ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 7 (2): 311-316, 2008
ISSN 1682-296X

Antagonistic Effects on Enteropathogens and Plasmid Analysis of Lactobacilli Isolated from Fermented Dairy Products

[1]A.A. Osuntoki, [1]O.R. Ejide and [2]E.A. Omonigbehin
[1]Department of Biochemistry, College of Medicine, University of Lagos, Nigeria
[2]Genetics Division, Nigerian Institute for Medical Research, Yaba, Lagos, Nigeria

Abstract: In order to reduce the incidence of foodborne disease which constitutes a major public health problem, we are focussing our attention on the exploitation of indigenous food grade lactic acid bacteria to improve the safety and hygiene of fermented products. In this study we isolated lactobacilli from four fermented dairy products and screened them for the production of antimicrobial substances by eliminating the effects of organic acids and hydrogen peroxide. Three foodborne bacterial pathogens, enterotoxigenic *Escherichia coli* (ETEC), *Salmonella typhimurium* and *Listeria monocytogenes* were used as indicators. Of the twelve *Lactobacillus* isolates obtained, six showed antagonistic effects against at least one of the pathogens. Three of the twelve isolates (one with detectable antimicrobial activity and two without) were found to harbour plasmids. Plasmid elimination by curing with ethidium bromide revealed no association between the antimicrobial activity and the plasmids. With these results it may be assumed that the antimicrobial effects of these isolates on the pathogens investigated were determined by their chromosomes. In conclusion we have identified indigenous strains of food grade lactobacilli with antimicrobial activity against clinically important bacterial enteropathogens.

Key words: *Lactobacillus*, fermented dairy products, antimicrobial activity, foodborne pathogens, plasmids

INTRODUCTION

Fermented products are a significant part of many indigenous diets. In Africa many of the products result from lactic acid fermentation and *Lactobacillus* species have been reported to be the dominant species (Molin, 2001; Sanni *et al*., 2002). The indigenous fermentation processes are natural or spontaneous i.e., attributable to chance inocula from the environment, the vessels used and/or the microbial flora on the substrates. Coupled with improper food handling, poor food hygiene and sanitation, these fermented products are exposed to spoilage organisms and pathogens which are foodborne disease risk factors (WHO, 1999). Illnesses arising from contaminated foods are major, global public health problems (King *et al*., 2000; D'Souza *et al*., 2004). In England and Wales, the number of cases annually is estimated to be above two million resulting in over twenty thousand hospitalizations and above seven hundred mortalities (Adak *et al*., 2002). The trend in the rest of the developed world is probably similar (Roucourt *et al*., 2003).The situation is worse in the developing world. Acute diarrhoea from infectious agents is the commonest single factor responsible for infectious disease morbidity and mortality worldwide and millions of children lose their lives annually to diarrhoea, over three million in the developing world alone, while many more suffer the impairment of nutritional status resulting from frequent diarrhoea episodes (Ribeiro, 2000; Sheth and Dwivedi, 2006; McFarland *et al*., 2006).

There are many obstacles hindering the control and treatment of infectious diseases. These include a marked increase in antibiotics resistant pathogens, emerging infectious diseases and a rise in the population of immunocompromised people with an accompanying rise in opportunistic infections. These problems cause a marked increase in antibiotics usage which is a key factor in the development of resistance and selection of resistant strains (Cristino, 1999; Moura *et al*., 2001). As long as antibiotics are used resistance will develop, though the rate of development and the population of organisms with resistance may vary (Cristino, 1999; Anderson, 1999).

The possibility of microbial resistance exceeding the present antibiotics development capabilities exists (Rolfe, 2000). Thus there is an urgent need to develop alternatives to antibiotics usage. In the case of diseases

Corresponding Author: Dr. Akinniyi Osuntoki, Department of Biochemistry, College of Medicine, University of Lagos, P.M.B. 12003, Lagos, Nigeria Fax: 234-1-5851432

caused by foodborne pathogens, a logical approach will be to reduce or eliminate the routes of food contamination. With fermented products, the use of protective cultures with antagonistic activity against undesired microbes in the fermentation will help to improve the processes as well as the safety and hygiene of the products.

