Controlled Gene Expression in *Bifidobacteria* by Use of a Bile-Responsive Element

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The promoter activity of the upstream region of the bile-inducible gene *betA* from *Bifidobacterium longum* subsp. *longum* NCC2705 was characterized. DNA fragments were cloned into the reporter vector pMDYAbfB, and the arabinofuranosidase activity was determined under different *in vitro* conditions. A segment of 469 bp was found to be the smallest operational unit that retains bile inducibility. The reporter activity was strongly affected by the presence of ox gall, cholate, and conjugated cholate, but not by other bile salts and cell-surface-acting compounds. Remarkably, this bile-inducible system was also active in other bifidobacteria containing *betA* homologs.

The organism *Bifidobacterium longum*, a commensal bacterium which is dominant in the gut microbiota of humans (7, 8, 21, 22), is one of the *Bifidobacterium* species for which health-promoting effects have been attributed to particular probiotic strains (3). In the intestine, bifidobacteria are exposed to bile salts, detergent-like biological molecules synthesized from cholesterol in the liver, whose major physiological function is to facilitate fat absorption during digestion. Apart from this, bile salts perform a key ecological role in the establishment of the intestinal microbiota, since they display antimicrobial activity, mainly through cell membrane damage caused by their amphipathic properties (13). Thus, only those microorganisms able to cope with physiological concentrations of bile salts will be able to survive or transiently colonize the gut. A common strategy used by enteric bacteria to counteract the deleterious effect of bile salts is the synthesis of bile efflux systems (14, 20). By modulating the expression of these transporters, bacteria are able to regulate the intracellular concentration of bile and diminish its biological damage.

Due to the limitations and the scarcity of effective molecular tools for bifidobacteria, functional genomics in this genus could not be exploited until very recently (16, 17, 25). While several authors have characterized gene regulation in intestinal lactobacilli under gastrointestinal conditions (i.e., pH and bile) (4, 11, 18, 23), and even some pH-inducible expression systems are available (12), inducible gene expression systems have not been developed in bifidobacteria yet. Furthermore, bifidobacteria are much more abundant than lactobacilli in the human gut; thus, they are thought to be a promising alternative for the local delivery of therapeutic agents. In this respect, some progress has been made using *B. longum* as a delivery vector of tumor-suppressing proteins (5, 24). However, to the best of our knowledge, bacterial delivery systems triggered by an intestinal signal to specifically release bioactive proteins into the intestine have not been developed yet.

Gene expression in bifidobacteria has been the subject of some studies, but only a few regulated promoters have been identified (26). During the course of our research on bile response of bifidobacteria, we have discovered a bile-inducible transporter, *BetA*, from *B. longum* NCC2705 (GenBank accession no. [AE014295.3](#)) (6). In the present work, we have characterized the upstream region of *betA* in order to explore its suitability for developing con-

trolled gene expression systems specifically induced in the intestinal tract.

Previous experiments have shown that the expression of the gene *betA* was induced more than 30 times when *B. longum* NCC2705 was grown in the presence of subinhibitory concentrations of bile, compared with conditions in which bile was absent (6). In order to find out if gene induction increased in a concentration-dependent manner, we grew *B. longum* strain NCC2705 in DeMan, Rogosa, Sharpe broth (MRS; Difco) supplemented with 0.25% L-cysteine (Sigma) (MRSc) at 37°C in a Mac500 anaerobic chamber (Don Whitley Scientific). Unless specified otherwise, these growth conditions were used throughout. Also, at least three biological replicates were carried out for all growth experiments described in the present study. Ox bile extract (ox gall; Sigma) was added to the medium at 0, 0.02, 0.10, and 0.14% (wt/vol), and *betA* expression was quantified by quantitative real-time PCR (qRT-PCR), using the primers BL0920F and BL0920R (Table 1), as previously described (6). We found that the expression of the gene increased linearly in this bile concentration range ($R^2 = 0.918$), *betA* was induced more than 90 times at the highest concentration tested and about 13 times at the lowest (see Fig. S1 in the supplemental material). These results seem to indicate that the expression of the gene is tightly regulated by the presence of bile and that it can be modulated by using increasing amounts of bile. It is worth mentioning that the bile concentrations used for this analysis are within the physiological range that can be found in the human intestine (2).

