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Study on Probiotic Lactic Acid Bacteria and Their Applications to New Functional Foods

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Summary

In the present mini review, we describe 1) a new screening system for selecting probiotic strains from lactic acid bacteria (LAB), mainly *Lactobacillus acidophilus* group bacteria, with strong adhesion to the human intestinal tract, 2) characteristics of immunostimulative oligo DNA motifs in LAB (*L. gasseri*) strains and a new evaluation system with a transfectant expressing porcine Toll-like receptor 9 for selection of immunostimulative LABs, and 3) characteristics of antimicrobial peptides (bacteriocins), especially gasericin A from *L. gasseri* LA39.

In the future, it is expected that superior functional foods containing more effective probiotic LAB will be developed by the use of our proposed mass screening system.

Lactobacillus (L.) acidophilus group lactic acid bacteria (LAB), *L. salivarius*, *L. casei*, *L. plantarum*, *L. fermentum*, *L. reuteri* and *L. brevis* have been the most common *Lactobacillus* species isolated from the human intestine. *L. acidophilus* LAB, isolated from the human feces or intestine, are thought to have beneficial effects on health and to be particularly useful probiotic bacteria. They have been used for many years in acidophilus milk and fermented milk products with the expectation that they will serve as probiotics by their survival and growth in the gastrointestinal tract.

It is thought that more than one hundred species and more than ten billion bacteria coexist in the human intestine. The intestinal microflora is thought to be an important constituent of the gut mucosal barrier. Human health is thought to be maintained by a balance of useful and harmful bacteria. Bacteria that live in the human intestine and control the balance of intestinal microflora and finally elicit physiological and beneficial effects on health of the host have recently been named "probiotics", and there has been intensive research on probiotics.

In this mini review, we describe 1) a new screening system that enables

evaluation of useful strains among *L. acidophilus* group LAB with strong adhesion to the human intestinal tract, 2) immunostimulation of AT motifs and CpG-like motif derived from genomic DNA from LAB and the development of a new evaluation system for useful bacteria involving the motifs by using a transfectant expressing porcine Toll-like receptor 9, and 3) new characteristics of antimicrobacterial peptides (bacteriocins), especially gasericin A with wide antibacterial spectra from *L. gasseri*.

1. Proposal of a Mass Screening System for Selecting Probiotic LAB with Strong Adhesive Activity to the Human Intestinal Tract

The adhesive activity of LAB to human intestine is thought to be one of the most important characteristics as probiotics, which show initial colonization and later proliferation in intestinal tract. The mechanism by which *L. acidophilus* group LAB adhere to the human gastrointestinal tract has been partially elucidated. We have revealed, from results of hemagglutination (HA) assays, the presence of several lectin-like proteins on the cell surfaces of *L. acidophilus* group LAB (Yamada *et al.*, 1994). Such proteinaceous components in surface layer proteins (SLPs) are thought to contribute to cell adhesion through their binding to carbohydrate portions of the colonic mucous layer.

We have proposed a new screening system that involves a combination of three steps using a rat colonic mucin (RCM)-micro plate assay, Carnoy's histochemical staining method and a carbohydrate probe binding assay. We have also recently proposed a new screening system by surface plasmon resonance (SPR) with the biosensor BIACORE.

1) First Step

Eight kinds of acidic and neutral O-glycosidic (mucin-type) sugar chains that combine with RCM have been reported by Slomiany *et al.* (1980). The chemical structures of those sugar chains in the rat have been reported to be similar to the structures of those isolated from human colonic mucin (HCM) (Podolsky, 1985). We proposed to use RCM for the screening assay instead of HCM for the first time. Takahashi *et al.* (1996) reported that the conventional HA assay is not a suitable method for selecting *L. acidophilus* strains with strong adhesion to the human intestinal tract.

We introduced a new screening method using polystyrene beads coated with RCM, but the method could not be used to screen B-group strains of *L. acidophilus* group LAB because of weak nonspecific binding of SLPs to polystyrene beads. Matsumura *et al.* (1999) reported a modified screening method using an RCM-coated microtiter plate without nonspecific reaction by improving blocking conditions. The method was shown to be useful as a first mass screening method for

evaluating the adhesiveness of *L. acidophilus* LAB to the human intestinal tract.

