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Brochocin-C, a New Bacteriocin Produced by *Brochothrix campestris*

GREGORY R. SIRAGUSA* AND CATHERINE NETTLES CUTTER

Roman L. Hruska U.S. Meat Animal Research Center, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 166, Clay Center, Nebraska 68933

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Brochothrix campestris **ATCC 43754 produces a bacteriocin inhibitory towards *Brochothrix thermosphacta*, lactobacilli, *Listeria* spp., and other gram-positive bacteria. This antimicrobial agent is heat stable, sensitive to proteases, catalase insensitive, and free of organic acids. No phage particles were detected by transmission electron microscopy. Muramidase activity was not detected in the preparations. On the basis of established criteria, the antimicrobial agent was classified as a bacteriocin and named brochocin-C.**

The microbial flora of foods includes bacteria that are either associated with fermentation, spoilage, or no defined activities in this ecological niche (3, 5, 7, 9, 10). This and other populations of bacteria (e.g., silage, vegetation, rumen, intestinal tract) offer a source of microorganisms which may synthesize bacteriocins as yet undescribed.

To date, only two species are assigned to the genus *Brochothrix*, namely, *Brocothrix thermosphacta* and *Brocothrix campestris* (13, 15). The genus *Brochothrix* is classified within the *Clostridium-Lactobacillus-Bacillus* branch (13) and is closely related to the genus *Listeria* (8, 14). The role of *B. thermosphacta* in the microbial progression of meats is well documented (3, 5); however, relatively little has been published concerning *B. campestris*. The biochemical activity profile of *B. campestris* closely resembles that of *B. thermosphacta*. Both of these species are nonpathogenic and generally will not grow in temperatures above 30°C. *B. campestris* will not grow in 8 or 10% NaCl nor ferment rhamnose. *B. thermosphacta*, however, grows in 8 and 10% NaCl and ferments rhamnose (15). *B. thermosphacta* ferments glucose to acidic end products including branched-chain fatty acids that are responsible for spoilage attributed to this species (2, 6). End products of *B. campestris* metabolism are not documented.

In this report, we describe a bacteriocin produced by *B. campestris* that inhibits several other species of gram-positive bacteria including isolates from vacuum-packaged beef and pork. This bacteriocin has been designated brochocin-C. To our knowledge, no reports of bacteriocin production by either member of the genus *Brochothrix* have been made.

(Portions of this research were presented previously [12].)

B. campestris ATCC 43754 was maintained at −20°C in 75% glycerol suspensions. *Brochothrix* spp. were cultured at 24°C in either tryptic soy broth plus 0.5% (wt/vol) yeast extract (TSBYE) or TSBYE plus 0.1 M MOPS (morpholinepropanesulfonic acid) buffer (pH 7.2) (TM broth) for 16 h (11). Key phenotypic traits of *B. campestris* were verified as described by Talon et al. (15). *Brochothrix* spp. isolates from the U.S. Meat Animal Research Center were obtained in this lab by the scheme of Gardner (4) by using streptomycin thallous acetate agar (STAA; Oxoid; Unipath, Ogdensburg, N.Y.). *Listeria monocytogenes* isolates include rough strains (indicated by an R prefix in Table 1). All lactobacilli and lactic acid bacterial strains were propagated in lactobacillus MRS or APT broth (Difco Laboratories, Detroit, Mich.) at 37°C. All other species were grown in TM broth at 37°C for 16 h before use.

Antimicrobial activity was assayed by methods described previously (11). Briefly, all lawn overlays were seeded with approximately 10^6 CFU of indicator organisms per ml. In situ antimicrobial activity was carried out by using the colony-spot-seeded lawn agar diffusion method with TM or MRS agar and the homologous semisoft agar overlays. Characterization of the activity was performed with concentrated cell-free protein preparations in the agar well diffusion method by using either TM or MRS top and bottom agars. The brochocin-C preparation was diluted serially in MOPS-buffered saline, and 20 μl of each dilution was added to an agar well. Plates were incubated at the respective temperatures for 16 h. Activity units are defined as the reciprocal of the highest dilution resulting in a clear zone of inhibition in the agar well diffusion assay (16).

