Multiple Antibiotic Production by *Erwinia herbicola*

Carol A. Ishimaru, Edward J. Klos, and Robert R. Brubaker

Graduate student and professor, respectively, Department of Botany and Plant Pathology, and professor, Department of Microbiology and Public Health, Michigan State University, East Lansing 48824. Present address of first author: Department of Plant Pathology, University of Nebraska, Lincoln 68583.

This study was supported in part by Public Health Service grant AI 13590 from the National Institute of Allergy and Infectious Diseases (R.R.B.) and by funds generously provided by E. J. K.

Journal article 11668 from the Michigan Agricultural Experiment Station.

Accepted for publication 11 December 1987.

ABSTRACT

Ishimaru, C. A., Klos, E. J., and Brubaker, R. R. 1988. Multiple antibiotic production by *Erwinia herbicola*. Phytopathology 78:746-750.

Cells of *Erwinia herbicola* strain C9-1 produced at least two antibiotics, termed herbicolin O and herbicolin I, when grown at 30 C in a chemically defined medium containing D-gluconate and L-asparagine as sources of energy. Herbicolin O inhibited growth of a wide variety of a limited number of tested procaryotes, including *Erwinia amylovora* strain 110, other erwiniae, *Enterobacter aerogenes*, *Escherichia coli*, *Serratia marcescens*, *Proteus* species, yersiniae, shigellae, salmonellae, and *Bacillus cereus*. In contrast, the antibacterial spectrum of herbicolin I was limited to *E. amylovora*, *B. cereus*, and *Staphylococcus aureus*. Mutants of *E. amylovora* were selected that exhibited resistance to herbicolin O but not herbicolin I and vice versa. In addition, antibacterial activity of herbicolin O, but not that of herbicolin I, was inhibited by L-histidine. An evidently related herbicolin was produced by *E. herbicola* strain 112Y. Treatment of immature pear fruit slices with whole cells of *E. herbicola* strain C9-1 or with partially purified herbicolins promoted significant reduction in severity of disease caused by *E. amylovora*. These findings are consistent with the suggestion that herbicolins serve to control fire blight naturally, providing a basis for further study of herbicolins' structure and mode of action.

Erwinia herbicola is a member of the Enterobacteriaceae found commonly in association with plants or as saprophytes from soil and water (12). Strains of *E. herbicola* capable of causing disease in humans have also been isolated and classified as *Enterobacter agglomerans* (8,22). Since *E. herbicola* can compete successfully with the indigenous flora of a variety of natural environments, it is not surprising that this organism produces potent antimicrobial agents. Some recently described examples are two peptide antibiotics termed herbicolins A and B that inhibit fungi (26) and sterol-requiring *Mycoplasma*, *Spiroplasma*, and *Ureaplasma* (10). Production and isolation of a novel β-lactam antibiotic by *E. herbicola* was also described (20).

Plant microbiologists interested in evaluating the ability of *E. herbicola* to promote biological control of fire blight, caused by related *E. amylovora*, have investigated the in vitro antibiosis of *E. amylovora* by *E. herbicola*. Such antagonism has been attributed to production of organic acids (21), hydroquinones (7), and bacteriocins (4). The latter are antibacterial enzymes characterized by their ability to kill only organisms closely related to that which produced the bacteriocin (19). In addition, *E. herbicola* strain 112Y was reported to produce a nonproteinaceous, bacteriocinlike substance, termed herbicolicin 112Y, which inhibited only *E. amylovora* and a few additional enteric bacteria (11,23).

Preliminary results indicated that in vitro antibiosis of *E. amylovora* is a common characteristic of *E. herbicola* strains isolated from Michigan apple orchards. One particular isolate, *E. herbicola* strain C9-1, produced very large zones of inhibition on lawns of *E. amylovora*. This marked activity was only observed on a chemically defined solid medium buffered at neutral pH (17). These findings are extended in this report, which provides evidence showing that cells of *E. herbicola* C9-1 produce at least two antibiotics distinguished by antibacterial spectra, differences among resistant mutants, rates of diffusion in agar, and inhibition of antibacterial activity by L-histidine. Furthermore, we show that *E. herbicola* 112Y also expresses antibiotic activity, and we demonstrate that these compounds from *E. herbicola* C9-1 exhibit significant in planta protection against disease caused by *E. amylovora*.

