Advanced Journal of Microbiology Research Vol. 2004
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research paper

Identification of exopolysaccharides-producing lactic acid bacteria from Burkina Faso fermented milk samples

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Accepted 31 December 2003

Spacer region between 16S and 23 S rRNA genes of thirteen lactic acid bacteria strains from Burkina Faso fermented milk samples were amplified by the polymerase chain reaction (PCR). *Lactobacillus delbrueckii, Lactobacillus acidophilus, Lactobacillus fermentum, Streptococcus thermophilus, Pediococcus spp, Leuconostoc mesenteroides subsp mesenteroides* were identified. The *Lactobacillus* group was the predominant bacteria. Plasmids identified ranged between 2000 and 4000 bp. Exopolysaccharides (EPS) production varied from 181 mg/l and 814 mg/l, monomer analysis showed that glucose and galactose were predominant.

Key words: Lactic acid bacteria, fermented milk, PCR, exopolysaccharides.

INTRODUCTION

Lactic acid bacteria (LAB) are widely employed in traditional fermented milk, in industrial fermentation processes and as starter cultures in the dairy industry. Some strains of lactic acid bacteria have importance in general health, providing a beneficial microflora in the intestinal tract (Salminen et al., 1993) and are able to synthesize exopolysaccharides (EPS) (Cerning, 1990; Sikkema, Oba, 1998; Cerning, Marshall, 1999; De Vuyst, Degeest, 1999; Ricciardi, Clementi, 2000).

Exopolysaccharides produced by lactic acid bacteria have gained increasing attention over the last few years because of their contribution to the reology and texture of food products (Cerning and Marshall, 1999).

Lactic acid bacteria play an important role in food fermentation, as the products obtained with their aid are characterized by hygienic safety, storage stability and attractive sensory properties.

Detecting and identifying various species of LAB with molecular methods are powerful alternatives to the traditional differentiation of bacteria. It is also important for quality control of dairy products. Previously, there have been several reports on species-specific probes for different LAB derived from ribosomal RNA (rRNA), especially 16S and 23S rRNA sequences (Hensiek et al., 1992; Hertel et al., 1993; Ehrmann et al., 1994). Barry et al., 1991 have shown that the ribosomal intergenic regions are more variable between bacteria species compared to 16S and 23 S rRNA genes.

The nature of fermented products is different from one region to another. This depends on the local indigenous microflora, which in turn reflected the climatic conditions of the area. Thus traditional fermented milk in region with a cold temperature climate contained mesophilic bacteria such as *Lactococcus* and *Leuconostoc spp.*, whilst thermophilic bacteria, which include mostly *Lactobacillus* and *Streptococccus*, prevailed in regions with a hot, subtropical or tropical climate (Thomas, 1985; Tamine and Robinson, 1988; Kurmann, 1994).

Burkina Faso milk production was estimated at 37392 tons of commercialised milk in 1998 (Réseau Documentaire d'Elevage, 1998). The Fulani of Burkina Faso, ferment their milk in calabashes, gourds, and clay pots. Burkina Faso population still produce unpasteurized

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fermented milk by traditional methods, since such milk product still enjoy loyal following in the communities.

To our knowledge, information does not exist on the Burkina traditional fermentative microflora. Thus we focus our study on the isolation and identification of exopolysaccharides producing LAB using DNA-based specific detection system by PCR and EPS analysis by HPLC.

MATERIALS AND METHODS

Sampling

Thirty samples of fermented milk were obtained from individual households in rural areas in northern Burkina. Samples were collected in sterile small bottles and stored in laboratory under refrigeration at 4°C until they were used in experiments.

Isolation of lactic acid bacteria

Serials dilutions of homogenized fermented milk samples in 0.1% peptone saline were used for microbial isolation with the following media: (a) MRS agar (De Man et al., 1960) (Fluka Biochemika 69966) incubated anaerobically for 48 h at 42°C for isolation of thermophilic *Lactobacilli* and *Streptococci*, (b) MRS agar incubated anaerobically for 48 h at 35°C for isolation of mesophilic *Lactobacilli* and *Leuconostoc*, (c) M17 agar (Terzaghi and Sandine, 1975) (Difco) incubated aerobically for 48 h at 30°C for isolation of *lactococci*, (d) Rogosa agar (Rogosa et al., 1951) (Difco) incubated anaerobically for 48 h at 35°C for isolation of lactobacilli.

Fifty isolates were obtained by random selection of slimy (exopolysaccharides producer) colonies (Smitinont et al., 1999) from all media used. These isolates were tested for exopolysaccharides production in modified MRS broth.

