JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Diverse Antimicrobial Killing by *Enterococcus faecium* E 50-52 Bacteriocin

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An effective bacteriocin was identified and characterized. Lactic acid bacteria were screened against *Campylobacter jejuni*. One bacteriocin producer, *Enterococcus faecium* (NRRL B-30746), was studied. The isolate was grown, and the bacteriocin was purified to single-band homogeneity. Biochemical traits indicated that the peptide was a Class IIa bacteriocin, and it was named E 50-52. The bacteriocin had a molecular weight of 3339.7 and an isoelectric point of 8.0. The minimal inhibitory concentrations of E 50-52 against *C. jejuni*, *Yersinia* spp., *Salmonella* spp., *Escherichia coli* O157:H7, *Shigella dysenteriae, Morganella morganii*, *Staphylococcus* spp., and *Listeria* spp. ranged from 0.025 to 32 μg/mL. In therapeutic broiler trials, oral treatment with E 50-52 reduced both *C. jejuni* and *Salmonella enteritidis* by more than 100 000-fold in the ceca, and systemic *S. enteritidis* was reduced in the liver and spleen. The wide range of antibacterial activity of bacteriocin E 50-52 against pathogens provides a promising alternative to antibiotics.

KEYWORDS: Antimicrobial polypeptide; bacteriocins; poultry; MIC

INTRODUCTION

Public concerns have been expressed over increasing frequencies of antibiotic resistance (AbR) among wide varieties of bacterial species. It has been suggested that agricultural antibiotic use hastens the appearance of AbR in humans (*1*). It has been further proposed that overuse and misuse of antibiotics pose a serious danger to public health and that phasing out of antibiotics for nonmedical use in animals would serve to reduce antimicrobial resistance (*2*). Although causality may still be controversial, the society continues to observe increases in AbR. The consequence of AbR has been reduced effectiveness or even ineffectiveness of therapeutic antibiotic treatment, resulting in previously preventable zoonotic diseases and deaths. In recent times, the pharmaceutical industry has made slow progress in creating new or alternative therapeutic drugs. The combination of a lack of new antibiotics and regulatory hurdles has slowed the discovery of new antimicrobials.

Bacteriocins are a class of antimicrobials meriting consideration as alternatives to classical antibiotic therapies (*3*). Class IIa bacteriocins are low-weight ribosomally synthesized polypeptides produced by a bacterium which creates pores in susceptible bacteria, causing the death of the target (*4*). The variety of such bacteriocins is described in decades-old literature and was initially anticipated to provide a substantial alternative to antibiotic therapeutics (*5–8*). Some literature does suggest that these antimicrobial polypeptides are active only against limited arrays of bacteria belonging to closely related species or genera of the bacteriocin-producing organism (*9–12*). Among the genus *Enterococcus,* numerous isolates have been described to produce bacteriocins (*13–17*). These bacteriocins are heterogeneous in structure and differ in ability to kill diverse bacteria. The enterococcal bacteriocins are diverse and may comprise the pediocin-like Class IIa and the leaderless Class IIc formats (*16*).

This report describes a novel bacteriocin, E 50–52, produced by *Enterococcus faecium* E capable of killing a wide variety of both Gram-negative and Gram-positive pathogens under in vitro and in vivo conditions. The diversity of this bactericidal activity described herein was predicated on the unique screening procedure used for its selection.

MATERIALS AND METHODS

Microbiology. Cecal contents from 376 commercial broilers were streak-plated on a de Man−Rogosa−Sharp (MRS) agar medium (Accumedia, Lansing, MI). The plates were incubated at 37 °C for 16–18 h. Three to five colonies were selected and purified from each

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10.1021/jf073284g CCC: $40.75 © 2008 American Chemical Society
Published on Web 02/23/2008

sample. Isolates of *Enterococcus* spp. were identified by using established criteria (*18*).

After colony isolation, isolates were tested as follows. About 10^7 colony-forming units (cfu) of each candidate isolate was suspended in normal saline, distributed onto MRS agar, and incubated at 37 °C for 24 h under normal atmosphere. An agar block containing confluent growth of the isolate (~0.5 cm^3) was aseptically cut and transferred to surface-plated (~10^7 cells) *Campylobacter jejuni* NCTC 11168 on brucella agar with 5% lysed blood. Because this medium contained only 0.1% dextrose, acid production was limited by the candidate antagonists. Plates were incubated at 42 °C for approximately 24–48 h under microaerobic atmosphere (5% O_2, 10% CO_2, and 85% N_2). Activity was evaluated by measuring the resulting diameters of growth inhibition in millimeters.

The isolate showing the largest zone of inhibition was subjected to 16S rRNA-DNA 500 bp sequence analysis by using the MicroSeq 500 system (ABI Inc., CA) for taxonomic resolution to the likely genus level and possible species level. The resulting sequence was analyzed by using the online tools of the Ribosomal Database Project RDPII (*19*), NCBI Blast Beta version (http://www.ncbi.nlm.nih.gov/BLAST), and Greengenes (*20*). Further resolution to the species level was accomplished by using the *sod*A-based PCR assay as reported to speciate members of the genus *Enterococcus* (*21*).

Bacteriocin Purification. *E. faecium* NRRL B-30746 was evaluated for bacteriocin production (*22*). The strain was grown in 6.0 L of brucella broth (Difco, Detroit, MI) at 37 °C for 16 h. The spent culture was centrifuged at 12 000*g* under refrigeration for 10 min to remove the cells. The supernatant was adjusted to pH 6.2 by adding 1 N NaOH to neutralize organic acids, and 130 U/mL catalase was added to remove hydrogen peroxide. Solid ammonium sulfate was added stepwise over 60 min to achieve 75% saturation. The mixture was stirred for an additional 30 min on a magnetic plate. The precipitated suspension was centrifuged for 30 min at 10 000*g* at 5 °C, and the supernatant was decanted. The pellet was dissolved in 3-(*N*-morpholino)propanesulfonic acid-buffered saline (pH 7.2) to 5% of the starting volume. The concentrated solution was dialyzed against phosphate buffer (0.1 M, pH 7.2), with two changes of buffer, at 5 °C overnight to produce a crude antimicrobial preparation (CAP). The CAP sample (480 mL) was filtered through a 0.22 μm filter (Millipore, Bedford, MA).

