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Plasmid-Associated Bacteriocin Production in *Lactobacillus* Strains Isolated from Some Traditional Fermented Foods

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Abstract: Earlier report has shown the *Lactobacilli* isolates from some traditional fermented foods, LABB (appam batter) and LABP (vegetable pickle) to produce bacteriocins, inhibitory to a variety of spoilage and pathogenic organisms. In the present study, the plasmid profile, their curing and transformation experiments were carried out to deduce if the bacteriocins are plasmid mediated. Both the strains harbored multiple plasmids ranging in size from 1.5 kb to more than 10 kb. Plasmid curing trials showed that a combination of acriflavin, ethidium bromide and novobiocin could induce loss of plasmid DNA in these strains, 10 and 6 kb plasmids in LABB while only a single plasmid of 10 kb in LABP. They were resistant to ciprofloxacin, colistin, gentamycin, nalidixic acid and streptomycin while LABB was resistant to norfloxacin as well. The cured strains became sensitive to ciprofloxacin, gentamycin and streptomycin as well as to its bacteriocin. Plasmid curing resulted in loss of their ability to produce bacteriocins as evidenced by the absence of both the inhibitory activity against indicator organism, *S. aureus* and the bacteriocin band in SDS-PAGE clearly indicating that the bacteriocin production was plasmid mediated. This was also confirmed by the transformation of non-producing LAB with the eluted 10kb plasmid which elicited inhibitory activity against indicator organisms as well as presence of the bacteriocin band in SDS PAGE corresponding to that of native strains.

Key words: Antibiotic resistance · Bacteriocins · Fermented foods · *Lactobacillus plantarum* · Plasmid profile · Plasmid curing · Transformation

INTRODUCTION

The application of bacteriocins from lactic acid bacteria in combination with traditional methods of preservation and proper hygiene could be effective in controlling spoilage and pathogenic bacteria in a variety of food products. However, a number of problems such as low production levels and instability in certain environments/foods need to be addressed. Recombinant DNA technology is currently being applied, to enhance production; to transfer bacteriocin genes to other species using cloning vectors [1, 2] as well as for mutation and selection of bacteriocin variants with increased and/or broader activity spectra [3]. The genetic determinants for bacteriocin production and for immunity to bacteriocin are usually associated with plasmid DNA [4]. Location of nisin gene varies among strains of *Lactococci.* Nisin production and immunity can be mediated by plasmid DNA in *L. lactis* subsp. *lactis*. However, some results indicate that either chromosomal DNA or a conjugal transposon mediates production [5]. Plasmid linkage of pediocin production, immunity, antibiotic resistance and sugar utilization has been reported in several strains of *Pediococci* [6]. The structural gene of plantaricin A was shown to be located on the chromosome [7]. Many *Lactobacillus* species possess one or more natural resident plasmids of various sizes. Phenotypes for bacteriocin production and

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immunity [8] and drug resistance [9, 10] have been related to extra-chromosomal DNA in several species of *Lactobacilli*. Plasmid-associated production of bacteriocin has been shown in a *Lactobacillus* strain without affecting the immunity, indicating the possibility of the immunity genes on the chromosomes [11]. Plasmid profiling and curing of Lactobacillus strains were employed to determine the genetic determinant of bacteriocin and resistance to various antibiotics [12]. Earlier, we have reported that the bacteriocins from *Lactobacillus* isolates, viz., LABB (MTCC 6160) from appam batter and LABP (MTCC 6161) from vegetable pickle, both being identified as *Lactobacillus plantarum* by Microbial Type Culture Collection (MTCC) at Institute of Microbial Technology, Chandigarh, India having a wide antimicrobial spectrum may have great potential as food biopreservative [13, 14]. The objective of this study was plasmid profiling and curing of these *Lactobacillus* strains to demonstrate the plasmid-associated bacteriocin production, antibiotic resistance and immunity. Additionally, transformation of a non-producing strain with the suspected plasmid has also been done to confirm the results.

MATERIALS AND METHODS

Chemicals: Analytical grade chemicals and dyes were obtained either from SRL, India or SD Fine Chemicals, India while molecular weight markers and bacteriological media were obtained from Sigma, USA and HiMedia, India respectively.

Bacterial Strains and Culture Conditions: The *Lactobacillus* isolates, LABB (MTCC 6160, *L. plantarum*) from appam batter and LABP (MTCC 6161, *L. plantarum)* from vegetable pickle were maintained on semi-solid MRS agar and grown in MRS broth. The indicator organism, *Staphylococcus aureus* (MTCC 737), procured from Microbial Type Culture Collection (MTCC) at Institute of Microbial Technology, Chandigarh, India was maintained at - 10°C on nutrient agar slants, sub-cultured monthly in Brain Heart Infusion broth and incubated at 37°C for 18 - 24 h.

