In these strains lactose is transported by the lac-PTS and hydrolyzed by P-β-galactosidase. The galactose 6-phosphate formed is further metabolized via the tagatose 6-phosphate pathway. In lactococci, operons encoding proteins involved in efficient lactose transport and metabolism are located on plasmids. Because of this and wide biotechnological applications of lactococci, lactose-positive strains have been extensively studied over the past decades (14, 51). lac-PTS genes can also be located on chromosomes, as has been described for Streptococcus mutans (41) and the non-LAB Staphylococcus aureus (6, 7, 42). Moreover, there is some indirect evidence that additional lac-PTS genes can also be located in the genomes of several L. lactis strains (3, 9, 10, 12, 46).

Besides the lac-PTS, there is only one other type of lactose transport system that has been described for L. lactis, namely, the lactose-H^+ symport permease (26). Two other known types of lactose transport systems, the lactose-galactoside antipporter and ABC protein-dependent lactose transporter, have been found in Streptococcus thermophilus (36, 37, 38) and in the non-LAB, gram-negative Agrobacterium radiobacter (57), respectively.

L. lactis IL1403, a plasmid-cured derivative of the IL594 strain (8), is essentially lactose negative and does not contain in its chromosome genes encoding proteins homologous either to PTS lactose permease or to P-β-galactosidase (4). Despite this, L. lactis IL1403 possesses another system that has been shown in several other bacteria to be involved in lactose assimilation, a lactose permease-β-galactosidase system. In IL1403 this system depends on proteins encoded by genes of the Leloir pathway operon: lacS, specifying a putative H^+-lactose symporter or γ-galactose-lactose antipporter; lacA, coding for thiogalactoside acetyltransferase; and lacZ, encoding β-galactosidase. The other genes of the Leloir pathway cluster encode proteins involved in galactose catabolism, such as Gam, GalK, GatT, and GalE (4).

In this study, we present a glimpse of the diversity of Lactococcus lactis subsp. lactis IL1403 β-galactosidase phenotype-negative mutants isolated by negative selection on solid media containing cellobiose or lactose and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and we identify several genes essential for lactose assimilation. Among these are ccpA (encoding catabolite control protein A), bglS (encoding phospho-β-glucosidase), and several genes from the Leloir pathway gene cluster encoding proteins presumably essential for lactose metabolism. The functions of these genes were demonstrated by their disruption and testing of the growth of resultant mutants in lactose-containing media. By examining the ccpA and bglS mutants for phospho-β-galactosidase activity, we showed that expression of bglS is not under strong control of CcpA. Moreover, this analysis revealed that although BglS is homologous to a putative phospho-β-glucosidase, it also exhibits phospho-β-galactosidase activity and is the major enzyme in L. lactis IL1403 involved in lactose hydrolysis.

Bacteria have evolved three different systems for the assimilation of the main milk sugar, lactose, which differ in their phosphorylation states, intermediate metabolites, and bioenergetics. They include the group translocation systems (13, 39) and the primary (15) and secondary (27, 37) transport systems.

During transport by the bioenergetically most efficient group translocation system, the lactose-specific phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) (lac-PTS), lactose is phosphorylated at the C-6 carbon, and the internalized lactose 6-phosphate is degraded into galactose 6-phosphate and glucose by phospho-β-galactosidase. It has been suggested that in some cases lactose 6-phosphate can be hydrolyzed by β-glycosidases specific for β-glucoside sugars, that is, by P-β-glycosidases (46). This seems to be supported by sequence similarities between P-β-galactosidase and P-β-glucosidase enzymes, both of which, according to the nomenclature of Henrissat (24), belong to family I of glycohydrolases.

In primary and secondary transport systems lactose is not phosphorylated, and after internalization, it is hydrolyzed by β-galactosidase, yielding glucose and galactose. Galactose is subsequently metabolized through the Leloir pathway. Uptake of lactose via primary transport systems depends on hydrolysis of ATP, which provides energy for translocation of the substrate by an ATPase. Secondary transport systems use the energy from solute gradients, and in sugar translocation different types of mechanisms are involved, such as symport, antiport, and uniport.

Lactococcus lactis is a lactic acid bacterium (LAB) used in the dairy industry as a starter culture. Some strains of this species are able to ferment lactose present in milk very rapidly.

* Corresponding author. Mailing address: Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5a, 02-106 Warsaw, Poland. Phone: (48) 22 592 12 23. Fax: (48) 22 658 46 36. E-mail: jacek@ibb.waw.pl.