© 1988 The American Phytopathological Society

MATERIALS AND METHODS

Bacteria. Origins of strains of *E. amylovora* used in this study were previously reported (16). *E. herbicola* strain 112Y, producer of the bacteriocin herbicolicin 112Y, was obtained from S. V. Beer (Cornell, NY). *E. herbicola* C9-1 was isolated from Michigan-grown apple *Malus malus* 'Jonathan' stem tissue. Other bacteria used in these experiments were obtained from preserved laboratory stocks maintained at −20 C (40% glycerol in 0.033 M of potassium phosphate buffer, pH 7.0 [phosphate buffer]).

Media. Before being used in experiments, bacteria were directly inoculated from stocks to the surface of slopes of Tryptose blood agar base (Difco Laboratories, Detroit, MI) and, depending on the species, incubated overnight at 26 or 37 C. A modification of the chemically defined morpholino-propane sulfonic acid (MOPS)-buffered medium of Neidhart et al (18) was used in most experiments. This MOPS, gluconate, asparagine medium (MGA) was prepared by substitution of potassium gluconate (10 mM) for glucose and addition of L-asparagine (10 mM), thiamine (10 μM), and nicotinic acid (10 μM); all of these reagents were sterilized by filtration. MGA was further supplemented with hypoxanthine (1 mM), L-methionine (1 mM), and calcium pantothenate (0.1 mM) for growth of *Escherichia coli* strain Φ and its bacteriocin-tolerant and -resistant mutants; different additions (6) were made for growth of *Yersinia pestis*. The more complex medium of Atherton et al (1), termed Atherton medium, was used as an overlayer in some experiments. The combination of Atherton medium as an overlay and MGA as a base layer supported growth of all organisms examined and expressed all antibiotics detected in MGA. Solid media were always prepared by addition of 1% (w/v) Bacto agar (Difco).

Production and assay of antibacterial activities. Antibiotics were produced in solid MGA medium by spotting 0.02 ml of a suspension of *E. herbicola* C9-1 or 112Y (10^8 cells/ml of phosphate buffer) on the agar surface. After incubation for 2 days at 30 C, cells of the resulting producer colony were killed by exposure to $CHCl_3$ vapor and overlaid with 5 ml of warm MGA agar seeded with about 2×10^6 indicator bacteria per milliliter. Antibacterial activity was recorded as formation of clear zones of inhibition after further incubation for 2 days at 30 C.

Liquid MGA was used to produce antibacterial compounds by inoculating a first transfer (10 ml/125 ml Erlenmeyer flask) at an optical density (620 nm) of 0.1. This culture was aerated at 200 rpm on a model G76 gyrotory water bath shaker (New Brunswick Scientific Co., Inc., New Brunswick, NJ) at 30 C until the organisms approached stationary phase. The latter then served as an inoculum for a second transfer in MGA (10 ml/125 ml Erlenmeyer flask), which was similarly used to inoculate a third culture of MGA (50 ml/500 ml Erlenmeyer flask). After aeration for 18 hr, the cells were harvested by centrifugation (10,000 *g* for 10 min at 4 C) and the supernatant fluid was decanted, sterilized by passage through a membrane filter (pore size of 0.22 μm; Millipore Corp., Bedford, MA), and concentrated 10-fold under reduced pressure at 40 C. In some experiments, the cells obtained by centrifugation were washed once in phosphate buffer, suspended in 2 ml of 0.05 M Tris HCl buffer, pH 7.8, and disrupted by treatment with an MSE ultrasonic probe (Instrumentation Associates, New York, NY) for 2 min at 15-sec intervals. Cellular debris was removed by centrifugation and the resulting cell-free extract was assayed for the presence of antibiotics.

Antibacterial activity was determined by application of 0.01 ml of the sample to the surface of MGA agar in petri dishes. After the liquid was absorbed, the plates were exposed to $CHCl_3$ vapor and then overlayered with indicator bacteria as described. Quantitative determinations were performed by testing samples diluted appropriately in phosphate buffer; units of activity were defined as the reciprocal of the highest dilution that inhibited growth of indicator bacteria.

Induction of antibacterial activities. The effect of UV light and mitomycin C on expression of antibacterial activities by *E. herbicola* C9-1 was determined by standard procedures. The role of UV light was assayed by first determining the dose required to lower the population to 37% survival. After irradiating a suspension initially containing 10^8 organisms per milliliter phosphate buffer to obtain this reduction, the bacteria were transferred to liquid MGA and cultivated to mid-log phase. Cells were removed by centrifugation and samples of supernatant fluid and cell-free extract, prepared as described, were assayed for antibacterial activity (14). The effect of mitomycin C was investigated by adding 0, 0.2, 1, or 10 μg per milliliter to cultures (25 ml of MGA/250 ml Erlenmeyer flask) of *E. herbicola* C9-1 previously grown for two transfers (13). The organisms were aerated for 5 hr at 30 C and then harvested by centrifugation; supernatant fluids and cell-free extracts were prepared and assayed as described.

