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Growth characteristics of *Lactobacillus brevis* KB290 in the presence of bile

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ABSTRACT

Live *Lactobacillus brevis* KB290 have several probiotic activities, including immune stimulation and modulation of intestinal microbial balance. We investigated the adaptation of *L. brevis* KB290 to bile as a mechanism of intestinal survival. Strain KB290 was grown for 5 days at 37 °C in tryptone–yeast extract–glucose (TYG) broth supplemented with 0.5% sodium acetate (TYGA) containing 0.15%, 0.3%, or 0.5% bile. Growth was determined by absorbance at 620 nm or by dry weight. Growth was enhanced as the broth's bile concentration increased. Bile-enhanced growth was not observed in TYG broth or with xylose or fructose as the carbon source, although strain KB290 could assimilate these sugars. Compared with cells grown without bile, cells grown with bile had twice the cell yield (dry weight) and higher hydrophobicity, which may improve epithelial adhesion. Metabolite analysis revealed that bile induced more lactate production by glycolysis, thus enhancing growth efficiency. Scanning electron microscopy revealed that cells cultured without bile for 5 days in TYGA broth had a shortened rod shape and showed lysis and aggregation, unlike cells cultured for 1 day; cells grown with bile for 5 days had an intact rod shape and rarely appeared damaged. Cellular material leakage through autolysis was lower in the presence of bile than in its absence. Thus lysis of strain KB290 cells cultured for extended periods was suppressed in the presence of bile. This study provides new role of bile and sodium acetate for retaining an intact cell shape and enhancing cell yield, which are beneficial for intestinal survival.

1. Introduction

Probiotics are defined by the Food and Agriculture Organization and the World Health Organization as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". It has been suggested that bacteria ingested as probiotics cannot affect the intestinal environment unless their populations reach a certain minimum level of between 10^6 and 10^8 CFU/g of intestinal content [24]; in order to accumulate in the intestinal tract, the administered bacteria must first survive the various conditions specific to the digestive system, such as low pH in the stomach and the presence of bile in the intestines. This is one of the most important properties of probiotics.

Bile secreted into the duodenal section of the small intestine is known to reduce the viability of probiotics, and the ability to tolerate bile is one of the requisite characteristics of successful probiotics. *Lactobacillus* and *Bifidobacterium* strains isolated from human intestines are the most widely used probiotics because they are expected to pass live through the intestinal tract, and many studies have examined bile tolerance in such lactic acid bacteria for the selection of successful probiotics [8,14].

Some strains derived from sources other than animal intestines also have the ability to tolerate bile. We recently showed that some plant-derived strains have high bile tolerance [19]. *Lactobacillus brevis* KB290 isolated from *suguki,* a traditional fermented vegetable product from the Kyoto region of Japan, has several probiotic activities, including stimulation of immune function [7,21,38] and improvement of gut health [27,29]. Strain KB290 can tolerate artificial digestive juice and bile salts because of its cell-bound exopolysaccharide (EPS) [34] and other factors. To expand the usefulness of this strain in the intestine, its tolerance to bile needs to be well characterized.

So far, several factors that influence bile tolerance in bacteria

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have been investigated. Comparing a bacterial strain that has high bile tolerance with a strain that has low or no tolerance can help to identify the factors that affect tolerance. However, comparisons of bile tolerance in the same bacterial species can be complicated by the high degree of differences in characteristics seen among strains of the same species. Thus, comparisons would be more effective if conducted with the same strain treated with certain modifications (e.g., altered culture conditions). Although the bile tolerance of some lactic acid bacteria and bifidobacteria has been shown to vary according to the components of the culture medium, such as the type of carbohydrate [30] and the presence or absence of Tween 80 [16], the relationship between culture conditions and bile tolerance is not yet fully understood.

Here, we investigated the growth characteristics of strain KB290 in the presence of bile by changing the following parameters: culture time, type of carbohydrate, and concentration of bile or other components of the culture medium.

2. Materials and methods

2.1. Strain and growth conditions

Lactobacillus brevis KB290 (Japan Collection of Microorganisms: JCM 17312) was maintained by subculturing 1% inoculum in MRS broth (Becton, Dickinson and Company, Sparks, MD, USA) for 18–24 h at 37 °C for static condition. The culture was stored at 4 °C between transfers and was subcultured once overnight in fresh culture medium before use.

Tryptone–yeast extract–glucose (TYG) broth was prepared containing 0.5% tryptone (Becton, Dickinson and Company), 0.5% yeast extract (Wako Pure Chemical Industries Ltd., Osaka, Japan), and 1.0% glucose supplemented with 1% sodium succinate and 1% sodium chloride. TYG broth or TYG supplemented with 0.5% sodium acetate (TYGA broth) was adjusted to pH 6.8 and was dispensed in 4-ml lots and sterilized by autoclaving at 121 °C for 15 min. The medium was inoculated with 1% (v/v) of a fresh overnight culture of the tested strain and then incubated at 37 °C.