A fragment of 573 bp and shorter fragments derived from it by deleting bases in the 5' region were cloned upstream from the reporter gene *abfB* (GenBank accession no. [AY259087](#)) in the plasmid pMDYAbfB (Fig. 1). This plasmid is a derivative of the reporter vector pMDY23 (10), in which the original *gusA* gene was

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TABLE 1 Vectors and primers used in this study

Vector or primer	Characteristics ^a	Reference or source
Vectors		
pMDY23	Sp ^r , general reporter vector, <i>gusA</i> reporter gene	10
pMDYAbfB	Sp ^r , general reporter vector, <i>abfB</i> reporter gene	This work
pMDYP573AbfB	pMDYAbfB derivative, 573-bp fragment cloned upstream of NsiI site	This work
pMDYP469AbfB	pMDYAbfB derivative, 469-bp fragment cloned upstream of NsiI site	This work
pMDYP367AbfB	pMDYAbfB derivative, 367-bp fragment cloned upstream of NsiI site	This work
pMDYP267AbfB	pMDYAbfB derivative, 267-bp fragment cloned upstream of NsiI site	This work
pMDYP167AbfB	pMDYAbfB derivative, 167-bp fragment cloned upstream of NsiI site	This work
Primers		
BL0920F	GCCGCCTCCCTGTTCAA	6
BL0920R	TAGGGCTGATCGTCCATGATTC	6
pMDY23AbfBF	TCTAGACCAATGCATACCACTCACAACAGCCAGTATTC (NsiI)	This work
pMDY23AbfBR	TCTAGACCAAGCTTTTATTAGCCGTGGAATTCACGCTG (HindIII)	This work
Prom573F	GCTCGTAGATCTGCCTCGCATGTTTCAGCTTCAC (BglII)	This work
Prom469F	TCAGGCAGATCTTATGCGGTAAGCCCAATC (BglII)	This work
Prom367F	TCAGGCAGATCTTACGAAGACCCCATCAAGGAG (BglII)	This work
Prom267F	TCAGGCAGATCTCAATCCACGAATGGCCCTATTC (BglII)	This work
Prom167F	TCAGGCAGATCTCTCACCATAGCCATCAACTTAC (BglII)	This work
Pro-T	GCGTTTTTCTGTGAGATGCATAGCATTTCTCTGTTGGTAACG (NsiI)	This work

^a Restriction enzyme sites are underlined, and the corresponding restriction enzyme is shown in parentheses.

replaced by *abfB* from *B. longum* (15). To do that, *abfB* was amplified with *Pfx* DNA polymerase (Invitrogen), using total DNA from *B. longum* NB667 as a template, and the primers pMDY23AbfBF and pMDY23AbfBR. The resulting PCR product was digested with NsiI and HindIII and ligated into pMDY23 vector, previously digested with the same enzymes, to yield pMDYAbfB. To our knowledge, this is the first vector using a bifidobacterial gene as a reporter. AbfB displays a very high arabinofuranosidase activity that can be monitored, by using *pNP*-arabinofuranoside as a synthetic substrate, through a wide *pNP* concentration range (1, 15). Afterwards, different DNA fragments, ranging from 573 bp to 167 bp, were cloned upstream from the NsiI site of pMDYAbfB (ATGCAT, containing the start codon of *abfB*). Fragments were amplified by PCR using the corresponding Prom forward primers and the reverse primer Pro-T (Table 1) and subsequently digested with the enzymes BglII and NsiI according to the manufacturer's instructions. The PCR products were cloned into the promoter reporter vector pMDYAbfB, similarly digested, to generate pMDYP573AbfB, pMDYP469AbfB, pMDYP367AbfB, pMDYP267AbfB, and pMDYP167AbfB. For plasmid construction details, see Fig. 1.

