A Proteome Reference Map and Proteomic Analysis of Bifidobacterium longum NCC2705*

Jing Yuan†‡¶, Li Zhu†‡¶, Xiankai Liu†‡¶, Ting Li‡, Ying Zhang‡, Tianyi Ying‡, Bin Wang¶, Junjun Wang‡, Hua Dong‡, Erling Feng‡, Qiang Li‡, Jie Wang**, Hongxia Wang**, Kaihua Wei**, Xuemin Zhang**, Cuifeng Huang‡, Peitang Huang‡, Liuyu Huang‡, Ming Zeng‡‡ and Hengliang Wang‡§§

A comprehensive proteomic study was carried out to identify and characterize proteins expressed by Bifidobacterium longum NCC2705. A total of 708 spots representing 369 protein entries were identified by MALDI-TOF-MS and/or ESI-MS/MS. Isoelectric point values estimated by gel electrophoresis matched closely with their predicted ones, although some discrepancies exist suggesting that post-translational protein modifications might be common in B. longum. The identified proteins represent 21.4% of the predicted 1727 ORFs in the genome and correspond to 30% of the predicted proteome. Moreover 95 hypothetical proteins were experimentally identified. This is the first compilation of a proteomic reference map for the important probiotic organism B. longum NCC2705. The study aimed to define a number of cellular pathways related to important physiological processes at the proteomic level. Proteomic comparison of glucose- and fructose-grown cells revealed that fructose and glucose are catabolized via the same degradation pathway. Interestingly the sugar-binding protein specific to fructose (BL0033) and Frk showed higher levels of expression in cells grown on fructose than on glucose as determined by semiquantitative RT-PCR. BL0033 time course and concentration experiments showed that the induction time and fructose concentration correlates to increased expression of BL0033. At the same time, an ABC (ATP-binding cassette) transporter ATP-binding protein (BL0034) was slightly up-regulated in cells grown on fructose compared with glucose. All of the above results suggest that the uptake of fructose into the cell may be conducted by a specific transport system in which BL0033 might play an important role. Molecular & Cellular Proteomics 5: 1105–1118, 2006.

The gastrointestinal tracts (GITs)1 of humans and other living organisms are colonized by a large, active, and complex community of microbes, collectively termed intestinal microbiota. Bifidobacterium represents the third most common genus of the microbiota after genera Bacteroides and Eubacteria (up to 3% of the total fecal microflora of adults) (1). Bifidobacterium is an obligate anaerobe in the Actinomycetales branch of the high-G + C Gram-positive bacteria (2). It plays an important role in maintaining a balance of normal intestinal flora by liberating lactic and acetic acids, which in turn prevent the colonization of potential pathogens. In addition, Bifidobacterium has been reported to up-regulate host immune function and contribute to cancer resistance (3). Currently bifidobacteria are extensively used in the food industry as health-promoting microorganisms. Much effort has been made to increase the diversity and number of Bifidobacterium species in the intestinal tract by supplying certain Bifidobacterium strains (probiotics) or food ingredients that can stimulate the growth of Bifidobacterium (prebiotics). The therapeutically efficacies of these practices have been confirmed by clinical treatment and prevention of gastrointestinal disorders (4). In recent years, bifidobacteria have been explored for their potential use in secreting proteins of biotechnological interest and for the delivery of pharmacologically active substances. These studies will benefit from a detailed knowledge of the proteome relating to Bifidobacterium metabolism.

Bifidobacterium longum is a strict fermentative anaerobe, deriving its principal source of energy from substrate level phosphorylation during glycolysis. In the human GIT, bifidobacteria function as scavengers in the large intestine and possess a wide range of catabolic pathways that confer a growth advantage where readily fermentable carbohydrates are in short supply (2, 5). The ability of Bifidobacterium spp. to survive and persist in this competitive environment is made possible by use of negative transcriptional regulation as a

---

1 The abbreviations used are: GIT, gastrointestinal tract; 2-D, two-dimensional; CAI, codon adaptation index; ABC, ATP-binding cassette; F6P, fructose 6-phosphate; F6PPK, F6P phosphoketolase; PMF, peptide mass fingerprinting; rDNA, ribosomal DNA; 2-DE, two-dimensional electrophoresis; ThDP, thiamine diphosphate; MurNAc, N-acetylmuramic acid; PEP, phosphoenolpyruvate; PTS, phosphotransferase system.
flexible control mechanism in response to nutrient availability and diversity as well as predicted genetic features such as exo- and endo-glycosyl hydrolases and high affinity oligosaccharide transporters. These features likely help \textit{B. longum} compete for uptake of structurally diverse oligosaccharides released from digestion of plant fibers. Several studies conducted on fructose-containing polymers as potential selective substrates for colonic bacteria have provided evidence that bifidobacteria are able to ferment these carbohydrates, particularly the short chains of \(\beta-(2\rightarrow1)\)-linked fructosyl units (6–10). Recently bifidobacteria were shown to possess only one pathway for the metabolism of glucose, the F6P phosphoketolase (F6PPK) pathway, otherwise known as the bifid shunt (11, 12). Analyses of fructose fermentation patterns suggested that \textit{B. longum} could use \(D\)-fructose as the sole carbon source (11, 12); however, the uptake mechanism of fructose into the cell and proteins regulated by fructose remained to be defined.

The 2.26-Mb genome of \textit{B. longum} strain NCC2705 was sequenced in 2002, predicting 1729 ORFs (2). The sequences were revised to gi:23464628 (containing 1727 ORFs) according to the National Center for Biotechnology Information (NCBI) website in 2005. Unlike genome studies, investigations at the proteomic level provide insights into protein abundance and/or post-translational modifications. It is also one of the best methods of investigating basic biological processes such as pathogenesis, physiology, and metabolic mechanisms (13, 14). In recent years 2-D electrophoresis has been used in proteomic studies of many bacteria, including \textit{Vibrio cholerae} (19–21), \textit{Escherichia coli} (25, 26), \textit{Brucella melitensis} (27, 28), \textit{Mycobacterium tuberculosis} (19–21), \textit{Escherichia coli} (13, 22–24), \textit{Helicobacter pylori} (25, 26), \textit{Bacillus subtilis} (27, 28), \textit{Lactococcus lactis} (30–33), \textit{Shigella flexneri} (34), \textit{Agrobacterium tumefaciens} (35), \textit{Vibrio cholerae} (36), and \textit{Brucella melitensis} (37).

Vitali \textit{et al.} (38) reported the identification of 136 proteins in \textit{Bifidobacterium infantis} B107 by using multidimensional chromatography and tandem mass spectrometry. To date, a two-dimensional reference map to correlate protein expression to physiological changes in \textit{B. longum} has not been generated.

In this study we focused on generating a base-line signature or cartographic reference of the \textit{B. longum} strain NCC2705 proteome by identifying ORF expression and investigating the mechanism of fructose translocation, uptake, and utilization.

**EXPERIMENTAL PROCEDURES**

\textbf{Strain, Medium, and Growth Condition—}\textit{B. longum} strain NCC2705 was kindly provided by Nestle Research Center (Lausanne, Switzerland). \textit{B. longum} NCC2705 were grown anaerobically at 37 °C in 400 ml of De Man-Rogosa-Sharpe broth (39) containing 0.05% L-cysteine or in modified Garches medium as described previously (4, 40). Anaerobic conditions were maintained by sparging the cultures with \(O_2\)-free \(N_2\) gas (5 ml min$^{-1}$). Cells were harvested at midexponential phase with an \(A_{600}\) of 0.9 corresponding to \(1.5 \times 10^8\) colony-forming units/ml. Cells were pelleted for 10 min at 8,000 \(\times g\) (Sigma 3K12 centrifuge, Nr.12150) and washed four times with 40 ml of ice-cold low salt buffer (3 mm KCl, 1.5 mm KH$_2$PO$_4$, 68 mm NaCl, and 9 mm NaH$_2$PO$_4$) (41).

