HERBICOLINS O, I AND 2C: NOVEL BETA-LACTAM ANTIBIOTICS PRODUCED BY *ERWINIA HERBICOLA* STRAIN C9-1

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To Dan and Nicky

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INTRODUCTION

Erwinia herbicola is a ubiquitous saprophyte that displays a diversity of interesting phenotypes; free living nitrogen fixation, ice nuclease production, and production of antibiotics or bacteriocins have all been reported. Another interesting attribute of this species is its *in vivo* ability to antagonize the development of the pathogen, *E. amylovora*, causal agent of fire blight on Rosaceous hosts. The detection of bacteriocin production in *E. herbicola* was met with great excitement; biological control of *E. amylovora* would, hopefully, be accentuated through the use of bacteriocin producing strains. However, the results obtained by this strategy have been inconsistent, and have not confirmed that bacteriocin production per se enhances the antagonism.

From studies on the characterization of a bacteriocin from *E. herbicola* strain 112Y, it is evident that this compound is not a bacteriocin in the classical sense; herbicolicin 112Y is not a protein. The nature of antimicrobial activities produced by *E. herbicola* was of interest to me. It seemed reasonable that the proper identification of antimicrobial activities produced by *E. herbicola* could aid our understanding of the types of interactions one might anticipate *in situ*. I pursued this interest by studying the antimicrobial activities produced by *E. herbicola* strain C9-1. Later, an understanding of the requirements for production and detection of antibiotics by strain C9-1 enabled the detection of antibiotic production in strain 112Y.

This dissertation is divided into four sections. The first is a literature review that provides background information on *E. herbicola* as an antagonist of *E. amylovora*, and summarizes the recent reports of antibiotics produced by members of the *Enterobacteraceae*. The second section is a preparation of a manuscript to be submitted for publication, which defines the requirements for multiple antibiotic production by *E. herbicola* strain C9-1. The third section is also a preparation of a manuscript to be submitted for publication. This paper presents procedures for the purification of three antibiotics (herbicolins) from broth culture of *E. herbicola* strain C9-1, the identification of these herbicolins as novel beta-lactam antibiotics, and the antimicrobial activity spectrum of each herbicolin. The fourth section presents results of preliminary investigations into the use of *E. herbicola* strain C9-1 and partially purified herbicolins as controls of *E. amylovora in vivo*.

SECTION I

LITERATURE REVEIW

Nomenclature

The Herbicola group of the genus Erwinia and Enterobacter agglomerans are collectively referred to as the Herbicola-Agglomerans group (9, 64). Bacteria in this group are typically yellow, peritrichously flagellate, nitrate reducing, Gram negative rods. Herbicola-Agglomerans group strains are ubiquitous; they have been isolated from plants, animals, soil and water (23, 40, 43, 90). Though most Herbicola-Agglomerans isolates are plant saprophytes, some isolates are human pathogens, which cause secondary and septicemic infections (66, 74). Other isolates are plant, insect or fungal pathogens (58). Some isolates are reported which fix nitrogen (77), produce bacteriocins (7, 39), antibiotics (70, 90) or ice nucleases (58). Anaerogenic and aerogenic isolates are included in the complex (29). Non-nitrate reducing and non-pigmented isolates have also been included. Because of its diversity, the Herbicola-Agglomerans complex has posed an, as yet, unresolved nomenclatural challenge to the systematic bacteriologist (9).

Taxonomy of the Herbicola-Agglomerans group is undertaken in Dye's classification of the genus, Erwinia. Dye subdivides Erwinia into the herbicola, the amylovora, the carotovora and the atypical erwinia groups (21, 22, 23, 24). Herbicola-Agglomerans taxonomy is specified in the herbicola group discussions (23). Dye has proposed that the nomenspecies: *Enterobacter agglomerans*, *Erwinia milletiae*, and *E. herbicola*, commonly used to name Herbicola-Agglomerans isolates, be combined into the one nomenspecies, *E. herbicola*; the other nomenspecies are considered synonyms for *E. herbicola*. Though Dye's proposals have been adapted by Lelliot and have subsequently appeared in the Eighth edition of Bergey's Manual (57), naming of Herbicola-Agglomerans complex isolates is still inconsistent. Plant microbiologists implement Dye's suggestions for naming Herbicola-Agglomerans strains, but clinical microbiologists prefer the nomenspecies *En. agglomerans* (10), citing the recommendations of Ewing and Fife (29). The 1980 Approved List of Bacterial Names accentuates this nomenclatural disparity; *En. agglomerans*, *E. herbicola*, and *E. milletiea* are all listed, without a reference to their proposed synonomy (79).

Numeric taxonomic studies (25, 40), protein electrophoretic patterns (63) and deoxyribonucleic acid homologies (9, 36) have determined that Herbicola-Agglomerans strains are highly related to other *Enterobacteraceae*, but that they are also inherently heterogenous. A recent DNA homology study of clinical and plant isolates has indicated that several distinguishable species and possibly more than one genus are represented in the Herbicola-Agglomerans complex (9). However, the authors have proposed that the renaming of Herbicola-Agglomerans isolates be postponed until more isolates have been examined. Until then, *E. herbicola* (Lohnis) Dye would be the appropriate nomenspecies for Herbicola-Agglomerans isolates (9, 23).

Genetics

Though few studies have been conducted, genetic investigations have corroborated that *E. herbicola* can accept and transfer a variety of plasmids. Conjugative transfer of R (13, 15, 38) and F'factors (12), and transformation with pBR322 (56) has been reported. F'lac+ has been transferred from *Es. coli* to lac- *E. herbicola* isolates. F'lac+ *E. herbicola* exconjugates can then donate lac+ to other *Erwinia* spp., *Escherichia*, and *Shigella* (12). In addition, lactose utilizing strains of *E. herbicola* have demonstrated that a novel sex factor, E factor, exists in both clinical and plant isolates. Exconjugates, obtained from an *E. herbicola* lac- and an *E. herbicola* lac+ mating, can donate lac+ to F'lac- *Es. coli* strains. E factor's existence was further deduced by plasmid compatibility experiments and from the insensitivity of *E. herbicola* lac+ donors to the F-specific bacteriophage, M13 (14). R-factor mediated multiple drug resistance has been transferred to *E. herbicola* from

Shigella flexneri and *Pseudomonas aeruginosa* (38). These findings hint to a potentially hazardous situation (13, 15); it has been inferred that agricultural usage of clinically important bactericides should be limited (37).

Indigenous plasmids have been correlated with specific phenotypes of *E. herbicola*. For example, yellow pigmentation and thiamine prototrophy are correlated with a ca. 350 Mdal plasmid; isolates cured of this plasmid by growth at supraoptimal temperatures (>36C) are white and auxotrophic for thiamine (34). Production of herbicolicin 112Y, a bacteriocin-like compound (discussed later), is correlated with a ca. 96 Mdal plasmid; transposon mutagenesis was used to generate selectable, non-producing mutants of *E. herbicola* strain 112Y (35). Nitrogen fixing strains of *E. herbicola*, obtained from the wheat rhizosphere, contain plasmids that have been correlated with the strains' ability to fix nitrogen. When nitrogen fixing strains were cured of their plasmid, nitrogen fixation was undetected. Furthermore, plasmids from five of these nitrogen fixing strains hybridized to a ^{32}P-labelled *nif*KDH probe (77).

Antagonism of plant disease

Yellow saprophytic bacteria, and their ability to prevent or delay plant disease, have been studied for more than fifty years. Their antagonism of the fire blight disease on apple and pear has been of particular interest.

Fire blight, caused by E. amylovora (Burr.) Winslow et al., is both a sporadic and a devastating disease on many Rosaceous plants. The greatest economic losses due to fire blight are reported in the pear and apple industries, and more recently, the ornamental industry. These losses are substantial; estimated losses due to fire blight for a given year can be in the millions of dollars. Fire blight control strategies depend on the removal of diseased plant material, and on the application of bactericides, such as streptomycin (93). However, the detection of streptomycin resistant E. amylovora isolates in California (65), Oregon and Washington (16), and the demonstration of genetic promiscuity in Erwinia species (13, 15, 17), have prompted consideration of alternative control strategies. For example, weather monitoring and early detection of the pathogen with a selective medium is used in California to both time and limit the number of streptomycin applications (64, 84). Another strategy, which has received a good deal of attention, but which has not been economically advantageous, is the biological control of fire blight with saprophytic bacteria; E. herbicola has received most of the attention in this regard.

In 1929, Shaw reported that Bartlett pear tissue was less susceptible to fire blight after it had been sprayed with a suspension of a yellow saprophytic bacterium. He conducted detailed studies on yellow saprophytic bacteria and named them, Flavobacterium (93), a name now synonymous with E. herbicola (23). Since this early report, numerous E. herbicola isolates and unidentified yellow saprophytic bacteria have been tested for similar effects (28, 42, 72, 85). From such studies, it has been determined that inoculation of host tissues with E. herbicola, prior to inoculation with E. amylovora, can reduce disease severity; inoculation of tissues with E. herbicola after inoculation with E. amylovora has been ineffectual. Disease severity has also been decreased when suspensions of E. amylovora and yellow saprophytic bacteria are mixed together prior to their inoculation onto the host; the decrease is proportional to the length of the premixing period (41).

One explanation for E. herbicola's antagonism of fire blight in blossoms has postulated that colonization of flowers by E. herbicola produces an acidic environment, which is unfavorable to growth of the pathogen (72). This hypothesis has developed from the following observations: E. herbicola produces acid from a variety of carbon sources in vitro (23); clearings around E. herbicola colonies, in lawns of E. amylovora, have been eliminated when the medium was buffered (30); when E. herbicola has been grown in a simulated nectar medium, and the cells removed, the spent medium (pH 4.2) does not support E. amylovora growth; spent nectar medium supports E. amylovora growth if the pH is neutralized and nitrogen and carbon sources are resupplied (72). If a similar pH reduction occurs in blossoms colonized by E. herbicola, growth of E. amylovora would be inhibited (30, 72). Critics of this hypothesis purport that the natural buffering capacity of plant tissues is sufficient to maintain a favorable pH, and that *in situ* acid accumulation has not been demonstrated (39).

Studies on fire blight resistance in pear suggest another explanation for the antagonism of fire blight by *E. herbicola* in pear tissue. When leaf sections of various pear varieties are placed onto lawns of *E. amylovora*, zones of inhibition have been detected around certain varieties (53). The inhibitor has been identified as hydroquinone; a beta-glucosidase in pear produces hydroquinone and D-glucose from arbutin, the major beta-glucoside in pear (49). Hydroquinone is produced by the plant in response to injury or pathogen invasion (48). It is toxic to *E. amylovora* at a concentration of 25 mg/ml. *E. herbicola* is insensitive to hydroquinone concentrations of 1 mg/ml (11). In addition, all tested *E. herbicola* isolates produce hydroquinone when grown in a defined medium containing arbutin as the sole carbon source (11). It has been proposed that *E. herbicola* contributes to the plant's hydroquinone level, and thereby, aids in protecting the plant from pathogen invasion (11). A similar scenario for apple is unsubstantiated, because few *E. herbicola* isolates produce phloretin from phlorezin, the major beta-glucoside found in apple, and phloretin's toxicity to *E. amylovora* is equivocal (37).

The possibility that *E. herbicola* effectuates an induced resistance response in the host plant has been investigated (42, 62, 92). A variety of tissues, including etiolated pear seedlings, pear stems, pear fruit, and apple fruit, were used in these studies. It has been determined that a 24 hour interval between application of an inducer (*E. herbicola*) and a challenger (*E. amylovora*) is more effective than a 0.5 hour interval in delaying disease development (62, 92). This effect of *E. herbicola* is considered non-specific, because similar results have been obtained using avirulent *E. amylovora* or *Pseudomonas tabaci* strains as inducers (42, 62). *Xanthomonas pruni*, however, is an ineffectual inducer (42). Cell sonicates from virulent and avirulent *E. amylovora* isolates also delay symptom development, but cell sonicates from yellow saprophytic bacteria (*E. herbicola*?) or *P. tabaci* do not (62). There is some evidence which suggests that a permanent resistance to fire blight can be induced by *E. amylovora* cell sonicates (62). Since cell sonicates do not inhibit *E. amylovora in vitro*, a host response appears to be involved. However, these limited observations have neither confirmed nor disproved that *E. herbicola* induces disease resistance in plant tissues.

