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DEVELOPMENT OF STRAIN DISCRIMINATIVE AMPLIFIED FRAGMENT LENGTH POLYMORPFIC DNA FOR BIFIDOBACTERIA. DESIGN OF STRAIN-SPECIFIC MARKERS

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ABSTRACT
Strain-discriminative differentiation of Bifidobacteria strains by help of Amplified Fragment Length Polymorphism (AFLP) with satisfactory discriminative power and reproducibility was developed. AFLP genotyping is based on restriction cleavage of DNA with enzyme couple Xho I and Taq I, specially designed adapters, preselective and selective PCR primers. AFLP derived fragments were used successfully as a source of strain-specific markers for one probiotic Bifidobacterium longum strain, and subsequently, based on their specific sequences - for design of strain-specific probe. It's specificity was confirmed upon 70 Bifidobacteria strains.

Keywords: Bifidobacteria, AFLP, strain-specific markers

Introduction
Several health-promoting effects are attributed to *Bifidobacteria* as a major part of the normal intestinal microbiota. Because of the probiotic properties are strain-specific, the use of reliable and discriminative molecular methods is very important. Various molecular techniques have been used to characterize *Bifidobacteria* spp. such as cell-wall and whole cell protein analysis, RAPD, ribotyping, various restriction fragment length polymorphism (RFLP) techniques, PFGE, etc. (2, 3, 11). RAPD is rapid, cheap and an easy performable method. However, amplification techniques, such as RAPD, have several drawbacks, such as reproducibility of the patterns between the laboratories (6). During the last two decades PFGE has been used for genotyping of various *Bifidobacteria* spp. such as *B. longum, B. bifidum, B. infantis, B. adolescentis, B. catenulatum,* ext (7, 8) and it appears to be one of the molecular techniques with the greatest discriminative power available. However, PFGE is labor-intensive and is difficult to adapt for automation. More recently AFLP approach has been developed and evaluated for genomic characterization of bacteria (4, 9). This technique is based on selective amplification with primers that recognize adapters ligated to the ends of DNA fragments derived after restriction with two endonucleases. AFLP produces highly discriminative and complex banding patterns and is not so time consuming and labor-intensive as PFGE.

The aim of this study was to develop AFLP technique appropriate for typing of *Bifidobacteria* spp. strains, as well as a new method for development of strain-specific DNA markers.

Materials and Methods

Bacterial strains
Bifidobacteria strains from faecal samples of twenty healthy donors were isolated in an anaerobic chamber (nitrogen 85%, carbogen dioxide 10%, and hydrogen 5%). Briefly, 1 g faecal sample was diluted ten times in BL broth (BBL), supplemented with 0.05% (w/v) L-cysteine. Appropriate ten-fold dilutions were plated onto BL agar. The strains were maintained in BL broth at –80˚C in the presence of 15% glycerol. Working cultures were prepared by three subsequent overnight transfers into BL broth at 37˚C. The strains were characterized at species level by help of API 50CH carbohydrate tests (API Products, Bio-Merieux, France), ARDRA with enzymes *Hae* III, *Alu* I (1), applying of species-specific PCR primers (5). The profiles received after ARDRA and species-specific PCR were compared with the corresponded patterns of reference strains. Type strains from American Type Culture Collection (ATCC) were used: *B. bifidum* ATCC 29521, *B. longum* ATCC 15707, *B. breve* ATCC 15700, *B. adolescentis* ATCC 15703, *B. animalis* ATCC 25527, *B. infantis* ATCC 15697, *B. catenulatum* ATCC 27539.

Amplified fragment length polymorphism
Chromosomal DNA was extracted and purified from overnight BL broth cultures according to the method described by Walter *et al*. (10). 50 ng DNA from every strain was cleaved with 1U of restriction enzyme *Taq* I at 55˚C for 2 h and after addition of 1 U of *Xho* I the incubation of the final volumes of 10 µl was continued at 37˚C for additional 2 h. The enzymes (Boehringer Mannheim) were inactivated at 80°C for 20 min. and the preparations were mixed with 10 µl ligase mix (1U of DNA ligase from Amersham Biosciences, 2 µl of buffer supplied (660 mM Tris-HCl, pH 7.6, 66 mM $MgCl_2$, 100 mM dithiothreitol, 0.66 mM ATP), 2.5

