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# A Preliminary Analysis of Secreted Proteins from *Bifidobacterium pseudocatanulatum* BP1 by Two-Dimensional Gel Electrophoresis

□ **Research Note** □

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**Abstract**

**Proteins secreted from bifidobacteria are believed to play important roles in human intestines via interacting with different host cells. In this respect, proteins secreted from *Bifidobacterium pseudocatanulatum* BP1, which has been rarely studied, were analyzed by two-dimensional gel electrophoresis (2DE). Using this approach, approximately 21 protein spots on a 2DE gel were detected and 10 of these spots were identified by mass spectrometry. Five spots were identified as hypothetical proteins and the remaining 5 spots were identified as a putative iron-siderophore binding lipoprotein, a short-chain dehydrogenase/reductase SDR, an exonuclease, cytochrome P450 hydroxylase, and a putative dehydrogenase. The identification of secreted putative iron-siderophore binding lipoprotein was highly interesting since it is an important protein that is involved in ferric iron uptake in pathogenic bacteria. This finding could accelerate studies on the probiotic effect of *Bifidobacterium* by explaining the competition between bifidobacteria and intestinal pathogens for ferric iron.**

**Key words:** *Bifidobacterium pseudocatanulatum*, secreted proteins, iron-siderophore binding lipoprotein, two-dimensional gel electrophoresis

## INTRODUCTION

The genus *Bifidobacterium* is a Gram-positive, heterofermentative, and strictly anaerobic bacteria (1). Bifidobacteria reside in human intestines and play important roles in human health such as inhibiting pathogenic bacteria (2), preventing diarrhea (3), alleviating constipation (4), and promoting immunomodulatory effects (5), which have been supported by many scientific studies (6-8).

There is currently a significant effort devoted to understanding and revealing novel roles of bifidobacteria in human intestines. As the results of these efforts, the genome sequences of two bifidobacterial strains, *B. longum* NCC2705 (GenBank accession no., AE014295) and *B. adolescentis* ATCC 15703 (AP009256), have been determined and the genome sequences of 12 strains including *B. longum* DJO10A (AABM00000000), *B. adolescentis* L2-32 (AAXD00000000), and *B. dentium* ATCC 27678 (ABIX00000000), etc. are under draft assembly or analysis (from http://www.ncbi.nlm.nih.gov/sites/entrez). Based on the analyses of genome sequences, it could be possible to predict many roles of bifidobacteria in the human gastrointestinal (GI) tract (1,9,10).

In relation to the interactions between microbes and host cells (or environments), there is evidence that suggests proteins secreted or exported from bifidobacteria are involved in carbohydrate metabolisms, cell adhesion, cell-cell communications, environmental sensing, etc. (1, 9,11). Based on the genome sequence of *B. longum* NCC2705, approximately 200 proteins with Sec-type signal sequences were predicted to be secreted from the bacteria (9). In this study, the proteins secreted from the *B. pseudocatanulatum* BP1 strain, which has been rarely studied, were analyzed by two-dimensional gel electrophoresis (2DE) and mass spectrometry.

## MATERIALS AND METHODS

### Bacterial strains

*B. pseudocatanulatum* BP1, previously identified by 16S rRNA gene sequencing, was isolated from a human stool sample. The strain was cultured in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, Hampshire, UK) at 37°C under anaerobic conditions.

### Protein preparation, separation and identification

*B. pseudocatanulatum* BP1 was inoculated into 500 mL BHI broth and cultured up to the early stationary phase. The culture broth was centrifuged twice at 7,000 $\times g$ for 30 min and the supernatant was filtered using a membrane filter (0.2 μm pore size; Sartorius Stedim Biotech, Aubagne Cedex, France). The same volume of a cold 10% trichloroacetic acid (TCA; Sigma, St. Louis, MO, USA) solution was added to the supernatant and

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stored at 4°C overnight. The proteins were recovered by centrifugation at 7,000×*g* for 1 hr, washed 3 times with 96% ethanol, and air-dried. The pellet was resuspended with 50 mM potassium phosphate buffer (pH 7.0) and dialyzed (Slide-A-Lyzer® Dialysis Cassettes, MWCO. 3,500; PIERCE, Rockford, IL, USA) against ultra pure water for 24 hr. The protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad, Hemel Hempstead, Hertfordshire, UK), according to the manufacturer's instruction. The protein sample was stored at -20°C.

