

Search for excellent probiotic lactic acid bacteria and their applications

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ABSTRACT

The adhesive activity is one of the most important characteristics of probiotic lactic acid bacteria (LAB) in the host's intestine. We have proposed a new screening system for LAB that involves a combination of three steps using a rat colonic mucin-micro plate assay, Carnoy's histochemical staining and a carbohydrate probe binding assay. We have recently proposed a new screening assay for searching adhesive LAB to human colonic mucin with a biosensor BIACORE. Special LABs which recognize human ABO blood type expressed in mucin covered with intestinal tracts were isolated and expected to prevent the adhesion of harmful bacteria.

We clarified characteristics of immunostimulative oligo DNA motifs in genomic DNA of LAB and developed a new evaluation system with a transfectant expressed swine Toll-like receptor 9 for selection for immunostimulative LABs. The selected CpG or AT motifs are expected to improve the allergy by changing the balance of Th1/Th2 lymphocyte. We clarified characteristics of antimicrobial bacteriocin: gassericin A from *L. gasseri* isolated from human intestine. Gassericin A was expected to apply in both biopreservation and prevention and treatment of mastitis through the inhibition of the growth of *Staphylococcus aureus*.

Finally, we have developed a new functional yogurt by using selected LAB which can expect continuous immunostimulation and antimicrobiological activity through strong affinity to intestinal mucosa.

INTRODUCTION

The *Lactobacillus* (*L.*) *acidophilus* group 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. gasseri* (B₁ subgroup of *L. acidophilus* group LAB), which is isolated with high frequency from human feces and intestine, is thought to have beneficial effects on health and particularly useful probiotic bacteria. The intestinal microflora is thought to be an important constituent of the gut mucosal barrier. 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.

MATERIALS AND METHODS

LAB strains were isolated from feces of human adults and infants by LB and MRS medium with incubation for 48 hr. The identification of LAB was done by analyses of 16S rDNA sequencing and carbohydrate consumption with API 50CH. The adhesion of LAB was analyzed by BIACORE (BIACORE company, Japan). Human colonic mucin (HCM) with different ABO blood types was obtained from colon cancer patients. HCM was immobilized to a center chip surface as ligand and eluted LAB cells as analyte. The adhesive activity was evaluated with the increasing resonance unit (RU) value.

Swine Peyer's patch was isolated from pig intestine. mRNA was isolated from total RNA and constructed cDNA by polymerase reaction. After reading the sequence, ORF of swine Toll-like receptor (sTLR) 9 was transfected into human HEK293 or CHO cells. Immunogenic LAB was isolated by mitogenic activity test. Several oligonucleotide motif (ODN) was prepared from genomic DNA of LAB by digestion of restricted enzymes. Immunoactive ODN was evaluated by FACS with a transfectant of sTLR9.

Bactericin activity was measured by agar well diffusion method. The chemical structure of Gassericin A isolated from *L. gasseri* LA39 was clarified by gene cloning and chemical analyses. The antimicrobial spectrum was determined for *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus* (SA). The antimicrobial activity of Gassericin A was tested for biopreservation and treatment of mastitis.

RESULTS AND DISCUSSION

1. Development of a mass screening system for selecting probiotic LAB with strong adhesive activity to the human intestinal tract

Although probiotics have several necessary and sufficient conditions such as tolerance to low pH and bile acid, the adhesive activity of LAB on intestinal tracts is thought to be one of the most important characteristics to express later many beneficial effects to the host through initial colonization and later proliferation in intestinal tract. 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. Such proteinaceous components (lectin-like protein) in the surface layer proteins (SLPs) is thought to contribute to cell adhesion through the binding to carbohydrate portions of human colonic mucin (HCM). We have proposed a new screening system that involves a combination of three steps using rat colonic mucin (RCM)-micro plate assay, Carnoy's histochemical staining method and carbohydrate probe binding assay.