Phenotypic and biochemical characterization of selected isolates

Gram staining, catalase activity, gas production from glucose, growth in NaCl 6.5% was determined according to methods for lactic acid bacteria (Roissart Luquet, 1994). Cells morphology was determined with cells grown in MRS broth for 35°C at 20 h by using phase-contrast microscopy.

Isolation, purification, and quantification of EPS

Selected isolates were grown in modified MRS broth (glucose 20 g/l was remplaced by lactose 75 g/l), cells were harvested by centrifugation for 10 min at 11 000 x g. Two volumes of cold ethanol were added to culture supernatants and stored overnight at 4°C. Precipitated material was collected by centrifugation (20 min at 2 500 x g) resuspended in demineralised water, and mixed with 2 volumes of cold ethanol. Samples were centrifuged 2500 x g and the pellets were dried at 100°C. The total carbohydrate content of the EPS was determined using the phenol-sulfuric acid procedure of Dubois et al. (1956).

Monosaccharides analysis

After complete hydrolysis of EPS (2 h in 1 M H_2SO_4 at 100°C in a sealed recipient) monosaccharide composition of the hydrolysate was determined by HPLC (using corogel 87C colum, and RI detector) with flow rate 0.6 ml/min (using Jasco pump PU-980) and the relative proportion of the peak (using Integrator SP4290) areas calculated to estimate the monomer composition.

Extraction of bacterial total DNA

Total DNA was extract from 10 ml of harvested cultures in the mid-log phase (OD_{600} of 0.5 -1). Cells were collected by centrifugation (3000 x g, 10 min, 4°C) and frozen for at least 1 h at -20°C. The thawed pellet was washed in 1 ml TES buffer (6.7% sucrose, 50 mM Tris-HCl, pH 8.0, 1mM EDTA) and resuspended in 300 µl STET buffer (8% sucrose, 5% Triton X-100, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA). Seventy-five microliters of lysis buffer (TES containing 1330 U ml^{-1} mutanolysine and 40 mg ml^{-1} lysosyme) was added and the suspension incubated at 37°C for 1 h. After addition of 40 µl preheated (37°C) 20% SDS in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and glass beads, cells were vortexed for 60 s and incubated at 37°C for 10 min, followed by 10 min incubation at 65°C. One hundred microliters of TE buffer was added and the lysate was extracted with 1 volume phenol/chloroform/isoamyl alcohol (49:49:1). Phase was separated by centrifugation (18000 x g, 5 min) using phase lock gel tubes (Eppendorf). The aqueous phase was carefully mixed with 70 µl 5M NaCl, 1 ml isopropanol, and DNA precipitated on ice for least 15 min. DNA was collected by centrifugation (20000 x g, 30 min, 4°C) and the pellet washed in ice-cold 70% ethanol. DNA was dried with DNA speed vac and resuspended in 100 µl TE. This DNA solution obtained was stored at 4°C.

PCR amplifications

PCR was performed in a DNA thermal cycler (Perkin Elmer cetus) with Sigma polymerase kit. A typical reaction mixture (50 µl) for PCR of the 16S-23S rRNA gene spacer region consisted of reaction buffer (end concentration 10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton), 200 µM each dNTP, 1 µM of each specific primer pair (Table1), 50 ng of bacterial DNA and 0.6 U of sigma DNA polymerase. The reaction tubes were overlayed with drops of mineral oil (Sigma). The amplification profile was 95°C for 30 s, 55°C for 30 s and 72°C for 30 s. This was repeated for 35 cycles. The program also included a preincubation at 92°C for 2 min before the first cycle and incubation at 72°C followed by a cooling step down to 4°C after the last cycle.

The presence of specific PCR products was determined by gel electrophoresis in 0.7% agarose gel containing ethidium bromide. Electrophoresis in Tris-borate-EDTA was performed at 100 volts for 30 min, and photographed under ultraviolet light illumination. DNA of references strains was included.

RESULTS AND DISCUSSION

Biochemical and morphological characteristics of LAB strains

Thirteen strains were selected and identified as exopolysaccharides producers. All the strains were Gram-positive, catalase negative. One strain produced gas from glucose, nine strains did not grew in 6.5% NaCl. Three strains were rods, five were cocobacilli, four were spherical and one was ovoid (Table 2).

Table 1. Specific primer used in this study.

