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Citrate metabolism and aroma compound production in lactic acid bacteria

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1. Abstract

The main activity of lactic acid bacteria (LAB) during fermentation is the catabolism of sugars present in food, producing lactic acid by homo- or heterofermentative pathways. In addition, these microorganisms also have the capability to metabolize other substrates, such as citrate. Citrate is present in fruit juices, milk and vegetables and is also added as a preservative to foods. Citrate fermentation by LAB leads to the production of 4-carbon compounds, mainly

*diacetyl, acetoin and butanediol, which have aromatic properties. One of these compounds, diacetyl is responsible for the buttery aroma of dairy products such as butter, acid cream and cottage cheese. In addition, it is an important component of the flavour of different kinds of chesses and yoghurt. Moreover, the CO₂ produced as a consequence of citrate metabolism contributes to the formation of "eyes" (holes) in Gouda, Danbo and other cheeses. Thus, the utilization of citrate in milk by LAB has a very positive effect on the quality of the end products. Therefore, the interest of the dairy industry in controlling citrate utilization by LAB has promoted research into the proteins and effectors controlling its metabolic pathway. In this chapter we summarize the current knowledge of citrate utilization by LAB. The transport of citrate and its metabolism to pyruvate, as well as further conversion to aroma compounds, is described and, the differences in the co-metabolism of citrate with glucose between homo or heterofermentative bacteria is discussed. In addition, the molecular mechanisms controlling expression of genes responsible for transport and conversion of citrate into pyruvate are presented, as are their correlation with the physiological function of citrate metabolism. To date, two different models of regulation have been described which are unique to LAB. In *Lactococcus lactis*, a specific transcriptional activation of the promoters controlling the cit operons takes place at low pH to provide an adaptative response to acidic stress. In *Weissella paramesenteroides*, the CitI transcriptional regulator functions as a citrate-activated switch allowing the cell to optimize the generation of metabolic energy. CitI, its operators and citrate transport and metabolic operons are highly conserved in several LAB. Therefore, this mechanism of sensing and response to citrate appears to have been conserved and propogated during the evolution of LAB.*

2. Introduction

Analysis of a large set of bacterial genomes has shown that, in spite of its high occurrence in nature, only a limited number of bacteria are able to ferment citrate [1]. Citrate is of course the key intermediate in the Krebs' cycle (tricarboxylic acid cycle) which is very widely spread in all major classes of aerobic organisms. However, some bacterial species possess alternative metabolic pathways for the metabolism of citrate under anaerobic or aerobic conditions. Citrate fermentation can be carried out in the presence of oxygen when bacteria lack, or possess a defective Krebs' cycle (such is the case of LAB), or in anaerobiosis, when this cycle is not completely functional. Citrate fermentation can produce a variety of metabolic products (Fig. 1) depending on the genus of the bacteria and the growth conditions. In all cases, citrate fermentation involves the transport of this compound through a specific membrane protein and its subsequent conversion into acetate and oxaloacetate catalyzed by the enzyme citrate lyase. In most bacteria including

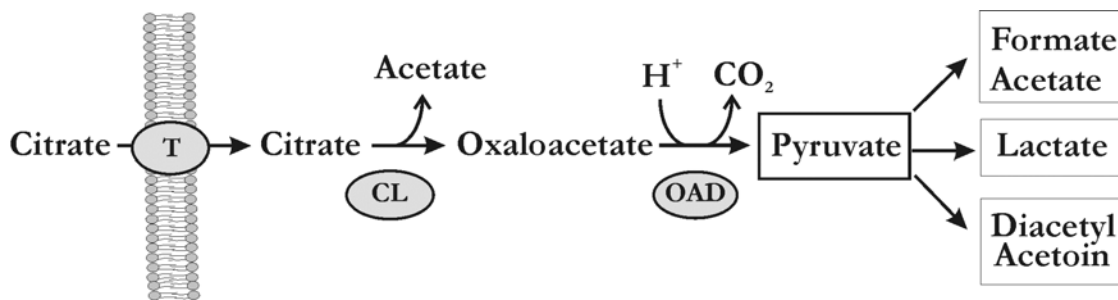


Figure 1. Citrate utilization pathways in bacteria and its end products. Citrate fermentation requires a specific membrane transporter (T), a citrate lyase activity (CL) and in most of the bacterial systems an oxaloacetate decarboxylase activity (OAD). The initial products of the pathway are acetate, CO₂ and pyruvate. The end products will be different depending on the micro-organism and the conditions of the fermentation (see details in the text).

LAB an oxaloacetate decarboxylase converts oxaloacetate into pyruvate, whose metabolism yields various compounds (Fig. 1). Among them, the aroma compound diacetyl has special interest for the dairy industry.

Strains of *Lactococcus lactis* subspecies *lactis* biovariety *diacetylactis* (*L. diacetylactis*), and some species belonging to *Leuconostoc* and *Weissella* genera are used as diacetyl producers by dairy industry. Thus, most of the knowledge of LAB citrate utilization has been derived from these dairy bacteria, and they will be the central topic of this review. In addition, in traditional cheeses *Enterococcus* species contribute to the aroma by fermenting citrate. Moreover, other bacteria such as *Lactobacillus plantarum* and *Oenococcus oeni* frequently use citrate present in the culture media and produce secondary fermentation in wine, beer and sausages. In these cases high concentration of diacetyl confers to the products a deleterious butter taste and affect negatively to the flavour of the product. Therefore, information regarding other LAB will be also presented in this chapter. Finally, the molecular organization and regulation of expression of the genes required for citrate degradation in these bacteria will be described.

3. The aroma compounds biosynthetic pathway from citrate

In LAB, citrate metabolism, like glucolysis, leads to the formation of pyruvate. The synthesis of pyruvate from citrate requires three steps: citrate transport by a permease, its conversion into oxaloacetate by citrate lyase (CL), and further conversion of oxaloacetate into pyruvate and CO₂ by the oxaloacetate decarboxylase (OAD) (Fig. 1 and 2). These three steps will be described in detail below. Metabolism of pyruvate can yield in LAB different end-products such as lactate, formate, acetate, ethanol and the aroma compounds

