Safety assessment of *Bifidobacterium longum* JDM301 based on complete genome sequences

Yan-Xia Wei, Zhuo-Yang Zhang, Chang Liu, Pradeep K Malakar, Xiao-Kui Guo

**Abstract**

**AIM:** To assess the safety of *Bifidobacterium longum* (*B. longum*) JDM301 based on complete genome sequences.

**METHODS:** The complete genome sequences of JDM301 were determined using the GS 20 system. Putative virulence factors, putative antibiotic resistance genes and genes encoding enzymes responsible for harmful metabolites were identified by blast with virulence factors database, antibiotic resistance genes database and genes associated with harmful metabolites in previous reports. Minimum inhibitory concentration of 16 common antimicrobial agents was evaluated by $E$-test.

**RESULTS:** JDM301 was shown to contain 36 genes associated with antibiotic resistance, 5 enzymes related to harmful metabolites and 162 nonspecific virulence factors mainly associated with transcriptional regulation, adhesion, sugar and amino acid transport. *B. longum* JDM301 was intrinsically resistant to ciprofloxacin, amikacin, gentamicin and streptomycin and susceptible to vancomycin, amoxicillin, cephalothin, chloramphenicol, erythromycin, ampicillin, cefotaxime, rifampicin, imipenem and trimethoprim-sulphamethoxazole. JDM301 was moderately resistant to bacitracin, while an earlier study showed that bifidobacteria were susceptible to this antibiotic. A tetracycline resistance gene with the risk of transfer was found in JDM301, which needs to be experimentally validated.

**CONCLUSION:** The safety assessment of JDM301 using information derived from complete bacterial genome will contribute to a wider and deeper insight into the safety of probiotic bacteria.

© 2012 Baishideng. All rights reserved.

**Key words:** Bifidobacterium longum; Safety assessment; Genome; Antibiotic resistance; Harmful metabolite; Virulence factor
INTRODUCTION

Bifidobacteria spp are high-GC content, Gram-positive bacteria which belong to the Actinobacteria branch and these species naturally colonize the gastrointestinal tract (GIT) of mammals, birds and insects. Scientists have determined the major probiotic properties of Bifidobacteria spp isolated from the human intestine and these properties include the strengthening of the intestinal barrier, modulation of the immune response and antagonism of pathogens.

Bifidobacterium spp has been reported to possess various glycosyl hydrolases (GH) and these hydrolases metabolize plant- or milk-derived oligosaccharides including nondigestible ones such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS). The capability to utilize nondigestible oligosaccharides confers a competitive advantage to Bifidobacterium spp in the human gut.

Bifidobacterium longum (B. longum) and various other bifidobacteria strains are often added to probiotic products in combination with other lactic acid bacteria (LAB). Through their long and safe history of application, LAB have acquired the status of “Generally Regarded As Safe” (GRAS), but the safety of Bifidobacteria and other LAB strains selected for probiotics still need to be carefully evaluated. The key safety aspects for use of bifidobacteria and other LAB strains in probiotics include antibiotic resistance, production of harmful metabolites and the potential for virulence. Antibiotic resistance in potential probiotic strains is not considered a risk factor unless resistance is transferred to pathogens or it renders the probiotic untreatable in very rare cases of infection.

Biogenic amines, D-lactic acid, azoreductases and nitroreductases produced by bifidobacteria and other LAB strains are potential health hazards and the safety of some of these compounds have been evaluated. Virulence genes may be present in commensal bacteria and absence of virulence in these bacteria needs to be proved on a case by case basis.

Probiotic agents are widely used in the food and drug industry and as more commercial probiotic products are being introduced in the market, it is timely to re-evaluate the safety of these probiotic products using the latest technology. Information from the complete genome sequences of Bifidobacteria will provide additional insight into the genetic basis for their safety. We sequenced the complete genome sequences of B. longum JDM301 (GenBank accession number CP001095) and the others were arranged by multiplex polymerase chain reaction (PCR). Gap closure was carried out by sequencing gap-spanning PCR products or clones using ABI 3730 xl DNA sequencers. Primer design and sequence assembly were performed by the Phred/Phrap/Consed software package. The locations of low-quality sequences in genome were verified by directly resequencing the PCR products spanning the low-quality sequences using the ABI 3730 xl DNA sequencers.

Statistical analysis

The genome sequences of Bifidobacteria except JDM301 were retrieved from GenBank at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) respectively. Potential open reading frames (ORF) were identified using Glimmer and ZCURVE. Using default settings. Clusters of orthologous group (COG) functional categories were used for functional classification of all genes in the genome sequences of JDM301 and the COGs. A BLAST analysis of the translations with GenBank’s nonredundant database was performed, which was followed by manual curation.

MATERIALS AND METHODS

Bacterial strains and growth conditions

JDM301 was isolated from commercial probiotic product and identified using a sequence analysis of its 16S rRNA gene. De Man-Rogosa-Sharpe (MRS) broth (Difco) supplemented with 0.05% L-cysteine HCl (Sigma) was used for cultivating JDM301. Cultures were incubated at 37 °C under anaerobic conditions.

Antibiotic susceptibility

Minimum inhibitory concentration (MIC) of 16 common
antimicrobial agents was evaluated by E-test (AB Biodisk, Solna, Sweden) including amoxicillin (0.016-256 mg/L), amikacin (0.016-256 mg/L), ampicillin (0.016-256 mg/L), bacitracin (0.016-256 mg/L), cephalothin (0.016-256 mg/L), ciprofloxacin (0.002-32 mg/L), cefotaxime (0.016-256 mg/L), chloramphenicol (0.016-256 mg/L), erythromycin (0.016-256 mg/L), gentamicin (0.016-256 mg/L), imipenem (0.002-32 mg/L), rifampicin (0.016-256 mg/L), streptomycin (0.016-256 mg/L), tetracycline (0.016-256 mg/L), trimethoprim-sulphamethoxazol (0.002-32 mg/L), and vancomycin (0.016-256 mg/L). Tests were done with MRS agar supplemented with 0.05% L-cysteine·HCl (Sigma) and were conducted in triplicate for each antibiotic. Cultures sub-inoculated into the MRS agar supplemented with 0.05% L-cysteine·HCl were incubated anaerobically at 37 ℃ for 24 h.

