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Genetic Characterization of the *Bifidobacterium breve* UCC 2003 *hrcA* Locus

Marco Ventura,[1,2]* Carlos Canchaya,[1] Valentina Bernini,[2] Antonio Del Casale,[3] Franco Dellaglio,[3] Erasmo Neviani,[2] Gerald F. Fitzgerald,[1] and Douwe van Sinderen[1]

Alimentary Pharmabiotic Centre and Department of Microbiology, Bioscience Institute, National University of Ireland, Western Road, Cork, Ireland[1]; Department of Genetic, Anthropology and Evolution, University of Parma, Parma, Italy[2]; and Dipartimento Scientifico e Tecnologico, University of Verona, Verona, Italy[3]

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The bacterial heat shock response is characterized by the elevated expression of a number of chaperone complexes and transcriptional regulators, including the DnaJ and the HrcA proteins. Genome analysis of *Bifidobacterium breve* UCC 2003 revealed a second copy of a *dnaJ* gene, named $dnaJ_2$, which is flanked by the *hrcA* gene in a genetic constellation that appears to be unique to the actinobacteria. Phylogenetic analysis using 53 bacterial *dnaJ* sequences, including both $dnaJ_1$ and $dnaJ_2$ sequences, suggests that these genes have followed a different evolutionary development. Furthermore, the *B. breve* UCC 2003 $dnaJ_2$ gene seems to be regulated in a manner that is different from that of the previously characterized $dnaJ_1$ gene. The $dnaJ_2$ gene, which was shown to be part of a 2.3-kb bicistronic operon with *hrcA*, was induced by osmotic shock but not significantly by heat stress. This induction pattern is unlike those of other characterized *dnaJ* genes and may be indicative of a unique stress adaptation strategy by this commensal microorganism.

Bifidobacteria have been shown to be the predominant species in the gastrointestinal tract (GIT) of infants and represent the third most numerous species encountered in the colon of adult humans. In the human GIT their presence has been associated with beneficial health effects (8, 13, 16, 21). Despite the generally accepted importance of bifidobacteria as probiotic components of human GIT microflora and their use in health-promoting foods, there is a paucity of information about their physiology, phylogenetic relationship, and underlying genetics (21).

We are interested in studying the stress response in bifidobacteria because probiotic bifidobacteria must resist adverse environmental conditions that are encountered not only during food preparation and storage but also in their natural environment (i.e., the GIT).

Recently, many genes induced upon exposure of bifidobacterial cultures to stressful conditions have been identified (15, 19, 20, 22–24). These include *groELS*, *dnaK*, *clpB*, *clp*, and *clpP* genes. The expression of the *dnaK* operon and *clpB* is induced by osmotic shock and severe heat stress but not by moderate heat stress (22, 24), suggesting an overlap between the osmotic shock and severe heat shock regulons. Interestingly, in bifidobacteria *clpB* and *dnaK* represent the first chaperone-encoding genes to be strongly induced after exposure to very high temperature (ΔT of 13 K). In contrast, maximal transcription of heat stress-induced genes such as *groESL* (20) and *clpC* (23) occurs upon moderate shock regimens (ΔT of 6 K). Notably, transcription of the latter two genes is not induced by osmotic stress (20, 23). Thus, at least two separate regulatory pathways for coping with different types and levels of stress are operating in bifidobacteria. The first pathway corresponds to the HspR regulon that protects cells from protein damage when bifidobacteria are exposed to severe heat and osmotic shocks, while a second pathway regulated by ClgR becomes active when bifidobacterial cells are exposed to moderate heat stress (23).

In *Bacillus subtilis*, in which the heat shock response has been investigated in great detail, the last gene of the *dnaK* operon was shown to encode the repressor of the *dnaK* and *groEL* operons. This genetic constellation also appears to be conserved in other *Firmicutes*. This gene is named *hrcA* (for *h*eat *r*egulation at *C*IRCE) and encodes a protein which binds to CIRCE (for *c*ontrolling *i*nverted *r*epeat [IR] of *c*haperone *e*xpression)-bearing DNA fragments (28). In the main representative genus of the group actinobacteria, *Streptomyces*, neither *hrcA* nor CIRCE is associated with the *dnaK* operon, but in these bacteria the *hrcA* gene is instead associated with a second copy of the *dnaJ* gene, which encodes a molecular chaperone involved in assisting the posttranslation protein folding processes (7).

In this report we describe the genetic characterization of the *Bifidobacterium breve* UCC 2003 *hrcA*-$dnaJ_2$ genes, which appear to represent a third pattern of controlled expression of chaperone-encoding genes in the genus *Bifidobacterium*.

Sequence analysis of *B. breve* UCC 2003 *hrcA* locus. The *B. breve* UCC 2003 genome sequence (S. Leahy, J. A. Moreno Munoz, M. O'Connell-Motherway, D. Higgins, G. F. Fitzgerald, and D. van Sinderen, unpublished data) contains two different *dnaJ*-like genes. The *dnaJ*-like gene that is found downstream of the *dnaK* gene (22) shares the highest degree of similarity to *dnaJ* of *B. subtilis* and was therefore designated $dnaJ_1$. The second *dnaJ*-like gene, designated here as $dnaJ_2$, is

* Corresponding author. Present address: Department of Genetics, Evolution, and Anthropology, University of Parma, Parma, Italy. Phone: 39 0521 905479. Fax: 39 0521 905604. E-mail: marco.ventura@unipr.it.

located adjacent to a gene sharing a high degree of similarity to *hrcA*.

The *B. breve* UCC 2003 *hrcA* operon is preceded by the *tal* and *tkt* genes, which encode a putative transaldolase and a transketolase, respectively, and followed by a gene encoding a hypothetical protein and the *bacA* gene, which encodes a putative bacitracin resistance protein (Fig. 1a). Comparison of the *B. breve* UCC 2003 *hrcA* and $dnaJ_2$ products with proteins deposited in the publicly available databases showed significant sequence similarity with the HrcA transcriptional repressor and DnaJ chaperones from other high-G+C gram-positive bacteria (e.g., *Mycobacterium* and *Streptomyces*).

The *B. breve* UCC 2003 *hrcA* gene encodes a putative protein of 365 amino acids, with a predicted molecular mass of 38.6 kDa and a sequence 42% identical to that of *Streptomyces albus* G, which is the only actinobacterial *hrcA* gene characterized to date (7). The predicted *B. breve* UCC 2003 HrcA protein contains a C-terminal helix-turn-helix domain which has been described as being highly conserved among HrcA homologs.

Furthermore, the deduced *B. breve* HrcA sequence contained the functionally unassigned motif TIRNDMAALE, which is highly similar to the consensus TIRN(EDY)MA(DA QV)LE derived from HrcA proteins of *Bacillus subtilis* (27), *Staphylococcus aureus* (12), and *Clostridium acetobutylicum* (11).

The alignment of *B. breve* $DnaJ_2$ sequence with those of other representative species allowed the identification of four distinct regions, which are typical of all members of the DnaJ family (Fig. 1b). The N-terminal 70 to 80 amino acids are referred to as the J domain, which is postulated to interact with the DnaK protein to stimulate its ATPase activity (26). The next region, which is rich in glycine and phenylalanine, is postulated to act as a flexible hinge necessary for the activation of the substrate binding properties of the DnaK protein (26). A third region found in the middle of the protein is characterized by four conserved repeats (marked a to d in Fig. 1b) containing the CxxCxGxG motif. This consensus is similar to the "Zink finger" motif and is believed to be involved in disulfide isomerase activity (4). The fourth domain comprises a region at the C-terminal end of the protein for which little structural or functional information is available (3).

