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Review article

MOLECULAR GENETICS OF AUTOCHTHONOUS LACTIC ACID BACTERIA

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Autochthonous strains of lactic acid bacteria (LAB) were isolated from homemade fermented milk products. These products were collected from specific ecological localities such as high mountains (above 1200 m above sea level), mountain plateaus, river valleys, islands, Adriatic coast, etc. Analysis of LAB from the collection of natural isolates revealed that they produce proteinases, bacteriocins and exopolysaccharides. It was also shown that some isolates of lactococci and lactobacilli produce two bacteriocins simultaneously. According to their antimicrobial and biochemical properties, most of the analysed bacteriocins in natural isolates of lactococci were class II bacteriocins. In addition some isolates produced both proteinase and bacteriocin. Biosynthesis of spe-

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cific proteinases was detected in natural isolates. Analysis of the *prt* gene organisation among natural isolates of LAB showed that four isolates of lactobacilli exibited *prt* genes organisation different from those described so far. Elucidation of the regulation of the genes encoding proteinases, bacteriocins and exopolysaccharides could facilitate the construction of specific starter cultures for production of autochthonous fermented milk products, i.e. fermented products with a geographical origin.

Key words: Autochthonous LAB, bacteriocins, exopolysaccharides, proteinases, plasmid

INTRODUCTION

Lactic acid bacteria (LAB) have been essential in food and feed fermentation for centuries. They have a very broad application in the food industry and agriculture. LAB significantly contribute to the nutritional value of the products as well as flavour, texture and others. Data is rapidly accumulating that some strains of LAB could be probiotics. Therefore, an increasing interest exists for dairy products containing specific bacterial species with potential health–improving properties (PORTIER *et al.*, 1993). The fermentation of milk with intestinal species of *Lactobacillus acidophilus* and/or *Bifidobacterium bifidum* is being applied for a number of products (DRIESSEN and BOER, 1989). These species are more and more connected with health–promoting effects (as probiotics) in the human and animal intestinal tract (HOLZAPFEL *et al.*, 1998). That is why probiotics are considered as starter cultures for the production of functional food. Research on the molecular genetics and genetic engineering of LAB has allowed precise elucidation of gene expression in these bacteria (TOPISIROVIĆ *et al.*, 1991; TOPISIROVIĆ, 1997).

Bearing in mind the importance of LAB in many aspects of human activity, genetic manipulation of these microorganisms has highly expanded in the last ten years. However, most attention was paid so far to genetic investigation of LAB strains that are routinely used in industrial processes. Very little is known about the genetic organisation of LAB from the natural environment. Therefore, a study of the genetic organisation of LAB isolated from the traditionally produced homemade fermented products would be very interesting because such LAB could be potentially a source of genes encoding different variants of, for instance, proteinases, bacteriocins or exopolysaccharides.

NATURAL ISOLATES OF LAB

A collection of LAB was made by isolating them from fermented products produced in specific ecological localities such as high mountains (above 1200 m above sea level), mountain plateaus, river valleys, islands, Adriatic coast, etc. Analysis of LAB from the collection of natural isolates revealed that they produce proteinases, bacteriocins, aromogenic substances and exopolysaccharides (BANINA *et al.*, 1987). In addition, a collection of human lactobacilli including vaginal ones was also made.

Special attention is nowadays paid to the molecular taxonomy of LAB especially to those isolated from the natural environment (VUKASINOVIĆ and TOPISIROVIĆ, 2000). For this purpose a set of techniques was used such as DNA-DNA hybridisation using specific probes, RFLP (Restriction Fragment Length Polymorphism), and different PCR approaches. Ribotyping is also used for molecular taxonomy that includes a combination of RFLP and hybridisation with labelled rRNA probe. In addition, species–specific primers were developed for PCR reaction that ensures precise molecular taxonomy of LAB. A combination of these approaches gave more precise classification of LAB up to the subspecies level. For instance, groups of *Lactobacilli* such as *Lb. casei*, *Lb. delbrueckii* and *Lb. acidophilus* were precisely analysed. In addition, phylogenetic links were established among different LAB species and subspecies that were difficult to make based on classical microbiological and biochemical techniques used so far (VANDAMME *et al.*, 1996; O'SULLIVAN, 1999).