More recently, we proposed a novel LAB screening assay by surface plasmon resonance (SPR) with a biosensor BIACORE using both carbohydrate probes and HCM of blood type A having an A-antigen trisaccharide structure [GalNAc α 1-3 (Fuc α 1-2) Gal β 1-]. 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 LAB and sugar chains in HCM of different blood types. The method has been shown to be quite useful for determining the interaction in real time between living bacterial cells and the human intestinal surface like an *in vivo* system without the requirement of any labeling process. We found a special LAB which can recognize and strongly bind to human blood type A antigen and was expected to show the strong competitive exclusion property of harmful bacteria by scrambling for the same binding points on intestinal mucosa. Recently, we also have started to search for the superior LAB in pig intestine to improve intestinal conditions and to decrease the usage of antibiotics in feeds by the same competitive exclusion principle through the addition of such probiotic adhesive LAB isolate from pig intestine. .

2. Construction of a potential immune assay system for the functional DNA from immunobiotic LAB by swine Toll-like receptor 9 transfectant

Mammalian Toll-like receptors (TLRs) represent a structurally conserved family of membrane receptors, which 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. To date, 13 TLRs have been identified and their ligands also found out except for TLR10.

TLR9 has been identified as a particular receptor for pathogenic bacterial DNA that contains a specific sequence pattern, unmethylated CpG dinucleotide (CpG motif). Recently, we have found the possibility that AT oligonucleotide (ODN) and CpG-like ODN from LAB, induced immunoactivation in Peyer's patches (Pps), which belong to a gut-associated lymphoid tissue (GALT), via TLR9.

For developing the mass screening system for immunobiotic, the TLR-expressing transfectants are expected to evaluate the putative relationship between TLRs and immunobiotic LAB as a valuable tool of immune screening system. To elucidate the role of TLR9 with AT ODN in the intestinal immunity, first, we isolated a cDNA encoding a TLR9 from swine Peyer's patch (Pps) and clarified the sequence, which are considered to be useful for human models. Next, we constructed a transfectant of swine TLR9 (sTLR9) with mammalian cells for the development of an immunoassay against ODN. We demonstrated that the transfectant recognizing not only CpG but also non-CpG ODN such as AT ODN from LAB resulted in the induction of NF- κ B activation by gene reporter assay, and later expression of mRNA and protein levels of several cytokines. Our study firstly shows that TLR9 is a certain receptor for not only CpG but also for non-CpG AT ODN and may help in understanding the intestinal immunoregulation mediated by immunobiotic LAB DNA through TLR9.

3. Cyclic bacteriocins, Gassericin A produced by *L. gasseri* and its application for biopreservation and medical treatment of mastitis.

Bacteriocins are antimicrobial proteins that act mainly against related bacterial species. Many probiotic LAB strains found in the human intestine have been shown to be bacteriocin producer. In our laboratory, 130 strains of *L. acidophilus* group LAB isolated from human feces were screened to identify bacteriocin-producing strains. The bacteriocin, Gassericin A, of *L. gasseri* strain LA39 isolated from feces of a human infant had the wide antibacterial spectra against the food-borne pathogenic bacteria such as *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus* (SA). Purification, cloning of its structural gene (*gaaA*) and sequencing of the complete primary chemical structure of gassericin A

revealed that this bacteriocin has a cyclic structure linking at N- and C-terminal amino acids and consisting of 58 amino acid residues.

Circular dichroism (CD) spectrum and D/L-amino acid analyses of Bacteriocin A clarified that two residues of D-Ala were contained in 18 Ala residues of the molecule and contribute special 3-D structure. The localization of these D-Ala residues in the molecule and cyclic mechanism at gene level are under progress in our laboratory. Recently, we applied to use Gassericin A to the biopreservation of high-fat foods such as custard cream because the bacteriocin is highly hydrophobic and it gave good results as fine food preservative. More recently, we found that Gassericin A prevented the growth of wild type of toxic SA isolated from mastitis milk and breast organs and also was quite useful for medical treatment of mastitis without any inflammation on cow.

Finally, we have developed a new functional yogurt by using selected probiotic LAB which can expect continuous immunostimulation by CpG or AT motifs and the expression of antimicrobiological activity by bacteriocin through strong affinity to intestinal mucosa.

IMPLICATIONS

The global market for functional foods is growing at a very fast rate, and probiotic products represent a potential growth area around the worlds. We have found several superior strains of probiotic LAB from the nature mainly human feces by our proposed and established screening systems. We are now planning to produce the functional foods and feeds using the selected LAB with strong and specific adhesive activity to host's intestinal mucosa which give continuous supply of immunoactive ODN and bacteriocin.

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