RESULTS

Comparative genomic analysis of Bifidobacteria

The predicted proteins of B. longum JDM301 were functionally categorized. The functional distribution of genes assigned to clusters of orthologous groups of proteins was relatively similar to the other Bifidobacteria, e.g., B. longum and B. adolescentis in the GIT and B. dentium in the oral cavity[34,35]. The top four functional categories in B. longum JDM301, namely, carbohydrate transport and metabolism, amino acid transport and metabolism, were identical with other Bifidobacteria[20,26].

Putative orthologues among B. longum strains were determined in a comparative study (Figure 1). Overall, 1265 proteins were conserved in all four B. longum strains (B. longum JDM301, B. longum NCC2705, B. longum DJO10A and B. longum ATCC15697). These proteins represent the “core” genome of B. longum, whereas 219 proteins are unique to B. longum JDM301. The most common functional distributions of the core proteins were these involved in housekeeping functions including amino acid transport and metabolism, translation, ribosomal structure and biogenesis, carbohydrate transport and metabolism and DNA replication, recombination and repair. Twenty-one percent of the core proteins were dedicated to carbohydrate and amino acid transport and metabolism, indicating the important roles of these proteins in Bifidobacteria.

Stability of the genome of B. longum JDM301

Horizontal gene transfer (HGT) events are responsible for introduction of alien genes, which may reinforce the adaptation of bacteria in their specific niches. Genes on plasmids, bacteriophages, genomic islands and IS are sensitive to HGT[51]. Twelve phage-related fragments were identified in the genome of B. longum JDM301[3], but no complete prophages were found. The JDM301 chromosome also possesses 15 complete or disrupted IS elements[9]. The number of IS element in JDM301 is relatively smaller than the other sequenced B. longum spp[34]. Another set of genes disseminated by HGT in Bifidobacteria is the CRISPR-related system. No CRISPR was discovered in the genome.

One complete type II restriction-modification (R-M) system and one incomplete type III R-M system were present in the genome of JDM301. A complete and incomplete type I R-M system was also identified in this genome. Two complete type II R-M systems and one type I R-M system were present in the genome of B. longum NCC2705, while one complete type II R-M system and type I R-M system were found in B. longum DJO10A.

Antibiotic resistance determinants

The antibiotic resistance genes in JDM301 were identified...
using ARDB (E < 1e-2, coverage > 70%)[18]. Homologs of the antibiotic resistance determinants for vancomycin, methicillin, tetracycline, chloramphenicol and trimethoprim were found in the genome of JDM301 (Table 1) and 6 putative resistance genes for vancomycin. B. longum JDM301 also possessed 5 putative bacitracin efflux pumps, 5 homologs of macrolide efflux proteins. Additionally, 7 putative multidrug resistance efflux pumps belonging to an ATP-binding cassette (ABC)-type transport system, a major facilitator superfamily transporter and resistance-nodulation-cell division (RND) family were found in the genome. The genome of B. longum JDM301 also contains 4 tetracycline resistance genes encoding for TetV, TetW, TetPB and TetQ. The gene for TetW shows a strong difference in G + C content (53.0%) compared to the average value of B. longum JDM301 (59.8%) genome and it is flanked by genes encoding for integrases, indicating that this region may have been acquired by HGT.

The antibiotic susceptibility of B. longum JDM301 to 16 antibiotics was determined by an E-test to probe the in silico analyses of the complete genome sequence. The results of the E-test are summarized in Table 2. The breakpoints for determining susceptibility were determined using accepted protocols[22-24]. B. longum JDM301 showed a high resistance to ciprofloxacin, amikacin and gentamicin, moderate resistance to streptomycin and bacitracin and were sensitive to tetracycline, vancomycin, amoxicillin, cephalothin, chloramphenicol, erythromycin, ampicillin, cefotaxime, rifampicin, imipenem and an antimicrobial compound, trimethoprim-sulphamethoxazol.

### Table 1 Putative antibiotic resistance genes identified in the genome of Bifidobacterium longum JDM301

| Antibiotics     | Antibiotic resistance genes | Product name                                      |
|-----------------|-----------------------------|--------------------------------------------------|
| Bacitracin      | BLJ_1636                    | ABC transporter-related protein                   |
|                 | BLJ_0984                    | ABC transporter-related protein                   |
|                 | BLJ_0923                    | ABC transporter-related protein                   |
|                 | BLJ_1105                    | Undecaprenyl pyrophosphate-phosphatase           |
| Vancomycin      | BLJ_0853                    | VanU                                             |
|                 | BLJ_1784                    | Dehydrogenase VanH                               |
|                 | BLJ_1084                    | Sensor protein VanSB                             |
|                 | BLJ_0707                    | VanS5                                            |
|                 | BLJ_0343                    | Histidine kinase VanKc3                          |
|                 | BLJ_0287                    | D-Ala-D-Lac ligase VanD                          |
| Multiple drugs  | BLJ_1090                    | ATP-binding protein                              |
|                 | BLJ_1457                    | LmrB                                             |
|                 | BLJ_0818                    | Multidrug export protein MepA                    |
|                 | BLJ_0769                    | Efflux transporter, RND family, MFP subunit      |
| Chloramphenicol | BLJ_1672                    | Chloramphenicol resistance protein               |
|                 | BLJ_1322                    | Chloramphenicol resistance protein               |
| Thiostrepton    | BLJ_0885                    | Thiostrepton-resistance methylase                |
| Penicillin      | BLJ_1301                    | Penicillin binding protein                       |
| Kusagymycin     | BLJ_2030                    | 3'-adenosylmethylene-6'-N'-adenosyl              |
|                 | [22-25]                     | (rRNA) dimethyltransferase                       |
| Tetracycline    | BLJ_0814                    | Tetracycline-resistance determinant tetV         |
|                 | BLJ_1245                    | TetW                                             |
|                 | BLJ_0594                    | Tetracycline resistance protein                  |
|                 | BLJ_1401                    | TetQ                                             |
| Carbomycin      | BLJ_1625                    | Carbomycin resistance protein                    |
| Sulfonamide     | BLJ_1629                    | Dihydropteroate synthase                         |
| Tetracenomycin C| BLJ_1624                    | Tetracenomycin C efflux protein                  |
| Trimethoprim    | BLJ_1657                    | dihydrofolate reductase                          |
| Macrolide       | BLJ_0925                    | Macrolide-efflux protein                         |
|                 | BLJ_1386                    | Macrolide-efflux protein                         |
|                 | BLJ_0819                    | Macrolide-efflux protein                         |
|                 | BLJ_0042                    | Macrolide-efflux protein variant                 |

