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Construction, characterization and exemplificative application of bioluminescent *Bifidobacterium longum* biovar *longum*

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ABSTRACT

The aim of this work was to construct a bifidobacterial biosensor that could be used to analyze the metabolic state of cells. We transformed by electroporation the human intestinal bacterium *Bifidobacterium longum* biovar *longum* with a vector (pGBL8b) containing the insect luciferase gene from a click beetle (*Pyrophorus plagiophthalamus*) and studied the basic parameters affecting light production in the bioluminescent phenotype. We detected a minimum of 4000 cells, which indicates that the insect luciferase expression in *Bifidobacterium longum* is extremely good, and a measurement requires only a few minutes of incubation in ambient oxygen conditions. A pH of 7.0 was optimal for incorporating the substrate D-luciferin, and the substrate saturation effect occurred at 125 μM. We employed bioluminescent *B. longum* for a quick test of the efficacy of different carbohydrates to preserve cell physiology under acidic conditions. The prebiotic compounds Actilight® and lactulose were the most active in preventing loss of intracellular ATP during incubation at pH 3. Glucose and inulin were less active, though still effective. In sum, our results show that bioluminescent *B. longum*, transformed with the pGBL8b plasmid, is a valuable tool for rapidly studying the physiological state of anaerobic bacterial cells under different environmental conditions.

1. Introduction

Probiotics are a fast-growing category of alimentary and pharmaceutical products predominantly constituting microbes of the genera *Lactobacillus* and *Bifidobacterium* (Del Piano et al., 2006). Whereas lactobacilli have a long history of industrial application, bifidobacteria are greatly limited in their use because most of their strains are sensitive to stresses such as oxygen and acidity (Gomes and Malcata, 1999). Only a few strains, mainly belonging to the species *Bifidobacterium animalis*, *B. longum* and *B. breve*, are successfully employed in dairy industry. Due to their promising health-promoting properties (Goktepe et al., 2006; Iwabuchi et al., 2007; Lahtinen et al., 2007), interest is increasingly being focused on creating conditions suitable for profitable industrial use of *Bifidobacterium* strains of human origin, belonging to species such as *B. adolescentis*, *B. bifidum* or *B. longum*. To gain this goal, new approaches are sought after for quick and efficient selection of new strains and growth conditions.

Insect luciferase is a commonly used reporter gene (Lewis et al., 1998; Wilson and Hastings, 1998), which catalyzes a reaction in which ATP, D-luciferin and O_2 are converted to AMP, CO_2, inorganic pyrophosphate and oxyluciferin, together with yellowish light production at 560 nm. Therefore, firefly luciferase catalyzes a bioluminescent reaction that depends stoichiometrically on ATP. ATP is involved in many essential metabolic processes, and its intracellular level provides a measure of the metabolic state of a cell. Quantification of light emission, i.e., bioluminescence, is one of the most sensitive real-time means of assessing cell conditions, metabolism included. For these reasons, luminescent reporter systems based on insect luciferase have been successfully employed for several analytical purposes in a number of microbes (Lahtinen et al., 2007; Loimaranta et al., 1998; Selbitschka et al., 2006; Virta et al., 1998).

Prebiotics are non-digestible food ingredients, which affect the host beneficially by selectively stimulating the growth and/or activity of resident bacteria considered beneficial for human health, e.g., bifidobacteria and lactobacilli (Gibson and Roberfroid, 1995). The use in association with probiotic bacteria of prebiotic carbohydrates, such as fructoligosaccharides (FOS), inulin, or lactulose, is increasing in foods and pharmaceutical preparations (Young, 1998). Moreover, there is increasing interest in identifying new potential prebiotic molecules (Borrelli et al., 2004; Tzortzis et al., 2005) and studying the effect of prebiotics on the physiology of bacterial cells (Saulnier et al., 2007).

