AEM
Journals.ASM.org

A Two-Component Regulatory System Controls Autoregulated Serpin Expression in *Bifidobacterium breve* UCC2003

Pablo Alvarez-Martin,[a]* Mary O'Connell Motherway,[a] Francesca Turroni,[a,b] Elena Foroni,[b] Marco Ventura,[b] and Douwe van Sinderen[a]

Alimentary Pharmabiotic Centre and Department of Microbiology, Bioscience Institute, National University of Ireland, Cork, Ireland,[a] and Laboratory of Probiogenomics, Department of Genetics, Biology of Microorganisms, Anthropology and Evolution, University of Parma, Parma, Italy[b]

This work reports on the identification and molecular characterization of a two-component regulatory system (2CRS), encoded by *serRK*, which is believed to control the expression of the ser_{2003} locus in *Bifidobacterium breve* UCC2003. The ser_{2003} locus consists of two genes, Bbr_1319 (*sagA*) and Bbr_1320 (*serU*), which are predicted to encode a hypothetical membrane-associated protein and a serpin-like protein, respectively. The response regulator SerR was shown to bind to the promoter region of ser_{2003}, and the probable recognition sequence of SerR was determined by a combinatorial approach of *in vitro* site-directed mutagenesis coupled to transcriptional fusion and electrophoretic mobility shift assays (EMSAs). The importance of the *serRK* 2CRS in the response of *B. breve* to protease-mediated induction was confirmed by generating a *B. breve serR* insertion mutant, which was shown to exhibit altered ser_{2003} transcriptional induction patterns compared to the parent strain, UCC2003. Interestingly, the analysis of a *B. breve serU* mutant revealed that the SerRK signaling pathway appears to include a SerU-dependent autoregulatory loop.

Bifidobacteria are high-G+C-content, Gram-positive microorganisms which are considered to constitute an important bacterial group among the microbiota of the human gastrointestinal tract (GIT) (47, 51, 55). In recent years, they have become a focus of scientific attention due to the perceived beneficial or probiotic action that they generate in their host (4, 22, 30, 58). This has catalyzed their exploitation as active ingredients of various commercial functional foods (43). The health-promoting effects attributed to (certain strains of) bifidobacteria include prevention of infection by pathogenic bacteria (59, 60), immunostimulatory (10) and anticarcinogenic capabilities (23), protection against infectious diarrhea (38), lowering of serum cholesterol (61), anti-inflammatory activity (17), and alleviation of lactose maldigestion (14). Understanding the mechanisms of probiosis is essential for their rational inclusion as probiotics in functional foods (20); however, only a small number of genetic loci have been implicated in the interaction between bifidobacteria and their host (8, 9, 34, 54, 56). Among them, the serpin (serine protease inhibitor)-encoding genes have been proposed to be involved in host-bacterium cross talk. Serpins represent a large class of protease inhibitors that are involved in regulating a wide spectrum of protease-mediated processes (13, 42). They are widely distributed in higher eukaryotic organisms but are also found in some viruses, where they appear to modulate virus-host interactions and viral infectivity (13). In addition, serpins have recently been identified in bacteria and *Archaea* and are thus present in all major domains of life (16, 18). Among members of the genus *Bifidobacterium*, serpin-encoding homologs are not widely distributed but are restricted to a small number of species in this genus (50). The physiological function of serpins produced by bacteria is not fully understood, and it has been hypothesized that intestinal bacteria, such as bifidobacteria, produce serpins to protect themselves against host-derived proteases, providing an advantage for survival in a highly complex and competitive environment (19, 51). Inhibition of human proteases, such as α-antitrypsin and human neutrophil elastase, by bifidobacterial serpins suggests an intriguing possibility that their release at the sites of intestinal inflammation is beneficial because it reduces exaggerated serine protease activity, which in turn may cause pathological tissue damage (19, 44, 48).

Two-component regulatory systems (2CRSs) are employed extensively in nature by microorganisms to modify their cellular physiology in response to alterations in environmental conditions (for reviews, see references 5, 11, 15, 27, 31, and 45). A 2CRS typically consists of a membrane-associated sensor protein or histidine protein kinase (HPK), which monitors one or more environmental parameters, and a cytoplasmic effector protein or response regulator (RR), which induces a specific cellular adaptive response. Although 2CRSs have previously been identified in different bifidobacterial genomes (26, 41, 57), their physiological function remains largely unknown. Just one bifidobacterial 2CRS, the *phoRP* system from *Bifidobacterium breve* UCC2003, has been functionally characterized and shown to regulate the response to phosphate limitation (2).

The presence of serpin-encoding genes in several bifidobacterial species as well as their induced transcription in response to different proteases has previously been described; however, the molecular mechanism driving this regulated expression has remained elusive. In this work, we report that the specific protease-mediated, transcriptional induction of the serpin-encoding gene

Received 31 May 2012 Accepted 20 July 2012
Published ahead of print 27 July 2012
Address correspondence to Douwe van Sinderen, d.vansinderen@ucc.ie.
* Present address: Pablo Alvarez-Martin, Nestlé Research Center, Vers-chez-les-Blanc, Lausanne, Switzerland.
P.A.-M. and M.O.M. contributed equally to this work.
Supplemental material for this article may be found at http://aem.asm.org/.

doi:10.1128/AEM.01776-12

TABLE 1 Bacterial strains and plasmids used in this work

Strain or plasmid	Genotype or phenotype	Reference or source
Strains		
B. breve		
UCC2003	Isolate from nursling stool	28
UCC2003-serR	pORI19-*tetW-serR* insertion mutant of UCC2003	This study
UCC2003-serU	pORI19-*tetW-serU*-containing gene insertion mutant of UCC2003	This study
UCC2003-serR-pPKserRK	*B. breve* UCC2003-serR containing *serRK* region in pPKCm vector	This study
UCC2003-serU-pPKserU	*B. breve* UCC2003-serR containing fusion of *serU* gene p44 promoter and cloned in pPKCm vector	This study
UCC2003-lacZ	pORI19-*tetW-lacZ* insertion mutant of UCC2003	M. O'Connell Motherway and D. van Sinderen (unpublished data)
E. coli		
EC101	*E. coli* JM101 with *repA* from pWV01 integrated in chromosome, Km^r	21
XL1-Blue	Δ(*mcrA*)*183*Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI*^{q}*Z*ΔM15 Tn*10* (Tet^r)]	Stratagene
Plasmids		
pNZ44	Cm^r, pNZ8048 containing constitutive p44 promoter from *Lactococcus lactis* chromosome	29
pNZ44-serR	Cm^r; pNZ44 derivative containing translational fusion of *serR*-containing DNA fragment to p44 promoter	This study
pNZ44-serU	Cm^r; pNZ44 derivative containing translational fusion of *serU*-containing DNA fragment to p44 promoter	This study
pQE30	Ap^r, IPTG-inducible promoter expression plasmid for N-terminal His tag fusions	Qiagen
pQE30-serR	Ap^r, pQE30 carrying the *serR* gene of *B. breve* UCC2003	This study
pQE30-serR-T	Ap^r, pQE30 carrying the truncated *serR* gene of *B. breve* UCC2003	This study
pAM5	pBC1-pUC19 Tet^r [*tetW*]	3
pORI19	Em^r *repA ori*$^+$; cloning vector	21
pORI19-serR	Internal 525-bp fragment of *serR* cloned in pORI19	This study
pORI19-serU	Internal 696-bp fragment of *serU* gene cloned in pORI19	This study
pORI19-tet-serR	Internal 525-bp fragment of *serR* and *tetW* cloned in pORI19	This study
pORI19-tet-serU	Internal 696-bp fragment of *serU* gene and *tetW* cloned in pORI19	This study
pUC18	Ap^r *lacZ*α; general cloning vector	62
pUC18:inter-serpin	Ap^r, pUC18 carrying the ser_{2003} promoter	This study
pUC18:inter-serpin-Mut1	Ap^r, pUC18 carrying mutation 1 at the ser_{2003} promoter	This study
pUC18:inter-serpin-Mut2	Ap^r, pUC18 carrying mutation 2 at the ser_{2003} promoter	This study
pUC18:inter-serpin-Mut3	Ap^r, pUC18 carrying mutation 3 at the ser_{2003} promoter	This study
pUC18:inter-serpin-Mut4	Ap^r, pUC18 carrying mutation 4 at the ser_{2003} promoter	This study
pNZ272	Cm^r, pSH71 derivative containing promoterless glucuronidase gene for promoter screening	35
pNZ272-inter-serpin	pNZ272 derivative carrying the ser_{2003} promoter	This study
pNZ272-inter-serpin Mut1	pNZ272 derivative carrying the ser_{2003} promoter with mutation 1	This study
pNZ272-inter-serpin-Mut2	pNZ272 derivative carrying the ser_{2003} promoter with mutation 2	This study
pNZ272-inter-serpin-Mut3	pNZ272 derivative carrying the ser_{2003} promoter with mutation 3	This study
pNZ272-inter-serpin-Mut4	pNZ272 derivative carrying the ser_{2003} promoter with mutation 4	This study
pPKCm	Cm^r, *E. coli-Bifidobacterium* shuttle vector	6
pPK-serU	pPKCM derivative carrying the p44 promoter and the *serU* gene	This study
pPK-serRK	pPKCM derivative carrying the p44 promoter and the *serRK* genes	This study

