Molecular Characterization of \textit{hsp20}, Encoding a Small Heat Shock Protein of \textit{Bifidobacterium breve} UCC2003\textsuperscript{V}\textsuperscript{†}

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Small heat shock proteins (sHSPs) are members of a diverse family of stress proteins that are important in cells to protect proteins under stressful conditions. Genome analysis of \textit{Bifidobacterium breve} UCC2003 revealed a single sHSP-encoding gene, which was classified as \textit{hsp20} gene by comparative analyses. Genomic surveillance of available genome sequences indicated that \textit{hsp20} homologs are not widely distributed in bacteria. In members of the genus \textit{Bifidobacterium}, this gene appears to be present in only 7 of the 30 currently described species. Moreover, phylogenetic analysis using all available bacterial and eukaryotic sHSP sequences revealed a close relationship between bifidobacterial HSP20 and the class B sHSPs found in members of the division \textit{Firmicutes}. The results of this comparative analysis and variation in codon usage content suggest that \textit{hsp20} was acquired by certain bifidobacteria through horizontal gene transfer. Analysis by slot blot, Northern blot, and primer extension experiments showed that transcription of \textit{hsp20} is strongly induced in response to severe heat shock regimens and by osmotic shock.

Bifidobacteria are considered one of the most common inhabitants of the distal tracts of the intestines of mammals. In humans, their presence in the colon is often associated with beneficial health effects (15). For this reason, various bifidobacterial strains are considered to be probiotic, often being added as viable bacteria into dairy products (e.g., yoghurt) or supplied in infant foods. However, despite the generally accepted importance of bifidobacteria as probiotic components of the human intestinal microflora, relatively little is known about their physiology, phylogenetic relationships, and underlying genetics (31).

The incorporation of bifidobacteria in such products requires that they survive industrial food manufacturing processes, such as starter handling and storage (freeze-drying, freezing, or spray-drying), while remaining viable during storage. This reinforces the need for robust bifidobacteria that also survive passage through the upper part of the digestive tract, while being able to compete with resident intestinal flora, preferably colonize the digestive tract and express specific functions under what probably are suboptimal growth conditions. However, bifidobacteria are subjected to stressful environmental challenges not only during industrial processes but also under natural conditions where their ability to quickly respond to stress is essential for survival.

In recent years, several genes have been identified (for example, \textit{groESL}, \textit{dnaK}, \textit{clpB}, \textit{clpC}, \textit{dnaJ2}, and \textit{clpP}) whose expression is induced upon exposure of bifidobacterial cultures to stressful conditions, particularly heat shock (reviewed in reference 30). The expression of the \textit{dnaK} operon and \textit{clpB} is induced by osmotic shock and severe heat stress, but not by moderate heat stress (33, 34), suggesting an overlap between the osmotic shock and severe heat shock regulons. Conversely, maximal transcription of various other heat stress-induced genes, such as \textit{groESL} and \textit{clpC}, occurs upon moderate heat shock regimens but is not induced by severe heat or osmotic stress (27, 32).

All the above-mentioned heat shock protein (HSP)-encoding genes are known to be involved in diverse processes, such as protein folding and protein turnover (for a review, see reference 30), and such functions appear to be highly conserved between prokaryotes and eukaryotes (2). Small heat shock proteins (sHSPs) are also important for an appropriate response to heat stress because of their capacity to prevent irreversible protein denaturation of heat-damaged proteins (13). This protein family is characterized by their low molecular mass, ranging between 12 and 30 kDa, and unlike large HSPs, the sHSPs are highly divergent in their primary sequence except for the presence of a conserved \(\alpha\)-crystallin domain (30). Furthermore, in contrast to the larger HSPs, which are usually active as well-defined oligomers, the sHSPs from different organisms exist as oligomers that vary in size and shape with some of them even displaying polydispersity (9, 13). According to the present model, sHSPs act as ATP-independent chaperones by binding to denatured proteins that have accumulated under stressful conditions, thereby maintaining them in a folding-competent state (5, 11). \(\alpha\)-Crystallin-containing sHSPs are widely distributed among all kingdoms from bacteria to animals. However, not all organisms have sHSP genes. In the case of bacteria, sHSPs may be absent in some species, e.g., \textit{Mycoplasma genitalium}, while in other species only a small number of sHSP-encoding genes are present, such as in the case of \textit{Escherichia coli}, which specifies just two.
types of sHSPs (13). In contrast, rhizobia possess a large set of genes specifying sHSPs (12).

In this report we describe the genetic characterization of the Bifidobacterium breve UCC2003 hsp20 gene, representing the first characterized sHSP-encoding gene in the genus Bifidobacterium.