Alternative Lactose Catabolic Pathway in Lactococcus lactis IL1403

Tamara Aleksandrzak-Piekarczyk, Jan Kok, Pierre Renault, and Jacek Bardowski

Department of Microbial Biochemistry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5a, 02-106 Warsaw, Poland; Department of Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands; and Laboratoire de Génétique Microbienne, Institut National de Recherches Agronomiques, 78352 Jouy-en-Josas Cedex, France

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Recently, it has been observed that in the presence of cellobiose, _L. lactis_ IL1403 shows a lactose-positive phenotype (1). The catabolite control protein A (CcpA), a member of the LacI-GalR family of bacterial repressors (55), has been shown to play a role in this phenomenon (1). CcpA acts as a global regulator of carbon catabolite repression (CCR) in low-GC gram-positive bacteria, and its regulatory functions have been characterized in detail in _Bacillus subtilis_ (16, 23, 32, 33, 34). To carry out its role, CcpA interacts with the corepressor, a serine-phosphorylated phosphocarrier protein (P-Ser_46^-HPr) (16). HP is one of the proteins constituting the PEP-PPTS, and after phosphorylation at its His-15 position by enzyme I (P-His_15^-phosphorylated phosphocarrier protein (P-Ser_46^-HPr)) (16), HP is involved in the transfer of the phosphoryl group to the sugar-specific enzyme II (39). CcpA in complex with P-Ser_46^-HPr has been shown to exert its function by binding to a cis-acting DNA site called the catabolite-responsive element (cre) (54), located in front of or within the CcpA-regulated genes and operons. CcpA alone also interacts with DNA, but this binding is nonspecific and very weak (32). In most cases CcpA acts as a repressor (22, 34), but some examples showed it also to mediate catabolite activation (19, 50).

In this paper we describe the identification of several mutants of _L. lactis_ IL1403 that exhibit a β-galactosidase-negative phenotype in the presence of cellobiose or lactose and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). We show that non-PTS-specific BglS and LacS proteins are involved in lactose and X-Gal utilization, respectively.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and media.** The _L. lactis_ exocherica coli strains and plasmids used in this study are listed in Table 1. _E. coli_ was cultivated at 37°C in Luria-Bertani (LB) medium to which 1.5% agar (Merk) or erythromycin (Em) (ampicillin (Amp) 100 µg/ml) was added when necessary. The _L. lactis_ strains were grown at 30°C in M17 broth (Difco Laboratories, Detroit, MI) or in a synthetic chemically defined medium (CDM) (35, 40) containing 1% of glucose (G-M17 or G-CDM), lactose (L-M17 or L-CDM), cellobiose (C-M17 or C-CDM), or galactose (Gal-CDM), as required. The use of CDM instead of the rich M17 eliminates the “M17 effect,” that is, residual _L. lactis_ growth even in the absence of any added sugar.

**Determination of β-galactosidase-positive phenotype.** For the induction of the β-galactosidase-positive phenotype, _L. lactis_ strains were cultivated in M-L17 or L-CMD broth with cellobiose added at the inductive concentration of 0.1% (CL-M17 or CL-CDM). β-Galactosidase activity in _L. lactis_ was detected by adding 80 µg/ml of X-Gal to C-M17, L-M17, C-CDM, or L-CDM agar plates. β-Galactosidase activity of _E. coli_ colonies was detected on LB agar plates containing 50 µg/ml of X-Gal and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Colonies of cells with a β-galactosidase-positive phenotype developed a blue color.

**DNA manipulation and transformation.** Molecular cloning, restriction enzyme analysis, and transformation of _E. coli_ were performed according to general procedures (43). _L. lactis_ cells were made competent and used for electroporation with pGhost9::ISSI as described by Holo and Nesi (25) and Wells et al. (56). Transformants were selected on GM17 agar plates containing 5 µg/ml Em, at 30°C, after 2 h of regeneration in GM17-saccharose (0.5 M) medium. Plasmid DNAs from _E. coli_ and _L. lactis_ strains were isolated using QIAGEN columns and protocol with the modification for _L. lactis_ that 5 mg/ml lysozyme was added to P1 buffer. Restriction and modifying enzymes were purchased from Fermentas (Lithuania) and used according to the recommendations of the manufacturer.

**Plasmid integration mutagenesis, Southern hybridization, and DNA rescue-chromatography.** Chromosomal DNA of _L. lactis_ IL1403 was randomly mutagenized by integration of the pGhost9::ISSI plasmid as described previously (30). To validate the method, three independent mutagenesis procedures were performed. Mutagenized cells were plated on M17 agar plates containing X-Gal, Em, and 1% of cellobiose or 1% of lactose and grown for 3 days at 37.5°C. Mutants forming white colonies, indicating their β-galactosidase-negative phenotype, were then selected.

To identify genes surrounding the pGhost9::ISSI integration site, total DNA was isolated from _L. lactis_ IL1403 cells growing exponentially in G-M17 with Em as described previously (21). EcoRI- or HindIII-generated DNA fragments (2 µg) were separated on 0.8% agarose gels and transferred onto a nylon Hybond-N+ membrane (Amersham International, United Kingdom) (43). Fluorescent probe labeling, hybridization, and detection were done with the ECL labeling and detection procedures (Amersham). The DNA probe containing the ISSI sequence comprised a 1,037-bp fragment amplified from pGhost9::ISSI by using the uni and pGh9 primers (Table 1).

