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Chromosomal location of the genetic determinants for bacteriocins produced by *Lactobacillus gasseri* K7

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The production of similar or even identical bacteriocins by different lactic acid bacteria is not a rare event. To take advantage of this finding, genetic determinants of the *Lactobacillus* K7 bacteriocins were tested for putative homologies with previously described bacteriocins of the *Lactobacillus acidophilus* group through polymerase chain reaction (PCR). Among specific primer pairs of seven known bacteriocins, derived from their respective sequences, only acidocin LF221 A and B primers amplified fragments in chromosomal DNA of K7 strain that revealed strong similarity over small regions of LF221 bacteriocins. Treatment of *Lactobacillus* K7 with ethidium bromide and mitomycin C was ineffective in generating non-bacteriocinogenic derivatives and had no impact on plasmid loss either. Classification studies elucidated *Lactobacillus* K7 as a member of the *Lactobacillus gasseri* species.

Keywords: Bacteriocin, *Lactobacillus gasseri*, *Lactobacillus acidophilus* group, PCR.

Lactic acid bacteria (LAB) colonize parts of the human and animal body, and environments where spontaneous fermentations of carbohydrate-containing substrates occur (Teuber et al. 1999). To survive and persist in such microbially diverse ecological niches, LAB produce various antibacterial substances, including bacteriocins. Since food safety and bioconservation have become an increasingly important concern all over the world, the application of bacteriocins or LAB that produce bacteriocins with wide range of inhibitory activity, has received great attention and many efforts have been directed towards isolation of new bacteriocins (Stiles, 1996; Cleveland et al. 2001). After the successful screening for bacteriocin producing strains, the characterization commonly begins with biochemical analyses. As a result of this approach all the research efforts are often in vain because gene alignments can reveal certain homologies or even identities between newly identified and previously described bacteriocin(s) (Remiger et al. 1996; Van Belkum & Stiles, 2000).

LAB commonly harbour plasmid-borne genetic determinants for bacteriocin production and for maintaining immunity of the producer cells to their bacteriocins (Klaenhammer, 1993). Yet, there have been reports suggesting that chromosomal determinants may be involved as well (Barefoot & Klaenhammer, 1983; Joerger & Klaenhammer, 1986; Kawai et al. 1998, 2000). Such statements most often implicate genome stability in curing experiments and/or the absence of plasmid DNA.

Up to the 1980s, most of the *Lactobacillus* species isolated from intestines were classified as *Lb. acidophilus,* based on their morphological and phenotypic characteristics. With the development of modern taxonomy based on molecular techniques, six distinct species within the group of previously termed *Lb. acidophilus* were identified and further separated into A (*Lb. acidophilus, Lb. amylovorus, Lb. crispatus, Lb. gallinarum*) and B (*Lb. gasseri, Lb. johnsonii*) DNA homology group (Johnson, 1980). The hypervariable 16S-23S intergenic spacer regions as well as 16S rRNA gene sequences were found to be sufficiently specific for differentiation between closely related *Lactobacillus* species, either by the use of species-specific PCR primers or by DNA-sequencing (Tannock et al. 1999; Kullen et al. 2000).

A wide inhibitory spectrum of *Lactobacillus* K7 was described previously (Bogovič Matijašić & Rogelj, 1999, 2000). The bacteriocin production studies were conducted and K7 bacteriocins were analysed for activity against different gram positive bacteria. The sensitivity of different *Clostridium* species such as *Cl. difficile, Cl. perfringens* and *Cl. tyrobutyricum* is particularly interesting. Due to the human origin of the strain, some probiotic properties of K7 strain such as resistance to low pH and bile were tested as well (Bogovič Matijašić & Rogelj, 2000). Here we report the preliminary characterization of K7 bacteriocins. We used the PCR and specific primers for some known bacteriocins

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Table 1. Primers used in our study, and their characteristics

