JOURNAL OF PURE AND APPLIED MICROBIOLOGY, December 2013. Vol. 7(4), p. 3201-3206

Molecular Characterization of *Lactobacillus plantarum* Isolates of Vegetables and Phyllosphere

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(Received: 08 February 2013; accepted: 26 March 2013)

The present study was conducted to know the biological and molecular variability among the *Lactobacillus plantarum* isolates. Different vegetables and phyllospheres were screened for *Lactobacillus* spp. Out of the fourteen isolates; ten were identified as *Lactobacillus plantarum* based on microscopic observation, biochemical tests and by using species specific primer. RAPD marker analysis was used to study the molecular diversity. Total of 37 bands were scored in RAPD analysis and 29 bands were found to be polymorphic. RAPD analysis depicted that ten isolates formed four clusters. Banding pattern of these isolates distinguished the isolates of different sources.

Key words: *Lactobacillus plantarum*, Molecular diversity and RAPD.

Lactobacillus is a genus under lactic acid bacteria described as a heterogeneous group of regular, non spore forming, Gram positive rods, found in a great variety of habitats such as plants and gastrointestinal tract1 (Amin *et al*., 2009). *Lactobacillus plantarum* is a heterofermentative, microaerophilic, Gram positive microorganism, with rod shaped morphology, occurring singly or in short chains. Numerous strains of *L. plantarum* have been isolated from different ecological niches including meat, fish, fruits, vegetables, milk, and cereal products. *L. plantarum* has been used as a starter culture in various food fermentation processes contributing to the organoleptic properties, flavour, and texture. Production of lactic acid and other antimicrobial compounds, by *L. plantarum* also contributes to the safety of the final products[2] (Todorov and Franco, 2010).

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Genetic diversity can be estimated at molecular level. One such method is protein analysis using electrophoresis or direct amino acid sequencing. Electrophoresis analysis of proteins has long been a valuable tool in systematic and population genetic studies of bacteria, plants and fungi. The biochemical characteristics are useful in distinguishing two different genera or species. Further, it can also identify different compounds produced by different strains[3] (Dodd *et al*., 1996). With the advent of molecular DNA techniques, several arbitrary primers based on randomly amplified polymorphic DNA (RAPD) technique has been used for typing and identification of number of closely related species of bacteria and assessment of genetic relationships. RAPD analysis is faster, technically less demanding and more economical than the other genomic typing methods like RFLP and AFLP. Further, this technique elucidates the biodiversity in a group of isolates[4] (Hansel *et al*., 1998). Hence, the biodiversity of *L. plantarum* isolated from different vegetables and leaf surfaces were checked using RAPD analysis.

MATERIALS AND METHODS

Isolation of *Lactobacillus* spp.

Isolation of *Lactobacillus* spp. from different vegetables and leaf surfaces viz., Cabbage, Cauliflower, Cucumber, Radish, Carrot, Okra, Capsicum, Brinjal, Tomato, Raddish leaves, Turmeric leaves, Jack leaves, Coriander leaves and Banana leaves was carried out by the method described by[5] (Kamble & Pathade, 2010). Healthy vegetable and leaf samples were collected in sterile beaker and then 10g of the sample was suspended in 100ml sterile water blank and shaken for 5 minutes and was serially diluted up to 10^{-7}. 1ml from 10^{-6} and 10^{-7} dilutions were plated out on MRS agar medium using pour plate method and incubated at 29°C for 48-72 h. The colonies formed on MRS medium were sub cultured into sterile MRS broth and incubated at 29°C for 72 h and then after growth of bacteria the broth was again plated on to petri plates containing sterile MRS medium for purification of the cultures. Purified cultures were preserved for further identification studies.

Identification of *Lactobacillus* spp.

Identification of the *Lactobacillus* upto generic level.

The tentatively identified *Lactobacillus* isolates were subjected to morphological and physiological characterization as listed below.

Morphological Characterization

Colony morphology studies on MRS medium and Microscopic observation

The *Lactobacillus* isolates form characteristic creamy white coloured round colonies on MRS agar media. Each isolate was streaked on petri plate containing MRS agar medium and incubated for 2 days. The *Lactobacillus* isolates were studied for cell morphology and gram reaction.

Biochemical tests

All the biochemical/physiological tests were conducted in duplicate for each isolate like Gelatin liquefaction, catalase , urease and motility test.

Identification of the *Lactobacillus plantarum* at species level using species specific primer.

The culture so identified as *Lactobacillus* was further studied at the species level by using species specific primer. Specific primer sequence was used for the identification of the *Lactobacillus* at the species level6 (Amin *et al*., 2009). This primer sequence was used to identify *Lactobacillus plantarum* isolated from different vegetables and leaf surfaces.

