Regulation of carbon catabolism in *Lactococcus lactis*.

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The *Lactococcus lactis* IL1403 is a lactose negative, plasmid free strain. Nevertheless, it is able to hydrolyze lactose in the presence of cellobiose.

In this work we describe identification of a gene involved in this process. The gene was found to be homologous to the sugar catabolism regulator, *ccpA*. The complete DNA sequence and analysis of the region encoding the *ccpA* gene is also presented.

1. INTRODUCTION

Lactic Acid Bacteria (LAB) are able to utilize a great number of sugars in fermentation processes. The most important carbon source for LAB grown in milk is lactose, because it is a main sugar in this medium. To be catabolized lactose has first to enter the bacterial cell. Lactose can be transported into the cell in two ways. One way, which is energetically preferable, is the PEP:PTS system (phosphoenolpyruvate-dependent phosphotransferase system). Using this system lactose is transported and simultaneously undergoes phosphorylation. Therefore, it enters the cell as phosphorylated molecule. Then, inside the cell, it is hydrolyzed by P-β-galactosidase into glucose and galactose-6-P [1]. The second way of lactose internalization occurs with the use of the permease and without phosphorylation of lactose. Inside a cell a non-phosphorylated lactose is cleaved into glucose and galactose by β-galactosidase.

The whole lactose catabolic pathway is one of the best known metabolic systems in LAB. Lactose operon consists of lacABCDEFGX genes [2], among which there are those coding for lactose transport and phosphorylation (*lacEF*), hydrolysis of lactose-6-P (*lacG*) and for tagatose pathway enzymes (*lacABCD*). Expression of the *lac* operon is regulated by the product of *lacR* gene, which is the transcriptional repressor. The *lacR* gene constitutes a monocistronic operon, which is divergently orientated to *lac* operon. A function of LacR protein depends on inducers such as tagatose-6-P and an intermediate of tagatose-6-P pathway. In the absence of lactose the LacR protein represses transcription of *lac* operon. In the presence of lactose inducers inactivate the LacR protein and therefore alleviate repression of *lac* genes.

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Some of the LAB, mainly from the genus *Lactococcus*, are also able to assimilate β-glucosides, which are sugars found in plants. This catabolic potential probably results from the fact that the natural niche of lactococci is plant environment.

The regulatory system involved in catabolism of β-glucosides relies on bgIR gene function. It was shown that BglR protein, which belongs to the BglG/SacY family of regulators is required for utilization of β-glucosides by *Lactococcus lactis* [3].

This protein acts as an antiterminator, which prevents RNA polymerase from stopping the transcription of β-glucosides operon. A bgIR mutant is not able to grow neither on salicin, arbutin, nor on esculin but is still able to grow on cellobiose [4].

In this work we show that on reach M17-agar medium supplemented with cellobiose and X-gal the wild type *Lactococcus lactis* IL1403 strain forms blue colonies. Since this strain normally does not grow on lactose as the only source of carbon, we suggest that the ability to hydrolyze X-gal is a cellobiose-inducible phenotype. Moreover, it seems that a system involved in cellobiose catabolism is different from that involved in arbutin, salicin and esculin assimilation. This observation led us to concentrate our work on identification and characterization of the gene responsible for cellobiose inducible lactose catabolism.

2. IDENTIFICATION OF THE GENE INVOLVED IN CELLOBIOSE-INDUCIBLE LACTOSE CATABOLISM

To identify the gene responsible for cellobiose inducible lactose catabolism, the plasmid integration mutagenesis system was used [5]. This system is efficient for lactococci and other Gram-positive bacteria. The plasmid pGhost that is used in this system is characterized by a thermosensitive replication, contains the ISS1 sequence and the erythromycin resistance marker which is used for selection (Figure 1). Integration of the plasmid occurs at non-permissive temperature (37°C) when plasmid replication is blocked. This random integration results in duplication of the ISS1 sequence. As a result of this duplication plasmid pGhost is flanked by two ISS1 sequences. Presence of unique restriction sites HindIII and EcoRI in the pGhost molecule is very useful for DNA cloning. The HindIII or EcoRI digestion of a DNA with integrated pGhost9:ISS1 plasmid leads to excision of the pGhost9:ISS1 with its right or left flanking fragment of chromosomal DNA respectively. The nucleotide sequences of pGhost9:ISS1 downstream or upstream regions can next be determined with primers corresponding to ISS1 or pGhost sequences.

This plasmid integration mutagenesis system was applied to isolate mutants of *Lactococcus lactis* IL1403 strain that do not exhibit cellobiose-inducible β-galactosidase positive phenotype. The selection was carried on reach M17-agar medium supplemented with cellobiose, X-gal and erythromycin. We expected to obtain white colonies of transformants, which had lost their ability to cellobiose-inducible lactose catabolism and blue colonies for all other transformants. The antibiotic selection excluded non-transformed *Lactococcus lactis* cells from growing.

After mutagenesis of *Lactococcus lactis* IL1403 strain, performed by random integration of pGhost9:ISS1 into its chromosomal DNA, 2000 colonies were obtained on the selective M17-agar medium with cellobiose and X-gal. Out of 2000 erythromycin resistant integrants 6 grew as white colonies. Integration sites in all white mutants were analyzed by Southern hybridization.
Southern hybridization patterns showed that 6 clones could be divided into 5 classes. After plasmid rescues from 5 integrants, pGhost9:ISS1 with flanking fragments of chromosomal DNA were introduced into *Escherichia coli* EC1000 strain. The chromosomal DNA fragments from all 6 white integrants were subsequently sequenced.