	5′-3′ Sequence (forward/reverse)	Tm (°C)	Expected length (bp)
acidocin A			
acA-231	TGG TGT GCA TTG TAC T	46·6	96
acA-326	TTG ATC GGC AAC GAT T	46·6	
acidocin B			
acB-1308	AGA TGC AGT GGC TTC T	49·2	73
acB-1380	CCA TGC AGG TAA TGT C	49·2	
gassericin A			
gaA-515	GAC CAC AGC GAA CAT T	49·2	102
gaA-616	AAT GAG GCA CCA GAA G	49·2	
gassericin T			
gaT-950	GGA GTA GGT GGA GCG ACA GT	61·4	126
gaT-1075	TCC ACC AGT AGC TGC CGT TA	59·4	
lactacin F			
laF-828	ACC TGC ATG TGC TGT A	49·2	70
laF-897	ACC TGT TGC AGC TGT A	49·2	
acidocin LF221 A			
LFA-185	GTT GCA GGA TCA TGT G	49·2	84
LFA-268	TGT TGC AGC TCC GTT A	49·2	
acidocin LF221 B			
LFB-1193	GGA GCT ACT CGC GGA GTA AG	61·4	70
LFB-1262	CTC CTA ACG CAC AGG CAG TC	61·4	

produced by related species to shortcut the commonly used approach of bacteriocin characterization and to avoid the needless work if the bacteriocin(s) produced by K7 are identical to bacteriocins already described.

Materials and Methods

Bacterial strains, culture media and growth conditions

Lb. gasseri K7 and *Lb. gasseri* LF221 are babies' faeces-isolates deposited in a Culture Collection at the Chair of Dairy Science, University of Ljubljana, Slovenia. *Lb. johnsonii* ATCC 11506, together with *Lb. gasseri* LF221, served as positive controls when necessary. *Lb. sakei* NCDO 2714 was applied as bacteriocin sensitive indicator strain. The strains were propagated in MRS broth (Merck, 64271 Darmstadt, Germany) under microaerophilic atmosphere (Genboxmicroaer, Bio-Merieux, France) at 37 °C, except for *Lb. sakei* which was grown at 30 °C.

DNA isolation

Chromosomal DNA was isolated from lactobacilli as described by Leenhouts et al. (1990) while small scale isolations of plasmid DNA were conducted according to the protocol of Klaenhammer (1984).

Primer selection and PCR program

Specific primers, amplifying segments of different bacteriocin-encoding genes of selected lactobacilli, were applied in PCR reactions. The bacteriocins produced by the members of *Lb. acidophilus* group were selected. Therefore, bacteriocin specific primers were constructed within the structural genes of acidocin A produced by *Lb. acidophilus* TK9201 (Kanatani et al. 1995), acidocin B of *Lb. acidophilus* M46 (Leer et al. 1995), gassericin A of *Lb. gasseri* LA39 (Kawai et al. 1998), gassericin T of *Lb. gasseri* SBT 2055 (Kawai et al. 2000), lactacin F of *Lb. acidophilus* 11088 (*Lb. johnsonii* ATCC 11506) (Muriana & Klaenhammer, 1991), and acidocins LF221 A and B of *Lb. gasseri* LF221 (Čanžek Majhenič, 1998). Primer sequences, melting temperatures (Tm's) and expected length of PCR amplicons are listed in Table 1.

PCR reactions were carried out in a Biorad thermocycler (Biorad, D-80901 München, Germany). Regarding the Tm of primers, fragments of bacteriocin genes were amplified through 35 cycles by two different programs (Table 2). PCR mixes (total volume 100 µl) were prepared as recommended in Pwo DNA polymerase kit (Boehringer, D-68305 Mannheim, Germany). DNA of *Lb. gasseri* LF221 and *Lb. johnsonii* ATCC 11506 served as positive controls for acidocins LF221 A/B and lactacin F.

Sequencing

PCR amplicons were recovered from PCR mixtures using the QIAquick PCR Purification Kit (Qiagen, D-40724 Hilden, Germany) and further subjected to sequencing analyses (Microsynth, CH-9436 Balgach, Switzerland).