MOLECULAR GENETICS AND GENETIC ENGINEERING OF LAB

During the last decade many efforts to develop various kinds of vectors for genetic manipulation of LAB have been made (KOK, 1991). Cloning vectors derived from lactococcal and lactobacillus replicons have been constructed. Plasmid pWV01 was isolated from *Lactococcus lactis* subsp. *cremoris* Wg2 to which the chloramphenicol acetyltransferase (*cat*) gene of pC194 and the erythromycin resistance (*erm*) gene of pE194 were integrated to generate plasmid pGK12 (KOK *et al.*, 1984). Vector pGK12 can also replicate in *E. coli* and *Bacillus subtilis* providing a vehicle that facilitates cloning in these two bacteria besides lactococci. A similar strategy was applied in the construction of genetically marked vectors pNZ12 and pCK1 that have replication functions of cryptic plasmid pSH71 isolated from *L. lactis* subsp. *lactis* NCDO712 (for review see LEENHOUTS and VENEMA, 1993). A small cryptic plasmid pA1 (2820 bp) was isolated from *Lactobacillus plantarum* A112 and used for the construction of cloning vector by integrating the *erm* gene into it. Resulting constructs pA1-1 (4.43 kb) and pA1-6 (3.55 kb) were able to replicate with high segregation stability in *L. lactis* and *Lb. plantarum* (VUJČIĆ and TOPISIROVIĆ, 1993; VUJČIĆ *et al.*, 1994). Plasmid pA1 was a basis for the construction of shuttle vector pA13 that contains the *erm* gene and *lacZ*(α) gene. Cloning DNA fragments in multiple restriction sites present in the *lacZ*(α) gene was facilitated by using blue/white selection of *E.coli* transformants. Further genetic manipulation was allowed by selection of Em^r transformants in both LAB and *E.coli*. The results showed that vector pA13 possesed high efficiency of transformation of LAB and had a broad host range (KOJIĆ *et al.*, 1999).

On the other hand, constructions of promoter-probe vectors containing a promoterless reporter gene facilitate the elucidation of gene expression in LAB. The widely used vectors of this kind harbour the promoterless *cat* genes. The first constructed vector was plasmid pGKV210 (VAN DER VOSSEN *et al.*, 1985). Improved versions of this vector such as pBV5030, pBN37 and pBN38 were constructed (BOJOVIĆ *et al.*, 1991; MILADINOV and TOPISIROVIĆ, 1997). Recently,

plasmids with other reporter genes have been constructed such as pNZ272 and pNZ273, harbouring promoterless *gusA* gene, as well as pKSB8, harbouring promoterless *luxAB* gene (DE VOS and SIMONS, 1994). Plasmids pGKV210 and pBV5030 were used for cloning of promoter sequences from the chromosomal DNA of a natural isolate of *Lb. paracasei* subsp. *paracasei* CG11 and *Lb. acidophilus* ATCC4356, respectively. The cloned promoter showed activity in three hosts (*E. coli, L. lactis* and *Lb. reuteri*) (ĐORĐEVIĆ *et al.*, 1994; ĐORĐEVIĆ *et al.*, 1997). Cloning of promoters and elucidation of their activity are very important, opening the possibility for construction of LAB in which the expression of cloned genes of interest could be precisely controlled.

BACTERIOCIN PRODUCTION IN NATURAL ISOLATES OF LAB

Lactic acid bacteria produce a variety of antimicrobial substances (LINDGREN and DOBROGOSZ, 1990). Apart from metabolic end products, some strains also secrete antimicrobial substances termed bacteriocins, which are proteinaceous substances characterised by a relatively narrow inhibitory spectrum and a bactericidal mode of action (TAGG *et al.*, 1976). On the basis of their chemical, structural and functional property bacteriocins could be divided into three classes (for review see NES *et al.*, 1996).