\textbf{Preparation of Whole Cell Protein Extract—}\textit{B. longum} NCC2705 cell pellets (about 0.30 g) were resuspended in 5 ml of lysis buffer (7 mm urea, 2 mm thiourea, 4% (v/w) CHAPS, and 50 mm DTT) containing complete protease inhibitors (Roche Applied Science). The cells were sonicated for 10 min on ice using a Sonifier 750 (Branson Ultrasonics Corp., Danbury, CT) with the following conditions: 2 s of sonication with a 2-s interval, set at 35% duty cycle. After adding 2.5 mg of RNase (Promega, Madison, WI) and 100 units of RNase (Promega), the cell lysate was incubated for 1 h at 15 °C to solubilize proteins and centrifuged for 20 min at 20,000 \(\times g\) to pellet the insoluble components. The supernatant was collected, and protein concentration was measured using the PlusOne 2-D Quant kit (Amersham Biosciences), and 1-mg aliquots were stored at –70 °C.

\textbf{Two-dimensional Polyacrylamide Gel Electrophoresis—IEF was performed by using IEF strips (18 cm; Amersham Biosciences). The first dimensional isoelectric focusing was carried out as described previously (42). For the second dimension vertical slab SDS-PAGE (12.5%) was performed for about 4 h at 30 mA/gel using a Bio-Rad Protean II Xi apparatus (Bio-Rad). The gels were stained with Coomassie Brilliant Blue G-250 (Amresco, Solon, OH) and were scanned with ImageScanner (Amersham Biosciences). Image analysis was carried out using ImageMaster 2D Platinum software (Amersham Biosciences). Images from two independent cultures were compared. The relative volume of each spot was determined from the spot intensities in pixel units and normalized to the sum of the intensities of all the spots of the gel. Proteins displaying at least 3-fold volume variations in response to glucose in all experiments were considered in this work.

\textbf{In-gel Protein Digestion and MALDI-TOF-MS—}The Coomassie-stained protein spots of interest were cut out, and in-gel protein digestion was performed as described previously (43). Peptides from digested proteins were resolubilized in 2 \(\mu l\) of 0.5% TFA. Peptide mass fingerprinting (PMF) measurements were performed on a Bruker Reflex$^{TM}$ III MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) working in reflectron mode with 20 kV of accelerating voltage and 23 kV of reflecting voltage. A saturated solution of \(\alpha\)-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% TFA was used for the matrix. A total of 2 \(\mu l\) of the matrix solution and sample solution were mixed in a 1:1 (v/v) ratio and applied onto the Score 384 target well. Mass accuracy for PMF analysis was 0.1–0.2 Da with external calibration, and internal calibration was carried out using enzyme autolysis peaks; resolution was 12,000. PeakClean (www.proteomics.com.cn/tools/PkClean/) was used to remove contaminants including matrix peaks, solvent peaks, and enzyme autolysis peaks.

\textbf{Nanospray ESI-MS/MS—}The peptide solution after in-gel protein digestion was collected, lyophilized, reconstituted in 30 \(\mu l\) of 30% acetonitrile containing 0.1% TFA, and then desalted using ZipTip C$_{18}$ pipette tips (Millipore, Bedford, MA). Electrospray ionization (ESI-MS/MS) was carried out with a hybrid quadrupole orthogonal acceleration tandem mass spectrometer (Q-TOF2, Micromass Ltd., Manchester, UK). The capillary voltage in MS and MS/MS experiments was set to an average of 900 V, and the sample cone voltage was 30 V. A microchannel plate detector was applied with 2200 V. The collision gas was argon with a pressure of 0.1 megapascal (Mpa), and collision energy was 50 V. Glu-Fibrinopeptide was used to calibrate the instrument in the MS/MS mode. MS/MS spectra were transformed using MaxEnt3 (Mass Lynx, Micromass Ltd.), and amino acid sequences were interpreted manually using PepSeq (BioLynx, Micromass Ltd.).

\textbf{Protein Identification—}Database searches were performed by using the software Mascot (Matrix Science Ltd.) licensed in-house.
The oligonucleotide primers used for cloning *B. longum* and Phosphoketolase Assay—

| Oligonucleotide | Nucleotide sequence (5' → 3') |
|-----------------|-------------------------------|
| xfp Primer 1    | CTGGCCTGAACCTTCCTCATGG<sup>a</sup> |
| xfp Primer 2    | AGCCGAGCCCGCGGTTAGC<sup>a</sup> |
| 16 S rDNA Primer 1 | TCCAGTTTGATGGCATGTTGC<sup>b</sup> |
| 16 S rDNA Primer 2 | GGGAAAGCCGTCATCTTACGA<sup>b</sup> |
| BL0033 Primer 1 | AGGTGGCACTTGGGAGCTCC |
| BL0033 Primer 2 | GGGCGCTGCGAAGGCTTGG |
| BL0034 Primer 1 | GTCAGGCAAGGAAGCCTAGG |
| BL0034 Primer 2 | TGCCTCCTTTCCGGTGTTTACG |
| frk Primer 1    | GCTCGGGCGAGCACTTGAGG |
| frk Primer 2    | AGATGGCCGGCGGTGAAGG |
| BL1340 Primer 1 | GTCGGTCTCATTACAACGGTCTCC |
| BL1340 Primer 2 | GCGGACAGGGTGAGATTAGC |
| BL1341 Primer 1 | GCGGCGTGGGACAATATCG |
| BL1341 Primer 2 | GCCAGGTCACGAGCTTCAAGG |
| glkA Primer 1   | CTACACCTTCCCTCCAGCAATCC |
| glkA Primer 2   | TCGCCGATCTTCCCGAGATGCC |
| tal Primer 1    | GACCTCATCGCCACAAAGACG |
| tal Primer 2    | AGCTAGAAGACACGAGAATCC |

<sup>a</sup> Primer specific to sequences within 16 S rDNA of *B. longum* NCC2705.

<sup>b</sup> Primer specific to sequences within 16 S rDNA of *B. longum* NCC2705.

Sequence was extracted from *B. longum* NCC2705 culture by using a Master-Pure RNA purification kit (Epicenter Technologies, Madison, WI). RNA concentrations were determined by spectrophotometry at 260 nm. Reverse transcription was carried out with Omniscript reverse transcription kit (Qiagen) with 2 μg of total RNA as the template. Specific target RT-PCR products were normalized to an established endogenous internal control transcript (a primer pair designed to amplify a fragment of 831 bp from the *B. longum* 16 S ribosomal DNA (rDNA) transcript (48), the expression of which is relatively constant in bacteria (49). The primers used for RT-PCR assays listed in Table I were designed to generate PCR products of comparable sizes. Negative controls consisted of reactions without primers, reverse transcriptase, and *Taq* polymerase (Promega) to confirm the absence of contaminating DNAs in the RNA preparations. The results were analyzed with Quantity One software (Bio-Rad) as described previously (50).