The world over, lactic acid bacteria are finding increasing use in the prevention, control and treatment of diseases and health maintenance (Reid, 1999; Anurada and Rajeshwari, 2005; Ishida-Fujii *et al*., 2007). However, In Africa, we are yet to fully explore the potentials of indigenous strains for health purposes.

This study is part of continuing efforts to explore the potentials of our indigenous microbial flora in developing fermented products with improved safety and benefits beyond nutritional provisions (Osuntoki *et al*., 1999, 2007). In the present study, we isolated indigenous *Lactobacillus* species from fermented dairy products and evaluated the inhibitory action against some clinically important enteropathogens. The essence was to identify candidate strains for use as protective cultures. The indigenous fermented dairy products were chosen because they are not subjected to pasteurisation or other heating processes that kill viable organisms and may denature labile antimicrobial substances present. Additionally, this eliminates the problem of acceptability, affordability and accessibility of potential products to the primary target populace expected to benefit from the research. The second objective was to determine the contribution of plasmids to the antagonistic activity.

MATERIALS AND METHODS

Sources of organisms: The *Lactobacillus* isolates were obtained from four Nigerian fermented dairy products; wara (an indigenous soft unripened cheese produced by adding leaves extracts of *Calotropsis procera* to whole cow milk), nunu (an indigenous yoghurt produced from skimmed cow milk) and two unpasteurised commercially available yoghurt. The indicator organisms; enterotoxigenic *Escherichia coli* (ETEC), *Salmonella typhimurium* and *Listeria monocytogenes* were clinical isolates from the culture collection of The Nigerian Institute for Medical Research.

Isolation and identification of lactobacilli: Samples taken from the products under aseptic conditions were serially diluted with 0.1% peptone water and plated on sterile MRS agar (De Man *et al*., 1960). The plates were incubated at 37°C for 48 h under microaerophilic conditions. To ensure purity, isolates were randomly picked and reinoculated on fresh sterile MRS agar (Oxoid, UK) plates under the previously stated conditions. Identification of the isolates were done based on the Bergey's manual of systematic bacteriology (Sneath, 1986) using these criteria: Gram stain reaction, microscopic and macroscopic morphological examination, absence of catalase and oxidase production, absence of spores and fermentation of different carbon sources.

Antimicrobial activity assay: The *Lactobacillus* isolate to be screened for activity was grown overnight in MRS broth and spotted onto the surface of sterile Bacteriocin Screening Medium (BSM) described by Tichaczek *et al*. (1992) which excludes inhibitory activity caused by organic acids and hydrogen peroxide (H_2O_2) by low sugar content and buffering and contains catalase to degrade the hydrogen peroxide. The plate was incubated at 37°C for 24 h under microaerophilic conditions. The indicator organism, grown overnight in brain heart infusion broth, was inoculated into brain heart infusion agar, Oxoid and used to overlay the overnight culture of the *Lactobacillus* isolate. The plate was incubated at 37°C overnight (16 h) and observed for clear halos around the *Lactobacillus* colony indicating inhibition of the growth of the pathogen. Sterile bacteriocin screening agar was used as negative control.

Plasmid screening: The lactobacilli isolated were screened for plasmids using the plasmid DNA isolation technique described by Zhou *et al*. (1990). The plasmid sizes were estimated by running the plasmid preparation alongside molecular mass markers prepared from *Escherichia coli* strain V517 (Macrina *et al*., 1978) on agarose gels.

Agarose gel electrophoresis: Horizontal electrophoresis was carried out on 0.8% agarose gel prepared in running buffer (TBE, in mmol L^{-1}: Tris 89, boric acid 89 EDTA 2; pH 8.0) at 100 v for 1 h. Stained in 0.5 mg L^{-1} ethidium bromide (EtBr).

Plasmid curing: The isolates found to harbour plasmids were treated with EtBr (20-40 mg L^{-1}) as previously described by Osuntoki *et al*. (2003). After which serial dilutions of the treated organisms were made and plated on fresh MRS agar to obtain colonies. Plasmid screening and the antimicrobial activity assay were repeated for each colony as described earlier in this study in order to detect cured organisms, mutants with altered activities and to correlate antagonism with plasmid possession.

