Bacteriocin-Like Substances from Tomato Race 3 Strains of *Xanthomonas campestris* pv. *vesicatoria*

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ABSTRACT

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Tomato race 3 (T3) strains of *Xanthomonas campestris* pv. *vesicatoria* are antagonistic in vitro to tomato race 1 (T1) strains of the bacterium. All T1 strains and 11 strains of other *X. campestris* pathovars tested were inhibited by T3 strains. Sensitivity of tomato race 2 (T2) strains was variable. No strains from other bacterial genera tested were inhibited. Cell-free filtrates from T3 strains were inhibitory to sensitive strains. The inhibitory activity of these filtrates was lost after treatment at temperatures above 80°C and with selected protease enzymes. However, treatment with trypsin or DNase had no effect on their activity. Seven cosmid clones from a genomic library of a T3 strain were selected for their ability to consistently inhibit a sensitive indicator strain in plate assays. Southern hybridization analysis placed these into three bacteriocin (BCN)-producing groups designated BCN-A, BCN-B, and BCN-C. The BCN-like groups could be differentiated by variations in inhibitory spectra and levels of activity in plate assays. Mutations that inactivated expression of each BCN group individually in a wild-type T3 strain had inhibitory activity confirming that multiple BCNs are present in the T3 strain. T3 strains were inhibitory to a sensitive indicator strain in tomato leaf tissue, but this effect was observed only when T3 strains were applied in advance of the sensitive strain. BCN-A was the major BCN-like substance involved in the suppression of the sensitive indicator strain in tomato leaf tissues.

Xanthomonas campestris pv. *vesicatoria* (Doidge) Dye causes bacterial spot disease on pepper (*Capsicum annuum* L.) and tomato (*Lycopersicon esculentum* Mill.). Strains of this bacterium can be placed into groups based on the host species attacked (i.e., tomato and pepper) and into races based on the disease response on differential pepper and tomato cultivars (12). The race composition of the pathogen is variable in nature. Race shifts can occur as a result of changes in plant genotypes (12), introduction of new races on seeds or transplants (19), application of copper-based bactericides that select for strains carrying the copper-resistance plasmid and associated *avrBs1* gene (23), and differences in strain competitiveness (10). An opportunity to study the competitive nature of strains of *X. campestris* pv. *vesicatoria* in the same populations was recently afforded. Prior to the early 1990s, only tomato race 1 (T1) strains of the bacterium were detected on tomato plants in Florida (3,4). In 1991, tomato race 3 (T3) strains of *X. campestris* pv. *vesicatoria* were first identified in two Florida locations (13). Within 4 years, T3 strains became dominant on tomato in commercial fields and were found in all areas of the state (10).

This race shift was particularly surprising because T1 strains were the only strains in Florida on tomato for the prior 30 years. Coincident with the change of race was the observation that T3 strains were inhibitory to T1 strains (6). Antibiosis is one of the factors commonly involved in microbial competition (29,30).

The objectives of this study were to determine the cause of the antibiosis of T3 strains against T1 strains, define the genetic determinants responsible, and determine if the antibiosis factors, in part, explain T3 strains' ability to compete more effectively than T1 strains.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All strains of *X. campestris* pv. *vesicatoria* were grown on nutrient agar (NA; Becton, Dickinson and Company, Sparks, MD) at 28°C. Strains of *Escherichia coli* were grown on Luria-Bertani (LB) medium (16) at 37°C. All strains were stored in capped tubes containing 2.0 ml of sterile tap water at room temperature or in nutrient broth (NB) containing 30% glycerol at –70°C. Antibiotics were used to maintain selection for resistance markers at the following concentrations: tetracycline (Tet), 10 μg/ml; rifamycin (Rif), 100 μg/ml; and spectinomycin (Spec), nalidixic acid (Nal), and kanamycin (Kan), 50 μg/ml.

Screening for antimicrobial activity. Screening of T3 strains for the production of inhibitory products was performed with a deferred assay (25). Strains to be tested for antagonism were grown on a shaker for 18 h at 28°C in tubes containing 4 ml of NB. The cells were pelleted and resuspended into sterile tap water. Suspensions were standardized to A_{600} = 0.3, equating to $\approx 3 \times 10^8$ CFU/ml. Twenty-five microliters was then spotted onto NA plates, five strains per plate, and incubated for 18 h at 28°C. Both a positive and a negative control were plated onto each test plate. A suspension of 3×10^7 CFU/ml of the indicator strain was sprayed over the surface of the plate with an aerosol spray unit (Sigma Chemical, St. Louis). A clear zone of inhibition around test colonies after 24 to 48 h was considered indicative of antagonism. The width of inhibition zones was measured as the distance from the edge of the test culture to the outer edge of the zone. Each treatment was replicated four times.

