Expression of the bglH Gene of *Lactobacillus plantarum* Is Controlled by Carbon Catabolite Repression

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A newly identified *bglH* gene coding for a phospho-β-glucosidase of *Lactobacillus plantarum* was isolated and expressed in *Escherichia coli*. The sequence analysis of the cloned DNA fragment showed an open reading frame encoding a 480-amino-acid protein with a calculated molecular mass of 53 kDa. The *bglH* gene was shown to be expressed on a monocistronic transcriptional unit. Its transcription was repressed 10-fold in *L. plantarum* cells grown on glucose compared to the β-glucoside salicin as a sole carbon source. A catabolite-responsive element (CRE) spanning from −3 to +11 with respect to the transcriptional start point was found, and its functionality was assessed by mutational analysis. In vitro and in vivo DNA binding experiments suggested the occurrence of a DNA-protein complex at the CRE site, which would mediate glucose repression of *bglH* expression.

Much effort has been devoted in most recent years to the understanding of regulation of carbon metabolism in gram-positive bacteria (11, 16, 20, 26, 28–31, 36). Carbon catabolite repression (CCR) has been extensively studied in *Bacillus subtilis*, and it has been shown to be different from the well-known CCR mechanism operating in *Escherichia coli* and other gram-negative bacteria, where the catabolite gene activator protein in complex with cyclic AMP activates transcription from catabolite repression-responsive operons (27). CCR in *B. subtilis* involves a negative regulatory mechanism characterized by the cis-acting catabolite-responsive element (CRE) and the CcpA trans-acting element belonging to the GalR-LacI family of bacterial regulatory proteins (13, 14, 17, 39, 40). Mutations in CRE sequences occurring in various carbon catabolite-responsive operons result in loss of glucose repression (17, 18). It has been recently reported that the negative control underlying catabolite repression in *B. subtilis* might represent a global regulatory mechanism for gram-positive bacteria (16, 29, 30). Immunological cross-reactivity experiments have recently shown the presence of the catabolite control protein CcpA in many gram-positive bacteria (20). Very recently CcpA-mediated catabolite repression has been reported also for *Lactobacillus casei* (25).

In this paper we report the cloning and expression in *E. coli* of the phospho-β-glucosidase-encoding *bglH* gene of *Lactobacillus plantarum*, one of the most widespread lactic acid bacteria in the environment and also widely used in fermented-food technology, where β-glucosidase activity is responsible for reduction of bitter flavor (10, 38). The *bglH* gene complemented a *bgl* mutation of *E. coli*. It was shown to be homologous to the *bglH* gene of *B. subtilis*, encoding a phospho-β-glucosidase active on aryl-β-glucosides such as salicin and arbutin and belonging to the *bglPH* operon, whose expression is regulated by a CcpA-mediated carbon catabolite repression (18, 19, 21). Our results indicate that *bglH* expression is negatively regulated by glucose and suggest that this control might be exerted by a CcpA-like mechanism.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *L. plantarum* B21, isolated from a brining stage of naturally ripened olives (5), was used throughout this study. *L. plantarum* was grown in MRS medium (Oxoid) supplemented with 2% glucose, 1% ribose, or 0.4% salicin. *E. coli* FA31 (ara rbs15 xy1 lacY1 mglP1 bglB208 bglY202) (6) was grown in TY or M9 minimal salts supplemented with 0.4% glucose or 0.4% salicin.

**DNA cloning and sequencing.** Total DNA from *L. plantarum* B21 was prepared as described previously (22), partially digested with *Sar*3Al, and size fractionated through a sucrose gradient as described previously (32). DNA fragments of 5 kb (average size) were ligated into the *bgl* operon, whose expression is negatively regulated by glucose and suggest that this control might be exerted by a CcpA-like mechanism.

**Primer extension analysis.** Total RNA from *L. plantarum* cells grown to mid-exponential phase was isolated as described by Leong-Morgenthaler and coworkers (23). Primer extension products were obtained by using oligonucleotide SPI (5′-GCCACCTGGTAAAGCGCC-3′), corresponding to the complement of nucleotide positions 351 to 331 (Fig. 1). The oligonucleotide was end labeled with [γ-32P]ATP and T4 polynucleotide kinase, and the reverse transcriptase reaction was performed as described previously (32). The extension products were separated by electrophoresis on 6% polyacrylamide urea sequencing gels (32) and were visualized and quantified by autoradiography on phosphor storage plates (PhosphorImager, Molecular Dynamics). As a reference, sequencing reactions were performed by the dideoxy-chain termination method (33) by using the same primer as in the primer extension experiments.

**Assay of β-glucosidase activity.** *Lactobacillus plantarum* cells (100 ml) were grown to mid-exponential phase in MRS supplemented with either 2% glucose or 0.4% salicin, washed twice with 150 mM NaCl, and resuspended in 1 ml of 50 mM phosphate buffer (pH 6.2). Appropriate aliquots of cell suspensions were added to 800 μl of 30 mM salicin in phosphate buffer. After 20 min of incubation at 30°C, the enzymatic reaction was stopped by adding 500 μl of 1 M Na2CO3. The production of saligenin from salicin was detected as described previously (34).

