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Research Article

Screening and Identification of Exopolysaccharide-producing Bifidobacteria from Infant Gut

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Abstract: Exopolysaccharide (EPS) produced from lactic acid bacteria has many bioactivities and the role of prebiotic as reported. However, being a well-knowen probiotic, there is little report about EPS-producing bifidobacteria. In this study, several bifidobacteria strains were isolated from baby feces with mupirocin lithium salt improved medium and evaluated for the production of EPS. Culturing strains with MRS medium containing 2% sucrose, one EPS-producing bifidobacteria strain A19 was screened by ethanol precipitation, dialysis, phenol-sulfuric acid method and other methods. By the physiological and biochemical characteristics, 16S rDNA and *tuf* gene sequence analysis, strain A19 was identified as *Bifidobacterium pseudocatenulatum*. It was a novel EPS-producing bifidobacteria and the EPS yield was 55.546 mg/L in MRS broth with 2% sucrose. It could be a potential available strain for microecologic products and food starters.

Keywords: Bifidobacteria, exopolysacharide, identification, screening

INTRODUCTION

In 1899, the French scholar Tissier isolated anaerobic gram-positive bacteria from feces of breast-fed infants at the first time. As for as the cell morphology, the end of bacteria was often biforked, hence it was named as bifidobacteria (*Bifidobacterium*). Bifidobacteria are typical beneficial bacteria in the human gut being a biochemical barrier in human gut. It can inhibit the growth of pathogenic and spoilage bacteria, promote lactose digestion and absorption, regulate intestinal flora, improve the metabolism of gut microbes and protein and resist infection of intestinal pathogens. Thus it plays a probiotic effect and its role in promoting human health far more than other lactic acid bacteria (De Vuyst *et al*., 2001).

Several lactic acid bacteria produce Exopolysaccharides (EPS) that are secreted into the growth media. And some of lactobacilli and bifidobacteria used as the probiotic bacteria produce EPS. In fact, the health-promoting effect of bifidobacteria may have a great relationship with its EPS produced by metabolic process (Prasanna *et al*., 2012). EPS can protect bifidobacteria and survive well in the intestine. Being a natural carbohydrate, EPS produced from *Bifidobacterium* can be used as a natural preservative and improve physiological and metabolic state of intestinal flora as a substrate for the intestinal microbial (Salazar *et al*., 2009, 2008). While, it also has variety of pharmacological effects, such as immune regulation, anti-tumor, anti-oxidation, removing toxins and hypoglycemic effect (Ren *et al*., 2011). Therefore, in this study, bifidobacteria isolated from the intestinal tract of healthy infants were evaluated for EPS-producing ability. By ethanol precipitation, dialysis, phenol-sulfuric acid method, several EPS-producing strains were found and of the highest-EPS-yield bifidobacterium was carried out for the taxonomic identification. The work laid the theoretical and practical basis for the understanding, adapting and utilizing *Bifidobacterium* EPS. These EPS-producing bifidobacteria were potential novel probiotic strain for food starters and bioecological agent to enhance the quality and functionality.

MATERIALS AND METHODS

Isolation of bifidobacteria strains: At first, 10 g infant feces was put into the flask with 90 mL sterile saline keeping aseptic operation and pipetting diluted to the 10^{-1}, then 0.5 mL mixed liquid was taken from the flask into Hungate anaerobic tubes containing 4.5 mL saline as 10^{-2} dilution. In the second, after 4 times dilution, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions were separately inoculated in three dishes containing MRS agar medium (Beijing Land Bridge Technology Co., Ltd., China) with mupirocin lithium salt, which was previously sterilized at 121°C for 20 min and clutured under anaerobic conditions at 37°C for 48 h. The anaerobic conditions were achieved by AnaeroGen™ Anaerobic generation system (OXOID, United Kingdom). Finally, plates containing 10-20 colonies were selected to pick suspected colonies. Almost every colony was picked and inoculated in a test tube with

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MRS broth medium. After anaerobic cultivation at 37°C for 24 h, all isolates were gram stained and observed under oil lens of biological microscope (Carl Zeiss Microimaging GmbH, Germany).

Cultivation of bifidaobacteria: The suspected colonies were separately streaked on MRS agar and incubated anaerobically at 37°C for 72 h to obtain pure inoculum. Then the strain inoculums were subsequently inoculated in hung ate anaerobic tube with MRS broth at 37°C for 24 h. Finally, bacterial cultures were stored in 70% (v/v) glycerol at -80°C.