Characterization of antimicrobial activities. Sensitivity to proteolytic enzymes was determined by diffusion in agar (5). A single off-center streak of *E. herbicola* C9-1 on solid MGA was incubated at 30 C for 48 hr. After exposure to $CHCl_3$ vapor, cells composing the streak were overlayered with 5 ml of warm MGA agar containing indicator bacteria. Next, a sterile filter paper strip (1 × 7 cm) previously soaked in a solution of trypsin or pronase (both 1 mg/ml phosphate buffer) or buffer alone was placed at right angles to the bacterial streak. After appropriate incubation at 30 C, sensitivity to proteases was suggested if the indicator grew next to the treated strip.

Effects of pH were determined by adjusting samples of concentrated supernatant fluids to pH values ranging from 2 to 12 with 1 N NaOH or 1 N HCl. The preparations were maintained at 4 C for 2 hr, neutralized with 1 M potassium phosphate buffer at appropriate pH, brought to constant volume with distilled water, and assayed for antibacterial activity.

Partial purification. Herbicolins at 1–5 units/ml were removed from concentrated supernatants by adjustment of samples to pH 3.0 followed by cation exchange chromatography (Dowex 50W ×4, 200–400 mesh, NH_4^+ cycle, equilibrated in 2.5 mM ammonium acetate buffer, pH 5.0). Activity was eluted with 20 mM ammonium acetate buffer, pH 5.0. Preparations obtained were desalted twice by the addition of activated charcoal (Darco G-60, 5 g/100 ml) followed by elution in 50% (v/v) ethanol:water (50 ml/g). The ethanol was removed under reduced pressure at 40 C, and herbicolins were resuspended in distilled, deionized water. The herbicolins obtained were designated as partially purified herbicolins. Final preparations contained 40 units/ml of herbicolin O and 60 units/ml of herbicolin I.

Pear fruit assays. Immature Bartlett pear fruits (2–3 cm in diameter) were collected in the spring and stored at 4 C for no longer than 4 wk. For assays, pears were peeled, sliced (5 mm), quartered, and then placed on sterile filter paper disks (Whatman No. 3) that had been saturated with sterile distilled water (0.1 ml). Pear slices were inoculated with 0.01 ml of sample (phosphate buffer, *E. herbicola* strain C9-1 [10^7 cfu/ml], or partially purified herbicolins). After 15 min, 0.01 ml of *E. amylovora* strain 110, O, HI, or OI (10^7 cfu/ml) was spotted onto the fruit. Petri dishes were sealed with Parafilm and placed in a 30 C incubator for 36 hr. Disease severity was rated on a scale of 1–5; 1 indicated no symptoms; 5 indicated the presence of ooze and complete blackening of the fruit. An average disease-severity rating was determined by observation of five pear slices per dish. These determinations were analyzed as a random complete block design.

RESULTS

Expression of activities. When MGA agar was used as the basal and overlay medium, zones of inhibition around colonies of *E. herbicola* C9-1 were consistently observed in lawns of a variety of gram-positive and gram-negative indicator organisms (Table 1). Addition of 1% yeast extract (Difco) to the overlay markedly reduced or eliminated the zones of inhibition, suggesting that one or more constituents of this natural product blocked antibacterial activity. All 18 strains of *Staphylococcus aureus* were inhibited by *E. herbicola* C9-1 in the presence of yeast extract; potential inhibition of antibacterial activity by this supplement could not be determined for these strains because yeast extract was required for growth.

Antibacterial activity was detected in concentrated culture supernatant fluids (40–100 units/ml), but not in cell-free extract. Titers of antibacterial activity after treatment with UV or mitomycin C were not significantly different from those of untreated controls, indicating that the inhibitory compounds are not inducible.

Resistant mutant phenotypes. Colonies of *E. herbicola* C9-1 produced at least two components active against cells of *E. amylovora* 110 in agar overlay as judged by the presence of a clear inner zone of inhibition surrounded by a larger hazy zone (Fig.

