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Mini-Review

Genetic organization and expression of citrate permease in lactic acid bacteria

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ABSTRACT. Citrate is present in many natural substrates, such as milk, vegetables and fruits, and its metabolism by lactic acid bacteria (LAB) plays an important role in food fermentation. The industrial importance of LAB stems mainly from their ability to convert carbohydrates into lactic acid and, in some species, like *Lactococcus lactis* and *Leuconostoc mesenteroides*, to produce C4 flavor compounds (diacetyl, acetoin) through citrate metabolism. Three types of genetic organization and gene locations, involving citrate metabolism, have been found in LAB. Citrate uptake is mediated by a citrate permease, which leads to a membrane potential upon electrogenic exchange of divalent citrate and monovalent lactate. The internal citrate is cleaved into acetate and oxaloacetate by a citrate lyase, and oxaloacetate is decarboxylated into pyruvate by an oxaloacetate decarboxylase, yielding a pH gradient through the consumption of scalar protons.

Key words: Lactic acid bacteria, Citrate permease, Citrate lyase, Citrate lyase ligase, Proton motive force, Open reading frame

INTRODUCTION

Citrate is abundant in nature, and it is a natural constituent of all living cells, being an important source of energy for bacteria. Citrate can be used as a carbon and as an energy source, under both aerobic and anaerobic conditions. Its utilization under aerobic conditions occurs via the tricarboxylic acid cycle, whereas various bacterial fermentation pathways are involved in citrate metabolism under anaerobic conditions (Bott, 1997). A limited number of lactic acid bacteria (LAB) are able to catabolyze carboxylic acids like citrate and malate. Lactic acid bacteria that use citrate play an important role in many dairy processes. In these bacteria, the co-metabolism of citrate and lactose leads to diacetyl and carbon dioxide production. Diacetyl is essential for the flavor of butter, fresh cheese and buttermilk. Carbon dioxide is responsible for cavity formation in certain types of cheese, like blue cheeses. The homofermentative *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* (*L. diacetylactis*), and the heterofermentative *Leuconostoc* species are the main LAB using citrate found in dairy starters. Studies made of citrate metabolism in *L. lactis* and *Leuconostoc mesenteroides* have revealed the same genetic pathway, composed of three steps for citrate conversion into pyruvate. The *cit* genes are positively regulated by pH in *Lactococcus* and by citrate in *Leuconostoc*. Energetically, in *Leuconostoc* the coupling between the citrate metabolic pathway and glycolysis is at the level of the redox state of the cell, and in *Lactococcus* it is at the level of the end product of glycolysis (Konings, 2002). We reviewed the genetic organization and expression of genes involved in citrate metabolism in LAB.

BIOENERGETICS AND MECHANISMS OF CITRATE UPTAKE IN THE LACTIC ACID BACTERIA

The mechanism of citrate metabolism in *L. lactis* and *L. mesenteroides* is a secondary proton motive force (PMF)-generating pathway. The anionic form of the acid is transported into the cell by an electrogenic secondary carrier generating an exchange with the end-product of the pathway (Marty-Teysset et al., 1995). The PMF results from a pH gradient and the generation of a membrane potential. The trans-membrane pH gradient is due to scalar proton consumption in the decarboxylation of oxaloacetate. The membrane potential-generating secondary transporters, malate permease (MleP) and citrate permease (CitP) involved in malolactic and citrolactic fermentations, translocate net negative charges across the membrane, and they catalyze a heterologous exchange of two structurally related precursor and product molecules, i.e., malate/lactate and citrate/lactate. Lactate is an inside substrate of MleP and CitP and malate could be an outside substrate of CitP (Marty-Teysset et al., 1995). Both MleP and CitP transport a range of 2-hydroxycarboxylates (symbol $R_1R_2COHCOOH$) with R substituents ranging in size from two hydrogens (glycolate) to acetyl and methyl groups (citromalate) for MleP and two acetyl groups (citrate) for CitP. The citrate metabolic pathway described in *Lactococcus* and *Leuconostoc* is a precursor/product exchange system. The product (lactate) results from the conversion of citrate and also from glucose metabolism, which explains why citrate uptake by *Lactococcus* and *Leuconostoc* is efficient only during lactate production from co-metabolism with a sugar. The use of citrate by *L. lactis* and *Leuconostoc* spp. strains is strongly dependent on the pH of the medium (Marjo et al., 1991). In *Leuconostoc* spp. strains, the growth rate is stimulated and CitP expression levels are increased when citrate is

