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Research Article

CHARACTERIZATION OF ADHESIVE DETERMINANTS IN SELECTED *LACTOBACILLI* STRAINS

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ABSTRACT
Lactobacilli are widely used in food and agricultural fermentations. For successful probiotic action, the lactobacilli must adhere to the gastro intestinal tract. The biochemical nature of the cell surface adhesive molecules of the lactobacilli was characterized. The adhesive properties of 32 lactobacilli isolates from gastrointestinal tract of rats and human beings were studied by cell surface hydrophobicity through bacterial adherence to hydrocarbons(BATH) and salting out aggregation test values(SAT) and adhesion to epithelial cells. The percent hydrophobicity, SAT value and adhesion index were studied for early log phase cells (6 hour) and stationary phase cells (18 hour). The percent hydrophobicity varied between 02 to 9.6 for 6 hour culture and 0.4 to 40.06 for 18 hour culture. The bacterial isolate, which exhibited the highest hydrophobicity, also exhibited the maximum adhesion index of 29.The SAT values of 6 hour old culture ranged between 1.1 to 2.1 and that of 18 hour old culture varied between 0.4 to 1.8. The isolates with lower SAT value showed higher adhesion index. The mode of adhesion was found to be calcium independent and lectin free. Enzymatic, physical and chemical treatment of the isolates revealed that the adhesive determinants present on the bacterial cell surface may be a lipoprotein complex, which is heat labile in nature. Enzymatic treatment reduced the cell surface hydrophobicity greatly. Treatment of the isolates with Sodium dodecyl sulfate (SDS) had no drastic effect on percent hydrophobicity.
The plasmid profile of the 32 isolates suggested that the presence of one larger plasmid in cultures numbering L1, L3, L5, L6, L19 and L24 of approximate size of 23.7 Kb. Cured variants of isolate L15 and isolate L1 were obtained by using ethidium bromide (6 microgram/mL). One larger plasmid of 23.7 Kb was found to be lost in isolate L1 and three larger plasmids of sizes 15 Kb, 23.7Kb and 33 Kb were found to be lost in isolate L15.The adhesive properties of cured variants like, the percent hydrophobicity, SAT value and adhesion index were reduced drastically. So, the adhesion may be plasmid mediated at least in the isolate L1 and L15.

Key Words: *Lactobacilli, Probiotics, Bath, Sat, Adhesion Index, Plasmid*

INTRODUCTION
Lactobacilli, one of the Lactic acid bacteria (LAB), are widely used in food and agricultural fermentations. Lactobacilli have a number of traits that make them particularly attractive to be used as probiotics. Most of the beneficial effects of probiotics are caused by the live microbial cells and their metabolites in the gastrointestinal tract. Hence, for successful implantation of ingested lactobacilli, bacteria must be viable within the GI tract and also have adhesive properties to avoid transient passage through the GI tract. The establishment of stable population involves a stepwise production of intestinal adhesion of the bacteria to the mucosal surface of the intestine, multiplication and subsequent colonization. Bacterial cell- intestinal epithelial cell interactions are mainly mediated through cell surface hydrophobicity. Biochemical characterization of the cell surface determinants responsible for bacterial adhesion is helpful for development of better colonizer. As all the phenotypes are determined by the genotype of the organisms, assessing the genetic determinants of adhesive property could help in genetic manipulation of the probiotics. So as to assess the biochemical and genetic determinants of lactobacilli, the present study was carried out in three phases. In the first phase, 32 lactobacilli isolates obtained from

GI tracts of albino rats and human beings are screened for their cell surface hydrophobicity through bacterial adherence to hydrocarbons (BATH), salting out aggregation test(SAT) and adhesion to isolated rat intestinal epithelial cells. In the second phase, selected lactobacilli strains were subjected to different enzymatic and chemical treatments to assess the biochemical nature of the cell surface determinants. In the third and last phase, plasmid profile of the isolates and curing experiments were performed to assess the genetic determinants of the cell surface adhesive property.

MATERIALS AND METHODS

The pure cultures of Lactobacillus strains obtained from a single colony were maintained in MRS broth and sub culturing was carried out at a regular interval of 15 days.