TABLE 1. Sensitivity of various bacteria to inhibitory activities produced in solid medium by strain C9-1 of *Erwinia herbicola*[a]

Indicator species	Strains tested (no.)	Sensitive (no.)
Erwinia amylovora	41	41
Erwinia herbicola	5	5
Erwinia carotovora	1	1
Escherichia coli	3	3
Enterobacter cloacae	1	1
Serratia marcescens	1	1
Enterobacter aerogenes	1	1
Yersinia pseudotuberculosis	1	1
Yersinia entercolitica	2	0
Yersinia pestis	2	2
Klebsiella pneumoniae	1	0
Proteus mirabilis	1	1
Proteus morganii	1	0
Proteus vulgaris	1	1
Pseudomonas aeruginosa	1	0
Pseudomonas fluorescens	1	0
Pseudomonas syringae	1	0
Bacillus megaterium	1	0
Bacillus cereus	2	2
Staphylococcus aureus	18	18

[a] Sensitivity is defined as the presence of a zone of inhibition on lawns of the indicator. Basal and overlay medium were morpholino-propane sulfonic acid (MOPS), gluconate, asparagine agar; yeast extract (1% w/v) was added to the overlayer for growth of *Y. pestis* and *S. aureus*.

1A). Large colonies of presumed resistant mutants arose in both regions of the seeded overlayer. These were isolated, purified, and assayed for sensitivity by the methods described above. Typical strains from the outer hazy zone, termed O mutants, retained sensitivity to at least one activity (Fig. 1B), which presumably accounts for the inner zone seen with the wild type. All mutants obtained from the inner zone, termed I mutants, exhibited a single large hazy zone (Fig. 1D) evidently caused by the activity that accounts for the outer zone seen with wild type. Use of O mutants in the same selective procedure yielded strains, termed OI mutants, that were insensitive to all antibacterial activities (Fig. 1E). It was also evident from these studies that cells of *E. herbicola* C9-1 produced one or more substances capable of inhibiting the antibacterial activity it produced. This effect was especially pronounced with I mutants where a zone of growth occurred over and immediately surrounding the producer colony (Fig. 1D).

Inhibition of antibacterial activity. The striking reduction in the size of antibacterial zones in the presence of yeast extract and the profuse growth of I mutants over the producer colony (Fig. 1D) indicated the presence of an inhibitor that could neutralize at least one of the antibiotics produced by *E. herbicola* C9-1. An attempt was made to identify this inhibitor by spreading 0.05 ml of concentrated supernatant fluid over the surface of a petri dish containing MGA agar. After absorption of liquid, 0.01-ml samples of neutralized solutions or suspensions of amino acids (10 mM) and purine or pyrimidine bases (5 mM) were placed on the agar surface. When dry, the plates were sterilized by treatment with $CHCl_3$ vapor, overlayered with warm MGA agar seeded with cells of *E. amylovora* 110 or its I and O mutants, and incubated for 48 hr at 30 C. No proliferation was observed on plates containing wild-type cells or O mutants, whereas intense growth of I mutants occurred over and adjacent to the area previously spotted with L-histidine. L-histidinol and dipeptides containing L-histidine in either N or C terminal position also inhibited antibacterial activity; D-histidine and histamine had no detectable effect. Although yeast extract prevented killing of wild-type or O mutants of *E. amylovora* 110, this effect was not duplicated by any individually tested amino acid or base.

When cells of *E. amylovora* 110 were tested as described in overlayers containing L-histidine, a single clear zone of reduced size was observed surrounding the producer colony (Fig. 1C). Colonies arising within this zone (termed HI mutants) were purified and again tested in the absence of L-histidine; patterns indistinguishable from those observed for I mutants (Fig. 1D) were obtained for HI mutants (not shown). Addition of L-histidine to the overlayer greatly reduced the area of inhibition detected in lawns of I mutants (Fig. 1F) or HI mutants (not shown); the pattern observed with O mutants (Fig. 1B) was not affected by added L-histidine (not shown).

Characterization of activities. Results obtained by assay of *E. amylovora* 110 and its mutants in the presence and absence of L-histidine indicated the existence of at least two antibacterial compounds. The first diffused away from the producer colony rapidly, where it produced a hazy outer zone of inhibition that was readily reversed by added L-histidine. The second diffused more slowly, and its activity was not reversed by L-histidine. Subsequent study showed that both of these activities were dialyzable, indicating molecular weights of 3,500 or less and that both activities were resistant to proteolytic enzymes. We thus termed the histidine-sensitive component in the outer zone herbicolin O and the histidine-resistant activity composing the inner zone herbicolin I. Herbicolin O was labile to acid (pH 3.5) and to base (pH 10), whereas herbicolin I was labile only to base (pH 10). Herbicolins were slightly soluble in ethanol and insoluble in butanol, chloroform, acetone, and isopropanol. Under acidic conditions herbicolins were extracted into butanol, which indicated that they are weak acids.