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the sample was mixed with Laemmli sample buffer (Bio-Rad), loaded on a ready gel (NuPAGE® Novex Bis-Tris gel, 10%; Invitrogen, Paisley, UK) and run at 150 V for 1 hr. The gel was stained with Bio-Safe™ Coomassie (Bio-Rad), according to the manufacturer's instruction.

For two-dimensional gel electrophoresis (2DE), 100 μg of protein sample was resuspended with a rehydration buffer (8 M urea, 2 M thiourea) and adjusted to a final volume of 360 μL. Forty μL of a 10-fold concentrated reswelling solution, which consisted of 8 M urea, 2 M thiourea, 20% CHAPS, 200 mM dithiothreitol, and 5% IPG buffer (pH 3～11 NL; GE Healthcare, Little Chalfont, UK) was added to the sample. The sample was loaded onto an IPG strip (Immobiline DryStrip, 18 cm, pH 3～11 NL; GE Healthcare). The strip was transferred to a strip-holder (pHaser Isoelectric Focusing System; Genomic Solutions, Holliston, MA, USA) and overlaid with non-conducting mineral oil. The isoelectric focusing was performed for 24 hr with a total of 80,000 V-h. The completed strip was stored at -80°C until further use. The second dimensional electrophoresis was performed on a 10% gel (280×230 mm gel plate). The mineral oil on the stored strip was removed by flowing ultra pure water and the strip was soaked in an equilibration buffer (14 mL GS tris-acetate equilibration buffer, 0.125 g SDS, 9 g urea, 7.5 mL glycerol, and ultra pure water up to 25 mL) with 0.2 g DTT and shaken for 30 min. The strip was then removed, soaked in another equilibration buffer with 0.625 g iodoacetamide and shaken for 30 min. The strip was placed in the middle of the prepared gel for the second dimensional electrophoresis. The cathode buffer consisted of 200 mM tris base, 200 mM tricine and 14 mM SDS and the anode buffer consisted of 25 mM tris-acetate buffer (pH 8.3). A voltage of 406 V, a current of 98 mA, a power of 39941 mW and a total run time of 4.5 hr were used as the running conditions.

After electrophoresis, the gel was fixed and stained with Sypro Ruby stain (Bio-Rad). The image was captured and analyzed using a ProXPRESS Proteomics Imaging System and a Perkin-Elmer imaging program. Targeted spots were removed from the gel by a ProPick spot-picking robot (Genomic Solutions) and transferred into a 96-well microtitre plate. In-gel digestion was performed using a ProGest Protein Digester (Genomic Solutions) at 37°C for 2.5 hr with sequencing grade porcine trypsin (Promega, Southampton, UK). The trypsin digests were analyzed by a MALDI/TOF (matrix-assisted laser desorption ionization/time-of flight) mass spectrometry system (Bruker, Coventry, UK) and the peptide mass fingerprints obtained from the system were analyzed by a Mascot program from Matrix Science (http://www.matrixscience.com) using the MSDB protein database.