RESULTS

***Lactobacillus* isolation:** A total of twelve lactobacilli were isolated from the four fermented dairy products employed. The isolates were 3 strains of *L. acidophilus*, 3 strains of

Table 1: *Lactobacillus* species isolated from the fermented dairy products

Laboratory code	Source	Species
LAA1	Wara	*L. acidophilus*
LAA2	Wara	*L. casei*
LAA3	Wara	*L. jensenii*
LAA4	Wara	*L. jensenii*
LAA5	Nunu	*L. fermentum*
LAA6	Nunu	*L. brevis*
LAA7	Nunu	*L. acidophilus*
LAA8	CY1*	*L. jensenii*
LAA9	CY1	*L. brevis*
LAA10	CY1	*L. salivarius*
LAA11	CY2#	*L. fermentum*
LAA12	CY2	*L. acidophilus*

*: Unpasteurised commercial yoghurt 1, #: Unpasteurised commercial yoghurt 2

L. jensenii, 2 strains each of *L. brevis* and *L. fermentum* and 1 strain each of *L. casei* and *L. salivarium* (Table 1).

Antimicrobial activity: Six of the twelve isolates (five from indigenous products and one from a commercial yoghurt) showed antimicrobial activity by inhibiting the growth of an indicator organism on BSM. Four of the isolates inhibited the growth of *L. monocytogenes* while *S. typhimurium* and ETEC were inhibited by two organisms each. Inhibitory action on *L. monocytogenes* was shown by isolates from wara only, with the highest activity (as seen from the zone of inhibition) shown by a strain of *L. casei* and the least by a strain of *L. jensenii*. Two of the isolates inhibited two of three indicator pathogens. These were a strain of *L. casei* with activity against *L. monocytogenes* and *S. typhimurium*. This organism had a higher activity on *Listeria*. The other was a strain of *L. jensenii* which in addition to inhibiting *L. monocytogenes* also inhibited ETEC, with a more pronounced action on ETEC. The only isolate from the commercial yoghurt with antagonistic action on the pathogens was a strain of *L. salivarius* which inhibited ETEC though with a lower activity than the *L. jensenii* described above. The spectrum of activity of the lactobacilli from the dairy products against the selected pathogens are shown on Table 2.

Plasmid screening: Plasmids were detected in three of the isolates, one with detectable antimicrobial activity and two without. The isolates harboured small plasmids between 3.8 and 5.5 kb in size (Fig. 1, 2, Table 3).

Plasmid curing and inhibitory activity: Treatment of the plasmid bearing isolates with EtBr successfully cured them. However, the loss of these plasmids did not alter the antagonistic activity of the isolates.

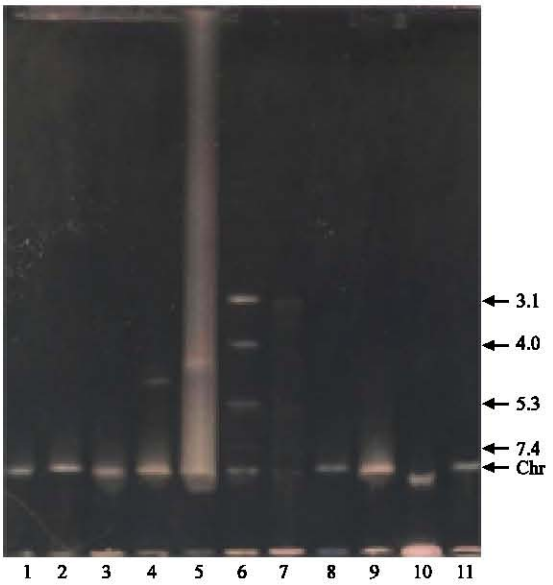

Fig. 1: Agarose gel electrophoresis of plasmid DNA: 1: LAA5, 2: LAA11, 3 LAA9, 4: LAA6, 5: LAA2, 6: 7: Markers, 8: LAA10, 9: LAA3, 10: LAA4, 11: LAA8, Chr- band of chromosomal DNA. The sizes (kb) of the molar mass markers are indicated on the right

