Production, Properties, and Morphology of Bacteriocins from *Erwinia chrysanthemi*

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ABSTRACT

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Sixteen of 18 *Erwinia chrysanthemi* strains produced bacteriocins. The 18 strains were divided into five types according to a scheme devised by using three strains as bacteriocin producers. The activity spectra of all but one of the bacteriocinogenic strains was limited to *E. chrysanthemi*. Bacteriocins from two strains were characterized further. These bacteriocins were induced with ultraviolet light and mitomycin C, precipitated with ammonium sulfate, and inactivated between 60 and 70 C; they were not affected by trypsin, DNase, or RNase. Bacteriocins from both strains were composed of a contractile sheath and core. Loose cores and empty contracted sheaths were observed in preparations from both bacteriocins.

Additional key words: defective phages, typing by bacteriocin production.

Bacteriocins are nonreplicating, proteinaceous antibiotics with specificity generally restricted to closely related bacterial strains and species. Small amounts are produced spontaneously in cultures of bacteriocinogenic strains, but the substances also can be induced by treating cells with ultraviolet light or mitomycin C. Because bacteriocins specifically kill the cells of sensitive bacteria, bacterial strains can be differentiated or typed by sensitivity patterns to bacteriocins (9,10).

Bacteriocins are classified into two basic groups: low and high molecular weight. Low molecular weight bacteriocins are trypsin-sensitive, thermostable, and not sedimentable, whereas those of high molecular weight are sedimentable, trypsin-resistant, thermolabile, and visible with the electron microscope as phagelike components (1).

Bacteriocin-producing strains of *Erwinia chrysanthemi* were reported by Hamon and Peron (5) but details were not given. This paper deals with production, properties, and morphology of bacteriocins from *E. chrysanthemi*.

MATERIALS AND METHODS

Bacterial strains. Strains Ech 9 and Ech 10 of *E. chrysanthemi* were isolated from tobacco leaves with barn rot in North Carolina; Ech 19–34, from different hosts, were obtained from the Plant Pathology Laboratory, Ministry of Agriculture, Fisheries and Food, Harpenden, England (Table 1). Species and strains of other bacteria were obtained from the collection maintained in the Department of Plant Pathology at North Carolina State University. The species and the number of strains of each were: *Agrobacterium tumefaciens*, 1; *Bacillus subtilis*, 1; *Corynebacterium michiganense*, 6; *E. carotovora* var. *carotovora*, 6; *E. carotovora* var. *atroseptica*, 1; *E. herbicola*, 4; *Escherichia coli*, 1; *Pseudomonas aeruginosa*, 1; *P. angulata*, 1; *P. coronafaciens*, 1; *P. lachrymans*, 1; *P. tabaci*, 1; *P. tomato*, 1; *Xanthomonas begoniae*, 1; *X. campestris*, 3; *X. phaseoli*, 1; and *X. vesicatoria*, 1. The names of some of the *Pseudomonas* and *Xanthomonas* species correspond to those in the respective addenda of Bergey's manual (2).

Media. Media used were nutrient agar (NA), nutrient broth (NB), yeast-dextrose-calcium carbonate (YDC) (11), and 0.7% water agar (WA). All strains of *E. chrysanthemi* were suspended in sterile water and maintained at room temperature in screw-cap test tubes. The strains were transferred to NA or YDC twice before use.

Bacteriocin detection. Bacteriocins were detected by the procedures of Echandi (4). Producer strains were either spot-seeded on NA plates and overlaid with the indicator strain or spot-assayed on NA plates surface-seeded with the indicator strain. Qualitative tests were conducted by spot-seeding a layer (25 ml per 9-cm diameter petri dish) of NA with 24-hr cultures of the test strains and incubating them for 48 hr at 20 C. Colonies were transferred from these master plates with a multipoint replicator with 4-mm diameter aluminum rods to fresh NA plates previously dried at 27 C for 3 days. After 48 hr, the colonies were killed by inverting the plates over 3 ml of chloroform until all the chloroform had evaporated (about 60 min). The plates, with the lids off, were then placed in a transfer chamber with continuous air circulation for another 60 min. Indicator strains (lawns) were prepared by adding 0.2 ml of a suspension (10^7 cells/ml in sterile distilled water) of the indicator strains to 4 ml of WA at 40 C and pouring the mixture over the bottom layer of agar. Inhibition zones around killed bacterial colonies after 24–48 hr were considered indicative of bacteriocin production.