## RESULTS AND DISCUSSION

Proteins secreted from *B. pseudocatanulatum* BP1 were separated by 1-dimensional SDS-PAGE and 2DE (Fig. 1). To exclude proteins that originated from the BHI broth, the protein sample was also subjected to SDS-PAGE as a negative control (Lane 1 in Fig. 1A). From the SDS-PAGE gel, approximately 11 protein bands from the culture supernatant of *B. pseudocatanulatum* BP1 were detected, whereas approximately 21 protein spots were distinguished on the 2DE gel. Among these 21 spots, 10 representative spots were subjected to spot-picking followed by in-gel digestion and MALDI-TOF mass spectrometry. Proteins were then identified

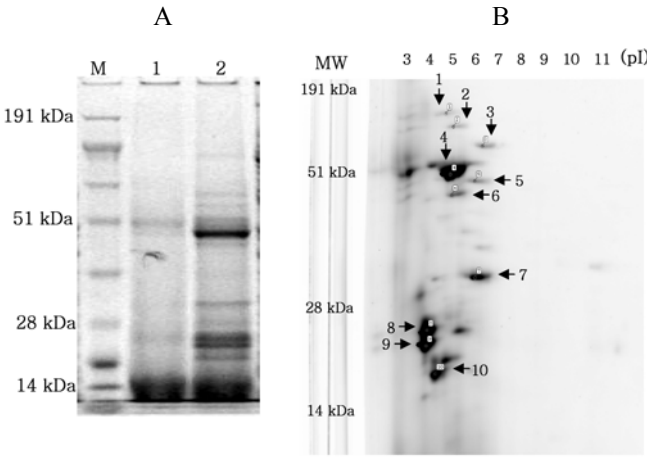


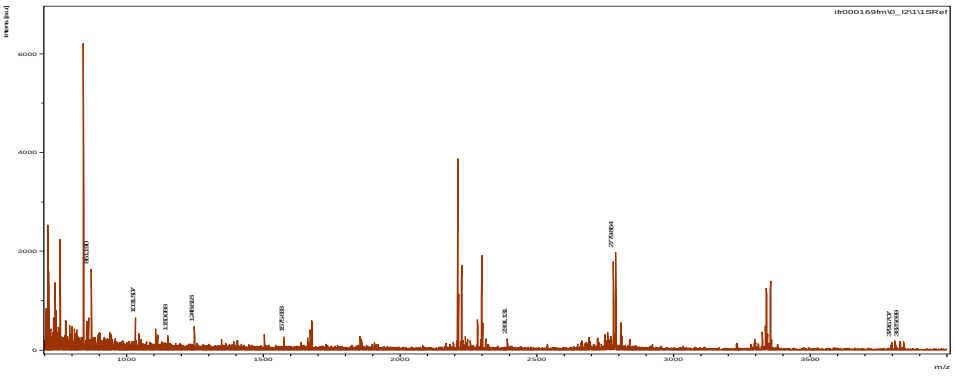

**Fig. 1.** One-dimensional SDS-PAGE (A) and two-dimensional gel electrophoresis (B) of proteins secreted from *B. pseudocatanulatum* BP1. Panel A: M, SeeBlue® Plus2 pre-stained standards (Invitrogen, Paisley, UK); lane 1, trichloroacetic acid (TCA) precipitate from the Brain Heart Infusion (BHI) broth; lane 2, TCA precipitate from the culture supernatant of *B. pseudocatanulatum* BP1.