Cell-free filtrates were screened for inhibitory activity. Test cultures were grown for 18 h in NB followed by centrifugation to pellet cells. The supernatants were then sterilized using low protein binding membrane filters (Gelman Sciences, Ann Arbor, MI) with a pore size of 0.2 μm and analyzed for antagonistic activity by the well-diffusion method (26). Ten-millimeter-diameter

wells were cut into NA plates containing 20 ml of the medium. Two hundred microliters of filtrate was applied to each well and allowed to diffuse for 12 h. Media were overlaid with 3.5 ml of 0.7% molten (45°C) water agar containing 200 µl of a 3 × 10^8 CFU/ml suspension of the indicator strain. Strain 91-106 (T1) was used as both the negative control and indicator strain for these studies. Plates were examined after 24 h at 28°C for zones of inhibition around the wells. Each treatment was replicated four times.

Sensitivity to heat and enzymes. Cell-free filtrates were assessed for their sensitivity to various enzyme and heat treatments. Temperature stability was determined by heating 200 ml of culture filtrate to 65, 80, or 100°C for 10, 20, or 30 min, respectively. After heat treatments, 200-µl samples were assayed for activity by the well-diffusion method. The enzymes and their buffers were pronase (Sigma) in 5 mM Tris-HCl (pH 7.3) with 10 mM $CaCl_2$; proteinase K (Boehringer Mannheim Biochemicals, Indianapolis) in 50 mM Tris-HCl (pH 7.3) with 5 mM $CaCl_2$; trypsin (Sigma) in 50 mM Tris-HCl (pH 7.9) with 5 mM $CaCl_2$; and DNase (Promega, Madison, WI) in 40 mM Tris-HCl (pH 7.9) with 10 mM NaCl, 6 mM $MgCl_2$, and 10 mM $CaCl_2$. Enzymes were tested at a final concentration of 1 mg/ml, except for pronase and trypsin which were tested at final concentrations of 100 and 200 µg/ml, respectively. The mixtures were incubated for 2 h at 37°C, except for samples containing trypsin, which were incubated at 24°C. Prior to assaying for antagonistic activity, preparations containing trypsin and proteinase K were treated with protease inhibitors. Trypsin was inhibited using type II-0 Ovoinhibitor (Sigma) at a final concentration of 1 mg/ml. Proteinase K was treated with 170 µg of phenylmethylsulfonyl fluoride (Sigma) per ml according to the manufacturer's instructions. After enzyme treatments, 200-µl samples were assayed for remaining activity by the well-diffusion method.

Test for bacteriophage activity. The possible involvement of bacteriophages was tested by determining if the inhibitor agent could be transferred in the absence of the bacterium by two different methods. In the first method, agar plugs of 2-mm diameter were removed from inhibition zones and crushed in 500 µl of NB. Fifty microliters of chloroform was added, and the mixture was vortexed vigorously and then centrifuged at 14,000 × *g* for 1 min. Fifty microliters of the top phase was removed and added to 450 µl of sterile deionized water. Five serial 10-fold dilutions were made. Five hundred microliters of each was added to 3.5 ml of melted 0.7% water agar (50°C) in tubes containing 200 µl of the indicator strain (75-3) and plated on the surface of an NA plate. Each dilution was plated in duplicate. After incubating for 24 h at 28°C, plates were examined for the presence of plaques. Cell-free filtrates with inhibitory activity were examined by electron microscopy for the presence of bacteriophage particles on Formvar-coated grids washed with distilled water and negatively stained with 1% uranyl acetate.

Another method was used to screen for chloroform-sensitive bacteriophages. A thin layer of agar was removed with a sterile loop from the surface of the zone between a T3 producing strain and a T1 sensitive strain, 91-106. The loop was quadrant streaked over the agar surface in a petri dish containing a lawn of a 24-h-old suspension of 91-106. The plates were assessed for the appearance of plaques after 24 h incubation at 28°C. A positive control was used in which similar plates were quadrant streaked with a T1 bacteriophage.

DNA manipulations and genetic techniques. Standard procedures were used for plasmid isolation, restriction endonuclease digestion, agarose gel electrophoresis, and Southern hybridizations (21). A cosmid library of purified DNA from *X. campestris* pv. *vesicatoria* strain 91-118 was screened. The library was constructed in the cosmid vector pLAFR3 (Tet^r) (24) and maintained in *E. coli* strain DH5α. Each cosmid clone was conjugated into *X. campestris* pv. *vesicatoria* strain ME90 (Rif^r, Kan^r) using triparental mating as described previously (2). Cosmid clones (Tet^r) were used as donor strains, strain ME90 as the recipient and pRK2073 ($Spec^r$) as the helper plasmid. All three strains were mixed on nutrient yeast-glucose agar (5) and incubated at 28°C for 24 h. The mixture was resuspended in sterile tap water and