2) Second Step

SLPs were prepared from cells of *L. acidophilus* LAB selected by mass screening in the above-described first step. The binding of lectin-like protein(s) in SLPs to the colonic mucus layer was confirmed by histochemical staining using human colon tissues fixed with Carnoy's fixative (Takahashi *et al.*, 1996).

3) Third Step

Saito *et al.* (2000) proposed a new method for prediction of the sugar-recognizing portion on the human mucosal layer by using selected bacteria with a commercial biotinylated carbohydrate probe (BCP) that has representative partial structures of the sugar chains constituting HCM. High levels of reactivity were detected in 19 strains with a trisaccharide probe that has an A-antigen structure [GalNAcα1-3 (Fucα1-2) Galβ1-]. The existence of cell-surface lectins was first clearly confirmed in the B_1 and B_2 subgroups of *L. acidophilus* group LAB. The evaluation method proposed in the report is also considered to be useful as an indicator for the selection of more effective probiotic LAB.

Thus, the proposed system for evaluating probiotic LAB that adhere to the human intestinal tract involves the following three steps: 1) mass screening by the RCM-coated micro plate assay, 2) confirmation of adhesion to HCM by histochemical staining and 3) prediction of the recognizing portion of the epitope sugar chain on HCM (Saito, 2004). The concept of this screening system is shown in Fig. 1.

Recently, there have been some reports on differences in the carbohydrate chains of glycolipids and HCM in people with different ABO blood types. However, there have been no studies on adhesion between *L. acidophilus* group LAB and sugar chains in HCM of different blood types. We recently proposed a novel LAB binding assay by surface plasmon resonance (SPR) with the biosensor BIACORE using both carbohydrate probes and HCM of blood type A having an A-antigen trisaccharide structure (Uchida *et al.*, 2004). The method has been shown to be useful for determining the interaction in real time between living bacterial cells and the surface of the human intestine like an *in vivo* system without the requirement of a labeling process.

We hope to use probiotic LAB selected through the screening system for the production of new functional foods in the near future.

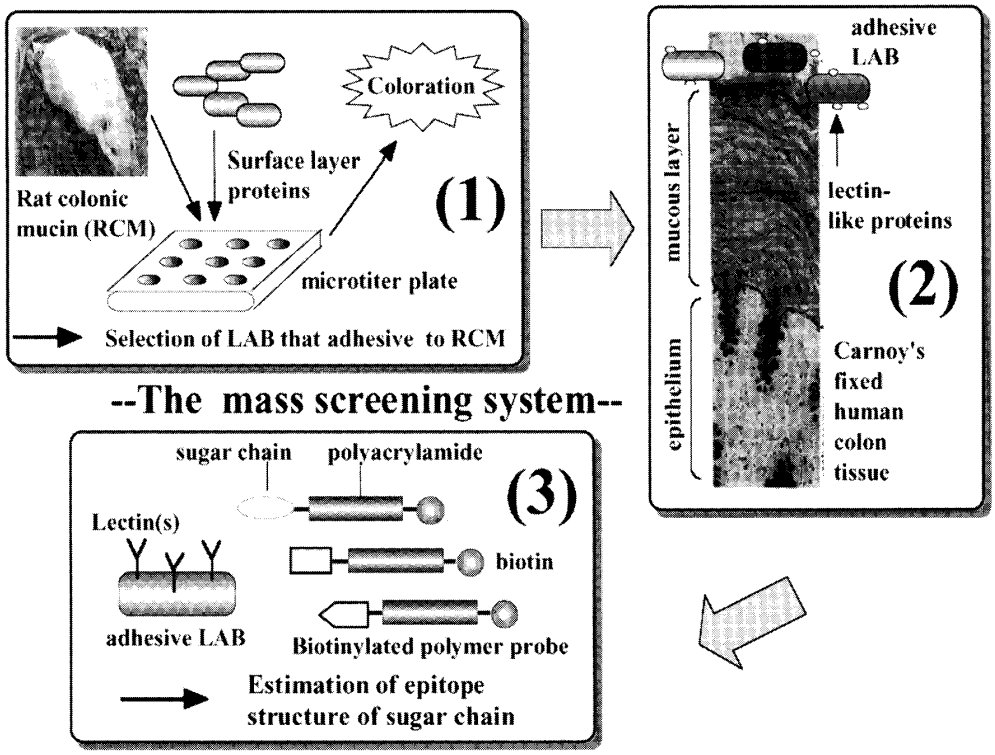

FIG. 1. A proposed mass screening system for identifying probiotic LAB with strong adhesion to human colonic mucosa.
RCM, rat colonic mucin ; SLP, surface layer protein ; LAB, lactic acid bacteria.

