48/48
B	26	47/48	47/47
C	26	45/48	26/45
D	26	39/48	25/39

[a] Tc^s, sensitivity to tetracycline; Ant$^-$, no inhibition of *E. amylovora* on GA plates.

FIG. 6. Southern blot analysis of genomic DNA isolated from kanamycin-resistant tetracycline-sensitive derivatives of Eh252 selected after marker exchange mutagenesis. Total DNA was cut with *Bam*HI and hybridized on transfer membranes with plasmid pRZ102 labeled with digoxygenin-dUTP as a probe. Strains (lanes): a, 10:12; b, A_1; c, A_2; d, B_1; e, B_2; f, C_1; g, C_2; h, C_4; i, C_5; j, C_6; k, C_7; l, C_8; m, C_9; n, D_1; o, D_2; p, D_4; q, D_5; r, D_8; s, D_9; t, B_3; u, 10:12; v, C_3; w, D_3; x, D_6; y, D_7; z, 10:12. The plus or minus sign below the letters indicates whether the strain did or did not inhibit *E. amylovora* on minimal medium. The arrows indicate the sizes of DNA fragments. Identical results were obtained when total DNA was cut with *Eco*RI (data not shown).

otic-producing derivatives had hybridizing bands similar to that of 10:12, no exchange recombination took place in those derivatives; they most probably acquired kanamycin resistance by independent transposition of the Tn*5*. Secondary transposition of the Tn*5* was found for 16 of the 23 derivatives analyzed; six of them (C_6, C_8, C_9, D_4, D_6, and D_8) carried up to three insertions. Only five derivatives (A_1, B_1, B_2, B_3, and C_4) carried a single Tn*5* insertion that was similar to that carried by 10:12.

To demonstrate a relation between antibiotic production and ability to control fire blight, three Ant^- derivatives from three independent marker exchange experiments, A_1, B_1, and C_4, and one Ant^+ derivative, D_3, were retained for further analysis. They were all prototrophic and grew as well as Eh252 in L broth and GA broth. The ability of these derivatives to suppress fire blight was then assessed by comparing the number of immature pear fruits infected that were treated with either Eh252 or one of these derivatives before inoculation with *E. amylovora*. No difference could be noted in the number of fruits infected that were treated with D_3 or Eh252 (data not shown). On the contrary, in the two experiments that were done, the number of fruits infected that were treated with A_1, B_1, or C_4 was similar to that of fruits treated with 10:12 or 17:22 and was always greater than that of fruits treated with Eh252 (Table 5). This indicates that in 10:12 Tn*5* insertion was responsible for the loss of antibiotic production and the decrease in efficiency in controlling fire blight. Thus, these data further support the hypothesis that antibiotic production by Eh252 is one of the mechanisms responsible for inhibition of development of *E. amylovora*.

TABLE 5. Infection of immature pear fruits treated with *E. herbicola* Eh252 or Ant⁻ derivatives and inoculated with *E. amylovora* Ea273[a]

Treatment	No. of infected fruits on the following day after inoculation:							
	Expt 1			Expt 2				
	3	4	5	3	4	5	6	7
Eh252	0	0	0	0	0	0	0	0
10:12	2	2	3	0	1	2	4	5
17:22	0	2	3	0	1	2	2	2
A1	1	3	3	1	1	2	2	2
B1	1	2	2	1	2	2	2	2
C4	ND	ND	ND	0	0	0	2	4
Buffer	5	6	6	2	5	5	6	6

[a] The concentrations of *E. amylovora* were 1.2×10^6 and 2×10^6 CFU/ml in experiments 1 and 2, respectively. The concentrations of *E. herbicola* were 2×10^5 to 3×10^5 CFU/ml in experiment 1 and 1.5×10^5 to 2×10^5 CFU/ml in experiment 2. Samples containing 50-μl suspensions of *E. herbicola* and *E. amylovora* were used per pear. Six halves of immature pear fruits were used per treatment. ND, not determined.

DISCUSSION

In this study, we investigated the possibility that the inhibitory compound produced by *E. herbicola* Eh252 was involved in the biological control of fire blight. We showed that Tn*5*-induced mutants that lost their ability to inhibit *E. amylovora* on minimal medium were less effective than the wild-type strain in reducing fire blight in immature pear fruits. Furthermore, analysis of marker exchange derivatives of Eh252 indicated that, in at least one mutant, the Tn*5* insertion was responsible for both the loss of antibiotic production and the decreased efficiency in reducing fire blight.