**RESULTS AND DISCUSSION**

**Predicted Proteome of *B. longum* NCC2705—Sequence analysis of the 2.26-Mb genome of *B. longum* NCC2705 revealed 1727 predicted ORFs. The theoretical 2-DE map of *B. longum* was constructed according to the genome annotation (at The Institute for Genomic Research website) showing the distribution of all the predicted proteins. To simulate protein mobility during 2-DE, the x axis was drawn on a linear scale to imitate protein mobility during isoelectric focusing gel electrophoresis, and the y axis was drawn on a logarithmic scale to represent migration during SDS-PAGE (Fig. 1) (13, 31, 35). Isoelectric point and molecular weight values were calculated for all of ORFs using the ExpaSy Protparam tool (us.expasy.org/tools/pi_tool.html). Acidic proteins with pl between 3 and 7 represented about 71.7% of the theoretical proteome, whereas 19% of the proteins displayed a pl greater than 9, and less than 9.3% displayed a pl between 7 and 9. This bimodal protein charge distribution is similar to those of other lactic acid bacteria (32, 33). Due to the relatively low levels and difficulty handling alkaline protein, our study focused on the description of cytosolic, acidic proteins, and the
Fig. 2. Two-dimensional gel electrophoresis of the whole cell proteins of *B. longum* and the identified spots. The identified spots are labeled on the integrated 2-DE map of pH 4–7, pH 4–5, and pH 4.4–5.5.
pH range of 4–7 was chosen as the standard analytical window.

Two-dimensional Gel Electrophoresis and Protein Identification—To obtain an overview of the protein distribution of *B. longum* NCC2705, a wide range IPG strip (18 cm) of pH 3–10 was used for the first dimensional separation of the whole cell proteins. The protein concentration of prepared sample was 9.02 μg/μl. After SDS-PAGE, the separated spots on the gel were visualized by Coomassie Brilliant Blue G-250 staining. The result of the pH 3–10 gel showed that most protein spots clustered in the pH range of 4–7. To resolve protein in the densely populated pH 4.0–5.5 zone, we analyzed whole cell protein using variable IPG strips of pH 4–7, pH 4.0–5.0, and pH 4.5–5.5. The numbers of spots detected in these Coomassie-stained gels were 1572, 585, and 916, respectively. The separate graphs of pH 4–7, pH 4.0–5.0, and pH 4.5–5.5 gels were merged to produce an artificial gel map of pH 4–7, comprising a total of 1847 detectable spots, approximately 2 times more than the 899 resolved spots detected on the actual pH 4–7 gel.

The 899 matched spots on the corresponding micro-preparative electrophoresis gels were excised and destained. After in-gel trypsin digestion, MALDI-TOF-MS and/or ESI-MS/MS were performed to identify proteins. Among the 899 protein spots processed, 708 spots were successfully identified, representing 369 protein entries by PMF; 14 spots representing 11 protein entries were reconfirmed by ESI-MS/MS (Supplemental Table 3). The identified spots are labeled on the integrated map of pH 4–7 (Fig. 2), and identified spots are listed in Supplemental Tables 1–3. Maps and information of all identified proteins are available at www.mpib-berlin.mpg.de/2D-PAGE and www.proteomics.com.cn.

Distribution of Proteins According to Cellular Role Categories and Localization—Experimentally identified proteins grouped into cellular roles are summarized in Fig. 3. Metabolism-related proteins, especially those related to energy metabolism, comprise a great part of the identified proteins. The significance of this observation might be that they are necessary for sustenance.

In this study, every processed spot on Coomassie-stained gels led to the identification of a corresponding protein. However, only 369 proteins were identified, accounting for ~22% of the predicted proteins. The possible explanations are that 1) many proteins are inherently difficult to be identified by these methods (low abundance proteins, hydrophobic proteins, and proteins with high or low molecular weight), or 2) *B. longum* NCC2705 proteins occurred at several locations of the 2-DE gels.

The cellular localizations of all 369 identified proteins predicted by PSORT Version 2.0 (www.psort.org) are listed in Supplemental Table 1. 320 proteins identified are cytoplasmic; 17 proteins were predicted in the cytoplasmic membrane, two proteins were predicted in the cell wall, and 30 spots had unknown cellular localization. Moreover eight detected proteins (BL0033, BL0077, BL0141, BL0352, BL1345, BL1386, BL1638, and BL1714) were predicted to have a signal peptide. Surprisingly four proteins were annotated as extracellular proteins suggesting that they might be interact tightly with cell surface components.

Extractoplastic proteins play critical roles in establishing and maintaining interactions between a microbe and its environment. Many cell wall surface proteins of Gram-positive bacteria are covalently anchored to the cell wall by a mechanism requiring a C-terminal anchoring motif, consisting of a conserved amino acid sequence Leu-Pro-Thr-Gly (LPXG, where X is any amino acid) (51). In bifidobacteria, extractoplastic proteins might mediate important host interactions, such as adhesion, nutrient availability, immune system modulation, and pathogen inhibition. Interestingly two proteins (spot 88, BL1132; and spot 747, BL1064) with unknown cellular location and one protein (spot 403, BL0603) predicted to localize to cytoplasmic membrane displayed a clear Gram-positive cell surface anchor motif. This evidence suggests that these proteins may be involved in host interactions.

CAI, GRAVY Value of Each Protein, and High Abundance Proteins—Synonymous codons are not generally used at equal frequencies. The degree of codon bias is related to both the content of the isoacceptor tRNAs and the level of gene expression as the result of selection to increase translational efficiency. Thus, highly expressed genes tend to predominantly use synonymous codons with the most abundant tRNA (“major” or preferred codons), whereas weakly expressed genes show a more frequent use of the “minor” or preferred synonymous codons (44, 52). Accordingly highly expressed proteins...
genes show a base composition that departs more strongly from that expected by a mutational equilibrium. CAI estimates the degree of synonymous codon adaptation in a coding region compared with the optimal usage. A value of 1.0 indicates the maximum codon use fit, and values <1.0 indicate use of less preferred codons. Fig. 4 compares the CAI distributions of genes coding for the proteins identified on the pH 4–7 gel with those of all genes. The genes with the highest CAI encode glycolytic enzymes or proteins of the translational apparatus. The proteins encoded by genes with a CAI value <0.5 account for 77.0% of the total proteins and only 55.2% of the currently identified proteins. All genes having a CAI value >0.75 were identified on the 2-D maps. These results demonstrated that proteins encoded by genes with a high CAI were abundant and easily identified. Interestingly three hypothetical proteins with a CAI value below 0.1 were also identified (spot 217, BL1039; spot 329, BL0667; and spot 7, BL0256). The abundance of all spots in the 2-D gel is listed in Supplemental Table 1, and the 50 most abundant proteins are labeled as b1–b50. Proteins with a CAI value above 0.5 are involved in energy metabolism, fatty acid and phospholipid metabolism, protein synthesis, and cellular processes. They represented a large part of the most abundant proteins. Translation elongation factor Tu and universal stress protein were the top two abundant proteins. Our results were similar to the results from E. coli (13), L. lactis (33), and B. subtilis (28).

Proteins with extended hydrophobic regions, such as membrane proteins, are difficult to detect under standard gel conditions. The overall hydrophobicity of a protein is helpful in predicting its solubility in a buffer. This parameter, expressed as the GRAVY index (52), was calculated for all identified and predicted proteins using CodonW software. Fifty-four of all identified proteins have a GRAVY index value above 0 with the highest being 0.433585. Seven proteins with high GRAVY index value are cytoplasmic membrane proteins. A comparison between identified proteins and total predicted proteins (Fig. 5) shows that hydrophobic proteins (with high GRAVY values) are not present among the identified proteins, suggesting that they are not represented in the gel. It is interesting to note that the very hydrophilic proteins are also missing from the identified protein, although they are assumed to dissolve easily in the buffer. This result is probably due to the low abundance of such hydrophilic proteins in B. longum NCC2705.