Primer	Species	Sequence 5'-3'	References
1	All *Lactobacillus spp..*	GTAAGG TGGCGATGTGTACCTCAAG	Heilig et al., 2002
2		CACCGCTACACATGGAG	
Lfpr FermII	*Lb. fermentum*	GCCGCCTAAGGTGGGACAGAT	Walter et al., 2000
		TTACCTAACGGTAAATGCGA	
Aci I	Lactobacilllus acidophilus	TCTAAGGAAGCGAAGGAT	(Tilsala-Timisjarvi ,
Aci II		CTCTTCTCGGTCGCTCTA	Alatossava , 1997
Del I	*Lactobacillus delbrueckii*	ACGGATGGATGGAGAGCAG	(Tilsala-Timisjarvi,
Del II		GCAAGTTTGTTCTTTCGAACTC	Alatossava, 1997)
	Pediococcus spp	GTAAAGTGGCGTGT GTACCTCAAG	(Heilig et al., 2002)
		CACCGCTACACATGGAG	
	Lactococcus	CTTTGAGTGATGCAATTGCATC	Ampe et al., 1999
		CACCGCTACACATGGAG	
Thr I	*Streptococcus thermophilus*)	ACGGAATGTACTTGAGTTTC	(Tilsala-timisjarvi,
ThrII		TTTGGCCTTTCGACCTAAC	Alatossava, 1997
	Leuconostoc	GATCCATCTCTAGGTGACGCCG	Ampe et al., 1999
		CACCGCTACACATGGAG	
LMMf	*Leuconostoc mesenteroides*	CCGTTACCCCTAAACCCCGAC	Moschetti et al., 2000
LMMr	subsp.*mesenteroides*	GACCAAATACAATAGGTTGCG	
	EPS plasmid	TTGTTCTCGAGATGGATATGGGATATAGC	Lamothe, 2000
		TATTCCCCTTTATTAATCTGATATGCCAAGG	

Table 2. Biochemical and morphological characteristics of lactic acid bacteria strains from Burkina Faso fermented milk samples.

Strains	Cell form	Cellular arrangement	Growth in 6.5% NaCl	Gram	Catalase	Gas from glucose
1 MR1	Rods	Chains and pairs	-	+	-	-
2 MR2	Cocobacilli	Pairs and chains	-	+	-	-
3 MR3	Cocobacilli	Chains	-	+	-	-
4 MR5	Cocobacilli	Chains	-	+	-	-
5 MR6	Cocobacilli	pairs	-	+	-	-
6 MR9	Rods	Chains and pairs	-	+	-	-
7 MR10	Ovoid	Chain and pairs	-	+	-	-
8 MR11	Rods	Chains and pairs	-	+	-	-
9 MR12	Cocobacilli	Chains and pair	-	+	-	-
10 MR14	Spherical	pairs	±	+	-	-
11 MR15	Spherical	chains	±	+	-	+
12 MR16	Spherical	pairs	±	+	-	-
13 MR17	Spherical	pairs	±	+	-	-

+ = positive, - = negative, ± = little response.

With PCR reaction, the lactic acid bacteria (Figure 1) cab be identified. Using the specific primers in Table 1, strains MR1, MR9, MR12 were identified as *Lactobacillus acidophilus*, whilw strains MR6, MR3, MR11 were identified as *Lactobacillus fermentum* (Figures not shown). The strain MR10 was identified as *Streptococcus thermophilus*, and strains MR14, MR16, MR17 were idenfied as *Pediococcus spp*. The strain MR15 was identified as *Leuconostoc mesenteroides subsp mesenteroides* (Figures not shown).

Table 3. Carbohydrates concentration, monomer composition of exopolysaccharides.

Exopolysaccharides producing strains	Carbohydrates (mg/l)	Monomer composition
1 MR1	219	100 % glucose
2 MR2	506	nd
3 MR3	322	99.18% galactose, 0.80% glucose
4 MR5	474	nd
5 MR6	713	2.95% glucose, 97.04% pentose
6 MR9	512	99.97% galactose, 0.03% pentose
7 MR10	814	nd
8 MR11	549	47.41% glucose, 52.57% rhamnose
9 MR12	181	81.95% glucose, 18.01% pentose
10 MR14	288	100%glucose
11 MR15	615	nd
12 MR16	559	93.92% glucose, 1.35% fructose, 5.27% pentose
13 MR17	357	32.58% mannose, 36.42% rhamnose, 30.41 %fructose

nd: not determined.

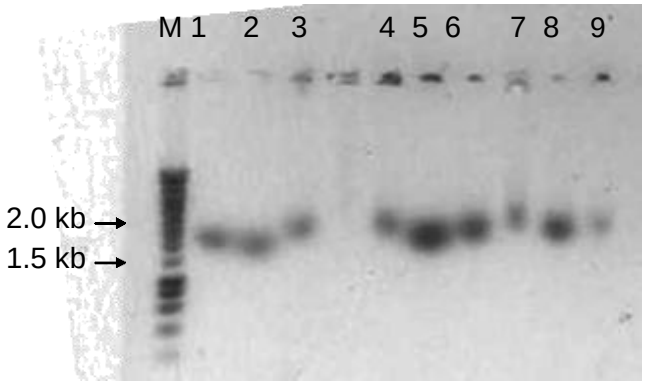

Figure 1. PCR products of amplified 16S-23S RNA gene spacer regions different LAB using primers for *Lactobacillus group*. Lanes: M, molecular weight marker; 1, MR1; 2, MR2; 3, MR3; 4, MR5; 5, MR6; 6, MR9; 7, MR11; 8, MR12).

