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Expression of *Lactobacillus pentosus* B96 bacteriocin genes under saline stress

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ABSTRACT

This research studied the influence of sodium chloride on bacteriocin activity of table olives' strain *Lactobacillus pentosus* B96. The strain was cultured in MRS under different NaCl concentrations (0, 4, 6 and 8%, in w/v). In MRS, maximum bacteriocin activity was achieved 9 h later. A medium containing 4 or 6% NaCl (w/v) increased the total bioactivity of the strain and an 8% NaCl reduced it. Real-time PCR was used to monitor the genetic expression of the bacteriocin genes *pln*A, *pln*B, *pln*C, *pln*E/F, *pln*J, *pln*K, *pln*N and plantaricin S. Cultured in MRS, plantaricin S reached its maximum expression during the lag phase while *pln*E/F expresses during the exponential phase. The presence of sodium chloride in the medium moved the maximum expression of plantaricin S to the stationary phase, independently of the concentration. 4% (w/v) of NaCl didn't affect the expression pattern of *pln*E/F while promotes the expression of *pln*N during both the lag and the exponential phases. More sodium chloride, 6% (w/v) maintained the expression of plnN in the pag phase but not in the exponential and moved *pln*E/F expression to the stationary phase. Plantaricin S, *pln*E/F and *pln*N over-expressed during the stationary phase in the higher sodium chloride concentration assayed, 8% (w/v). The relative expression level of *pls*A was 1000-fold higher than that of the *pln*E/F and *pln*N genes and even the *ldh*D constitutive gene used. Under our conditions, expression of *pln*A, *pln*B, *pln*C, *pln*J and *pln*K genes was not observed.

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1. Introduction

Several authors consider bacteriocin production to be an important factor in strain establishment because it helps increase the quality and safety of fermented foods (Ruíz-Barba et al., 1994; Leroy and De Vuyst, 1999a; Liu et al., 2008). As table olives are one of the most important fermented foods in world trade (Garrido-Fernández et al., 1997), there is interest in their lactic acid bacteria (LAB) producing bacteriocins (Delgado et al., 2001; Leal et al., 2002; Bordons et al., 2010).

Bacteriocin titers are influenced by environmental factors (Leal-Sánchez et al., 2002; Delgado et al., 2005). Conditions during natural fermentations, such as uncontrolled pH and suboptimal temperatures, usually stimulate bacteriocin production (Moretro et al., 2000). Although NaCl and $NaNO_3$, which occur in many natural environments, usually inhibit both growth and bacteriocin production (Leroy and De Vuyst, 1999b; Moretro et al., 2000; Verluyten et al., 2004), a certain level of osmotic stress seems to favor bacteriocin release (Uguen et al., 1999).

Lactobacillus pentosus B96 was isolated from fermenting Portuguese green olives of the *Azeiteira* cultivar that had been prepared according to the Spanish style. *L. pentosus* B96 bacteriocin production was stimulated at suboptimal temperature (22 °C) and by moderate NaCl stress (0.65 mol l^{-1}) (Delgado et al., 2005). Although bacteriocin activity of *L. pentosus* B96 is presumably due to the expression of plantaricin S, this strain possesses not only plantaricin S operon genes, but also the genes *pln*A, *pln*B, *pln*C, *pln*E/F, *pln*J, *pln*K and *pln*N (Delgado et al., 2005; Bordons et al., 2010).

Although DNA microarray technology has been used to study the overall transcriptional response of lactobacilli under certain conditions (Hüfner et al., 2008), reverse transcription real-time quantitative polymerase chain reaction, RT qPCR (Bustin et al., 2009), is a useful technique for studying the genetic expression levels of a limited number of genes. RT qPCR has been used to monitor stress related genes in wine LAB species *Oenococcus oeni* (Beltramo et al., 2006; Olguin et al., 2009). Bacteriocin genes have also been studied by RT qPCR: Vaughan et al. (2004) studied the transcriptional response of different bacteriocin genes in a *Lactobacillus sakei*, and Ramiah et al. (2007) studied the genetic expression of adhesion proteins and one bacteriocin of the probiotic *Lactobacillus plantarum* 423.

The aim of this work was to study the transcriptional response of the bacteriocin genes present in *L. pentosus* B96 under the effect of different salt (NaCl) concentrations. The expression of these genes was quantified by means of reverse transcription real-time quantitative polymerase chain reaction (RT qPCR). The total

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bacteriocin activity was also monitored to evaluate its correlation with the expression of the related genes.

2. Materials and methods

2.1. Growing conditions and sampling

L. pentosus B96 was propagated in MRS (Difco, Franklin Lakes, NJ, USA). A control medium without added salt and 3 different NaCl concentrations of 4, 6 and 8% (w/v) were used. *L. pentosus* B96 growth in each medium was measured beforehand to establish equivalences between the conditions in each growth phase (data not shown). Three different samples were collected during the lag, exponential and stationary phases of each of the saline media.

Strain B96 was cultured overnight in MRS to obtain an appropriate inoculum (absorbance 1.9–2.0). 1% inoculation was performed in 250 ml bottles. An additional sample of each medium was collected 1 h after inoculation to check that there was no loss of viability. The incubation was done at 28 °C under a CO_2 atmosphere. All assays were performed in duplicate and the growth was monitored by plate counts on MRS and absorbance measurements at 600 nm.