Distribution of *hrcA* and *dnaJ* genes across bacterial genomes. In order to assess the distribution of the *hrcA-dnaJ* operon across bacteria we surveyed 65 sets of available bacterial genomic data, including those for actinobacteria, *Firmicutes*, fusobacteria, chloroflexi, chlorobia, and cyanobacteria for the presence of the *hrcA* and *dnaJ* genes as well as their genomic locations (Table 1). The genomes of members of the group actinobacteria contained two copies of *dnaJ* genes, where $dnaJ_2$ is always flanked by the *hrcA* gene, whereas $dnaJ_1$ is located within the *dnaK* operon. Notably, only the *Streptomyces avermitilis* genome contains a third copy of the *dnaJ* gene ($dnaJ_3$), which is placed within a second copy of the *dnaK* operon. In the available genomes of *Firmicutes* a single copy of the *dnaJ* gene is present, which is always located within the *dnaK* operon. *Proteobacteria* possess the same genetic constellation as *Firmicutes* except for certain genera (*Ralstonia*, *Sinorhizobium*, and *Thermosynechoccus*), in which the *hrcA* gene is not flanked by a DnaJ-encoding gene.

Notably, other bacteria like the cyanobacterium *Synechocystis* sp. possess two DnaJ homologs, although neither of these is located in the vicinity of the *hrcA* gene.

The *hrcA* gene is uniformly present in all gram-positive bacteria studied to date (Table 1). However, the chromosomal location of the *hrcA* gene differs in gram-positive bacteria with low G+C content, where it is associated with the *dnaK* operon, in contrast to the *hrcA* position in high-G+C genomes, where is associated with $dnaJ_2$. A *hrcA* homolog is also present in *Synechocystis* species and *Leptospira interrogans* (Table 1), both of which, according to 16S rRNA phylogeny (1), diverged at an early stage from the common ancestor of all gram-positive bacteria and *Proteobacteria*.

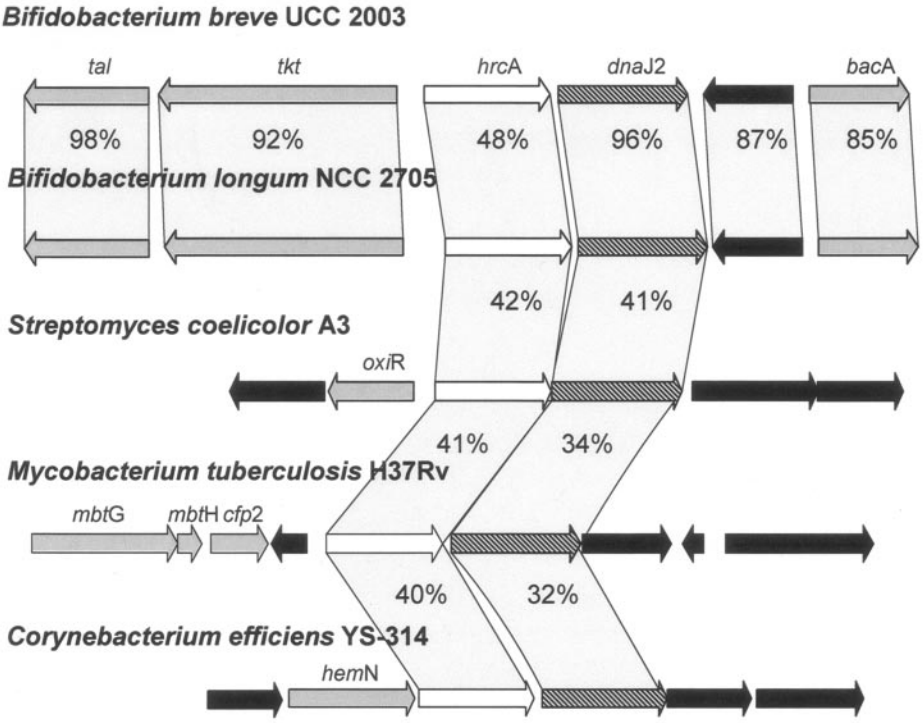
Hence, we can conclude that the *hrcA-dnaJ*$_2$ genetic constellation is unique and typical of the members of the group actinobacteria.

Phylogenetic analysis based on DnaJ sequences. The DnaJ sequence fulfills all prerequisites to be a suitable phylogenetic marker, such as very high genetic stability and a wide distribution. It has been applied to infer phylogeny in the genera *Deinococcus-Thermus* (2), *Mycobacterium* (10), and *Legionella* (9). In order to investigate the phylogeny of high-G+C and low-G+C gram-positive bacteria using DnaJ sequences, various DnaJ sequences were retrieved, including $DnaJ_1$ and $DnaJ_2$, from databases. A total of 53 sequences were thus obtained, including those from actinobacteria, *Firmicutes*, *Proteobacteria*, and cyanobacteria. Phylogeny calculations, including distance calculations and generation of phylogenetic trees, were performed using the PHYLIP package, employing default parameters (e.g., Dayhoff PAM matrix) and with the ProtDist module (5).

Moreover, since our main target was to study the phylogeny of bifidobacteria, a fragment of the $dnaJ_2$ gene from 17 different bifidobacterial species was obtained and subsequently sequenced using a PCR-based strategy employing two primers, DNAJ2-uni (5′-CTCGGCCAGATGATGAC-3′) and DNAJ2-rev (5′-CTGAGCTT[C/G]GTGGGAATC-3′), based on two conserved regions (positions 541 to 558 and positions 1009 to 1027) from the *B. breve* UCC 2003 $dnaJ_2$ gene (Fig. 1b). The reaction mixture (50 μl) contained 25 ng DNA template, which was derived from the protocol described in a previous study (17); 20 mM Tris-HCl; 50 mM KCl; 200 μM of each deoxynucleoside triphosphate; 50 pmol of each primer; 1.5 mM $MgCl_2$; and 1 U *Taq* DNA polymerase (Gibco BRL, United Kingdom). Each PCR cycling profile consisted of an initial denaturation step of 5 min at 95°C, followed by amplification for 30 cycles as follows: denaturation (30 s at 95°C), annealing (30 s at 52°C), and extension (1 min at 72°C). The resulting amplicons were separated on a 1.5% agarose gel, followed by an ethidium bromide staining. PCR fragments were purified using the PCR purification spin kit (QIAGEN, West Sussex, United Kingdom) and were subsequently sequenced.