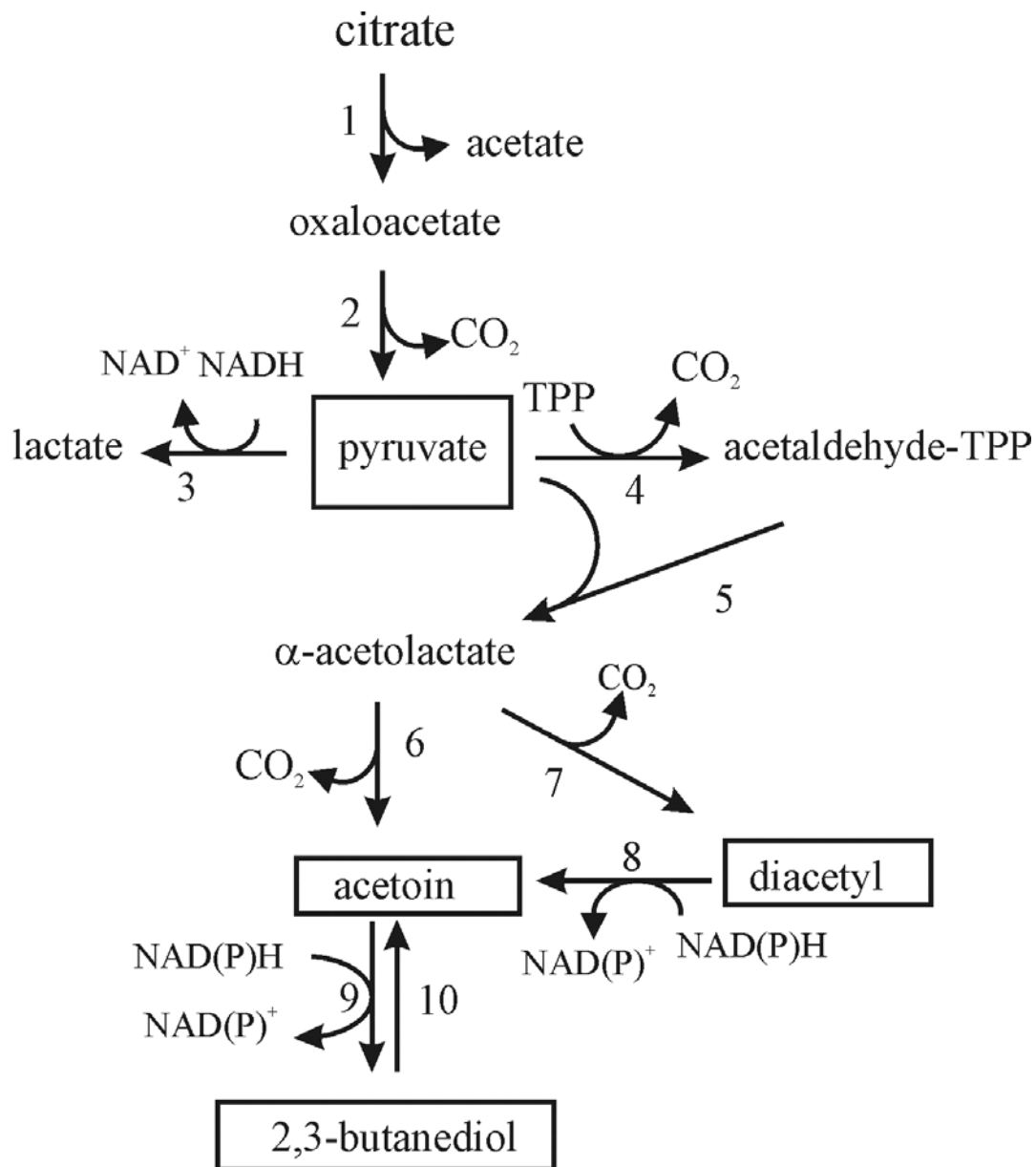


Figure 2. C₄-compound biosynthetic pathway in LAB. The degradation pathways in the genera *Lactococcus* and *Leuconostoc*. Enzymes involved in the pathway: 1, citrate lyase; 2, oxaloacetate decarboxylase; 3, lactate dehydrogenase; 4, pyruvate decarboxylase; 5, acetolactate synthase; 6, acetolactate decarboxylase; 7, non enzymatic oxidative decarboxylation of α-acetolactate; 8 and 9, diacetyl acetoin reductase; 10, 2,3-butanediol dehydrogenase; TPP, thiamine pyrophosphate.

of four carbons (C₄ compounds) diacetyl, acetoin y butanediol ([2] and Figs. 1 and 2). In the early 1980s, it was established that citrate is required specifically for efficient diacetyl and acetoin synthesis in LAB (reviewed in [3]). Also it was postulated, that in these bacteria the C₄ compounds are produced to eliminate toxic amounts of pyruvate, which are produced under particular

growth conditions (reviewed in [3]). Similarly, excess pyruvate could occur in mutant strains deficient in lactate dehydrogenase, the enzyme which catalyzes the conversion of citrate into lactate, since it was observed that in these microorganisms high amounts of diacetyl are formed (reviewed in [2]).

The metabolic biosynthetic pathway from citrate to diacetyl was revealed in *L. diacetylactis* by use of nuclear magnetic resonance (NMR) techniques [4, 5]. Ramos et al. [5] demonstrated that the main route of diacetyl synthesis is via the intermediary α -acetolactate (Fig. 2). α -Acetolactate synthase (α -ALS) is the key enzyme in the synthesis of C₄ compounds by catalyzing the condensation of two pyruvate molecules to generate α -acetolactate. Studies carried out with different strains of *L. lactis* showed that α -ALS has a low affinity for pyruvate (K_m between 30 to 50 mM), and an optimal pH of 6.0. Therefore, synthesis of α -acetolactate is favoured under conditions of pyruvate excess and acidic pH, which has been shown to increase production of C₄ aroma compounds [6, 7]. Once synthesized, α -acetolactate is unstable and is readily decarboxylated to acetoin by α -acetolactate decarboxylase (α -ALD), or by non-enzymatic decarboxylation (in the presence of oxygen) to diacetyl. Acetoin can be also synthesized from diacetyl by diacetyl reductase (DAR) [8]. This enzyme also possesses an acetoin reductase activity, which yields 2,3 butanediol from acetoin, being the reverse reaction catalyzed by 2,3 butanediol dehydrogenase (BDH). The DAR of *L. lactis* requires NADH, while its counterpart in *Leuconostoc* uses NADPH. Thus, the balance between the end products of citrate fermentation (diacetyl, acetoin and 2,3 butanediol) will depend on the redox state of the cells [9]. Once synthesized, these C₄ compounds are secreted without requiring specific transporters.

4. Citrate transporters

The limiting step for the utilization of extracellular citrate is the requirement for specific transporters, which facilitate its entrance into the cells. Biochemical characterization of citrate permeases has shown that citrate can be taken up by diverse mechanisms and they have been classified into different families. The bacterial citrate transporters occur in four different families (Table 1) which have been named: the metabolite:proton symporters (MHS); the citrate:cation symporters (CCS); the 2-hydroxycarboxylate transporters (2-HCT); and the citrate:metal symporters (CitMHS).

The MHS family, which belongs to the major facilitator superfamily (MFS) of transporters, includes permeases utilized by enterobacteriaceae during aerobic citrate fermentations (Table 1). These transporters are the CitH of *Klebsiella pneumoniae* and the CitAs of *Escherichia coli* and *Salmonella typhimurium*. They are symporters, which transport one molecule of citrate and three protons.

Table 1. Families that include citrate secondary transporters in bacteria.

MHS family (Metabolite:H⁺ symporters, TC 2.A.1.1.6.)				
Transporter	Micro-organisms	Gene location	Protein function	Ref.
CitH (444 aa)	<i>K. pneumoniae</i>	Chromosome	Citrate ²⁻ / H ⁺ symport	10
CitA (431 aa)	<i>E. coli</i>	plásmid, transposon	Citrate ²⁻ / H ⁺ symport	11
CitA (434 aa)	<i>S. typhimurium</i>	Chromosome	Citrate ²⁻ / H ⁺ symport	12
CCS family (Citrate:cation symporters, TC 2.A.24)				
Transporter	Microorganisms	Gene location	Protein function	Ref.
CitT (487 aa)	<i>E. coli</i>	Chromosomal	Citrate ²⁻ / succinate ⁻ antiport	13
CitT (479 aa)	<i>H. influenza</i>	Chromosomal	Citrate ²⁻ / succinate ⁻ antiport	14
YfIS (478 aa)	<i>B. subtilis</i>	Chromosomal	Di-Tricarboxilate transport	15
2-HCT family (2-Hydroxy-carboxylate transporters, TC 2.A.47)				
Transporter	Micro-organisms	Gene location	Protein function	Ref.
CitP (443 aa)	<i>W. paramesenterodes</i> J1	Plasmid pCITJ1	Citrate ²⁻ / lactate ¹⁻ antiport	16
CitP (442 aa)	<i>L. lactis</i> CRL264	Plasmid pCIT264	Citrate ²⁻ / lactate ¹⁻ antiport	17
CitP (442 aa)	<i>L. lactis</i> NCDO176	Plasmid pCT176	Citrate ²⁻ / lactate ¹⁻ antiport	18
CitP (443 aa)	<i>Lc. mesenteroides</i> 19D	Plasmid pCit19D	Citrate ²⁻ / lactate ¹⁻ antiport	19
CitP (441 aa)	<i>Lc. lactis</i> NZ6070	Plasmid pCT71	Citrate ²⁻ / lactate ¹⁻ antiport	20
CitS (445 aa)	<i>K. pneumoniae</i>	Chromosomal	Citrate ²⁻ / Na ⁺ symport	21
CimH (450 aa)	<i>B. subtilis</i>	Chromosomal	Citrate ²⁻ / H ⁺ symport	22
CitMHS family (citrate:metal symporters TC 2.A.11)				
Transporter	microorganisms	Gene location	Protein function	Ref.
CitM (433)	<i>B. subtilis</i>	Chromosomal	Citrate ²⁻ - Mg ²⁺ transport	23
CitH (426)	<i>B. subtilis</i>	Chromosomal	Citrate ²⁻ - Mg ²⁺ transport	24
CitQ (438)	<i>B. subtilis</i>	Chromosomal	Citrate ²⁻ - Mg ²⁺ transport	24
CitM (467)	<i>S. mutans</i>	Chromosomal	Citrate ²⁻ - Fe ³⁺ transport	25
CitH (465)	<i>En. faecalis</i>	Chromosomal	Citrate ²⁻ - Ca ²⁺ transport	26

(*) Only systems of citrate transport, and not the members of these families associated to the transport of other compounds, will be mentioned.