from *B. breve* UCC2003 is subject to autoregulatory control mediated by a 2CRS, here designated SerRK.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *B. breve* UCC2003 and its derivatives were grown at 37°C in de Man-Rogosa-Sharpe (MRS) medium or reinforced clostridial medium (RCM) (Oxoid, Hampshire, England) as standing cultures supplemented with 0.05% cysteine or on RCM agar plates containing 1.5% (wt/vol) agar under anaerobic conditions in a modular atmosphere-controlled system (Davidson & Hardy Ltd., Dublin, Ireland). *Escherichia coli* strains were grown in LB medium under aerobic conditions on a rotary shaker (150 rpm) at 37°C or plated on LB agar plates. Where appropriate, media were supplemented with ampicillin (Amp; 100 μg ml^{-1}), erythromycin (Er; 100 μg ml^{-1}), chloramphenicol (Cm; 20 μg ml^{-1}), kanamycin (Kn; 50 μg ml^{-1}), or tetracycline (Tet; 10 μg ml^{-1}) for *E. coli* and Cm (2 μg ml^{-1}) or Tet (10 μg ml^{-1}) for *B. breve*.

DNA techniques and transformation. The general procedures used for DNA manipulation were previously described (39) unless otherwise specified. Restriction enzymes and T4 DNA ligase were obtained from Roche (Roche Diagnostics, East Sussex, United Kingdom) and used according to the manufacturer's instructions. PCRs were performed using *Taq* PCR master mix (Qiagen GmbH, Hilden, Germany). Synthetic oligonucleotides were synthesized by MWG Biotech AG (Ebersberg, Germany) and are described in Table S1 in the supplemental material. PCR products were purified using the High-Pure PCR product purification kit (Roche). Plasmid DNA was introduced into *E. coli* and *B. breve* by electrotransformation as previously described (28). Plasmid DNA was ob-

tained from *B. breve* and *E. coli* using the QIAprep spin plasmid miniprep kit (Qiagen GmbH, Hilden, Germany). An initial lysis step was performed using 30 mg ml^{-1} of lysozyme for 30 min at 37°C as part of the plasmid purification protocol for *B. breve*.

Bioinformatics. Sequence data were obtained from the Artemis-mediated (37) genome annotations of *B. breve* UCC2003 (34). Database searches were performed using the nonredundant sequence database accessible at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov) using TBLASTN, BLASTX, and BLASTP (1). Sequence analysis was performed using Clone Manager Professional Suite (Sci-Ed Central), and multiple local alignments were carried out with ClustalW software (46).

SerR overexpression in *B. breve*. In order to achieve SerR overexpression in *B. breve* UCC2003, the complete coding region of the *serR* gene, including its presumed ribosomal binding site, was amplified by PCR from chromosomal DNA of *B. breve* UCC2003 using the primer combination SerR-for and SerR-rev (see Table S1 in the supplemental material). The resulting PCR product was restricted at the unique PstI and XbaI sites, which had been incorporated at the 5′ end of the forward and reverse primers, respectively; ligated into the similarly digested pNZ44 vector; and introduced into *E. coli* XL1-Blue by electroporation. The expected structure of the resulting plasmid, pNZ44-serR, was verified by sequence analysis prior to its transfer to *B. breve* UCC2003 by electroporation.

Transcriptome analysis. DNA microarrays containing oligonucleotide primers representing each of the 1,864 annotated genes of the genome of *B. breve* UCC2003 (34) were obtained from Agilent Technologies (Palo Alto, CA). Overnight cultures of *B. breve* UCC2003 with pNZ44 or pNZ44-serR were used to inoculate 50 ml of MRS broth. Such cultures were then incubated at 37°C until an optical density at 600 nm (OD_{600}) of 0.5 was reached, at which point cells were harvested by centrifugation at 8,000 × *g* for 1 min at room temperature and immediately frozen prior to RNA isolation. Methods for cell disruption, RNA isolation, RNA quality control, cDNA synthesis, and indirect labeling were used as described previously (63). Labeled cDNA was hybridized using the Agilent Gene Expression hybridization kit (catalog no. 5188-5242) as described in the Agilent Two-Color Microarray-Based Gene Expression Analysis v4.0 manual (publication number G4140-90050). Following hybridization, the microarrays were washed as described in the manual and scanned using Agilent's DNA G2565A microarray scanner. The scans were converted to data files with Agilent's Feature Extraction software (version 9.5).

DNA microarray data were processed as previously described (12, 52). Differential expression tests were performed with the Cyber-T implementation of a variant of the *t* test (25). A gene was considered differentially expressed between a test condition and a control when an expression ratio of >3 or <0.33 relative to the result for the control was obtained with a corresponding *P* value that was equal to or less than 0.001. Final data presented are the averages of 2 independent array experiments. The array data were deposited in the GEO database under accession number GSE37835.

EMSA. In order to obtain the full *serR* gene or a 5′-truncated *serR* (here designated *serR-T*, lacking codons 1 through 114 of the full-length *serR* gene) from *B. breve* UCC2003, PCR amplification was performed using primer combination serR-p30F and serR-p30R or serR-CterF and serR-CterR, respectively (see Table S1 in the supplemental material). The DNA fragments encompassing *serR* or *serR-T* were restricted with BamHI and HindIII and ligated to pQE30 digested with the same enzyme combination. The ligation mixtures were introduced into *E. coli* XL1-Blue by electrotransformation, and transformants were selected based on Amp resistance. Amp^r transformants were shown by restriction and sequence analysis to contain the expected plasmids, which were designated pQE30:serR and pQE30:serR-T. Crude cell extracts were prepared as follows: overnight cultures of *E. coli* XL1-Blue independently harboring pQE30:serR and pQE30:serR-T were diluted 1:50 into fresh LB medium supplemented with 100 μg/ml of Amp and grown at 37°C with vigorous shaking. At an OD_{600} of 0.6, expression of the relevant recombinant protein was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG). Growth was continued for 4 h, and cells from 200 ml of culture were collected by centrifugation (10 min, 8,000 × *g*, 4°C) in an Avanti J-20 XP centrifuge (Beckman Coulter, Mijdrecht, The Netherlands). The pellets were washed with 50 ml of buffer A (50 mM $NaHPO_4$, 300 mM NaCl, 10 mM imidazole, 3.5% glycerol, 1 mM β-mercaptoethanol, pH 8.0) and stored at −80°C for future use. Cell extracts were prepared using 106-μm glass beads and a Mini-BeadBeater-8 cell disrupter (Biospec Products, Bartlesville, OK). After homogenization, the glass beads and cell debris were sedimented by centrifugation (30 min, 20,000 × *g*, 4°C) and the supernatant containing the cytoplasmic fraction was retained. DNA fragments representing different portions of the ser_{2003} promoter region were prepared by PCR using IRD800-labeled primer pairs (MWG Biotech; see Table S1 in the supplemental material). Electrophoretic mobility shift assays (EMSAs) were performed as described previously (53). Following electrophoresis, the presence and mobility position of the fluorescent PCR products in the gel were detected using an Odyssey infrared imaging system (Li-Cor Biosciences UK Ltd., Cambridge, United Kingdom) and captured using the supplied software Odyssey V3.0.