The potential use of bacteriocins and bacteriophage as controls of plant pathogenic bacteria has been considered (86). Since *E. herbicola* is apparently antagonistic to fire blight development, and production of bacteriocins (7, 39) and bacteriophage (27) of *E. herbicola* have been reported, the biological control of fire blight with bacteriocins and bacteriophage has appeared plausible. Indeed, some success, as measured by reductions in blossom blight severity, is afforded by inoculating apple blossoms with strains of *E. herbicola* which produce bacteriocin-like antibiotics (5, 6). Production of bacteriocin-like antibiotics is considered incidental to the results obtained, because non-producing strains have also reduced disease severity (5, 6). Temperate bacteriophage of *E. herbicola*, which also lyse *E. amylovora*, can affect blight development *in vitro* (27). When pear fruit slices are inoculated with lysogenic *E. herbicola* isolates, prior to their inoculation with the pathogen, blight symptoms are absent; when inoculated with non-lysogenic *E. herbicola* isolates, symptoms are merely delayed (27).

Antibiotics produced by Enterobacteria

Most commercially important antibiotics are produced synthetically or semi-synthetically from fermentation products of actinomycetes and fungi. Bacteria, other than a few *Bacillus* species, have been considered insignificant sources of antibiotics. However, improved detection methods have recently enabled the discovery of an abundance of novel bacterially produced antibiotics (83). Of special interest are the antibiotics produced by Gram-negative bacteria; in addition to their potential for commercial application, the question of their ecological significance has been posed (20). A discussion of some of the antibiotics produced by members of the Enterobacteraceae follows.

Bacteriocins. Bacteriocins are a specific class of antibiotics, characterized by their proteinaceous composition and by their limited antimicrobial spectra; only those species which are closely related to the producer are affected (68). Bacteriocins have been detected in both Gram-negative and Gram-positive bacteria, including the plant related genera: Erwinia, Agrobacterium, Pseudomonas, Bacillus, Corynebacterium and Xanthomonas. Excellent reviews concerning bacteriocin production, isolation, characterization, and genetics are available (18, 19, 46, 68). Salient features of bacteriocin production include the following: they are induced in complex media by SOS-system inducing mutagens, such as, mitomycin C or ultraviolet light; they are plasmid encoded gene products; they are produced in early and mid exponential growth phases.

Colicins. Most of our knowledge regarding bacteriocins has evolved from studies on colicins, the bacteriocins produced by Es. coli. Gratia was the first to note the specific bactericidal affect of Es. coli strain V on another Es. coli strain. Jacob et al. named these inhibitors, bacteriocins (68). Since then, approximately 20 colicins have been discovered. They have been classified into one of two groups, A or B, depending on cross resistance and tolerance of their producers to other colicins (18, 19). Colicin resistant strains have lost the receptor for that colicin. Colicin tolerant strains retain the receptor for that colicin, but are insensitive to its action (18, 19).

Colicins classified in Group A are colicins A, E1, E2, K, L, N, S4 and X (19). Binding of group A colicins to sensitive cells requires energy (51). In addition, a functional _bfe_ gene product is required for lethal binding of colicins E1, E2 and E3 (19). The _bfe_ gene encodes for a 60K outer membrane protein, which is also the vitamin B 12 receptor (89). Colicin K, bacteriophage T6 and nucleoside uptake all require the _tsx_ gene product (45).

Colicins classified as group B colicins are B, D, G, H, I , M, S, V and Q (18). Group B colicins require a functional _ton_ B gene product (18), which is an essential iron transport protein (33). Mutants lacking a functional _ton_ B gene product are resistant to all group B colicins and the bacteriophage TI and phi 80 (18). Proteins that are produced in iron deficient growth media, such as, the _Feu_ B (44), the _Feu_ A (71) or the _ton_ A (51) gene products, are required for lethal binding of certain colicins (18).

The ability to produce colicins is determined by genetic information located on plasmids called, col factors (68). Col factors are classified as Group I or Group II, on the basis of their size (46). Group A colicins, with one exception, are encoded for by the small (ca. 5 Mdal) Group I col factors; group B colicins are encoded for by the larger (ca. 60-100 Mdal) group II col factors. Group II col factors resemble F and I plasmids, in that they are self-transmissible. Increases in col factor copy number have, in some cases, been observed after induction of colicin production. (Summarized in reference 46.)

Bacteriocin-like antibiotics. Antibiotics which inhibit only those species that are closely related to their producer, but which are non-proteinaceous, have been called bacteriocin-like antibiotics. Agrocin 84 (54, 55) and herbicolicin 112Y (6, 81) are examples of this class of antibiotics. Agrocin 84 is produced by *Agrobacterium radiobacter* var. *radiobacter* strain K84 (55). It specifically inhibits *A. radiobacter* strains that contain a nopaline type TI plasmid; the TI plasmid also confers virulence to the strain (87). Agrocin 84 is a phosphoamidate of an adenine deoxyarabinoside (73), which inhibits DNA synthesis (61). Production of agrocin 84 has been correlated with a 30 Mdal plasmid (26, 80).

Herbicolicin 112Y is a bacteriocin-like antibiotic produced by *E. herbicola* strain 112Y (81). Herbicolicin 112Y is especially inhibitory to *E. amylovora*. Strains outside of the *Enterobacteraceae* have been insensitive to herbicolicin 112Y (50). Herbicolicin 112Y is heat and acid stabile, base labile, protease and nuclease resistant, ethanol soluble and of low molecular weight (< 800). The highest titers of herbicolicin 112Y are obtained after late logarithmic growth of the producer (81). As was previously stated, herbicolicin 112Y production has been correlated with a ca. 96 Mdal plasmid (35). More than one gene product may be involved in herbicolicin 112Y production (35).

Microcins. Bacteria, primarily members of the Enterobacteraceae, have recently been reported to produce low (<5,000) molecular weight peptide antibiotics (2, 4). These antibiotics have been termed, microcins (2). Microcins are constituitively produced and non-inducible (47). They are produced in minimal media after late logarithmic growth of the producer (2, 4). They are, as most antibiotics are, secondary products of metabolism that require more than one gene product for production (4). The activity spectra of microcins includes bacteria unrelated to the producer strain, but microcins from Es. coli, for example, are most active against other Enterobacteraceae (2). Since microcin production and detection has required a minimal growth medium, growth of some indicators is poor; sensitivity of Gram-positive bacteria to microcins has been inconclusive in some studies (4). However, the sensitivity of Staphylococcus and Bacillus to certain microcins has been demonstrated (2). Generally, microcins are resistant to extremes in pH and temperature, and to nucleases and to proteases; individual microcins are resistant to some, if not all, of these conditions (2, 4).

All microcins that have been studied require at least one plasmid determined gene product for synthesis. The plasmids involved in microcin production are called M plasmids (3). Additional gene products, some of which may be chromosomally determined, are also required for microcin production. For example, synthesis of microcin B17 requires seven gene products, three of which are of chromosomal origin (47). Immunity of a producer to its microcin is also plasmid encoded (4). M plasmid size and cross resistance patterns of microcin producers have been used to group microcins into five classes: A, B, C, D or E (4, 47).

Information on the mode of action of certain microcins is available. Microcin A15 is an analogue of L-methionine, whose action is reversed by L-methionine and precursors of L-methionine. Microcin A15 inhibits homoserine-O-transsuccinylase, the first enzyme in biosynthesis of methioinine (1). Microcin B 17 is an inhibitor of DNA replication (4). Microcin C7 inhibits protein synthesis (4).

Some previously described bacteriocins may be more appropriately called microcins (20). Colicin V, for example, has been an anomaly in colicin classification, because it is non-inducible, and of low molecular weight colicins (32). Other bacteriocins that have been suggested to be microcins are produced by <u>Staphylococcus</u>, <u>Lactobacillus</u> and <u>Streptococcus</u> (20). Also, bacteriocin-like antibiotics from Salmonella typhimurium (8) and a gonococcal inhibitor produced by Es. coli (76) may be microcins (20). The supposition is that microcins represent a link in a continuum between classical bacteriocins and classical broad spectrum, amino acid derived, beta-lactam antibiotics (20).

Beta-lactam antibiotics. In addition to producing bacteriocins and microcins, bacteria also produce beta-lactam antibiotics (83). Bacterially produced beta-lactam antibiotics are a recent discovery, made possible by the development of indicators that are highly sensitive to beta-lactam antibiotics (83). Beta-lactam antibiotics all contain a characteristic four membered heterocycle, called a beta-lactam ring. In many cases, the beta-lactam ring is fused to another heterocylce to form a bicylic compound. Penicillin and cephalosporin are well known beta-lactam antibiotics (91). Tabtoxin, produced by Pseudomonas tabaci, is a beta-lactam that has been shown to be a plant toxin, involved with the virulence of the producer (82). The plant related genera: Agrobacterium, Erwinia, Xanthomonas and Pseudomonas reportedly produce beta-lactam antibiotics (20, 70, 83).

Many bacterially produced beta-lactams have novel structures, sensitivities to beta-lactamases, and antimicrobial spectra (83). Monocyclic beta-lactams called, monobactams, have been isolated from Gluconabacter, *Acetobacter*, *Chromobacter* and *Agrobacterium* (83, 88). Monobactams from *Agrobacterium* are unique to other monobactams; they contain a substituted aromatic residue attached to the beta-lactam ring (83). Another class of bacterially produced beta-lactams are the monolactones, produced by *Bacillus* (69), *Pseudomonas* and coryneform bacteria (83, 88). Monolactones contain oxygen instead of nitrogen in the beta-lactam ring. Monolactones are more sensitive to beta-lactamases than are monobactams (83). Cephalosporins and deacetyl-cephalosporins are produced by *Xanthomonas* and *Flavobacterium* (78). These are bicyclic beta-lactams, which are resistant to beta-lactamases produced by Gram-positive bacteria. Carbapenems are another class of beta-lactams, produced by *Serratia* and *Erwinia* (70).

Erwinia antibiotics. There are at least four antibiotics that have been isolated from *Erwinia*. Two of these antibiotics, herbicolin A and herbicolin B, are produced in a minimal growth medium by *E. herbicola* strain A111 (90). Herbicolins A and B are antifungal (90). They also inhibit sterol requiring *Mycoplasma*, *Ureaplasma* and *Spiroplasma* (31). Herbicolin A is a beta-hydroxymirystic acid substituted peptide antibiotic (90). It has been suggested that herbicolins A and B act by disrupting the lipid bilayer of membranes after complexing with cholesterol (31). The evidence for this proposal has been two-fold: activity of herbicolins is reversed by sterols, such as cholesterol; herbicolins A and B are cytolytic to human blood (31, 90).

Another antibiotic isolated from *Erwinia* is the carbapenem, SQ 27,860. This antibiotic is produced by two *Erwinia* isolates, identified as belonging to the carotovora and herbicola groups. SQ 27,860 is an unstable bicyclic beta-lactam, whose isolation was possible only after derivitization to its p-nitrobenzyl ester. SQ 27,860 is a broad spectrum antibiotic; it inhibits both Gram-positive and Gram-negative bacteria (70).

L-cycloserine is produced by a strain of *E. uredovora* (76). *E. uredovora* belongs to the Herbicola group of *Erwinia*, and is pathogenic on uredia of *Puccinia graminis* f. sp. *tritici* (57). This is the first report of naturally occurring L-cycloserine (75). The well known antibiotic, D-cycloserine, has been isolated from *Streptomyces* and *Pseudomonas fluorescens* (67). D-cycloserine inhibits alanine racemase and D-Ala-D-Ala-synthetase (67). L-cycloserine has the same mode of action, but its potency is greatly reduced (75).

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SECTION II

MULTIPLE ANTIBIOTIC PRODUCTION BY
ERWINIA HERBICOLA STRAIN C9-1

INTRODUCTION

Erwinia herbicola (Lohnis) Dye is a common saprophyte of plants, soil and water (13). Strains of *E. herbicola* that are pathogenic to humans have also been described (21); clinically obtained *E. herbicola* strains are usually named by the synonym, *Enterobacter agglomerans* (9). *E. herbicola* is among those *Enterobacteraceae* which have recently been reported to produce antibiotics. Herbicolins A and B are peptide antibiotics produced by *E. herbicola* strain A111. These antibiotics inhibit fungi (24) and sterol requiring *Mycoplasma*, *Spiroplasma* and *Ureaplasma* (11). An herbicola group *Erwinia* produces the novel beta-lactam antibiotic, SQ 27,860 (19).

