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Expression of the *Lactobacillus plantarum malE* Gene Is Regulated by CcpA and a MalR-Like Protein[§]

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***Lactobacillus plantarum* is commonly used in the food industry as a starter in various fermentations, especially in vegetable fermentations, in which starch is a common substrate. This polysaccharide, which is obtained from potatoes or corn and is hydrolysed mainly to maltose and glucose by acids or enzymes, is commercially used for the production of lactate by lactic acid fermentation. In this study, we describe the regulation of *malE* gene expression in *L. plantarum*. This gene, located in a 7-gene cluster, probably organized in an operon, encodes a putative maltose/maltodextrin-binding protein. We studied the expression of *malE* in *L. plantarum* LM3 (wild type) and in LM3-2 (*ccpA1*), which carries a null mutation in the *ccpA* gene, encoding the catabolite control protein A (CcpA). In the presence of glucose, expression of the MalE protein was higher in the mutant strain as compared to that in the wild-type strain. Transcription of the *malE* gene was induced by maltose and regulated by a CcpA-mediated carbon catabolite repression. Further, we isolated strains carrying mutations in 2 genes, *lp_0172* and *lp_0173*, whose deduced amino acid sequences share significant identity with MalR, a regulator of the maltose operon in several gram-positive bacteria. A double mutant exhibited glucose-insensitive *malE* transcription, while absence of the functional Lp_0172 open reading frame had no effect on *malE* expression.**

***Keywords*:** maltose/maltodextrin, *Lactobacillus plantarum*, catabolite control protein A, MalR

Maltose/maltodextrin utilisation has been extensively studied in gram-negative bacteria (Schwartz, 1987). The *Escherichia coli* maltose regulon consists of 10 genes encoding proteins responsible for the uptake and metabolism of maltosaccharides. *lamB* is involved in the uptake of maltodextrins into the periplasmic space, while *malE*, *malF*, *malG*, and *malK* are responsible for the transport of these substrates into the cell. Maltodextrin degradation is achieved by the action of 4 enzymes: an amylomaltase, a maltodextrin phosphorylase, a maltodextrin glucosidase, and an amylase, encoded by *malQ, malP*, *malZ*, and *malS*, respectively (Pugsley and Dubreuil, 1988; Tapio *et al*., 1991; Schneider *et al*., 1992; Watson *et al*., 1997; Dippel and Boos, 2005). The complex network regulation for this system involves the specific transcriptional activator MalT (Richet and Raibaud, 1989) and the global regulator Mlc, a repressor that controls *malT* expression in a glucose transport-dependent fashion (Decker *et al*., 1998; Schiefner *et al*., 2005). MalT is also activated by maltotriose (Raibaud and Richet, 1987) and inhibited by MalK, the ATP-hydrolysing subunit of the ATP-binding cassette (ABC) transport system (Joly *et al*., 2004). *malT* and genes encoding the maltodextrin ABC transporter components are also subjected to carbon catabolite repression (Richet and Søgaard-Andersen, 1994; Richet, 2000). Recently, the effects of cytoplasmic glucose and glucokinase on the activity and expression of MalT have been reported (Lengsfeld *et al*., 2009).

In contrast to the MalT-dependent positive regulation in *E. coli*, maltose metabolism is negatively controlled by the MalR protein in *Streptococcus pyogenes* (Shelburne *et al*., 2008), *S. pneumoniae* (Nieto *et al*., 1997, 2001), *Clostridium butyricum* (Goda *et al*., 1998), *Streptomyces coelicolor* (van Wezel *et al*., 1997), and *S. lividans* (Schlösser *et al.*, 2001). MalR belongs to the LacI-GalR family of transcriptional regulators. Disruption of the *malR* gene resulted in a constitutive glucose-insensitive *malE* transcription in *S. coelicolor*. In other gram-positive bacteria, such as *Staphylococcus xylosus* (Egeter and Bruckner, 1995) and *Lactococcus lactis* (Andersson and Rådström, 2002), MalR is regarded as an activator, due to its suggested activating effect on the maltose transport system. Recently, Gopal *et al*. (2010) have demonstrated that Lmo2128, the *Listeria monocytogenes* orthologue of the *Bacillus subtilis* YvdE, is a positive regulator of the maltose/maltodextrin uptake system.

