Characterisation of ropy slime-producing Lactobacillus sakei using repetitive element sequence -based PCR

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Abstract

Eighteen earlier well characterised Lactobacillus sake strains representing different slime production capabilities in vacuum-packaged meat products were analysed using repetitive element sequence-based PCR (rep-PCR). The single primers BOXA1R and RW3A and the primer pair REP1R-Dt & REP2R-Dt were evaluated for their applicability in L. sake genotyping. The five different patterns produced by RW3A were least revealing, with the discriminatory power equalling to ribotyping. BOXA1R and REP-primer pair both produced six different banding patterns and the combination of these results yielded seven different rep-types. Rep-PCR was concluded to have approximately the same discriminatory power as randomly amplified polymorphic DNA (RAPD) analysis, but it was inferior to pulsed-field gel electrophoresis (PFGE). However, if the results of rep-PCR and RAPD were combined, the discrimination was comparable PFGE, with the exception that within Ribogroup I the non-slime-producing strains were not distinguished from weak slime producers. It was concluded that the combination of the two PCR-based typing techniques, rep-PCR and RAPD, would be a valuable tool in large scale contamination studies at the meat processing plants, since results can be obtained rapidly and fewer isolates need further analysis by PFGE.

Key words: slime-producing L. sake; rep-PCR, contamination studies

1. Introduction

Ropy slime-producing Lactobacillus sake strains are potent spoilage organisms associated with vacuum-packaged cooked meat products (Korkeala et al., 1988; Mäkelä et al., 1992). The spoilage is manifested by formation of very unpleasant-looking, slimy glucose-galactose polysaccharide on the product surfaces. This spoilage type has caused considerable economic hardship for several meat processing plants in Finland. During the worst years, as many as ten different producers suffered from contamination with ropy slime-producing L. sake strains (Björkroth and Korkeala, 1996).

In addition to phenotyping, ropy slime-producing L. sake strains have been characterised using ribotyping (Björkroth and Korkeala, 1996), randomly amplified polymorphic DNA (RAPD) and SmaI and ApaI macrorestriction analysis (Björkroth et al., 1996). Based on ribotypes, the strains can be divided into four main groups of which the Group 1 contains the most potent slime producers. Within the Group 1, macrorestriction analysis employing pulsed-field gel electrophoresis (PFGE) has been the only technique distinguishing all non-slime-producing mutants from the slime-producers (Björkroth et al., 1996). With the help of restriction endonuclease analysis (REA) we have also studied the effectiveness of a commercial biopreservative to prohibit the growth of ropy slime-producing L. sake strains (Björkroth and Korkeala, 1998). These strains were found to possess striking competitive feature, while being capable of growing despite the presence of high numbers of the biopreservativein the packages.

By employing molecular typing techniques, we have gained knowledge of this spoilage form. Also the future studies associated with the growth control and biopreservation will rely on molecular techniques. Despite the variety of molecular tools used, a rapid and repeatable technique, suitable for cost-efficient characterisation of many isolates has been lacking. PFGE is simply too expensive and slow to be used in large scale analysis. Repetitive element sequence-based PCR (rep-PCR) employs primers that are targeted to highly conserved interspersed repetitive sequences in the bacterial genome (Versalovic et al., 1991). These sequences are characterised by a length of 20-400 bp, presence throughout the entire genome but rarely within open reading frames and widespread occurrence among bacterial species (Stern et al., 1984; Wenzel and Herrmann, 1988; Hulton et al., 1991; Martin et al., 1992). Due to the defined primer sequences, high stringency amplification conditions can be applied in rep-PCR as opposed to RAPD analysis which employs

short arbitrary primers with low stringency PCR conditions. This study was set out to evaluate the suitability of rep-PCR for strain typing of ropy slime-producing L sake.

2. Materials and methods

2.1. L. sake strains

Seventeen L sake strains originating in spoiled vacuum-packaged meat products and representing different slime production capabilities were characterised using rep-PCR. L. sake ATCC 15521^T was used as a reference strain. The details of the phenotypic and genetic characteristics previously observed by using inoculated pack studies, ribotyping, RAPD and PFGE are presented in Table 1. Maintenance and culturing of L. sake strains were performed as described previously (Björkroth and Korkeala, 1996).

2.2. Isolation of chromosomal DNA and rep-PCR

The cultures were grown overnight in 10 ml MRS broth (Oxoid, Basingstoke, UK) at 30^{o}C. Cells were harvested from 1 to 1.5 ml by centrifuging for 2 min at full speed (about 15 000 x g) in a Biofuge A bench centrifuge (Heraeus Sephatec GmbH, Osterode am Kalkberg, Germany) to provide a 15 mg pellet (wet weight). Chromosomal DNA was isolated according to the method by Pitcher et al. (1989), with modifications described by Björkroth and Korkeala (1996).