Sequence analysis of B. breve UCC2003 hsp20 locus. The B. breve UCC2003 genome sequence (S. Leahy, M. O’Connell-Motherway, J. A. Moreno Munoz, D. Higgins, G. F. Fitzgerald, and D. van Sinderen, unpublished data) contains a single gene encoding an sHSP, which was termed hsp20 on the basis of its calculated molecular mass (18.73 kDa). When the deduced amino acid sequence of hsp20 was compared with sequences in the GenBank and EMBL databases, it was found to contain an α-crystallin domain (Fig. 1). Therefore, the B. breve UCC2003 HSP20 belongs to the α-crystallin-type sHSP family. The amino acid sequences of members of the sHSP family do not exhibit high levels of homology, typically seen for the high-molecular-weight HSPs (e.g., DnaK, GroEL, ClpC, and ClpB) (13). Instead, sHSPs display moderate similarity to the α-crystallin domain, while their N and C termini tend to be much more variable (Fig. 1). Notably, a hydropathy profile of the deduced amino acid sequence of B. breve UCC2003 hsp20 revealed a strong hydrophilic region that is a characteristic trait found in the C-terminal end of members of this family protein (AXXXXGXL), located at the C-terminal end of the α-crystallin
FIG. 2. Phylogenetic tree obtained using the sHSP protein sequences. Bootstrap values are reported for a total of 1,000 replicates. The bifidobacterial (Bif.) HSP20 sequences are shown in bold type and grouped with class B. The principal bacterial groups are indicated. Moreover, the sHSPs identified in plants are indicated. The scale bar indicates phylogenetic distances.
domain (3) (Fig. 1). Notably, the α-crystallin domain of the HSP20s of the members of Bifidobacterium longum group contains a domain (PAAEXXXXSDXXCACAS) that is absent in the homologous proteins from other bacteria (Fig. 1). Bioinformatic analyses suggest that this motif is a disordered region, i.e., lacks a fixed three-dimensional (3D) structure. Generally, such disordered regions have diverse functional roles, including cell signaling, molecular recognition, and interactions of protein with nucleic acids (4), and this region in the HSP20 of B. breve UCC2003 as well as in the homologous proteins in the other analyzed bifidobacteria may for example be involved in dimerization.

**Structural investigation of HSP20 from B. breve UCC2003.** In order to corroborate these findings, a structural investigation of B. breve UCC2003 HSP20 was performed. Since B. breve UCC2003 HSP20 exhibits the highest level of sequence identity (25%) to the *Triticum* HSP16.9 sequence (PDB entry 1gme), this protein was selected to serve as the template for homology modeling. A theoretical monomeric structure for B. breve UCC2003 was built via homology modeling from FFAS (16), and the 3D model of B. breve HSP20 was obtained via the SWISS-model server (22). The predicted B. breve UCC2003 HSP20 monomer is composed of a complete α-crystallin domain flanked by a variable hydrophobic N-terminal region and a short C-terminal extension. The α-crystallin domain consists of a β-sandwich comprised of two antiparallel β-sheets, followed by a short C-terminal extension and a long loop extending from the β-sandwich (see Fig. S1 in the supplemental material). The structural prediction of HSP20 highlights the amino acid residues that in homologous proteins have been experimentally demonstrated to have essential roles within the substrate binding site. Molecular chaperones bind to exposed hydrophobic patches on denatured protein substrates using various strategies to provide hydrophobic binding sites (17). The sHSP chaperone mechanism appears to involve temperature-regulated exposure of hydrophobic binding sites and the formation of large sHSP-substrate complexes (1, 11). Two putative binding sites were identified in HSP16.9 (25). The equivalent positions for these two putative binding sites in the HSP20 of B. breve UCC2003 are located at the C-terminal hydrophobic groove in the α-crystallin domain that is represented by the conserved amino acid residues Ile163 and Ile165 (Fig. 1) and at the N-terminal arm, which corresponds to Asp44 (Fig. 1). Compared to the 3D structure of *Triticum* HSP16.9, the predicted 3D model of B. breve UCC2003 HSP20 possesses a longer dimerization loop, represented by amino acid residues 84 to 122. The amino acid residues that have been shown to play a crucial role in the dimerization of HSP16.9 (14, 25) are located at an equivalent position, i.e., Glu114 and Arg122, in HSP20 of B. breve UCC2003, which supports its presumptive function as the bifidobacterial HSP20 dimerization domain.

