Production of bacteriocin by a novel *Bacillus* sp. strain RF 140, an intestinal bacterium of Caspian Frisian Roach (*Rutilus frisii kutum*)

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(Received 10 Aug 2008; revised version 19 Apr 2009; accepted 27 Apr 2009)

Summary

Bacteriocins are proteinaceous antibacterial compounds that exhibit bactericidal activity against species closely related to the producer strain. The aim of this research was to investigate the production of bacteriocin by *Bacillus* spp. isolated from intestinal bacterial flora of the Caspian Frisian Roach (*Rutilus frisii kutum*). A bacteriocin produced by the bacterium *Bacillus cereus* strain RF 140 was identified. The antimicrobial activity started at the exponential growth phase and maximum activity was at the stationary growth phase. A crude bacteriocin obtained from culture supernatant fluid was inhibitory to indicator strains, including *Listeria monocytogenes*, *Clostridium perfringens*, and several species of *Bacillus*. Bacteriocin was stable at 80°C, but the activity decreased and was lost when the temperature reached 100 and 121°C, respectively. It was resistant to the proteolytic action of papain, catalase and amylase, but sensitive to proteinase K, pronase E and trypsin. Maximum bacteriocin activity was observed in the pH 4-9. This study indicates the importance of the bacteriocin produced by *B. cereus* strain RF 140 against food-borne pathogenic microorganisms such as *L. monocytogenes* and *C. perfringens*, and presents a potential for use as a biopreservative in food.

Key words: *Rutilus frisii kutum*, *Bacillus cereus*, Bacteriocin, *Listeria monocytogenes*, *Clostridium perfringens*

Introduction

Bacteriocins are proteinaceous antibacterial compounds that exhibit bactericidal activity against species closely related to the producer strain (De Vuyst and Vandamme, 1994). Bacteriocins from a variety of Gram-positive species have been biochemically and genetically characterized, including *Staphylococci* (Navaratna *et al*., 1998; Oliveira *et al*., 1998) and coryneform bacteria (Valdes-Stauber *et al*., 1991; Motta and Brandelli, 2002). The latter bacteriocins have been grouped into four distinct classes defined by Chen and Hoover (2003). Bacteriocins belonging to class I, also called the lantibiotics, are characterized by the presence of unusual thioether amino acids, generated through post-translational modification. Class II bacteriocins represent small (610 kDa), heat-stable and membrane-active peptides. Bacteriocins belonging to class III consist of large (s30 kDa) and heat labile proteins, while class IV represents complex bacteriocins that contain essential lipid or carbohydrate moieties in addition to a protein component (Ahern *et al*., 2003). The genus *Bacillus* includes a variety of species with a history of safe use in industry. Commercial products that are currently obtained from *Bacillus* spp. include enzymes, antibiotics, amino acids and

insecticides (Motta *et al*., 2007). The potential of *Bacillus* species to produce antibiotics has been recognized for more than 50 years and peptide's antibiotics represent the predominant class. Many bacteriocins or bacteriocin-like inhibitory substances (BLIS) in the genus *Bacillus* have been reported. Examples are cerein, produced by *B. cereus* Gn105 (Naclerio *et al*., 1993) and cerein 7 produced by *B. cereus* Bc7 (Oscariz *et al*., 1999). The first is a compound with a molecular mass of 9 kDa which displays antimicrobial activity against a wide range of *B. cereus* strains without affecting the growth of other tested Gram-positive bacteria. Cerein 7 as a class II bacteriocin with a molecular mass of 3.94 kDa inhibits a wide range of Gram-positive bacteria (Oscariz and Pisabarro, 2000).

A number of *Bacillus* strains obtained from the kutum Roach (*Rutilus frisii kutum*) have been screened for the production of antimicrobial substances against *L. monocytogenes* ATCC 11915 as the indicator strain. In our previous study, *B. cereus* strain RF 140 showed the highest activity against *L. monocytogenes* ATCC 11915 and was chosen as an active strain and subjected to further examination. This microorganism is a Gram-positive spore-forming bacterium, presenting catalase- and lecitinase-positive reactions. Taxonomic classification of isolates was done using API 50 CHB fermination strips (bioMerieux, Marcy I'Etoile, France). Except for the presence of a subterminal spore, all physiological and morphological characteristics were identical to *B. cereus* PTCC 1247 (Clauss and Berkeley, 1986). This paper describes the antimicrobial spectrum and some properties of the antimicrobial substance produced by *B. cereus* RF140.

