Characterization of an oxaloacetate decarboxylase that belongs to the malic enzyme family

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Abstract The *citM* gene from *Lactococcus lactis* CRL264 was demonstrated to encode for an oxaloacetate decarboxylase. The enzyme exhibits high levels of similarity to malic enzymes (MEs) from other organisms. CitM was expressed in *Escherichia coli*, purified and its oxaloacetate decarboxylase activity was demonstrated by biochemical and genetic studies. The highest oxaloacetate decarboxylation activity was found at low pH in the presence of manganese, and the K_m value for oxaloacetate was 0.52 ± 0.03 mM. However, no malic activity was found for this enzyme. Our studies clearly show a new group of oxaloacetate decarboxylases associated with the citrate fermentation pathway in gram-positive bacteria. Furthermore, the essential catalytic residues were found to be conserved in all members of the ME family, suggesting a common mechanism for oxaloacetate decarboxylation.

Keywords: Citrate fermentation; Lactic acid bacteria; Malic enzyme; Oxaloactetate decarboxylase

1. Introduction

Malic enzymes (MEs) catalyze the reversible oxidative decarboxylation of L-malate to produce pyruvate and carbon dioxide, coupled to the reduction of NAD^+ or $NADP^+$. These enzymes also require the presence of divalent cations (most commonly Mg^{2+} or Mn^{2+}) for their catalytic activity. Three classes of MEs (EC1.1.1.38, EC1.1.1.39, and EC1.1.1.40) have been defined on the basis of their coenzyme specificity and their capability to decarboxylate oxaloacetate. MEs have been found in representatives of all of the major biological divisions and participate in diverse metabolic pathways such as photosynthesis, lipogenesis, and energy metabolism. In eukaryotes, cytoplasmic, mitochondrial and chloroplastic isoforms have been identified and crystal structures of several MEs have been reported [1]. The prokaryotic MEs described so far are fewer in number and more diverse in structure than their eukaryotic counterparts. MEs have been characterized from *Bacillus stearothermophilus* [2], *Sulfolobus solfataricus* [3], *Rhizobium meliloti* [4,5], *Corynebacterium glutamicum* [6] and *Streptococcus bovis* [7].

In *Escherichia coli*, the deletion or mutation of the two paralogous genes encoding MEs (*sfcA* and *b2463*) resulted in severe growth defects when this microorganism was grown in the presence of C_4 dicarboxylic acid [8,9]. In *Bacillus subtilis*, it is possible to identify four paralogous genes encoding MEs (*maeA*, *malS*, *ytsJ and mleA*) but only *ytsJ* is required for efficient growth on malate as well as on other TCA intermediates [10].

Recently, we have cloned and sequenced the clusters involved in the citrate metabolism pathway from *Weissella paramesenteroides* J1 [11] and *Lactococcus lactis* CRL264 [12]. Transcriptional analysis showed that in *W. paramesenteroides*, the genes encoding the citrate permease (*citP*), the citrate lyase complex (*citDEF*) and a putative ME (*citM*) are transcribed in a polycistronic unit (*citMCDEFGRP*) thus constituting the *cit* operon. The expression of this operon is increased in the presence of citrate [11]. In *L. lactis* CRL264, the citrate utilization genes are organized in two different operons: a chromosomal *cit* operon including the genes *citM-CDEFXG* (in a molecular organization similar to *W. paramesenteroides*) and a plasmidic operon including the *citP* gene. In this case, the transcription of both operons is coordinately induced under acidic conditions [12–14].

In this paper, we describe the characterization of the *L. lactis* oxaloacetate decarboxylase (product of the *citM* gene), including some unique properties. By amino acid sequence comparison to other MEs, we could identify a closed group of oxaloacetate decarboxylases associated to the citrate pathway in gram-positive bacteria.

2. Materials and methods

2.1. Bacterial strains and growth media

Escherichia coli DH5α (Bethesda Research Laboratories) was used as a general cloning host, *E. coli* M15 [pREP4] (QIA express) was used for expressed His_6-CitM and *E. coli* EJ1321 [*galK2*, *pck*, *dme*, *tme*, Str^r] [8] was used for complementation studies. *E. coli* cells were grown aerobically at 37 °C in LB medium and transformed as previously described [15]. Complementation test was performed in minimal medium M9 [15] supplemented with 0.4% succinate, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and adjusted at initial pH 5.5. Cultures were incubated at 30 °C with shaking and growth was monitored by measuring the absorbance at 600 nm in a Beckman DU640 spectrometer. *L. lactis* subsp. *lactis* biovar. diacetylactis CRL264 (*lac*$^+$, *pro*$^+$, *cit*$^+$) [14] and *L. lactis* subsp. *cremoris* MG1363 (*lac*$^-$, *cit*$^-$) [16] cells were grown in batch cultures at 30 °C without