Initially, we were interested in advancing the use of *E. herbicola* as a biological control agent of the plant pathogenic bacterium, *E. amylovora*, causal organism of fire blight on Rosaceous plants (3, 20). *In vitro* antibiosis of *E. amylovora* by *E. herbicola* has been attributed to acid (20), hydroquinone (8), and bacteriocin (4) production. Bacteriocins are characterized by their limited antimicrobial activity spectra; only strains closely related to the producing strain are inhibited (18). *E. herbicola* strain 112Y has been reported to produce a bacteriocin called, herbicolicin 112Y (12, 22).

Herbicolicin 112Y inhibits only *E. amylovora* and a few other *Enterobacteraceae*. Unlike most bacteriocins, herbicolicin 112Y is not a protein.

Preliminary results indicated that *in vitro* antibiosis of *E. amylovora* is a common characteristic of *E. herbicola* strains obtained from Michigan apple orchards. One particular isolate, *E. herbicola* strain C9-1, produces very large zones of inhibition in *E. amylovora* lawns. Such zones were only observed when a defined and buffered medium was used in the assays. The purpose of this report is to demonstrate that the major antibacterial activity produced by *E. herbicola* stain C9-1 is not a bacteriocin, to demonstrate that more than one antibacterial activity is present, and to demonstrate that at least one antibacterial activity is antagonized by histidine. Furthermore, we demonstrate antibiotic production in *E. herbicola* strain 112Y.

MATERIALS AND METHODS

Bacteria. *Erwinia amylovora* isolates were obtained from various culture collections, as previously reported (16). *E. herbicola* strain 112Y, producer of the bacteriocin, herbicolicin 112Y, was generously donated by S. V. Beer. *E. herbicola* strain C9-1 was isolated from Michigan grown apple *Malus malus* 'Jonathan' stem tissue. Other bacteria used in these experiments were obtained from culture collections available in the Department of Microbiology and Public Health, Michigan State University, East Lansing, MI. All bacteria were stored in glycerol buffer solutions at -20C. Prior to use, bacteria were streaked onto tryptose blood agar (TBA) slants, and incubated for 24-48 hr at 26C or 37C.

Media. The medium used throughout these experiments was the morpholinopropane sulfonic acid-buffered (MOPS) synthetic medium of Neidhart et al. (17). This medium was prepared without glucose, and supplemented with 10 mM potassium gluconate, 10 mM asparagine, 0.10 mM thiamine and 0.10 mM nicotinic acid. It was referred to as MOPS, gluconate, asparagine (MGA) medium. For growth of *Escherichia coli* strain ϕ and its derivatives, MGA was further supplemented with 1 mM hypoxanthine, 1 mM L-methionine and 0.1 mM calcium-pantothenate. MGA was supplemented further, as previously described (7), for growth of Yersinia pestis.

Another medium (Atherton medium), which had been described by Atherton et al. for detecting the antimicrobial activities of D-alanine derivatives (1), was used as an overlay medium in some experiments. Though some bacteria did not grow in MGA, Atherton medium supported the growth of all strains tested. Except for the addition of L-alanine and the omission of L-histidine, Atherton medium was prepared as suggested.

In all cases, solid medium was obtained by the addition of 1% (w/v) Bacto agar.

Detection and production of antibacterial activities. Samples (0.01 ml) were applied to MGA solid medium, dried, exposed to chloroform vapors, and then overlayed with 5 ml of molten medium, that was seeded with about 10^7 indicator cells. After incubating 24-48 hr at 30C, antibacterial activity was indicated by a clearing in the overlay lawn. Inoculum for overlays was obtained by washing a TBA slant of the indicator with phosphate buffer (0.033M potassium phosphate buffer, pH 7.0), followed by appropriate dilutions in phosphate buffer. Units of antibacterial activity were defined as the reciprocal of the highest dilution which inhibited growth of the indicator. The indicators routinely used were E. amylovora strain 110, derivatives of strain 110, and Es. coli strain ϕ.

For production of antibacterial activities in solid medium, 0.02 ml of a 10^8 colony forming units (cfu)/ml suspension of strain C9-1 or strain 112Y in phosphate buffer was spotted off-center on MGA medium. After incubating for 48 hr at 30C, the producer colony was killed with chloroform vapors, and assayed for activity, as described above.

Strain C9-1 antibacterial activities were also produced in MGA liquid medium. Ten ml of MGA liquid medium, contained in a 125 ml Erlenmeyer flask, was inoculated to an optical density (O.D.) of 0.1 (620 nm) with strain C9-1 cells, obtained from a TBA slant washing. Cells were grown at 30C with aeration (200 rpm an a model G76 gyratory water bath shaker). At late exponential phase, the cells were used as inoculum for a second broth culture. In a similar manner, this second culture was used to inoculate 50 ml of MGA medium, contained in a 500 ml Erlenmeyer flask. After 18 hr of growth, cells were pelleted by centrifugation (17,000 x g for 10 min at 4C). The supernatant was removed, sterilized by passage through a 0.2 micron filter unit, and concentrated (10 fold) under reduced pressure at 40C. This concentrate was referred to as the extracellular fraction. The pellet that had been obtained from centrifugation of the broth was washed, and then resuspended in 2 ml of 50 mM Tris-hydrochloride buffer, pH 7.8. The cells were sonicated at 15 second intervals for a total of 2 minutes sonication time; an ultrasonic probe (Instrumentation Associates, New York, N.Y.) was used to disrupt the cells. Preparations were kept on ice throughout the sonication procedure. Cellular debris was removed by centrifugation. The supernatant obtained was designated the intracellular fraction. All samples obtained from broth were frozen (-20C) between assays. During assays, samples were kept on ice.

Ultraviolet (UV) light induction of strain C9-1 antibacterial activities was attempted using the methods of Hu et al (15). This procedure involved the construction of an UV light kill curve to determine a 37% survival dosage for _E. herbicola_ strain C9-1. After irradiating, the surviving population was transferred to fresh MGA liquid medium, and grown until it had reached mid exponential growth stage. At this time, the broth was centrifuged. Using the methods described above, an intracellular and an extracellular fraction was obtained from the pelleted cells and the supernatant, respectively.

The methods of Hu and Brubaker (14) were used to test the inducibility of strain C9-1 antibacterial activities by mitomycin C. Twenty-five ml MGA broth cultures of strain C9-1 in 250 ml Erlenmeyer flasks were grown to mid or to late exponential growth stage. They were then centrifuged, and the pelleted cells used to inoculate fresh MGA medium to an O.D. of 0.1. At this time, mitomycin C (0, 0.2, 1, or 2 ug) was added to the broths. The cultures were incubated with aeration for 5 hr at 30C, and then centrifuged. The pelleted cells and the supernatant were developed in the same manner as those obtained from the UV light induction experiments.

Characterization of antimicrobial activities. Sensitivity to proteolytic enzymes was evaluated by previously described methods (5). A single off-center streak of strain C9-1 on MGA solid medium was incubated at 30C for 48 hr. After killing the cells with chloroform vapors, a sterile strip of Whatman no. 3 filter paper (1 x 7 cm) that had been soaked in a test solution was placed perpendicular to the bacterial streak. This assemblage was then assayed for antibacterial activity. Sensitivity to proteolytic enzymes was indicated when the indicator grew within the zone of inhibition next to the treated strip. Test solutions included phosphate buffer, trypsin and protease; enzyme concentrations used were 1 mg/ml.

To obtain molecular weight information, concentrated supernatants were dialized in 50 mM Tris-hydrochloride buffer, pH 7.8. One ml samples were secured in dialysis tubing that had a molecular weight cutoff of 3500. The bags were suspended in 10 or 1000 ml buffer for 18 hr at 4C. The dialysate from the 10 ml buffer treatment and the contents of both dialysis bags were assayed for antibacterial activity.

Effects of pH were assayed by adjusting aliquots of concentrated supernatants to pH 2, 3, 4, 6, 8, 10, and 12 with 1 N NaOH or 1 N HCl. Samples were stored at 4C for 2 hr. After neutralization with 1 M phosphate buffer, samples were brought up to a constant volume with distilled H_2O, and assayed for antibacterial activity.

RESULTS

Detection of antibiotic production. When MGA was used as the basal and the overlay medium, zones of inhibition around colonies of *E. herbicola* strain C9-1 were consistently observed in lawns of Gram-positive and Gram-negative indicators (Table 1). Addition of yeast extract (1% w/v) to the overlay medium reduced or eliminated the zone of inhibition in all cases. The only possible exception was *Staphylococcus aureus*, but this could not be examined directly; *S. aureus* required yeast extract for growth in MGA. All *S. aureus* isolates tested, including a penicillin resistant, beta-lactamase producing strain, were sensitive to at least one antibiotic produced by strain C9-1.

Antibacterial activity was detected in the extracellular fraction, but not in the intracellular fraction obtained from broth culture of strain C9-1. The activity spectrum of the extracellular fraction was similar to that produced in solid medium. Again, addition of yeast extract into the overlay medium reduced the zone of inhibition size in all cases.

Detection of multiple antibiotic production. Two distinct zones of inhibition in lawns of *E. amylovora* strain 110 were observed around strain C9-1 colonies. A

Table 1. Activity spectrum of the antibacterial activities produced in MGA solid medium by a colony of *Erwinia herbicola* strain C9-1.[a]

Indicator	no. tested	no. sensitive
Erwinia amylovora	41	41
E. herbicola	5	5
E. carotovora pv. *carotovora*	1	1
Escherichia coli	3	3
Enterobacter cloacae	1	1
Serratia marcescens	1	1
Aerobacter aerogenes	1	1
Yersinia pseudotuberculosis	1	1
Y. entercolitica	2	0
Y. pestis[b]	2	2
Klebsiella pneumonia	1	0
Proteus mirabilis	1	1
P. morgani	1	0
P. vulgaris	1	1
Pseudomonas aeruginosa	1	0
P. flourescens	1	0
P. syringae var. *syringae*	1	0
Bacillus megaterium	1	0
B. cereus	2	2
Staphylococcus aureus[c]	18	18

[a] Basal and overlay medium were MGA, which is described in text.

[b] MGA overlay medium was supplemented as described in ref. 7

[c] Yeast extract (1% w/v) was added to the overlay.

clear inner zone, extending 2-3 cm from the center of the colony, was surrounded by a 0.5-2 cm hazy zone (Figure 1A). From each region, ten colonies of spontaneous insensitive mutants of strain 110 were selected, and streaked for single colonies three times on TBA. These mutants were classified as E. amylovora strain O or strain I, to designate their selection from the outer and inner region, respectively. The sensitivity of these mutants to strain C9-1 antibacterial activities differed from that of strain 110's (Figure 1). Another class of mutants was obtained from strain O, using the same procedure as described above. This class of mutants, designated strain 12, were insensitive to all strain C9-1 antibacterial activities produced in MGA agar medium (Figure 1).

The striking reduction in the size of the zone of inhibition by yeast extract suggested the presence of one or more inhibitors of antibiotic activity. To identify such an inhibitor, 0.05 ml of concentrated supernatant was spread over MGA agar, dried, and then various amino acid (10 mM) and base (5 mM) solutions were spotted (0.01 ml). After it was dried and exposed to chloroform vapors, the agar was overlayed with MGA seeded with E. amylovora strains 110, O, or I, or E. herbicola strain 112Y, and the cells incubated for 48 hr at 30C. Reversal of antibiotic activity was observed wherever the indicator grew. Where L-histidine had been spotted, an obvious reversal of inhibition was seen in lawns of E. herbicola strain 112Y

Figure 1. Patterns of inhibition zones in lawns of *E. amylovora* strain 110 and derivatives of strain 110, produced around colonies of *E. herbicola* strain C9-1. (a) *E. amylovora* strain 110. (b) *E. amylovora* strain 0. (c) *E. amylovora* strain 110 grown with histidine (10 mM) added to the overlay. (d) *E. amylovora* strain I. (e) *E. amylovora* strain 12. (f) *E. amylovora* strain I grown with histidine.

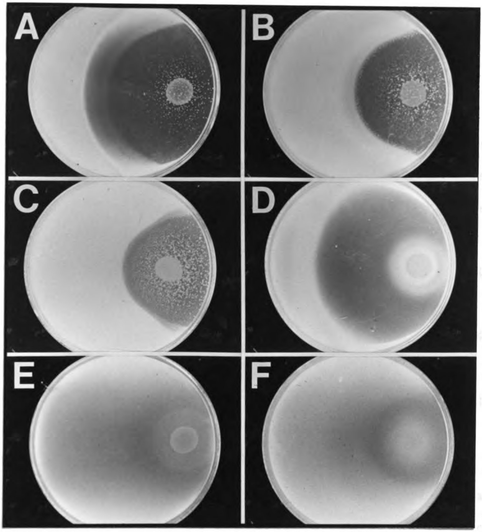

Figure 1.

and *E. amylovora* strain I. L-Histidinol and dipeptides containing L-histidine, in either the N or the C terminus position, also reversed activity; D-histidine and histimine did not. Though yeast extract had reversed activity in lawns of *E. amylovora* strain 110 and strain 0, the same results were not obtained with any of the amino acids and bases tested.