The DNA rescue-cloning procedure applied was as described previously (1, 30). Briefly, total DNA isolated from mutants was cut with EcoRI or HindIII, self-ligated, and cloned in _E. coli_ EColi1000 (28). Clones containing pGhost9::ISSI linked to its flanking chromosomal DNA fragments (EcoRI [left] or HindIII [right]) were selected on LB plates containing Em. Rescued fragments were sequenced directly from pGhost9::ISSI by using the didonucleotide chain termination method (42, 44) with primers pISS1/H and pISS1/E (Table 1).

The sequences obtained were analyzed and compared to the _L. lactis_ IL1403 genome sequence database (4) by using the National Center for Biotechnology Information BLAST network service and standard parameters (2).

**Stabilization of the _L. lactis_ IL1403 cpoD mutant.** In our previous study (1), the cpoD mutant (IBB595pG) and the nucleotide sequence of the cpoD gene (GenBank accession number AF106673) were obtained by applying pGhost9::ISSI mutagenesis (30). Since pGhost9::ISSI possesses a thermosensitive replicon, this mutation could be stably maintained only at the nonpermissive temperature of 37°C. As this temperature is not physiological for lactococci, the pGhost9::ISSI-generated mutation was stabilized by excision of the integrative plasmid, leaving a single copy of the ISSI in the plasmid insertion site (30). Southern hybridization and DNA sequence analysis were used to verify the correct chromosomal structure of the stable cpoD mutant, named IBB550.

**Enzyme assays.** _L. lactis_ cells were grown overnight at 30°C in CDM containing the appropriate sugar. A sample (20 ml) of culture was collected by centrifugation and resuspended in 1 ml Z buffer (31). Crude extracts were obtained by vortexing three times for 1 min each at high speed (Mini Bead Beater MB-8) with glass beads (106-µm diameter; Sigma) at 1-min intervals, during which the cells were kept on ice. Cellular debris and glass beads were removed by centrifugation for 10 min at 8,000 rpm. The activities of β-glycosidases in cell extracts were determined by using the chromogenic substrates p-nitrophenyl β-D-glucopyranoside, o-nitrophenyl β-D-glucopyranoside, o-nitrophenyl β-D-galactopyranoside, p-nitrophenyl β-D-gentiobiose, and o-nitrophenyl β-D-cellobiose. P-β-galactosidase activity was assayed with o-nitrophenyl β-D-galactopyranoside-6-phosphate at 30°C, as described by Miller (31). All substrates were obtained from Sigma. As a control for enzyme specificity, p-nitrophenol o-β-galactopyranoside-6-phosphate and p-nitrophenol o-β-glucopyranoside-6-phosphate (kind gifts from J. Thompson) were used. Protein concentrations were determined using the Bio-Rad protein assay (5).

**Sugar utilization.** Growth tests were performed in CDM supplemented with various sugars, using a Microbiology Reader Analyser (Bioscreen C; Labystems, Finland) to measure changes in absorbance of the culture at 600 nm. Sugar fermentation patterns were determined by using the API 50CH test as specified by the manufacturer (API-BioMerieux, Marcy l’Etoile, France) after 3, 6, and 48 h of incubation.

**Construction of cpoB, bgIS, galK, galT, galE, and lacZ mutants through disruption of pJIM2374.** Mutants were created by single crossover between pJIM2374 harboring the respective internal DNA fragment of the cpoB, bgIS, galK, galT, galE, or lacZ gene and the chromosomal copy of each of these genes. The internal fragments were amplified using the appropriate “for” and “rev” primer pairs (Table 1). The PCR-generated DNA fragments were then ligated with the pGEM-T vector and transferred into _E. coli_ TG1. The resultant plasmids were isolated from Amp’ cells that were unable to hydrolyze X-Gal. The orientation of inserts in pGEM-T was checked with the primer 1224 and the appropriate reverse primer. Next, isolated plasmid DNA was digested with Sall and SacI, followed by ligation in pJIM2374 digested with the same restriction enzymes and transformation of _E. coli_ TG1. The resulting hybrid plasmids comprising pGEM-T, pJIM2374, and internal fragments of the appropriate genes were isolated. The pGEM-T was removed by excision with NcoI, and the rest was self-ligated and transformed into _E. coli_ Repa strain, because pJIM2374 lacked replication functions. Subsequently, the plasmids were isolated from the resultant Em’ transformants and transferred into _L. lactis_ wild-type strain. Homologous recombination was induced by 10^-9 to 10^-8 in G-M17-Em medium of the overnight culture of the lactococcal strain harboring pJIM2374 with the internal fragment of the appropriate gene. Diluted cultures were incubated for 3 h in G-M17 at 37°C. Integrants containing pJIM2374 in the appropriate gene in the chromosome were selected at 37°C on G-M17 agar plates.
containing Em. The physiological effect of gene disruption in the resultant mutants was examined.