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pmol *Xho* I adapter, 25 pmol *Taq* I adapter and incubated at 20˚C overnight. The adapter oligonucleotide sequences were 5'-CTCGTAGACTGCGTACC-3' and 5'-TCGAGGTACGCAGTC-3' (for *Xho* I sites), and 5'-CGGTCAGGACTCATC-3' and 5'-GACGATGAGTCCTGAC-3' (for *Taq* I sites). Tenfold dilution was performed with 0.1x TE buffer (10 mM Tris-HCl, pH 8, 0.1 mM EDTA) and 1 µl was used for preselective PCR. Preselective PCR was performed in 10 µl reaction mixtures containing 1 µl of 10x PCR buffer II (Perkin Elmer), 1.5 mM $MgCl_2$, 0.2 mM concentration of dNTPs and 0.25 U of Amply*Taq* DNA polymerase (Perkin Elmer). The primers used were 5'-GACTGCGTACCTCGAG-3' for *Xho* I adapter with 0.25 µM concentration and 5'-GATGAGTCCTGACCGA-3' for *Taq* I adapter with 1.25 µM concentration, respectively. The preselective PCR conditions were as follows: 30 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min. After 50 times dilution of the samples with 0.1x TE, 2 µl of the dilutions were used for the selective PCR in 10 µl volumes containing 1 µl of 10x PCR buffer II (Perkin Elmer), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 U of Amply*Taq* DNA polymerase (Perkin Elmer) and primers with selective base –**T** at the 3` ends. The use of these selective primers showed higher discriminatory power than after using the primers ending with other (no T) nucleotide bases. The selective PCR consisted of 13 cycle touch-down phase with decrease of the annealing temperature from 65°C to 56°C at step 0.7°C. After that 30 cycles were performed at the preselective PCR conditions. The samples were denaturated with an equal quantity of loading solution (95% (v/v) formamide, 20 mM EDTA, pH 8.0, 0.05% (w/v) xylene cyanol) at 95˚C for 5 min and immediately cooled on ice. A volume of 3 µl was applied to a 4% (3% bis-acrilamide) 40 cm long 0.4 mm polyacrylamide gel containing 8 M urea (Amersham) and 0.5x TBE buffer. The power was maintained constant at 55W and temperature was about 50°C. The gel was stained with the silver staining kit (Promega) according to the instruction of the producer. After drying the gels were photographed with a digital camera and analyzed by help of GelCompar 4.0 software. Dice coefficient and UPGMA method were used for the genetic similarity evaluation. PCR 100 bp low ladder (Sigma) was used as a molecular weight standard.

Development of strain-specific DNA markers

The target strain was the probiotic strain *B. longum* 1/20. The first stage from the process of strain-specific marker development was preparative AFLP for *B. longum* 1/20 with initial DNA quantity of 1 µg. A set of selective AFLP processes were performed using different combination of selective bases. The received AFLP fragments were used for preparation of AFLP library by help of cloning kit (Promega). The ligase reaction for insertion of fragments to the hydrolyzed by *Sma I* plasmid pGEM and all other reactions were performed according to the instructions of the supplier of the cloning kit.

After electroporation and inoculation the white colonies were picked up and cultivated in TPY broth. The AFLP fragments were reamplified directly from the colonies using the corresponding selective PCR primers and AFLP conditions except the initial denaturation was increased to 95°C for 5 min. The received PCR fragments were amine-bonded at 5'-ends and covalently fixed onto the 96 well microplates by help of Corning DNA-BIND Surface kit according to the supplier's instructions. The produced DNA arrays from AFLP fragments were hybridized with hydrolyzed DNA mix from 68 different *Bifidobacterium* strains. The combined DNAs were hydrolyzed by the same endonucleases used in AFLP – *Xho I* and *Taq I*. The received DNA fragments after hydrolysis were labeled with Cy5 dye (Amersham) as it was explained by the producer. The hybridization was performed using hybridization buffer from AlkPhos Direct kit (Amersham) with addition of 0.7 M NaCl. The concentration of labeled DNA fragments was 10 ng/ml and they were denaturized at 100°C for 5 min. The hybridization was performed overnight at 65°C. The first- and second-washing buffers were from the AlkPhos Direct kit. The fluorescence of the wells on microplates was read on Perkin Elmer fluorescence meter using 620 nm excitation and 670 nm emission light length. The AFLP fragments which correspond to these wells where the fluorescence signal is zero were selected for approval of their strain-specificity by help of hybridization on the individual DNA spots from many different *Bifidobacterium* strains.