Eh252 produced a compound that inhibited *E. amylovora*, was sensitive to proteases, and exhibited a narrow host range of activity. These are characteristics shared by protein antibiotics such as bacteriocins and microcins. However, since the compound produced by Eh252 has been only partially characterized, we referred to it as an antibiotic. Production of antibiotics that inhibit *E. amylovora* seems to be a common characteristic of strains of *E. herbicola*. Indeed, in two studies (16, 60), each involving more than 300 strains of *E. herbicola*, 17 and 60% of the strains tested, respectively, inhibited *E. amylovora* on minimal medium. In addition, in both studies 50 to 60% of the antibiotic-producing strains examined, like Eh252, no longer inhibited *E. amylovora* in the presence of histidine. Interestingly, El-Goorani and Beer found that all of their strains that produced an antibiotic inactivated by histidine also reduced fire blight in immature pear fruits (16). Thus, production of inhibitory compounds similar to that of Eh252 might be a characteristic well distributed among *E. herbicola* strains. Nevertheless, the antibiotic produced by Eh252 is different from the antibiotics produced by other strains of *E. herbicola* that have been previously described. Like Eh252, three strains of *E. herbicola*, Eh318 (2), Eh112Y (28), and C9-1 (61), produce compounds that inhibit growth of *E. amylovora* on plates and suppress fire blight development in immature pear fruits. However, the antibiotic of Eh318 is inactivated by a combination of arginine and histidine, whereas the antibiotic of Eh252 is inactivated by histidine alone (61). Eh112Y produces an antibiotic that is inactivated by histidine and seven other amino acids (62) and, in contrast to Eh252, is not sensitive to proteases. C9-1 produces two inhibitory compounds called herbicolins O and I, that are also resistant to proteolytic enzymes, but, like the antibiotic from Eh252, herbicolin O is inactivated by histidine (28). Finally, the two peptide antibiotics produced by *E. herbicola* A111, called herbicolins A and B, are also different from the antibiotic produced by Eh252, since A111 inhibits yeasts and filamentous fungi but not bacteria (59).

Kanamycin-resistant derivatives of Eh252 were easily obtained after infection of Eh252(pTROY9) with λ Tn*5* bacteriophage lysates. However, the best results were obtained using unusually high multiplicities of infection (around 30). This could be due to a characteristic of Eh252, which does not allow readily adsorption or infection by λ derivatives. Salmond et al. (43), who reported transposon mutagenesis of *Erwinia* sp. with λ derivatives as vector, noted that the frequency of antibiotic-resistant derivatives obtained after mutagenesis could vary 100-fold among different *Erwinia* strains. Alternatively, this high multiplicity of infection could indicate that plasmid pTROY9 did not supply enough LamB (the λ receptor protein) for all of the bacteriophages to find a receptor. This, in turn, could reflect either that the pTROY9 was unstable or that the *lamB* gene, which is expressed from an IS-3 promoter, was poorly expressed in Eh252. De Vries et al. (15) had already noticed that *E. coli* ECG10, from which the *lamB* gene was cloned to construct pTROY9, had only 1/10 the efficiency of λ phage adsorption of HB101, from which it was derived.

Infection of Eh252 with λ Tn*5* gave rise to mostly single insertions of Tn*5* in the genome of *E. herbicola*. Indeed, reversion to prototrophy, for all the auxotrophic mutants tested, was strictly correlated with sensitivity to kanamycin (data not shown). In contrast, 16 of the 23 derivatives isolated after marker exchange mutagenesis had several copies of Tn*5*. The limited phosphate medium used for the marker exchange might have led to this high frequency of secondary transposition of the Tn*5*, since at least one gene whose product is involved in Tn*5* transposition, *himA* (5), is induced in *E. coli* K-12 by limited-phosphate conditions (56).

Three independent mutants that did not inhibit *E. amylovora* on GA medium were isolated. Only two of them, 10:12 and 17:22, had single Tn*5* insertions. Since none of the three indigenous plasmids isolated from these two mutants hybridized with pRZ102, at least one gene involved in antibiotic production in Eh252 is not harbored by those plasmids. Only one protein antibiotic (microcin H47) produced by a strain of the family *Enterobacteriaceae* has been shown to be coded by chromosomal genes (33). However, as discussed by Lavina et al. (33), other protein antibiotics from *K. pneumoniae* and *E. coli* could also be chromosome encoded. Likewise, we cannot rule out the possibility that in Eh252 other genes involved in antibiotic production might be plasmid borne or that a megaplasmid went undetected.

The immature pear fruit assay used to compare efficiency in controlling fire blight between the Ant⁻ mutants and Eh252 is the only assay that allows studies of the interaction between *E. amylovora* and *E. herbicola* in the laboratory. In contrast to *E. amylovora*, *E. herbicola* does not multiply outside the area of the fruit where it has been introduced (3, 17). Thus, treatment of immature pear fruits with a strain of *E. herbicola* that is an effective biocontrol agent restricts the development of *E. amylovora* only in and around the well (where both bacteria were introduced) and does not prevent production of exudate at the extremities of the fruit. Therefore, we considered that *E. amylovora* was inhibited only when no exudate was produced in or around the well. Since the quantity of exudate produced per pear was variable, each