Primers

Forward(planF)-5'-CCgTTTATgCggAACACCTA-3'
Reverse(planRev)-5'-TCgggATTACCAAACATCAC-3'

DNA extraction protocol (Reagents and preparation of reagents)

DNA extraction protocol followed was according to7 Sambrook *et al*., 1989. The DNA was dissolved in TE buffer.

PCR amplification conditions

PCR reactions were performed in a final volume of 25 µl containing 30 ng of template DNA, 0.75 µl of 2mM dNTPs each, 2.5µl of 10X taq buffer, 0.36µl of 3unit/µl of Taq DNA polymerase, 3 µl of 10 picomole primer. Amplifications were achieved in MWG-Biotech primus thermocycler with the program consisting initial denaturation of 94°C for 5 min followed by 30 cycles each consisting of denaturation at 94°C for 30 sec, primer annealing temperature at 50°C for 45sec, primer extension at 72°C for 1 min, and a final extension of 72°C for 10 min. These reactions were repeated to check the reproducibility of the amplification.

RAPD analysis

PCR amplification conditions

PCR reactions were performed in a final volume of 25 µl containing 30 ng of template DNA, 0.75 µl of 2mM dNTPs each, 2.5µl of 10X taq buffer, 0.36µl of 3unit/µl of Taq DNA polymerase, 3 µl of 10 picomole primer. Amplifications were achieved in MWG-Biotech primus thermocycler with the program consisting initial denaturation of 94°C for 3 min followed by 35 cycles each consisting of denaturation at 94°C for 1 min, primer annealing temperature at 37°C for 1 min, primer extension at 72°C for 3 min, and a final extension of 72°C for 10 min. These reactions were repeated to check the reproducibility of the amplification.

Selection of primers

To choose the RAPD primers that can amplify informative sequences, Primer screening was carried out using DNA obtained from the *Lactobacillus plantarum* isolates, Out of 13 primers screened, finally 6 primers producing sharp, intense bands were selected for the RAPD analysis (Table 1).

Analysis of RAPD data

The bands were manually scored '1' for the presence and '0' for the absence and the binary data were used for statistical analysis. The scored band data (presence or absence) was subjected to cluster analysis using STATISTICA.

RESULTS AND DISCUSSION

Isolation and Identification

Fourteen isolates were isolated from different sources and named based on the sources (Table 2). All *Lactobacillus* isolates formed Characteristic round creamy white coloured colonies on MRS medium. *Lactobacillus* isolates were further examined for their Gram's reaction and shape. All the isolates were found Gram positive and rod shaped. All isolates scored negative to gelatin liquefaction, catalase test, urease test and motility test (Table 3).

Table 1. RAPD primers with sequences chosen for analysis

Sl.No	Primer No.	Sequence
1	Random primer 1	5'-GAG AGC CAA C-3'
2	Random primer 2	5'-GTT TCG CTC C-3'
3	Random primer 3	5'-GTA GTC ATA T-3'
4	Random primer 4	5'-AAG AGC CCG T-3'
5	Random primer 5	5'-GGC TGC TGG C-3'
6	Random primer 6	5'-CCC GTC AGC A-3'
7	Random primer 7	5'-GAA CGG ACT C-3'
8	Random primer 8	5'-GGT GCG GGA A-3'
9	Random primer 9	5'-TTG GAG GGC A-3'
10	Random primer 10	5'-CTT CCG TCA A -3'
11	Random primer 11	5'-TGC TCT GCC C-3'
12	Random primer 12	5'-GGT GAC GCA G-3'
13	Random primer 13	5'-TCG CTG GGA C-3'

Identification of the *Lactobacillus plantarum* at species level

The *Lactobacillus* isolates from different sources, which showed positive for morphological, physiological and biochemical tests were tested with specific primer for species identification. The isolates were amplified with species specific primer and identified as *L. plantarum* with PCR product of 300bp. Out of fourteen cultures isolated only

Table 2. Lactobacillus isolates from different sources

Sl. No	Isolates	Sources
1	CAB	Cabbage
2	CAF	Cauliflower
3	CUM	Cucumber
4	RAD	Radish
5	CAR	Carrot
6	OKR	Okra
7	TUR	Turmeric leaves
8	JAK	Jack leaves
9	COR	Coriander leaves
10	BAN	Banana leaves
11	CAP	Capsicum
12	BRI	Brinjal
13	TOM	Tomato
14	RADL	Radish leaves