### Table 2 Minimum inhibitory concentration values of 16 antibiotics for Bifidobacterium longum JDM301

| Antibiotics     | Minimum inhibitory concentration (mg/L) |
|-----------------|----------------------------------------|
| Ciprofloxacin   | > 32                                   |
| Amikacin        | > 256                                  |
| Gentamicin      | > 256                                  |
| Bacitracin       | 26.67                                  |
| Streptomycin    | 170.67                                 |
| Vancomycin      | 0.9                                    |
| Amoxicillin     | 0.064                                  |
| Cephalothin     | 1.33                                    |
| Chloramphenicol | 0.25                                    |
| Erythromycin    | 0.04                                    |
| Ampicillin      | 0.058                                  |
| Cefotaxime      | 0.19                                    |
| Rifampicin      | 0.074                                  |
| Tetracycline    | 8                                      |
| Imipenem        | 0.19                                    |
| Trimethoprim-sulphamethoxazol | 1.83 |

Putative enzymes for harmful metabolites

Genes encoding enzymes responsible for harmful metabolites, including beta-glucosidase (GS), arylsulphatase (AS), beta-glucuronidase (GN), nitroreductase (NR), azoreductase (AR), D-lactate dehydrogenase (DLD), amino acid decarboxylase (AD) and conjugated bile salt hydrolase (CBSH) were searched for in the genome of B. longum JDM301. Two GS genes (BLJ_1280, BLJ_1540) and one CBSH gene (BLJ_0948) were found in the chromosome of B. longum JDM301. Homologs of DLD (BLJ_1306, BLJ_1436) and NR (BLJ_1980) were also discovered in the genome. Enzymes involved in putative-ly harmful metabolites, AR, GN, AD and AS were not found in JDM301 genome.

