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Molecular characterization of *Lactobacillus casei* strains

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Abstract

Three different molecular techniques were used to characterize bacterial strains isolated from dairy products and phenotypically classified as *Lactobacillus casei*. Some strains were taxonomically identified as *Lb. casei* and *Lb. paracasei* using 23S rRNA-targeted probes specific for *Lb. casei* and *Lb. paracasei*. Chromosomal DNA was analyzed by pulsed-field gel electrophoresis. *Lb. paracasei* strains had a variable number of *Sfi*I fragments harboring rRNA genes, while *Lb. casei* strains showed more homogeneous patterns. The chromosome size of *Lb. paracasei* ATCC 334 was evaluated to be 2.17 Mb.

Keywords: *Lactobacillus casei*; Pulsed-field gel electrophoresis; Chromosomal analysis; Ribotyping

1. Introduction

Lactobacillus casei is a group of heterogeneous lactic acid bacteria, able to colonize various natural and man-made environments. Strains of this species are of great interest as starters to improve food quality, and able to maintain the microbiological balance of several habitats [1]; moreover *Lb. casei* is present in Grana cheese mostly as secondary microflora [2]. The taxonomy of the *Lb. casei* group is still under study; genetic tests, such as DNA-DNA hybridization, may give a proper classification of strains, but not a quantitative evaluation of the homology among strains of the same species [3].

A number of molecular techniques are now available, allowing more precise taxonomic classification and differentiation of lactic acid bacteria isolates at strain level [4,5].

The aim of this work was the taxonomic classification of four previously selected strains using the 23S rRNA directed oligonucleotide probes available for the differentiation between *Lb. casei* and *Lb. paracasei* [6] and the strain typing of *Lb. casei* and *Lb. paracasei* by means of genomic fingerprinting and pulsed field gel electrophoresis (PFGE) analysis.

2. Materials and methods

2.1. Strains and culture conditions

The strains (5Mn373, 3So129, 4St304, 1St252) used in this study were isolated from Grana cheeses of various Po Valley factories, at different ripening times, phenotypically identified as *Lb. casei* [2] and analyzed for plasmid profile [7]. They were maintained in stock collection at −80°C and, when re-

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quired, subcultured in MRS medium (Oxoid). The type strain *Lb. casei* ATCC 393 and *Lb. paracasei* ATCC 334 were also employed as reference. Three other strains (3Mn351, 6Me211 and 4St293) isolated from Grana cheese as well [2], and previously showing to have an identical phenotypic and plasmid pattern, were investigated by PFGE analysis.

2.2. Chromosomal DNA preparation

Cultures were grown overnight at 32°C in MRS medium up to 0.6 OD_{560}. DNA was prepared according to the Marmur procedure [8] and digested with *Hin*dIII restriction endonuclease according to the supplier's instructions (Boehringer Mannheim, Germany).

2.3. Preparation of genomic DNA in situ in agarose blocks

Genomic DNA in agarose blocks was obtained from 4.5 ml exponential phase culture, serially washed in 50 mmol l^{-1} EDTA, 0.5% SDS, sterile milliQ water and 1 mol l^{-1} NaCl, 10 mmol l^{-1} Tris pH 7.6, then resuspended in 600 μl of the last solution, mixed with 600 μl of 2% low melting point agarose (Sigma) and solidified in moulds. Cells were lysed in situ by 6 mmol l^{-1} Tris chloride pH 7.6, 1% Sarcosyl, 10 mg ml^{-1} lysozyme, 50 U ml^{-1} mutanolysin, 28 μg ml^{-1} RNase for 18 h at 37°C. Restriction digestion of DNA was achieved using 40 U of the following enzymes according to the suppliers' instructions: *Sma*I (Promega) at 30°C; *Bgl*I at 37°C; *Sfi*I at 50°C (Boehringer).

2.4. Pulsed-field gel electrophoresis

DNA fragments were separated in 0.9–1.1% (w/v) agarose gels using TBE (45 mmol l^{-1} Tris, 45 mmol l^{-1} boric acid (pH 8.3) and 1 mmol l^{-1} sodium EDTA) in a CHEF MAPPER BioRad apparatus over a range of migration conditions. Different pulse times (1–20; 1–5; 40–80 s) were adopted to obtain optimal separations of fragments ranging in size from 50 to 240, 8 to 100 and 200 to 800 kb respectively. *Sma*I digested genomic DNA from *Lactococcus lactis* MG 1363 [9] was used as molecular size standard.

2.5. Probe labeling

Two oligonucleotide probes complementary to a species-specific region of the 5′-terminus of the 23S rRNA genes for *Lb. casei/Lb. rhamnosus* and *Lb. paracasei*, described by Hertel et al. [7], were purchased from Primm Srl (Milan, Italy) and labelled with DIG Oligonucleotide 3′-End Labeling Kit (Boehringer), according to the manufacturer's instructions.