Sensitivity of species other than *E. amylovora* to the herbicolins was determined by assay in the presence or absence of L-histidine in overlayers composed of Atherton medium. As shown in Table 2, herbicolin O had a broad activity spectrum, whereas that of herbicolin I was limited to *E. amylovora*, *S. aureus*, and one strain of *Bacillus cereus*.

Bacteriocin activity. *Escherichia coli* Ϙand its mutants known to be resistant or tolerant to group A and B colicins (9) were also used as indicators of herbicolins produced by colonies of *E. herbicola* C9-1. All strains were sensitive to herbicolin O and resistant to herbicolin I. In addition, small variable zones of inhibition were observed during growth with L-histidine that could be eliminated if $FeCl_3$ (20 μM) was included in the overlayer. Mutant *ivt* (colicin I tolerant) and *cir* (colicin I resistant) organisms were not inhibited

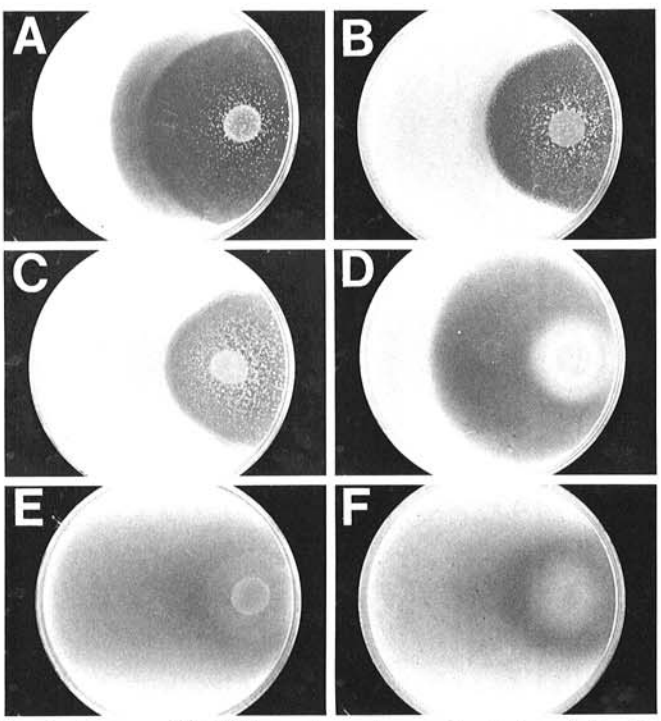

Fig. 1. Patterns of inhibition observed around colonies of *Erwinia herbicola* C9-1 grown on morpholino-propane sulfonic acid, gluconate, asparagine (MGA) agar following addition of MGA agar overlayers containing **A,** *Erwinia amylovora* 110; **B,** O mutants of *E. amylovora* 110; **C,** *E. amylovora* 110 plus L-histidine (10 mM); **D,** I mutants of *E. amylovora* 110; **E,** IO mutants of *E. amylovora* 110; and **F,** I mutants of *E. amylovora* plus L-histidine (10 mM).

TABLE 2. Sensitivity of various bacteria to the herbicolins present in supernatant fluids of *Erwinia herbicola* C9-1[a]

Indicator species	Strains tested (no.)	Sensitive[b] (no.)	
		Herbicolin 0	Herbicolin I
Erwinia amylovora	4	4	4
Erwinia carotovora	1	1	0
Enterobacter aerogenes	2	2	0
Escherichia coli	2	1	0
Serratia marcescens	1	1	0
Proteus mirabilis	1	1	0
Proteus vulgaris	2	2	0
Yersinia entercolitica	1	0	0
Yersinia pestis	1	1	0
Yersinia pseudotuberculosis	1	1	0
Shigella dysenteriae	1	1	0
Salmonella typhimurium	1	1	0
Pseudomonas aeruginosa	1	0	0
Bacillus cereus	2	2	1
Bacillus megaterium	1	0	0
Staphylococcus aureus	2	2	2

[a] Concentrated supernatants, obtained from liquid MOPS, gluconate, asparagine (MGA) cultures of *E. herbicola* strain C9-1, contained 60 units/ml of herbicolin 0 and 40 units/ml of herbicolin I. Atherton medium was used for the overlayer; MGA composed the basal medium.

[b] Herbicolin 0 was defined as L-histidine antagonized activity. Herbicolin I was the residual activity.

by this activity. This finding would be anticipated if *E. herbicola* C9-1 also produced colicin I or a related activity.

