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Deoxyribonuclease activities in *Lactobacillus delbrueckii*

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Abstract

DNase activity was examined in the extracellular and subcellular fractions of six non-transformable strains belonging to *Lactobacillus delbrueckii* subsp. *lactis* (*L. lactis*) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) and compared with the activity present in *Lactobacillus johnsonii* NCK 65, a transformable strain of *Lactobacillus*. In the extracellular fraction of the *L. delbrueckii* strains, a common protein band of 36 kDa was detected, while a band of 29 kDa was found in the same fraction of *L. johnsonii*. No nuclease activity was detected in the cytoplasmic fraction of this strain, indicating that the localization of the DNase activity could be a key factor in the uptake of foreign DNA.

Key words: Nuclease – *Lactobacillus delbrueckii* – DNase activity

Introduction

The *Lactobacillus delbrueckii* group is one of the few thermophilic lactic acid bacteria involved in milk fermentations. *Lactobacillus delbrueckii* subsp. *lactis* (*L. lactis*) is the starter lactobacillus for cheeses with high cooking temperatures, and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) is a component in yogurt (Mäyrä-Mäkinen and Bigret, 1998). Despite the importance of these microorganisms no reliable transformation methods were described. The inability of *L. delbrueckii* to accept and maintain exogenous DNA may be attributed to the presence of DNase activity since one of the major roles of DNases is to protect the cell from assault by foreign DNA (the most obvious sources of which are conjugative plasmids and phage genomes). Nucleases may also have a role providing the cell with nucleosides and phosphates for growth (Bickle, 1993).

Recently Caso and Suarez (1997) reported the presence of DNase activity for *Lactobacillus plantarum*. However, it was not related to the transformability of this group. To investigate the role that nucleases may have in the ability of uptaking exogenous DNA by *L. delbrueckii*, we compared the DNase activity in selected strains of this species with the nuclease activity present in *Lactobacillus johnsonii* (*L. johnsonii*) a transformable group of this genus. The comparative study of the subcellular fractions with DNase activity indicates that the localization of the nuclease activity could be an important factor in uptaking of exogeneous DNA. The deoxyribonuclease activity of *L. lactis* CNRZ 326, a strain that harbors a restriction-modification system (Auad *et al.*, 1998), was also analyzed. Selection of strains with low DNase activities for transformation may be a key to achieve the acquisition of heterologous DNA by *L. delbrueckii*.

Materials and methods

Microorganisms and culture conditions. The strains of *L. lactis*, *L. bulgaricus* and *L. johnsonii* used in this study and their characteristics are described in Table 1. Cultures of lactobacilli were incubated at 37 °C in Lactobacilli MRS broth (De Man *et al.*, 1960).

Extracellular (EC) fraction. The EC fraction was routinely obtained from 16-hour cultures. For *L. bulgaricus* B1, samples from 6, 8, 10, 24, and 48 hours were also taken. After centrifugation (6000 xg, 5 min), the supernatants were neutralized with 5 N sodium hydroxide (pH 8) and filtered through a cellulose acetate membrane filters with a pore size of 0.2 µm (Gelman

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Table 1. *Lactobacillus* strains used in this work.

Strain	Characteristics	Reference
L. delbrueckii subsp. *bulgaricus* B1	Non-defined	This work
L. delbrueckii subsp. *lactis* CRL[a] 1212	Non-defined	This work
L. delbrueckii subsp. *bulgaricus* CNRZ[b] 1004	Cured of prophage mv4	Cluzel *et al.*, 1987
L. delbrueckii subsp. *lactis* CRL 934	Indicator strain for bacteriophages	Auad *et al.*, 1997
L. delbrueckii subsp. *lactis* CNRZ 326	Indicator strain for bacteriophages; R^+/M^+	Cluzel *et al.*, 1987
L. delbrueckii subsp. *lactis* CNRZ 700 (LKT)	Indicator strain for bacteriophages	Cluzel *et al.*, 1987
L. johnsonii NCK[c] 65	Plasmid-free; transformable	Allison *et al.*, 1994

[a] CRL denotes Centro de Referencia para Lactobacilos, Tucumán, Argentina.
[b] CNRZ denotes Centre National de Recherches Zootechniques, Jouy -en-Josas, France.
[c] NCK denotes North Caroline Klaenhammer, North Caroline, USA.