A two-step method for purification of bacteriocin E 50-52 was developed by using batch ion-exchange and hydrophobic-interaction chromatography. The above dialyzed CAP was added to the ion exchange SP Sepharose Fast Flow IEX material (Amersham Bioscience, Piscataway, NJ) at a ratio of 500:1 (v/v). After gentle mixing, the entire suspension was incubated at room temperature for 1 h without agitation. The SP Sepharose with the bound peptide was washed twice with 20 mM Na_2HPO_4 at pH 4.5 and centrifuged at 12 000*g* for 15 min at 4 °C, and the supernatant was decanted. A total of 900 mL of an eluting buffer consisting of 20 mM Na_2HPO_4 and 0.2 M NaCl, pH 4.5, was added to the pellet. The mixture was incubated at room temperature for 1 h. The sorbent was removed by centrifugation (12 000*g*, 15 min at 4 °C), and the supernatant was analyzed for protein concentration and antimicrobial activity.

After ion-exchange chromatography, the supernatant was mixed with the hydrophobic-interaction chromatographic material Octyl Sepharose 4 Fast Flow (Amersham Bioscience) at a ratio of 100:1 (v/v). This mixture was incubated at room temperature for 1 h, washed twice with 15 mM K_2HPO_4, pH 5.5, and centrifuged at 10 000*g* for 20 min at 4 °C. The resulting pellet was combined with 700 mL of elution buffer (25 mM Tris-HCl and 15 mM K_2HPO_4, pH 4.5). The materials were incubated at room temperature for 1 h, and after incubation, the sorbent was removed by centrifugation (10 000*g*, 20 min at 4 °C). The concentration of the bacteriocin in the supernatant was measured at 215 nm (*23*). The solution was titrated, and anti-*C. jejuni* activity of the bacteriocin was determined in 10 μL spot tests onto plated *C. jejuni* NCTC 11168 (*24*).

Bacteriocin Characterization. The molecular mass of the peptide was initially estimated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) (*25*). The purified bacteriocins and contaminants were separated in a 1.5% polyacrylamide gel (9 × 12 cm) by using 1% SDS in Tris-glycine buffer. After electrophoresis at 100 mA for 4 h, the gel was fixed with a solution containing 15% ethanol and 1% acetic acid for 30 min and then washed with distilled water for 4 h. To estimate the molecular mass of the separated protein fractions, the gel was stained with a solution containing 0.15% Coomassie Brilliant Blue R-250 (Sigma, St. Louis, MO) in 40% ethanol and 7% acetic acid. The stained gel was washed sequentially with phosphate-buffered saline (pH 7.2) for 1.5 h and then with deionized water for 3 h. To define the peptide band mediating anti-*Campylobacter* activity, the renatured gel containing the separated peptides was placed in a Petri dish and overlaid with semisolid brucella agar (0.75% agar) seeded with cells of *C. jejuni* NCTC 11168. The plate was incubated under microaerobic conditions for 48 h at 42 °C. Bacteriocin activity among the separated proteins was demonstrated by observing a zone of *Campylobacter* growth inhibition surrounding the bacteriocin protein (*26*).

The crude preparation and purified bacteriocin were also placed on isoelectric focusing gels (pH 3.1–10.0) (Novex, San Diego, CA). The gel was run at 100 V for 1 h, 200 V for 2 h, and 500 V for 30 min in XCM II Mini-Cell per company instructions (Novex). The gel was washed with distilled water for 30 s without fixation and stained with Coomassie Brilliant Blue R-250 (Sigma) to determine the isoelectric point (p*I*) of the bacteriocin and its ability to inhibit the growth of the *C. jejuni* test strain, as described above.

The amino acid sequence of the purified bacteriocin was determined by Edman degradation by using a 491cLC automatic sequencer (Applied Biosystems, Middletown, CT). The bacteriocin was hydrolyzed in 6 M HCl under vacuum at 110 °C for 72 h. The molecular mass was determined by mass spectrometry by using a Voyager-DERP instrument (Perkin-Elmer, Salem, MA). The matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) system was used along with matrix 2-cyano-hydroxycinnamic acid per manufacturer's instruction. After biochemical determination of the primary amino acid sequence, the predicted physical characteristics were analyzed (*6*) by utilizing Protean of the DNASTAR (Madison, WI) software. The primary amino acid sequence was entered into BLAST (*27*) to search for proteins of similar sequence.

Bioassays. The effect of enzymes, temperature, and pH on bacteriocin E 50-52 activity was determined. A total of 10 μL of each of the following enzymes was transferred to separate tubes containing 20 μL of bacteriocin: beta-chymotrypsin, 100 mg/mL; proteinase K, 200 mg/mL; papain, 60 mg/mL; lysozyme, 75 mg/mL; and lipase, 100 mg/mL (Sigma-Aldrich Corp., St. Louis, MO). After 3 h of incubation at 37 °C, the mixture of bacteriocin and enzyme was analyzed for antimicrobial activity by using the spot test described above. The untreated bacteriocin served as positive control.

To study thermo-stability, a 2 mg/mL solution of E 50-52 bacteriocin (volume 10 mL in glass tube) was boiled in a water bath for 15 min, cooled in an ice bath, and assessed for its antimicrobial activity by using the spot test described above. A total of 2 mg/mL of bacteriocin was used to evaluate the effect of pH. Drops of 10 mM NaOH or 10 mM HCl were added to 2 mL of the sterile bacteriocin solution to test stability at pH of 3−10. Samples were incubated at 37 °C for 2 and 24 h and at 90 °C for 20 min. After incubation, samples were adjusted to a pH of 7.2 by addition of 4 mM sterile phosphate buffer and analyzed for antimicrobial activity by using the spot test described above.

Serial 2-fold titrations of the purified bacteriocin in sodium phosphate buffer (pH 7.0) were performed. A sample of 10 μL of each titration was pipetted onto brucella agar previously surface-plated with cells of *C. jejuni* NCTC 11168 or the other target pathogens (isolate listings provided in Supporting Information). Plates were cultivated at 42 °C under microaerobic atmosphere to facilitate *Campylobacter* growth, at 28 °C for *Yersinia* spp., and at 37 °C under normal atmospheric condition for the other pathogens tested. Activity of E 50-52 was expressed as minimal inhibitory concentrations (MICs, μg/mL), documenting the highest dilution at which a visible zone of inhibition of pathogen growth appeared. All assays were conducted in duplicate and provided a comparison for sensitivity. *Staphylococcus* spp. test strains 1–13 and 15–27, *E. coli* O157:H7 Ya61, *Streptomyces aureus*, and *Shigella dysenterae* were isolated from humans.