Materials and Methods

Bacterial strains and media

B. cereus strain RF140, isolated from intestinal bacterial flora of the Kutum Roach (*Rutilus frisii kutum*), was used. The strain was stored at -80°C before use. Prior to use, the frozen bacteria were thawed at room temperature and inoculated in Tryptic Soy Broth (TSB, Merck, Germany).

Indicator strains listed in Table 1 are laboratory stock obtained from different sources and were kept frozen in 20% (v/v) glycerol at -20°C. The organisms were propagated in appropriate media and temperature, as indicated in Table 1.

Bacterial growth and production of crude bacteriocin

To produce bacteriocin, bacteria were grown in TSB medium at 25°C on an orbital shaker at 125 rpm for desired times. The cells were subsequently removed by centrifugation at 8000 × g for 10 min. The

Table 1: Antimicrobial activity of bacteriocin produced by *Bacillus cereus* RF 140 on indicator organisms

Indicator organism	Medium*	Temperature (°C)	Inhibition zone (mm)
Bacillus cereus ATCC 9634	BHI	37	18
Bacillus coagulans	BHI	37	14
Bacillus subtilis ATCC 9372	BHI	37	22
Clostridium perfringens ATCC 3624	RCM	37	14
Corynebacterium fimi NCTC 7547	BHI	37	12
Corynebacterium glutamicum ATCC 14752	BHI	37	10
Enterobacter aerogenes	BHI	37	-
Escherichia coli ATCC 25922	BHI	37	-
Lactobacillus acidophilus ATCC 4356	MRS	30	-
Lactobacillus casei	MRS	30	-
Lactobacillus plantarum ATCC 8014	MRS	30	-
Listeria monocytogenes ATCC 7644	BHI	37	16
Pseudomonas aeruginosa	BHI	37	-
Pseudomonas fluorescens	BHI	37	-
Salmonella Enteritidis ATCC 13076	BHI	37	-
Staphylococcus aureus ATCC 25923	BHI	37	-

* BHI: brain hearth infusion, MRS: de Man-Rogosa-Sharpe agar and RCM: reinforced clostridial medium

supernatant was filter-sterilised using 0.45-µm filters (Millipore) to produce a cell free culture supernatant (CFS) and tested for antimicrobial activity using indicator strains. The determination of growth was carried out as described by Motta and Brandelli (2002). Bacterial growth was developed at 25°C on a rotary shaker. At 4 h intervals, the optical density (OD) of the cultures was measured at 600 nm with a Hitachi U-1100 spectrophotometer (Hitachi, Tokyo, Japan).

Assay of antagonistic activity

The antimicrobial activity was detected by agar disk diffusion assay and was tested against all indicator strains. An aliquot of 50 µl CFS was applied on disks (6 mm) on agar plates previously inoculated with each individual indicator strain suspension, which corresponded to a 0.5 McFarland turbidity standard solution. Plates were incubated 24 h at optimal temperature of the test organism. The bacteriocin titer was determined by the serial two-fold dilution method previously described by He *et al*. (2006).

Effect of proteolytic enzymes, heat and pH on antimicrobial activity

In order to determine the biological nature of the antimicrobial activity of bacteria, CFS of *B. cereus* strain RF140 was tested for its sensitivity to the proteolytic enzymes. One ml samples of 1 ml of CFS were treated at 37°C for 1 h with 2 mg ml^{-1} final concentration of the following enzymes: papain, trypsin, proteinase K, pronase E and α-amylase (Sigma). Chemicals were added to the CFS and the samples were incubated for 1 h at 25°C before being tested for antimicrobial activity (Table 2).

To determine the sensitivity of the bacteriocin activity to the temperatures, samples of CFS were incubated at a particular temperature for specific times (Table 3). The effect of pH on bacteriocin activity was determined by adjusting the pH of the CFS with diluted HCL and NaOH (Table 3). Samples were incubated for 2 h at 37°C, readjusted to pH 7 and assayed as described above. These assays were performed using strain *L. monocytogenes* ATCC 11915 as the indicator bacterium.