**Phylogenetic analyses of bifidobacterial HSP20.** In order to assess the distribution of *hsp20* homologs across bacteria, we surveyed currently available genomic data, representing prokaryotes and eukaryotes, for the presence of sHSP-encoding genes. The results of such an analysis revealed that sHSP-encoding genes are not uniformly present in all bacteria studied so far. The distribution of sHSP-encoding genes and in particular *hsp20* is expected to be a consequence of an organism’s evolutionary history of acquiring *hsp20* through either a vertical or horizontal transfer mechanism.

Alignment of the α-crystallin domain of identified sHPSs was performed using ClustalW and resulted in an unrooted neighbor-joining phylogenetic tree (Fig. 2). sHSP class A and class B clustered separately, suggesting that these sHSP subfamilies may either have diverged after being evolved from one ancestral gene or have formed by convergent evolution from more than one ancestral gene (Fig. 2). The bifidobacterial HSP20 clusters together with all classical sHSPs from class B, indicating that the bifidobacterial HSP20 exists exclusively as monodisperse oligomers, i.e., the protein exists simultaneously as oligomers of the same size, as previously reported for many of the other protein members of the same phylogenetic cluster (6, 8). Notably, bifidobacterial HSP20 does not form a monophyletic group within the sHSPs from other high-G+C gram-positive bacteria; instead, HSP20 branches together with the sHSP sequences of members of the division Firmicutes (Fig. 2). This phylogenetic inconsistency of the hsp20 gene product can be explained by assuming that the bifidobacterial *hsp20* gene has evolved from a Firmicutes hsp20 ancestor via lateral gene transfer. Other findings that strengthen this hypothesis are represented by the lower G+C% content of the bifidobacterial *hsp20* genes compared to the average of their genome sequences (e.g., between 2.66% to 3.59% lower than the G+C average value) and by the different codon usage bias for the *hsp20* gene (data not shown). In fact, factorial correspondence analysis of codon usage of bifidobacterial open reading frames (ORFs), which was performed according to previous reports (7), displayed a different codon usage bias for the *hsp20* gene (data not shown). All together, these data point to the possibility that the bifidobacterial *hsp20* gene was acquired by horizontal gene transfer.

**Conservation of the hsp20 gene across Bifidobacterium genomes.** The structural organization and location of the *hsp20* locus in the chromosome of *B. breve* UCC2003 and other bacteria is schematically displayed in Fig. 3. This comparative analysis shows that the most similar protein to the predicted *B. breve* HSP20 is the assumed homologous protein from *B. longum* (20) and from *Bifidobacterium adolescentis* (NCBI accession number NC_008618), while homology levels of ≥34% were still observed between the predicted *B. breve* HSP20 protein and the HSP20 protein of much less related low-G+C gram-positive bacteria. At the DNA level also, significant sequence homology was still detectable between the various bifidobacterial HSP20-encoding genes, in contrast to the flanking ORFs, which were shown to be variable (Fig. 3). The high variability of the *hsp20* locus is also confirmed by the high variability of the currently sequenced *hsp20* sequences within the genus *Bifidobacterium*. Using a PCR strategy employing two primers Hsp20-uni-univ (5′-GAGGGTTGGCCGCAACA-3′) and Hsp20-rev-univ (5′-GTAGCTGGCGTGATG-3′) based on two conserved regions (positions 66 to 82 and positions 401 to 417) from the *B. breve* UCC2003 *hsp20* gene, we were able to amplify this gene in only three bifidobacterial species, which include *B. longum* biotype longum, *B. breve*, and *B. adolescentis*. Thus, in order to determine whether other bifidobacteria also contain *hsp20* homologs, amplified *B. breve* UCC2003 *hsp20* DNA was hybridized to genomic DNA from 34 bifidobacterial strains representative of 30 different species following a slot...
blot hybridization procedure (18) (Fig. 4a). Interestingly, using different hybridization stringency conditions, only *B. breve*, *B. longum* biotype longum, *B. longum* biotype infantis, *B. adolescentis*, *Bifidobacterium bifidum*, and *Bifidobacterium catenulatum* yielded a positive hybridization signal (Fig. 4a). This clearly suggests that homologs of the *hsp20* gene are not widely distributed in bifidobacterial genomes. These findings are similar to those described in other bacterial species where the distribution of sHSP-encoding genes is not uniformly present and is apparently restricted to a few species within the same genus (13).

Many species of bacteria contain several genes encoding sHSPs (12, 13). To determine whether the members of the genus *Bifidobacterium* also contain multiple copies of the *hsp20* gene, the amplified *hsp20* DNA, p-*hsp20*, was hybridized to genomic DNA digested with EcoRI or EcoRV of *B. breve*, *B. longum* biotype longum, *B. longum* biotype infantis, *B. adolescentis*, *Bifidobacterium bifidum*, and *Bifidobacterium catenulatum* by the method of Sambrook et al. (18). All investigated bifidobacteria yielded one single band of different sizes in all strains tested (Fig. 4), suggesting that only a single copy of the *hsp20* gene is present in these genomes.