When *E. amylovora* strain 110 was grown with histidine, spontaneous insensitive mutants were obvious in the single clear zone of inhibition that surrounded the producer colony (Figure 1). Some of these mutants were selected, as described above, and designated strain HI mutants. No obvious differences were evident between the zone of inhibition produced by a colony of strain C9-1 in lawns of strain HI or strain I. The addition of histidine to the overlay greatly reduced the zone of inhibition produced in lawns of strain HI and strain I (Figure 1); sensitivity of strain 0 was not affected by histidine (Figure 1).

Escherichia coli strain ϕ and mutants of this strain that had been described as resistant or tolerant to various group A or group B colicins were also used as indicators (10). All *Es. coli* strain ϕ mutants were sensitive to a histidine reversible antibiotic produced in agar medium by colonies of strain C9-1. When grown with histidine, there remained small (less than 1.5 cm) and highly variable zones of inhibition; depending on the strain, one, two or three rings of inhibition were observed around the producer. In addition, rings of indicator growth were observed in the region of the inhibition zones. These rings of growth indicated inhibitors of antibacterial activity were concomitantly produced. The resulting patterns of growth and inhibition were not correlated with insensitivity to group B or group A colicins. In some cases, iron affected the observed pattern of the inhibition zones. When grown with histidine and with iron, a colicin I tolerant (ivt) strain and a colicin I resistant (cir) strain were insensitive to all strain C9-1 produced antibacterial activities; when grown with histidine, but without the addition of iron to MGA, small zones of inhibition were observed.

Characterization of herbicolins O and I. The activity spectrum of antibiotics found in concentrated supernatants of strain C9-1 was assessed with and without histidine in the overlay medium (Table 2). These results indicated that at least two antibiotics were present. A histidine antagonized, broad spectrum antibiotic was detected, and given the name herbicolin O. Another antibiotic, that was designated, herbicolin I, was also detected. Herbicolin I was not antagonized by histidine, and had a narrow activity spectrum, which included *E. amylovora* and *S. aureus* (Table 2). In these studies, Atherton medium was used as the overlay medium, and MGA was used as the basal medium. Atherton medium was not used as both the basal and overlay

Table 2. Effect of histidine on the antibacterial activity spectrum of *Erwinia herbicola* strain C9-1 antibiotics contained in concentrated supernatants.[a]

Indicator	no. tested	no. sensitive without histidine	no. sensitive with[b] histidine
Erwinia amylovora	4	4	4
E. carotovora	1	1	0
Enterobacter aerogenes	2	2	0
Escherichia coli	2	1	0
Serratia marcescens	1	1	0
Proteus mirabilis	1	1	0
P. vulgaris	2	2	0
Yersinia entercolitica	1	0	0
Y. pestis	1	1	0
Y. pseudotuberculosis	1	1	0
Shigella dysenteria	1	1	0
Salmonella typhimurium	1	1	0
Pseudomonas aeruginosa	1	0	0
Bacillus cereus	2	2	1
B. megaterium	1	0	0
Staphylococcus aureus	2	2	2

[a] A double agar diffusion assay was used for detecting antibiotic activity; the basal medium was MGA; the overlay was Atherton medium. Concentrated supernatants, that were obtained from MGA broth cultures of *E. herbicola* strain C9-1, contained 60 units/ml of histidine antagonized antibacterial activity and 40 units/ml of antibacterial activity that was not antagonized by histidine. Units of activity were defined as the reciprocal of the highest dilution of sample which inhibited *E. amylovora* strain 110.

[b] L-Histidine (10 mM) was added to the overlay medium.

medium, because under those conditions herbicolin activities were reduced or eliminated.

By growing *E. amylovora* strains 110, 0 and I, with and without histidine, initial characterization of herbicolins 0 and I was accomplished. Herbicolin 0 was labile to acid (pH < 3.5) and to base (pH > 10). Herbicolin I was labile to base (pH > 10) only. Both herbicolins were insensitive to protease and to trypsin. The inductive methods tested did not increase the titers of herbicolins 0 and I obtained from supernatants. Antibacterial activity was not induced in the intracellular fraction either. All antibacterial activity that was present in a concentrated supernatant was dialyzable, which indicated that herbicolins 0 and I had molecular weights of less than 3500.

Antibiotic production in E. herbicola strain 112Y. *E. herbicola* strain 112Y also produced antibiotics in MGA agar medium (Table 3). Yeast extract reduced the size of the zone of inhibition in most cases; histidine did not. The sensitivities of select *E. amylovora* strains to the antibacterial activities produced by *E. herbicola* strain 112Y and strain C9-1 (Table 4) indicated that the antibacterial activities of these two strains differed.

Table 3. Activity spectrum of antibacterial activities produced by a colony of *Erwinia herbicola* strain 112Y grown on MGA solid medium.[a]

Indicator	no. tested	no. sensitive
Erwinia amylovora	3	3
E. carotovora	1	1
Escherichia coli	2	2
Klebsiella pneumonia	1	0
Pseudomonas aeruginosa	1	0
Bacillus megaterium	1	0
B. cereus	1	1
Staphylococcus aureus[b]	2	2

[a] Conditions used were the same as those in Table 1.

[b] Yeast extract (1% w/v) was added to the MGA overlay medium for growth of *S. aureus*.

Table 4. Comparison of the antibacterial activities produced in MGA agar medium by colonies of *Erwinia herbicola* strain C9-1 and strain 112Y.[a]

Indicators			Producer strain	
Strain	source	description	C9-1	112Y
E. herbicola 112Y	S. V. Beer	produces herbicolicin 112Y	+	-
E. herbicola C9-1	this report	produces herbicolin O[b] and herbicolin I[c]	-	-
E. amylovora 273	S. V. Beer	wild type	+	+
E. amylovora 336	S. V. Beer	insensitive to herbicolicin 112Y	+	-
E. amylovora 110	D. Ritchie	wild type	+	+
E. amylovora I	this report	insensitive to herbicolin I	+	+
E. amylovora O	this report	insensitive to herbicolin O	+	+
E. amylovora 12	this report	insensitive to herbicolin O and herbicolin I	-	-

[a] Conditions used were the same as those in Table 1. (+) = zone of inhibition present; (-) = no zone of inhibition present.

[b] The histidine antagonized antibacterial activity produced by strain C9-1

[c] The antibacterial activity produced by *E. herbicola* strain C9-1 which was not antagonized by histidine.

DISCUSSION

E. herbicola strain C9-1 produces at least two antibiotics, herbicolins O and I, *in vitro*. This conclusion is supported by the antimicrobial activity spectra of the antibiotics, by the specificity of histidine antagonism, and by the detection of two classes of herbicolin insensitive mutants in *E. amylovora*. The possibility exists that *E. herbicola* strain C9-1 produces more than two antibiotics, because cross resistance to similar antibiotics cannot be excluded. Herbicolin O is a broad spectrum, histidine antagonized antibiotic. It is labile to acid and to base. Herbicolin I has a much narrower activity spectrum than does herbicolin O, is labile to base, and is not antagonized by histidine. Both herbicolin O and I are low molecular weight (less than 3500) compounds. They are insensitive to proteases and are not inducible by mitomycin C or ultraviolet light. Additional uncharacterized antibacterial activities may be produced in MGA agar medium by a colony of *E. herbicola*. This possibility is suggested by the complex patterns of inhibition zones in lawns of *Es. coli* strain ϕ and its colicin tolerant and resistant derivatives.

An unexpected result of our experiments is the detection of antibiotic production by *E. herbicola* strain

112Y. Strain 112Y was included as a control in our screenings to determine if strain C9-1 and strain 112Y activities were the same. In fact, differences between their antibacterial activities were observed. However, an as yet undefined relationship is suggested by the observation that an E. amylovora strain that has been selected for insensitivity to herbicolins O and I is also insensitive to all antibacterial activity produced by strain 112Y (Table 4).

Detection and production of microcins, the low molecular weight peptide antibiotics produced by certain Enterobacteraceae, has required minimal media (2). This requirement limits the indicators tested, because not all indicators grow well in minimal media. Detection of herbicolins O and I also requires a defined, minimal medium. This has necessitated that a minimal medium be used for production; diffusible inhibitors in the production medium could obscure detection of antibacterial activity. We have found the combination of MGA as the basal medium and Atherton medium as the overlay medium supported growth of all indicators, and afforded detection of herbicolins O and I. When Atherton medium is used as the overlay and the basal medium, activity of both herbicolins is reduced.

This is the first report of a histidine antagonized antibiotic being produced by E. herbicola. Though its reversal by histidine suggests a mode of action for herbicolin O may involve histidine metabolism or uptake, other explanations should also be considered. For example, histidine is known to inactivate penicillin, a beta-lactam antibiotic, by nucleophilic attack of the beta-lactam ring (6). Recently, a number of novel bacterially produced beta-lactams have been reported (23).

The definitive enumeration and characterization of antibiotics produced by *E. herbicola* strain C9-1 will require their purification. Our results have afforded an understanding of the multiplicity and lability of the antibiotics produced by this strain. This will presumably aid further studies regarding herbicolin purification, mode of action, genetics of production, and relationship to antibiotics produced by *E. herbicola* strain 112Y.

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SECTION III

HERBICOLINS O, I AND 2C: NOVEL BETA-LACTAM ANTIBIOTICS PRODUCED BY *ERWINIA HERBICOLA* STRAIN C9-1

INTRODUCTION

Erwinia herbicola (Lohnis) Dye is a member of the *Enterobacteraceae* that has been isolated as a saprophyte from plants, animals, water and soil (6). Strains that are pathogenic to humans have also been reported (13). *E. herbicola* is among those *Enterobacteraceae* that have been reported to produce antibiotics. The peptide antibiotics, herbicolin A and herbicolin B are produced by *E. herbicola* A111 (24). The novel beta-lactam, SQ 27,860, is produced by an herbicola group *Erwinia* (12). We have reported the detection of multiple antibiotic production by *E. herbicola* strain C9-1. These antibiotics have been referred to as herbicolins O and I. Herbicolin O is a broad spectrum, histidine reversible antibiotic; herbicolin I has a narrow activity spectrum, and is not antagonized by histidine.

In this report, we present a procedure for the purification of three herbicolins from broth culture of *E. herbicola* strain C9-1. We also present physiochemical data which identifies these three herbicolins as novel beta-lactam antibiotics.

MATERIALS AND METHODS

Bacteria. *Erwinia herbicola* strain C9-1 was isolated from apple *Malus malus* 'Jonathan' stem tissue. *E. amylovora* strain 110 and derivatives of strain 110, that had been selected for insensitivity to herbicolins (previously described), were routinely used for detection of antibacterial activity. Other bacteria used were obtained from culture collections located in the Microbiology and Public Health Department, Michigan State University, East Lansing, MI. All bacteria were stored in phosphate buffered glycerol at -20C. Bacteria were streaked onto Tryptose Blood Agar Base (TBA) slants, and incubated 48 hr at 26C, prior to use.

Media. The morpholinopropane sulfonic-acid buffered medium of Neidhart et al. (10), which was modified by the omission of glucose and the addition of gluconate (10 mM), asparagine (10 mM), thiamine (0.01 mM) and nicotinic acid (0.01 mM), was used for routine detection and production of herbicolins. Modified Neidhart medium was referred to as MGA. Atherton medium (2), modified by the addition of L-alanine and omission of L-histidine, was used as an overlay medium for determination of the antimicrobial activity spectra of herbicolins.

Biological assays. Preparations (0.01 ml) of herbicolins were spotted on the surface of MGA solid medium, dried, exposed to chloroform vapors, and overlayed with 5 ml molten overlay medium, which contained about 10^7 indicator cells. The cells were incubated at 30C for 48 hr. The presence or absence of a zone of inhibition in the indicator lawn was then noted. Antibacterial activity (units/ml) was defined as the reciprocal of the highest dilution of a preparation that produced a clearing in an indicator lawn.