TABLE 1. Strains and plasmids used in study

Strain or plasmid	Relevant characteristics	Source or reference[a]
Xanthomonas campestris pv. *vesicatoria*		
75-3	T1 strain, BCN sensitive	RES
91-118	T3 strain, BCN producing	RES
91-118R	Rif^r	This study
91-106	T1 strain, BCN sensitive	RES
91-106R	Rif^r	This study
ME90	Rif^r Kan^r	Bonas et al. (2)
ME-A	$BCN^-A^-B^+C^+$	This study
ME-B	$BCN^-A^+B^-C^+$	This study
ME-C	$BCN^-A^+B^+C^-$	This study
Escherichia coli		
HB101	F^- rec A	BRL
DH5α	F^- rec A	BRL
C2110	Nal^r	BRL
Plasmids		
pLAFR3	tet^r *rlx*$^+$ RK2 replicon	Staskawicz et al. (24)
pRK2073	$Spec^r$ tra^+ mob^+	Figurski and Helinski (8)
pHoKmGus	Kan^r Amp^r *tnp A*$^-$ contains promoterless β-glucuronidase gene	BJS
pXV120	pLAFR3 cosmid clone from *X. campestris* pv. *vesicatoria* strain 91-118 (BCN-C^+)	Tudor (27)
pXV442	pLAFR3 cosmid clone from *X. campestris* pv. *vesicatoria* strain 91-118 (BCN-B^+)	Tudor (27)
pXV501	pLAFR3 cosmid clone from *X. campestris* pv. *vesicatoria* strain 91-118 (BCN-A^+)	This study
pXV519	pLAFR3 cosmid clone from *X. campestris* pv. *vesicatoria* strain 91-118 (BCN-A^+)	Tudor (27)
pXV344	pLAFR3 cosmid clone from *X. campestris* pv. *vesicatoria* strain 91-118	This study
pXV699	pLAFR3 cosmid clone from *X. campestris* pv. *vesicatoria* strain 91-118 (BCN-B^+)	This study
pXV711	pLAFR3 cosmid clone from *X. campestris* pv. *vesicatoria* strain 91-118 (BCN-A^+)	This study
pXV717	pLAFR3 cosmid clone from *X. campestris* pv. *vesicatoria* strain 91-118	This study
pXV754	pLAFR3 cosmid clone from *X. campestris* pv. *vesicatoria* strain 91-118 (BCN-A^+)	This study
pXV878	pLAFR3 cosmid clone from *X. campestris* pv. *vesicatoria* strain 91-118	This study
pXV933	pLAFR3 cosmid clone from *X. campestris* pv. *vesicatoria* strain 91-118	This study

[a] RES, R. E. Stall, University of Florida, Gainesville, FL, BRL, Bethesda Research Laboratories, Gaithersburg, MD; BJS, B. J. Staskawicz, University of California, Berkeley.

plated onto NA containing rifamycin, kanamycin, and tetracycline. Purified transconjugants were transferred onto NA plates and screened for antagonism against strain 91-106 as described previously. Seven hundred fifty clones, each containing 15 to 30 kb of insert DNA, were screened for antagonistic activity. Transconjugants surrounded by clear zones of inhibition were selected for further study. Expression of bacteriocin (BCN) activity of selected transconjugants also was tested in four other recipients (T1 strains) and against several other indicator strains.

Clones with BCN activity in T1 strains were used in cross-hybridization experiments with each other. Insertion mutagenesis of BCN clones was performed using the Tn3HoHo derivative, pHoKmGus (provided by D. Dahlbeck and B. Staskawicz, University of California, Berkeley). The protocol used was as follows: *E. coli* strain HB101 (pHoKmGus) was transformed with plasmid DNA isolated from a BCN clone. Transformants (Tet^r Kan^r) were then mated with *E. coli* C2110 (Nal^r) using pRK2073 as the helper plasmid (2). Matings were plated on LB agar plates amended with tetracycline, kanamycin, and nalidixic acid. Insertion derivatives were mobilized into *X. campestris* pv. *vesicatoria* strain ME90 as described previously and screened for loss of BCN activity against strain 91-106. Confirmation of insertions was made by restriction endonuclease analysis.