Site-directed mutagenesis. The ser_{2003} promoter region (170 bp; identified based on the identical and characterized ser_{210B} promoter region of *B. breve* 210B described by Turroni et al. [50]) was PCR amplified using chromosomal DNA of *B. breve* UCC2003 as a template and primer combination pUC-interF and pUC-interR (see Table S1 in the supplemental material), primers which had restriction sites incorporated at their 5′ ends. The generated DNA fragment was cut with EcoRI and XbaI and ligated into the similarly digested vector pUC18 (Table 1). The ligation mixture was introduced by electroporation into *E. coli* XL1-Blue, and transformants were selected based on Amp resistance. The expected structure of the resulting plasmid, pUC18-interSer, was verified by restriction and sequence analysis prior to its use as a probe for the site-directed mutagenesis. *In vitro* site-directed mutagenesis of the putative SerR binding site was carried out using primer combinations Mut-1F/Mut-1R, Mut-2F/Mut-2R, Mut-3/Mut-3R, and Mut-4F/Mut-4R, respectively (Table S1) and the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Sequence analysis of the resulting plasmids was performed to confirm the presence of the specific mutations.

Transcriptional fusions and GusA assays. The ser_{2003} promoter region, as well as the site-directed mutated versions of this region (see above), were amplified by PCR using primer combination pNZ272-InterF/pNZ272-InterR (see Table S1 in the supplemental material), which contained EcoRI and BglII restriction sites at their 5′ ends, respectively. Amplicons were digested with EcoRI and BglII and cloned upstream of the promoterless *gusA* gene present in the similarly restricted pNZ272 reporter vector (Table 1). Ligation mixtures were independently introduced by electroporation in *E. coli* XL1-Blue competent cells. The resulting plasmids, once verified by restriction and sequence analysis, were then transferred to *B. breve* UCC2003 by electroporation. GusA activity assays in *B. breve* UCC2003 were carried out in triplicate by independent assay as previously described by Cronin et al. (6) with the following modifications: cells were grown in MRS medium to an OD_{600} of approximately 0.4 to 0.5, at which point ser_{2003} transcription was induced by the addition of pancreatic elastase (final concentration of 1 mg/ml).

Construction of *serR* and *serU* insertion mutants. The insertion mutants were constructed using a previously described method (33). An internal fragment of the *serR* gene (between codons 26 and 201) and one of the *serU* gene (from codon 164 to codon 336) were amplified by PCR using *B. breve* UCC2003 chromosomal DNA as a template and the oligonucleotide primers serRKOF and serRKOR and serpinKOF and serpinKOR, respectively (see Table S1 in the supplemental material). The two generated amplicons were restricted with PstI and XbaI (which had been incorporated in the PCR primers); individually cloned into similarly

restricted pORI19, an Ori$^+$ RepA$^-$ integration plasmid (21); and introduced into *E. coli* EC101 by electroporation. The expected structures of the recombinant plasmids, designated pORI19-serR and pORI19-serpin, were confirmed by restriction mapping and sequencing. The *tetW* gene, amplified by PCR using pAM5 plasmid DNA as the template (3) and primers tetWf and tetWr (Table S1), thereby incorporating a SalI site, was cloned into the SalI-cut pORI19-serR and pORI19-serpin plasmids (flanking the previously inserted fragment) to generate plasmids pORI19-tet-serR and pORI19-tet-serpin. The latter plasmids were introduced into *E. coli* EC101 harboring pNZ-M.BbrII-M.BbrIII to facilitate methylation (33), and the resulting methylated pORI19-tet-serR and pORI19-tet-serpin were then independently introduced into *B. breve* UCC2003 by electroporation. Mutants were selected on reinforced clostridial agar (RCA) plates supplemented with Tet, and their expected integrated position in either the *serR* or the *serU* gene was checked by PCR. A single colony of each of the *B. breve* UCC2003 derivatives containing either a disrupted *serR* or *serU* gene was selected and designated *B. breve* UCC2003-serR or *B. breve* UCC2003-serU, respectively.

Complementation experiments. For the construction of the complementation plasmids pPK-serRK and pPK-serU, DNA fragments encompassing *serRK*, including its native promoter region, and the coding sequence of *serU* were generated by PCR amplification from chromosomal DNA of *B. breve* UCC2003 using KOD DNA polymerase and primer combination serRKF and serRKR or SerU-for and SerU-rev, respectively (see Table S1 in the supplemental material). The *serRK*-containing amplicon was digested with NotI and XbaI and ligated to similarly digested pPKCM1. The *serU* amplicon was digested with PstI and XbaI and ligated to similarly digested pNZ44. The ligations were introduced into *E. coli* XL1-Blue by electroporation, and the resulting plasmids were verified by sequence analysis and designated pPK-serRK and pNZ44-serU. Plasmid pNZ44-serU was used as a template for PCR using p44F and SerU-rev in order to amplify *serU* together with the p44 promoter. This amplicon was digested with NotI and ligated to similarly digested pPKCm1 before transformation into *E. coli* XL1-Blue. The integrity of the resulting plasmid, designated pPK-serU, was verified by restriction and sequence analysis. Finally, the plasmids pPK-serRK and pPK-serU were introduced by electroporation into *B. breve* UCC2003-serR and *B. breve* UCC2003-serU strains, to generate strains *B. breve* UCC2003-serR-pPKserRK and UCC2003-serU-pPKserU, respectively.

Measurement of protease-mediated transcriptional induction of the ser_{2003} genes by qRT-PCR. Cultures of *B. breve* UCC2003, the mutant strains UCC2003-serR and UCC2003-serU, and their complemented derivatives UCC2003-serR-pPKserRK and UCC2003-serU-pPKserU were grown at 37°C in MRS medium to an OD_{600} of 0.5, after which enzyme treatment was initiated by the addition of plasmin (3 mg/ml), papain (0.5 mg/ml), kallikrein (0.55 mg/ml), or trypsin (0.25 mg/ml) (all enzymes were obtained from Sigma, Italy). Cultures were maintained at 37°C for 90 min, after which samples (30 ml) were briefly centrifuged to harvest cells, which were used for RNA isolation as described previously (57) and then treated with DNase (Roche). A set of quantitative reverse transcriptase PCR (qRT-PCR) primers was used to target *sagA* (the first gene of the ser_{2003} operon), and the reference genes *atpD*, *tufA*, *rpoB*, *ldh*, *pdxS*, *gluC*, and *uvrD/Rep* (49). Primer design criteria were based on a desired melting temperature (T_m) between 58 and 60°C and an amplicon size of approximately 100 bp. qRT-PCR was performed using the CFX96 system (Bio-Rad, CA). PCR products were detected with SYBR green fluorescent dye and amplified according to the following protocol: one cycle of 95°C for 3 min, followed by 39 cycles of 95°C for 5 s and 66°C for 20 s. The melting curve was 65°C to 95°C with increments of 0.5°C/s. Each PCR mix contained the following: 12.5 μl 2× SYBR SuperMix Green (Bio-Rad), 1 μl of cDNA dilution, and each of the forward and reverse primers at an 0.5 μM concentration; nuclease-free water was added to obtain a final volume of 20 μl. In each run, negative controls (no cDNA) for each primer set were included. The threshold cycle ($2^{-\Delta\Delta CT}$) method (24) was used to calculate relative changes in gene expression determined from real-time quantitative PCR experiments. Results were calculated from at least two independent RNA extractions. Statistical analysis of the obtained data was performed using the CFX Manager software (Bio-Rad).

Microarray data accession number. The array data were deposited in the GEO database under accession number GSE37835.

RESULTS

Overexpression of the response regulator SerR in *B. breve* UCC2003 leads to increased transcription of the serpin-encoding gene *serU*. The *B. breve* UCC2003 genome harbors a single serpin-like gene (34), here designated *serU* (Bbr_1320), whose deduced amino acid sequence shows a high level (>90%) of similarity with serpin proteins encoded by other bifidobacteria (e.g., *B. breve* 210B, *Bifidobacterium longum* subsp. *longum* NCC2705, *B. longum* subsp. *longum* DJO10A, and *Bifidobacterium longum* subsp. *infantis* ATCC 15697), although in the case of the serpin from *Bifidobacterium dentium* Bd1 and *B. dentium* ATCC 27679 the similarity level falls to 44% (50). The SerU sequence contains, in addition to the functional domains of the serpin superfamily (PFAM00079), domains identified in alpha-1 antitrypsin, antithrombin, and angiotensinogen.