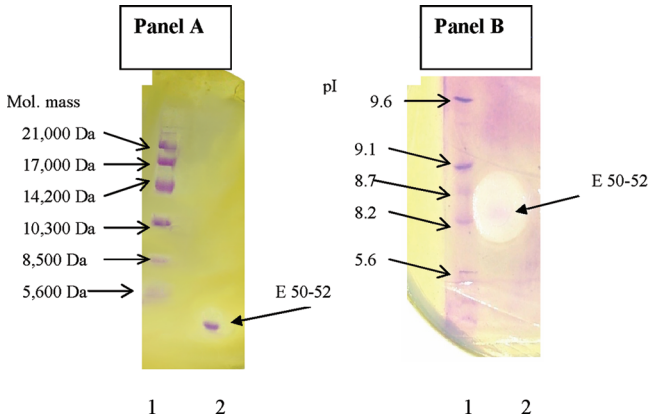

Figure 1. Direct detection and characterization of bacteriocin E 50-52 by SDS-PAGE and iso-electro focusing. (**A**) The PAGE gel was overlaid with agar inoculated with *Campylobacter jejuni* to determine which band(s) corresponded to the antimicrobial activity, molecular weight, and p*I*. Lane 1 shows molecular weight markers with a range of 5600–21 000 Da (Amersham Pharmacia Biotech; 5600, 8500, 10300, 14200, 17000, and 21000 Da). The band in lane 2, containing bacteriocin E 50-52, demonstrated the antimicrobial activity; the zone of growth inhibition (arrow) was at a mass of about 4000 Da. (**B**) Lane 1 contained iso-electro focusing standards (Protein Test Mixture, p*I* Marker Proteins, Serva). The band in lane 2 containing bacteriocin E 50-52 demonstrated antimicrobial activity. The protein band inducing the zone of *C. jejuni* growth inhibition (arrow) migrated to a p*I* of about 8.5. The other bands did not show antimicrobial activity.

Table 1. Assays To Demonstrate Purified Antimicrobial Compound as a Bacteriocin[a]

(A) Effect of Enzymes and Temperature on Antimicrobial Activity of Moiety E 50-52

treatment	activity[b]
beta-chymotrypsin	−
proteinase K	−
papain	−
lysozyme	+
lipase	+
100 °C, 15 min	+

(B) Effect of pH and Temperature on Activity of Moiety E 50-52

pH	20 min at 90 °C	2 h at 37 °C	24 h at 37 °C
3.0	+	+	+
5.0	+	+	+
6.2	+	+	+
7.0	+	+	+
8.4	+	+	+
9.1	−	−	−

[a] −, absence of activity after treatment with enzymes or exposure to temperature; +, presence of activity. [b] Activity determined by a 10 μL spot test with *C. jejuni* NCTC 11168 as indicator strain.

Feed Emendation, Poultry Challenge, and in Vivo Assays. A sample of 500 mg of purified bacteriocin E 50-52 was mixed into a 25 mL solution of 0.8 M K_2HPO_4/L containing 1.25 g of completely dissolved poly-vinyl-pyrrolidone (PVP) powder. The bacteriocin−PVP solution was thoroughly mixed with 100 g of ground maize to produce a highly concentrated treated feed. This feed was used to prepare the treated−commercial feed containing 100 g of concentrate feed mixed with 1900 g of commercial feed to produce feed having a bacteriocin concentration of 250 mg/kg.

Approval for the following animal experiments was provided by the Institutional Animal Care and Use Committee (PMS-03-03, Control of *Campylobacter* in Poultry Production). Chick-treatment experiments were conducted by challenging four separately caged groups of day-of-hatch birds ($n = 10$ per group) with equal numbers of $\sim 10^6$ cfu *C. jejuni* isolates B1 and L4 per bird (41) held in isolation. These four groups comprised (1) birds provided unmedicated feed throughout life, (2) birds provided 125 mg of bacteriocin E 50-52/kilogram feed from day four to seven, (3) birds provided 62.5 mg of bacteriocin E 50-52/kilogram feed from day four to seven, and (4) birds provided 31.2 mg of bacteriocin E 50-52/kilogram feed from day four to seven. All birds were sacrificed at day 15, ceca were aseptically excised, and cfu of *C. jejuni* and lactic acid bacteria/gram cecal content was enumerated on Campylobacter-Cefex and MRS agar, respectively.

The adult-bird (35−41 day old broilers) experiments were conducted by using commercially grown broilers that were environmentally colonized by *C. jejuni*. Four groups of 7−10 birds were held in separated rooms and reared on solid floors. The 35 day old birds were challenged with 0.2 mL suspension of $\sim 10^{10-11}$ cfu *Salmonella enteritidis* 92 Rif^R. The rifampicin resistant *S. enteritidis* isolate had been created by sequential passages on media containing increasing levels of rifampicin. Strain *S. enteritidis* 92 Rif^R was cultured overnight in Endo's medium containing 100 μg/mL rifampicin. The adult birds were provided feed and water ad libitum. The three treated groups of broilers were provided 12.5 mg of bacteriocin E 50-52/liter of drinking water for either one, two, or three days. Birds in the untreated control group were killed when they were 41 days old, whereas the birds in the treated groups were killed when they were 39, 40, or 41 days old. Ceca, liver, and spleen were aseptically removed, and dilutions of individual homogenates were plated onto (1) MRS agar, (2) Campylobacter−Cefex agar, or (3) Endo agar with 100 μg of rifampicin/milliliter to estimate levels of lactic acid bacteria, *C. jejuni*, and *S. enteritidis*, respectively. Resulting *Campylobacter*-characteristic colonies were counted after microscopic examination and latex agglutination assay confirmation. Arithmetic estimates of the number of organisms were transformed into $\log_{10}$ values. Means and standard deviations for each experimental group were determined.