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Abbreviations: ME, malic enzyme; IPTG, isopropyl-β-D-thiogalactopyranoside; OAA, oxaloacetic acid; LDH, L-lactate dehydrogenase

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shaking. M17 broth medium (Oxoid) supplemented with 0.5% glucose (M17G) was adjusted at initial pH 5.5 or 7.0 with HCl acid [13]. Streptomycin (5 μg ml^{-1}), kanamycin (25 μg ml^{-1}), ampicillin (100 μg ml^{-1}) and erythromycin (1 and 100 μg ml^{-1} for *L. lactis* and *E. coli*, respectively) were added to the medium when necessary.

2.2. Cloning, expression and complementation

The open reading frame corresponding to CitM from *L. lactis* CRL264 was amplified by PCR using a forward primer (5′-AGGAAAGAGGATCCATGGTTGATTTTAATAAAG-3′) containing a *Bam*HI restriction site and a reverse primer (5′-TCCAGAAAAGCTTGTCGTTAAACCAG-3′) containing a *Hin*dIII restriction site. The amplified DNA fragment was ligated to the *Bam*HI and *Hin*dIII sites of pQE30 expression vector (Qiagen) giving the pQE264 plasmid. Thus, this plasmid has the *citM* gene under control of the T5 promoter recognized by the *E. coli* RNA polymerase. To control the expression of *citM* from the pQE264 plasmid, EJ1321 was transformed with the plasmid pREP4 carrying the *lacI* gene. The new strain EJ1321 (pREP4) was transformed successfully with plasmid pQE264 (strain EJ264) or the vector pQE30 (strain EJ30).

To construct the pHSO1 plasmid, a 1.7 kb DNA fragment containing the *citM* gene from *L. lactis* CRL264 under control of its own promoter (P*cit*) [12] was amplified by PCR employing the primers (5′-ATGTAGAGAAGCTTCAAAAAAATAATGCACTCC-3′) and (5′-ATAGCGGATCCCAATAATAATTACAATCATCAG-3′) containing *Hin*dIII and *Bam*HI restriction sites, respectively. This product was purified, digested with *Hin*dIII and *Bam*HI and cloned in the same sites of the pAK80 vector [17]. This plasmid was subsequently transformed into strains IL1403 and MG1363 as previously described [18].

To determine the oxaloacetate decarboxylase activity in cell extracts of *L. lactis*, 100 ml of culture was grown until mid-exponential phase. The cells were then harvested by centrifugation and resuspended in ice-cold buffer A (sodium acetate–acetic acid buffer, pH 4.4, and 3 mM $MnCl_2$) containing 1 mM PMSF and 10% glycerol. Total protein extracts of *L. lactis* were prepared by passing cells three times through a French Pressure Cell at 10 000 lb/in.^{2} and cell debris was removed by centrifugation at 20 000 rpm for 25 min. The soluble fraction was used for oxaloacetate decarboxylase assays.

2.3. Purification of CitM

High level of soluble recombinant His tagged CitM protein was recovered when *E. coli* M15 (pREP4, pQE264) cells were grown in LB at 37 °C until $O.D._{600\,nm} = 0.6$ and induced by addition of 0.5 mM IPTG during 6 h at 20 °C with gentle shaking (25 rpm). Then, 500 ml of culture was harvested by centrifugation and resuspended in ice-cold buffer A containing 1 mM PMSF and 10% glycerol. The cells were lysed through a French Press and cell debris was removed by centrifugation as described. After adding 150 mM NaCl, CitM was purified from the soluble fraction by affinity chromatography using a nickel-NTA column according to the protocol recommended by Novagen. CitM was eluted at 100 mM imidazole. The purified enzyme was then dialyzed in buffer A containing 20% glycerol and stored at −80 °C for further studies. Protein concentration was determined by Lowry method using bovine serum albumin as standard.

2.4. Decarboxylation of oxaloacetate

Oxaloacetic acid, $MnCl_2$, L-lactate dehydrogenase (LDH) from rabbit muscle, sodium acetate, acetic acid, $NAD(P)^+$, and NAD(P)H were from Sigma. L-malate and all other chemicals and reagents were obtained from commercial sources and were of high purity. The oxaloacetic acid (OAA) decarboxylation was monitored following the decrease in the absorbance of enolic form of OAA [19]. λ_{max} for enolic OAA was obtained from the absorption spectrum between 240 and 340 nm. In the reaction conditions tested (50 mM sodium acetate–acetic acid buffer, pH 4.4, 10 mM $MnCl_2$, and 1 mM OAA), the λ_{max} for OAA was 280 nm and the effective extinction coefficient was 1.47 cm^{-1} mM^{-1}. When a divalent ion was added a spontaneous decarboxylation of OAA was detected, and its rate increased in acidic conditions and at high temperature.