Extraction of exopolysaccharide: The single colonies on plates were inoculated in 100 mL MRS broth containing 2% sucrose. Twenty four hour grown cultures were centrifuged at 8000 g for 10 min in Allegra X-15R centrifuge (Beckman Coulter, Inc. USA) to remove cells. EPS were precipitated from supernatants by adding 3 volumes of anhydrous ethanol and the mixture was stored overnight at 4°C. After ethanol precipitation and another centrifugation at 8000 g for 10 min, precipitates were resuspended in distilled water and dialyzed using dialysis tubes (molecular weight cut-off 8 to 14 kDa, Beijing Solarbio Science and Technology Co., Ltd., China). Solutions were prepared for the further yield determination after dialysis for 48 h against water, which was, removed each 8 h (Xu *et al*., 2011).

Determination of exopolysaccharide yield: The crude EPS obtained above was diluted with distilled water to 50.0 mL and then the EPS content was detected by phenol-sulfuric acid method with glucose as standard.

The glucose standard curve was prepared for the quantitative determination, according Dubois *et al*. (1956) with some modifications. Briefly, 4.00 mg glucose was dissolved in water to make 100 mL. Then 4.0, 12.0, 20.0, 28.0 mg/L glucose solution were made by diluting with distilled water to 2.0 mL, respectively. 1.0 mL of 6% phenol and 5.0 mL sulfuric acid 95% (v/v) were added quickly and shake up after 10 min standing, then absorbance at 490 nm was measured using YU-1810 UV-visible spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., China). (2.0 mL) of distilled water used as a blank in the same conduct. The standard curve was drawn with concentration of EPS as the horizontal axis and absorbance values as the vertical axis.

For EPS yield determination, weighed accurately 0.1 mL of EPS samples and diluted to 2.0 mL with distilled water, then added quickly with 1.0 mL of 6% phenol and 5.0 mL of sulfuric acid 95% (v/v) and shake up after 10 min standing and then absorbance at 490 nm was measured. The concentration of EPS was determined in triplicate and 2.0 mL distilled water used as blank. The EPS content of each sample was calculated by the standard curve.

Identification of high-yield EPS-producing strain determination of physiological and biochemical characteristics: The physiological and biochemical characteristics of the highest yield-producing strain were examined using standard procedures (Dong and Cai, 2001). Gram staining, bacterial morphology, enzyme reaction, oxidase reaction and other characteristics were investigated. The utilization of some carbon sources, such as erythritol, D-arabinose, L-arabinose, inositol, mannitol, sorbitol and α-methyl-D-mannose glycosides, by the strain was performed based on Bergey's Manual of Systematic Bacteriology (Kandler and Weiss, 1986).

PCR amplification and sequence analysis: The highest EPS yielding strain was subjected the 16S rDNA sequencing. The forward primer was 27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and the reverse primer was 1541R 5'-CTACGGCTACCTTGTTACGA-3'. PCR reaction mixture was of 100 μL and the amplifying conditions were as follows: denaturized at 95°C for 5 min, amplified for 30 cycles under the following conditions (95°C for 1 min, 57°C for 1 min, 72°C for 1 min), then finally extended at 72°C for 5 min. The PCR products were sent to a Biotechnology Company (Beijing Haocheng Mingtai Technology Co., Ltd., China) for sequencing.

The *tuf* gene sequencing was also applied for the identification. The forward primer was Bif-1: 5'-gAgTACgACTTCAACCAg-3' and the reverse primer was Bif-2: 5'-CAggCgAggATCTTggT-3'. PCR reaction mixture was of 50 μL and the amplifying conditions were as follows: denaturized at 95°C for 5 min, amplified for 30 cycles under the following conditions (95°C for 30 sec, 52°C for 30 sec, 72°C for 2 min), then finally extended at 72°C for 10 min. The PCR products were sent to the same Biotechnology Company for sequencing.

The obtained 16S rDNA sequence and *tuf* sequence, respectively, were manually corrected and subsequently aligned to sequences in GenBank Database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The aligned 16S rDNA sequences and *tuf* sequence of related species were retrieved from the NCBI nucleotide database. Phylogenetic and distance analysis of the aligned sequences was performed by the program MEGA (version 4.1). The resulting unrooted tree topologies were evaluated by bootstrap analysis of Un-weighted Pair Group Method arithmetic averages clustering Algorithm (UPGMA).