RESULTS

The inhibitory bacterial isolate was identified as *E. faecium* NRRL B-30746 (E 50-52) and deposited under the authority of the Budapest treaty at the ARS Culture Collection in Peoria, IL. *E. faecium* B-30746 is a facultative Gram-positive cocci. The isolate grew on nutrient agar producing irregular-shaped edges. The colonies were approximately 2 mm in diameter after microaerobic cultivation for 24 h at 37 °C and conformed to other specified taxonomic criteria (*17*). Under microscopy, the cells were 2 × 2 μm Gram-positive cocci and were observed in pairs or short chains. The isolate grew on MRS agar in the presence of 6.5% NaCl and 40% bile at 45 °C. The cells grew poorly at 10 °C and grew in MRS broth at a pH of 9.6. The cells lacked catalase activity and were nonmotile. Additional biochemical properties of the isolate were determined by the EN-COCCUS test system (Lachema, Brno, Czech Republic). The cells showed characteristic hydrolysis of arginine and hippurate, fermentation of lactose, arabinose, and mannitol, nonfermentation of sorbitol, melibiose, rafinose, and melizitose, use of 0.4% tellurite, and non-use of 0.01% tetrazolium. They grew in blood medium, resulting in α-hemolysis, and on MRS agar at 37 °C under microaerobic or aerobic atmospheres. Cells produced red-colored colonies on *Enterococcus* agar. 16S rRNA sequencing and *sod*A-based PCR test resulted in the *E. faecium* classification (*20*).

Biochemical purification of the bacteriocin from *E. faecium* NRRL B-30746 fermentation increased purity from the culture supernatant to 84.7% after ammonium sulfate precipitation, dialysis, ion-exchange chromatography, and hydrophobic-interaction chromatography. These purification steps increased the anti-*Campylobacter* activity/milliliter approximately 400-

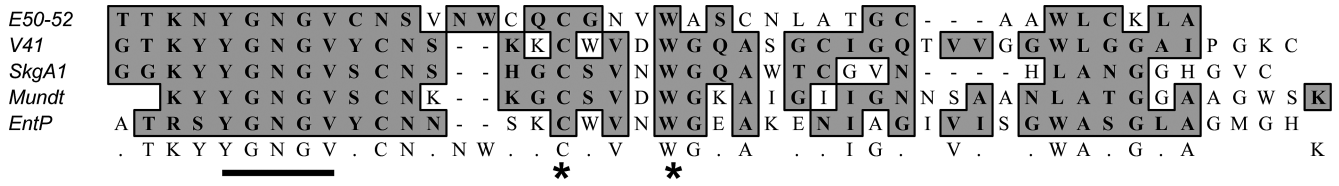

Figure 2. Primary amino acid sequence of the E 50-52 bacteriocin and alignments following BLAST. The purified peptide bacteriocin E 50-52 was purified by chromatography and subjected to protein sequencing. The amino acid sequence of the peptide is presented with conserved bacteriocin Class II sequences underlined. The primary amino acid sequence was entered into the BLAST analysis (*27*) to search for short, nearly exact protein matches in the NCBI database. Class II sequences are underlined or asterisked.

Table 2. Distributions of in Vitro MIC of Bacteriocin E 50-52 against *Campylobacter jejuni*, *Yersinia* spp., *Staphylococcus* spp., and *Listeria* spp

	MICs (μg/mL)
Campylobacter jejuni ($n = 98$)	
1	0.025
11	0.05
17	0.1
21	0.2
14	0.4
15	0.8
9	1.6
8	3.2
2	6.4
Yersinia enterocolitica ($n = 24$) and *Yersinia pseudotuberculosis* ($n = 2$)	
8	0.156
8	0.312
9	0.625
1	1.25
Staphylococcus aureus ($n = 26$) and *Staphylococcus epidermidis* ($n = 4$)	
3	0.2
17	0.4
10	0.8
Listeria monocytogenes ($n = 10$) and *Listeria* spp. ($n = 6$)	
6	2
3	4
7	8
1	32

fold over the starting culture supernatant. **Figure 1**, **panel A**, provides the E 50-52 molecular mass estimation as less than 5000 or 6000 Da. The zone of inhibition surrounding this purified protein defines its anti-*Campylobacter* activity. **Figure 1**, **panel B**, provides an estimation of the p*I* of this protein at a pH of about 8.5. Again, a zone of inhibition surrounding the bacteriocin was observed. The MALDI-TOF mass spectrometric analysis indicated a molecular mass for E 50-52 of 3339.7 ± 0.3 Da. Accession number P85148 for protein enterocin E 50-52 produced by *E. faecium* NRRL B-30746 was provided by UniProt Knowledgebase.

Table 1 provides data on the response of E 50-52 to selected enzymes, time/temperature exposure, and pH-activity ranges which were indicative of Class IIa bacteriocin characteristics. Loss of anti-*Campylobacter* activity was observed after incubation with each of the three protease enzymes tested. Continued activity following exposure to both lysozyme and lipase enzymes further suggested that E 50-52 was not structurally related to the substrates of these enzymes. Activity of the polypeptide after exposure to 100 °C for 15 min and after exposure to pH values of 3.0−8.4 was consistent with previous Class II bacteriocin data. Bacteriocin E 50-52 was ultimately inactivated at a pH of 9.1 under the three time/temperature conditions tested.

Figure 2 compares the amino acid sequences of the E 50-52 bacteriocin and other related bacterially produced antimicrobial

Table 3. In Vivo Activity of Bacteriocin E 50-52 Provided in Feed from Four to Seven Days Post-hatch To Treat Broilers Challenged with Equal Numbers of 10^6 cfu *Campylobacter jejuni* Isolates B1 and L4 on Day of Hatch[a]

		$\log_{10}$ cfu/g in cecal contents[b]	
bird numbers	therapeutic regimen	*C. jejuni*[c]	lactic acid bacteria[d]
7	bacteriocin-free feed (control group)	8.40 ± 0.47 a	9.51 ± 0.07a
10	feed containing bacteriocin E 50-52 (0.125 g/kg)	not detected b	9.52 ± 0.36a
10	feed containing bacteriocin E 50-52 (0.0625 g/kg)	not detected b	8.73 ± 1.20b
10	feed containing bacteriocin E 50-52 (0.0312 g/kg)	not detected b	8.55 ± 1.15b