2.5. Enzyme activity assay

Enzymatic assays were performed in a Spectronic Unicam spectrophotometer at 30 °C using 10 mm path length and 1 ml cells. CitM activity was determined following the OAA decarboxylation in standard conditions (50 mM sodium acetate–acetic acid buffer, pH 4.4, 10 mM $MnCl_2$, 1 mM OAA, and 2.5 μg of enzyme) by measuring the decreases in the A_{280}. The reported OAA decarboxylase activity was corrected considering the spontaneous decarboxylation of OAA catalyzed by the divalent metal ion.

Table 1
Inhibition of oxaloacetate decarboxylase activity by different compounds

	(%) Inhibition	
	0.5 mM	2 mM
Malate	23	65
Fumarate	2	21
Succinate	8	23
α-Keto-glutarate	6	11
Citrate	24	24
Oxalate	80	93
Malonate	84	92
Pyruvate	1	8
[a] Ni^{2+}	30	40
[a] Ca^{2+}	5	30
[a] Zn^{2+}	100	100
[a] Cu^{2+}	100	100
NADH	50	100
NAD^+	50	100

The enzymatic activity measurements were carried out in 50 mM sodium acetate–acetic acid buffer, pH 4.4, 1 mM oxaloacetate and 10 mM Mn^{2+}, except in (a) where 1 mM Mn^{2+} was used. In all cases, the same amount of enzyme (2.5 μg) was added.

The optimal pH was determined in 50 mM sodium acetate–acetic acid buffer (1 mM oxaloacetate and 10 mM $MnCl_2$) ranging from 3.0 to 6.0 pH units. A Michaelis constant for OAA was determined varying the OAA concentration and keeping the Mn^{2+} concentration at saturating conditions (10 mM). The experimental dates were evaluated by the Michaelis Menten equation, by non-linear regression. The effect of different metals, inhibitors and substrate concentrations on the oxaloacetate decarboxylase activity was tested by inclusion of the appropriate amounts of each compound in the assay mixture, as indicated in Fig. 2 and Table 1.

Pyruvate production from OAA decarboxylation in the reaction catalyzed by CitM was analyzed through the following enzymatic assay. First, an OAA decarboxylation assay was run during a minute in standard conditions. Then, the reaction mixture was adjusted to a final concentration of 200 mM NaCl, 0.2 mM NADH, pH 7.0. The presence of pyruvate was monitored by disappearance of NADH at 340 nm by adding 5 U ml^{-1} LDH, pH 7.0.

One unit of the enzymatic activity was defined as the amount of enzyme catalyzing the decarboxylation of 1 μmol of OAA per min, at 30 °C, under the assay conditions. Specific activity was expressed in units per milligram of protein. Data are reported as means of triplicate experiments ± S.E.

The oxaloacetate decarboxylase activity in cell extracts was determinated in 50 mM sodium acetate–acetic acid buffer, pH 4.4, 10 mM $MnCl_2$, and 1 mM OAA was used.

2.6. Immunoblot analysis

Rabbit polyclonal antiserum against *L. lactis* CitM was generated using the purified His-tagged CitM protein as antigen (Bioterio FCByF, UNR). Rabbits were immunized subcutaneously with 100 μg of purified protein. Polyclonal antibodies raised against CitM were purified [15] and used at a 1:1000 dilution.

For Western blot analysis, cells were lysed by French press as described and samples containing 30 μg of total protein were loaded onto a sodium dodecyl sulfate electrophoresis gel [15]. Then, electrophoretic transfer of polypeptides from SDS–PAGE gels to nitrocellulose membranes was carried out using a Bio-Rad minigel apparatus. The CitM-antibodies complex was visualized using Goat anti-rabbit IgG (H+L)-AP conjugate antibodies from Bio-Rad and *p*-nitro blue tetrazolium (ICN) plus 5-Br-4-Cl-3-indolyl phosphate (Sigma) as alkaline phosphatase substrates.

To quantify the levels of protein from Western blots, densitometry was performed by scanning the photographs and performing an

analysis with an ImageQuant V.5 software (Molecular Dymanics, 1999).