Putative virulence factors

Published reports of rare infections involving Lactobacilli or Bifidobacteria are available and the potential virulence of Lactobacilli or Bifidobacteria used as probiotics should be assessed[15]. Putative virulence genes of B. longum JDM301 were determined by BLAST analysis of the VFDB[19]. A total of 141 homologs of virulence factors were identified in the genome of JDM301, including 28 sugar-binding transcriptional regulators, 20 genes associated...
| Query     | Identity | Subject                                                                 | Predicted functions                                                                 |
|-----------|----------|-------------------------------------------------------------------------|---------------------------------------------------------------------------------------|
| BLJ_1089  | 24.9     | VFG0934 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase                 | ABC-type amino-acid transporter peri                                            |
| BLJ_1835  | 26.36    | VFG0934 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase                 | ABC-type amino-acid transporter peri                                            |
| BLJ_0323  | 29.3     | VFG0934 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase                 | ABC-type amino-acid transporter peri                                            |
| BLJ_1476  | 22.11    | VFG2578 6 kDa early secretory antigenic target exsA                     | ABC-type amino-acid transporter peri                                            |
| BLJ_0992  | 32.98    | VFG0869 AatC ATB binding protein of ABC transporter                     | ABC-type amino-acid transporter peri                                            |
| BLJ_1080  | 34.81    | VFG0869 AatC ATB binding protein of ABC transporter                     | ABC-type amino-acid transporter peri                                            |
| BLJ_1968  | 37.3     | VFG0869 AatC ATB binding protein of ABC transporter                     | ABC-type amino-acid transporter peri                                            |
| BLJ_0770  | 37.43    | VFG0869 AatC ATB binding protein of ABC transporter                     | ABC-type amino-acid transporter peri                                            |
| BLJ_0026  | 35.71    | VFG1404 apfC                                                          | ABC-type amino-acid transporter peri                                            |
| BLJ_0136  | 28.73    | VFG2218 ATPase VirB11 homolog                                          | ABC-type amino-acid transporter peri                                            |
| BLJ_0880  | 25.18    | VFG1402 ATP-binding protein FecE                                      | ABC-type amino-acid transporter peri                                            |
| BLJ_0787  | 47.92    | VFG0077 ATP-dependent Clp protease proteolytic subunit                  | ABC-type amino-acid transporter peri                                            |
| BLJ_0786  | 53.8     | VFG0077 ATP-dependent Clp protease proteolytic subunit                  | ABC-type amino-acid transporter peri                                            |
| BLJ_0948  | 37.66    | VFG2216 Bile salt hydrolase                                             | ABC-type amino-acid transporter peri                                            |
| BLJ_1243  | 22.97    | VFG2224 Conjugal transfer protein trag                                 | ABC-type amino-acid transporter peri                                            |
| BLJ_0551  | 26.54    | VFG1108 Conserved hypothetical protein                                  | ABC-type amino-acid transporter peri                                            |
| BLJ_1951  | 29.85    | VFG1269 Cyclosisin secretion ATP-binding protein                        | ABC-type amino-acid transporter peri                                            |
| BLJ_1925  | 32.31    | VFG1269 Cyclosisin secretion ATP-binding protein                        | ABC-type amino-acid transporter peri                                            |
| BLJ_1863  | 45.5     | VFG0079 Endopeptidase Clp ATP-binding chain C                           | ABC-type amino-acid transporter peri                                            |
| BLJ_1465  | 56.77    | VFG0079 Endopeptidase Clp ATP-binding chain C                           | ABC-type amino-acid transporter peri                                            |
| BLJ_0713  | 30.12    | VFG0925 Ferric enterobactin transport                                  | ABC-type amino-acid transporter peri                                            |
| BLJ_1872  | 25.51    | VFG2225 GDP-mannose-4,6-dehydratase                                    | ABC-type amino-acid transporter peri                                            |
| BLJ_1334  | 32.49    | VFG1399 glnA1                                                          | ABC-type amino-acid transporter peri                                            |
| BLJ_0624  | 62.11    | VFG1399 glnA1                                                          | ABC-type amino-acid transporter peri                                            |
| BLJ_1834  | 29.47    | VFG0313 Glucose/galactose transporter                                   | ABC-type amino-acid transporter peri                                            |
| BLJ_1926  | 30.02    | VFG1557 HlyB protein                                                   | ABC-type amino-acid transporter peri                                            |
| BLJ_1477  | 56.12    | VFG1855 Hsp60, 60k heat shock protein HtpB                               | ABC-type amino-acid transporter peri                                            |
| BLJ_0064  | 26.21    | VFG1397 hspX                                                           | ABC-type amino-acid transporter peri                                            |
| BLJ_1444  | 40.85    | VFG1563 Hypothetical protein                                            | ABC-type amino-acid transporter peri                                            |
| BLJ_1606  | 27.78    | VFG1593 Hypothetical protein                                            | ABC-type amino-acid transporter peri                                            |
| BLJ_1640  | 30.81    | VFG1593 Hypothetical protein                                            | ABC-type amino-acid transporter peri                                            |
| BLJ_0011  | 22.16    | VFG1604 Hypothetical protein                                            | ABC-type amino-acid transporter peri                                            |
| BLJ_1513  | 26.3     | VFG1604 Hypothetical protein                                            | ABC-type amino-acid transporter peri                                            |
| BLJ_1846  | 27.67    | VFG1604 Hypothetical protein                                            | ABC-type amino-acid transporter peri                                           |
| BLJ_0337  | 44.25    | VFG1630 Hypothetical protein                                            | ABC-type amino-acid transporter peri                                            |
| BLJ_0336  | 44.38    | VFG1630 Hypothetical protein                                            | ABC-type amino-acid transporter peri                                            |
| BLJ_1500  | 23.53    | VFG1963 Hypothetical protein Gj1435c                                     | ABC-type amino-acid transporter peri                                            |
| BLJ_1169  | 24.64    | VFG1963 Hypothetical protein Rv0981                                     | ABC-type amino-acid transporter peri                                            |
| BLJ_0708  | 36.8     | VFG1963 Hypothetical protein Rv0981                                     | ABC-type amino-acid transporter peri                                            |
| BLJ_0802  | 28.83    | VFG1824 Hypothetical protein Rv3133c                                     | ABC-type amino-acid transporter peri                                            |
| BLJ_1357  | 30.46    | VFG1824 Hypothetical protein Rv3133c                                     | ABC-type amino-acid transporter peri                                            |
| BLJ_1113  | 32.41    | VFG1824 Hypothetical protein Rv3133c                                     | ABC-type amino-acid transporter peri                                            |
| BLJ_0835  | 32.42    | VFG1824 Hypothetical protein Rv3133c                                     | ABC-type amino-acid transporter peri                                            |
| BLJ_0859  | 27.93    | VFG1206 Iron(II) ABC transporter, ATP-binding protein                   | ABC-type amino-acid transporter peri                                            |
| BLJ_0348  | 28.13    | VFG1206 Iron(II) ABC transporter, ATP-binding protein                   | ABC-type amino-acid transporter peri                                            |
| BLJ_0530  | 29.29    | VFG1206 Iron(II) ABC transporter, ATP-binding protein                   | ABC-type amino-acid transporter peri                                            |
| BLJ_2016  | 35.81    | VFG1206 Iron(II) ABC transporter, ATP-binding protein                   | ABC-type amino-acid transporter peri                                            |
| BLJ_1875  | 36.19    | VFG1627 IS100 transposase; transposase ORFA                           | ABC-type amino-acid transporter peri                                            |
| BLJ_1249  | 37.55    | VFG1627 IS100 transposase; transposase ORFA                           | ABC-type amino-acid transporter peri                                            |
Wei YX et al. Safety assessment of Bifidobacterium longum JDM301

Table 4 Putative genes associated with adhesion identified in the genome of Bifidobacterium longum JDM301

| Locus_tag | Pfam number | Product name |
|-----------|-------------|--------------|
| BLI_1932  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_0112  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_1284  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_1240  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_0131  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_1604  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_1686  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_1964  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_1994  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_1996  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_2001  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_0288  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_0021  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_0345  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_0414  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_0522  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_0523  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_0524  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_0012  | pfam07174   | Hypothetical protein BLI_0012 |
| BLI_1801  | pfam05738   | LPXTG-motif protein cell wall anchor |
| BLI_0140  | pfam07811   | TadE family protein |

Table 4 Putative genes associated with adhesion identified in the genome of Bifidobacterium longum JDM301

| Locus_tag | Pfam number | Product name |
|-----------|-------------|--------------|
| BLI_1932  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_0112  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_1284  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_1240  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_0131  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_1604  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_1686  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_1964  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_1994  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_1996  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_2001  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_0288  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_0021  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_0345  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_0414  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_0522  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_0523  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_0524  | pfam01547   | Family 1 extracellular solute-binding protein |
| BLI_0012  | pfam07174   | Hypothetical protein BLI_0012 |
| BLI_1801  | pfam05738   | LPXTG-motif protein cell wall anchor |
| BLI_0140  | pfam07811   | TadE family protein |

with iron, amino acid and sugar transport, 5 transposases, and 2 glutamine synthetase related to plasminogen (Pig)-binding (Table 3).