A Global View of Glycolysis and Energy Metabolism—Bifidobacteria are reported to possess only one route for the metabolism of glucose, the F6PPK (or bifid shunt) pathway (11, 12). With the exception of glucokinase, the remaining eight enzymes of the bifid shunt were identified in this study, including the xylulose-5-phosphate-fructose-6-phosphate phosphoketolase (BL0959; EC 4.1.2.7; CAI, 0.769), glucose-6-phosphate isomerase (BL0279; EC 5.3.1.9; CAI, 0.792), transaldolase (BL0715; EC 2.2.1.12; CAI, 0.769), transketolase (BL0716; EC 2.2.1.11; CAI, 0.725), a probable ribose-5-phosphate isomerase (BL1623, EC 5.3.1.6, CAI 0.684), ribulose-phosphate 3-epimerase (BL0753; EC 5.1.3.1; CAI, 0.610), acetate kinase (BL0969; EC 2.7.2.1; CAI, 0.701), and L-lactate dehydrogenase (BL0710; EC 1.1.1.27; CAI, 0.580). Moreover we also identified 12 enzymes involved in the Embden-Meyerhof pathway.

Preliminary studies of the biochemistry, genetics, and regulation of these essential enzymes in the nutritionally important bifidobacteria showed that phosphoketolases are key thiamine diphosphate (ThDP)-dependent enzymes of bifid shunt. Phosphoketolase links several metabolic routes to the bifid shunt, including N-acetylhexosamine fermentation, galactose catabolism (the Leloir pathway), and peptidoglycan biosynthesis. Similar results have been reported in other microorganisms (10, 11, 53). In bifidobacteria, there is evidence for the existence of two distinct F6P phosphoketolase enzymes (34, 35): a specific enzyme (F6PPK) solely for F6P found in humans, such as Bifidobacterium dentium, and a dual substrate xylulose-5-phosphate/F6P phosphoketolase (Xfp; pl 5.0; 92,469 Da) found in animals, such as Bifidobacterium globosum. The dual specificity xylulose 5-phosphate/F6P phosphoketolase is encoded by the gene xfp, which was first described in Bifidobacterium animalis subsp. Lactis by Meile et al. (45). Native Xfp was predicted to be a homohexamer.
with a molecular mass of 550,000 Da. The subunit size upon SDS-PAGE (90,000 Da) matches the predicted size (92,469 Da) calculated from the amino acid sequence of the isolated gene (named \textit{xfp}), which encodes 825 amino acids (11). In the genome of infant-derived NCC2705 strain, a single copy of \textit{xfp} is identified at locus BL0959.

Interestingly 21 spots of Xfp (molecular weight, 92,697; pI, 5.06) differing in charge and mass were identified in the present work by MALDI-TOF and ESI-MS/MS (see Supplemental Table 3). The proteins were distributed into pH ranges 4.2–4.4 and 6.0–6.2 in the gel. To study the regulation and expression of the gene coding for the enzyme, we cloned and sequenced the ORF (2475 bp; 825 amino acids) from \textit{B. longum} NCC2705 and ATCC15707. After analyzing homology to sequences in GenBank™ using the Basic Local Alignment Search Tool (BLAST) available on the National Center for Biotechnology Information server, we found that the \textit{xfp} sequences from \textit{B. longum} NCC2705 and ATCC15707 are very similar to the 10 \textit{xfp} coding sequences from other bifidobacteria. The Xfp amino acid sequence from \textit{B. longum} NCC2705 and ATCC15707 contains a ThDP-dependent enzyme signature sequence YG\textit{XX}5P\textit{XX}3V\textit{XX}2I\textit{GDGE} (amino acids 165–184), which closely matches the consensus [LIVMF]-G\textit{XX}5P\textit{XX}4V\textit{IX}-\textit{GDG-[GSAC]} (PROSITE PS00187). (In the PROSITE database, ambiguities are indicated by listing the acceptable amino acids for a given position between square brackets.) Another motif (GDGX\textit{XX}24–27\textit{NN}), which is common to ThDP-binding enzymes like acethydroxy acid synthases, transketolases, E1 (decarboxylase) components of 2-ketoacid and acetoin dehydrogenases, and others, is also present in \textit{B. longum} NCC2705 Xfp in a modified form, GDGE\textit{XX}30D (amino acids 181–215). In \textit{B. longum} ATCC15707, the motif is present in Xfp in conserved form, GDGE\textit{XX}30N. The peptide sequence of the Xfp protein from NCC2705 derived by ESI-MS/MS (Fig. 6) indicates that Xfp has a mutation in amino acid position 215 resulting in Asp instead of Asn. This modification corresponds to an increase in the global negative charge due to the acidic residue (Asp\textsuperscript{215}). Modified Xfp showed activity similar to that of the conserved Xfp from ATCC15707. The enzymatic activities in cell extracts were 22 and 24 nmol min\textsuperscript{-1} mg of protein\textsuperscript{-1}, respectively. However the cDNA sequence of \textit{xfp} obtained by RT-PCR was identical to the original gene (data not shown). We speculate that the natural variant might likely be formed by post-translational modification, which was confirmed with further investigation. We suggest that the structural variants of Xfp could be used to provide information for the structure, property, and function of the enzyme.

Furthermore several additional enzymes involved in the bifid shunt were also distributed in three to 10 adjacent spots, varying in charge. They were subjected to a physiologically relevant post-translational modification. Further experiments are in progress to identify the nature of the modification.

Bifidobacteria colonize at the lower GIT, an environment poor in mono- and disaccharides because they are consumed by the host and by microflora in the upper GIT. As previous studies suggested, \textit{B. longum} utilizes a variety of plant-derived dietary fibers, such as arabinoxylans and gums (2, 54). We identified 44 proteins assigned to the carbohydrate transport metabolism category, four of which were related to oligosaccharide hydrolysis: BL0682 (spot 293, xylan esterase), BL0978 (spot 758, β-galactosidase, EC 3.2.1.23), and BL0982/BL1573 (spots 732/513, isoamylase, EC 3.2.1.68); two other proteins involved in the fermentation of nondigestible dietary carbohydrates were identified: BL0673 (spots 714, 716, and 727; ATP-binding protein of ATP-binding cassette A

![Proteomic Analysis of B. longum NCC2705](Molecular & Cellular Proteomics 5.6 1111)
(ABC) transporter for sugars) and BL1656 (spots 261, 313, and 812; phosphoglycerate mutase, EC 5.4.2.1).

**Amino Acid Metabolism—** *B. longum* has genes involved in synthesis of at least 19 amino acid from NH₃, and major biosynthetic precursors including phosphoenolpyruvate, oxaloacetate, oxoglutarate, and fumarate provided by a partial Krebs cycle lacking fumarase, oxoglutarate dehydrogenase, and malate dehydrogenase (2). These genes have been predicted in the genome of *B. longum* NCC2705. In the present map, 101 proteins (about 32% of all functional proteins identified) related to amino acid metabolism were identified according to the results of a search in the database of the Kyoto Encyclopedia of Genes and Genomes (www.kegg.com/kegg/pathway.html). We identified a majority of proteins corresponding to amino acid metabolism pathway, urea cycle, and metabolism of amino groups.

Transamination reactions have recently attracted attention because they are the first step for the synthesis of important flavor or aroma compounds in amino acid catabolism pathways (55). We found five aminotransferases: AspC (aspartate transaminase, EC 2.6.1.1, BL1286), HisC (histidinol-phosphate transaminase, EC 2.6.1.9, BL1296), SerC (probable phosphoserine transaminase, EC 2.6.1.52, BL1660), IlvE (probable Branched-chain-amino-acid transaminase, EC 2.6.1.42, BL0852), and probable aminotransferase (aspartate transaminase, EC 2.6.1.1, BL0783). These enzymes possess the catalytic potency to deaminate aspartate, histidinol phosphate, branched-chain amino acids, and other amino acids. But we did not detect the proteins related to lysine degradation.