3. Results

3.1. Complementation of the malic enzyme-deficient E. coli strain EJ1321

E. coli employs two different mechanisms for the synthesis of phosphoenolpyruvate from C_4 compounds such as oxaloacetate, succinate or malate. One pathway involves the ATP-dependent action of PEP-carboxykinase (*pck*). The other route utilizes the decarboxylation of C_4 dicarboxylic acid to pyruvate, catalyzed by MEs (*dme*, *tme*) [8]. The EJ1321 strain is a triple mutant (*dme*, *tme*, *pck*) unable to grow in minimal medium supplemented with malate or succinate because it cannot convert C_4 dicarboxylic acid into C_3 compounds (Fig. 1). However, this mutant strain could be complemented by the product of *citM* gene restoring its capability to grow in minimal medium with succinate as sole carbon source.

In accord with our hypothesis, the EJ264 cells (EJ1321 carrying the *citM* gene in pQE264 plasmid, see Section 2) were able to grow in minimal medium supplemented with succinate (Fig. 1). The EJ30 cells (control strain EJ1321 carrying the plasmid vector pQE30) were unable to grow in the same conditions. Thus, CitM restores the ability to grow on C_4 dicarboxylic acid, suggesting that this enzyme could generate pyruvate from OAA (Fig. 1). In this way, in these experimental conditions, pyruvate could be generated from the combined action of succinate dehydrogenase, malate dehydrogenase (*mdh*) and OAA decarboxylase (*citM*), converting succinate to malate, malate to OAA, and OAA to pyruvate, respectively. Although malic activity could not be excluded in this experiment, this activity has not been reported in *L. lactis* [7].

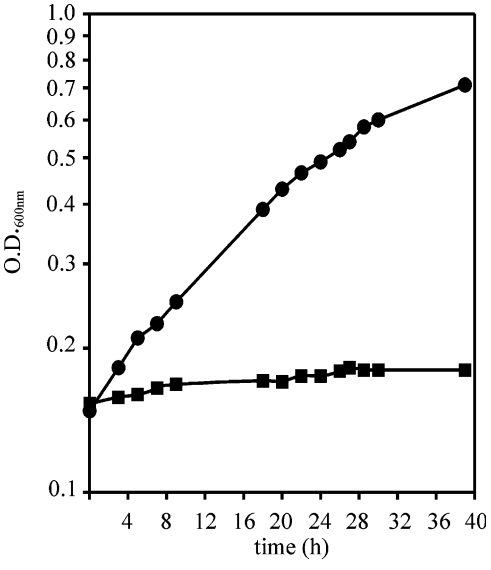

Fig. 1. Growth curves of *E. coli* EJ1321 (*dme*, *tme*, *pck*) transformed with plasmid pQE264 (strain EJ264, ●) or pQE30 (strain EJ30, ■). Cells were grown in minimal medium M9 with succinate as sole carbon source.

3.2. Biochemical characterization:

The *citM* gene was expressed in *E. coli* M15 and the CitM protein was purified as a His-tagged protein. The activity of this purified recombinant product was determined by measuring the rate of OAA decarboxylation.

(i) *Kinetic parameters*. The *L. lactis* CitM catalyzed specific oxaloacetate decarboxylation. The kinetic constants (K_m and V_{max}) of the purified enzyme related to oxaloacetate decarboxylation were calculated. We determined a K_m value for oxaloacetate of 0.52 ± 0.03 mM (Fig. 2A) with a maximum specific activity of 30.04 ± 1.51 U/mg, k_{cat} of 20.26 s^{-1} and k_{cat}/K_m of 39.0 s^{-1} mM^{-1}.

(ii) *Divalent cations requirements*. Malic enzymes need an essential divalent metal ion, which plays dual functional roles in catalysis and in structural stability [1]. The decarboxylase activity of CitM was demonstrated to be dependent on the presence of Mn^{2+} (maximum activity was observed in the presence of 10 mM Mn^{2+}). In the absence of divalent cations, or in the presence of 10 mM EDTA, no activity could be measured. Replacement of the catalytically essential Mn^{2+} by other metal ions leads to loss of activity. The effect of several metal ions on the activity was analyzed, Mg^{2+} and Ni^{2+} but not Ca^{2+}, Zn^{2+} or Cu^{2+} can also act as cofactors of the reaction in the absence of Mn^{2+} (data not shown). In the presence of 1 mM Mn^{2+}, these cations produce inhibition of the decarboxylation activity but complete inhibition was found with either Cu^{2+} or Zn^{2+} (Table 1).

(iii) *Effect of pH and temperature*. The pH activity profile of the bacterial enzyme was determined and yielded a bell-shaped curve (Fig. 2B). The optimum pH for the OAA decarboxylation activity was 4.4, using 50 mM sodium acetate–acetic acid buffer, 1 mM oxaloacetate and 10 mM $MnCl_2$. No measurable activity was detected at pH values above 6.5 and below 3.0. When the activity was measured at various temperatures, the highest specific activity of the enzyme was obtained at 50 °C and no measurable activity could be detected above 58 °C.