Production of herbicolins by *E. herbicola* 112Y. *E. herbicola* 112Y, producer of the bacteriocinlike compound herbicolicin 112Y, expressed antibacterial activity on solid MGA (Table 3). In most cases, the size of inhibitory zones was reduced by incorporation into overlayers of yeast extract but not L-histidine. Patterns of resistance in selected *E. amylovora* strains indicated that the antibacterial activities produced by *E. herbicola* strains C9-1 and 112Y differed by at least the lack of a histidine-reversible activity, namely herbicolin O in the latter (Table 4).

Immature fruit assays. The effects of *E. herbicola* C9-1 and of its partially purified herbicolins in inhibiting severity of disease are presented in Tables 5 and 6, respectively. The virulence of *E. amylovora* strains 110, I, O, and OI were not significantly different in pear fruit assays.

DISCUSSION

E. herbicola C9-1 produced at least two antibiotics in vitro, termed herbicolin O and I, as judged by comparison of antimicrobial spectra, specificity of antagonism by L-histidine, and isolation of two classes of herbicolin-insensitive mutants of *E. amylovora* 110. Herbicolin O was a broad spectrum antibiotic that was antagonized by L-histidine and destroyed by extremes of pH. In contrast, herbicolin I exhibited a narrow antibacterial spectrum, was not inhibited by L-histidine, and was sensitive only to alkaline conditions. Both herbicolins were low molecular weight molecules that were protease-resistant, noninducible by UV or mitomycin C, and inhibitory to growth of bacteria unrelated to their producer. Accordingly, herbicolins O and I are clearly not bacteriocins. The possibility exists, of course, that *E. herbicola* C9-1 may produce additional herbicolins. Similarly, bacteriocins as yet uncharacterized may also be expressed in agar medium as judged by the complex patterns of inhibition observed in lawns of *Escherichia coli* Φ and its colicin-tolerant and -resistant derivatives.

An unanticipated result of these determinations was detection of antibiotic rather than bacteriocin production by *E. herbicola* 112Y. Further study will be necessary to demonstrate that *E. herbicola* 112Y does not exhibit herbicolicin activity. However, results of the present work indicate that herbicolicin 112Y of others (11,23) and herbicolin I described here may be closely related. For example, *E. amylovora* strain 336, an herbicolicin 112Y-resistant mutant, was also insensitive to herbicolin I but was inhibited by herbicolin O (Table 4). Similarly, both herbicolin I and antibacterial activity from *E. herbicola* 112Y inhibited only *E. amylovora*, *S. aureus*, and *B. cereus* in the presence of yeast extract (Table 3), and both structures were heat stable, alkali labile, protease resistant, and of low molecular weight (23). Initial consideration of the activity from *E. herbicola* 112Y as a bacteriocin may have reflected its limited spectrum of activity and requirement for full expression in minimal fully buffered medium. These limitations of detection are also similar to those of certain microcins (2) and β-lactam antibiotics (24). Since herbicolin O-resistant and herbicolin I-resistant mutants of *E. amylovora* 110 remained partially sensitive to *E. herbicola* 112Y, the possibility remains that the latter expresses an additional antibiotic. This activity was not detected when yeast extract was present in the overlayer (Table 4) but, unlike herbicolin O, was not inhibited by L-histidine.

Several investigators have reported that inoculation of host tissues with *E. herbicola* or unidentified yellow saprophytic bacteria prior to infection with *E. amylovora* correlated with reductions in severity of disease (3,15,21,25). Our results from pear fruit assays suggest that cells of *E. herbicola* C9-1 can also

TABLE 3. Antibacterial activity produced by colonies of strain 112Y of *Erwinia herbicola* grown on solid MGA medium

Indicator species	Strains tested (no.)	Sensitive[a] (no.)	
		Without yeast extract	With yeast extract
Erwinia amylovora	3	3	3
Erwinia carotovora	1	1	0
Escherichia coli	2	2	0
Klebsiella pneumoniae	1	0	0
Pseudomonas aeruginosa	1	0	0
Bacillus megaterium	1	0	0
Bacillus cereus	1	1	1
Staphylococcus aureus	2	NG[b]	2

[a] Yeast extract (1%) was added to MGA overlayer medium.
[b] NG = no growth.