In this report, we have investigated the expression of the *Lactobacillus plantarum malE* gene, which encodes a homologue of the *E. coli* and group A *Streptococcus* maltose binding protein (Shelburne *et al*., 2007). This protein is the extracellular component of an ABC-transport system, anchored to the cytoplasmatic membrane. *L. plantarum* is one of the most prevalent lactic acid bacteria found in vegetable matter and in human intestinal tract, and exhibits a high probiotic activity (Hooper and Gordon, 2002; Botes *et al*., 2008; Ramiah *et al*., 2008). For this reason it is largely used in fermentation technology, especially in vegetable fermentations, in which starch is a

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common substrate (Hu *et al*., 2009; Yang *et al*., 2010). Starch is one of the main sources of carbohydrates for the colon microbiota (Wang *et al*., 1999); therefore, maltose utilisation genes are critical for survival in this specialized habitat. Knowledge of the molecular mechanism governing maltose metabolism in *L. plantarum* is important for the optimisation of vegetable fermentations and for understanding how *L. plantarum* adapts to the intestinal environment.

Here, we show that MalE expression is induced by maltose and is negatively regulated by glucose. Using mutational analysis we show that such repression requires the catabolite control protein A (CcpA) (Warner and Lolkema, 2003) and the *lp_0173* gene product, which shows a high degree of identity with the MalR repressor identified in other microorganisms. The *malR* gene is expressed on a 3-cistron transcriptional unit that also includes the *lp_0172* and *agl1* genes, which encode a putative transcriptional regulator and an alpha-glucosidase, respectively.

Materials and Methods

Bacterial strains and culture conditions

L. plantarum LM3 and LM3-2 (Muscariello *et al*., 2001) were used throughout this study. *L. plantarum* was grown in the MRS medium (prepared without a carbon source) containing 2% glucose, 1% maltose or 0.4% salicin. When needed, erythromycin (5 μg/ml) or chloramphenicol (10 μg/ml) was added to the MRS medium. The *E. coli* Top10 F'{F' [(*lac*I^q Tn10 (Tet^R)] *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) 80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G} strain was used for plasmid cloning (Invitrogen, USA).

Surface proteins and SDS-PAGE

LM3 and LM3-2 cells were grown to exponential phase (OD_{595}=0.7) at 30°C in 100 ml of MRS broth containing 2% glucose or 1% maltose, washed with PN buffer (50 mM phosphate buffer; pH 6.5 and 100 mM NaCl) and then resuspended in 2 ml of the same buffer containing 550 mM sucrose and 5 mg/ml lysozyme. After 120 min of incubation at 30°C and centrifugation for 20 min at 16,000×g, the pellets were resuspended in 2 ml of PN buffer, French-pressed (3 cycles, at 1,500 psi), and centrifuged twice for 10 min at 10,000×g. Supernatants were ultracentrifuged for 1 h at 120,000×g. Pellets were then resuspended in 50 μl of PN buffer. The protein concentration was determined by the Bradford method, using the Protein Assay kit (Bio-Rad, USA). Twenty micrograms of proteins was subjected to SDS-PAGE (10%).

Protein identification by peptide mass fingerprinting

For protein identification mass spectrometry data was searched against the NCBI_nr database using the MASCOT search algorithm (http://www.matrixscience.com/), and the parameters were described previously (Castaldo *et al*., 2006).

Primer extension analysis

Total RNA was isolated from *L. plantarum* cells grown to mid-exponential phase on MRS medium containing 2% glucose, 1% maltose, or 0.4% salicin, as described previously (Muscariello *et al*., 2001). Primer extension products of *malE* transcripts were obtained using the oligonucleotide mal2 (5′-CTATTACCACAACCTACGAGC G-3′). The experiments were performed as described previously (Muscariello *et al*., 2001).

Construction of *L. plantarum* LM3 mutant strains

To obtain an in-frame deletion in the *lp_0172* gene, plasmid pJM2 was constructed as follows. Firstly, a 624 bp fragment, corresponding to the 5′-end of the *lp_0172* gene and spanning 4 codons in the coding region, was cloned into the *Hin*dIII-*Bam*HI sites of the pJDC9 integrating vector (Chen and Morrison, 1988), yielding plasmid pJM1. Then, a 510 bp DNA fragment spanning nucleotides 501 to 1,011 of the *lp_0172* coding region was cloned into the *Bam*HI-*Eco*RI sites of the pJM1 vector, yielding plasmid pJM2. In the PCR reactions oligonucleotides malR5 (5′-CCCAAGCTTCGTTTGGCCGTACTTA TTAGGCC-3′) and malR6 (5′-CGCGGATCCGATCGTTGCCATCA TGATCCCC-3′) were used to amplify the 624 bp fragment; malR7 (5′-CGCGGATCCCAAGCCGGAACGGATGCAAGCCC-3′) and malR8 (5′-CCGGAATTCGACTTGATTTGGTGGACGGAATGG-3′) were used to amplify the 510 bp fragment. Electroporation of *L. plantarum* cells with the integration plasmid pJM2 was performed as previously reported (Muscariello *et al*., 2001). Integration events were selected for growth in the presence of erythromycin, and further analysed by PCR.