Plasmid Isolation and Analysis: Plasmids were isolated from *Lactobacilli*, LABB and LABP using the previous method [15] and the presence of plasmids was checked by 0.7% agarose gel electrophoresis in a submerged horizontal gel apparatus using Tris-borate [16] at 100 V for 5 h. Gels were stained for 30 min with 0.5 µg/ml of ethidium bromide, destained for 15 min in distilled water and then visualized under UV light on a transilluminator and photographed. Size of plasmids was determined with the help of a calibration curve prepared using log MW (kb) of the standard molecular size markers, ë DNA and Hind III digest (Sigma) run concurrently with their respective mobility.

Plasmid Curing: Curing trials were conducted using different concentrations of ethidium bromide (20, 30, or 40 µg/ml) along with acriflavine at 2.5, 5 or 10 µg/ml and/or novobiocin at 2.5, 5 or 10 µg/ml to determine if bacteriocin production was plasmid-linked. The overnight cultures of LABB and LABP were inoculated into MRS broth containing curing agents as above. Cultures were incubated at 37°C for 48 h, transferred into fresh broth containing the appropriate curing agents and incubated again. The transfer process was repeated several times. Cultures, which survived the highest concentrations of the curing agents were diluted, plated on MRS agar and incubated at 30°C for 36-48 h under anaerobic conditions to obtain well-developed colonies. After replica plating, the colonies were overlaid with soft agar containing *S. aureus* as indicator organism and incubated once again for 12-15 h and checked for zones of growth inhibition. The colonies not depicting any inhibitory zones against the indicator were considered as cured colonies. These colonies were purified on MRS agar and propagated in MRS broth. Plasmid DNA from both the parental (LABB and LABP) and cured ($LABB_C$ and $LABP_C$) strains was isolated and the presence of plasmid was checked as described above.

Antibiogram of *Lactobacillus* Isolates LABB and LABP: *Lactobacillus* isolates were inoculated into MRS broth individually and incubated for 24 h. About 25 ml of MRS agar was seeded with the cultures of *Lactobacillus* isolates, 10^6 cfu/ml, mixed well and poured in to sterile petri-plates and stored at 4°C for 1 h to solidify the media. OCTA-discs (8 antibiotics in a single ring) were placed and pressed on the top of the agar plates and kept again at 4°C for 1 h. The plates were incubated at 37°C over night. The zone of inhibition was measured in terms of diameter (mm). Resistance was defined as the absence of a growth inhibition zone around the discs. The cured strains were also examined for antibiotic sensitivity to find out whether the loss of plasmid confers with sensitivity to antibiotics to which the parental strains were resistant.

Isolation of the Bacteriocin from *Lactobacillus* Isolates: Partially purified bacteriocins were obtained from both the parental and cured LABB and LABP cultures by cell adsorption-desorption technique and subjecting the same to gel filtration column chromatography using Sephadex G25. The active fraction was subjected to membrane-filtration through 1 kDa MW cut off (Pall Microsep Centrifugal Device) to remove very low molecular weight contaminants. The antimicrobial activity of the bacteriocin preparation by cell adsorption-desorption was determined after adjusting the pH to 5.0 against *S. aureus* as indicator organism, by agar well diffusion assay as described previously [13]. Briefly, seventy-five micro liter of sample was loaded to the wells of Trypticase soya agar (0.75%) plates seeded with overnight culture of test organism at a concentration of 10^7 cfu/ml. The plates were incubated at 30°C for 16-18 h after pre-incubation at 4°C for 4 h and examined for zones of inhibition around the wells.

SDS-PAGE Analysis: The partially purified bacteriocin preparations from both parental and cured strains as above were subjected to electrophoresis by Tris-Tricine method of SDS-PAGE [17] to find out if plasmid curing led to loss of bacteriocin peptide. It was performed in a vertical slab gel apparatus with stacking gel containing 6% acrylamide and 0.5% bisacrylamide; spacer gel of 10% acrylamide and 0.5% bisacrylamide and separating gel containing 16% acrylamide and 0.5% bisacrylamide. Electrophoresis of the bacteriocin samples and molecular weight markers (26.6, 17.0, 14.2, 6.5, 3.5 and 1.06 kDa) were carried out at 50 V for 12 h. The gels were stained with coomassie brilliant blue G [13].