Purification of herbicolins. The pooled activities contained in two 10 l fermentations of strain C9-1 were used as the source of herbicolins for all of the tests described. Cells of strain C9-1 were grown for 30 hr at 30C with aeration in fermentor vessels containing 10 l MGA medium (New Brunswick model FS305 fermentor). Inoculum for each fermentor vessel was 400 ml of strain C9-1 grown in MGA medium with aeration at 30C (10% v/v) to late logarithmic growth stage. Supernatants of the fermentations, obtained by centrifugation (9,000 x g for 30 minutes), were pooled, and concentrated to 2 l under reduced pressure at 40C. This concentrate was divided into various sized aliquots, and frozen at -20C.

Herbicolins were removed from supernatants by cation exchange chromatography. A sample of concentrated supernatant (50 ml) was adjusted to pH 2.5 with 5 N H_2SO_4, and then quickly added to a 5.5 cm x 10 cm column of Dowex 50W x 4 (200-400 mesh) that was in the NH_4^+ form. The resin had been equilibrated in 2.5 mM ammonium acetate buffer, pH 5. The column was washed with 3 l of 2.5 mM ammonium acetate buffer (500 ml/hr), and the eluate discarded. Herbicolins were eluted with 3 l of 20 mM ammonium acetate buffer, concentrated to dryness under reduced pressure at 40C, and then resuspended in 15 ml of distilled water. This preparation of herbicolins was placed in a -20C freezer for 48 hr. The resulting precipitate (which did not contain herbicolin activity) was removed by centrifugation (20,000 x g for 20 minutes). The supernatant was removed, divided into 1 ml aliquots, and stored at -20 C. These preparations of herbicolins retained activity for at least 30 days.

Herbicolins were further purified by low pressure (flash) chromatography. Using methanol as a solvent, a 100 ml flash chromatography column (J. T. Baker Chemical Co., Phillipsburg, NJ) was packed with 30 g octydecyl (C_{18}) bonded phase sorbent (particle size 40 microns). By gradually increasing the concentration of water in the solvent, the column was finally equilibrated in 0.1% (v/v) trifluoroacetic acid (TFA): water. Herbicolins (0.5-1 ml of a 300 mg/ml solution) that had been obtained from cation exchange chromatography were applied to the column. The column was sequentially rinsed (50 ml at a time) with solvent which contained 0, 5, 10, 20 and 50% methanol: water; both the water and the methanol contained 0.1% TFA (v/v). Flow rates were maintained between 4 and 7 ml/min.

Fractions (5 ml) were collected, and immediately adjusted to a pH of 3.5-4.0 with a few drops of 2% NH_4OH. Absorbance at 215 nm and antibacterial activity of each fraction was determined. Fractions with activity were pooled, concentrated under reduced pressure to a syrup, and brought up to 2 ml with distilled, deionized water. If the pH of the sample was less than 3.5, the pH was adjusted as described above.

After flash chromatography, herbicolins were separated by high pressure liquid chromatography (HPLC). A Zorbax C8 semi-preparative column, 25.0 cm x 9.4 mm (Dupont Instruments) was equilibrated in acetonitrile:water:TFA (5:94.9:0.1). Samples (0.1 ml) were injected. A Waters model 6000A solvent delivery system was used to elute herbicolins isocratically, at a flow rate of 1.5 ml/min. Absorbance at 215 nm was monitored by a Gilson model HM UV-VIS dual beam spectrophotometer. All peaks were collected, adjusted to pH 3.5-4.0 with 2% NH_4OH, and pooled with like peaks from separate HPLC runs. After concentrating to 1/10 volume under reduced pressure, antibacterial activity was assayed. Samples with activity were resuspended in distilled, deionized water, and lyophilized. This lyophilization procedure was repeated two more times.

To obtain homogeneous preparations, herbicolins were further purified by instant thin layer chromatography (ITLC). Herbicolins obtained from HPLC were applied to several ITLC-SA papers (Gelman Sciences Inc., Ann Arbor, MI, 20 x 20 cm), and developed in acetonitrile:water (80:20) to a solvent front of 13 cm. A strip on each side of the ITLC paper was removed, and sprayed with ninhydrin reagent (2% w/v ninhydrin in butanol, with 2% v/v acetic acid). Ninhydrin positive regions were eluted with water, passed through a 0.2 micron filter unit, and concentrated to dryness under reduced pressure. Herbicolins were dissolved in 1 ml deionized, distilled water, lyophilized, and frozen (-20C).

During their purification, herbicolins obtained after cation exhange chromatography were kept on ice or placed in a cold cabinet (4C). Whenever the preparations were not being subjected to purification, they were frozen (-20C).

Characterization of herbicolins. Herbicolins that had been obtained after HPLC were used as a source of herbicolins for characterization of acid, base, heat, and beta-lactamase stabilities. To determine pH labilities, samples of each herbicolin were adjusted to various pHs with 0.5 N H_2SO_4 and 2% NH_4OH. After 2 hours, samples were neutralized with 1 M potassium phosphate buffer (pH 7.0), brought up to a constant volume, and then assayed for herbicolin activity. Heat stability was tested by placing samples of herbicolins into sealed lyophylization tubes, and then incubating the tubes at 95C for 2 h. Samples were cooled, and assayed for activity. Herbicolin samples that were not heated served as controls. Sensitivity to beta-lactamase was determined with Penicillinase 1 (Sigma

Chemical Co.). To samples (0.02 ml) that contained approximately 20 units/ml of herbicolin activity, 0.01 ml of penicillinase 1 in distilled water (0.2 units of cephalosporin hydrolase activity) was added. After incubation for 1 hr at 26C, antibiotic activity was assayed. Results were compared to those obtained from herbicolin samples that had been treated with water only.

Herbicolins obtained from HPLC were also used for determination of the antimicrobial activity spectrum of each antibiotic. To determine the relative sensitivity of various indicators, herbicolins were serially diluted in distilled water, and then assayed for antibacterial activity.

Highly purified herbicolins, that were obtained after ITLC, were used for ^{1}H-nuclear magnetic resonance (NMR) analysis, amino acid analysis, amino acid sequencing, ultraviolet (UV) light absorption, and mass spectrometry.

Herbicolins were prepared for ^{1}H-NMR by dissolving 0.7 mg of sample in 0.5 ml deuterium oxide (99.7%; Aldrich Chemical Co.). After lyophilization, the deuterium-exchanged samples were dissolved in 99.97% (0.3 ml) deuterium oxide (Aldrich Chemical Co.). Spectra were collected on a Bruker WM-250 instrument with suppression of the solvent (HDO) resonance signal.

Fast atom bombardment mass spectrometry (FAB-MS) of intact, underivatized herbicolins was used to obtain molecular weight and chemical formula information. A glycerol matrix (0.01 ml) containing about 0.01 mg of lyophilized herbicolin was directly applied to a stainless steel probe. The instrument used was a Varian Mat CH5-DF double-focusing mass spectrometer that had been modified for FAB by attachment of a B11NF saddle field fast atom source. The fast atom gun was operated to give 8.6 keV Xe atoms (99.9995% ^{12}xenon gas, Matheson) (1).

The amino acid composition and sequence of each herbicolin was determined at the Protein Sequencing Facility, Dept. of Biological Chemistry, University of Michigan, Ann Arbor, MI. Samples were sequenced using a manual partitioning technique; Edman degradations were liberated as phenylthiohydantoin-amino acids (21). The amino acid composition of herbicolin hydrolysates was analyzed by HPLC of the resulting phenylthiocarbamyl (PTC) derivatives (21).

UV absorbance spectra of herbicolins (0.5 mg) in distilled, deionized water were recorded on a Varian Cary-219 spectrophotometer.

RESULTS

Isolation of herbicolins. Due to their pH labilities, the purification of herbicolins by ion exchange chromatography necessitated that the resin form and elution buffer be carefully selected. When Dowex 50 was used in the ammonium form, and eluted with ammonium acetate buffer, the column was maintained at the favorable pH of 5.0. This form of the resin excluded most cations present in concentrated supernatants. Therefore, prior desalting of the concentrated supernatants was unnecessary. All antibacterial activities present in concentrated supernatants were retained by the resin. After elution, herbicolin activity was detected in the 20 mM eluate only. A 90% recovery of non-histidine reversible activity was achieved by these methods. However, recovery of histidine reversible activity was much less; a 30-50% recovery was common. The loss of histidine reversible activity was presumably caused by the acidic conditions required for retention of herbicolins on the resin.

Flash chromatography of herbicolins obtained from cation exchange chromatography removed most of the residual ammonium acetate, and separated herbicolin activity into three peaks. The first two peaks, which overlapped, were eluted with 10% methanol. These peaks contained a histidine sensitive and a histidine insensitive activity. The third peak eluted at the end of the 20% methanol elution and the beginning of the 50% methanol elution. This third peak contained only histidine insensitive activity.

Herbicolins were further purified by HPLC (Figure 1). For purposes of illustration, peaks of activity detected by flash chromatography were pooled to show HPLC separation of all three antibiotics. In practice, the third peak of activity obtained from flash chromatography was not pooled with the first two peaks, and was purified by separate HPLC runs. The antibiotics obtained were designated, herbicolin O, herbicolin I and herbicolin 2C.

The herbicolins purified through HPLC were found to produce single antibiotic peaks in subsequent HPLC runs, but also contained some HPLC solvent residues. These contaminants were removed by ITLC. Herbicolins purified through ITLC each gave a single ninhydrin positive spot. After elution from ITLC-SA papers, the herbicolins obtained were again subjected to HPLC for demonstration of single peaks in solvents of varied acetonitrile concentrations. From 500 ml of supernatant, 0.8, 0.7, and 0.7 mg of herbicolins 2C, I and O, respectively, were obtained by purification through the ITLC step. The purified herbicolins so obtained were tan-colored powders.

<u>Antimicrobial activity of herbicolins.</u> Herbicolins I and 2C had indistinguishable antimicrobial activity spectra

Figure 1. HPLC UV absorption profile (215 nm) of herbicolins obtained by flash chromatography. A Zorbax C8 semi-preparative column (25.0 cm x 9.4 mm) was eluted isocratically with acetonitrile: water:TFA (5:94.9:0.1) at a flow rate of 1.5 ml/min. (a) herbicolin 0. (b) herbicolin I. (c) herbicolin 2C.

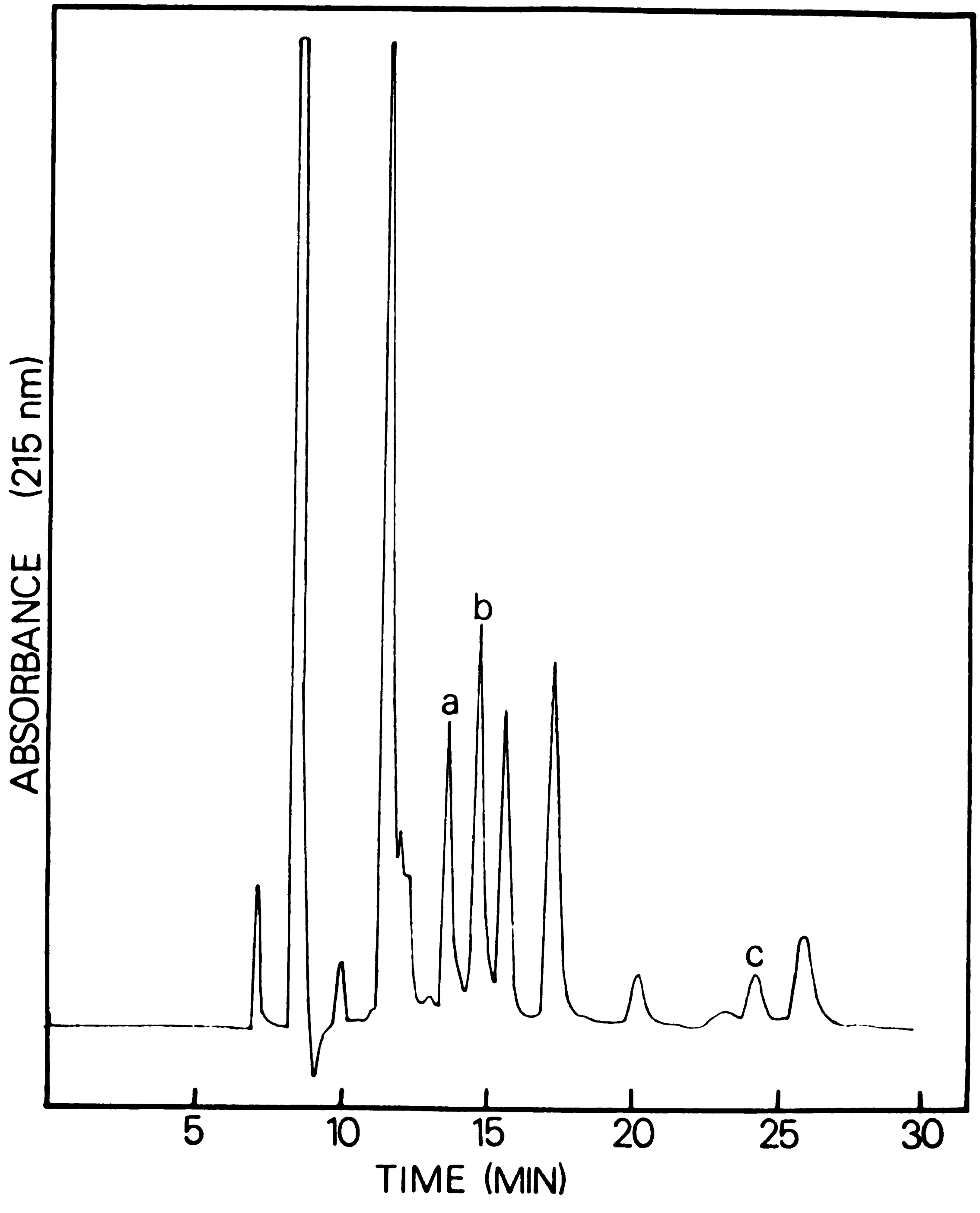

Figure 1.