2.6. Southern blotting and hybridization

PFGE gels were treated with 250 mmol l^{-1} HCl for 15 min, gently shaken and transferred to nylon membranes (Boehringer) by Southern blotting [10]. DNA was bound to membranes by ultraviolet cross-linking. Membranes were prehybridized for 2 h at 68°C in a buffer containing 5 × SSC (20 × SSC: 3 mol l^{-1} NaCl; 0.3 mol l^{-1} Na-citrate pH 7), 1% blocking reagent (Boehringer), 0.1% *N*-laurylsarcosyne, 0.02% sodium dodecylsulfate (SDS).

The probe was then added and hybridization was allowed to proceed overnight at 42°C. The membrane washing steps were: two times in 2 × SSC, 0.1% SDS for 5 min at room temperature and two times in 0.1 × SSC, 0.1% SDS for 15 min at 53°C. Chemiluminescent detection was performed by the Boehringer kit, according to the manufacturer's instructions.

3. Results

3.1. Taxonomic recognition and ribotyping

*Sfi*I digested genomic DNA of the considered strains, separated by PFGE, were transferred on a nylon membrane by Southern blot. The hybridization performed employing an oligonucleotide probe complementary to 23S rRNA species-specific region of *Lb. paracasei*, recognized the type strain ATCC 334 and demonstrated that the strains 5Mn373 and 3So129 belong to *Lb. paracasei* species.

Moreover, this technique showed that the genomic DNA of these three strains contained three to five *Sfi*I fragments harboring *rrn* gene(s), located in different positions (Fig. 1). A subsequent hybridiza-

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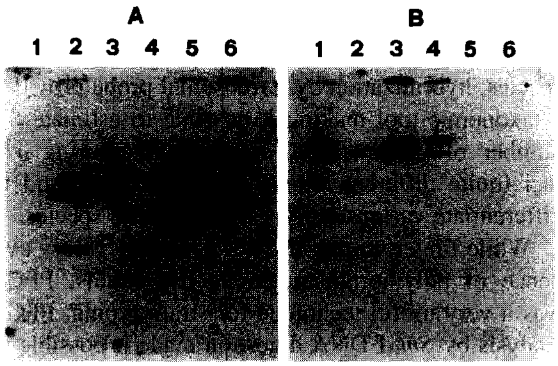

Fig. 1. Southern blot of *Lb. casei* DNA hybridized with 23S rRNA-targeted oligonucleotide probes. DNA samples were digested with *Sfi*I. In (A) *Lb. paracasei*-specific 23S rRNA oligonucleotide probe was used, in (B) *Lb. casei / rhamnosus*-specific 23S rRNA oligonucleotide probe. Lanes: 1 = *Lb. casei* ATCC 393; 2 = *Lb. paracasei* ATCC 334; 3 = 4St304; 4 = 1St252; 5 = 3So129; 6 = 5Mn373.

tion utilizing the oligonucleotide probe group-specific for a variable region of 23S rRNA of *Lb. casei / Lb. rhamnosus* showed that the remaining strains ATCC 393, 4St304 and 1St252 belong to one of these species; as strain 4St304 fermented rhamnose [2], it was ascribed to the *Lb. rhamnosus* species. Moreover, in *Lb. casei* ATCC 393, 4St304 and 1St252

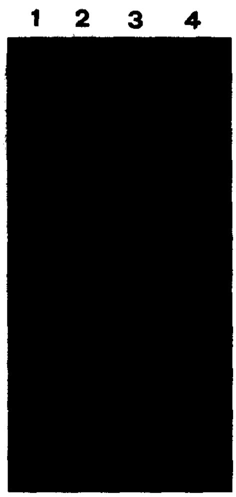

Fig. 2. PFGE patterns of *Sma*I digested genomic DNA of *Lb. casei* and *Lb. paracasei* strains. Lanes: 1 = 4St304; 2 = 1St252; 3 = 5Mn373; 4 = 3So129. Running conditions: electrophoresis was performed on a 1.1% agarose gel for 20 h at 11°C, with a pulse time increasing linearly from 1 to 3 s, operating at 6 V cm^{-1}.