Sciences, Michigan). For SDS-PAGE assays, the supernatants were concentrated to 1/50 of the original volume in cellulose dialysis sacks with a nominal molecular weight cut-off of 12 kDa (Sigma) using carboxymethylcellulose (BDH Chemicals Ltd., Poole, England).

Preparation of cell-free extracts and subcellular fractions. Cells from 16-hour cultures (5 ml) were centrifuged and washed twice in 1 volume of TEDA buffer (0.1 M Tris-HCl, pH 7.5, 0.001 M EDTA, 0.001 M dithiotreitol, 0.001 M sodium azide) as described by Caso and Suárez (1997), resuspended in 300 µl of the same buffer, and disrupted by glass beads (diameters, 0.1–0.11 mm). Finally, the extracts were cleared by centrifugation (16000 × g, 5 min). The subcellular fractionation of strains was carried out according to Caso and Suárez (1997).

Deoxyribonuclease activity tests. DNase activity was detected in agarose gels by mixing 10 µl of the supernatant (previously neutralized and filtered), cell-free extract or subcellular fraction with 0.5 µg of the DNA used as substrate with DNase buffer (Tavares and Sellsted, 1997) in a final volume of 20 µl. The reaction mixtures were incubated at 37 °C for 2–16 hours and the DNA hydrolysis was visualized in a 1% (w/v) agarose gel. DNA from coliphage lambda (Gibco, BRL) or from pGK12 (Kok et al., 1984) purified according to Birnboim and Doly (1979) and Wizard DNA Clean-Up System (Promega) were utilized as substrates. For the analysis of nuclease activity in *L. lactis* CNRZ 326, DNA from bacteriophages LL-H and lb539 obtained as previously described (Auad *et al.*, 1997), and from plasmid pIL253 (Chopin and Simon, 1988) was also used.

Identification of DNases by polyacrylamide gels. The detection of deoxyribonucleases was carried out mainly according to Rosenthal and Lacks (Rosenthal and Lacks, 1977) and Tavares and Sellstedt (1997), with minor modifications: salmon sperm DNA (Gibco, BRL) was added denatured since no difference was observed between native or denatured DNA in the detection; after renaturation of the proteins the gel was incubated at 37 °C. Molecular masses were determined by comparison with molecular mass standards. The standards were purchased from Sigma and contained α-lactalbumin (molecular weight, 14,200), trypsin inhibitor (21,100), trypsinogen (24,000), carbonic anhydrase (29,000), glyceraldehyde-3 phosphate dehydrogenase (36,000), ovalbumin (45,000), and bovine serum albumin (66,000).

DNase activity of cell extracts and subcellular fractions was detected in native polyacrylamide gels since no activity was observed in SDS-PAGE. When indicated 8% instead of 10% acrylamide was used. Protein concentration was determined according to Bradford (1976). To determine the effect of calcium on nuclease activity, 2 mM $CaCl_2$ was added to the developing buffer. Because SDS precipitates with calcium, in SDS-PAGE assays, gels were previously incubated in the developing buffer without calcium for 2 hours (Tavares and Sellsted, 1997). In native gels, considering that no SDS was used, calcium was added to the developing buffer immediately after rinsing the gel.

Results and discussion

DNase activity in the extracellular (EC) fraction

DNase activity tests in agarose gel electrophoresis revealed that both coliphage lambda and plasmid pGK12 were degraded by the extracellular fraction of the *L. lactis*; *L. bulgaricus* and *L. johnsonii* strains tested (Fig. 1 and Table 2). However, even though all strains were processed at similar optical densities, *L. lactis* CRL 934 showed a weak nuclease activity on both substrates. These results suggest that the strains analyzed secreted extracellular DNases capable of digesting linear, open circular and covalently closed circular (ccc) DNA. SDS-PAGE of concentrated EC fraction in gels containing DNA allowed, after enzyme renaturation and staining of nonhydrolysed substrate, the detection of DNase

