Search for Protein Adhesin Gene in *Bifidobacterium Longum* Genome Using Surface Phage Display Technology

A. N. Shkoporov, E. V. Khokhlova, L. I. Kafarskaia, K. A. Pavlov, V. V. Smeianov*, J. L. Steele*, and B. A. Efimov

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A fragment of the nucleotide sequence encoding polypeptide binding to HT-29 epithelial cells was cloned from VMKB44 *Bifidobacterium longum* genome library using surface phage display technology. Sequencing of this polypeptide consisting of 26 amino acid residues showed that it is an extracellular fragment of a large BL0155 transmembrane protein belonging to the ABC transport protein superfamily. The genes encoding homologues of this protein were detected in genomes of not only bifidobacteria of different species, but also in many other enteric commencals and pathogens.

Key Words: *bifidobacteria; HT-29; adhesion; adhesin; phage display*

Bifidobacteria are gram-positive nonsporulating anaerobic bacteria, belonging to one of the most important genera among mutualistic human enteric microorganisms, characterized by the positive effect on human gastrointestinal physiology and health status in general. Due to this bifidobacteria are widely used as the basis for manufacturing of bacterial probiotics. On the other hand, the mechanisms of colonization of human large intestine with bifidobacteria are little studied. In light of this, the search for adhesion and colonization factors in bifidobacteria is an important problem.

The most prevalent experimental model for evaluation of adhesion characteristics is coculturing of bifidobacteria with CaCo-2 and HT-29 tissue epithelium-like cultures [4]. It was demonstrated not once that some of bifidobacterial strains can adhere to CaCo-2 cells and to the mucus produced by HT-29 strain. It was found that adhesion is calcium-independent and is mediated by a protein factor present on intact cells and in culture supernatant. Moreover, it was shown that adherent strains of bifidobacteria dose-dependently inhibited adhesion and invasion of enteropathogenic bacteria *E. coli* (EPEC), *Salmonella typhimurium*, and *Yersinia pseudotuberculosis* in CaCo-2 cells [1,5].

We searched for protein adhesin genes in the *Bifidobacterium longum* VMKB44 strain genome using the surface phage display technology.

MATERIALS AND METHODS

The surface phage display technology is based on obtaining (by the gene engineering methods) translation fusions of polypeptides encoded in the bacterial genome with glycoprotein pVIII in the bacteriophage M13 capsid [3]. Expression of chimerical proteins of this kind on the surface of recombinant bacteriophages makes is possible to select adherent clones, isolate them in pure culture, and identify the cloned sequence.

Bifidobacteria were cultured in TPY medium. *Escherichia coli* TG1 strain was cultured in LB and 2xYT media (Amresco). For phagemid selection, ampicillin (100 μg/ml) was added to the media. Helper bacteriophage R408 was cultured by infecting *E. coli* TG1 culture. Cells HT-29 were cul-

Russian State Medical University, Federal Agency for Health care and Social DevelopmentRussian Ministry of Health, Moscow, Russia; *University of Wisconsin-Madison, USA. ***Address for correspondence:*** a.shkoporov@gmail.com. A. N. Shkoporov

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tured at 37°C and 5% CO_2 in DMEM with 10% FCS, 100 U/ml penicillin G, and 100 μg/ml streptomycin.

Bifidobacterial genome DNA for the library construction was isolated by the modified Stahl method [6]: 5 ml bifidobacteria culture was incubated overnight, centrifuged (3 min, 13,000*g*), washed in 1 ml TES buffer (100 mM Tris hydrochloride, pH 8.0, 50 mM NaCl, and 70 mM EDTA), and resuspended in 500 μl of the same buffer, to which 25% sucrose, 30 mg/ml lysozyme, and 70 U/ml mutanolysin were added. The suspension was incubated on a water bath at 42°C for 1 h. Subsequent stages were carried out in accordance with the original protocol.