Although the ability to adhere to the intestinal wall has been one of the selection criteria for probiotics and also a characteristic of commensal bacteria in the intestine, adhesion is also considered to be a significant step in the initial pathogen infections. Thus, predicted proteins for adhesion of JDM301 were also included in the analysis of virulence. A total of 21 predicted proteins for adhesion were identified in JDM301 (Table 4). A large number of predicted surface and extracellular proteins were identified in JDM301, which may be involved in the bacterium-host interaction as in other LAB[27]. A total of 217 proteins with probable Sec-type signal peptides were identified by the tool, Signal P[28]. The genome of JDM301 also harbors 18 copies of extracellular solute-binding protein (SBP, pfam01547) which is predicted to bind oligosaccharides (SBP family 1) as a component of the ABC transporter complex.

**DISCUSSION**

As more probiotic strains are used in the food and drug industry, more attentions should be paid to the safety of strains used as probiotics. Thus, the safety of LAB used as probiotics need to be reassessed using the latest technology. B. longum JDM301, is a commercial probiotic strain used in many probiotic products sold in China. Analysis of the genome of JDM301 reveals several potential risk factors needing further experimental validation, including a tetracycline resistance gene (tetR) with the risk of transfer, and the genes associated with harmful metabolites.
**Bifidobacteria** were considered free of phage infection until prophage-like elements were identified in the genomes of *B. longum* NCC2705, *B. longum* DJO10A and *B. breve* UCC2003. Absence of complete prophages is important for the stability of genomes and for industrial applications of probiotic bacteria. Absence of complete prophages and scarcity of IS element may play important roles in generating genome stability of *B. longum*. Another set of genes disseminated by HGT in *Bifidobacteria* is the CRISPR-related system (CASS), which is involved in defense against phages and plasmids. No CRISPR was discovered in the genome. R-M systems are diverse and widespread in nature and they are considered as barriers to HGT, e.g., in transformation and phage infection. The diversity of R-M systems in *B. longum* JDM301 may be significant to the stability of genome and its use in industry compared with the other two *B. longum* strains.

*B. longum* JDM301 was not resistant to tetracycline as the minimum inhibitory concentration (8.0 mg/L) was not higher than the breakpoint value (8.0 mg/L). However, the MIC for *B. longum* strains ranges from 0.5 to 2 mg/L in a report. Thus, further experiments may be needed to determine the microbiological breakpoint. The *tetW* (BLJ_1245) gene encodes for a ribosomal protection protein and *tetW* genes were responsible for acquired tetracycline resistance in human *B. longum* strains. The rest of the tetracycline resistance genes found in *B. longum* JDM301 were *tetV* (BLJ_0814), *tetQ* (BLJ_1401) and *tetPB* (BLJ_0594). The gene *tetW* encodes for a tetracycline efflux pump and the genes *tetQ* and *tetP* encode for ribosomal protection proteins. Further experiments are needed to confirm whether the *tetW* gene in the chromosome of *B. longum* JDM301 is a transferable antibiotic resistance determinant and responsible for resistance to tetracycline in human *B. longum* strains.

The MIC of *B. longum* JDM301 to bacitracin was 26.7 mg/L, which indicated a moderate resistance. A previous report indicated that *B. longum* strains were susceptible to bacitracin. A total of 7 putative bacitracin resistance genes were identified, including 6 genes encoding for ABC transporters and 1 for an uncharacterized bacitracin resistance protein. These genes may be responsible for the resistance to bacitracin.

The resistances to ciprofloxacin, amikacin, gentamicin and streptomycin and susceptibility of *JDM301* to vancomycin, amoxicillin, cephalothin, chloramphenicol, erythromycin, ampicillin, cefoxatime, rifampicin, and an antimicrobial compound, trimethoprim-sulphamethoxazole were consistent with reported findings. However, there are discrepancies between the phenotype and the genotype. *B. longum* JDM301 was sensitive to vancomycin and chloramphenicol but the genome contained vancomycin and chloramphenicol resistance genes. Further analysis will be needed to determine this discrepancy.

Several cases of D-lactic acidosis associated with consumption of LAB in patients with short bowel syndrome were reported, implying that bacteria used as probiotics should be screened for the ability to generate D-lactate. In this study, two homologs of DLD genes were identified in the genome of JDM301. Since there were no reported cases of D-lactic acidosis caused by bifidobacteria, the activities of these homologous DLDs in bifidobacteria may be low so that the amount of lactate produced is insufficient to cause D-lactic acidosis.

Although biogenic amines (BA) play an important physiological role in mammals, a high amount of BA in the diet may have a variety of toxic effects. The main BA contained in food and beverages includes histamine, tyramine, putrescine, and cadaverine, some of which are associated with toxicological characteristics of food poisoning. The decarboxylase activities of histidine, tyrosine, and ornithine were reported in lactobacilli and the capabilities might be strain-dependent rather than species-dependent. Therefore, BA production, especially thylamine and tyramine, must be carefully evaluated for individual strains.

Bacterial enzymes, such as GN, GS, NR, AR and AS, play important roles in the metabolism of carcinogens and other toxicants in the intestine. Homologs of GS are common in sequenced *Bifidobacteria* genomes where GS and GN facilitate the absorption of a variety of toxicants and may contribute to the development of colon cancer. The link between *Bifidobacteria* and the genotoxic enzyme activities of intestinal microflora has been reported, with *Bifidobacteria* inhibiting the activity of some genotoxic enzymes. NR activity is common in oral bacteria and it plays an important role in bacterial nitrate reduction. Although NR activities have been reported in *Bifidobacteria*, the activity of this enzyme is lower than the NR activity of other gut bacteria.