Selection of Non-Producing LAB: To explicitly reveal the phenotype of the 10kb plasmid, it is essential to transform this plasmid in a non-producing and antibiotic sensitive LAB. As both the parental isolates, identified as *L. plantarum,* were resistant to streptomycin (30 µg/ml) and cured ones were turned out to be sensitive, this antibiotic sensitivity of the strains was used as the criteria for the selection of strain for the transformation purpose. Accordingly, a streptomycin sensitive LAB strain, not having the ability to inhibit the indicator strain, *S. aureus* and not harboring any 10 kb resident plasmid was selected for transformation experiment.

Plasmid DNA Elution: Plasmids were isolated from *Lactobacilli*, LABB and LABP using the previous method [15] and run on 0.7% agarose gel electrophoresis using Tris-borate [16] at 100 V for 5 h. Gels were stained for 30 min with 0.5 µg/ml of ethidium bromide, destained for 15 min in distilled water and then visualized under UV light on a transilluminator and the 10kb plasmid was cut from the gel using the sharp blade and the plasmid was eluted. This procedure of elution was done repeatedly to get enough plasmid (concentration as high as 200ng) for use in the transformation experiment.

Transformation of Non-Producer Strains: Competent cells of non-producing LAB were prepared using calcium chloride and were transformed with 10kb plasmid obtained from either LABB or LABP as transforming agent following the standard procedure [18]. The transformed strains were obtained from MRS agar plates containing streptomycin (30 µg/ml) and subsequently sub-cultured in MRS broth having streptomycin for plasmid isolation. These transformed strains were also subjected for isolation of bacteriocin by cell adsorption-desorption method to deduce the antibacterial activity against the indicator strain, *S. aureus* as described above. Partial purification of bacteriocins from these transformed strains was also carried out as described above.

RESULTS AND DISCUSSION

Earlier, we have reported that the bacteriocins isolated from *Lactobacillus* isolates LABB and LABP may be of much interest as biopreservatives based on their spectrum of activity [13], while the amino acid sequences of these bacteriocins are not determined yet. Recently, these bacteriocins were shown to be better antibacterial in combination with nisin than when used alone in liquid and food systems [14]. In the present study, we report the presence of multiple plasmids of varied size and plasmid-associated bacteriocin production, antibiotic resistance and immunity in these isolates.

The plasmid profiles of the *Lactobacillus* isolates depicted LABB harboring 6 plasmids, viz., 2 mega plasmids of about 23 and >23 kb and 4 other plasmids of 10, 6, 2.3 and 1.5 kb, while LABP harboring 5 plasmids, viz., 2 mega plasmids of about 23 and >23 kb and 3 other plasmids of 10, 2.3 and 1.5 kb (Fig. 1a). The curing trials with various curing agents showed that a combination of ethidium bromide (40 µg/ml) with acriflavine (5 µg/ml) and novobiocin (5 µg/ml) cured both the *Lactobacillus* isolates after repeated passages leading to the loss of plasmids 10 and 6 kb in case of LABB, while only a single plasmid of 10 kb was lost in LABP (Fig. 1a). At higher concentrations of acriflavine or novobiocin (10 µg/ml), these strains failed to grow.

Table 1: Antibiogram of *Lactobacillus* isolates by antibiotic sensitivity OCTA-discs

		Inhibition zone (mm)	
Antibiotics	Concentration (µg/ml)	LABB	LABP
Amikacin (Ak)	10	02	0
Ampicillin (A)	25	16	25
Ceftriaxzone (Ci)	30	30	28
Cefuroxime (Cu)	30	23	25
Cephalexin (Cp)	30	18	22
Cephaloridine (Cr)	30	02	0
Cephradine (Cv)	30	22	25
Chloramphenicol (C)	25	30	30
Ciprofloxacin (Cf)	10	0	0
Cloxacillin (Cx)	5	22	25
Colistin (Cl)	10	0	0
Co-Trimazine (Cm)	25	20	30
Co-Trimoxazole (Co)	25	25	19
Erythromycin (E)	10	15	20
Gentamicin (G)	30	02	02
Lincomycin (L)	10	04	17
Mecillinam (Mc)	33	30	30
Nalidixic acid (Na)	30	0	0
Nitrofurantoin (Nf)	300	20	25
Norfloxacin (Nx)	300	0	15
Penicillin (P)	2	20	25
Streptomycin (S)	10	0	0
Tetracycline (T)	10	22	30

The antibiogram of these two strains were determined using OCTA-discs of Himedia, India. Both the *Lactobacillus* isolates were found to be resistant to amikacin, cephaloridine, ciprofloxacin, colistin, gentamycin, nalidixic acid and streptomycin (Table 1) while LABB was resistant to norfloxacin as well. The cured strains were found to be sensitive to ciprofloxacin, gentamycin and streptomycin as well as to their bacteriocins.