The genetic determinants governing bacteriocin synthesis were shown to be plasmid encoded in various lactococcal species (MØRTVEDT and NES, 1990; VAN BELKUM *et al.*, 1989), whereas in other members of LAB, such as *Lb. plantarum* and *Lb. helveticus*, genetic determinants of bacteriocins are located on the chromosome (JOERGER and KLAENHAMMER, 1986; DIEP *et al.*, 1994). Production of more than one bacteriocin was demonstrated in *L. lactis* subsp. *cremoris* and *Carnobacterium piscicola* as well as in *L. lactis* subsp. *lactis* (van BELKUM *et al.*, 1991; QUADRI *et al.*, 1994; GAJIĆ *et al.*, 1999b). A bacteriocin producing natural isolate *Lactococcus lactis* subsp. *lactis* biovar. diacetylactis S50 was isolated from homemade butter. Genetic analysis revealed that genes encoding bacteriocin production and immunity to it were located on a very large (approx. 290 bp) plasmid (KOJIĆ *et al.*, 1991a).

The most interesting bacteriocin–producing natural isolate *L. lactis* subsp. *lactis* BGMN1–5 was isolated from homemade semihard cheese produced in Zabrđe village situated close to the seacoast in the bay Boka Kotorska (Adriatic Sea, Yugoslavia). The strain BGMN1–5 produced two bacteriocins designated bacteriocin 501 (approx. 20 kDa) and bacteriocin 513 (approx. 10 kDa), a cell wall–bound proteinase and showed a clumping phenotype. Plasmid curing resulted in the derivative BGMN1–501 that retained the ability to produce bacteriocin 501 and proteinase, and showed clumping phenotype, but had lost the ability to produce bacteriocin 513. Another derivative BGMN1–513 retained only the ability to produce bacteriocin 513, but concurrently lost the ability to produce bacteriocin 501 and proteinase, as well as the clumping phenotype. Production of bacteriocin 501 by BGMN1–501 was more pronounced in a chemically defined medium (CDM) containing casitone than in rich broth GM17. Production of bacteriocin 513 by

BGMN1–513 was the same regardless of the growth media. Bacteriocin 501 retained activity within the pH range of 3 to 12 or after treatment at 100°C for 2 h. However, the activity was gradually lost at pH 2 and only about 25% of the original activity was retained when bacteriocin 501 was autoclaved at 121°C for 20 min. According to these characteristics both bacteriocins could be classified as class II bacteriocins (GAJIĆ *et al*, 1999a;. GAJIĆ *et al*, 1999b)

Among 60 lactobacilli isolated from homemade cheeses of different geographic origins that are produced in a traditional way seven were bacteriocin producers. One of them, *Lb. paracasei* subsp. *paracasei* BGSJ2-8 (isolated from the semihard homemade white cheese produced in Sjenica, Pešter plateau, Yugoslavia) producing bacteriocin SJ, was analysed in detail. Besides bacteriocin production, isolate BGSJ2-8 is also a producer of proteinase similar to the PI–type of lactococcal proteinases and exhibits an aggregation phenotype. Bacteriocin SJ is a thermostable proteinaceous substance of small molecular weight (approx. 5 kDa). It retained activity after treatment for 1 h at 100°C and showed activity in the pH range from 2 to 11. Bacteriocin SJ has a narrow antimicrobial spectrum inhibiting the growth of closely related species. Plasmid curing resulted in loss of both bacteriocin production and immunity. Plasmid profiles of Bac^+ and Bac^- derivatives differed among themselves (ARSENIJEVIĆ *et al.*, 1999).

Analysis of LAB isolated from the semihard homemade white cheese produced in Bukovica village located on Durmitor mountain, Montenegro, Yugoslavia revealed that among the isolates there were lactobacilli that were resistant to nisin in the range from 1000 IU up to 10.000 IU (BANINA *et al.*, 1996). These isolates are very interesting from an industrial point of view since nisin is used as an additive to various foods in over forty countries in the world. Thus, the natural isolates resistant to nisin could be a very valuable material for construction of starter cultures, which will be functional in the presence of nisin.