[a] Groups of 7–10 birds each were held in isolation, individual birds were sacrificed, and cecal contents were sampled at 15 days of age. To determine lactic acid bacteria, the cecal content from three broilers of each group was plated onto MRS agar. [b] Mean $\log_{10}$ cfu within a column followed by different letters denote significantly different treatment effects. [c] Mean $\log_{10}$ cfu of birds per group ± standard deviation. [d] Mean $\log_{10}$ cfu of three birds per group ± standard deviation.

peptides. The consensus Class II N-terminal pediocin-box sequence YGNGV (*28, 29*) was found from positions 5−9 within the 39-amino-acid E 50-52 molecule and was predicted to be highly hydrophilic. The remaining portion of the molecule was predicted to be hydrophobic with low surface probability that could potentially form an amphipathic helix or sheet structure. The estimated molecular weight of 4125 Da was slightly greater than the electrophoretically determined size with a predicted p*I* of 8.0 that was close to the experimentally determined p*I*. The peptide was composed of 50% polar residues with six cysteines, 36% nonpolar residues, and 5% basic residues (K). Following a BLAST of the E 50-52 peptide sequence, the molecule most closely resembled bacteriocins enterocin P (*14*) and mundticin (*15, 30*), produced by *E. faecium* and *Enterococcus mundtii*, respectively. The other closely related bacteriocins were sakacin G (*31*) and divercin V41 (*32*), produced by *Lactobacillus sake* and *Carnobacterium divergens*, respectively.

Bacteriocin E 50-52 was initially selected for antimicrobial activity against *C. jejuni* NCTC 11168. The bacteriocin was further tested against 98 additional strains of *C. jejuni*, with MIC values ranging from 0.025 to 6.4 μg/mL (**Table 2**). The strains tested were derived from a diverse sampling of Russian poultry (*33*) as well as isolates from human gastroenteritis. The MICs of 89.6% of the isolates were less than 1.6 μg of E 50-52 per milliliter.

In vitro testing of *Yersinia* spp. further revealed the extent of microbiocidal activity of E 50-52 (**Table 2**). Against *Y. enterocolitica*, the MIC values ranged from 0.312 to 1.25 μg/mL for the 24 isolates tested. These clinical *Y. enterocolitica* isolates were part of the culture collection maintained at the

Table 4. Log_{10} cfu of Lactic Acid Bacteria *Campylobacter jejuni*, and *Salmonella enteritidis* per Gram of Cecal Homogenate[a]

				log_{10} cfu per bacterial group[b]				
						S. enteritidis		
duration (d) of treatment	milligram of E 50-52 per bird	bird number	bird age in days	lactic acid bacteria	C. jejuni	cecum	liver	spleen
		7	41	8.40 ± 0.11 a	8.00 ± 0.90 a	7.48 ± 0.76 a	8.76 ± 0.15 a	7.68 ± 0.32 a
1	3.56	10	39	8.32 ± 0.15 a	2.68 ± 0.74 b	1.76 ± 1.25 b	6.94 ± 0.45 b	6.99 ± 0.45 b
2	7.28	10	40	8.15 ± 0.93 a	2.15 ± 0.34 b	ND b	6.83 ± 0.67 b	6.57 ± 0.28 b
3	10.8	10	41	8.15 ± 0.08 a	2.90 ± 0.23 b	ND b	4.58 ± 1.00 c	4.57 ± 0.38 c

[a] Bacteriocin E 50-52 administered in drinking water (12.5 mg of E 5052 per liter) to grouped commercial broilers environmentally infected with *Campylobacter* spp. and experimentally challenged with $\sim 10^{10-11}$ cfu *Salmonella enteritidis* 92 Rif^R. [b] Mean log_{10} cfu ± standard deviations among colonized birds. Values within a column followed by different superscripted letters denote significantly different treatment effects. ND, not detected.

SRCAMB. MIC testing against 30 isolates of *Staphylococcus* spp. and 16 isolates of *Listeria* spp. was conducted (**Table 2**). Lethality was quite uniform among both *S. aureus and S. epidermidis* isolates, with MIC values ranging from 0.2 to 0.8 μg/mL. Among the 16 *Listeria* spp. isolates tested, we observed the greatest range of MIC values. Among the 10 isolates of *L. monocytogenes* tested, the MIC range was from 2 to 8 μg/mL. Five additional isolates (*L. grayi*, *L. innocula*, and *L. ivanovi*) yielded similar results. One isolate of *L. denitrificans* had the uniquely high MIC of 32 μg/mL. MICs for limited numbers of *E. coli* O157:H7 ($n = 1$), *Morganella morganii* ($n = 1$), *Salmonella* spp. ($n = 4$), and *Shigella dysenteriae* ($n = 1$) were observed to be <1.6 μg/mL. The susceptibility of such a wide array of pathogens is notable in the bacteriocin literature.

Next, we assessed the potentials of using bacteriocin E 50-52 as a therapeutic treatment in young chicks colonized with *C. jejuni*. **Table 3** provides the results from these in vivo experiments. Day-of-hatch birds (7−10 chicks per group) were colonized with *C. jejuni* isolates B1 and L4. This nonpathological commensal relationship resulted in very high levels of the organism colonizing the ceca. Non-inoculated levels of endogenous lactic acid bacteria were associated with all bird groups. The three treated groups of chicks were given bacteriocin from day four to seven, the birds were killed, and cecal content was plated on day 15. All three durations of bacteriocin E 50-52 treatment eliminated detectable ($<10^2$/g) levels of *C. jejuni*, whereas the untreated control birds were colonized with $10^{8.40 \pm 0.47}$ cfu/g of cecal content (Z-test, $p < 0.001$). Levels of lactic acid bacteria were essentially indistinguishable between the bacteriocin-fed and the bacteriocin-free groups of birds (Z-test, $p = 0.29$).

The final in vivo study was conducted by using mature broilers that were commercially reared. These birds, environmentally contaminated with *C. jejuni*, were challenged with very high levels of *S. enteritidis* when they were 35 days old. Bacteriocin was provided in drinking water (12.5 mg of E 50-52 per liter), and birds having free access to drinking water were sampled at 39, 40, and 41 days of life, providing the different mean levels of bacteriocin per bird reported in **Table 4**. The data indicated that the numbers of lactic acid bacteria were essentially unaltered by the bacteriocin regimen ($p = 0.26$). The numbers of *C. jejuni* in the ceca of treated birds were reduced by more than 99.999% by the various bacteriocin regimens ($p < 0.001$). Similarly, the numbers of *S. enteritidis* were also reduced significantly ($p < 0.001$) within the treated broiler ceca. Although the numbers were also reduced in the liver ($p < 0.001$) and spleen ($p = 0.03$), the magnitude of reductions in the treated birds was not as great as that observed within the ceca. Reductions among the systemically disseminated *S. enteritidis* mediated by oral bacteriocin were noteworthy.