**Stress Proteins—** *B. longum* is moderately aerotolerant and encodes homologs of enzymes that repair oxidative damage. The enzymes responsible for minimizing the toxicity of active oxygen species were predicted in the genome of *B. longum* NCC2705; NADH peroxidase and superoxide dismutase were absent, but a NADH oxidase was present (2). Under our experimental conditions we found alkyl hydroperoxide reductase (*ahpC*, BL0615), a protein that can reverse oxidative damage to proteins and lipids. We hypothesize that AhpC might play an important role in reversing oxidative damage to proteins.

Heat shock proteins corresponding to DnaK (BL0520), GroEL (BL0002), and GroES (BL1558) can also be induced by salt stress, mild acid treatment, and UV irradiation in *B. longum* NCC2705. They were identified as highly abundant proteins in this study.

Bifidobacteria are a dominant population of the human intestinal microflora. One of the most important metabolic activities is deconjugation of bile salts, which occurs naturally in human intestines. The responsible enzyme, cholyglycine hydrolase (BL0796, EC 3.5.1.24), which catalyzes the hydrolysis of glycine- and/or taurine-conjugated bile salts into amino acid residues and deconjugated bile salts (bile acids), was identified in our study. It might play a role in *B. longum* resistance to toxic levels of bile salts in the gastrointestinal environment. This enzyme may be medically relevant because in recent years the possibility of using bile salt deconjugation by lactic acid bacteria to decrease serum cholesterol levels in hypercholesterolemic patients or to prevent hypercholesterolemia in individuals with normal cholesterol levels has received increasing attention (56).

**Peptidoglycan Precursor Assembly and Nucleotide Metabolism—** Nine key enzymes identified in the present map, *glmU*, *nagA*, *ddIA*, *glmA1*, *glmA2*, *murA*, *murB*, and *murF*, are involved in the synthesis of a cytoplasmic peptidoglycan precursor (UDP-MurNAc-pentapeptide) (33, 57). MurA (BL1267, UDP-N-acetylg glucosamine 1-carboxyvinyltransferase) and MurB (BL1561, UDP-N-acetylenolpyruvoylglucosamine reductase) are likely to catalyze the synthesis of UDP-MurNAc from UDP-GlcNAc. MurD (BL1321, UDP-N-acetyl muramoyl-l-alanine-β-glutamate ligase) and MurF (BL1319, UDP-N-acetylmuramoylalanine-γ-glutamyl-2,6-diaminopimelate-β-calycolyc-β-alanine) can add α-glutamate and the dipeptide β-Ala-β-Ala, respectively, to form the UDP-MurNAc-pentapeptide. However, we did not detect MurC and MurE.

*B. longum* has all genes required for pyrimidine and purine nucleotides biosynthesis from glutamine. The present map contains 29 enzymes involved in the synthesis of purine (15 proteins) and pyrimidine (14 proteins) nucleotides. As a dairy fermentative bacterium, bifidobacteria need trace amounts of free purine bases in the medium (guanine, hypoxanthine, and guanosine (58)). These purines positively regulate the enzymes required for de novo synthesis of precursors for ATP and GTP, but we did not identify these proteins. Therefore, we hypothesize that De Man-Rogosa-Sharpe medium provided the cell with the necessary amount of purine bases and/or nucleotides, which stimulated the growth of Bifidobacterium.

**Discrepancies of Theoretical Prediction and Experimental Results—** From the statistical results, proteins with pl 4–6 and of 10–70 kDa are more likely to be identified on the 2-D gel. The gel-estimated pl and molecular weight analyzed by using the ImageMaster 2D Platinum software matched well with those obtained from the theoretical predictions. Discrepancies between gel-estimated and theoretical masses might result from post-translational proteolytic processing and modification. These phenomena have been reported in similar studies (34, 59). Different from their theoretical pl values, several proteins appeared in the relatively acidic region (such as spots 60, 321, and 525) or in the alkaline region (such as spots 86, 484, and 792). This might result from the cleavage of acidic regions or alkaline regions and phosphorylation.

Proteomic analysis enables a global inspection of post-translational modifications as many of these events are manifested in changes of charge or molecular weight. Post-translational modifications are known to play an important role in eukaryotes, whereas less is known about their role in bacterial physiology. In this experiment, post-translational modifications appeared to be common in *B. longum* NCC2705. One
hundred and fifteen proteins identified appeared in more than one spot on the gel. These proteins are listed in Supplemental Tables 1 and 2, and nine proteins were identified in more than 10 distinct spots. They include elongation factor Tu (BL1097), chaperone (BL0002), xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (BL0959), transketolase (BL0716), ATP synthase β chain (BL0357), LacZ (BL0978), pyruvate kinase (BL0988), transaldolase (BL0715), and elongation factor G (BL1098). Another 11 proteins were identified from more than five spots: cholesterylglycine hydrolase (BL0796), DppA2 (BL1386), DnaK protein (BL0520), phosphoglycerate kinase (BL0707), 6-phosphogluconate dehydrogenase, decarboxylating II (BL0444), ketol-acid reductoisomerase (BL0531), trigger factor chaperone (BL0947), acetate kinase (BL0969), enolase (BL1022), RNA polymerase α-E factor (BL1357), and UDP-glucose 4-epimerase (BL1671). These 20 proteins, accounting for 30.8% of the total proteins identified, represent a large part of the most abundant proteins. We speculate that many of them should be biologically modified. The versatility of modification might reflect the need for its growth functions.

Hypothetical and Conserved Hypothetical Proteins Identified as Abundant Proteins—Ninety-five proteins annotated as “hypothetical protein” including 38 conserved hypothetical protein and 57 proteins with unknown functions were identified that should be considered as real proteins. The presence of these proteins is interesting because up to now no function has been assigned to them. Proteome studies focusing on the presence of these proteins during growth phase, stress, or other processes may give insight into their cellular roles (35).

Proteomes of B. longum Strain NCC2705 Grown on Fructose and Glucose—Bifidobacterium spp. can survive and persist in a competitive environment of the human gastrointestinal tract. Several studies have proved that bifidobacteria are able to ferment fructose-containing polymers in particular the short chains of β-2→1-linked fructosyl units (6–10). Although analyses of fructose fermentation patterns revealed that B. longum could use β-fructose as a sole carbon source, the events following the uptake of fructose into the cell and proteins regulated by fructose remained to be defined.

To identify the catabolic route allowing β-fructose fermentation, we first compared the proteomic profile of B. longum strain NCC2705 grown on fructose or glucose. Their 2-DE patterns are highly similar, and many landmark spots have counterparts. In comparison with glucose catabolism, the enzymes of bifid shunt in fructose catabolism were identified in this study, indicating that intracellular fructose and glucose are catabolized via the same degradation pathway. However, we observed a greater than 3-fold variation for the 18 identified proteins (Table II and Fig. 7A). These spots showed clear differences in intensity or position possibly caused by amino acid exchanges and/or small deletions. The possibility that some of the variants are due to different post-translational modifications cannot be excluded. To investigate the involvement of the carbohydrate substrate on expressed genes and the encoded proteins, we cultivated B. longum NCC2705 in modified Garches medium containing either β-glucose (2 g/liter) or fructose (2 g/liter) as the sole carbon source. Total RNAs were used as the template for expression analysis of interesting genes by RT-PCR (Fig. 7B). Comparison with an
internal control (16 S rDNA) provided a semiquantitative measurement of the level of these genes expression. The intensities of the bands provided an approximation of their relative abundance. Overall the results of RT-PCR confirmed up- and down-regulation in the transcription level of interesting genes in cells grown on fructose and glucose, which were consistent with their protein expression.