Spot assays were used for qualitative and quantitative determinations of bacteriocins in liquid suspension. Lawns were prepared by adding 0.2 ml of the indicator strain (10^7 cells/ml) to 4 ml of WA at 40 C and pouring the mixture over the bottom layer. Bacteriocin preparations were filter-sterilized (0.45-μm Millipore HA membrane), spotted (0.025 ml) on the lawns, and read after 24–48 hr at 20 C. Bacteriocin titers were expressed in arbitrary units (AU); an AU was the reciprocal of the highest dilution causing visible inhibition of the lawn. An activity index (AI) for quantitative evaluations among samples was made by rating the intensity (I) of the inhibition zone from 0–5 (0 = no inhibition, 5 = complete inhibition). The I rating of each dilution was multiplied by the negative log of the dilution and summed for each sample.

Bacteriocin induction. Ultraviolet (UV) light and mitomycin C were used as bacteriocin inducers. Strains Ech 25 and Ech 33 were grown on YDC slants and transferred into 100 ml of NB in 500-ml flasks on a reciprocal shaker for 24 hr at 20 C. Bacteria were collected by centrifugation (11,000 rpm, 10 min in a Sorval GSA rotor; Sorvall Centrifuge, Newton, CT 06470) and resuspended in 0.85% NaCl; 10 ml of the suspension then was dispensed into 9-cm diameter petri plates. Three plates of each strain were placed 20 cm

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from a UV light (UVS-11, Mineralight UV Lamp Company, San Gabriel, CA 93705) for 0, 30, 60, 120, 180, 240, and 300 sec. Then, 5 ml from each plate was added to 5 ml of double-strength NB and incubated in the dark for 4 hr at 20 C. Cellular debris and unlysed cells were removed by centrifugation (11,000 rpm, 10 min), and the supernatant liquid was filter-sterilized and assayed.

Mitomycin C induction was done by incubating strains Ech 25 and Ech 33 in 50 ml of NB in 250-ml flasks placed on a reciprocal shaker for 18 hr at 20 C. Mitomycin C (Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178) was added at a final concentration of 0.5, 1, or 2.5 μg per milliliter to 10-ml samples of a suspension of each strain, and the mixture was shaken in the dark at 20 C. Cellular debris was removed by centrifugation, and bacteriocin activity was assayed after 4 and 8 hr of incubation as described previously.

Bacteriocin purification. Strains Ech 25 and Ech 33 were grown on YDC slants at 27 C for 24 hr; a loopful was seeded into 1 L of NB in a 2-L flask and incubated on a reciprocal shaker for 24 hr at 20 C. Mitomycin C induction was done as described, using a concentration of 1 μg per milliliter.

Preliminary trials indicated that bacteriocins induced by mitomycin C were most effectively precipitated when filtrates were brought to 40% of saturation with ammonium sulfate (248 g/L) for at least 1 hr. Solutions were allowed to stand overnight at 5 C. After centrifugation (11,000 rpm for 20 min in a Sorval GSA rotor), the ammonium sulfate precipitate was resuspended in 0.02 M KH_2PO_4-K_2HPO_4 buffer, pH 7.0, and the insoluble debris was removed by low-speed centrifugation (5,000 rpm for 10 min in a Sorval SS-34 rotor). The supernatant was again centrifuged (28,000 rpm for 2.5 hr in a Beckman Type 30 rotor), resuspended in phosphate buffer, and centrifuged at low speed. The supernatant (1 ml) was layered on a linear gradient of 100–400 mg sucrose per milliliter in 0.02 M phosphate buffer and centrifuged (23,000 rpm for 2.5 hr in a Beckman SW-25.1 rotor). UV absorbing fractions (280 nm) were identified with a density gradient fractionator (Model 640, ISCO, Lincoln, NE 68505) and absorbance monitor (ISCO, Model UA-2) and assayed for bacteriocin activity. The bacteriocin in fractions with activity were further purified by differential centrifugation and sucrose density gradient centrifugation as described previously. Purified bacteriocins collected from the second gradient were used for determinations of heat and enzyme sensitivity and for electron microscopy.