(iv) *Effect of substrate-analogue compounds on the OAA decarboxylase activity*. Oxalate (dead-end analogue of enolpyruvate) and malonate (dead-end analogue of oxaloacetate/pyruvate), produced high inhibition on the activity (Table 1). Malate and other intermediates of the TCA cycle produced some inhibition (Table 1). Furthermore, we found inhibition on the activity of CitM in the case of fumarate and succinate, both characterized as positive effectors of many MEs [1]. None of the following compounds exerted a significant effect on the CitM activity: pyruvate,glucose-6-phosphate, arginine, proline, and alanine (data not shown). By contrast to other MEs with OAA decarboxylase activity, the presence of dinucleotides did not increase the affinity of enzyme for OAA [20]. Neither NAD^+ nor $NADP^+$ were required for OAA decarboxylation reaction at any of the pH values measured. In fact, we determined that dinucleotides inhibited the reaction. When either 0.50 mM NAD^+ or 0.50 mM NADH was added, the specific activity was reduced by approximately 50% (Table 1). Similar results were found in the presence of $NADP^+$ and NADPH (data not shown).

(v) *Conversion of OAA to pyruvate by CitM*. The presence of pyruvate in the reaction mixture after the action of CitM

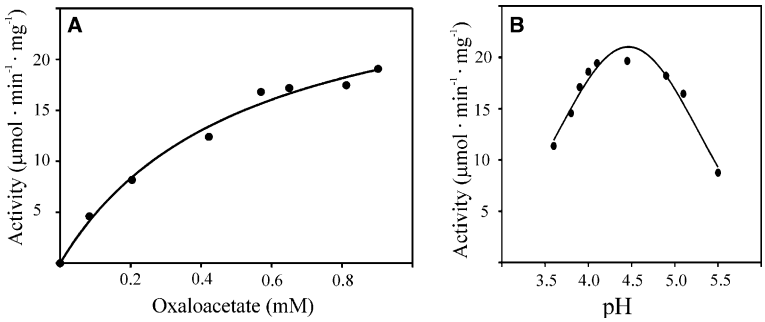

Fig. 2. (A) Oxaloacetate decarboxylase activity versus substrate concentration. Data were obtained from activity measurements carried out in 50 mM sodium acetate–acetic buffer, pH 4.4, and 10 mM Mn^{2+} at the oxaloacetate concentrations indicated. (B) pH dependence of the oxaloacetate decarboxylase activity. Data were obtained from activity measurements carried out in 50 mM sodium acetate–acetic buffer, 1 mM oxaloacetate, and 10 mM Mn^{2+} at the pH indicated in the figure. In all cases the same amount of enzyme (2.5 μg) was added.

on oxaloacetate was determined as indicated in Section 2. Once the oxaloacetate decarboxylation had occurred, NADH and LDH were added to the reaction mixture. The presence of pyruvate was confirmed by the rapid reduction of the A_{340} as a consequence of the NADH consumption during the reduction of pyruvate to lactate. In order to determine if the OAA decarboxylation reaction was reversible, we measured the appearance of OAA absorbance at 280 nm in the presence of 50 mM Tris–Cl, pH 7.0, 30 mM sodium pyruvate, 10 mM $NaHCO_3$, 8 mM NH_4Cl and 10 mM $MgCl_2$ or $MnCl_2$ [6]. However, no signal could be detected.

(vi) *Malic enzyme activity*. CitM did not exhibit malate oxidative decarboxylation activity although different assay conditions were tested. This enzymatic activity was analyzed by determining the rate of pyruvate formation from malate, following the increase of absorbance at 340 nm [6,21]. We employed different concentrations of Mg^{2+}, Mn^{2+}, malate, pH, NAD^+ and $NADP^+$.

3.3. *Expression of CitM in* L. lactis

In lactic acid bacteria, the first intermediate in citrate breakdown, oxaloacetate, results from the activity of the citrate lyase complex [14]. The *citM* gene from *W. paramesenteroides* [11] and *L. lactis* [12] and *mae* gene from *Lc. mesenteroides* [22] were described as potentially encoding the oxaloacetate decarboxylase. In previous reports, we demonstrated that in *L. lactis* CRL264 the expression of *citM* is induced by natural acidification of the external medium [14]. To analyze the synthesis of CitM in the wild type strain CRL264, we performed immunoblot experiments using purified anti-CitM antibodies. As shown in Fig. 3A, the presence of a specific immunoreactive band (with the predicted molecular weight, 40 kDa) was found in cell extracts of *L. lactis* CRL264 grown either at pH 5.5 or at pH 7.0 (lanes 1 and 2). The signal corresponding to this band was quantified and a five times higher intensity was found when the cells of *L. lactis* CRL264 were grown at acidic pH (Fig. 3A) in agreement with our previous transcriptional studies [12].