**Replacement of the ccpA mutated gene with its wild-type copy.** In order to complement the ccpA mutation of strain IBBS50, the ccpA gene with its putative promoter region was amplified using pepQfor and trxBrev primers (Table 1), cloned into pGEM-T, and transferred into *E. coli* TG1. The resultant plasmid DNA was isolated, digested with Sall, ligated to pJIM2374 digested with the same restriction enzyme, and transferred into *L. lactis* IBBS50.

**RESULTS**

Cellulbiose induces a β-galactosidase-positive phenotype in the lactose-negative *L. lactis* strain IL1403. Our previous results have shown that *L. lactis* IL1403 plated on M17 agar medium supplemented with cellulose and X-Gal forms blue colonies (1). Here we tested whether various other sugars, including monosaccharides, disaccharides, β-glucosides, pentoses, and hexoses (ribose, galactose, glucose, fructose, mannose, mannotol, sorbitol, N-acetylglucosamine, arbutin, esculin, salicin, maltose, lactose, saccharose, trehalose, rafinose, melibiose, xylose, arabinose, and glycerol) could induce the same phenotype. None of the 20 sugars tested allowed *L. lactis* IL1403 to develop blue colonies on X-Gal plates, suggesting that only cellulbiose induces a metabolic pathway that confers the same phenotype. None of the 20 sugars tested allowed *L. lactis* IL1403 to develop blue colonies on X-Gal plates, suggesting that only cellulbiose induces a metabolic pathway that confers the same phenotype. None of the 20 sugars tested allowed *L. lactis* IL1403 to develop blue colonies on X-Gal plates, suggesting that only cellulbiose induces a metabolic pathway that confers the same phenotype. None of the 20 sugars tested allowed *L. lactis* IL1403 to develop blue colonies on X-Gal plates, suggesting that only cellulbiose induces a metabolic pathway that confers the same phenotype. None of the 20 sugars tested allowed *L. lactis* IL1403 to develop blue colonies on X-Gal plates, suggesting that only cellulbiose induces a metabolic pathway that confers the same phenotype. None of the 20 sugars tested allowed *L. lactis* IL1403 to develop blue colonies on X-Gal plates, suggesting that only cellulbiose induces a metabolic pathway that confers the same phenotype. None of the 20 sugars tested allowed *L. lactis* IL1403 to develop blue colonies on X-Gal plates, suggesting that only cellulbiose induces a metabolic pathway that confers the same phenotype.

To test this assumption, *L. lactis* IL1403 was cultivated in CDM broth supplemented either with a low (0.01%) concent-
The text discusses the isolation and characterization of β-galactosidase-negative phenotype mutants of *L. lactis* IL1403 grown in C-M17. It mentions the use of chromosomal DNA as a control for the isolation of mutants. The article describes the growth of *L. lactis* IL1403 in CDM supplemented with 1% lactose, 0.01% cellobiose, and 1% lactose with 0.01% cellobiose, and the optical density at 660 nm is measured. The text also mentions the use of lactose (black line) and 0.01% cellobiose (gray line) as controls.

TABLE 2. Proteins encoded in the *L. lactis* IL1403 genome matching the predicted products of DNA regions interrupted by pGhost9::ISS1 integration in the β-galactosidase-negative mutants and their growth in L-CDM and CL-CDM media

<table>
<thead>
<tr>
<th>Gene (hits)</th>
<th>Function</th>
<th>Location on IL1403 chromosomal DNA</th>
<th>Growth on lactose&lt;sup&gt;c&lt;/sup&gt;</th>
<th>With cellobiose&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Without cellobiose</th>
</tr>
</thead>
<tbody>
<tr>
<td>ccpA (2)</td>
<td>Catabolite control protein A</td>
<td>1605930-1606928</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>yheB (4)</td>
<td>Conserved hypothetical protein</td>
<td>741571-742494</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>lacS (1)</td>
<td>Lactose permease</td>
<td></td>
<td>awt</td>
<td>awt</td>
<td>awt</td>
</tr>
<tr>
<td>ydgC (1)</td>
<td>Amino acid permease</td>
<td>361431-362810</td>
<td>awt</td>
<td>awt</td>
<td>awt</td>
</tr>
<tr>
<td>ynhH (1)</td>
<td>Unknown protein</td>
<td>1378866-1379273</td>
<td>awt</td>
<td>awt</td>
<td>awt</td>
</tr>
<tr>
<td>ydhB (1)</td>
<td>Hypothetical protein</td>
<td>371163-371975</td>
<td>awt</td>
<td>awt</td>
<td>awt</td>
</tr>
<tr>
<td>yfa (1)</td>
<td>Unknown protein</td>
<td>1150456-1151043</td>
<td>awt</td>
<td>awt</td>
<td>awt</td>
</tr>
<tr>
<td>yrcA (1)</td>
<td>P-β-glucosidase</td>
<td>1721677-172311</td>
<td>awt</td>
<td>awt</td>
<td>awt</td>
</tr>
<tr>
<td>UPlbp (1)</td>
<td>Fructose-1,6-bisphosphatase</td>
<td>254043-255965</td>
<td>awt</td>
<td>awt</td>
<td>awt</td>
</tr>
<tr>
<td>UPltopA (1)</td>
<td>DNA topoisomerase 1</td>
<td>1254565-1256697</td>
<td>awt</td>
<td>awt</td>
<td>awt</td>
</tr>
<tr>
<td>UPydhD (1)</td>
<td>Hypothetical protein</td>
<td>373134-373424</td>
<td>awt</td>
<td>awt</td>
<td>awt</td>
</tr>
</tbody>
</table>

<sup>a</sup> UP, integration of the plasmid occurred in the upstream noncoding region of the gene.