TABLE 4. Comparison of the antibacterial activities produced in solid MGA medium by strains C9-1 and 112Y of *Erwinia herbicola*

Producer strain[a]		Indicator strains		
C9-1	112Y	Strain	Source	Description
+[b]	−	*Erwinia herbicola* 112Y	S. V. Beer	Produces herbicolicin 112Y
−	−	*E. herbicola* C9-1	This report	Produces herbicolin 0 and herbicolin I
+[b]	+	*E. amylovora* 273	S. V. Beer	Wild type
+[b]	−	*E. amylovora* 336	S. V. Beer	Insensitive to herbicolicin 112Y
+[b]	+	*E. amylovora* 110	D. Ritchie	Wild type
+[b]	+	*E. amylovora* (I mutant)	This report	Insensitive to herbicolin I
+	+	*E. amylovora* (0 mutant)	This report	Insensitive to herbicolin 0
−	−	*E. amylovora* (10 mutant)	This report	Insensitive to herbicolin 0 and herbicolin I

[a] + = Zone of inhibition present; − = no zone of inhibition present.
[b] Zone of inhibition reduced by addition of L-histidine into overlayer.

TABLE 5. Effect of strain C9-1 of *Erwinia herbicola* on severity of fire blight caused by wild-type and herbicolin insensitive mutants of *Erwinia amylovora* 110 in pear Bartlett fruit slices[x]

Treatment	Mean disease-severity rating[y]
Buffer[z]; *E. amylovora* 110	5.0 a
Buffer; *E. amylovora* OI	4.9 a
Buffer; *E. amylovora* HI	4.6 a
Buffer; *E. amylovora* O	4.7 a
E. herbicola; *E. amylovora* 110	1.8 c
E. herbicola; *E. amylovora* OI	1.5 c
E. herbicola; *E. amylovora* HI	2.7 b
E. herbicola; *E. amylovora* O	2.7 b

[x] 2- to 3-cm immature pear fruit were peeled, sliced (3 mm), and quartered.
[y] Average disease severity rating of five slices per petri dish, with three replications; 1 = no symptoms; 5 = ooze and complete blackening of tissues. Numbers with the same letter were not significantly different ($P = 0.05$).
[z] 0.033 M potassium phosphate buffer, pH 7.0.

TABLE 6. Effect of partially purified herbicolins[y] on severity of fire blight caused by wild-type and herbicolin insensitive mutants of *Erwinia amylovora* 110 in pear Bartlett fruit slices

Treatments	Mean disease-severity rating[z]
Buffer; *E. amylovora* 110	5.0 a
Buffer; *E. amylovora* OI	4.4 a
Buffer; *E. amylovora* HI	4.9 a
Buffer; *E. amylovora* O	4.8 a
Herbicolins; *E. amylovora* 110	1.9 c
Herbicolins; *E. amylovora* OI	2.4 c
Herbicolins; *E. amylovora* HI	4.5 a
Herbicolins; *E. amylovora* O	3.4 b

[y] Herbicolins were purified by cation exchange chromatography (see Materials and Methods). Preparations contained 40 units/ml of herbicolin O and 60 units/ml herbicolin I at 20 C.
[z] As defined in Table 5.

antagonize development of fire blight in immature pear fruit (Table 5); partially purified herbicolins had a similar effect (Table 6). This phenomenon was probably mediated by the applied herbicolins per se because mutation of *E. amylovora* 110 to herbicolin resistance decreased the ability of these structures to control disease. However, resistance to both herbicolin O and herbicolin I did not decrease the degree of control obtained (Tables 5 and 6). This may be due to the nature of herbicolin resistance in *E. amylovora* OI or, alternatively, to some other consequence of multiple herbicolin insensitivity such as slower growth in vitro. Considered together, these results suggest that herbicolins alone may not account for the full protective effect achieved with *E. herbicola* C9-1 or its partially purified antibiotics. The data presented here provide an initial evaluation of the stability and potential in vivo activity of the multiple activities produced by *E. herbicola* C9-1. We have now purified herbicolins O and I to homogeneity and have proposed a structure for the latter (*unpublished*).