The CCS family includes the CitT of *E. coli* which transports citrate under anaerobic conditions, and its proposed mechanism of action involves an antiport of citrate and succinate, the end-product of citrate metabolism in this bacterium [27].

The 2HCT family includes transporters of dicarboxylic and tricarboxylic acids [1]. Among the citrate transporters of this family are included the well characterized *K. pneumoniae* CitS permease, and the CitP permeases of *Lactococcus*, *Leuconostoc* and *Weissella* (Table 1). The transporters of Klebsiella and of LAB utilize a different mechanism to take up citrate. CitS is a symporter which uses a Na²⁺-gradient to transport citrate, whereas CitP is responsible for the antiport of H-citrate²⁻ and lactate¹⁻ generating a membrane potential (see below). In LAB the genes encoding the CitP citrate permease were identified in plasmids (Table 1 and Fig. 3). The *citP* genes so far identified have a 99% sequence homology, suggesting a recent acquisition of this gene by horizontal transfer [28]. These genes are located in unrelated plasmids in *L. lactis*,

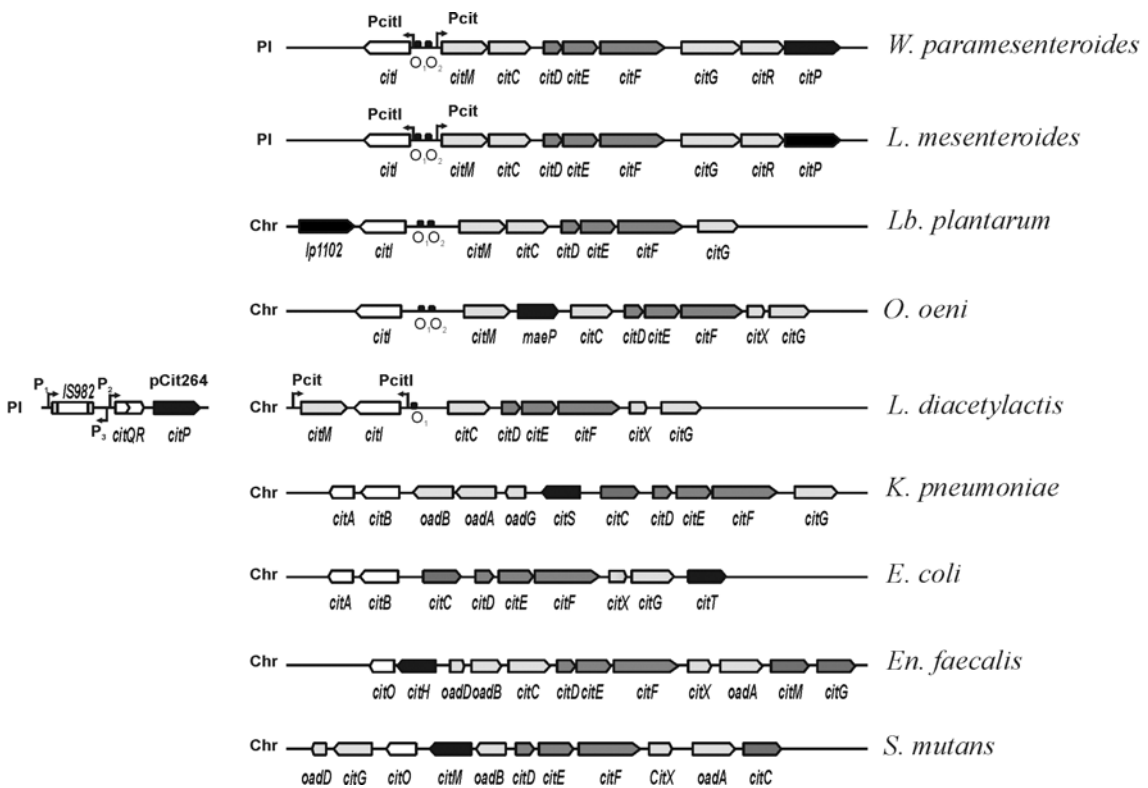


Figure 3. Physical organization of genes encoding proteins involved in citrate transport and conversion into pyruvate. The *cit* genes of the indicated bacteria are depicted. Genes encoding proteins, which regulate expression of *cit* genes are also shown (see details in the text). Genes with various functions are indicated by boxes: black, citrate transport; grey, citrate metabolism and white, regulation of gene expression. Plasmidic (PI) or chromosomal (Chr) location of the *cit* clusters is indicated. O_1 and O_2 , operators of the CitI regulator. The O_2 operator TTTTAAA-AA-TTTAAAA with perfect matched inverted repeats, corresponds to a high affinity site, whereas the O_1 operator TTTTAAA-AA-TTTGAAC corresponds to the site recognised with less affinity by the regulator.

W. paramesenteroides and *Lc. mesenteroides*. In *W. paramesenteroides* the genes encoding OAD and CL constitute, with *citP*, the *citMCDEFXG* operon ([29] and Fig. 3). In *L. lactis* the coding genes of the enzymes implicated in citrate metabolism are located in the chromosome (Fig. 3) and only *citP*, included in the *citQRP* citrate transport system operon[30], is located in a plasmid [17].

The CitMHS family constitutes a new group of citrate transporters that have been recently described (Table 1). This group of permeases is associated with the aerobic metabolism of citrate and includes the transporters CitM and CitH of *Bacillus subtilis* [24]. Both permeases transport citrate complexed with metal ions; CitM takes up citrate in complex with Mg^{2+} , whereas CitH transports the complex of citrate and Ca^{+2} . To this family also belong citrate

permeases of LAB, CitM of *Streptococcus mutans* [25] and CitH of *Enterococcus faecalis* [26]. Although the enterococcal CitH shares 75% sequence homology with the streptococcal CitM, the uptake of citrate complex is different in these micro-organisms. In *En. faecalis* citrate is co-transported with Ca^{2+} (also Mn^{2+} but not Fe^{2+} or Fe^{3+}), whereas for *S. mutant* it has been proposed that citrate is transported complexed to Fe^{3+} (also Mn^{+2} but not Ca^{2+} or Mg^{2+}). In contrast to LAB carrying CitP transporters, in *S. mutans* and *En. faecalis* the genes encoding either the CitM or CitH permeases are located in the chromosome associated to the citrate fermentation clusters (Table 1 and Fig. 3). These chromosomal gene clusters for citrate transport and metabolism are also detected in *K. pneumoniae* citS and *E. coli* citT (Fig. 3).

Two further systems of citrate transporters in *Lactobacillus* and *Oenococcus* have not been fully characterized. However, two genes exist in the *O. oeni* chromosome, that by homology encode organic acid transporters, which could be the citrate transporters of this micro-organism. The *maeP* located within the citrate metabolism *cit* (*citM-maeP-citCDEFXG*) cluster (Fig. 3) and the unlinked *yaeP*, whose expression is induced by the presence of citrate in the growth medium [31]. In *Lb. plantarum*, upstream of the gene cluster encoding CL and OAD, and with divergent polarity, is located the *lp-1102* gene (Fig. 3), whose product has homology to cation transporters and that could be the citrate permease of this bacteria.