RESULTS AND DISCUSSION

Microbiological characteristics of isolates: A total of 22 suspected bifidobacteria strains were isolated. The purity of each strain was ensured by the further streak

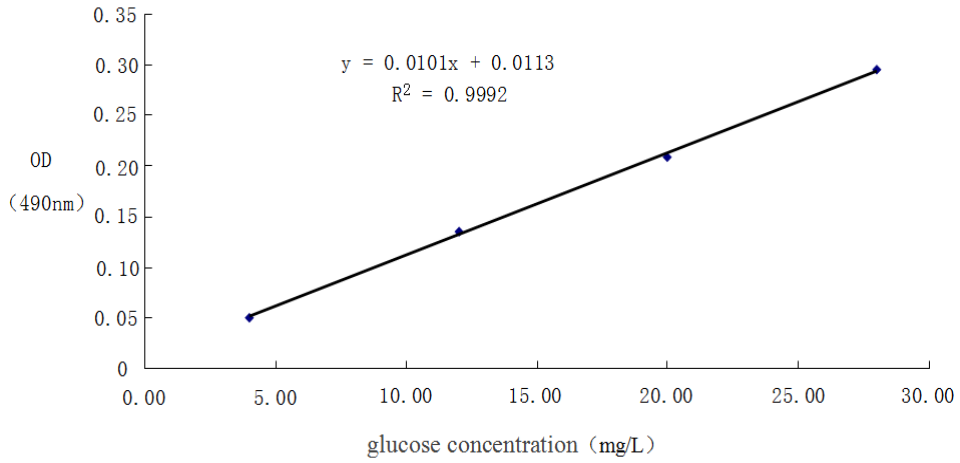

Fig. 1: The standard curve of glucose used in phenol-sulfuric acid method for measuring the yield of exopolysaccharide

Table 1: Exopolysaccharide production of different strains of bifidobactia

Strains	EPS content (mg/L)	Strains	EPS content (mg/L)
A19	55.546	A21	30.924
A12	44.972	A16	27.148
A3	48.346	A22	11.432
A17	40.648		

cultivation and the observation by microscopic morphology. Their cell morphology was mainly rodlike and Y-shaped and colony morphology was large, smooth and transparent.

Exopolysaccharide production: Phenol-sulfuric acid method was used to measure the content of EPS using glucose as standard. There was a good linearity and the correlation coefficient was higher than 0.9992. EPS content was calculated according to the regression equation based on the standard curve shown in Fig. 1 and then converted with dilution ratio. There are only 5 strains producing EPS and the yield of each strain was shown in Table 1.

The visual inspection of bacterial colonies on agar plates is most probably the easiest method among many different EPS screening methods. This method is unable to detect bacteria that produce low amounts of EPS, unless they are very ropy. The partial purification of EPS through precipitation with ethanol and its photometrical quantification is another quick screening method. In this study, the extract used the examine exopolysaccharide content as the screening method. This method was effective and accurate and unsuccessfully avoided some producing low yield EPS strains failing to be chosen. In addition, the screening methods used in this experiment is simple, reliable and having low equipment requirements. It is suitable for initial screening a large number of species. As the result, EPS was produced in a range from 11.432 to 55.546 mg/L. Among them, EPS yield of strain A19 was the highest. Therefore, strain A19 was chosen for the further identification, in which classical microbiological classification methods and gene involved molecular biology methods.

Itentification of the highest EPS yield strain: The strain A19 was identified to genus by traditional classification methods. The identification results are shown in Table 2. The result showed that strain A19 was a gram-positive, rod-shaped and belonged to *Bifidobacterium pseudocatenulatum*.

16S rDNA and tuf sequencing: 16S rDNA gene was a commonly used material for bacterial species identification. The content was large and its molecular size is moderate (approximately 1.5 Kb). Besides, it is highly conserved and present in all organisms, which reflects the difference between the different species. *Tuf* sequence analysis is proper to classify strains with close genetic relationship (Lee *et al*., 2004). Because *tuf* gene (elongation factor gene) has strong variability and low homologous rate, a small difference in strain can be accurately identified and classified (Wang *et al*., 2011). Moreover, *tuf* gene sequence analysis is highly reliable and rigorous, so it was applied for the identification and classification of strain A19.