In this report, we describe the construction of a *Bifidobacterium longum* strain containing a bioluminescence reporter gene for high

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light emission. We characterized the conditions affecting the light emission and, finally, tested the system by studying the role of different prebiotic and non-prebiotic carbohydrates in protecting bacterial cells at low pH. To our knowledge, this is the first functional bioluminescence reporter gene system ever described for a bifidobacterial strain.

2. Materials and methods

2.1. Plasmids, bacterial strains, and media

Table 1 lists the bacterial strains and plasmids used in this study. *Bifidobacterium* strains were grown at 37 °C in MRS (Difco, Detroit, MI,) supplemented with 0.05% L-cysteine hydrochloride (cMRS) in an anaerobic chamber (80% N_2, 10% CO_2, 10% H_2). *Escherichia coli* was cultivated at 37 °C in SOB medium (Bacto-tryptone (Difco) 20 g l^{-1}, Bacto-yeast extract (Difco) 5 g l^{-1}, 1 M $MgCl_2$ 10 ml l^{-1}, NaCl 0.5 g l^{-1}, 1M KCl 2.5 ml l^{-1}, pH 7). Antibiotics (supplied by Sigma, St. Louis, MO) were administered at the following concentrations: *E. coli*, 200 μg erythromycin (Em) ml^{-1}, 10 μg kanamycin ml^{-1}, 6-μg chloramphenicol (Cm) ml^{-1}, *Bifidobacterium*, 10 μg Cm ml^{-1}.

2.2. Vector construction

A DNA fragment of 3 kb, containing the luciferase gene *lucGR* of *Pyrophorus plagiophthalamus* and the chloramphenicol acetyl transferase gene under a phage T5 promoter, was amplified from vector pCSS962 (Lampinen, 1995). For this reaction, we used DyNAzyme EXT DNA Polymerase (Finnzymes Oy, Espoo, Finland) and primers LucKpnF (5′-TGCGTAAGGAGAA*GGTACC*GCATCA-3′) and LucKpnR (5′-GGCT*GGTACC*TTGTGTGGAATTGTGA-3′), which contained the restriction site *Kpn*I (underlined). After restriction with *Kpn*I, the PCR product was ligated in the *Kpn*I site of plasmid pGOSBif33 (Guglielmetti et al., 2007) and cloned into *E. coli* VE7108 to obtain vector pGBL8b (Fig. 1). Ligation reaction was performed overnight in 10 μl with 2.5 U of T4 ligase (Fermentas, Vilnius, Lithuania) at 15 °C. *E. coli* transformation was performed by electroporation according to conventional methods (Sambrook and Russell, 2001).

2.3. Electroporation of bifidobacteria

Electrocompetent *Bifidobacterium* cells were prepared as described in (Guglielmetti et al., 2007). Electroporation was carried out with a MicroPulser Electroporator (Biorad, Milano, Italy), set at 12.5 kV/cm and employing a 2-mm cuvette (time constants obtained between 3.9 and 4.2 ms).

Table 1
Bacterial strains and plasmids used in the study

Bacterial strains or plasmids	General information	Reference
Strains		
E. coli VE7108	Gram negative host of pGOSBif33	Guglielmetti et al. (2007)
B. longum NCC2705	From Nestlè culture collection; whole genome available (GenBank: NC_004307)	Schell et al. (2002)
B. longum MIML3	Human adult intestinal isolate	This work
B. longum MIML4	Isolated from commercial fermented milk	This work
B. animalis subsp. *lactis* Bb12	Probiotic commercial strain	Ouwehand et al. (2004)
Plasmids		
pCSS962	Source for the cassette T5 promoter-*lucGR-cat*	Lampinen (1995)
pGOSBif33	*E. coli* VE7108-*Bifidobacterium* shuttle vector	Guglielmetti et al. (2007)
pGBL8b	*lucGR*	This work