The *serU* gene is organized in a manner identical to that determined for *B. breve* 210B (50) (Fig. 1a): *serU* is preceded by a gene, here designated *sagA* (serpin-associated gene) (Bbr_1319), which encodes a membrane-associated protein and which together with *serU* forms a bicistronic operon, here designated ser_{2003}. Furthermore, two genes, here designated *serR* (Bbr_1318) and *serK* (Bbr_1317), which constitute a response regulator and a histidine protein kinase, respectively, of a predicted two-component regulatory system (2CRS), were identified immediately upstream of the *sagA* gene of both *B. breve* UCC2003 and 210B. This genetic organization is only partly conserved among the various bifidobacterial genomes (50): for example, both genes of the 2CRS as well as *sagA* are partially or completely absent in the *B. longum* strains, while in the case of *B. dentium* the *sagA* gene is absent but the 2CRS-encoding genes are present. Although the expression of bifidobacterial serpin genes has previously been shown to be induced following treatment with specific proteases (50), the role, if any, of the *serRK* 2CRS in the regulation of this expression in *B. breve* has not been investigated. In order to determine the possible involvement of *serRK* in the regulation of ser_{2003} transcription, a *B. breve* UCC2003 SerR-overexpressing derivative was generated by cloning the *serR* gene into the high-copy-number pNZ44 vector (to generate pNZ44-serR), which carries a strong constitutive promoter (see Materials and Methods) (29). Microarray analysis showed that the level of *serR* transcription in *B. breve* UCC2003 (pNZ44-serR) was 203-fold higher than that in the *B. breve* control strain containing the empty pNZ44 vector. Furthermore, from a total of 1,864 identified open reading frames of *B. breve* UCC2003 (34), microarray analysis showed a significantly increased transcriptional activity for 19 genes (>3-fold; $P \leq 0.001$), while 21 genes exhibited significantly decreased levels of transcription (<3-fold; $P \leq 0.001$) (Table 2). Among the genes with the highest level of increased transcription was *sagA* (119-fold), which is located adjacent to the *serRK* locus. Although *sagA* and the adjacent *serU* gene are organized as an operon (50), the level of transcriptional increase of the serpin-encoding gene was just 4.8-fold (Table 2). However, when these microarray results were investigated by qRT-PCR (see Materials and Methods), an equal

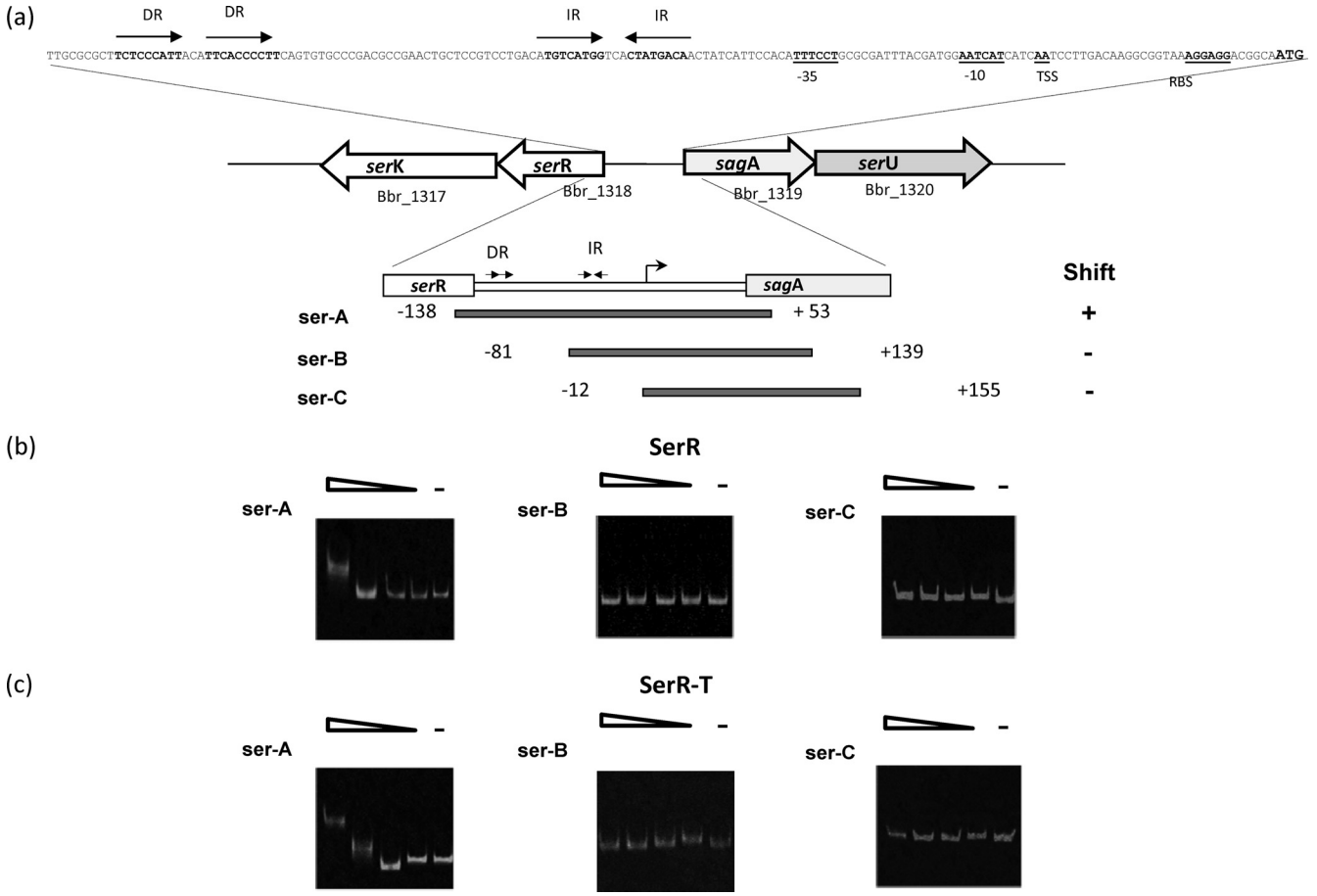

FIG 1 (a) Schematic representation of the ser_{2003} gene locus, promoter region, and DNA fragments used in the gel mobility shift assays; the numbers indicate the ends of the fragments relative to the transcriptional start site. Direct repeats (DRs) and inverted repeats (IRs) are indicated; −10 and −35 hexamers, the transcriptional starting site (TSS), and the ribosomal starting site (RBS) are underlined. (b and c) Electrophoretic mobility shift assays carried out using ser_{2003} promoter fragments and cell crude extracts containing the full SerR protein (b) or its 5′-truncated fragment SerR-T (c). Gradients correspond to 2, 1, 0.5, and 0.25 μg of protein content. Cell extract from *E. coli* harboring the empty vector pQE30 was used as a negative control.

level of upregulation for these genes was observed (data not shown). The obtained microarray data provided a first view of global gene expression changes upon SerR overexpression. However, such changes may not necessarily be due to direct transcriptional effects of the two-component system, since they may also have been caused by secondary or indirect effects, for example, as a result of stress due to protein overexpression. In order to address this issue, further experiments were carried out.

SerR specifically binds the ser_{2003} promoter. In order to obtain evidence for the possible regulatory role of the *serRK* 2CRS with regard to ser_{2003} transcription, the ability of SerR and an N-terminally truncated version, which includes the predicted SerR DNA binding domain (residues 171 to 217), to bind to the ser_{2003} locus promoter region was analyzed by electrophoretic mobility shift assay (EMSA). A DNA region containing the promoter (from position −343 to +50, relative to the presumed transcriptional start site [50] [Fig. 1a]) was prepared by PCR using IRD800-labeled primers. EMSA was carried out using crude cell extracts obtained from *E. coli* strains harboring plasmid pQE30-serR or pQE30-serR-T, while a cell extract from *E. coli* harboring the empty vector pQE30 was used as a negative control. The obtained EMSA results revealed that in contrast to the negative control, crude cell extracts obtained from *E. coli* expressing either the full-length SerR protein or its truncated version SerR-T were capable of a specific interaction with the ser_{2003} promoter region (Fig. 1b and c, ser-A panels). Additional control EMSAs, in order to probe the specificity of the DNA-protein interaction, were performed using the *pstS* (Bbr_1682) promoter (2), and as expected, this promoter region was not bound by either SerR or SerR-T (results not shown). These binding results, which demonstrate a physical interaction between the SerR protein and the ser_{2003} promoter region, support the notion of a direct regulatory role of SerR in ser_{2003} transcription, as suggested by our microarray data.

