Bacteriocin Production by Corynebacterium michiganense

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Journal Series Paper No. 4702 of the North Carolina Agricultural Experiment Station, Raleigh.

The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Experiment Station of the products named, nor criticism of similar ones not mentioned.

The author thanks R. Overman for his excellent technical assistance.

Accepted for publication 21 August 1975.

ABSTRACT

ECHANDI, E. 1976. Bacteriocin production by Corynebacterium michiganense. Phytopathology 66: 430-432

Corynebacterium michiganense, the causal organism of bacterial canker of tomato, produced bacteriocins in solid media and to a lesser extent in liquid media. Fifty-five of the 96 isolates tested produced bacteriocins as evidenced by the formation of inhibition zones on double-layer agar plates. The 55 bacteriocinogenic strains, were initially classified in 12 groups, and subsequently reclassified in four principal groups. One representative strain was selected from each of the four groups for further study, and to develop a typing scheme. Two of the four selected strains produced thermolabile trypsin-resistant bacteriocins, but bacteriocins from the other two strains were thermostable and trypsin-sensitive. Typing of *C. michiganense* isolates with the four selected strains permitted differentiation of 10 types which included 96% of the isolates. No correlation was found between bacteriocin type and virulence to tomato plants.

Additional key words: bacterial canker of tomato, typing by bacteriocin production.

Bacteriocins are highly specific proteinaceous antibacterial substances produced by many bacterial species. They differ principally from bacteriophages in that they are not capable of reproducing in host bacterial cells (1). Genetic determinants of bacteriocins exist as extra-chromosomal elements analogous to those associated with temperate bacteriophages. They replicate with bacterial chromosomes, and are maintained as long as the bacteriocinogenic strain exists. Bacteriocins are present in small amounts in cultures of bacteriocinogenic strains; presumably, they are produced by spontaneous lysis of cells. Many agents have been used to induce bacteriocins: among these are ultraviolet light and mitomycin C; however, not all bacteriocins are inducible (4).

Bacterial isolates from distinct sources can be differentiated or typed by their sensitivity patterns to bacteriocins (3). Typing by bacteriocin production has been very useful in epidemiological studies of human pathogens (3). Nevertheless typing with bacteriocins has been little used in studies of plant pathogenic bacteria.

Bacteriocins of phytopathogenic bacteria have been little studied and have not been previously reported in phytopathogenic *Corynebacterium* species. This paper deals with properties of bacteriocins from *C. michiganense,* and their use in establishing a typing scheme for this pathogen.

MATERIALS AND METHODS.—*Corynebacterium michiganense* isolates were purified by a single-colony transfer. Only isolates that produced typical bacterial canker symptoms in tomato plants were used. Source and number of isolates were: Western North Carolina, 78; Southern Cal. 1; Merced, Cal. 1; Berkeley, Cal. 1; Rochester, N. Y., 1; Cheyenne, Wyo. 1; American Type Culture Collection, 1; Plant Pathology Lab. Ministry of Agriculture, Fisheries and Food, England, 12. Isolates were maintained on yeast-dextrose-calcium carbonate agar slants at 4 C.

The following media were used: (i) Potato semisynthetic agar (PSA) and potato semisynthetic broth (PS) (2); (ii) Yeast-dextrose-calcium carbonate agar (YDC); (iii) 0.7% water agar (WA); (iv) nutrient broth (NB). PSA, PS, and NB were adjusted to pH 7.2 with 0.1 M NaOH before autoclaving (2).

Bacteriocins were produced and detected by the procedures of Vidaver et al. (5). A layer of PSA (25 ml) in a 9-cm diameter petri dish was spot-seeded on the surface with four or 10 different test isolates that had grown for 24 hours at 28 C on YDC Slants. During the initial phase of this work, large petri dishes (14 cm diameter) containing about 50 ml of PSA in the bottom layer were seeded with 20 different test isolates, the plates inverted, and the bacterial growth incubated for 48 hours at 24 C. Colonies were transferred from these master plates with a multipoint replicator with 4-mm diameter aluminum rods, to fresh PSA plates previously dried for 3-5 days at 27 C (5). After incubation for 48 hours at 24 C, bacterial cells were killed by inverting the plates and exposing them to the vapor from 3 and 5 ml of chloroform, respectively, until all chloroform had evaporated (about 60 minutes), and kept with the lids off for another 60 minutes in a Microvoid transfer chamber with continuous air circulation. Indicator isolates or lawns, in log phase of growth (A 530 nm, approximately 0.3) were diluted in NB and 0.3 ml of the suspension added to 4 and 8 ml of 0.7% melted water agar (40 C), respectively, and poured over the bottom layer of agar. The plates were reincubated upside down for 48 hours at 24 C. Inhibition zones in the

lawn were measured from the periphery of the original colony to the outside edge of the inhibition zone with a vernier caliper.