LITERATURE CITED

1. Atherton, F. R., Hall, M. J., Hassall, C. H., Lambert, R. W., and Ringrose, P. S. 1979. Phosphonopeptides as antibacterial agents: Rationale, chemistry, and structure-activity relationships. Antimicrob. Agents Chemother. 15:677-683.
2. Baquero, F., and Moreno, F. 1984. The microcins. FEMS Microbiol. Lett. 23:117-124.
3. Beer, S. V. 1981. Towards biological control of fire blight. (Abstr.) Phytopathology 71:859.
4. Beer, S. V., and Vidaver, A. K. 1978. Bacteriocins produced by *Erwinia herbicola* inhibit *E. amylovora*. Page 75 in: Abstracts of the Third International Congress of Plant Pathology. W. Laux, ed. Munich, Deutsche Phytomedizinische Gesellschaft. Göettingen, Germany.
5. Brubaker, R. R., and Surgalla, M. J. 1961. Pesticins. I. Penticin-bacterium interrelationships, and environmental factors influencing activity. J. Bacteriol. 82:940-948.
6. Burrows, T. W., and Gillett, W. A. 1966. The nutritional requirements of some *Pasturella* species. J. Gen. Microbiol. 45:333-345.
7. Chatterjee, A. K., Gibbins, L. N., and Carpenter, J. A. 1968. Some observations on the physiology of *Erwinia herbicola* and its possible implications as a factor antagonistic to *Erwinia amylovora* in the "fire-blight" syndrome. Can. J. Microbiol. 15:640-642.
8. Ewing, W. H., and Fife, M. A. 1972. *Enterobacter agglomerans* (Beijerinck) comb. nov. (The *herbicola*-lathyri bacteria). Int. J. Syst. Bacteriol. 22:4-11.
9. Ferber, D. M., Fowler, J. M., and Brubaker, R. R. 1981. Mutations to tolerance and resistance to pesticin and colicins in *Escherichia coli*. J. Bacteriol. 146:506-511.
10. Freund, E. A., and Winkelman, G. 1984. Activity of herbicolin A against *Mycoplasma*, *Acholeplasma*, *Ureaplasma*, and *Spiroplasma* species. Antimicrob. Agents Chemother. 26:112-114.
11. Gantotti, B. V., Kindle, K. L., and Beer, S. V. 1981. Transfer of the drug-resistance transposon Tn*5* to *Erwinia herbicola* and the induction of insertion mutations. Curr. Microbiol. 6:377-381.
12. Graham, D. C., and Hodgkiss, W. 1967. Identity of gram negative, yellow pigmented, fermentative bacteria isolated from plants and animals. J. Appl. Bacteriol. 30:175-189.
13. Hu, P. C., and Brubaker, R. R. 1974. Characterization of pesticin: Separation of antibacterial activities. J. Biol. Chem. 249:4749-4753.
14. Hu, P. C., Yang, G. C. H., and Brubaker, R. R. 1972. Specificity, induction, and absorption of pesticin. J. Bacteriol. 112:212-219.
15. Isenbeck, M., and Schulz, F. A. 1985. Biological control of fireblight (*Erwinia amylovora* (Burr.) Winslow et al.) on ornamentals. Control of the pathogen by antagonistic bacteria. Phytopathol. Z. 113:324-333.
16. Ishimaru, C., and Klos, E. J. 1984. New medium for detecting *Erwinia amylovora* and its use in epidemiological studies. Phytopathology 74:1342-1345.
17. Ishimaru, C., and Klos, E. J. 1984. Antibiotic production in *Erwinia herbicola* strain C9-1. (Abstr.) Phytopathology 74:881.
18. Neidhardt, F. C., Bloch, P. L., and Smith, D. F. 1974. Culture medium for enterobacteria. J. Bacteriol. 119:736-747.
19. Nomura, M. 1967. Colicins and related bacteriocins. Annu. Rev. Microbiol. 21:257-284.
20. Parker, W. L., Rathnum, M. L., Wells, J. S., Jr., Trejo, W. H., Principe, P. A., and Sykes, R. B. 1982. SQ 27,860, a simple carbapenem produced by species of *Serratia* and *Erwinia*. J. Antibiot. 35:653-660.
21. Riggle, J. H., and Klos, E. J. 1972. Relationship of *Erwinia herbicola* to *Erwinia amylovora*. Can. J. Bot. 50:1077-1083.
22. Schneierson, S. S., and Bottone, E. J. 1973. *Erwinia* infections in man. Crit. Rev. Clin. Lab. Sci. 4:341-355.
23. Stein, J. I., and Beer, S. V. 1980. Partial purification of a bacteriocin from *Erwinia herbicola*. (Abstr.) Phytopathology 70:469.
24. Sykes, R. B., Parker, W. L., and Wells, J. J. 1982. β-lactam antibiotics produced by bacteria. Pages 115-124 in: Int. Conf. Trends Antibiot. Res. H. Umezawa, A. L. Demain, T. Hata, and C. R. Hutchinson, eds., Int. Acad., Tokyo.
25. Thompson, S. V., Schroth, M. N., Moller, W. J., and Reil, W. O. 1976. Efficacy of bacteriocides and saprophytic bacteria in reducing colonization and infection of pear flowers by *Erwinia amylovora*. Phytopathology 66:1457-1459.
26. Winkelman, G., Lupp, R., and Jung, G. 1980. Herbicolins—new peptide antibiotics from *Erwinia herbicola*. J. Antibiot. 33:353-358.