Mutant selection and plasmid analysis

For selection of mutants lacking bacteriocin activity, *Lactobacillus* K7 was propagated at 37 °C for 24 h in MRS

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Table 2. PCR conditions used for amplification of acidocin A (acA), acidocin B (acB), gassericin A (gaA), lactacin F (laF), gassericin T (gaT), acidocins LF221 A (LFA) and B (LFB) gene fragments

	acA, acB, gaA, laF, LFA	gaT, LFB
Initial step	94 °C/3 min	94 °C/3 min
Denaturation	94 °C/1 min	94 °C/1 min
Annealing	45 °C/30 s	58 °C/30 s
Elongation	72 °C/30 s	72 °C/30 s

broth containing various amounts of ethidium bromide (0–30 µg/ml; Boehringer) or mitomycin C (0·25 µg/ml, Sigma Aldrich, D-D-82024 Taufkirchen, Germany), respectively. After incubation, cultures were diluted serially and plated on M17 agar (Merck, D-64271 Darmstadt, Germany) to obtain clearly distinctive colonies (up to 60 colonies per plate). When colonies grew up to 2 mm in diameter (after 48 h at 37 °C in microaerophilic conditions), plates were overlaid with 4 ml MRS soft agar, seeded with 100 µl of an overnight culture of *Lb. sakei* indicator strain. After 24 h incubation at 30 °C, single colonies were examined for halos formation. Randomly selected colonies were analysed for plasmid DNA profile as described by Klaenhammer (1984).

Identification

Lactobacillus K7 has been identified by the PCR amplification and sequencing of the V2-V3 region of the 16S rRNA gene. Primers (HDA1 and HDA2) and the PCR program used were as previously described (Tannock et al. 1999). PCR amplicons were further analysed for nucleotide sequences (Microsynth).

Results

Bacteriocinogenic strain *Lactobacillus* K7 was tested for the presence of known bacteriocin genes of *Lb. acidophilus* members. Chromosomal and plasmid DNAs of K7, respectively, were analysed in PCR reactions with specific primers of seven bacteriocins by two different PCR programs (Tables 1 & 2). PCR with lactacin F specific primers (laF-828, laF-897) generated a fragment of 70 bp of the lactacin F-encoding gene when amplifying DNA of *Lb. johnsonii* ATCC 11506 as positive control. No products were amplified when the DNA of the investigated strain K7 was used as a template in the PCR with the same primer pair. The acidocin LF221 A (LFA-185, LFA-268) and acidocin LF221 B (LFB-1193, LFB-1262) specific primers were able to generate PCR amplicons of 84 bp and 70 bp, respectively, when chromosomal DNA of K7 was applied as target DNA. The same sized DNA fragments were obtained with DNA from *Lb. gasseri* LF221 (control; Fig. 1). On the other hand, no amplification was observed when these primers were employed with the plasmid DNA of *Lactobacillus* K7. Moreover, specific primers of acidocin A, acidocin B, gassericin A and gassericin T, applied to either chromosomal or plasmid DNA of *Lactobacillus* K7, did not generate any PCR products. The results obtained suggested similarities at the gene level between LF221 acidocins and K7 bacteriocins only. Therefore, sequencing of amplicons generated in PCR reaction with chromosomal DNA of K7 and acidocin LF221 A and B primers, respectively, was performed. When sequenced DNA fragments of K7 were aligned to the corresponding regions of acidocin LF221 A and LF221 B genes, 100% homologies were noted among the DNA sections compared.

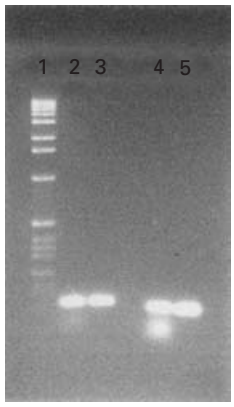

Fig. 1. PCR amplicons generated by either LFA or LFB specific primers. Lane 1, 1 kb DNA ladder; lanes 2 and 3, PCR products (84 bp) amplified with LFA specific primers on chromosomal DNA of LF221 and K7; lanes 4 and 5, PCR products (70 bp) amplified with LFB primers on chromosomal DNA of LF221 and K7.