Once all of the constructs were obtained, the vectors were introduced into *B. longum* NCC2705 cells by electrotransformation, according to Álvarez-Martín et al. (1), and the reporter activity was determined under different *in vitro* conditions. Previous experiments have shown that cholate, a primary bile salt, strongly induces *betA* expression (data not shown). Protein extracts of *B. longum* NCC2705 transformants grown in the absence of cholate and in the presence of 0.06% cholate, harboring pMDYAbfB and the derived plasmids, were obtained as previously described (1), and the arabinofuranosidase activity was determined on cell extracts using *pNP*-arabinofuranoside. Briefly, cells from 10 ml of cultures were harvested by centrifugation, washed twice with 100 mM potassium phosphate buffer (pH 6.8), and resuspended in 2 ml of the same buffer. The cell suspensions were transferred to tubes containing glass beads (acid washed, $\leq 106 \mu\text{m}$ in diameter;

Sigma), and cells were lysed using a FastPrep FP120 cell disrupter (Thermo Savant). Following 30 s of disruption at a speed of 4.5, samples were held on ice for 1 min, and this step was repeated 3 times. Cell debris was removed by centrifugation, the protein content of cell extracts was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce), and the enzymatic activity was measured according to Margolles and de los Reyes-Gavilán (15) (Fig. 2). The constructs containing the two larger DNA fragments (469 and 573 bp) displayed some arabinofuranosidase activity in the absence of cholate that was increased several times when cholate was present in the medium (Fig. 2). The remaining shorter versions displayed low or almost undetectable activity, similar to that displayed by the construct containing the control plasmid (pMDYAbfB), and did not show any induction. The arabinofuranosidase activity of *B. longum* NCC2705 extracts coming from cells without vector was negligible.

Since the *B. longum* NCC2705 derivatives containing pMDYP469AbfB and pMDYP573AbfB showed similar reporter activity, we considered the 469-bp DNA fragment to be the shortest functional unit that retains bile inducibility. Thus, the arabinofuranosidase activity was determined in the presence of subinhibitory concentrations of different bile salts commonly found in human bile (19), as well as in the presence of other known membrane- and cell-wall-damaging agents, in order to ascertain whether the bile-responsive element was also affected by other compounds acting on the cell surface (Fig. 3). In this respect, there are remarkable findings showing that some bacteria sense the effect of bile on the cell surface, by using two-component regulatory systems or other specific cell surface sensors for the perception of external stimuli, able to trigger gene expression upon contact with the compounds (9, 18). Therefore, we also tested the effect of some detergents (SDS and Tween 80), antibiotics acting on the cell wall (bacitracin and vancomycin), ionophores (valinomycin), and cholesterol. Previous growth experiments were performed to determine the higher compound concentrations that did not affect growth rate (data not shown), and the corresponding subinhibi-

FIG 1 Schematic representation of the constructs used in this work. (A) Main features of the *betA* upstream region (unpublished data) deduced after the analysis with Softberry and Neural Network Promoter Prediction. Pin-like symbols represent potential inverted repeats. (B) Sizes of the different fragments of the *betA* upstream region. (C) Construction of pMDY23-derived vectors.

tory concentrations were used in this experiment. Different percentages of cecum juice were included as well in this experiment. Ox gall, cholate, glycocholate, and taurocholate, but not other bile salts, strongly induced the arabinofuranosidase activity (more than 25 times with cholate and taurocholate) (Fig. 3). Finally, all of

the other compounds did not significantly affect the reporter activity, suggesting that this 469-bp DNA fragment is specifically responding to bile salts (mainly cholate and conjugated salts) but not to other compounds acting on the cell surface.