<sup>b</sup> awt, the mutant grows in the same manner as *L. lactis* IL1403 (its growth in CL-CDM was regarded as “+,” and that in L-CDM was regarded as “+/−”); ++, very good growth; --, slight inhibition of growth; --−, lack of growth.

<sup>c</sup> Cellobiose was used at the inducing concentration of 0.01%.
0.29 ± 0.02/h) reduced on glucose (Fig. 2A) and cellobiose (Fig. 2B), respectively. In both strains cellobiose induced lactose catabolism, but the ccpA-deficient strain responded better, growing 1.5-fold faster (0.06 ± 0.002/h versus 0.04 ± 0.01/h) and showing a shorter lag phase than IL1403 (Fig. 2C). Unexpectedly, IBB550 was able to ferment lactose even in the absence of cellobiose (Fig. 2D), with a similar growth rate (0.06 ± 0.003/h).

The phenotype of IBB550 was also tested on lactose-containing M17 or CDM agar plates supplemented with X-Gal. Surprisingly, strain IBB550 was able to hydrolyze X-Gal in both media, resulting in blue colonies.

To test whether these phenotypic changes were indeed due to ccpA inactivation and not to an additional, unidentified mutation, the ccpA gene was disrupted by single-crossover recombination in the wild-type IL1403 strain. This mutant was found to exhibit the same properties as those described previously for IBB550 (data not shown). In addition, the ccpA mutation in IBB550 was complemented by the wild-type copy of ccpA cloned into pJIM2374. Strain IBB550(pJIM2374) regained the wild-type phenotype (data not shown).

**P-β-galactosidase is elevated in L. lactis IBB550.** The effect of the ccpA mutation on the enzymatic activities of β-galactosidase, P-β-galactosidase, β-glucosidase, P-α-galactosidase, and P-α-glucosidase was examined in L. lactis IL1403 and IBB550. P-β-galactosidase activity was detected in both strains. None of the other enzymes was detected in either of the strains grown in the presence of all the sugars tested.

The level of P-β-galactosidase activity in IL1403 was relatively low when it was grown in the presence of glucose (Table 3) and was 19-fold higher on cellobiose-lactose. P-β-galactosidase activity was only about threefold higher in IBB550 than in IL1403.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Enzymatic activity (nmol/min/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL1403 (wild-type)</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.1 ± 3.35</td>
</tr>
<tr>
<td>Cellobiose-lactose</td>
<td>123.73 ± 15.69</td>
</tr>
<tr>
<td>Lactose</td>
<td>276 ± 36.7</td>
</tr>
<tr>
<td>Galactose</td>
<td>19.95 ± 2.53</td>
</tr>
<tr>
<td>Galactose-cellobiose-lactose</td>
<td>51.06 ± 2.35</td>
</tr>
</tbody>
</table>

* All sugars were used at the concentration of 1%; however, in media containing multiple sugars, the cellobiose concentration was 0.01%.

b Mean values and standard deviations from at least three independent experiments are given.

—, unable to grow.

ND, not determined.
grown in the presence of glucose and was six- to ninefold higher on cellobiose-lactose, lactose, or cellobiose.

**Physiological characterization of the other L. lactis IL1403 β-galactosidase-negative phenotype mutants.** To test whether the inability to hydrolyze X-Gal on C-M17 plates correlates with the expected inability to catalyze cellobiose-inducible fermentation of lactose, the 31 mutants obtained in the second round of pGhost9::ISS1 mutagenesis were grown in CL-CDM. Unexpectedly, 27 of them were able to grow in CL-CDM in the same manner as the wild-type strain, whereas growth of the other four mutants was significantly impaired. These four mutants, which were unable to grow on lactose as the sole carbon source, were found to be inactivated in the lactS gene, retained full ability to grow in CL-CDM, but in comparison to IL1403 its capability of growth in L-CDM was reduced (Table 2).

**Isolation of β-galactosidase-negative phenotype mutants in the ccpA background.** Since the IBB550 strain exhibited an increased ability to hydrolyze lactose and loss of the cellobiose-inducible β-galactosidase phenotype (C-M17–X-Gal plate) while demonstrating a lactose-inducible β-galactosidase phenotype (L-M17–X-Gal plate), an attempt was made to elucidate which system is involved in this phenomenon. Therefore, IBB550 was randomly mutagenized using pGhost9::ISS1.

