ISSN 0976 – 3333

Available Online at www.ijpba.info.

International Journal of Pharmaceutical & Biological Archives 2011; 2(4):1095-1099

ORIGINAL RESEARCH ARTICLE

Influence of Growth Conditions on Production of Klebicin K and Raoultellin L, Two Antimicrobial Substances against Gram-Negative Pathogens

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Received 02 May 2011; Revised 10 Jul 2011; Accepted 18 Jul 2011

ABSTRACT

Klebicin K and raoultellin L are two bacteriocins produced by *Klebsiella ozaenae* K and *Raoultella terrigena* L, respectively. They possess antimicrobial activity against foodborne pathogens belonging principally to the coliform group. The influence of culture medium, temperature, pH, NaCl concentration and atmosphere on production of these substances was evaluated. Both of these bacteriocins could be produced in different growth media, under a pH range from 6 to 7.5, NaCl concentrations from 0.5 to 2.5% and either aerobiotic or anaerobiotic conditions. Production of klebicin K and raoultellin L occurred at 25°C and 37°C, but ceased at 42°C. However, the growth of producer strains was not reduced, thus indicating that there is no obvious correlation between numbers of culture cells and bacteriocin production. Our results show that klebicin K and raoultellin L production are stable in relation to the most common environmental factors tested. This characteristic is important in evaluating the biopreservative potential of these bacteriocins and constitutes the first step to their purification.

Keywords: Antimicrobial substance, bacteriocins, growth conditions, foodborne pathogens, Enterobacteriaceae, klebicin K, raoultellin L.

INTRODUCTION

Bacteriocins are of interest as potential replacements for traditional antibiotics and as natural food preservatives [1,14]. Today, applied research on bacteriocins aiming towards their application is focused on bacteriocins produced by lactic acid bacteria (LAB). These substances generally inhibit only Gram-positive bacteria. Since Gram-negative agents such as *Escherichia*, *Pseudomonas* and *Salmonella* are only rarely sensitive to LAB bacteriocins [3], antimicrobial substances effective against these pathogens need to be studied.

Although bacteriocins of different enteric bacteria have been studied, colicins were the first to be identified and have since served as a model system for biochemical and genetic investigations, followed by the microcins [2,3,8].

Colicins E1, E4, E7, E8, K and S4, well as microcin J25 and 24 showed to be the most effective bacteriocins against to *E. coli* O157: H7, an enterohemorrhagic shiga toxin strain, cause of hemolytic uremic syndrome and hemorrhagic colitis. These studies reveal the importance of strategies to control this pathogen, since antibiotics increases the release of shiga toxin, contributing to the increased level of virulence of the strain [5,9,20,22].

Other studies have shown that administration of colicin and microcin-producing bacteria in the gut of cows has reduced the levels of enteric pathogens in animals, possibly by preventing the acquisition of new pathogenic strains. Incidentally, the colicins E and B are already being marketed by PBS Animal Health and Horse Health USA, for the prevention and control strains of *E. coli* pathogenic for piglets and newborn foals, respectively [9].

Microcins have been presented as potential alternatives to quinolone antibiotics, inhibitors of DNA gyrase. The microcin L and J25, both produced by *E. coli*, exhibit strong antagonistic activity against strains of *Salmonella enterica* serotype Typhimurium and Enteritidis, causative

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agents of diarrheal disease in humans [19,21]. Microcin E294, produced by *Klebsiella pneumoniae*, induces morphological and biochemical changes typical of apoptosis in human tumor cells, presenting potential as an anti-cancer drug [11].

In a previous study, conducted by our group, 44 strains of Gram-negative bacteria, mostly belonging to the coliform group were isolated from salads, cheeses and meat products marketed in the city of Rio de Janeiro. These strains were identified and tested for antimicrobial production. Two strains (4.5%) exhibited a remarkable inhibitory activity against the indicator strain, *Escherichia coli* LMIFRJ. Producer strains were identified as *Klebsiella ozaenae* and *Raoultella terrigena* [6].