PROTEINASE PRODUCTION IN NATURAL ISOLATES OF LAB

The ability to produce cell wall-associated proteinases is a very important feature of LAB. These proteinases, encoded by the *prt* genes, catalyse the initial steps in hydrolysis of milk proteins providing the cell with the amino acids that are essential for growth of LAB. Proteinases together with peptidases and the peptide–transport system enable the efficient growth of LAB in protein–rich media (KUNJI *et al.,* 1996). In addition, development of flavour during cheese ripening depends on the activities of proteinases and peptidases. Casein degradation by proteinases and peptidases results in liberation of both desirable and undesirable flavour peptides (VISSER, 1993; KOK, 1993). The cell wall–associated proteinases have been intensively investigated in various lactococcal strains (GASSON, 1983; DE VOS *et al.*, 1989; KEMPLER and MCKAY, 1979). Considering their caseinolytic specificity, lactococcal cell wall–associated proteinases have been divided into two groups. The PI–type proteinases predominantly hydrolyse β–casein. On the other hand, the PIII–type proteinases cause degradation of α_{S1}– and κ–caseins in addition to β–casein (KOK and VENEMA, 1988). In all lactococcal strains studied thus

far, except for *L. lactis* subsp. *cremoris* BC101, proteinase genes are located on plasmids of different sizes (KOK and VENEMA, 1988; NISSEN–MEYER *et al.*, 1991). The proteinase encoding DNA region of *L. lactis* subsp. *cremoris* Wg2 has been cloned and sequenced (KOK *et al.*, 1985). Interestingly, in close proximity to the proteinase gene (*prtP*), another oppositely orientated gene, named *prtM*, was located. The *prtM* gene codes for a membrane–located lipoprotein that is essential for the activation of the proteinase. Removal of the *prtM* gene resulted in the elimination of proteolytic activity, but synthesis and secretion of the proteinase were not affected (HAANDRIKMAN, 1990). An identical genetic organisation of the proteinase gene region of *L. lactis* subsp. *cremoris* SK11 was found (VOS *et al.*, 1989a).

The lactococcal proteinase genes are transcribed from a regulatory region within a 340 bp *Cla*I DNA fragment (KOK *et al.*, 1988). The AT-rich 340 bp *Cla*I DNA fragments of Wg2 and SK11 contain a long inverted repeat, possibly forming a stem-loop structure. Primer extension studies revealed that the transcriptional start sites of both the *prtP* and *prtM* promoters are located within this putative stem-loop structure. Due to the presence of five additional base pairs in the corresponding DNA fragment from SK11, a much more stable stem-loop structure in SK11 than in Wg2 was proposed (VOS *et al.*, 1989a; VOS *et al.*, 1989b). The differences in stability of these putative stem-loop structures from different lactococcal strains seem to coincide with differences in regulation of proteinase production. A recent report showed that the extent of proteinase biosynthesis in *L. lactis* is regulated by the components of the medium in which the bacteria grew. Specific dipeptides were found to be involved in the control of transcription initiation of proteinase genes (MARUGG *et al.*, 1995).

