Folia Microbiol. **48** (1), 56–58 (2003) **http://www.biomed.cas.cz/mbu/folia/**

Evidence for Chromosomal Determination of Fungicidal Activity in Strains of *Lactobacillus brevis* and *Lactobacillus fermentum* Isolated from Fermented Foods

A.A. OSUNTOKI[a], G.O. GBENLE[a], D.K. OLUKOYA[b]

[a]*Department of Biochemistry, College of Medicine, University of Lagos, Lagos, Nigeria*
[b]*Genetics Division, Nigerian Institute of Medical Research, Yaba, Lagos, Nigeria*

Received 12 April 2001
Revised version 30 July 2002

ABSTRACT. The genetic basis of the fungicidal activity of strains of *Lactobacillus brevis* and *L. fermentum* isolated from indigenous fermented foods was determined. A 5.5-kb plasmid was isolated from *L. brevis* while *L. fermentum* was found to harbor no plasmid. Plasmid curing indicated no correlation between the plasmid and the fungicidal activity of the *Lactobacillus* species. The fungicidal activity of the isolated organisms can be supposed to be mediated by the chromosome. No antibiotic resistance genetic markers were detected on the plasmid and hence it was classified as cryptic.

The ability of microorganisms to inhibit growth of other microbes has been known for a long time; this property is of importance particularly in the development of starter cultures which result in fermented foods with extended shelf life and improved safety by retarding the growth of contaminant spoilage and pathogenic organisms (Belicová *et al*. 1999; Lauková *et al*. 1999, 2001; Pantev *et al*. 2002). *Lactobacillus* species are often involved in food and industrial fermentations. Selected strains of *Lactobacillus* are utilized as starter cultures for meat, vegetables, dairy and bakery products (Chassy 1985).

Plasmids harbored by *Lactobacillus* spp. were studied and correlated with commercially useful properties like proteolytic activity (Kok and Venema 1988), milk coagulation (Morelli *et al.* 1986), bacteriocin production (Mortvedt and Nes 1990; Olasupo *et al*. 1994) and drug resistance (Lin *et al*. 1996; Fons *et al*. 1997). Some other isolated plasmids were found to be cryptic (Lee-Wickner and Chassy 1985).

In our previous study we aimed to develop starter cultures for use in the production of indigenous fermented foods and we found the production of fungicidal agents by *Lactobacillus* species (Osuntoki *et al*. 1999). Here we describe this biotechnologically important property in relation to the presence of chromosomal or extra-chromosomal genetic factors.

MATERIAL AND METHODS

Bacterial strains, media and growth conditions. The fungicidal *Lactobacillus* isolates used were *L. brevis* and *L. fermentum* strains isolated from 'wara' (an indigenous soft unripened cheese) and 'ugba' (fermented African oil bean), respectively. *L. plantarum* without fungicidal activity was used as negative control. The lactobacilli were maintained in MRS broth (de Man *et al.* 1960) containing 50 % glycerol (*V*/*V*) and stored at –20 °C. Prior to experiments, the organisms were cultivated on MRS agar (*Oxoid*, UK) for 1 d at 37 °C under anaerobic conditions using a disposable gas generating kit (Anaerobier System; *Oxoid*).

Antibiotic sensitivity testing. The antibiotic susceptibility patterns of the fungicidal isolates were determined using the disc-diffusion technique.

Mueller–Hinton broth (MH broth, *Oxoid*) was inoculated with 1 colony per mL of the organism and incubated for 1 d at 37 °C. This was diluted 10^4 fold with sterile saline. Sterile cotton swabs dipped in the dilute broth were used to inoculate MH agar (*Oxoid*) plates. The inoculated plates were allowed to dry and antimicrobial discs (*Oxoid*) were placed on the agar surface. The plates were incubated for 1 d at 37 °C and the presence of inhibition zones were observed. The antibiotic discs used and the concentrations are shown on Table I.

Plasmid DNA isolation. Plasmid DNA was isolated according to Anderson and McKay (1983). The molar mass of the isolated plasmid was estimated by running alongside molar-mass markers prepared from *Escherichia coli* strain V517 (Macrina *et al*. 1978) on agarose gel.

Agarose gel electrophoresis. Horizontal electrophoresis was carried out on 0.75 % agarose gel prepared in running buffer (TBE, in mmol/L: Tris 89, boric acid 89, EDTA 2; pH 8.0) for 5 h at 100 V. The gel was stained in 0.5 mg/L ethidium bromide and photographed using a red filter.