Marker exchange was performed using triparental matings with BCN^- insertion mutants (Tet^r Kan^r) as donors, 91-118R (Rif^r) as the recipient and pRK2073 as the helper plasmid. The rifamycin-resistant 91-118R strain was previously isolated by plating 10^9 cells of wild-type 91-118 onto NA amended with rifamycin, followed by selection of resistant colonies with wild-type BCN and pathogenicity characteristics. Transconjugants were selected on NA amended with tetracycline, kanamycin, and rifamycin. They were then serially transferred daily for 10 days onto NA amended with kanamycin and rifamycin. At the end of the transfer schedule, dilutions were plated onto a phosphate medium (NA containing 10 g of NaH_2PO_4 per liter) containing kanamycin and rifamycin. Single colonies were screened on a medium containing all three antibiotics. Colonies that were unable to grow on this medium (Tet^s Kan^r Rif^r) were selected to represent those in which a successful recombination event had occurred with concomitant loss of the introduced plasmid (pLAFR3). These were then tested for loss of BCN activity. Confirmation was obtained by probing marker exchange mutants with a probe derived from the transposable element used to generate the mutants.

Plant assays. Leaflets of 6-week-old seedlings of the tomato cultigen Florida 7060 were infiltrated with bacterial suspensions of both the producer and the indicator strains with a hypodermic syringe as described previously (10). Prior to inoculation, strains were grown in NB for 18 h, harvested by centrifugation, and resuspended in sterile tap water. Suspensions of producer and sensitive strains were infiltrated into fully expanded leaflets at the same time or into the same area with the producer strain inoculated 12 h prior to the sensitive indicator strain. Concentrations of the producer strains and indicator strains were 5×10^7 and 5×10^6 CFU/ml, respectively. Each treatment consisted of three replications. Plants were incubated between 24 and 28°C. The indicator strain populations in leaflets of each treatment were quantified by removing 1-cm^2 leaf disks from inoculated areas, macerating in 1 ml of sterile tap water, and plating 10-fold dilutions onto NA amended with the appropriate antibiotic. Populations were determined at 12- to 24-h intervals. Each experiment was repeated at least twice with three replicates per treatment.

RESULTS

Screen for antimicrobial activity. None of the *X. campestris* pv. *vesicatoria* T3 strains were sensitive to antagonism caused by the T3 producer-strain 91-118 (Table 2), whereas all *X. campestris* pv. *vesicatoria* strains classified as T1 were inhibited by strain 91-118. Zones of inhibition ranged from 6 mm against strain 6107 to 1 mm against strain BV55. T2 strains were variable in their sensitivity to inhibition by strain 91-118. Twelve of the twenty-four pathovars of *X. campestris* screened were sensitive to antagonism by strain 91-118. None of the other bacterial genera tested were sensitive (Table 2). Sterile culture filtrates of *X. campestris* pv. *vesicatoria* strain 91-118 were inhibitory to indicator strain 75-3.

Sensitivity to heat and enzymes. Antagonistic activity against strain 75-3 (sensitive) was detected with cell-free filtrates held at 65°C for 30 min. However, when filtrates were held at temperatures of 80°C and above for 10 min or longer, activity was lost. Treatment of culture filtrates with pronase and proteinase K resulted in the inactivation of antagonism. Treatment of filtrates with trypsin and DNase had no effect on activity.

Test for antagonism due to bacteriophage. No plaques were observed in lawns after 24 h of incubation. Thus, the inhibitory agent had not replicated in the indicator strain. No phage-like structures were identified in culture filtrates of strain 91-118 by

TABLE 2. Diameter of inhibition zones caused by tomato race 3 strains of *Xanthomonas campestris* pv. *vesicatoria* against other bacterial strains

Strain	Race	Diameter of inhibition zone (mm)[a]
X. campestris pv. *vesicatoria*		
91-108, 91-106, 92-40, 92-51, 92-47, 93-29, 91-111, 80-5, 94-1, 93-1, 1712, 90-60, 92-17, 92-16, 91-66, XV14, 89-8, 89-10, 75-3, 90-40, 87-44, 87-21, 1522, 1520, 87-35, 1521	T1	2–4
90-21, 87-13	T1	1
6107	T1	6
10, MM-E, BA28-1, BV55, 0350A	T2	1–3
92-48, 9, 18, 19, MM-G, 90-20, 0226A	T2	0
1483, 1484, 91-118	T3	0
X. campestris		
pv. *alfalfae*	–	3
pv. *amoraceae*	–	0
pv. *begoniae*	–	3
pv. *campestris*	–	0
pv. *carotae*	–	0
pv. *celebensis*	–	3
pv. *corylina*	–	0
pv. *dieffenbachiae*	–	2
pv. *fici*	–	0
pv. *glycines*	–	3
pv. *graminis*	–	3
pv. *holcicola*	–	2
pv. *juglandis*	–	0
pv. *maculifoliigardeniae*	–	0
pv. *pelargonii*	–	1
pv. *phaseoli*	–	2
pv. *phaseoli* var. *fuscans*	–	0
pv. *physalidicola*	–	0
pv. *pisi*	–	0
pv. *poae*	–	1
pv. *strelitzia*	–	0
pv. *taraxici*	–	3
pv. *translucens*	–	0
pv. *vitians*	–	1
Agrobacterium tumefaciens	–	0
Erwinia stewartii	–	0
Pseudomonas syringae		
pv. *syringae*	–	0
pv. *tomato*	–	0
Xanthomonas fragariae	–	0
Stenotrophomonas maltophilia	–	0
Clavibacter michiganensis	–	0
subsp. *michiganensis*	–	0
subsp. *sepedonicus*	–	0
subsp. *insidiosus*	–	0
Clavibacter rathayi	–	0

