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Characterization of Novel Anti-rotavirus Protein from *Bifidobacterium* sp. BORI

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A novel anti-rotaviral bifidobacteria has been isolated from Korean and the active material has been identified and characterized.

Human rotavirus has been the leading cause of diarrhea and acute gastroenteritis in infant and young children. *Bifidobacterium* is one of the most common bacteria in human large intestine and regarded to have the useful effects on human health. We have screened for antirotaviral bifidobacteria and *Bifidobacterium longum* BORI was found to have the most potent inhibitory activity on rotavirus infection. The infection inhibitory activity of this bifidobacteria has been attributed to one protein, which has been purified and named as BORI.

Accordingly, we cloned its gene based on the internal amino acid sequence and over-expressed it in *Escherichia coli*. It was about 59 KDa and highly homologous to protease derived from bifidobacteria. Newly found rotavirus infection-inhibitory protein can be used in the functional dairy food and might contribute to the prevention of rotaviral illness and any other pathogenic viruses in the near future. This bifidobacteria was applied to the infant probiotic product named as JIGEUNEOG BIFIDUS BABY.

Introduction

Acute diarrheal diseases are still a major health problem throughout the world among children younger than 5 years of age in developing countries. Acute diarrhea can be caused by many different agents including parasites, bacteria and viruses. Rotavirus is the leading cause of infantile gastroenteritis worldwide and is responsible for approximately 20 % of diarrhea-associated death in children under 5 years of age [1]. The outcome of infection is more devastating in developing countries, where an estimated 600,000 deaths occur annually, and surviving children may fail to thrive. The live-attenuated rhesus rotavirus (RRV)-tetravalent vaccine (TV), the first rotavirus vaccine licensed, was withdrawn from the market because of a temporal association with intussusception [2]. These unexpected disadvantages emphasize the need to better understand the mechanisms underlying rotavirus pathogenesis and the needs of a new therapy.

A number of studies have been carried out on the effect of several probiotic species on treatment and

prevention of intestinal infections. Bifidobacteria and other lactic acid bacteria are thought to have a protective effect against an intestinal disease. Bifidobacteria are the most common bacteria and considered to be one of the most beneficial probiotic organisms. The underlying mechanism of these therapeutic effects is still unclear [3].

In this study, the rotavirus infection inhibitory protein was cloned from *Bifidobacterium longum* BORI and over-expressed in *Escherichia coli.* We will study the characterization and the inhibitory mechanism of anti-rotavirus protein.

Bacterial strains and plasmid

Bifidobacteria strains used in this study are from BIFIDO Co., Ltd. All of them were isolated from healthy infants and adults with various age and sex. *Bifidobacterium* was grown anaerobically at 37℃ in *Lactobacilli* MRS broth (Hardy diagnostics, CA, U.S.A.) supplemented with 0.05%(w/v) of L-cystein HCl. The anaerobic condition has been established by using ANOXOMAT WS8000 system (MART Microbiology BV, Lichtenvoorde, The Netherlands). As recipient strains for transformation, *E. coli* DH5α was used and cultivated in Luria-Bertaini liquid medium (Hardy diagnostics, CA, U.S.A.). All the bacterial strains used in this study were stored in 15% (v/v) glycerol stock at -70℃ and sub-cultured periodically. All the enzymes for molecular work were purchased from Promega (Madison, WI, U.S.A.) and the chemicals were from Sigma-Aldrich Co., (St. Louis, MO, U.S.A.) if not indicated otherwise. For the cloning of antirotaviral protein, pUC19 and *E. coli* DH5a were used. *E. coli* DH5a was cultivated in LuriaBertani broth. Over-expression system pMAL-c2 (NEB, U.S.A.) was used according to the manufacturer's recommendation.

General cloning technique

Plasmid isolation, cloning, PCR amplification, protein purification and SDS PAGE were performed according to Sambrook et al.[4]. Sequencing reaction was carried out using ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit and the sequence was read by ABI PRISM 377 autosequencer (Applied Biosystems, CA, U.S.A.).

Rotavirus culture and assay of its inhibitory activity

The MA104 cells wre cultured in DMEM containing 10% FBS, 1% antibiotic-antimycotics, and 3.5g/l sodium bicarbonate (CO2 incubator, 37℃). As a nonactivated virus, the Wa rotavirus(400ul) was activated with 20ul of 0.1mg of trypsin per ml at 37 for 0.5h [5 ,6]. Washed MA104 cell (1.5 * 10^6 cells/25 cm^2 flask) monolayers were then inoculated with the activated rotavirus at a multiplicity of infection of 1 to 5, incubated for 2h, and then aspirated. Next, the cells were washed twice, refed with DMEM containing trypsin, and grown until a cytopathic effect was visible, usually within 3-5 days. Quantifying the rotavirus

in the given sample was performed according to an endpoint dilution assay. The assoy of the inhibitory activity of the Bifidobacteria and their components was based on the inhibition of rotavirus-induced cytopathogenicity. Briefly, 50ul of 10^{-3}-diluted Wa virus ($1*10^3$ pfu) was infected into 100ul of MA104 cells ($3*10^5$ cells/ml) containing 50ul of the sample. The cells were then grown until a cytopathic effect was visible and the inhibitory activity of the sample towards the rotavirus infectivity was measured.