Protein determinations were performed by using the BCA protein assay system with bovine serum albumin as the protein standard (Pierce Chemicals, Rockford, Ill.).

The antimicrobial activity of the spent cell-free supernatant of *B. campestris* ATCC 43754 TSBYE culture (16 h, 24°C) was concentrated by 75% ammonium sulfate fractionation as described previously (11) and further characterized. The pH of the concentrated supernatant extract (CSE) was adjusted to 7.2. The effect of various proteases and catalase on the antimicrobial agent was determined by adding the respective enzyme (1 mg/ml) to the CSE (1:1 [vol/vol]), incubating the mixture for 1 h at 37°C, and assaying it in the agar well diffusion assay against *B. thermosphacta* ATCC 11509. Lysozyme or muramidase activity in the CSE was assayed by the method of Cornett et al. (1). Heat stability of the CSE was determined as described previously (11). Volatile fatty acid content was determined by high-performance liquid chromatography (HPLC) analysis as described by Varel and Pond (17).

B. campestris ATCC 43754 was found to inhibit strains of *B. thermosphacta* and *Listeria* spp. in colony-spot-seeded lawn assays (data not shown). When in situ activity was observed, ammonium sulfate protein extracts of cell-free supernatant fluids from *B. campestris* ATCC 43754 cultures were prepared to determine whether the antimicrobial activity was proteinaceous and to assess its range of activity (see Table 1). Brochocin-C was incubated with the following

* Corresponding author.

TABLE 1. Inhibitory spectrum and relative sensitivities of selected bacteria to brochocin-C bacteriocin produced by *B. campestris* ATCC 43754

Target species	Source[a]	Strain[b]	Reciprocal of highest inhibitory dilution[c]
Brochothrix campestris	ATCC	43754	R
Brochothrix thermosphacta	MARC, VP beef	15 strains	2–4
	GG, beef	15 strains	4–16
	ATCC	11509	8
	RT, pork	2 strains	8–16
	RT, beef	1 strain	16
Carnobacterium divergens	ATCC	35677	128
Carnobacterium gallinarum	ATCC	49517	4
Carnobacterium mobile	ATCC	49516	32
Enterococcus durans	ATCC	19432	2
Enterococcus faecalis	ATCC	19433	32
Enterococcus faecium	ATCC	19434	32
Enterococcus hirae	ATCC	9790	16
	MARC	C311	16
Kurthia gibsonii	ATCC	43195	16
Kurthia zopfii	ATCC	33403	16
Lactobacillus acidophilus	MARC	2 strains	1–32
Lactobacillus casei	ATCC	393	32
	MARC	YIT 003	1
Lactobacillus fermentum	MARC	No. 36	16
Lactobacillus helveticus	MARC	1 strain	1
Lactobacillus plantarum	MARC	NCDO 352	1
	MARC	YIT 0001	1
Lactobacillus delbrueckii subsp. *lactis*	ATCC	4797	128
Listeria grayi	ATCC	19170	4
Listeria innocua	ATCC	33090	4
Listeria monocytogenes	HH	R5	4
	HH	R1/2c	4
	HH	SV 1/2a	2
	HH	R4	2
	HH	R1	4
	HH	R3	2
	ATCC	19113	16
	ATCC	15313	8
	MARC	V7	2
	MARC	Scott A	2
Listeria murrayi	ATCC	25401	8
Listeria welshimeri	ATCC	35897	4
Pediococcus acidilactici	MARC	1 strain	2
Pediococcus spp.	MARC	3 strains	4–8
Unidentified LAB[d]	MARC, VP pork	5 strains	32–128
	MARC, VP beef	25 strains	2–128
	MARC, VP beef	2 strains	R
	MARC, rumen	2 strains	R
	MARC, silage	2 strains	R
Bacillus subtilis	ATCC	6051	R
Clostridium perfringens	ATCC	13124	R
Leuconostoc carnosum	ATCC	49367	R
Micrococcus flavus	ATCC	10240	R
Staphylococcus aureus	ATCC	12598	R
	ATCC	25923	R
Gram-negative spp.[e]		7 Strains	R

[a] ATCC, American Type Culture Collection, Rockville, Md.; MARC, U.S. Meat Animal Research Center, Clay Center, Nebr.; GG, Gordon Greer, Agriculture Canada, Lacombe, Alberta, Canada; HH, Herbert Hof, Institute of Medical Microbiology and Hygiene, Mannheim, Germany; RT, Regine Talon, Institut Nationale Recherche Agronomique, Theix, France; VP, vacuum packaged.