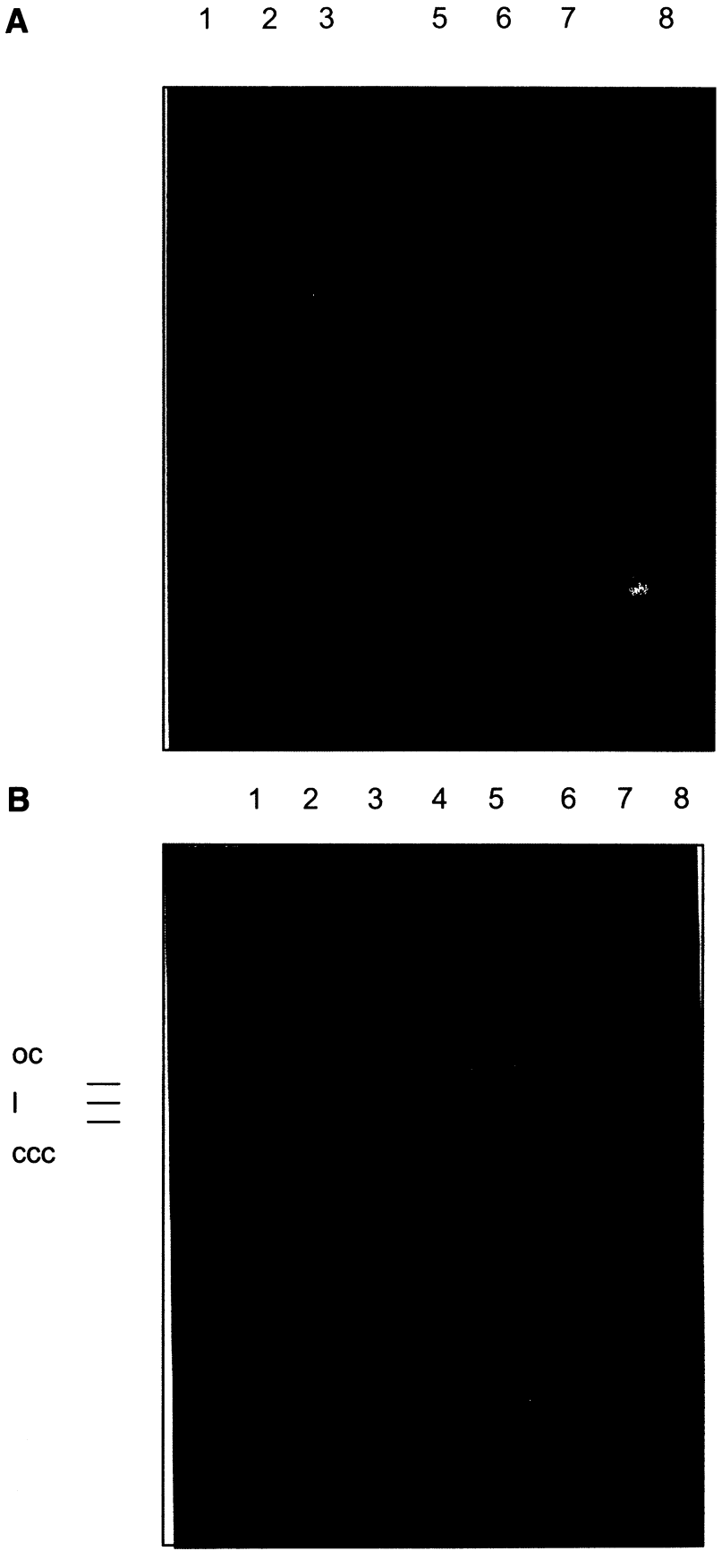

Fig. 1. Detection ef extracellular DNases in *L. lactis*, *L. bulgaricus* and *L. johnsonii*. Aliquots of 10 µl of the supernatant were added to DNA substrates and incubated for 2 hours at 37 °C with DNase buffer. A) 0.5 µg of lambda DNA; and B) 0.5 µg of pGK12. Lanes: 1 – control; 2 – *L. bulgaricus* B1; 3 – *L. bulgaricus* CNRZ 1004; 4 – *L. lactis* CRL 1212; 5 – *L. lactis* CRL 934; 6 – *L. lactis* CNRZ 326; 7 – *L. lactis* CNRZ 700 (LKT); 8 – *L. johnsonii* NCK 65. The covalently closed circular (ccc), open circular (oc) and linear (l) forms of pGK12 are indicated.