Both the LABB and LABP possessed almost a similar plasmid profile (Fig. 1a) except for 6.0 kb band which was absent in LABP strain. This similar plasmid profiles is not feasible if LABB being *L. acidophilus* and LABP, *L. casei* as reported earlier [13, 14], because there are much systematic differences with *L. acidophilus* and *L. casei*. Moreover, cell-wall proteins extraction from these *Lactobacillus* isolates and its analysis as per the previous procedure [19] revealed a similar profile except for a prominent band at 45 kDa in LABB being absent in LABP (data not shown). The antibiogram of both the strains were also almost similar except for LABB being additionally resistant to norfloxacin (Table 1). This led us to refer these isolates to Microbial Type Culture Collection (MTCC) at Institute of Microbial Technology, Chandigarh, India for identification. Both the organisms have been identified as *Lactobacillus plantarum* and designated LABB as *L. plantarum* with accession number MTCC 6160 and LABP as *L. plantarum* with accession number MTCC 6161. Some of the strains of *Lactobacillus plantarum* possess profiles appearing to be similar in both number of plasmids harbored and molecular mass of these plasmids, however, it is not necessarily true that all those plasmids are identical [20]. The bacteriocins produced by these strains are differing in the molecular mass (as indicated by SDS-PAGE of partially purified bacteriocins and detection of activity on the gel in situ) and antibacterial activity against various indicator organisms [13].

To determine the possible linkage of bacteriocin production with plasmid DNA in the *Lactobacillus* isolates plasmid-curing experiments were conducted as plasmid linked functions are generally ascertained by curing experiments using curing agents such as ethidium bromide, acriflavine, acridine orange, novobiocin, etc. In our studies, the strategy of combining ethidium bromide, acriflavine and novobiocin could cure both the *Lactobacillus* isolates of 10 kb plasmid after repeated passages. Novobiocin was found to be very useful for curing of plasmids in these isolates as observed by others [20, 21].

The cell adsorption-desorption preparation of bacteriocin from the cured strains of both the isolates showed loss of antimicrobial activity against *S. aureus* in agar well diffusion assay (Fig. 2a), while a similarly partially purified bacteriocin preparations indicated absence of bacteriocin band when run on SDS-PAGE (Fig. 3) as against parental strains confirming the loss of bacteriocin production in these cured strains. From these results, it can be inferred that the genetic determinants for bacteriocin production, immunity and resistance to certain antibiotics were encoded by the 10 kb plasmid in case of LABP, while one can only assume that a similar 10 kb plasmid could be involved in case of LABB as well because of an additional loss of 6 kb plasmid during curing. It has been reported that both the antimicrobial compound production and immunity determinants were encoded by an 8.8 kb plasmid in a *L. casei* strain of vegetable origin [22], while there was a plasmid-associated production of bacteriocin in a lactobacillus strain without affecting the immunity, indicating the possibility of the immunity genes on the chromosomes

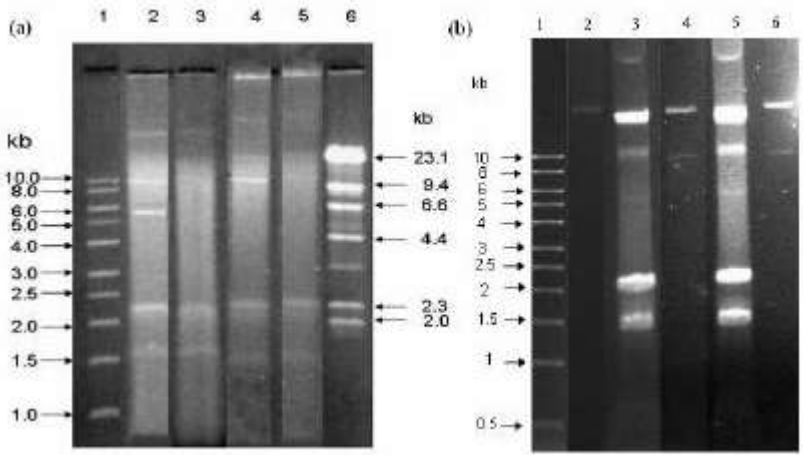

Fig. 1: Plasmid profile of (a) parental and cured cells of the lactobacillus isolates, LABB and LABP: Lane 1 – DNA ladder 1kb; Lane 2 – LABB; Lane 3 – $LABB_C$; Lane 4 – LABP; Lane 5 – $LABP_C$; Lane 6 – ? DNA Hind III digest; (b) parental and transformed strains containing 10kb plasmid of parental cells of the lactobacillus isolates, LABB and LABP: Lane 1 – DNA ladder 1kb; Lane 2- strains chosen for transformation; Lane 3 – LABP; Lane 4 – transformant having 10kb of LABP; Lane 5 – LABB; Lane 6 – transformant having 10kb plasmid of LABB.