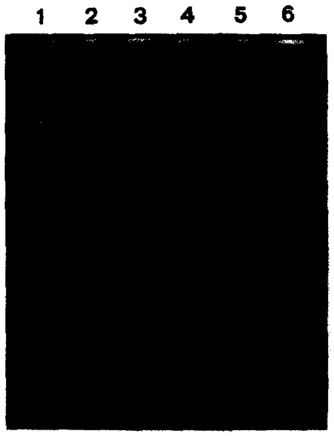

Fig. 3. PFGE patterns of *Sfi*I digested genomic DNA of *Lb. casei* and *Lb. paracasei* strains. Lanes: 1 = *Lb. casei* ATCC 393; 2 = *Lb. paracasei* ATCC 334; 3 = 4St304; 4 = 1St252; 5 = 3So129; 6 = 5Mn373. Running conditions: electrophoresis was performed on a 0.9% agarose gel for 24 h at 11°C, with a pulse time increasing linearly from 1 to 20 s, operating at 4.5 V cm^{-1}.

strains, only two *Sfi*I fragments containing *rrn* gene(s) were found.

3.2. Genomic fingerprinting

Restriction patterns of *Hin*dIII digested genomic DNA from four *Lactobacillus* strains (5Mn373, 3So129, 4St304 and 1St252) and the two type strains *Lb. casei* ATCC 393 and *Lb. paracasei* ATCC 334 showed some polymorphism, difficult to interpret (data not shown).

3.3. PFGE analysis

Further analyses were carried out to differentiate *Lactobacillus* strains by the use of rare-cutting restriction enzymes to digest chromosomal DNA and

Table 1
Sizes of *Sfi*I restriction fragments of genomic DNA from *Lb. paracasei* ATCC 334

Pulse time (s)	Fragment size (kb)
1–5	18.4, 23.5, 31, 42.6
1–20	62.6, 80.7, 101, 101, 109.5, 124, 126, 130
40–80	220, 446.8, 558
	Genome size [a] (kbp) 2175

[a] Sum of the sizes of the individual fragments.

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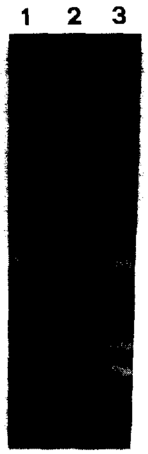

Fig. 4. PFGE patterns of *Sfi*I digested genomic DNA of *Lb. casei* strains. Lanes: 1 = 3Mn351; 2 = 6Me211; 3 = 4St293. Running conditions: PFGE was performed on a 0.9% agarose gel for 20 h at 11°C, with a pulse time increasing linearly from 40 to 80 s, operating at 6 V cm^{-1}.

PFGE to separate DNA fragments. The restriction enzymes *Sma*I, *Bgl*I and *Sfi*I were utilized, as they recognize G + C rich sequences. Restriction patterns obtained by *Bgl*I digestion showed some differences among the strains, detectable by densitometric analysis only (data not shown). *Sma*I digestion patterns of the two species *Lb. casei* and *Lb. paracasei*, analyzed by PFGE and densitometric analysis, revealed further differences among the strains, while *Sfi*I digestion revealed a marked polymorphism among the strains of the same species. The PFGE patterns are shown in Figs. 2 and 3. PFGE migrations carried out at three different pulse times, allowed to estimate that the chromosomal size of ATCC 334 was 2.17 Mb (Table 1).

Three other strains previously identified as *Lb. casei* (3Mn351, 6Me211 and 4St293), analyzed by PFGE, showed a clear polymorphism; this result is noteworthy as these strains were phenotypically indistinguishable and all of them harbored a single 25 MDa plasmid (Fig. 4).

4. Discussion

A deeper typing of strains previously identified as *Lb. casei* was obtained by the molecular techniques employed in this study. Species-specific oligonucleotide probing made it possible to discriminate between *Lb. casei / rhamnosus* and *Lb. paracasei* species; hybridization by a ribosomal probe provided a taxonomic tool making it possible to estimate the number of chromosomal fragments harboring *rrn* loci (quite different among the eubacteria), and to differentiate various biotypes of the same species.

While fingerprinting did not give satisfactory evidence of polymorphism among the strains, PFGE was a very useful technique for strain typing. PFGE analysis by *Sma*I DNA digestion made it possible to differentiate the strains into two groups with similar patterns, corresponding to *Lb. casei / rhamnosus* and *Lb. paracasei* species.

Subsequent analysis of *Sfi*I patterns by PFGE showed further genome variations, so demonstrating that also by PFGE alone it is possible to type the strains. The observed differences in the patterns may be due partly to point mutations produced by endonuclease sites in different locations of the genome, partly to chromosomal rearrangements such as deletions, insertions and inversions.

In the present research, PFGE carried out at different electrophoretic conditions made it possible to estimate for the first time the apparent genome size of *Lb. paracasei* species; the type strain ATCC 334 showed a chromosome size of about 2.17 Mb, which is in accordance with the sizes already determined for other lactic acid bacteria [11]. PFGE analysis was further useful to discriminate among three strains isolated from Grana cheese, as well, all harboring a single 25 MDda plasmid and physiologically indistinguishable.

Acknowledgements

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