5. Conversion of citrate into pyruvate

The CL is an enzymatic complex, which catalyzes the conversion of citrate in acetate and oxaloacetate in a multi-reaction process (Fig. 4). This reaction is the initial step in all citrate fermentation pathways known in bacteria. The holoenzyme has been purified and characterized in several species, but most of the studies have been carried out with the *K. pneumoniae* enzyme [27]. In this micro-organism the CL consists of three different subunits, α (54.7 kDa), β (31.4 kDa) and γ (11.4 kDa), associated in a stoichiometric relationship of $\alpha_6\beta_6\gamma_6$ (550 kDa). The α and β subunits are responsible for the catalytic activity of CL. The γ subunit contains the prosthetic group triphosphoribosyl-diphospho-coenzyme A, which lacks any intrinsic enzymatic activity, and acts as acyl transporter protein (ACP) [27]. Figure 4 shows a scheme of the steps catalyzed by each subunit. In the first step the α -subunit (acetyl-ACP:citrate ACP-transferase) catalyzes the interchange between the acetyl group (linked to the prosthetic group of the enzyme) and the citryl group to generate acetate and citryl-S-ACP. Subsequently, the β -subunit (citryl-S-ACP oxaloacetate-lyase) catalyzes the regeneration of acetyl-S-ACP, releasing oxaloacetate from the citryl-S-ACP. The catalytic activity of CL requires the activation of the prosthetic group and for this, it is necessary to convert its thiol group to an acetyl

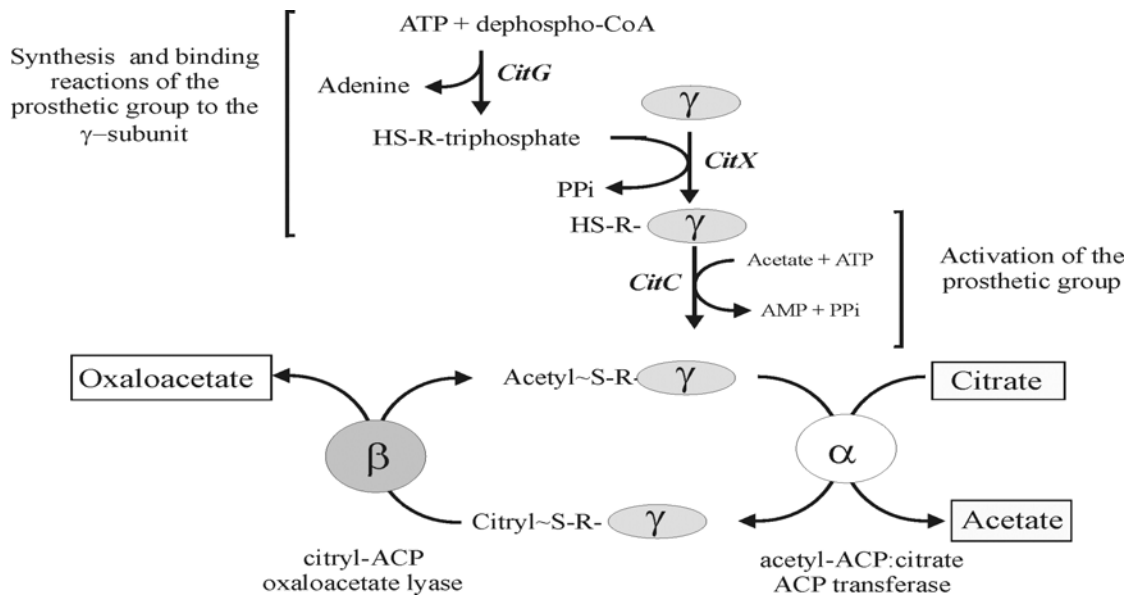


Figure 4. Reaction catalyzed by the citrate lyase complex and reactions involved in the synthesis and activation of its prosthetic group. CL converts citrate into oxaloacetate and pyruvate in a process consisting of two hemi reactions. The first is catalyzed by the acetyl-ACP:citrate ACP-transferase (α -subunit), while the second is driven by citryl-S-ACP oxaloacetate-lyase (β -subunit). The γ -subunit has the prosthetic group linked to serine 14 and acts as an acyl transporter protein. The steps of biosynthesis and linking of the prosthetic group (catalyzed by CitG and CitX, respectively) to the γ -subunit are also shown. The activation of the prosthetic group is produced by acetylation, catalyzed by the enzyme acetate:SH-citrate lyase ligase (CitC). HS-R represents the prosthetic group triphosphoribosyl-dephospho-coenzyme A.

thioester. This enzymatic reaction is catalyzed by the acetate:SH-citrate lyase ligase (CitC) in the presence of acetate and ATP [27]. Studies in *E. coli* and *K. pneumoniae* showed that a protein named CitX (20.4 kDa) catalyzes the formation of the holo-ACP by linking the prosthetic group triphosphoribosyl-dephospho-coenzyme A to the apo-ACP. Furthermore, this prosthetic group is synthesized from ATP and dephospho-coenzyme A in a process catalyzed by the protein CitG (25.2 kDa) [32]. In *E. coli*, these two latter proteins were designated: CitX: apo-citrate lyase phosphoribosyl-dephospho-coenzyme A transferase, CitG: triphosphoribosyl-dephospho-coenzyme A synthase. In this chapter CitC, CitX and CitG will be referred to as accessory proteins (required for the activity of CL).

CL and its coding genes have been investigated in LAB. CL has been purified from a *Lc. mesenteroides* 195 strain and its activity established [33]. The coding genes have been cloned and characterized from a genomic library of *Lc. mesenteroides* 195 [33], from a plasmid belonging to the J1 strain of acetyl *W. paramesenteroides* [16, 29], and also from the chromosome of

L. diacetylactis CRL264 [34]. In the genomes of these LAB, as in *K. pneumoniae*, it is possible to find *citD*, *citE* and *citF* genes (Fig. 3), which encode the γ -, β - and α -subunits of the CL complex. Genomic analysis has shown that in *O. oeni*, *En. faecalis* and some species of *Lactobacillus* (i.e. *acidophilus* and *casei*) the CL coding genes are present. However, the analysis of *L. lactis* genomes revealed that only the strains belonging to subsp. *lactis*, and not those of subsp. *cremoris*, possess CL. This correlates with the fact that *L. cremoris* is not able to metabolize citrate. Comparison of the amino acid sequences of CitC, CitE and CitF from *L. diacetylactis* with their homologues in others micro-organisms revealed the existence of an elevated conservation for the three CL subunits in bacteria. Moreover, the analysis identified a conserved region in all the sequenced γ -subunits. This region, with the sequence 9-AGTLE \underline{S} SDV-17, includes the residue serine 14, to which the prosthetic group 2'-(5'-phosphoribosyl)-3'-dephospho-CoA [32] is bound. Flanking the *L. diacetylactis* CL genes are *citC* (upstream) and, *citG* and *citX* (downstream) (Fig. 4), which encode the accessory proteins responsible, respectively, for the biosynthesis, the assembly, and the acetylation of the prosthetic group of the enzyme. *CitX* and *citG* can be found as independent genes, e. g. *L. lactis* or *O. oeni*, or as part of one gene (named *citG**), as occurs in *Lc. mesenteroides*, *W. paramesenteroides* and *K. pneumoniae*. For this last case, the product of the hybrid gene is a single, bi-functional polypeptide, which possesses both enzymatic activities (CitX activity in the N-terminal and CitG activity in the C-terminal).

The second step of citrate metabolism, catalyzed by OAD, is the decarboxylation of oxaloacetate synthesised by CL, generating pyruvate and CO₂. The best characterized bacterial oxaloacetate decarboxylase is the enzyme of *K. pneumoniae*, a biotinylated multi-enzymatic complex attached to the membrane (Fig. 5), and encoded by the *oadGAB* genes (Fig. 3). OAD is composed of two integral membrane subunits β and γ . (encoded by *oadB* and *oadG*). The α -subunit (encoded by *oadA*) is the decarboxylase and it is composed of two domains α and δ . The δ -domain is the biotin acceptor and contains the prosthetic group which binds to the [1-C]-carboxyl group of oxaloacetate in a reaction catalyzed by the α -domain. Finally, decarboxylation of the carboxybiotin group is coupled to Na⁺ translocation by the β -subunit. Thus, OAD couples the exergonic reaction of decarboxylation of oxaloacetate to pyruvate with the electrogenic transport of sodium ions out of the cell, which generates an electrochemical gradient of Na⁺ ($\Delta\mu_{\text{Na}^+}$) through the membrane.