In this study, using software MEGA4.1, 16S rDNA and *tuf* sequences phylogenetic tree of A19 and some other related species was made by neighbor-joining method after 1000 times repeating similarity calculation. The result of 16S rDNA sequencing showed strain A19 was *Bifidobacterium pseudocatenulatum* (Fig. 2). Phylogenetic analysis of *tuf* gene sequencing also revealed that strain A19 was clustered with *Bifidobacterium pseudocatenulatum*. Its phylogenetic tree based on other bacterial *tuf* sequences from GenBank was shown in Fig. 3.

Table 2: Physiological and biochemical identification results of strain A19

Project	Result	Project	Result	Project	Result
Cell morphology	Rod	Gram stain	+	Catalase	-
Oxidase	-	Sporulation	-	45°C growth	-
pH 4.5 growth	-	pH9.6 growth	-	Glucose fermentation and gas production	-
API 50CH					
Contrast	-	Inositol	-	Melezitose	+
Glycerin	-	Mannitol	-	α-methyl-D-glucoside	+
Erythritol	-	Sorbitol	-	Starch	-
D-arabinose	-	Glycogen	-	α-methyl-D-mannoside	-
L-arabinose	+	Raffinose	+	β-methyl-D-xyloside	-
D-ribose	+	Gentiobiosyl	-	N-acetyl-glucosamine	-
D-xylose	+	Amygdalin	-	D-turanose	+
L-xylose	-	Arbutin	-	D-lyxose	-
Adon alcohol	-	Esculin	-	D-tagatose	-
Xylitol	-	Salicin	-	D-fucose	-
D-galactose	+	Cellobiose	+	L-fucose	-
D-glucose	+	Maltose	+	D-arabitol	-
D-fructose	+	Lactose	+	L-arabinitol	-
D-mannose	+	Melibiose	+	Gluconate	-
L-sorbose	-	Sucrose	+	2-keto-gluconate	-
L-rhamnose	-	Trehalose	-	5-keto-gluconate	-
Dulcitol	-	Inulin	-		