The phylogenetic tree based on DnaJ sequences shows low bootstrap scores and long branch lengths for only a very few nodes and species. In view of this, their usefulness for phylogenetic studies is limited. However, despite this caveat it should be noted that the two DnaJ homologs of actinobacteria did not form a monophyletic group in this tree, but instead the $DnaJ_2$ proteins of actinobacteria branched together with the DnaJ sequences of *Firmicutes* and *Proteobacteria*, while the

a)



b)

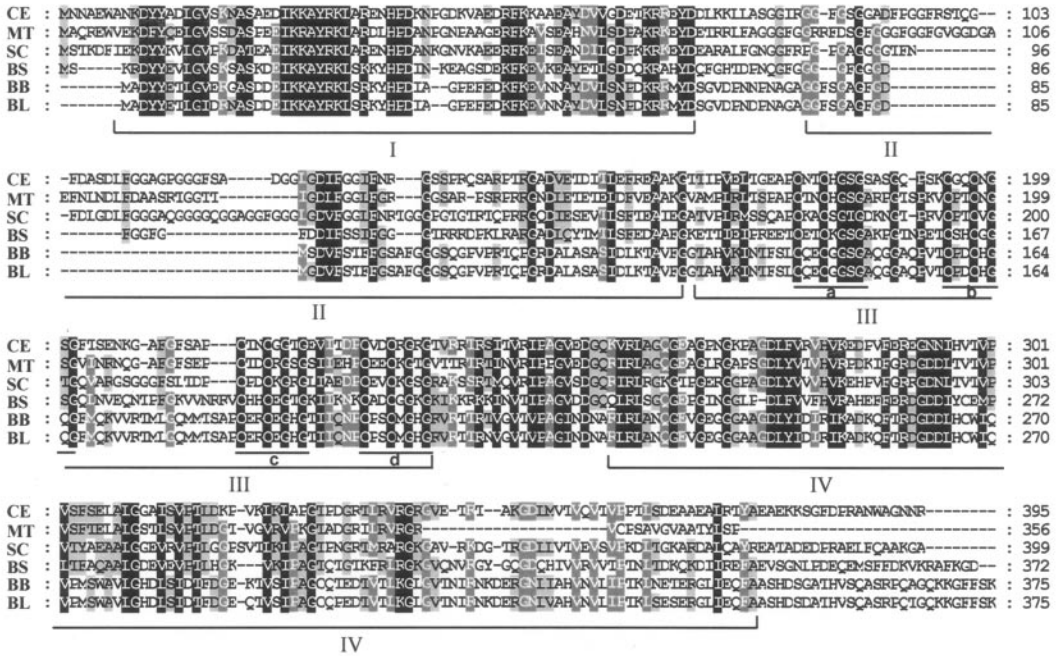


FIG. 1. (a) Comparison of the *hrcA* locus in *B. breve* UCC 2003 with corresponding loci in various other actinobacteria. Each arrow indicates an open reading frame. The length of arrows is proportional to the length of the predicted open reading frames. Corresponding genes are marked with the same color or pattern. The putative function of the proteins is indicated above each arrow (see text for explanation). Black arrows indicate hypothetical open reading frames. The amino acid identity with respect to *B. breve* UCC 2003 protein sequences is indicated as a percentage. (b) Alignment of the amino acid sequence for $DnaJ_2$ proteins from gram-positive bacteria. CE, *Corynebacterium efficiens* YS-314; MT, *Mycobacterium tuberculosis* H37Rv; SC, *Streptomyces coelicolor* A3; BL, *B. longum* NCC 2705; BB, *B. breve* UCC 2003; BS, *B. subtilis*. The various conserved amino acid regions are indicated and explained in the text.

TABLE 1. Distribution of *hrcA* and *hrcA-dnaJ*$_2$ or *hrcA-dnaK* operon genetic constellation in various bacteria

Group	Organism	Database no.	*hrcA*	*hrcA-dnaJ*$_2$	*hrcA-dnaK* operon
Actinobacteria	*Bifidobacterium longum* DJO10A	ZP_00120370.1	+	+	−
Actinobacteria	*Bifidobacterium longum* NCC2705	NP_695900.1	+	+	−
Actinobacteria	*Brevibacterium linens* BL2	ZP_00381522.1	+	+	−
Actinobacteria	*Corynebacterium diphtheriae*	NP_940058.1	+	+	−
Actinobacteria	*Corynebacterium efficiens* YS-314	NP_738799.1	+	+	−
Actinobacteria	*Corynebacterium glutamicum* ATCC 13032	CAF20632.1	+	+	−
Actinobacteria	*Kineococcus radiotolerans* SRS30216	ZP_00198394.1	+	+	−
Actinobacteria	*Leifsonia xyli* subsp. *xyli* strain CTCB07	YP_062381.1	+	+	−
Actinobacteria	*Mycobacterium avium* subsp. *paratuberculosis* strain k10	Q73XZ5	+	+	−
Actinobacteria	*Mycobacterium tuberculosis* H37Rv	NC_000962	+	+	−
Actinobacteria	*Mycobacterium tuberculosis* CDC1551	NP_216889.1	+	+	−
Actinobacteria	*Nocardia farcinica* IFM 10152	YP_117634.1	+	+	−
Actinobacteria	*Streptomyces avermitilis* MA-4680	NC_003155	+	+	−
Actinobacteria	*Streptomyces coelicolor* A3(2)	CAB66232.1	+	+	−
Actinobacteria	*Propionibacterium acnes* KPA171202	YP_055626.1	+	+	−
Actinobacteria	*Thermobifida fusca*	ZP_00293589.1	+	+	−
Firmicutes	*Acholeplasma laidlawii*	AAM43820.1	+	−	+
Firmicutes	*Bacillus anthracis* strain Sterne	YP_021184.1	+	−	+
Firmicutes	*Bacillus cereus* ATCC 10987	NP_980687.1	+	−	+
Firmicutes	*Bacillus clausii* KSM-K16	YP_175153.1	+	−	+
Firmicutes	*Bacillus licheniformis* DSM 13	YP_079885.1	+	−	+
Firmicutes	*Bacillus sphaericus*	CAA76661.1	+	−	+
Firmicutes	*Bacillus subtilis*	NP_390424.1	+	−	+
Firmicutes	*Clostridium tetani* E88	AAO36533.1	+	−	+
Firmicutes	*Clostridium thermocellum* ATCC 27405	ZP_00314236.1	+	−	+
Firmicutes	*Enterococcus faecalis* V583	NP_815028.1	+	−	+
Firmicutes	*Exiguobacterium* sp. strain 255-15	ZP_00182778.2	+	−	+
Firmicutes	*Geobacillus kaustophilus* HTA426	YP_148356.1	+	−	+
Firmicutes	*Lactobacillus acidophilus*	YP_194110.1	+	−	+
Firmicutes	*Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC BAA-365	ZP_00387602.1	+	−	+
Firmicutes	*Lactobacillus gasseri*	ZP_00046571.1	+	−	+
Firmicutes	*Lactobacillus johnsonii* NCC 533	NP_965280.1	+	−	+
Firmicutes	*Lactobacillus plantarum* WCFS1	CAD64400.1	+	−	+
Firmicutes	*Lactobacillus sakei*	CAA06939.1	+	−	+
Firmicutes	*Listeria monocytogenes*	NP_464997.1	+	−	+
Firmicutes	*Moorella thermoacetica* ATCC 39073	ZP_00330048.1	+	−	+
Firmicutes	*Oceanobacillus iheyensis* HTE831	NP_692888.1	+	−	+
Firmicutes	*Pediococcus pentosaceus* ATCC 25745	ZP_00323327.1	+	−	+
Firmicutes	*Staphylococcus aureus* subsp. *aureus* MRSA252	YP_041051.1	+	−	+
Firmicutes	*Staphylococcus epidermidis* ATCC 12228	NP_764821.1	+	−	+
Firmicutes	*Streptococcus agalactiae* 2603V/R	NP_687131.1	+	−	+
Firmicutes	*Streptococcus mutans* UA159	AAN57865.1	+	−	+
Firmicutes	*Streptococcus pneumoniae* TIGR4	ZP_00403006.1	+	−	+
Firmicutes	*Streptococcus pyogenes* MGAS10394	YP_060809.1	+	−	+
Firmicutes	*Streptococcus suis* 89/1591	ZP_00332240.1	+	−	+
Firmicutes	*Streptococcus thermophilus* LMG 18311	AAV61733.1	+	−	+
Firmicutes	*Tetragenococcus halophilus*	Q93R26	+	−	+
Firmicutes	*Thermoanaerobacter tengcongensis* MB4	NP_622605.1	+	−	+
Proteobacteria	*Xanthomonas axonopodis* pv. citri strain 306	AAM36389.1	+	−	+
Proteobacteria	*Xanthomonas campestris* pv. campestris strain ATCC 33913	NP_636844.1	+	−	+
Proteobacteria	*Xanthomonas oryzae* pv. oryzae KACC10331	YP_200668.1	+	−	+
Proteobacteria	*Xylella fastidiosa* Dixon	ZP_00039265.2	+	−	+
Proteobacteria	*Xylella fastidiosa* Temecula1	NP_779567.1	+	−	+
Proteobacteria	*Methylococcus capsulatus* strain Bath	YP_114292.1	+	−	+
Proteobacteria	*Geobacter sulfurreducens* PCA	NP_951093.1	+	−	+
Proteobacteria	*Anaeromyxobacter dehalogenans* 2CP-C	ZP_00398344.1	+	−	+
Proteobacteria	*Sinorhizobium meliloti* 1021	CAC41570.1	+	−	−
Proteobacteria	*Ralstonia solanacearum* GMI1000	CAD16341.1	+	−	−
Cyanobacteria	*Thermosynechococcus elongatus* BP-1	NP_681550.1	+	−	−
Cyanobacteria	*Synechocystis*. sp. strain PCC 6803	NC_000911	+	−	−
Spirochaetes	*Leptospira interrogans*	AAC35414.1	+	−	+
Spirochaetes	*Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130	YP_000507.1	+	−	+
Fusobacteria	*Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586	AAL94322.1	+	−	+
Chloroflexi	*Dehalococcoides ethenogenes* 195	YP_182107.1	+	−	+
Chlorobia	*Chlorobium tepidum* TLS	NP_662369.1	+	−	+