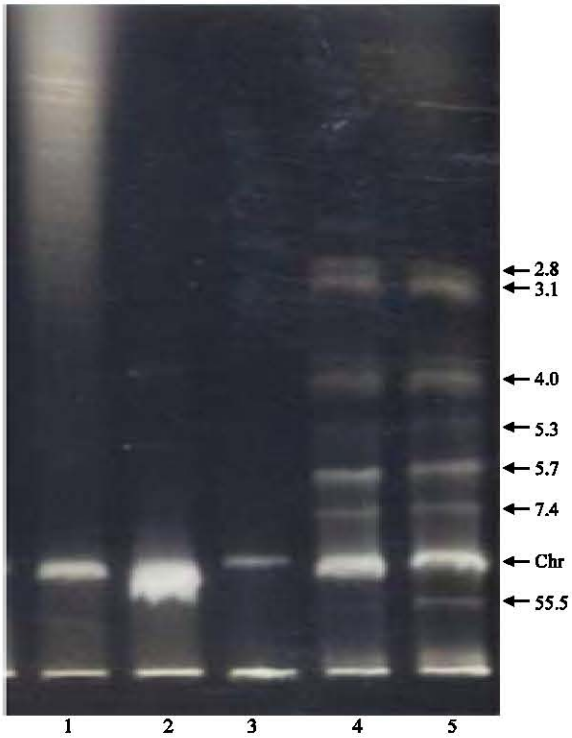

Fig. 2: Agarose gel electrophoresis of plasmid DNA: 1: LAA7, 2: LAA12, 3: LAA1, 4,5: Markers, Chr-band of chromosomal DNA. The sizes (kb) of the molar mass markers are indicated on the right

Table 2: Inhibitory activity of *Lactobacillus* isolates against selected foodborne pathogens

Source of isolate	Lab No.	Enterotoxigenic *Escherichia coli*	*Salmonella typhimurium*	*Listeria monocytogenes*
Wara	LAA1	-	-	+ (3.1±0.4)
	LAA2	-	+ (4.3±0.2)	+ (7.6±0.3)
	LAA3	+ (4.2±0.4)	-	+ (2.8±0.0)
	LAA4	-	-	+ (5.0±0.2)
Nunu	LAA5	-	+ (3.0±0.2)	-
	LAA6	-	-	-
	LAA7	-	-	-
CY1	LAA8	-	-	-
	LAA9	-	-	-
	LAA10	+ (2.7±0.3)	-	-
CY2	LAA11	-	-	-
	LAA12	-	-	-

+: Inhibitory activity observed, -: No detectable inhibitory activity. Values in bracket show the mean zones of inhibition ±SD in mm for triplicates measurements

Table 3: Plasmids profiles of the lactobacilli isolates from the fermented dairy products

Isolate	No. of detected plasmids	Size of plasmids (kb)
LAA1	0	-
LAA2	1	4.3
LAA3	0	-
LAA4	0	-
LAA5	0	-
LAA6	2	4.5, 4.7
LAA7	0	-
LAA8	0	-
LAA9	0	-
LAA10	0	-
LAA11	0	-
LAA12	2	3.8, 5.5

DISCUSSION

The present study observed growth inhibitory activity in some strains of *Lactobacillus* isolated from fermented dairy products though a screening medium which eliminates antimicrobial effects due to pH and H_2O_2 activity was used. This reveals the production of antimicrobial substances by these strains. Lei and Jacobsen (2004) reported that antimicrobial activity by lactic acid bacteria from African fermented foods in many previous studies were due to the production of lactic acid and low pH. Hydrogen peroxide (H_2O_2) has also been reported to enact antimicrobial effects (Pericone *et al*., 2000; Batdorj *et al*., 2007). The virulence potential of pathogens is reduced by inhibitory or antagonistic activity. Thus the antimicrobials producing isolates may prevent or reduce the extent of food contamination and the gastrointestinal tract infections caused by susceptible pathogens.

The results of this study are quite significant because the pathogens inhibited by the lactobacilli are of clinical importance. Reports implicate ETEC and *S. typhimurium* as being amongst the major agents responsible for the high morbidity and death in GIT infections (Ljungh, 1999; King *et al*., 2000). *L. monocytogenes* is responsible for sporadic epidemics. It is credited with a high case mortality rate and the highest rate of hospitalization, above 90%, for known foodborne pathogens. Certain populations; pregnant women, their foetuses and immunocompromised people are particularly vulnerable (Vazquez-Boland *et al*., 2001; Jemmi and Stephen, 2006; Gandhi and Chikindas, 2007). The contemporary means of management of the diseases caused by these pathogens include antibiotics therapy which is a selector for resistant strains. Present study therefore shows a potential application of indigenous strains of lactobacilli in ameliorating a major global health problem and reducing antibiotics usage.