Among the approximately 1,000 integrants grown on L-M17 plates supplemented with X-Gal, 100 were unable to hydrolyze X-Gal. Southern hybridization was used to show that 51 of these mutants had undergone a unique integration of pGhost9::ISS1 (data not shown), and these clones were further analyzed by sequencing of insertion sequence extremities and by growth tests.

Eleven DNA regions in strain IBB550 were hit more frequently, strongly indicating their possible involvement in lactose and/or X-Gal metabolism (Table 4). In seven of these, ISS1 had inserted into DNA regions II, III, IV, VIII, IX, X, and XI, comprising genes coding for hypothetical proteins or proteins of unknown functions. Among them, DNA region II, which contains the ydhB and ydhC genes, was knocked out most frequently, namely, six times. Other DNA regions that underwent frequent mutations were regions III and IV, in which the yfgQ and yveH genes, each disrupted three times, were identified. Out of 11 regions, only 4 (regions I, V, VI, and VII) comprised genes coding for proteins homologous to proteins with assigned functions, among them the bglS gene (region VI, two clones), coding for a putative P-β-glucosidase, and the operon containing genes encoding proteins of the Leloir pathway (region I, 8 clones).

**TABLE 4. Multiple-hit DNA regions interrupted by pGhost9::ISS1 integration in double β-galactosidase-negative phenotype mutants of the IBB550 (ccpA) strain**

<table>
<thead>
<tr>
<th>Region (hits)</th>
<th>Gene (hits)</th>
<th>Function</th>
<th>Chromosomal localization of plasmid insertion</th>
<th>Growth on lactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (8)</td>
<td>galE (1)</td>
<td>UDP-glucose 4-epimerase (EC 5.1.3.2)</td>
<td>2054147–2055127</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>galT (1)</td>
<td>Galactose-1-phosphate uridylyltransferase (EC 2.7.7.10)</td>
<td>2058844–2060325</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>galK (2)</td>
<td>Galactokinase (EC 2.7.1.6)</td>
<td>2060499–2061698</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>galM (1)</td>
<td>Aldose 1-epimerase (EC 5.1.3.3)</td>
<td>2061725–2062744</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>lacS (2)</td>
<td>Lactose permease</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>yugA (1)</td>
<td>Transcription regulator</td>
<td>2064540–2065274</td>
<td>+/-</td>
</tr>
<tr>
<td>II (6)</td>
<td>ydhB (2)</td>
<td>Hypothetical protein</td>
<td>371163–371975</td>
<td>awt</td>
</tr>
<tr>
<td></td>
<td>ydhC (4)</td>
<td>Hypothetical protein</td>
<td>371975–372952</td>
<td>awt</td>
</tr>
<tr>
<td>III (3)</td>
<td>yfgQ (3)</td>
<td>Hypothetical cation-transporting ATPase</td>
<td>568648–570975</td>
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<tr>
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</table>

a UP, integration of the plasmid occurred in the upstream noncoding region of the gene.

b Integrants were cultivated in L-CDM. awt, the mutant grows in the same manner as the parental, ccpA-deficient strain (its growth in L-CDM is regarded as “+ + ”); +/-, impairment of growth; −, lack or severe inhibition of growth.

c ND, not determined.
Growth tests of the ccpA double mutants. To better characterize the effect of the pGhost9::ISS1 mutations and to check whether the β-galactosidase-negative phenotype is linked to the expected loss of lactose-fermenting ability, growth of the majority of the ccpA-derived double mutants was examined in L-CDM (data not shown). In contrast to expectations, only four out of all the integrants tested displayed a complete inability to grow in this medium. These were mutants with disrupted galE, galT, or bgI5 genes and a clone containing ISS1 in the noncoding region upstream of yebF (Table 4). Either the other integrants retained full lactose-fermenting ability, or their growth was variously impaired (Table 4).

The roles of bgI5, galK, galT, and galE genes were further studied by making stable single-crossover knockouts of each of them. In the same way, the lacZ gene, encoding a putative β-galactosidase and the only gene from the Leloir cluster not hit during pGhost9::ISS1 mutagenesis, was inactivated. Growth of the resulting strains in L-CDM revealed that all of them had the same growth defects as the corresponding pGhost9::ISS1 integrants. In comparison to L. lactis IL1403, the growth of the lacZ mutant in CL-CDM was reduced, although the double mutant (lacZ ccpA) grew in the same manner as IBB550. The bgI5 single mutant was unable to grow in CL-CDM (data not shown).

The bgI5 mutant has lower P-β-galactosidase activity. Disruption of the bgI5 gene in both IL1403 and IBB550 resulted in an approximately 2.5-fold reduction of P-β-galactosidase activity in G-CDM, compared to their respective parental strains containing intact bgI5 (Table 3). The obtained values were low but measurable, indicating that BglS is not the only enzyme with P-β-galactosidase activity in L. lactis IL1403.