(Table 1). This similarity was also seen when E. amylovora 110 and its derivatives were tested for sensitivity to herbicolins (Table 2). Herbicolin O inhibited both Gram-positive and Gram-negative bacteria (Table 1). While many members of the Enterobacteraceae were sensitive to herbicolin O, only E. amylovora was sensitive to herbicolins I and 2C. These observations were consistent with those previously reported. Due to the limited supply and instability of purified herbicolins, the sensitivity of an indicator species was defined in activity (units/ml) instead of a minimum inhibitory concentration (MIC) (Table 1).

Physical characterization. After treatment with penicillinase 1, no antibacterial activity was detected in preparations of herbicolins O, I, or 2C. This indicated that herbicolins contained a beta-lactam ring. The base lability of the antibiotics was consistent with this conclusion (4). The lability of herbicolins to various environmental parameters is summarized in Table 3. All three herbicolins were soluble in water, slightly soluble in methanol, and insoluble in chloroform or in butanol. At pH < 3.5, herbicolins from concentrated supernatants were extractable with butanol, which indicated that they, like the carbapenem, SQ 27,860, were weak acids (12). Herbicolins O, I and 2C had maxima at 235 nm in UV their absorbance spectra.

A structure for herbicolin I (Figure 2) was deduced

Table 1. Activity (units/ml) of purified herbicolins 0, I and 2C detected by various indicator species.[a]

Indicator	Herbicolin 0	Herbicolin I	Herbicolin 2C
Erwinia amylovora 110	4[b](80)[c]	40(40)	40(40)
E. herbicola 112Y	4(200)	0	0
E. carotovora pv. _carotovora_	20(40)	0	0
Escherichia coli ϕ	20(ng[d])	0	0
Salmonella typhimurium	4(ng)	0	0
Proteus vulgaris	8(400)	0	0
Serratia marcescens	2(40)	0	0
Pseudomonas aeruginosa	0(0)	0	0
Staphylococcus aureus	0(ng)	20(ng)	20(ng)
Bacillus cereus	0(160)	0	0

[a] Activity (units/ml) was defined as the reciprocal of the highest dilution of a preparation which produced a clearing in the indicator lawn. Herbicolin preparations tested were obtained, as described in materials and methods, by a combination of ion exchange chromatography, flash chromatography, and HPLC. Samples (0.01 ml) were spotted on MGA solid medium, and then overlayed with 5 ml of molten MGA seeded with about 10^7 indicator cells.

[b] Overlay medium was Atherton medium (see text).

[c] Numbers in parenthesis represent the activity (units/ml) detected when the overlay medium was MGA.

[d] MGA medium did not support growth of the indicator.

Table 2. Sensitivity of *Erwinia amylovora* strain 110 and its derivatives to herbicolins purified through reversed-phase HPLC.[a]

	herbicolin		
Erwinia amylovora strain[b]	0	I	2C
110	+[c]	+	+
I-1	+	-[d]	-
HI-1	+	-	-
0-1	-	+	+
12	-	-	-

[a] Herbicolins were obtained by a combination of cation exchange chromatography, flash chromatography and HPLC, as described in the materials and methods section. Samples (0.01 ml) of herbicolins containing 20 units/ml were spotted on MGA solid medium and overlayed with 5 ml of MGA seeded with about 10^7 indicator cells. Cells were then incubated for 48 hr at 30C.

[b] *E. amylovora* strains were characterized by their insensitivities to herbicolins produced in MGA agar by a colony of *E. herbicola* strain C9-1 (previously described): wild type (strain 110); insensitive to non-histidine reversed herbicolins (strains HI-1 and I-1); insensitive to histidine reversed herbicolins (strain 0-1); insensitive to all antibacterial activity produced by strain C9-1 (strain 12).

[c] A clearing in the indicator lawn was observed (+).

[d] A clearing in the indicator lawn was not observed (-).

Table 3. Summary of herbicolin characteristics.[a]

	herbicolin		
Characteristic	0	I	2C
beta-lactamase sensitive	+	+	+
base labile (pH > 10)	+	+	+
acid labile (ph < 3.5)	+	-	-
heat labile (95C for 2 hr)	+	-	+
histidine antagonized	+	-	-
Rf[b]	3.4	4.6	4.8

[a] See materials and methods for conditions of assays.

[b] Rf determined in acetonitrile:water (80:20) on instant thin layer chromatography (ITLC)-SA papers, detected by ninhydrin.

from amino acid analysis, amino acid sequencing, ^{1}H-NMR analysis and FAB-MS. Amino acid analysis indicated that herbicolin I contained equimolar amounts of valine and an uncommon amino acid. Sequencing of herbicolin I revealed the N-terminus was occupied by the uncommon amino acid; valine was in the second position. As reasoned from the expected hydrolysis products of intact herbicolin I, the uncommon amino acid was identified as 3-[(2-amino-2-carboxy)ethylamino]-3-carboxy-2-oxoazetidine.

Molecular weight and chemical formula information was obtained from the FAB mass spectrum of intact herbicolin I. The pseudomolecular ions: $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$, appeared at m/z 317, 339 and 355, respectively. Peak matching provided a chemical formula ($C_{12}H_{21}N_4O_6$) for $[M+H]^+$ with a calculated mass of 317.14610; the observed mass (317.14461) deviated from the calculated mass by only -4.6 millimass units.

^{1}H-NMR spectra of herbicolin I, obtained on a homogeneous sample and a sample that contained ammonium acetate, corroborated assignment of all resonance signals (Figure 3). Chemical shifts were determined on the spectrum of homogeneous sample, since the chemical shift of some signals was affected by the ammonium acetate. Valine protons were assigned to a 6H multiplet at δ 0.87-0.97, a 1H multiplet at δ 2.05-2.23, and a 1H doublet ($J = 7$ Hz) at δ 4.0. In the spectrum obtained on samples with ammonium acetate, a broad signal at δ 4.08-4.18 was

Figure 2. Proposed structure of herbicolin I. Wavy lines indicate predicted amide linkages, which, when cleaved, would yield valine and the uncommon amino acid (intact) 3-[(2-amino-2-carboxy)ethylamino]-3-carboxy-2-oxoazetidine. Stereochemistry was not determined.

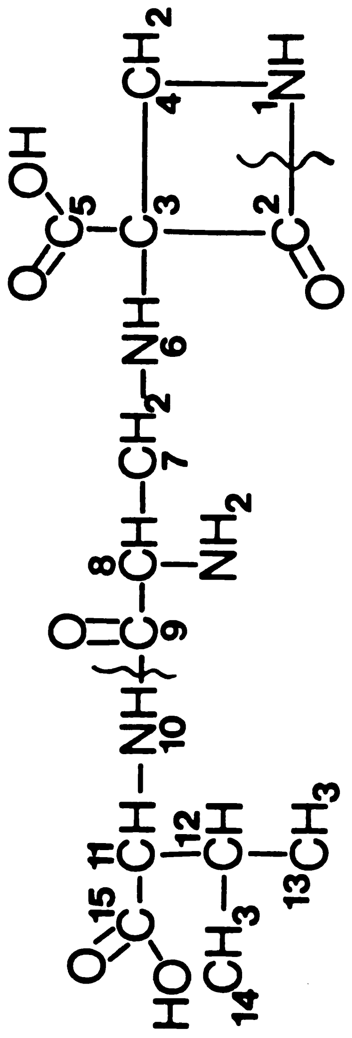

Figure 2.

observed. In the spectrum obtained on homogeneous herbicolin, this broad signal correlated with a 1H triplet (J = 6 HZ) at δ 4.14 that was observed after resolution enhancement of the spectrum, by applying suitable Laurentzian and Gaussian window functions. The broad, unresolved signal indicated that the corresponding proton was on a carbon to which an amino group was attached. The triplet indicated that the proton was coupled to methylene protons. The methylene protons were assigned to a 2H doublet of doublets (J = 9+6) at δ 3.67 and 3.70, evident in the resolution enhanced spectrum. The slight difference between the chemical shifts of these methylene protons simplified the resonance signals for that ABX system; the signals due to the major splitting dominated the spectrum. Consequently, the smaller resonance signals were not detectable above the background noise. The only remaining signal was a 2H multiplet at δ 3.58-3.62. This signal appeared as a 2H singlet at δ 3.70 in the ammonium acetate containing sample. Based on the published ^{1}H-NMR spectra of other beta-lactams (9), these signals were assigned to the C4 position of the beta-lactam ring.

Complete structures for herbicolins O and 2C were not elucidated, but their inactivation by beta-lactamase indicated that they also contained a beta-lactam ring. Methylation at the C4 position of the beta-lactam (Figure 5) was proposed to account for the presence of a 3H doublet (J = 6.2) at δ 1.25, and the lack of a 2H multiplet at δ

Figure 3. 250-MHz ^{1}H-NMR spectra of herbicolin I in D_2O. (a) Herbicolin I preparation which contained ammonium acetate (Ac). (b) Laurentz-Gauss resolution enhanced spectrum of homogeneous herbicolin I.

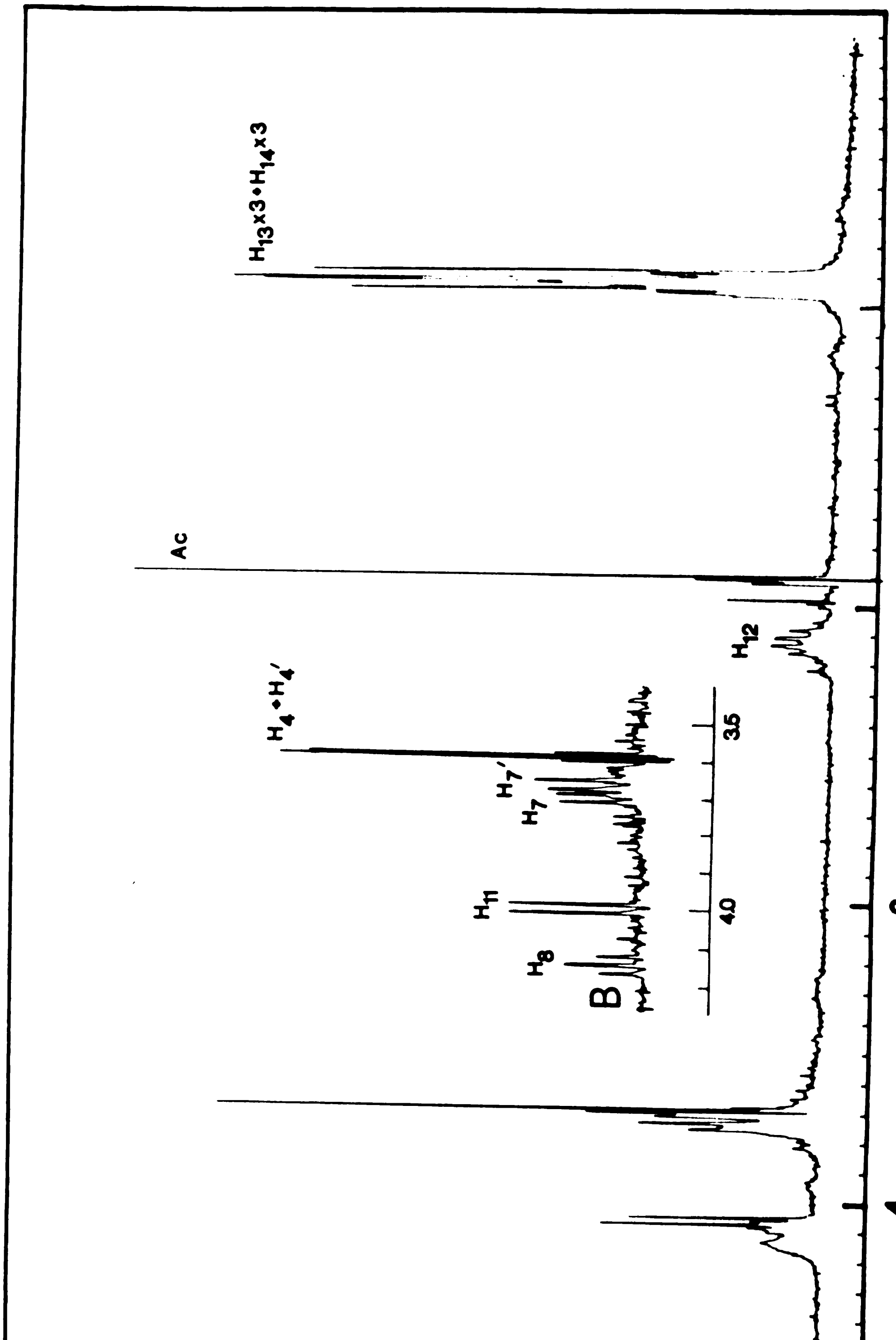

Figure 3.