Interestingly one striking difference was that a sugar-binding protein specific to fructose (BL0033, probable solute-binding protein of ABC transporter system possibly for sugars) had a higher expression level in the cells grown on fructose than on glucose. In comparison with glucose catabolism, the intensity of this protein increased more than 10-fold. Five isoforms differing in charge were identified by MALDI-TOF and ESI-MS/MS (see Supplemental Table 3). In addition, isolated RNAs from cells grown in the presence of fructose and glucose were used as the templates for BL0033 expression analyses by RT-PCR. The results showed a significant quantity of expression of BL0033 mRNA (Fig. 7, A and B) in fructose catabolism.

As for proteins in phosphorylation of sugar, we identified a possible fructokinase (Frk, BL1339, protein in PtkB family of sugar kinases) responsible for the incorporation of intracellular fructose in two culture patterns. At the same time, the results of semiquantitative RT-PCR measurement demonstrated a higher level of frk transcription in fructose medium, suggesting that frk expression is subject to glucose-mediated repression and/or fructose induction. Our results agree with that of Caescu et al. (10), who suggest that fructokinase is the enzyme that is necessary and sufficient for the assimilation of fructose into this catabolic route in B. longum A10C.

Unfortunately we failed to identify glucokinase (glkA, EC 2.7.1.2) in cells grown on fructose or glucose. The RT-PCR results also showed a lower level of glkA transcription in cells grown on fructose or/and on glucose (data not shown). Proteins involved in phosphorylation of glucose are often regulators of catabolism of certain carbon (energy) sources. The catabolite repression of genes has already been reported for Bifidobacterium spp. (60). Thus, we thought GlkA was a low abundance regulator of sugar catabolism in B. longum NCC2705. Interestingly we identified a second gene putatively encoding a glucokinase (glk, EC 2.7.1.2) located 1.5 kb upstream of the frk locus in B. longum NCC2705 grown on fructose or glucose; the two genes (glk and frk) are simply separated by the gene encoding a putative regulator of the ROK (Repressor, ORF, Kinase) family. Thus, we deduced there were two glucokinases (GlkA and Glk) responsible for glucose phosphorylation, and an Frk was responsible for fructose phosphorylation in B. longum NCC2705.

Further Investigation of BL0033—At present, no studies on BL0033 have been reported. To further investigate the expression of BL0033, we compared the proteomes and analyzed the transcription level of cells grown on fructose or glucose after 8 h (early exponential phase), 13 h (midexponential phase), and 16 h (end of exponential phase) according to the bacterial growth curves. The ratios of BL0033 to 16 S rDNA (RT-PCR band intensities, in arbitrary units) were determined as a function of the carbohydrate substrate and time of growth. As depicted in Fig. 8, A and B, the effect of fermentation time on expression of BL0033 was very clear. We observed a consistent up-regulation of intensity for these proteins in 2-D maps with time of growth; the volumes (%) of these spots were 13.9492% (8 h), 15.0830% (13 h), and 18.2231% (16 h). These experiments demonstrate a significantly higher level of BL0033 transcription and expression in cells grown on fructose than in those grown on glucose.

We then performed concentration series experiments with B. longum NCC2705 cells grown in modified Garches medium supplemented with fructose containing 1, 2, 3, or 4 g/liter, respectively. With increasing concentration of fructose, we observed a consistent up-regulation for these proteins in 2-D maps; the volumes (%) of these spots were 11.1889% (at 1 g/liter), 13.2891% (at 2 g/liter), 15.1148% (at 3 g/liter), and 18.0024% (at 4 g/liter) (Fig. 9A). At the same time, an obvious increase of expression quantities of BL0033 was measured by RT-PCR (Fig. 9B). The sequence of BL0033 has a periplasmic sugar binding motif (PFAM PF00532), suggesting that it is sugar-responsive. These data strongly demonstrate that BL0033 is induced by fructose and plays a key role in the transport pathway of fructose.

Sugar can cross the cytoplasmic membrane by three different mechanisms: passive diffusion, facilitated diffusion, or active transport. In addition, several active transport mecha-
nisms in cells allow sugar incorporation. 1) Carbohydrates can be transported by the PEP:sugar PTS, which is involved in both translocation and phosphorylation of carbohydrate. 2) Sugars can be carried over by an ATP-driven system where transport is driven by energy released from the hydrolysis of high energy phosphate bonds. 3) Saccharides can be transported by metal symport or proton symport, which couples substrate translocation with the incorporation of a proton or cation. In bifidobacteria, only two transport systems have been characterized, a PEP:glucose PTS (61) and a lactose symport (62). Although the ability of \textit{Bifidobacterium} spp. to grow on fructose as a unique carbon source has been demonstrated (40), the enzyme(s) needed to incorporate fructose into a catabolic pathway has hitherto not been defined. A bioinformatic analysis of \textit{B. longum} genome sequences revealed neither a fructose-specific PEP-PTS, which would deliver phosphorylated fructose to the cell, nor a complete sucrose utilization gene cluster. However, fascinatingly we can conclude that the protein encoded by BL0033 may play an important role in the transport pathway of fructose and is induced by fructose. These results were exiting and significant because little was known about the mechanism of fructose transport in \textit{B. longum} NCC2705. These findings may document that fructose was not carried over by a PEP:fructose PTS. The uptake of fructose into the cell probably involves a specific transport system.

Surprisingly another ORF, which encodes an ATP-binding protein of an ABC transporter (BL0034) and flanks downstream of BL0033 in the NCC2705 genome, showed slight up-regulation in cells grown on fructose compared with glucose. We speculate that BL0033 and BL0034 are responsible for fructose uptake together in \textit{B. longum} NCC2705. The mechanism of regulation of fructose translocation and the increase of fructose uptake still need to be characterized.

\textbf{Conclusions—}Bifidobacteria are predominant commensal bacteria in the intestinal microflora and exert various beneficial effects on human health. Hence they are widely used as active ingredients in functional dairy-based products. Although bifidobacteria have been studied for over a century, a lack of genetic tools and uniformity among studies have pre-
vented a comprehensive and coherent view of their biosynthetic capabilities. Our proteome analysis of *B. longum* NCC2705 extends previous studies in regard to the physiological characteristics, supports the hypothesis formulated by Schell et al. (2) of the adaptation to the human gastrointestinal tract, and more importantly confirms the expression of a large number of proteins related to their habitat (2).

In this study, reference maps of *B. longum* with variable pH gradients (such as pH 3–10, pH 4–7, pH 4.5–5.5, and pH 4–5) were constructed. A total of 899 spots were processed, and 708 spots representing 369 protein entries were identified by MALDI-TOF-MS and/or ESI-MS/MS. We described a significant number of cellular pathways (a global view of glycolysis and energy metabolism, amino acid metabolism, peptidoglycan precursor assembly, and nucleotide metabolism) related to important physiological processes at the proteome level. At the same time, CAI, GRAVY value of each protein identified, and high abundance proteins also were analyzed. The most abundant proteins include energy metabolism components, periplasmic ABC transporter proteins, and fatty acid and phospholipid metabolism enzymes, and other proteins involved in protein synthesis and cellular processes represent a large proportion. Ninety-five hypothetical proteins were experimentally identified. Maps and information about all identified proteins, which will be helpful for subsequent physiological studies of *B. longum*, are available at www.mpib-berlin.mpg.de/2D-PAGE and www.proteomics.cn.