TABLE 1. *Erwinia chrysanthemi* strains and host plant origins

Strain code no.[a]	Host plant origin	
Ech 9	Tobacco	*Nicotiana tabacum*
Ech 10	Tobacco	*N. tabacum*
Ech 19 (Sa 453)	Carnation	*Dianthus caryophyllus*
Ech 20 (a 518)	Carnation	*D. caryophyllus*
Ech 21 (a 568)	Carnation	*D. caryophyllus*
Ech 22 (a 1065)	Corn	*Zea mays*
Ech 23 (a 1066)	Corn	*Z. mays*
Ech 24 (a 1385)	Dahlia	*Dahlia* sp.
Ech 25 (a 1490)	Dieffenbachia	*Dieffenbachia picta*
Ech 26 (a 1514)	Dieffenbachia	*D. picta*
Ech 27 (a 1515)	Dieffenbachia	*D. picta*
Ech 28 (a 1516)	Dieffenbachia	*D. picta*
Ech 29 (a 1955)	Dahlia	*Dahlia* sp.
Ech 30 (a 1956)	Dahlia	*Dahlia* sp.
Ech 31 (a 2308)	Dieffenbachia	*D. picta*
Ech 32 (a 2309)	Chrysanthemum	*Chrysanthemum maximum*
Ech 33 (a 2347)	Corn	*Z. mays*
Ech 34 (a 2348)	Corn	*Z. mays*

[a]Strains Ech 19–34 were obtained from the National Collection of Plant Pathogenic Bacteria, Ministry of Agriculture, Fisheries and Food, Harpenden, England (NCPPB). Strain code numbers given by the NCPPB appear in parentheses.

TABLE 2. Bacteriocin typing of 18 strains of *Erwinia chrysanthemi*

Bacteriocin typing pattern	Bacteriocin-producing strains			Strains in each type	Total strains per type
	Ech 10	Ech 25	Ech 33		
A	+[a]	+	+	Ech 19, Ech 20, Ech 21, Ech 22, Ech 23, Ech 24, Ech 29, Ech 30, Ech 34	9
B	+	−	+	Ech 25, Ech 26, Ech 27, Ech 28, Ech 31	5
C	−	+	+	Ech 9	1
D	−	+	−	Ech 10	1
E	−	−	−	Ech 32, Ech 33	2

[a]+ = bacteriocin-sensitive; − = bacteriocin-insensitive.