[a] Width of inhibition zone measured as the distance from the edge of the test culture to the outer edge of the zone. Represents an average of four replicates. Indicator strain applied by spray to plates.

electron microscopy. No plaques from the zone were observed with the quadrant streaking method, but visible plaques were observed with the positive bacteriophage control.

Screen of cosmid library. Cosmid clones mobilized into *X. campestris* pv. *vesicatoria* strain ME-90 conferred antagonistic activity when tested against the indicator strain 91-106. These clones were divided into three groups based on cross-hybridization experiments: BCN-A consisted of pXV501, pXV519, pXV711, and pXV754; BCN-B consisted of pXV442 and pXV699; and BCN-C consisted solely of pXV120. No activity was detected in the *E. coli* strain in which the library was maintained.

Differences between BCN groups. Differences between in vitro inhibitory activity of a sensitive strain containing each clone were observed among the three groups (Fig. 1A). The strain containing the BCN-A clones consistently produced the largest zones of inhibition, even larger than those of the wild-type T3 strain, whereas BCN-C activity consistently produced the smallest zones of inhibition. Activity in nonconcentrated cell-free filtrates occurred only with the clones for the BCN-A group (Fig. 1B).

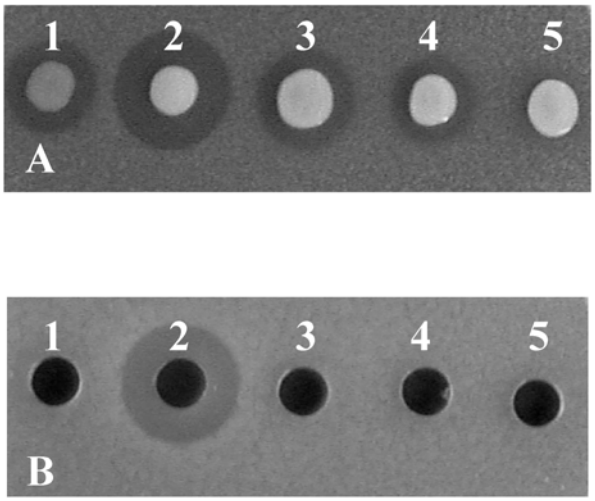

Fig. 1. Assays of bacteriocin (BCN)-like activity against *Xanthomonas campestris* pv. *vesicatoria* strain 91-106. Included in the assays were **A,** viable cell cultures and **B,** cell-free supernatants. Plate designations were as follows: lane 1, 91-118 (BCN$^+$); lane 2, pXV519 in ME90 (BCN-A$^+$); lane 3, pXV442 in ME90 (BCN-B$^+$); lane 4, pXV120 in ME90 (BCN-C$^+$); and lane 5, pLAFR3 in ME90.

TABLE 3. Differential inhibition of selected *Xanthomonas campestris* pv. *vesicatoria* indicator strains by the three identified groups of bacteriocin (BCN)-like activity (BCN-A, BCN-B, and BCN-C)[a]

Indicator strain	Tomato race	BCN-A	BCN-B	BCN-C
92-51	T1	–	+	+
M103B	T1	++	+	+
91-106	T1	++	+	+
91-108	T1	++	+	+
91-111	T1	+	–	–
92-40	T1	–	+	–
93-29	T1	++	+	+
XV14	T1	++	–	–
10	T2	+	–	–
XV56	T2	+	–	–
19	T2	+	–	–
92-48	T2	+	–	–
91-118	T3	–	–	–

[a] Width of inhibition zones measured as the distance from edge of test culture to the outer edge of the zone (millimeters). Measurements were used to assign three phenotypes which were scored as noninhibition (–), weak inhibition (zone diameters less than or equal to 4 mm) (+), and strong inhibition (zone diameters greater than 4 mm) (++). Each treatment was replicated four times. Strains were provided by R. E. Stall, Plant Pathology Department, University of Florida, Gainesville.

The BCN groups had differential inhibition patterns against selected *X. campestris* pv. *vesicatoria* indicator strains (Table 3). All T1 strains tested were susceptible to at least one of the BCN groups, whereas T2 strains were sensitive only to BCN-A. Some T1 strains, such as strain 91-106, which was used as the standard indicator strain for most experiments, were sensitive to all three groups. A wild-type T3 strain was not sensitive to any of the BCN-like activities.