Rep-PCR analysis was performed according to the method of Versalovic et al. (1991), with minor modifications and carefully observing factors affecting reproducibility (Tyler et al., 1997). Ready-To-Go PCR Beads™ (Pharmacia Biotech, Uppsala, Sweden) were used for PCR reactions. Two opposing degenerate primers REP1R-Dt (5'-IIINCGNCGNCATCNGGC-3') (N=A, T, C or G; I=iosine) and REP2R-Dt (5'-NCGNCTTATCNGGCCTAC-3') and two single oligonucleotide primers BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3') and RW3A (5'-TCGCTCAAAACAACGACACC-3') were evaluated using different annealing temperatures in amplifications with respect their applicability in L. sake genotyping. The aforementioned primers were selected for evaluation because they had been successfully used for typing other gram-positive bacteria (Jordens et al., 1995; Koeuth et al., 1995; Jersek et al., 1996; Cotter et al., 1997; Malamathum et al., 1998). All primers were synthesised by Pharmacia Biotech (Vantaa, Finland). Amplifications were performed in a PTC-200 thermal cycler (MJ research, Watertown, MA, USA) and the

PCR conditions for different primers were the following: for REP1R-Dt & REP2R-Dt 35 cycles of 30 s at 90°C, 1 min at 40°C and 8 min at 65 °C; for BOXA1R 30 cycles of 30 s at 90°C, 1 min at 52°C and 8 min at 65 °C; and for RW3A 45 cycles of 30 s at 90°C, 1 min at 45°C and 8 min at 65 °C. Each amplification included an initial denaturation of 7 min at 95°C and a final extension of 16 min at 65°C. The sample volume of 25 μl contained 100 ng of DNA and 50 pmol of each primer. Amplification products were electrophoresed in 2.0% (w/v) agarose gels (SeaKem I.D.N.A, FCM BioProducts, Rockland, ME, USA) in 1 x TAE buffer (Amresco, Solon, OH, USA) at 80 V for 5 h. Gels were stained for 1 h in 1.0 l of distilled water containing 0.5 mg of ethidium bromide, destained for 1 h in distilled water and photographed using standard procedures (Sambrook et al., 1989). DNA molecular weight markers II and VI (Boehringer Mannheim GmbH, Mannheim, Germany) were used as a fragment size marker. The reproducibility of the method was verified by repeating all amplifications from two isolates of the same strain a minimum of two times. Additionally, amplifications from the same isolate were repeated twice. The banding patterns were analysed visually. Strains were classified as different subtypes if a difference in the size of two or more fragments was observed. Faint bands were included in the fingerprint only if they were detected reproducibly from two isolates of the same strain.

3. Results

Primer RW3A produced 1-15 fragments of size 500-5500 bp and generated five different banding patterns among the 18 strains characterised. Its discriminatory power was equal to the ribotyping. REP-primers generated 5-13 fragments of size 200-4500 bp and faint bands were frequently observed. Primer BOXA1R generated 5-17 fragments of size 1000-9500 bp (Fig. 1). Both the REP -primer pair and the primer BOXA1R produced six different banding patterns. However, their discriminatory power differed between certain Ribogroup I strains (Table 1). Neither of the primers distinguished the slime-producing strains within group I from non-slime-producers. The best discrimination was achieved by combining the results of the primers BOXA1R and REP1R&2R-Dt, which yielded seven different subtypes (Table 1). The combined results divided the Ribogroup I strains in three different subtypes, but still the slime-producers were not distinguished from non-slime-producers. Reproducibility of the banding patterns by the primers RW3A and BOXA1R was excellent and that of the REP primer pair of moderate level.

4. Discussion

In the present study, two single oligonucleotide primers and one primer pair, all based on interspersed repetitive sequences, were evaluated for their ability to genotype a well characterised set of L. sake strains. To the authors' knowledge, this was the first attempt to characterise lactobacilli using rep-PCR. The discriminatory power of the individual primers tested was only of moderate level. When the results of two primers (BOXA1R and REP1R&2R-Dt) were combined, the level of discrimination was approximately equal to the RAPD analysis, but still inferior to PFGE (Björkroth et al., 1996). However, when the results of the two PCR-based methods, rep-PCR and RAPD, were combined, the resulting subtypes agreed reasonably well with the subtypes generated by PFGE (Table 1). Only the four non-slime-producing strains in ribogroup I (9, 10, 11, 12) were missclassified to belong to the same subtype (I) with the two strains possessing weak slime production capacity (7, 8).