Plasmid curing. Sodium dodecyl sulfate (SDS; 0.002–5 %) and ethidium bromide (ETB; 20–40 mg/L) were used as curing agents. 10^2–10^4 cells per mL were inoculated into a series of tubes containing MRS broth (*Oxoid*) and various concentrations of the curing agents. The inoculated tubes containing SDS were incubated for 3 d at 37 °C. Those containing ETB were incubated for 1 d at 37 °C, after which three consecutive transfers into fresh MRS broth (*Oxoid*) containing the same concentration of ETB were made every day. After incubation, serial dilutions were made from the tubes and plated on MRS agar. Assay of antifungal activity was done by agar overlay bioassay (Osuntoki *et al*. 1999): Bacteriocin screening medium (Tichaczek *et al*. 1992) was inoculated with the test organism and incubated anaerobically for 1 d at 37 °C. The inoculated plate was overlaid with 4 % (*W*/*V*) Sabouraud dextrose agar (SDA; *Oxoid*), seeded with *Aspergillus niger* spores (indicator organism), incubated for 1 d at 37 °C and examined for zones of inhibition. The antibiotic susceptibility patterns and the presence of plasmid DNA were also determined. The plasmid-free fungicidal isolate was treated in the same way to eliminate any effect(s) arising from possible mutagenic activity of the curing agent.

RESULTS AND DISCUSSION

The fungicidal strain of *L. brevis* was found to harbor a 5.5-kb plasmid designated pLB01 while the fungicidal strain of *L. fermentum* and *L. plantarium* (control) were found not to harbor any plasmid (Fig. 1). Two plasmid-curing methods were used – SDS had no effect on our strain of *L. brevis* while 30 mg/L ETB produced mutants which had lost pLB01. However, the loss of pLB01 had no effect on the fungicidal activity of the organism.

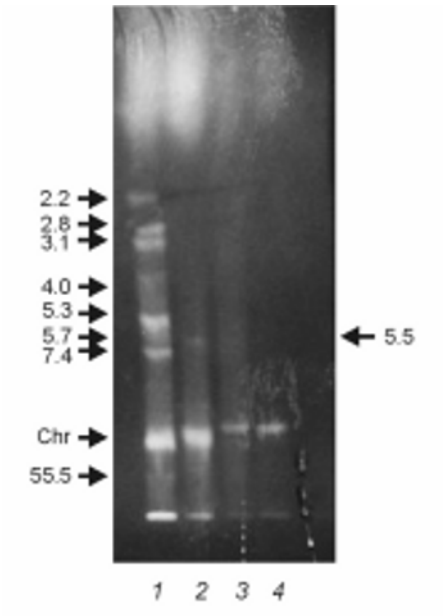

Fig. 1. Agarose gel electrophoresis of plasmid DNA; *1* – *E. coli* V517, *2* – *L. brevis*, *3* – *L. fermentum*, *4* – *L. plantarum*; Chr – band of chromosomal DNA, molar-mass markers in kb.

The antibiotic sensitivity profiles of the two fungicidal *Lactobacillus* species were similar, being sensitive to the same antibiotics, though there were differences in the degree of sensitivity to some of the antibiotics used. The organisms were also resistant to the same three antibiotics (Table I). The curing of pLB01 had no effect on the antibiotic sensitivity profile of the *L. brevis* strain. No effect was also observed on the *L. fermentum* control strain, which was subjected to the same conditions so as to eliminate effects arising from the possible mutagenic action of the curing agent.

In the production of many indigenous fermented foods fermentation is carried out by the existing flora on the substrate. The processes are thus prone to microbial contamination. One of the key roles of lactic acid bacteria in natural fermentation is the inhibition of competing flora which can include spoilage organisms and potential or real pathogens (Schillinger and Lucke 1989).

Our local fermented foods are prone to contamination by fungi, which reduce the acceptance quality of the foods. Since it is generally accepted that the ability of microbes to inhibit other microbes evolved as a strategy to reduce competition and increase the chances of survival, we reasoned that some of the *Lactobacillus* species in our indigenous fermented foods would have developed the ability to inhibit common fungal contaminants. However, only 2 % of 200 isolates screened showed antifungal activity against the indicator organism, *A. niger* (Osuntoki *et al.* 1999). The two fungicidal isolates were screened for plasmids because the observation of an unusual phenotype in well characterized bacteria species often indicate plasmid determinism (Stanisich 1988). Plasmids isolated from

Table I. Antibiotic sensitivity of the fungicidal *Lactobacillus* isolates[a]

Antibiotic	μg	*L. brevis*	*L. fermentum*
Ampicillin	10	+++	+
Chloramphenicol	10	++	++
Cloxacillin	10	R	R
Erythromycin	5	++	+++
Gentamicin	10	+	+
Penicillin	1[b]	R	R
Streptomycin	10	R	R
Tetracycline	10	+	++

[a]R – resistant; sensitivity: + – low (diameter of inhibition zone <15 mm), ++ – medium (20–25), +++ –high (26–30).
[b]IU.

bacteria have a potential application as genetic vectors (Chassy 1985), their transfer and movement can be monitored by the possession of selectable markers; such markers include antibiotic resistance genes which were reported in various *Lactobacillus* species (West and Warner 1985; Lin *et al.* 1996; Fons *et al.* 1997). The results of plasmid screening and study of plasmid-cured strain suggested that the genes for the fungicidal activity are not plasmid-borne. Likewise, no plasmid determining antibiotic resistance was detected. The plasmid harbored by the *L. brevis* remains cryptic because no determinable function was found to be associated with it. Though the question of why this gene is not found expressed in a greater number of isolated organisms arises, our results indicate that the gene(s) for the fungicidal activity are located on the chromosome of the *Lactobacillus* strains.