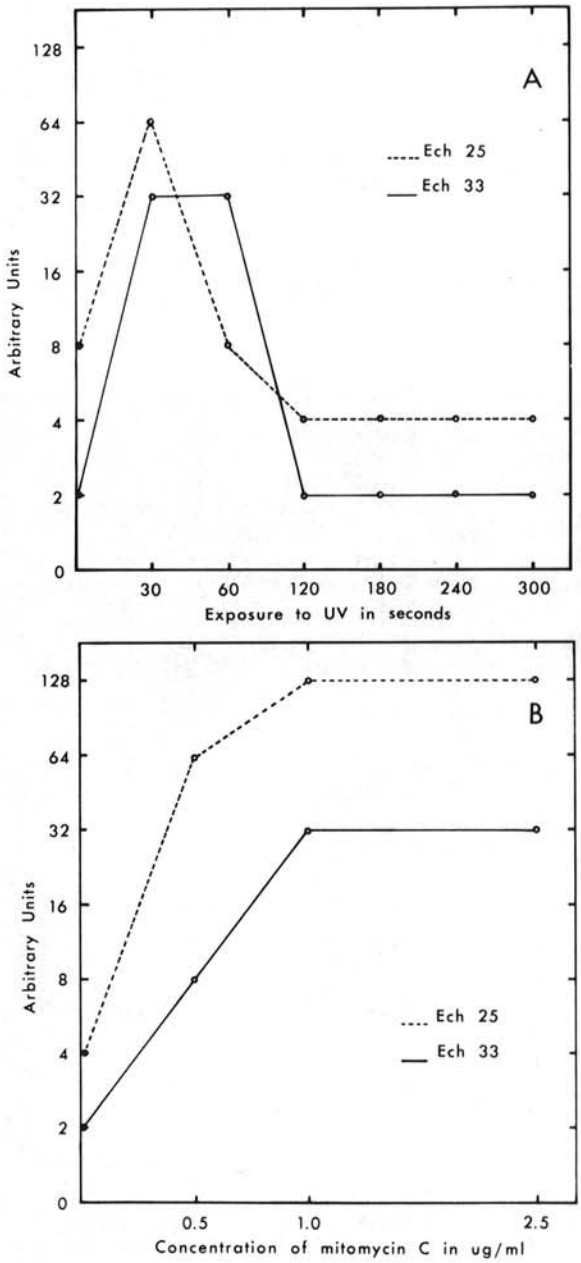

Fig. 1. A, Bacteriocin induction of *Erwinia chrysanthemi* strains Ech 25 and Ech 33 with ultraviolet irradiation. Bacteriocin concentration is expressed in arbitrary units (AU). **B,** Bacteriocin induction of *E. chrysanthemi* strains with mitomycin C. Bacteriocin concentration is expressed in AU.

Sensitivity to heat and enzymes. Bacteriocin sensitivity to heat was determined in thin-walled test tubes containing 1 ml of purified preparations of bacteriocins from Ech 25 and Ech 33 in 0.02 M phosphate buffer. These were immersed for 10 min in a water bath at 30, 40, 50, 60, 70, and 80 C, then placed immediately in crushed ice and assayed for bacteriocin activity.

Purified preparations of bacteriocins from Ech 25 and Ech 33 were tested for sensitivity to trypsin, pancreatic ribonuclease A (RNase), and deoxyribonuclease I (DNase) (Sigma Chemical Co.). Preparations were mixed with 100 μg of enzyme per milliliter, allowed to stand for 1 hr at room temperature, and assayed for bacteriocin activity.

Electron microscopy. Purified bacteriocins from Ech 25 and Ech 33 in 0.02 M phosphate buffer, pH 7.0, were placed on Formvar-coated grids, washed with distilled water, negatively stained with 1% uranyl acetate, and examined with a Siemens electron microscope. Particles were measured from prints; a replica grating (2,160 lines/mm, Ernest F. Fullam, Inc., 900 Albany-Shaker Road, Latham, NY 12110) was used as a magnification standard.

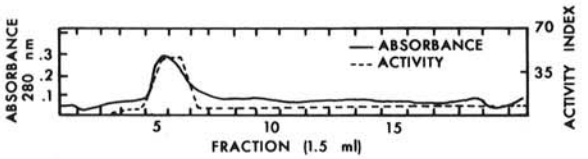

Fig. 2. Ultraviolet absorbance (280 nm) and bacteriocin activity of fractions from a sucrose density gradient column layered with partially purified bacteriocin (Ech 33) after centrifugation (23,000 rpm for 3 hr). Bacteriocin activity was measured by rating the intensity (I) of the inhibition zone from 0–5 (0 = no inhibition, 5 = complete inhibition). The I rating of each dilution was multiplied by the negative log of the dilution and summed for each sample.