To examine the carbohydrate preference of strain KB290 in the presence or absence of bile, tryptone–yeast extract (TY) broth was dispensed into 4-ml lots and sterilized by autoclaving at 121 °C for 15 min. Fructose, lactose, sucrose, and xylose were each dissolved in distilled water to a concentration of 10% (w/v) and then sterilized by passage through a filter (0.2-μm, Advantec, Tokyo, Japan). We prepared each medium by adding one of these carbohydrates to the TY broth to a final concentration of 1.0% (v/v).

2.2. Determination of growth in the presence of bile

Bacto Oxgall (Becton, Dickinson and Company) was dissolved in distilled water and sterilized by autoclaving at 121 °C for 15 min. Sterilized oxgall solution was added to the broths to a final concentration of 0.15%, 0.3%, and 0.5% (v/v). A bile concentration of 0.3% is considered suitable in medium for selecting probiotic bacteria for human use [9,10]. Each broth was inoculated with 1% of a fresh overnight broth culture and then incubated at 37 °C. After incubation, bacterial growth was determined by measuring the culture absorbance at a wavelength of 620 nm (OD_{620}) with a Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, NY, USA) against an uninoculated broth blank [16]. Bile resistance was expressed as a percentage of growth of the control (growth in bile-free broth) [17].

The growth yield of cells generated per mole of glucose consumed (the molar growth yield for glucose, Y_G) is widely used to assay the molecular energetics of biochemical pathways [23]. The molar growth yield was calculated by dividing the dry weight of cells by the molar mass of consumed glucose. For the measurement of dry weight, cells were collected by centrifugation (1800 × *g*, 20 min, 4 °C), washed once with distilled water, and dried at 105 °C for 4 h.

Viable cells after culture in broth with or without bile were counted by plating samples of the appropriate dilution in 0.85% NaCl solution onto MRS supplemented with agar (1.6%). All plates were then incubated at 35 °C or 37 °C for 2 days.

2.3. Analyses of carbohydrates and fermentation end-products

Culture supernatants were stored at −20 °C before analyses for substrates and end-products. Residual glucose in the broth was measured enzymatically by using a Glucose CII test kit (Wako). Lactate in the broth after culture was quantified with an F-kit (Boehringer, Mannheim, Germany). Bile removal from media was determined with a total bile acid test kit (Wako): subtracting the amount in the spent broth from that in the uninoculated broth yielded the amount removed by the cells.

2.4. Hydrophobicity of cells grown on different carbon sources

Cell hydrophobicity was determined by using the methods of Morata de Ambrosini et al. [26], with some modifications. Washed cells were suspended in water to an OD_{620} of about 1.0. *n*-Hexadecane (1.0 ml) was added to the cell suspension (1.2 ml). After a 10-min pre-incubation at 30 °C, the solution was mixed by vortexing for 60 s and left to stand for about 15 min until the hydrocarbon phase rose completely to the top. The OD_{620} of the aqueous phase was then measured. Hydrophobicity was expressed as the percentage decrease in absorbance after mixing according to the formula: $100 \times [1 - (OD_{620}$ after mixing/OD_{620} before mixing)].

2.5. Electron microscopy

For scanning electron microscopy, the culture was fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 h at room temperature. After being washed 5 times with phosphate buffer, cells were postfixed for 30 min with 2% OsO_4 in the same buffer, washed 5 times, and then dehydrated in a graded series (50, 70, 80, 90, 100%) of ethanol. Bacterial cells were dried by Freeze drying (JFD-320; JEOL, Tokyo, Japan) and coated with osmium. Specimens were examined with a scanning electron microscope (JSM-7600F; JEOL). These procedures were carried out in NARO Institute of Animal Health (Ibaraki, Japan).

2.6. Cell lysis

Lysozyme resistance was performed by using a modified version of the method of [33]. Cells were grown in TYGA broth with or without 0.3% bile for 5 days, and then washed twice with 0.85% NaCl. Cells were suspended in 0.06 M phosphate buffer (pH 6.2) supplemented with 1% NaCl [12] and then incubated with 100 μg/mL chicken lysozyme (Sigma–Aldrich, St. Louis, MO, USA) at 37 °C. Cell lysis was determined by the decrease in absorbance at a wavelength of 620 nm compared with that of untreated cells [18].

Autolysis of the tested strains was assessed as follows. Cells were washed twice with water, resuspended at an OD_{620} of 0.35–37 in 0.1 M potassium phosphate (pH 7.0), and incubated at 37 °C. To measure the leakage of cell materials as a result of autolysis, cell suspensions were centrifuged (10 min at 8000 × *g*) and the clear supernatant was recovered. The supernatant was checked for the presence of UV-absorbing materials by reading the absorbance at 260 nm with Nano Vue (GE Healthcare, Buckinghamshire, UK).