By Western blotting, we also explored the expression of CitM in the strain *L. lactis* MG1363. We found that this strain presents undetectable levels of expression of CitM (Fig. 3, lane 3). Taking into account these results, we decided to complement this strain. For this reason, we constructed the plasmid pHSO1, which allows the expression of *citM* driven by its own promoter region (Section 2). The CitM deficient MG1363 strain was transformed with the plasmid pHSO1 and the cells were grown in M17G either at pH 5.5 or at 7.0. As shown in Fig. 3A, the MG1363/pHSO1 strain expressed the *citM* gene (lanes 4 and 5) and its expression increased when the cells were grown at initial pH 5.5 (lane 4).

The oxaloacetate decarboxylase activity was analyzed in cell extracts from cultures of the strain MG1363/pHSO1 grown at pH 5.5 and 7.0. We found more than fourfold higher activity

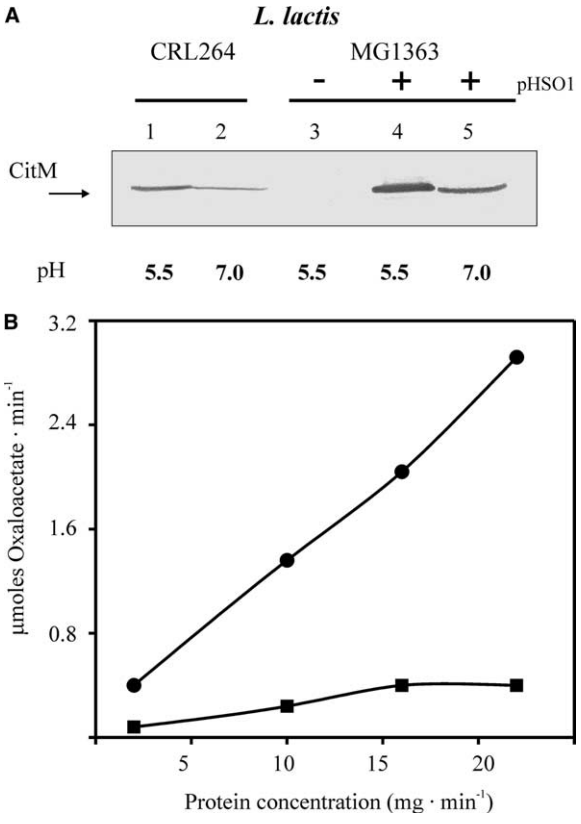

Fig. 3. (A) Expression of *citM* gene in *L. lactis* strains was analyzed by Western blot using purified antibodies anti-CitM. Aliquots containing 30 μg of total protein from extracts of the indicated strain were loaded in each well. (B) OAA decarboxylase activity determined in cell extracts of *L. lactis* MG1363/pHSO1 grown at pH 5.5 (●) and 7.0 (■). Enzymatic activities are indicated as oxaloacetate decarboxylation (μmol min^{-1}).

when the cells were grown at pH 5.5 (Fig. 3B). No significant oxaloacetate decarboxylase activity was found in the parental strain of *L. lactis* MG1363.

All these experiments clearly indicate that *citM* encodes the oxaloacetate decarboxylase involved in citrate metabolism in *L. lactis*.

4. Discussion

MEs are widely distributed enzymes involved in different metabolic pathways. They catalyze the oxidative decarboxylation of malate to yield pyruvate and CO_2, with concomitant reduction of either NAD^+ or $NADP^+$ in the presence of divalent cations. Catalysis by MEs generally proceeds in two steps, the dehydrogenation of malate to produce OAA and decarboxylation to produce pyruvate. In this way, some MEs are also able to catalyze the decarboxylation of oxaloacetate. In bacteria, the function of ME has been poorly explained.

In order to provide further insights into the role of the CitM protein from *L. lactis* CRL264, we searched databases at the websites NCBI and TIGR Institute using the Blastp facility (www.ncbi.nlm.nih.gov and www.tigr.org). This protein was found to be strongly homologous to MEs distributed in prokaryotic and eukaryotic organisms. The amino acid sequence of *L. lactis* CitM was compared with 56 bacterial homologous sequences retrieved from NCBI and TIGR data banks. A multiple sequence alignment of bacterial putative MEs was generated using ClustalW [23]. This alignment revealed highly conserved regions previously described in MEs [1,24]. According to the crystallographic structure of the human mitochondrial NAD^+ dependent ME, CitM contains conserved active site residues: Tyr residue (Y36) and Lys residue (K91) are proposed to be involved in the general acid–base mechanism. The Glu (E133) and Asp (D134 and D159) residues are required for the divalent cation-binding site [14]. Furthermore, CitM is predicted to include two pyridine nucleotide-binding domains (Rossmann fold) that are highly conserved