Results and discussion

The strategies for cloning rotavirus infection inhibitory protein show in Figure 1. Several bifidobacteria were isolated from healthy korean, the cytosol and cell wall components were fractionated by sonication, and the inhibitory activities of the bifidobacteria fractions on rotavirus infection investigated (Table 1). The cytosol components of most of the bifidobacteria exhibited higher inhibitory activity than their respective cell wall components. Among them, the cytosol component of *B. sp* AR-81 showed the most potent inhibitory activity. *B. sp* AR-81 was determined to *Bifidobacterium longum* by 16S rDNA analysis. After the purification of anti-rotavirus infection protein, to clone its gene from *B. sp* AR-81, We sequenced internal amino acids of its protein (data not shown). Degenerate PCR primer was designed to amplify an interest gene based on the results. We also checked the GenBank Database based on several amino acids. Therefore, a gene of anti-rotaviral protein was cloned and sequenced. It is about 59 KDa and highly homologous to a protease derived from bifidobacteria. The gene was subcloned into pMAL-c2 vector to over-express in *Escherichia coli* and the expression of its gene was confirmed by SDS-PAGE (Figure. 2). We will study the characterization of anti-rotaviral protein. It showed dipeptidase activity.

Newly found rotavirus infection-inhibitory protein was named BORI and can be used in the functional dairy food and might contribute to the prevention of rotaviral illness and any other pathogenic viruses in the near future.

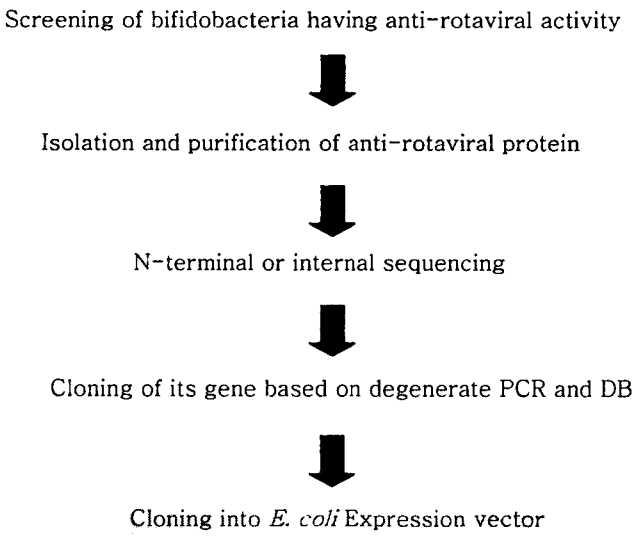

Figure 1. The strategies for cloning rotavirus infection inhibitory protein gene from Bifidobacteria

Table 1. Anti-rotaviral activity of several bifidobacteria strains.

Bifidobacterium	Inhibition rate (%)					
	cytosolic components (mg/ml)			cell walls (mg/ml)		
	0.0005	0.002	0.010	0.0005	0.002	0.010
B-47	16.7	16.7	66.6	0	0	16.7
B-81	**0**	**33.3**	**100**	**0**	**28.6**	**16.7**
B-179	0	33.3	83.3	0	16.7	33.3
KK-11	0	16.7	66.6	16.7	16.7	16.7
KK-12	0	16.7	83.3	16.7	16.7	16.7
B. longum[a]	0	14.3	14.3	0	14.3	14.3
B. infantis[a]	0	28.6	42.9	0	14.3	14.3
L. acidophilus[a]	0	14.3	42.9	14.3	14.3	14.3

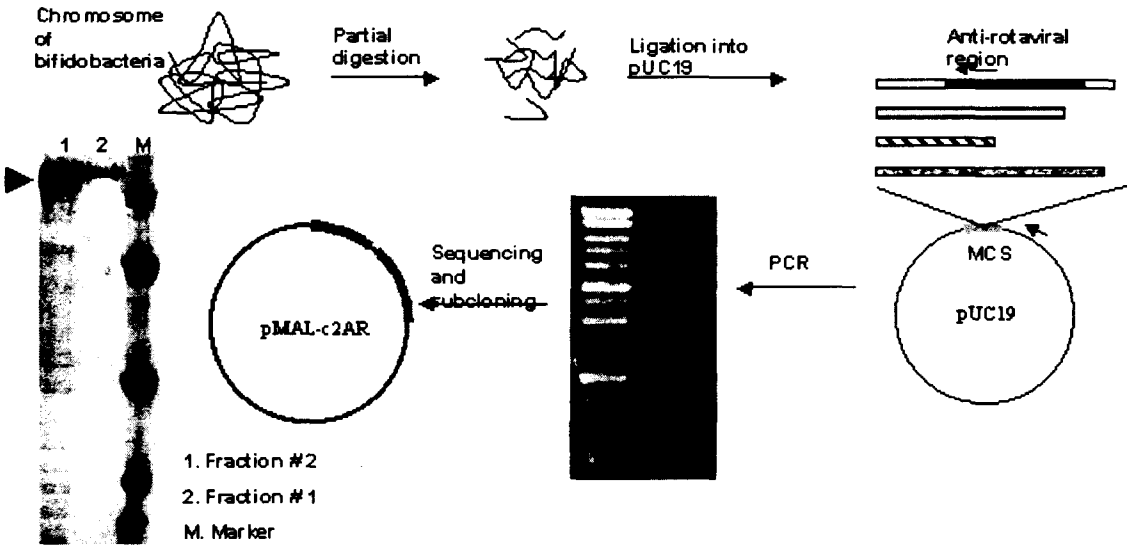

Figure 2. The construction of anti-rotaviral protein expression vector and its over-expression in Escherichia coli

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