present in the medium (Marty-Teysset et al., 1996), whereas growth stimulation by citrate was not observed in *L. lactis*, while the low constitutive expression of citrate transport increased at low pH (López et al., 1998; Garcia-Quintans et al., 1998). The co-metabolism of citrate and glucose increases the specific rate and molar growth yield of *L. mesenteroides* and the citrate alkalinizes the external pH (Marty-Teysset et al., 1996). In *Leuconostoc*, lactate is produced from citrate during citrolactic fermentation, and CitP catalyzes a precursor/product exchange, while in *Lactococcus*, the pyruvate produced from citrate is not the main precursor of lactate, which is produced via the homofermentative pathway. In *Leuconostoc*, glucose and citrate fermentation results in a growth advantage relative to growth on glucose alone, because of a metabolic shift in the heterofermentative pathway for glucose. In the absence of citrate, acetyl-phosphate formed from glucose is reduced to ethanol, which balances the redox equivalents from the phosphoketolase pathway. In the presence of citrate, the redox equivalents are shuttled to pyruvate that is produced from citrate, yielding lactate, and acetyl-P is converted into acetate via the acetate kinase pathway, resulting in the generation of one ATP per mole of acetyl-P.

GENETIC ORGANIZATION AND EXPRESSION OF CITP IN LACTIC ACID BACTERIA

The CitP sequences available in the database are from: *Lactococcus lactis* subsp. *lactis*, *Leuconostoc lactis*, *Weissella paramesenteroides*, and *Leuconostoc mesenteroides* subsp. *mesenteroides* 19D. These CitPs are composed of 442, 441, 442, and 443 amino acids, respectively; they are highly homologous (>98% identity) with a hydrophobic N-terminal domain, and all of them are plasmid-encoded. The loss of the plasmid harboring CitP encoding DNA, by curing experiments, resulted in the interruption of citrate uptake and therefore in diacetyl production, which proves that the citrate transport system is a crucial step. Restoration of this activity is experimentally possible, as observed in a plasmid-free strain *L. lactis* IL1403 (cit^-), which was rendered a diacetyl producer (Bourel et al., 1996), upon its transformation with plasmid pFL3 containing the lactococcal *citP* gene, under control of the *S. pneumoniae polA* promoter (Magni et al., 1994). The genetic organization and genomic location of CitP have been established in *L. diacetylactis*, *W. paramesenteroides*, and *L. mesenteroides* subsp. *cremoris* (*L. cremoris*) (Figure 1). In lactococcal plasmid pCIT264, *citP* is included in the *citQRP* operon, in which *citP* codes for a CitP, *citR* codes for a regulatory protein, CitR, and *citQ* codes for a leader peptide, CitQ (López de Felipe et al., 1995). In *L. diacetylactis*, transcription of *citQRP* operon is driven mainly from promoter P1, yielding a 2.9-kb mRNA regulated at the post-transcriptional level by processing that occurs in a complex secondary structure (López de Felipe et al., 1995). Similar processing has been found in the heterologous *E. coli* (Drider al., 1998), and RNases responsible for this processing were identified by using mutant strains deficient in endoribonucleases (RNase E and RNase III) and exoribonucleases (PNPase and RNase II). The cleavage located at the CitR Shine-Dalgarno sequence is ascribed to RNase III, which seems therefore to play a major role in the control of CitP expression (Drider et al., 1999). In *L. lactis*, the existence of RNase III was reported for the first time by Drider et al. (1999), and its structural gene was cloned and expressed in *E. coli* (Drider al., 2002). Moreover, transcription of the *citQRP* operon is induced by acid stress since the mRNA species detected in the 5' end region were 14-fold more abundant at pH 4.5 than at pH 6.5 (Garcia-Quintans et al., 1998). At low pH, *L. diacetylactis* relieves the inhibition due to lactate accumulation by a citrate meta-