In order to identify which sequences within the promoter region are required for SerR binding, three IRD800-labeled amplicons, namely, ser-A, ser-B, and ser-C, which encompass different sections of the promoter region, were generated (Fig. 1a). The results obtained from the corresponding EMSAs showed that the SerR binding site is localized within a fragment between positions −138 and −81 (relative to the transcriptional start site of ser_{2003}), which contains an imperfect direct repeat (DR) sequence (DR_{ser}; TTCTCCCATT-3N-TTCACCCC TT), suggesting that this DR plays a role in SerR binding. Interest-

TABLE 2 Summary of gene expression changes in *B. breve* UCC2003 upon SerR overexpression[b]

Expression category and locus tag	Putative function	SerR overexpression[a]
Increased expression		
Bbr_1318	Bbr_1318; two-component response regulator, reverse	203.05
Bbr_1319	Bbr_1319; conserved hypothetical membrane-spanning protein	119.77
Bbr_1656	Bbr_1656; sugar ABC transporter, permease protein	9.89
Bbr_1657	Bbr_1657; sugar-binding protein of ABC transporter system 2045106:2046023, reverse; MW, 33,668	8.01
Bbr_1658	Bbr_1658; sugar-binding protein of ABC transporter system 2046044:2047339, reverse; MW, 45,772	7.91
Bbr_1655	*bgl3*; beta-glucosidase	6.55
Bbr_0249	Bbr_0249; ABC1 family protein kinase	6.32
Bbr_0107	*cebF*; cellobiose/cellotriose transport system permease protein F 155651:156772, forward; MW, 41,448	5.76
Bbr_1464	Bbr_1464; conserved hypothetical membrane-spanning protein 1827409:1828293, reverse; MW, 31,617	5.45
Bbr_0106	*cebE*; cellobiose/cellotriose binding protein	5.24
Bbr_1320	Bbr_1320; neuroserpin precursor	4.82
Bbr_0108	*cebG*; cellobiose/cellotriose transport system permease protein 156780:157727, forward; MW, 33,983	4.16
Bbr_1293	Bbr_1293; myosin-cross-reactive antigen	3.38
Bbr_1434	Bbr_1434; ATP-binding protein of ABC transporter system	3.37
Bbr_0109	*bgl1*; beta-glucosidase	3.35
Bbr_1433	Bbr_1433; ATP-binding protein of ABC transporter system	3.27
Bbr_1291	Bbr_1291; short-chain dehydrogenase	3.25
Bbr_1506	Bbr_1506; cyclopropane-fatty-acyl-phospholipid synthase	3.16
Bbr_1710	*rbsK5*; ribokinase	3.04
Reduced expression		
Bbr_1892	Bbr_1892; PTS system, IIC component	452.50
Bbr_1893	Bbr_1893; PTS system, IIB component	194.45
Bbr_1894	Bbr_1894; PTS system, IIA component	190.63
Bbr_1898	*nrdF*; ribonucleoside-diphosphate reductase beta chain	13.02
Bbr_1891	Bbr_1891; transcriptional regulator, GntR family	12.95
Bbr_1899	*nrdE*; ribonucleoside-diphosphate reductase alpha chain	9.45
Bbr_0273	Bbr_0273; ATP-binding protein of ABC transporter system	5.91
Bbr_0274	Bbr_0274; permease protein of ABC transporter system	5.19
Bbr_0925	Bbr_0925; permease MFS superfamily	4.95
Bbr_1501	Bbr_1501; ATP-binding protein of ABC transporter	4.76
Bbr_0195	Bbr_0195; conserved hypothetical membrane-spanning protein	3.92
Bbr_1500	Bbr_1500; conserved hypothetical membrane-spanning protein	3.84
Bbr_0939	Bbr_0939; solute binding protein of ABC transporter	3.82
Bbr_0285	*lacZ2*; beta-galactosidase	3.54
Bbr_1446	*nrdG*; anaerobic ribonucleoside-triphosphate reductase activating protein	3.53
Bbr_0940	Bbr_0940; narrowly conserved hypothetical protein	3.41
Bbr_1558	Bbr_1558; permease protein of ABC transporter system	3.39
Bbr_0734	Bbr_0734; sensory transduction protein kinase	3.34
Bbr_0116	*malQ1*; 4-alpha-glucanotransferase	3.30
Bbr_0039	*trxB1*; thioredoxin reductase/thioredoxin/glutaredoxin family	3.25
Bbr_1843	Bbr_1843; narrowly conserved hypothetical membrane-spanning protein	3.08

[a] Expression ratios presented have a Bayesian *P* value of <0.001 according to the Cyber-T test (25).
[b] Abbreviations: MW, molecular weight; PTS, phosphotransferase; MFS, major facilitator superfamily.

ingly, this fragment does not contain the inverted repeat (IR) that had previously been suggested to play a regulatory role in ser_{2003} transcription (50).

Directed mutagenesis of DR_{ser} affects SerR binding activity. In order to verify the presumed role of DR_{ser} as a recognition sequence for SerR binding to the ser_{2003} locus promoter region, mutated versions of this region in which the sequence of the DR was changed were generated and then individually tested for their ability to be bound by SerR. *In vitro* site-directed mutagenesis was performed (see Materials and Methods), generating four different plasmids, pUC18-BSMut1, pUC18-BSMut2, pUC18-BSMut3, and pUC18-BSMut4, which each harbor a differently mutated version of the putative SerR binding site (Fig. 2). These plasmids were used as the templates to amplify the mutated DR-containing fragments, which were then used for EMSAs. The results reveal that SerR retains the capacity to interact with the mutated binding site, although apparently with decreased efficacy compared to that obtained with the nonmutated binding site, as these mutated fragments were much less affected in their mobility by the presence of SerR than was the DNA fragment containing the unmutated DR_{ser} (Fig. 2).

In order to relate these observed binding differences to SerR-directed transcriptional activation of the ser_{2003} promoter, transcriptional fusions were constructed by cloning the wild-type ser_{2003} promoter and the four mutated derivatives upstream of the promoterless *gusA* reporter gene in pNZ272 (see Materials and

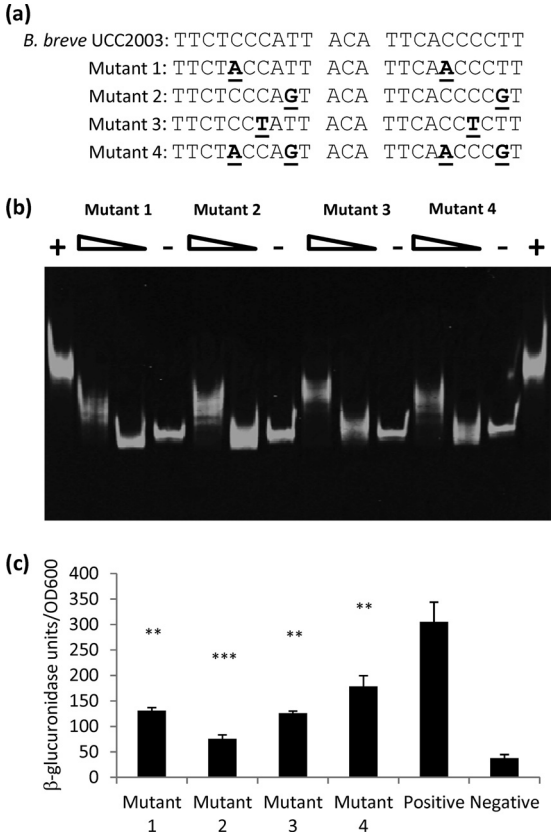

FIG 2 (a) Schematic representation of the different mutations performed in the ser_{2003} locus promoter. (b) Gel mobility shift assays carried out with crude cell extracts containing SerR and various mutated versions of the ser_{2003} promoter region (see panel a); as a positive control, the wild-type ser_{2003} promoter region was used. (c) GusA assays testing protease-mediated induction of the ser_{2003} promoter of *B. breve* UCC2003 harboring plasmid pNZ272-BSMut1, pNZ272-BSMut2, pNZ272-BSMut3, or pNZ272-BSMut4; *B. breve* UCC2003 harboring pNZ272 with the wild-type ser_{2003} promoter or without any insert was used as a positive or a negative control, respectively. Data correspond to mean values of three independent experiments, and they were compared by an unpaired *t* test analysis. Error bars correspond to the standard deviations. **, $P \leq 0.01$; ***, $P \leq 0.001$.

Methods). *B. breve* UCC2003 strains harboring these *gusA* fusion plasmids, designated pNZ272-ser, pNZ-ser-Mut1, pNZ-ser-Mut2, pNZ-ser-Mut3, and pNZ-ser-Mut4, were used for GusA assays following protease-mediated induction. Pancreatic elastase treatment clearly induced the wild-type ser_{2003} locus promoter activity. In contrast, the mutated promoters did in all cases exhibit significantly lower β-glucuronidase (GUS) activity than did the wild-type promoter (Fig. 2). Although the number of point mutations present in DR_{ser} does not show an inverse correlation with the obtained GUS activity level, since mutant 4 (harboring 4 mutations) exhibited a higher level of activity than did the other 3 mutants (harboring just 2 mutations), these results confirm the regulatory role of DR_{ser} in protease-mediated transcriptional induction of the ser_{2003} promoter and concur with the presumed role of SerR in directing this induction.