Since the bgI5 mutants were not able to grow in lactose media, these mutants were also grown in CDM supplemented with galactose, a sugar previously shown to be unable to induce catabolic repression (29). P-β-galactosidase activity was lower in the bgI5 mutant than in IL1403 (Table 3). However, in the presence of galactose together with lactose and inducing amounts of cellubiose, P-β-galactosidase activity reached 51 nmol/min/mg in IL1403 but decreased to 10 nmol/min/mg in the bgI5 mutant. Moreover, the level of P-β-galactosidase activity in galactose-lactose-cellubiose-growing bgI5 mutant cells was decreased in comparison to that in cells grown in a medium containing only galactose. This indicates that no more P-β-galactosidase activity was induced by the presence of lactose and that the obtained value was due to the presence of galactose. These observations are consistent with the complete inability of bgI5 mutant cells to grow in lactose-containing media. In the bgI5 mutants growing in the presence of cellubiose, the P-β-galactosidase activity was reduced approximately 10-fold in comparison to their parental strains, although it was still detectable, reaching a mean level of 24.5 nmol/min/mg. These data suggest that although several genes may encode P-β-galactosidase activity in L. lactis IL1403, BglS is the major enzyme involved in lactose hydrolysis.

DISCUSSION

L. lactis IL1403 is a plasmid-cured strain that is unable to assimilate lactose, although after prolonged incubation it begins to utilize this sugar slowly (Fig. 1). Moreover, it was found to be able to assimilate lactose faster after induction by cellolbiose (Fig. 1) or in the absence of functional CcpA (Fig. 2). An additional, remarkable feature of this strain is its ability to hydrolyze the lactose analog X-Gal in the presence of cellubiose. These results suggest that a cryptic lactose utilization system exists in L. lactis IL1403. The emergence of chromosomally encoded alternative lactose degradation pathways in Lactococcus might be a good method for adaptation from the plant environment to milk. Plants are believed to constitute the primary habitat for these bacteria, whereas milk is believed to be a secondary one. Cellubiose, a β-glucoside plant sugar, can serve as a constitutive signal for preadaptation of lactococcal cells to growth in a lactose-containing environment such as milk. Acquiring such a cellubiose-inducible catabolic potential might have provided an opportunity to settle in another environment and gain an evolutionary advantage over other bacteria.

In this article, we characterize the genes involved in both lactose and cellubiose-inducible lactose metabolisms in IL1403 and its ccpA mutant, IBB550. By random pGhost9::ISS1 mutagenesis of IL1403 and IBB550 and screening for inactivation of the β-galactosidase-positive phenotype, several mutants were obtained that had lost their β-galactosidase-positive phenotype but unexpectedly retained the ability to assimilate lactose. It is possible that the affected genes code for proteins that are involved only in X-Gal metabolism or are not involved in sugar metabolism, whereas the β-galactosidase-negative phenotype is caused by side effects (e.g., starvation or a response to environmental changes such as cultivation of mutants at 37°C). It is also possible that the β-galactosidase-negative phenotype is caused by mutations other than those due to pGhost9::ISS1 integration (e.g., spontaneous mutations in the IL1403 genome). Among all the pGhost9::ISS1 integrants tested, only insertions of the plasmid in the lacS, bgI5, galE, galT, yebF, and yebB genes led to clear inhibition of growth of mutants in cellubiose-lactose- and/or lactose-containing media (Tables 2 and 4). This result strongly suggests that proteins encoded by these genes are directly or indirectly involved in lactose assimilation in L. lactis strains. However, it should be taken into account that the physiological effects obtained in the tested mutants might also be due to the polarity of mutations introduced into the downstream genes.

Establishing the inability of the lacS mutant to hydrolyze X-Gal appeared to be a highly promising result with respect to understanding lactose catabolism in L. lactis IL1403. Indeed, the lacS gene is linked to the gene cluster of the Leloir pathway, which, in combination with the chromosomally encoded lac genes, has been shown to be engaged in lactose metabolism in several bacteria. Up to now, among Lactococcus strains such a permease–β-galactosidase system has been described only for L. lactis NCDO2054, a strain fermenting lactose slowly. In L. lactis NCDO2054 lactose is transported by a lactose permease–H+ symporter (26) and is subsequently cleaved by β-galactosidase (53). The fact that this permease has a much higher affinity for galactose than for lactose (26, 47, 48) could explain the poor ability of this strain to grow in lactose-containing media. The genes of the Leloir cluster have not yet been studied in L. lactis IL1403, but the predicted genes are also associated with genes needed for lactose assimilation, such as lacZ (β-galactosidase) and lacA (thiogalactoside acetyltransferase) (4). Furthermore, the lacS gene of IL1403 is almost
identical to that of *L. lactis* NCDO2054 but also to *galP* of the lactose-negative *L. lactis* strain MG1363 (18). Both permeases belong to the same subfamily (TC 2.A.2.3 [http://www-biology.ucsd.edu/~msaier/transport/]), which includes permeases specific for galactose transport. However, the MG1363 Leloir gene cluster does not contain genes for β-galactosidase and thiogalactoside acetyltransferase. This might explain why MG1363, despite possessing the same permease as the lactose-positive *L. lactis* lactose-negative mutant, and that its function is limited to the transport of galactosides to glucose 6-phosphate and the respective aglycons (4, 49). Indeed, according to the *L. lactis* IL1403 genome sequence (4), *bglS* gene, encoding P-β-galactosidase, had been inactivated. Genes for such hydrolases are mostly associated with operons encoding β-glucoside-specific PTSs, and the products of those genes are involved in the cleavage of C-6-phosphorylated β-glucosides to glucose 6-phosphate and the respective aglycons (45, 49). Indeed, according to the *L. lactis* IL1403 genome analysis also showed that some consensus *cre* sites are present in the neighborhood of sugar catabolism genes, suggesting the involvement of Cre in CCR of these genes.