[b] The actual strain code or the number of strains tested is shown.

[c] Reciprocal of the highest dilution of crude brochocin-C (3.6 mg/ml of total protein) resulting in a clear zone of inhibition. R indicates strains which were not inhibited (i.e., were resistant to brochocin-C both in this assay and the colony spot method).

[d] LAB, lactic acid bacterium. All LAB isolates are gram-positive, catalase-negative, oxidase-negative, rod-shaped bacteria capable of growth at 37°C under 8% CO_2 and isolated on either ATP or MRS agar; they were presumptively identified as either *Lactobacillus* or *Leuconostoc* spp.

[e] Gram-negative species include *Salmonella typhimurium* ATCC 14028, *Escherichia coli* ATCC 25922, *E. coli* O157:H7, *Shigella sonnei* ATCC 9290, *Citrobacter freundii* ATCC 8090, *Acinetobacter baumannii* ATCC 19606, and *Pseudomonas fluorescens* ATCC 13525.

proteases (all enzymes were purchased from Sigma Chemical Co., St. Louis, Mo., unless noted otherwise; catalog numbers are indicated in parentheses): α-chymotrypsin (C-4129), ficin (F-6008), proteinase-K (P-0390), papain (P-4762), trypsin (T-8253), and pronase (Calbiochem; 53702). The antimicrobial agent synthesized by *B. campestris* ATCC 43754 was sensitive to all of the proteases tested. The activity was unaffected by the addition of catalase (Sigma catalog no. C-10), thereby eliminating the possibility that the antimicrobial activity was due to hydrogen peroxide. HPLC analysis of the concentrated activity revealed no organic acid end products which may cause inhibition (data not shown). No lysozyme or muramidase-like activity was detected in the concentrated activity. Transmission electron microscopy (phosphotungstic acid-negative staining) of the concentrated agent both with and without indicator cells revealed no bacteriophage or phage subparticles. On the basis of the evidence presented here, the antimicrobial agent produced by *B. campestris* was classified as a bacteriocin and named brochocin-C.

Aliquots of brochocin-C heated at 100°C for 0, 5, 10, 20, or 40 min showed no decline in activity when assayed against *B. thermosphacta* ATCC 11509. The spectrum of activity of brochocin-C is presented in Table 1. All *Brochothrix* spp. strains (34 strains) were sensitive to the effects of brochocin-C. Thirty bacterial isolates from vacuum-packaged beef and pork were highly sensitive to brochocin-C. Bacterial isolates from corn silage and rumen fluid were resistant to brochocin-C (Table 1). The gram-negative species tested were resistant to brochocin-C under the conditions described.

To our knowledge, brochocin-C is the first bacteriocin produced by a member of the genus *Brochothrix* to be described. This information expands the generic range of gram-positive bacteria which produce bacteriocins. The significance of bacteriocin production by *B. campestris* in nature is unknown; however, it is likely that this activity offers the organism some competitive advantage in its niche. *B. campestris* was originally isolated from soil and grass (15) and has not been documented to be a part of food microflora. However, brochocin-C inhibits bacteria that are taxonomically similar to the producing organism including *B. thermosphacta*, *Lactobacillus* spp., *Carnobacterium* spp., *Listeria* spp., *Enterococcus* spp., *Pediococcus* spp., and *Kurthia* spp., all of which have been found associated with foods and meat. Brochocin-C is similar to other bacteriocins from some gram-positive bacteria in that it apparently does not inhibit gram-negative bacteria under standard assay conditions (7).

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