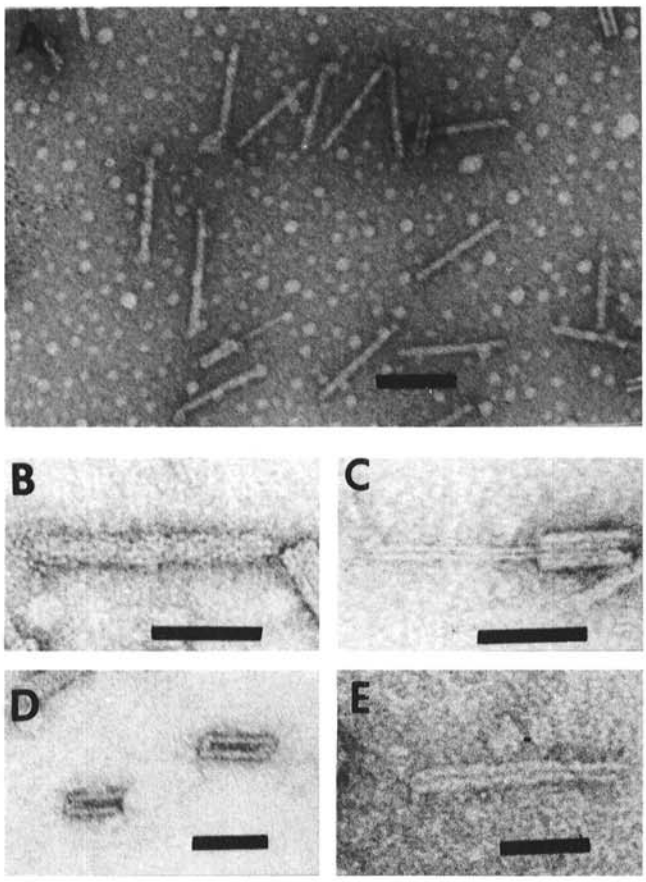

Fig. 3. Bacteriocin particles from purified preparations of *Erwinia chrysanthemi* negatively stained with 1% uranyl acetate. **A,** phage taillike particles from Ech 33; **B,** extended rods; **C,** contracted particles; **D,** empty sheaths; **E,** loose cores. Bar equals 100 nm in A and 50 nm in B–E.

RESULTS

E. chrysanthemi bacteriocins were detected when strains were assayed as producers against the same strains as indicators. Bacteriocinogenicity was presumed when inhibition zones formed around indicator colonies. Size, clarity, and consistency of the inhibition zones in bacteriocin assays were profoundly affected by concentration of agar in the NA medium, incubation temperature for growth of producer colonies, and bacterial concentration of the lawn. Relatively large, clear, and consistent zones were obtained with 15 g of agar per liter in NA, 2 days' incubation at 20 C, and a lawn seeded with 0.2 ml of a bacterial suspension (10^7 cells/ml in sterile distilled water) in 4 ml of WA at 40 C.

Bacteriocin inhibition zones were assayed for bacteriophages by crushing agar plug samples (about 3 mm^3), suspending the samples in NB, and spotting or incorporating the suspension with the indicator strains in the agar overlay. No bacteriophage plaques were observed in any of the samples.

When the 18 strains of *E. chrysanthemi* were assessed as bacteriocin producers against the same strains as bacteriocin indicators, 16 produced bacteriocins active against some or most *E. chrysanthemi* strains. Strains Ech 10, Ech 25, and Ech 33 produced bacteriocins active against 14, 11, and 15 strains, respectively, and had a reaction pattern that facilitated classification of the 18 strains into five types: A, B, C, D, and E (Table 2). Most strains were type A or B. Type A strains were from carnation (Ech 19, Ech 20, Ech 21), corn (Ech 22, Ech 23, Ech 34), and dahlia (Ech 24, Ech 29, Ech 30). Type B strains were from dieffenbachia only (Ech 25, Ech 26, Ech 27, Ech 28, Ech 31). Type C or D strains were from tobacco (Ech 9, Ech 10), and type E strains were from chrysanthemum (Ech 32) and corn (Ech 33). Typing patterns remained consistent in four experiments conducted over several months. The *E. carotovora* var. *atroseptica* strain and one of the *E. herbicola* strains were sensitive to the bacteriocin from strain Ech 33. All other bacteria were insensitive to the bacteriocins from Ech 10 and Ech 25.

UV irradiation and mitomycin C treatment of strains Ech 25 and Ech 33 enhanced production of bacteriocins. Exposure to UV irradiation for 30 or 60 sec induced higher titers of bacteriocins than did exposure for 120, 180, 240, or 300 sec (Fig. 1). Exposure to either 1 or 2.5 μg per milliliter of mitomycin C for 8 hr induced higher titers of bacteriocins than did exposure to 0.5 μg or exposure to any of the three concentrations for 4 hr (Fig. 1).

Ammonium sulfate-precipitated bacteriocins from Ech 25 and Ech 33 lost more than half their activity when stored for 2 wk or longer in 0.01 M tris buffer (pH 7.0), 0.02 M phosphate buffer (pH 7.0), or 0.85% NaCl (pH 7.0) at 4 C. Other methods of storage, such as lyophilization and freezing at −20 and −100 C, did not prevent loss of activity. However, addition of 0.2 mg per milliliter of bovine serum albumin prevented loss of activity for 1 mo or longer.

Bacteriocins from Ech 25 and Ech 33 were inactivated between 60 and 70 C. Treatment with trypsin, RNase, and DNase had no effect on bacteriocin activity of the two strains.

Maximum bacteriocin activity (AI = 45–55) in the sucrose density gradient was associated with the UV (280 nm) absorbing region in fractions four, five, and six (Fig. 2). Preparations of Ech 25 from these fractions had numerous filamentous and arched rod-shaped particles resembling flagella fragments. Nearly all of these were removed after the second cycle of sucrose density centrifugation, as determined by electron microscopy.