**Transcriptional analysis of *hsp20***. In bifidobacteria, transcription of many stress genes is known to be induced by heat or osmotic shock (27, 28, 32–35). In order to determine whether the *B. breve* UCC2003 *hsp20* gene is induced following heat or osmotic shock, a slot blot hybridization procedure was employed. The mRNA used in these experiments was isolated from *B. breve* UCC2003 cultures grown for different lengths of time at temperatures ranging from 37°C to 50°C (Fig. 5) or in medium containing NaCl at a concentration of 0.7 M, which was previously shown to be the most effective concentration to induce bifidobacterial chaperones (33, 34) (Fig. 5). RNA isolation and slot blot hybridizations were performed by the method of Ventura et al. (26).

Based on the intensity of the hybridization signal, the highest expression level of the *hsp20* gene was shown to occur following exposure to 50°C for 150 min or incubation for 150 min in a medium containing 0.7 M NaCl (Fig. 5), conditions which increased *hsp20* mRNA levels approximately 28- and 25-fold, respectively (Fig. 5b). These levels of induction were significantly higher (from 3- to 10-fold) than those observed after 25 or 50 min of incubation at 50°C or in a medium containing 0.7 M NaCl (Fig. 5a). Notably, the experimental procedure used in
this study was identical to that applied in previous studies describing heat or osmotic induction of chaperone-encoding proteins (27, 28, 32–35). The observed level of hsp20 induction was higher than those of the clpB and dnaK genes (28, 29) and therefore represents the highest induction level of any stress-induced gene in bifidobacteria described thus far (30), suggesting that hsp20 is a major player in coping with heat and osmotic stress in bifidobacteria.

The transcriptional regulation of hsp20 was analyzed by Northern blotting using p-hsp20 as a probe. Total mRNA was isolated from B. breve UCC2003 grown at 37°C, following heat shock at 43°C or 50°C or following osmotic shock at 0.7 M NaCl. Northern blot analysis revealed two hybridization signals corresponding to a 750-nucleotide- and 650-nucleotide-long mRNA species, respectively (Fig. 5d), which is the expected size of a monocistronic transcript that would encompass only the hsp20 gene (523 bp). In agreement with the slot blot hybridization results, it was shown that transcription of the hsp20 gene of B. breve UCC2003 was activated following exposure to 50°C or following osmotic stress (i.e., exposure to 0.7 M of NaCl) for 150 min (Fig. 5). Northern analysis of the DNA sequences surrounding the hsp20 gene using RNA extracted from unstressed and heat-shocked cells did not reveal any specific transcripts, indicating that the pldB gene and the hypothetical genes are neither transcriptionally linked to the hsp20 locus nor expressed to a detectable level under the conditions used (Fig. 5d).

Analysis of the nucleotide sequence of the hsp20 locus revealed that the gene was flanked at its 3' end by two inverted repeats (ΔG of −10.7 and −10.6 kcal) that may function as rho-independent transcriptional terminator structures (Fig. 5).

The transcription start site of the hsp20 locus was determined by primer extension analysis using mRNA isolated from cultures grown at 37°C or heat shocked at 43°C or 50°C for 150 min or following osmotic shock at 0.7 M NaCl for 150 min (Fig. 6). Primer extension of the 5' end of the hsp20 mRNA transcript was performed following a protocol described in a previous study (28) and employing the synthetic oligonucleotide hsp20-prom1 (5'-CATCAAAGCGGGAAACATTG-3'). The primer extension result was confirmed using a second primer, hsp20-prom2 (5'-CTCAAGGGTATGTGCCTCTC-3') (data not shown). Multiple transcriptional start points were observed preceding the hsp20 gene using mRNA extracted from cultures subjected to osmotic or heat shock. In fact, three transcription start sites were identified at −81 bp, which corresponds to a very weak extension signal, at −92 bp, and at −117 bp relative to the start site of the coding sequence (Fig. 6a). However, the analysis of the putative promoter regions of the hsp20 gene did not reveal sequences with obvious similarity to the consensus −10 and −35 hexamers, which may suggest that the expression of hsp20 of B. breve 2003 is under the control of an alternative sigma factor (Fig. 6a and b). The upstream region of the hsp20 gene of B. breve UCC2003 and those of B. longum biotype longum NCC 2705 and B. adolescentis ATCC 15703 were aligned in an attempt to identify a putative promoter or regulatory elements. As shown in Fig. 6, no obvious promoter sequence can be deduced from the four aligned sequences, although it was obvious that the promoter region contains various stretches of conserved sequences which could serve as regulatory elements. The ribosomal binding site region and the transcriptional start site(s) were conserved in all bifidobacterial sequences examined.