$DnaJ_1$ sequences of actinobacteria formed a separate phylogenetic branch (Fig. 2).

This suggests that the DnaJ of *Firmicutes* and *Proteobacteria* and $DnaJ_2$ of actinobacteria originated from a common ancestor, which possessed two copies of DnaJ-encoding genes, and that a differential loss of gene copies did occur in *Firmicutes-Proteobacteria* and actinobacteria, resulting in the current *dnaJ* gene distribution. Of note, the close phylogenetic relationship between DnaJ of *Firmicutes* and $DnaJ_2$ of actinobacteria is further illustrated by a high degree of synteny observed in the *hrcA* locus of these bacteria, in which the $dnaJ_2$ genes of actinobacteria and the *dnaJ* genes of *Firmicutes* are located near the *hrcA* gene.

Finally, the overall similarity of DnaJ proteins roughly resembles that exhibited by HrcA sequences (1) and provides an alternative hierarchical order among gram-positive bacteria.

Conservation of $dnaJ_2$ gene across *Bifidobacterium* genomes. Due to the apparent high variability of $dnaJ_2$ sequences within the genus *Bifidobacterium* we were able to identify this gene by PCR amplification in only 17 species of the 30 currently described species in the genus *Bifidobacterium* (21). Thus, in order to determine whether other bifidobacteria also contain $dnaJ_2$ homologs, the amplified $dnaJ_2$ DNA was hybridized to genomic DNA digested with EcoRI or HindIII of 30 different bifidobacterial species according to the method of Sambrook et al. (14). All investigated bifidobacteria yielded one single band of different sizes in all strains used (Fig. 3 and data not shown). This clearly suggests that a $dnaJ_2$-like gene is present in most, if not all, bifidobacterial genomes.

Kinetics of *hrcA* transcription following heat-osmotic stresses. Expression of the *hrcA* locus in *S. albus* G (7) is induced by heat stress. To determine if the induction of the *hrcA* gene occurs upon exposure to stressful conditions in *B. breve* UCC 2003, slot blot hybridization was used to analyze total RNA isolated from *B. breve* cultures following exposure for up to 150 min to temperatures ranging from 20°C to 50°C and to NaCl concentrations of 0.5 M and 0.7 M (Fig. 4a and b). RNA isolation and slot blot hybridizations were carried out according to the method of Ventura et al. (19, 24).

Based on the strength of the hybridization signal, the strongest expression of the *hrcA* gene occurred upon osmotic shock, whereas exposure to high temperatures (43°C or 50°C) or low temperature (20°C) did not appear to significantly increase the level of *hrcA* transcription (Fig. 4a and b). Densitometric analysis of Northern slot blots revealed that the levels of *hrcA* mRNA were increased at least 4.5-fold in cells that were subjected to osmotic stress for 150 min compared to unstressed cells (Fig. 4c). Furthermore, an amount of total RNA identical to that used in the induction experiment was slot blotted and hybridized using the 16S rRNA gene as a probe, in a control experiment according to the protocol described previously (23). The strength of the hybridization signal was of a similar intensity for all the slots, thus confirming the uniformity of RNA samples employed in the induction experiments (data not shown).

Transcription analysis of the *hrcA* locus. Transcription of the *hrcA B. breve* locus was investigated by Northern blotting with two different probes encompassing *hrcA* and $dnaJ_2$ genes, respectively. Total mRNA was isolated from *B. breve* UCC 2003 grown at 37°C, following heat shock at 43°C and 50°C or following osmotic shock at 0.7 M NaCl. Northern blot hybridizations were carried out according to the method of Ventura et al. (18). Northern blot analysis revealed a major hybridization signal corresponding to a 2.3-kb mRNA (Fig. 4d and e). This transcription pattern was also revealed for $dnaJ_2$, where the size of the $dnaJ_2$-hybridizing band was the same as that of the *hrcA*-hybridizing band, which suggests that the two genes are transcribed as a single bicistronic mRNA (data not shown). No *hrcA* mRNA was detected at 37°C, whereas *hrcA* transcripts accumulated after osmotic upshift, with maximum mRNA levels being observed about 150 min after the NaCl concentration had been increased to 0.7 M. Analysis of the nucleotide sequence of the *hrcA* locus revealed that the $dnaJ_2$ gene was flanked at its 3′ end by two inverted repeats (ΔG of −25.6 and −12.2 kcal) that may function as rho-independent transcriptional terminator structures (Fig. 4d).

Determination of the transcription initiation site. The transcription start site of the *hrcA* locus was determined by primer extension analysis using mRNA isolated from cultures grown at 37°C or heat shocked at 43°C or at 50°C for 150 min or following osmotic shock at 0.5 M and 0.7 M NaCl for 150 min. The 5′ end of the *hrcA* RNA transcript was determined following a protocol described in a previous study (18) and employing the synthetic oligonucleotide hrcA-prom (5′-CATTG CGAATGGTGGCAGAG-3′). Two transcription start sites were identified 41 bp and 44 bp upstream of the *hrcA* translation initiation codon using mRNA extracted from cultures subjected to osmotic shock (Fig. 5). Analysis of the putative promoter region of the *hrcA* reveals a potential sigma-70-promoter-like sequence resembling the consensus −10 and −35 hexamers. The DNA spacing between the −10 and −35 regions is exceptionally long, being 21 bp rather than the canonical 17 (25). The −35 hexamer of the *hrcA* promoter is located within a palindromic sequence (IR).