This system constitutes a primary mechanism of energy generation that has been studied in depth [27]. Analysis of the genomes of various LAB indicate that a homologue of the *K. pneumoniae* OAD is present in *S. mutans*

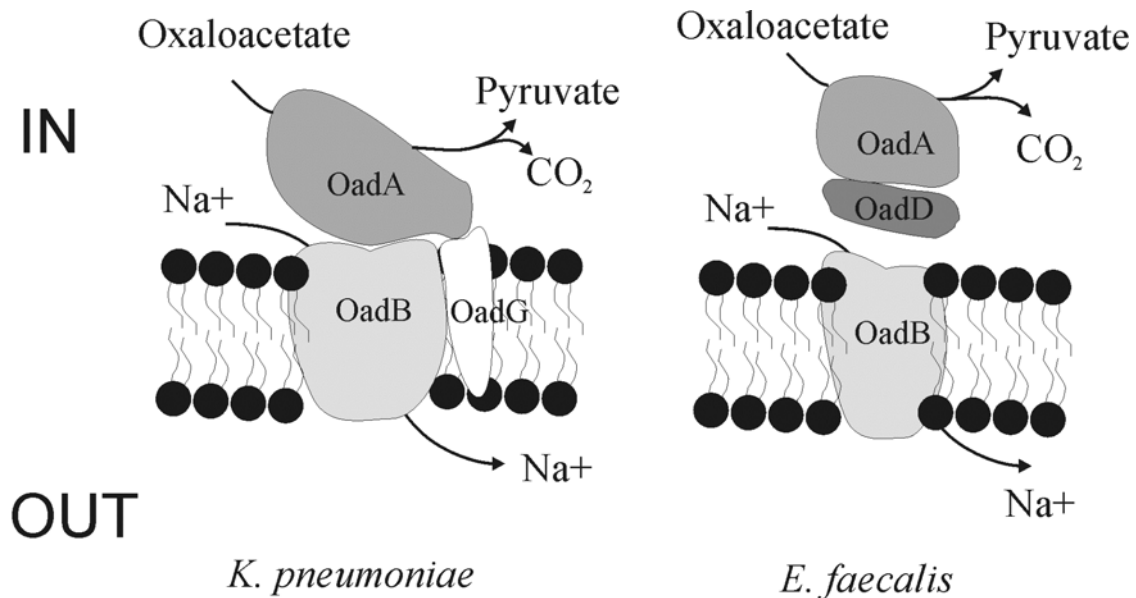


Figure 5. Oxaloacetate decarboxylase complex. Left, the membrane biotin decarboxylase from *K. pneumoniae*; Right, putative oxaloacetate decarboxylase present in *En. faecalis* and other LAB.

and *En. faecalis* (Fig. 5) as well as in *Lactobacillus sakei* and *Lb. casei*. In micro-organisms belonging to *Firmicutes*, the *oadA*, *oadB* and *oadD* genes, encode the α -, β - and δ -subunits of the oxaloacetate decarboxylase. The physiological role of the OAD complex remains to be studied in this group of micro-organisms, and it should be noted that in *Firmicutes* the two functional domains of the α -subunit from *K. pneumoniae* are divided in two different subunits.

Upstream of the genes encoding CL in *W. paramesenteroides*, *Lc. mesenteroides*, *L. diacetylactis*, *Lb. plantarum* and *O. oeni* (Fig. 3), there is a gene (named *citM* for *Weissella* [29] or *mae* [33, 35] for the other LAB), which encodes a 40 kDa polypeptide [16, 33, 35]. This polypeptide, by amino acid homology, belongs to the malic enzyme family (MEF) and could be the OAD of these bacteria. Moreover, the co-transcription of *citM* with CL genes was detected in *Leuconostoc*, *Weissella* and *L. lactis* [29,33, 34], supporting that CitM could play the role of a soluble OAD.

The product of the *citM* gene from *L. diacetylactis* was expressed and biochemical studies demonstrated that this enzyme has specific oxaloacetate decarboxylase activity [36]. The comparative analysis among members of the MEF revealed that the CitM from various LAB contain conserved active site residues such as the Tyr residue (Y36) and Lys residue (K91) proposed to be involved in the general acid-base mechanism (Fig. 6). Furthermore, the Glu(E133) and Asp (D134 and D159) residues required for the divalent cation-

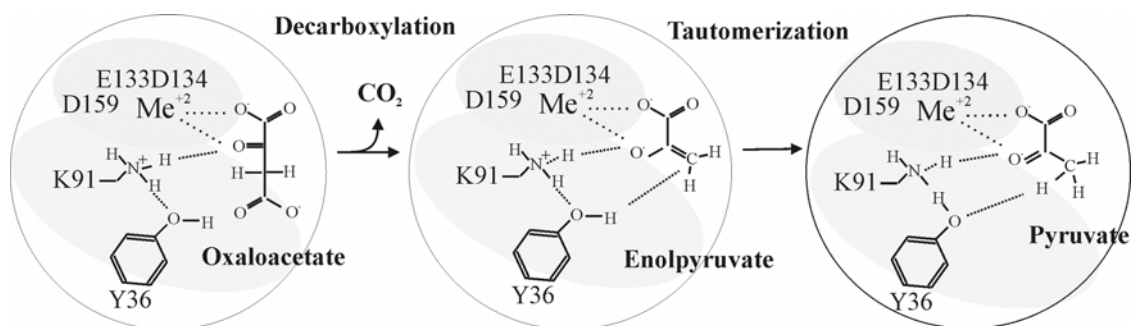


Figure 6. Putative catalytic mechanism for the soluble oxaloacetate decarboxylase (*citM* product) associated with citrate metabolism. Conserved residues are indicated (see details in the text).

binding site, and two Rossmann Domains (GXGXXG), are highly conserved in all members of the MEF. These functional conservations indicate a common mechanism for oxaloacetate decarboxylation.

6. Regulation of citrate transport and conversion into pyruvate

The genes involved in the transport and conversion of citrate into pyruvate are grouped together in the chromosomes of different micro-organisms (Fig. 3). In *K pneumoniae* *oadGAB* genes, which encode the OAD complex, the *citS* gene encoding for the citrate transporter, and the regulatory genes *citAB*, constitute an operon. Adjacent to this operon, and with divergent polarity, is transcribed the *citCDEFG* operon which is responsible for the synthesis of the proteins involved in the first step of citrate metabolism [27]. The *citA* and *citB* gene encodes the CitA and CitB proteins, which constitute a signal transduction two component system (TCS). In this system CitA is the sensor kinase, while CitB is the response regulator [27]. The system regulates its own expression as well as the expression of the *citS* gene and the *citCDEFG* and *oadGAB* operons (Fig. 3). Transcription is stimulated by the presence of citrate, low oxygen levels, the presence of sodium ions and alternative carbon sources other than citrate in the culture medium. The citrate fermentation genes are subject to catabolite repression exerted by the cAMP receptor protein Crp [27]. In *E. coli* the genes encoding the CitT permease (*citT*), the CL complex and its accessory proteins (*citCDEFXG*) as well as the TCS (*citAB*), are also grouped in the chromosome ([13] and Fig 3), and their expression presumably is also controlled by the TCS. This mechanism of regulation is also functional in *B. subtilis*, in which the CitST TCS (a member of the CitAB family) is involved in the regulation of the expression of genes encoding the citrate transporters required for the utilization of citrate in aerobic conditions [37,38,39].

The above quorum sensing molecular mechanism of regulation of citrate utilization does not appear to exist in LAB and we have described two different transcriptional regulatory circuits for these micro-organisms. In *W. paramesenteroides*, upstream of the *citMCDEFGRP* operon (which encodes CitM, CL and CitP), is located, with divergent polarity, the *citI* gene ([16,29] and Fig. 3). Transcription of the operon and *citI* from P_{cit} and P_{citI} promoters is induced upon addition of citrate to the growth medium [29]. Complementation experiments revealed that the presence of *citI* in *cis* and in *trans* resulted in an increased transcription from P_{cit} [29]. Characterization of CitI by DNA-protein interaction and *in vitro* transcriptional studies revealed that the protein is a transcriptional activator directly controlled by citrate and specifically involved in citrate metabolism, which induces its own transcription and that of the *cit* operon [40]. CitI is able to bind co-operatively to two operators O1 (high affinity) and O2 (low affinity) located between P_{citI} and P_{cit} (Fig. 3). Moreover, citrate increases the affinity of CitI to these operators, as well as enhancing the formation of P_{cit}-RNA polymerase (RNAP)-CitI ternary complex.