CBSH mediates microbial bile tolerance and enhances microbial survival in the intestine. Metagenomic analyses demonstrated that CBSH activity is enriched in the human gut microbiome, and has the potential to greatly influence host physiology. In *Bifidobacterium* spp. and *Lactobacillus* spp., CBSH activity is also common and nearly all *Bifidobacteria* species and strains have bile salt hydrolase activities. However, bile salt hydrolase activity releases free bile acids which are harmful to the human body and may act as mutagens. Recommendations have been made for absence of bile salt transformation capacity in bacteria added to food. However, it is noteworthy that the evidence for harmful effects is inconclusive so far and bile salt deconjugation activity may play a role in reducing human serum cholesterol. Given the huge CBSH pool in intestinal microflora, the CBSH activities of the small number of additional bacteria consumed as probiotics can be ignored.

Putative genes for Plg-binding proteins, DnaK (BLJ_0123) and glutamine synthethase (BLJ_0624 and BLJ_1324) were found in the JDM301 genome, whereas these proteins play a role in the interaction with human epithelial cells. The protein DnaK has been shown to be present on the surface of pathogens, such as *Neisseria meningitidis*. The glutamine synthethases BLJ_0624 and BLJ_1324 had a 62.11% and 32.49% similarity to the glutamine synthethases in *Mycobacterium tuberculosis* H37Rv.
The authors thank Dr. Hua-Jun Zheng from Shanghai-Institute of Microbiology and Molecular Biology for kindly providing us assistance in data analysis.

Bifidobacterium longum DM301 is a commercial strain used widely in China and other countries, and its safety and health benefits have been extensively studied. The strain exhibits potential benefits in treating various diseases, including but not limited to gastrointestinal disorders, immune modulation, and anti-inflammatory effects.

In conclusion, the study provides valuable insights into the genomic characteristics of B. longum DM301, which could contribute to further understanding of its probiotic potential and aid in the development of targeted probiotic products.
complete genome sequences. Through bioinformatics analysis of the genome sequences, the authors found that although the strain was safe based on phenotype, the information derived from complete bacterial genome sequences revealed some putative unfavourable genes that should be paid attention to.

**Applications**

The study provides a comprehensive assessment on potential risk factors of a probiotic strain based on complete genome sequences. The information related to biosafety derived from the genome of JDM301 will contribute to a wider and deeper insight into the safety of probiotic bacteria.

**Peer review**

This is a very nice and comprehensive study assessing the genomic stability, potential of antibiotic resistance, virulence and production of harmful metabolites. This adds valuable information to current knowledge about probiotics.