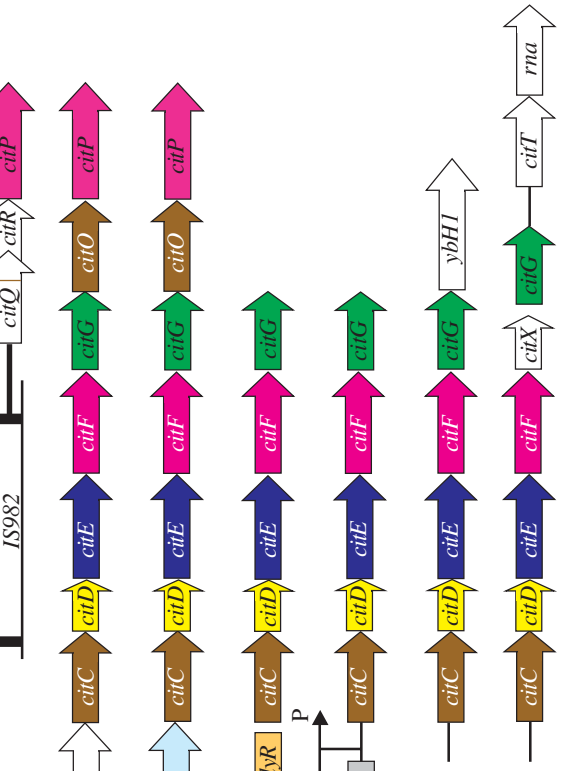

Figure 1. Physical map of the citrate fermentation gene cluster in lactic acid bacteria and eubacteria. In *Weissella paramesenteroides* and *Leuconostoc mesenteroides* var. *cremoris*, divergent transcript is indicated by promoters P1 and P2. Dashed lines correspond to the T + A-rich region. Transcription of *citQRP* operon in *Lactococcus diacetylactis* is driven from promoters P1 and P2 and from promoter P2' in *E. coli*.

bolic pathway induction, making the cells more resistant (Magni et al., 1999). In *L. mesenteroides*, the existence of the *citQRP* operon is not established, although the DNA sequence analysis of the upstream and adjacent regions of the *citP* gene showed a *citR* disrupted by several mutations (Vaughan et al., 1995). In *W. paramesenteroides*, the *citP* gene is included in a plasmidic cluster named *citMCDEFGRP*, which is transcribed as an 8.8-kb polycistronic mRNA (Martin et al., 1999, 2000). As in *L. diacetylactis*, the *citP* gene is immediately preceded by an open reading frame (ORF) encoding a 30-kDa polypeptide termed CitR, whose C-terminal domain is extremely similar (97%) to Lactococcal CitR; but its N-terminal domain is homologous to esterases and peroxidases. Furthermore, two ORFs, *citM* and *citI*, encoding for an NAD-dependent malic enzyme and a putative regulatory protein have been identified upstream of *citC*. In *L. cremoris*, *citP* is chromosomally located within a cluster of eight genes named *maecitCDEFGOP*. Two transcripts of 5.2 kb (*citmaeCDEFG*) and 4 kb (*citGOP*), presumably resulting from the processing of a larger transcript, were detected only when cells were grown in the presence of citrate (Bekal et al., 1998; Bekal-Si-Ali et al., 1999). Two ORFs referred to as *mae* and *clyR*, whose products are exactly similar to those of *citM* and *citI*, were identified upstream of *citC*. The *clyR* and *citI* genes are transcribed divergently from the *citmaeCDEFG* and *citMCDEFG* clusters. The putative regulatory proteins ClyR (312 amino acids) and CitI (322 amino acids) belong to the SorC transcriptional regulator family, and the DNA region between the start of *citI* and *citM* or *clyR* and *mae* contains two putative promoters with an extraordinarily high A + T content (80%). The effect of *citI* gene on expression of *cit* operon was studied in *E. coli*, in which *cis* or *trans* addition of the *citI* gene increased the activity of the *cit* promoter, showing that transcriptional regulation of citrate utilization is through an activator (Martin et al., 2000). Furthermore, gel shift and footprinting assays revealed that CitI recognizes at least three operator sites placed in the *citI-citM* intergenic DNA promoter region (Sender et al., 2002). In *K. pneumoniae*, the transport of citrate is catalyzed by three secondary carriers, called CitS, CitH and CitW. CitS is a Na^+-dependent citrate carrier that catalyzes the electroneutral transport of $HCit^{2-}$, using ΔpNa and ΔpH as driving forces (Pos and Dimroth, 1996). CitH is a Na^+-independent transporter that catalyzes the $Hcit^{2-}$ in symport with protons (Van der Rest et al., 1991), and it is assumed that CitH is functional under oxic growth conditions. The function of CitW remains to be determined.