Bacterial Adherence to Hydrocarbons

Bacterial adherence to hydrocarbons (BATH) was carried out, as suggested by Rosenberg (1981). The bacterial cultures grown in MRS broth for 6 hours and 18 hours were used. The cells were harvested by centrifugation at 12,000 rpm for 10 min. The washed cells were suspended in Phosphate Urea Magnesium sulphate (PUM) buffer and the absorbance of cells was adjusted to an absorbance of 1.4 to 1.6 at 400 nm. To 1.2 mL of bacterial suspension, 0.2 mL of hexadecane was added. After 10 minutes of preincubation at 30^{O}C, the two phases were mixed under controlled conditions for different intervals of time (30 sec, 60 sec and 120 sec). The phases were allowed to separate for 15 minutes. The aqueous phase was carefully removed and its light absorbance was measured by using spectrophotometer. The fraction of adherent cells was taken as the percent decrease in absorbance of the aqueous phase after mixing and phase separation as compared with that of original suspension. The percent cell surface hydrophobicity in terms of adherence to hexadecane was expressed.

Salting Out Aggregation Test (SAT)

Salting out aggregation test (SAT) was carried out, as suggested by Lindhal *et al*., (1981). Sodium phosphate (0.02M,pH 6.8) was used to dilute a solution of 4M ammonium sulphate. Serial dilution was made using ammonium sulphate concentration differing from 4.0 to 0.2 M differing by 0.1M per dilution. The bacterial cultures were grown for 6 hours and 18 hours. The cells were harvested by centrifugation and washed once and suspended in 0.02 M sodium phosphate buffer pH 6.8. A bacterial suspension of 25 microliters (5x109 bacterial cells/mL in sodium phosphate buffer pH6.8) was mixed with an equal volume of salt solution on glass depression slides. The bacterial salt solution mixture was gently rocked for two minutes and visual reading was performed against a black background. A reaction causing optimal aggregation was regarded as positive reaction.

Screening of Lactobacilli for Adhesion Index

Adhesion index experiment was carried out by following the method described by Wadstrom *et al.*, (1987). This method involves 2 steps. First, isolation of epithelial cells from rat small intestine and the second is cell adhesion assay. The epithelial cells were isolated from male rat small intestine. The intestine was incubated in EDTA buffer and the cells were suspended in Krebs Henseleit buffer. The bacterial cells were adjusted to 10^7 cells and incubated at 25^{O}C for 20 minutes. The cells were stained and examined microscopically. Adhesion index was calculated as a value of the number of bacteria attached to approximately 20 epithelial cells.

Enzymatic and Chemical Treatments

0.02 M Phosphate buffer saline (pH 7.4), 0.2 N HCl, 1 N NaOH,0.01 M $CaCl_2$, 1%Sodium dodecyl sulphate, 0.5M D-Mannose, 0.1% EDTA, protease solution, trypsin solution, Lipase solution and bacterial suspension(5×10^9 cfu/mL) were prepared. The treatment of bacterial suspension with different enzymes was carried out as described by Ofek *et al*., (1981). Divalent cation treatment was carried out as per the procedure of Suegara *et al*.,(1975) and treated with mannose.

Plasmid DNA Isolation

Anderson and Mckay (1983) were followed for plasmid isolation. The bacterial inoculation was done with 10 mL of lysis broth tubes with fresh overnight cultures and incubated at 37^{O}C for 4 hours. The cells were harvested by centrifugation and transferred to 1.5 mL Eppendorf tube. The cells were added with lysozyme and then treated with SDS and further treated with phenol. Then extraction was done with chloroform - isoamylalcohol. Final dissolution of plasmid DNA was carried out with Tris-EDTA buffer. The plasmid DNA was visualized with 0.6% agarose in Tris-Acetate-EDTA buffer (pH 8.0).

Cured Variants of Lactobacilli Strains

Chemical mutagen (Ethidium bromide 0.5 mg /mL stock solution) and elevated temperature induced curing was carried out. Aliquots of 5 mL MRS broth were incubated with actively growing cultures. Ethidium bromide was added to a final concentration of 6 microgram/mL. Then the cultures were incubated at 39^{O}C for 48 hours. Then plating was done and colonies were selected on BCP-MRS agar and the suspected cured variants were picked up and transferred to MRS broth and plasmid isolation was carried out.