The phagemid library was constructed using *B. longum* VMKB44 strain genome DNA fragments, obtained by incomplete hydrolysis of genome DNA by DNAse I. Genome DNA (60 μg) was treated with 800 ng DNAse I in a buffer containing 10 mM Tris hydrochloride (pH 8.0), 10 mM $MnSO_4$, 0.1 mM $CaCl_2$ for 10 min at 15°C. After incubation, the reaction was stopped by adding EDTA to a final concentration of 100 mM and warming at 65°C for 10 min. The resultant DNA preparation was extracted with a phenol/chloroform/isoamyl alcohol mixture, precipitated with ethanol, and treated with bacteriophage T4 DNA polymerase (Sibenzyme) in buffer solution with a mixture of deoxynucleoside triphosphates (0.2 mM) for the formation of "blunt ends". Vector DNA pG8SAET was treated with *Bst*SNI restriction endonuclease (Sibenzyme) in the buffer solution from the same reagent kit and then with alkaline phosphatase (Sibenzyme). Vector DNA was extracted, re-precipitated with ethanol, and mixed with insertion DNA in a 1:5 molar ratio. Ligation was carried out using a Ready-To-Go Ligation Kit (GE-Amersham). The ligation products were used for transformation of 10 aliquots of electrocompetent *E. coli* TG1 cells isolated as described previously [3]. Transformed culture was incubated for 18 h in 100 ml LB medium with ampicillin.

A 6-ml aliquot collected from the transformed culture incubated overnight was infected with 4×10^{11} plaque-forming units of R408 helper bacteriophage. The culture was then mixed with semisolid agar LB (0.5%) and distributed in dishes with LB/ampicillin medium. After 24-h incubation, phage particles were washed from semisolid agar into medium LB, sterilized by filtration, and stored at -70°C. The phage library "panning" procedure was carried out using methanol-fixed confluent HT-29 culture washed in PBS in a 25-cm^2 culture flask. Phage stock (5 ml) was applied onto a surface of fixed cells and after 18-h incubation at 4°C free phage particles were removed by repeated washing in PBS. The remaining adherent phage particles were eluted for 5 min with 2 ml buffer of the following composition: 50 mM sodium citrate and 150 mM NaCl (pH 2.1). The eluate was directly neutralized by adding 0.2 ml 1 M Tris hydrochloride (pH 8.0) and used for infection of strain TG1. The resultant culture was re-infected with the helper phage and the whole cycle of phage library amplification was repeated as described previously. After three "panning" cycles 30 transduced colonies were selected at random and their DNA inserts were analyzed by PCR and sequencing as described previously [3].

Homology search in the GenBank nucleotide and amino acid sequences database was carried out using BLAST-N and BLAST-X online software (http://www.ncbi.nlm.nih.gov/blast/). Protein transmembrane topology was analyzed using TMHMM online algorithm (http://www.cbs.dtu.dk/services/TMHMM/).

RESULTS

The genes encoding protein adhesins in *Bifidobacterium longum* VMKB44 strain genome were detected by the surface phage display method. To this end, genome library of VMKB44 strain in pG8SAET phagemid vector containing about 4×10^6 clones with inserts of 100 to 3000 n. p. was created. According to the previously determined criteria [2,7], this library size is sufficient for presentation of all open reading frames of the bacterial genome in fusions with the pVIII protein and signal peptide genes in the vector.

After 3 panning cycles of recombinant phage particles on HT-29 cell culture monolayer surface, the number of viral particles binding to epithelio-

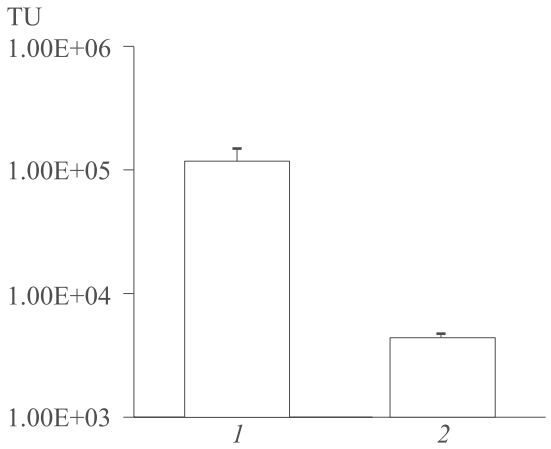

Fig. 1. Analysis of adhesion characteristics of pure cultures of wild type bacteriophages (*2*) with Blap-1 peptide presented on the surface (*1*). *1*) $1.14\times10^5\pm3.178\times10^3$ TU; *2*) $4.4\times10^3\pm2.64\times10^2$ TU.