REFERENCES

ANDERSON D.G., MCKAY L.L.: Simple and rapid method for isolating large plasmid DNA from lactic streptococci. *Appl.Environ. Microbiol*. **46**, 549–552 (1983).

BELICOVÁ A., KRAJČOVIČ J., DOBIAS J., EBRINGER L.: Antimutagenicity of milk fermented by *Enterococcus faecium. Folia Microbiol*. **44**, 513–518 (1999).

CHASSY B.M.: Prospects for improving economically significant *Lactobacillus* strains by genetic technology. *Trends Biotechnol*. **3**, 273–275 (1985).

FONS M., HEGE T., LADIRE M., RAIBAUD P., DUCLUZEAU R., MAGUIN E.: Isolation and characterization of a plasmid from *Lactobacillus fermentum* conferring erythromycin resistance. *Plasmid* **37**, 199–203 (1997).

KOK J., VENEMA G.: Genetics of proteinases of lactic acid bacteria. *Biochimie* **70**, 475–488 (1988).

LAUKOVÁ A., CZIKKOVÁ S., BURDOVÁ O.: Anti-staphylococcal effect of enterocin in Sunar® and yogurt. *Folia Microbiol*. **44**, 707–712 (1999).

LAUKOVÁ A., VLAEMYNCK G., CZIKKOVÁ S.: Effect of enterocin CCM 4231 on *Listeria monocytogenes* in Saint-Paulin cheese. *Folia Microbiol*. **46**, 157–160 (2001).

LEE-WICKNER L.J., CHASSY B.M.: Characterization and molecular cloning of cryptic plasmids isolated from *Lactobacillus casei. Appl. Environ.Microbiol.* **49**, 1154-1161 (1985).

LIN C.F., FUNG Z.F., WU C.L., CHUNG T.C.: Molecular characterization of a plasmid-borne (pTC82) chloramphenicol resistance determinant (cat-TC) from *Lactobacillus reuteri* G4. *Plasmid* **36**, 116–124 (1996)

MACRINA F.L., KOPECKO D.J., JONES K.R., AYERS D.J., MCCOWEN S.M.: A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. *Plasmid* **1**, 417–420 (1978).

DE MAN J.C., ROGOSA M., SHARPE M.E.: A medium for cultivation of lactobacilli. *J.Appl.Bacteriol.* **23**, 130–135 (1960).

MORELLI L., VESCOVO M., COCCONCELLI P.S., BOTTAZZI V.: Fast and slow milk-coagulating variants of *Lactobacillus helveticus* HLM1. *Can.J.Microbiol*. **32**, 758–760 (1986).

MORTVEDT C.I., NES I.F.: Plasmid-associated bacteriocin production by a *Lactobacillus sake* strain. *J.Gen.Microbiol*. **136**, 1601–1607 (1990).

OLASUPO N.A., OLUKOYA D.K., ODUNFA S.A.: Plasmid profiles of bacteriocin-producing *Lactobacillus* isolates from African fermented foods. *Folia Microbiol*. **39**, 181–186 (1994).

OSUNTOKI A.A., OLUKOYA D.K., GBENLE G.O., OMONIGBEHIN E.A.: Isolation of *Lactobacillus* species with antifungal activity from Nigerian fermented foods. *Nig.Quart.J.Hosp.Med*. **9**, 314–316 (1999).

PANTEV A., KABADJOVA P., DALGALARRONDO M., HAERTLÉ T., IVANOVA I., DOUSSET X., PRÉVOST H., CHOBERT J.-M.: Isolation and partial characterization of an antibacterial substance produced by *Enterococcus faecium. Folia Microbiol*. **47**, 391–400 (2002).

SCHILLINGER U., LUCKE F.K.: Antibacterial activity of *Lactobacillus sake* isolated from meat. *Appl.Environ.Microbiol*. **55**, 1901–1906 (1989).

STANISICH V.A.: Identification and analysis of plasmids at the genetic level. *Meth.Microbiol.* **21**, 11–47 (1988).

TICHACZEK P.S., NISSEN-MEYER J.N., NES I.F., VOGEL R.F., HAMMES W.P.: Characterization of bacteriocin curvacin A from *Lactobacillus curvatus* LTH 174 and sakacin P from *L. sake* LTH 673. *Syst.Appl.Microbiol*. **15**, 460–468 (1992).

WEST C.A., WARNER P.J.: Plasmid profiles and transfer of plasmid-encoded antibiotic resistance in *Lactobacillus plantarum*. *Appl. Environ.Microbiol.* **50**, 1319–1321 (1985).