by comparison to a protein database (see materials and methods) (Fig. 1B and Table 1). Using this analysis, five protein spots were identified as hypothetical proteins (spot no. 2, 4, 5, 7, 10) including hypothetical protein SCO3365 (spot no. 4) (GenBank accession no., NP_627573), which is a protein found in the genome sequence of *Streptomyces coelicolor* A3(2) (GenBank accession no., AL645882). Spot no. 3 was identified as the short-chain dehydrogenase/reductase SDR, which is a superfamily that consists of more than 3,000 members and has a wide-range of different substrates including sugars, alcohols, xenobiotics, etc (12). Spot no. 6 was matched with an exonuclease, which was predicted to be a secretory protein in the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). Spots no. 8 and 9 were identified as a cytochrome P450 hydroxylase and a putative dehydrogenase, respectively. Among the spots analyzed in this study, none were of interest except for spot no. 1, which was identified as a putative iron-siderophore binding lipoprotein (GenBank accession no., CAB53324) from *S. coelicolor* A3(2) and was definitively predicted to be a secretory protein in the SignalP 3.0 server. Although the peptide sequences from spot no. 1 had a 12% similarity to the putative lipoprotein, three peptide fragments matched well with the lipoprotein sequence (Fig. 2B). Furthermore, another peptide fragment from the spot had a 17% sequence similarity with a siderophore-interacting protein (GenBank accession no., YP_056885) from *Propionibacterium acnes* KPA171202 (data not shown). If this protein spot is confirmed to be an iron-siderophore binding lipoprotein, this finding could have a significant impact because an iron-siderophore uptake system has not yet been found in *Bifidobacterium* spp. even though some strains produce iron-siderophores (13). The iron-acquiring systems of pathogenic bacteria are very important machineries for bacterial infections and virulence in iron-limited environments, such as human intestines, where the pathogens use iron-siderophore for uptake of transferrin or lactoferrin, an iron-binding glycoproteins. In these systems, iron-siderophore binding lipoproteins play critical roles in Gram-positive bacteria because the ferrisiderophores (iron-siderophore complexes) are first recognized and bound by lipoproteins (14,15). In this respect, if *Bifidobacterium* spp. produces iron-siderophore binding lipoproteins, it could be used as a probiotic by competing with pathogenic bacteria in human intestines for acquisition of limited iron sources. Currently, the gene

**Table 1.** Identification of proteins secreted from *B. pseudocatenulatum* BP1

| Spot no. | Hit score | Protein ID |
|---|---|---|
| 1 | 37 | Putative iron-siderophore binding lipoprotein |
| 2 | 54 | Hypothetical protein |
| 3 | 52 | Short-chain dehydrogenase/reductase SDR |
| 4 | 48 | Hypothetical protein SCO3365 |
| 5 | 38 | Hypothetical protein |
| 6 | 34 | Exonuclease |
| 7 | 49 | Hypothetical protein |
| 8 | 53 | Cytochrome P450 hydroxylase |
| 9 | 40 | Putative dehydrogenase |
| 10 | 48 | Hypothetical protein |

**A**



**B**

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  1 mllrttrtkp wrrlaaalsa aalgvgllag cgsdsddpad eagggtpaaa gafpvtveha
 61 fgttkidkap ervvsvgytd dqtvlafgik pvgmvdqypn pagqspdint qwpwvkdkwg
121 dtkpevvmkn gdtgpnfeki aalrpdliva vyseidqaay dklskiaptv grtkgekepf
181 sapwqdnalh iakalgkaee gekmvadiqg kldaakqahp efadktavvl swykdsvapf
241 tstdvrgrlv tgigfkyete idkvaggdfy ttlspervdl vdvdrvfvin dkadqdalkk
301 felftnldav kngkvsylld segpavgaai sqgtllsmpy avdelvksag
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**Fig. 2.** MALDI/TOF mass spectrum (A) of peptide fragments from protein spot no. 1 and sequence coverage (B). The protein sequence matched a putative iron-siderophore binding lipoprotein from *Streptomyces coelicolor* A3(2). Matched peptides are shown in bold and underlined.

encoding the protein identified as a putative iron-siderophore binding lipoprotein is being investigated using a polymerase chain reaction (PCR) method with degenerate primers that target the matched peptide sequences (Fig. 2B).

Bifidobacteria are one of the most beneficial bacteria for human health and the genus has been studied by many research scientists. The genome sequences of the genus have been extensively analyzed and are currently still being examined. Based on the results from this work, it is possible to predict their physiology and roles in human intestines. However, to reasonably understand these roles we need to analyze the proteins that are secreted from this genus, which will ultimately interact with human cells, intestinal microbiota, and nutrients after secretion (9). In a recent paper, 17 proteins were detected from the culture supernatant of a *Bifidobacterium longum* strain by 2DE gel (16). Similarly, in this study, approximately 21 protein spots were detected on a 2DE gel and 10 spots were analyzed by mass spectrometry. Among these 10 spots, one protein spot was identified as a putative iron-siderophore binding lipoprotein. Due to the potential impact of this protein, it is being examined in further detail. This study is preliminary but relevant in regards to identifying proteins secreted from bifidobacteria that could be relevant to human health.