Inactivation of *lp_0172* and *lp_0173* by homologous recombination was carried out with the pUM1 plasmid, which was constructed as follows. Two fragments of 1017 (UP) and 1015 (DOWN) bp, localised upstream and downstream from the *lp_0172* gene, respectively, were amplified from *L. plantarum* chromosomal DNA by PCR and cloned into the *Hin*dIII-*Pst*I and *Bam*HI-*Eco*RI sites of pUC18Ery, respectively (van Kranenburg, 1997). UP includes the *lp_0170* and *gplF2* genes, and DOWN corresponds to the *lp_0173* locus. In the PCR reactions the oligonucleotides malR1 (5′-CCCAAGCTTCGGATGA CAATCAAGTCGGAACC-3′) and malR2 (AAACTGCAGCTGCGC ACAAAATCGGCACAG-3′) were used to amplify the UP fragment; malR3 (5′-CGCGGATCCGCCATTCCGTCCACCAAATCAAGTCC 3′) and malR4 (5′-CCGGAATTCGGTCGATTCAGCCACCGACAA CCG-3′) were used to amplify the DOWN fragment. The resultant pUM1 plasmid was used to transform *L. plantarum* as described previously (Muscariello *et al*., 2001). Recombinant clones were selected as described above.

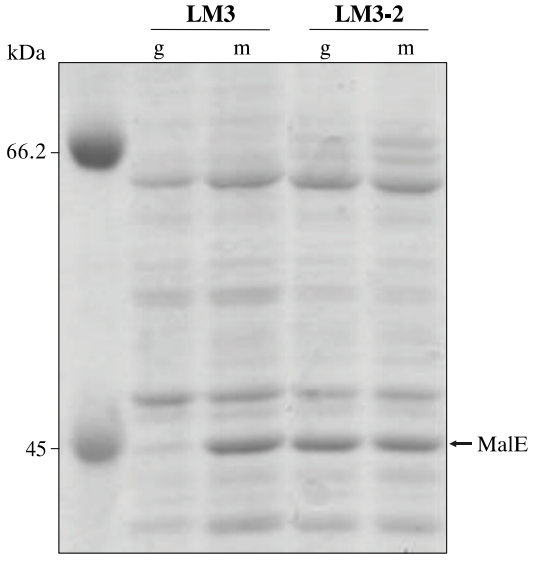


Fig. 1. SDS-PAGE analysis of surface proteins from LM3 (wild type) and LM3-2 (*ccpA1*) strains grown on glucose (g) or maltose (m). Molecular weight markers are indicated.

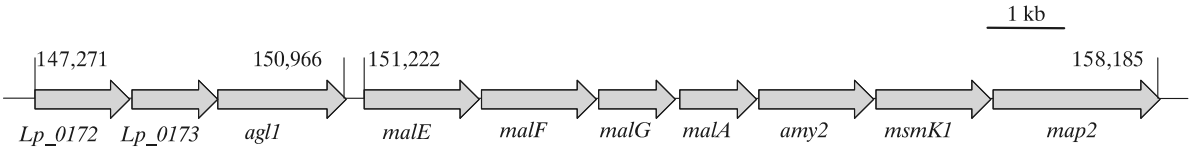


Fig. 2. Genetic organisation of the *L. plantarum* WCFS1 gene clusters involved in maltose/maltodextrin transport and metabolism. Arrows represent the orientation of each individual ORF and gene designations are indicated below the arrows. The numbers at the ends of each cluster indicate their positions in the chromosome.