Mutagenesis. Transposon insertion mutations in clones were obtained for the three BCN groups. Using these, marker exchange mutants for each of the wild-type loci in strain 91-118 were obtained and designated ME-A, ME-B, and ME-C. These mutants retained detectable levels of BCN activity (data not shown).

Plant assays. The population size of a sensitive strain in planta at 48 h after inoculation into tomato leaves was approximately 10^4 CFU/cm^2 less when a T3 strain (91-118) was applied 12 h prior to the exact same area of the leaf (Fig. 2). However, this reduction was not observed when the indicator strain and producer strain were inoculated simultaneously. The wild-type T1 strain did not significantly inhibit growth of the indicator strain in similar experiments, although relative to the water control, growth was slightly reduced.

Clones corresponding to each of the BCN groups were individually mobilized into *X. campestris* pv. *vesicatoria* strain ME90 and tested for their ability to inhibit the sensitive indicator T1 strain (91-106) in the tomato genotype 7060 when applied 12 h before the indicator strain. Both the wild-type T3 strain and the strain containing the BCN-A clone reduced the growth of the indicator strain by approximately 100- and 10,000-fold, respectively, over a 72-h period (Fig. 3). In contrast, the population size of the indicator strain increased in the presence of a wild-type T1 (BCN$^-$) strain and strains with BCN-B and BCN-C clones. The T3 strains with single mutations at each of the BCN loci were tested for their ability to inhibit a sensitive T1 strain when applied to tomato

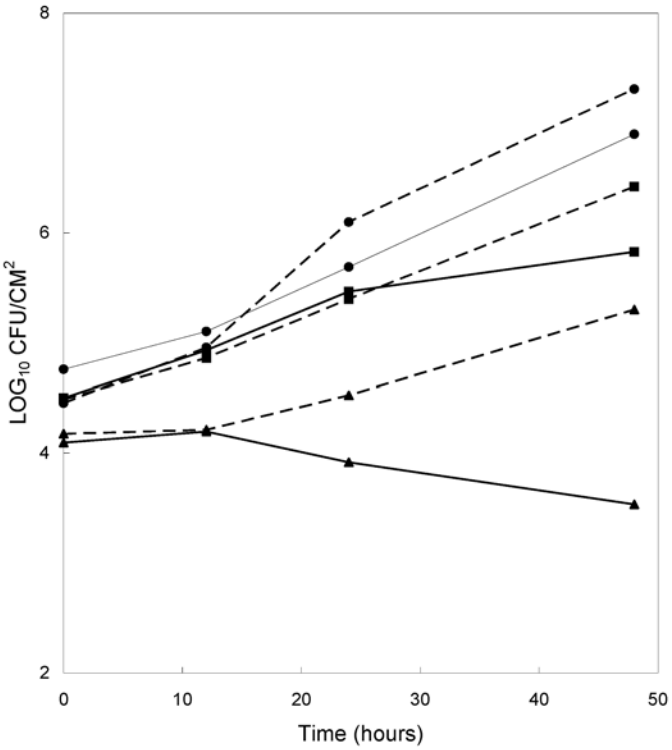

Fig. 2. Time course of growth of *Xanthomonas campestris* pv. *vesicatoria* indicator strain 91-106 in tomato cultigen 7060 when inoculated at the same time (dashed line) or 12 h after treatments (solid line). Bacterial populations in leaves were sampled every 12 to 24 h for 48 h following infiltration with sterile tap water (●), 75-3 (BCN$^-$) (■), and 91-118 (BCN$^+$) (▲). Results represent means of three independent experiments.

leaflets 12 h earlier. Growth of the indicator strain was restricted in the presence of the wild-type T3 strain and marker exchange strains containing mutations at the BCN-B and BCN-C loci (Fig. 4). However, a wild-type T3 strain mutated at the BCN-A locus only slightly inhibited the growth of the sensitive strain.

DISCUSSION

The production of an antagonistic compound against selected strains of *X. campestris* pv. *vesicatoria* was a trait that was uniformly found among the T3 strains tested. The substance causing antagonism was secreted extracellularly and was diffusible through agar media. In reports in which in vitro antagonism was attributed to metabolic by-products, the producer culture was always autosensitive (20). The opposite is true of BCN production (14,29). In the case of the observed antagonism, strain 91-118 was not sensitive to its own antagonistic agent, indicating that the phenomenon is unrelated to production of a toxic nonspecific metabolic by-product. Inactivation of the antagonistic properties of culture filtrates of strain 91-118 at temperatures of 80°C, and with pronase and proteinase K, indicates the involvement of a biologically active protein component, a characteristic shared by most BCNs.