Analysis of the partial nucleotide sequences obtained revealed that in two mutants the same gene was mutated. The two, above-mentioned sequences show the homology to the CcpA protein from *Bacillus subtilis*.

Figure 1. Scheme of DNA mutagenesis and DNA cloning with pGhost9:ISS1 integration plasmid (modified from [6]).
Figure 2. The nucleotide and amino acid sequences of the ccpA gene. Potential ribosome binding site (RBS), -10 and -35 promoter elements, and terminator (inverted repeats) are underlined. A 14-bp sequence with homology to cre consensus sequence is bolded and HTH (helix-turn-helix) element is indicated in gray.
The nucleotide sequence of ccpA gene (Gene Bank accession number AF106673) contains 999 bp that corresponds to 333 amino acids (Figure 2). The gene is preceded by a putative ribosome binding side RBS (AGAGG) located 10 bp upstream of the methionine start codon. The putative promoter consists of -10 box (TATAAT) at 226 bp and a -35 box (TTGAAA) at 203 bp. We also identified a putative cre sequence, which differs by 1 bp from the cre consensus sequence. (Table 1). This sequence is upstream of putative ccpA and is overlapping the promoter region. The CcpA protein contains also HTH (helix-turn-helix) element, which is the DNA binding domain. The ccpA gene is followed by a transcriptional terminator (inverted repeats) at 1297 bp.

The location of ccpA gene in chromosomal DNA was further characterized. It was found that there is the pepQ gene coding for peptidase immediately upstream of the ccpA gene. The pepQ gene is divergently oriented in respect to ccpA.

The putative pepQ promoter consists of -10 box (TAGAAT) at 153 bp and a -35 box (GTGATT) at 175 bp. The trxB gene coding for thioredoxin reductase, transcribed in the same direction, follows ccpA. The putative trxB promoter was identified. It consists of -10 box (AATAAT) at 1361 bp and -35 box (TTGACA) at 1338 bp.

Table 1
Comparison of Lactococcus lactis ccpA cre sequence with the cre consensus

<table>
<thead>
<tr>
<th>cre sequence</th>
<th>Consensus sequence</th>
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<tbody>
<tr>
<td>TA</td>
<td>TGA<em>A</em>CG<em>T</em>TCA</td>
</tr>
<tr>
<td>Lactococcus lactis ccpA</td>
<td>TGAAAGGTTTACA</td>
</tr>
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3. CONCLUSIONS

We identified a gene implicated in cellobiose inducible lactose catabolism. The gene that was found is homologous to the ccpA gene from Bacillus subtilis. CcpA protein from Lactococcus lactis reveals 96% identity and 97% similarity with the CcpA protein from Lactococcus lactis subsp. cremoris [7]. The high homology (more then 48% amino acids identity) is also observed with CcpA proteins from: Lactobacillus casei, Streptococcus mutans, Listeria monocytogenes, Bacillus subtilis, Bacillus megaterium, Staphylococcus xylosus and Thermocactinomycetes sp.

Some other proteins, which are homologous to CcpA from Lactococcus lactis, are members of the LacI Family of regulators, like: DepA - transcriptional regulator from Bacillus subtilis, RbsR - transcriptional regulator from Thermatoga maritima, RbsR - ribose operon represor from Bacillus subtilis.

These results strongly suggest that the CcpA protein play a role in cellobiose-inducible lactose catabolism in Lactococcus lactis. Moreover, it is possible to speculate that the CcpA from Lactococcus lactis is involved in carbon catabolite repression.

Three elements had been reported to be necessary for carbon catabolite repression of amyE gene in Bacillus subtilis. One was the CcpA protein, another was P-Ser-HPr, which interacts with CcpA. The third one - an operator-like, cis acting element in front of the regulated gene...
is catabolite responsive element, cre [8]. It is already known that CcpA protein binds to the cre, which is present in most operons sensitive to carbon catabolite repression in Gram-positive bacteria. This interaction occurs in the presence of P-Ser-HPr. Therefore binding of CcpA to the cre elements is probably mediated by an allosteric interaction between P-Ser-HPr and CcpA [9]. HPr can be phosphorylated at Ser-46 by the ATP-dependent, metabolite activated protein kinase (HPr kinase). The activity of HPr kinase is positively regulated by fructose-1,6-bisphosphate (FBP).

The P-Ser-HPr is formed when the cell takes rapidly metabolisable carbon source. The role of CcpA protein in carbon catabolite regulation in Lactococcus lactis is still not well elucidated. However, we demonstrated that CcpA is involved in growth of Lactococcus lactis on lactose in the presence of cellobiose. The ccpA sequence analysis shows that the CcpA protein consists of 333 amino acids. Similarly to its counterparts from other Gram-positive bacteria the lactococcal CcpA protein contains the HTH motif, which is essential for binding to the DNA of regulated genes and interacts with the cre sequences [10]. The presence of the cre sequence upstream of the ccpA and overlapping the putative promoter region suggests the autoregulation of the ccpA gene. Moreover, location of the cre between ccpA and pepQ genes hints that the expression of the latter gene can be regulated by CcpA protein.

The results presented in this work can be promising for future applications in respect to the possibility of improvement of dairy starters through genetic modifications. The ccpA gene takes part in the carbon catabolite regulation and increasing or decreasing of carbon catabolism could become an industrial, biotechnological advantage.

REFERENCES