2. Construction of a Potential Immune Assay System for the Functional DNA from Immunobiotic Lactic Acid Bacteria by Swine Toll-like Receptor 9 Transfectant

Mammalian Toll-like receptors (TLRs) are a structurally conserved family of membrane receptors that have homology to the *Drosophila* Toll system. TLRs play an essential role in the innate recognition of pathogen-associated molecular patterns (PAMPs) and in the triggering of adaptive immunity in higher organisms. Eleven mammalian TLRs and their ligands except for that of TLR10 have so far been identified, as shown in Fig. 2 (Akira and Takeda, 2004). TLR9 has been shown to be a specific receptor for pathogenic bacterial DNA that contains a specific sequence pattern, unmethylated CpG dinucleotide (Hemmi *et al.*, 2000). After the discovery of TLR9, possible molecular mechanisms in immune responses through pathogenic bacterial DNA have been rapidly revealed in mice. We have studied specific effector molecules and their receptor targets. Results of our recent studies have suggested that AT oligonucleotide (ODN) and CpG-like ODN from LAB induce immunoactivation in Peyer's patches (Pps), which belong to a gut-associated lymphoid tissue (GALT), via TLR9 (Kitazawa *et al.*, 2001 ; 2003).

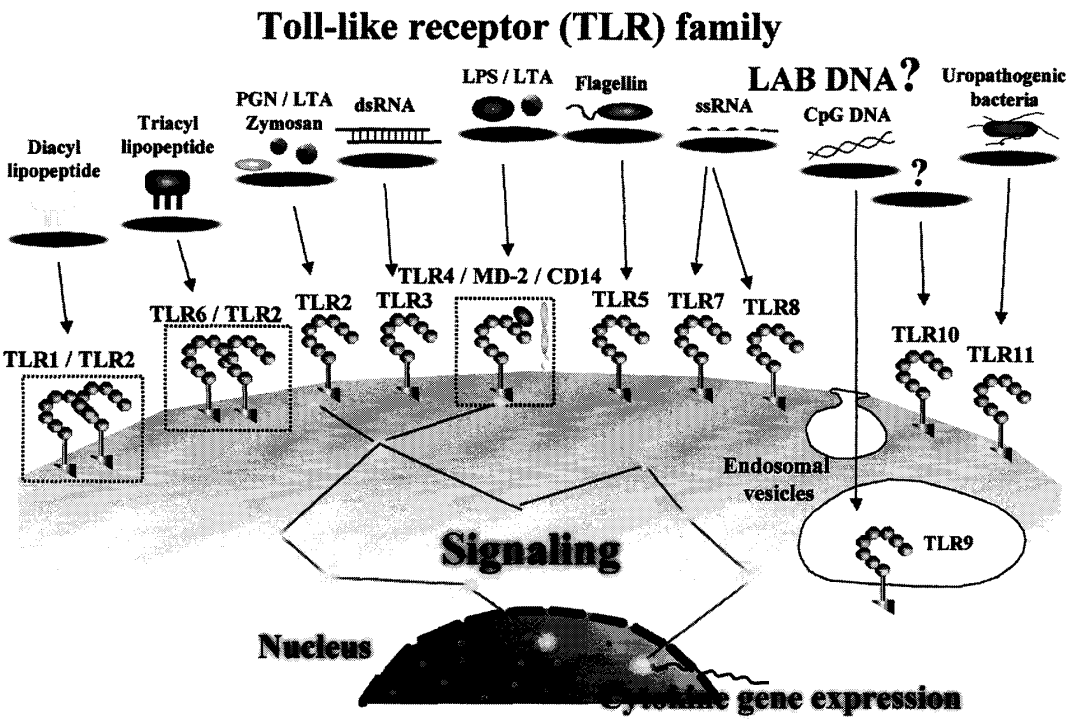

FIG. 2. Toll-like receptor families and their ligands.