Evaluation of strain-specificity of the selected AFLP fragments

The selected AFLP fragments with zero fluorescence signals to DNA mix from 68 *Bifidobacterium* strains were labeled with thermostable alkaline phosphatase according to the instructions of the AlkPhos Direct kit (Amersham). The NBT/BCIP mix was used for signal development after hybridization. The DNA from every strain was isolated and purified according to Walter *et al*. 2000. DNAs were denaturized under alkaline conditions and spotted onto N+ membrane according to the instruction of the producer (Amersham). The selected and labeled AFLP fragments after denaturation at 100°C for 5 min were used as probes to the spotted on the membrane Bifidobacteria DNAs.

Results and Discussion

Evaluating different genotyping techniques, several criteria have to be taken into account: discriminatory power, reproducibility, interpretability, rapidity, easy way of the

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performance, and the cost. AFLP (**Fig. 1**) was the most discriminative strain typing method, revealing the highest number of strain-specific profiles for *Bifidobacterium* strains comparing to Pulsed Field Gel Electrophoresis (PFGE) and RAPD techniques. There was not one successfully discriminated isolate with PFGE, which was indistinguishable by AFLP (data not shown). Even more, the developed AFLP could differentiate closely related strains indistinguishable by PFGE.

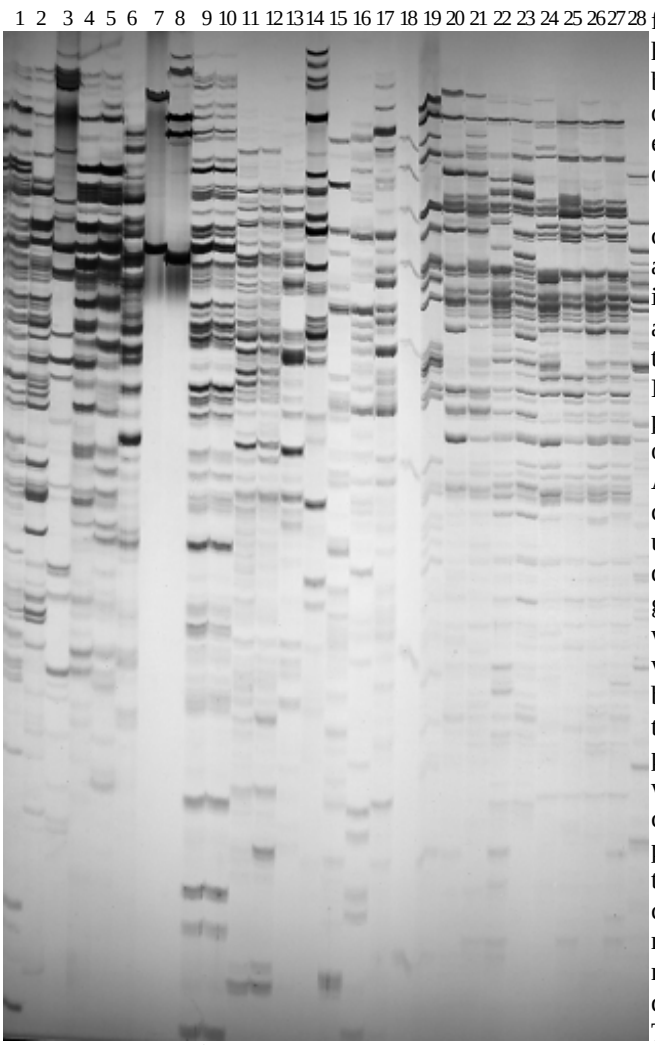

Fig. 1. AFLP image after silver staining. Lane 18 – PCR low ladder 100 bp molecular weight marker, lanes 1 to 6 – *B. breve* strains, lanes 7 to 8 – *Lb. bulgaricus* strains, lanes 9 to 17 – *B. longum* strains, line 19 – *B. infantis* strain, lines 20 to 27 – *B. bifidum* strains, line 28 *B. adolescentis* strains

As pointed out by Janssen et al. (4), AFLP appears to be highly influenced by the species under study, and optimization of the method is needed for each species. Therefore, we initially searched for an appropriate set of two restriction enzymes whose application resulted in a sufficiently high number of fragments covering a range of 100-1000 bases. This is a higher molecular range compared with the classical AFLP using enzymes *EcoR* I and *Mse* I. Our attempts were inspired by the necessity to obtain a better image with the silver staining. In order to obtain better resolution between fragments with this enlarged molecular range the percentage of bis-acrylamide was decreased to 3%. In previous experiments we found that the profiles of strains with a GC content higher than 50% as *genus Bifidobacterium* were better distributed on the AFLP gel, covering the desired size range of 100-1000 bases, with the enzymes used in the present study than those obtained with *EcoR* I and *Mse* I.