Figure 4. 250-MHz ^{1}H-NMR spectra of herbicolins O and 2C in D_2O. (a) Herbicolin O. (b) Herbicolin 2C.

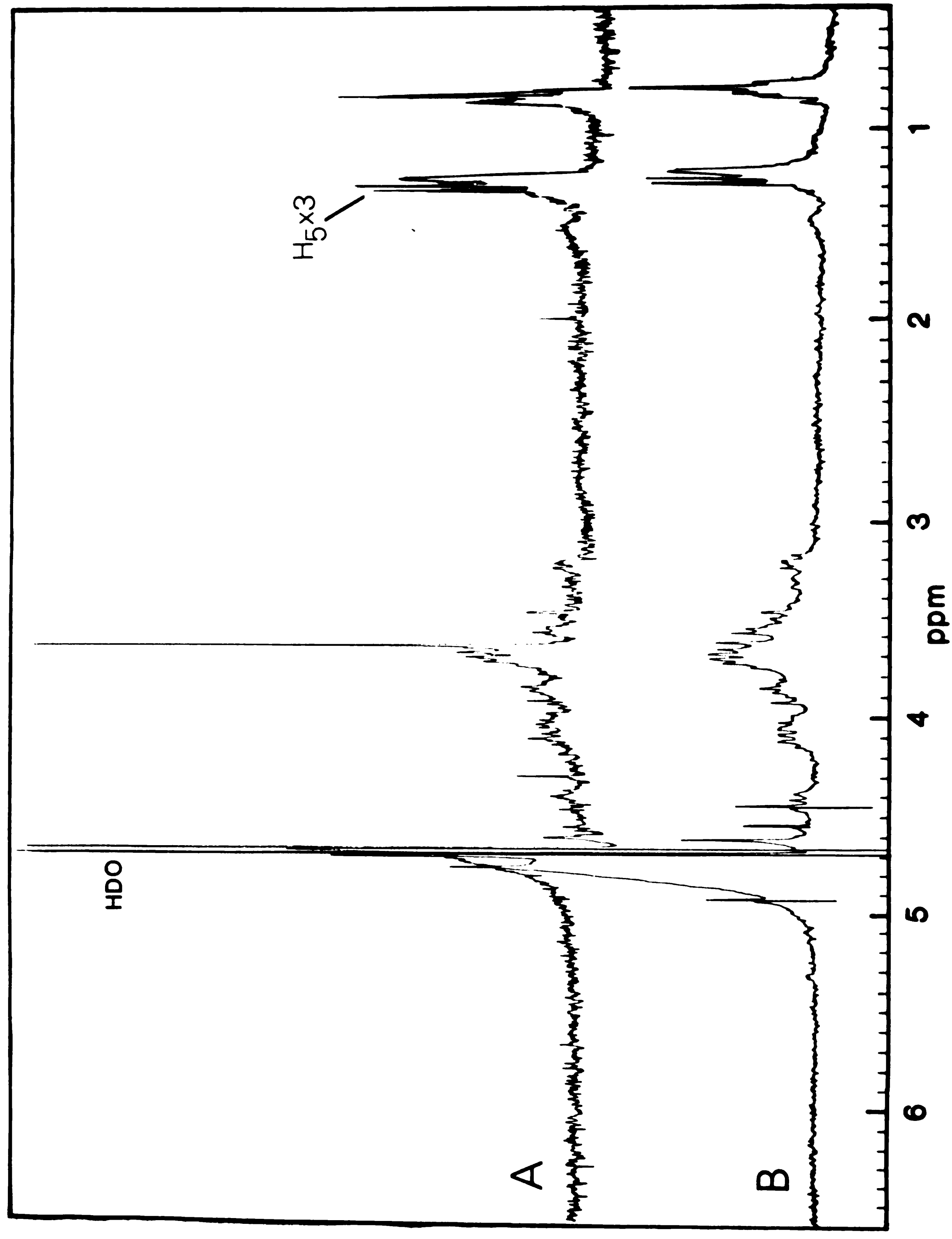

Figure 4.

3.58-3.62 in the ^{1}H-NMR spectra of herbicolins O and 2C (Figure 4). Methylation at this position was also suggested by the fact that the doublet at δ 1.25 was shifted upfield in ^{1}H-NMR spectra of biologically inactive herbicolins O and 2C. The 3H singlet at δ 3.65 in the spectrum of herbicolin O always appeared as a singlet, even after resolution enhancement. This indicated that the signal was due to equivalent protons. Its chemical shift was indicative of a methyl ester linkage. Since no other obvious differences were seen between the spectra of herbicolins O and 2C, the singlet at δ 3.65 suggested that herbicolin O was a methyl ester of 2C. However, FAB-MS of herbicolins O and 2C did not support this hypothesis; in the FAB-mass spectrum of herbicolin O, an [M+14] pseudomolecular ion, indicative of the methylation of herbicolin 2C, was not found. Amino acids were not detected in either herbicolin O or herbicolin 2C.

Figure 5. Proposed partial structure of herbicolins O and 2C. R and X were not elucidated.

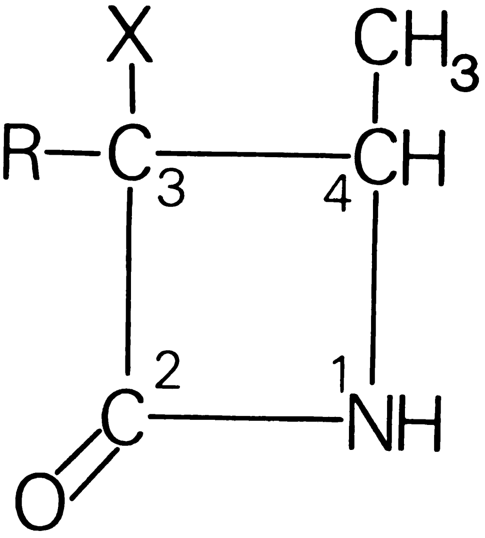

Figure 5.

DISCUSSION

Erwinia herbicola strain C9-1 produces three detectable antibiotics in broth culture. We have isolated these antibiotics to homogeneity through a combination of cation exchange, low pressure, high pressure and instant thin layer chromatography. The criteria for purification were the appearance of single peaks in various HPLC solvent systems and consistent physiochemical analysis of the antibiotics. The antibiotics are given the trivial names: herbicolin O, herbicolin I and herbicolin 2C. The antimicrobial activity spectra of herbicolins I and 2C are indistinguishable (Tables 1 and 2); of the species tested, these two antibiotics inhibited only E. amylovora and Staphylococcus aureus. Other species may be affected at higher concentrations of the antibiotics. Herbicolin O is a broad spectrum, histidine reversible antibiotic (Table 1). These descriptions are consistent with those reported for crude herbicolins.

We propose that the structural moiety common to all three herbicolins is a beta-lactam ring. This conclusion is ascertained from the sensitivity of herbicolins O, I and 2C to Penicillinase 1. The relative sensitivities of herbicolins to Penicillinase 1 were not determined. The base lability of herbicolins is consistent with the base lability of other beta-lactams (4).

Due to sensitive detection methods, reports of bacterially produced beta-lactam antibiotics are now common (20). Classical beta-lactam antibiotics, such as cephalosporins (15), and a variety of novel beta-lactams are produced by bacteria (11, 12, 23). Monobactams constitute a class of novel bacterially produced beta-lactams that have varied antibacterial activity and sensitivities to beta-lactamases (19). Unlike most beta-lactam antibiotics, which are bicyclic, monobactams are monocyclic compounds that contain 2-oxoazetidine-1-sulfonic acid (8). Since herbicolins O, I, and 2C are weak acids, they can be distinguished from the strongly acidic monobactams. A comparison of published physical-chemical data of other beta-lactam antibiotics with those obtained from herbicolins indicates that herbicolins do not contain a fused beta-lactam ring (5, 12). To our knowledge, herbicolins O, I and 2C, therefore, represent a family of novel beta-lactam antibiotics.

The structure of herbicolin I (Figure 2) was deduced from amino acid analysis, amino acid sequencing, ^{1}H-NMR analysis, UV light absorbance, and FAB-MS. These same parameters did not elucidate complete structures for herbicolins O and 2C. Herbicolins O and 2C appear to be structurally related (Figure 3), but their antimicrobial activities are different (Table 1 and 2). Though supporting evidence from FAB-MS is lacking, ^{1}H-NMR suggests herbicolin O and 2C differ by a methyl group, which may be ester linked. Partial structures (Figure 5) for these antibiotics are proposed in an attempt to show their possible relationship to herbicolin I. It is known that methoxy and methyl additions to the beta-lactam ring significantly affect the biological activity of other beta-lactam antibiotics (7).

The biological activity of beta-lactam antibiotics has been correlated with the reactivity of the beta-lactam ring; electronic and steric effects created by ring substitution can result in destabilization of the beta-lactam ring (9, 18). Highly reactive beta-lactam rings, in some cases, have greater antimicrobial activity and increased base and acid lability (4, 14). The pH labilities and antibacterial activity of herbicolins O, I and 2C suggest that they contain reactive beta-lactams. Herbicolin O is the most labile of the three herbicolins, has the broadest activity spectrum, and is the only herbicolin whose activity was reversed by histidine (Table 3). This antagonism may reflect a mechanism for action of herbicolin O that involves histidine metabolism or uptake. Alternatively, it may reflect an inactivation of herbicolin O in a manner analogous to the inactivation of some penicillins; nucleophilic addition to the beta-lactam carbonyl by imidazoles has been reported (3).

Even though they are beta-lactam antibiotics, it can not be assumed that herbicolins O, I and 2C have the same mode of action as penicillin; that is, that they inhibit penicillin binding proteins (16). Tabtoxin, which contains a beta-lactam (17), is an inhibitor of glutamine synthetase in plants (22). Also, monobactams have low affinities for essential penicillin binding proteins (8). In addition to the mode of action, the stereochemistry and ecological significance of herbicolins O, I, and 2C is yet to be determined.

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SECTION IV

IN VIVO ANTAGONISM OF *ERWINIA AMYLOVORA* BY *ERWINIA HERBICOLA* STRAIN C9-1 AND HERBICOLINS O, I and 2C

INTRODUCTION

The biological control of *Erwinia amylovora* (Burr) Winslow et al., causal organism of fire blight on Rosaceous hosts, has been investigated as an alternative to chemical control strategies (1, 11). Indeed, some success, as measured by reductions in disease severity, have been obtained when the biological control agent used was *E. herbicola* (Lohnis) Dye or unidentified yellow saprophytic bacteria. Such reductions have been greatest when the biological control agent was inoculated prior to, or together with, the pathogen (6, 7, 10, 12). Though reductions have been procurred through biological control strategies, chemical control of the pathogen is still a more dependable and economically advantageous strategy (1, 11).