Finally, in order to test the suitability of the vector to be used in

FIG 2 Arabinofuranosidase activity of total protein extracts of *B. longum* NCC2705 derivatives, containing the vectors with the *betA* upstream regions with different sizes or the empty vector (control). The bars indicate the results from protein extracts from cells grown in the absence (dark gray) or presence (gray) of 0.06% cholate. AU, arbitrary units of arabinofuranosidase activity. One unit of enzyme activity was defined as the amount of protein that releases 1 nmol of *p*NP per min.

different bifidobacterial hosts, we transformed pMDYP469AbfB into *Bifidobacterium breve* NCIMB8807, *Bifidobacterium pseudolongum* subsp. *pseudolongum* LMG11571, *Bifidobacterium animalis* subsp. *lactis* IPLAIC4, *Bifidobacterium longum* subsp. *infantis* CECT4551, *Bifidobacterium adolescentis* LMG10502, and *Bifidobacterium pseudocatenulatum* M115. We were able to detect induction of arabinofuranosidase activity, in the presence of cholic acid, in *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, *B. breve*, *B. pseudocatenulatum*, and *B. adolescentis*. However, the activity was extremely low in *B. pseudolongum* and *B. animalis*, two species belonging to the *B. pseudolongum* phylogenetic group that seem to be less evolutionarily adapted to the human intestinal tract (21). In addition, the presence of cholic acid did not increase the reporter activity in these two species (Fig. 4). It is worth mentioning that *betA* homologs are present in *B. breve*, *B. longum* subsp. *infantis*, *B. pseudocatenulatum*, and *B. adolescentis*, but not

FIG 3 Arabinofuranosidase activity of total protein extracts of *B. longum* NCC2705 containing pMDYP469AbfB in the presence of potential inducers. AU, arbitrary units; GC, glycocholate; TC, taurocholate; GDC, glycodeoxycholate; TDC, taurodeoxycholate; CJ, cecum juice.

FIG 4 Arabinofuranosidase activity of total protein extracts of *Bifidobacterium* strains from different species, containing pMDYP469AbfB, grown in the presence of 0.025% (black) or 0.05% (gray) cholic acid. The y axis indicates the fold increase of arabinofuranosidase activity compared with activity in the absence of cholic acid.

in species belonging to the *B. pseudolongum* group (6), thus suggesting that the signal able to trigger gene induction is only sensed in those bifidobacterial hosts containing *betA* orthologs.

In conclusion, we have characterized a bile-responsive DNA element located upstream from the *betA* gene of *B. longum* NCC2705. The minimal functional unit needed for gene inducibility seems to be located between 367 and 469 bp upstream of the coding region. The inclusion of this DNA fragment in delivery vectors, in order to target bioactive proteins to the intestine, is a very promising application. To the best of our knowledge, bacterial vectors specifically activated in the presence of intestinal signals have not been developed yet. Our next step is to validate the application of this system under *in vivo* conditions.

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REFERENCES

- Alvarez-Martín P, Flórez AB, Margolles A, del Solar G, Mayo B. 2008. Improved cloning vectors for bifidobacteria, based on the *Bifidobacterium catenulatum* pBC1 replicon. *Appl. Environ. Microbiol.* 74:4656–4665.
- Begley M, Gahan CG, Hill C. 2005. The interaction between bacteria and bile. *FEMS Microbiol. Rev.* 29:625–651.
- Bergogne-Bérézin E. 2000. Treatment and prevention of antibiotic associated diarrhea. *Int. J. Antimicrob. Agents* 16:521–526.
- Bron PA, et al. 2004. Genetic characterization of the bile salt response in *Lactobacillus plantarum* and analysis of responsive promoters *in vitro* and *in situ* in the gastrointestinal tract. *J. Bacteriol.* 186:7829–7835.
- Cronin M, et al. 2010. Orally administered bifidobacteria as vehicles for delivery of agents to systemic tumors. *Mol. Ther.* 18:1397–1407.
- Gueimonde M, Garrigues C, van Sinderen D, de los Reyes-Gavilán, CG, Margolles A. 2009. Bile-inducible efflux transporter from *Bifidobacterium*