Table 3. Biochemical and physiological characters of Lactobacillus isolates

Sl.No	Biochemical tests	Result
1	Gelatin liquefaction	-
2	Catalase	-
3	Urease	-
4	Motility	-

'-'= Negative

Table 4. Oligonucleotide primers that showed genetic variation among the *Lactobacillus plantarum* isolates

Primers	No. of amplified fragments	No. of polymorphic bands shared	unique	No. of Monomorphic bands
Random primer 1	5	4	0	1
Random primer 4	6	3	1	2
Random primer 5	5	2	0	3
Random primer 6	6	5	0	1
Random primer 8	9	8	1	0
Random primer 10	6	4	1	1
Total	37	26	3	8
Percentage	100	75.67	8.10	21.62

Table 5. Dissimilarity index of Lactobacillus plantarum isolates based on RAPD analysis

	CAB	CAF	CUM	RAD	CAR	OKR	TUR	JAK	COR	BAN
CAB	0									
CAF	3.61	0								
CUM	3.46	3.32	0							
RAD	3.61	3.16	3.32	0						
CAR	2	3	3.16	3.32	0					
OKR	3.74	3.87	3.46	4.12	3.16	0				
TUR	2.83	3.87	3.16	3.61	2.83	3.46	0			
JAK	2.65	3.16	3.32	3.16	2.24	3.61	3.32	0		
COR	2.45	3	3.16	3.32	1.41	3.46	2.83	1.73	0	
BAN	3.61	3.74	3.61	4.47	3.32	2.65	3.87	3.46	3.32	0

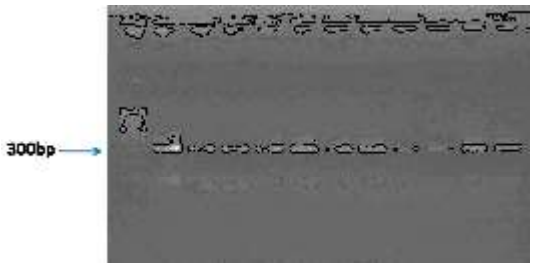

Fig. 1. Gel profile of *Lactobacillus plantarum* amplified using species specific primer. M: marker lane, s: standard culture and 1-10 represent isolates from different sources. (No. 1: Cabbage, no. 2: Cauliflower, no. 3: Cucumber, no. 4: Radish, no. 5: Carrot, no. 6: Okra, no. 7: Turmeric leaves, no. 8: Jack leaves, no. 9: Coriander leaves and no. 10: Banana leaves).

ten cultures were amplified with the specific primer and identified as *L. plantarum* (Fig. 1). This result is in conformity with the results obtained by8 Amin *et al*. who isolated and identified *L. plantarum* from plants using the same species specific primer. Out of sixty isolates from fresh vegetables 14 of them were confirmed as *L. plantarum*[9]. Sawitzki *et al*., also isolated and identified *L. plantarum* from naturally fermented artisanal sausage using species specific primers (16/Lpl and LbP11/LbP12). Out of ten isolates five isolates were confirmed as *L. plantarum*.

RAPD characterization

A total of 37 RAPD bands produced from the selected 6 primers were used for fingerprinting and for estimation of genetic diversity among ten isolates of *L. plantarum* (Fig. 2). The number of bands scored for each primer varied from 1 to 9 with an average of 6.16 bands per primer. Out of 37 amplification bands, 8 bands (21.62%) were monomorphic, 3 bands (8.10%) were unique and 26 bands (75.67%) were shared polymorphic, which were informative in revealing the relationship among the bacterial isolates (Table 4). Similarly[10] Nigatu *et al*. evaluated forty one type and reference strains of *Lactobacillus* using their randomly amplified polymorphic DNA (RAPD) band profiles. Developed bands for each strain were distinct and enabled discrimination. All of the strains were clearly differentiated at and below the 72 % similarity value. Species discrimination might be possible making use of the distinctly polymorphic bands amplified specific to a strain. Similar result was reported by[11] Kumar *et al*. who carried out RAPD analysis in seventy isolates of Bacillus thuringiensis isolated from cotton fields. Different random decamer primers were used for RAPD amplification, which generated a total of 1935 fragments; of these 1865 (96.38 %) were polymorphic and 68 monomorphic (3.51 %).