cytes increase. After the first panning cycle, the total quantity of eluted bacteriophage was 10^4 transducing units (TU), after cycle 2 it was 1.2×10^5 TU, and after cycle 3 it reached 3×10^5 TU. Analysis of insert DNA in phagemid clones obtained after panning cycle 3 showed that about 90% eluted clones contained an insert of the same molecular weight. Hence, specific enrichment of the phage pool with the same insert type was detected. Sequencing of three clones selected at random showed that all inserts had an identical nucleotide sequence of 79 b. p. Insert DNA reading frame coincided with the reading frames of vector-encoded signal peptide at the 5'-terminal and gene pVIII (also vector-coded) at the 3'-terminal. These facts attest to nonrandom selection of this insert in three panning cycles.

In order to confirm the adhesion properties of this 26-amino-acid peptide, which we called Blap-1, we studied the capacity of the corresponding clone of recombinant bacteriophages to interaction with HT-29 cells in comparison with empty phagemid vector packed in wild type capsid. This experiment showed that incorporation of this polypeptide in surface pVIII protein of bacteriophage M13 significantly increased its adhesion characteristics (Fig. 1).

The search for amino acid residues homologous to this polypeptide fragment in the GenBank database (Fig. 2) revealed a 100% identical site in a large transmembrane protein (1263 amino acid residues, NP_695374), encoded by BL0155 open reading frame of *Bifidobacterium longum* NCC2705 genome. This protein is characterized by the presence of Smc and FtsX conservative domains, belonging to COG1196 and pfam02687 protein families, characteristic of ATP-binding proteins involved in the sister chromatide segregation during mitosis (Smc) and of proteins with a transmembrane permease function (FtsX). Despite this disagreement, the common structural characteristics of this hypothetical protein classify it as an ATP-binding transmembrane protein belonging to the ABC transporting protein superfamily and presumably participating in lipoprotein export.

The next stage of the study was selection of amino acid sequences homologous to BL0155 gene product of *B. longum* NCC2705 in the GenBank database. It was found that homologous amino acid sequences were encoded by open reading frames from genomes of *Bifidobacterium longum* DJO10A (ZP_00121322, 95% identity), *Bifidobacterium adolescentis* ATCC15703 (YP_908948, 64% identity), *Clostridium barlettii* DSM16795 (ZP_02211237, 29% identity), and by genes of many other obligate commencals of human gastrointestinal tract, such as *Eubacterium siraeum*, *Ruminococcus obeum*, *Enterococcus faecalis*, *Peptostreptococcus micros*, and *Lactobacillus salivarius*. Interestingly, homologous genes were also found in genomes of obligate gastrointestinal pathogens, for example, *Lysteria monocytogenes* 4bF2365 (29% homology). It is noteworthy that the amino acid sequence site corresponding to Blap-1 peptide is removed in the