Bacteriocins from Ech 25 and Ech 33 appeared to be composed of phage taillike particles consisting of an inner core surrounded by a contractile sheath (Fig. 3). Intact, extended particles measured 15 × 120 nm (average of 36 particles); relatively few of these were observed in Ech 25 preparations. Contracted particles consisted of a contracted sheath or "head" (15 × 50 nm, average of 80 particles) and an inner core or "tail" (8 × 120 nm, average of 80 particles). These were approximately the same length as intact extended

particles and predominated in both preparations. Loose cores and empty contracted sheaths occurred in preparations of both bacteriocins and occasionally were aggregated end-to-end.

DISCUSSION

Our typing scheme illustrates the possible use of bacteriocins in typing and infrasubspecific classification of *E. chrysanthemi.* Strains from the same host were classified either in different types or in the same type with other strains, with the exception of those from dieffenbachia, which were all type B and formed a homologous group. The predominance of type A strains (50%), however, could be a disadvantage; further investigations into the bacteriocin-producing ability of other strains might lead to isolation of a strain or strains to subdivide this type.

Bacteriocin synthesis in *E. chrysanthemi* occurs spontaneously but can be enhanced by exposure to UV light or mitomycin C. Addition of bovine serum albumin preserves biologic activity of induced purified bacteriocin preparations for at least 1 mo.

High molecular weight bacteriocins are mostly phage-related, thermolabile, and resistant to trypsin (9). The bacteriocins from Ech 25 and Ech 33 are of high molecular weight and can be classified as "defective" phages (8). They resemble pyocin R, produced by *P. aeruginosa,* which is formed by a double hollow cylinder consisting of a contractile sheath and a core (6). They also resemble morphologically the bacteriocin from *E. carotovora* reported by Itho et al (7) and Campbell and Echandi (3).

The sheath diameter of the extended bacteriocin particles from strains Ech 25 and Ech 33 was 15 nm and the core diameter was 8 nm. Hollow sheaths and loose cores in purified preparations suggested that some particles either were damaged during the purification process or were incompletely synthesized during induction. Which particles were responsible for bacteriocinogenic activity or if the hollow sheaths or loose cores had antibacterial activity is not known.

LITERATURE CITED

1. BRADLEY, D. E. 1967. Ultrastructure of bacteriophages and bacteriocins. Bacteriol. Rev. 31:230-314.
2. BUCHANAN, R. E., and N. E. GIBBONS, eds. 1974. Bergey's Manual of Determinative Bacteriology. 8th ed. Williams & Wilkins, Baltimore. 1,268 pp.
3. CAMPBELL, P., and E. ECHANDI. 1979. Bacteriocin production in *Erwinia carotovora.* Phytopathology 69:1-A3.
4. ECHANDI, E. 1976. Bacteriocin production by *Corynebacterium michiganense.* Phytopathology 66:430-432.
5. HAMON, Y., and Y. PERON. 1961. Les proprietes antagonistes reciproques parmi les Erwinia. Discussion de la position taxonomique de ce genre. C. R. Acad. Sci. 253:913-915.
6. ISHII, S., Y. NISHI, and F. EGAMI. 1965. The fine structure of a pyocin. J. Mol. Biol. 13:428-431.
7. ITOH, Y., K. IZAKI, and H. TAKAHASHI. 1978. Purification and characterization of a bacteriocin from *Erwinia carotovora.* J. Gen. Appl. Microbiol. 24:27-39.
8. LOTZ, W. 1976. Defective bacteriophages: The phage tail-like particles. Prog. Mol. Subcell. Biol. 4:53-102.
9. REEVES, P. 1972. The bacteriocins. Molecular Biology, Biochemistry, and Biophysics. Vol. 11. Springer-Verlag, New York. 142 pp.
10. TAGG, J. R., A. S. DAJANI, and L. W. WANNAMAKER. 1976. Bacteriocins of gram-positive bacteria. Bacteriol. Rev. 40:722-756.
11. WILSON, E. E., F. M. ZEITOUN, and D. L. FREDERICKSON. 1967. Bacterial phloem canker, a new disease of Persian walnut trees. Phytopathology 57:618-621.