**REFERENCES**

1. Ventura M, Canchaya C, Tauch A, Chandra G, Fitzgerald GF, Chater KF, van Sinderen D. Genomics of Actinobacteria: tracing the evolutionary history of an ancient phylum. *Microbiol Mol Biol Rev* 2007; 71: 495-548
2. Marchal ML, Pavan S, Kleebezezem M. Towards understanding genomic modes of probiotic action. *Curr Opin Biotechnol* 2006; 17: 204-210
3. Schell MA, Kamaritzou M, Snel B, Vilanova D, Berger B, Pessi G, Zwhalen MC, Desiere F, Bork P, Delley M, Pridmore RD, Arigoni F. The genome sequence of Bifidobacterium longum reflects its adaptation to the human gastrointestinal tract. *Proc Natl Acad Sci USA* 2002; 99: 14422-14427
4. Sela DA, Chapman J, Adey F, Kim JH, Genes E, Whitehead TR, Lapidus A, Rokhsar DS, Lebrilla CB, German JB, Price NP, Richardson PM, Mills DA. The genome sequence of Bifidobacterium longum subsp. infantis reveals adaptations for milk utilization within the infant microbiome. *Proc Natl Acad Sci USA* 2008; 105: 18964-18969
5. Borrelli SP, Hampes WP, Holzapfel W, Marteau P, Schrezenmeir J, Vaara M, Valtonen V. Safety of probiotics that contain lactobacilli or bifidobacteria. *Clin Diagn Lab Dis* 2003; 36: 775-780
6. O’Brien J, Crittenden R, Arthur C, Ouwehand A, Seppo Salminen. Safety evaluation of probiotics. *Trends in Food Sci Technol* 1999; 10: 418-424
7. McBain AJ, Macfarlane GT. Investigations of bifidobacterial ecology and oligosaccharide metabolism in a three-stage complex microbial rumen culture system. *Scand J Gastroenterol Suppl* 1997; 222: 32-40
8. Ruiz-Moyano S, Martín A, Benito MJ, Casquete R, Serradilla MJ, Córdoba MD. Safety and functional aspects of pre-selected lactobacilli for probiotic use in Iberian dry-fermented sausages. *Meat Sci* 2009; Epub ahead of print
9. Wei YX, Zhang ZY, Liu C, Zhu YZ, Zhu YQ, Zheng H, Zhao GP, Wang S, Guo XQ. Complete genome sequence of Bifidobacterium longum JDM301. *J Bacteriol* 2010; 192: 4076-4077
10. Benson DA, Karch-Mizarchi I, Lipman DJ, Ostell J, Wheeler DL. GenBank. *Nucleic Acids Res* 2008; 36: D25-D30
11. Delcher AL, Harmon D, Kasif S, White O, Salzberg SL. Improved microbial gene identification with GLIMMER. *Nucleic Acids Res* 1999; 27: 4636-4641
12. Guo FB, Ou HY, Zhang CT. ZCURVE: a new system for recognizing protein-coding genes in bacterial and archaeal genomes. *Nucleic Acids Res* 2003; 31: 1780-1789
13. Bose M, Barber RD. Prophage Finder: a prophage loci prediction tool for prokaryotic genome sequences. In *Silico Biol* 2006; 6: 223-227
14. Grissa I, Vergnaud G, Pourcel C. The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats. *BMC Bioinformatics* 2007; 8: 172
15. Lowe TM, Eddy SR. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res* 1997; 25: 955-964
16. Gai H, Hain T, Chakrabarty T. GenomeVis: visualizing microbial genomes. *BMC Bioinformatics* 2004; 5: 198
17. Chen L, Yang J, Yu J, Yao Z, Sun L, Shen Y, Jin Q. VFDB: a reference database for bacterial virulence factors. *Nucleic Acids Res* 2005; 33: D325-D328
18. Liu B, Pop M. ARDB—Antibiotic Resistance Genes Database. *Nucleic Acids Res* 2009; 37: D443-D447
19. Ventura M, Furroni F, Zommer A, Foroni E, Giubellini V, Bottacin C, Canchaya C, Claesson MJ, Fe H, Mantzourani M, Mulas L, Ferrari A, Gao B, Delledonne M, Henriassat B, Coutinho P, Oggoni M, Gupta RS, Zhang Z, Beighton D, Fitzgerald GF, O’Toole PW, van Sinderen D. The Bifidobacterium dentium Bd1 genome sequence reflects its genetic adaptation to the human oral cavity. *PloS Genet* 2009; 5: e1000785
20. Barrangou R, Brizzens EP, Traeger LL, Luqasto JR, Richards M, Horvath P, Cöveyt-Monvoisin AC, Leyer G, Rendulic S, Steele JL, Broadbent JR, Oberg T, Dudley EG, Schuster S, Romero DA, Roberts RF. Comparison of the complete genome sequences of Bifidobacterium animals subsp. lactis DSM 10140 and Bi-04. *J Bacteriol* 2009; 191: 4144-4151
21. Philippe H, Douady CJ. Horizontal gene transfer and phylogenetics. *Curr Opin Microbiol* 2006; 9: 498-505
22. Ammors MS, Flörez AB, Mayo B. Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. *Food Microbiol* 2007; 24: 559-570
23. Delgado S, Flörez AB, Mayo B. Antibiotic susceptibility of Lactobacillus and Bifidobacterium species from the human gastrointestinal tract. *Curr Microbiol* 2005; 50: 202-207
24. Mouabreack C, Giavini F, Butel MJ, Doucet-Populaire F. Antimicrobial susceptibility of bifidobacteria. *J Antimicrob Chemother* 2005; 55: 38-44
25. D’Aimmo MG, Modesto M, Bivati V. Antibiotic resistance of lactic acid bacteria and Bifidobacterium spp. isolated from dairy and pharmaceutical products. *Int J Food Microbiol* 2007; 115: 39-42
26. Wilson JW, Schurr MJ, LeBlanc CL, Ramamurthy R, Buchanan KL, Nickerson CA. Mechanisms of bacterial pathogenicity. *Postgrad Med* 2002; 78: 216-224
27. von Ossowski I, Reunanen J, Satokari R, Resterlund S, Kankainen M, Huitihien T, Tynkynen S, Salminen S, de Vos WM, Palva A. Mucosal adhesion properties of the probiotic Lactobacillus rhamnosus GG SpaCBA and SpaFED. *Appl Environ Microbiol* 2010; 76: 2049-2057
28. Emmanuelsson O, Brunak S, von Heijne G, Nielsen H. Locating proteins in the cell using TargetP, SignalP and related tools. *Nat Protoc* 2007; 2: 953-971
29. Ventura M, Lee JH, Canchaya C, Zink R, Leahy S, Moreno-Munoz JA, O’Connell-Motherway M, Higgin D, Fitzgerald GF, O’Sullivan DJ, van Sinderen D. Prophage-like elements in bifidobacteria: insights from genomics, transcription, integration, distribution, and phylogenetic analysis. *Appl Environ Microbiol* 2005; 71: 8692-8705
30. Brussow H. Phages of dairy bacteria. *Annu Rev Microbiol* 2001; 55: 283-303
31. Touchon M, Rocha EP. Causes of insertion sequence abundances in prokaryotic genomes. *Mol Biol Evol* 2007; 24: 969-981
32. Godde JS, Bickerton A. The repetitive DNA elements called CRISPRs and their associated genes: evidence of horizontal transfer among prokaryotes. *J Mol Evol* 2006; 62: 718-729
33. O’Driscoll J, Heiter DF, Wilson GF, Fitzgerald GF, Roberts R, van Sinderen D. A genetic dissection of the LjAI restriction cassette reveals insights on a novel bacteriophage resistance system. *BMC Microbiol* 2006; 6: 40
34. FEEDAP. Prepared by the Panel on Additives and Products or Substances used in Animal Feed on the Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. *EFSA* 2008; 732: 1-15
35. Masco L, Van Hoorde K, De Brandt E, Swings J, Huys G.
Antimicrobial susceptibility of Bifidobacterium longum from strains of human, animals and probiotic products. *J Antimicrob Chemother* 2006; 58: 85-94

Scott KP, Melville CM, Barbosa TM, Flint HJ. Occurrence of the new tetracycline resistance gene tet(W) in bacteria from the human gut. *Antimicrob Agents Chemother* 2000; 44: 775-777

Nakata S, Arakawa C, Kohira R, Fujita Y, Fuchigami T, Mugishima H. A case of D-lactic acid encephalopathy associated with use of probiotics. *Brain Dev* 2010; 32: 691-694

Bongaerts G, Bakkeren J, Severijnen R, Sperl W, Willem H, Naber T, Wevers R, van Meurs A, Tolboom J. Lactobacilli and acidosis in children with short small bowel. *J Pediatr Gastroenterol Nutr* 2000; 30: 288-293

Uchida H, Yamamoto H, Kikuchi Y, Fujino J, Ishimaru Y, Ikeda H. D-lactic acidosis in short-bowel syndrome managed with antibiotics and probiotics. *J Pediatr Surg* 2004; 39: 634-636

Garai G, Dueñas MT, Irastorza A, Martín-Alvarez PJ, Moreno-Arrabas MV. Biogenic amines in natural cider. *J Food Prot* 2006; 69: 3006-3012

Silla Santos MH. Biogenic amines: their importance in foods. *Int J Food Microbiol* 1996; 29: 213-231