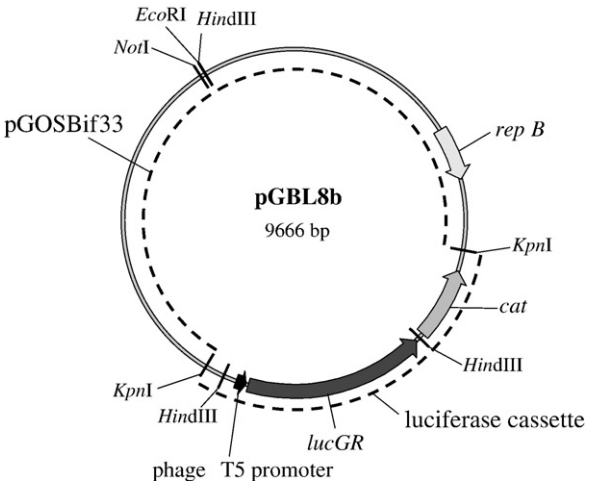


Fig. 1. Map of reporter vector pGBL8b.

2.4. Plasmid extraction

Plasmid DNA was extracted from bifidobacteria by means of a Qiagen kit (Qiagen, Valencia, CA) with an additional incubation step in 20 mg/ml lysozyme at 37 °C for 2 h, performed before beginning the Qiagen kit protocol.

2.5. ARDRA and BOX analyses

Genotyping of recombinant and wild-type *B. longum* cells was carried out by ARDRA and BOX analyses. ARDRA was performed according to Ventura et al. (2001). In detail, Taq Polymerase was used to amplify the 16 S rRNA gene by PCR using the primers P0 (5′-GAAGAGTTTGATCCTGGCTCAG-3′) and P6 (5′-CTACGGCTACCTTGTTACGA-3′). Each PCR mixture (50 μl) contained a reaction mix of 20 mM Tris–HCl, 50 mM KCl, 200 μM of each deoxynucleoside triphosphate, 0.5 μM of each primer, 1.5 mM $MgCl_2$ and 1.5 U of Taq polymerase (Fermentas). Each PCR cycling profile consisted of an initial denaturation time of 2 min at 95 °C followed by an amplification for 35 cycles of denaturation (30 s at 95 °C), annealing (45 s at 55 °C) and extension steps (2 min at 72 °C). The PCR was completed with an elongation period (7 min at 72 °C). The resulting amplicons were digested for 3 h at 37 °C in 15-μl volumes of incubation buffer (Fermentas) containing 2 U of the restriction enzyme MboI (Fermentas). The resulting digestion products were visualised under UV-light (260 nm) after 3% agarose gel electrophoresis.

BOX analysis was done with the primer BOXA1R (5′-CTACGGCAAGGCGACGCTGACG-3′) according to Masco et al. (2003). PCR mix was as for ARDRA analysis with 1 μM of the only BoxA1 primer. The PCR profile consisted of an initial denaturation time of 5 min at 95 °C followed by an amplification for 35 cycles of denaturation (60 s at 94 °C), annealing (60 s at 45 °C) and extension steps (5 min at 72 °C). The PCR was completed with an elongation period (10 min at 72 °C). PCR products were visualized through 2% gel electrophoresis.

2.6. Light emission measurements

To measure the light emission from *B. longum* cells, we dispensed samples in a 96-well white microtiter plate in quadruplicate, immediately added the substrate D-luciferin (Sigma, St. Louis, MO) to the cell suspensions, and measured the luminescence signal with a luminometer (Chameleon Multilabel Detection Platform, Hidex OY, Turku, Finland) under aerobic conditions. All bioluminescence measurements were performed at 37 °C in a constant mode. The maximum in light emission was measured after about 16–18 min from