Only one out of the five isolates from the non pasteurised commercial yoghurt showed inhibitory activity while five out of the seven isolates from the indigenous products were active. Though the precise reasons are not known, it may be as a result of the wild strains' adaptation to survival and competition with other microbes under the non sterile processes of natural fermentation.

The antimicrobials produced were not associated with plasmids. Plasmid curing which resulted in the loss of the plasmids harboured by some of the isolates did not alter the pattern of inhibitory activity. In addition, no plasmids were found in most of the lactobacilli with antimicrobial activity. These are suggestive of the antimicrobial activities being chromosomally mediated. Chromosomal location may be an advantage in the exploitation of these *Lactobacillus* strains because plasmid losses can occur due to selection pressure, plasmid instability or plasmids incompatibility.

In conclusion, we have isolated and identified lactobacilli strains from indigenous fermented products with antimicrobial effects on some clinically important foodborne bacterial pathogens. This reveals potential applications of indigenous *Lactobacillus* strains as protective cultures for the improvement of the microbial safety of fermented foods and reduction in the incidence of illnesses arising from food contamination which is a major global public health concern.

REFERENCES

Adak, G.K., S.M. Long and S.J. O' Brien, 2002. Intestinal infection: Trends in indigenous foodborne disease and deaths, England and Wales: 1992 to 2000. Gut, 51 (6): 832-841.

Anderson, R.M., 1999. The pandemic of antibiotic resistance. Nat. Med., 5 (2): 147-149.

Anurada, S. and K. Rajeshwari, 2005. Probiotics in health and disease. J. Indian Acad. Clin. Med., 6 (1): 67-72.

Batdorj, B., V. Trinetta, M. Dalgalarrondo, H. Prevost, X. Dousset, I. Ivanova, T. Haertle and J.M. Chobert, 2007. Isolation, taxonomic identification and hydrogen peroxide production by *Lactobacillus delbrueckii* subsp. lactis T31, isolated from Mongolian yoghurt: Inhibitory activity on food-borne pathogens. J. Applied Microbiol., 103 (3): 584-593.

Cristino, J.M., 1999. Correlation between consumption of antimicrobials in humans and development of resistance in bacteria. Int. J. Antimicrob. Agents, 12 (3): 99-202.

De Man, J.C., M. Rogosa and M.E. Sharpe, 1960. A medium for cultivation of lactobacilli. J. Applied Bacteriol., 23: 130-135.

D'Souza, R.M., N.G. Becker, G. Hall and K.B.A. Moodie, 2004. Does ambient temperature affect foodborne disease? Epidemiology, 15 (1): 86-92.

Gandhi, M. and L. Chikindas, 2007. Listeria: A foodborne pathogen that knows how to survive. Int. J. Food Microbiol., 113 (1): 1-15.

Ishida-Fujii, K., R. Sato, S. Goto, X.P. Yang, S. Hirano and M. Sato, 2007. Prevention of pathogenic *Escherichia coli* infection in mice and stimulation of macrophage activation in rats by oral administration of probiotic *Lactobacillus casei* I-5. Biosci. Biotechnol. Biochem., 71 (4): 866-873.

Jemmi, T. and R. Stephen, 2006. *Listeria monocytogenes*: Food-borne pathogen and hygiene indicator. Rev. Sci. Tech., 25 (2): 571-580.

King, J.C., R.E. Black, M.P. Doyle, K.L. Fritsche, B.H. Halbrook, O.A. Levander, S.N. Meydani, W.A. Walker and C.E. Woteki, 2000. Foodborne illnesses and nutritional status: A statement from an American Society for Nutritional Sciences working group. J. Nutr., 130 (10): 2613-2617.

Lei, V. and M. Jacobsen, 2004. Microbiological characterization and probiotic potential of koko and koko sour water, African spontaneously fermented millet porridge and drink. J. Applied Microbiol., 96 (2): 384-397.

Ljungh, A., 1999. Bacterial infections of the small intestine and colon. Curr. Opin. Gastroenterol., 15 (1): 43.