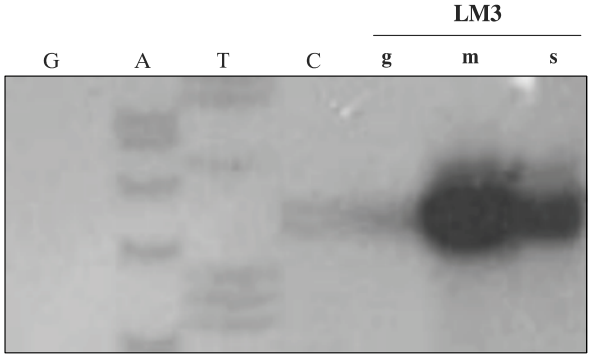
Results

Analysis of *L. plantarum* surface proteins

We analysed the electrophoretic pattern of surface proteins extracted from *L. plantarum* LM3 (wild type) and LM3-2 (*ccpA1*), which carries a null mutation in the *ccpA* gene (Muscariello *et al*., 2001), grown to the stationary phase on glucose. We found a protein with an apparent molecular mass of 45 kDa, whose expression was higher in the mutant strain than in the wild-type strain (Fig. 1). Using in-gel tryptic digestion and MALDI-TOF analysis, this protein was identified as MalE, a maltose/maltodextrin-binding protein, belonging to an ABC-transport system. The surface proteins extracted from cells grown in the MRS medium containing maltose were also analysed by SDS-PAGE. In the presence of maltose, MalE expression was induced in the wild-type strain, but not in the LM3-2 (Fig. 1). These results show that disruption of the *ccpA* gene results in constitutive expression of MalE, suggesting that the CcpA protein is involved in the negative regulation of MalE expression.

Transcriptional analysis of the *malE* gene

Sequence analysis of the *L. plantarum* WCFS1 genome (GenBank: A2935263) shows that the *malE* gene is located in a 7-gene cluster probably organized in an operon, which includes maltose/maltodextrin ABC transporters (*malF*, *malG*, and *malA*), an alpha-amylase (*amy2*), a multiple sugar ABC transporter (*msmK1*), and a maltose phosphorylase (*map2*) (Fig. 2) (Kleerebezem *et al*., 2003). On the basis of the high sequence identity between LM3 and WCFS1, as assessed by