DISCUSSION

The case for employing bacteriocins as safe and effective antimicrobial agents has already been presented (*34*). These compounds have either intentionally or unintentionally been added via food fermentations over the past millennia as part of human strategy to preserve foods. After having consumed bacteriocins for this duration, there is no evidence for concern related to product safety. Consumers continue to seek natural food products free of artificial additives, and bacteriocins meet this need.

Food safety and preservation through bacteriocin application is consistent with consumer desires for natural food products, and bacteriocins can be used in food-producing animals. As bacteriocins have been approved for use in human foods, it seems logical to expect the same approval for animal feeds. *Enterococci* has long been employed as part of intentionally added fermenting bacteria to dairy and meat products (*35*). Studies pertaining to Class IIa bacteriocins produced by *Enterococcus* spp. have been reported and thoroughly reviewed (*9, 16*). *Enterococcus* spp. are an essential part of the lactic acid bacterial flora that occupy the intestinal tract of healthy animals. Likewise, this niche can also be occupied by antibiotic-resistant strains (*36*), and concerns over transmission of such resistance exist (*37*). However, because the mechanisms of bacterial killing by bacteriocins are substantially different than those involved in antibiotic-mediated killing, increased concern over bacteriocin use seems unwarranted. The present article further extends the potentials for application of such a bacteriocin produced by *E. faecium* NRRL B-30746 (E 50-52) for controlling pathogens within the animal intestinal tract to reduce food contamination.

Enumeration of the lactic acid bacteria was very similar in the ceca of both treated and untreated groups of chickens (**Tables 3** and **4**). This was demonstrated by using both described therapeutic treatment protocols. In one case, an extended delay of eight days after bacteriocin treatment was imposed, and in the second case, we sampled one day after treatment. The bacteriocin nisin does not alter the gut bacterial composition because bacteriocins are inactivated by the proteases contained in the intestinal tract (*38, 39*). Resistance of micro-organisms to bacteriocins has been recognized, but the mechanism is different from antibiotic resistance. Monitoring quantities of lactic acid bacteria did not indicate a qualitative difference in the intestinal microflora of the treated animals. It seems possible that bacteriocin E 50-52 was lethal for some portion of the lactic acid bacterial flora, while the remaining resistant flora proliferated or were maintained at the nontreated levels one day after treatment. Additional studies will be needed to determine whether qualitative changes in these bacterial populations occur. Although bacteriocin treatment did not disrupt levels of lactic acid bacteria, we did observe a significant reduction in the levels of intestinal and systemic *Salmonella*

enteritidis and intestinal *Campylobacter jejuni* in the chicken host after bacteriocin treatment.

Class IIa bacteriocins range in size from 37 to 48 amino acids in length and have a conserved N-terminal consensus sequence of YGNGVxCxxxxCxV (*40*). The amino acid sequence of bacteriocin E 50-52 was consistent with this Class IIa criterion. Bacteriocin produced by both *Lactobacillus salivarius* NRRL B-30514 (*26*) and *Paenibacillus polymyxa* NRRL B-30509 (*24*)were also categorized as Class IIa. Similar to that of these bacteriocins, the bactericidal activity of E 50-52 was quite diverse and extended to both Gram-positive and Gram-negative pathogens. The bacteriocins most closely related to E 50-52 were enterocin P produced by *E. faecium* (*14*) and mundticin produced by *E. mundtii* (*15, 30*). These inhibited Gram-positive organisms; they are enterocins of the pediocin-like family designated Class II.1 (*29, 41*). The specific amino acid sequences of our bacteriocin differed from previously published sequences and potentially conferred the diverse killing capacities at relatively low MIC which we report.

Bacteriocins are known to destabilize the target organism membranes, causing pore formation and lysis. Nisin and pediocin are thought to work in this manner but are modestly capable of permeablilizing Gram-negative pathogen membranes. Shelburne et al. (*42*) reported that the bacteriocin subtilosin A had activity against both Gram-negative and Gram-positive bacteria. Their report indicated MIC against limited numbers of Gram-negative bacteria as <100 μg/mL. Overall, the predicted structure of bacteriocin E 50-52 resembles the Class II.1 molecules (*29, 41*) originally classified as Class IIa (*10*). The N-terminal hydrophilic cationic portion for initial binding to the bacterial cell or anionic liposomes contains the Asn5 crucial for full activity (*43*). There was, however, a N-for-D substitution in the hinge region of the E 50-52 molecule that is more common to Class II.2 bacteriocins (*43*). The C-terminal portion is more hydrophobic, and there is an amphiphilic α-helix that contains the tryptophan (W) residue which stabilizes a hairpin-like structure (*44*) and potentially should recognize the cognate immunity protein (*45*). Consequently, E 50-52 contains the requisite characteristics of highly active, bacterially derived, antimicrobial peptides, although why it is active against both Gram-negative and Gram-positive organisms remains under study.

The MICs for the Gram-negative pathogens were generally <1 μg of E 50-52 per milliliter. This was particularly notable given the multispecies killing observed among the diverse genera *Yersinia* spp., *Salmonella* spp., and *Staphylococcus* spp. Of further importance was the applicability of the bacteriocin to our chicken model. Both *C. jejuni* and *S. enteritidis* are human pathogens and are quite refractile in the chicken intestinal tract and in the liver/spleen. Our data demonstrate the applicability of bacteriocin as a therapeutic treatment in poultry to resolve such infections. Another observation was related to the significant reductions of *S. enteritidis* in the systemic liver and spleen infection foci. The oral administration of E 50-52 resulted in the reductions reported at these extra-intestinal sites. The physiology/pharmacology of this therapeutic bacteriocin remains for further explanations and studies. Our route of administration may not be optimum for such parenchymal infection. Alternative bacteriocin treatment protocols for systemic infections are being studied and have been reported (*46*).