Cluster analysis and genetic dissimilarity matrix of 10 *L. plantarum* isolates

The Cluster analysis based on 37 RAPD bands revealed that the ten *L. plantarum* isolates examined clustered at a linkage distance of about 3 units on the dendrogram with isolate of CAB and isolate of BAN spanning the extremes. The dendrogram (Fig. 3) has clearly depicted that all the 10 *L. plantarum* isolates formed four clusters. Among four clusters two major groups are formed, isolates CAB, CAR, COR, JAK, TUR and CAF formed the first group, isolates CUM, RAD, OKR and BAN formed the second group. Analysis of the clustering pattern indicates that isolates from vegetables and leaf surfaces are present in extreme positions. The dissimilarity index of *L. plantarum*

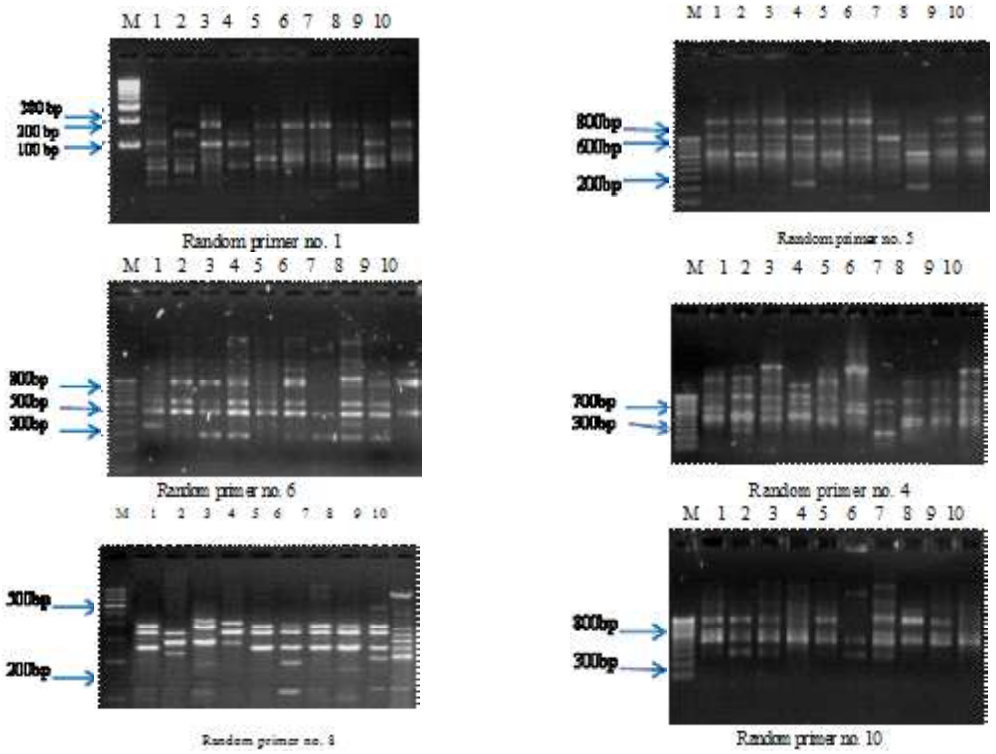

Fig. 2. RAPD gel profile of Lactobacillus plantarum isolates generated using 10-mer random primers (m: marker lane and lane 1 to 10 represent isolates from different sources)

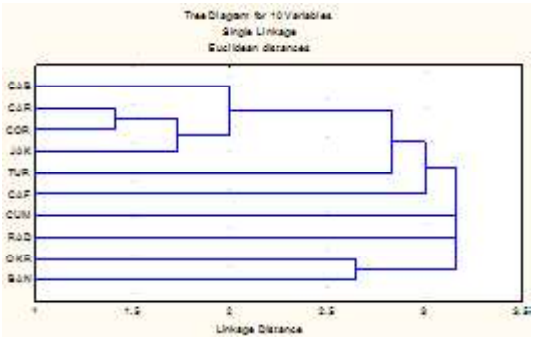

Fig. 3. Dendrogram based on RAPD profile of 10 Lactobacillus plantarum isolated from different sources.

isolates are presented in (Table 5). Similar result was obtained by[12] Hamza *et al*. who evaluated the genetic diversity of One hundred and sixty isolates of lactic acid bacteria (LAB) isolated from ten samples of traditional soured milk. Earlier,[13] Congo *et al*. evaluated diversity of 72 isolates of *Lactobacillus plantarum*, previously identified from different raw vegetables and fruits, based on phenotypic (Biolog System) and genotypic (randomly amplified polymorphic DNA-polymerase chain reaction, RAPD-PCR, and amplified fragment length polymorphism, AFLP) approaches. Eight clusters were formed at the similarity level of 92% based on Biolog System analysis. At 2.5 linkage distance, a high polymorphism was found and several sub clusters were formed with both analyses.

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