Table 2: Effect of enzymes and chemicals on bacteriocin activity

Treatment	Concentration	Residual activity (%)
Enzymes		
Trypsin	2 mg ml^{-1}	0
Papain	2 mg ml^{-1}	100
Proteinase K	2 mg ml^{-1}	0
Pronase E	2 mg ml^{-1}	0
α-amylase	2 mg ml^{-1}	100
Catalase	2 mg ml^{-1}	100
Organic solvents		
Acetone	10% (v/v)	100
Chloroform	10% (v/v)	92
Dimethyl sulfoxide	10% (v/v)	100
Ethanol	10% (v/v)	92
Methanol	10% (v/v)	100
Ethyl ether	10% (v/v)	100
Xylol	10% (v/v)	100
EDTA	10 mmol l^{-1}	83
Trichloroacetic acid	100 mg ml^{-1}	0
Sodium deoxycholate	1 mg ml^{-1}	100
Sulphobetaine 14	1 mg ml^{-1}	92
Tween 20	10% (v/v)	100
Tween 80	10% (v/v)	92

Table 3: Effect of heat and pH on bacteriocin activity

Treatment	Residual activity (%)
Heat at	
4°C/30 d	100
50°C/30 min	100
70°C/30 min	100
80°C/30 min	100
100°C/15 min	30
121°C/5 min	0
pH	
2	60
4	100
6	100
8	100
9	100
10	72
12	0

Results

Growth and bacteriocin production

B. cereus strain RF 140 was aerobically incubated in TSB at 25°C on a rotary shaker. Cell growth reached the stationary phase at 4 h of cultivation (Fig. 1). Kinetics of bacteriocin production showed that its synthesis and/or secretion started at the first determination (4 h) in the exponential phase of growth and maximum activity was

observed at the early stationary phase of growth (3000 AU ml^{-1}). Afterward, the bacteriocin activity slowly decreased (Fig. 1). The number of viable cells (CFU ml^{-1}) and pH were also determined during growth of the strain RF 140 (result not shown). The values of CFU ml^{-1} correlate well with OD (r^2 value of 0.98). The pH values increased from 7.5 to 9.5 at the end of incubation.

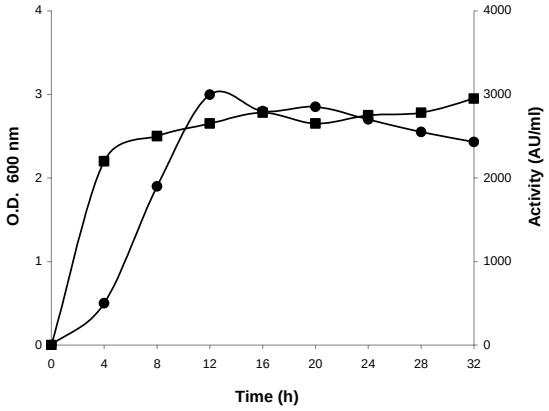

Fig. 1: Production of antimicrobial activity during growth of *Bacillus cereus* RF140 in TSB medium. (■) Bacterial growth and (●) antibacterial activity at the indicated times. Each point represents the mean of three independent experiments

Inhibitory spectrum of bacteriocin

CFS of a culture of *B. cereus* strain RF 140 was tested for antimicrobial activity against several Gram-positive and Gram-negative bacteria (Table 1). Inhibitory effect was observed on several Gram-positive bacteria such as *C. perfringens*, *B. cereus* and *L. monocytogenes*. However, it had no effect on growth of other tested and Gram-positive (*Lactobacillus casei*, *L. plantarum*, *Staphylococcus aureus*) and Gram-negative (*Enterobacter aerogenes*, *Escherichia coli*, several *Pseudomonas* species, *Salmonella enteritidis*) bacterial species.