Proteinase-producing strains were detected among natural isolates of both lactococci and lactobacilli. The most interesting strain among natural isolates of lactococci that produce proteinase was *L. lactis* subsp. *lactis* BGIS29. This strain is isolated from the soft homemade white cheese produced in Ilijaš, Bosnia and Herzegovina, former Yugoslavia. It produces a PI-type proteinase. The presence of casitone in chemically defined medium (CDM) has a specific influence on the regulation of the proteinase activity in BGIS29. The regulatory region of the *prt* genes of BGIS29 was cloned and sequenced. Results showed distinct differences in the nucleotide sequences of the *prt* regulatory region of BGIS29 in comparison to those of *L. lactis* subsp. *cremoris* SK11 and Wg2. Potential secondary structures of the *prt* regulatory regions in these three strains appeared to be topologically different. Transcriptional gene fusion with the *E. coli* β–glucuronidase gene (*gusA*) was used to study medium-dependent expression of both *prtP* and *prtM* promoters. The results showed that the activities of both promoters were controlled by casitone at the transcriptional level, but also that their regulation occurred in a different manner. The activity of the *prtP* promoter gradually decreased with an increase of casitone in the growth medium (0.1% to 2%) reaching minimal activity in the presence of 2% casitone. The activity of the *prtM* promoter also decreased with increasing casitone concentrations in the growth medium. However, it retained sig-

nificant activity even in the presence of 2% casitone (TOPISIROVIĆ *et al.*, 1993; MILADINOV and TOPISIROVIĆ, 1996; MILADINOV *et al.*, 1999).

In contrast to lactococci, the proteolytic systems of lactobacilli have been much less studied. Comparison of different lactobacilli have indicated heterogeneity of cell wall–associated proteinases within the *Lactobacillus* genus. Some *Lb. helveticus* and *Lb. delbrueckii* subsp. *bulgaricus* strains have been reported to contain two different proteinases. The genes encoding proteinases have been cloned and sequenced from *Lb. paracasei* subsp. *paracasei, Lb. delbrueckii* subsp. *bulgaricus* (the *prtB* gene) and *Lb. helveticus* (the *prtH* gene) (HOLCK and NAES, 1992; GILBERT *et al.*, 1996; PEDERSON et al. 1999). Lactobacilli isolated from different natural sources were screened for the presence of cell envelope-associated proteinases (Prt^+ strains). Among 75 tested mesophilic isolates of lactobacilli 17 were Prt^+. All Prt^+ strains were producers of a serine-type proteinase. Most of them degraded only β–casein such as *Lb. paracasei* subsp. *paracasei* strains BGLI17, BGLI18 and BGHN14 as well as *Lb. rhamnosus* BGEN1. Only *Lb. divergens* BG742 cleaved all three α_{s1}–, β– and κ–caseins. Interestingly, the results showed that the proteinase genes of *Lactobacillus paracasei* subsp. *paracasei* strains BGLI17, BGLI18 and BGHN14 are most probably chromosomally located (KOJIĆ *et al.*, 1995).

One of the interesting Prt^+ isolates was *Lb. acidophilus* BGRA43. It was selected among a set of human origin isolates of *Lb. acidophilus* strains for the highest growth rates and antagonistic effect against both Gram–positive and Gram–negative bacteria. *Lb. acidophilus* BGRA43 produces an extracellular proteinase. Whole cells efficiently hydrolysed all three major casein fractions (α_{S1}–casein, β–casein and κ–casein) in 3 h at 37^0C wherein the highest activity was observed towards α– and β–casein fractions. Under optimal conditions (pH 6.5 and 45^0C), BGRA43 proteinase completely hydrolysed all three casein fractions within 3 h of incubation. The proteolytic activity of BGRA43 was partially inhibited only by serine protease inhibitors. Thus, it appears that the BGRA43 proteinase belongs to some other class of LAB proteinases (TOPISIROVIĆ *et al.*, 1997; BANINA *et al.*, 1998).

The organisation of the *prt* genes regulatory regions of 20 different *Lactobacillus* isolates was studied. The *prt* gene organisations in these isolates were analysed using PCR primers specific for the *prtM* and *prtP* genes of lactococci. Only four *Lactobacillus* isolates had different types of *prt* gene organisation from that already found in lactococci. Natural isolates of *Lb. paracasei* had high similarity of *prt* gene organisation to that described in lactococci, i.e. both the *prtP* and *prtM* genes were present. In addition, the expression of the *prt* genes in *Lb. paracasei* isolates was regulated by casitone as observed in lactococci. The regulatory region of the *prt* genes of *Lb. paracasei* BGHN14 (isolated from homemade semi-hard cheese produced in Herceg Novi, Montenegro, Yugoslavia that had been kept in the olive oil for one year) was cloned and sequenced (KOJIĆ *et al.*, 1991b). The results revealed that this region contains both *prtM* and *prtP* promoters, but is 35 bp shorter in comparison to that in lactococci. The nucleotide sequence missing in