Disruption and complementation of the *serR* gene in *B. breve* UCC2003. In order to verify and further understand the regulatory connection between the *serRK*-encoded 2CRS and protease-mediated induction of the ser_{2003} operon, an insertion mutant in the *serR* gene was constructed (designated UCC2003-serR). In addition, this mutant strain was complemented by introducing a plasmid containing the complete 2CRS *serRK*, yielding *B. breve* UCC2003-serR-pPKserRK (see Materials and Methods). Wild-type, mutant, and complemented strains were analyzed for their ability to induce the ser_{2003} promoter by different proteases. qRT-PCR experiments were carried out in triplicate (Fig. 3). For the wild-type strain *B. breve* UCC2003, different proteases were shown to induce various levels of transcription of the ser_{2003} locus (as measured by qRT-PCR targeting the *sagA* gene), varying from 70-fold induction with papain to 170-fold induction following exposure to trypsin, thus confirming a protease-specific transcriptional induction. In contrast, transcriptional induction of ser_{2003} was significantly reduced in *B. breve* UCC2003-serR, thereby providing further evidence that SerR regulates expression of ser_{2003}. Protease-mediated induction of ser_{2003} transcription in the *B. breve* UCC2003-serR mutant strain was restored to levels observed for the wild-type strain *B. breve* UCC2003 when plasmid pPKserRK was introduced into UCC2003-serR, thus demonstrating complementation of the phenotype of the latter mutant (Fig. 3). Attempts to achieve complementation of UCC2003-serR using a plasmid that contained just *serR* resulted in a partial restoration of protease-mediated ser_{2003} transcription (results not shown). This partial complementation is likely due to a polar effect of the *serR* insertion mutation on *serK* expression, thus implicating the latter gene in ser_{2003} transcriptional regulation.

The SerRK signaling pathway involves *serU* and suggests protease-mediated ser_{2003} autoregulation. In order to investigate the role, if any, of the *serU* gene in protease-mediated induction of the ser_{2003} operon, a strain that carried a disrupted *serU* gene was created (designated UCC2003-serU; see Materials and Methods). Interestingly, protease-mediated transcriptional induction of ser_{2003} was significantly reduced in strain UCC2003-serU, while it was restored to wild-type levels in UCC2003-serU, in which the *serU* gene was present on plasmid pPKserU (Fig. 3). These results clearly indicate that SerU regulates ser_{2003} transcription and thus its own expression through an autoregulatory mechanism.

DISCUSSION

Members of the serpin superfamily are widely distributed throughout all kingdoms of life, including the *Eukarya*, *Prokarya*, *Archaea*, and certain viruses (18, 42). Nevertheless, their presence in the genus *Bifidobacterium* is not ubiquitous and, in fact, appears to be restricted to strains belonging to the species *B. breve*, *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, *Bifidobacterium longum* subsp. *suis*, *Bifidobacterium cuniculi*, *Bifidobacterium scardovia*, and *B. dentium* (50). Very little is known about the physiological function of serpins in bacteria beyond their protease-inhibiting activity, although previous work has shown that serpin genes of different bifidobacterial species and subspecies (*B. breve*, *B. longum* subsp. *infantis*, and *B. longum* subsp. *longum*) are highly induced when bacterial cultures are exposed to various proteases (19, 36, 50). Some of these proteases can be found in the human gut; therefore, this serpin activity can be employed by bifidobacteria to protect themselves against host-derived proteases and to provide an advantage for survival in a highly complex and competitive environment. In contrast, other gut-associated bifidobacterial species, such as *Bifidobacterium adolescentis*, *Bifidobacterium catenulatum*, and *Bifidobacterium bifidum*, do not contain a serpin-encoding gene in their genomes, suggesting that these species

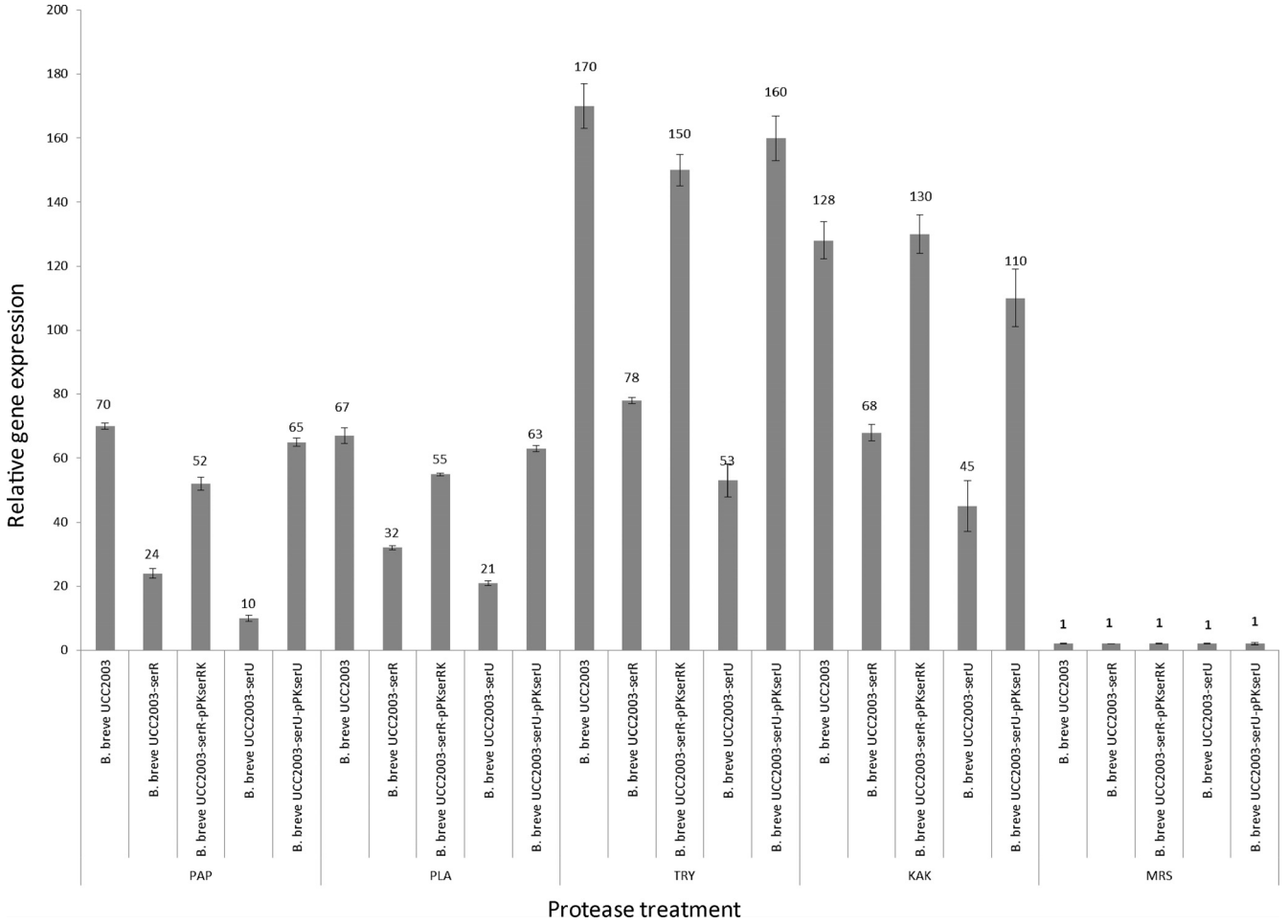

FIG 3 The relative transcription levels of ser_{2003} in *B. breve* UCC2003 and various mutant and complemented strains upon serine protease treatment versus growth in MRS-based medium as analyzed by quantitative reverse transcriptase PCR assays. The histograms indicate the relative amounts of ser_{2003} mRNAs for the specific samples. The cDNAs were generated from RNA collected following exposure of the bifidobacterial cultures, for 90 min, to kallikrein (KAK), papain (PAP), plasmin (PLA), or trypsin (TRY) or left untreated (MRS; taken as a value of 1).

may exert an alternative way of protection or may not need protection. The activation of the bifidobacterial serpin-encoding genes seems to be very specific: in strains of the subspecies *B. longum* subsp. *infantis* and *B. longum* subsp. *longum*, the highest level of induction is reached in response to human elastase and, although to a lower degree, to papain and pancreatic elastase, respectively (19). In contrast, in the *B. breve* 210B strain the highest induction was shown to occur following exposure to papain (50). The reason for the differences in the patterns of serpin gene induction between these two closely related bifidobacterial species may be linked to the different ecological niches of these bifidobacterial species, a notion that is consistent with the finding that there is no significant induction of the serpin gene of *B. dentium* Bd1 in response to any of the serine proteases examined (50).