- longum* NCC2705, conferring bile resistance. Appl. Environ. Microbiol. 75:3153–3160.
7. Haarman M, Knol J. 2005. Quantitative real-time PCR assays to identify and quantify fecal *Bifidobacterium* species in infants receiving a prebiotic infant formula. Appl. Environ. Microbiol. 71:2318–2324.
 8. Hill JE, et al. 2010. Improvement of the representation of bifidobacteria in fecal microbiota metagenomic libraries by application of the cpn60 universal primer cocktail. Appl. Environ. Microbiol. 76:4550–4552.
 9. Jordan S, Hutchings MI, Mascher T. 2008. Cell envelope stress response in Gram-positive bacteria. FEMS Microbiol. Rev. 32:107–146.
 10. Klijn A, et al. 2006. Construction of a reporter vector for the analysis of *Bifidobacterium longum* promoters. Appl. Environ. Microbiol. 72:7401–7405.
 11. Kullen MJ, Klaenhammer TR. 1999. Identification of the pH-inducible, proton-translocating F1F0-ATPase (*atpBEFHAGDC*) operon of *Lactobacillus acidophilus* by differential display: gene structure, cloning and characterization. Mol. Microbiol. 33:1152–1161.
 12. Kullen MJ, Klaenhammer TR. December 2000. Acid-inducible promoters for gene expression. Patent WO/2000/078922.
 13. Kurdi P, Kawanishi K, Mizutani K, Yokota A. 2006. Mechanism of growth inhibition by free bile acids in lactobacilli and bifidobacteria. J. Bacteriol. 188:1979–1986.
 14. Lin J, et al. 2005. Bile salts modulate expression of the CmeABC multidrug efflux pump in *Campylobacter jejuni*. J. Bacteriol. 187:7417–7424.
 15. Margolles A, de los Reyes-Gavilán CG. 2003. Purification and functional characterization of a novel alpha-L-arabinofuranosidase from *Bifidobacterium longum* B667. Appl. Environ. Microbiol. 69:5096–5103.
 16. O'Connell-Motherway M, et al. 2008. Characterization of ApuB, an extracellular type II amylopullulanase from *Bifidobacterium breve* UCC2003. Appl. Environ. Microbiol. 74:6271–6279.
 17. 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.
 18. Pfeiler EA, Azcarate-Peril MA, Klaenhammer TR. 2007. Characterization of a novel bile-inducible operon encoding a two-component regulatory system in *Lactobacillus acidophilus*. J. Bacteriol. 189:4624–4634.
 19. Ridlon JM, Kang DJ, Hylemon PB. 2006. Bile salt biotransformations by human intestinal bacteria. J. Lipid Res. 47:241–259.
 20. Thanassi DG, Cheng LW, Nikaido H. 1997. Active efflux of bile salts by *Escherichia coli*. J. Bacteriol. 179:2512–2518.
 21. Turroni F, van Sinderen D, Ventura M. 2009. Bifidobacteria: from ecology to genomics. Front. Biosci. 14:4673–4684.
 22. Turroni F, et al. 2009. Microbiomic analysis of the bifidobacterial population in the human distal gut. ISME J. 3:745–751.
 23. Whitehead K, Versalovic J, Roos S, Britton RA. 2008. Genomic and genetic characterization of the bile stress response of probiotic *Lactobacillus reuteri* ATCC 55730. Appl. Environ. Microbiol. 74:1812–1819.
 24. Xu YF, et al. 2007. A new expression plasmid in *Bifidobacterium longum* as a delivery system of endostatin for cancer gene therapy. Cancer Gene Ther. 14:151–157.
 25. Yasui K, et al. 2009. Improvement of bacterial transformation efficiency using plasmid artificial modification. Nucleic Acids Res. 37:e3.
 26. Zomer A, et al. 2009. An interactive regulatory network controls stress response in *Bifidobacterium breve* UCC2003. J. Bacteriol. 191:7039–7049.