BGHN14 forms a potential stem–loop structure suspected to be involved in casitone–dependent regulation of *prt* gene expression in *L. lactis* subsp. *lactis* strains SK110, Wg2 and BGIS29 (SPASOJEVIĆ *et al.*, 1999).

EXOPOLYSACCHARIDE PRODUCTION IN NATURAL ISOLATES OF LAB

Many bacteria including LAB are able to produce exopolysaccharides (EPS), which give them a mucous phenotype. Results showed that the monomeric composition of EPS differs considerably among bacteria (CERNING 1990). Analysis of natural isolates of mesophilic lactobacilli revealed that 15 of them produce exopolysaccharides (EPS) (ĆIRIĆ *et al.*, 1990). One of them, *Lb. casei* strain CG11, was isolated from homemade semihard white cheese produced in Adrovići village, Montenegro, Yugoslavia. Results showed that CG11 produces EPS in either rich or basal minimal medium in the presence of most sugars but not in the presence of fructose. The strain CG11 has the ability to coagulate milk very fast giving a slimy curd. In basal minimal medium supplemented with glucose, it produces a neutral heteropolysaccharide consisting predominantly of glucose (about 75%) and rhamnose (about 15%). Plasmid curing experiments revealed that exopolysaccharide-producing ability of the strain CG11 is linked to a plasmid of approx. 30 kb (KOJIĆ *et al.*, 1992; CERNING *et al.*, 1994).

CONCLUSION

Elucidation of the molecular genetics of autochthonous LAB gave an opportunity for us to make a genetically defined collection of natural isolates that could be used for construction of specific starter cultures for fermented food products. Such starter cultures could be used for the production of fermented products with a geographical origin. In addition, genetically characterised natural isolates of LAB could eventually be a source of genes for construction of new starter cultures or for the improvement of existing ones by using genetic engineering. This is especially interesting since LAB are treated as GRAS microorganisims (Generally Regarded As Safe). In addition, bearing in mind that increasing attention exists for dairy products containing specific bacterial species with potential health–improving properties, selection of natural isolates from humans that could be used as probiotics is extremely interesting. Such isolates could be a basis for construction of starter cultures for production of novel, functional food.

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MOLEKULARNA GENETIKA AUTOHTONIH BAKTERIJA MLEČNE KISELINE

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Izvod

Autohtoni sojevi bakterija mlečne kiseline (BMK) su izolovane iz fermentisanih mlečnih proizvoda radjenih u domaćoj radinosti. Fermentisani mlečni proizvodi su sakupljani sa specifičnih ekoloških lokaliteta kao što su visoke planine (iznad 1200 m nadmorske visine), planinske visoravni, rečne doline, ostrva, obala Jadranskog mora, itd. Analiza kolekcije prirodnih izolata BMK je pokazala da izolati proizvode proteinaze, bakteriocine i egzopolisaharide. Pokazano je, takodje, da neki izolati laktokoka i laktobacila proizvode istovremeno dva različita bakteriocina. Pored toga, odredjeni broj izolata proizvodi i proteinaze i bakteriocine. Nadjeno je da prirodni izolati sintetišu specifične proteinaze. Analiza organizacije *prt* gena u prirodnim izolatima BMK je pokazala da četiri laktobacila poseduju organizaciju *prt* gena koja se razlikuje od do sada opisanih. Izučavanje regulacije gena koji kodiraju proteinaze, bakteriocine ili egzopolisaharide može olakšati konstrukciju specifičnih starter kultura za proizvodnju autohtonih fermentisanih mlečnih proizvoda, tj. fermentisanih proizvoda sa geografskim poreklom.

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