Since the *L. lactis* IL1403 genome does not contain any gene coding for P-β-galactosidase (4), it was surprising to find that this strain is capable of hydrolyzing the C-6-phosphorylated β-glucosides to glucose 6-phosphate and the respective aglycons (45, 49). Indeed, according to the *L. lactis* IL1403 genome analysis also showed that some consensus *cre* sites are present in the neighborhood of sugar catabolism genes, suggesting the involvement of Cre in CCR of these genes.

The hypothesis that the main lactose transport system in *L. lactis* IL1403 is a β-glucoside-specific PTS is strengthened by the fact that β-galactosidase-negative phenotype mutants were obtained through pGhost9::ISX1 mutagenesis, in which the *bglS* gene, encoding P-β-galactosidase, had been inactivated. Genes for such hydrolases are mostly associated with operons encoding β-glucoside-specific PTSs, and the products of those genes are involved in the cleavage of C-6-phosphorylated β-glucosides to glucose 6-phosphate and the respective aglycons (45, 49). Indeed, according to the *L. lactis* IL1403 genome analysis also showed that some consensus *cre* sites are present in the neighborhood of sugar catabolism genes, suggesting the involvement of Cre in CCR of these genes.

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reaction can be understood, as both P-β-galactosidase and P-β-glucosidase exhibit high sequence similarities that allow them to be classified as belonging to family I of glycosylhydrolases (24). Simons et al. (46) have similarly observed that L. lactis mutated in the lacG gene (encoding a P-β-galactosidase) is still capable of slow growth on lactose, and this growth has been proposed to likewise depend on P-β-glucosidase activity. Here, we showed that disruption of bglS in both IL1403 and IBB550 led to complete inability of the mutants to grow in all lactose-containing media. Additionally, when cultivated in galactose-cellobiose-lactose-containing medium, no more P-β-galactosidase was induced by the presence of lactose in the bglS mutant (Table 3). Thus, we postulate that in L. lactis IL1403 the metabolisms of β-glucosides (cellobiose) and β-galacto-sides (lactose) are interconnected and that after internalization by the β-glucoside-specific PTS (presumably involving CelB), lactose-P is hydrolized by BglS. Despite the occurrence of several P-β-glucosidase genes in the IL1403 chromosome, it seems that BglS is the major enzyme involved in lactose hydrolysis. Nevertheless, it is not the only enzyme possessing P-β-galactosidase activity. The bglS gene is preceded by three cre sites, all deviating from the cre consensus by two mismatches. We suggest that bglS is probably only weakly or not at all regulated by CcpA, because when measured under repressive conditions (in glucose-containing medium), P-β-galactosidase activities in wild-type IL1403 and IBB550 are not very different (Table 3).

Other mutants severely impaired in lactose fermentation were affected in genes of the Leloir pathway. We postulate that lactose-P, internalized by the β-glucoside-specific PTS, is cleaved by BglS into glucose-P and galactose but not to glucose and galactose-P. Then, galactose is further metabolized by the enzymes of the Leloir pathway. The tagatose 6-phosphate pathway, which would deal with the catabolism of galactose-6-P (52), is absent in IL1403 (4).

Based on the results presented above, a putative model of lactose- and lactose-cellobiose-coupled sugar metabolism was made, in which the key elements are the proteins of the β-glucose-specific PTS (Fig. 3). In the presence of glucose IL1403 is unable to assimilate lactose due to repression by CcpA. Inactivation of ccpA results in derepression of the β-glucose-specific PTS transport system, thus enabling the IL1403 strain to import and grow on lactose. Moreover, the availability of cellobiose activates this PTS transporter, and IL1403 is able to grow on cellobiose and lactose. Internalized lactose is phosphorylated and subsequently split by BglS into galactose and glucose-P, the former of which is further metabolized through the Leloir pathway, while glucose-P enters glycolysis. Based on the results obtained with the bglS mutant, which shows a low level of P-β-galactosidase activity, it may be speculated that another P-β-glucosidase is encoded in the IL1403 genome. It is also proposed in this model that internalization of X-Gal occurs independently of the above-described PTS sugar transport system, via LacS.

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