Our data clearly implicate the 2CRS *serRK* of *B. breve* UCC2003 in the transcriptional regulation of the ser_{2003} locus. The transcriptome results combined with qRT-PCR analyses demonstrate increased transcription of the ser_{2003} locus when the response regulator SerR is overexpressed. In order to further substantiate SerRK involvement, we generated two independent insertion mutant strains, *B. breve* UCC2003-serU and *B. breve* UCC2003-serR, and demonstrated that ser_{2003} induction was significantly reduced in both mutants, although this effect was more pronounced in the former mutant, where the *serU* gene is interrupted, compared to the latter strain, which carried a disrupted *serR* gene. These data clearly suggest an autoregulatory role for SerU and perhaps the existence of an additional SerRK-independent but SerU-dependent regulatory mechanism that controls protease-mediated transcription of the ser_{2003} locus, although further research will have to substantiate this notion. The identification of the specific signal(s) that is sensed by the SerRK system will be the key to uncovering how this signal is generated by the protease exposure. Such signals may be specific peptides generated following protease-mediated degradation of cell envelope proteins or cell wall-anchored structures or may even be degradation products of *serU* itself, thus providing an explanation for the observed positive autoregulatory loop, which ensures further production of SerU when the encountered proteolytic activity is higher than the provided serpin-mediated protease inhibition.

Recently, Turroni and coworkers (50) performed a comparison of the promoter regions of *ser* genes from different bifidobacteria, including *B. breve* 210B, *B. breve* UCC2003, *B. longum* subsp.

longum NCC2705, *B. longum* subsp. *longum* DJO10A, *B. longum* subsp. *infantis* CCUG52486, and *B. longum* subsp. *infantis* ATCC 55813, finding a number of DNA motifs, including the putative −10 and −35 hexamers and a nearly perfect inverted repeat (IR) (TGTCATGG-3N-CTATGACA). These elements are also conserved in the ser_{2003} promoter sequence, but in addition a direct repeat (DR) (TTCTCCCATT-3N-TTCACCCCTT), located upstream of the IR, was found, and it may also have a regulatory role in serpin expression. The presence of this DR was investigated in the promoter regions of genes previously showing an increased transcriptional activity by microarray analysis, but it was not found in any case. In order to clarify the role of the IR and DR, EMSAs between SerR and fragments encompassing different elements of the promoter region were carried out. Our results show that the presence of the DR, but not of the IR, is necessary for SerR binding to the promoter. Both repeats are absent from the *B. dentium* Bd1 and *B. dentium* ATCC 27679 genomes, suggesting a different regulatory mechanism for the serpin gene in this bifidobacterial species. Other response regulator proteins, such as PhoR in *Corynebacterium glutamicum*, have also been shown to bind a DR (40); for instance, the DNA binding targets of response regulators belonging to the largest subfamily (i.e., the OmpR family) are typically direct repeats, whereas response regulator proteins belonging to the second largest family (the NarL family) typically bind to inverted repeats. This difference in the types of DNA sequences that are targeted by these two response regulator families reflects a fundamentally different organization of the bound RR dimers (7). SerR belongs to the LuxR family, a very heterogeneous family, in which the DNA binding sequences are not fully characterized but in many cases have been identified as IRs (32). The role of DR_{2003} in SerR binding capacity was further investigated by mutagenesis experiments combined with EMSAs. The results demonstrated that SerR was able to bind the mutated binding site, though with decreased binding efficiency, which is then thought to diminish SerR's activity as a transcriptional activator, an assumption that was corroborated by GUS assays performed under protease induction conditions. These results confirm the role of DR_{2003} in the transcriptional regulation of the ser_{2003} operon and further advance our understanding of serpin gene expression in *B. breve*.

The results described in this work allow us to conclude that SerRK is a 2CRS that controls the transcriptional induction of the ser_{2003} locus from *B. breve* UCC2003 following specific protease treatment. Data derived from the analysis of a *B. breve-serU* mutant indicate that the SerRK signaling pathway includes a SerU-dependent autoregulatory feedback. However, the molecular mechanism driving this regulated expression, as well as the specific signals sensed by the SerRK 2CRS, remains elusive. Further investigation, with particular emphasis on the *in vivo* biological role of the serpin in the gut, is ongoing in order to elucidate these questions.

ACKNOWLEDGMENTS

The Alimentary Pharmabiotic Centre is a research center funded by Science Foundation Ireland (SFI), through the Irish Government's National Development Plan. We and our work were supported by SFI (grants 02/CE/B124 and 07/CE/B1368), a postdoctoral fellowship from the Spanish Ministry of Science and Innovation (MICINN) to P.A.-M., and an HRB postdoctoral fellowship (grant PDTM/20011/9) awarded to M.O.M. This work was also financially supported by a FEMS Advanced Fellowship 2011 and an IRCSET Embark postdoctoral fellowship to F.T.

REFERENCES

1. **Altschul SF, et al.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**:3389–3402.
2. **Alvarez-Martin P, et al.** 2012. A conserved two-component signal transduction system controls the response to phosphate starvation in *Bifidobacterium breve* UCC2003. Appl. Environ. Microbiol. **78**:5258–5269.
3. **Alvarez-Martin P, O'Connell-Motherway M, van Sinderen D, Mayo B.** 2007. Functional analysis of the pBC1 replicon from *Bifidobacterium catenulatum* L48. Appl. Microbiol. Biotechnol. **76**:1395–1402.
4. **Bisanz JE, Reid G.** 2011. Unraveling how probiotic yogurt works. Sci. Transl. Med. **3**:106ps41. doi:10.1126/scitranslmed.3003291.
5. **Crépin S, et al.** 2011. The Pho regulon and the pathogenesis of *Escherichia coli*. Vet. Microbiol. **153**:82–88.
6. **Cronin M, Knobel M, O'Connell-Motherway M, Fitzgerald GF, van Sinderen D.** 2007. Molecular dissection of a bifidobacterial replicon. Appl. Environ. Microbiol. **73**:7858–7866.
7. **de Been M, Bart MJ, Abee T, Siezen RJ, Francke C.** 2008. The identification of response regulator-specific binding sites reveals new roles of two-component systems in *Bacillus cereus* and closely related low-GC Gram-positives. Environ. Microbiol. **10**:2796–2809.
8. **Fanning S, et al.** 2012. Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection. Proc. Natl. Acad. Sci. U. S. A. **109**:2108–2113.
9. **Fukuda S, et al.** 2011. Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature **469**:543–547.
10. **Furrie E, et al.** 2005. Synbiotic therapy (*Bifidobacterium longum*/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial. Gut **54**:242–249.
11. **Gao R, Mack TR, Stock AM.** 2007. Bacterial response regulators: versatile regulatory strategies from common domains. Trends Biochem. Sci. **32**:225–234.
12. **García de la Nava GJ, van Hijum SAFT, Trelles O.** 2003. PreP: gene expression data pre-processing. Bioinformatics **19**:2328–2329.
13. **Gettins PG.** 2002. Serpin structure, mechanism, and function. Chem. Rev. **102**:4751–4804.
14. **He T, et al.** 2008. Effects of yogurt and bifidobacteria supplementation on the colonic microbiota in lactose-intolerant subjects. J. Appl. Microbiol. **104**:595–604.
15. **Hoch JA.** 2000. Two-component and phosphorelay signal transduction. Curr. Opin. Microbiol. **3**:165–170.
16. **Huntington JA.** 2011. Serpin structure, function and dysfunction. J. Thromb. Haemost. **9**:26–34.
17. **Imaoka TS, et al.** 2008. Anti-inflammatory activity of probiotic *Bifidobacterium*: enhancement of IL-10 production in peripheral blood mononuclear cells from ulcerative colitis patients and inhibition of IL-8 secretion in HT-29 cells. World J. Gastroenterol. **14**:2511–2516.
18. **Irving JA, et al.** 2002. Serpins in prokaryotes. Mol. Biol. Evol. **19**:1881–1890.
19. **Ivanov D, et al.** 2006. A serpin from the gut bacterium *Bifidobacterium longum* inhibits eukaryotic elastase-like serine proteases. J. Biol. Chem. **281**:17246–17252.
20. **Kullen MJ, Klaenhammer TR.** 2000. Genetic modification of intestinal lactobacilli and bifidobacteria. Curr. Issues Mol. Biol. **2**:41–50.
21. **Law J, et al.** 1995. A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. J. Bacteriol. **177**:7011–7018.
22. **Leahy SC, Higgins DG, Fitzgerald GF, van Sinderen D.** 2005. Getting better with bifidobacteria. J. Appl. Microbiol. **98**:1303–1315.
23. **Le Leu RK, Hu Y, Brown IL, Woodman RJ, Young GP.** 2010. Synbiotic intervention of *Bifidobacterium lactis* and resistant starch protects against colorectal cancer development in rats. Carcinogenesis **31**:246–251.
24. **Livak KJ, Schmittgen TD.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta}C_T$ method. Methods **25**:402–408.
25. **Long AD, et al.** 2001. Improved statistical inference from DNA microarray data using analysis of variance and a Bayesian statistical framework. Analysis of global gene expression in *Escherichia coli* K12. J. Biol. Chem. **276**:19937–19944.