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## REFERENCES

1. Klijn A, Mercenier A, Arigoni F. 2005. Lessons from the genomes of bifidobacteria. *FEMS Microbiol Rev* 29: 491-509.
2. Gueimonde M, Margolles A, de los Reyes-Gavilán CG, Salminen S. 2007. Competitive exclusion of enteropathogens from human intestinal mucus by *Bifidobacterium* strains with acquired resistance to bile–A preliminary study. *Int J Food Microbiol* 113: 228-232.
3. Yan F, Polk DB. 2006. Probiotics as functional food in the treatment of diarrhea. *Curr Opin Clin Nutr Metab Care* 9: 717-721.
4. Amenta M, Cascio MT, Di Fiore P, Venturini I. 2006. Diet and chronic constipation. Benefits of oral supplementation with symbiotic zir fos (*Bifidobacterium longum* W11 + FOS Actilight). *Acta Biomed* 77: 157-162.
5. Medina M, Izquierdo E, Ennahar S, Sanz Y. 2007. Differential immunomodulatory properties of *Bifidobacterium longum* strains: relevance to probiotic selection and clinical applications. *Clin Exp Immunol* 150: 531-538.
6. Sanz Y, Nadal I, Sánchez E. 2007. Probiotics as drugs against human gastrointestinal infections. *Recent Patents Anti-Infect Drug Disc* 2: 148-156.
7. Boirivant M, Strober W. 2007. The mechanism of action of probiotics. *Curr Opin Gastroenterol* 23: 679-692.
8. Ventura M, O'Connell-Motherway M, Leahy S, Moreno-Munoz JA, Fitzgerald GF, van Sinderen D. 2007. From bacterial genome to functionality; case bifidobacteria. *Int J Food Microbiol* 120: 2-12.
9. Schell MA, Karmirantzou M, Snel B, Vilanova D, Berger B, Pessi G, Zwahlen MC, Desiere F, Bork P, Delley M, Pridmore RD, Arigoni F. 2002. The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. *Proc Natl Acad Sci USA* 99: 14422-14427.
10. De Dea Lindner J, Canchaya C, Zhang Ziding, Neviani E, Fitzgerald GF, van Sinderen D, Ventura M. 2007. Exploiting *Bifidobacterium* genomes: The molecular basis of stress response. *Int J Food Microbiol* 120: 13-24.
11. MacConaill LE, Fitzgerald GF, van Sinderen D. 2003. Investigation of protein export in *Bifidobacterium breve* UCC2003. *Appl Environ Microbiol* 69: 6994-7001.
12. Persson B, Kallberg Y, Oppermann U, Jörnvall H. 2003. Coenzyme-based functional assignments of short-chain dehydrogenase/reductase (SDRs). *Chem Biol Interact* 143-144: 271-278.
13. O'Sullivan DJ. 2004. Isolated bifidobacteria that produce siderophores which inhibit growth of *Lactococcus lactis*. *US Patent* 6746672.
14. Crosa JH. 1997. Signal transduction and transcriptional and posttranscriptional control of iron-regulated genes in bacteria. *Microbiol Mol Biol Rev* 61: 319-336.
15. Dale SE, Sebulsky MT, Heinrichs DE. 2004. Involvement of SirABC in iron-siderophore import in *Staphylococcus aureus*. *J Bacteriol* 186: 8356-8362.
16. Sánchez B, Champomier-Vergès MC, Anglade P, Baraige F, de los Reyes-Gavilán CG, Margolles A, Zagorec M. 2008. A preliminary analysis of *Bifidobacterium longum* exported proteins by two-dimensional electrophoresis. *J Mol Microbiol Biotechnol* 14: 74-79.

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