The predicted promoter regions of *hrcA* genes from two bifidobacterial strains (*B. breve* UCC 2003 and *Bifidobacterium longum* NCC 2705) were aligned in an attempt to identify putative regulatory elements (Fig. 5c). For completeness, we determined by inverse PCR the putative promoter region of the *hrcA* gene from a more distantly related *Bifidobacterium* species (*Bifidobacterium animalis* subsp. *animalis*) following a protocol described previously (14, 23). The alignment of these promoter sequences revealed that the putative −10 region as well as the −35 box was highly conserved in these three bifidobacterial sequences. Moreover, the IR sequence was conserved among all three bifidobacteria examined (Fig. 5c). Interestingly, this inverted repeat resembled a regulatory structure termed CIRCE (TTAGCACTC N_9 GAGTGCTAA) (28), which has been demonstrated to be the operator sequence of the heat shock protein regulator (HrcA) in both low- and high-G+C gram-positive bacteria (6, 28). A preliminary screening of the *B. longum* NCC 2705 and *B. breve* UCC 2003 genome sequence for the consensus sequences (ATTAGC ACTC N_9 GAGTGCTAAT) revealed the presence of this IR in the promoter region of a classical stress-induced gene such as *groES* (21).

Conclusions. We identified and characterized a second *dnaJ* homolog which is organized with the *hrcA* gene in an apparently bicistronic operon in the *B. breve* UCC 2003 genome. Phylogenetic analysis using DnaJ proteins from low- and high-

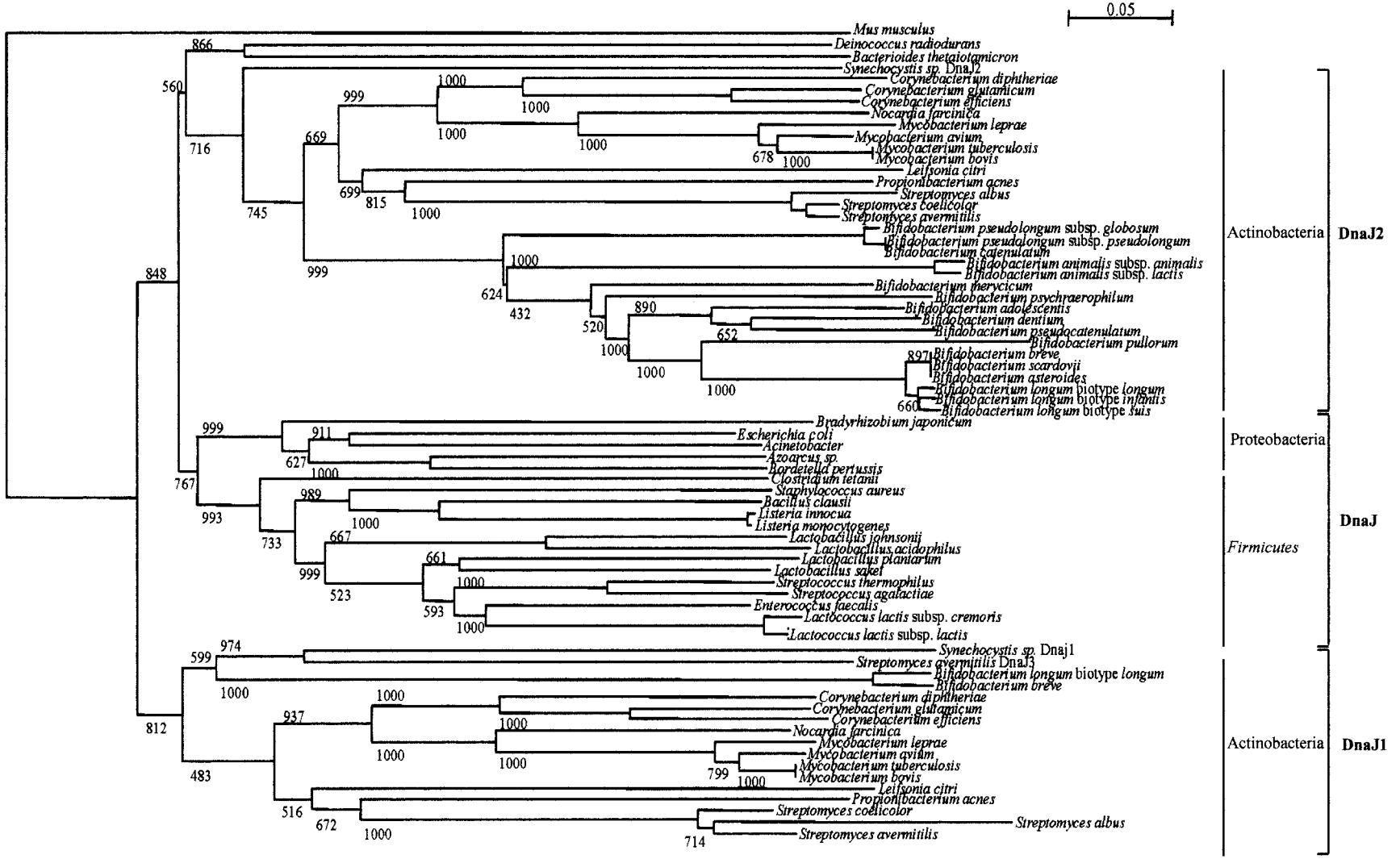


FIG. 2. Phylogenetic tree obtained using DnaJ homologs from various bacteria. The bar scale indicates phylogenetic distances. Bootstrap values are reported for a total of 1,000 replicates. The $dnaJ_1$ and $dnaJ_2$ gene sequences are indicated. The principal bacterial groups are indicated.