Based on the the above results, we have proposed the following model of regulation for CitI [40]. In the absence of citrate, CitI would bind to the operator sites stimulating the RNAP to form complexes on both promoters. This would result in low expression levels of the citrate fermentation enzymes as well as of the citrate permease P, which would catalyze the uptake of citrate when this compound becomes available in the external environment. Once transported inside the cell, citrate would bind to CitI enhancing the regulator affinity for its DNA operators and resulting in increased RNAP recruitment at P_{cit} and P_{citI}. This would result in transcriptional activation from both promoters. Activation of P_{cit} results in coordinated synthesis of the citrate fermentation enzymes and breakdown of citrate. The levels of *citI* and *cit* mRNAs *in vivo* are approximately 2.5- and 20-fold higher in cultures grown in the presence of citrate. Consequently, it seems that a small increase in CitI transcription could account for the large increase of the *cit* mRNA detected in *W. paramesenteroides* growing in a citrate-supplemented medium. The *Weissella* genetic organization of *citI* and the *cit* operon, and identical CitI operators, are also present in species of *Leuconostoc*, *Lactobacillus* and *Oenococcus* (Fig. 3). Consequently, it is probable that the mechanism of regulation of gene expression mediated by Cit I and citrate active in *Weissella* is also functional in these bacteria. Supporting this hypothesis, the transcription of the *Leuconostoc cit* operon is induced by the presence of citrate in the growth medium [41].

A *citMCDEFXG* (*cit*) operon occurs in the *L. diacetylactis* chromosome which encodes CitM and CL. This operon does not include the citrate permease *citP*; this latter gene is part of the *citQRP* operon which is located in a plasmid ([30] and Fig. 3). The *citQRP* operon is transcribed from the P1

promoter located 1 kb upstream of *citQ* [30], whereas the P_{cit} promoter located upstream of *citM* drives transcription of the *cit* operon ([34] and Fig. 3). A *citI* gene is also present in the lactococcal chromosomal *cit* region, with divergent polarity to the *cit* operon, but located between *citM* and *citC* (Fig. 3). An O1 operator is present upstream of the P_{citI} promoter, indicating that the regulator could control its own synthesis. However, an O2 operator for CitI does not exist upstream of P_{cit}. This could be the reason why transcription from P_{cit}, as for P1 [42], is not affected by the presence of citrate in the growth medium [34]. Interestingly, in this bacterium transcription from promoters P_{cit} and P1 is induced by growth under acidic conditions [34, 43]. Thus, it seems that when *L. lactis* cells detect a decrease in external pH, transcription of *citQRP* and *citMCDEFXG* operons increases in synchrony. As a consequence citrate is efficiently transported and converted into pyruvate. In addition, expression of the *citMCDEFXG* operon could trigger another circuit of transcriptional regulation mediated by CitI. We have detected that synthesis of *citI* mRNA also increases upon growth at acidic pH (unpublished results). This would result in increased levels of CitI, which could complex with the incoming citrate. The CitI-citrate complex, once bound to O1, would recruit RNAP, activating transcription from the P_{citI} promoter. It is worth noting that *citI* is located between the CitM and CL coding genes, and that the *cit* transcript includes the sequence complementary to the *citI* mRNA. Therefore, under steady state acidic conditions transcription of *citI* could down regulate expression of the CL coding genes. This could take place by collision of convergent rounds of transcription from P_{cit} and P_{citI} or by an antisense mechanism.

In addition to the transcriptional regulation provoked by acidic stress, there exist several mechanisms of regulation that affect the lactococcal *citQRP* citrate transport operon. As stated above in, *L. lactis* the operon is transcribed from the pH dependent P1 promoter (Fig. 3). In addition, it seems that introduction of the 1 kb insertion sequence *IS982* has generated a transcriptional promoter, named P2 (Fig. 3), which supports expression of the operon in *E. coli* [44]. In the citrate transport operon, only the citrate permease structural gene is required for citrate transport [45]. The *citQ* and *citR* genes seem to be involved in regulation of expression of CitP. Expression of *citQ* has been confirmed by *in vivo* analysis of translational fusions and by detection of the 3.5 kDa small CitQ polypeptide after synthesis by *in vitro* transcription and translation (unpublished results). Analysis of the influence of CitR on expression of CitP performed in *L. lactis* and *E. coli* indicate that CitR is a repressor, which controls the levels of CitP without affecting mRNA levels [30,46]. The 3'-region of *citQ* and the 5'-region of *citR* overlap and form a complex secondary structure. Analysis of the fate of the *citQRP* mRNA revealed that in *L. diacetylactis* and in *E. coli* the *citQRP* transcripts are

subjected to endonucleolytic cleavage at this location [30, 47]. Two endoribonucleases RNase III and RNase E catalyze the specific cleavages within the secondary structure, respectively upstream and at the ribosomal binding site of CitR [46,48]. In *L. lactis* the enzymes involved in the processing have not been identified. However, RNase III is ubiquitous and the enzyme and its activity on double stranded regions located in stem-loop structures have been identified in *L. lactis* [46, 49]. Determination of the genome sequence of various gram-positive bacteria has not shown the existence of a homologue of *E. coli* *rne* which encodes RNase E. However, results from our and other laboratories support that RNase E activity exists in *L. lactis* and *B. subtilis*. Therefore, it seems that analogues but not homologous to *E. coli* RNase E exist in gram-positive bacteria. The overall results allowed us to postulate the following mechanism of post-transcriptional regulation of *citQRP* expression. CitQ is a small peptide whose translation could compete with the formation and processing of the secondary structure by endoribonucleases, thereby modulating the level of the post-transcriptional regulator CitR, which controls translation of CitP.

7. Bioenergetics of citrate metabolism

Most of the knowledge on the metabolic pathways involved in citrate metabolism has been derived from LAB of dairy origin, although citrate itself does not always support their growth. This claim is based on the inability of certain LAB to grow in batch cultures to which citrate was added as the sole energy source. Nevertheless, most LAB are able to co-metabolize citrate in the presence of another energy source, such as glucose or lactose. Co-metabolism of citrate and sugar is of importance for food quality, as citrate and sugar are both present in fermented food products, such as during the fermentation of milk that naturally contains 8 to 9 mM of citrate. It is well documented that *L. diacetylactis* and species of *Leuconostoc* (such as *lactis* and *mesenteroides*) and *W. paramesenteroides* contribute, during the ripening of cheese, to the production of aroma (generated by the C₄ compounds synthesized from citrate and glucose) as well as to the formation of the “eyes” produced by the CO₂ released during citrate fermentation [3, 50].

In many dairy products significant number of enterococci are present. For example in many cheeses, such as Comté, Cebreiro, Mozzarella, Kefalotyri, Serra, Manchego, Feta, and Teleme, enterococci comprise a major part of the fresh cheese microbiota, and in some cases they are the predominant microorganisms in the fully ripened product. It has been concluded in many reports that enterococci may have an important role in cheese production, contributing to the ripening and quality of the mature products. Moreover, some researchers have suggested that enterococci may play a role in the development of the aroma and flavour of many cheeses, probably due to citrate catabolism

(reviewed in [51]). However, in contrast, to the extensive studies performed with *L. diacetylactis* and *Leuconostoc*, the utilization of citrate by *Enterococci* has not been systematically investigated until recently (reviewed in [51]).