GENETIC ORGANIZATION AND EXPRESSION OF CITRATE LYASE IN LACTIC ACID BACTERIA

Different mechanisms of regulation of bacterial citrate lyase (CL), such as configurational changes, reversible covalent modification by acetylation/deacetylation, and phosphorylation/dephosphorylation, have been reported. The CL (EC 4.1.3.6) of *L. lactis* and *Ln. mesenteroides* were shown to form a functional complex (Mr 585,000) of three proteins: an acyl carrier protein [ACP] (γ-subunit) carrying a prosthetic group; a citrate:acetyl-ACP transferase (α-subunit, EC 2.8.3.10) and a citryl-S-ACP lyase (β-subunit, EC 4.1.3.34) in a stoichiometric relationship of 6:6:6. The structures and the mechanism of action are similar to those of the CL of *K. pneumoniae* (Subramanian and Sivaraman, 1984; Antranikian and Giffhorn, 1987). The structure of the prosthetic group of CL purified from *Klebsiella* is a 5-phosphoribosyl-dephospho-co-enzyme A (CoA) attached by its ribose-5-phosphate moiety via a phosphodiester linkage to a serine residue of the ACP. The synthesis and attachment of the prosthetic group involve two

reactions. A triphosphoribosyl-dephospho-CoA synthase (ATP:dephospho-CoA 5'-triphosphoribosyl transferase) catalyses via an α-1,2-glycosidic linkage between ATP and dephospho-CoA the formation of the prosthetic group precursor 2'-(5"-triphosphoribosyl)-3'-dephospho-CoA (Figure 2, reaction 2). The second reaction (Figure 2, reaction 3), which consists of the transfer of the prosthetic group precursor to apo-ACP, is catalyzed by a holo-ACP synthase (Schneider et al., 2000a,b). The CL is active only if the thioester residue of the prosthetic group linked to its acyl carrier protein (γ-subunit) is acetylated. This activation is catalyzed by an acetate: SH-CL ligase (CLL, EC 6.2.1.22), which converts HS-ACP with ATP and acetate into the acetyl-S-ACP (Figure 2, reaction 4) (Schmellenkamp and Eggerer, 1974). The breakdown of citrate to acetate and oxaloacetate involves two consecutive steps. The α-subunit exchanges the acyl group for a citryl group to form the citryl-S-ACP (Figure 2, reaction 5). Lastly, the β-subunit cleaves citryl-S-ACP into oxaloacetate and regenerates the acyl-S-ACP (Figure 2, reaction 6) (Dimroth and Eggerer, 1975). In LAB, the genes encoding for CLL and CL are part of a cluster designed as *citCDEFG*. The proteins deduced from these *citC*, *citD*, *citE*, *citF* are CLL, and the subunits γ, β, and α of CL, respectively. CitC catalyzes the ATP-dependent acetylation of the phosphoribosyl dephospho-CoA