The mechanism(s) by which yellow saprophytic bacteria antagonize disease development are equivocal. More is known of the *in vitro* antibiosis of *E. amylovora* by saprophytic bacteria. This antibiosis has been attributed to acid (5, 10), hydroquinone (4), bacteriocin (2, 3), and, more recently, antibiotic production by *E. herbicola*. The ability to produce bacteriocins appears to be incidental to the reductions observed *in vivo*; non-producing mutants of *E. herbicola* bacteriocin producing strains also reduce disease severity (3). Induced resistance by saprophytic bacteria has been proposed to account for some of the reductions obtained (8, 12). However, a thorough examination is yet to be conducted in this regard.

E. herbicola strain C9-1 has been demonstrated to produce antibiotics *in vitro*. These antibiotics are called herbicolins. Three herbicolins: O, I and 2C, have been isolated from broth culture of *E. herbicola* strain C9-1. The herbicolins O, I and 2C have been identified as novel monocyclic, beta-lactam antibiotics. Herbicolin O activity is reversed by the addition of L-histidine to the detection medium. The detection of multiple antibiotic production by *E. herbicola* strain C9-1 was aided by the selection of spontaneous herbicolin insensitive mutants of *E. amylovora* strain 110. From these selections, mutants of *E. amylovora* were obtained which were insensitive to either herbicolin O, or herbicolins I and 2C, or insensitive to all three herbicolins.

The purpose of this report is to determine if *E. herbicola* strain C9-1 can effectuate a reduction in disease severity *in vivo*, to determine if partially purified herbicolins can reduce disease severity *in vivo*, and to determine if insensitivity to herbicolins affects the results obtained.

MATERIALS AND METHODS

Bacteria. *Erwinia herbicola* strain C9-1 was isolated from fire-blighted apple *Malus malus* 'Jonathan' stem tissue. *E. amylovora* and derivatives of this strain (table 1) were used as pathogens. All bacteria were stored in phosphate buffer (0.33 M potassium phosphate buffer, pH 7.0) glycerol solutions at -20C. Bacteria were cultured on tryptose blood agar base (TBA) slants for 48 hr before use.

Preparation of inoculum. Cells, that were obtained from broth culture of *E. herbicola* strain C9-1 and *E. amylovora* strains 110 and 12, served as inoculum for greenhouse experiments. Twenty-five ml of culture medium (Difco nutrient broth, supplemented with 0.5% w/v glucose and 0.25% w/v yeast extract) was inoculated with cells, obtained from a TBA slant washing, to an optical density (620 nm) of 0.1. Cells were grown with aeration to late logarithmic growth phase. At this time, they were used to inoculate fresh medium (200 ml contained in a 2 l flask) to an optical density of 0.1. These broths were grown, as described above, to an optical density of 2.0. Cells were then pelleted by centrifugation (9500 x g for 20 min), washed in phosphate buffer, and resuspended in phosphate buffer to an optical density of 0.05. Viable cell counts of these suspensions were obtained by plating (0.01 ml)

Table 1. Description of *Erwinia amylovora* strain 110 and its derivatives.

E. amylovora strain	description
110	sensitive to herbicolins O, I and 2C
HI	insensitive to herbicolins I and 2C
O	insensitive to herbicolin O
12	insensitive to herbicolins O, I and 2C

dilutions of the cells onto TBA contained in petri dishes.

Inoculum for pear fruit assays was prepared by washing cells (5 ml phosphate buffer) off the surface of a 24 hr TBA slant culture of each strain. These washings were diluted with phosphate buffer to an optical density of 0.05.

Preparation of herbicolins. Herbicolins were obtained from broth culture of *E. herbicola* strain C9-1, as previously described. The medium of Neidhart et al. (9), prepared without glucose, and supplemented with gluconate (10 mM), asparagine (10 mM), thiamine (0.01 mM) and nicotinic acid (0.01 mM), was used for production of herbicolins. Herbicolins were removed from concentrated supernatants by cation exchange chromatography (Dowex 50W x 4, 200-400 mesh, NH_4^+ cycle, equilibrated in 2.5 mM ammonium acetate buffer, pH 5.0). Herbicolins were eluted with 20 mM acetate buffer. Preparations obtained were desalted by the addition of activated charcoal (Darco G-60, 5 g/100 ml). After stirring for 12 hr at 4C, the mixture was filtered. Herbicolins were eluted from the charcoal with 50% (v/v) ethanol: water (50 ml/g). After stirring for 2 hr at 4C, the charcoal was removed by filtration. The ethanol was removed under reduced pressure at 40C, and herbicolins were resuspended in distilled, deionized water. This desalting procedure was then repeated. The herbicolins obtained were designated partially purified herbicolins. These preparations contained 40 units/ml of herbicolin O and 60 units/ml of herbicolins I and 2C. Activity (units/ml) was defined as the reciprocal of the highest dilution of a preparation to inhibit _E_. _amylovora_ strain 110, when the indicator was grown with and without histidine (10 mM) in the overlay medium; overlay and basal medium was modified Neidhart medium, solidified by addition of 1.5% (w/v) Bacto agar; samples (0.01 ml) were spotted.

Pear fruit assays. Immature 'Bartlett' pear fruit (2-3 cm in diameter) were collected, and stored at 4C. For assays, fruit were peeled, sliced (3 mm), quartered, and then placed on sterile filter paper disks (Whatman 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, or partially purified herbicolins. After 15 minutes, 0.01 ml of _E_. _amylovora_ strain 110, O, HI or 12 (10^7 cfu/ml) was spotted onto the fruit. The petri dishes were sealed with parafilm, and placed in a 30C 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. From five pear slices/dish, an average disease severity rating was determined. Each experiment was repeated three times. The experiments were analyzed as a randomized complete block design.

Potted apple tree assays. One-year-old dormant apple _Malus_ _malus_ 'Ida Red' trees, that had been grafted onto M109 rootstalks, were pruned to yield five buds/tree, and placed in a greenhouse. When four leaves/terminal were evident, trees were inoculated to run-off with suspensions of *E. herbicola* strain C9-1, streptomycin (50 ppm), or phosphate buffer; treatments were applied with a hand held atomizer. Thirty minutes later, trees were inoculated with *E. amylovora strains* 110 or 12, or phosphate buffer. After inoculations were completed, a plastic bag was loosely fitted over the trees to cover the terminals, and secured with a twist tie. Bags were removed 24 hr later. Disease severity ratings, recorded as the number of blighted terminals/tree, were assessed at 10 and 25 days after inoculation. Treatments were replicated three times in each experiment; the experiment was repeated five times. The experiment was analyzed as a randomized complete block design.

RESULTS AND DISCUSSION

At 10 days, the disease severity ratings on apple trees that had been inoculated with E. herbicola strain C9-1 were significantly lower than those of trees inoculated with the pathogen alone (Table 2). This reduction was seen when the pathogen was an herbicolin sensitive strain and when the pathogen was an herbicolin insensitive strain. At 25 days, however, a reduction in disease severity was only seen in trees that had been inoculated with the herbicolin insensitive, E. amylovora strain 12. When treated with buffer, disease severity ratings of trees inoculated with E. amylovora strain 110 were not significantly different than those of trees inoculated with E. amylovora strain 12 (Table 2).

Similar results were obtained by using pear fruit as the host tissue (Tables 3 and 4). When E. amylovora strain 110, 12, HI or O was the pathogen, prior inoculation of fruit with E. herbicola strain C9-1 was correlated with significant reductions in disease severity, as compared to those of fruit inoculated with a pathogen alone (Table 3). This reduction was significantly less in fruit inoculated with E. amylovora strain HI or O. Similar results were obtained by applying partially purified herbicolins to fruit (Table 4).

Table 2. Effect of _Erwinia herbicola_ strain C9-1 on fire blight disease severity of potted apple 'Ida Red' trees.[a]

Treatment[c]	Mean disease severity rating[b]	
	10 days[d]	25 days[e]
Buffer; buffer[f]	0 c	0 b
Buffer; _E. amylovora_ 110[g]	3.1 a	3.4 a
Buffer; _E. amylovora_ 12	2.5 ab	3.0 a
Streptomycin; _E. amylovora_ 110	0.4 c	0.4 b
Streptomycin; _E. amylovora_ 12	0.1 c	0.1 b
E. herbicola; buffer	0 c	0 b
E. herbicola; _E. amylovora_ 110	1.9 b	3.0 a
E. herbicola; _E. amylovora_ 12	0.5 c	0.9 b

[a] One year old grafted (M109 rootstocks) potted trees grown under greenhouse conditions.

[b] Disease severity rated as the number of terminals blighted/5 terminals. Numbers in a column with the same letter were not significantly different ($P=0.05$).

[c] Treatments applied at 4 leaves/terminal stage. Order of treatment application is indicated by the sequence in which they are written. Treatments were sprayed until run-off with a hand-held atomizer.

[d] Disease severity at 10 days after inoculation.

[e] Disease severity at 25 days after inoculation.

[f] 0.033 M potassium phosphate buffer, pH 7.0.

[g] Suspensions of bacteria contain 10^7 colony forming units/ml.

Table 3. Effect of *Erwinia herbicola* strain C9-1 on fire blight disease severity of pear 'Bartlett' fruit slices.[a]

Treatment[b]	Mean disease severity rating[c]
Buffer; buffer[d]	1.4 c
Buffer; *E. amylovora* 110	5.0 a
Buffer; *E. amylovora* 12	4.9 a
Buffer; *E. amylovora* HI	4.6 a
Buffer; *E. amylovora* 0	4.7 a
E. herbicola; buffer	1.0 c
E. herbicola; *E. amylovora* 110	1.8 c
E. herbicola; *E. amylovora* 12	1.5 c
E. herbicola; *E. amylovora* HI	2.7 b
E. herbicola; *E. amylovora* 0	2.7 b

[a] 2-3 cm immature pear fruit were peeled, sliced (3 mm), and quartered.

[b] 0.01 ml of each was treatment applied in order of the sequence written. Bacterial suspensions contained 10^7 colony forming units/ml.

[c] Average disease severity rating of 5 slices/petri dish, with replications (see text for experimental design); 1 = no symptoms; 5 = ooze and complete blackening of tissues. Numbers with the same letter were not significantly different (P = 0.05).

[d] 0.033 M potassium phosphate buffer, pH 7.0.

Table 4. Effect of partially purified herbicolins[a] on fire blight disease severity of pear 'Bartlett' fruit slices.[b]

Treatments[c]	Mean disease severity rating[d]
Buffer[e]; *Erwinia amylovora* 110[f]	5.0 a
Buffer; *E. amylovora* 12	4.4 a
Buffer; *E. amylovora* HI	4.9 a
Buffer; *E. amylovora* 0	4.8 a
Herbicolins; *E. amylovora* 110	1.9 c
Herbicolins; *E. amylovora* 12	2.4 c
Herbicolins; *E. amylovora* HI	4.5 a
Herbicolins; *E. amylovora* 0	3.4 b

[a] Herbicolins were purified by cation exchange chromatography (see text). Preparations contained 40 units/ml of herbicolin O and 60 units/ml herbicolin I and 2C (see text).

[b] Immature pear fruit (2-3 cm diameter) were peeled, sliced (3 mm), and quartered.

[c] 0.01 ml of each treatment was applied; order of application is indicated by the sequence written.

[d] Average disease severity was obtained on 5 pear slices/petri dish, with replications; 1 = no symptoms present; 5 = ooze and complete blackening of the tissues.

[e] 0.033 M potassium phosphate buffer, pH 7.0.

[f] Bacterial suspensions contained 10^7 colony forming units/ml.

Considered together, these results suggest that inoculation of host tissues with E. herbicola strain C9-1, prior to inoculation with the pathogen, can reduce disease severity. These reductions are probably not due to an induced resistance response of the host, because of the brevity between treatments (less than 30 min). An interesting result of these experiments was the finding that insensitivity to herbicolins O, I and 2C does not confer an advantage to the pathogen. In fact, the greatest reductions in disease severity were observed when E. amylovora strain 12 was the pathogen. Some advantage is conferred by insensitivity to individual herbicolins (Tables 3 and 4). A correlation between herbicolin production and E. herbicola's antagonism of disease is suggested by the fact that application of partially purified herbicolins, or cells of E. herbicola, yields similar pathogen strain dependent results. A reasonable approach to elucidating a mechanism for this antagonism may involve deciphering the nature of herbicolin insensitivity in E. amylovora and the mode of action of herbicolins O, I and 2C.

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