Macrina, F.L., D.J. Kopecko, K.R. Jones, D.J. Ayers and S.M. McCowen, 1978. A multiple plasmid-containing *Escherichia coli* strain: Convenient source of size reference plasmid molecules. Plasmid, 1 (3): 417-420.

McFarland, L.V., G.W. Elmer and M. McFarland, 2006. Meta-analysis of probiotics for the prevention and treatment of acute pediatric diarrhea. Int. J. Probiotics Prebiotics, 1 (1): 63-76.

Molin, G., 2001. Probiotics in foods not containing milk or milk constituents, with special reference to *L. plantarum* 299 V^{1-3}. Am. J. Clin. Nutr., 73 (2): 380S-385S.

Moura, L.N., E. Neumann, L.Q. Vieira and J.R. Nicoli, 2001. Protection by *Lactobacillus acidophilus* UFV-H2B20 against experimental oral infection with *Salmonella enterica* subsp. *Enterica* ser. *typhimurium* in gnotobiotic and conventional mice. Braz. J. Microbiol., 32 (1): 66-69.

Osuntoki, A.A., D.K. Olukoya, G.O. Gbenle and E.A. Omonigbehin, 1999. Isolation of *Lactobacillus* species with antifungal activity from Nigerian fermented foods. Nig. Qt. J. Hosp. Med., 9 (4): 314-316.

Osuntoki, A.A., G.O. Gbenle and D.K. Olukoya, 2003. Evidence for chromosomal determination of fungicidal activity in strains of *Lactobacillus brevis* and *Lactobacillus fermentum* isolated from fermented foods. Folia Microbiol., 48 (1): 56-58.

Osuntoki, A.A., E.A. Omonigbehin, S.O. Gbenebitse and U.E. Mojiminiyi, 2007. Prevention of salt induced hypertension in rats by oral administration of *Lactobacillus acidophilus* yoghurt. Nig. Food J., 25 (1): 184-189.

Pericone, C.D., K. Overweg, P.W.M. Hermans and J.N. Weiser, 2000. Inhibitory and bactericidal effects of hydrogen peroxide production by *Streptococcus pneumoniae* on other inhabitants of the upper respiratory tract. Infect. Immun., 68 (7): 3990-3997.

Reid, G., 1999. The scientific basis for probiotic strains of *Lactobacillus*. Applied Environ. Microbiol., 65 (9): 3763-3766.

Ribeiro, H. Jr., 2000. Diarrheal disease in a developing nation. Am. J. Gastroenterol., 95: S14-S15.

Rolfe, R.D., 2000. The role of probiotic cultures in the control of gastrointestinal health. J. Nutr., 130 (2): 396S-402S.

Roucourt, J., G. Moy, K. Vierk and J. Schlundt, 2003. The present state of foodborne disease in OECD countries. World Health Organization, Geneva, Switzerland.

Sanni, A.I., J. Morlon-Guyot and J.P. Guyot, 2002. New efficient amylase-producing strains of *Lactobacillus plantarum* and *L. fermentum* isolated from different Nigerian traditional fermented foods. Int. J. Food Microbiol., 72 (1-2): 53-62.

Sheth, M. and R. Dwivedi, 2006. Complementary foods associated diarrhea. Indian J. Pediatr., 73 (1): 61-64.

Sneath, P.H.A., 1986. Bergey's Manual of Systematic Bacteriology. Vol. 2. The Williams and Wilkins Co, Baltimore.

Tichaczek, P.S., J.N. Nissen-Meyer, I.F. Nes, R.F. Vogel and W.P. Hammes, 1992. Characterization of bacteriocin curvacin A from *Lactobacillus curvatus* LTH 1174 and sakacin from *L. sake* LTH 673. Syst. Applied Microbiol., 15: 460-468.

Vazquez-Boland, J.A., M. Kuhn, P. Berche, T. Chakraborty, G. Dominguez- Bernal, W. Goebel, B. Gonzalez-Zorn, J. Wehland and J. Kreft, 2001. *Listeria* pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev., 14 (3): 584-640.

WHO (World Health Organization), 1999. Removing obstacles to healthy development-World Health Organization report on Infectious diseases. WHO, Geneva, Switzerland.

Zhou, C., Y. Yang and A.Y. Jong, 1990. Mini-prep in ten minutes. Biotechniques, 8 (2): 172-173.