The antimicrobial substance produced by the strain of *K. ozaenae* was sensitive to all three proteolytic enzymes used, whereas that produced by the strain of *R. terrigena* was sensitive to two of the enzymes tested, suggesting that these substances may be bacteriocins. Although the action spectrum is similar, the bacteriocins (klebicin K and raoultellin L) were shown to be different. Both were able to inhibit important pathogens associated with foods such as strains of *E. coli*, *Klebsiella* and *Salmonella*, including those multi-antibiotic resistant [6].

Since some studies have reported that production of bacteriocins is dependent on the culture conditions, such as medium composition, pH, temperature and atmosphere [13,18], the present work aimed to determinate the influence of growth conditions on klebicin K and raoultellin L production. This information will be useful for further purification of these promising bacteriocins aiming their potential use against food borne pathogens.

MATERIALS AND METHODS

Bacterial strains and growth conditions:

Klebsiella ozaenae K and *Raoultella terrigena* L were isolated from salad samples that were available for sale in arbitrarily chosen commercial establishments in the city of Rio de Janeiro, Brazil. *Escherichia coli* LMIFRJ was used as the indicator in bacteriocin production assays. Strains were grown in TSB (trypticase soy broth, Himedia, São Paulo, Brazil) at 37°C for 18 h. When necessary, the medium was supplemented with either 15 g/L or 6 g/L agar (solid and soft agar, respectively). Stock cultures were maintained at - 20°C in TSB containing 40% (v/v) glycerol.

Bacteriocin production assay:

The agar-spot assay was performed as described by[7], with minor modifications. The producer strains, *Klebsiella ozaenae* K and *Raoultella terrigena* L, were grown in 5 ml of TSB for 18 h at 37°C. Five microliters of culture (approximately 5.0 x 10^6 cells) were spotted onto medium plates for analysis. After 18 h at 37°C, the bacteria were killed by exposure to chloroform vapor and the plates were sprayed with the indicator strain culture *Escherichia coli* LMIFRJ (0.3 ml of a previously grown culture in 3 ml of TSB soft agar). The plates were further incubated at 37°C for 18 h and the diameters (in mm) of the inhibition zones were measured.

Influence of growth conditions on bacteriocin production:

To evaluate the effect of the culture medium on bacteriocin production, the producer strains were grown as previously described, and 5 µl of culture were spotted onto the surface of plates containing 25 ml of the following solid media: BHI, CAS (casamino acids 15 g/L [Merck, Darmstadt, Germany]) with 5g/L NaCl), Casoy (Isofar, Rio de Janeiro, Brazil), HI (heart infusion, Himedia, São Paulo, Brazil), MH (Müller-Hinton, Himedia, São Paulo, Brazil), NA (nutrient agar, Himedia, São Paulo, Brazil) and TSA (trypticase soy agar, Oxoid, Hampshire, England).

The influence of the initial pH was determined by adjusting the CAS medium with 1N HCl or 1N NaOH, to achieve pH values of 6.0, 6.5, 7.0, 7.5 and 8.0 before spotting the producer strains. The plates were incubated at 37°C for 18 h. The effect of the growth temperature on bacteriocin production was evaluated by incubating CAS medium plates at 25°C, 37°C and 42°C for 18 h, after spotting the producer strains. The influence of salt was determined by growing the producer strains on CAS medium plates with NaCl concentrations ranging from 0.5 to 30 g/L. The plates were incubated for 18h at 37°C. The effects of aeration conditions on bacteriocin activity were evaluated after incubation of CAS plates spotted with the producer strains at 37°C, both aerobically and anaerobically. The anaerobic atmosphere was created using the AnaeroGen atmosphere generation system (Oxoid Ltd., Hampshire, England).

In all the analyses, after killing the producer strains with chloroform vapor, the indicator strain *E. coli* LMIFRJ was added as previously described.

Statistical analysis:

For all significance tests, *p* values < 0.05 were considered statistically significant. The unpaired T test was performed to analyze the aeration effect. One-way ANOVA with Tukey's post-test was used to assess differences in the other parameters studied. The statistical analyses were performed using the GraphPad Prism software, version 5.00 for Windows (GraphPad Software, San Diego, California, USA). At least three replicates of each experiment were performed.