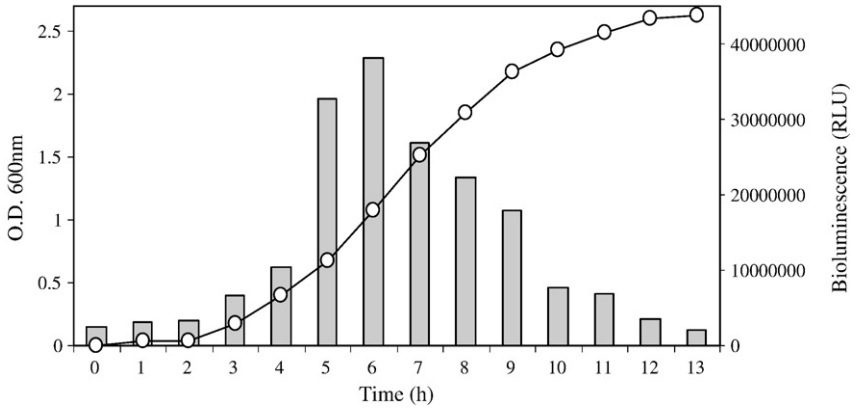


Fig. 2. Growth curve of recombinant *B. Longum* NCC2705 as measured by optical density and bioluminescence. The growth of *B. longum* cells in cMRS containing 10 μg/ml chloramphenicol was monitored by turbidity (O.D. at 600 nm; curve) and bioluminescence (relative light units, RLU; bars) after the addition of 125 μM D-luciferin at pH 7. The values are the means of three independent experiments conducted in quadruplicates. The variation between the replicates was less than 10%.

the addition of D-luciferin. Light emission was studied as a function of the number of cells by diluting the cell suspensions and by measuring both bioluminescence and colony forming units (CFU) in the samples. To optimize light emission from *B. longum*, we considered various parameters. The effect of pH was studied by adding a constant amount (125 μM) of D-luciferin to a 0.1-M phosphate-citrate buffer, adjusted to pHs ranging from 4.5 to 7.0. A bioluminescence reaction was started by adding 50 μl of substrate buffer to a 200-μl sample of living bacterial cells in the microtiter wells. The effect of the D-luciferin concentration was studied similarly on a range of 5–250 μM at pH 7.

2.7. Testing of carbohydrates at low pH

We tested six different carbohydrates: glucose, lactose, inulin (Sigma), lactulose (Dorom, Milano, Italy), lactitol (Sigma), and Actilight® 950P (Giulio Gross, Cinisello Balsamo, Italy). Early-log, bioluminescent *B. longum* cells were suspended in glycine-HCl buffer at pH 3 (approximately 10^8 cells/ml), supplemented with 20 g/l of each carbohydrate. The samples were incubated anaerobically for 2 h, followed by a light emission measurement at each 60 min, as described above, using a 250-μM D-luciferin solution at pH 7. Cells were also counted on cMRS agar plates, after anaerobic incubation for 4 days at 37 °C.

3. Results and discussion

This study sought to construct a bifidobacterial luminescent biosensor that could be used for a quick analysis of the metabolic state of cells under different conditions. To construct a luminescent *Bifidobacterium* strain, we electroporated *B. longum* NCC2705 with vector pGBL8b, which contains the luciferase gene (*lucGR*) from *P. plagiophtalamus* under the control of a T5 phage promoter. The strain *B. longum* NCC2705 is the first *Bifidobacterium* strain with a complete, annotated genome (Schell et al., 2002), and it is known to be transformable (Klijn et al., 2006). We obtained 81 colonies, corresponding to a transformation rate of 2×10^2 clones/μg of recombinant DNA. All recombinant isolates displayed a luminescent phenotype in the presence of D-luciferin. Though the insect luciferase reporter gene system has been demonstrated suitable for many applications in a number of microbes (Lahtinen et al., 2007; Loimaranta et al., 1998; Selbitschka et al., 2006; Virta et al., 1998), such a system has not yet been constructed for bifidobacteria, partly because of difficulties in manipulating the DNA of these microrganisms and partly because of the misconception that aerobic reporter proteins would not work in anaerobic bacteria. However, our results showed that only a few minutes of incubation in ambient oxygen conditions were enough to detect bioluminescence in anaerobically cultivated bifidobacteria.