Transport and citrate metabolism in *L. lactis* as well as in some species of the *Leuconostoc* genus, constitute a secondary system of proton-motive force generation (PMF) [1], in which both components of PMF, trans-membrane potential ($\Delta\Psi$) and proton gradient (ΔpH), are generated in two different stages. The $\Delta\Psi$ is produced during the antiport catalyzed by CitP of one molecule of divalent citrate, that enters into the cell, and one molecule of monovalent lactate, that is excreted [1]. The result of this process is the cellular uptake of one negative net charge. The ΔpH arises as a consequence of the consumption of protons during citrate degradation and also to the fact that the end-products (lactate, acetate, CO_2 , diacetyl, acetoin, etc.) of this catabolism are less acidic than citrate [1]. It is well documented that citrate metabolism provokes alkalinization of the cytoplasm by consumption of intracellular protons, and of the external medium by excretion of less acidic compounds [1]. Although in both micro-organisms, it was shown that they are capable of generating PMF through this metabolism, it was not enough to support growth with citrate as the only source of energy. However, the PMF generated could contribute to the growth enhancement during co-metabolism with sugars as well as surviving acidic stress conditions.

The co-metabolism of glucose and citrate produces different physiological effects in homofermentative and heterofermentative LAB. As we describe in detail below, in homofermentative LAB citrate utilization has a protective effect against acid stress, whereas in heterofermentative LAB “citrolactic” fermentation generates one extra mol of ATP per mol of citrate.

Bacteria from the genera *Lactococcus* and *Enterococcus* as well as *Lb. plantarum* degrade sugars (such as glucose or lactose) by a homofermentative pathway forming lactic acid as the main end product (Fig. 7). During the co-metabolism of one of these sugars with citrate, large amounts of pyruvate are generated which is converted to the production of the C_4 aroma compounds [51]. *L. diacetylactis* metabolizes citrate present in milk producing high concentrations of diacetyl. In these conditions the metabolism of glucose produces lactic acid, which is excreted from the cell in exchange for citrate by the antiporter CitP. By means of the homofermentative pathway this organism catalyzes the conversion of glucose into lactate producing 2 moles of ATP per mol of glucose metabolized. The NAD^+ consumed in the first steps of the pathway is regenerated during the conversion of pyruvate to lactate maintaining the redox balance. In the presence of glucose and citrate, each mol of citrate produces one mol of pyruvate without generating NADH. This produces an intracellular excess of pyruvate that is diverted to the synthesis of

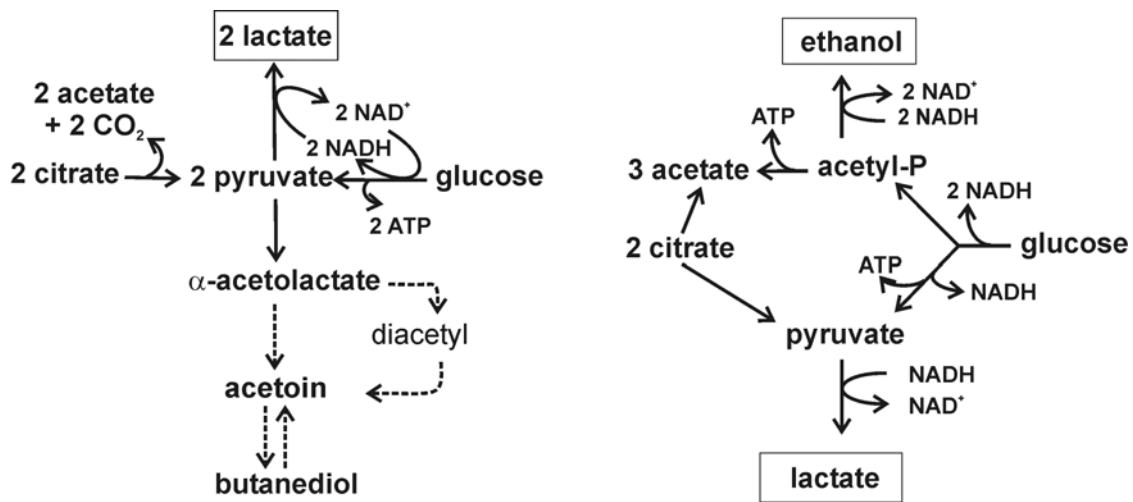


Figure 7. Co-metabolism of citrate and glucose in LAB. Left, homofermentative bacteria. Right, heterofermentative bacteria.

α-acetolactate and subsequently to the production of the C₄ aroma compounds. The end-products of this co-metabolism are lactate diacetyl, acetoin y butanediol. In *L. lactis*, the utilization of citrate results in a growth advantage which is evident when the medium has low pH values [34, 43, 52]. In these conditions, the presence of citrate provides an efficient use of glucose and also allows the cells to resist the high concentrations of lactate produced as a metabolic by-product. This detoxification effect has been attributed to the efficiency of the citrate/lactate antiport catalyzed by CitP [52], which increases its activity during the co-metabolism of glucose and citrate [43, 52]. The role of citrate metabolism in *Enterococci* is still unclear. Analysis of metabolic characteristics of 129 *En. faecalis*, *En. faecium* and *En. durans* strains indicated strain-to-strain variation with regard to citrate metabolism. However, the majority of the *En. faecalis* strains were able to utilize citrate [53, 54]. Interestingly, analysis of sugar and citrate metabolisms in *En. faecalis* and *En. durans* has revealed that in some strains citrate can be utilize in co-metabolism with lactose and not with glucose [53, 55]. In these strains citrate can only be utilized when all of the glucose in the medium is consumed. Therefore, it has been postulated that citrate utilization is controlled by catabolic repression [55, 56]. However, this mechanism has not yet been studied at the molecular level. In addition, it has been shown that *En. faecium* is able to co-metabolize glucose and citrate, generating energy production and C₄ aroma compounds Most of the citrate is consumed during the stationary phase, indicating that energy generated by citrate metabolism is used for maintenance by this host [57].

Glucose metabolism in *Leuconostoc*, *Oenococcus* and *Lactobacillus* strains takes place through the pentose phosphate pathway giving origin to heterolactic fermentation characterized by lactate, ethanol and acetate as end

products (review in [58] and Fig. 7). In this case the main part of the acetylphosphate formed during glucose degradation is reduced to ethanol through the phosphoketolase pathway, regenerating NAD^+ consumed previously. However, the presence of citrate together with glucose provides a growth advantage and provokes a shift of the sugar to the heterofermentative route. When citrate is present, the NAD^+ is regenerated through pyruvate reduction to lactate. The acetylphosphate is therefore converted to acetate by means of the acetate kinase enzyme, generating an extra mol of ATP [1]. Under these conditions of co-metabolism, the name of citrolactic fermentation is adequate, since there exists a direct relationship between the substrate (citrate) and the end product (lactic acid). On the other hand, the production of C_4 compounds in *Leuconostoc* only takes place in conditions of pyruvate accumulation, for example at low pHs and in the presence of citrate. In these conditions inhibition of LDH occurs, which results in higher levels of pyruvate.

8. Enzymes and coding genes involved in the conversion of pyruvate into aroma compounds

The first step of this pathway is catalyzed by α -ALS. Marrug and coworkers [59] determined the sequence of the *als* gene encoding α -ALS from *L. lactis* DSM20384. Numerous NMR studies, performed in strains of *E. coli* carrying the wild type or mutant *als* genes from *L. lactis*, corroborated that this gene encodes the α -ALS activity in *Lactococcus*. The native enzyme is a dimer composed of two monomers of 62 kDa, the expected molecular mass for the inferred product of the *als* gene. The enzyme uses thiamine pyrophosphate as cofactor and, in concordance with this, a binding motif specific for the cofactor was detected [59]. The *L. lactis* α -ALS is also present in other Gram-positive bacteria and other procaryotic organisms [59]. Moreover, acetohydroxyl acid synthase (AHAS) also occurs in *L. lactis*, which has significant homology with lactococcal α -ALS at the amino acid level. Interestingly, AHAS is a bifunctional enzyme, which yields acetohydroxybutyrate by condensation of pyruvate and ketobutyrate, and also catalyzes the conversion of pyruvate into α -acetolactate like α -ALS. A transcriptional analysis has revealed that the *als* gene from *L. diacetylactis* DSM20384 is transcribed from a promoter localized 63 nucleotides upstream from transcriptional initiation site, generating a 2 kb monocistronic mRNA [7]. The measurements of α -ALS levels in cultures of *L. diacetylactis* C17 grown in aerobic, anaerobic or lactose-limiting conditions indicated that *als* gene expression is constitutive [7]. A detailed transcriptional analysis of *als* expression in cultures grown on different physiologic conditions has never been made. However, the acid tolerance response of *L. cremoris* MG1363 has been investigated by proteomic analysis, after challenging for a

short period at pH 3.5 [60], and an increase of the Als levels was observed after acid stress. This result deserves further study to reveal whether expression of *als* is induced at acid pH. The lactococcal *als* gene is located upstream of (though not adjacent to) the *cit* operon encoding OAD and CL. This suggests that during evolution the genes required for the conversion of citrate into α -acetolactate have been acquired as a genetic cluster.