RESULTS AND DISCUSSION

The percent hydrophobicity, SAT value and adhesion index were studied for early log phase cells (6hours) and stationary phase cells (18 hours). The percent hydrophobicity of the 32 isolates of 6 h old cultures varied between 0.2 to 9.6 percent and the percent hydrophobicity of the 32 isolates of 18 hour old cultures varied between 0.4 to 40.06 percent. The isolate L1 which exhibited the highest hydrophobicity (40.06) showed highest adhesion index of 29. The isolate L19 which exhibited the lowest percent hydrophobicity (0.4%) showed the lowest adhesion index of 5. The SAT values of 32 isolates of 6 h old cultures ranged between 1.1 to 2.1 and that of 18 h old cultures varied between 0.4 to 1.8. The isolate L12 of 18 h old culture, which exhibited the lowest SAT value, had an adhesion index of 20, whereas the isolate L18 with a SAT value of 1.8 showed adhesion index of 11 and L19 with a SAT value of 1.8 showed an adhesion index of 5. The adhesion index of 32 isolates of 6 hours old cultures ranged between 3 to 18 and that of 18 hours old cultures ranged between 5 to 29. The stationary phase cells (18 h old) adhered better than that of early log phase cells (6 h old). Similar results have already been reported by Rosenberg *et al*., (1986).

The percent hydrophobicity correlated well with the corresponding adhesion index in most of the cases except the isolate L17 of 18 hour old culture with a percent hydrophobicity of 23.7 exhibited an adhesion index of 8. Barrow et al., (1980) reported that *L.acidophilus* cells to a pig squamous epithelial cell were in the range of 1.5 tp 12.7, for L.*Salivarius* (7.92 to 9.5) and for *L.fermentum* (7.4 to 28.6). This suggested that hydrophobicity is not the only force mediating adhesion; rather adhesion is a complex process. The SAT values correlated well with percent hydrophobicity in most of the isolates. The isolate of L18 of 18 hours old culture with 9.2 percent hydrophobicity exhibited a SAT value of 1.8. Since bacterial surface hydrophobicity is a delicate interplay of charged and uncharged groups, polar and non polar groups which are expressed in different degrees and densities on the surface, the higher the salt concentration used in SAT may alter the structure on the surface may be responsible for the higher values.

The calcium and mannose treatments in the selected lactobacilli isolates did not produce any major effect. This showed calcium independent adhesion may be involved in the isolates screened. No lectin like interactions involving mannose was noticed. Treatments with trypsin, protease, lipase, heat and HCl reduced the percent hydrophobicity greatly. The adhesive determinants on the bacterial cell surface may be lipoprotein complex, which may be heat labile in nature. Similar LTA-protein complex mediated adhesions were reported by Conway *et al*., (1985) and Sherman and Savage (1986). SDS treatment did not produce any drastic effect on the percent hydrophobicity of the isolates studied. So, the adhesive determinants may be tightly bound to the cell surface.

The plasmid profile of the 32 isolates suggested that the presence of one larger plasmid in cultures numbering L1, L3, L5, L6, L19 and L24 of approximate size of 23.7 Kb. The presence of larger plasmid DNA in lactobacilli was reported earlier by Lin and Savage (1985). Seven plasmids in isolate L15 of approximate sizes 4.7 Kb, 6.3 Kb, 7.1Kb, 12.5Kb, 15 Kb, 23.7Kb and 33.4 Kb. Two plasmids in isolate L32 of sizes 4 Kb and 6.5 Kb and in isolate L8, four plasmids of 4 Kb, 6.5Kb, 6.7 Kb and 23.7 Kb sizes.
Cured variants of isolate L15 and isolate L1 were obtained by using ethidium bromide (6 microgram/mL). One larger plasmid of 23.7 Kb was found to be lost in isolate L1 and three larger plasmids of sizes 15 Kb, 23.7Kb and 33 Kb were found to be lost in isolate L15.
The adhesive properties of cured variants like, the percent hydrophobicity, and SAT value and adhesion index were reduced drastically. So, the adhesion may be plasmid mediated at least in the isolate L1 and L15. Similar results of better adhesion of lactobacilli containing larger plasmids were earlier reported by Lin and Savage (1985). Further confirmation of the results could be obtained by using a larger population of isolates for studying the adhesive properties which may be encoded by the plasmids.

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