We compared an arbitrarily selected clone to the wild-type strain. The recombinant strain grew in cMRS supplemented with 10 μg/ml chloramphenicol at a rate similar to that of the wild type and exhibited the same morphology in optical microscopy, indicating that the level of the luciferase expression in recombinant *B. longum* keeps the cells physiologically viable. The electrophoretic plasmid profile of the clone showed two bands; restriction analysis showed that one band corresponded to the cryptic plasmid of *B. longum* NCC2705 (pBLO1) and the other to pGBL8b (data not shown). ARDRA and BOX analyses were performed to investigate possible genetic heterogeneity between the wild type and the luminescent clone. These analyses are commonly used to genetically type microbes and generally demonstrate inter- and intra-specific discriminating power, respectively (Masco et al., 2003; Tzortzis et al., 2005). The analyses, however, did not help differentiate between the wild type and recombinant strain (data not shown 1, only for referees). Two other *B. longum* biovar *longum* strains were also successfully transformed, one of human origin and the other isolated from commercial fermented milk (Table 1, data not shown).

3.1. Characterization of light emission

Light emission measured as a function of cell growth revealed that bioluminescence drops drastically when cells reach the mid-log phase

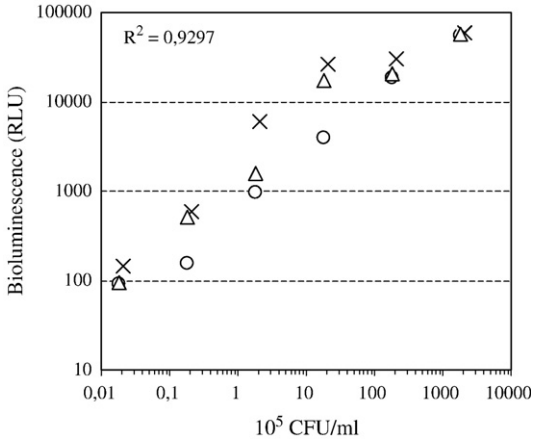


Fig. 3. Linear relationship between bacterial cells and bioluminescence. Samples from early-logarithmic growth-phase bacteria were serially diluted, and light emission (relative light units, RLU) was determined after the addition of 125 μM D-luciferin at pH 7. The number of CFU was also determined on agar plates from the same samples. Each symbol indicates the results of one independent experiment. R^2=linear regression coefficient, calculated for all measurements.

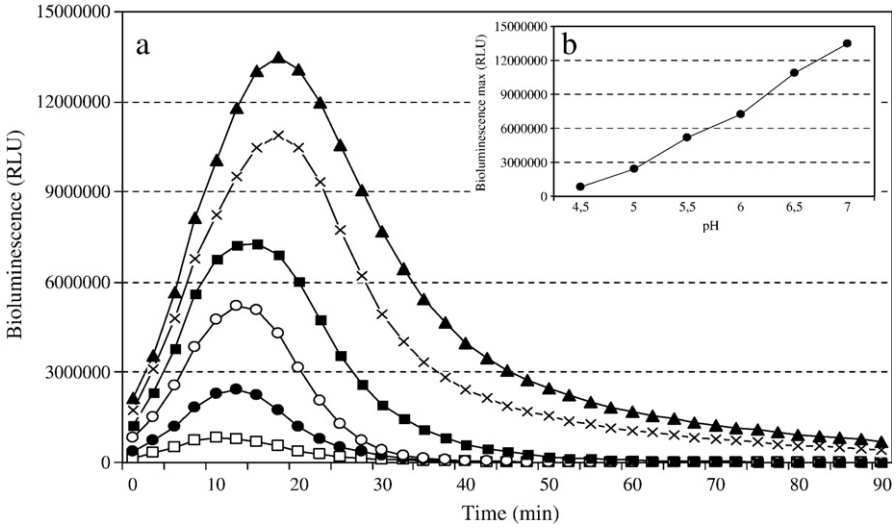


Fig. 4. Light emission as a function of time and pH. Bioluminescence (in relative light units, RLU) of early-log phase *B. longum* cells was determined at pH 4.5 (□), 5 (●), 5.5 (○), 6 (■), 6.5 (x) and 7 (▲) after the addition of 125 μM D-luciferin (a). (b) Highest light emission as a function of pH. The values are the means of two independent measurements, conducted in quadruplicate. The variation between the measurements was less than 10%.