group of CL. Contrarily to *L. lactis*, in *L. cremoris* and *W. paramesenteroides*, the *citMCDEFGRP* operon is unequivocally induced by citrate at the transcriptional level, independently of the pH of the medium (Bekal-Si-Ali et al., 1999; Martin et al., 2000). Northern blot analysis revealed an mRNA transcript of 8.8 kb starting upstream of *citM* and ending downstream of *citP*; this full-length transcript of 8.8 kb is subjected to a processing taking place in four different secondary structures, leading to synthesis and expression of various proteins at suitable concentrations (Martin et al., 2000). The function of CitG is not yet established in LAB, although sequence alignment showed that its C and N terminal domains are similar to the C-terminal domain of CitX and the N-terminal domain of CitG from *K. pneumoniae*. In this bacterium, the reactions of the tricarboxylic acid cycle are operative and the presence of citrate synthase requires a strict regulation of CL activity to avoid futile cycling between citrate fermentation and the L-glutamate biosynthetic pathway. After citrate depletion from the growth medium or upon transfer from an anaerobic citrate medium to an aerobic glucose medium, L-glutamate synthesis is ensured from oxaloacetate and acetyl-CoA via citrate only if the citrate fermentation pathway is turned off or partially turned off. The intracellular L-glutamate concentration controls these pathways by modulating the activity of the CL complex (Antranikian and Giffhorn, 1987; Antranikian and Gottschalk, 1989). In *K. pneumoniae* the *citCDEFG* cluster is located divergent to *citS* (Figure 1). Remarkably, in *E. coli* a *citCDEFXG* exists and displays high similarity to the CL cluster from *K. pneumoniae*, but the *E. coli* CL cluster contains an additional ORF named *citX* (Schneider et al., 2002). In both bacteria, the *citC* operon is regulated by a two-component regulatory system called CitA-CitB (Meyer et al., 1997) or DpiB-DpiA (Ingmer et al., 1998). Recently, it was demonstrated that CitG functions as ATP:dephospho-CoA 5'-triphosphoribosyl transferase) and CitX functions as 2'-(5"-triphosphoribosyl)-3'-dephospho-CoA transferase) (Schneider et al., 2002).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The DNA sequence of *L. lactis* IL1403 genome (Bolotin et al., 2001) did not reveal any evident ORF matching with oxaloacetate decarboxylase. Conversion of oxaloacetate to pyruvate is likely carried out by the product of *mae*, since a high level of sequence similarity exists