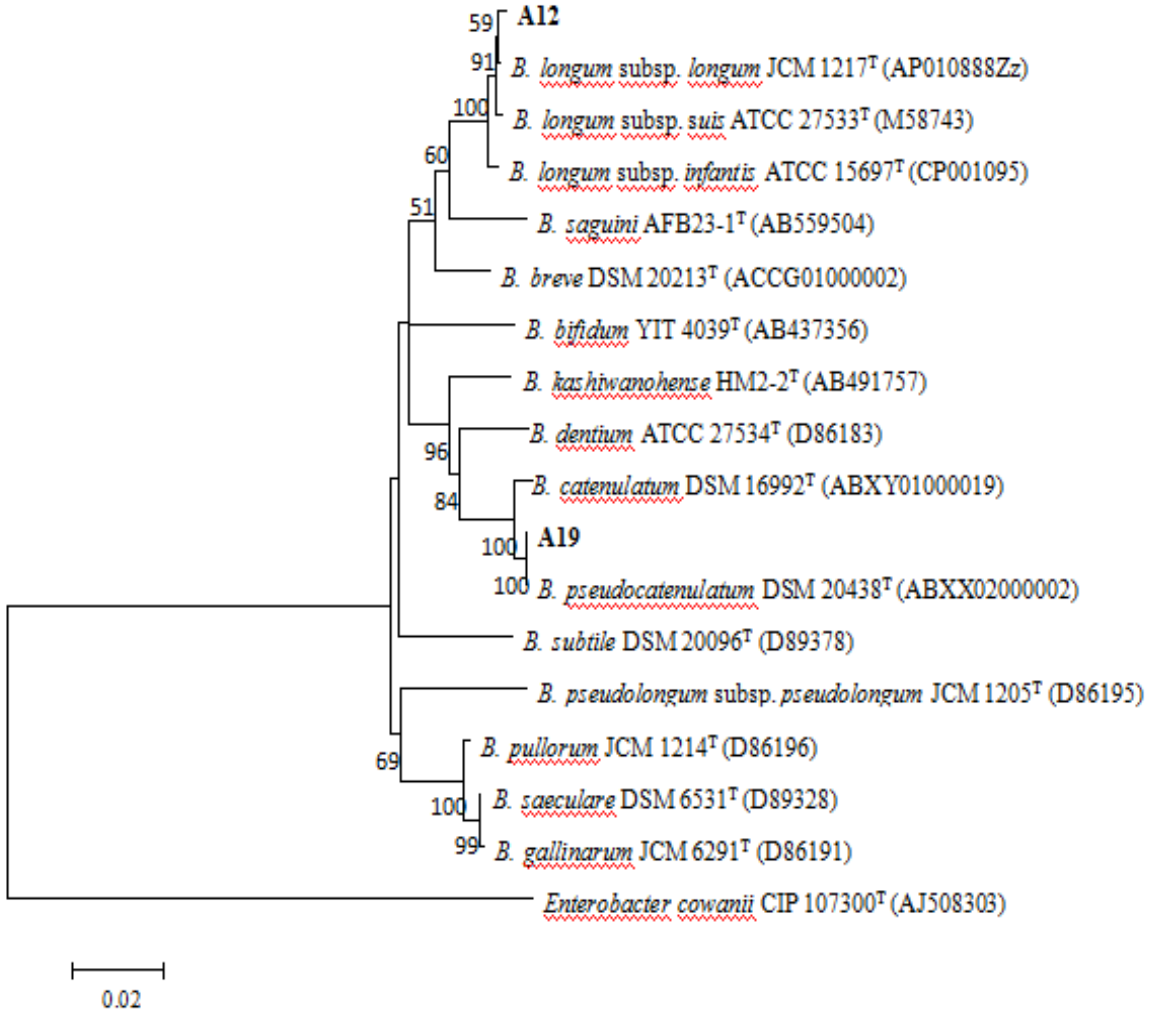

Fig. 2: Phylogenetic tree of strain A19 based on 16S rDNA sequence
Phylogenetic tree node only shows bootstrap values greater than 50%; ^{T}: The type strain; *B.*: *Bifidobacterium*

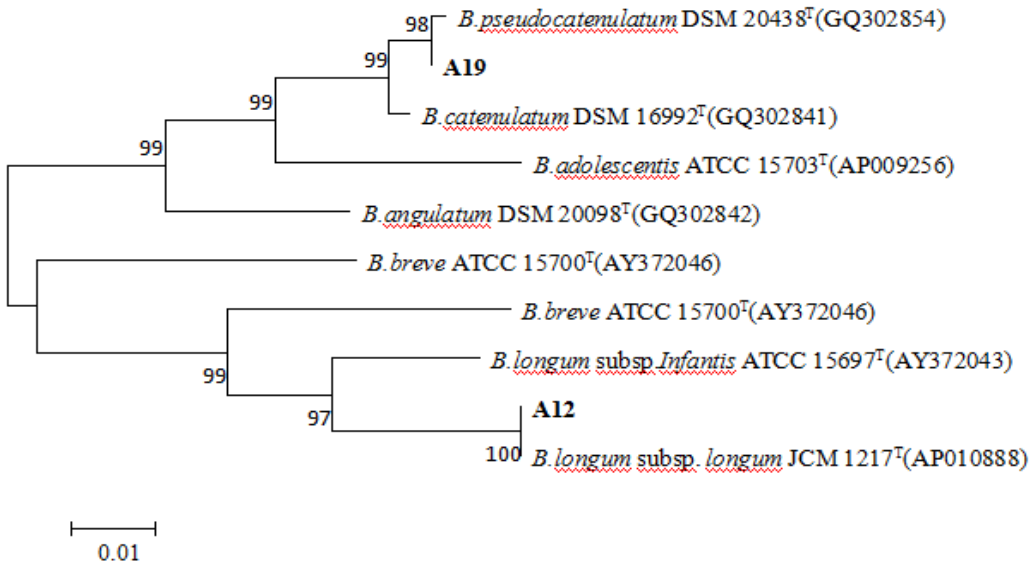

Fig. 3: Phylogenetic tree of strain A19 based on *tuf* sequence
Phylogenetic tree node only shows bootstrap values greater than 50%; ^{T}: The type strain; *B.*: *Bifidobacterium*

CONCLUSION

There are several reports about EPS produced by *Bifidobacterium longum* and *Bifidobacterium animalis*. In this study, one EPS-producing *Bifidobacteriim pseudocatenulatum* screened, which was not reported before. In spite, the further separation and purification for structure analysis and function evaluation. The strain A19 could be a novel potential probiotic strain for food starters and bioecological agent.

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