Table 2. Deoxyribonuclease activities in *Lactobacillus* strains

Lactobacillus strain		DNase activity				
		Extracellular fraction		Cell-free extract		
	DNA Substrate	minus Ca^{++}	plus Ca^{++}	cell wall	membrane	cytoplasm
L. bulgaricus B1	Salmon sperm *	+	+	+	+	+
	Lambda	nd	+	+	+	+
	pGK12	nd	+	+	+	+
L. lactis CRL 1212	Salmon sperm *	–	+	+	+	+
	Lambda	nd	+	+	+	+
	pGK12	nd	+	+	+	+
L. bulgaricus CNRZ 1004	Salmon sperm *	–	+	+	+	+
	Lambda	nd	+	+	+	+/–
	pGK12	nd	+/–	+	+	+/–
L. lactis CRL 934	Salmon sperm *	–	+	+	+	+
	Lambda	nd	+/–	+	+	+
	pGK12	nd	+/–	+	+	+
L. lactis CNRZ 326	Salmon sperm *	–	+	+	+	+
	Lambda	nd	+	+	+	+/–
	pGK12	nd	+	+	+	+
L. lactis CNRZ 700 (LKT)	Salmon sperm *	–	+	+	+	+
	Lambda	nd	+	+	+	+
	pGK12	nd	+	+	+	+
L. johnsonii NCK 65	Salmon sperm *	–	+	+/–	+	–
	Lambda	nd	+	+	+	+/–
	pGK12	nd	+/–	+	+	–

* Used as substrate in polyacrylamide gels; –: no activity detected; +: nuclease activity detected; +/–: weak activity detected; nd: not determined

activity in the gel as zones of clearing. A band with an molecular mass of approximately 36 kDa was observed in all *L. delbrueckii* strains analyzed after 2 hours of incubation in the detection buffer and the hydrolysis increased with time reaching its maximum at 24 hours. This activity required magnesium and calcium (Fig. 2). Nuclease activity of *L. delbrueckii* strains was not detected below 30 °C probably because the strains are thermophilic. The DNase activity detected was alkalophilic since no degradation was observed at acid pH (data not shown). *L. johnsonii* did also degrade both linear and ccc DNA but showed a weak DNase activity on pGK12. In SDS-PAGE gels the presence of a DNase with an molecular weight of approximately 29 kDa (Fig. 2) was determined indicating that this nuclease could prefer linear rather than circular DNA.

A calcium-independent nuclease was also detected in the *L. bulgaricus* B1 EC fraction with an molecular weight of approximately 30 kDa. The secretion of the calcium-independent nuclease in *L. bulgaricus* B1 was detected from the beginning with the exponential phase (6 hours) and the extent of degradation increased with culture age reaching its maximum during the late stationary phase (48 hours) (data not shown). These results indicate that the production of this nuclease might be growth-linked and it is steadyly maintained during the stationary phase.

DNase activity in cell-free extracts

High levels of nuclease activity were detected by agarose gels in cell-free extracts of the strains analyzed, including *L. johnsonii* NCK 65 (data not shown). DNase activity in the cell-free extracts and subcellular fractions was detected in native polyacrylamide gels but not in gels containing SDS indicating that the enzymes present in the extract fail to renature after SDS removal. This suggests that the enzymatic activity detected in the EC fraction is different from those detected in the cell-free extract since the former renaturates properly under similar conditions. Clear bands were observed after 20 minutes of incubation at 37 °C and the activity increased with time incubation, reaching extensive hydrolysis after 24 hours (Fig. 3). The apparent native molecular mass of DNases for the strains analyzed was larger than 66 kDa. They could represent large molecular mass complexes as described for other nucleases (Roberts and Halford, 1993).