The results of many works on biological functions of dairy LAB have contributed to the application of LAB as functional foods and supplements in the worldwide market. Recently, the term "immunobiotics", that promote health through activation of intestinal immunity compared to those with strictly local immunity, has been proposed and expected for an appropriate evolutionary development (Clancy, 2003). For developing the functional foods using immunobiotic LAB exerted immunomodulatory effects via TLRs, the TLR-expressing transfectants are expected to evaluate the putative relationship between TLRs and immunobiotic LAB as a valuable tool of immune screening system. The swine is thought to be more useful than the mouse as a human model for basic and applicable studies because of high homologues in tissue level. However, the basic study of the swine immunity remains largely unknown compared to murine and humans. Therefore, for the purpose of development of an immune screening system as a human model, we focused on swine TLRs playing key roles in intestinal immune responses that might be triggered by immunobiotic LAB.

To elucidate the role of TLR9 with AT ODN in intestinal immunity, we first isolated a cDNA encoding a TLR9 from swine Pps, which are considered to be useful as human models. Total RNA was isolated from the Pps of adult swine intestines. The complete open reading frame (ORF) of sTLR9 contains 3093bp coding and deduced 1030 amino acid residues with a calculated molecular mass of 115.7 kDa (Shimosato *et al.*, 2003). Next, we constructed a transfectant of swine

TLR9 with mammalian cells for the development of an immunoassay against ODN. We demonstrated that the transfectant recognized not only CpG but also non-CpG ODN such as AT ODN from LAB resulted in the induction of NF-κB activation by a gene reporter assay (Shimosato *et al.*, 2004). Furthermore, TLR9 was detected in the follicle-associated epithelium (FAE) including M cells as well as antigen-presenting cells such as dendritic cells (DCs) in Pps. These findings indicate that the TLR9-positive cells in Pps provide the host defense with the ability to respond to a variety of ODNs from the immunobiotic LAB. Our study firstly demonstrate that TLR9 is a receptor for not only CpG but also non-CpG AT ODN and may help in understanding the intestinal immunoregulation mediated by immunobiotic LAB DNA through TLR9 for the development of "immunobiotic foods" for the prevention of infectious and allergic diseases.

3. Identical Cyclic Bacteriocins, Gassericin A Produced by *Lactobacillus gasseri* LA39 and Reutericin 6 Produced by *L. reuteri* LA6

Bacteriocins are antimicrobial proteins that act mainly against related bacterial species. The bacteriocins of LAB and bacteriocin-producing LAB strains isolated from humans and foods are expected to be useful as food-preservatives and probiotics for preventing the growth of harmful bacteria.

Lactobacillus acidophilus group of LAB is found in the human intestine and is widely used in fermented milk products. Many strains have been shown to be bacteriocin producers. In our laboratory, 130 strains of *L. acidophilus* group LAB isolated from human feces were screened to identify bacteriocin-producing strains (Itoh *et al.*, 1995; Kawai *et al.*, 1997). The bacteriocin "gassericin A" of *L. gasseri* LA39 isolated from feces of a human infant had wide antibacterial spectra against food-borne pathogenic bacteria such as *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus* (Itoh *et al.*, 1995). Purification, cloning of its structural gene (*gaaA*, accession number AB007043) and sequencing of the complete primary chemical structure of gassericin A (Kawai *et al.*, 1998a; 1998b) revealed that this bacteriocin has a cyclic structure linking N- and C-terminal amino acids (molecular weight of 5,652 Da) and consisting of 58 amino acid residues (Table 1 and Fig. 3).

The hetero-fermentative *L. reuteri* LA6, which was isolated from feces of the same human infant, produced the bacteriocin "reutericin 6" (Itoh *et al.*, 1995). Its molecular weight, primary amino acid and DNA sequences, and cyclic structure were found to be identical to those of gassericin A (Kawai *et al.*, 2001). However, reutericin 6 had weaker activity and a narrower spectrum than those of gassericin A. The amounts and patterns of potassium-ion efflux from indictor cells and liposomes were different. Circular dichroism (CD) spectra of purified bacteriocins did not coincide. By D-, L-amino acid composition analysis, two