(Fig. 2). At the early-logarithmic stage of their growth, *B. longum* recombinant cells emit bioluminescence at a level directly proportional to the number of CFU (Fig. 3). The same luciferase gene cassette we employed in this work (*cat* and *lucGR* genes under phage T5 promoter) was reported to allow detection of fewer than 100 cells of recombinant *E. coli* and about 30,000 cells of *Streptococcus mutans* (Loimaranta et al., 1998). In this study, the minimum number of cells detected over the background number was about 4000, indicating that the phage T5 promoter controlling the insect luciferase expression is extremely high in *B. longum* NCC2705.

Our data revealed that the pH of the D-luciferin solution is important for the light emission of early-log phase bifidobacteria. Several studies have shown that under slightly acidic conditions the substrate for the luciferase reaction is efficiently incorporated in prokaryotic cells. For instance, pH 5 was reported to be the optimum for *E. coli* (Wood and DeLuca, 1987) and *Bacillus subtilis* (Lampinen, 1995) and pH 6 for *Streptococcus mutans* (Loimaranta et al., 1998). In our experiments, unexpectedly pH 7 produced the highest intensity of bioluminescence (Fig. 4), probably as a consequence of a different structure of the cytoplasmatic membrane in bifidobacteria. Also in *Staphylococcus aureus* the optimal pH for luciferin incorporation was reported to be close to neutrality (Tenhami et al., 2001).

We observed that the substrate saturation effect occurred at a concentration above 125 μM (Fig. 5). In subsequent experiments, we used bacterial cells at early-exponential growth and a 250-μM D-luciferin solution at pH 7.

3.2. Role of prebiotics in acid stress tolerance

In order to evaluate the suitability of the novel biosensor in the responsive monitoring of the metabolic state of cells, we employed bioluminescent *B. longum* for a quick test of the efficacy of different

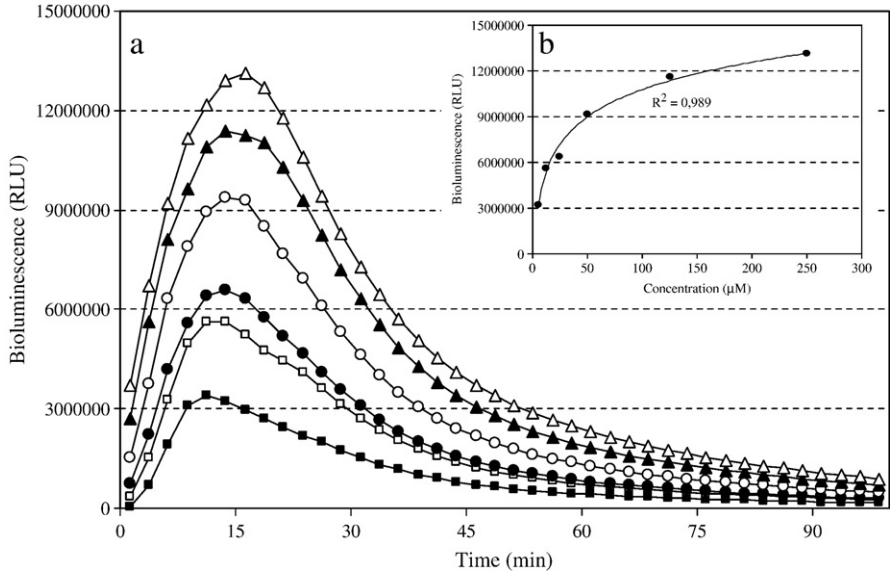
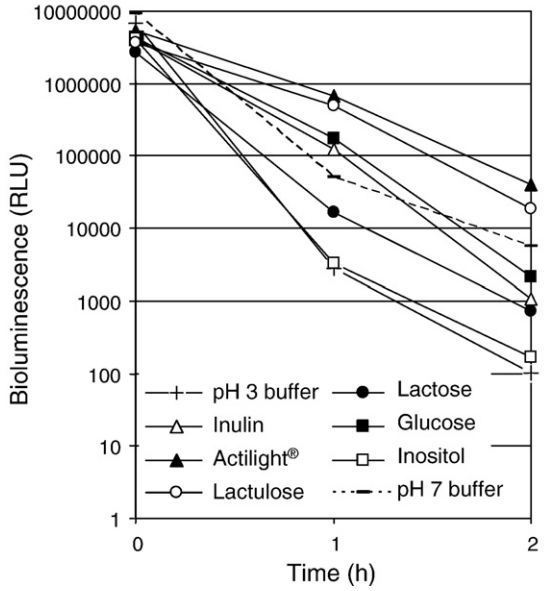