RESULTS AND DISCUSSION

The production of both bacteriocins was measured in terms of the size of inhibition zones against the indicator strain *Escherichia coli* LMIFRJ. In an initial analysis, seven culture media were compared to evaluate bacteriocin production. The influence of the growth media is shown in (**Table 1)**.

For both strains, HI and NA media generated the smallest inhibition zones (*p* < 0.05). No significant differences in the inhibition zones were detected in richer media such as BHI, MH, Casoy and TSA. However, the two strains seemed to produce visibly larger inhibition zones in 15 g/L casamino acids with 5 g/L NaCl (CAS medium). The inhibitory activity of klebicin K and raoultellin L produced on CAS medium against *E. coli* LMIFRJ is show in (**Figure 1)**. Similar results were described by [9] that also reported that the production of the bacteriocin microcin E492, produced by a *Klebsiella pneumoniae* strain, was enhanced using casamino acids medium. Although casamino acids are also a mixture of carbon sources, this medium allows easier use of the nitrogen source. Since CAS medium gave rise to satisfactory production of both bacteriocins assessed in this study, it was used in subsequent experiments, with the aim of investigating other parameters that might influence their production.

Klebicin K and raoultellin L production was evaluated on CAS agar medium plates at different temperatures (Table 1). For both strains, bacteriocin activity was observed at 25°C and 37°C, with slightly higher production at 37°C. On the other hand, at 42°C, there was total absence of bacteriocin production, although the growth of the producers was not reduced, suggesting that there is no obvious correlation between the culture growth and bacteriocin production. Absence of correlation with a bacteriocin produced by *Leuconostoc mesenteroides* E131, a Gram-positive bacterium [4]. When this microorganism was grown in lower pH value than optimum for growth, the bacteriocin production was enhanced. On the contrary, bacteriocin production was favored when the micro-organism was grown at temperatures close to the optimum for growth.

In relation to pH, similar results were noticed. For the producer strains, growth in CAS medium with initial pH ranging from 6.0 to 7.5 appears not to affect bacteriocin production, however, this situation changed when the initial pH was 8.0. Under this condition, the producer strains were able to grow, but klebicin K and raoultellin L production was significantly reduced ($p < 0.05$). Similar behavior has been observed in relation to other bacteriocins, such that the optimum pH for cell growth did not correlate with the temperature or pH requirements for maximum bacteriocin activity [12,15,19].

Bacteriocin production capability in the presence of NaCl was also evaluated. Strains were grown in CAS medium with NaCl concentrations ranging from 0 (medium without added salt) to 3%. Production of klebicin K seemed not to be affected by NaCl concentration from 0.5 to 2.5%, but it was significantly reduced ($p < 0.05$) when the producer strain was grown in the absence of salt and at 3.0% NaCl concentration. Regarding raoultellin L production, NaCl concentrations from 0.5 to 3.0% did not affect its production, but when *R. terrigena* L was grown in the absence of salt, a slight reduction was noticed (**Table 1**). In relation to aeration conditions, Klebicin K and raoultellin L production was not affected by anaerobiosis. The differences observed in the inhibition zones under aerobic and anaerobic conditions were not significant ($p > 0.05$).

Although bacteriocins produced by Gram-negative bacteria have not been studied to as great an extent as the bacteriocins produced by Gram-positive bacteria, different applications have been suggested for colicins and microcins. According to [9], administration of bacteriocins of the microcin and colicin classes reduced the levels of enteric pathogens in animals, thus possibly preventing acquisition of pathogenic strains. Other examples include their potential use as food preservatives, as antitumor agents and for controlling diarrheal diseases caused by enteropathogenic bacteria [5,8,11,16].

Our group has demonstrated that klebicin K and raoultellin L were able to inhibit several Gram-negative strains isolated from foods. These strains belonged to the genera *Escherichia*, *Enterobacter*, *Klebsiella*, *Raoultella*, *Salmonella*, *Serratia* and *Yersinia*, and including antibiotic multiresistant strains [6]. These data highlight the potential application against foodborne pathogens by

klebicin K and raoultellin L, since many Gram-negative baceriocins are active only against intrageneric bacterial strains [8,23].