26. **MacConaill LE, Butler D, O'Connell-Motherway M, Fitzgerald GF, van Sinderen D.** 2003. Identification of two-component regulatory systems in *Bifidobacterium infantis* by functional complementation and degenerate PCR approaches. Appl. Environ. Microbiol. **69**:4219–4226.
27. **Mascher T, Helmann JD, Unden G.** 2006. Stimulus perception in bacterial signal-transducing histidine kinases. Microbiol. Mol. Biol. Rev. **70**: 910–938.
28. **Mazé A, O'Connell-Motherway M, Fitzgerald GF, Deutscher J, van Sinderen D.** 2007. Identification and characterization of a fructose phosphotransferase system in *Bifidobacterium breve* UCC2003. Appl. Environ. Microbiol. **73**:545–553.
29. **McGrath S, Fitzgerald GF, van Sinderen D.** 2001. Improvement and optimization of two engineered phage resistance mechanisms in *Lactococcus lactis*. Appl. Environ. Microbiol. **67**:608–616.
30. **McNulty NP, et al.** 2011. The impact of a consortium of fermented milk strains on the gut microbiome of gnotobiotic mice and monozygotic twins. Sci. Transl. Med. **3**:106ra106. doi:10.1126/scitranslmed.3002701.
31. **Mitrophanov AY, Groisman EA.** 2008. Signal integration in bacterial two-component regulatory systems. Genes Dev. **22**:2601–2611.
32. **Nasser W, Reverchon S.** 2007. New insights into the regulatory mechanisms of the LuxR family of quorum sensing regulators. Anal. Bioanal. Chem. **387**:381–390.
33. **O'Connell Motherway M, O'Driscoll J, Fitzgerald GF, van Sinderen D.** 2009. Overcoming the restriction barrier to plasmid transformation and targeted mutagenesis in *Bifidobacterium breve* UCC2003. Microb. Biotechnol. **2**:321–332.
34. **O'Connell Motherway M, et al.** 2011. Functional genome analysis of *Bifidobacterium breve* UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. Proc. Natl. Acad. Sci. U. S. A. **108**:11217–11222.
35. **Platteeuw C, Simons G, de Vos WM.** 1994. Use of the *Escherichia coli* beta-glucuronidase (*gusA*) gene as a reporter gene for analyzing promoters in lactic acid bacteria. Appl. Environ. Microbiol. **60**:587–593.
36. **Roberts TH, Hejgaard J, Saunders NF, Cavicchioli R, Curmi PM.** 2004. Serpins in unicellular Eukarya, Archaea, and Bacteria: sequence analysis and evolution. J. Mol. Evol. **59**:437–447.
37. **Rutherford KJ, et al.** 2000. Artemis: sequence visualization and annotation. Bioinformatics **10**:944–945.
38. **Saavedra JM, Bauman NA, Perman JA, Yolken RH, Oung I.** 1994. Feeding of *Bifidobacterium bifidum* and *Streptococcus thermophilus* to infants in hospital for prevention of diarrhoea and shedding of rotavirus. Lancet **344**:1046–1049.
39. **Sambrook J, Russell DW.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
40. **Schaaf S, Bott M.** 2007. Target genes and DNA-binding sites of the response regulator PhoR from *Corynebacterium glutamicum*. J. Bacteriol. **189**:5002–5011.
41. **Schell M, et al.** 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.
42. **Silverman GA, et al.** 2001. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. J. Biol. Chem. **276**:33293–33296.
43. **Stanton C, Ross RP, Fitzgerald GF, van Sinderen D.** 2005. Fermented functional foods based on probiotics and their biogenic metabolites. Curr. Opin. Biotechnol. **16**:198–203.
44. **Steck N, et al.** 2011. *Enterococcus faecalis* metalloprotease compromises epithelial barrier and contributes to intestinal inflammation. Gastroenterology **141**:959–971.
45. **Stock AM, Robinson VL, Goudreau PN.** 2000. Two-component signal transduction. Annu. Rev. Biochem. **69**:183–215.
46. **Thompson JD, Higgins DG, Gibson TJ.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids. Res. **22**:4673–4680.
47. **Turroni F, Ribbera A, Foroni E, van Sinderen D, Ventura M.** 2008. Human gut microbiota and bifidobacteria: from composition to functionality. Antonie Van Leeuwenhoek **94**:35–50.
48. **Turroni F, et al.** 2009a. Exploring the diversity of the bifidobacterial population in the human intestinal tract. Appl. Environ. Microbiol. **75**: 1534–1545.
49. **Turroni F, et al.** 2011. Global genome transcription profiling of *Bifidobacterium bifidum* PRL2010 under in vitro conditions and identification of reference genes for quantitative real-time PCR. Appl. Environ. Microbiol. **77**:8578–8587.
50. **Turroni F, et al.** 2010. Characterization of the serpin encoding gene of *Bifidobacterium breve* 210B. Appl. Environ. Microbiol. **76**:3206–3219.
51. **Turroni F, et al.** 2009b. Microbiomic analysis of the bifidobacterial population in the human distal gut. ISME J. **3**:745–751.
52. **van Hijum SAFT, et al.** 2005. A generally applicable validation scheme for the assessment of factors involved in reproducibility and quality of DNA-microarray data. BMC Genomics **6**:77. doi:10.1186/1471-2164-6-77.
53. **Van Sinderen D, et al.** 1995. *comK* encodes the competence transcription factor, the key regulatory protein for competence development in *Bacillus subtilis*. Mol. Microbiol. **15**:455–462.
54. **Ventura M, et al.** 2007. Genomics of actinobacteria: tracing the evolutionary history of an ancient phylum. Mol. Biol. Rev. **71**:495–548.
55. **Ventura M, Canchaya C, Fitzgerald GF, Gupta RS, van Sinderen D.** 2007. Genomics as a means to understand bacterial phylogeny and ecological adaptation: the case of bifidobacteria. Antonie Van Leeuwenhoek **91**:351–372.
56. **Ventura M, et al.** 2009. The *Bifidobacterium dentium* Bd1 genome sequence reflects its genetic adaptation to the human oral cavity. PLoS Genet. **5**:e1000785. doi:10.1371/journal.pgen.1000785.
57. **Ventura M, Foley S, Bruttin A, Canchaya C, Brüssow H.** 2002. Transcription mapping as a tool in phage genomics: the case of the temperate *Streptococcus thermophilus* phage Sfi21. Virology **296**:62–76.
58. **Ventura M, et al.** 2009. Genome-scale analyses of health-promoting bacteria: probiogenomics. Nat. Rev. Microbiol. **7**:61–71.
59. **Wang KY, et al.** 2004. Effects of ingesting *Lactobacillus*- and *Bifidobacterium*-containing yogurt in subjects with colonized *Helicobacter pylori*. Am. J. Clin. Nutr. **80**:737–741.
60. **Wang Y, Jones PJH, Ausman LM, Lichtenstein AH.** 2004. Soy protein reduces triglyceride levels and triglyceride fatty acid fractional synthesis rate in hypercholesterolemic subjects. Atherosclerosis **173**:269–275.
61. **Xiao JZ, et al.** 2003. Effects of milk products fermented by *Bifidobacterium* longum on blood lipids in rats and healthy adult male volunteers. J. Dairy Sci. **86**:2452–2461.
62. **Yanisch-Perron C, Vieira J, Messing J.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
63. **Zomer A, et al.** 2009. An interactive regulatory network controls stress response in *Bifidobacterium breve* UCC2003. J. Bacteriol. **191**:7039–7049.