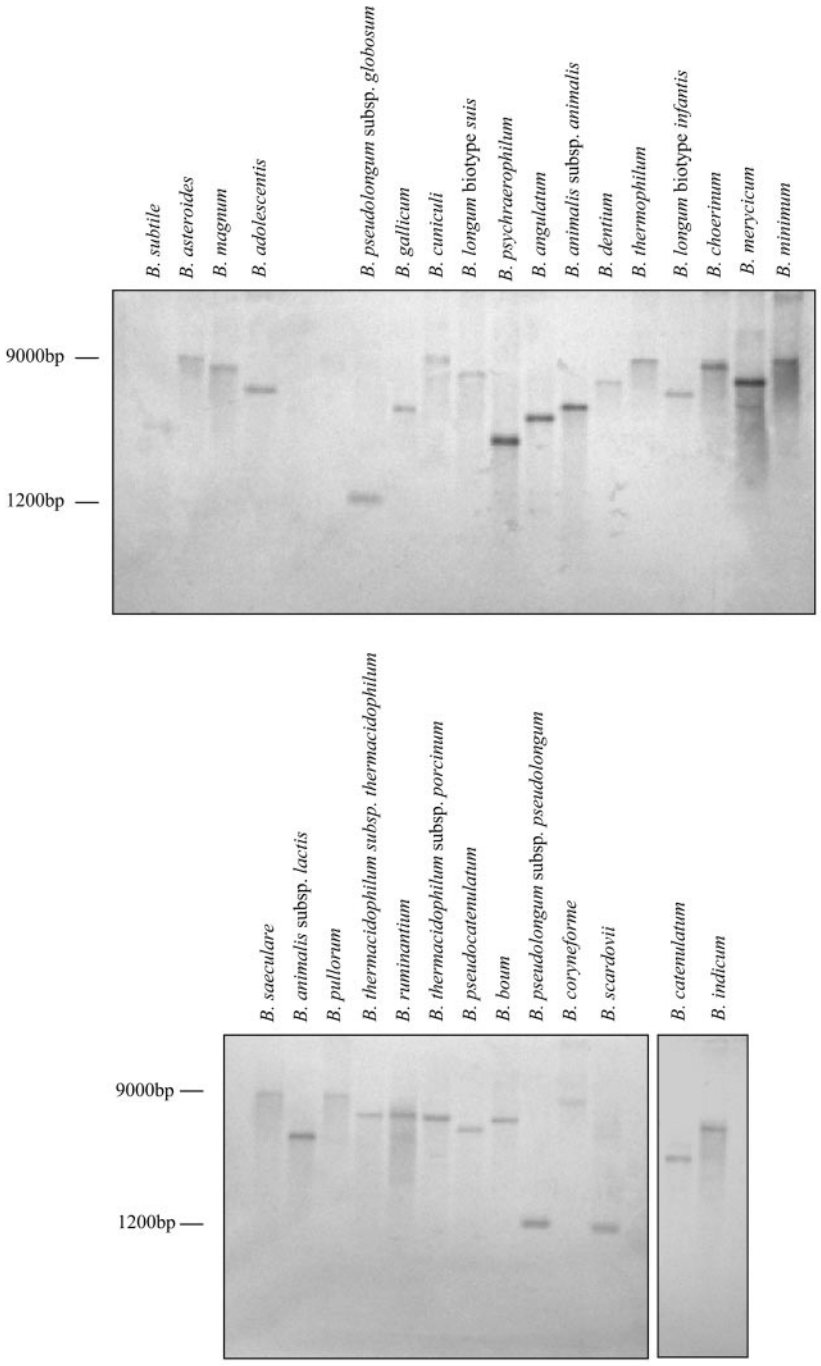


FIG. 3. Southern hybridization of EcoRI-digested genomic DNAs of *Bifidobacterium* species using the $dnaJ_2$ gene fragment as probe. The sizes of two markers are indicated.

G+C gram-positive bacteria revealed a close and specific relationship between the actinobacterial $dnaJ_2$ gene and the *dnaJ* gene of *Firmicutes* and *Proteobacteria*. This may indicate that the $dnaJ_1$ gene of actinobacteria has evolved from an ancient form which existed before the evolutionary split of actinobacteria from *Firmicutes* or that a differential loss of *dnaJ* gene copies occurred during the evolution of actinobacteria and *Firmicutes*.

The presence of two *dnaJ* genes may be an indication that the encoded proteins have different physiological functions either alone or in association with DnaK, a theory that is consistent with the differential expression patterns of the two *dnaJ* genes. The presence of the two bifidobacterial *dnaJ* genes where both are activated under osmotic stress but only one ($dnaJ_1$) is activated by high temperature may be a reflection of the fact that these bacteria normally grow in habitats like the

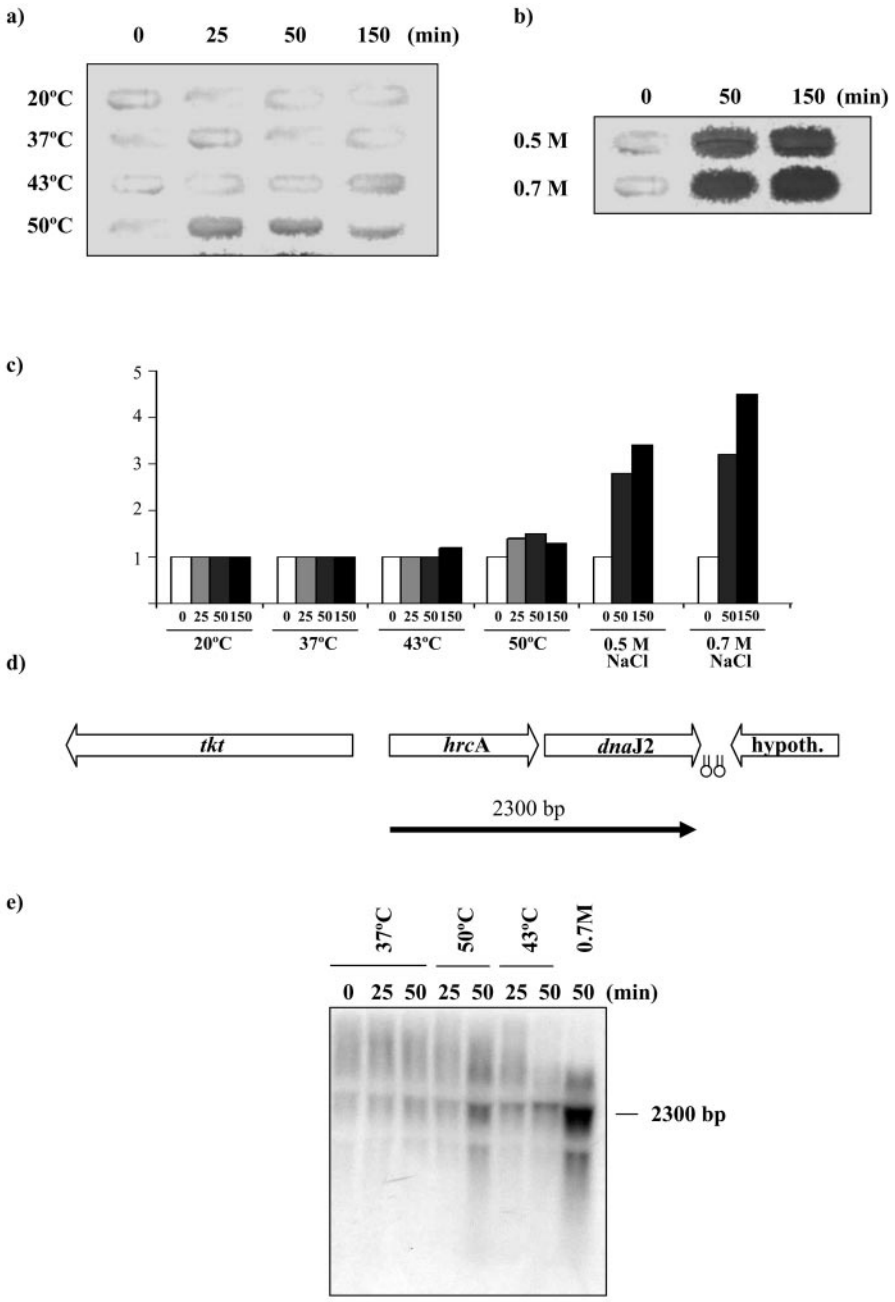


FIG. 4. Transcriptional regulation of the *B. breve* UCC 2003 *hrc*A operon. (a and b) Slot blot hybridization using RNA extracted from cells incubated up to 150 min in a range of temperatures or NaCl concentrations. (c) Quantitative representation of the induction levels of the *hrcA* mRNA transcripts. The numbers below each bar show the time in minutes for which each temperature or osmotic shock was applied. (d) Position of the transcripts with respect to the *hrcA* locus map. The estimated size of the mRNA is indicated in base pairs. Hairpins indicate possible rho-independent terminators. (e) Northern blot analysis of *B. breve* UCC 2003 *hrcA* locus performed using total mRNA isolated from cultures grown at 37°C, 43°C, or 50°C and under hyperosmotic conditions using an NaCl concentration of 0.7 M for the time indicated above each lane.