Spot assays were used to detect bacteriocins produced by isolates grown in PS broth. Serial ten-fold dilutions of centrifuged culture fluid supernatant were spotted (0.01 ml) with a hypodermic syringe with a 0.13-mm inside diameter needle (26-gauge), on plates previously seeded with the indicator isolate. The plates were incubated for 48 hours at 24 C and growth inhibition of the indicator isolate was compared with growth in broth controls.

Heat sensitivity of bacteriocins from the four strains was determined by exposing 48-hour PSA plates with colonies at 78-82 C for 15 minutes. After being cooled for about 60 minutes colonies were over-layered with an indicator isolate and incubated for 48 hours at 24 C. Inhibition zones were compared with nonheated controls.

Sensitivity of bacteriocins from the four strains to trypsin was determined by placing a drop of trypsin (approximately 100 μg) on the edge of 48-hour colonies on PSA. Once the drops had dried, plates were overlayered with the indicator isolate and incubated for 48 hours at 24 C. Inhibition zones were compared with untreated controls.

Ultraviolet induction of bacteriocins from the four strains was determined by irradiating 48-hour PSA plated colonies for periods ranging from 30 seconds to 5 minutes with UV light at 254 nm; irradiated plates were kept in the dark and incubated again 48 hours at 24 C before overlaying with the indicator isolate. Inhibition zones were compared with unirradiated controls.

Induction of bacteriocins with mitomycin C was examined in the four strains by incubating a PS bacterial suspension containing about 10^7 cells/ml with 1 μg/ml of mitomycin C. Cultures were incubated for 24 hours at 24 C. Five ml samples were removed from each culture and centrifuged at 10,000 *g* for 10 minutes. Serial ten-fold dilutions of supernatant culture fluids were spotted (0.01 ml) with a hypodermic syringe with a (26-guage needle), on plates seeded with the indicator isolate. Spotted plates were incubated for 48 hours at 24 C and growth inhibition of the indicator isolate was compared with growth in PS controls with and without mitomycin C.

RESULTS.—*Corynebacterium michiganense* bacteriocins were observed during attempts to isolate temperate bacteriophages by spotting culture supernatant fluids on homologous and heterologous isolates. When bacteriocinogenicity was determined by testing all isolates as producers against all isolates as indicators, 55 of the 96 *C. michiganense* isolates tested were bacteriocinogenic. Bacteriocinogenicity in these tests was evidenced by the formation of inhibition zones around bacterial colonies. Inhibition zones varied from clear and distinct to turbid and diffuse (Fig. 1). There were 12 groups of isolates with similar reaction patterns. As techniques were refined and consistency of reaction pattern was stressed, the 12 groups were reduced to four represented by strains 11F, 15-2, 170, and 1379. Strain 15-2 inhibited all isolates except five. Only one isolate inhibited itself when used as indicator. No plaques were formed when agar plugs taken from inhibition zones of the four strains were assayed for bacteriophages by crushing, suspending in NB, and spotting or incorporating the suspension with the indicator isolate in the agar overlay. Bacteriocins reaction to heat and trypsin were variable. Heat-sensitive bacteriocins were not sensitive to trypsin, and trypsin-sensitive bacteriocins were not sensitive to heat (Table 1). Bacteriocins from the four strains were not induced by ultraviolet light or mitomycin C. Bacteriocin production in liquid medium by the four strains was very low; it was not detected in the first ten-fold dilution. Lytic zones produced by