Not all *X. campestris* pv. *vesicatoria* strains were equally sensitive to inhibition by T3 strains. There are four distinct groups within the bacterial spot of tomato pathogens (11,22). These groups are designated A through D. Strains belonging to these groups can be differentiated phenotypically and genetically. Sensitivity to the BCN-like compounds secreted by T3 strains represents another way that they differ as shown in Table 2.

The pathovars of *X. campestris* that were screened were also differentially sensitive to antagonism by T3 strains. A number of DNA homology groups were described within *Xanthomonas* spp. (28), some of which contain pathovars that attack related hosts, whereas others do not share this similarity. The sensitive pathovars from this study did not fit into a single homology group; therefore, this common trait is contrary to their genetic relatedness. Xanthomonad species were the only bacteria inhibited by the putative BCN. The compound therefore has a very narrow inhibitory spectrum, a characteristic shared by BCNs.

The antagonism of T3 strains to other strains of the bacterium was demonstrated to be associated with at least three different regions of DNA encoding for inhibitory activity. Four additional clones were isolated from the T3 genomic library; however, because of inconsistencies of antagonism in plate assays, they were not characterized further. Similar inconsistencies were observed in the BCN-like activity of *Pseudomonas solanacearum* (9). Marker exchange mutants of a wild-type strain at each of the three loci were still antagonistic, further confirming the production of compounds, which are expressed independently of each other.

The production of multiple BCN-like compounds by a single bacterial strain is rare (29) but has been reported to occur in bacteria such as *X. campestris* pv. *glycines* and *Leuconostoc* spp. (1,7,18). The former (7) was based on findings that the BCNs (of a single strain) directed toward the different indicator strains were variably sensitive to protease and heat treatments. However, these activities were neither resolved nor demonstrated to occur independently. In another study, five cosmid clones conferring BCN-like activity were isolated from an *X. campestris* pv. *glycines* cosmid library (1). Cross-hybridization experiments with these clones revealed the presence of multiple BCN-like activities within the single strain. However, the activities were not separated and differences between them were not examined further. Interestingly, a

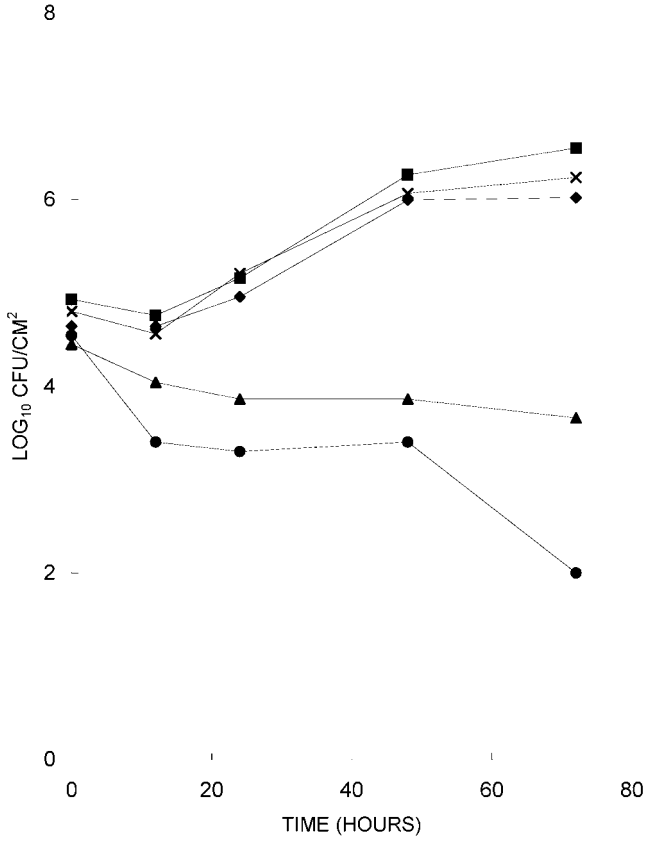

Fig. 3. Time course of multiplication of *Xanthomonas campestris* pv. *vesicatoria* indicator strain 91-106 in tomato cultigen 7060. Bacterial populations in leaves were sampled every 12 to 24 h for 72 h following infiltration with wild-type T1 strain 75-3 (BCN$^-$) (■), T3 strain 91-118 (BCN) (▲), pXV519 (BCN-A) (●), pXV442 (BCN-B) (♦), and pXV120 (BCN-C) (×). All plasmids were transferred to *X. campestris* pv. *vesicatoria* strain ME90 prior to testing.