GIT of animals where no significant temperature changes occur, whereas the osmotic conditions of the GIT are greatly variable due to the diet composition. This is corroborated by the fact that the number of chaperone-encoding genes involved in heat shock protection in actinobacteria that live in isothermal niches (e.g., the GIT of mammals) is smaller than that found in actinobacteria which are found in environments exposed to higher thermal fluctuations (e.g., soil or plant).

HrcA represents the third identified bifidobacterial transcriptional regulator, which would tune the expression of stress-induced genes. In fact, bifidobacterial genes (e.g., *clpB* and *dnaK* genes) which are highly induced upon exposure to severe heat shocks or osmotic stresses have been shown to be regulated by the transcriptional repressor protein HspR (24). Bifidobacterial genes (e.g., *clpP* and *clpC* genes) which are strongly induced upon moderate heat treatments were shown

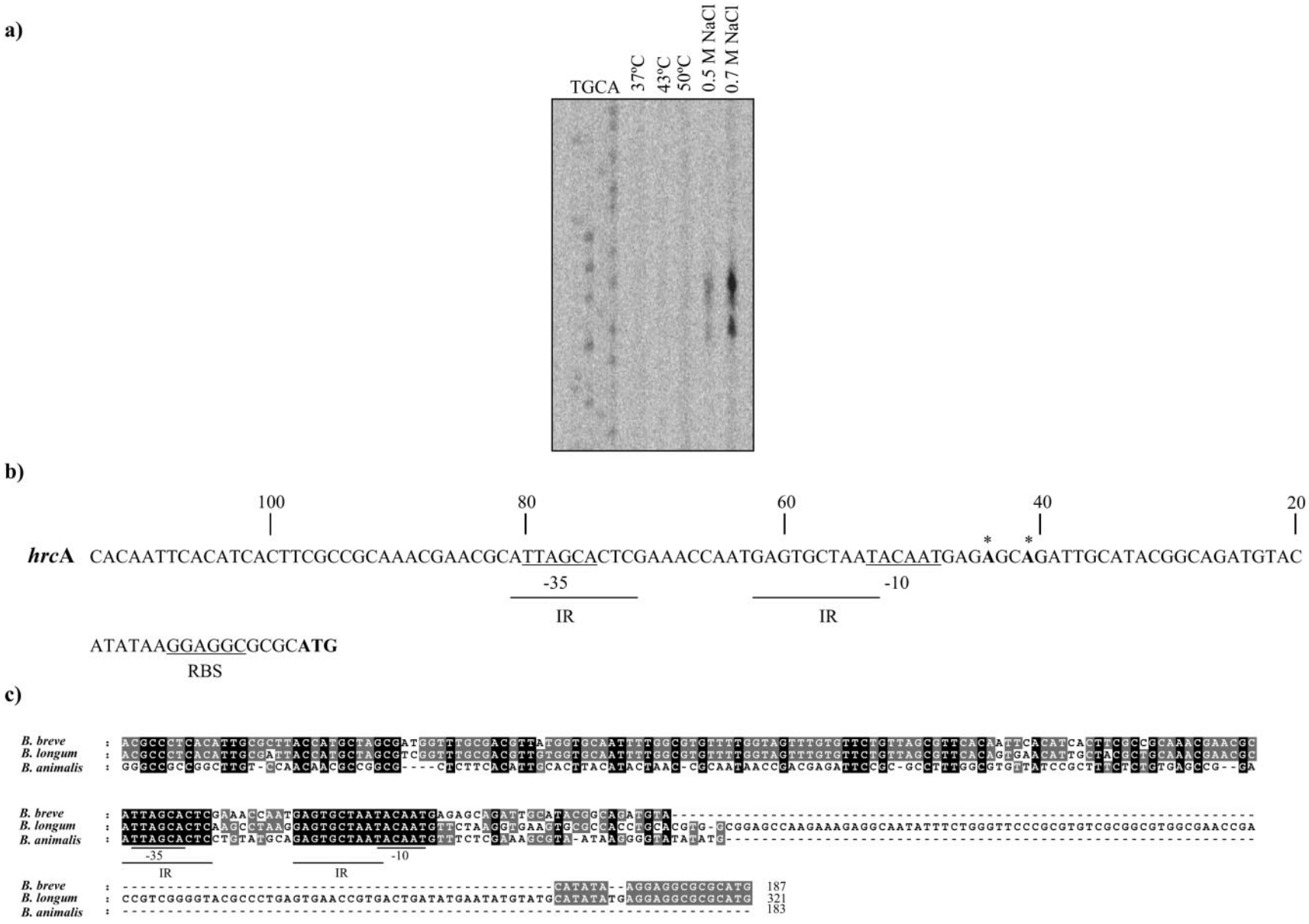


FIG. 5. Primer extension analysis of the putative promoter sequences for the *hrcA* gene using an oligonucleotide targeting the 5′ ends of the *hrcA* gene (a). The experiment was performed using cells grown at either 37°C, 43°C, or 50°C or 0.5 M of NaCl or 0.7 M of NaCl. (b) Putative promoter sequences for the *hrcA* gene of *B. breve* UCC 2003. Underlined sequences denote the putative −10 and −35 hexamers; boldface type with an asterisk denotes the transcription start point, and boldface type without an asterisk indicates the start codon. IR indicates putative regulator sequences. RBS, ribosome binding site. (c) Comparison of the promoter sequences of three *hrcA* homologs from different bifidobacterial species. Shaded nucleotides indicate that the base is present in more than two of the inspected sequences.

to be regulated by the transcriptional activator ClgR in conjunction with a so-far unidentified cofactor (23; unpublished data). The expression pattern of *hrcA* appears to be unique. In fact, bifidobacterial *hrcA* may represent an exception to the rule since, unlike other organisms, the *hrcA* and $dnaJ_2$ genes are significantly induced upon osmotic stress rather than heat stress. Although the expression of the *B. breve hrcA* gene was shown to be osmoregulated, it will remain to be determined whether its activity may still be thermoregulated. Thus, additional work will be necessary in order to verify this hypothesis and to exploit the CIRCE regulon.

Nucleotide sequence accession numbers. The nucleotide sequence data regarding the *hrcA* locus of *B. breve* UCC 2003 have been deposited in GenBank under accession number DQ144724. Furthermore, the GenBank accession numbers for $dnaJ_2$ gene sequences generated in this study are as follows: *B. animalis* subsp. *lactis* DSM 10140, DQ144709; *B. animalis* subsp. *animalis* ATCC 25527, DQ144713; *B. suis* LMG 10738, DQ144710; *B. psychraerophilum* LMG 21775, DQ144711; *B. pseudolongum* subsp. *pseudolongum* LMG 11595, DQ144712; *B. dentium* LMG 11585, DQ144714; *B. adolescentis* LMG 11579, DQ144715; *B. catenulatum* LMG 11043, DQ144716; *B. infantis* LMG 8811, DQ144717; *B. pseudolongum* subsp. *globosum* LMG 11614, DQ144718; *B. merycicum* LMG 11341, DQ144719; *B. scardovii* LMG 11571, DQ144720; *B. pseudocatenulatum* LMG 11593, DQ144721; *B. asteroides* LMG 10735, DQ144722; *B. pullorum* LMG 21816, DQ144723.