Supporting Information Available: Specific isolate identification and corresponding MICs and MALDI-TOF mass spectrometry of bacteriocin E 50-52. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Smith, D. L.; Harris, A. D.; Johnson, J. A.; Silbergeld, E. K.; Morris, J. G., Jr. Animal antibiotic use has an early but important impact on the emergence of antibiotic resistance in human commensal bacteria. *Proc. Natl. Acad. Sci.* **2002**, *99*, 6434–6439.

(2) Werner, G.; Bronzwaer, S. Ensuring prudent use of antimicrobials in human medicine in the European Union. www.eurosurveillance.org/em/v12n01/1201–225.asp, 2005.

(3) Joerger, R. D. Alternatives to antibiotics, bacteriocins, antimicrobial peptides and bacteriophages. *Poult. Sci.* **2003**, *82*, 640–647.

(4) Papagianni, M. Ribosomally synthesized peptides and antimicrobial properties: Biosynthesis, structure, function, and applications. *Biotechnol. Adv.* **2003**, *21*, 465–499.

(5) Gratia, A. Sur un remarquable exemple d'antaonisme etre deux souches de coilbacille. *Compt. Rend. Soc. Biol.* **1925**, *93*, 1040–1041.

(6) Mattick, A. T. R.; Hirsch, A. A powerful inhibitory substance produced by group N streptococci. *Nature* **1947**, *154*, 551.

(7) Papavassiliou, J. Biological characteristics of colicine X. *Nature* **1961**, *190*, 110.

(8) Whitehead, H. R. A substance inhibiting bacterial growth, produced by certain strains of lactic streptococci. *Biochem. J.* **1933**, *27*, 1793–1800.

(9) Drider, D.; Fimland, G.; Hechard, Y.; McMullen, L. M.; Prevost, H. The continuing story of class IIa bacteriocins. *Microbiol. Mol. Biol. Rev.* **2006**, *70*, 564–582.

(10) Klaenhammer, T. R. Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol. Rev.* **1993**, *12*, 39–85.

(11) Riley, M. A.; Wertz, J. E. Bacteriocins, evolution, ecology, and application. *Annu. Rev. Microbiol.* **2002**, *56*, 117–137.

(12) Tagg, J. R.; Dajani, A. S.; Wannamaker, L. W. Bacteriocins of Gram-positive bacteria. *Bacteriol. Rev.* **1976**, *40*, 722–756.

(13) Aymerich, T.; Holo, H.; Havarstein, L. S.; Hugas, M.; Garriga, M.; Nes, I. F. Biochemical and genetic characterization of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bacteriocins. *Appl. Environ. Microbiol.* **1996**, *62*, 1676–1682.

(14) Cintas, L. M.; Casaus, P.; Havarstein, L. S.; Hernandez, P. E.; Nes, I. F. Biochemical and genetic characterization of enterocin P, a novel sec-dependent bacteriocin from Enterococcus faecium P13 with a broad antimicrobial spectrum. *Appl. Environ. Microbiol.* **1997**, *63*, 4321–4330.

(15) Kawanmoto, S.; Shima, J.; Sato, R.; Eguchi, T.; Ohmsomo, S.; Shibato, J.; Horikoshi, N.; Taeshita, K.; Sameshima, T. Biochemical and genetic characterization of mundticin KS, an antilisterial peptide produced by *Enterococcus mundtii* NFRI 7393. *Appl. Environ. Microbiol.* **2002**, *68*, 3830–3840.

(16) Nes, I. F.; Diep, D. B.; Holo, H. Bacteriocin diversity in *Streptococcus* and *Enterococcus*. *J. Bacteriol.* **2007**, *189*, 1189–1198.

(17) Siragusa, G. Production of bacteriocin inhibitory to *Listeria* species by *Enterococcus hirae*. *Appl. Environ. Microbiol.* **1992**, *58*, 3508–3513.

(18) Holt, J. G.; Krieg, N. R.; Sneath, P. H. A.; Staley, J. T.; Williams, S. T. Enterococci. *Bergey's manual of determinative bacteriology*, 9th ed.; Lippincott Williams & Wilkins: Philadelphia, 2000; p528.

(19) Cole, J. R.; Chai, B.; Marsh, T. L.; Farris, R. J.; Wang, Q.; Kulam, S. A.; Chandra, S.; McGarrell, D. M.; Schmidt, T. M.; Garrity, G. M.; Tiedje, J. M. The Ribosomal Database Project (RDP-II): Previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res.* **2003**, *1*, 442–443.

(20) DeSantis, T. Z.; Hugenholtz, P.; Keller, K.; Brodie, E. L.; Larsen, N.; Piceno, Y. M.; Phan, R.; Andersen, G. L. NAST: A multiple sequence alignment server for comparative analysis of 16S rRNA genes. *Nucleic Acids Res.* **2006**, 34:W394-9.; http://greengenes.lbl.gov/.

(21) Jackson, C. R.; Fedorka-Cray, P. J.; Barrett, J. B. Use of a genus- and species-specific multiplex PCR for identification of enterococci. *J. Clin. Microbiol.* **2004**, *42*, 3558–3565.

(22) Muriana, P. M.; Luchansky, J. B. Biochemical methods for purification of bacteriocins. In *Bacteriocins of Lactic Acid Bacteria*; Hoover D. G., Steenson L. R., Eds.; Academic Press: New York, 1993; pp 41–61..

(23) Prioult, G.; Turcotte, C.; Labarre, L.; Lacroix, C.; Fliss, I. Rapid purification of nisin Z using specific monoclonal antibody-coated magnetic beads. *Int. Dairy J.* **2000**, *10*, 627–633.

(24) Svetoch, E. A.; Stern, N. J.; Eruslanov, B. V.; Kovalev, Y. N.; Volodina, L. I.; Perelygin, V. V.; Mitsevich, E. V.; Mitsevich, I. P.; Pokhilenko, V. D.; Borzenkov, V. N.; Levchuk, V. P.; Svetoch, O. E.; Kudriavtseva, T. Y. Isolation of *Bacillus circulans* and *Paenibacillus polymyxa* strains inhibitory to *Campylobacter jejuni* and characterization of associated bacteriocins. *J. Food Prot.* **2005**, *68*, 11–17.

(25) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.