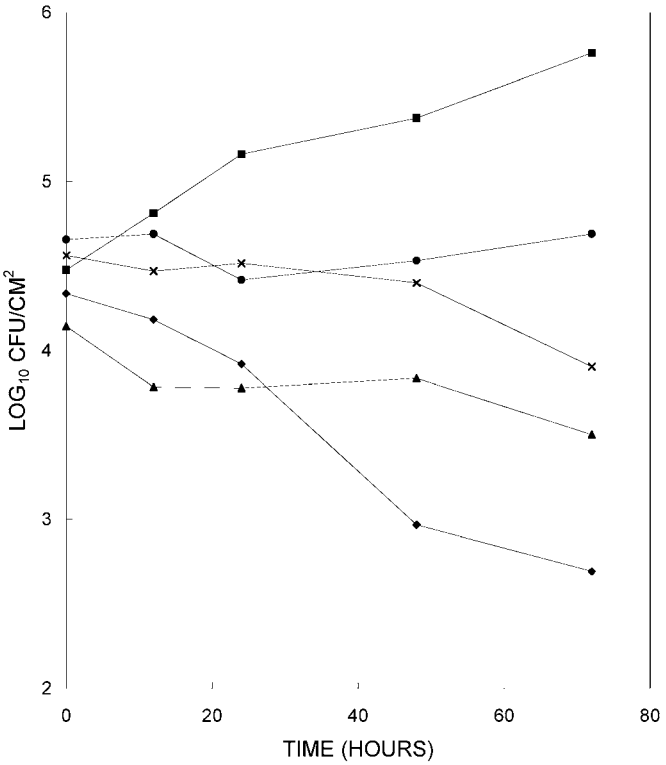

Fig. 4. Time course of multiplication of *Xanthomonas campestris* pv. *vesicatoria* indicator strain 91-106 in tomato cultigen 7060. Bacterial population in leaves were sampled every 12 to 24 h for 72 h following infiltration with strains 75-3 (T1) (BCN$^-$) (■), 91-118 (T3) (BCN$^+$) (▲), ME-A (BCN-B$^+$C$^+$) (●), ME-B (BCN-A$^+$C$^+$) (♦), and ME-C (BCN-A$^+$B$^+$) (×).

strain of *X. campestris* pv. *glycines* that produced BCNs (7) was inhibitory to some strains of *X. campestris* pv. *vesicatoria*.

T3 strains were inhibitory to T1 indicator strains in leaves of a susceptible tomato genotype, providing evidence of a direct selective advantage for T3 strains in the infection court. The coexistence of multiple races within a single leaf lesion in the field was documented (17), an indication that site competition may occur between two strains in a susceptible host. Reduction in the indicator strain population level was comparable to the reduction in populations achieved in the hypersensitive response of tomato line Hawaii 7998 to T1 strains (31). Similar growth levels of both producer and indicator strains independently in tomato leaves indicate that inhibition of the indicator strains is not the result of a plant effect. Antagonism, however, only occurred when T3 strains were preemptive. These results prompt speculation that the major use of these types of compounds in biocontrol strategies would be prophylactic rather than curative (29). In a recent study, a nonpathogenic T3 strain of *X. campestris* pv. *vesicatoria* was shown to reduce the severity of bacterial spot disease in the field when applied prophylactically (15).

Results of in planta antagonism studies indicate that BCN-A expression is an essential and dominant component in the suppression of a sensitive indicator strain in tomato leaf tissues. When expressed singly in a T1 strain, BCN-A inhibited indicator strain populations to an even greater extent than the wild-type T3 strain. However, the other two BCN-like compounds were insufficient on their own. Further evidence that BCN-A was the dominant component involved was obtained when a T3 mutant insertionally inactivated at this locus exhibited a much reduced inhibitory effect to the BCN-sensitive strain in vitro and in vivo. Inhibition of an indicator strain in tomato leaf tissues by BCN-A, coupled with the confirmation that it is produced in plant tissues, provides strong correlative evidence that BCN-A could play a major role in mediating the population dynamics between *X. campestris* pv. *vesicatoria* strains in tomato leaf tissues.

This study and others (10) have provided some initial insights as to the potential use of BCN-like producer strains in the biological control of bacterial spot disease on tomato. The lack of effective control measures to manage this disease justifies further investigation of this strategy, perhaps involving a more thorough screen for these types of compounds. In this case, identification of a compound with activity against T3 strains would be particularly useful.

A strain capable of producing several compounds against competitors likely has a selective advantage in natural ecosystems. In fact, the gradual increase of T3 strains of *X. campestris* pv. *vesicatoria* in tomato populations in Florida (10) may be a reflection of this phenomenon. Until 1991, T3 strains were not found in Florida; however, in field surveys over the following 4 years, T3 strains became increasingly predominant, and as of 1995 represented some 55% of the total strains isolated from tomato fields. Given that all T1 strains tested in the present study were susceptible to at least one of the BCN-like activities and that the T3 strains have increased in prevalence compared with T1 strains in Florida, it is very tempting to speculate that the antagonism between these strains has played a role in this race shift. In addition, no other obvious factors commonly associated with race shifts within populations could be identified in this situation (10).

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