The level of reproducibility of rep-analysis correlated well with the annealing temperatures of the primers tested. The single primers BOXA1R and RW3A produced highly reproducible results and also had the high optimal annealing temperatures of 52^{o}C and 45^{o}C, respectively. The optimal annealing temperature for the REP -primer pair was found to be as low as 40^{o}C and as a consequence, faint bands with low reproducibility were frequently observed. The difference in the reproducibility of the primers tested may reflect the occurrence and distribution of different interspersed repetitive sequences in the lactobacilli genomes. Gillings and Holley (1997) suggested that rep-PCR performed on non-enterobacterial targets may not necessarily be directed at genuine repetitive sequences when primers originating in repetitive sequences of enterobacterial species are used. They considered the method to be a variant of RAPD analysis. Repetitive extragenic palindromes (REP) were originally described in Gram-negative enteric bacteria, Escherichia coli and Salmonella typhimurium (Stern et al., 1984). BOXA1R and RW3A, on the other hand, are derived from BOX elements of Streptococcus pneumoniae (Martin et al., 1992) and RepMP3 sequences of Mycoplasma pneumoniae (Wenzel and Herrmann, 1988), respectively. The results of our study suggest that BOX and RepMP3 sequences seem to occur in the lactobacilli genomes although their presence should be confirmed

using DNA probe hybridisation. Based on the reasonably low discriminating capacity of the primers BOXA1R and RW3A, the sequences in question may be present in rather low numbers.

The results of the present study suggest that adequate level of discrimination among L. sake strains can be achieved by using the combination of rep-PCR and RAPD analysis. The problems in reproducibility can be overcome for the most part by the parallel analysis of two isolates from each strain when primers with lower reproducibility are used. As compared to time-consuming and laborious PFGE, the definite advantages of PCR-based typing methods are the rapid and easy performance and fairly inexpensive cost. Therefore, they are particularly suitable for large-scale analyses, such as contamination studies in manufacturing plants. The number of strains requiring PFGE analysis can be substantially decreased, when only a small subset of isolates will require further discrimination.

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Table 1. Details of the earlier determined phenotypic and genetic characteristics of the L. sake strains used in the present study and the different genetic subtypes generated by rep-PCR analysis using three different primers

Strain	Slime production capability[1]	Ribo-group[2]	Bacterial type[3]	rep-PCR				RAPD[4] + rep -type
				BOXA1R	REP1&2R-Dt	RW3A	rep-type	
1	+++	I	I	B1	R1	W1	I	I
2	+++	I	I	B1	R1	W1	I	I
3	+++	I	II	B2	R1	W1	II	II
4	++	I	IV	B1	R2	W1	III	III
5	++	I	IV	B1	R2	W1	III	III
6	++	I	IV	B1	R2	W1	III	III
7	+	I	V	B1	R1	W1	I	IV
8	+	I	V	B1	R1	W1	I	IV
10	-	I	VI	B1	R1	W1	I	IV
11	-	I	VII	B1	R1	W1	I	IV
12	-	I	VII	B1	R1	W1	I	IV
13	+++	II	VIII	B3	R3	W2	IV	V

14	+++	II	VIII	B3	R3	W2	IV	V
15	+	III	IX	B4	R4	W3	V	VI
16	+	III	IX	B4	R4	W3	V	VI
17	++	IV	X	B5	R5	W4	VI	VII
18[5]	-	-	XIV	B6	R6	W5	VII	VIII

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[1] As determined by Björkroth et al. (1996); categories are based on the amount of slime produced: negative (-), some (+), moderate (++), and abundant (+++)

[2] As determined by Björkroth and Korkeala (1995)

[3] As determined by Björkroth et al. (1996) by combining the results of ribotyping, PFGE and RAPD. Bacterial type is virtually the same as PFGE-type.

[4] As determined by Björkroth et al. (1996)

[5] L. sake strain ATCC 15521^{T}

Figure captions

Fig. 1. Patterns produced by primer BOXA1R showing the following rep-types: lanes 1, 2, 3, 4, 7, 8, 9, 11, 12, 13 and 14, type B1; lane 6, type B2; lanes 16 and 17, type B3; lanes 18 and 19, type B4; lane 21, type B5; lane 22, type B6; lanes 5, 10, 15 and 20, molecular weight marker II.