half pear could only be used as a qualitative test. With quantitative use of the test (the amount of exudate per fruit as a measure of the level of inhibition), no correlation was found between the abilities of different strains of *E. herbicola* to inhibit *E. amylovora* in fruits and their abilities to inhibit *E. amylovora* in flowers (58). Since this immature pear fruit assay is a qualitative test, the number of fruits used to compare the ability of different strains of *E. herbicola* to inhibit *E. amylovora* should be as large as possible. However, the time needed for the appearance of exudate on an infected fruit depends on the physiological state of the pear and on the relative humidity in the experimental box (55). All of the pears chosen for one experiment were at a similar physiological state. But the degree of humidity in our experiments could not be controlled, and thus only the results from pears contained in the same experimental box could be compared. This explains why in some experiments treatments were applied to only six pears. Since the susceptibility of the fruits decreases during storage, the concentration of the suspension of *E. amylovora* had to be adjusted between different series of experiments such that all of the fruits treated with buffer were infected within 3 days. Although the number of fruits infected that were treated with the same concentration of the same strain differed in all three series of experiments (Fig. 5; Tables 3 and 5), the relative efficacy of the strains was not changed. Indeed, in all experiments Eh252 provided better control of fire blight than did either one of the Ant$^-$ mutants.

The non-antibiotic-producing mutants of Eh252 were not as effective as the wild-type strain in suppressing fire blight development in immature pear fruits, suggesting that antibiotic production is one of the mechanisms involved in the biocontrol of fire blight by Eh252. Since only two mutants with possibly the same Tn*5* insertion were analyzed, antibiotic production and the ability to reduce fire blight might not be causally related but might be the result of a pleiotrophic mutation. This, however, is unlikely, since independent spontaneous mutants of Ea273 that are resistant on petri dishes to the antibiotic produced by Eh252 are not as inhibited as the sensitive wild-type strain in immature pear fruits treated with Eh252 (53, 55). Together, these results indicate that antibiotic production and the ability to reduce fire blight are causally related; thus, antibiotic production by Eh252 is a determinant in the biological control of fire blight in immature pear fruits.

Although several authors have suggested that production of antibiotics by *E. herbicola* could be involved in biological control of fire blight (17, 27, 28, 61), others have disagreed, since no inhibitory compound could be detected for some strains that are effective in the orchard. This was the case for Eh252 (2), until R. S. Wodzinski (63) showed that Eh252 inhibited *E. amylovora* in minimal medium in the absence of histidine. It has also been reported that Tn*5*-induced mutants of *E. herbicola* Eh112Y that lost the ability to produce antibiotic were as effective as the wild-type strain in protecting immature pear fruits from fire blight (2). Perhaps, as shown for Eh252, the role of this antibiotic is masked by another mechanism(s) when high populations of bacteria are used. Alternatively, Eh112Y might produce an inhibitory compound other than herbicolacin Eh112Y that has not yet been detected. Both hypotheses would be in agreement with the report that culture filtrates of Eh112Y reduce fire blight incidence on ornamental trees (27).

The non-antibiotic-producing mutants of Eh252 still retained some ability to suppress fire blight development, indicating that factors other than antibiotic production are involved in biocontrol of fire blight. A similar situation has been described in other systems where production of an antibiotic has been involved in biological control. Mutants of *A. radiobacter* K84 that do not produce agrocin 84 still reduce disease caused by the pathogen *Agrobacterium tumefaciens* (13). Similarly, Tn*5*-induced mutants of *Pseudomonas fluorescens* 2-79 that do not produce phenazine, an antibiotic involved in the inhibition of some pathogenic fungi, still suppress take-all caused by *Gaeumannomyces graminis* var. *tritici* (48). The only exception concerns a Tn*5*-induced mutant of *E. herbicola* B247 that, in contrast to the wild-type strain, does not inhibit at all the development of *Puccinia recondita* f. sp. *tritici* on wheat leaves (31). This same mutant, however, still suppresses *Fusarium culmorum*, although not to the same extent that the wild-type strain does (31).

Mechanisms other than antibiosis have been proposed to explain the inhibition of *E. amylovora* by different strains of *E. herbicola*. These include production of toxic aglycones by hydrolysis of arbutin or phloridzin (11, 25), induction of a phytoalexinlike compound (38), increase in acidity of the medium caused by the growth of the epiphyte (22, 40), and competition for nitrogen (3, 40). In the case of Eh252, electron microscopy showed that this strain grows like *E. amylovora* Ea273 on the stigmatic surfaces of apple flowers (24). This observation implies that site competition could be a mechanism for inhibition of *E. amylovora* in planta. Eh252 (55) and *E. amylovora* (49) were also shown to possess an iron uptake system, which in the case of *E. amylovora* could be involved in pathogenicity (46, 50). Thus, as demonstrated in the biological control of soil pathogens (23), competition for iron could be another mechanism involved in inhibition of *E. amylovora* on flowers.

In this study, we showed that production of an antibiotic by Eh252 was a major determinant of the biological control of fire blight. The role of the antibiotic, however, was masked by other unidentified mechanisms when large populations of *E. herbicola* were used. Genetic analysis of the antibiotic production by Eh252 is currently under way.

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