Garai G, Dueñas MT, Irastorza A, Moreno-Arrabas MV. Biogenic amine production by lactic acid bacteria isolated from cider. *Let Appl Microbiol* 2007; 45: 473-478

Benno Y, Mitsuoka T. Impact of Bifidobacterium longum on human fecal microbiota. *Microb Immunol* 1992; 36: 883-890

Kim Y, Lee D, Kim D, Cho J, Yang J, Chung M, Kim K, Ha N. Inhibition of proliferation in colon cancer cell lines and harmful enzyme activity of colon bacteria by Bifidobacterium adolescentis SPM0212. *Arch Pharm Res* 2008; 31: 468-473

Choi SS, Kang BY, Chung MJ, Kim SD, Park SH, Kim JS, Kang CY, Ha NJ. Safety assessment of potential lactic acid bacteria Bifidobacterium longum SPM1205 isolated from healthy Koreans. *Microbiol* 2005; 43: 493-498

Jones BV, Blegen MC, Hill C, Gali YC, Marchesi JR. Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome. *Proc Natl Acad Sci USA* 2008; 105: 13580-13585

Tanaka H, Doessburg K, Iwasaki T, Miera I. Screening of lactic acid bacteria for bile salt hydrolase activity. *J Dairy Sci* 1999; 82: 2530-2535

Yankaskovaova V, Huys G, Vanamney M, Vael C, Klare I, Romond MB, Entenza JM, Moreillon P, Wind BD, Knol J, Wiertz E, Pot B, Vaughan EE, Kahlmeter G, Geossens H. Biosafety assessment of probiotics used for human consumption: recommendations from the EU-PROSAFE project. *Trends in Food Sci and Technol* 2008; 19: 102-114

Nagengast FM, Gruben MJ, van Munster IP. Role of bile acids in colorectal carcinogenesis. *Eur J Cancer* 1995; 31A: 1087-1070

Martese P, Gerhardt MF, Myara A, Bouvier E, Trivin F, Rambaud JC. Metabolism of Bile Salts by Alimentary Bacteria During Transit in the Human Small Intestine. *Micro Ecol in Heal and Dis* 1995; 8: 151-157

Jones ML, Chen H, Ouyang W, Metz T, Prakash S. Microencapsulated Genetically Engineered Lactobacillus plantarum 80 (pCBH1) for Bile Acid Deconjugation and Its Implication in Lowering Cholesterol. *J Biomed Biotechnol* 2004; 2004: 61-69

Knaust A, Weber MV, Hammerschmidt S, Bergmann S, Froesch M, Kurzai O. Cytosolic proteins contribute to surface plasmon laser transmission in Neisseria meningitidis. *J Bacteriol* 2007; 189: 3246-3255

Lähteenmäki K, Kauresa P, Korhonen TK. Bacterial plasminogen activators and receptors. *FEMS Microbiol Rev* 2001; 25: 531-552

Candela M, Miccoli G, Bergmann S, Turroni S, Vitali B, Hammerschmidt S, Brüggi P. Plasminogen-dependent proteolytic activity in Bifidobacterium lactis. *Microbiology* 2008; 154: 2457-2462

Candela M, Bergmann S, Mici S, Vitali B, Turroni S, Eikmanns BJ, Hammerschmidt S, Brüggi P. Binding of human plasminogen to Bifidobacterium. *J Bacteriol* 2007; 189: 5929-5936

Candela M, Centanni M, Fiori J, Biagi E, Turroni S, Orrico C, Bergmann S, Hammerschmidt S, Brüggi P. DNAK from Bifidobacterium animalis subsp. lactis is a surface-exposed human plasminogen receptor upregulated in response to bile salts. *Microbiology* 2010; 156: 1609-1618

Luck SN, Turner SA, Rajakumar K, Sakellaris H, Adler B. Ferric dicarboxylic transport system (Fec) of Shigella flexneri 2a YSH600 is encoded on a novel pathogenicity island carrying multiple antibiotic resistance genes. *Infect Immun* 2001; 69: 6012-6021

Satin B, Del Giudice G, Dell’Bianca V, Dusi S, Laudanna C, Tonello F, Kelleher D, Rappuoli R, Montecucco C, Rossi F. The neutrophil-activating protein (HP-NAP) of Helicobacter pylori is a protective antigen and a major virulence factor. *J Exp Med* 2000; 191: 1467-1476

Verbeelen C, Dufréne YF. Direct measurement of Mycobacterium-fibroconnectin interactions. *Integr Biol (Camb)* 2009; 1: 296-300

Lee JH, Karamychev VN, Kozyavin SA, Mills D, Pavlov AR, Pavlova NV, Polouchine NN, Richardson PM, Shakhova VV, Slesarev AI, Weimer B, O’Sullivan DJ. Comparative genomic analysis of the gut bacterium Bifidobacterium longum reveals loci susceptible to deletion during pure culture growth. *BMCMicrobiology* 2008; 9: 247

Galliot O, Pellegrini E, Bregenholt S, Nair S, Berche P. The Cip7 sereine protease is essential for the intracellular parasitism and virulence of Listeria monocytogenes. *Mol Microbiol* 2000; 35: 1286-1294

Alfaleh K, Anabrees J, Bassler D. Probiotics reduce the risk of necrotising enterocolitis in preterm infants: a meta-analysis. *Neonatology* 2010; 97: 93-99

McFarland LV. Meta-analysis of probiotics for the prevention of antibiotic associated diarrhea and the treatment of Clostridium difficile disease. *Am J Gastroenterol* 2006; 101: 812-822

Mach T. Clinical usefulness of probiotics in inflammatory bowel diseases. *J Physiol Pharmacol* 2006; 57 Suppl 9: 23-33

Parveen S, Malik KA, Ah Kang S, Kim HY. Probiotics and their fermented food products are beneficial for health. *J Appl Microbiol* 2006; 100: 1171-1185