We also compared the proteomic profile of *B. longum* strain NCC2705 grown on fructose or glucose; results indicated that intracellular fructose and glucose are catabolized via the same degradation pathway. However, sugar-binding proteins specific to fructose (BL0033) had a 10-fold higher expression level in cells grown on fructose than on glucose. Five BL0033 isoforms differing in charge were identified by MALDI-TOF and ESI-MS/MS. We can conclude that the protein encoded by BL0033 is induced by fructose and may play an important role in the transport pathway of fructose. Moreover an ATP-binding protein of an ABC transporter (BL0034) was slightly up-regulated in cells grown on fructose than on glucose. We imagine that BL0033 and BL0034 together play a role in fructose uptake in *B. longum* NCC2705. These results were exciting and significant; little was previously known about the mechanism of fructose transport in *B. longum* NCC2705. These findings may document that fructose was not carried over the cytoplasmic membrane by a PEP:fructose PTS. The uptake of fructose into the cells most likely occurs via a specific transport system. Furthermore the expressed quantities of *frk* and Frk had significantly increased under these conditions, suggesting that *frk* expression is subject to glucose-mediated repression and/or fructose induction.

Acknowledgments—We are indebted to Nestle Research Center for kindly providing *B. longum* strain NCC2705 and helpful information. We are grateful to Peter R. Jungblut for advice on compiling this paper and Xiangyang Dong for technical assistance and helpful discussions.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

REFERENCES

1. Bezkorovainy, A. (2001) Probiotics: determinants of survival and growth in the gut. *Am. J. Clin. Nutr.* 73, 399S–405S

2. Schell, M. A., Karmirantzou, M., Snel, B., Vilanova, D., Berger, B., Pessi, G., Zwahlen, M. C., Desiere, F., Bork, P., Delley, M., Pridmore, R. D., and Arigoni, F. (2002) The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. *Proc. Natl. Acad. Sci. U. S. A.* 99, 14422–14427

3. Ishibashi, N., and Yamazaki, S. (2001) Probiotics and safety, *Am. J. Clin. Nutr.* 73, 465S–470S

4. Gagnon, M., Kheale, E. E., Lee, B. G., and Fliss, I. (2004) In vitro inhibition of Escherichia coli O157:H7 by bifidobacterial strains of human origin. *Int. J. Food Microbiol.* 92, 69–78

5. Yin, X., Chambers, J. R., Barlow, K., Park, A. S., and Wheatcroft, R. (2005) The gene encoding xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (xfp) is conserved among *Bifidobacterium* species within a more variable region of the genome and both are useful for strain identification. *FEMS Microbiol. Lett.* 246, 251–257

6. Ehrmann, M. A., Korakli, M., and Vogel, R. F. (2003) Identification of the gene for beta-fructofuranosidase of *Bifidobacterium lactis* DSM10140(T) and characterization of the enzyme expressed in Escherichia coli. *Curr. Microbiol.* 46, 391–397

7. Imamura, L., Hisamitsu, K., and Kobashi, K. (1994) Purification and characterization of p-fructofuranosidase from *Bifidobacterium infantis*. *Biol. Pharm. Bull.* 17, 596–602

8. Margolies, A., and de los Reyes-Gavilan, C. G. (2003) Purification and functional characterization of a novel α-L-arabinofuranosidase from *Bifidobacterium bifidum* B667. *Appl. Environ. Microbiol.* 69, 5096–5103

9. Moller, P. L., Jorgensen, F., Hansen, O. C., Madsen, S. M., and Stoogad, P. (2001) Intra- and extracellular β-galactosidases from *Bifidobacterium bifidum* and *B. infantis*: molecular cloning, heterologous expression, and comparative characterization. *Appl. Environ. Microbiol.* 67, 2276–2283

10. Caesu, C. I., Vidal, O., Krzewinski, F., Artene, V., and Bouquelet, S. (2004) Bifidobacterium longum requires a fructokinase (Frk; ATP:p-fructose 6-phosphotransferase, EC 2.7.1.4) for fructose catabolism. *J. Bacteriol.* 186, 6515–6525

11. Veerkamp, J. H. (1969) Catabolism of glucose and derivatives of 2-deoxy-2-amino-glucose in *Bifidobacterium bifidum* var. *pennsylvanicus*. *Arch. Biochem. Biophys.* 129, 257–263

12. de, V. W., Gerbrandy, S. J., and Stouthamer, A. H. (1967) Carbohydrate metabolism in *Bifidobacterium bifidum* and *B. infantis*: molecular cloning, heterologous expression, and comparative characterization. *Appl. Environ. Microbiol.* 57, 415–425

13. Link, A. J., Robison, K., and Church, G. M. (1997) Comparing the predicted and observed properties of proteins encoded in the genome of *Escherichia coli* K-12. *Electrophoresis* 18, 1259–1313

14. Cash, P. (2003) Proteomics in medical microbiology. *Electrophoresis* 24, 1187–1201

15. Fountoulakis, M., Takacs, B., and Langen, H. (1998) Two-dimensional map of basic proteins of *Haemophilus influenzae*. *Electrophoresis* 19, 761–766

16. Fountoulakis, M., Juravvivle, J. F., Roder, D., Evers, S., Berndt, P., and Langen, H. (1998) Reference map of the low molecular mass proteins of *Bacteroides thetaiotaomicron*.
Haemophilus influenzae. *Electrophoresis* 19, 1819–1827

17. Fontoulakis, M., Langen, H., Evers, S., Gray, C., and Takacs, B. (1997) Two-dimensional map of Haemophilus influenzae following protein enrichment by heparin chromatography. *Electrophoresis* 18, 1193–1202

18. Cash, P., Argo, E., and Bruce, K. D. (1995) Characterisation of Haemophilus influenzae proteins by two-dimensional gel electrophoresis. *Electrophoresis* 16, 135–148

19. Rosenkranz, I., King, A., Wellingh, K., Moniante, M., Moertz, E., and Andersen, P. (2000) Towards the proteome of Mycobacterium tuberculosis. *Electrophoresis* 21, 3740–3756

20. Schmidt, F., Donahoe, S., Hagens, K., Mattow, J., Schaible, U. E., Kaufmann, S. H., Aebersold, R., and Jungblut, P. R. (2004) Complementary analysis of the Mycobacterium tuberculosis proteome by two-dimensional electrophoresis and iso-coded affinity tag technology. *Mol. Cell. Proteomics* 3, 21–42

21. Sonnenberg, M. G., and Belisle, J. T. (1997) Definition of Mycobacterium tuberculosis culture filtrate proteins by two-dimensional polyacrylamide gel electrophoresis, N-terminal amino acid sequencing, and electrospray mass spectrometry. *Infect. Immun.* 65, 4515–4524

22. Pasquali, C., Frutiger, S., Wilkins, M. R., Hughes, G. J., Appel, R. D., Bairoch, A., Schaller, D., Sanchez, J. C., and Hochstrasser, D. F. (1996) Two-dimensional gel electrophoresis of Escherichia coli homogenates: the Escherichia coli SWISS-2DPAGE database. *Electrophoresis* 17, 547–555

23. VanBogelen, R. A., Sankar, P., Clark, R. L., Bogan, J. A., and Neidhardt, F. C. (1992) The gene-protein database of *Escherichia coli*: edition 5. *Electrophoresis* 13, 1014–1054

24. VanBogelen, R. A., Abshire, K. Z., Moldover, B., Olson, E. R., and Neidhardt, F. C. (1997) Escherichia coli proteome analysis using the gene-protein database. *Electrophoresis* 18, 1243–1251