2.2. Detection of antimicrobial activity: diffusion assay

Samples of 4 ml were centrifuged at 8500 rpm for 10 min (Biofuge primo, Heraeus). Supernatant was filter-sterilized and conserved at 4 °C until assays were performed. A diffusion assay was used to detect bacteriocin activity. The plates were prepared by pouring 20 ± 1 ml of MRS agar. To evaluate the inhibitory spectrum, 5 μl of each filtered supernatant sample were laid in drops over MRS agar. After 30 min, plates were covered with a soft overlay agar (10 ± 0.5 ml) seeded with 100 μl of an overnight culture of the sensitive indicator bacterium (liquid MRS, 28 °C, 10% CO_2 pressure). After a suitable incubation (overnight 28 °C, 10% CO_2 pressure), the diameter of each inhibition zone was measured twice in perpendicular directions with a 0.1 mm precision calliper and with the plate resting on a colony counter with amplification and illumination. Each assay was performed in duplicate. Indicator strains used were *Weissella paramesenteroides* DSM20288^T and two *L. pentosus* 5E5A7 and 7L3 previously isolated from table olives (Bordons et al., 2010).

2.3. RNA extraction

L. pentosus B96 cells were harvested by centrifugation, frozen in liquid nitrogen and kept at −80 °C until RNA extraction. Total RNA extractions were performed using Roche RNeasy kit according to the manufacturer's instruction (Roche, Mannheim, Germany). Purified RNAs were suspended in 50 μl of 0.1% DPC (dimethylpyrocarbonate)-treated water. RNA concentrations were calculated by measuring absorbance at 260 nm using Thermospectronic Genesys 10 UV Spectrophotometer (Thermo Fisher Scientific, Maryland, USA).

2.4. RT qPCR primer design

In a previous work, *pln*A, *pln*B, *pln*C, *pln*E/F, *pln*J, *pln*K, *pln*N and plantaricin S were the detected genes on *L. Pentosus* B96 (data not shown). Nucleotide sequences of *L. plantarum* WCFS1 (NC_004567) bacteriocin genes were used to design the primers except for bacteriocin S *pls*A gene sequence (Stephens et al., 1998) that was obtained from *L. plantarum* accession number Y15127. The sequences were obtained from the National Center for Biotechnology Information (NCBI). Primers were designed to be about 18–22 bases long, to contain over 50% G/C and to have a melting temperature (T_m) above 60 °C (see Table 2). The length of the PCR products ranged from 92 to 107 bp. Primer3 software (http://frodo.wi.mit.edu/primer3/) was used to select primer sequences. Secondary structures and dimmer formation was analyzed with Oligo Analyzer software (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/) from Laboratorios Conda (Madrid, Spain). Previously designed *pln*C PCR primers (Diep et al., 1996) were directly used for this gene. *L. plantarum ldh*D gene, coding for lactate dehydrogenase, was used as the internal control with the primers described by Fiocco et al. (2009).

2.5. Reverse transcription and RT qPCR

cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, USA) as recommended. The absence of chromosomal DNA contamination was confirmed by real-time PCR. RT qPCR was performed in 25 μl final volume containing 5 μl of cDNA dilution, 1 ml of each primer at appropriate concentration (see Table 2), 5.5 μl of RNAse free-water and 12.5 μl of SYBR Green Master Mix (Applied Biosystems, Foster City, USA). Amplifications were carried out using an ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, USA) with an initial step at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min and 72 °C for 30 s. An additional step starting between 90 and 60 °C was performed to establish a melting curve and to verify the specificity of the RT qPCR reaction for each primer pair. The efficiencies of amplifications were calculated using the formula $E = [10^{(1/-s)} - 1] \times 100$, where "s" is the slope of the standard curve with several dilutions of cDNA (Beltramo et al., 2006). In the present study, the threshold value was automatically determined by the

Table 1
Optical density and total population of *Lactobacillus pentosus* B96 cultured on MRS supplemented with different NaCl (w/v) concentrations. Values corresponding to time 0 were estimated from values of the inoculum, an overnight MRS culture of *L. pentosus* B96, just before the inoculation. Data shown are mean values ($n = 2$).

Time (hours)	Optical density (600 nm)				Total population (log cfu/ml)			
	MRS	4% NaCl MRS	6% NaCl MRS	8% NaCl MRS	MRS	4% NaCl MRS	6% NaCl MRS	8% NaCl MRS
0	0.020	0.020	0.020	0.020	5.28	5.28	5.28	5.28
1	0.037	0.025	0.033	0.050	5.18	5.13	5.20	5.22
3	0.082	0.013			5.22	5.18		
6	0.359				5.31			
9	1.045		0.102		5.36		5.91	
12	1.547				6.67			
24	2.123	1.768		0.593	8.98	6.06		5.06
30			1.127				8.32	
36	2.109			0.641	9.12			7.91
48	2.158				9.07			
60		2.046				8.16		
72			1.679				8.81	
78				1.280				8.28

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