**Gel retardation analysis.** Gel retardation experiments were performed as described previously (37). Cell extracts were obtained by resuspending exponential cultures in buffer containing 20 mM Tris HCl (pH 8.0), 100 mM KCl, 1 mM EDTA (pH 8.0), 0.5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride, followed by grinding with glass beads. Binding was carried out for 15 min at room
temperature in 20 mM Tris HCl (pH 8.0)–100 mM KCl–5 mM MgCl₂–1 mM EDTA (pH 8.0)–0.5 mM dithiothreitol–10% glycerol–0.02% Nonidet P-40, 2 μg of bovine serum albumin–1 μg of poly(dI-dC). A synthetic oligonucleotide called CRE1 (5’-GGGGCAGCTATTGTACCTATTGATGACGGCGAC-G3’) and its complement CRE2 were annealed to form a 40-bp fragment (CREs) containing the bglH sequence spanning from nucleotides (nt) 184 to 211 (Fig. 1). Oligonucleotide CRE3 (5’-GGGGCAGCTATTGTACCTATTGATGACGGCGAC-G3’) and its complement CRE4 were used to construct a 40-bp fragment (CREb) containing three mismatches in the CRE sequence (underlined).

In vivo footprinting. L. plantarum cells were grown overnight in MRS medium supplemented with 0.4% salicin or 2% glucose. Cells were then diluted 1:100 and grown to mid-exponential phase. Methylation was performed by adding freshly diluted dimethyl sulfate (DMS; Aldrich) to a final concentration of 0.1% for 3 min at 30°C with shaking. The methylation reaction was stopped by adding an equal volume of ice-cold saline phosphate buffer (150 mM NaCl, 40 mM K₂HPO₄, 2 mM KH₂PO₄ [pH 7.2]). Cells were harvested by centrifugation at 10,000 x g for 10 min and washed twice with saline phosphate buffer. Chromosomal DNA was purified as described previously (22). Contaminating RNA was removed by treatment with RNases A and T₁, followed by polyethylene glycol precipitation (32).

Breakage points of the modified DNAs were revealed by a primer extension method adapted from that of Brewer and coauthors (3) as follows. A linear PCR using Taq polymerase was performed on chromosomal DNAs. Primers SP1 (see above) and SP2 (5’-GGGGCAGCTATTGTACCTATTGATGACGGCGAC-G3’), corresponding to nucleotide positions 22 to 44 (Fig. 1), were used to probe the top and bottom strands, respectively. End labeling was performed with [γ-³²P]ATP and T4 polynucleotide kinase as described previously (32). Primer extension reactions were carried out in a volume of 20 μl containing 150 ng of chromosomal DNA, 0.5 pmol of ³²P-end-labeled oligonucleotide, 2 μl of 10% Taq polymerase reaction buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 20 mM MgCl₂, 0.2% [wt/vol] gelatin), and each deoxynucleoside triphosphate at a final concentration of 0.1 μM. The DNA was denatured by incubating the samples at 95°C for 5 min, followed by addition of 1 U of Taq polymerase. A program of 40 cycles, each consisting of 1 min of denaturation at 94°C, 5 min of annealing at 63°C, and 2 min of chain elongation at 72°C, was used for the amplification procedure. Four microtiers of formamide dye mixture was added to the samples, and the extension products were separated by electrophoresis on 6% polyacrylamide urea sequencing gels.

Sequence analysis of the 2.7-kb fragment of pRM11 showed the presence of an open reading frame hereafter called bglH (Fig. 1). Nucleotide sequence accession number.

**RESULTS**

**Cloning and sequencing of the bglH gene from L. plantarum B21.** Total DNA from L. plantarum B21 was partially cleaved with Sau3A1 and fractionated through a sucrose gradient, and fragments with an average size of 5 kb were ligated to the pKK232 vector in the BamHI restriction site. Competent cells of E. coli FA31 (unable to grow on salicin as a sole carbon source due to the bglB208 mutation) were transformed with λgt11 from the source due to the presence of a phosphorylating system was found in extracts of L. plantarum.

Sequence analysis of the 2.7-kb fragment of pRM11 showed the presence of an open reading frame hereafter called bglH (Fig. 1), coding for a 480-amino-acid protein with a calculated molecular mass of 53 kDa. The transcriptional start point, located at nt −42 relative to the putative translational start, and the promoter sequence (Fig. 1) were identified by primer extension analysis performed on total RNA from L. plantarum cells grown on salicin (Fig. 2, lane 2). A putative terminator structure was found downstream of the stop codon (Fig. 1). A bglH-specific transcript of 1.5 kb was detected by Northern analysis (data not shown), indicating that the bglH gene is expressed on a monocistronic transcriptional unit.