25. Bumann, D., Meyer, T. F., and Jungblut, P. R. (2001) Proteome analysis of the common human pathogen Helicobacter pylori. *Proteomics* 1, 473–479

26. McAtee, C. P., Lim, M. Y., Fung, K., Velligan, M., Fry, K., Chow, T., and Berg, D. E. (1998) Identification of potential diagnostic and vaccine candidates of Helicobacter pylori by two-dimensional gel electrophoresis, sequence analysis, and serum profiling. *Clin. Diagn. Lab. Immunol.* 5, 537–542

27. Bernhardt, J., Volker, U., Volker, A., Antelmann, H., Schmid, R., Mach, H., and Hecker, M. (1997) Specific and general stress proteins in *Bacillus subtilis*—a two-dimensional protein electrophoresis study. *Microbiology* 143, 999–1017

28. Buttner, K., Bernhardt, J., Scharf, C., Schmid, R., Mader, U., Eymann, C., Antelmann, H., Volker, A., and Hecker, M. (2001) A comprehensive two-dimensional map of cytosolic proteins of *Bacillus subtilis*. *Electrophoresis* 22, 2908–2935

29. Regula, J. T., Ueberle, B., Boguth, G., Gorg, A., Schnolzer, M., Herrmann, K., and Frank, R. (2000) Towards a two-dimensional proteome map of *Mycobacterium pneumoniae*. *Electrophoresis* 21, 3765–3780

30. Drews, O., Weiss, W., Rel, G., Parlar, H., Wait, R., and Gorg, A. (2000) High protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. *Humangenetik* 26, 231–243

31. Drews, O., Weiss, W., and Sharp, M. (1960) A medium for the cultivation of *Lactobacillus* sp. *J. Appl. Bacteriol.* 23, 130–135

32. Klose, J. (1975) Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. *Humangenetik* 26, 231–243

33. Teixeira-Gomes, A. P., Cloeckaert, A., Bezard, G., Dubray, G., and Zygmont, M. S. (1997) Mapping and identification of Brucella melitensis proteins by two-dimensional electrophoresis and mass sequencing. *Electrophoresis* 18, 156–162

34. Krezwinski, F., Brassart, C., Gavini, F., and Bouquelet, S. (1997) Glucose and galactose transport in *Bifidobacterium bifidum* DSM 20082. *Curr. Microbiol.* 35, 175–179

35. Pearson, W. R., and Lipman, D. J. (1988) Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. U. S. A.* 85, 2444–2448

36. Racker, E. (1962) Fructose-6-phosphate phosphoketolase from *Acetobacter xylinum*. *Methods Enzymol.* 5, 276–280

37. Matsuki, T., Watanabe, K., Tanaka, R., Fukuda, M., and Oyaizu, H. (1999) Distribution of bifidobacterial species in human intestinal microflora examined with 16S rRNA-gene-targeted species-specific primers. *Appl. Environ. Microbiol.* 65, 4506–4512

38. Desjardin, L. E., Hayes, L. G., Sohaskey, C. D., Wayne, L. G., and Eisenach, K. D. (2001) Microaerophilic induction of the α-crystallin chaperone protein homologue (hspX) mRNA of *Mycobacterium tuberculosis*. *J. Bacteriol.* 183, 2929–2936

39. Hurdado, C. A., and Rachubinski, R. A. (2002) Isolation and characterization of YIBEM1, a gene required for cell polarization and differentiation in the dimorphic yeast *Yarrowia lipolytica*. *Eukaryot. Cell* 1, 526–537

40. Antelmann, H., Yamamoto, H., Sekiguchi, J., and Hecker, M. (2002) Stabilization of cell wall proteins in *Bacillus subtilis*: a proteomic approach. *Proteomics* 2, 591–602

41. Buttny, K., Bernhardt, J., Scharf, C., Schmid, R., Mader, U., Eymann, C., Antelmann, H., Volker, A., and Hecker, M. (2001) A comprehensive two-dimensional map of cytosolic proteins of *Bacillus subtilis*. *Electrophoresis* 22, 2908–2935

42. Regula, J. T., Ueberle, B., Boguth, G., Gorg, A., Schnolzer, M., Herrmann, R., and Frank, R. (2000) Towards a two-dimensional proteome map of *Mycobacterium pneumoniae*. *Electrophoresis* 21, 3765–3780

43. Drews, O., Weiss, W., Rel, G., Parlar, H., Wait, R., and Gorg, A. (2002) High pressure effects step-wise altered protein expression in *Lactobacillus sanfranciscensis*. *Proteomics* 2, 765–774

44. Drews, O., Rel, G., Parlar, H., and Gorg, A. (2004) Setting up standards and a reference map for the alkaline proteome of the Gram-positive bacterium *Lactococcus lactis*. *Proteomics* 4, 1293–1304

45. Anglade, P., Demey, E., Labas, V., Le Caer, J. P., and Chich, J. F. (2000) Towards a proteomic map of *Lactococcus lactis* NCDO 763. *Electrophoresis* 21, 2546–2549

46. Guillot, A., Gitton, C., Anglade, P., and Mistou, M. Y. (2003) Proteomic analysis of *Lactococcus lactis*, a lactic acid bacterium. *Proteomics* 3, 337–354

47. Liao, X., Ying, T., Wang, H., Wang, J., Shi, Z., Feng, E., Wei, K., Wang, Y., Zhang, X., Huang, L., Su, G., and Huang, P. (2003) A two-dimensional proteome map of *Shigella flexneri*. *Electrophoresis* 24, 2864–2882

48. Rosen, R., Sacher, A., Shechter, N., Becher, D., Buttner, K., Biran, D., Hecker, M., and Ron, E. Z. (2004) Two-dimensional reference map of Agrobacterium tumefaciens proteins. *Proteomics* 4, 1061–1073

49. Coelho, A., de Oliveira, S. E., Faria, M. L., de Carvalho, D. P., Soares, M. R., von Kruger, W. M., and Bisch, P. M. (2004) A proteome reference map for Vibrio cholerae El Tor. *Proteomics* 4, 1491–1504

50. Teixeira-Gomes, A. P., Cloeckaert, A., Bezard, G., Dubray, G., and Zygmont, M. S. (1997) Mapping and identification of Brucella melitensis proteins by two-dimensional electrophoresis and mass sequencing. *Electrophoresis* 18, 156–162

51. Vitali, B., Wasinger, V., Brigidi, P., and Guilhaum, M. (2005) A proteomic view of Bifidobacterium infantis generated by multi-dimensional chromatography coupled with tandem mass spectrometry. *Proteomics* 5, 1859–1867
Proteomic Analysis of *B. longum* NCC2705

A., Mann, M., Jeno, P., and Boucherie, H. (1999) Two-dimensional gel protein database of *Saccharomyces cerevisiae* (update 1999). *Electrophoresis* 20, 2280–2298

60. Trindade, M. I., Abratt, V. R., and Reid, S. J. (2003) Induction of sucrose utilization genes from *Bifidobacterium lactis* by sucrose and raffinose. *Appl. Environ. Microbiol.* 69, 24–32

61. Degnan, B. A., and Macfarlane, G. T. (1993) Transport and metabolism of glucose and arabinose in *Bifidobacterium breve*. *Arch. Microbiol.* 160, 144–151

62. Krzewinski, F., Brassart, C., Gavini, F., and Bouquelet, S. (1996) Characterization of the lactose transport system in the strain *Bifidobacterium bifidum DSM 20082*. *Curr. Microbiol.* 32, 301–307