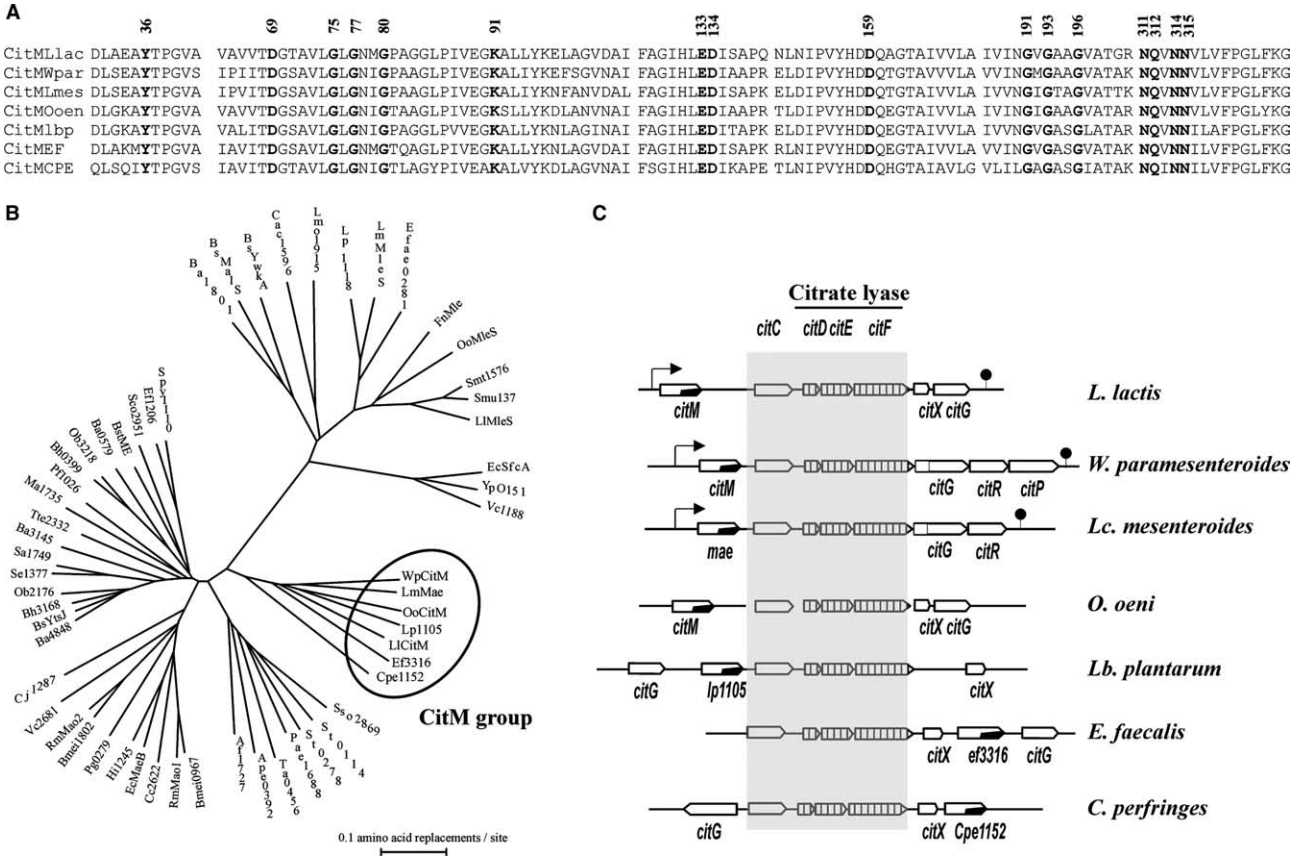

Fig. 4. (A) Multiple sequence alignment of seven MEs associated to citrate fermentation cluster. The sequences were aligned with ClustalX program. The numbers and amino acids in bold correspond to conserved residues present in CitM of *L. lactis*. (B) The phylogenetic analysis of 57 MEs available in the database shows a close group of CitM related enzymes (black circle). NCBI Entrez protein data bank: *S. solfataricus*, Sso2869; *Sulfolobus tokodaii*, St0114, St0278; *Pyrobaculum aerophilum*, Pae1688; *Thermoplasma acidophilum*, Ta0456; *Aeropyrum pernix*, Ape0392; *Archaeoglobus fulgidus*, Af1727; *Brucella melitensis*, Bmei0967, Bmei1802; *Sinorhizobium meliloti*, RmMao1, RmMao2; *E. coli*, EcMaeB, EcSfcA; *Caulobacter crescentus*, CC2622; *Haemophilus influenzae*, HI1245; *Porphyromonas gingivalis*, PG0279; *Vibrio cholerae* VC2681, VC1188; *Yersinia pestis*, Ypo151; *Campylobacter jejuni*, Cj1287c; *B. subtilis*, YtsJ, MalS, YwkA: *B. anthracis* Ba4848, Ba3145, Ba0579 Ba1801; *B. halodurans*, Bh3168; Bh0399; *B. stearothermophilus*, BstME; *Oceanobacillus iheyensis*, Ob2176, Ob3218; *Staphylococcus aureus*, Sa1749; *Staphylococcus epidermidis*, Se1377; *Thermoanaerobacter tengcongensis*, Tte2332; *Methanosarcina acetivorans*, Ma1735; *Pyrococcus furiosus*, Pf1026; *Streptomyces coelicolor*, Sco2951; *Enterococcus faecalis*, Ef1206, Ef3326; *E. faecium*, Efa0281; *Streptococcus pyogenes*, Spy1110; *Clostridium acetobutylicum*, Cac2596; *C. perfringes*, Cpe1152; *Listeria monocytogenes*, Lmo1915; *Lactobacillus plantarum*, Lp-1105, Lp-1118; *Leuconostoc mesenteroides*, LmMae, LmMle; *Fusobacterium nucleatum*, FnMle; *Oenococcus oeni*, OeCitM, OeMle; *W. paramesenteroides*, WpCitM; *L. lactis*, LlCitM, LlMleS. TIGR data bank: *Streptococcus mitis*, Smt.1576, *S. mutants*, Smu.137. Mle: malolactic enzyme. (C) Gene context analysis of each gene belonging to the phylogenetic CitM cluster. All members of this cluster are physically associated to genes involved in the citrate fermentation pathway.

(66-VVTDGTAVLGLGNMG-80 and 184-LKIVINGVGAAG V-AT-199) (Fig. 4A). The presence of these conserved residues suggests that CitM shares the same catalytic mechanism for the decarboxylation of the oxaloacetate than MEs. Nevertheless, other changes in essential amino acids could be the reason for the loss of the capability to reduce malate by this enzyme.