Table 1. Reported Primary Amino Acid Sequences and Origins of Cyclic Bacterioins

bacterioicin	origin	sequence	date of publication
enterocin AS-48	*Enterococcus feacalis* S-48	MAKEFGIPAAVAGTVLNVVEAGGWVTTIVSILTAV GSGGLSLLAAAGRESIKAYLKKEIKKKGKRAVIAW	1994
gassericin A	*Lactobacillus gasseri* LA39	IYWIADQFGIHLATGTARKLLDAMASGASLGTAFAA ILGVTLPAWALAAAGALGATAA	1998
microcin J25	*Escherichia coli* AY25	GGAGHVPEYFVGIGTPISFYG	1999
reutericin 6	*Lactobacillus reuteri* LA6	IYWIADQFGIHLATGTARKLLDAMASGASLGTAFAA ILGVTLPAWALAAAGALGATAA	2001
circulain A	*Clostridium beijerinckii* ATCC25752	VAGALGVQTAAATTIVNVILNAGTLVTVLGIIASIA SGGAGTLMTIGWATFKATVQKLAKQSMARAIAY	2003

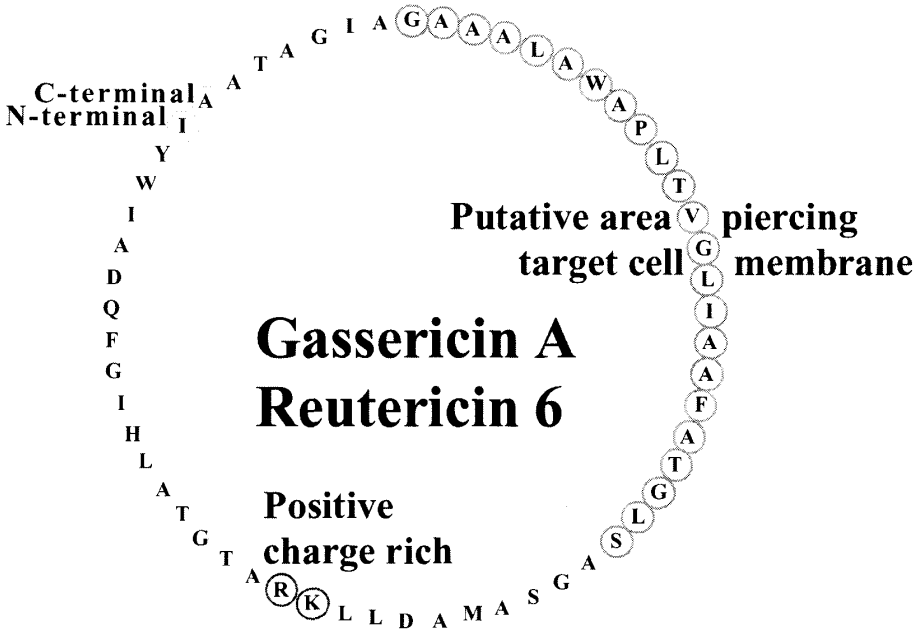

FIG. 3. The primary structure of gassericin A and reutericin 6.

residues of D-Ala were detected in 18 Ala residues of gassericin A and one residue was detected in reutericin 6 (Kawai *et al.*, 2003; 2004).

There have been no reports on the different characteristics of native primary identical bacteriocins such as gassericin A and reutericin 6, although bacteriocins with the same primary structure have been reported in different other LAB species. These findings indicate that characteristics of native bacteriocins produced by wild lactobacilli strains having the same structural genes are influenced by a difference in D-amino acid contents in the molecules. The research on characteristics and D-/L-amino acid analysis of peptides and proteins expressed by identical genes and prepared from wild microorganisms having the same genes are needed in future.

We have tried to elucidate by using molecular methods the mechanisms underlying the appearance of D-Ala residues and cyclization in the two bacteriocins.

Conclusions

In 2002, the genomic DNA sequence of *L. gasseri* strain ATCC33323 has been opened to the public as the first robotic strain by JGI (Doe Joint Genome Institute, US Department of Energy) on the Internet Web (URL: http://www.jgi.doe.gov/). We can expect the arrival of new time when useful probiotic LABs can be selected by screening from only DNA information in the future. The global market for functional foods is growing at a very fast rate, especially

probiotic products involve a potential growth area around the worlds. We hope to find superior strains of probiotics from nature by our study and to propose more useful functional probiotic foods (such as yogurt) in the future.

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