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5′-AAAATTGGTATATATAAATCATGTGATGTATTTTAATTAACTTATCAATCTTTATATAAA

   TTGGTATAAAGGTTGATGGACCAATAAATCCTAACGTTTTTAAGCGTTAGGATTTATTTT
                                                    -35
   TGGTATGATTCTGTTAACGTTAACATTACGAAAACGCTTGCGAAAGCGTTTGCACAGTTG
            WWTGNAARCGNWWWCAWW      AACGCAAACGTTTGCGTCTGCA
            * *** ***********       *****   **   **** ****
                  CRE                     MalR-box
      -10
   CATAATAAATTATGGTAAATGAATTAAGATACTTCGCAAGGAGGAAGCACTATGAGTACT-3′
                  +1
```

Fig. 3. Primer extension analysis of *malE* mRNA (upper panel). The extension products were obtained by using total RNA extracted from *L. plantarum* LM3 cells grown to the exponential phase on glucose (g), maltose (m) or salicin (s). *malE* promoter region (lower panel). The putative CRE sequence and the MalR binding site are boxed, together with the CRE consensus sequence and the operator of *S. pneumonia malM* gene, respectively. Asterisks represent identical bases (N=any base; W=A or T; R=A or G). Putative -35 and -10 elements and the start codon for MalE are underlined. Transcription start site is indicated by +1.

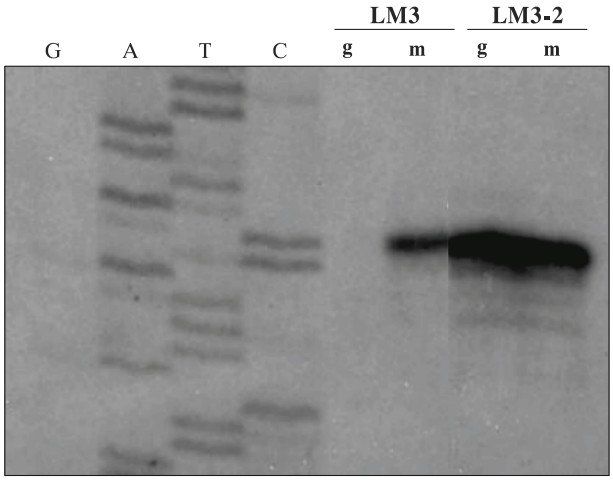

Fig. 4. Transcriptional analysis of *malE* in LM3 and LM3-2 strains grown on glucose (g) or maltose (m).

comparative genome hybridisation analysis (Siezen *et al*., 2006), transcriptional studies and DNA amplifications were performed with oligonucleotides designed from the WCFS1 genome sequence.

Primer extension analysis of *malE*-specific mRNA was performed using total RNA extracted from LM3 cells, grown in the presence of glucose, maltose or salicin. This analysis allowed the identification of the transcriptional start site and the promoter sequence, and led to the observation that *malE* expression is repressed by glucose and induced by maltose (Fig. 3): the abundance of *malE*-specific mRNA was greater in cells grown in the presence of salicin, a non-repressing carbon source, than in the presence of glucose; *malE*-specific mRNA levels were highest in cells grown in the presence of maltose. A primer extension analysis was also performed on total RNA extracted from LM3-2 cells grown on glucose or maltose. In this strain, *malE* transcription was no longer repressed by glucose and levels of transcription were similar in cells grown on glucose and maltose (Fig. 4).

Effects of mutations in *lp_0172* and *lp_0173*

MalR, which belongs to the LacI-GalR protein family, is a regulator of the maltosaccharide pathway in some Gram-positive bacteria. Using *in silico* analysis, 2 putative *L. plantarum* open reading frames (ORFs), Lp_0172 and Lp_0173, containing 339 and 320 amino acids, respectively, were recently identified (Francke *et al*., 2008). These ORFs exhibit homologies to MalR from several gram-positive bacteria; they are encoded by genes expressed on a 3-cistron transcriptional unit, upstream *malE*. *agl1*, the last gene of this putative operon, encodes an alpha-glucosidase (Fig. 2). Lp_0172 was found to share 34% and 33% amino acid sequence identity with MalR from *S. pneumoniae* and *L. lactis*, respectively, while Lp_0173 exhibited 43% and 25% identity with MalR from *Lactobacillus acidophilus* and *S. pneumoniae*, respectively. Both putative transcriptional regulators possess an amino-terminal helix-turn-helix domain and a sugar-binding domain found in proteins of the LacI family (Weickert and Adhya, 1992).

To determine the functions of Lp_0172 and Lp_0173 and their possible role in *malE* regulation, we first isolated a mutant strain carrying an in-frame deletion in the *lp_0172* gene, which removed 48% of the *lp_0172* coding region (corresponding to amino acids 5-163). Such a mutation would not have a polar effect on *lp_0173* and *agl1* expression. In order to generate this mutation, we used a 2-step homologous recombination process. A mutated copy of the *lp_0172* gene carrying the in-frame deletion, was cloned into the pJDC9 integrating vector, yielding plasmid pJM2, which was electroporated into *L. plantarum* LM3. Clones in which the homologous recombination event had occurred were selected as erythromycin-resistant strains. Integration events arising from a single cross-over yielded clones carrying both wild type *lp_0172* and its rearranged copy, as confirmed by PCR. To cure the cells of pJM2, 1 recombinant clone was grown without selection for 50 generations, and homologous recombination events were screened for loss of erythromycin resistance. For further analysis, we chose a strain carrying only the *lp_0172* rearranged copy, hereby named LM3-M1. LM3 and LM3-M1 exhibited similar growth rate on glucose (1 h^{-1}) and maltose (0.8 h^{-1}).

To examine the phenotype of LM3-M1, primer extension analysis was performed using RNA extracted from cells grown on glucose or maltose. No significant differences were found in *malE* expression in LM3 and LM3-M1 cells grown under the same conditions, indicating that absence of the functional Lp_0172 ORF had no effect on *malE* expression (Fig. 5).