Fig. 5. Decay of light emission of early-log phase *B. longum* cells at pH 7 with different D-luciferin concentrations: 5 (■), 12.5 (□), 25 (●), 50 (○), 125 (▲) and 250 (Δ) μM (a). (b) Highest light emission as a function of D-luciferin concentration. R^2 refers to the logarithmic regression coefficient. The values are the means of three independent measurements conducted in quadruplicate. The variation between the replicates was less than 10%.

A



B

	Viable count (log cfu/ml) Treatment time (h)		
	0	1	2
No carbohydrate	7.5	< 1	/
Glucose	7.5	2.1	1.1
Lactose	7.5	1.7	< 1
Inositol	7.5	< 1	/
Inulin	7.5	2.3	1.1
Actilight® 950P	7.5	2.2	1.4
Lactulose	7.5	2.1	< 1
pH 7	7.5	7.5	6.8

Fig. 6. Effect of different carbohydrates on light emission (A) and cultivability (B) of *B. longum* NCC2705 incubated at pH 3. A: Light emission is shown as curve of luminescence decay. The "0" point refers to the first measurement and corresponds to a technical time of about 5 min after the addition of the bacterial cells to the cell suspension. Measurements are the average of at least three independent experiments, conducted in quadruplicate for each test molecule. The variation between the measurements was less than 10%. B: Cultivability was determined through agar plate counts. The results are shown as the average of two independent experiments.

carbohydrates to preserve cell physiology under acidic conditions. In bifidobacteria and other anaerobic bacteria, intracellular pH is maintained primarily by the activity of the F1F0-ATPase (Matsumoto et al., 2004; Sanchez et al., 2006; Ventura et al., 2004). Even slightly more efficient carbon utilization by bacterial cells can lead to an enhanced ATP production, which the F1F0-ATPase could exploit for extruding protons. This assumption is supported by the observed overproduction of enzymes involved in carbohydrate catabolism and complex carbohydrates utilization by cells under acidic stress (Sanchez et al., 2006). The above consideration validates the selection of an optimal carbon source for improving bacterial resistance to low pH.

We incubated early-log phase cells of recombinant *B. longum* in glycin-HCl buffer at pH 3 for 2 h in the presence of six different carbohydrates and then measured the decrease in bioluminescence and cultivable cells by agar plate count (Fig. 6). Because most *B. longum* strains tolerate acid poorly (Matsumoto et al., 2004), up to 99.9% of cells were not cultivable after only 1 h of incubation at pH 3. The cultivable cell number increased slightly in the presence of the carbohydrates glucose, lactose, inulin, Actilight®, and lactulose, compared to inositol or no carbohydrates. However, the cell counts differed only slightly and did not help determine which carbohydrate was the most effective in protecting bifidobactrial cells at low pH.

In contrast, bioluminescence measurements indicated a wider heterogeneity in the capabilities of the various carbohydrates to prevent light decay at pH 3 (Fig. 6). Such a marked difference between the two techniques may depend on the low sensitivity of the agar plate count, resulting from the number of cultivable cells diminishing too fast at pH 3. Moreover, under stress many bacterial cells lose their cultivability though they remain alive and metabolically active (Tenhami et al., 2001). In contrast, the luciferase biosensor can monitor in real-time the physiological state of all cells, because it correlates sensitively to intracellular ATP. Moreover, a bioluminescence reporter system is much less time-consuming and laborious to apply than a conventional agar plate count.