Effect of enzymes, heat and pH on antimicrobial activity

The bacteriocin was sensitive to proteinase K, pronase E and trypsin. Catalase had no effect on the antimicrobial activity, indicating that the inhibitory activity is not due to hydrogen peroxide production (Table 2). The effect of several chemicals on the antimicrobial activity was also evaluated (Table 2). The bacteriocin lost its activity after treatment with trichloroacetic acid (TCA). Antimicrobial activity was not affected by treatment with organic solvents, or detergents.

To assess the heat stability, the bacteriocin was incubated for 30 min at different temperatures and residual activity was measured (Table 3). It was stable at 80°C, however the activity gradually decreased with the increase in temperature. The residual activity was 30% after incubation at 100°C/15 min, and total loss of activity was observed after incubation at 120°C/5 min. Storage of bacteriocin at low temperatures (4°C) does not alter its activity. In order to examine the pH stability of bacteriocin, its activity was assessed following incubation at different pH (Table 3). Bacteriocin was active in a wide range of pH, as full activity was retained at pH values between 4 and 9. Activity reduced to 60% at pH 2, while it severely altered at pH over 9, with a total loss of activity at pH 12.

Discussions

In the present study, we examined the production of bacteriocins by *B. cereus* strain RF 140 isolated from intestinal bacterial flora of the kutum Roach (*Rutilus frisii kutum*). It was found that an antibacterial substance produced by *B. cereus* RF 140 is proteinaceous, possibly a bacteriocin or a bacteriocin-like substance. The bacteriocin-like substance produced by *B. cereus* RF 140 exhibited the pronounced antagonistic activities against various species of Gram-positive bacteria, which was consistent with the reported bacteriocin-like substances produced by other strains of *Bacillus* (Bizani and Brandelli, 2002; He *et al*., 2006; Motta *et al*., 2007). Inhibitory activity was observed on several Gram-positive bacteria, including important pathogenic and spoilage microorganisms such as *C. perfringens*, *B. cereus* and *L. monocytogenes*.

Some bacteriocins from Bacillus present a narrow antimicrobial spectrum. Oscariz and Pisabarro (2000) isolated and identified cerein 7, a bacteriocin produced by *B. cereus* Bc7 that was inhibitory for *Listeria* spp. and

other Gram-positive bacteria. Coagulin, a bacteriocin-like substance produced by *B. coagulans* I4, has been reported (Hyronimus *et al*., 1998). It presents a broad spectrum of antibacterial activity, inhibiting strains of the same species as the producer strain. In addition, a *B. brevis* strain isolated from kimchi produces a bacteriocin active against a broad spectrum of bacteria, including some pathogens and food spoilage microorganisms and some yeast strains (Mah *et al*., 2001).

The pH of the growth medium of *B. cereus* strain RF 140 was in the range of 8-9.5. An increase in pH during cultivation is often associated with proteolytic microorganisms, and proteolytic activity could be harmful to antimicrobial peptides (He *et al*., 2006). Slow decrease of the bacteriocin activity in the later stationary growth phase indicates that the bacteriocin is relatively sensitive to extracellular proteases due to the partial digestion of the antagonistic compound by proteolytic enzymes released from the cells. In agreement with this fact, the antagonistic compound produced by *B. cereus* RF140 was sensitive to proteinase K, pronase E and trypsin, while it was not affected by α-amylase, papain or any of the organic solvent used, suggesting a proteinaceous nature of inhibitory compound. Other bacteriocins produced by *Bacillus* such as coagulin I4 and cerein 8 A are often more resistant to extracellular proteases (Hyronimus *et al*., 1998; Bizani and Brandelli, 2002).

In the evaluation of bacteriocin properties, its inhibitory activity was sensitive to microbial proteases and lost with TCA treatment, additional evidence that a peptide moiety is associated with activity. Decrease and total loss of activity were observed after incubation at 100 and 121°C, respectively. However, activity was maintained at 80°C, indicating the inhibitory compound was relatively heat stable. Bacteriocins have gained importance as natural biopreservatives for control of spoilage and pathogenic organisms in foods. This study indicated that the bacteriocin produced by *B. cereus* strain RF 140 might be useful to biological control of pathogenic and spoilage microorganisms such as *L. monocytogenes* and *C. perfringens* in foods. Nevertheless, purification of an antibacterial substance remains to be undertaken in the near future.

Acknowledgement

This work received financial support from Tarbiat Modares University.

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