(26) Stern, N. J.; Svetoch, E. A.; Eruslanov, B. V.; Perelygin, V. V.; Mitsevich, E. V.; Mitsevich, I. P.; Pokhilenko, V. D.; Levchuk, V. P.; Svetoch, O. E.; Seal, B. S. Isolation of a *Lactobacillus salivarius* and purification of its bacteriocin that is inhibitory to *Campylobacter jejuni* in the chicken gastrointestinal system. *Antimicrob. Agents Chemother.* **2006**, *50*, 3111–3116.

(27) Altschul, S. F.; Madden, T. L.; Schaffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D. J. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* **1997**, *25*, 3389–3402.

(28) Cotter, P. D.; Hill, C.; Ross, R. P. Bacteriocins: Developing innate immunity for food. *Nat. Rev. Microbiol.* **2005**, *3*, 777–88.

(29) Franz, C. M.; van Belkum, M. J.; Holzapfel, W. H.; Abriouel, H.; Galvez, A. Diversity of enterococcal bacteriocins and their grouping in a new classification scheme. *FEMS Microbiol. Rev.* **2007**, *31*, 293–310.

(30) Bennik, M. H.; Vanloo, B.; Brasseur, R.; Gorris, L. G.; Smid, E. J. A novel bacteriocin with a YGNGV motif from vegetable-associated *Enterococcus mundtii*: Full characterization and interaction with target organisms. *Biochim. Biophys. Acta* **1990**, *14*, 47–58.

(31) Simon, L.; Fremaux, C.; Cenatiempo, Y.; Berjeaud, J. M. Sakacin g, a new type of antilisterial bacteriocin. *Appl. Environ. Microbiol.* **2002**, *68*, 6416–20.

(32) Metivier, A.; Pilet, M. F.; Dousset, X.; Sorokine, O.; Anglade, P.; Zagorec, M.; Piard, J. C.; Marion, D.; Cenatiempo, Y.; Fremaux, C. Divercin V41, a new bacteriocin with two disulphide bonds produced by *Carnobacterium divergens* V41: Primary structure and genomic organization. *Microbiology* **1998**, *144*, 2837–2844.

(33) Stern, N. J.; Bannov, V. A.; Svetoch, E. A.; Mitsevich, I. P.; Volozhantsev, N. V.; Gusev, V. V.; Perelygin, V. V. Distribution and characterization of *Campylobacter* spp. from Russian poultry. *J. Food Prot.* **2004**, *67*, 239–245.

(34) Cleveland, J.; Montville, T. J.; Chikindas, M. L. Bacteriocins: Safe food preservatives of the future. *Int. J. Food Microbiol.* **2001**, *71*, 1–20.

(35) Franz, C. M.; Stiles, M. E.; Schleifer, K. H.; Holzapfel, W. H. Enterococci in foods—a conundrum for food safety. *Int. J. Food Microbiol.* **2003**, *88*, 105–122.

(36) Welton, L. A.; Thal, L. A.; Perri, M. B.; Donabedian, S.; McMahon, J.; Chow, J. W.; Zervos, M. J. Antimicrobial resistance in enterococci isolated from turkey flocks fed virginiamycin. *Antimicrob. Agents Chemother.* **1998**, *42*, 705–708.

(37) Rice, L. B. Emergence of vancomycin resistant enterococci. *Emerging Infect. Dis.* **2001**, *7*, 183–187.

(38) Heinemann, B.; Williams, R. The inactivation of nisin by pancreatin. *J. Dairy Sci.* **1966**, *49*, 312–313.

(39) Jarvis, B.; Mahoney, R. R. Inactivation of nisin by alpha-chymotrypsin. *J. Dairy Sci.* **1969**, *52*, 1448–1450.

(40) Eijsink, V. G.; Axelsson, L.; Diep, D. B.; Havarstein, L. S.; Holo, L. S.; Nes, I. F. Production of class II bacteriocins by lactic acid bacteria; An example of biological warfare and communication. *Antonie Van Leeuwenhoek* **1998**, *81*, 639–654.

(41) Fimland, G.; Johnsen, L.; Dalhus, B.; Nissen-Meyer, J. Pediocin-like antimicrobial peptides (class IIa bacteriocins) and their immunity proteins: Biosynthesis, structure, and mode of action. *J. Pept. Sci.* **2005**, *11*, 688–96.

(42) Shelburne, C. E.; An, F. Y.; Dholpe, V.; Ramamoorthy, A.; Lopatin, D. E.; Lantz, M. S. The spectrum of antimicrobial activity of the bacteriocin subtilosin A. *J. Antimicrob. Chemother.* **2007**, *59*, 297–300.

(43) Fimland, G.; Pirneskoski, J.; Kaewsrichan, J.; Jutila, A.; Kristiansen, P. E.; Kinnunen, P. K.; Nissen-Meyer, J. Mutational analysis and membrane-interactions of the beta-sheet-like N-terminal domain of the pediocin-like antimicrobial peptide sakacin P. *Biochim. Biophys. Acta* **2006**, *1764*, 1132–1140.

(44) Fimland, G.; Eijsink, V. G.; Nissen-Meyer, J. Mutational analysis of the role of tryptophan residues in an antimicrobial peptide. *Biochemistry* **2002**, *41*, 9508–9515.

(45) Johnsen, L.; Fimland, G.; Nissen-Meyer, J. The C-terminal domain of pediocin-like antimicrobial peptides (class IIa bacteriocins) is involved in specific recognition of the C-terminal part of cognate immunity proteins and in determining the antimicrobial spectrum. *J. Biol. Chem.* **2005**, *280*, 9243–9250.

(46) Sosunov, V.; Mischenko, V.; Eruslanov, B.; Svetoch, E.; Shakna, Y.; Stern, N.; Majorov, K.; Sorokoumova, G.; Selishcheva, A.; Apt, A. Antimycobacterial activity of bacteriocins and their complexes with liposomes. *J. Antimicrob. Chemother.* **2007**, *59*, 919–925.

Received for review November 9, 2007. Revised manuscript received January 8, 2008. Accepted January 9, 2008. Funds for the conduct of this research were provided by the U.S. State Department (ISTC #1720p), the U.S. Department of Agriculture (CRIS 6612-32000-034-00), and the State Research Center for Applied Microbiology and Biotechnology.

JF073284G