Regarding the effects of carbohydrates to prevent luminescence dacay at pH 3, more in detail, inositol had no significant effect on the intracellular metabolic state during incubation at pH 3 (Fig. 6). *B. longum* NCC2705 fermented poorly inositol (Table 2), whose fermentation by bifidobacteria depends on the strain (Sakata et al., 2002). Inositol is a carbocyclic polyol, whose prebiotic significance is not well known. Bifidobacteria have been reported to grow poorly on inulin and to prefer to degrade shorter fractions such as fructooligosaccharides (Falony et al., 2006; Louis et al., 2007; Ryan et al., 2005). Although an inulinase gene is present in the genome of *B. longum* NCC2705 (β-fructofuranosidase, accession number NP_695334, 25), *B. longum* NCC2705 fermented inulin to a limited degree (Table 2). The effect of this polysaccharide on bacterial cells at pH 3 was similar to that of glucose. The mechanism by which inulin can preserve intracellular ATP in *B. longum* cells at pH 3 remains unknown. However, we can speculate that the inulin-binding ability of *B. longum* NCC2705 inulinase may promote the attachment of cells to inulin and result in the protection of bacteria from low pH or other environmental stresses. Rossi et al. (2005) showed that only eight *Bifidobacterium* strains out of 55 could grow on inulin. They described the bifidogenic effect of this molecule in batch cultures containing fecal slurries. They hypothesized (Rossi et al., 2005) that this effect was the result of cross-feeding due to the release of shorter molecules from inulin by primary degraders. Our results suggest that it would be interesting to investigate also the inulin's possible prebiotic role, independent of its fermentation by bacterial strains.

We found no solid correlation between light emission and the ability of bacterial cells to ferment carbohydrates (Fig. 6 and Table 2).

Table 2
Broth culture pH after growth of *Bifidobacterium longum* NCC2705 in TPY medium without sugar (pH 7.2), anaerobically, at 37 °C for 4 days, in presence of 20 g/l of reported carbohydrate

Substrate	pH
Glucose	3.99
Lactulose	3.99
Actilight®	4.15
Lactose	4.44
Inositol	5.43
Inulin	6.50

Glucose and lactose are preferential carbon sources for *B. longum* (Parche et al., 2006), but these compounds made intracellular ATP available only to a limited degree compared to Actilight® and lactulose, which were the most active in preventing intracellular ATP loss during cell incubation at pH 3. Actilight® is a commercial product comprising a mix of short-chain fructooligosaccharides (1-kestose, nystose, and fructosylnystose; FOS) and produced from sucrose by the activity of a fructosyltransferase enzyme. Lactulose (4-*O*-β-D-galactopyranosyl-D-fructofuranose) is a keto analog of lactose and has for decades been used as a laxative agent. Lactulose resists gastrointestinal transit and reaches the large intestine, where it reportedly acts bifidogenically (Bouhnik et al., 2004). Our experiments with luminescent *B. longum* indicate that, under acidic stress condition, bifidogenic prebiotics such as FOS or lactulose can sensitively improve cell physiology. Our results are consistent with the use of these compounds in symbiotic food products, in order to help bifidobacteria to survive gastric transit. To our knowledge, this is the first study in which a probiotic microorganism of intestinal origin has been evaluated in real-time experiments together with putative prebiotic molecules, some of which are clearly beneficial to the metabolism of the organism.

In conclusion, luminescent *B. longum*, transformed with the pGBL8b plasmid encoding the insect luciferase *lucGR* gene, can be applied to monitor the intracellular metabolic state in real-time under different environmental conditions. The reporter system in this study enabled rapid, nondestructive analysis of cells and will now make possible to pursue in-depth studies of the specific gene expression of bifidobacteria in real-time.

Acknowledgements

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