Temperature, NaCl concentration, pH and atmosphere are relevant factors when considering the potential of a substance as a biopreservative. Except for growth at 42°C, which eliminated the ability of *K. ozaenae* K and *R. terrigena* L to produce the bacteriocin, their production seemed to be relatively stable with regard to other factors. This knowledge about the best production conditions for klebicin K and raoultellin L will be useful for further experiments involving purification of these bacteriocins, which is needed in order to evaluate their biotechnological application.

Table 1: Effect of the growth conditions on the production of klebicin K and roultellin L.

		Inhibition zones (mm)	
		Klebsiella ozaenae K	*Raoultella terrigena* L
Growth medium	BHI	25.8 ± 2.7	20.7 ± 3.2
	CAS	32.0 ± 1.0	26.0 ± 0.0
	Casoy	20.0 ± 1.7	20.0 ± 0.0
	HI	09.0 ± 1.0	09.0 ± 1.0
	MH	21.5 ± 0.7	21.0 ± 0.0
	NA	10.3 ± 0.6	09.7 ± 0.6
	TSA	22.3 ± 2.1	24.5 ± 2.1
Temperature	25°C	23.0 ± 2.0	23.0 ± 1.7
	37°C	23.7 ± 1.5	25.0 ± 2.0
	42°C	-	-
pH	6.0	24.7 ± 2.0	24.3 ± 1.1
	6.5	23.0 ± 1.4	23.5 ± 2.1
	7.0	23.5 ± 0.7	23.5 ± 0.7
	7.5	21.0 ± 2.6	21.7 ± 4.0
	8.0	15.3 ± 2.1	14.0 ± 1.7
NaCl concentration	0.0%	21.3 ± 0.6	18.7 ± 2.1
	0.5%	24.5 ± 0.7	24.5 ± 0.7
	1.0%	25.7 ± 1.5	22.3 ± 0.6
	1.5%	25.0 ± 1.0	24.0 ± 2.0
	2.0%	24.3 ± 1.1	22.7 ± 1.1
	2.5%	24.7 ± 0.6	23.0 ± 1.0
	3.0%	17.0 ± 0.0	19.5 ± 2.1
Atmosphere	Aerobiosis	24.5 ± 0.7	23.5 ± 1.6
	Anaerobiosis	26.5 ± 0.7	21.5 ± 1.6

The numbers represent the means and standard deviations of the diameters of inhibition zones (in mm) from three independent experiments; -, absence of bacteriocin production. *Escherichia coli* LMIFRJ was used as indicator strain.

Fig 1: Agar spot Assay

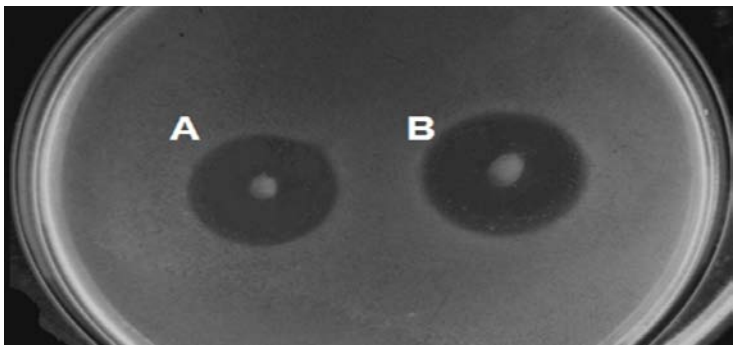

Fig 1: Agar-spot assay demonstrating the production of klebicin K (A) and raoultellin L (B) on CAS medium. The inhibitory activity against the indicator strain *E. coli* LMIFRJ is represented by the clear zone of inhibition around producer strains growth (central spots).

ACKNOWLEDGEMENTS

This work was supported by a research grant from Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) to J. S. Nascimento.

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