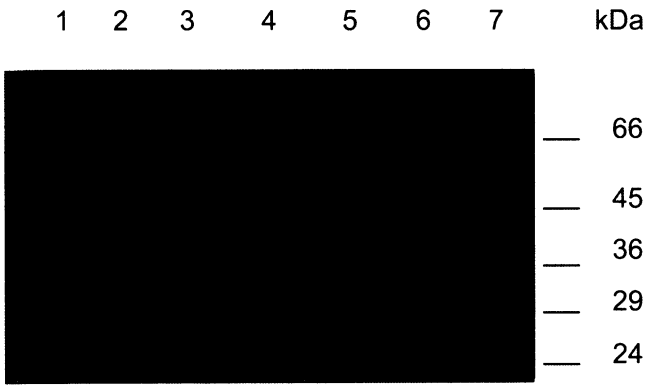

Fig. 2. Detection of extracellular DNases in 8% SDS-PAGE gels containing denatured DNA after 2 hours of incubation. Gels were incubated from 2 to 24 hours in developing buffer with calcium. Lanes: 1 – L. *bulgaricus* B1; 2 – *L. bulgaricus* CNRZ 1004; 3 – *L. lactis* CRL 1212; 4 – *L. lactis* CRL 934; 5 – *L. lactis* CNRZ 326; 6 – *L. lactis* CNRZ 700 (LKT); 7 – *L. johnsonii* NCK 65.

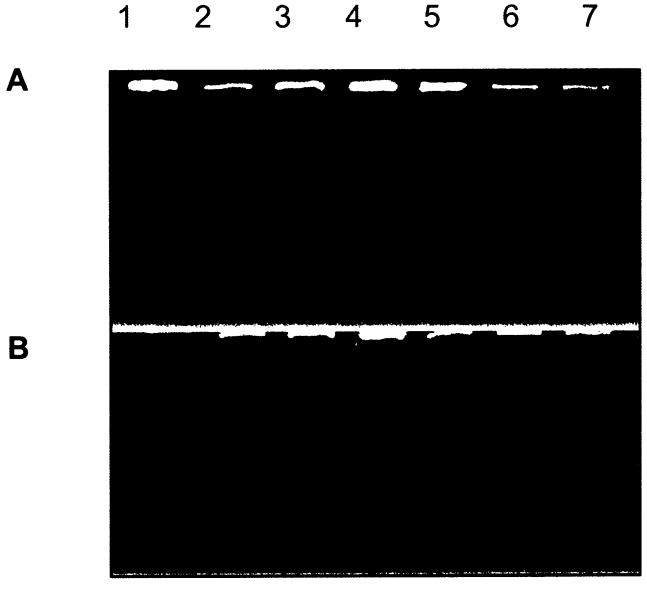

Fig. 3. Detection of DNases in cell-free extracts. Native polyacrylamide gels were incubated at 37 °C without shaking for 1 (A) to 24 hours (B). Lanes: 1 – *L. bulgaricus* B1; 2 – *L. bulgaricus* CNRZ 1004; 3 – *L. lactis* CRL 1212; 4 – *L. lactis* CRL 934; 5 – *L. lactis* CNRZ 326 ; 6 – *L. lactis* CNRZ 700 (LKT); 7 – *L. johnsonii* NCK 65.

Comparative analysis of nuclease activity in subcellular fractions

Agarose gel analysis showed different levels of DNase activity in cell wall (CW), membrane fraction (MF) and cytoplasmic fraction (CF) of the strains tested. CW and MF from all strains showed the highest level of activity. The substrate (lambda DNA) was fully degraded after 2 hours at 37 °C (data not shown). In contrast, high

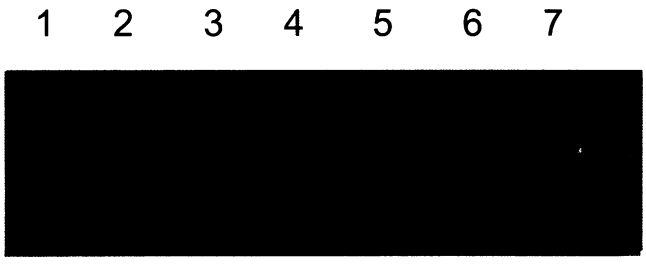

Fig. 4. Nuclease activity in the cytoplasmatic fraction (CF) after 16 hours of incubation with calcium. Equivalent amounts of protein (15 μg) were loaded in native polyacrylamide gels and incubated for 1 to 24 hours with calcium. Lanes: 1 – *L. bulgaricus* B1; 2 – *L. bulgaricus* CNRZ 1004; 3 – *L. lactis* CRL 1212; 4 – *L. lactis* CRL 934; 5 – *L. lactis* CNRZ 326; 6 – *L. lactis* CNRZ 700 (LKT); 7 – *L. johnsonii* NCK 65.