Among the 13 isolates, 11 had EPS plasmids using the EPS-specific primers in PCR reaction. The carbohydrates concentrations of EPS (Table 3) were between 814 mg/l for strain MR10 and 181 mg/l for strain MR12.

The major monosaccharide (carbohydrate) resulting from acid hydrolysis of the strains EPS was glucose. Other peaks of galactose, rhamnose on the HPLC chromatogram were obtained (Table 3).

The strains isolation and indentification showed diversity of lactic acid bacteria group in Burkina fermented milk. *Leuconostoc, Streptococcus*, *Lactobacillus, Pediococcus* were isolated from the fermented milk. Among the strains characterized, *Lactobacillus* genus was the dominant, consisting of *L. fermentum, L. acidophilub, L. delrueckii.* These bacteria have been previously been isolated from fermented food (Hammes and Vogel, 1995). *L. fermentum* produces both isomers of lactic acid (kandler and Weiss, 1986). *Lactobacillus plantarum* was isolated in Zimbabwe fermented milk (Mutukumira, 1996), in Tanzania fermented milk (Isono et al., 1994) in Cameroon fermented milk (Jiwoua and Millière, 1990).

Generally the EPS genes are encoded on large plasmids (>20 kb) that can be transferred from one strain to another one. (Van Kranenburg et al., 1997, 1998, 2000). *Lactococcus lactis* NIZO B40 strain produces a phosphopolysaccharide, 12 kb gene cluster contains 14 coordinately expressed genes, RXABCDEFGHIJKL, localised on a 40 kb plasmid is specific for EPS production (Van kranenburg et al., 1997).

Carbohydrates concentration are high compare with results obtained by Cerning et al., 1988; Cerning, 1990 of EPS with *L. lactis* subsp. *lactis* (100-600 mg /l). Other authors obtained higher concentrations of EPS high than those obtained in this study (Van de Berg et al., 1995; De Vuyst and Degeest, 1999).

After hydrolysis HPLC analysis showed that dominant neutral sugars were galactose and glucose (Table 3). Our results were similar to those of several authors who demonstrated that galactose and glucose were the neutral sugars occurring frequently in bacteria exopolysaccharides (De Vuyst et al., 1998; Cerning, 1990, 1995, Nakajima et al., 1990; Grobben et al., 1995; Gruter et al., 1993).

The monosaccharides occurring most frequently in the various exopolysaccharides from lactic acid bacteria are glucose and galactose (Cerning, 1990, 1995), but rhamnose (Cerning et al., 1986, 1988; Nakajima et al., 1990, 1992; Gruter et al., 1993; Grobben et al., 1995), mannose (Petit et al., 1991) fructose (Manca de Nadra et al., 1985) arabinose and xylose (Cerning et al., 1988, 1992) or sugar derivatives such as N-acetylgalactosamine (Doco et al., 1990; Petit et al., 1991)

and N-acetylglucosamine (Cerning et al., 1994) are also found.

Lactic acid bacteria play an important role in food fermentation, as the products obtained with their aid are characterized by hygienic safety, storage stability and attractive sensory properties. Since starter cultures are blended empirically for the desired characteristics of the final product, maintenance of the optimal strain balance throughout the fermentation process is important.

EPS in their natural environment are thought to play a role in the protection of the microbial cell against desiccation, phagocytosis, phage attack, antibiotics or toxic compounds, predation by protozoans, osmotic stress, adhesion to solid surfaces, and in cellular recognition. In food industry, microbial exopolysaccharides are used as thickeners or viscosifiers, stabilizing or emulsifying agents, and as gelling and water-binding agents or texturizers (Sutherland, 1994). The functional properties of exopolysaccharides are influenced by their primary structure (Sutherland, 1994).

Our results demonstrate the diversity of lactic acid bacteria in traditional fermented milk in Burkina Faso. The milk samples contained several species lactic acid bacteria and PCR specific primers were able to identify them. These strains can be use as starter culture with predictable characteristics and contribute to the development of small-scale and commercial production of fermented milk with stable, consistent quality.

ACKNOWLEDGMENTS

We thank Moussa Sanogo and Dr Ismael H N Bassolé for help in DNA extraction, PCR, and electrphoresis.

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