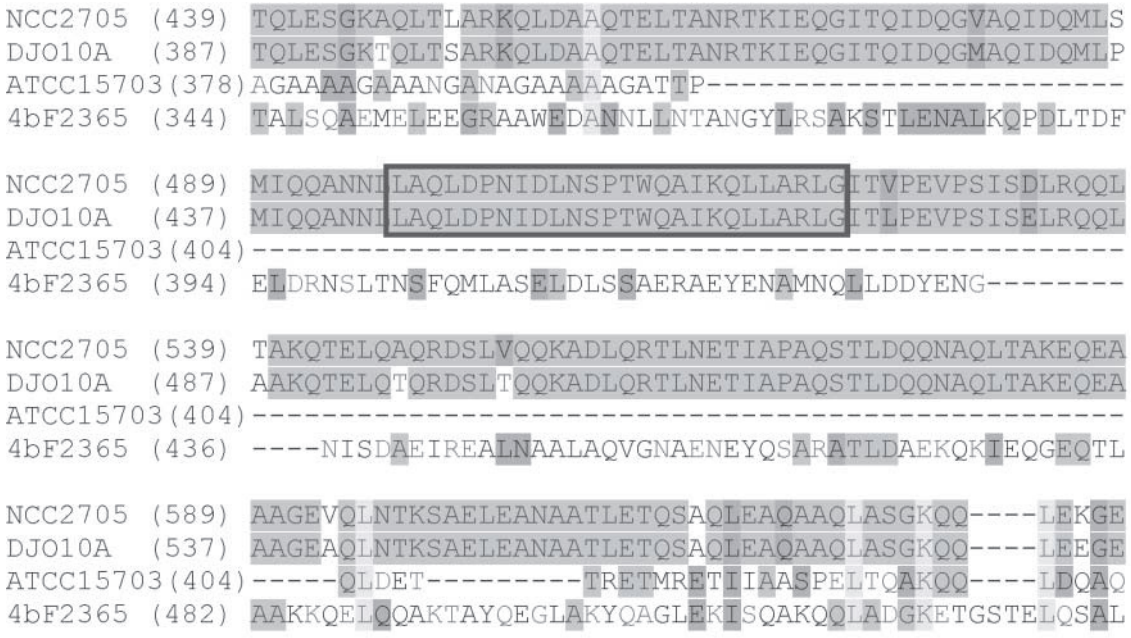

Fig. 2. Alignment of amino acid sequences of NCC2705 *B. longum* protein BL0155 and its homologs from *B. longum* DJO10A, *B. adolescentis* ATCC15703, and *L. monocytogenes* 4bF2365. The black rectangle shows the area corresponding to Blap-1 peptide.

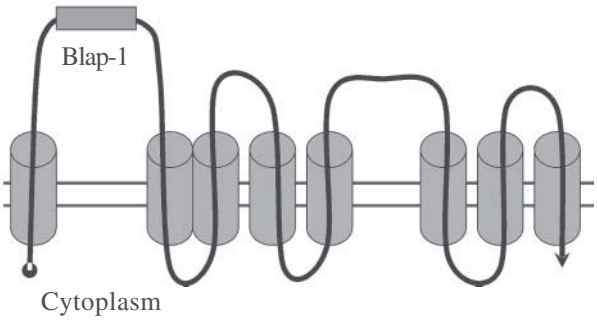

Fig. 3. Transmembrane topology of NCC2705 *B. longum* protein BL0155, predicted using the TMHMM algorithm. Similar topology is characteristic of homologous proteins from *B. adolescentis* ATCC15703 and *L. monocytogenes* 4bF2365. Cylinders represent hydrophobic transmembrane α-helix, the rectangle is an extracellular strand site including the Blap-1 peptide.

majority of proteins homologous to BL0155. The respective polypeptide fragment was detected only in proteins encoded by *B. longum* DJO10A and *L. monocytogenes* 4bF2365 genomes (Fig. 2).

Analysis of transmembrane topology of BL0155 protein and its homologues using the TMHMM algorythm showed that these proteins are characterized by intracytoplasmic location of the N-terminal and the presence of 8 transmembrane sites and two large extracellular loop domains. One of these domains includes the Blap-1 peptide fragment, which fact serves as an additional evidence of its role as the adhesin (Fig. 3).

Hence, DNA fragment cloned from *B. longum* VMKB44 genome library encodes Blap-1 polypeptide capable for adhesion on HT-29 cell surface. This peptide is a component of the extracellular loop of a large transmembrane protein (*B. longum* genome BL0155 open reading frame product). Structural organization of this protein indicates that it belongs to the ABC transport protein superfamily. Close homologues of this protein with similar transmembrane topology are present in many other human enteric symbionts.

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