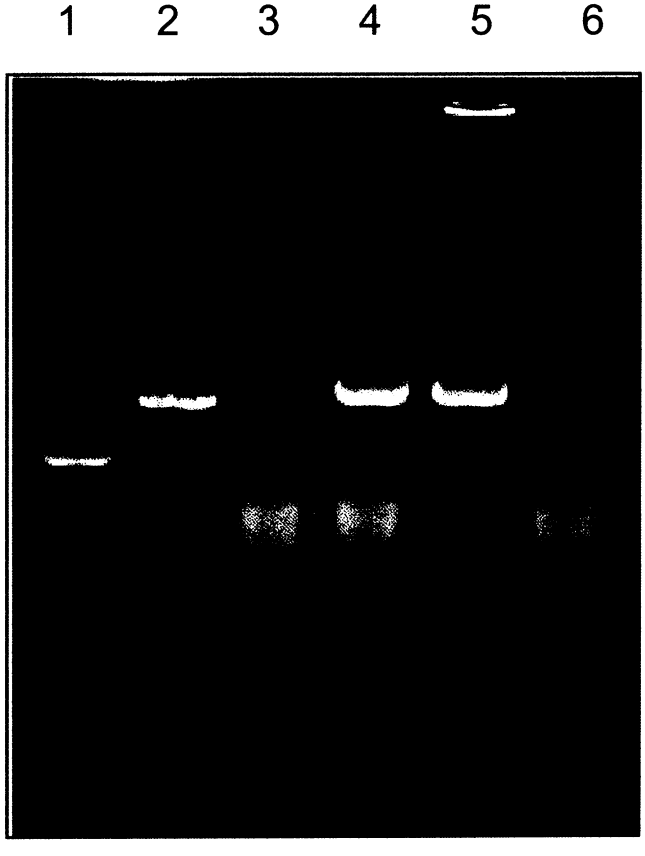

Fig. 5. Nuclease activity of the cell-free extract of *L. lactis* CNRZ 326. Lanes: 1 – Plasmid pIL253 used as control; 2 – cut with *Eco*RI; 3 – cell-free extract; 4 – pIL253 incubated with the extract at 4 °C during 30 minutes; 5 – pIL253 incubated with the heat-treated (100 °C, 10 min) extract at 37 °C during 30 minutes; and pIL253 incubated with the extract at 37 °C during 30 minutes (Lane 6).

DNase levels were only found in the CF of strains CRL 1212 and CRL 934, but weak activities in strains CNRZ 1004, CNRZ 326 and NCK 65. Similar results were observed when pGK12 was used as substrate, indicating

weak activity in the cytoplasm of strains CNRZ 1004 and NCK 65 (data not shown). Native polyacrylamide gels with equivalent amounts of protein (15 µg) demonstrated the presence of DNase activity in CW and MF fractions of all the strains analyzed; as well as in the cytoplasmic fractions of the *L. delbrueckii* strains, but not in the cytoplasmic fraction of *L. johnsonii* (Fig. 4).

Analysis of DNase activity of L. lactis CNRZ 326

Previously evidence for a restriction-modification (R/M) system in *L. lactis* CNRZ 326 was reported (Auad *et al.,* 1998). The analysis of DNases in this strain showed variable levels of activity on lambda, pGK12 (Fig. 1) and pIL253 DNA (Fig. 5). Furthermore, nuclease activity was detected on DNA from bacteriophages LL-H and lb539, although no discrete bands were observed (data not shown). Discrete bands were detected on pGK12 and pIL253 (Fig. 5) when the cell-free extract was heat-treated (100°C, 10 min). This could indicate the presence of more than one enzyme with nuclease activity and suggest that the endonuclease encoded by the R/M system would act on ccc DNA to produce the substrate (linear molecules of DNA) for unspecific nucleases.

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