Lactobacillus K7 was found to harbour one plasmid designated as pK7. To induce the plasmid curing, *Lactobacillus* K7 was grown in the presence of two curing agents. No mutants deficient in bacteriocin(s) K7 production were detected during examination of 4340 single colonies (2742 after treatment with ethidium bromide and 1598 after treatment with mitomycin C). Neither ethidium bromide nor mitomycin C were able to provoke the generation of non-bacteriocinogenic derivatives. Since all the colonies examined retained bacteriocin activity, 30 colonies were randomly selected for plasmid DNA isolations. The plasmid profile of tested isolates was identical to the plasmid profile of the non-treated *Lactobacillus* K7.

A sequenced portion (about 200 bp) of 16S V2-V3 region of K7 was aligned to V2-V3 sequences of *Lactobacillus* isolates held in GenBank. Comparisons clearly revealed that K7 strain is a member of the *Lb. gasseri* species.

Discussion

Lb. gasseri K7 was shown previously to produce bacteriocin(s) (Bogovič Matijašić & Rogelj, 2000). The aim of presented work was to find out whether the genetic determinants of *Lb. gasseri* K7 bacteriocins express any similarities

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to the genes of some already known bacteriocins produced by the members of *Lb. acidophilus* group. Additionally, plasmid curing was performed in order to examine whether the eventual loss of plasmid DNA has any impact on bacteriocin production. PCR programs were optimized for selected primer pairs. Only primers specific for LF221 acidocins amplified fragments of bacteriocin genes in chromosomal DNA of K7, that in size and nucleotide sequence corresponded to the DNA amplicons generated with DNA from LF221 (positive control). This result is not surprising, considering that production of a certain proteinaceous antimicrobial by LAB is not necessarily linked to one organism (Van Belkum & Stiles, 2000). If not just identical, the bacteriocins produced by different strains can reveal high homology, as was shown for gassericin A and acidocin B, which differ in three nucleotides inside the structural gene coding 91 amino acids long bacteriocin (Leer et al. 1995; Kawai et al. 1998). To identify the degree of similarity between K7 and LF221 bacteriocins, the complete nucleotide sequence of K7 bacteriocins must be determined in the first place and compared with the nucleotide sequences of LF221 acidocins. It is important to stress that although K7 and LF221 strains are both of human origin and belong to the *Lb. gasseri* species, they differ in other characteristics such as the plasmid profile, RAPD profile, growth characteristics, the level of bacteriocin(s) production, the optimal conditions for growth and bacteriocin production (Bogovič Matijašić, 1997; Bogovič Matijašić & Rogelj, 1999, 2000; Bogovič Matijašić et al. 1998, 2001).

In LAB, bacteriocin production and sensitivity have often been associated with plasmid DNA (Klaenhammer, 1993). Results of PCR reactions in our study however confirm chromosomal location of K7 bacteriocins, at least two of which were found to be similar to acidocins LF221 A and B, respectively. Yet, plasmid DNA of K7 might still carry genetic determinants for other bacteriocin(s) that were not detected in our study. As neither ethidium bromide nor mitomycin C were able to provoke formation of non-bacteriocinogenic derivatives and/or plasmid-loss in treated colonies of K7, we can not exclude the possibility that a plasmid pK7 harbours bacteriocin genes, different from those for LF221 acidocins. Therefore, additional genetic studies might identify if K7 strain produces more than two bacteriocins and where the genes are located.

The results presented confirmed the usefulness of a PCR based approach as the first step in the characterization of and screening for bacteriocin(s)-producing bacteria, avoiding, to some extent, time-consuming and expensive methods, such as protein isolation and amino-acid sequencing. As the bacteriocins produced by *Lb. gasseri* K7 were found to be similar to acidocins LF221 A and B, produced by another human *Lb. gasseri* isolate, the nucleotide sequences of LF221 bacteriocin genes will serve as a starting point in the further genetic characterization. Similar bacteriocins are particularly interesting for the investigation of the mechanism of action and immunity as well as elucidation of the inhibitory spectrum. These will be our future research targets.

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