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REFERENCES

1. **Ahmad, S., A. Selvapandiyan, and R. K. Bhatnagar.** 1999. A protein-based phylogenetic tree for Gram-positive bacteria derived from *hrcA*, a unique heat-shock regulatory gene. Int. J. Syst. Bacteriol. **49:**1387–1394.
2. **Bustard, K., and R. S. Gupta.** 1997. The sequences of heat shock protein 40 (DnaJ) homologs provide evidence for close evolutionary relationship between the *Deinococcus-Thermus* group and cyanobacteria. J. Mol. Evol. **45:**193–205.
3. **Caplan, A. J., D. M. Cyr, and M. G. Douglas.** 1993. Eukaryotic homologs of *Escherichia coli* DnaJ: a diverse protein family that functions with Hsp70 stress proteins. Mol. Biol. Cell **4:**555–563.
4. **De Crouy-Chanel, A., M. Kohiyama, and G. Richarme.** 1995. A novel function of *Escherichia coli* chaperone DnaJ. J. Biol. Chem. **270:**22669–22672.
5. **Felsenstein, J.** 1989. PHYLIP—phylogeny inference package (version 3.2). Cladistics **5:**164–166.
6. **Georgopoulos, C., K. Liberek, M. Zylicz, and D. Ang.** 1994. Properties of the heat shock proteins of *Escherichia coli* and the autoregulation of the heat shock response, p. 209–249. *In* R. I. Morimoto, A. Tissieres, and C. Georgopoulos (ed.), The biology of heat shock proteins and molecular chaperones. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
7. **Grandvalet, C., G. Rapoport, and P. Mazodier.** 1998. *hrcA*, encoding the repressor of the *groEL* genes in *Streptomyces albus* G, is associated with a second *dnaJ* gene. J. Bacteriol. **180:**5129–5134.
8. **Lievin, V., I. Pfeiffer, S. Hudault, F. Rochat, D. Brassart, J. R. Neeser, and A. L. Servin.** 2000. *Bifidobacterium* strains from resident infant human gastrointestinal microflora exert antimicrobial activity. Gut **47:**646–652.
9. **Liu, H., X. Huang, Y. Kawamura, and T. Ezaki.** 2003. Use of the *dnaJ* gene for the detection and identification of all *Legionella pneumophila* serogroups and description of the primers used to detect 16S rDNA gene sequences of major members of the genus *Legionella*. Microbiol. Immunol. **47:**859–869.
10. **Morita, Y., S. Maruyama, H. Kabeya, A. Nagai, K. Kozawa, M. Kato, T. Nakajima, T. Mikami, Y. Katsube, and H. Kimura.** 2004. Genetic diversity of the *dnaJ* gene in the *Mycobacterium avium* complex. J. Med. Microbiol. **53:**813–817.
11. **Narberhaus, F., K. Giebeler, and H. Bahl.** 1992. Molecular characterization of the *dnaK* gene region of *Clostridium acetobutylicum*, including *grpE*, *dnaJ*, and a new heat shock gene. J. Bacteriol. **174:**3290–3299.
12. **Ohta, T., K. Saito, M. Kuroda, K. Honda, H. Hirata, and H. Hayashi.** 1994. Molecular cloning of two new heat shock genes related to the Hsp70 genes in *Staphylococcus aureus*. J. Bacteriol. **176:**4779–4783.
13. **Ouwehand, A. C., S. Salminen, and E. Isolauri.** 2002. Probiotics: an overview of beneficial effects. Antonie Leeuwenhoek **82:**279–289.
14. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
15. **Schmidt, G., and R. Zink.** 2000. Basic features of stress response in three species of bifidobacteria: *B. longum*, *B. adolescentis*, and *B. breve*. Int. J. Food Microbiol. **55:**41–45.
16. **Tannock, G. W.** 1994. The acquisition of the normal microflora of the gastrointestinal tract, p. 1–16. *In* S. A. Gibson (ed.), Human health: the contribution of microorganisms. Springer, London, United Kingdom.
17. **Ventura, M., R. Reniero, and R. Zink.** 2001. Specific identification and targeted characterization of *Bifidobacterium lactis* from different environmental isolates by a combined multiplex-PCR approach. Appl. Environ. Microbiol. **67:**2760–2765.
18. **Ventura, M., C. Canchaya, V. Meylan, T. R. Klaenhammer, and R. Zink.** 2003. Analysis, characterization, and loci of the *tuf* genes in *Lactobacillus* and *Bifidobacterium* and their direct application for species identification. Appl. Environ. Microbiol. **69:**6908–6922.
19. **Ventura, M., C. Canchaya, D. van Sinderen, G. F. Fitzgerald, and R. Zink.** 2004. *Bifidobacterium lactis* DSM 10140: identification of the *atp* (*atpBEFHAGDC*) operon and analysis of its genetic structure, characteristics, and phylogeny. Appl. Environ. Microbiol. **70:**3110–3121.
20. **Ventura, M., C. Canchaya, R. Zink. G. F. Fitzgerald, and D. van Sinderen.** 2004. Characterization of the *groEL* and *groES* loci in *Bifidobacterium breve* UCC 2003: genetic, transcriptional, and phylogenetic analysis. Appl. Environ. Microbiol. **70:**6197–6209.
21. **Ventura, M., D. van Sinderen, G. F. Fitzgerald, and R. Zink.** 2004. Insights into the taxonomy, genetics and physiology of bifidobacteria. Antonie Leeuwenhoek **86:**205–223.
22. **Ventura, M., R. Zink. G. F. Fitzgerald, and D. van Sinderen.** 2005. Gene structure and transcriptional organization of the *dnaK* operon of *Bifidobacterium breve* UCC 2003 and its application in bifidobacterial tracing. Appl. Environ. Microbiol. **71:**487–500.
23. **Ventura, M., G. F. Fitzgerald, and D. van Sinderen.** 2005. Genetic and transcriptional organization of the *clpC* locus in *Bifidobacterium breve* UCC 2003. Appl. Environ. Microbiol. **71:**6282–6291.
24. **Ventura, M., J. G. Kenny, Z. Zhang, G. F. Fitzgerald, and D. van Sinderen.** 2005. The *clpB* gene of *Bifidobacterium breve* UCC 2003: transcriptional analysis and first insights into stress induction. Microbiology **151:**2861–2872.
25. **Wagner, R.** 2000. Transcription regulation in prokaryotes, p. 16–22. Oxford University Press, London, United Kingdom.
26. **Wall, D., M. Zylic, and C. Georgopoulos.** 1994. The NH_2-terminal 108 amino acids of the *Escherichia coli* DnaJ protein stimulate the ATPase activity of DnaK and are sufficient for lambda replication. J. Biol. Chem. **269:**5446–5451.
27. **Wetzstein, M., U. Volker, J. Dedio, S. Lobau, S. Zuber, M. Schiesswohl, C. Herget, M. Hecker, and W. Schumann.** 1992. Cloning, sequencing, and molecular analysis of the *dnaK* locus from *Bacillus subtilis*. J. Bacteriol. **174:**3300–3310.
28. **Zuber, U., and W. Schumann.** 1994. CIRCE, a novel heat shock element involved in regulation of heat shock operon *dnaK* of *Bacillus subtilis*. J. Bacteriol. **176:**1353–1363.