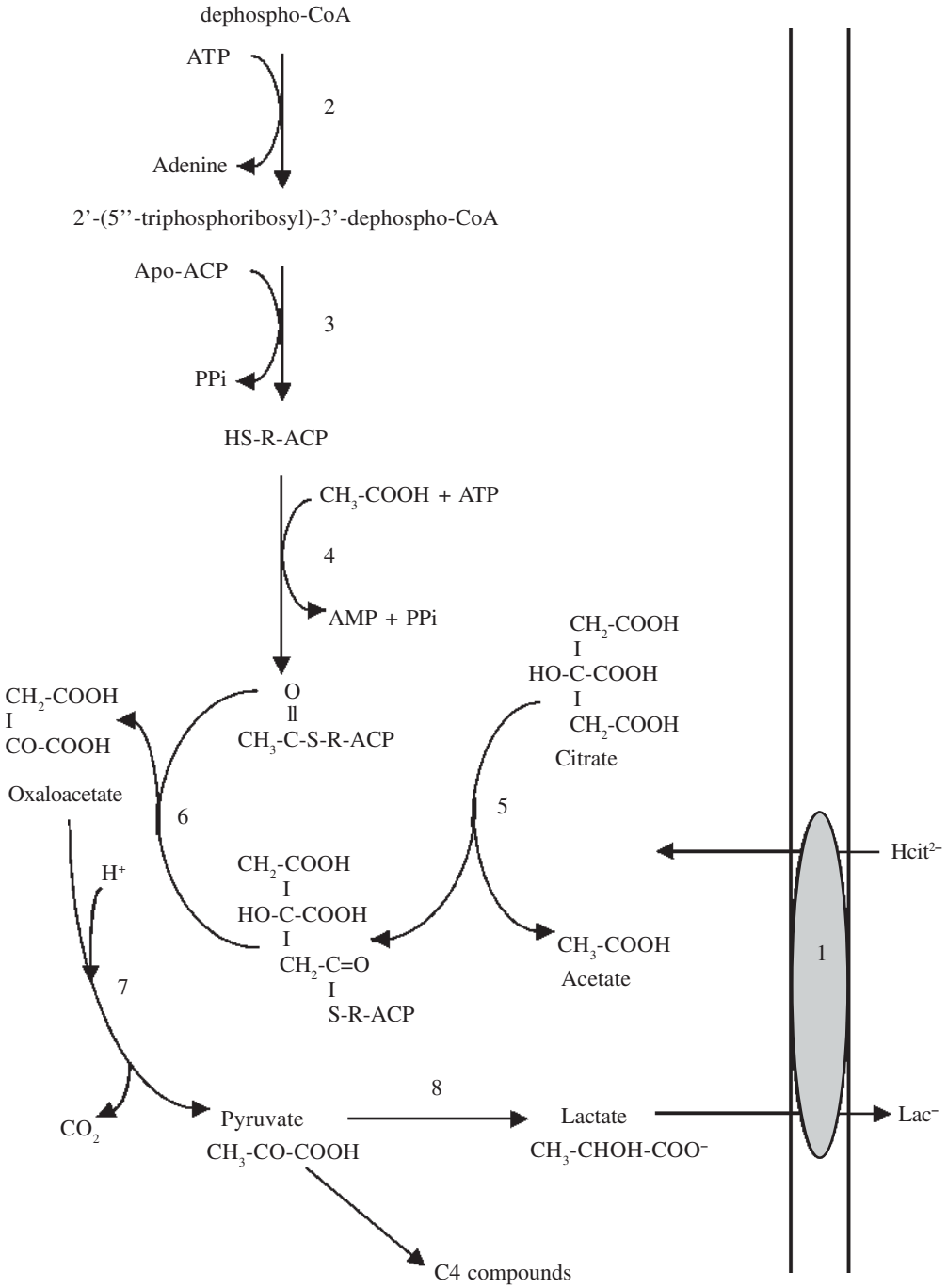

Figure 2. Citrate metabolic pathway in lactic acid bacteria is catalyzed by citrate permease (1), ATP:dephospho-CoA 5'-triphosphoribosyl transferase (2), holo-acyl carrier protein (ACP) synthase (3), acetate: SH-citrate lyase ligase [EC 6. 2. 1. 22] (4), citrate lyase α-subunit citrate:acetyl-ACP transferase [EC 2. 8. 3. 10] (5), citrate lyase β-subunit citryl-S-ACP lyase [EC 4. 1. 3. 34] (6), oxaloacetate decarboxylase (7), lactate dehydrogenase (8). R: prosthetic group of the γ-subunit ACP. CoA = co-enzyme.

between the *mae* product and malic enzyme from *Bacillus stearothermophilus*, an enzyme that catalyzes decarboxylation of oxaloacetate. To confirm this hypothesis, we suggest a study of the impact of the *mae* product on diacetyl production using a strain deficient in *mae* ORF. In this mini-review, we have mentioned that CitP of *L. cremoris* is chromosomally located; this is the first report on such location in LAB. Furthermore, the CitP of *L. cremoris* appears to be slightly different from those described in the literature.

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