We constructed a phylogenetic tree using DDBJ homology search (www.ddbj.nig.ac.jp). The *L. lactis* CitM was found in a phylogenetically closed group belonging to gram-positive bacteria of low G + C DNA (cluster *Bacillus Clostridium*) (Fig. 4B, in black circle). In order to investigate each member of this cluster in more detail, the genetic context of each gene was analyzed. Using sequenced genomes, we found that each ME in this group is functionally linked to genetic clusters involved in the metabolism of citrate. As shown in Fig. 4C, each member of the *citM* cluster is coupled with *citDEF* (citrate lyase) and accessories genes (*cit(X)G* and *citC*, required for the biosynthesis of the prosthetic group and active enzyme) that split citrate into OAA and acetate. Thus, the idea that the OAA decarboxylase activity involved in the citrate pathway is encoded by *citM* in *L. lactis* is also supported by the phylogenetic studies and analysis of the gene context in these microorganisms [25].

The biochemical characterization of CitM demonstrated that this enzyme catalyzes the specific decarboxylation of OAA to produce pyruvate. The presence of a divalent cation was essential for the decarboxylation reaction and no activity could be measured in the presence of EDTA. In common with other enzymes of this type, millimolar concentrations of Mn^{2+} elicit the best activity; the physiological significance of this observation remains to be seen whereas most cells contain millimolar Mg^{+2}, such levels of Mn^{2+} are not normally encountered. The hypothesis that the CitM is an OAA decarboxylase is supported by the inhibition results obtained when the assays were performed in the presence of structural analogous of oxaloacetate such as malonate and oxalate (Table 1). It has been reported that some MEs are able to catalyze OAA decarboxylation and the presence of dinucleotide increases the affinity of the enzyme for OAA [20]. However, we observed that $NAD(P)^+$ inhibits the decarboxylase activity.

Evidence from biochemical and genetic data demonstrates the role of *citM* in lactic acid bacteria. Also, we identified the presence of CitM in *L. lactis* CRL264 by Western blot (Fig. 3A) and we determined oxaloacetate decarboxylase activity in cell extracts of the *citM* deficient *L. lactis* MG1363 carrying *citM* in the plasmid pHSO1 (Fig. 3B). In conclusion, we show that CitM is involved in the decarboxylation of oxaloacetate in *L. lactis*.

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