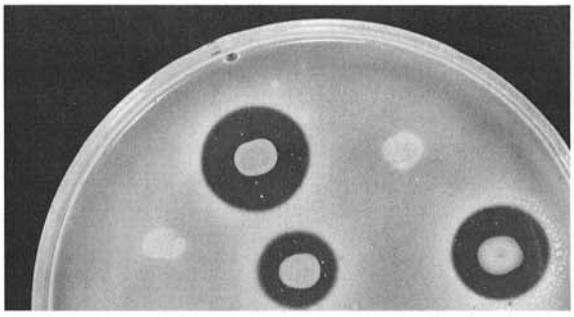

Fig. 1. Bacteriocin reaction of five *Corynebacterium michiganense* isolates to an indicator strain (of *C. michiganense*) layered over the colonies in water agar. Colonies were initially grown 72 hours on potato semisynthetic agar and killed with chloroform before the indicator strain was introduced. Three isolates produced the clear inhibition zones which indicate the presence of the bacteriocin(s) to which the indicator strain is sensitive; two isolates did not.

TABLE 1. Properties of bacteriocins from selected *Corynebacterium michiganense* strains

Producer strains	Bacteriocin sensitivity [a] to:		Production of bacteriocins in liquid medium[c]	Characteristics of lytic zones	
	Heat[b]	Trypsin[b]		Size (mm)[d]	Type[e]
11F	+	−	+	0.5 - 4.0	C/T
15-2	−	+	+	1.0 - 4.0	C
170	+	−	±	0.5 - 4.0	C/T
1379	−	+	±	0.5 - 3.0	C/T

[a]Symbols: + = sensitive; − = resistant.
[b]Sensitivity at 78-82 C for 15 minutes or to droplets containing approximately 100 μg of trypsin.
[c]Symbols: + = lytic ring around the drop; ± = slight thinning of the indicator isolate in the location where the drop was placed.
[d]Sizes differ with indicator, temperature of incubation, age of culture, amount of inoculum and concentration of indicator lawn.
[e]Types differ with indicator; C = clear; C/T = turbid in some indicators.

TABLE 2. Bacteriocin typing of 96 isolates of *Corynebacterium michiganense*. Bacteriocin types and number of isolates per type

Bacteriocin Producer strains	Bacteriocin typing patterns (Types A to J) as defined by indicator isolates:									
	14-4 (Type A)	15-6 (Type B)	23-1 (Type C)	13-3 (Type D)	16-7 (Type E)	36 (Type F)	870 (Type G)	15-2 (Type H)	12049 (Type I)	1574 (Type J)
11F	+[a]	+	+	−	−[a]	−	−	−	−	−
15-2	+	+	+	+	+	+	+	−	−	−
170	+	+	−	+	+	−	−	+	−	−
1379	+	−	−	+	−	+	−	+	+	−
Number of isolates /type	12	19	12	1	5	20	6	16	1	4

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

bacteriocins varied in size and type. The most critical variables were: incubation temperature, age of cultures, amount of inoculum, and concentration of the lawn.

On the basis of lytic reaction pattern of all isolates tested against strains 11F, 15-2, 170, and 1379, it was possible to differentiate *C. michiganense* isolates that were otherwise indistinguishable. As a result, a bacteriocin typing scheme with 10 types was assembled (Table 2). More isolates were placed in types F, B, and H than in any others. Only four isolates failed to react comprising about 4% of the 96 tested. In five separate experiments, as many as 10 colonies of the same isolate were picked from the same plate and typed; typing patterns were similar in the five experiments. Stock cultures typed over several months also retained similar type patterns.

DISCUSSION.—Bacteriocins from *C. michiganense* can be placed in the two groups suggested by Bradley (1). Those from strains 15-2 and 1379 could be classified as low molecular weight, thermostable, trypsin-sensitive bacteriocins; 11F and 170 could be classified as high molecular weight, thermolabile, trypsin-resistant bacteriocins. Ultraviolet light and mitomycin C were ineffective in inducing greater amounts of bacteriocins under the conditions employed. It is possible that some agent could be found to induce *C. michiganense* strains to produce larger quantities of bacteriocins.

Bacteriocin types were not correlated with virulence to tomato plants or cultural characteristics of *C. michiganense*. A similar finding was reported by Vidaver et al. (5) on *Pseudomonas* spp.

The bacteriocin-typing scheme reported here serves to illustrate the possible uses of bacteriocins in the subgroup classification and in epidemiological studies of *C. michiganense*. Now it should be possible to study bacteriocin types of *C. michiganense* and correlate them with distribution of bacterial canker of tomato. It may also be possible to monitor seasonal development of single or mixed bacteriocin types where bacterial canker recently has been introduced, or has become more intense.

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