A strain, carrying a null mutation in the *lp_0172* gene, with a predictable polar effect on the expression of *lp_0173* and *agl1*, was then isolated by a double-step homologous recombination process. The entire *lp_0172*-coding region, except for the last 31 bp, was replaced by a DNA fragment carrying the *ery*-antibiotic resistance cassette. A double homologous recombination event occurred in 15% of the recombinant clones, as detected by PCR. One of these clones, hereby named LM3-M2, was chosen for further analysis. LM3 and LM3-M2 exhibited similar growth rates on glucose (1 h^{-1}) and maltose (0.8 h^{-1}).

To examine the phenotype of LM3-M2, primer extension analysis was performed using RNA extracted from cells grown on glucose or maltose. Figure 5 shows that the amount of

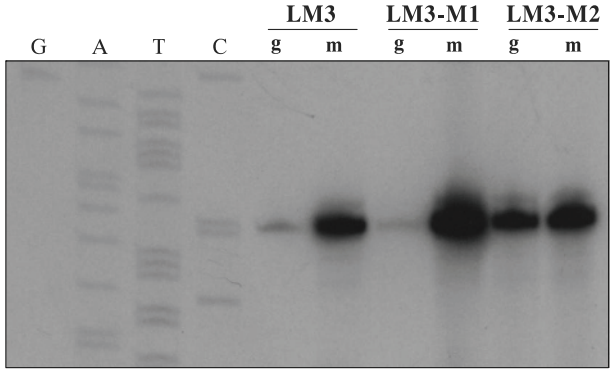

Fig. 5. Effects of *lp_0172* and *lp_0173* gene mutations on *malE* expression. Primer extensions were performed using total RNA extracted from LM3, LM3-M1 and LM3-M2 strains grown on glucose (g) or maltose (m).

extension products obtained from LM3-M2 grown on glucose was greater than that obtained from LM3 cells grown under the same conditions. Moreover, induction by maltose was lost in this mutant strain (Fig. 5).

Analysis of putative CcpA- and MalR-binding sites

The carbon catabolite element (CRE) is an 18-bp consensus sequence for binding of CcpA in the promoter regions of genes and operons regulated by catabolite repression (Miwa *et al*., 2000). A putative CRE sequence, which contained 2 mismatches with respect to the consensus sequence described by Miwa and colleagues, was found within the *malE* gene, between nucleotides -46 and -64 (Fig. 3).

Analysis of multiple sequence alignments between the *malE* promoter region and the MalR-binding sequences identified in other Gram-positive bacteria, showed the presence of a putative MalR-binding site (Fig. 3), overlapping the -35 sequence and sharing 68% identity with the operator of the *S. pneumoniae malM* gene (Nieto *et al*., 1997, 2001).

Discussion

In the present work we have analysed the expression of the *malE* gene in *L. plantarum*, one of the most predominant species in the human intestine where starch is an important source of carbon and energy for the microbiota. First, we showed that the expression of MalE, an *L. plantarum* surface protein, is induced by maltose and repressed by glucose. Its differential expression in *L. plantarum* LM3 (wild type) and LM3-2 (*ccpA1*) grown on glucose indicates that the CcpA protein is required for glucose-mediated repression. Similarly, transcriptional analyses reveal that *malE* expression is induced by maltose and regulated by a CcpA-mediated carbon catabolite repression; analysis of the *malE* regulatory region shows the presence of a CRE sequence, which is a putative binding site for the CcpA protein. Thus, CcpA is involved in the regulation of genes required for maltose/maltodextrin utilisation, and functions as a positive regulator in group A *Streptococcus* (Shelburne *et al*., 2008) and as a negative regulator in *Lactobacillus casei* (Monedero *et al*., 2008). In contrast, glucose-mediated repression of the genes required for maltose/maltodextrin uptake is independent of CcpA in *Listeria monocytogenes* (Gopal *et al*., 2010). We isolated 2 strains with mutations in the *lp_0172* and *lp_0173* genes, whose deduced amino acid sequences show significant sequence identity with MalR, a regulator of maltose operons in several Gram-positive bacteria. The 2 genes, located immediately upstream *malE*, are expressed on a 3-cistron transcriptional unit. Transcriptional analysis of the LM3-M1 mutant, which carries an in-frame deletion in the *lp_0172* gene, shows that *malE* expression is still induced by maltose and repressed by glucose. In contrast, the LM3-M2 strain, which carries a mutations in both ORFs, exhibits glucose-insensitive *malE* transcription, that is no longer induced by maltose. Taken together, these results indicate that the LM3-M2 phenotype is due to loss of the *lp_0173* gene product, suggesting that besides CcpA, Lp_0173 (MalR-like protein) may also be required for the regulation of *malE* expression.

In group A *Streptococcus*, transcription of the maltodextrin utilisation genes is regulated by competitive binding of the repressor MalR and the activator CcpA (Shelburne *et al*., 2008). It has been previously demonstrated that CcpA is required for glucose-mediated repression of the *mal1* operon in *L. casei*, where the location of the *malR* gene upstream the *mal1* operon, also points to a MalR-dependent regulation (Monedero *et al*., 2008).

This study suggests that in *L. plantarum* transcriptional regulation of *malE* requires the binding of both CcpA and Lp_0173 to the *malE* promoter region. Lp_0173 may interact with the sequence located immediately downstream CRE. To our knowledge this is the first study to show a negative regulation of the maltose operon by CcpA and a MalR-like protein.

Acknowledgements

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