A β-glucosidase activity able to hydrolyze salicin only in the presence of a phosphorylating system was found in extracts of both L. plantarum and the E. coli strain carrying plasmid pRM11, thus showing that bglH encodes an aryl phospho-β-glucosidase (data not shown).
Glucose repression of \textit{bglH} expression. A primer extension analysis was performed on total RNA from \textit{L. plantarum} cells grown in MRS medium supplemented with ribose (Fig. 2, lane 1), salicin (lane 2), or glucose (lane 3). The relative amount of the extension products showed a 10-fold reduction of the \textit{bglH}-specific mRNA in cells grown on glucose, as quantified by autoradiography on phosphor storage plates. No detectable difference in the amount of the \textit{bglH}-specific mRNA was found in cells grown on ribose or salicin.

Enzyme data were in agreement with the results of the primer extension. β-Glucosidase activity was analyzed by assaying the hydrolysis of salicin in whole cells of \textit{L. plantarum} grown in MRS medium supplemented with 2% glucose or 0.4% salicin. The presence of glucose in the growth medium caused a 60-fold decrease in the ability of cells to take up and hydrolyze salicin compared to cells grown in the presence of salicin. The specific activities were 7.6 and 464 mmol of product formed per min per 10^{10} cells grown in the presence of glucose and salicin, respectively.

Computer analysis of the \textit{bglH} sequence. A computer search showed the presence of three putative CRE sequences within the \textit{bglH} gene (Fig. 1). The CRE sequence overlapping the transcriptional start site, and spanning from nt 190 to nt 203 (Fig. 1), contained one mismatch with respect to the consensus sequence (17), while the other two, spanning from nt 1215 to 1228 and from nt 1549 to 1562 (Fig. 1), showed two mismatches each.

Using the FASTA program, we found that the predicted BglH protein has significant sequence identity (60%) with the BglH protein of \textit{B. subtilis}, a phospho-β-glucosidase active on aryl-β-glucosides such as salicin and arbutin (21), and with the BglB protein of \textit{E. coli} (53%).

Recognition of the \textit{bglH} CRE sequence by \textit{L. plantarum} protein extracts. Gel retardation experiments were performed to check whether the CRE sequence is involved in the binding of a putative CcpA-like protein. A 40-bp DNA fragment, containing the \textit{bglH} CRE sequence overlapping the transcriptional start site, was used as a probe in binding assays with protein extracts from \textit{L. plantarum} cells grown in catabolite-repressing conditions (2% glucose). Figure 3 shows the appearance of a retarded complex when 0.5 µg of protein extract was added to the \textsuperscript{32}P-labeled CREa fragment (see Materials and Methods) (lane 2). This binding was specifically counteracted by addition of increasing amount of unlabeled CREa fragment (Fig. 3, lanes 3 and 4). In contrast, the occurrence of a retarded complex obtained with the CREb fragment (see Materials and Methods), containing three mismatches in the CRE sequence, was fourfold less efficient and was not specifically counteracted by addition of unlabeled CREb fragment, as quantified by autoradiography on phosphor storage plates (Fig. 3, lanes 5 to 8).

In vivo analysis of the CRE sequence. An in vivo footprinting analysis was performed to test whether the CRE sequence overlapping the transcription start site is involved in the regulation of \textit{bglH} expression. Chromosomal DNA of \textit{L. plantarum} cells grown on glucose or on salicin was methylated with DMS during exponential growth. The analysis was focused on G residues protected from DMS attack during growth in the presence of glucose. Figure 4 shows the occurrence of protection of the G residue in position 191 of the top strand (Fig. 4A, lane 1) and of the G residue in position 202 of the bottom strand (Fig. 4B, lane 2). Both G residues protected from the DMS attack belong to the CRE sequence.

DISCUSSION

We have identified and characterized the \textit{L. plantarum} phospho-β-glucosidase-encoding \textit{bglH} gene by complementation of
an E. coli strain carrying a mutation in the bglB gene. The predicted BglH protein showed significant sequence identity with BglH of B. subtilis (60%) and BglB of E. coli (53%), two phospho-β-glucosidases active on aryl-β-glucosides such as salicin and arbutin. Northern analysis indicated that bglH is transcribed on a monocistronic unit, while in E. coli and in B. subtilis the genes coding for BglB and BglH are organized in operons coding also for specific permeases (21, 24). The expression of these operons responds to transcriptional antiterminator-mediated regulation (15, 19, 35), whose cis element is the so-called RAT (ribonucleic acid terminator) sequence, highly conserved among gram-positive and gram-negative bacteria (2, 28). A BglR protein, belonging to the BglG family of transcriptional antiterminators, has been found in Lactococcus lactis (1). A computer search for RAT sequences within the transcriptional antiterminators, has been found in many Gram-positive bacteria (2, 28). A BglR protein, belonging to the BglG family of transcriptional antiterminators, is involved in carbon catabolite repression of the gene, whose position 13 of the CRE affected carbon catabolite repression of the operon, to our knowledge the result shown here represents the first in vivo protection data obtained for a CRE sequence highlighting the potential functional CRE sequence-function correlation for B. subtilis and E. coli. We thank A. La Volpe for helpful discussion, S. Gargano and E. Patriarca for critical reading of the manuscript, M. Valenzi for computer assistance, and C. Sole and P. Villano for technical assistance. This work was supported by MIRAFA, Piano Nazionale Biotecnolo-

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