Conclusions. We identified and characterized an hsp20 homolog in the B. breve UCC2003 genome, and its predicted protein was shown through 3D model prediction and bioinformatic analyses to belong to the α-crystallin or small heat shock chaperone family. Phylogenetic analysis using HSP20 proteins from low- and high-G+C gram-positive bacteria revealed a close and specific relationship between the bifidobacterial HSP20 and homologous proteins in Firmicutes. This finding, taken together with the analysis of their G+C content and the
bias of their codon usage, suggests that the bifidobacterial hsp20 gene was acquired through a horizontal transfer event.

Interestingly, the kinetics of activation of the hsp20 gene was shown to be similar to that of other HSP-encoding genes, such as dnaK or clpB, which are also strongly induced upon exposure to severe heat shock and osmotic stress. Notably, in B. breve UCC2003, the observed level of activation of the hsp20 gene upon heat or osmotic shock being the highest so far reported among chaperone-encoding genes in this microorganism suggests that HSP20 represents an important protein player to cope with heat and osmotic stress in certain bifidobacteria. The sHSP-encoding genes have shown to be induced by heat stress in other bacteria, such as Mycobacterium tuberculosis (10), Deinococcus radiodurans (21), Clostridium

Fig. 5. Transcriptional regulation of the B. breve UCC2003 hsp20 locus. (a) Slot blot hybridization using RNA extracted from cells incubated for up to 150 min at a range of temperatures or in 0.7 M NaCl. (b) Quantitative display of the induction levels of the hsp20 mRNA transcripts. The different degrees of shading of the bars corresponds to the various times (0 to 150 min) for which heat and osmotic shocks were applied. (c) Positions of the transcripts with respect to the hsp20 locus map. The estimated size of the mRNA is indicated in base pairs. Hairpins indicate possible rho-independent terminators. hypoth., hypothetical. (d) Northern blot analysis of B. breve UCC2003 hsp20 locus performed using total mRNA isolated from cultures grown at 37°C, 43°C, or 50°C, and under hyperosmotic conditions using a NaCl concentration of 0.7 M for the time indicated above each lane (1, 0 min; 2, 50 min; 3, 150 min).
acetobutylicum (19), and Streptomyces albus (24). However, the expression pattern of the B. breve UCC2003 hsp20 gene appears to be unique compared to the expression patterns of hsp20 homologs from the above-mentioned bacteria in which sHSP-encoding genes are not induced upon exposure of the cells to osmotic stress (13). Furthermore, slot blot and Southern blot hybridization experiments revealed that the distribution of hsp20 within members of the Bifidobacterium genus is limited to classical human intestinal species, which due to the ecological niche where they live are normally not exposed to significant temperature changes, whereas they may encounter frequent changes in osmotic conditions as a consequence of diet composition. All together, these results suggest that HSP20 in bifidobacteria, in contrast to the previously described sHSPs, has acquired an osmotic functionality as a consequence of the adaptation of bifidobacteria to the human intestinal ecological niche.

Furthermore, some sHSPs, such as the HSP18 of Streptomyces albus or HSP20 of D. radiodurans, are known to be repressed via inverted repeat sequences bound by repressor proteins during normal growth (21, 23, 24). No obvious inverted repeat sequences were found in the putative promoter of the hsp20 gene of B. breve UCC2003. Nevertheless, transcriptional regulation cannot be excluded for the bifidobacterial hsp20 gene, and the lack of obvious similarities to vegetative −35 and −10 hexamers may suggest that an alternative sigma factor is involved in the regulation of this gene in a manner similar to that shown for certain other sHSPs (21). Notably, the genome of B. breve UCC2003 contains two rpoE paralogs coding for a putative σ54, although no data are currently available in the public domain regarding their role in coping with different stress challenges (30). Furthermore, no clear alternative promoter sequences could be identified in the promoter region of the hsp20 gene or in the genome of B. breve UCC2003. Thus, further studies will be necessary in order to verify the possibility of alternative sigma factor regulation of hsp20 and dimerization of the HSP20 protein in bifidobacteria.

Nucleotide sequence accession number. The nucleotide sequence data regarding the hsp20 locus of B. breve UCC2003 have been deposited in GenBank under accession number EF071947.

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