Silver staining gives the advantage over the classical AFLP, that all of the amplified fragments are visualized on the gel, not only those incorporating the labeled primer annealed to the adaptor to six-cutting enzyme site. This fact helps to increase the number of fragments on the gel. Both methods - PFGE and AFLP offer the possibility to select the best restriction enzymes in order to obtain better discrimination. However, AFLP has an additional advantage – the option to combine information from subsets of amplicons, using different selective primers. PFGE could display the whole genome DNA fragmented on the gel, but considering their very high molecular weight, some differences between the fragments with apparently equal molecular weight could not be revealed. This risk is considerably smaller with the much shorter AFLP fragments. AFLP had the potential to distinguish very closely related strains which were identical by PFGE. Because of the complex high number banded low-molecular patterns AFLP would be beneficial for monitoring the genetic stability. Genetic events related to small changes in the genome DNA may not always be revealed by PFGE, considering the fact that PFGE might not detect resolution between fragments with distance of several base pairs, in contrast to AFLP. The applicability of AFLP was compared to PFGE because PFGE is considered to be a gold standard for molecular typing at strain level, showing great polymorphism between isolates and excellent reproducibility. On the basis of this study, we could conclude that AFLP combining the advantages of a higher discriminatory power, rapidity, and less labor consumption is successful alternative to PFGE. By help of the developed AFLP method *Bifidobacterium* spp. strains can be successfully identified.

AFLP derived fragments were used successfully as a source of strain-specific DNA markers towards one probiotic Bifidobacteria strain. In order to study the strain-specificity of certain AFLP fragment it is necessary to prove the uniqueness of the fragment towards many strains from the same bacterial species. Searching such fragment we used DNAs from 68 different Bifidobacterium strains which were hydrolyzed

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with the same enzymes– *TaqI* и *XhoI* because other enzymes could break strain-specific sequences. If certain AFLP derived fragment from the targeted strain contains strain-specific sequences it will not hybridize with any fragments in the DNA from the rest strains. Thus, in the corresponding well in the DNA arrays from AFLP fragments the fluorescence signal will be zero. The fragments demonstrating lack of any hybridization signal were selected for further approval of their specificity. Following the upper procedure seven strain-specific fragments for *B. longum* 1/20 were found and the conformation of strain-specificity of one of them is shown on **Fig. 2**. On the figure the lack of any hybridization with dot-blotted DNAs from other 68 Bifidobacterium strains is clearly demonstrated. Simultaneously, the selected strain-specific fragment proved strong hybridization signal with DNA from *B. longum* 1/20 isolated from different sources.

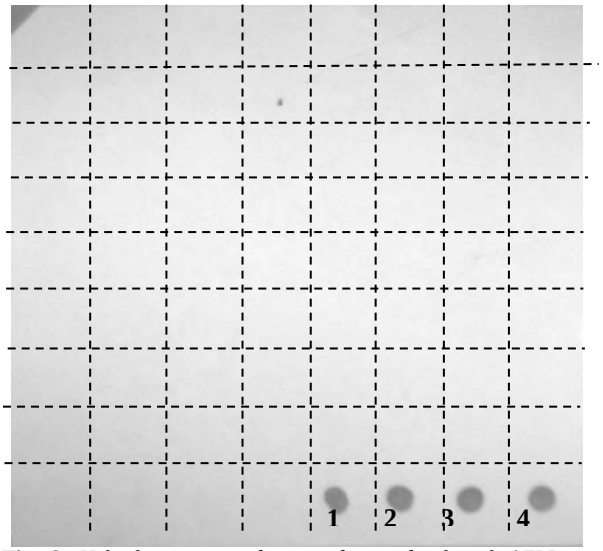

Fig. 2. Hybridization test for specificity of selected AFLP derived fragment from *B. longum* 1/20 against DNA dot-blots from 68 different *Bifidobacterium* strains. Cell 1 – total DNA isolated from fermented milk product containing *B. longum* 1/20, cells 2 and 4 – DNA from *B. longum* 1/20, cell 3 – hydrolyzed with XhoI and TaqI DNA from *B. longum* 1/20. Every other cell contains dot-blotted DNA from one of the rest 68 different *Bifidobacterium* strains

Conclusions

The developed AFLP method for DNA typing of Bifidobacterium strains proved excellent discriminative power and reproducibility. By help of the developed AFLP method *Bifidobacterium* strains can be successfully identified.

The developed method for design of strain-specific DNA markers could be applied to every strain with beneficial properties. The derived specific DNA fragments could be applied as strain-specific probes in order to evaluate the presence of certain strain with probiotic properties in complex microbial matrixes.

Acknowledgments

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