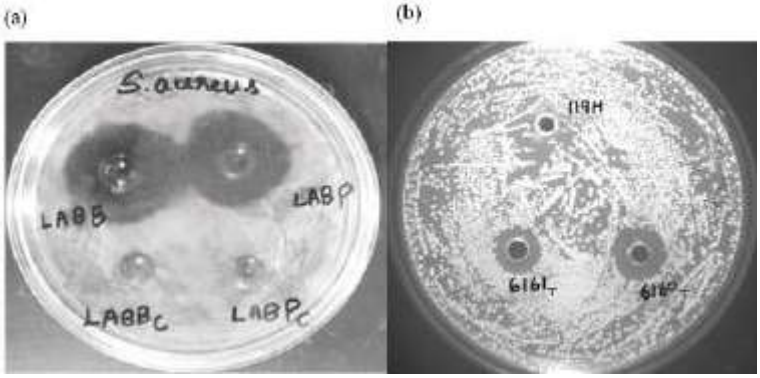

Fig. 2: Agar well diffusion assay showing (a) absence of bacteriocin activity in cured LABB and LABP; (b) presence of bacteriocin activity in transformed strains (having 10kb plasmid from either LABB or LABP and absence of bacteriocin activity in non-producer LAB strain

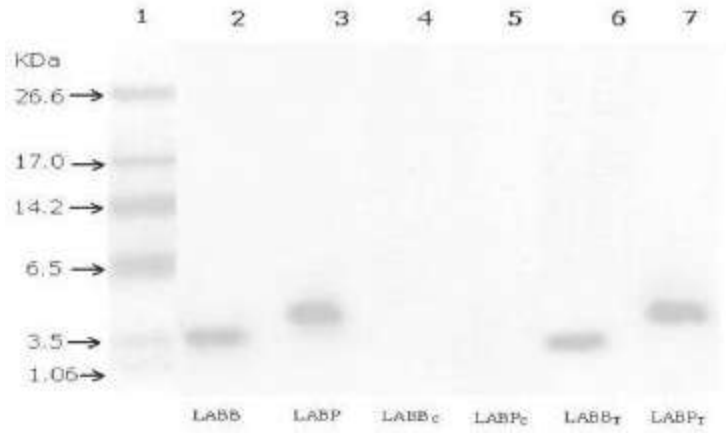

Fig. 3: Tris-tricine SDSPAGE showing the absence of bacteriocin in cured LABB and LABP and presence of bacteriocin in transformed strains with 10kb plasmid: Lane 1 – Standard MW markers; Lane 2 – LABB; Lane 3 – LABP; Lane 4 – $LABB_C$; Lane 5 - $LABP_C$; Lane 6 – $LABB_T$; Lane 6 – $LABP_T$

[11]. However, presently, we have demonstrated the plasmid mediated bacteriocin production, antibiotics resistance and immunity, albeit interrogation could be raised for LABB as the plasmid viz., 6kb apart from 10kb was also lost. Moreover, it has been recently reported that a *L. plantarum* strain produced a bacteriocin of about 2.5 kDa and is encoded by a plasmid of approximately 5.5 kb in size [21].

To confirm that the contribution was from the 10 kb, transformation experiment was carried out. This 10kb plasmid was eluted from the agarose gel and transformed into a streptomycin-sensitive LAB (not harboring 10 kb plasmid). The transformed LAB could grow on the streptomycin agar plate and could inhibit the indicator strain *S. aureus*. The parental strain chosen for transformation had only one plasmid viz., 23kb and after transformation it showed the inserted 10kb plasmid also (Fig. 1b). The partially purified bacteriocin preparations from the transformed strains containing 10kb plasmid of either of the isolates revealed inhibitory activity against *S. aureus* in agar well diffusion assay as compared to non-transformed ones (Fig. 2b). The bacteriocin preparations from both the transformed strains, LABB transformant and LABP transformant when run on SDS-PAGE showed the peptide band related to bacteriocin bands of the respective native strains (Fig. 3). It may be concluded that the production of bacteriocin, antibiotic resistance and immunity of these *Lactobacillus* isolates (*L. plantarum* strains) were plasmid mediated.

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