The *aldB* gene from *L. lactis* subsp. *lactis* NCDO2118 was cloned and the characterisation of the enzymatic activity of its product confirmed that this gene encodes α -ALD [61]. AldB plays a dual role, catalyzing the second step of the acetoin biosynthetic pathway, and regulating the pool of α -acetolactate in the cell during branched chain amino acid (BCAA) metabolism. This second role is very important as these BCAA are essential for protein synthesis, and consequently the expression of the enzyme is strictly regulated at both transcriptional and post-transcriptional levels [6, 61, 62]. The *aldB* is located (Fig. 8) downstream of genes encoding the enzymes involved in the biosynthesis of BCAA (leucine, valine and isoleucine) and precedes *aldR* gene encoding a putative regulator, whose function has not been completely elucidated [61]. The transcriptional analysis of *aldB* expression revealed that this gene forms an operon with *leu*, *ilv* and *aldR* genes. Interestingly, *aldB* is transcribed from three different promoters: P1, P2 and P3. Isoleucine represses transcription from P2 and leucine affects transcription from P1 by attenuation. In addition, α -ALD is allosterically activated by leucine [6]. Goulpin-Feullerat *et al.* [62] showed that expression of *aldB* is controlled at the post-transcriptional level by a secondary structure, which blocks the ribosomal binding site of the gene and interferes with AldB synthesis. This structure only exists in the *aldB* transcripts synthesized from P1 and P2. The expression from P3 is not affected by the presence of amino acids in the growth medium. However, transcriptional activation of P3 by factors connected with citrate metabolism has not been investigated. The overall regulation could have evolved to increase significantly the levels of AldB, without having an impact in the BCAA biosynthesis and deserves further investigation. The sequence comparison of the products of *aldB* from *L. lactis* strains NCDO2118 [61], MG1363 [63] and IL1403 [35] revealed that they are almost identical polypeptides, each of 236 amino acids, and that this class of enzymes is highly conserved in Gram-positive and Gram-negative bacteria. Furthermore, the *aldC* gene occurs in strain IL1403, whose product may be an isoenzyme of α -ALD. In *L. cremoris*, the *aldB* gene is also located adjacent to the *ilv* operon and may be subject to the same regulation of expression. Interestingly, in the *L. cremoris* SK11 strain, there exists an extra-chromosomal copy of *aldB* (98% identity) located on plasmid pSK11B [64], in addition to a chromosomal copy of *aldC*, which could be acting as an ALD.



Figure 8. Organization and transcription of the *L. diacetylactis* *leu-ilmv-ald* cluster. The genes and promoters are depicted. See details in the text.

DAR catalyzes two stages of citrate metabolism: it converts diacetyl into acetoin and also reduces acetoin to 2, 3-butanediol. The conversion of 2,3-butanediol into acetoin is catalyzed by BDH, and in *L. diacetylactis* two proteins seem to possess this enzymatic activity as well as DAR activities [8]. Rattray, et al. characterized the DAR of *Lc. pseudomesenteroides* encoded by the plasmidic *butA* gene [65,66]. Bioinformatic analysis performed using the *butA* gene of *Lc. pseudomesenteroides*, allowed us to identify the *butA* gene in the IL1403 genome, whose product shares 81% homology with the ButA from *Leuconostoc*. Preceding the *L. diacetylactis* IL1403 *butA* is located the *butB* gene, whose product is annotated as BDH in the lactococcal genome data bank, and belongs to the Zn-containing alcohol dehydrogenase superfamily PTHR11695. The genes *butB* and *butA* are also linked in the *L. cremoris* (MG1363 and SK11) chromosomes. However, in other species of LAB, such as *Lc. pseudomesenteroides* [66] *butA* is carried on plasmids, which could facilitate its horizontal transfer. The two DARs purified from lactococcal cells, both have less affinity for 2,3-butanediol than for diacetyl and acetoin as substrate and, both have an optimal alkaline and acidic pH, respectively, for its BDH and DAR activities [8]. Therefore, it is tempting to postulate that at acidic pH an increase of 2,3-butanediol results, though still allowing significant amounts of acetoin; this has been observed in *L. diacetylactis* strains [5]. This effect may contribute to the regulation of the NADH/NAD ratio by the reversible inter-conversion of acetoin and 2,3-butanediol. A *dar* gene exists in the MG1363 and SK11 chromosomes (but not in IL1403 genome) whose product has 46% identity with that of *butA*. Inactivation of *dar* in the chromosome of *L. cremoris* MG1363 [67] resulted in a decrease of the DAR activity, confirming the enzymatic identity of the gene product. The existence of three DAR in *L. cremoris* subsp., together with its lack of CL and OAD, could contribute to the lower production of acetoin compared to *L. diacetylactis*. Moreover, the *L. lactis* subsp. is preferred as an industrial starter culture, since these flavours compounds are very valuable in improving food quality.

It should be noted that transcriptional analyses of DAR enzymes have not been yet investigated. Thus, several factors remain to be elucidated such as the control of expression of the coding genes, as to demonstrate whether *butBA* constitute an operon, and if really *dar* is the second gene of a putative operon formed by seven genes.

9. Conclusions and perspectives

The biochemistry and bioenergetics of citrate transport and its subsequent conversion into pyruvate is well understood in most of dairy LAB. It has been demonstrated in the *L. lactis* homofermentative LAB that citrate metabolism is involved in the adaptative response to acidic stress and it contributes to homeostasis. However, the mechanism of activation of the *cit* promoter at acidic pH is still unknown. No evident operators required for binding of specific regulators have been detected in the promoter regions. Remarkably, no stress sigma factor of the RNAP is found in the *L. lactis* genome, which necessarily points to an alternative regulatory mechanism for lactococcal *cit* promoters, which has still to be elucidated.

In species of *W. paramesenteroides* and *Leuconostos* and probably other heterofermentative LAB, citrate metabolism is mainly devoted to energy generation and it is activated at the transcriptional level by the CitI regulator and citrate. The role, if any, of the lactococcal CitI in the induction of the *cit* operon at acidic pH requires further investigation.

Synthesis of aroma compounds from citrate *via* pyruvate is commercially important for the dairy and other food industries, but there is a gap between the knowledge of the biochemistry of the C₄-compound biosynthetic pathway and a complementary understanding of the genetics and regulation of the pathway. The recent determination of the entire genomes of various LAB provides a valuable tool to identify genes encoding enzymes that potentially could be involved in the pathway, and even to postulate pathways *in silico*, as for example has been done for the citrate catabolism of *Lb. casei* [68]. This will contribute in the near future to a better understanding of citrate utilization by species of *Enterococcus* and *Lactobacillus*.

Finally, due to the importance of diacetyl production to the dairy industries, the use of metabolic engineering has been investigated, which has allowed the generation of recombinant *L. lactis* strains able to overproduce diacetyl from glucose (reviewed in [69] and see details in chapter 9). However, as far as we know there is not any product on the market containing these bacteria either in Europe or in USA, due to current regulations on the use of genetically manipulated micro-organisms and also, to the fact that these strains, lacking the citrate transporter, do not produce very high levels of diacetyl. Presumably, transfer of the plasmid encoding the citrate permease to the *L. lactis* strains will significantly increase their ability to produce diacetyl. This strategy is showing promising results in the generation of a food-grade *Lactobacillus casei* diacetyl producer strain [70]. It is expected that this, and other approaches will be used to generate diacetyl overproducing strains in other LAB such as *Enterococci*.

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